# BIOREGENERATIVE LIFE-SUPPORT SYSTEMS FOR CREWED MISSIONS TO THE MOON AND MARS

EDITED BY: Cyprien Verseux, Jean-Pierre Paul de Vera, Natalie Leys and Lucie Poulet
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# BIOREGENERATIVE LIFE-SUPPORT SYSTEMS FOR CREWED MISSIONS TO THE MOON AND MARS

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# Editorial: Bioregenerative life-support systems for crewed missions to the Moon and Mars

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Editorial on the Research Topic
Bioregenerative Life-Support Systems for Crewed Missions to the Moon and Mars

The present decade may see the beginning of a sustainable human presence on the Moon; the next may be that of humankind's first steps on Mars. Such at least is the goal of the leading space agencies (ISECG, 2018), and private companies—most publicized, SpaceX—have stated related objectives (Musk, 2017).

Humans, of course, need a habitable environment and a wealth of consumables to survive: food, water, oxygen and possibly medication, to name a few. As missions get longer and more remote, providing all these consumables from Earth becomes unrealistic: launch costs, travel times, and risks of failure are critical obstacles. Bioregenerative life-support systems (BLSS) are a highly promising way of addressing this limitation, even more so if they can be combined with *in situ* resource utilization (ISRU). In the present Research Topic, this is illustrated by Berliner et al., who argue for an integrated biomanufacturing plant for resource production and recycling on Mars. They also present associated challenges, goals, and example systems.

Despite extensive research performed over the last few decades, no BLSS project has reached enough maturity to significantly increase the autonomy of even a small-sized base on the Moon or Mars. Experience gained from long-running BLSS projects (e.g., ESA's MELiSSA project; Lasseur et al., 2010; Walker and Granjou, 2017) shows that their development is a long-term process. Pragmatic efforts are thus needed presently for BLSS to be ready when Moon and Mars missions would benefit from them. This Research Topic aimed at stimulating such efforts.

Lunar and Martian BLSS will most likely include plants, which are necessary for food production. In addition, they provide air revitalization and water purification capabilities (e.g., Wheeler, 2010), and could be used for other functions including, for instance, pharmaceutical production (McNulty et al., 2021). Accordingly, nine contributions to this

Research Topic focus on plant cultivation. Johnson et al. review NASA's work toward the development of plant chambers for supplemental, fresh food production in space. Such chambers could be used in early missions, before on-site production covers the crewmembers' entire nutritional needs. Another facility, this one at the University of Naples, is described by Pannico et al. (with a higher focus on its atmosphere control system): the Plant Characterization Unit, an environmentally-controlled chamber for investigations on BLSS higher plant compartments. Poulet et al. describe major challenges for space crop production, as identified by the Kennedy Space Center, as well as NASA's efforts to overcome them. Medina et al. and Schuerger each focus on one of these challenges. Medina et al. give an overview of the available knowledge, and its gaps, on the influence of gravity levels below 1 g on the early development of plants. Schuerger argues that pests and phytopathogens, common in terrestrial agriculture, will be a concern in plant-supported missions to the Moon and Mars; he therefore outlines a first-order integrated pest management program. Tack et al. introduce an additional challenge: their results suggest that average ionizing radiation levels at the surface of Mars could reduce plant productivity (but not germination), although technical difficulties made conclusions hard to draw. Handy et al. describe, rather than challenges, some opportunities: those brought by plant growth promoting bacteria. They identify promising ones, isolated from a crop production system aboard the ISS. Finally, two articles pertain to the use of lunar and Martian regolith as plant growth substrates. In the first, Duri et al. describe regolith simulants previously used for cultivation experiments, review these experiments, and discuss solutions aimed at improving the suitability of simulants (and, possibly, of actual lunar and Martian regolith) for agriculture. In the second, Peyrusson presents preliminary results which suggest that hydrogels could improve the water retention of Martian regolith, thereby fostering germination and growth under low irrigation regimes.

Whether or not they are directly associated with plants, microorganisms are other likely components of lunar and Martian BLSS. The roles they could fulfill include waste processing, food production, atmosphere regeneration, the production of drugs, fuels, biomaterials and various industrially useful chemicals, metal leaching, and food processing for taste improvement (e.g., Hendrickx and Mergeay, 2007; Horneck, 2008)—in some cases after genetic engineering (see for instance Cockell, 2011; Montague et al., 2012; Menezes et al., 2014; Verseux et al., 2016). A number of microbial species have been proposed and the complexity of the targeted applications, as well as the variety of microbial metabolisms, make it hard to select rationally the microorganisms to be used. Averesch provides insights: he compares microbial systems which could be suitable for ISRU on Mars and sketches some classification schemes. He suggests, for instance, that microbial systems can be sorted based on carbon conversion: on whether carbon is directly converted from an inorganic state to end products, or first fixed by primary producers and then used as a substrate for secondary producers. An example of the latter case is provided by Cestellos-Blanco et al. They present a process aimed at producing PHB (a biodegradable polyester whose material properties resemble that of polyethylene, and which can be 3D-printed) from Mars's atmospheric $CO_2$ in two steps, each carried by a separate bacterium: $CO_2$ is first used as feedstock to generate acetate, which then serves as a substrate for PHB production.

While in the example given above the carbon fixer is an acetogen, most microbial primary producers under consideration are photosynthetic. Six articles of the present Research Topic treat of such organisms. Fahrion et al. review experiments performed with photobioreactors, over the past three decades, in view of developing BLSS for human space exploration. They also identify gaps in knowledge. Two articles focus on *Limnospira indica* (formerly *Arthrospira* sp. PCC8005), the cyanobacterium included in MELiSSA (Hendrickx et al., 2006). In the first, Poughon et al. describe a mass-balanced mechanistic model which can describe and predict its growth in photobioreactors of various scales. In the second, Sachdeva et al. compare the effects of three different nitrogen sources (nitrates, and the prominent nitrogen forms in non-nitrified urine: urea and ammonium) on its oxygen production rates, in a ground demonstrator where the cyanobacterium revitalizes the air breathed by a mouse. Results should help in assessing whether the nitrification of urine fed to cyanobacteria can be skipped, which could reduce the complexity of the MELiSSA loop. Detrell writes on the potential of the eukaryotic microalga *Chlorella vulgaris* (recently sent to the ISS for experiments on life support; Detrell et al., 2020) as a BLSS component for food production and air revitalization, as well as on the associated challenges. Cycil et al. exposed five microalgal species, considered for food production and air revitalization, to low total pressures (down to 80 hPa) of high-$CO_2$ atmospheres. The goal was to compare the organisms' tolerance for hypobaric conditions: relying on lower-than-ambient pressures in photobioreactors could help relax engineering constraints, and consequently the costs, of microalgal cultivation on the Moon or Mars (Kanervo et al., 2005; Verseux et al., 2021). Finally, Matula et al. assess the impact of rapid temperature variation on the oxygen production of temperate and psychrotolerant microalgae. Results will help in assessing the feasibility of using culture media as a heat sink in crewed spacecraft, thereby coupling air revitalization with temperature control.

While carbon is certainly central to BLSS, as illustrated by the many articles in this Research Topic which focus on organisms capable of its fixation, nitrogen is another key element (see, e.g., Loader et al., 1997). Its recycling will most likely require microorganisms, and so may its fixation from Mars's atmospheric $N_2$: though abiotic fixation with the Haber-Bosch process is being considered as well, the associated upmass and energy consumption are high. Three articles address this theme.

One is that of Sachdeva et al., already mentioned above. Another is that of Langenfeld et al., who review approaches considered for nitrogen fixation and recycling in BLSS. The third is that of Verbeelen et al., who discuss nitrogen recovery from urine waste streams (the main source of nitrogen in BLSS waste) and detail the compartment which, within MELiSSA, performs such functions.

Microbial capabilities on the Moon and Mars can go beyond basic life support. In addition to the PHB production process described by Cestellos-Blanco et al., this is exemplified by Kozyrovska et al.: they discuss potential applications of kombucha microbial communities beyond Earth that range from the synthesis of health-promoting compounds to the production of clothing materials.

Plants, bacteria and microalgae are not the only organisms considered for lunar or Martian BLSS. Examples of proposed elements include fungi (Cortesão et al., 2020), insects (Li et al., 2015), and fish. Przybyla addresses the last. He discusses the prospects of space aquaculture, reviews experiments with fish in low Earth orbit, and describes Lunar Hatch: a project whose contributors assess the feasibility of sending fish eggs to the Moon for on-site hatching.

A number of concepts for BLSS elements have been described, here and elsewhere, and promising proofs-of-concept have been obtained. The next steps are highly challenging. Efforts are nonetheless underway to test the integration of different elements, or to assess the cost-efficiency of maturing BLSS technologies. Garcia-Gragera et al., for instance, report recent results from MELiSSA's Pilot Plant, a ground-based demonstrator whose focus is currently on the integration of three elements: a nitrifying packed-bed bioreactor, an air-lift photobioreactor for *L. indica*, and an animal isolator with rats as a mock-up crew. McNulty et al. present an assessment, based largely on equivalent system mass (a single metric accounting for mass, volume, power, cooling and crew-time requirements; Levri et al., 2003), of different strategies for the purification of monoclonal antibodies. More broadly, they discuss paths toward the development on-site pharmaceutical production systems, as well as approaches to their evaluation. Finally, Irons and Irons propose a framework to quantify the sustainability of BLSS, after pointing out that, while BLSS are often seen as enablers of sustainability beyond Earth, ways of formally quantifying sustainability are lacking.

When proposing this Research Topic, we hoped that it would both provide an overview of the field and lessons from past efforts, as well as introduce innovative concepts and new results that may find their way into mission designs. Thanks to the enthusiasm of our colleagues who submitted manuscripts, and to the diligence of the solicited reviewers, we hope that readers from the broader scientific community will find that these objectives have been met.

## Author contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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# Optimizing Nitrogen Fixation and Recycling for Food Production in Regenerative Life Support Systems

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Nitrogen (N) recycling is essential for efficient food production in regenerative life support systems. Crew members with a high workload need 90–100 g of protein per person per day, which is about 14 g of N, or 1 mole of N, per person per day. Most of this N is excreted through urine with 85% as urea. Plants take up N predominantly as nitrate and ammonium, but direct uptake as urea is possible in small amounts. Efficient N recycling requires maintenance of pH of waste streams below about 7 to minimize the volatilization of N to ammonia. In aerobic reactors, continuous aerobic conditions are needed to minimize production and volatilization of nitrous oxide. N is not well recycled on Earth. The energy intensive Haber–Bosh process supplies most of the N for crop production in terrestrial agriculture. Bacterial fixation of dinitrogen to ammonium is also energy intensive. Recycling of N from plant and human waste streams is necessary to minimize the need for N fixation. Here we review approaches and potential for N fixation and recycling in regenerative life support systems. Initial estimates indicate that nearly all the N from human and plant waste streams can be recovered in forms usable for plants.

**Keywords: nitrogen, nitrogen recycling, regenerative life support, nitrogen fixation, nitrogen on Mars**

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## INTRODUCTION

Human habitation on extraterrestrial surfaces presents a challenge due to their distance from Earth and inhospitable conditions for life. Earth's moon has virtually no atmosphere, and the thin atmosphere of Mars is dominated by carbon dioxide ($CO_2$) with small amounts of nitrogen (N) and oxygen (Owen et al., 1977). Because of the thin atmospheres of the Moon and Mars, there will be minimal protection from dangerous short-wave radiation from the Sun and incoming meteorites, meaning that early missions will likely take place in largely closed habitats and must rely on supplies brought from Earth to sustain crew members (CMs). This creates a supply dependency, which can lead to problems if unforeseen challenges arise during extended missions. International space agencies have funded decades of research to promote the development of advanced life support systems for CMs to maintain self-sustainability by producing food and oxygen (Wheeler, 2010). Although optimizing plant growth has been a large research topic, little work has been funded to study nutrient recycling. A related study nearly 25 years ago (discussed later) was funded in the United States by the National Aeronautics and Space Administration (NASA) to model mass balance of N in a closed system (Loader et al., 1997).

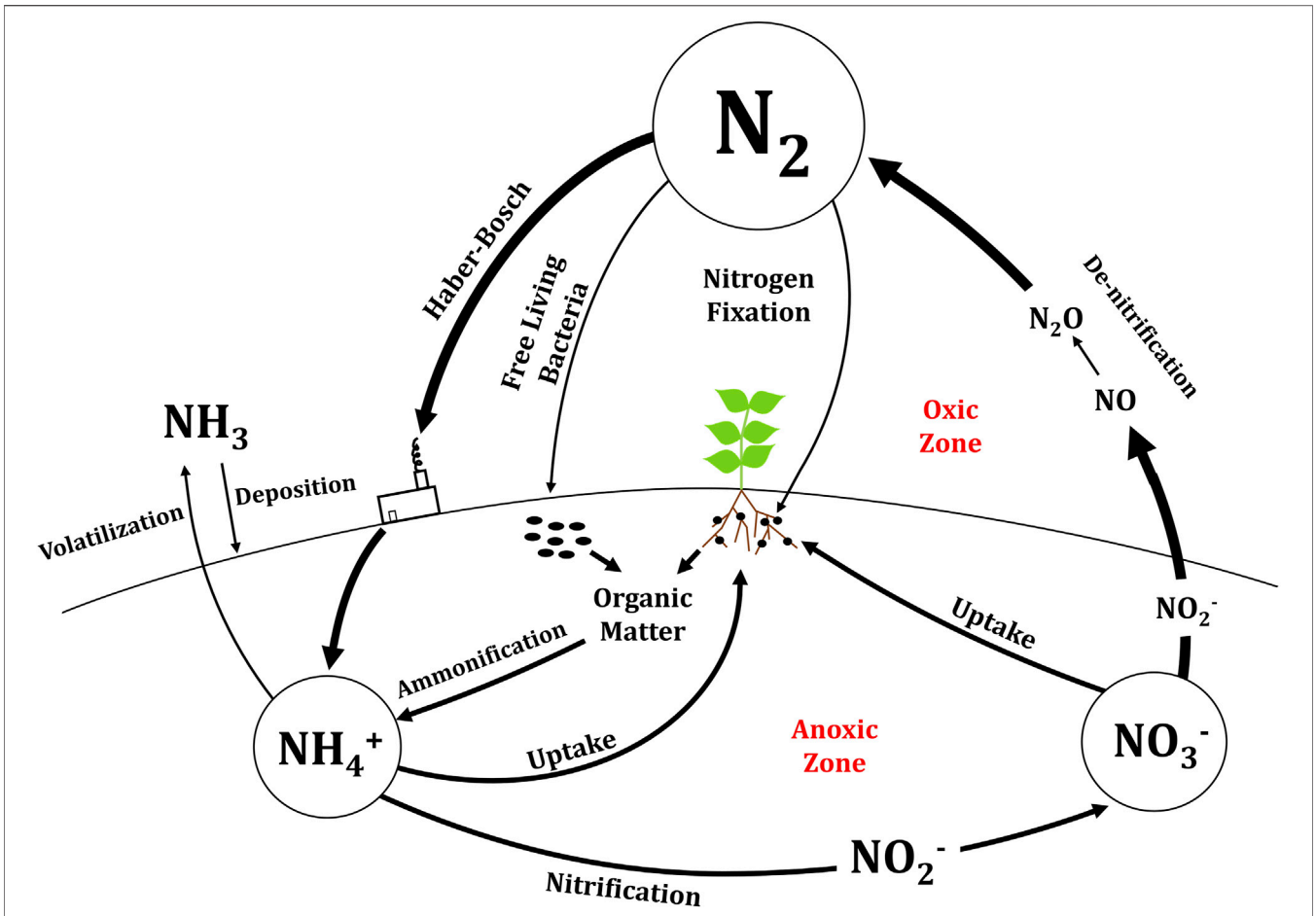


**FIGURE 1 |** Conversions between nitrogen forms in the nitrogen cycle on Earth. The long convex line represents the surface of Earth with anoxic conversions below ground and oxic conversions above ground.

In the short term, bioavailable N must either be shipped from Earth or fixed from atmospheric dinitrogen gas ($N_2$). We expect it to be highly cost effective to recycle N. N is likely present in small amounts of Lunar and Martian regolith in addition to being a small component of the Martian atmosphere (Kerridge, 2001). Existing surface N may come from the solar wind or from carbonaceous chondrite meteorites, which have been found to contain amino acids (Cronin and Pizzarello, 1983). Changes in atmospheric composition over time may have resulted in higher or lower levels of N in the past, which may now be locked away in untapped deposits (Klingler et al., 1989; Gebauer et al., 2020). These N reserves represent a possible source for N that may be mined in the future once colonies are established on extraterrestrial surfaces. This review focuses on *in situ* resource utilization of N by fixing it directly from the atmosphere.

Recycling becomes paramount, especially as mission duration and scope increase. N is an essential plant macronutrient that presents a rich opportunity for recycling due to its well-understood transformations between organic and inorganic forms. N recycling must rely on integrated waste collection, separation, and processing components. Waste must be collected and source separated for successful recovery of N from human excreta. Urea must then be removed and concentrated from the liquid phase before being efficiently hydrolyzed to ammonium to minimize volatilization as ammonia gas and maximize N recovery. Plant and human waste can be proportionally fed into a combination of aerobic and anaerobic digesters to promote nitrification and mineralization. These processes produce ammonium and nitrate that can be used as a N source for plants, which produce food, completing the cycle. Optimizing every step in the cycle decreases the amount of $N_2$ that must be recovered from the atmosphere through bacterial N fixation. Here we review approaches for recycling N and progress on microbial N fixation for advanced life support systems.

## Fixing Nitrogen

The N cycle on Earth is shown in **Figure 1**. The atmosphere of Earth is about 79% inert $N_2$. $N_2$ is fixed into bioavailable ammonium by archaea and bacteria (either free-living or plant-associated) and by the Haber–Bosch process, with the biological and industrial processes each accounting for approximately 50% of globally fixed N. Both approaches are energy intensive and require large amounts of either adenosine

triphosphate (ATP) or fossil fuels, respectively (Fowler et al., 2013). This fixed N can be rapidly taken up by plants and assimilated into proteins (Xu et al., 2012).

## Nitrogen Transformations

Ammonium that has been fixed from the atmosphere can be converted to nitrate by naturally occurring nitrifying bacteria or through industrial production. Biological nitrification occurs across two steps when *Nitrosomonas* spp. oxidize ammonium to nitrite and *Nitrobacter* spp. oxidize nitrite to nitrate (Gee et al., 1990). Plants can then uptake either ammonium or nitrate. N is transferred from plants to animals as proteins. After hydrogen, carbon, and oxygen, N comprises the highest quantity of all essential nutrients on a mole basis in both plants and animals. This is because N is a major component of proteins, as well as nucleic acids, chlorophylls, and defensive compounds (Mu et al., 2016; Stein and Klotz, 2016).

When proteins degrade in mammalian cells, the N is converted to urea and is discarded by the organism through urine and sweat. Waste products also include other nitrogenous compounds in small amounts such as creatinine, ammonium bicarbonate, and ammonium citrate (Verostko et al., 2004). Urea is a stable molecule that will hydrolyze unaided with a half-life of 3.6 years to form two molecules of ammonia and one molecule of carbonic acid (Zerner, 1991). The enzyme urease, both free and in bacteria, catalyzes the conversion of urea to ammonia above pH five and increases the reaction rate by four orders of magnitude compared to unaided hydrolysis (Amtul et al., 2002; Udert et al., 2003). Urease is found in both bacteria and plants, but not in animals. Animals must excrete urea as a waste product before it builds up to toxic concentrations. Urea is the most common N fertilizer applied to crops with almost 60% of all N fertilizers being urea (Davis et al., 2016). Urea applied to the soil is hydrolyzed by urease in naturally occurring bacteria for plant uptake or is taken up in small amounts through plant cells by transporters, where it is later hydrolyzed and assimilated into proteins (Wang et al., 2008).

## Atmospheric Losses

In addition to waste products from living organisms, decomposition of dead organisms and organic matter by decomposers (detritivores, bacteria, and fungus) releases organic N as ammonium and nitrate back into the soil. N that has been energetically fixed to ammonium and nitrate can be lost back to the atmosphere through abiotic and biotic processes, respectively. Ammonium can volatilize to ammonia gas under alkaline conditions and escape to the atmosphere. Oxidation of ammonium by ammonia-oxidizing microorganisms produces a hydroxylamine intermediate that can be oxidized to nitric oxide, then to nitrite, and then to nitrate or reduced to nitrous oxide and $N_2$ (Soler-Jofra et al., 2021). The bacterial process of annamox can also oxidize ammonium to $N_2$ using either nitric oxide or nitrite as an oxidant while producing water (Stein and Klotz, 2016). While both nitrification and denitrification can produce nitrous oxide, the major products of nitrification are nitrite and nitrate, and the major product of denitrification is $N_2$ (Heil et al., 2016; Wrage-Mönnig et al., 2018).

## Extraterrestrial Nitrogen Cycle

The atmosphere on Earth is dominated by N (79% $N_2$ gas), but the Martian atmosphere is dominated by $CO_2$ and contains less than 2% $N_2$ gas by volume (Mahaffy et al., 2013). In space or extraterrestrial surfaces, industrial $N_2$ fixation will not initially be feasible due to the large amount of energy and infrastructure needed. Instead of industrial fixation, early inhabitants must either bring N reserves from Earth or fix N using bacteria. Bringing large initial N stocks from Earth as a N gas ($N_2$ or ammonia) or a solid (nitrate salt) can be challenging due to transport difficulties and overall N percentage. For example, ammonia gas is 82% N on a mass basis, while salts have a much lower percentage. Frequent resupply is currently impractical with extended mission durations, and bacterial N fixation represents the most feasible option. Recovery of N with minimal volatilization is essential to reduce the resources (launch mass/equivalent system mass) necessary to support bacterial N fixation. The volumetric and chemical demands of a biological N fixation system to support a manned mission are expected to be significant, even with high N recovery in fixation, composting, and human waste integration. An infrastructure capable of efficient N recovery at each step will allow N fixation to focus mainly on replenishing unrecovered N and will drastically reduce volumetric, chemical, and energetic demands required for fixation.

An overview of N recycling on Mars is provided in **Figure 2**. $N_2$ from the Martian atmosphere will be fixed by N-fixing microbes in bioreactors. Biological fixation is still possible even with the much lower percentage of atmospheric N on Mars compared to Earth (Klingler et al., 1989). This step transforms $N_2$ into organic N in the form of proteins. These proteins must be degraded to plant-available N (ammonium) through anaerobic digestion. Small amounts of N are lost during anaerobic digestion as gaseous and recalcitrant forms. Plants may be grown hydroponically or in media and must be fed a combination of ammonium and nitrate to overcome toxicity issues/physiological disorders. Half of the plant biomass is edible, while the other half is inedible and must be composted in an aerobic digester. This aerobic digestor can also take in hydrolyzed urea from human urine and output nitrate for plants, but minor gaseous losses are again possible. Feces can also be composted in a digester to recover N that is fed back to plants. This simple overview shows how recycling of N can greatly reduce the demand for atmospheric N fixation.

Loader et al. (1997) appear to be the first group to publish a simple model for mass balance of N for regenerative life support. The authors envisioned fixing N from the life-support module atmosphere with an aquaculture unit providing additional protein. Their model suggests that denitrification might be a 12% gaseous loss. A well-designed plant production system with adequate aeration should have minimal denitrification. They did not include an anaerobic reactor, make mention of N recycling from urine, and did not include loss as recalcitrant N. While their separate nitrification and aerobic bioreactors produce nitrate and ammonium, our

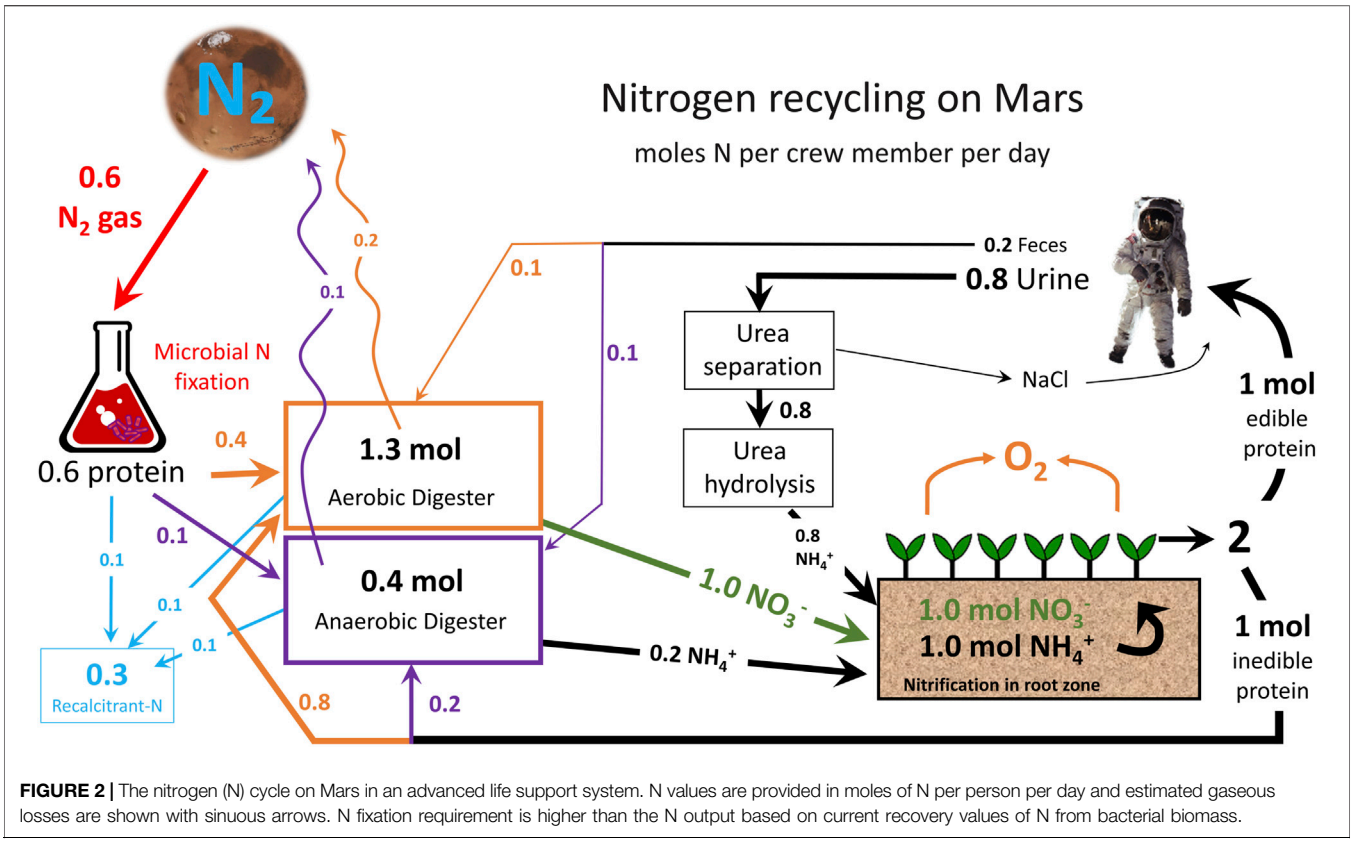


**FIGURE 2 |** The nitrogen (N) cycle on Mars in an advanced life support system. N values are provided in moles of N per person per day and estimated gaseous losses are shown with sinuous arrows. N fixation requirement is higher than the N output based on current recovery values of N from bacterial biomass.

**TABLE 1 |** Daily nitrogen (N) replenishment requirements for a crew member (CM) in an advanced life support system.

| Component | N requirement (N $CM^{-1}$ $d^{-1}$) | |
|---|---|---|
| | g | mol |
| Urine (L)<br>N in urine (g $L^{-1}$) | 1,500<br>8.1 | |
| Total N from urine | **12.2** | **0.87** |
| Fecal matter (g)<br>Dry mass (%)<br>N in feces (%) | 123<br>25<br>5–7 | |
| Total N from feces | **1.8** | **0.13** |
| Daily CM N loss/replenishment | **14** | **1** |
| Plant harvest index (%) | 50 | |
| Total plant N requirement | **28** | **2** |
| N loss after bacterial fixation (%) | 20 | |
| N fixation requirement | **35** | **2.5** |

*Values for N are presented as grams and as moles in bold. Units for all other assumptions are provided in the first column. Harvest index is the percent of total plant biomass in edible food. Here, it is assumed that N is allocated equally between edible and inedible plant biomass.*

model specifies separate bioreactors to produce bioavailable N. Their model estimated similar values for plant uptake, harvest index, and human N requirements.

## NITROGEN REQUIREMENTS

Adult humans excrete about the same amount of N as they ingest on a daily basis. The daily N requirement is determined by the N required to replenish spent proteins and nucleic acids. This can be calculated based on replenishment of excreted N as shown in **Table 1** or based on the requirements of ingested nitrogen.

### Requirements Based on Excreted Nitrogen

About 90% of the nitrogen discarded by humans is found in urine (Heinonen-Tanski and van Wijk-Sijbesma, 2005). CMs are estimated to produce on average 1.5 kg urine $CM^{-1}$ $d^{-1}$ (Anderson et al., 2018). The concentration of urea in typical adult human urine is 13.4 $gL^{-1}$ with ammonium salts contributing 4.1 $gL^{-1}$ and organic compounds contributing 5.4 $gL^{-1}$ (Putnam, 1971). The total N concentration across the urine waste products equates to 8.1 $gL^{-1}$ or 12.2 gN $CM^{-1}$ $d^{-1}$. Daily fecal excretion is estimated to be about 123 g $CM^{-1}$ $d^{-1}$. Feces is about 5–7% N on a dry mass basis, which represents 25% of the wet mass (Harder et al., 2019). N lost in solid excreta therefore represents an additional requirement of 1.8 gN $CM^{-1}$ $d^{-1}$. This equates to a daily N replacement of about 14 g N, or 1 mole of N, per person per day.

### Requirement Based on Ingested Nitrogen

A CM is expected to consume about 3,000 kcal (12,550 kJ) per day due to high mission workloads (Cooper et al., 2011). If 16% of the daily intake is protein, and protein provides a gross energy of

5.1 $kcalg^{-1}$ protein, a daily intake of about 94 g protein would be required (Hormoz, 2013; Anderson et al., 2018). The daily N requirement is therefore 15 $gN\,CM^{-1}\,d^{-1}$assuming 16% N in protein. These values are based on food exported from Earth (Anderson et al., 2018). Ideally, this caloric intake would be split between initial food supply and food grown in transit or on extraterrestrial surfaces. Mission costs increase when the initial supply is increased (Cahill and Hardiman, 2020), and optimal food production reduces dependence on food resupply. The calculated N requirement based on both crew input and output is about 14 $gN\,CM^{-1}\,d^{-1}$.

## Total Nitrogen Required

Assuming 14 gN per CM per day, four CMs over a standard 967-days mission would require 54 kg of N to replace that lost to urine excretion (Anderson et al., 2018).

However, about half of the N in plant biomass is not in an edible form. Harvest index is the ratio of edible to total biomass and varies from 20% in peanut (*Arachis hypogaea*) and sweet potato (*Ipomoea batatas*) to 90% in lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea*), with an average value of 50% for common crops (Wheeler et al., 2003; Anderson et al., 2018). This means that the N required to grow the food is doubled to 28 $gN\,CM^{-1}\,d^{-1}$. This must come primarily from ammonium and nitrate.

### Nitrogen-Fixing Bacteria

Without recovery, capture of N must come directly and entirely from the atmosphere. On Earth, this process is split between microbial production and Haber-Bosch industrial production. The energy and mass requirements to fix N using the Haber-Bosch process are likely prohibitively expensive. A more efficient approach, especially on a small scale, is to use bacterial fixation through the enzyme nitrogenase. This can be accomplished by fixing N from the atmosphere of an enclosed life support system or from the Martian atmosphere, although the lower partial pressure of $N_2$ must be considered (Klingler et al., 1989; Mahaffy et al., 2013).

#### *Nitrogenase*

Nitrogenase uses ATP to reduce $N_2$ to two molecules of ammonia, consuming electrons in the process (Newton, 2007). ATP required to fix $N_2$ can be produced through consumption of reduced carbon compounds or light reactions, depending on chemotrophic or phototrophic metabolism (Soundararajan et al., 2019). At a biologically relevant pH, ammonia is in the protonated ammonium form and is assimilated to glutamate by glutamate and glutamine synthetase for use in proteins (Nagatani et al., 1971). N fixation efficiency of nitrogenase is largely determined by oxygen concentrations with anaerobic conditions leading to the highest efficiency (Mortenson, 1978).

#### *Cyanobacteria*

Plant-associated bacteria, such as rhizobia in legumes, have long been known to be productive N fixers (Franche et al., 2009). They form associations with plant roots and form nodules, which provide the host plant with N. Growing non-leguminous plants in regenerative life support systems requires free-living N-fixing bacteria, such as heterotrophic *Azotobacter* spp. or photosynthetic cyanobacteria (Meeks and Elhai, 2002; Inomura et al., 2017). Early work with cyanobacteria in regenerative life support systems was carried out at NASA Ames by Packer et al. (1986). Major cellular components, such as protein, glycogen, and sugars, were higher when the cyanobacteria *Nostoc muscorum* were grown under white light compared to blue light. They also characterized extreme oxygen sensitivity to nitrogenase as stated above. Cyanobacteria have continued to be a promising resource for regenerative life support systems, though recent work has focused on producing carbon compounds such as ethanol instead of fixing N (Zhou and Gibbons, 2015). Cyanobacteria are capable of N fixation under aerobic conditions and would be valuable for integration with oxygen-containing components of life support systems (Stal, 2015). Combining cyanobacterial means of N fixation with anaerobic methods can add to life support system versatility.

#### *Purple Non-sulfur Bacteria*

Multiple species of purple non-sulfur bacteria (PNSB) are also ideal for N fixation, especially because of their accumulation of compounds beneficial for plants and their ability to switch between multiple metabolic methods (Sakarika et al., 2020). *Rhodopseudomonas palustris* TN110, a strain of a widespread PNSB, was shown by Sakpirom et al. (2017) to contain all three nitrogenase isozymes (Mo–Fe, V–Fe, and Fe–Fe) and released the highest concentration of ammonium among 235 tested isolates. This makes *R. palustris* a valuable asset to a potential biological N fixation system and has been the basis for the N fixation and recovery systems described below.

## Recovery of Nitrogen from Bacteria

Once fixed by bacteria, N must be transformed into bioavailable forms for plant uptake. When applied to the root-zone, both living and dead PNSB cells have been shown to increase edible biomass across many species (Sakarika et al., 2020). In addition to supplying N, it is possible that PNSB may indirectly promote plant growth by promoting the growth of other microorganisms which release plant growth promoting substances (PGPS), such as auxins. Kondo et al. (2008) found reduced growth of spinach (*S. oleracea*) and komatsuna (*Brassica rapa* var. *perviridis*) when applying PNSB to sterilized soil, demonstrating a possible interaction with other soil microorganisms. Rice (*Oryza sativa*) yield was enhanced when a biofertilizer comprising of PNSB at a concentration of $10^8$ cells $g^{-1}$ was applied at 0.75 $kgha^{-1}$, although no distinction was made between growth promotion due to release of N or other PGPS (Kantachote et al., 2016). Substitution of PNSB for synthetic N did not significantly change the fruit mass of tomato, but malic and phosphoric acid content were increased, contributing to higher fruit quality (Kondo et al., 2010).

Few studies have quantified the recovery of N from PNSB for use as a fertilizer. The most closely related studies have focused on determining quantities and rates of ammonia production in ruminants. Hyper-ammonia-producing (HAB) gram-positive bacteria isolated from the intestines of ruminants can ferment amino acids to ammonia (Bento et al., 2015). Eschenlauer et al. (2002) reported ammonia production rates of 1.8–19.7 nmol ammonia per mg of protein per min from HAB grown on

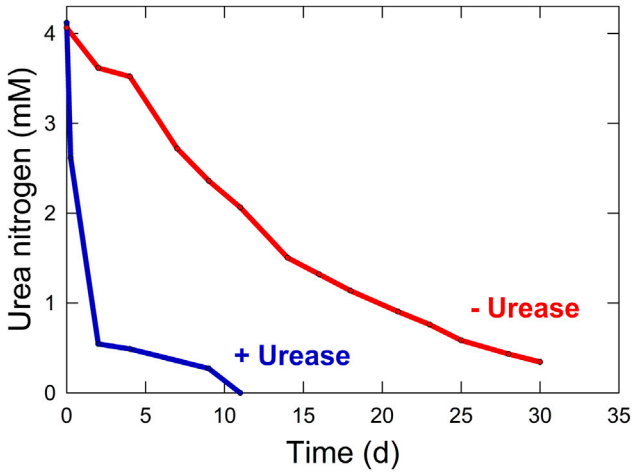


**FIGURE 3 |** Rate of urea hydrolysis to ammonium with (3.2 enzyme units $L^{-1}$) and without (0 enzyme units $L^{-1}$) the addition of urease from Jack bean (*Canavalia ensiformis*).

trypticase. The ammonia production rate of HAB in ruminants varies considerably based on diet and has been found to be higher *in vitro* than *in vivo* (Taghavi-Nezhad et al., 2014). Capture of ammonia under acidic conditions will lead to protonation, preventing loss of N through volatilization.

## Nitrogen Requirement Without Recycling

Preliminary studies from our laboratory indicate 80% of total N from *R. palustris* can be recovered as ammonium if the pH is rigorously controlled at 7. If we assume 28 gN $CM^{-1}$ $d^{-1}$ from plants combined with an 80% recovery efficiency from PNSB this leads to a final total of 35 gN $CM^{-1}$ $d^{-1}$ that must be fixed by bacteria. This number can be significantly reduced if N is recycled.

# OPTIMIZING NITROGEN RECOVERY

Optimizing N recovery involves increasing the efficiency of recovery from excreta and maximizing the recovery of bioavailable N (ammonium and nitrate) from anaerobic and aerobic digestion of human and plant wastes. Minimizing volatile losses and/or maximizing recovery of volatiles further improves N recovery. Selecting for crop cultivars with a high harvest index minimizes inedible plant materials and helps to improve the recovery efficiency.

## Nitrogen Recovery from Urine

The simplest method to apply N to plants from urine is direct application following sterilization. Direct application of urine has been shown to improve plant growth compared to no fertilizer controls, but its use is complicated by low levels of bioavailable N, odor, and salt build-up in the substrate (Salisbury et al., 1997; Pandorf et al., 2019). N must be extracted from the urea in urine and transformed to bioavailable forms to alleviate these issues. This consists of four steps: sterilization, volume reduction, stabilization, and recovery (Maurer et al., 2006). Many methods have been developed to accomplish these tasks, though they vary in simplicity, expense, volume requirements, and efficiency.

### Nitrogen Forms in Urine

The N from protein degradation is converted into urea, which is the dominant N form in the waste stream. Urea comprises over one half of the total dissolved solids and 75–90% of the discarded N in human urine (Rose et al., 2015; Simha and Ganesapillai, 2017). The remaining N is bound in other N-containing compounds such as creatinine and ammonium salts. Urea excretion from the human body varies based on diet, with the average adult human producing about 500 L of urine per year, equating to about 20 g urea $CM^{-1}$ $d^{-1}$ (Lind et al., 2001; Amtul et al., 2002). Urea can be fed directly to plants in the root zone, but it is taken up very slowly. Unaided urea hydrolysis is slow, but will eventually proceed due to bacterial contamination (Elliot, 1986). Supplementing the growing media with small amounts of urease has the potential to significantly increase the rate of hydrolysis (**Figure 3**).

### Sterilization

Urine exits the body as a sterile liquid (Simha, 2013). Pathogens from unhealthy humans and fecal contamination from incomplete source separation compromise sterility (Höglund et al., 2002; Santos et al., 2004). Sterilization involves processes to eliminate pathogens from the waste stream to reduce contamination of downstream products. Urine may be sterilized by heat, pressure, or ultraviolet light, but these methods require energy. Alternatively, storage at low pH for weeks to months can inactivate viruses, inhibit pathogens, combat the rise in pH due to urea hydrolysis, and reduce ammonia volatilization (Hellström et al., 1999; Patel et al., 2020). If acidic conditions are maintained, no N is lost during the sterilization process.

### Volume Reduction and Water Recovery

The volume of urine must be condensed following sterilization to both recycle water and collect the valuable metabolites. Evaporation energy requirements to remove water can be minimized by vapor-compression distillation to generate additional heat for evaporation and to recover 85% of the energy used compared to standard evaporation (Wood, 1982). Urea can also be separated from the liquid phase through freeze/thaw cycles. When urine begins to freeze, the ice that forms has minimal solutes. These solutes, such as urea, remain dissolved in the unfrozen portion. Removal of the distilled frozen water effectively concentrates the dissolved solids while concurrently purifying water for other uses. Lind et al. (2001) found that freezing and melting urine samples could concentrate more than 80% of the original N into a much lower volume. Reverse osmosis may also be used to remove water from urine and concentrate solutes. Ammonia recovery during reverse osmosis has been found to be about 70%, but higher recoveries may be possible if acidification is used to

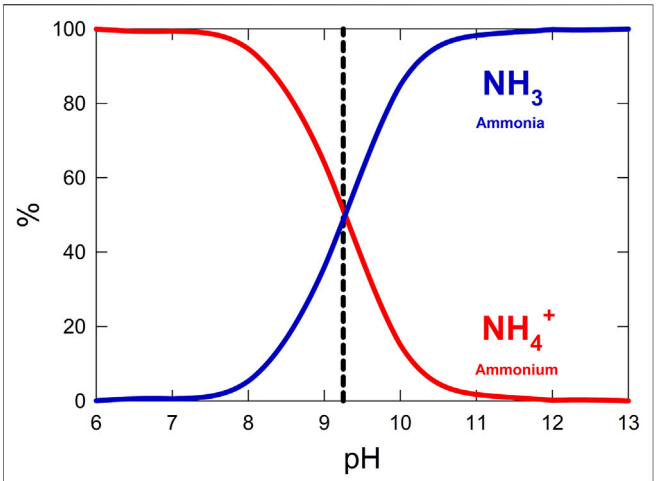


**FIGURE 4 |** Percent abundance of ammonia and ammonium across a range of pH values in a closed system. The pKa of ammonium is shown as a dashed line.

prevent volatilization (Thörneby et al., 1999). An 80% recovery rate means 16 g urea $CM^{-1}$ $d^{-1}$ can be recovered as ammonium following volume reduction.

### Stabilization

Stabilization of condensed urine is essential to minimize volatilization of ammonium to ammonia gas. Released ammonia causes unpleasant odors and becomes difficult to capture in the gas phase. When urea is hydrolyzed in water, ammonium, $CO_2$, and hydroxide are released, causing the pH of the solution to increase. Alkaline pH causes ammonium to deprotonate and volatilize to ammonia gas. Acidification is therefore necessary to both maintain neutral pH for optimal microbial activity in an aqueous system and prevent the loss of ammonia gas as shown in **Figure 4** (Hellström et al., 1999; Maurer et al., 2006). $CO_2$ must be vented or additionally recycled during this step to prevent build-up to toxic levels.

## Recent Recovery Systems

Recovering urea and/or ammonium from urine is the most important step in N recovery from human waste. The pH must be rigorously controlled during the processes to prevent escape of ammonia gas. Current systems take the urea in urine and convert it to ammonium, nitrate, or a mix that can be fed to plants as their principal N sources. A recent example is the two-step Valorization of Urine Nutrients in Africa (VUNA) process where aeration is used for oxidation of organic matter and nitrification, converting half the ammonium in the urine into nitrate, a step that is completed when the pH has fallen to 6.20–6.25. At this point, the VUNA process uses vacuum distillation for recovery of water and a solution that is rich in ammonium nitrate (Udert and Wächter, 2012; Fumasoli et al., 2016).

### Ammonium Recovery

Although energy consumption is high for recovery, volume reduction of urine can reduce the energy requirement by 58% (Maurer et al., 2003). Tun et al. (2016) studied filters for reducing water volume and concentrating ammonium from urine using membrane distillation following acidification. They found a combination PTFE/PP filter reduced the amount of ammonium transferred in relation to water to as low as $6.91 \times 10^{-5}$ g-N/g-$H_2O$. Some of these processes have been implemented in long-term studies to quantify N recovery from urine for simulated space missions. Beler-Baykal et al. (2011) utilized the natural conversion of urea to ammonium over six weeks to transform the N in urine into a bioavailable form before passing it through a column of clinoptilolite for separation to recover about 86% of the original N. An additional benefit of the use of clinoptilolite was the elimination of salinity from the waste stream, a valuable attribute to eliminate salt stress in plants. Simha et al. (2018) used a regenerative activated carbon column to achieve 90% recovery of urea from urine concentrate after multiple passes through their system. Fu et al. (2016) studied a 105 days crewed simulated closed ecosystem and were able to recover 20.5% of N from urine using simple distillation. However, this recovered N was stored instead of fed to plants, which were fertilized with pre-formed plant minerals stored from the beginning of the study.

### Nitrification

Recent work has used synthetic microbial communities to complete nitrification directly in fresh diluted urine. This method bypasses the separate aerobic digestion step where nitrification may normally be present. Though previous stabilization steps still apply, Christiaens et al. (2019) were able to achieve N production rates of 29 mg nitrate $L^{-1}$ $d^{-1}$ in 10% diluted urine even under high sodium pressures. Udert and Wächter (2012) developed a system combining distillation with nitrification to completely recover nutrients from urine. Controlling pH for optimal nitrification led to 97% N recovery in the form of ammonium nitrate. Feng et al. (2008) showed up to 95% of the original ammonium could be converted to nitrate under high dissolved oxygen levels with rigorous solution pH control. These processes present alternative methods that may save space and resources in comparison to separate dedicated bioreactors.

Significant nitrification occurs in all agricultural soils and there is great potential to enhance nitrification in the root zone in a regenerative system. Controlled watering would optimize aeration, and the process could be supplemented by adding nitrifying organisms.

### Current Recovery Work

We are currently optimizing small scale bioreactors to convert the urea in urine into ammonium. A peristaltic pump is used to move solution from a bottom reservoir to the top of the column where it flows through perlite under continuous recirculation (**Figure 5**). Initial results show that ammonia volatilization is minimized when the pH is controlled near neutral (**Figure 6**). Our diagram assumes that we can achieve 100% recovery of ammonium from urine (**Figure 2**).

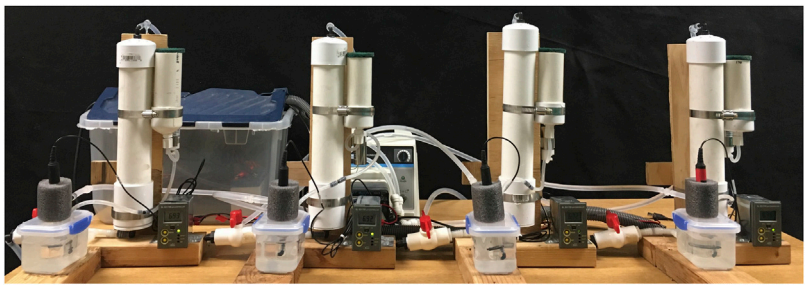

**FIGURE 5 |** Recirculating columns controlled at pH 7 with perlite as a substrate for the conversion of urea into ammonium.

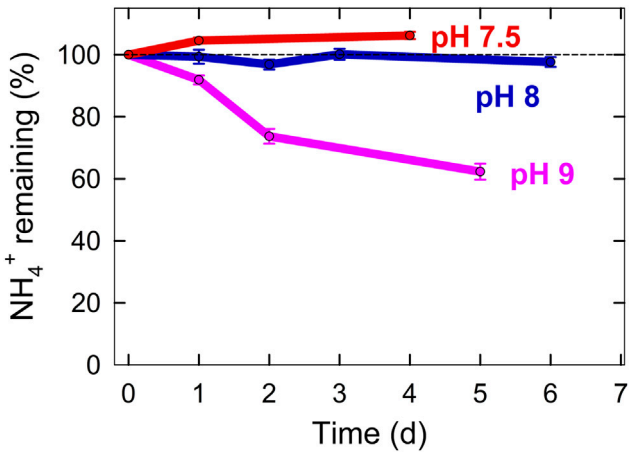


**FIGURE 6 |** Ammonia gas volatilization over time determined by the amount of ammonium remaining in solution controlled at 3 pH values.

## NITROGEN RECOVERY FROM INEDIBLE PLANT BIOMASS AND SOLID WASTE

The most bioavailable forms of N for plants are ammonium and nitrate. Their interaction in plant nutrition is complex, and optimal ratios may vary among species. Plants commonly show no deficiencies or toxicities when only fed nitrate as a nitrogen source, but they often exhibit toxicity effects when ammonium is the sole source (Britto and Kronzucker, 2002; Miller et al., 2007; Esteban et al., 2016). This may be due to nutrient imbalances caused by a preference for ammonium uptake over nitrate uptake leading to pH imbalance (Imas et al., 1997). Savvas et al. (2006) studied ammonium as a nitrogen source from 0 to 30% of total nitrogen and found 30% ammonium produced plants with the highest dry mass, though all treatments appeared healthy with no noticeable deficiencies or toxicities. Yield among some species can decrease as the fraction of N from ammonium is increased beyond 25% (Britto and Kronzucker, 2002). In a previous NASA funded project at Utah State University, Hooten (1998) found no decrease in the yield of wheat under 80% ammonium as an N source when the pH in the hydroponic solution was rigorously controlled at pH 6. If this approach can be expanded to other crops, it would significantly decrease the amount of N that must be nitrified.

No plant is completely edible (100% harvest index). Roots, stems, and in the case of fruiting plants, leaves, are inedible biomass and frequently discarded on Earth. This inedible plant waste must be digested to recover the N. Composting plant material relies on bacteria to convert the unusable biomass (proteins) into useful nutrients (ammonium and nitrate). Heat, moisture, and sometimes oxygen are required to sustain the microbial breakdown. Methane, nitrous oxide, and ammonia are all released as byproducts by microbes during the composting process (Brown et al., 2008). The N in ammonia must be captured to optimize recovery efficiency. This may be achieved by capturing the gas and bubbling it through an acidic solution to protonate the ammonia to ammonium. Current field composting studies of plant material indicate poor N recovery. Hartz et al. (2000) studied over 30 compost amendments ranging from animal manure to plant yard waste. N recovery in manure was about 15–16% while N recovery in plant waste was only 1–2%. Chalk et al. (2013) reviewed recent literature on N recovery in compost and found a maximum recovery of N from manure to be 38% and N recovery form plant waste to range from 8–26%. The composting process is also slow in addition to being inefficient.

### Digestion of Wastes

Alternative and more efficient means of composting involve both aerobic and anaerobic digestion. These processes are currently being investigated and implemented on Earth for food waste recycling to both produce useable compost and generate clean energy. Both digestion procedures are more rigorous, efficient, and contained composting methods to capture the maximum number of useable byproducts possible with minimal wasted energy or heat losses.

#### Aerobic Digestion

Aerobic digestion utilizes aerobic microorganisms to break down proteins and oxidizes N to nitrate while releasing $CO_2$ and water. The digestion process requires constant agitation and aeration to maintain aerobic conditions and promote microbial degradation of waste (Layden et al., 2007). Aerobic digestion is commonly used in waste water treatment facilities on Earth to stabilize fecal matter, food wastes, and microbial bio-solids (Khalili et al., 2000).

**TABLE 2 |** A relative comparison of aerobic and anaerobic digestion.

| | Aerobic digestion | Anaerobic digestion |
|---|---|---|
| Community structure | Aerobic heterotrophs and autotrophic nitrifying bacteria | Fermenting bacteria, acetogens, and methanogens |
| Oxygen input | Fully aerobic | Strictly anaerobic |
| Digestion time | Short | Long |
| Energy inputs | High | Low |
| Nitrogen product | Nitrate | Ammonium |
| Byproducts | Carbon dioxide, water, stabilized recalcitrant biomass | Carbon dioxide, methane, stabilized recalcitrant biomass |

*Aerobic digestion is faster, but higher energy inputs are required. Byproducts from both reactors can be combusted for use as a fuel source.*

Additional volatile losses of nitrous oxide present a considerable recovery challenge and can add an additional 20% daily loss of N (**Figure 2**). The product is a stable material (no objectionable odor, acceptable and slow decay rate, disinfected) that can facilitate conversion of Martian regolith into soil. During NASA's Breadboard project, potato yield of plants grown with liquid effluent from aerobic digestion of inedible plant biomass were within 10% of control yields (Mackowiak et al., 1997). Recalcitrant nutrients accumulated over time, but final potato composition was comparable to control tubers. While N was not specifically studied, this project illustrates the compatibility of aerobic digestion effluent with crops in regenerative life support systems.

### Anaerobic Digestion

Anaerobic digestion also uses anaerobic microorganisms to break down wastes into ammonium while releasing methane as a byproduct. The process can be dry or wet depending on the level of moisture. Dry fermentation anaerobic digestion has higher methane yields, lower water inputs, and reduced energy requirements compared to wet fermentation (Luning et al., 2003). The generated methane can be used as a clean energy source and the ammonium can be fed to plants or into additional nitrification digesters to be converted to nitrate. Microbes, much like plants, often show toxicity symptoms and reproductive challenges at high levels of ammonium. This feedback loop poses challenges for anaerobic digestion if it is to sustain a continuous ammonium output without negatively impacting microbial populations (Shapovalov et al., 2020). The remaining bio-solids from digesters in advanced life support systems can be added to plant substrates for slow, extended release of nutrients or tilled into the Martian regolith to begin developing a soil base for future *in situ* agriculture. This recalcitrant N can be 10% from anaerobic digestion on a daily basis that must be replaced through bacterial fixation.

### Digestion Comparisons

A visual comparison of aerobic and anaerobic digestion is shown in **Table 2**. Aerobic digestion is much faster than anaerobic digestion, but it requires higher energy inputs to maintain adequate oxygen levels. A combination of these two systems allows production of both nitrate and ammonium for plant fertilizers. Their input quantities can be manipulated to produce the desired ratios of both N species. An important consideration in both anaerobic and aerobic digestion is the use of batch or continuous throughput methods. Batch digestion involves adding wastes, processing through digestion over a set time period, and removing the desired byproducts before restarting the process. Continuous throughput methods would have an initial waste supply and continue adding additional waste while simultaneously removing byproducts for fuel or fertilizers. Most research has focused on the efficiency of methane produced from both systems. Batch systems with dual solid and liquid phases have shown increased methane production compared to continuous systems when unwanted contaminants were separated from bulk waste prior to digestion (Chowdhury and Fulford, 1992; Zhang et al., 2013). Ammonium is likely to follow this trend as another valuable microbial byproduct.

A combination of both methods will likely be the best approach due to the inevitable buildup of recalcitrant bio-solids that must eventually be eliminated from the digesters. This method is termed semi-continuous and involves coordinated input of waste and removal of byproducts over a set time frame before cleaning and restarting the digester. The process is similar to industrial aquaponics systems on Earth that use continuous throughput mineralization tanks to convert organic fish wastes into inorganic N forms useable by plants.

## Nitrogen Recovery from Bacteria

The biomass of N-fixing bacteria is rich in nutrients essential for plant growth. They can serve as a valuable N source and fertilizer supplement if applied to plants. Although little work has been done to quantify the release of N from N-fixing bacteria, quantitative studies have compared plant harvest parameters in response to applications of live and dead cells. Sakarika et al. (2020) compiled data from many studies to compare effects of PNSB used as a biofertilizer. The application of PNSB generally increased crop yield, but there was no difference in the effects of living or dead cells. Tomato yield and lycopene content were significantly enhanced from the application of both living and dead cells (21–98% and 42–50%, respectively) (Lee et al., 2008).

### Culturing Purple Non-sulfur Bacteria

The N fixation rates of most PNSB are high. *Rhodopseudomonas palustris* is a diazotroph that possesses three different nitrogenase enzymes and photoheterotrophic metabolic capability. It also has high metabolic versatility and is capable of photoheterotrophic, diazotrophic, photoautotrophic, and non-diazotrophic metabolism (Soundararajan et al., 2019). Since $CO_2$ fixation and N fixation compete for ATP, carbon sources can be

provided to maximize N fixation (Larimer et al., 2004). *Rhodopseudomonas palustris NifA** has been genetically engineered to have reduced inhibition of nitrogenase at high ammonium concentrations (Adessi et al., 2012). This strain is typically cultured under photoheterotrophic conditions to maximize biomass N content. Although photons provided from sunlight are an option to drive bacterial photosynthesis, this would require a system that utilizes solar fiber optics and concentrating mirrors, which has a higher equivalent system mass than a system that utilizes light-emitting diodes (LEDs) and photovoltaics (Hardy et al., 2020). LED systems can be designed to output specific wavelengths of photons, and *R. palustris* appears to be able to use photons with wavelengths between 400 and 900 nm for photosynthesis with relatively similar efficiencies (Soundararajan et al., 2019). Acetate or wastewater organics are provided as a readily available food source along with ample $N_2$ gas. Maximizing N biomass output will raise demand for available organic compounds, but these compounds can be obtained from waste, heterotrophic metabolism of *R. palustris* may stand to be an overall benefit.

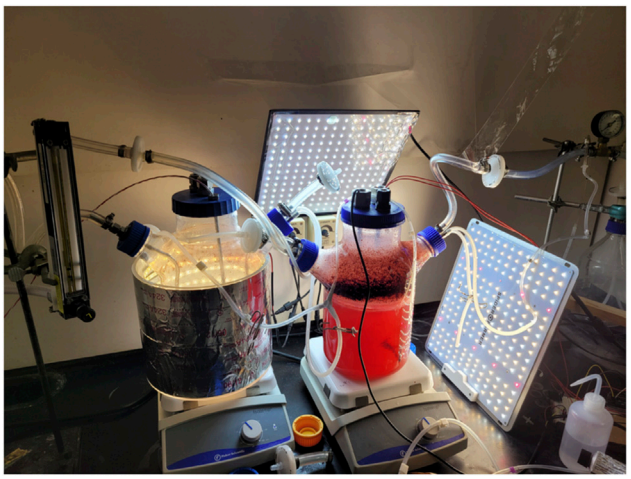

**FIGURE 7 |** Photobioreactors used for nitrogen (N) recycling. Wastewater reactor (left) obtains N from atmospheric dinitrogen gas and wastewater while fixing reactor (right) obtains N from atmospheric gas alone.

### Bacterial Nitrogen Sources

*Rhodopseudomonas palustris* can obtain N through urease-facilitated urea hydrolysis or N fixation via nitrogenase (Malofeeva, 1979). This suggests that N remediation and fixation may occur simultaneously. In non-engineered strains, nitrogenase activity would decrease during urease catabolism as ammonium levels rise and prevent nitrogenase expression (Adessi et al., 2012). It is unlikely that urease or its activity inhibits *R. palustris NifA** given its nitrogenase desensitization to elevated ammonium concentrations. Since urease activity is not limited by ATP, whereas nitrogenase activity is, it is likely that nitrogenase would be deprioritized under energy limiting conditions. Urea degradation can occur if urea is readily available. Once urease catalysis ceases, N fixation will become the primary means of N acquisition, though both processes can occur simultaneously if urea and $N_2$ are present. Remediation of N via urea hydrolysis can directly serve to treat wastewater, and N losses can be compensated by nitrogenase activity. This forms a valuable part of the Martian N cycle in which N fixation and remediation can occur in tandem. The major barrier to this process and the implementation of such a system is the removal of inhibitory components, such as those present in sludge or wastewater soap content. Preparing wastewater for integration with N fixation and recovery systems will therefore likely demand considerable upstream processing.

### Optimizing *R. palustris* Growth

In regenerative life support systems, N-fixing bacterial biomass would serve as a principal N source for crops. Within cells, most of the N is contained in amino acids. Bacterial cells must be broken down by other microbes which mineralize the organic N to ammonium. The pH of this process must be controlled to both allow for an optimal bacterial environment and prevent the volatilization of ammonia gas. Culturing *R. palustris* is optimized when it is provided with an input of $N_2$ (80%) and $CO_2$ (20%) to facilitate N fixation and pH control. The system must also account for lighting conditions, stirring mechanisms, heating, and the energy required to extract and replenish media during and after harvest. Our current culture system (**Figure 7**.) has a total power demand of approximately 100 $WL^{-1}$ of media to provide these conditions, from which approximately 0.1 and 0.05 g of N per liter day can be produced from acetate and wastewater media, respectively. We have demonstrated the N recovery process from bacteria can take about 6 weeks without the intentional inoculation of additional microbes and recover 80% of the N. Eventual recovery systems will use both anaerobic and aerobic digesters to achieve desired products and implement specific bacterial strains with high digestion capabilities to increase both the speed and recovery efficiency of biological N fixation.

## CONCLUSION

N recycling is essential to efficient regenerative life support. Recycling of N is complex and requires many steps, but can be accomplished with high efficiency. Microbial systems can recover N from the atmosphere, and well-designed bioreactors can efficiently recover N from inedible plant waste, urine, and feces. Efficient N recovery can reduce the amount of atmospheric $N_2$ that must be fixed by nearly 10-fold (**Table 1** compared to **Figure 2**). N losses will inevitably occur, but recycling systems can minimize losses and maximize self-sustainability for long-term space missions.

## Future Research

Many areas of N recycling in an advanced life support system are prime candidates for future research to increase N

recovery. Urea hydrolysis requires acidic conditions to avoid volatile ammonia losses. Further work is needed to improve both the speed and recovery efficiency of N as ammonium. The extent to which composting can occur in the root zone is also not well studied. The requirement for nitrification is minimized if plants can be fed higher levels of ammonium. Optimization of both speed and efficiency are paramount to this effort. Improving N fixation efficiency can reduce the resources required for bacterial N fixation. Future research will seek to achieve 100% N recovery for a fully regenerative life support system.

## AUTHOR CONTRIBUTIONS

NL, PK, and TW contributed to the original draft of the manuscript. CC, LS, and BB guided the editing process, literature search, and design of figures.

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# Space Aquaculture: Prospects for Raising Aquatic Vertebrates in a Bioregenerative Life-Support System on a Lunar Base

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The presence of a human community on the Moon or on Mars for long-term residence would require setting up a production unit allowing partial or total food autonomy. One of the major objectives of a bioregenerative life-support system is to provide food sources for crewed missions using *in situ* resources and converting these into the food necessary to sustain life in space. The nutritive quality of aquatic organisms makes them prospective candidates to supplement the nutrients supplied by photosynthetic organisms already studied in the context of space missions. To this end, it is relevant to study the potential of fish to be the first vertebrate reared in the framework of space agriculture. This article investigates the prospects of space aquaculture through an overview of the principal space missions involving fish in low orbit and a detailed presentation of the results to date of the Lunar Hatch program, which is studying the possibility of space aquaculture. A promising avenue is recirculating aquaculture systems and integrated multi-trophic aquaculture, which recycles fish waste to convert it into food. In this sense, the development and application of space aquaculture shares the same objectives with sustainable aquaculture on Earth, and thus could indirectly participate in the preservation of our planet.

**Keywords: space exploration, fish, aquaculture, bioregenerative life-support system, European seabass, moon, mars, lunar hatch**

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## INTRODUCTION

Space agencies are currently considering plans to build bases on the Moon or eventually on Mars, establishing a community of *Homo sapiens* outside the Earth for the first time. Such a project places humanity at the dawn of an unprecedented adventure, which will involve subsisting in a hostile environment devoid of a local trophic chain of nourishment. Like most Earth's organisms, *Homo sapiens* is composed predominantly of water. Indeed, water is an essential element for human survival, and its presence on a celestial body is a prerequisite for sustainable settlement there. Beyond this, a balanced nutritional intake (proteins, lipids, and carbohydrates) is necessary for basic needs and daily activity. The provision of essential needs will depend on the population size and the duration of stay, and will also require a technical and economic model that allows the supply of food and water. One promising avenue to respond to this constraint is a bioregenerative life-support system (BLSS), which would allow partial food self-sufficiency by deploying strategies for water recycling, aquaponics, and food-production systems.

## Photosynthetic Organisms as Food Sources in a Bioregenerative Life Support System

From the first plants sent into space in 1960 with Sputnik 2 to the current experiments underway at the International Space Station (ISS), the physiological responses of several terrestrial plants under microgravity conditions have been studied for their potential to develop "astrocultures" intended to feed future residents of a space base (Zabel et al., 2016). The environment for cultivation would be different on the Moon (Zeidler et al., 2017) or on Mars (Bamsey et al., 2009; Kiss, 2014) and plant selection and cultivation strategy would have to be adapted for the available local nutrients. A project led by NASA is running experiments on plants in low orbit in a small plant-growth chamber called Veggie carried on the ISS. In 2015, Veggie provided the first lettuce at an edible size entirely produced in real microgravity conditions (Khodadad et al., 2020). The list of candidate plants for cultivation under BLSS conditions includes more than a dozen species: wheat, rice, soybean, peanuts, sweet pepper, carrots, tomatoes, coriander, lettuce, radish, squash, onion, and garlic (Liu et al., 2008; Paradiso et al., 2012; El-Nakhel et al., 2019). These cereals, vegetables, and fruits could provide carbohydrates, phosphorus, pre-vitamin A, vitamins $B_1$, $B_6$, $B_9$, and C; however, the protein and fat provided by vegetal sources are often negligible compared to animal sources.

Aside from terrestrial food candidates, aquatic sources have the capacity to provide nutritional compounds required for balanced health. For example, aquatic cyanobacteria, could be produced in bioreactors to supply biological resources. Cyanobacteria are able to fix carbon dioxide from the exhalation of organisms and transform nitrogen waste from various physiological activities (Baque et al., 2014). These organisms are likely to be easier to cultivate on Mars than on the Moon because of the presence in the Martian atmosphere of carbon dioxide and different forms of nitrogen (Verseux et al., 2016), meaning they would not be dependent on the importation of sources of carbon or carbohydrates to the site as would be the case on the Moon. The most well-known edible photosynthetic cyanobacteria on Earth, *Arthrospira platensis* (formerly known as spirulina), is a credible candidate for cultivation in space. This food source is the subject of study in the framework of the European MELiSSA project consortium (Lasseur et al., 1996; Poughon et al., 2009) to feed astronauts. Moreover, *Arthrospira platensis* could be used as an indirect food source for humans by feeding fish such as trout, tilapia or sturgeon (Olvera-Novoa et al., 1998; Palmegiano et al., 2005; Flores et al., 2012). The nutritional contribution of *Arthrospira platensis* is mainly concentrated in its supply of proteins (Sarma et al., 2008), iron and pigment precursors of vitamin A, and antioxidants (Dartsch, 2008), but it only contains marginal amounts of essential fatty acids (ω 6) and lacks of the polyunsaturated fatty acid ω3.

Microalgal life forms are another potential food source–they are highly diversified and offer a wide range of physiological strategies and proximate composition. To date, a few microalgae strains have been studied in low orbit for experiments in a space environment, including *Sphaerocystis* sp., a Chlorophyceae (polar/permafrost green algae), which spent 530 days on a panel outside the ISS in the BIOMEX experiment, in temperatures ranging from −20°C in the dark to 47.2°C in the light (de Vera et al., 2019). *Chlorella* sp., well described in the scientific literature, is the favored algae species for a space mission. This microalgae, as well as other families of marine and freshwater microalgae, are aquaculture study subjects, in particular for water purification by eliminating dissolved and suspended matter in water (Yang et al., 2019), the fixation and sequestration of carbon dioxide (Guo et al., 2015; Gales et al., 2020), as feed for aquatic invertebrates such as copepods, and also as a food supplement for humans (Hader et al., 2006; Niederwieser et al., 2018; Yang et al., 2019).

Many strains of marine microalgae that can be cultivated in aquaculture offer a complete nutritional contribution of proteins, vitamins and especially PUFA, ω3/ω6 and alpha-linoleic acid (ALA), the precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are essential elements for proper body functioning, in particular for heart health, vision and brain function. These essential fatty acids are not produced directly by the human body: ALA, EPA, DHA sources are mainly provided by the ingestion of marine organisms such as microalgae, shellfish or finfish.

## Space Aquaculture: A Relevant Source of Complementary Nutrition

Resupplying a base in space from Earth on a weekly basis is neither economically nor technologically feasible (a trip to the Moon takes 4–7 days, and to Mars 5–8 months). A short-term solution is to provide processed and prepackaged space food. However, lyophilized conservation is unstable, especially concerning essential nutrients such as potassium, calcium, vitamin D, and vitamin K, which is involved in muscle and bone maintenance. The micronutrients most sensitive to storage degradation are vitamins A, C, $B_1$, and $B_6$ after one year at ambient temperature (Cooper et al., 2017). A possible nutrition strategy for space bases could be to couple local fresh production with supplies brought by cargo spaceships.

Providing fresh, nutritious and safe food is imperative for the success of a manned base on Moon or Mars. Recent studies have shown that food energy needs during a spaceflight are similar to those required on Earth. If energy intake is reduced, the human body is subjected to physiological stress causing cardiovascular deconditioning, bone demineralization, muscle atrophy and immune system deficiency. Moreover, microgravity exposure reduces the nitrogen balance in an astronaut's body. This results in a 30% reduction in protein synthesis (Stein, 2001). A study of previous manned missions in low orbit monitored the crew's physical performance consuming food commonly used in space missions and showed that an increase in carbohydrates (from plants) and a decrease in animal protein and fat can disturb the diet balance (Gretebeck et al., 1994). Ideally, a fresh animal-based food source should be included in the diet of space residents.

Seafood is one of the healthier animal products for human nutrition. Its nutritional merits and protective benefits have been abundantly described over the last century. Like wild fish, aquaculture fish sequester digestible proteins and essential amino acids, lipids, including essential polyunsaturated fatty acids (PUFAs), essential vitamins and minerals in their

**TABLE 1 |** Micronutrients and human nutritional benefits found in fresh fish (adapted from Tacon et al., 2020).

| Nutrient class | Fish nutrients | Human health benefits |
|---|---|---|
| Proteins | Amino acids | High digestibility–Muscle repair–Cell, enzyme, hormone composition–Neural and digestive functions |
| | Taurine | Most abundant amino acid in human brain, muscle, retina–Essential for renal, retinal and cardiac activity |
| Lipids | Phospholipids | Cell membrane regeneration–Energy source for metabolism–EPA precursor |
| | EPA/DHA (ω3) | Blood clotting prevention–Artery flexibility–Anti-inflammatory–Cardiovascular disease protection–Macular degeneration protection–Cognitive function–Dementia prevention |
| Vitamins | A | Vision–Tissue, skin, bones–Cell communication–Heart, lungs, kidneys–cancer prevention |
| | D | Calcium, phosphorus absorption and regulation–Bone mineralization, osteoporosis shield–Immune function–Anti-inflammatory–Hypertension regulation |
| | E | Antioxidant–PUFA assimilation–Anti-inflammatory–Immune function–Cancer prevention–Cardiovascular protection |
| | B (Choline) | Neurotransmitter–Mood, memory–Muscle control–Nervous system–Fatty liver prevention |
| | B9 (Folic acid) | Cell renewal–Brain maintenance–Cardiovascular protection and cancer prevention - Depression risk protection–Cognitive function |
| | B12 | Red blood formation–Neurological function–DNA synthesis–Cardiovascular protection–Cognitive function |
| Minerals | Calcium | Bones–Muscle activity–Nerve pulse transmission–Osteoporosis protection–Weight management–Hypertension regulation and cardiovascular protection |
| | Copper | Blood cells–Immune function–Energy production–Cardiovascular disease protection–Dementia–Muscle function |
| | Iodine | Thyroid protection from radiation–Protein synthesis–Blood regulation |
| | Iron | Oxygen transportation in blood–Muscle metabolism–Hormone synthesis |
| | Magnesium | Enzymatic and metabolic system–Hypertension regulation–Bone development–Heart rhythm–Osteoporosis prevention |
| | Selenium | Thyroid hormone metabolism–DNA synthesis–Cardiovascular disease prevention |
| | Zinc | Immune function–DNA synthesis–Macular degeneration protection |

muscles. Vitamins are precursors of molecules that are essential coenzymes for enzyme catalysis. When the synthesis of coenzymes is not included in an organism's genetic heritage (this is the case for *Homo sapiens*), their natural synthesis must be achieved by the ingestion of living cells. These cells are provided by a diet of plants or animals. In addition to micronutrients, farmed marine, brackish and freshwater fish can sequester ALA (PUFA precursor), EPA or DHA from their diet (Tocher, 2015). Several aquaculture fish have the physiological capability to produce EPA and DHA (ALA chain elongation) and store these essential compounds (Morais et al., 2015; Gregory et al., 2016). The micronutrients commonly found in fish and their health benefits are presented in **Table 1** (Tacon et al., 2020).

At the beginning of the 1980s, the first study on the possibility of space aquaculture emphasized the shared points between recirculating aquaculture systems (RAS) and BLSS (Hanson, 1983). Yet although aquaculture seems to offer a relevant solution for manned long-term missions (Bluem and Paris, 2003), almost four decades later, no significant innovative solutions have been proposed for space exploration. This may be due to the international strategy of developing low orbit science over the last 30 years with the ISS program, to the detriment of more complex and ambitious projects such as trips to the Moon or Mars involving long-term stays.

## Why Raise Aquatic Organisms in Space?

Hydrogen and oxygen are abundant in the Universe, and water molecules are everywhere in the solar system. Sub-glacial liquid water has been detected on many rocky planets such as Mars, Mercury, and Venus (Liu, 2019; McCubbin and Barnes, 2019). There is evidence of the presence of an internal ocean on icy moons such as Enceladus (Cadek et al., 2016) and Europa (Kalousova et al., 2016). Recent research has indicated the presence of water molecules on rocky exoplanets from other solar systems in our galaxy (Olson et al., 2020). Water is the main *in situ* resource required for a planetary mission, both for long-term human settlement or astrobiology considerations; however, most observations have revealed that this water has high mineral content or is close to brine due to geological mineralization (Orosei et al., 2018). It would need to be purified to use as a source for water of drinking quality, yet it could be primarily used for rearing marine organisms such as algae, invertebrates, or fish.

Today, producing protein from farmed animals (poultry, cattle, or sheep) in low gravity does not seem feasible. A large surface area is needed for livestock rearing, which would directly compete with human space, and costly synthetized air reconditioned from precious *in situ* resources such as lunar or planetary water or gas produced by BLSS biotechnology would be reserved for the human residents' artificial atmosphere. Due to their poikilothermic physiology, fish require five to twenty times less energy than mammals, and around three times less oxygen, as well as generate less carbon dioxide emissions, which is an important consideration for BLSS gas exchange management.

Another issue is waste management. With terrestrial animals such as pigs, chickens, goats, or cows, feces collection is not easy to solve. However, in aquatic vertebrate production, all dissolved compounds and particulate matter are sequestered in the water and can be easily treated and removed from the system or converted by another organism.

Lastly, compared to terrestrial farmed animals, aquaculture is commonly viewed as playing a major role in improving global food security on Earth because the feed conversion ratio (FCR: the feed biomass necessary to provide to a farmed organism to obtain a weight increase of 1 kg) for fish is drastically lower than for land vertebrates. The FCR for different aquaculture organisms compared to that of the main farmed land animals is shown in **Figure 1**. Protein and calorie retention from aquaculture production is comparable to livestock production (Fry et al.,

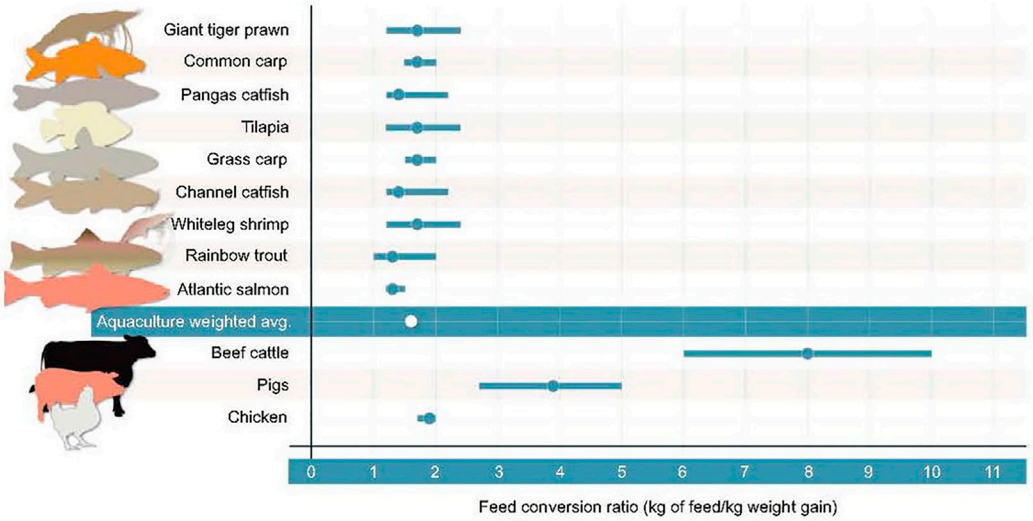


**FIGURE 1 |** Feed conversion ratios for selected aquaculture species compared to main terrestrial farmed species. Dots represent means and bars indicate the range. Lower values represent higher feed conversion and productivity (from Fry et al., 2018).

2018). All aquatic vertebrates exhibit better feed efficiency, which implies less feed to produce in a BLSS and to manage on the Moon or Mars.

Gas management in lunar or Martian bases will probably be the main challenge for engineers in the next decade. On Earth, the atmosphere sequesters a stock of oxygen, and its continuous production is provided by oceanic and terrestrial photosynthetic organisms. Before the Industrial Revolution, carbon dioxide production was balanced with oxygen consumption. Today, even with the rise in $CO_2$ emissions, oxygen is not a limited source. In contrast, in a closed system in an extreme environment such as the Moon or Mars, oxygen is not available in its basic form and must be produced. Hence, it is a precious molecule and it is of particular interest to include low oxygen consumers–and consequently, low carbon dioxide producers–in a BLSS. Compared to animals that breathe air, fish, and more generally aquatic organisms, have the lowest oxygen requirement and are the lowest producers of carbon dioxide (**Figure 2**). In fish, carbon dioxide production from respiration is dissolved, concentrated and stored in the water column. Fish have been shown to maintain their oxygen consumption under conditions of elevated $CO_2$ partial pressure (Ishimatsu et al., 2008). The dissolved $CO_2$ from RAS effluent could be used directly by an aquatic photosynthetic organism such as algae. Collecting $CO_2$ emitted from fish and dissolved in the water column and directing it to a secondary biological system without an additive process would be a huge advantage for BLSS gas management.

In contrast to farmed poultry and mammals, aquatic organisms would also be protected from cosmic rays by the water environment, which is an intrinsic radiation shield. The first life forms on Earth developed in a brackish ocean with a salinity of around 10 mg/L (Quinton, 1912). Complex life emerged from the Earth's oceans when the atmospheric layer had not yet been totally formed by the respiration of

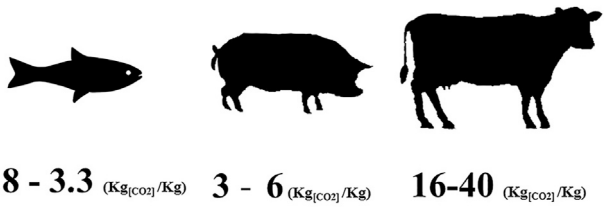


**FIGURE 2 |** Fish carbon dioxide production (kg produced per kg of biomass) compared with terrestrial animals reared for protein and lipid sources.

microorganisms (stromatolites, bacteria and microalgae) and volcanic activity. The thin atmosphere exposed the Earth's surface to intense cosmic radiation. The hypothesis that water played a role as a radiation shield in the appearance of aquatic life is strong and plausible. In connection with the development of space aquaculture, further experiments would be needed to determine the integrity or splitting of a heavy charged particle from cosmic radiation entering the water of an aquaculture tank.

Transporting any type of animal in a space mission would subject them for several minutes to hypergravity between 4 and 8 g (unit of acceleration due to gravity) depending on the space engine. But hypergravity conditions are not unknown for oceanic fish such as the bluefin tuna (*Thunnus thynnus*). In one stress experiment, the force required for maximal acceleration was measured in this species. The associated hypergravity applied to the tuna was around 3 g for a few seconds (Dubois et al., 1976). No experiments have been conducted on aquaculture fish, but the natural acceleration caused by an escape behavior has been recorded as between 1 and 3 g.

Another argument in favor of finfish as candidates for space aquaculture is that as opposed to other reared vertebrates and

humans, in the water column they can move vertically as well as horizontally. Fish use a ballast system, the swim bladder, and otolith sensitivity to move in a volume of water, experiencing gravity but also buoyancy. In the ocean, fish are already in microgravity conditions due to water density and Archimedes' principle. Thus, altered gravity should not interfere with swimming behavior during the lifecycle of a fish. Experiments have revealed that a fish in microgravity during a space mission orients its swimming direction and body position according to the position of the light in the module without losing the ability to feed or affecting social behavior. Fish movement can also be correlated with spaceship rotation (Ibsch et al., 2000; Anken et al., 2002).

Indeed, astronauts train underwater as this is the best way to imitate the weightless conditions found in space. The suits they wear in the training pool are designed to provide neutral buoyancy (like a fish's swim bladder) to simulate the microgravity experienced during spaceflight (Otto F.Trout, 1969). Spaceflight analog missions are conducted underwater in NASA's Extreme Environment Mission Operations (NEEMO), involving multi-hour activities at a depth of 19 m (Koutnik et al., 2021). While the hypothesis that the variation in space gravity will not drastically disturb the fish from a physical, behavioral or welfare point of view is plausible, this remains to be tested in experiments on aquaculture fish species.

## Ornamental Fish as a Model for Understanding Human Physiology in Space

The zebrafish *Danio*, the medaka *Oryzias*, and the swordtail fish *Xiphophorus* have been frequently boarded on space missions as models for understanding human gravitational sensations, due to the homology with human morphological and physiological systems. These species have proved the most suited vertebrate animals for basic gravity research. The gravity-sensing system in vertebrates from fish to humans has the same basic structure. Although aquarium fish are not aquaculture fish, space missions over the last five decades have provided useful results on fish physiology, behavior and well-being in microgravity (Lychakov, 2016).

The earliest spaceflight with fish occurred on July 28, 1973. Two fingerlings and fifty embryonated eggs of the mummichog (*Fundulus heteroclitus*) were launched by a Saturn 1B rocket. The Apollo service module joined Skylab 3 and the fish were positioned in a plastic bag filled with seawater. This American space mission preferred the mummichog, a small saltmarsh killifish, to goldfish for this experiment. This species was not well known or described at that time, but it became the first "fishonaut". For three days, swimming in loops and circles was observed for the two fingerlings, but they gradually returned to normal swimming. The fish acclimation period was comparable to that for a human crew during a first spaceflight. This observation suggested that the vestibular function (the otolith for fish–the inner ear for humans) probably plays the same sensory role in microgravity. The *Fundulus heteroclitus* eggs carried aboard the Skylab station in low orbit hatched successfully during the mission with a very good hatching rate (96%). The hatched fry displayed normal swimming behavior in contrast to the first hours in microgravity for the fingerlings (Baumgarten, 1975). Fish embryos in microgravity develop a physiological strategy to compensate for the unusual environment, and the larvae formed were already adapted to microgravity, as evidenced by the lack of looping behavior.

In 1975, during nine days of the manned Apollo-Soyuz MA-161 mission, a group of 21-day-old juvenile mummichogs were exposed to real microgravity, and similar irregular swimming was observed. Fish eggs were also boarded ($n$ = 100/samples at 32 hpf [hours post-fertilization], 66 hpf, and 128 hpf stages; pre-liftoff fertilization times) and were subjected to post-flight hatching rate evaluation back on Earth. The juveniles were evaluated using light orientation tests, and no significant differences were observed in behavior, suggesting an adaption capability to the space environment. The embryo hatching rate was 75%, and hatching date monitoring showed that the three earliest stages of egg batches carried on Apollo-Soyuz hatched at 15 days (normal hatching rate is 21 days), much sooner than the latest stage batch and earlier than the control batches at 1 g. Apparently, the development of young eggs was faster under microgravity, but the embryos exhibited no abnormalities resulting from development in a zero-gravity environment. The eyes, heart, nerves, and bones were found to be the same in the flight group as in the control group. There was no evidence of calcium deficiency, except in the shorter hatching-time group (Hoffman et al., 1977).

In July 1994, the 17th Columbia space shuttle mission STS-65 boarded Japanese medaka (*Oryzia latipes*) for 15 days of spaceflight in the second International Microgravity Laboratory (IML-2). These ornamental fish laid eggs, and normal hatching was observed in space, with the results showing that medaka fertilization and embryonic development was not significantly impaired by altered gravity (Ijiri, 1998).

Probably the most impressive aquatic closed-loop experiment in low orbit and a successful demonstration of an aquatic trophic chain in space, in the 1990s, a German team from Ruhr University Bochum and the German Aerospace Centre (DLR) developed the Closed Equilibrated Biological Aquatic System (CEBAS) with fresh water, containing small aquarium fish (*Xiphophorus hellerii*), water snails (*Biomphalaria glabata*), aquatic plants (*Ceratophyllum dermersum*), and aquatic microorganisms. The ground-based demonstration showed that a filter system was able to keep a closed artificial aquatic ecosystem stable for several months and to eliminate waste products deriving from degraded dead fish without a decrease in oxygen concentration to less than 3.5 mg/I at 25°C (Blum et al., 1994; Blum et al., 1995). Then in January 1998, during the Endeavour space shuttle mission STS-89 to the MIR station, aquarium swordtail fish (*Xiphophorus helleri*) were exposed to 9 days of microgravity, with 200 juveniles and four pregnant adult fish carried in a mini CEBAS module (10 L) (Blum et al., 1994). The aim of this aquatic mini-module (**Figure 3**) was to record the behavior of an artificial ecological closed loop in low orbit and verify the hypothesis that aquatic life is not affected by exposure to space conditions using a complementary organism. The female fish were retrieved in good physiological condition, adult and

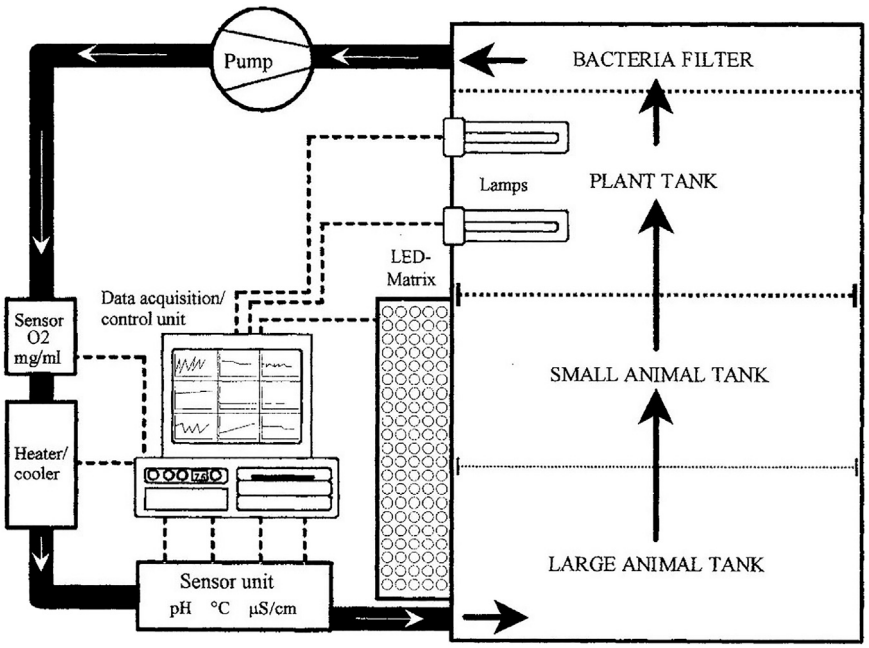


**FIGURE 3 |** Aquatic CEBAS module diagram demonstrating the trophic chain concept (extract from Bluem et al., 2000).

juvenile fish had a survival rate of about 33%, and almost 97% of the snails had survived and produced more than 250 neonates in microgravity (Bluem et al., 2000). During the spaceflight, the vertebrates were video-recorded for behavioral analysis and no aberrant looping or spinning behavior was observed. Immediately after landing back on Earth, the adult fish swam vertically, head upward, to the top of their habitat, strongly beating the caudal and pectoral fins. This was due to empty swim bladders not used during the spaceflight and reuse acclimation on Earth (Anken et al., 2000; Bluem et al., 2000; Rahmann and Anken, 2002).

In April 1998, another population of swordtail fish and four adult wild marine fish oyster toadfish (*Opsanus tau*) flew with the space shuttle STS-90 mission, hosted in the Neurolab facility. After 16 days in real microgravity, fish brain synaptic contacts were compared to a control population at 1 g on Earth. Spaceflight yielded an increase in synaptic contacts within the vestibular nucleus indicating a compensation processes for neonates swordtail fish (Ibsch et al., 2000). Results revealed a gravity compensation process and the role of the fish lateral line associated to the fish brain for appropriate swimming behavior (Anken et al., 2002).

The Vestibular Function Experiment Unit (VFEU) aboard STS-95's SpaceHab again hosted two oyster toadfish as experimental subjects. The fish were electronically monitored to determine the effect of gravitational changes on the otolith system. The freely moving fish provided physiological signals of the otolith nerves. Measurements of afferent and efferent responses were made before, during, and post-flight (Boyle et al., 2001).

In January 2003, four medaka eggs laid on Earth in an artificially controlled environment were launched by the Columbia space shuttle during the STS-107 mission. For the control, four eggs in the same condition remained on the ground. No difference was observed in the time of development. In the ground experiment, the embryos were observed to rotate in the egg membrane, whereas in flight they did not rotate. One egg hatched 8 days after the mission launch in the flight unit, while four eggs hatched in the ground unit. In the flight unit, the fry was observed with its back usually to the camera and little swimming movement suggest. The results shown no appreciable difference in the time course of development between space- and ground-based embryos. (Niihori et al., 2004). The hatched medaka larva, embryos and the crew from the space mission tragically never returned to Earth alive due to the accident during the space shuttle's reentry in the atmosphere.

In 2007, dry eggs of the ornamental killifish the redtail notho (*Nothobranchius guentheri*) were placed into cotton-cloth bags, then into plastic Petri dishes, and fastened on the outer side of the ISS. The aim of the Biorisk-MSN mission was to expose dry incubated eggs to low orbit radiation. Unfortunately, no data is available concerning the resistance of the fish eggs as the equipment had no temperature sensor and the plastic dishes reached 95°C, deforming the plates, and the eggs died due to the high temperature and vacuum contact (Baranov et al., 2009).

To study the fish response at early stage to microgravity, two missions using medaka fish were performed on ISS, in 2012 and 2014. Each time a Soyuz rocket sent 24 juveniles medaka (6 weeks after hatching, 16 mm) with the objective of rearing this population in the Aquatic Habitat (AQH) on the Kibo section of the ISS. Medaka fish in space and control fish from the same family on Earth were filmed. The movies showed that the fish became adapted to life under microgravity although despite an unusual swimming

**TABLE 2 |** Studies of ornamental fish used as a physiological model in low orbit missions. References to major missions are noted with numbers in brackets: [1] Baumgarten, 1975; [2] Proshchina, 2021; [3] Hoffman, 1977; [4] Ijiri, 1998; [5] Anken, 2000; [6] Anken, 2002; [7] Boyle, 2001; [8] Niihori, 2004; [9] Baranov et al., 2009; [10] Chatani, 2015; Murata, 2015; [11] Chatani, 2016.

| Year | Fish species | Mission | Low orbit station | Duration | Fish stage | Embryos | Study aim |
|---|---|---|---|---|---|---|---|
| 1973 | Mummichog *Fundulus heteroclitus* | Apollo [1] CSM-117 | *Skylab 3* 558 orbits | 59 days | 2 fingerlings (post-hatching) | Fish eggs | Video Hatching and normal behavior |
| 1974 | Zebrafish *Brachidanio rerio* | Soyuz 16 [2] | | 5 days 22 h | – | Five somites | Weightlessness |
| 1975 | Mummichog *Fundulus heteroclitus* | MA-161 [3] | *Apollo-Soyuz* | 9 days | 28 juveniles (21 days old) | 32, 66, 128, 216, 336 hpf | Return Hatching and behavior analysis |
| 1975 | Mummichog *Fundulus heteroclitus* | Cosmo782 [2] | *Bion* | 19 days | – | Fish eggs | Fixation after landing |
| 1976 | Zebrafish *Brachidanio rerio* | Soyuz 21 [2] | *Salyut* 5 | 7.5 days | – | Medium gastrula | Fixation after landing |
| 1976 | Zebrafish *Brachidanio rerio* | Soyuz 22 [2] | *Salyut* 5 | 49 days | – | Late gastrula | Fixation weightlessness |
| 1994 | Medaka *Oryzias latipes* | STS-65 [4] Columbia | IML-2 | 15 days | 4 adults Fertilization in space | 43 laid eggs, 8 hatched | 30 hatched after landing |
| 1998 | Swordtail fish *Xiphophorus helleri* | STS-89 [5] *Endeavour* | MIR | 8 days | 4 pregnant adults 200 juveniles | – | Video–Return–behavior |
| 1998 | Swordtail fish Oyster toadfish *Opsanus tau* | STS-90 [6] *Columbia* | Neurolab CEBAS | 16 days | 4 toadfish 150–700 g | – | Vestibular compensation Synapse formation |
| 1998 | Oyster toadfish *Opsanus tau* | STS-95 [7] *Discovery* | SpaceHab | 9 days | 2 adults | – | Vestibular monitoring while swimming |
| 2003 | Medaka *Oryzias latipes* | STS 107 [8] *Columbia* | AHAB *Aquatic habitat* | | | 4 embryonated eggs | Hatching launch + 8 days |
| 2007 | Redtail notho *Nothobranchius guentheri* | Soyuz [9] BioRisk-MSN | ISS | 406 days | – | Dry fertilized eggs | Outside exposure failed |
| 2011 | Goldenfish + Oyster toadfish *Opsanus tau* | STS-135 (last mission) *Atlantis* | Aquatic animal experiment unit | 12 days | 6 juveniles | Fertilized eggs | Otolith removed–eggs returned |
| 2012 | Medaka *Oryzias latipes* | Soyuz [10] TMA-06M | ISS *Kibo Module* | 60 days | 24 juveniles | – | Fish behavior gene fluo-signals |
| 2014 | Medaka *Oryzias latipes* | Progress [11] M-22M | ISS *Kibo Module* | 8 days | 24 larvae | Hatching in low orbit | Fluorescence microscopy |

behavior. In addition, a mating behavior was observed under microgravity at day 33 and was not different from that on the Earth, indicating microgravity environment doesn't disturb fish reproduction. The aquarium fish used for this experiment have fluorescent osteoclast cells, which makes them easier to observe. An osteoclast is a type of bone cell that breaks down bone tissue and responsible for bone loss. After 47 days in space, the fish tended to stay still in the tank. After 56 days, the mission fish group had normal growth compared to a terrestrial control. For fish in microgravity impairment of some physiological functions was accompanied by the activity of osteoclasts and a slight decrease in mineral density and vertebral bones. (Chatani et al., 2015; Murata et al., 2015; Chatani et al., 2016). Historical space missions involving ornamental fish are listed in **Table 2**.

## Missions With Aquaculture Fish in Low Orbits

Very few missions involving aquaculture fish have been carried out to date (**Table 3**). In one of these, the common carp (*Cyprinus carpio*)—considered a very important aquaculture species in many countries–was chosen as a model for a sensor motor experiment by Japanese university teams and the Japan Aerospace Exploration Agency (JAXA). Two colored carp (16 months old, 26 cm and 263–270 g) were carried to the American SpaceLab in 1992. One of the two carp was given a labyrinthectomy (the otolith was removed). For both fish, swimming behavior and dorsal light response was studied and compared. As observed during the first space missions with small fish, the normal carp was unstable (associated with a kind of space motion-sickness) for the first three days, then finally recovered its Earth-based swimming behavior. The fish whose otolith was removed two months before showed a normal dorsal light response 22 h after launch, and disruption for the next two days as with the normal carp. Unfortunately, the recovery process for the fish with the removed otolith could not be evaluated due to a technical issue, but these observations provided evidence of a sensory-motor disorder during the early phase of adaption to microgravity in aquaculture fish (Mori et al., 1996). The change in body weight was monitored from two days before launch to four days after landing. Both fish recorded a weight loss around 12% in low

**TABLE 3 |** Studies of aquaculture fish as models for sensory motor, reflex experiments and trophic chain demonstrations in low orbit missions. References to major missions are noted with numbers in brackets: [12] Mori, 1994 [13] Sebastian, 2001 [14] Anken, 2016.

| Year | Fish species | Mission | Low orbit station | Duration | Fish stage | Embryos | Study aim |
|---|---|---|---|---|---|---|---|
| 1992 | Common carp *Cyprinus carpio* | STS-47 [12] *Endeavour* | Space Lab-J ML2 | 8 days | 2 carp (263–270 g) | – | Sensory motor experiment |
| 1993 | Tilapia *O. mossambicus* | STS-55 [13] *Columbia* | Space Lab D-2 | 9 days | Larvae (post-hatching) | – | Vestibuloocular reflex test |
| 1997 | Tilapia *O. mossambicus* | STS-84 [13] *Atlantis* | MIR SMM-06 | 10 days | Larvae (post-hatching) | – | Vestibuloocular reflex test–video |
| 2007 | Tilapia *O. mossambicus* | Soyuz-U [14] | Foton M3 | 12 days | 26 larvae (12 mm) | – | Video–vestibular organ–enzymatic activity |
| 2013 | Tilapia *O. mossambicus* | Soyuz 2 *1a* | BION-M1 | 30 days | All fish died | – | Equipment failure |

orbit after 14 days of fasting. No conclusion can be made as a fasting replicate on the ground was not available (Mori et al., 1994).

During space shuttle missions STS-55 (1993) and STS-84 (1997), tilapia *Oreochromis mossambicus* larvae that had not yet developed the roll-induced static vestibuloocular reflex were exposed to microgravity for 9–10 days. Young larvae (11–14 days after hatching) already exhibited the vestibuloocular reflex on the 1993 mission. Back on Earth, a vestibuloocular reflex test (fish were turned around their longitudinal axis at an angle of 15, 30, and 45°) showed that eye movement and reflex were not affected by exposure to microgravity during the two space missions (Sebastian et al., 2001).

The OMEGAHAB (Aquatic Habitat) is a closed artificial ecosystem that was sent into orbit for 13 days on board the Russian satellite FOTON-M3 in 2007. The goal of the mission led by the German Space Agency was to investigate the possibility of designing a trophic chain in real microgravity using the photosynthetic flagellate *Euglena gracilis* as an oxygen producer and larvae of tilapia *Oreochromis mossambicus* as a consumer. This freshwater and brackish species is a popular aquaculture fish, with worldwide production of around 15,000 tons per year. In the 2007 experiment, 26 small larvae (approx. 12 mm in length) in the flagellate aquarium were studied in low orbit to increase knowledge about the development of the vestibular organs and enzymatic activity. The best fish survival rate (42%) ever achieved in a German experiment was recorded. Conditions of real microgravity during spaceflight induced a larger than normal otolith compared to a control maintained at 1 g. This could result in a difference in the ability to sense gravity (Anken et al., 2016). In a same ground unit, the photosynthetic producers supplied sufficient amounts of oxygen to a fish compartment with 35 larval cichlids (Hader et al., 2006). Historical space missions involving aquaculture fish are listed in **Table 3**.

## Feeding Fish in Space: Integrated Multi-Trophic Aquaculture

If fish were farmed on a space base, sending aquaculture feed from Earth to Moon or Mars would make no sense from an economic or lifecycle analysis point of view. Aquatic systems contain a large diversity of species with different roles in nutrient cycles and biomass conversion that contribute to ecosystem balance. Photosynthetic organisms (algae, phytoplankton), invertebrates (crustaceans, mollusks, zooplankton), vertebrates (fish, amphibians), and microorganisms interact in a complex trophic web. By associating different complementary species such as fish, filter feeders, detritivores and primary producers, integrated multi-trophic aquaculture (IMTA) provides an innovative possibility for BLSS on the Moon or Mars.

The nutritional profile of fish is closely linked to their diet quality. In aquaculture, this can be easily adjusted by ensuring a fish feed formulation that includes organisms that synthesize or sequester proteins, lipids of interest (e.g., EPA or DHA), vitamins and minerals. These aquatic organisms can be cultivated separately in a chain (from algae to invertebrates to fish) exclusively with fish waste as a fertilizer or using other available waste from human activities, such as exhaled carbon dioxide, space agriculture byproducts, or residents food waste.

In the framework of sustainable aquaculture on Earth, researchers are studying trophic webs using closed or semi-closed aquatic systems that reuse fish nutrients dissolved in the water column or fish fecal matter as a fertilizer or food source for another aquatic organism. In an IMTA system, microalgae or macroalgae cultivation is easy using fish tank effluents, as the N/P ratio fits the requirements of algae: the increasing algae biomass assimilates nitrogen and phosphorus forms (Pagand et al., 2000). To return treated water back to the fish tank, it can be cleaned so it is safe for fish growth and welfare (Mladineo et al., 2010). Moreover, fish farm effluent is a suitable media for cultivating *Nannochloropsis gaditana*, a marine algae with a high PUFA content (Dourou et al., 2018). Several studies have reported the possibility of feeding aquaculture fish with microalgae (mostly marine) included in the fish feed formulation. Several microalgae strains have been tested successfully (they do not alter growth kinetics or organoleptic quality) with fish feed made up of 20–40% of microalgae: C*rypthecodinium* sp., *Phaeodactylum* sp. (Atalah et al., 2007) and *Schizochytrium* sp. (Ganuza et al., 2008; Stuart et al., 2021) have been tested for the seabream and amberjack diet; *Tetraselmis* sp. (Tulli et al., 2012), and *Isochrysis* sp. (Tibaldi et al., 2015) for European seabass; *Nanofrustulum sp*. for salmon, common carp and schrimps

(Kiron et al., 2012); and *Tetraselmis* sp. and *Isochrysis* sp. for cod (Walker and Berlinsky, 2011).

The modern feed form for aquaculture fish is dried pellets with less than 10% moisture. However, a study has shown that feeding fish using a moist formulation, such as algae or aquatic worms, with a water content around that of the natural prey profile in oceans, did not affect fish growth parameters and in fact increased resistance and immune protection (Przybyla et al., 2014). Thus, photosynthetic or invertebrate aquatic organisms produced in a Moon or Mars greenhouse could be fed directly to aquaculture fish with no transformation process. Researchers are exploring these alternatives to preserve wild fish stocks currently used for aquaculture fish feed (e.g., processed into fish meal and fish oil). Other algae sources with higher integration rates in feed formulations are the focus of future studies, while research is also investigating new types of aquatic prey compatible with fish feed, such as jellyfish (Marques et al., 2016).

The algae cultivated in an IMTA system, as well as fish effluent, can also be a feed source for invertebrates, mollusks (Li et al., 2019), and sea cucumbers (Chary et al., 2020). A team from NASA is studying the possibility of using invertebrate production systems to purify water while growing protein-rich species as food/feed sources. Aquatic species such as copepods or mussels should grow rapidly, offer good protein content and have low mass for launch requirements (Brown et al., 2021). In the ocean, copepods and mussels are the favored natural prey of fish (especially seabream) and can be used as live feed for aquaculture fish. This production could also serve as food for the human crew. Thus, aquatic invertebrates and microalgae could play a key role in a trophic chain on a space base.

In a recirculating aquaculture system, particulate matter is composed mainly of feces, mucus and bacterial clusters. This waste is easy to separate and remove from the RAS. Some copepods can use this media as feed, but another invertebrate is being studied for its ability to reduce this particulate matter and convert it into valuable biomass: the aquatic worm (Galasso et al., 2020). Polychaeta are detritivores and can be a feed source of interest for fish. Aquatic worms cultivated in an RAS can convert fecal matter into useful fatty acids for fish feed (Kicklighter et al., 2003; Bischoff et al., 2009; Palmer et al., 2014). Other synergies might also be possible: for example, *Caenorhabditis elegans* is a small terrestrial nematode already studied in space as a model for ageing in microgravity, as 35% of *C. elegans* genes have human homologs (Honda et al., 2014). This nematode could thus be both cultivated and observed in space in a BLSS.

In wild environments on Earth, a fish's diet is composed of its own congener, algae or invertebrates. Ground-based experiments have evaluated Nile tilapia as a bioregenerative sub-process for reducing solid waste potentially encountered in a space aquaculture system (Gonzales, 2009). The Tilapia feed formulation consisted of vegetable, bacterial, or food waste. Sulfur, nitrogen, protein, carbon and lysine content of waste residues were assimilated, sequestered and recycled in Tilapia muscle. Although Tilapia's specific growth rate from population fed with different fibrous waste were widely inferior (1.4—89.8 mg/day$^{-1}$) compared to the control population (281.6 mg/day$^{-1}$), the Tilapia's survival rate was not different. These results suggest additional research to improve feed formulation composed with fibrous residues (Gonzales and Brown, 2007).

When considering formulating aquaculture fish feed on a space base using exclusively aquatic organisms cultivated in an IMTA system, it is essential to determine the digestive efficiency of the fish feed. A recent study highlighted the extreme flexibility of European seabass to feed formulations without fish meal and fish oil. In the experiment, fish were given several formulations containing 85% plant sources and 15% alternative sources (yeast, insects, and processed animal protein or *Arthrospira platensis*). Zootechnical results showed that three formulations resulted in a growth equal to fish fed with a traditional commercial formulation including a wild fish source. The bacterial community in the fish digestive tract adapted to the new formulation composed of alternative protein and lipid sources, and bacterial diversity was not altered (Perez-Pascual et al., 2020). This plasticity is probably common to other fish species, allowing a promising avenue to test new innovative formulations for aquaculture fish using exclusively BLSS raw matter sources such as cyanobacteria, plants, algae, and invertebrates.

## Applicability and Limitations of a Space Aquaculture System

Like the systems for other types of food sources being studied for a future BLSS, such as those to produce microalgae and higher plants (Tikhomirov et al., 2007), the design of a space aquaculture system (SAS) is subject to various parameters, including the location in the Solar System. The size of the SAS would depend on the number of residents to feed, the other food sources necessary based on nutritionist's recommendations, the space available on the lunar base, water availability and quality, the energy available for this activity, and the duration the BLSS will need to operate. One scenario might be to provide around 250 g of fish per person per week. The volume of the tank for rearing the fish should also be correlated to the fish growth rate and the frequency at which the fish are harvested. The diversity of fish species allows possibilities to be imagined such as using the area under the floor of the lunar base for flat fish, for example, or a tank that is not connected to the crew's living area.

On the Moon as on Earth, an aquaculture system requires water circulation. While the energy needed to pump water in an SAS with lunar gravity (one-sixth of Earth's gravity) is yet to be defined, maintaining a set water temperature will have an energy cost. Within a window of tolerance depending on the species, fish growth directly depends on the water temperature (Handeland et al., 2008). In a context of 14 days of Sun exposure and 14 days of darkness, the latter period will require warming the water to maintain the growth rate. Thus the thermal profile of the selected species will be one of the parameters to consider. This aspect will have a direct impact on the total energy required for an acceptable growth yield in the SAS.

Although fish have a low oxygen uptake compared to other vertebrates (**Figure 2**), a regular supply is required. Oxygen dissolution in the water from hydroxyl extraction and oxygen from the regolith and/or from photosynthesis in plants cultivated in the BLSS must be synchronized with the biological demands of the fish. This requires the capacity to regularly collect, store and

dissolve oxygen in the water column. The oxygen data from the CEBAS experiment on the STS-89 and STS-90 missions was analyzed to model this concept. Results based on the experimental MINI-MODULE (8.6 L) showed different periods of oxygen accumulation and depletion in the aquatic habitat in plants (oxygen producer) and snails (oxygen consumer). Simulations from ground-based models predict the oxygen concentration and can be adapted for other species (Drayer and Howard, 2014). A trend has to be defined between the volume of oxygen instantly available or stored and the demand of aquatic consumers. This highlights the importance of an oxygen buffer tank linked to a feedback control mechanism (possibly remotely controlled from Earth) in case of a lack of oxygen. Another aspect to monitor is bacterial development inside the system. An axenic environment cannot be considered as bacteria play an essential role in all stages of a balanced ecosystem. Yet bacteria activity affects the nutrient budget and oxygen measurement and availability (Konig et al., 2001). All these parameters will drive the size of the SAS and the fish biomass allowed in an extreme environment such as the Moon.

Another issue to consider is aquatic biomass extraction in the space environment. Harvesting cells such as microalgae is a current challenge, today handled using vacuum and flocculation (Barrut et al., 2012). The development of harvesting tools is required for different aquatic organisms in a limited and constrained space. Regardless of the organism, extraction is necessary when the biomass has reached its optimum growth to avoid uncontrolled water degradation and increased oxygen consumption by microorganisms that would endanger fish production.

The time needed for fish management on a lunar base also depends on the size of the SAS. Current technology developed for RAS drastically reduces the time necessary to maintain the system. Most of the tasks can be automated, such as starting and cleaning the biofilter, monitoring water parameters (Konig et al., 2001), and regulating the water. Fish feeding is a time-consuming task, but this can also be automated. Fish are able to adapt to self-feeding devices (Coves et al., 1998; Di-Poi et al., 2008), which contribute to the social interaction of the population (Chen et al., 2002). As in plant production systems (Bamsey et al., 2009), several automated SAS actions could be carried out remotely from a control room on Earth. A daily routine (visual checking of the system and fish behavior and non-automated actions) could be considered to involve around 1 h every 12 h for a closed loop system composed of 16 tanks (1 $m^3$) and 8 $kg/m^3$ of fish biomass (based on personal experience).

The energy available to power the SAS will also determine its design. A ground-based greenhouse simulation for food production with lunar constraints is necessary to study and understand gas flow management, organism interactions, and all related parameters necessary to maintain a stable and balanced ecosystem.

## Studying the Feasibility of Sending Aquaculture Fish Embryos to the Moon: The Lunar Hatch Program

In research underway since 2019, the Lunar Hatch program is investigating the feasibility of shipping embryonated aquaculture fish eggs to space for programmed hatching in a lunar BLSS. The hatched larvae would then be fed with local resources and reared until they reached an appropriate size for human consumption. The aim of the study is proof of concept based on experimental data collected first in ground-based trials, followed by test missions in low orbit, and concluding with a real flight to space, perhaps leading to the hatching of the first vertebrate on the Moon.

The program focuses on the viability of European seabass (*Dicentrarchus labrax*) for such a project, by analyzing the potential effects on embryos of a Moon journey and the associated environmental changes. Water found on celestial bodies in the Solar System have a saline or hypersaline profile. The choice of the European seabass in the Lunar Hatch program was based on the fact it is a marine organism with an appreciated taste, and its physiology and behavior have been abundantly described. A secondary water source for fish aquaculture could also be considered such as recycled water from a greenhouse or non-potable water from technical process or human activities. The diversity of aquaculture fish species allows the appliacation of many potential "fishonauts", depending on the primary or secondary water resource available *in situ* (fresh or salt water). Other aquaculture species could equally be considered for rearing in space, such as trout, flat fish or shrimp.

As mentioned, in the 1970s, spaceflight tests were carried out at the egg stage with ornamental fish (**Table 2**). The choice of eggs as the biological stage for space travel is relevant for several reasons. A low volume of water is required for egg incubation, so the initial launch biological payload could be less than 1 kg for around 900 future larvae. In aquaculture nurseries, European seabass egg density in the water column is around one egg per milliliter. Unlike the larval or adult stages, the embryogenesis phase is suitable for a spaceflight because embryo development does not require human intervention for several days (the duration of embryogenesis depends on the species). Although embryogenesis involves intense metabolic activity for the development of the future larva, the low biomass and the chorion limit catabolite emission as well as the self-pollution of water during the journey. This would allow either long manned spaceflights with no need for maintenance from the crew, or simply the transport of fish eggs using an automated cargo ship.

Compared to normal conditions in land-based aquaculture production, during a spaceflight fish embryos would be initially subjected to atypical acoustic and mechanical vibrations caused by launcher motors and acceleration in the atmosphere. The effects of this are under study in the framework of the Lunar Hatch program (supported by the French National Institute for Ocean Science, Ifremer) using a standard qualification test commonly employed in the space industry. In a recent experiment, a vibration exciter mimicked the conditions of a SOYUZ-2/FREGAT launch on a population of fish embryos (**Figure 4**).

In this test, two triplicates ($n = 300$) of embryos of aquaculture species (European seabass and meagre in two separate experiments) were submitted to the acoustic and mechanical environment of a launch for 10 min at one-third and two-thirds of their development. The hatching rate was then compared to a control triplicate ($n = 300$). No significant differences were observed on the hatching rate for either species whatever the

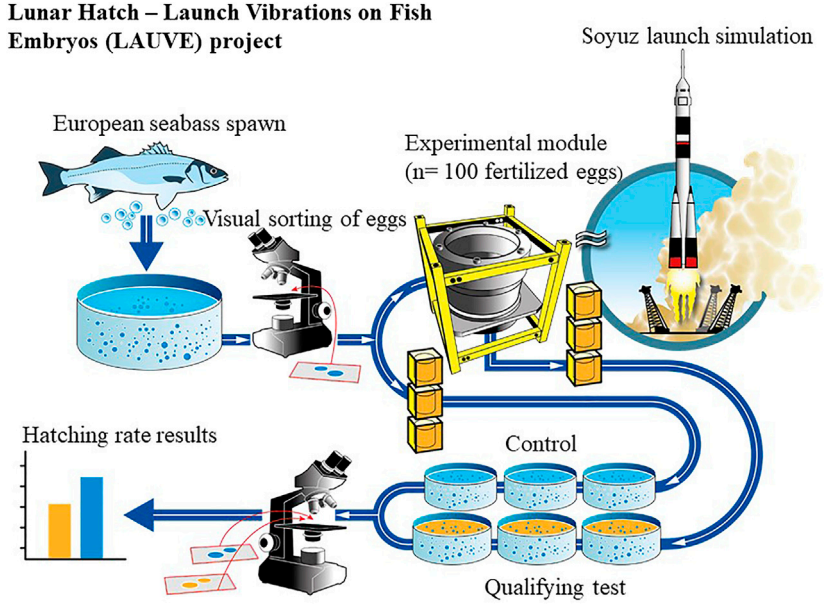


**FIGURE 4 |** Protocol for the acoustic and mechanical vibrations qualifying test on European seabass embryos (from Przybyla et al., 2020).

stage of development when the embryos were exposed to the conditions (**Figure 5**).

These encouraging results indicate the egg robustness of two major aquaculture species. A credible hypothesis to explain these results is that the success of the global aquaculture industry is based on the selection of aquatic species for robustness criteria to actions such as unusual and stressful handling–especially at an early lifecycle stage–such as sorting, sampling, transfer from aquarium to tank, or long transport by road or air. The aquaculture sector has selected the most biologically flexible strains with the most interesting nutritional profile for economic reasons. The resulting robustness could benefit space programs–it would not be surprising if other aquaculture species also successfully pass this qualifying test.

Beyond intense vibrations, understanding the influence of hypergravity and microgravity on embryonic development is essential to evaluate the feasibility of space aquaculture. Previous studies on ornamental aquarium fish can provide some information on fish behavior and physiology in space that may be useful.

Hypergravity is experienced during rocket take-off, an acceleration phase that lasts about 10 min at 4–8 g, depending on the launcher motors. This situation was tested on swordtail fish and medaka otoliths (Anken et al., 1998; Ijiri et al., 2003; Brungs et al., 2011; Anken et al., 2016) and larvae bone development (Aceto et al., 2015; Chatani et al., 2015), but its effects on early ontogeny (hatching capability) are as yet poorly described. A recent research showed that six month exposition at 5 g can induce vertebral curvatures and asysmetric otoliths (Chatani et al., 2019). However, the duration of exposure to hypergravity during a launch to the Moon or Mars will be about 10 min, the time to extract the embryos from the Earth's attraction. Ongoing experiments are exploring the ability of aquaculture finfish embryos to develop in these conditions. It is credible to posit that hypergravity applied to a water reservoir may be less felt by a submerged embryo. In contrast to poultry eggs stored in air, the water density surrounding fish eggs may reduce the acceleration force on the chorion.

Following the initial conditions of rocket vibrations and acceleration, a situation of microgravity appears beyond an altitude of 110 km. During the entire evolution of life on Earth, the development of all organisms took place under constant gravity conditions in different media (air/water). It should be noted that in the ocean, fish embryos are already in a kind of microgravity compared to terrestrial organisms due to Archimedes' principle and other physical phenomena. This is why, to simulate partial microgravity, astronaut training exercises are carried out in a swimming pool. A study has found that embryos of *Xenopus* (an aquatic frog) are able to adjust to microgravity environments until hatching through an adaptation mechanism and strategy (Black et al., 1995). Might this capability be common to other aquatic organisms, including fish embryos? Supported by the French space agency (CNES), the Lunar Hatch program plans to study the embryo behavior of European seabass in hypergravity and microgravity in the Gravitational Experimental Platform for Animal Models (GEPAM), a European Space Agency platform to test different gravity environments on animals (Bonnefoy et al., 2021).

Exposure to radiation during the space journey will be the last environmental change investigated in future Lunar Hatch program studies: this is probably the parameter with the most impact on fish embryo biology. Knowledge about the effects of space radiation on a variety of organisms has increased over the last decades: for bacteria (Leys et al., 2009), plant and mammalian cells (Arena et al., 2014), and amphibians (Fuma et al., 2014). A ground-based study on the influence of radiation on fish immediately post-hatching was carried out on the ornamental zebrafish (*Danio rerio*), in which eggs were irradiated with doses ranging from 1 to 1,000 $mSv.d^{-1}$ for 20 days (Simon et al.,

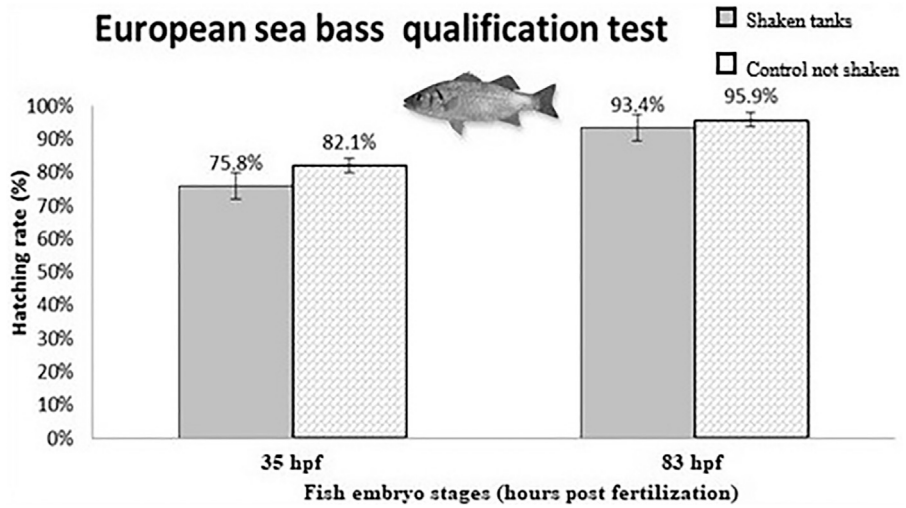


**FIGURE 5 |** European seabass embryo hatching rate following acoustic and mechanical vibrations from a simulated Soyuz launch qualification test (from Przybyla et al., 2020).

2011). At the stage of 3 days post-hatching, no significant difference in mortality was observed between irradiated eggs and the control. The maximum daily dose was 100 times greater than the total dose astronauts were subjected to during the Apollo 11 mission. These results are consistent with a study in which no significant difference in mortality was observed between 0.8 mGy (the threshold recommended to protect ecosystems) and 570 mGy delivered per day, but the radiation exposure induced accelerated hatching for both doses and a decrease in yolk bag diameter for the highest dose (Gagnaire et al., 2015). In contrast, another study exposing zebrafish embryos to 1, 2.5, 5, 7.5, and 10 mGy of gamma radiation at 3 hpf showed that increasing gamma radiation increased DNA damage, decreased hatching rate, increased median hatching time, decreased body length, increased mortality rate, and increased morphological deformities (Kumar et al., 2017). A higher total dose but spread over time therefore seems to be less harmful than a single high dose concentrated in the early stages of development. Gagnaire et al. also found abnormal development of the spine for individuals subjected to 570 $mGy.d^{-1}$. These research results on a small fish provide useful information for countermeasures that would need to be implemented on a lunar base. Fish and crew should be protected to reduce cosmic ray damage. Fish embryos could benefit from progress in countermeasure technology developed for humans, but it would be valuable to conduct experiments on the impact of different particles and charges (separate and cumulative) from cosmic radiation on the candidate fish.

## CONCLUSION

The Lunar Hatch program is investigating the prospects of lunar aquaculture based on a circular food system using a selected species at a specific stage of the lifecycle. It may be of interest to investigate other aquaculture species for other targeted planets or other lifecycle development stages. In the case of the Moon, it is so close to Earth that rearing adults for reproduction would not be worthwhile: a regular shipment of fertilized eggs for monthly generation would avoid costly fish-spawning management on the lunar base. For a more distant destination such as Mars, the embryo stage would be realistic for the first part of the mission, but the total flight would be longer than the duration of embryogenesis. In this case, larval development would need to be considered during the multi-month journey. For farther destinations, studies would need to determine the possibility of rearing broodstock to control the entire biological lifecycle in space.

Space aquaculture would provide a valuable food source in addition to those already studied for long-term missions. The diversity of nutrients provided by fish and the benefits for human metabolism may help in the challenges of space medicine, in particular the prevention of cancer caused by long-term exposure to radiation. The activity of fish farming itself could have positive psychological and cognitive effects. Reports about plant-growth chambers on manned missions have described the psychological benefits of working with living organisms in space. An investigation involving social scientists could be conducted to better understand the possible positive benefits of human–animal interaction in space. Vertebrates may recall basic human activities and provide a psychological umbilical cord with the Earth.

Modern recirculating aquaculture systems share many characteristics with the closed bioregenerative life-support systems planned for space. Progress in aquaculture technology on land and in space can feed into each other. For example, developments that allow space aquaculture systems to recover and convert waste molecules into edible food could be deployed on Earth to increase food availability while avoiding waste discharge in the environment and preserving biodiversity. Joint efforts to design such waste

conversion systems will be applicable above all to human activities on Earth.

Like other aspects of BLSS, while space aquaculture is close to being a reality, it is highly dependent on the water and energy available *in situ*. At the turn of the 20th century, the Russian father of astronautic science Konstantin Tsiolkovsky wrote: "Earth is the cradle of humanity, but one cannot remain in a cradle forever." Plants and animals are part of the human biosphere and food chain. Space exploration will likely be more successful if humans leave the cradle with a part of their own biosphere and their knowledge of agricultural science, including aquaculture.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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# Use of Photobioreactors in Regenerative Life Support Systems for Human Space Exploration

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There are still many challenges to overcome for human space exploration beyond low Earth orbit (LEO) (e.g., to the Moon) and for long-term missions (e.g., to Mars). One of the biggest problems is the reliable air, water and food supply for the crew. Bioregenerative life support systems (BLSS) aim to overcome these challenges using bioreactors for waste treatment, air and water revitalization as well as food production. In this review we focus on the microbial photosynthetic bioprocess and photobioreactors in space, which allow removal of toxic carbon dioxide ($CO_2$) and production of oxygen ($O_2$) and edible biomass. This paper gives an overview of the conducted space experiments in LEO with photobioreactors and the precursor work (on ground and in space) for BLSS projects over the last 30 years. We discuss the different hardware approaches as well as the organisms tested for these bioreactors. Even though a lot of experiments showed successful biological air revitalization on ground, the transfer to the space environment is far from trivial. For example, gas-liquid transfer phenomena are different under microgravity conditions which inevitably can affect the cultivation process and the oxygen production. In this review, we also highlight the missing expertise in this research field to pave the way for future space photobioreactor development and we point to future experiments needed to master the challenge of a fully functional BLSS.

**Keywords: space exploration, bioregenerative life support systems, microalgae, photobioreactors, air revitalization, cyanobacteria**

## INTRODUCTION

Human space exploration aims to go farther into space and crewed missions are planned to Moon and Mars. The European Space Agency (ESA) as well as the National Aeronautics and Space Administration (NASA) plan human missions to Mars in the coming decades (Hufenbach et al., 2014; Anderson et al., 2019). The space travelers need oxygen, fresh water and nutritional food to survive on such space missions (MacElroy and Bredt, 1984) and the supply has to become independent from Earth. In order to minimize the resupply needs and the embarked mass, the recycling process will have to include food production coupled to oxygen ($O_2$) and water ($H_2O$) recovery that entails the use of at least one biological compartment for producing edible biomass. This challenge can be solved by the development of a bioregenerative life support system (BLSS),

that meets the needs at least for a part of food supply to the crew and secures air, water as well as safe waste recycling (Gitelson et al., 1976).

This review will focus on the bioprocess of microbial photosynthesis and, in particular, on photobioreactors (PBRs) used in space for BLSS. Moreover, we focus on the process of air revitalization, meaning the efficient removal of carbon dioxide ($CO_2$) and production of $O_2$. The goal of this study is to give an overview of the experiments that have been conducted with liquid cultures of photosynthetic microbes and PBRs for application in space, and to highlight similarities and common challenges. Hereby, the last 30 years will be focused. Additionally, missing data and suggestions on future experiments will be discussed. The following sections describe the general requirements for life support systems and the current state of the art to how a BLSS can be developed using different techniques and organisms.

## OXYGEN REQUIREMENTS AND CARBON DIOXIDE LIMITS IN SPACE HABITATS

In our daily lives, $O_2$ is freely available to us because our atmosphere functions as an infinite buffer tank for $O_2$ produced by Earth's photosynthetic biosphere. However, when humanity wants to explore space, closed spacecraft do not have an endless supply of $O_2$. Due to the human respiration process, the $O_2$ level decreases while $CO_2$ and water vapor ($H_2O_{vapor}$) increase inside a closed habitat over time. The typical respiratory rate for a healthy adult at rest is 12–18 breaths per minute or roughly 20,000 breaths a day (Crockett et al., 2018). In spacecraft environments, it is considered that one standard 82 kg crew member consumes 0.82 kg $d^{-1}$ $O_2$ and produces 1.04 kg $d^{-1}$ $CO_2$ and 1.85 kg $d^{-1}$ $H_2O_{vapor}$ during intravehicular activities. Based on these values, the respiratory quotient (mole $CO_2$ produced per mole $O_2$ consumed) is 0.92, but the respiratory quotient varies depending on the physical workload, diet and individual metabolism (Anderson et al., 2018). When the partial pressure of $CO_2$ in the air becomes too high, it becomes toxic to humans. For example, a maximum value for $CO_2$ of $\leq$0.52 kPa (0.52% per volume or 5,200 ppm) is allowed on the International Space Station (ISS) (Anderson et al., 2018) and even 0.50% (5,000 ppm) $CO_2$ in a habitat is thought to have a negative impact to human health over longer periods. In order to keep the risk of headache development under 1%, the average value over 7 days should not exceed ∼0.33% $CO_2$ (Law et al., 2014). Consequently, a system is needed to remove excess $CO_2$ and provide a sufficient partial $O_2$ pressure. The partial $O_2$ pressure depends on the total cabin pressure and should range between 18 and 23.1 kPa or 21–50% per volume, respectively (Lange et al., 2003; Swickrath and Anderson, 2012; Anderson et al., 2018). On the ISS, the total pressure is set at 101.3 kPa (with 21% $O_2$), so it resembles the pressure on Earth (101.325 kPa on sea level). But other pressure regimes are also suitable for manned habitats. When using atmospheres consisting of mixed gasses (e.g., $N_2/O_2$ mixtures), total pressures between 48.0 and 102.7 kPa are acceptable. In early space missions, almost pure $O_2$ atmospheres at total pressure of 34.5 kPa were used. Due to the increased fire hazard under pure oxygen atmospheres, nowadays spacecraft use gas mixtures similar to our atmosphere (Lange et al., 2003; Anderson et al., 2018).

## PHYSICOCHEMICAL AIR REVITALIZATION IN SPACE HABITATS

To date all crewed spacecraft solely rely on physicochemical methods of air revitalization. In the first years of crewed spaceflight, different physicochemical methods were tested. Early space missions like Mercury, Gemini, the Apollo Command Module and the Apollo Lunar Module used lithium hydroxide (LiOH) canisters that remove $CO_2$ by converting atmospheric $CO_2$ to lithium carbonate ($Li_2CO_3$) (Winton et al., 2016). The canisters have to be replaced after usage because the chemical reaction is irreversible. Additionally, different molecular sieves were used to remove $CO_2$ and $H_2O_{vapor}$. $O_2$ storage was usually accomplished via high pressure liquid $O_2$ or chemically bound $O_2$.

On the ISS, a Carbon Dioxide Removal Assembly (CDRA), an Oxygen Generation Assembly (OGA) and a Carbon Dioxide Reduction Assembly (CRA), are currently used to maintain a suitable gas balance. These systems use regenerable absorbent materials (in CDRA), water electrolysis (Eq. 1) (in OGA) and the Sabatier reaction (Eq. 2) (in CRA) to remove $CO_2$ and produce potable water ($H_2O_{potable}$) (Mansell et al., 2011; Knox and Stanley, 2015; Takada et al., 2019).

$$2H_2O \rightarrow 2H_2 + O_2 \quad (1)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (2)$$

The methane produced in the CRA is vented into space. Considering a respiratory coefficient near than unity, the mol quantities of $O_2$ to produce and $CO_2$ to remove are almost equal. This means that the stoichiometric eqs. (1, 2) show that there is a global hydrogen imbalance. Either it must be compensated by hydrogen resupply or excess $CO_2$ has to be removed by absorption to materials that end up as trash. As a consequence, substantial amounts of carbon and other elements are lost over time (Mansell et al., 2011; Knox and Stanley, 2015). So even nowadays, all of the methods used for air revitalization are physicochemical and not regenerative (Junaedi et al., 2011; Tobias et al., 2011). They use a lot of consumables and produce a lot of waste and are therefore only applicable for near-by (LEO) missions with easy resupply from Earth (Daues, 2006).

## BIOREGENERATIVE AIR REVITALIZATION IN SPACE HABITATS

Possible solutions to this problem are photosynthetic biological systems using plants, algae and cyanobacteria for air revitalization and carbon recycling coupled to production of edible biomass. The photosynthetic activity of these organisms

captures $CO_2$ and $H_2O$ to produce edible biomass and $O_2$. The development of a BLSS to ensure the autonomous air supply of space crews on distant and long-duration missions, became a long time goal of many space agencies (Lasseur et al., 2010). The first ideas on this topic were already developed around 1900 (Salisbury et al., 1997) and the development process has already started in the 1960s before the first human flew to space. Many different attempts to obtain such a BLSS with efficient biological air revitalization were conducted during the second half of the 20th century. Especially the soviet space program investigated different approaches using microalgae and higher plants very early (Hooke et al., 1986; Niederwieser et al., 2018).

Additionally, several test sites were developed on Earth. Some examples of large ground based studies are the BIOS-I project, where the algae *Chlorella* provided $O_2$ for one human and the BIOS-III project that inhabited three crew members; plants and green algae performed at the BIOS facility in Krasnoyarsk, Siberia, Russia (Kirensky et al., 1968; Gitelson et al., 1976; Gitelson, 1992). The NASA Biomass Production Chamber project was a closed greenhouse designed to grow crops on a small area (20 $m^2$), that was successfully operated for over 1,200 days (Wheeler et al., 1996). Another example is the Biosphere-2 project (in Oracle, AZ, United States), which tried to mimic the terrestrial biosphere by inhabiting eight crew members together with various plants (Allen and Nelson, 1997). A recent example is Lunar Palace 1, that investigated a system consisting of plants, insects and three crew members for 105 days (Fu et al., 2016). ESA has initiated the development of its own Micro-Ecological Life Support System in 1988, called MELiSSA, combining physicochemical technologies with microbial and plant conversion of organic and inorganic waste into nutrients, and biological air revitalization (Lasseur et al., 2010). In addition to carbon, hydrogen and $O_2$ balances, the MELiSSA loop considers the recycling of nitrogen, which in turns incorporates food production by biological compartments coupled to the other balances. The MELiSSA loop consists of five interconnected compartments including two PBRs. Compartment III holds a nitrifying culture to produce nitrate for compartments IVa (cyanobacteria) and IVb (higher plants), which use the nitrate, excess $CO_2$ from the crew and light to produce edible biomass, potable water and $O_2$ (Gòdia et al., 2002). The photosynthetic cyanobacterium *Limnospira indica*, formerly known as *Arthrospira* sp. PCC8005 (Nowicka-Krawczyk et al., 2019) is used as part of the $O_2$ and edible biomass production in one of the PBRs (Poughon et al., 2020).

# PHOTOSYNTHETIC MICROORGANISMS AS CATALYSERS FOR AIR REVITALIZATION IN SPACE

As mentioned above, air revitalization can be achieved by photoautotrophic organisms like cyanobacteria, algae or plants. In general, algae and cyanobacteria have many benefits compared to land plants for $CO_2$ removal, and $O_2$ and biomass production. Due to these benefits, unicellular photosynthetic organisms are investigated as key component of life support systems for a long time (Averner et al., 1984; Niederwieser et al., 2018).

## Oxygen Production and Carbon Fixation

The light intensity is a key factor for $O_2$ production in photosynthetic organisms. On the surface of Earth, the light intensity has a maximum of 2,500 $\mu E\ m^{-2}\ s^{-1}$ (full sunlight, $\mu E$ = $\mu$mol photons) and on average, a daily dosage of 60 mol photons $m^{-2}$ is reached (Melis et al., 1998). Cyanobacteria like *Limnospira* spp. are very potent $O_2$ producers. For example, ponds in southeastern California release about 16.8 t of $O_2$ $ha^{-1}\ yr^{-1}$ while fixating 6.9 t of $CO_2$ $ha^{-1}\ yr^{-1}$. In comparison, trees produce about 2.5–11 t of $O_2$ $ha^{-1}\ yr^{-1}$ and fixate 1–4 t of $CO_2$ $ha^{-1}\ yr^{-1}$ (Henrikson, 2010). It has been found, that *Limnospira* cultures are about 2.5 times more productive in tropical environments and under these warm and humid conditions, a pond surface of approximately 80 $m^2$ would meet the oxygen needs of one crew member. It was stated that the use of modern photobioreactors that e.g., control temperature, humidity, $CO_2$ influx and illumination could reduce the needed surface significantly (Verseux et al., 2015).

In BIOS-I, the experimental data proposed that 20 L or respectively, an illuminated surface of 8 $m^2$ of *Chlorella* culture is needed to supply one crew member. In comparison, the BIOS-3 experiment investigated how big a plant compartment has to be to sufficiently provide oxygen for one human and found that the surface would need to be 30 $m^2$ (Gitelson, 1992). Javanmardian and Palsson (1992) showed that a 200 L PBR (calculated from a 600 mL prototype) containing *Chlorella vulgaris* is able to meet the gas exchange needs of one human and they proposed that the system could be downsized to a 20 L *Chlorella vulgaris* tank reactor if equipped with an optimized illumination system and regime. In addition, it was shown and experimentally demonstrated that the $O_2$ production and $CO_2$ consumption of a 83 L PBR with *Limnospira indica* is adaptable via the light intensity and can meet the needs of a three rat compartment (∼5–10% of the $O_2$ requirements of one human) in the MELiSSA Pilot Plant facility at the Universitat Autònoma de Barcelona. In this experiment, the rats were kept alive for several months through gas exchange with the PBR (Alemany et al., 2019).

## Biomass Production

Algae and cyanobacteria are also more efficient for edible biomass production in many cases, as they need less surface or volume for biomass production than higher plants, can be continuously harvested, and are fully comestible without complex food preparation and with reduced waste production. Wheeler et al. (2003) presented biomass productivity data (edible dry weight biomass per area and day) of different plants and showed that wheat plants are able to produce up to 12.6 g $m^{-2}$ $d^{-1}$ and soybeans up to 6 g $m^{-2}$ $d^{-1}$. In comparison, in open ponds, *Limnospira indica* yields up to 15 g $m^{-2}$ $d^{-1}$ (dry weight) (Jimenez et al., 2003) and *Spirulina* sp. LEB-18 can yield up to 69 g $m^{-2}$ $d^{-1}$ (dry weight) (Morais et al., 2009). Helisch et al. (2020) also reported high biomass production rates (1.3 g $L^{-1}$ $d^{-1}$ (dry weight per volume and day) for *Chlorella vulgaris* in a microgravity capable PBR (see **Table 1**,

**TABLE 1 |** PBR ground experiments with the eukaryotic algae *Chlorella vulgaris*.

| Hardware | Gas exchange | Volume | Light intensity | Mode | Duration | Results | Authors |
|---|---|---|---|---|---|---|---|
| PBR | Hollow fiber cartridges | 600 mL | 0.6 mW/cm$^2$ (usable light) ≈ 27.6 μE m$^{-2}$ s$^{-1}$ | Batch and continuous | >2 months | The measured oxygen production rate under continuous operation (4–6 mmol/L*h) meets the expectations | Javanmardian and Palsson (1992) |
| PBR with a vertical rectangular slab-shaped illumination chamber | Hollow fiber cartridges | 70–340 mL, depending on the experiment and the number of illumination chambers | 25 mW/cm$^2$ (on each LED plate) ≈ 1,150 μE m$^{-2}$ s$^{-1}$ via LED | Continuous | 10 days | The general performance of the hollow fiber PBR was comparable to the PBR using a sparging system, but the oxygen production rate was decreased | Lee and Palsson (1995) |
| Plate PBR with automated control system | Unknown | 1.5 L | 2 LED panels with 117–143 μE m$^{-2}$ s$^{-1}$ | Continuous | 6 months | A completely closed water cycle could be achieved in the biological system containing multiple organisms. A sufficient gas exchange was also achieved. | Tong et al. (2011, 2012) |
| Plate PBR with automated control system | Unknown | 1.5 L | 150, 300, and 350 μE m$^{-2}$ s$^{-1}$ | Continuous | 192 days | *C. vulgaris* can be used as an emergency system in case of high plant system problems. Additionally, the $CO_2$ and $O_2$ concentrations could be kept in a good range. | Li et al. (2013) |
| Raceway PBR | Microgravity-capable membrane | 650 mL | 200–300 μE m$^{-2}$ s$^{-1}$ | Repeated batch mode | 188 days | Achievement of biomass growth up to a maximum of 12.2 g/l. The bioreactor works on Earth and is ready to be tested in Space (PBR@LSR) | Helisch et al. (2020) |

light intensity: 200–300 μE m$^{-2}$ s$^{-1}$). In addition, Zhang et al. (2018) observed generation times between 13 and 28 h for several cyanobacterial species at a light intensity of 30 μE m$^{-2}$ s$^{-1}$. Therefore, harvesting can be done continuously or several times a week, even under low light intensities. On the other hand, typical staple crop plants like rice, wheat or potato, usually take several months until they are harvestable (Watson et al., 2018). In fact, there are large discrepancies between the reported biomass volumetric productivities values. This is due to the fact that microalgae cultures are generally limited by light energy supply so that the production rate depends on the illuminated surface and the light intensity. Therefore, the productivities primarily depend on culture design (illuminated surface versus culture volume) and operational variable (light energy flux). A departure value of light energy yield by photosynthetic systems is in the order of magnitude of 20 mol of photons per mol of carbon fixed (Cornet and Dussap, 2009; Poughon et al., 2020). This is consistent with the maximum value of 69 g m$^{-2}$ d$^{-1}$ reported by Morais et al. (2009) with a maximum possible light intensity before the appearance of photo inhibition.

## Use of Biomass as Food Supplement

Also, the full biomass of several microalgae is edible producing no waste like non-edible roots, stems or leaves. For example, the algae *Chlorella vulgaris* is rich in protein (up to 58%) and contains all essential amino acids (Becker, 2007). It is also rich in unsaturated fatty acids, carotenoids, dietary fibers, vitamins, and minerals (Safi et al., 2014). The cyanobacterium *Limnospira* is also known for its great nutritive value including proteins of high quality, many minerals, vitamins and phytopigments (Farag et al., 2015). It was shown by Morist et al. (2001) that *Limnospira indica* can be used to produce healthy and nutritious food using different methods like freeze-drying, spray-drying and pasteurization. In addition, the consumption of *Limnospira* has beneficial features for space travelers because of its antioxidant and anti-inflammatory treats (Wu et al., 2016). Furthermore, it was shown that *Limnospira indica* is highly irradiation resistant (survival up to at least 6400 Gy of gamma rays) and therefore no negative effects from the increased ionizing irradiation in space are to be expected (Badri et al., 2015).

As algae and cyanobacteria can be cultivated axenic or defined xenic in bioreactors, they do not contribute possibly harmful microorganisms to the microbiome of a spacecraft (Helisch et al., 2020). In comparison, several crops need parts of their own microbiome for optimal growth and contain a risk of catching molds, when grown in spacecraft simulations (Saleem et al., 2019; Fahrion et al., 2020).

Despite the many advantages, using cyanobacteria and algae also has some disadvantages. For instance, cyanobacteria and algae contain less fiber then the edible biomass of plants and too few carbohydrates for a complete diet (Lehto et al., 2006). The nucleic acid content is too high in some microorganisms and some are also too high in certain minerals, which can have a negative effect on the health of the crew members (Averner et al., 1984). Lastly, nutrition from algae and cyanobacteria alone

also has a negative impact on certain social aspects. Because photosynthetic microorganisms are very nutrient dense, only a few grams are usually ingested. Preparing, consuming and sharing food is impaired in this case, however, it was shown that the social aspect of eating is important for our mental and physical well-being (Chappuis et al., 2020). Furthermore, being around plants has a positive effect on the mental wellbeing of humans (Lehto et al., 2006). Ideally, a system connecting both should be aimed for as combining a cyanobacterial or algae PBR with a higher plant compartment increases the variety of the consumed food and was also shown to increase the recycling rates of a BLSS, and will also provide system redundancy for the essential $CO_2$ removal and $O_2$ production for the crew members (Gros et al., 2003).

## DEVELOPMENT OF BIOLOGICAL AIR REVITALIZATION SYSTEMS FOR SPACE

The development of biological air revitalization in space is a tedious process. Even before a bioreactor is functional, several key aspects need to be addressed. One of the first tests is to investigate whether the target organism is able to survive a space upload, exposure and return. Different space exposure experiments were launched to address questions of survivability and resistance mechanisms of terrestrial microorganisms to microgravity and ionizing irradiation (Sancho et al., 2007; Onofri et al., 2012; de Vera et al., 2019). Additionally, the organisms are investigated for their growth kinetics, metabolic pathways and genetic stability, in engineered and space cultivation conditions. These primal experiments usually use liquid batch cultures. In a next step, a suitable kinetic model for all important parameters (e.g., in the case of cyanobacteria: $O_2$ production, $CO_2$ uptake, biomass production, use of nutrients, etc.) has to be developed and tested (Poughon et al., 2020). Only if the organism suits all requirements, experiments with fed-batch or continuous liquid cultures can start. For these tests, suitable PBR hardware has to be created. Sterility, biocompatibility, sealing and proper mixing are some of the requirements here. In addition, sufficient pumps for nutrient delivery and removal of biomass and products are needed (Ai et al., 2008). Depending on the organism, different pH, temperature and nutrient control systems have to be installed. For phototrophic organisms, adequate illumination and gas transfer is necessary. Typically, specific membranes are used frequently to improve the transfer of gasses like $CO_2$ and $O_2$ to and from the liquid phase (Sarbatly and Suali, 2013).

As soon as a bioreactor and its control system are set up, the interconnection to other compartments of the BLSS has to follow.

### Development of Space PBRs on Earth

Most of the research for biological air revitalization is executed on Earth. **Tables 1–3** give an overview of the different ground experiments that were performed in the last 30 years for the development of PBRs for $O_2$ production with *Chlorella vulgaris, Limnospira indica* and microalgae in combination with nitrifying communities providing the necessary nitrogen source for algal growth. The hardware and conditions that were used and the corresponding achievements are shown. The volumes for the experiments with *Chlorella vulgaris* were in lab scale and range between 70 mL and 1.5 L. Different PBR shapes were used, and several interesting discoveries were made. Javanmardian and Palsson (1992) showed that the calculated $O_2$ production and growth rate fits the experimental value and Li et al. (2013) concluded that a *Chlorella vulgaris* PBR can be used as an emergency $O_2$ production system, in case of failure of a plant unit. Four of the five experiments shown in **Table 1** persisted for over 2 months. Therefore, longer durations were covered, especially when compared to the experiments in **Table 2**. The ground experiments with *Limnospira indica* in continuous mode were mainly conducted in cylindrical PBR. With exception of the experiment described in Farges et al. (2009), only the batch cultures were grown in rectangular flasks or Erlenmeyers. Models and their application to the set up were investigated in several experiments. For example, Alemany et al. (2019) validated a model for a two-compartment system and Cornet and Dussap (2009) tested the functionality of different hardware setups and defined a model to predict the $O_2$ and biomass productivities of *Limnospira indica*. **Table 3** displays three experiments using an interconnected system of nitrifying compartments to microalgae. It is an important step in the development of a functional BLSS to use nitrified urine as nitrogen source for algae and cyanobacteria. In an already quite sophisticated approach, Gòdia et al. (2002) showed the successful combination of a nitrifying bioreactor with a PBR containing *Limnospira indica*. The two other experiments showed that microalgae can be grown successfully on different nitrogen sources.

The bioreactors developed for space used artificial light (e.g., halogen lamps or LEDs) with varying intensities and different designs of hardware were used, e.g., plate, cylindrical and rectangular bioreactor forms (**Tables 1–3**). The volumes used are generally in lab to pilot scale and range from 100 mL (Cornet and Dussap, 2009) up to 83 L (Alemany et al., 2019). Batch as well as continuous modes were tested. Also, the durations of the experiments vary widely (a few hours up to 4 years). *Limnospira indica* was the most often used organism in these experiments (**Table 2**). In many experiments, the gas exchange between the liquid and gaseous phase was accomplished by airlift systems. However, due to minimal gravity in space, this aeration system does not work and has to be replaced by e.g., membrane-aerated hardware (Wagner et al., 2015). Literature research revealed additional PBR research for space applications with other microorganisms. One interesting example is the ModuLES project with the green alga *Chlamydomonas reinhardtii* grown in a membrane-aerated plate-type PBR. Two liters of culture were grown continuously and the system was successfully tested in two parabolic flight campaigns (Wagner et al., 2015).

Additionally, many experiments using higher plants as photosynthetic organisms have been done, but these approaches are not covered in these tables.

### Testing Space PBRs in Space Flight

So far, micro-gravity experiments in space only contributed small parts to the development of different life support systems. Space experiments are very expensive, need extensive

**TABLE 2 |** PBR ground experiments with *Limnospira indica*.

| Hardware | Gas exchange | Volume | Light intensity | Mode | Duration | Results | Authors |
|---|---|---|---|---|---|---|---|
| Batches: rectangular PBR, cont.: cylindrical PBR | Airlift | Batches: 1 L and 4 L, cont.: 7 L | Batch: via white fluorescent lamps (20 W), continuous: via halogen lamps (20 W) | Batch and continuous | Unknown | A model to couple radiant light transfer and growth kinetics is proposed | Cornet et al. (1995) |
| PBR, 2 identical cylinders | External loop airlift | 77 L | Batch: 95 W/m$^2$ ($\approx$ 437 $\mu$E m$^{-2}$ s$^{-1}$), cont.: 133 W/m$^2$ ($\approx$ 611.8 $\mu$E m$^{-2}$ s$^{-1}$) via white halogen lamps (20 W) | Batch and continuous | 400 h (16.66 days) | Scaling up a 7 L to 77 L PBR was successful and the developed model was applicable | Vernerey et al. (2001) |
| PBR, 2 identical cylinders | External loop airlift | 77 L | Incident light flux 133 W/m$^2$ ($\approx$ 611.8 $\mu$E m$^{-2}$ s$^{-1}$) via white halogen lamps (20 W) | Continuous | Unknown | Three food preparation methods can be used to process *L. indica* to constitute a safe food source | Morist et al. (2001) |
| Cylindrical PBR | Airlift | 5 L | Batch: 88 W/m$^2$ ($\approx$ 404.8 $\mu$E m$^{-2}$ s$^{-1}$), continuous: 122 W/m$^2$ ($\approx$ 561.2 $\mu$E m$^{-2}$ s$-1$) and 150 W/m$^2$ ($\approx$ 690 $\mu$E m$^{-2}$ s$^{-1}$) via white halogen lamps (20 W) | Batch and continuous | Batch: $\sim$14 days, continuous: $\sim$50 days | The nutrient uptake rates of Zn, Fe, Mn, Mg, Cu, and K by *L. indica* have been successfully characterized | Cogne et al. (2003a) |
| Cylindrical PBR | Airlift | 5 L | 20–230 W/m$^2$ ($\approx$ 92–1,058 $\mu$E m$^{-2}$ s$^{-1}$) | Continuous | Unknown | The metabolic network of *L. indica* was characterized and revealed some interesting constraints | Cogne et al. (2003b) |
| Cylindrical PBR | Mixing and headspace | 132 mL | Via halogen lamp | Batch | Up to 400 h | *L. indica* could be grown in a newly designed PBR. Pressure, pH, temperature and cell density were monitored online | Cogne et al. (2005) |
| One-cylindrical PBR | Hollow fiber membrane | 31.8 L (calculated from measures in the reference) | 300 $\mu$E m$^{-2}$ s$^{-1}$ | Continuous | 7 days | Successful operation of the bioreactor and suitable control mechanisms could be demonstrated | Ai et al. (2008) |
| Rectangular PBR | Headspace with tubing and Peltier condensers, aeration via tube | 600 mL (batch) | Via red and blue LEDs, two different LED panels | Batch and continuous | Up to 3.500 h | Red LEDs cause the same biomass productivity in a PBR with *L. indica*, but with a much lower energy consumption | Farges et al. (2009) |
| Eight different PBRs, different volumes, shapes, illumination | Depending on the PBR, mostly airlift | 0.1–77 L | Photon fluxes between 30 and 1,600 $\mu$E m$^{-2}$ s$^{-1}$ | Batch and continuous | Cont. cultures: at least six residence times | Successful presentation of an analytical formula to predict the productivities of *L. indica* in different PBRs | Cornet and Dussap (2009) |
| Erlenmeyer flasks (batch), cylindrical PBR | Unknown | 250 mL and 2 L | 43 $\mu$E m$^{-2}$ s$^{-1}$ (batch), 140 $\mu$E m$^{-2}$ s$^{-1}$ (PBR) | Batch and continuous | Up to 50 days (PBR) | Urea seems to be a better nitrogen source than $NH_4^+$. Pulse feeding might help to avoid inhibitory effects | Deschoenmaeker et al. (2017) |
| Cylindrical double jacketed PBR | Purging with $N_2$, stirring with turbine | 2 L | 60 $\mu$E m$^{-2}$ s$^{-1}$ (batch) and 300 $\pm$ 50 $\mu$E m$^{-2}$ s$^{-1}$ (PBR), radially illuminated | Batch and continuous | 90 days | $NH_4^+$ salts (instead of expensive $NO_3^-$ salts) can be used to commercially grow *L. indica* | Sachdeva et al. (2018a) |
| Erlenmeyer flasks (batch) and cylindrical double jacketed PBR | Purging with $N_2$, stirring with turbine | 250 mL (batch), 2 L (PBR) | 60 $\mu$E m$^{-2}$ s$^{-1}$ (batch) and 300 $\pm$ 50 $\mu$E m$^{-2}$ s$^{-1}$ (PBR), radially illuminated | Batch and continuous | 7 days | Demonstration of potential of using urea and nitrite salts, as cheaper alternatives to nitrate salts | Sachdeva et al. (2018b) |

*(Continued)*

**TABLE 2 |** Continued

| Hardware | Gas exchange | Volume | Light intensity | Mode | Duration | Results | Authors |
|---|---|---|---|---|---|---|---|
| PBR consisting of two glass cylindrical tubes | External-loop airlift | 83 L (55 L illuminated volume) | Varying depending on experiment | Continuous | 30 and 50 days | A mathematical model to describe a two compartment system is successfully demonstrated. ("crew" = rats and PBR providing $O_2$) | Alemany et al. (2019) |
| Flat, cylindrical PBR | Hollow fibers | 2.6 L | 20, 35, and 50 W/m$^2$ (≈ 92, 161, 230 μE m$^{-2}$ s$^{-1}$), via LED | Quasi-batch | 27 days | No excessive shear stress is applied to the bacteria, the model is applicable | Chapuis et al. (2020) |

**TABLE 3 |** PBR ground experiments on algae and cyanobacteria, fed by a nitrifying culture.

| Organisms | Hardware | Gas exchange | Volume | Light intensity | Mode | Duration | Results | Authors |
|---|---|---|---|---|---|---|---|---|
| *Limnospira indica* PCC 8005, fed by *Nitrosomonas europaea* ATCC 19178, *Nitrobacter winogradsky* ATCC 14123 | PBR, 2 identical cylinders | External loop airlift | 7 L and 77 L | white halogen lamps (20 W), between ≈ 100–400 W/m$^2$ (≈ 460–1,840 μE m$^{-2}$ s$^{-1}$) | Continuous | 4 years, many different experiments and conditions | The separately operated as well as the interconnected bioreactors were successfully run in a continuous way | Gòdia et al. (2002) |
| Axenic *Limnospira indica,* fed by 12 different nitrifying inocula | Two membrane bioreactors (nitrifying community), 96-well plate (*L. indica*) | Air pump | 8 L (bioreactor), 0.3 mL (*L. indica*) and 0.8 L (*L. indica*) | 200 μE m$^{-2}$ s$^{-1}$ (0.3 mL batch of *L. indica*), 160 μE m$^{-2}$ s$^{-1}$ (0.8 L batch of *L. indica*) | Batch and continuous | 0.8 L batches of *L. indica*: 10 days, bioreactor up to 180 days (diagram) | *Limnospira indica* grew with high rates on the nitrified urine and yielded a high biomass protein content. *Nitrobacter* spp. became the dominant species in the nitrite oxidizing community | Coppens et al. (2016) |
| Different microalgae species, fed by commercially available nitrifying activated sludge | Plexiglas, gastight PBR | Airlift | 4 L | 300 μE m$^{-2}$ s$^{-1}$ | Semi-continuous | 180 days | The biological oxidation of all nitrogen sources in urine was successful and is a promising treatment for nutrient recovery of waste water | Muys et al. (2018) |

preparation and the precious crew-time of astronauts (Leys et al., 2004). Nevertheless, some experiments on liquid culturing and photobioreactors have already been performed in space (**Table 4**). Depending on the scientific question, many different experimental setups were used, i.e., liquid cultivation using different tube and flask designs up to fully assembled bioreactors (Poughon et al., 2020). Most conducted experiments aimed for very fundamental questions like how liquid cultures respond to microgravity and space radiation. The earliest record for algae flown to space dates back to 1960. In this 25 h experiment on Korabl-Sputnik 2, the algae were grown on agar in the dark and in liquid culture under periodic artificial illumination. Since some cells survived the flight and were able to grow and reproduce, it was concluded that algae can perform their basic physiological and photosynthetic functions in orbit (Semenenko and Vladimirova, 1961). More elaborate experiments were conducted in the years afterward, using different photosynthetic organisms on space stations (Mir, ISS) and on free flying return capsules. In the 1960s, 1970s, and 1980s, common organisms were *Chlorella pyrenoidosa*, *Chlorella vulgaris* and *Chlamydomonas reinhardtii* (Niederwieser et al., 2018). In 1987, the cyanobacterium *Nostoc sp.* PCC7524 and a plastid mutant of the eukaryotic alga *Euglena gracilis* flew on board a Long March II Chinese rocket to space. In this experiment, the plastid mutant alga was the $O_2$ consumer and CO2 producer and the cyanobacterium produced $O_2$ and consumed $CO_2$, respectively. It could be shown, that both organisms survived the 4.5 days in space and that some of the cyanobacteria grew under illumination (Dubertret et al., 1987). The used hardware of this early experiment is shown in **Figure 1**. This experiment is often referred to as the origin of MELiSSA (Lasseur and Mergeay, 2021, accepted for publication). In the later years, there were also some approaches with cyanobacteria like *Nostoc commune* var. *sphaeroides* (Wang et al., 2004), *Anabaena siamensis* (Wang et al., 2006), and *Limnospira indica* (Ilgrande et al., 2019). The latter was used in the Arthrospira-B experiment which was launched to the ISS in 2017 and which was the first approach to allow online measurements of the $O_2$ production rate as well as growth rate in space. Additionally, the cultures were kept axenic over the entire duration of over 1 month (Poughon et al., 2020). To date, this experiment is the most sophisticated successful approach to run an instrumented photobioreactor onboard a space station (**Figure 1**).

Although several experiments using photobioreactors in space were conducted, many of them were unsuccessful or the data were never reported in publications (Niederwieser et al., 2018). The

**TABLE 4 |** PBR space flight experiments with algae and cyanobacteria (~ last 30 years, only published ones, chronologically listed).

| Organism | Vehicle | Hardware | Volume | Light intensity | Mode | Duration | Results | Authors |
|---|---|---|---|---|---|---|---|---|
| *Nostoc sp.* PCC7524 and a plastid mutant of *Euglena gracilis* | Long March 2 | Dialysis bags that allow for gas exchange | 3 mL per culture | 0.3 W bulb | 1 batch | 4.5 days | Fixation of the cells was successful, some of the *Nostoc* cells germinated in the microgravity conditions | Dubertret et al. (1987) |
| *Chlorella vulgaris* LARG-1 | Bion-9 (Cosmos, 2044) | Three-component aquatic system | Unknown | Unknown, but it was illuminated | 1 batch | 13 days | Microscopy revealed differences in organelle-organization between space and ground samples but there was so significant difference in growth | Popova et al. (1989) |
| *Nostoc sphaeroides* Kütz | Shenzhou-II | Closed chambers | 85 mL | 2,200 Lux, 12-h-dark/12-h-light cycle | 1 batch | 6 days 15 h | A high growth rate was observed for the space samples exposed to microgravity | Wang et al. (2004) |
| *Anabaena siamensis* FACHB 799 | Chinese retrievable satellite | small bioreactor | 200 mL | 15 $\mu E\ m^{-2}\ s^{-1}$ | 1 batch | 15 days | Growth in space was slower, but after return, the space cultures grew at a higher rate. After a few generations, both cultures grew at the same rate | Wang et al. (2006) |
| *Euglena gracilis* with *Oreochromis mossambicus* (cichlid fish) | Foton M2 | Cylindrical prototype with two connected bioreactors | 1.45 L (*E. gracilis*) and 1.26 L (fish) | Via red LEDs (emission peak at 625 nm) | Continuous | 15 days | The oxygen production of *E. gracilis* gradually decreased in the first 9 days and increased afterward. Seven of the 35 fish died | Häder et al. (2006) |
| *Chlorella pyrenoidosa* FACHB 415 and *Bulinus australianus* (snail) | Chinese retrievable satellite and Shenzhou-II | Culture chambers | 85 mL algal culture in 120 mL chamber | 35 $\mu E\ m^{-2}\ s^{-1}$ | 1 batch | Satellite: 15 days; spacecraft: 6 days 15 h | Satellite: The algae survived but became a little lower in number, the snails died (probably from CO2 intoxication), spacecraft: the average *Chlorella* concentration decreased | Wang et al. (2008) |
| *Euglena gracilis* with *Oreochromis mossambicus* (cichlid fish) | Foton M3 | Polycarbonate cylinder with adjacent compartments | Unknown | Via three pairs of high-power red LEDs | Continuous | 12 days | The oxygen level in the tank decreased a little more than expected. 11 out of 26 fishes survived the flight | Strauch et al. (2008) |
| *Euglena gracilis* Z | Shenzhou-8 | Double culture chamber, separated by biofoil | 11 mL | 500 $\mu E\ m^{-2}\ s^{-1}$ | 1 batch | 17 days, fixation after 40 min (other samples failed) | First report on microgravity-induced changes at the transcriptional level of an unicellular eukaryotic organism | Nasir et al. (2014), Preu and Braun (2014) |
| *Limnospira indica* PCC8005 | ISS | Cylindrical PBR with flat membrane liquid and gaseous phase | 60 mL | 35 and 45 $\mu E\ m^{-2}\ s^{-1}$ | Batches (14, 6, 8, 6 days) | 5 weeks | Generally successful, but some technical difficulties. First dynamic growth of cyanobacteria in space and the gas and biomass model was shown to be applicable | Poughon et al. (2020) |

first experiment with in-flight analysis on *Chlorella sorokiniana* in liquid medium for 30 days failed because the reactor had a leak and exposed the algae to vacuum (Ward et al., 1970). A more recent unsuccessful experiment is the Eu:CROPIS project described in Hauslage et al. (2018). In this life support system experiment, *Euglena gracilis* was used in combination with a tomato plant and different bacteria (*Nitrosomonas*, *Nitrobacter*) to produce $O_2$ and edible biomass. Unfortunately, the flight experiment did not result in usable data due to a technical failure. Another example is the PBR@LSR project. In this project, a PBR inoculated with *Chlorella vulgaris* was brought to the ISS in 2019. The proposed experiment time was 6 months, but the experiment had to be terminated after a few weeks due to technical issues (Keppler et al., 2018). **Table 4** shows a selection of space flight experiments using photosynthetic unicellular organisms in illuminated test chambers (PBRs) that have been in space over the last 30 years. Since not all of the experiments in space have been successful, not all space experiments were published so that only experiments published in peer reviewed papers that could be used as references are shown.

## DISCUSSION

In this section, we highlight the different challenges in context of PBR for space applications in order to give an overview of

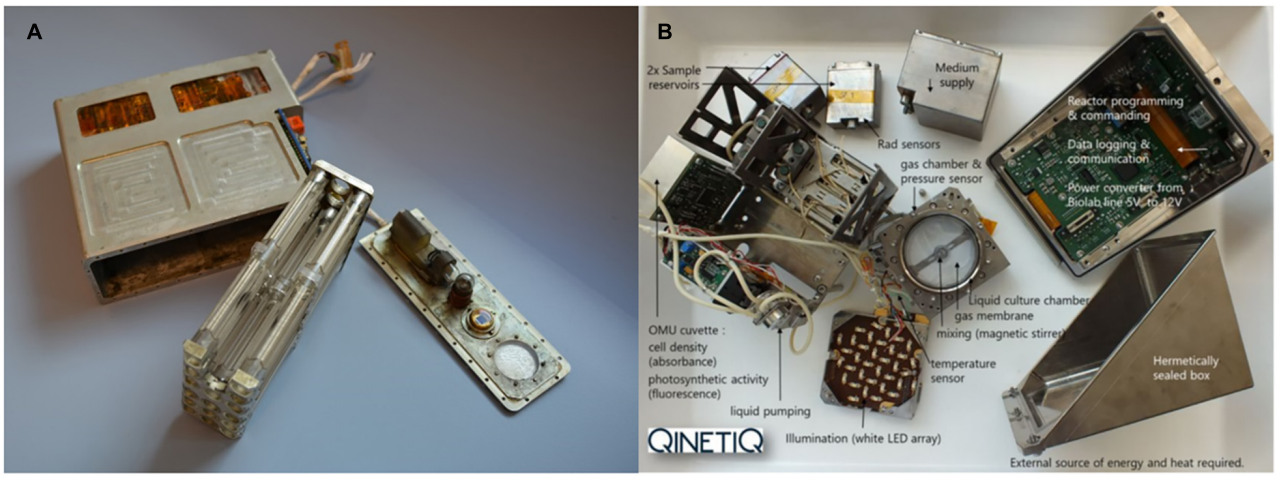


**FIGURE 1 | (A)** *Nostoc* sp./*Euglena gracilis* container of the first space flight experiment associated with MELiSSA (Dubertret et al., 1987), courtesy of ESA. **(B)** *Limnospira indica* hardware from the ArtEMISS-B experiment (Poughon et al., 2020, original source: QINETIQ).

knowledge gaps and problems that already occurred or might arise in the future of BLSS research.

## Safety and Reliability – Robustness, Resilience, and Redundancy

The safety and reliability of a life support system is of utmost importance. In order to avert fatal incidents, several back-up facilities and control mechanisms have to be installed and the system has to be monitored consistently. All possible scenarios have to be calculated and evaluated beforehand to avoid failure, because failure can be fatal for the crew (Bartsev et al., 1996). For example, a failure in $O_2$ production has to be intercepted by an emergency system before a drop in the $O_2$ concentration of the cabin occurs. A high degree of redundancy has to be achieved. Physicochemical emergency back-up systems, plant compartments and different PBRs could be put in parallel that can be uncoupled from each other. And not all bioreactors need to be operated in long duration continuous production, but a regime of alternating batches or operation and downtime of bioreactors could be implemented, if shown to be advantageous for operation, harvesting or maintenance. In addition, reliable mathematical models for the bioreactors are essential to keep all processes predictable (Vernerey et al., 2001). This is a highly strategic point when the recycling efficiencies of different elements and compounds are coupled and intertwined as it is the case for BLSS, such as MELiSSA. In this case, the action on an operational variable has distributed consequences at several points of the recycling system, calling for an intelligent control strategy based on knowledge models taking into account the dynamic exchanges between the different parts of the recycling system. The other important point for life support systems for space is that the buffer tanks generally have a minimal capacity entailing an online control strategy. The criteria for reliability, availability, maintainability and supportability (RAMS) engineering have to be applied in the BLSS research.

## Gas Exchange and $O_2$/$CO_2$ Balance Between Consumer and Producer

As mentioned, on average, one human needs ∼0.82 kg of $O_2$ and produces ∼1.04 kg of $CO_2$ per day. Depending on the activity level, the ratio of exhaled $CO_2$ to consumed $O_2$, i.e., the respiratory coefficient, can vary (Anderson et al., 2018). On the other hand, the $O_2$ production of algae and cyanobacteria can be characterized by a photosynthetic coefficient, describing the ratio of produced moles of $O_2$ per consumed moles of $CO_2$. This ratio is dependent on the organism (and its biochemical composition) and the nutrient substrate (e.g., the nitrogen source). The stoichiometric eqs. 3, 4 [simplified from Cornet et al. (1998)] show this dependence for *Limnospira indica* on the examples ammonium ($NH_3$) and nitrate ($NO_3^-$ or here: $HNO_3$) as nitrogen sources. Solving the stoichiometric equations reveals that the photosynthetic coefficient for ammonium is ∼1.0 and for nitrate ∼1.4.

$$CO_2 + 0.528H_2O + 0.173NH_3 + nh\nu \rightarrow CH_{1.575}O_{0.459}N_{0.173} + 1.034O_2 \quad (3)$$

$$CO_2 + 0.701H_2O + 0.173HNO_3 + nh\nu \rightarrow CH_{1.575}O_{0.459}N_{0.173} + 1.381O_2 \quad (4)$$

Importantly, it must be outlined that photosynthetic growth stoichiometry has no degree of freedom when the composition of nitrogen source (or its degree of reduction) is fixed so that the photosynthetic coefficient is only depending and linked to the culture conditions. The number of photons required to fix 1 mol of carbon is depending on the culture conditions. Away from photo inhibition conditions, a typical value is $n$ = 20 mol photons per mol of carbon fixed (Cornet and Dussap, 2009; Poulet et al., 2020).

In order to avoid an imbalance in gas composition, a system has to be developed to combine the respiratory quotient of the crew members and the photosynthetic coefficient of the

microalgae. In some experiments, successes were achieved (see section "Photosynthetic Microorganisms as Catalysers for Air Revitalization in Space"). However, gas exchange in space is much more complex, due to the lower or lack of gravity. There is still very little information about the gas, water and solute transport in microgravity in living organisms. Moreover, microgravity conditions strongly modify the environment of the chemical and biochemical processes, e.g., implying lack of sedimentation and impaired gas and liquid phase separation. Consequently, transport is limited to diffusion causing an increase of boundary layer thickness and therefore a significant decrease of mass and heat transfer coefficients. This can cause problems with pumping and the mineral availability for the cultures and has to be elucidated more thoroughly (Klaus et al., 1997).

## *In situ* Resource Utilization and Light as an Energy Source

Another challenge is the complete closure of a BLSS. So far, no loop has an efficiency of 100%, which means that all tested life support systems still rely on external addition of different substances like carbonate or trace elements, etc. For example, the 105 days long Lunar Palace 1 experiment (plants, insects, and three crew members) reached a full oxygen and water recycling but only 20.5% nitrogen recovery from urine and 55% of the food was regenerated. In this approach, physico-chemical and biological processes were combined (Fu et al., 2016). Some substances are either difficult to find in the space environment or it is very costly and time consuming to convert them into a usable form. Therefore, space habitats for humans have to be fully functional under the specific conditions and have to rely on the materials available around and only a small amount of material brought from Earth. For example, lunar regolith, mars soil and $CO_2$ in the Martian atmosphere are promising substances to be used for *in situ* resource utilization (ISRU) (Montague et al., 2012; Muscatello and Santiago-Maldonado, 2012).

The usage of photoautotrophic organisms helps to overcome parts of the material problems because their main energy source is light. But so far, only experiments using artificial light (e.g., halogen lamps or LEDs) have been flown (**Table 4**) which means that the naturally available solar energy is not used directly so far. One of the main reasons is that the natural light intensities and spectral energy distributions available in space are not compatible with the needs of the photosynthetic organisms. The intensity of sun light depends on the distance from the sun and the irradiation spectrum in deep space consists of a different wavelength composition than the irradiation we experience on ground due to absorption of light in the Earth's atmosphere (Cockell and Horneck, 2001). Besides that, the ISS, Moon and Mars surface are eclipsed for 50% of the time and the day and night cycles, e.g., on the moon are very different from Earth. For example, one lunar night is as long as 18 Earth days (Alvarado et al., 2021; Xie et al., submitted)[1]. Also, the intensity of natural light sources is much more difficult to be controlled than artificial light sources. Therefore, approaches where solar power is used to store energy in batteries as fuel for LEDs with suitable light characteristics, that can be used in PBRs and greenhouses, are desired.

## Scaling Up

So far, the research on the PBR part of the BLSS is the most sophisticated area. But also in the PBR research, most experiments were done in lab up to pilot scale (100 mL up to 83 L, **Tables 1–4**) and these volumes were not sufficient to provide 100% of the $O_2$ need of a crew member (Javanmardian and Palsson, 1992; Alemany et al., 2019). So even if a small lab scale bioreactor is successful, scaling-up procedures have to follow to achieve the needed production rates for a BLSS, which vary strongly depending on the used organisms (Vernerey et al., 2001). In order to develop a reliable system, the PBR needs to represent a well-balanced combination of a relatively small, but sufficient volume and high productivity via usage of proper illumination in high cell density cultures.

## Connecting Multiple Bioreactors and Closing the Loop

Only a few experiments involve bioreactors that are connected to other life support compartments like the crew or a waste recycling compartment. Consequently, many challenges remain in this research area and the connection between the different systems has to be elucidated more. Additional experiments on the nitrifying community and the other parts of the waste treatment (e.g., thermophilic anaerobic bacteria to produce volatile fatty acids out of waste) have to be conducted in space and further developed on ground (Lasseur et al., 2010). Some unidentified problems might arise in connected bioreactors. Cross-contamination and cross-talking of the organisms by quorum sensing molecules between the different compartments might disturb the system on a long-term scale (Mastroleo et al., 2013). However, also in axenic bioreactors, there is missing knowledge about cell-cell communication and biofilm formation in space.

## Long Duration Cultivation in Engineered Bioreactors and Space Conditions

The following question has to be answered: does a long-term cultivation in engineered bioreactors under space conditions have an effect on the microorganisms? A predictable and stable growth rate of the culture is essential for the performance of a PBR. Early stress signals have to be monitored to avoid culture failure in space and adequate countermeasures have to be developed. For this, low-dose prolonged irradiation and (simulated) microgravity experiments are needed. The genetic stability over multiple generations has to be addressed, e.g., the mutation rates, differences in gene expression and epigenetic effects like changes in DNA methylation patterns. So far, not enough experiments on long term conduction of photobioreactors were done, to answer that question.

[1] Xie, G., Zhang, Y., Yang, J., Yu, D., Ren, M., Qiu, D., et al. (submitted). *Adaptation to real 1/6 g Moon Gravity Contributes To Plant Development And Expeditious Acclimation To Super-Freezing*. Chongqing, Research Square. doi: 10.1007/s00204-016-1744-5

The effect of cosmic irradiation and reduced gravity on the oxygen production rate and nutritive value of the photosynthetic microorganisms has to be investigated. In plant experiments, it was reported that low doses of ionizing irradiation can cause an increase in growth rate (Sax, 1963; Upton, 2001), but for algae and cyanobacteria very limited data are available so far. Planel et al. (1987) presented a hormesis effect for the cyanobacterium *Synechococcus lividus* when irradiated with 1.49 mGy per year. If proven for BLSS relevant organisms, such hormesis effects might even be useful for the BLSS productivity.

Most space experiments with living organisms have only been done in LEO so far. There are very few reports of microbial experiments that went out of LEO (Horneck et al., 2010). One of the rare examples is the Chinese lunar chang'e 4 lander that brought organisms (plants, yeast and fruit fly eggs) farther into Space (Xie et al., submitted, see text footnote 1). Therefore, future investigations need to be beyond LEO, and e.g., in Moon orbit or on the Moon surface. Also, insightful investigations remain to be systematically done in order to detail and understand the mechanisms of interaction of zero-gravity conditions and different levels of intracellular organization and metabolic regulation. By example, it is well-known that gravitropism is an important phenomenon for higher plants growth and roots development. Similar effects are likely to occur even in simpler prokaryotic organisms linked to dissolved gas exchanges (namely $O_2$ and $CO_2$) between intracellular level and the culture environment.

### Remote Commanding, Monitoring, Reporting, and Data Exploitation

During literature research, it became apparent, that the published data often do not include all needed information. Especially the light intensity inside the culture, temperature and pH data are often missing.

Furthermore, the data of space experiments are deficient and often difficult to compare. For example, different sizes for different missions were used (**Table 4**) and the space experiments were mainly to investigate on exposure, survival and simplified processes. Also, only very few experiments with on-board monitoring can be found in the literature [e.g., ArtEMISS-B described in Poughon et al. (2020)]. Real-time bioprocess monitoring has to be achieved to obtain reproducible and reliable results.

## CONCLUSION

Bioregenerative life support systems are complex networks of biological and physicochemical transformations, including bioreactors, whose functioning are influenced by space environment like reduced gravity levels and increased doses of ionizing radiation. As a consequence, several biological and physical key bioreactor processes have to be controlled and adapted to these altered conditions by, for example, the usage of membrane-aerated PBRs. In practice, recent flown experiments have shown the challenge linked to the deployment of a successful PBR in space. Further knowledge is therefore needed to improve the necessary success rate that will allow continuous operation at a larger scale.

In general, more long-term continuous experiments should be conducted and all important parameters (temperature, gas exchange rates, light intensity, concentration of nutrients and biomass production) have to be monitored online, allowing remote bioreactor control from Earth, to reduce the dependence on the crew. Even though many promising experiments on photobioreactors for space applications were conducted, the development of a sufficient life support system still depends on an interconnected, continuously running loop system with a maximum closure.

## AUTHOR CONTRIBUTIONS

JF and NL performed the literature research and writing of the first draft. FM, C-GD, and NL contributed to reviewing and editing. All authors contributed to the article and approved the submitted version.

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# Choice of Microbial System for *In-Situ* Resource Utilization on Mars

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Various microbial systems have been explored for their applicability to *in-situ* resource utilisation (ISRU) on Mars and suitability to leverage Martian resources and convert them into useful chemical products. Considering only fully bio-based solutions, two approaches can be distinguished, which comes down to the form of carbon that is being utilized: (a) the deployment of specialised species that can directly convert inorganic carbon (atmospheric $CO_2$) into a target compound or (b) a two-step process that relies on independent fixation of carbon and the subsequent conversion of biomass and/or complex substrates into a target compound. Due to the great variety of microbial metabolism, especially in conjunction with chemical support-processes, a definite classification is often difficult. This can be expanded to the forms of nitrogen and energy that are available as input for a biomanufacturing platform. To provide a perspective on microbial cell factories that may be suitable for Space Systems Bioengineering, a high-level comparison of different approaches is conducted, specifically regarding advantages that may help to extend an early human foothold on the red planet.

**Keywords: Mars, carbon-fixation, bioproduction, synthetic biology, microbial bioprocess, bio-regenerate life-support system, in-situ resource utilization**

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## INTRODUCTION

A multitude of different bioregenerative life-support systems, have been proposed for *in-situ* resource utilization in Space and at destinations across the Solar System, such as Luna and Mars. Approaches draw from all domains of life, employing microbes as well as higher organisms, with applications ranging from production and recovery of resources, (e.g. generation of oxygen, food and materials, biomining, wastewater recycling), to providing shelter and protection, as far as generation of energy and even terraforming (Kalkus et al., 2018; Llorente et al., 2018; Hastings and Nangle, 2019; Lopez et al., 2019; Shunk et al., 2020; Volger et al., 2020). Here, the focus is on the deployment of microorganisms in bioreactors for bioproduction of feedstocks for consumables and durable goods to sustain and extend an initial presence on Mars. Special attention is given to the disruptive impact of Synthetic Biology on advancing these capabilities (Menezes et al., 2015a; Menezes et al., 2015b). Molecular pharming by use of plants has recently been discussed elsewhere (Mcnulty et al., 2020).

Any biological system for application in white biotechnology (the implementation of biotechnology in the industrial sphere) must rely on initial source(s) of carbon and nitrogen, as

**Abbreviations:** BES, bio-electrochemical system; BLSS, bio-regenerative life-support system; ISRU, *in-situ* resource utilization; ISS, International Space Station; MES, microbial electrosynthesis; PPB, purple phototrophic bacteria.

major constituents of biomass (besides oxygen). While critical for the economic viability of a process on Earth (Clomburg et al., 2017; Averesch and Krömer, 2018), this becomes even more imperative in a scenario where resources are strictly limited: organics are present in the Martian regolith (parts-per-billion) (Eigenbrode et al., 2018), most accessible, however, is inorganic carbon in the form of $CO_2$ and molecular nitrogen ($N_2$) in the atmosphere (Nangle et al., 2020a; Berliner et al., 2020). In addition, Space Technology has the requirements of being light, low maintenance/robust, and safe. Especially when implementing biomanufacturing for contingency mitigation, (i.e., on-demand production as means to reduce up-mass rather than merely a backup and subsidiary function), response time becomes critical. Further requirements strongly depend on the target product. For example, in case of pharmaceuticals needed to treat unexpected medical conditions but which cannot be stocked due to low shelf-life, a rapid response (production rate) can be vital. For other products, like materials, efficiency (production yield) may be the most important characteristic in order to deliver the required quantities within the spatial and resource constraints of the outpost. When using microbial fermentation as means to supplement a healthy diet and enhance palatability, quality (production titer/purity) is of foremost importance. This has implications for the process and choice of microbial cell factory, often on a case-by-case basis.

## From Autotrophy to Heterotrophy—Impact on Process Parameters and Complexity

While chemical carbon capture and utilization technologies are rapidly advancing (Ho et al., 2019), biology still offers the highest flexibility to fix inorganic carbon in order to make it available as a means of life-support and for further conversion into chemical products (Lee et al., 2019; Chen et al., 2020b; Löwe and Kremling, 2021). For a differentiation on process-level, it is most meaningful to distinguish autotrophic systems into phototrophs and chemotrophs. The former can be microalgae, cyanobacteria, or purple phototrophic bacteria. On Mars, the reduced intensity of natural sunlight (≈60% of Earth's maximum solar irradiance) has wide ranging implications for application of these organisms to bio-ISRU. To achieve high productivity, it would be imperative to concentrate photons by focusing natural lighting, or to rely on artificial lighting to achieve sufficient photon flux density at the desired wavelength (blue and red range) (Pattison et al., 2018). Both approaches have trade-offs in terms of equivalent system mass (Bugbee et al., 2020), and either further complicates the already complex design of photobioreactors (Johnson et al., 2018). If footprint and productivity constraints are relaxed (considering semi-open cultivation-systems like domed and pressurized ponds instead of photobioreactors), however, it is a straight forward way to generate initial biomass, which can serve as foundation for further biology (Billi et al., 2021; Verseux et al., 2021). Light-independent, lithoautotrophy can fix carbon by relying on chemically provided reducing power. On Mars, this could either directly be electricity through microbial electrosynthesis in bio-electrochemical systems (Moscoviz et al., 2016; Abel et al., 2020; Chen et al., 2020a), or indirectly by means of hydrogen or (organoautotrophically) formate, both of which can also be generated electrochemically (Kracke et al., 2020; Abel and Clark, 2021). Use of other electron donors like, e.g., sulphide, sulphur and iron (II) is theoretically possible, but technically less feasible (crustal materials from Mars are in principle able to support lithotrophic growth (Milojevic et al., 2021), but mining and purifying these in quantities that could support biotechnological processes is likely not viable). A special case is methylotrophy, where the C1-carbon also serves as source of reducing power (chemoorganotrophy). Several ways to obtain hydrogen and methane from $CO_2$ and $H_2O$ are being considered for ISRU on Mars (the primary purpose being propellant for the return journey),[1] as methane does exist on Mars (Webster et al., 2018) but concentrations are much too low (parts-per-billion) to be readily utilisable (unless minable reservoirs are found). Processes relying on gas or electricity as input generally require more sophisticated process setup. Further, these processes are often limited by mass transfer of the poorly soluble gases (Geinitz et al., 2020). Some also present an explosion hazard. Integrated setups can, however, avoid explosive gas mixtures and achieve significant production rates (Kracke et al., 2020; Sahoo et al., 2021). To completely avoid these problems, methane could be further converted to methanol (Zakaria and Kamarudin, 2016; Latimer et al., 2018)—albeit poor conversion efficiency is a century old unsolved problem. Not as troubled by the fundamental predicament of high activation energy combined with high activity of the CH-bonds in methane, biological methods could remedy this (Averesch and Kracke, 2018; Bjorck et al., 2018). Alternatively, methanol could be directly synthesized catalytically (Basile and Dalena, 2017)[2]. Another option and a form of chemoorganoautotrophy is utilization of formate not only as electron donor but also carbon-source (Abel and Clark, 2021). All these approaches, however, represents an additional process-step and therefore require auxiliary infrastructure. A biological equivalent to a liquid intermediate-substrate that can serve as unified feedstock for various heterotrophic microbial cell factories is acetate. As alternative to methanol carbonylation[3], numerous bioprocesses have emerged that sequestrate $CO_2$ into acetate and it is considered a next-generation biotech-feedstock (Kiefer et al., 2021). Processes with chemoorganoheterotrophic organisms using soluble substrates can achieve high productivities and allow for streamlined process design. The utilization of a defined feedstock also has the advantage of more consistent and predictable process performance, which

[1]Chemical synthesis of methane (I) via coupling of water-electrolysis or sulphur–iodine cycle and Sabatier reaction: "$2\ H_2O + CO_2 \rightarrow 2\ O_2 + CH_4$" (Clark, 1997; Ying et al., 2017) (II) as a by-product of solid oxide electrolysis followed by methanation: "$CO_2 + H_2O \rightarrow CO + H_2 + O_2$," "$CO + 3\ H_2 \rightarrow CH_4 + H_2O$" (Biswas et al., 2020; Hecht et al., 2021) or (III) via methanogenesis in an integrated BES: "$2\ H_2O + CO_2 \rightarrow 2\ O_2 + CH_4$" (Kracke et al., 2020).

[2]Chemical synthesis of methanol from synthesis gas or carbon dioxide and hydrogen, overall reaction: "$CO_2 + 3\ H_2 \rightarrow CH_3OH + H_2O$."

[3]Chemical synthesis of acetate from methanol and carbon monoxide: "$CH_3OH + CO \rightarrow CH_3COOH$."

can be problematic when using crude biomass like e.g., cyanobacterial lysate. A basic overview of the different options for carbon-flow is presented in **Figure 1** and **Table 1** gives an indication of the advantages and drawbacks on process-level, depending on the type of metabolism of the deployed microbe.

## From Substrates to Products—Impact of Synthetic Biology

Genetic tractability is highly desirable for an organism to serve as a versatile chassis for a microbial cell factory that can be tailored toward production of a variety of useful compounds. Hitherto genetic engineering has been adopted more widely with heterotrophic microbes, judging by their dominance in industrial-scale processes and employment as model organisms for genetic studies (Papagianni, 2012). This is likely owed to their more straight forward maintenance, facilitating domestication: their high metabolic rates accelerate cultivation and hence genetic interventions. The requirement for high productivity is likely also an economic "selection-pressure." In reverse, this may explain the scarcity of genetic tools for autotrophs. Further, especially phototrophs are often more complex organisms with genetic traits like polyploidy and cellular differentiation, (e.g. filamentous, heterocysts, etc.) and/or exotic defense systems (they cannot just "outgrow"), complicating the modification of their genotype (Riley and Guss, 2021). This applies in particular to cyanobacteria and algae (Hitchcock et al., 2020). Nevertheless, recent developments in Synthetic Biology, like CRISPR-technology, have narrowed that gap (Behler et al., 2018). Genetic engineering of chemolithotrophs can be more straightforward as many of them are mixotrophic and can also be cultivated on complex carbon-sources. A wide substrate-range also makes organisms attractive for bioproduction in an ISRU scenario, in particular when a mixed feedstock, derived from waste-biomass or cyanobacterial lysate, is intended to support growth and production (Verseux et al., 2016; Billi et al., 2021). The borders are blurry, as some autotrophs, like, e.g., *Cupriavidus necator*, can also function heterotrophically or may not even satisfy the strict definition of autotrophy (utilization of inorganic carbon-sources), like methanotrophs. Recently there has also been rapid development in engineering heterotrophic hosts toward artificial autotrophy and methylotrophy, based on natural as well as synthetic pathways for fixation of carbon (Gleizer et al., 2019; Liang et al., 2020; Liu et al., 2020; Satanowski et al., 2020; Wang et al., 2020; Scheffen et al., 2021). This may accelerate the adoption of C1-carbons as feedstocks in white biotechnology, which consequently could also warrant more profound applicability of microbial systems to ISRU and *vice versa*.

## Source of Nitrogen in Light of Carbon-Metabolism

Diazotrophy is ubiquitous among microbes and fixation of nitrogen exists in combination with all modes of carbon and energy metabolism considered in **Figure 1**. Biological nitrogen-fixation has, however, an excessive energy demand (16 ATP per $N_2$), which translates to low growth-rates and/or high demand for reduced carbon-sources, the latter inevitably accompanied by significant evolution of carbon dioxide. Heterotrophic fixation of nitrogen would thus be at the cost of net carbon-fixation, translating into the demand to significantly increase the dimensions of associated infrastructure and inputs. In addition, it creates a dependency on a supply chain. Therefore, fixation of nitrogen may be best served with a separate, dedicated chemical process, (e.g. Haber-Bosch) or conducted in combination with low-intensity bio-ISRU, like carbon-fixation by photoautotrophs, (e.g., cyanobacteria, **Figure 1** route (V)), if availability of power or space permits. The latter would yield a combined (C-N) feedstock, rich in amino sugars like glucosamine, a building block of the peptidoglycan in bacterial biomass (Averesch and Rothschild, 2019).

For production of bulk-compounds that do not contain nitrogen, (e.g. polyesters) N-input may not even be needed, if

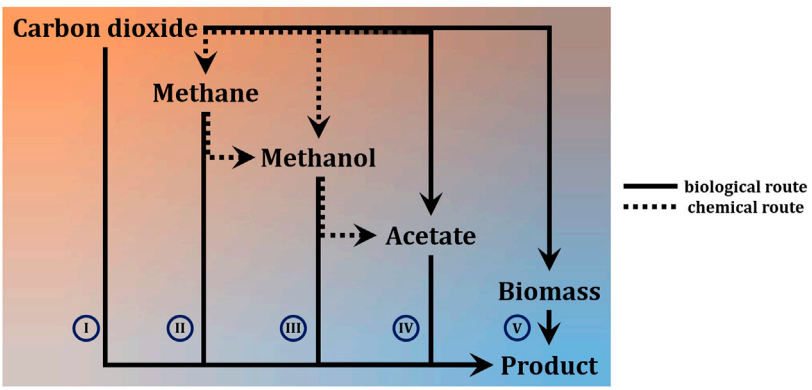


**FIGURE 1 |** High-level differentiation of approaches for conversion of carbon dioxide from Martian atmosphere into carbon-based products via microbial ISRU. Solid lines are bio-based processes, dashed arrows indicate involvement of catalytic (chemical) steps. The fading dashed line indicates a combined chemical-biological approach, rather than alternatives (I) single-step process from inorganic carbon directly to target compound (II)—(IV) processes that rely on significant auxiliary input, (e.g. chemical conversion of $CO_2$ to $CH_4$ or bioconversion of $CO_2$ to $CH_3COOH$) (V) two-step processes via intermediate with broad bio-availability.

**TABLE 1 |** Qualitative comparison of processes based on the type of metabolism of microbial cell factories to support an initial foothold on Mars in an ISRU scenario. Limited to production of basic compounds (small-, as well as macromolecules but excluding biomanufacturing i.e., physical structures) with pure cultures. Green shading represents an advantage, red shading a disadvantage, yellow a draw. Gray shading indicates non-decision due to great diversity of process factors preventing categorization. It should be noted that the different decision factors do not necessarily have the same weight.

| Metabolism of employed organism \ Process characteristics | | Substrate versatility | Product flexibility | Response time | System robustness | Scalability / potential for intensification | Footprint (space demand) | Required input and maintenance | Complexity and safety requirements |
|---|---|---|---|---|---|---|---|---|---|
| Autotrophic | light-dependent * | nominal | limited | high | case-dependent | scenario-dependent | modest – substantial | modest – substantial | minimal – substantial |
| | light-independent # | mediocre | case-dependent | medium | case-dependent | intricate / constricted | modest | modest | medium – substantial |
| Heterotrophic $ | | extensive | extensive | low | high | significant / considerable | minimal | substantial | minimal – medium |

[a]*strictly limited to phototrophy*
[b]*lithotrophy, including carboxidotrophy, cathodic electro-fermentation (Chen et al., 2020b), and, exceeding the strict definition of autotrophy, methanotrophy*
[c]*including mixotrophs that can also operate in other chemotrophic and organotrophic modes.*

an efficient circular recycling system is realized. The same is true for food—as long as nitrogen is effectively recovered from the organic waste (Beckinghausen et al., 2020), little supplemental input should be required.

## Considerations Beyond Metabolism

In addition to genetic tractability and high metabolic rate, it is desirable that the designated microbial cell factories are robust. Considering the increasing metabolic flexibility on product as well as substrate side, owed to advances in metabolic engineering, the general resilience of the organism may become the more decisive factor for selection of a microbial cell factory for application in Space Technology. Particularly relevant in order to compromise intermittency and unexpected down-time is for example the resistance of spore-forming species to vacuum and desiccation (Horneck, 1993), as well as tolerance of certain cyanobacteria toward high perchlorate concentrations (Billi et al., 2021), or the radiation resistance of certain bacteria (Krisko and Radman, 2013) and fungi (Shunk et al., 2020).

Nevertheless, despite all advances in Synthetic Biology, there may in some cases not be a choice: for complex products, like therapeutic proteins or products derived from plant-pathways, (e.g. natural products, where enzymes require post-translational modifications for activity) (Kayser and Averesch, 2015), almost exclusively only heterotrophs can be used, as these capabilities are limited to eukaryotic organisms, like yeasts (Nielsen, 2013).

Lastly, process intensification is critical in order to conserve precious resources, like water, shifting importance of substrate availability to auxiliary resources and sizing of infrastructure and secondary support processes. Because the same principles and constraints apply on Mars as on Earth, the synergistic integration of metabolic and process engineering can advance this (Woodley, 2017) to successfully integrate biomanufacturing into mission design. The extent to which this is possible of course strongly depends on the microbes and their unique metabolism, but also other factors, like preference to grow in biofilms, to produce by-products that reduce surface tension thus resulting in foaming, or resilience in the face of stressors like shear-force and sudden changes of environmental parameters.

## Organisms With High Potential for Bio-ISRU on Mars

For production of biomass, the diazotrophic and spore-forming cyanobacterium species *Anabaena*, which is tolerant to Mars-like growth-conditions (Verseux et al., 2021), holds great promise. *Synechocystis* sp. PCC 6803, is also able to grow in high-$CO_2$ atmospheres (Murukesan et al., 2016)—albeit not nitrogen-fixing, it is much more amenable to genetic engineering (Sebesta et al., 2019), can switch between auto- and heterotrophy and naturally produces bio-polyesters. It may therefore be attractive as a system for production of basic resources and/or food supplements.

Hydrogen oxidizing bacteria like *Cupriavidus necator* and/or acetogens can directly convert $CO_2$ to products. The mixotroph *C. necator* also utilizes substrates like sugars and organic acids (formate, acetate), and is an excellent producer of bio-polyesters (Raberg et al., 2018). An extensive library of proven genetic tools allows its engineering into microbial cell factories for a wide range of products, including high-performance polymers, as well as expansion of its substrate range (Heinrich et al., 2018; Nangle et al., 2020b). Anaerobic (homo)acetogens (like, e.g. *Sporomusa ovata*) have a lower metabolic rate and are less genetically tractable, but also have a remarkable substrate range in addition to their unique reductive acetyl-CoA pathway, the latter making them attractive to directly convert CO and/or $CO_2$ to organic acids and alcohols (Schuchmann and Müller, 2016; Su et al., 2020). Certain PPBs are even more flexible in terms of carbon- and energy-source (chemo-, auto-/heterotrophic), capable of anoxygenic photosynthesis, oxidize carbon monoxide, and are in addition also diazotrophic. Some species can be genetically engineered, (e.g. *Rhodospirillum rubrum* or *Rhodopseudomonas palustris*), and have previously been used for production of fuels and advanced bio-polyesters (Heinrich et al., 2016; Doud et al., 2017). Many of these species have also been studied in BES for potential to drive metabolism bio-electrochemically and perform MES (Nevin et al., 2011; Bose et al., 2014; Liu et al., 2016), to intensify process operations and increase efficacy.

Methanotrophs stand apart from most other C1-utilizing microbes and rarely accept other substrates (except for

methanol). Of interest for application in bio-ISRU are mostly aerobic species, in particular of type I and II as they exceed rates of anaerobic methanotrophs by orders of magnitude, albeit at reduced efficiency, (i.e. carbon-yield) (Averesch and Kracke, 2018). In particular alkalophilic and halotolerant *Methylomicrobium* species could simplify axenic cultivation by abolishing the need for strictly sterile conditions, as contamination is unlikely. These methanotrophs and also certain methylotrophs, like Methylobacterium extorquens and *Bacillus* species can to some extent also be genetically engineered (Kalyuzhnaya et al., 2015).

Many *Bacillus* species also grow on various sugars and organic acids. In particular *B. subtilis*, a very versatile model-organism, industrially employed for protein-production, as well as in metabolic engineering (Averesch and Rothschild, 2019), is strongly being considered and studied for application in Space Synthetic Biology, due to the resistance of its spores (Horneck, 1993). It also has a rapid metabolism and growth rate, exceeded by few other organisms. *Vibrio natrigens* is an emerging workhorse of white biotechnology, able to use a wide range of common organic molecules as its sole source of carbon and energy (Austin et al., 1978); its biggest advantage, however, being a doubling-time just under 10 min (Thoma and Blombach, 2021). It's exceptionally fast metabolism alone may warrant employment for specialty applications in Space Synthetic Biology, potentially in combination with adaptive evolution, which strongly depends on rate of replication (Dragosits and Mattanovich, 2013). Another organism highly attractive for vital special applications is the yeast *Pichia pastoris*, a methylotrophic eukaryote most valued for production of recombinant proteins (Schwarzhans et al., 2017).

Topics not closely discussed here are biohydrometallurgy and bioremediation for extraction and recovery of rare Earth elements, precious metals, as well as perchlorate removal, since their primary purpose is not directly related to the conversion of carbon. Nevertheless, they should at least be briefly mentioned as they are important strategies for bio-ISRU. Several microorganisms have been utilized in proof-of-concept experiments for biomining on Earth, as well as the proving-ground of the International Space Station (Johnson, 2014; Schippers et al., 2014; Jerez, 2017; Loudon et al., 2018; Cockell et al., 2020; Volger et al., 2020; Cockell et al., 2021). In particular, *Acidithiobacillus ferroxidans*, *Cupriavidus metallidurans*, *Shewanella oneidensis* and *Sphingomonas desiccabilis* are promising, most of these species performing chemolithotrophic leaching.

## CONCLUSION

There is likely no single "golden" solution that serves all purposes and covers all needs in any situation. Most likely several complementary systems will have to be advanced toward technological readiness, to cover all applications and for reasons of redundancy. A three-fold approach could make most sense: Diazotrophic cyanobacteria could be utilized to continuously produce biomass as a form of unspecific/universal feedstock from crude Martian resources. Versatile (mixotrophic) natively or artificially chemoautotropic strains could be deployed in parallel to rapidly produce a variety of bulk compounds from flexible and readily available C1-feedstocks. In order to provide for unusual and speciality products needed on short notice, a few highly specialized heterotrophs could be employed. A biofoundry, (i.e. automated infrastructure for engineering and analytics of biological systems) (Holowko et al., 2020), if such can be accommodated in the mission design, may even allow these be constructed at destination as the need arises, based on design-plans transmitted from Earth.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

NA conceived and composed the manuscript.

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# Towards a Biomanufactory on Mars

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A crewed mission to and from Mars may include an exciting array of enabling biotechnologies that leverage inherent mass, power, and volume advantages over traditional abiotic approaches. In this perspective, we articulate the scientific and engineering goals and constraints, along with example systems, that guide the design of a surface biomanufactory. Extending past arguments for exploiting stand-alone elements of biology, we argue for an integrated biomanufacturing plant replete with modules for microbial *in situ* resource utilization, production, and recycling of food, pharmaceuticals, and biomaterials required for sustaining future intrepid astronauts. We also discuss aspirational technology trends in each of these target areas in the context of human and robotic exploration missions.

**Keywords: space systems bioengineering, human exploration, *in situ* resource utilization, life support systems, biomanufacturing**

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## 1 INTRODUCTION

Extended human stay in space or upon the surface of alien worlds like Mars introduces new mission elements that require innovation (Musk, 2017); among these are the biotechnological elements (Menezes et al., 2015a; Menezes et al., 2015b; Nangle et al., 2020a) that support human health, reduce costs, and increase operational resilience. The potential for a Mars mission in the early 2030s (Drake et al., 2010) underscores the urgency of developing a roadmap for advantageous space biotechnologies.

A major limiting factor of space exploration is the cost of launching goods into space (Wertz and Larson, 1996). The replicative capacity of biology reduces mission launch cost by producing goods

**FIGURE 1 |** Artist's rendering of a crewed Martian biomanufactory powered by photovoltaics, fed *via* atmospheric ISRU, and capable of food and pharmaceutical synthesis (FPS), *in situ* manufacturing (ISM), and biological loop closure (LC). Artwork by Davian Ho.

on-demand using *in situ* resources (Rapp, 2013), recycling waste products (Hendrickx et al., 2006), and interacting with other biological processes for stable ecosystem function (Gòdia et al., 2002). This trait not only lowers initial launch costs, but also minimizes the quantity and frequency of resupply missions that would otherwise be required due to limited food and pharmaceutical shelf-life (Du et al., 2011) on deep space missions. Biological systems also provide robust utility *via* genetic engineering, which can provide solutions to unforeseen problems and lower inherent risk (Menezes et al., 2015a; Berliner et al., 2019). For example, organisms can be engineered on-site to produce a pharmaceutical to treat an unexpected medical condition when rapid supply from Earth would be infeasible (McNulty et al., 2021). A so-called "biomanufactory" for deep space missions (Menezes, 2018) based on *in situ* resource utilization and composed of integrated biologically-driven subunits capable of producing food, pharmaceuticals, and biomaterials (**Figure 1**) will greatly reduce launch and resupply cost, and is therefore critical to the future of human-based space exploration (Menezes et al., 2015a; Nangle et al., 2020a).

## 2 FEASIBILITY, NEEDS, AND MISSION ARCHITECTURE

The standard specifications for Mars exploration from 2009 (Drake et al., 2010) to 2019 (Linck et al., 2019) are not biomanufacturing-driven (Berliner et al., 2019) due to the novelty of space bioengineering. Here, we outline biotechnological support to produce food, medicine, and specialized construction materials on a long-term mission with six crew-members and surface operations for ~500 sols (a Martian sol is ~40 min longer than an Earth day) flanked by two interplanetary transits of ~210 days (Miele and Wang, 1999). We further assume predeployment cargo that includes *in situ* resource utilization (ISRU) hardware for Mars-ascent propellant production (Sanders, 2018), which is to be launched from Earth to a mission site. Additional supplies such as habitat assemblies (Hoffman and Kaplan, 1997; Cohen, 2015), photovoltaics (Landis, 2000; Landis et al., 2004), experimental equipment, and other non-living consumables (Benton, 2008) will be included.

The proposed biomanufactory would augment processes for air generation and water and waste recycling and purification—typically associated with Environmental Control and Life Support Systems (ECLSS) (Gòdia et al., 2002; Hendrickx et al., 2006)—since its needs overlap but are broader, and drive a wider development of an array of ISRU, *in situ* manufacturing (ISM), food and pharmaceutical synthesis (FPS), and loop closure (LC) technologies (**Figure 2**).

Food, medicine, and gas exchange to sustain humans imposes important ECLSS feasibility constraints (Yeh et al., 2005; Yeh et al., 2009; Weber and Schnaitmann, 2016). These arise from a crewmember (CM) physiological profile, with an upper-bound metabolic rate of ~11–13 MJ/CM-sol that can be satisfied through prepackaged meals and potable water intake of 2.5 kg/CM-sol (Liskowsky and Seitz, 2010; Anderson et al., 2018). Sustaining a CM also entails providing oxygen at 0.8 kg/CM-sol and recycling the 1.04 kg/CM-sol of $CO_2$, 0.11 kg of fecal and urine solid, and 3.6 kg of water waste within a habitat kept at ~294 K and ~70 kPa. Proposed short duration missions lean heavily on chemical processes for life support with consumables sent from Earth (Drake et al., 2010). As the length of a mission increases, demands on the quantity and quality of consumables increase dramatically. As missions become more complex with longer surface operations, biotechnology offers methods for consumable production in

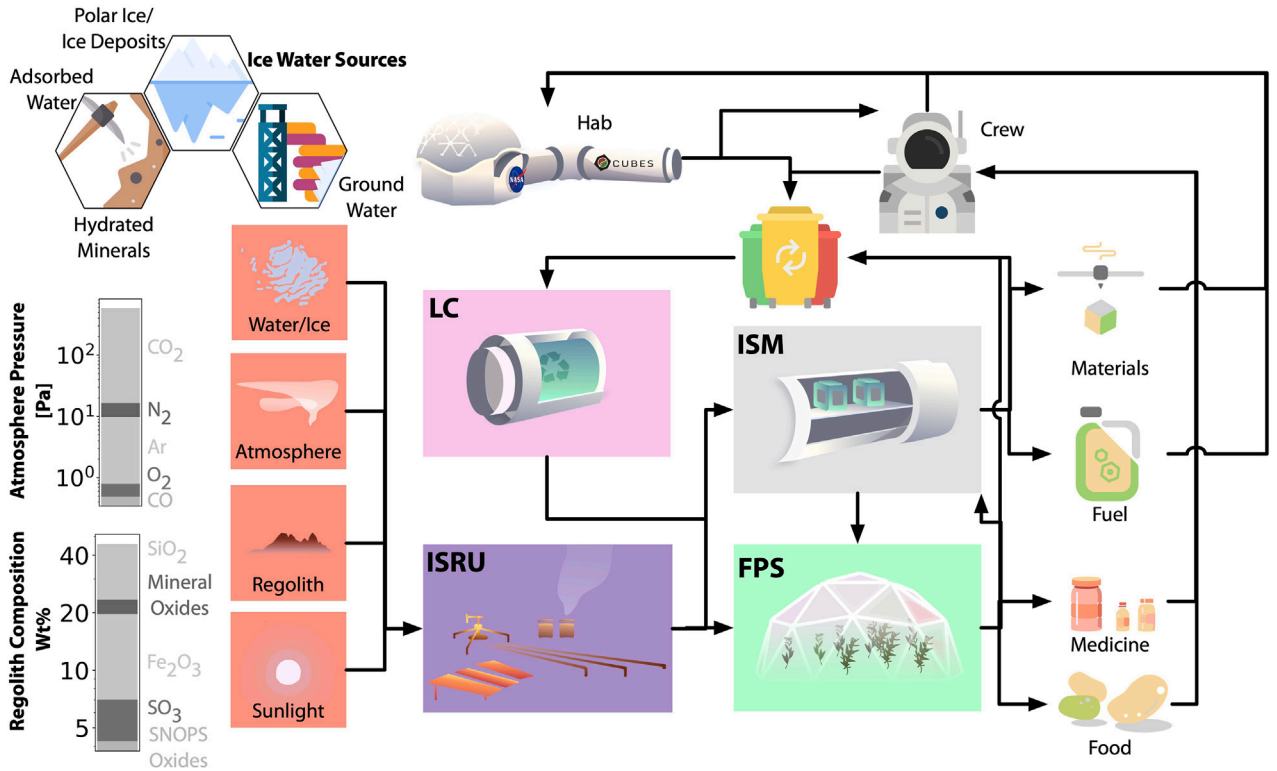


**FIGURE 2 |** Proposed surface operations are drawn from inventories of *in situ* resources (red) such as ice, atmosphere, regolith, and sunlight. Atmospheric feedstocks of carbon and nitrogen are biologically fixed *via* the ISRU (*in situ* resource utilization) biomanufactory components (including abiotic processes, purple), providing the source of biopolymer manufacturing *via* the ISM (*in situ* manufacturing) component (grey) and food *via* the FPS (food and pharmaceutical synthesis) component (green), which are used for astronaut consumption and utilization during mission operations. Waste from each of these elements is collected and fed into the LC (loop closure) element (pink) to maximize efficiency and reduce the cost of supply logistics from Earth.

the form of edible crops and waste recycling through microbial digestion (Hendrickx et al., 2006). Advancements in biomanufacturing for deep space exploration will ensure a transition from short-term missions such as those on the ISS that are reliant on single-use-single-supply resources to long-term missions that are sustainable.

## 2.1 Biomanufactory Systems Engineering

Efficiency gains in a biomanufactory come in part from the interconnection (**Figure 2**) and modularity of various unit operations (**Figures 3–6**) (Crowell et al., 2018). However, different mission stage requirements for assembly, operation, timing, and productivity can lead to different optimal biomanufactory system configurations. A challenge therefore exists for technology choice and process optimization to address the high flexibility, scalability, and infrastructure minimization needs of an integrated biomanufactory. Current frameworks for biomanufacturing optimization do not dwell on these aspects. A series of new innovations in modeling processes and developing performance metrics specific to ECLSS biotechnology is called for, innovations that can suitably capture risk, modularity, autonomy, and recyclability. Concomitant invention in engineering infrastructure will also be required.

# 3 FOOD AND PHARMACEUTICAL SYNTHESIS

An estimated ~ 10,000 kg of food mass is required for a crew of six on a ~ 900 days mission to Mars (Menezes et al., 2015a). Food production for longer missions reduces this mission overhead and increases food store flexibility, bolsters astronaut mental health, revitalizes air, and recycles wastewater through transpiration and condensation capture (Vergari et al., 2010; Kyriacou et al., 2017). Pharmaceutical life support must address challenges of accelerated instability [ ~ 75% of solid formulation pharmaceuticals are projected to expire mid-mission at 880 days (Menezes et al., 2015a)], the need for a wide range of pharmaceuticals to mitigate a myriad of low probability medical risks, and the mismatch between the long re-supply times to Mars and often short therapeutic time windows for pharmaceutical treatment. Pharmaceutical production for longer missions can mitigate the impact of this anticipated instability and accelerate response time to unanticipated medical threats. In early missions, FPS may boost crew morale and supplement labile nutrients (Khodadad et al., 2020). As mission scale increases, FPS may meet important food and pharmaceutical needs (Cannon and Britt, 2019). A biomanufactory that focuses on oxygenic photoautotrophs, namely plants, algae and cyanobacteria, enhances simplicity, versatility, and synergy with intersecting life

support systems (Gòdia et al., 2002; Wheeler, 2017) and a Martian atmosphere has been shown to support such biological systems (Verseux et al., 2021). While plant-based food has been the main staple considered for extended missions (Drake et al., 2010; Anderson et al., 2018; Cannon and Britt, 2019), the advent of cultured and 3D printed meat-like products from animal, plant and fungal cells may ultimately provide a scalable and efficient alternative to cropping systems (Cain, 2005; Pandurangan and Kim, 2015; Hindupur et al., 2019).

FPS organisms for Mars use must be optimized for growth and yields of biomass, nutrient, and pharmaceutical accumulation. Providing adequate and appropriate lighting will be a challenge of photoautotrophic-centric FPS on Mars (Massa et al., 2007; Kusuma et al., 2020). Developing plants and algae with reduced chloroplast light-harvesting antenna size has the potential to improve whole-organism quantum yield by increasing light penetration deeper into the canopy, which will reduce the fraction of light that is wastefully dissipated as heat and allow higher planting density (Friedland et al., 2019). Developing FPS organisms for pharmaceutical production is especially complicated, given the breadth of production modalities and pharmaceutical need (e.g., the time window of intervention response, and molecule class) (McNulty et al., 2021). Limited-resource pharmaceutical purification is also a critically important consideration that has not been rigorously addressed. Promising biologically-derived purification technologies (Werner et al., 2006; Mahmoodi et al., 2019) should be considered for processing drugs that require very high purity (e.g., injectables).

Developing FPS growth systems for Mars requires synergistic biotic and abiotic optimization, as indicated by lighting systems and plant microbiomes. For lighting, consider that recent advancements in LED efficiency now make LEDs optimal for crop growth in extraterrestrial systems (Hardy et al., 2020). The ideal spectra from tunable LEDs will likely be one with a high fraction of red photons for maximum production efficiency, but increasing the fraction of shorter wavelength blue photons could increase crop quality (Johkan et al., 2010; Kusuma et al., 2021). Similarly, higher photon intensities increase production rates but decrease production efficiency. Understanding the associated volume and power/cooling requirement tradeoffs will be paramount to increasing overall system efficiency.

For microbiomes, consider that ISS open-air plant cultivation results in rapid and widespread colonization by atypicaly low-diversity bacterial and fungal microbiomes that often lead to plant disease and decreased plant productivity (Khodadad et al., 2020). Synthetic microbial communities (SynComs, **Figure 3A**) may provide stability and resilience to the plant microbiome and simultaneously improve the phenotype of host plants *via* the genes carried by community members. A subset of naturally occurring microbes are well known to promote growth of their plant hosts (Hassani et al., 2018), accelerate wastewater remediation and nutrient recycling (Nielsen, 2017), and shield plant hosts from both abiotic and biotic stresses (Caddell et al., 2019), including opportunistic pathogens (Bishop et al., 1997; Ryba-White et al., 2001; Leach et al., 2007). While SynCom design is challenging, the inclusion of SynComs in life support systems represents a critical risk-mitigation strategy to protect vital food and pharma resources. The application of SynComs to Mars-based agriculture motivates additional discussions in tradeoffs between customized hydroponics versus regolith-based farming, both of which will require distinct technology platforms and applied SynComs.

## 3.1 FPS Integration Into the Biomanufactory

Our biomanufactory FPS module has three submodules: crops, pharmaceuticals, and functional foods (**Figure 3**). The inputs to all three submodules (**Figure 3**) are nearly identical in needing $H_2O$ as an electron donor, $CO_2$ as a carbon source, and light as an energy source, with the required nitrogen source being organism-dependent (e.g., *Arthrospira platensis* requires nitrate). $H_2O$, $CO_2$, and light are directly available from the Martian environment. Fixed nitrogen comes from the biomanufactory ISRU module. The submodules

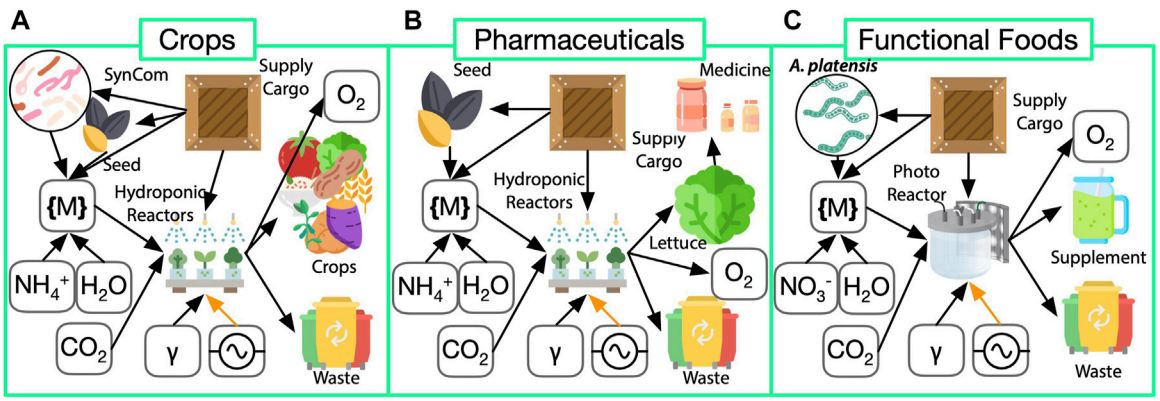


**FIGURE 3 |** FPS (green in **Figure 2**) system breakdown for biomanufactory elements of **(A)** crops, **(B)** a biopharmaceutical, and **(C)** functional food production. In all cases, growth reactors require power (electrical current symbol ⏦) and light (γ). **(A)** Crop biomass and oxygen gas ($O_2$) are produced from hydroponically grown plants using seeds and the set of media elements ({M}) derived from supply cargo. The reactor is also supplied with an ammonium ($NH_4^+$) nitrogen source and $CO_2$ carbon source from ISRU processes. **(B)** In a similar fashion, medicine can be produced from genetically modified crops such as lettuce **(C)** Functional foods such as nutritional supplements are produced *via* autotrophic growth of *Arthrospira platensis*. In all cases, biomass is produced, collected, and inedible biomass is distributed to the LC module for recycling. Orange lines indicate additional power supply to the system.

output $O_2$, biomass, and waste products. However, the crop submodule (**Figure 3A**) chiefly outputs edible biomass for bulk food consumption, the pharmaceutical submodule (**Figure 3B**) synthesizes medicines, and the functional foods submodule (**Figure 3C**) augments the nutritional requirements of the crop submodule with microbially-produced vitamins (e.g., vitamin $B_{12}$). These outputs will be consumed directly by crew-members, with waste products entering the LC module for recycling.

All submodules will have increased risk, modularity, and recyclability relative to traditional technological approaches. Increased risk is associated with biomass loss due to lower-than-expected yields, contamination, and possible growth system failure. Increased modularity over shipping known pharmaceuticals to Mars derives from the programmability of biology, and the rapid response time of molecular pharming in crops for as-needed production of biologics. Increased recyclability stems from the lack of packaging required for shipping food and pharmaceuticals from Earth, as well as the ability to recycle plant waste using anaerobic digestion.

At a systems integration level, FPS organism care will increase the crew time requirements for setup, maintenance, and harvesting compared to advance food and pharmaceutical shipments. However, overall cost impacts require careful scrutiny: crop growth likely saves on shipping costs, whereas pharmaceutical or functional food production on Mars may increase costs relative to shipping drugs and vitamins from Earth.

# 4 *IN SITU* MATERIALS MANUFACTURING

Maintaining FPS systems requires cultivation vessels/chambers, support structures, plumbing, and tools. Such physical objects represent elements of an inventory that, for short missions, will likely be a combination of predeployment cargo and supplies from the crewed transit vehicle (Drake et al., 2010). As mission duration increases, so does the quantity, composition diversity, and construction complexity of these objects. The extent of ISM for initial exploration missions is not currently specified (Drake et al., 2010). Nevertheless, recent developments (Owens et al., 2015; Moses and Bushnell, 2016; Owens and De Weck, 2016) imply that ISM will be critical for the generation of commodities and consumables made of plastics (Carranza et al., 2006), metals (Everton et al., 2016), composite-ceramics (Karl et al., 2020), and electronics (Werkheiser, 2015) as mission objects, with uses ranging from functional tools (Grenouilleau et al., 2000) to physical components of the life-supporting habitat (Owens et al., 2015).

Plastics will make up the majority of high-turnover items with sizes on the order of small parts to bench-top equipment, and will also account for contingencies (Prater et al., 2016). Biotechnology—specifically synthetic biology—in combination with additive manufacturing (Rothschild, 2016) has been proposed an a critical element towards the establishment of offworld manufacturing (Snyder et al., 2019) and can produce such polymeric constructs from basic feedstocks in a more compact and integrated way than chemical synthesis, because microbial bioreactors operate much closer to ambient conditions than chemical processes (Malik et al., 2015). The versatility of microbial metabolisms allows direct use of $CO_2$ from Mars' atmosphere, methane ($CH_4$) from abiotic Sabatier processes (Hintze et al., 2018), and/or biologically synthesized $C_2$ compounds such as acetate, as well as waste biomass.

A class of bioplastics that can be directly obtained from microorganisms (Naik et al., 2008) are polyhydroxyalkanoates (PHAs). While the dominant natural PHA is poly (3-hydroxybutyrate) (PHB), microbes can produce various co-polymers with an expansive range of physical properties (Myung et al., 2017). This is commonly accomplished through co-feeding with fatty acids or hydroxyalkanoates, which get incorporated in the polyester. These co-substrates can be sourced from additional process inputs or generated *in situ*. For example the PHA poly-lactic acid (PLA) can be produced by engineered *Escherichia coli* (Jung et al., 2010), albeit to much lower weight percent than is observed in organisms producing PHAs naturally. PHA composition can be modulated in other organisms (Rehm, 2010). The rapid development of synthetic biology tools for non-model organisms opens an opportunity to tune PHA production in high PHB producers and derive a range of high-performance materials.

Before downstream processing (melting, extrusion/molding), the intracellularly accumulating bioplastics need to be purified. The required degree of purity determines the approach and required secondary resources. Fused filament fabrication 3D-printing, which works well in microgravity (Prater et al., 2016; Prater et al., 2018), has been applied for PLA processing and may be extendable to other bio-polyesters. Ideally, additive manufacturing will be integrated in-line with bioplastics production and filament extrusion.

## 4.1 ISM Integration Into the Biomanufactory

**Figure 4** depicts the use of three organism candidates from genera *Cupriavidus*, *Methylocystis*, and *Halomonas* that can meet bioplastic production. This requires a different set of parameters to optimize their deployment, which strongly affects reactor design and operation. These microbes are capable of using a variety of carbon sources for bioplastic production, each with a trade-off. For example, leveraging $C_2$ feedstocks as the primary source will allow versatility in the microbe selection, but may be less efficient and autonomous than engineering a single organism like *Cupriavidus necator* to use $CO_2$ directly from the atmosphere. Alternatively, in the event that $CH_4$ is produced abiotically for ascent propellant (Musk, 2017), a marginal fraction of total $CH_4$ will be sufficient for producing enough plastic without additional hardware costs associated with ISRU $C_2$ production. Relying on *Halomonas* spp. in combination with acetate as substrate may allow very rapid production of the required bioplastic, but substrate availability constraints are higher than for $CH_4$ or $CO_2/H_2$. A terminal electron acceptor is required in all cases, which will almost certainly be $O_2$. Supplying $O_2$ safely without risking explosive gas mixtures, or wasting the precious resource, is again a question of reactor design and operation. Certain purple non-sulfur alphaproteobacteria (e.g., *Rhodospirillum rubrum* (Brandl et al., 1989; Heinrich et al., 2016) and

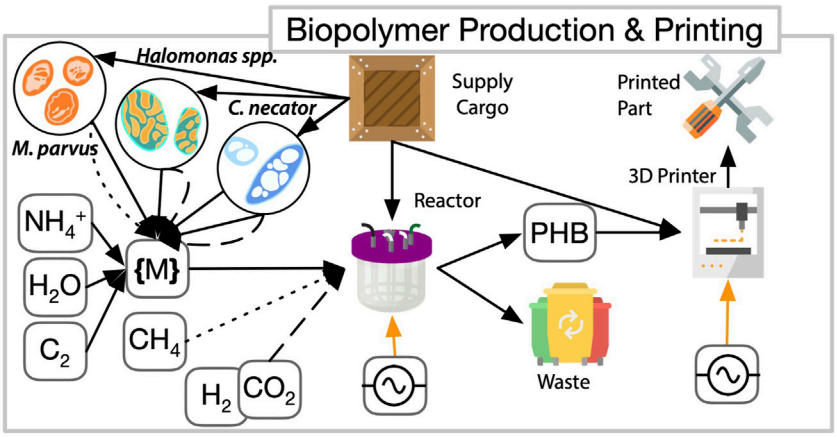


**FIGURE 4 |** ISM (grey in **Figure 2**) systems breakdown for biomanufactory elements of biopolymer production and 3D-printing. 3D printed parts are fabricated from bioproduced plastics. Biopolyesters such as PHB, along with corresponding waste products, are formed in cargo-supplied reactors with the aid of microorganisms. A variety of available carbon feedstocks can serve as substrates for aerobic auto-, hetero-, or mixotrophic microorganisms such as *Cupriavidus necator*, *Methylocystis parvus* and *Halomonas* spp. All three microbes are capable of using $C_2$ feedstocks (like acetate, indicated by solid line), while *C. necator* and *Methylocystis* can also use $C_1$ feedstocks. The former utilizes a combination of $CO_2$ and $H_2$ (large dotted line), while only *M. parvus* can leverage $CH_4$ (small dotted line).

*Rhodopseudomonas palustris* (Doud et al., 2017; Touloupakis et al., 2021)) also feature remarkable substrate flexibility and can produce PHAs (Averesch, 2021).

Bioplastic recovery and purification is a major challenge. To release the intracellular compound, an osmolysis process (Rathi et al., 2013) may be employed with the halophile (Tan et al., 2011; Chen et al., 2017). However, the transfer of cells into purified water and separation of the polyesters from the cell debris, potentially through several washing steps, may require substantial amounts of water. An alternative and/or complement to the common process for extraction of PHAs with halogenated organic solvents, is to use acetate or methanol as solvents (Anbukarasu et al., 2015; Aramvash et al., 2018). This is applicable independent of the organism and the inputs can be provided from other biomanufactory modules.

The high crystallinity of pure PHB makes it brittle and causes it to have a narrow melting range, resulting in warp during extrusion and 3D-printing. Such behavior places operational constraints on processing and hampers applications to precision manufacturing (Marchessault and Yu, 2005). Workarounds may be through additives, biocomposite synthesis, and copolymerization. However, this ultimately depends on what biology can provide (Müller and Seebach, 1993). There is a need to advance space bio-platforms to produce more diverse PHAs through synthetic biology.

ISM of biomaterials can reduce the mission cost, increase modularity, and improve system recyclability compared to abiotic approaches. In an abiotic approach, plastics will be included in the payload, thereby penalizing up-mass at launch. As with elements of FPS and ISRU, ISM increases flexibility and can create contingencies during surface operations, therefore reducing mission risk. The high modularity of independent plastic production, filament formation, and 3D-printing allows for a versatile process, at the cost of greater resources required for systems operations. Overall, this maximizes resource use and recyclability, by utilizing mission waste streams and byproducts for circular resource management.

## 5 *IN SITU* RESOURCE UTILIZATION

Biomanufacturing on Mars can be supported by flexible biocatalysts that extract resources from the environment and transform them into the complex products needed to sustain human life. The Martian atmosphere contains $CO_2$ and $N_2$ (Menezes et al., 2015a). Water and electrolytically produced $O_2$ and $H_2$ are critical to mission elements for any Mars mission. It is very likely that the expensive and energy-intensive Sabatier plants (Clark and Clark, 1997; Meier et al., 2017; Hintze et al., 2018) for $CH_4$ production will be available per Design Reference Architecture (DRA 5.0) (Drake et al., 2010). While a Haber-Bosch plant could be set up for ammonia production, this is neither part of the current DRA (Drake et al., 2010) nor exceptionally efficient. Thus, for a biomanufactory, we must have carbon fixation reactors to fix $CO_2$ into feedstocks for non-methanotrophs, and have nitrogen fixation reactors to fix $N_2$ to fulfill nitrogen requirements for non-diazotrophs. Trace elements and small-usage compounds can be transported from Earth, or in some cases extracted from the Martian regolith. In the case where power is provided from photocollection or photovoltaics, light energy will vary with location and season, and may be critical to power our bioreactors.

Although photosynthetic organisms are attractive for FPS, a higher demand for carbon-rich feedstocks and other chemicals necessitates a more rapid and efficient $CO_2$ fixation strategy. Physicochemical conversion is inefficient due to high temperature and pressure requirements. Microbial electrosynthesis (MES), whereby reducing power is passed

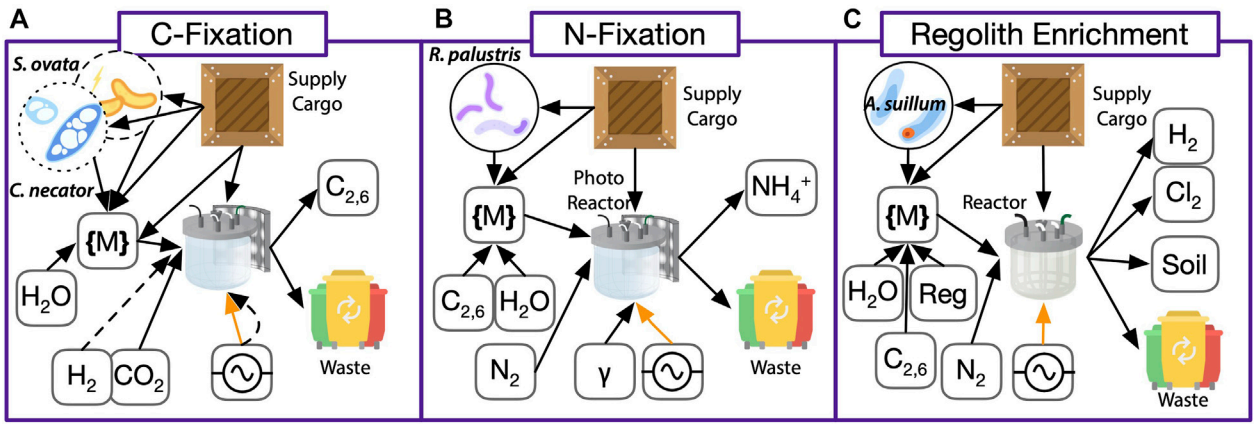


**FIGURE 5 |** ISRU (purple in **Figure 2**) system breakdown of biomanufactory elements. **(A)** Carbon fixation with the autotrophic bacteria *Sporomusa ovata* or *Cupriavidus necator* through electrosynthesis or lithoautotrophic fixation of $C_1$-carbon (cathodes or $H_2$ as the electron donor). **(B)** Microbial nitrogen fixation with diazotrophic bacteria like *Rhodopseudomonas palustris* growing photoheterotrophically **(C)** Regolith (Reg) enrichment using the perchlorate-reducing microbe *Azospira suillum*. Black lines represent material and energy flows related to biological consumption and production. Orange lines indicate additional power supply to the system.

from abiotic electrodes to microbes to power $CO_2$ reduction, can offer rapid and efficient $CO_2$ fixation at ambient temperature and pressure (Abel and Clark, 2020). MES can produce a variety of chemicals including acetate (Liu et al., 2015), isobutanol (Li et al., 2012), PHB (Liu et al., 2016), and sucrose (Nangle et al., 2020b), and therefore represents a flexible and highly promising ISRU platform technology (Abel et al., 2020).

Biological $N_2$-fixation offers power- and resource-efficient ammonium production. Although photoautotrophic $N_2$ fixation with, for example, purple non-sulfur bacteria, is possible, slow growth rates due to the high energetic demand of nitrogenase limit throughput (Doloman and Seefeldt, 2020). Therefore, heterotrophic production with similar bacteria using acetate or sucrose as a feedstock sourced from electromicrobial $CO_2$-fixation represents the most promising production scheme, and additionally benefits from a high degree of process redundancy with heterotrophic bioplastic production.

Regolith provides a significant inventory for trace elements (Fe, K, P, S, etc.) and, when mixed with the substantial cellulosic biomass waste from FPS processes, can facilitate recycling organic matter into fertilizer to support crop growth. However, regolith use is hampered by widespread perchlorate (Catling et al., 2010; Cull et al., 2010; Navarro-González et al., 2010), indicating that decontamination is necessary prior to enrichment or use. Dechlorination can be achieved *via* biological perchlorate reduction using one of many dissimilatory perchlorate reducing organisms (Byrne-Bailey and Coates, 2012; Davila et al., 2013; Wetmore et al., 2015; Bywaters and Quinn, 2016). Efforts to reduce perchlorate biologically have been explored independently and in combination with a more wholistic biological platform (Llorente et al., 2018). Such efforts to integrate synthetic biology into human exploration missions suggest that a number of approaches should be considered within a surface biomanufactory.

## 5.1 ISRU Integration Into the Biomanufactory

A biomanufactory must be able to produce and utilize feedstocks along three axes as depicted in **Figure 5**: $CO_2$-fixation to supply a carbon and energy source for downstream heterotrophic organisms or to generate commodity chemicals directly, $N_2$-fixation to provide ammonium and nitrate for plants and non-diazotrophic microbes, and regolith decontamination and enrichment for soil-based agriculture and trace nutrient provision. ISRU inputs are submodule and organism dependent, with all submodules requiring water and power. For the carbon fixation submodule (**Figure 5A**), $CO_2$ is supplied as the carbon source, and electrons are supplied as $H_2$ or directly *via* a cathode. Our proposed biocatalysts are the lithoautotrophic *Cupriavidus necator* for longer-chain carbon production [e.g., sucrose (Nangle et al., 2020b)] and the acetogen *Sporomusa ovata* for acetate production. *C. necator* is a promising chassis for metabolic engineering and scale-up (Nangle et al., 2020b), with *S. ovata* having one of the highest current consumptions for acetogens characterized to date (Logan et al., 2019). The fixed-carbon outputs of this submodule are then used as inputs for the other ISRU submodules (**Figures 5B,C**) in addition to the ISM module (**Figure 2**). The inputs to the nitrogen fixation submodule (**Figure 5B**) include fixed carbon feedstocks, $N_2$, and light. The diazotrophic purple-non sulfur bacterium *Rhodopseudomonas palustris* is the proposed biocatalyst, as this bacterium is capable of anaerobic, light-driven $N_2$ fixation utilizing acetate as the carbon source, and has a robust genetic system allowing for rapid manipulation (Doloman and Seefeldt, 2020; Abel et al., 2020). The output product is fixed nitrogen in

the form of ammonium, which is used as a feedstock for the carbon-fixation submodule of ISRU along with the FPS and ISM modules. The inputs for the regolith enrichment submodule (**Figure 5C**) include regolith, fixed carbon feedstocks, and $N_2$. *Azospira suillum* is a possible biocatalyst of choice due to its dual use in perchlorate reduction and nitrogen fixation (Bywaters and Quinn, 2016). Regolith enrichment outputs include soil for the FPS module (in the event that solid support-based agriculture is selected instead of hydroponics), $H_2$ that can be fed back into the carbon fixation submodule and the ISM module, chlorine gas from perchlorate reduction, and waste products.

Replicate ISRU bioreactors operating continuously in parallel with back-up operations lines can ensure a constant supply of the chemical feedstocks, commodity chemicals, and biomass for downstream processing in ISM and FPS operations. Integration of ISRU technologies with other biomanufactory elements, especially anaerobic digestion reactors, may enable (near-)complete recyclability of raw materials, minimizing resource consumption and impact on the Martian environment (MacElroy and Wang, 1989; Pogue et al., 2002).

## 6 LOOP CLOSURE AND RECYCLING

Waste stream processing to recycle essential elements will reduce material requirements in the biomanufactory. Typical feedstocks include inedible crop mass, human excreta, and other mission wastes. Space mission waste management traditionally focuses on water recovery and efficient waste storage through warm air drying and lyophilization (Yeh et al., 2005; Anderson et al., 2018). Mission trash can be incinerated to produce $CO_2$, CO, and $H_2O$ (Hintze et al., 2013). Pyrolysis, another abiotic technique, yields CO and $H_2$ alongside $CH_4$ (Serio et al., 2008). The Sabatier process converts $CO_2$ and CO to $CH_4$ by reacting with $H_2$. An alternate thermal degradation reactor (Caraccio et al., 2013), operating under varying conditions that promote pyrolysis, gasification, or incineration, yields various liquid and gaseous products. The fact remains however, that abiotic carbon recycling is inefficient with respect to desired product $CH_4$, and is highly energy-intensive.

Microbes that recover resources from mission wastes are a viable option to facilitate loop closure. Aerobic composting produces $CO_2$ and a nutrient-rich extract for plant and microbial growth (Ramirez-Perez et al., 2007; Ramirez-Perez et al., 2008). However, this process requires $O_2$, which will likely be a limited resource. Hence, anaerobic digestion, a multi-step microbial process that can produce a suite of end-products at lower temperature than abiotic techniques ($\sim$35–55°C compared to $\sim$500–600°C, an order of magnitude difference), is the most promising approach for a Mars biomanufactory (Meegoda et al., 2018; Strazzera et al., 2018) to recycle streams for the ISM and FPS processes. Digestion products $CH_4$ and volatile fatty acids (VFA, such as acetic acid) can be substrates for polymer-producing microbes (Myung et al., 2015; Chen et al., 2018). Digestate, with nutrients of N, P, and K, can be ideal for plant and microbial growth (Möller and Müller, 2012), as shown in **Figure 6**. Additionally, a $CH_4$ and $CO_2$ mixture serves as a biogas energy source, and byproduct $H_2$ is also an energy source (Schievano et al., 2012; Khan et al., 2018).

Because additional infrastructure and utilities are necessary for waste processing, the extent of loop closure that is obtainable from a treatment route must be analyzed to balance yield with its infrastructure and logistic costs. Anaerobic digestion performance is a function of the composition and pretreatment of input waste streams (crop residuals, feces, urine, end-of-life bioproducts), as well as reaction strategies like batch or continuous, number of stages, and operation conditions such as organic loading rate, solids retention time, operating temperature, pH, toxic levels of inhibitors ($H_2S$, $NH_3$, salt) and trace metal requirements (Rittmann and McCarty, 2001; Schievano et al., 2012; Aramrueang et al., 2016; Liu et al., 2018; Meegoda et al., 2018; Strazzera et al., 2018). Many of these process parameters exhibit trade-offs between product yield and necessary resources. For example, a higher waste loading reduces water demand, albeit at the cost of process efficiency. There is also a potential for multiple co-benefits of anaerobic digestion within the biomanufactory. Anaerobic biodegradation of nitrogen-rich protein feedstocks, for example, releases free $NH_3$ by ammonification. While $NH_3$ is toxic to anaerobic digestion and must thus be managed (Rittmann and McCarty, 2001), it reacts with carbonic acid to produce bicarbonate buffer and ammonium, decreasing $CO_2$ levels in the biogas and buffering against low pH. The resulting digestate ammonium can serve as a fertilizer for crops and nutrient for microbial cultures.

### 6.1 LC Integration Into the Biomanufactory

FPS and ISM waste as well as human waste are inputs for an anaerobic digester, with output recycled products supplementing the ISRU unit. Depending on the configuration of the waste streams from the biomanufactory and other mission elements, the operating conditions of the process can be varied to alter the efficiency and output profile. Open problems include the design and optimization of waste processing configurations and operations, and the identification

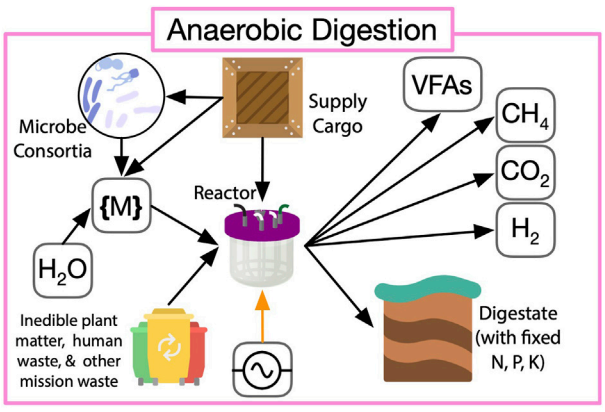


**FIGURE 6 |** LC-based (pink in **Figure 2**) anaerobic digestion of mission waste such as inedible plant matter, microbial biomass, human, and other wastes produce methane, volatile fatty acids (VFAs), and digestate rich with key elemental nutrients (N, P, K), thereby supplementing ISRU operation.

of optimal end-product distributions based on a loop closure metric (Benvenuti et al., 2020) against mission production profiles, mission horizon, biomanufacturing feedstock needs, and the possible use of leftover products by other mission elements beyond the biomanufactory. A comparison with abiotic waste treatment strategies (incineration and pyrolysis) is also needed, checking power demand, risk, autonomy, and modularity benefits.

# 7 DISCUSSION AND ROADMAP

Biomanufactory development must be done in concert with planned NASA missions that can provide critical opportunities to test subsystems and models necessary to evaluate efficacy and technology readiness levels (TRLs) (Mankins, 1995). **Figure 7** is our attempt to place critical elements of a biomanufactory roadmap into this context. We label critical mission stages using Reference Mission Architecture (RMA)-S and RMA-L, which refer to Mars surface missions with short ( ~ 30 sols) and long ( > 500 sols) durations, respectively.

Reliance on biotechnology can increase the risk of forward biological contamination (Piaseczny et al., 2019). Beyond contamination, there are ethical issues that concern both the act of colonizing a new land and justifying the cost and benefits of a mission given needs of the many here on earth. Our roadmap begins with the call for an extensive and ongoing discussion of ethics (**Figure 7** Ⓐ). Planetary protection policies can provide answers or frameworks to address extant ethical questions surrounding deep-space exploration, especially on Mars (Rivkin et al., 2020; Tavares et al., 2020). Critically, scientists and engineers developing these technologies cannot be separate or immune to such policy development.

## 7.1 Autonomous Martian Surface Missions

**Figure 7** Ⓑ denotes the interconnection between current Martian mission objects (Mars InSight Landing Pres, 2018; Mars Science Laboratory L, 2012; Baldwin et al., 2016; MAVEN Press Kit, 2013; Mars Reconnaissance Orbit, 2006; Mars Express: A Decade of Observing the Red Planet, 2013; Mars Odyssey Arrival Pres, 2001) and Earth-based process development elements for a biomanufactory (**Figures 3–6**). Together with *en route* autonomous surface missions (Mars Helicopter/Ingenuity, 2020; Mars 2020 Perseverance La, 2020) (**Figure 7** Ⓒ), these missions provide a roadmap for continued mission development based on landing location biosignatures (Bussey and Hoffman, 20162016; Vago et al., 2017). The biomanufactory (**Figures 3–6**) will require ample water in media, atmospheric gas feedstocks, and power that can be bounded by measurements from autonomous missions. Upcoming sample return missions offer an opportunity to shape the design of ISRU processes such as regolith decontamination from perchlorate and nitrogen enrichment for crop growth. Additional orbiters (Jedrey et al., 2016) and lander/rover pairs (**Figure 7** Ⓗ) have been planned and will aid in the selection of a landing site for short term Martian exploration missions (**Figure 7** Ⓙ, Ⓚ). Such locations will be determined based on water/ice mining/availability (McKay et al., 2013) as depicted in **Figure 7** Ⓘ. These missions can be deployed with specific payloads to experimentally validate biomanufactory elements. Low TRL biotechnologies can be flown as experimental packages on upcoming rovers and landers, offering the possibility for TRL advancement of biology-driven subsystems. Planning for such testing will require coordination with, and validation on, ISS and satellite payloads (**Figure 7** Ⓔ), for instance, to understand the impact of Martian gravity, to contrast levels of radiation exposure, and so on.

## 7.2 Artemis Operations

The upcoming lunar exploration missions, Artemis (Smith et al., 2020) and Gateway (Crusan et al., 2019), provide additional opportunities for integration with Earth-based biomanufactory development. Early support missions (**Figure 7** Ⓓ, Ⓕ) will provide valuable experience in cargo predeployment for crewed operations, and is likely to help shape logistics development for short-term (**Figure 7** Ⓙ, Ⓚ) as well as long-term Mars exploration missions (**Figure 7** Ⓜ) when a biomanufactory can be deployed. Here we present a subset of Artemis efforts as they relate to mission elements with opportunities for testing and maturing biomanufacturing technology. Although ISRU technologies for the Moon and Mars will be sufficiently distinct due to different resource availabilities, crewed Artemis missions (**Figure 7** Ⓕ, Ⓖ) provide a testing ground for crewed Mars bioprocess infrastructure. Later Artemis missions (**Figure 7** Ⓛ) also provide a suitable environment to test modular, interlocked, scalable reactor design, as well as the design of compact molecular biology labs for DNA synthesis and transformation. Since these technologies are unlikely to be mission critical during Artemis, their TRL can be increased and their risk factors studied through in-space evaluation.

The Artemis missions also provide a testbed to evaluate the space-based evolution of microbes and alterations of seedstocks as a risk inherent to the biological component of the biomanufactory. This risk can be mitigated by incorporating backup seed and microbial freezer stocks to reset the system. However, ensuring that native and/or engineered traits remain robust over time is critical to avoid the resource penalties that are inherent to such a reset. Consequently, while optimal organisms and traits can be identified and engineered prior to a mission, testing their long-term performance on future NASA missions prior to inclusion in life support systems will help to assess whether engineered traits are robust to off-planet growth, whether microbial communities are stable across crop generations, and whether the *in situ* challenges that astronauts will face when attempting to reset the biomanufacturing system are surmountable. Quantifying these uncertainties during autonomous and crewed Artemis missions will inform tradeoff and optimization studies during the design of an enhanced life support system for Martian surface bio-operations.

## 7.3 Human Exploration of Mars

Crewed surface operations of ~ 30 sols by four to six astronauts are projected (Drake et al., 2010) to begin in 2031 (**Figure 7** Ⓙ),

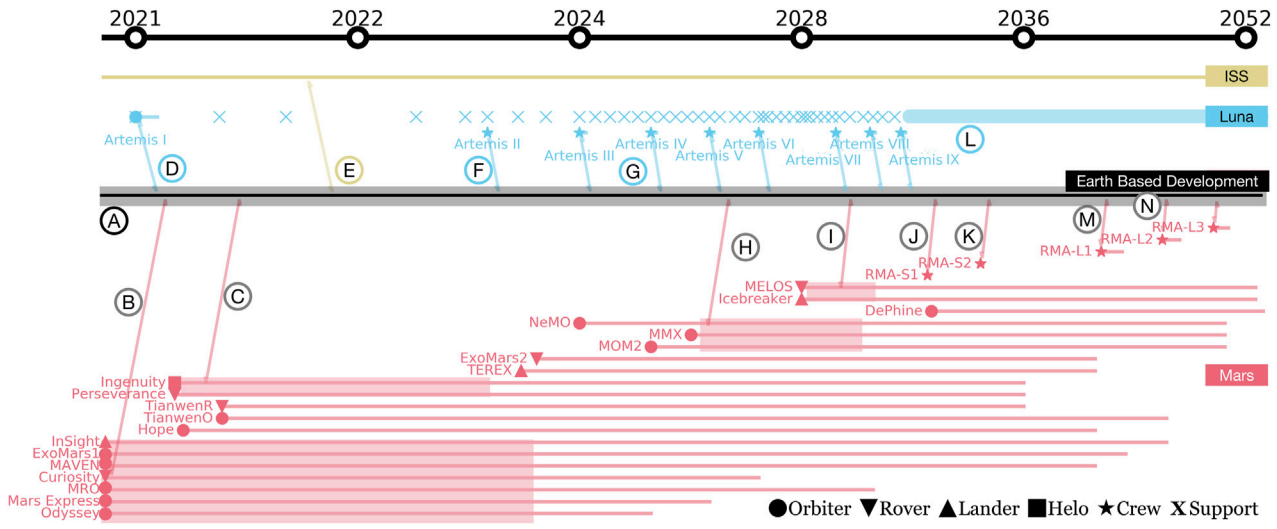


**FIGURE 7 |** Proposed roadmap from 2021 to 2052 in $\log_2$-scale time of Earth-based developments (black) and their relationships to ISS (gold), lunar (blue), and Martian (red) missions. Missions range in status from currently operational, to enroute, planned, and proposed. Reference Mission Architecture (RMA)-S is a 30-sol mission, and RMA-L are missions with more than 500 sols of surface operations. RMA-L1 is the mission target for deployment of a biomanufactory. An arrival at target location is denoted with a symbol to indicate its type as orbiter, rover, lander, helicopter, support, or crewed operations. Circled letters are colored by location and correspond to specific milestones or opportunities for biomanufactory development.

with an additional mission similar in profile in 2033 (**Figure 7** Ⓚ). Given the short duration, a mission-critical biomanufactory as described herein is unlikely to be deployed. However, these short-term, crewed missions RMA-S1, S2 provide opportunities to increase the TRL of biomanufactory elements for ~ 500 sol surface missions RMA-L1 (**Figure 7** Ⓜ) in ~ 2040 and RMA-L2 (**Figure 7** Ⓝ) in ~ 2044. Building on the abiotic ISRU from early Artemis missions, we propose that RMA-S1 carry experimental systems for C-and-N-fixation processes such that a realized biomanufactory element can be properly scaled (**Figure 5**). Since RMA-S1, S2 will be crewed, regolith process testing becomes more feasible to be tested onsite on the surface of Mars, than during a complex sample return mission. Additionally, while relying on prepacked food for consumption, astronauts in RMA-S1 will be able to advance the TRL of platform combinations of agriculture hardware, crop cultivars, and operational procedures. An example is growing crops under various conditions (**Figure 3A**) to validate that a plant microbiome can provide a prolonged benefit in enclosed systems, and to determine resiliency in the event of pathogen invasion or a loss of microbiome function due to evolution. Additionally, the TRL for crop systems can be re-evaluated on account of partial gravity and/or microgravity.

The RMA-S1 and RMA-S2 crews will be exposed for the first time to surface conditions after interplanetary travel, allowing for an initial assessment of health effects that can be contrasted to operations on the lunar surface (**Figure 7** Ⓕ), and that may be alleviated by potential biomanufactory pharmaceutical and functional food outputs (**Figures 3B,C**). The RMA-S1 and RMA-S2 mission ISRU and FPS experiments will also provide insight into the input requirements for downstream biomanufactory processes. ISM technologies such as bioplastic synthesis and additive manufacture (**Figure 4**) can be evaluated for sufficient TRL. Further, loop closure performance for several desired products can also be tested. This will help estimate the impact of waste stream characteristics changes on recycling (Brémond et al., 2018).

## 7.4 Moving Forward

We have outlined the design and future deployment of a biomanufactory to support human surface operations during a 500 days manned Mars mission. We extended previous stand-alone biological elements with space use potential into an integrated biomanufacturing system by bringing together the important systems of ISRU, synthesis, and recycling, to yield food, pharmaceuticals, and biomaterials. We also provided an envelope of future design, testing, and biomanufactory element deployment in a roadmap that spans Earth-based system development, testing on the ISS, integration with lunar missions, and initial construction during shorter-term initial human forays on Mars. The innovations necessary to meet the challenges of low-cost, energy and mass efficient, closed-loop, and regenerable biomanufacturing for space will undoubtedly yield important contributions to forwarding sustainable biomanufacturing on Earth. We anticipate that the path towards instantiating a biomanufactory will be replete with science, engineering, and ethical challenges. But that is the excitement—part-and-parcel—of the journey to Mars.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

AJB, JMH, AJA, and APA conceived the concept based on the Center for the Utilization of Biological Engineering in Space (CUBES) that was established by AJB, BB, DSC, DCD, AM, SN, RMW, PY, CSC, KAM, LCS, AAM, and APA. All authors wrote and edited the manuscript with grouped efforts led by JMH, AJA, AJB, MJM, NJHA, SSG, and GM.

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REVIEW
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# *Chlorella Vulgaris* Photobioreactor for Oxygen and Food Production on a Moon Base—Potential and Challenges

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A base on the Moon surface or a mission to Mars are potential destinations for human spaceflight, according to current space agencies' plans. These scenarios pose several new challenges, since the environmental and operational conditions of the mission will strongly differ than those on the International Space Station (ISS). One critical parameter will be the increased mission duration and further distance from Earth, requiring a Life Support System (LSS) as independent as possible from Earth's resources. Current LSS physico-chemical technologies at the ISS can recycle 90% of water and regain 42% of $O_2$ from the astronaut's exhaled $CO_2$, but they are not able to produce food, which can currently only be achieved using biology. A future LSS will most likely include some of these technologies currently in use, but will also need to include biological components. A potential biological candidate are microalgae, which compared to higher plants, offer a higher harvest index, higher biomass productivity and require less water. Several algal species have already been investigated for space applications in the last decades, being *Chlorella vulgaris* a promising and widely researched species. *C. vulgaris* is a spherical single cell organism, with a mean diameter of 6 µm. It can grow in a wide range of pH and temperature levels and $CO_2$ concentrations and it shows a high resistance to cross contamination and to mechanical shear stress, making it an ideal organism for long-term LSS. In order to continuously and efficiently produce the oxygen and food required for the LSS, the microalgae need to grow in a well-controlled and stable environment. Therefore, besides the biological aspects, the design of the cultivation system, the Photobioreactor (PBR), is also crucial. Even if research both on *C. vulgaris* and in general about PBRs has been carried out for decades, several challenges both in the biological and technological aspects need to be solved, before a PBR can be used as part of the LSS in a Moon base. Those include: radiation effects on algae, operation under partial gravity, selection of the required hardware for cultivation and food processing, system automation and long-term performance and stability.

**Keywords: life support system, Moon base, photobioreactor, oxygen and food production, microalgae**

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**Abbreviations:** ACLS, Advanced Closed-Loop System; CCA, Carbon Dioxide Concentration Subsystem; CDRA, Carbon Dioxide Removal Assembly; CRA, Carbon Dioxide Reprocessing Subsystem; ESM, Equivalent System Mass; FPA, Flat Panel Airlift; IRS, Institute of Space Systems (*Institut für Raumfahrtsysteme*); ISRU, *In-situ* Resources Utilization; ISS, International Space Station; LSR, Life Support Rack; LSS, Life Support System; OGA, Oxygen Generation Assembly; PBR, Photobioreactor; PC, Phyisico-chemical; SRA, Sabatier Reactor Assembly.

## INTRODUCTION

The International Space Station (ISS) has been continuously inhabited for over twenty years. The Life Support System (LSS) on board the station is in charge of providing the astronauts with oxygen, water and food. For that, Physico-Chemical (PC) technologies are used, recycling 90% of the water and recovering 42% of the oxygen ($O_2$) from the carbon dioxide ($CO_2$) that astronauts produce (Crusan and Gatens, 2017), while food is supplied from Earth.

Space agencies currently plan missions beyond Low Earth Orbit, with a Moon base or a mission to Mars as potential future scenarios (ESA Blog 2016; ISEGC 2018; NASA 2020). The higher distance from Earth of a lunar base, compared to the ISS, might require the production of food *in-situ*, to reduce the amount of resources required from Earth. PC technologies are not able to produce food, which can only be achieved using biological organisms. Several candidates are currently being investigated, with a main focus on higher plants (Kittang et al., 2014; Hamilton et al., 2020) and microalgae (Detrell et al., 2020b; Poughon et al., 2020).

Microalgae, like higher plants, produce $O_2$ and edible biomass through photosynthesis by consuming $CO_2$, nutrients and water. Compared to higher plants, microalgae provide a higher harvest index, biomass productivity and light exploitation, and consume less water (Degen 2003; Schmid-Staiger et al., 2009). However, algae cannot be used as our unique nutrition source, due to their high protein content. To ensure a balanced diet, algae can only substitute part of the human daily consumption, thus they can complement either higher plant technologies or food supplied from Earth. The amount of microalgae recommended will depend on the species and how they have been cultivated, with some estimations achieving a recommended maximum of 35% (Belz et al., 2014).

The selection of the algal species will play an important role in the design of the technology required and the performance of the system. Two microalgae are widely used on Earth as food supplement or biofuel source among others: *Chlorella vulgaris* and *Limnospira indica* (*Spirulina*). Both species have been widely studied for space applications. *Chlorella* is a spherical unicellular eukaryotic green algae (**Figure 1**), while *Spirulina* is a filamentous multicellular prokaryotic cyanobacteria (also called blue-green algae). The main advantages of *Chlorella* vs. *Spirulina* are its simple shape and its adaptability to a wide range of cultivation conditions, making it very robust. However, *Chlorella*'s thick cell wall does not allow the human body to assimilate the nutrients inside the cell, requiring a cell wall breakdown process before human consumption (Mason 2001), which is not required with *Spirulina*. Both candidates present advantages and disadvantages, and both have the potential to be used for space applications. This paper focuses on the use of *Chlorella vulgaris*, based on the experience at the Institute of Space Systems (IRS) at the University of Stuttgart, Germany. *Chlorella* has a mean diameter of 6 μm (Yamamoto et al., 2004). It can grow in a wide range of pH and temperature levels (Ackerman 2007) and $CO_2$ concentrations (Powell et al., 2009). It also shows a high resistance to cross contamination (Lakaniemi Aino-Maija et al., 2012), making it a perfect candidate for long-duration cultivation.

The use of microalgae for space applications requires the design of the equipment for a controlled cultivation, a Photobioreactor (PBR). Besides the physical containment provided by the reactor chamber itself, the system will require other subsystems like lighting, nutrient supply, gas exchange, thermal control, growth medium control, harvesting and processing (Storhas 2000). The PBR will need to provide the required environment for the microalgae to perform as required, considering the constrains and requirements of a space missions, for example power consumption limitations. The size of the required PBR will depend on several parameters: the algal species, the cultivation parameters and the intended amount of oxygen or biomass production rate per day.

This paper first looks at the current state-of-the-art, with focus on research of microalgae for space applications, looking as well to the currently used PC technologies for air management, in Chapter 2. A trade-off analysis of a PC system and a hybrid system (including PC technologies and a microalgae PBR) is evaluated in Chapter 3, justifying the potential of a PBR for a Moon base. Chapter 4 identifies the challenges and open questions that still need to be solved, before a PBR can become a reality in a lunar base.

## CURRENT STATE-OF-THE-ART

Recycling LSS technologies have already been used in space in several missions, for water recycling, $CO_2$ removal and $O_2$ production, among other tasks. Several experiments, looking at food production have already taken place in space, but such a technology is still not available as part of the LSS. This chapter focuses on the current state-of-the art of PC recycling technologies for air management onboard the ISS and the research carried out on microalgae for space applications.

### PC Technologies for Air Management

The main tasks considered within the air management system for this paper are the $CO_2$ removal, $CO_2$ reduction and $O_2$ production, since those tasks can also be fulfilled using microalgae. Several technologies onboard the ISS and short-term research plans are available from different space agencies, mainly NASA, ESA, JAXA, and Roscosmos.

The main NASA technologies for air management are the Carbon Dioxide Removal Assembly (CDRA), the Sabatier Reactor Assembly (SRA) and the Oxygen Generation Assembly (OGA). CDRA is a regenerative $CO_2$ absorption system based on zeolite sorbent beds. Currently, further technologies are being investigated to substitute CDRA, for example using amine sorbents, which should be tested in the coming years onboard the ISS. The SRA processes the $CO_2$, consuming hydrogen and producing water and methane. It was operating on the ISS until end of 2017, and NASA currently plans an upgrade and return to the ISS. The OGA produces oxygen by water electrolysis. The system is currently in use onboard the ISS

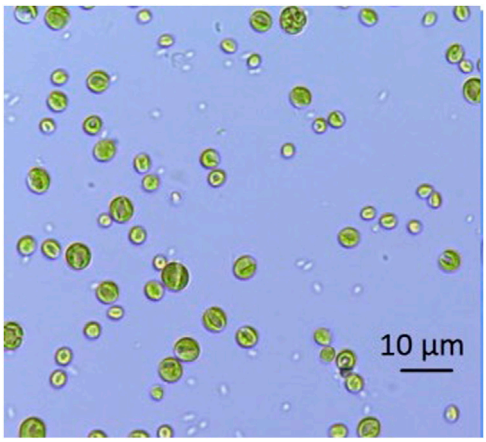


**FIGURE 1 |** Microscope picture of *Chlorella vulgaris* cells, cultivated at IRS.

and NASA is planning an upgrade based on operational experience. (Shaw et al., 2020).

The Russian technologies onboard the ISS include *Vozdukh* for $CO_2$ extraction, also a zeolite sorbent system, and *Elektron* for oxygen production through electrolysis. JAXA future plans include a low temperature Sabatier catalyst, and an amine-based $CO_2$ removal. (Anderson et al., 2016).

The Life Support Rack (LSR), also known as Advanced Closed-Loop System (ACLS), is an European technology, launched to the ISS in September 2018. The LSR is composed by the Carbon Dioxide Concentration Subsystem (CCA), an amine-based $CO_2$ extraction system, the Carbon Dioxide Reprocessing Subsystem (CRA), a Sabatier reactor, and the Oxygen Generation Subsystem (OGA), a fixed alkaline electrolyzer. All technologies are integrated in one Rack and capable to provide in nominal operations for three crew members. (Witt et al., 2020).

## Microalgae Research

The potential of using microalgae for space applications has been considered since the beginning of human spaceflight. Several experiments have taken place both on Earth and in space. Those experiments have either evaluated the potential contribution of microalgae to LSS loop closure, tested the required technology for space applications or investigated the effects of the space environment on microalgae cells.

During the 1960's, several experiments with closed compartments on Earth with several living organisms cohabitating with humans took place independently in the United States (Gitelson and Lisovsky 2003) and in Russia (Kirensky et al., 1968). For example, one of the test platforms used, which included microalgae among other organisms, was Bios-3. It had a 315 $m^3$ compartment for three inhabitants, with experiments lasting up to 90 days (Gitelson and Lisovsky 2003). In Europe, since the 1990's, the MELiSSA (Micro-Ecological Life Support System Alternative) project is also aiming to test LSS closure on Earth. The European Space Agency (ESA) initiative is based on a system with separate compartments, each with a specific

**FIGURE 2 |** 6 L Flat Panel Airlift (FPA) Reactor from the company Subitec®. The system is illuminated with halogen lamps. The system allows the cultivation of *Chlorella vulgaris* with growth rates of up to 4 g/l/d.

living organism contributing to the recycling pathway. One of the five compartments for MELiSSA contains *Spirulina,* which uses the nutrients produced by its predecessor compartment to produce oxygen and biomass for the crew (Lasseur et al., 2010). Experiments on Earth focused on long-term cultivation for space applications have been carried out at the Institute of Space Systems (IRS)—University of Stuttgart since 2010. Their research includes the longest reported experiment with *Chlorella* for space applications lasting over six years in a Flat Panel Airlift (FPA) reactor from the company Subitec® (Buchert et al., 2012; Helisch et al., 2016; Helisch et al., 2020), **Figure 2**, and two experiments lasting over 180 days in a microgravity adapted reactor (Keppler et al., 2018; Helisch et al., 2020), **Figure 3**. The research at IRS also includes the development of hardware for space applications, looking for example at the development of a lighting unit and evaluating its effects on the performance of the PBR.

Those experiments on Earth have focused on the performance of the algae, the design of the PBR and its interaction with other technologies under Earth conditions. Besides this research, about 50 experiments have already been carried out in space, mostly focusing on biological aspects. Those experiments generally had a short duration (several days) and have used different algal species, including amongst others *Chlorella vulgaris* (Niederwieser et al., 2018). The first experiments took place during the 1960's, exposing *Chlorella* to space conditions in a photosynthetically inactive state, followed by a cultivation back on Earth (Semenenko and Vladimirova 1961; Shevchenko et al., 1967; Ward and Phillips

**FIGURE 3 |** PBR@LSR reactor chamber (Detrell et al., 2019a). The PBR@LSR experiment included two microgravity-adaptet reactors, with a total capacity of 650 ml algae suspension.

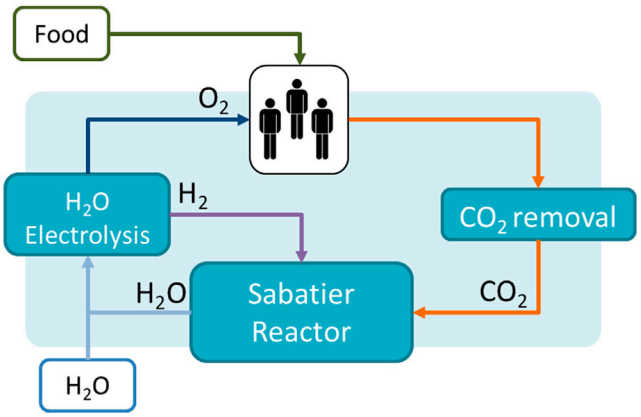


**FIGURE 4 |** PC LSS for Air Management. The system is based on the current LSR technology on board the ISS.

1968; Antipov et al., 1969). The first actual cultivation of *Chlorella* in space took place in the 1970's, lasting two weeks (Moskvitin and Vaulina 1975). Other experiments, including not only algae but also other organisms, have been flown over the last decades, for example the Closed Equilibrated Biological Aquatic System (CEBAS) (Blüm 2003), Omegahab (Anken 2008), and Closed Aquatic Ecosystem (CAES) (Wang et al., 2008). Only two experiments have focused to date on the PBR technology in microgravity conditions, artemiss with 30 days's cultivation of *Spirulina* (Poughon et al., 2020) and PBR@LSR with two week's cultivation of *Chlorella* (Detrell et al., 2020b).

## POTENTIAL–MICROALGAE LSS TECHNOLOGY FOR A LUNAR BASE

To evaluate the effects of including a PBR in the LSS, the Equivalent System Mass (ESM) can be used. It considers not only the mass of the system, but also the influence of its volume, required power, cooling and crew time. Equivalency factors, specific for a mission scenario can be used to transform all terms in mass-equivalent (Anderson et al., 2018). Adding a PBR will certainly increase the system mass, but will reduce the amount of food supplied from Earth. At a certain mission duration, the system with a PBR will become most favourable.

A comparison of a PC and a hybrid system's ESM is carried out by Detrell 2021. The PC system is based on current technologies for $CO_2$ removal, $CO_2$ reduction and $O_2$ production (**Figure 4**). As a reference technology the LSR is used, since it is the latest full technology brought to the ISS for air management. The LSR has a total mass of 715 kg, a volume of 1.8 $m^3$ and requires a power of 2.1 kW, providing for three astronauts (Kappmaier et al., 2016; Matthias 2018). The system requires an addition of 0.47 kg/d of water per person. This water is spitted though electrolysis into $O_2$ and $H_2$, used by the crew and by Sabatier reactor respectively. In the PC LSS, the food will entirely be brought from Earth. The hybrid system includes the same PC technologies and a PBR system (**Figure 5**). A PBR system for a lunar base is still not available,

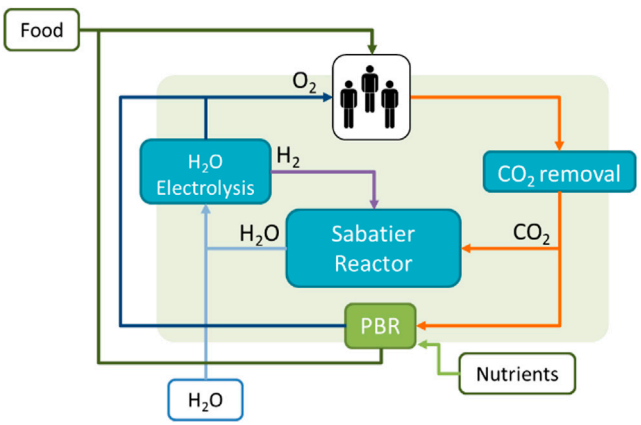


**FIGURE 5 |** Hybrid LSS: PC + PBR. The system includes the technologies based on the LSR and the addition of a PBR, which can provide oxygen and food. Nutrients need to be added to the system.

and thus a first estimation for the main sizing parameters is done using laboratory data. Current experiments at IRS have shown that *Chlorella vulgaris* can be cultivated to provide growth rates up to 4 g/l/d. Variations in the growth rate will have a high impact on the system sizing and thus the ESM. Although growth rates of 4 g/l/d have been reported in several experiments, maintaining the system at those levels for long-periods of time in space conditions (that is at reduced gravity and higher space radiation dose) still needs to be demonstrated. Therefore, the study considers a range of reasonable growth rates, between 2 and 4 g/l/d. The algae volume required also depend on the PBR goal, i.e., the amount of food or oxygen that it is expected to be provided. Although first estimations, based on its composition, suggest up to 35% of the daily mass food consumption could be substituted by algae (Belz et al., 2014), such a diet and its potential side effects have still not been reported. In the study, two scenarios are considered for a first estimation, a PBR sized to satisfy 10 and 30% of the daily consumption, which would require a PBR system between 50 and 100 L per person (Detrell 2021). The

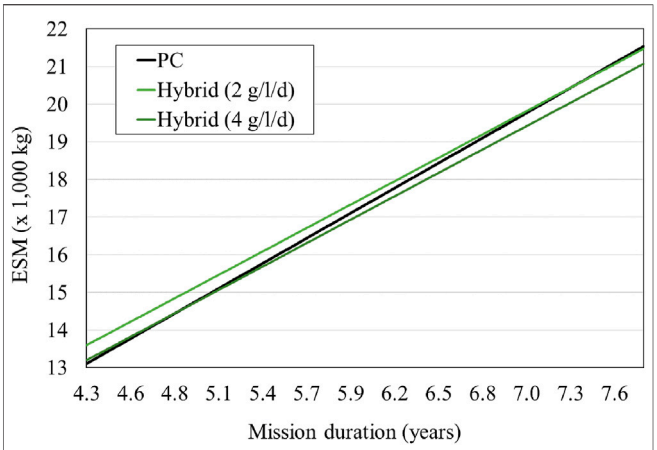


**FIGURE 6 |** ESM comparing PC and Hybrid LSS with microalgae 10% food supply. The PC ESM is based on the LSR published data, while the PBR is based on the IRS laboratory data. A growth rate between 2 and 4 g/l/d is realistic according to current experiments and would provide an ESM that is more favorable for mission durations between 4.8 and 7.3 years (Detrell 2021).

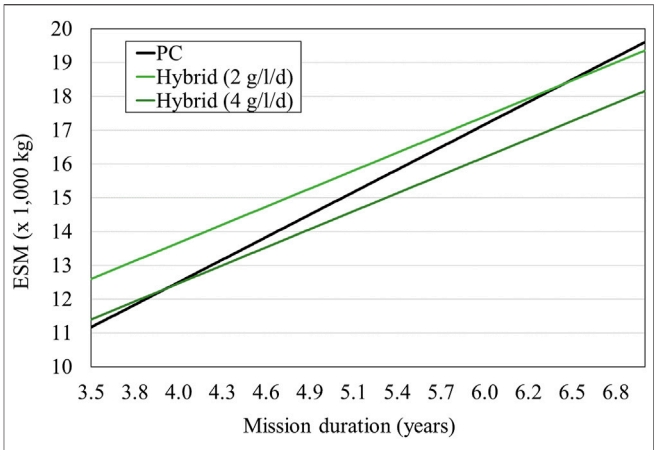


**FIGURE 7 |** ESM comparing PC and Hybrid LSS with microalgae 30% food supply. The PC ESM is based on the LSR published data, while the PBR is based on the IRS laboratory data. A growth rate between 2 and 4 g/l/d is realistic according to current experiments and would provide an ESM that is more favorable for mission durations between 4.0 and 6.5 years (Detrell 2021).

addition of the PBR will reduce the amount of food that needs to be brought from Earth, but will add the need of nutrients. **Figures 6**, **7** show the results of the ESM per crew member. The break-even-point at which a PBR will be more favourable occurs at missions lasting between 4.0 and 6.5 years for a 30% food supply, and 4.8 and 7.3 years for a 10% food supply (Detrell 2021). Those mission durations are quite high, but reasonable for scenarios such as a permanently crewed Moon base.

The ESM of the PBR system could potentially be reduced in a Moon-base scenario with *In-Situ* Resources Utilization (ISRU). A PBR requires a high amount of water, as a medium support for the algae inside the reactor chamber. If the water could be obtained directly on the Moon surface (Honniball et al., 2021), or even the materials to build the reactor chamber (Schleppi et al., 2021), a PBR ESM would be reduce and thus it would be favourable in even shorter mission durations.

Besides the ESM, other parameters such as technology readiness should also be considered. The PC systems have been widely used in a relevant environment for long periods of time. For a PBR technology, still more research and testing is needed, requiring a research effort during the coming years, for it to become a real competitive option to PC technologies. Even if the PBR can only produce part of the required food, it will reduce the amount of food stored for long periods of time, which could have a significant impact on its nutritional value. This aspect will become even more relevant for missions further away, for example to Mars, where resupply intervals will increase substantially (Drysdale et al., 2003).

## CHALLENGES–OPEN QUESTIONS

According to current research, a *Chlorella* PBR shows a big potential as a biological component in a lunar base to produce oxygen and fresh food supplement. The research at IRS since 2010 has been focused on the usage of microalgae, particularly *Chlorella vulgaris*, for space applications. The research has focused mainly in two aspects: the long-term stable cultivation and the system design under space conditions. Regarding the PBR design, several studies and experiments have been taken place at IRS considering all potential scenarios, including the microgravity experiment PBR@LSR, launched to ISS in 2019, and Moon/Mars photobioreactor preliminary studies. At IRS cultivation techniques have been investigated, allowing a successful over 6 years non-axenic cultivation, under Earth condition. During this research, several challenges or open questions that need to be solved, before such a component can become a reality, have been identified. Those include the influence of lunar environmental conditions, technical and biological aspects.

## Influence of Moon Conditions

No experiments with microalgae have been carried out on the Moon, thus no previous experience exist on the influence of its environment on the algae and the performance of a PBR. The main two aspects to be considered are space radiation and partial gravity.

### Space Radiation

The experiments with microalgae carried out to date in space were in Low Earth Gravity, i.e., under the Earth's magnetosphere protection (Niederwieser et al., 2018). Those experiments have shown different results on the effects of space conditions at cell level, still showing that cultivation under space conditions is possible. A series of experiments showed that *Chlorella* can survive continuous exposure to ionizing radiation while maintaining more than 90% of its original photosynthetic capacity, higher than other species (Rea et al., 2008). Space

radiation levels on the Moon will be considerably higher. It is possible to partially test the effects of certain radiation on Earth, however, the long-term effects of space radiation need to be tested *in-situ*, in the lunar surface. The location of the PBR within the lunar base will play an important role in the amount of radiation received. If the PBR is integrated within the habitable structure of the station, the system will count with the same level of radiation protection than the crew.

### Reduced Gravity

*Chlorella* is an immobile unicellular organism. Gravity causes sedimentation within the reactor, which is avoided by creating a continuous movement of the algae-suspension. The lunar gravity is 1/6th of Earth's gravity, which will have an effect on the movement of the cells and the gas within the liquid phase, and could eventually have an effect at cell level. The latter can be investigated in the laboratory using a clinostat. The experiments on microgravity have not shown an effect in the algae performance due to lack of gravity. Thus, no effects with partial gravity are expected. The major influence of reduced gravity will be on the system design, requiring a reactor and other subsystems to work as expected under partial gravity. Hardware design and testing in low gravity can be achieved through Computational Fluid Dynamics (CFD) simulations (Detrell et al., 2019b) and lunar-gravity parabolic flight campaign experiments (Pletser et al., 2012), before testing the system *in-situ* in the lunar environment.

## Technical Challenges

The environmental conditions on the Moon explained in the previous section will have an influence on the technology, for example the reduced gravity level will have an influence on the movement of fluids within the reactor. Besides that, other challenges, inherent to the technology itself and the long-term performance also need to be addressed. The main challenges are related to the reactor design, the lighting system, the harvesting and processing unit and the scaling up and automation of the entire process/system.

### Reactor Geometry

Several types of reactor geometries are currently being used on Earth, from open ponds to high complex geometry reactors (Płaczek et al., 2017). For space applications, a closed system will be required, since avoiding contamination of the system will be a must. For that reason, systems such as open ponds can be discarded.

A high volumetric efficiency (up to 4 g/l/d with *Chlorella*) can be obtained with a high complex reactor geometry, with the Flat Panel Airlift (FPA) from the company Subitec®, **Figure 2**. The air is introduced in the bottom of the reactor and bubbles up in a gravity environment. The complex geometry creates swirls in the sub-chambers of the reactor, ensuring a proper mixing of the algae and providing a homogenous availability of nutrients, $CO_2$ and light. However, the high complex geometry requires an even more complex maintenance. The Subitec® reactor geometry is optimized for an Earth gravity environment, but it can be adapted to lunar conditions, so it provides the same movement as on Earth with 1/6th of gravity (Detrell et al., 2019b). Other reactor types, such as tubular reactors, are easier to build and maintain, but are less efficient with growth rates below 0.1 g/l/d (Martin et al., 2020).

A raceway microgravity adapted reactor, with a FEP membrane for gas exchange and a pump to ensure algae-suspension circulation was designed and used for the PBR@LSR experiment (Detrell et al., 2020a). The growth rates obtained by this design were considerably lower, 0.42 g/l/d (Helisch et al., 2020). An important limitation of this type of reactor is the gas transfer rate of the membrane. While the use of airlift-based reactors was not possible for this experiment due to microgravity conditions, it can be advantageous under lunar reduced gravity conditions.

A trade-off between efficiency and complexity will be required. Volumetric efficiency plays a major role in the selection process if the entire system mass (including reactors and required water to fill them) needs to be brought from Earth. The possibility of using lunar resources, e.g., water from the Moon surface or materials to build the reactors *in-situ*, might make it possible to consider the use of simpler geometries. In that case, energy requirements and maintenance effort shall also be considered in the geometry selection.

Several reactor geometries could be used for a lunar base, and mission-related parameters, such as the ISRU will play an important role in the reactor geometry selection. The design of the selected geometry will need to consider lunar gravity levels, as explained in section *Reduced Gravity*.

### Light/Energy Availability

The usage of direct sunlight is highly dependent on the lunar base location. A base on the lunar equator would experience 14-days-long nights, in which case artificial lighting would be required. Although it is possible to locate a base in areas with high illumination rates, for example on the rims of certain craters in the poles, with sunlight availability over 90% of the time, an artificial lighting system could still be more advantageous. This would allow a better control and adaptability of the lighting to the growth stages and could be used as a non-invasive control tool.

However, power availability is generally a constrain for space systems, thus the lighting system needs to be efficient in terms of energy. Several experiments on Earth have focused on the effect of specific wavelengths and their effect on the cultivation (Blair et al., 2014; Lysenko et al., 2021). Blue and red LEDs, which represent the two main peaks of the light absorption spectrum for *Chlorella vulgaris,* have been satisfactorily used in laboratory experiments (Bretschneider et al., 2016; Keppler et al., 2017). A lighting system, including more LEDs at different wavelengths could be used to reproduce more precisely the absorption spectrum of *Chlorella* and would be required to allow a non-invasive control (Martin et al., 2020). Experiments with *Chlorella* use typically photon flux densities of 200–300 μmol photons/$m^2$/s (Helisch et al., 2020).

The effects of the different lighting concepts and the potential of non-invasive control still need to be further researched. It will be necessary to evaluate the long-term effects on the algae cells and their performance, while reducing the required energy for the

lighting. The non-invasive control requires a deep knowledge of the culture and its reaction to different lighting spectrums.

### Harvesting and Processing

The produced biomass in the reactor needs to be extracted from the system and processed to edible biomass, including a cell wall breakdown process for *Chlorella*.

The harvesting process requires the separation of the algae biomass from the growth medium, which can be further used in the PBR. Solid-liquid separation technologies are widely used for biomass harvesting in microalgae systems on Earth, with sedimentation, centrifugation and filtration being the main used processes (Singh and Patidar 2018). A key element for space applications is to obtain a high efficient harvesting system, that requires low energy, and does not compromise the biomass to be used as food source. Sedimentation is to expected to occur under lunar gravitational conditions, but it is a slow process. Centrifugation has a high recovery and is a fast process, but requires high amounts of energy and might cause cell damage due to high shear forces. Filtration also provides a high recovery efficiency without the high shear forces, however membranes/filters need to be cleaned or exchanged due to fouling/clogging. Other technologies, like microfluidic systems (Hønsvall et al., 2016) or electrophoresis (Pearsall et al., 2011), should also be considered for a lunar base, but have currently only been tested at small scale on the laboratory. Further research, and scaling up the system is still required.

There are several potential processes for processing, which are known on Earth, including high pressure homogenisation (Halim et al., 2013), freeze drying (Grima et al., 1994), microwave irradiation, and ultrasound (McMillan et al., 2013), but none has been developed or even evaluated for space applications yet. A major problematic of all those methods is the high energy required. As an alternative to direct human consumption, other alternatives such as fish feeding (Carneiro et al., 2020) or enhancing plant growth (Michalak et al., 2016) could also be considered.

### Scale-Up and Automation

The experiments with microalgae for space applications carried out to date have been small experiments, with a couple of litres, while a full PBR for a LSS will require about 100 L per person. A modular PBR system would allow avoiding scaling up effects and at the same time provide redundancy (Xu et al., 2009). In case of contamination occurring at one module, the rest could continue working. Stand-by modus reactors, ready to substitute a failed reactor, could be provided. A scale-up strategy, defining a modular system, ensuring efficient use of resources (e.g., common sensor unit), will be required.

A PBR experiment is generally well monitored and followed by the scientists, in some cases requiring their interaction when off-nominal situations occur. A LSS PBR should be able to work fully automated, to reduce crew time but also the risk of contamination associated with human interaction. The system will require sensors that are reliable for long periods of time or can easily be exchanged/recalibrated with minimal effort. For the system to react in off-nominal situation, those need to be fully understood and easily identifiable with the sensors in the system.

## Biological Challenges

Besides the technological challenges, biological challenges need to be addressed as well. These mainly relate to the long and stable performance of the algae, looking both at the culture composition (axenic/non-axenic) and the long-term cultivation effects.

### Non-Axenic Cultivation

Microalgae cohabit with other living organisms on Earth. However, for space applications, the use of a closed PBR is preferred to an open system, to allow control of the population inside the PBR. That allows an axenic cultivation, which would require a complex hardware to ensure no other organisms enter the system during the required interactions for biomass extraction and nutrient insertion. A closed PBR also allows the use of a defined and well-established ecosystem, with the microalgae as dominant species. This has the potential to prevent the invasion of other species or suppress their upsurge during cultivation (Zhang et al., 2018). The use of a defined non-axenic culture has been discussed in several publications, with some results showing growth promotion (Cho et al., 2015; Ramanan et al., 2016), while others report system contamination (Wang et al., 2013). No evidence has been found in literature of a stable axenic cultivation for long periods of time, over years (Detrell et al., 2020a). Ensuring an axenic cultivation for long periods of time would require a highly complex system and procedures. An interaction with the exterior of the PBR will be required for air exchange, nutrient insertion and biomass. To ensure the axenicity in the PBR, it is necessary that the system is able to ensure that no other organisms can enter the system or that treatments (for example use of antibiotics) can be applied to selectively eliminate those (Mustapa et al., 2016). But the usage of a non-axenic cultivation comes with its challenges too. It is crucial not only to guarantee the predominance of the microalgae over time, but also of the associated community. If the biomass is to be used as a food source, it is required to ensure that it is edible and no organisms harmful for humans have entered the system. Microbial community analyses have shown the prevalence of *Chlorella* as the main species in non-axenic cultivation experiments (Haberkorn et al., 2020). A modular approach and proper analysis would allow to identify and reject any running reactor that might not fulfil the requirements for human consumption. Automated flow cytometry with advanced data analysis relying on phenotypic fingerprinting could contribute to a continuous monitoring of the microbial community (Haberkorn et al., 2021). The use of flow cytometry in a known non-axenic culture can enable the understanding of population dynamics and their response to external events.

### Long-Term Cultivation

A PBR for a LSS will need to work continuously for long-periods of time, since it would be responsible for providing part of the food and oxygen required by the crew. However, most of the experiments carried out for space applications to date have lasted only a few days or weeks (Niederwieser et al., 2018; Helisch et al., 2020). Three long-term cultivation laboratory experiments on non-axenic cultivation for space applications have been reported so far: a Subitec® FPA

reactor experiment lasting over 6 years (Buchert et al., 2012; Helisch et al., 2016; Helisch et al., 2020), and two microgravity adapted reactor experiments over 180 days (Keppler et al., 2018; Helisch et al., 2020). Those experiments have demonstrated the feasibility of long-term cultivation and performance of the PBR. Besides the long-term performance of the culture, related to the non-axenic cultivation already mentioned, changes at cell level over time, influences in the composition, as well as biofilm formation are the main concerns for long-term cultivation. As mentioned in *Light/Energy Availability*, the lighting system can have a high influence on the culture. Thus, this can be used to influence the composition of the biomass. Similarly, the nutrient supply, including composition and supply interval will also have an impact in the culture.

Long-duration cultivation increases the probability of biofilm formation, which can be caused by direct adhesion of cells, biological deposits (e.g., extracellular polysaccharides–EPS) or cellular debris. The biofilm formation can result in an inhomogeneous availability of nutrients and dispersion of light energy influx, influencing the PBR performance. Some of the parameters showing a high influence on biofilm formation include light intensity and temperature, availability of carbon, nitrogen and phosphorous, as well as stress response to bacteria and mechanical forces (Wang et al., 2013; Helisch et al., 2016). Further long-term experiments are required to further understand, prevent or minimise the effects of biofilm formation, before a PBR can be used long-term in a LSS.

## CONCLUSION

The use of microalgae for space applications has been widely investigated for several decades. One of the potential candidate species is *Chlorella vulgaris*, due to its robustness and adaptability. The feasibility of a microalgae based system depends both on biological and technological aspects. Besides the cultivation strategies, the required hardware, the Photobioreactor, will play an important role.

Several experiments with microalgae have already taken place in space. Those experiments were mostly short (several weeks) and small (several milliliters). Although those experiments have provided highly valuable results, further research is still required. Microalgae for a LSS will need to work continuously for long periods of time and will require a much higher volume.

A first estimation for a *Chlorella* PBR, based on laboratory data, suggests a reactor size of 100 L per person, to fulfil 30% of the human daily food requirements. The PBR would reduce the amount of required food supplied from Earth, but would require the addition of the PBR system itself, with a certain mass, but also other resources such as power or nutrients. A comparison of the PC LSR technology, with the same system including a PBR, shows that in terms of ESM, the PBR would be more favorable in missions lasting at least four years. This is a plausible scenario for a lunar base.

The work carried out at IRS serves as an initial base for the PBR research for a lunar base, but several challenges have been identified, which require further research, before a PBR can be used as part of a LSS in a lunar base. The main identified challenges include the higher space radiation and lower gravity on the Moon surface, as well as technical and biological aspects. A PBR design for the Moon does not exist yet, and several aspects need to be consider for the design: the reactor geometry (which will define volumetric efficiency, and needs to be adapted to lunar gravity), the lighting system (that will be defined by the power requirement) and the harvesting and processing (which shall allow the continuous cultivation and food production). Modularity and system automation (particularly looking at sensors and off-nominal scenarios) will also be crucial during the technology design. The design should also consider biological aspects, like the biofilm formation and its effects, and the effects of a non-axenic cultivation, to ensure a long-term stable performance.

## AUTHOR CONTRIBUTIONS

GD is the main and only author of this paper.

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# Production of PHB From $CO_2$-Derived Acetate With Minimal Processing Assessed for Space Biomanufacturing

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Providing life-support materials to crewed space exploration missions is pivotal for mission success. However, as missions become more distant and extensive, obtaining these materials from *in situ* resource utilization is paramount. The combination of microorganisms with electrochemical technologies offers a platform for the production of critical chemicals and materials from $CO_2$ and $H_2O$, two compounds accessible on a target destination like Mars. One such potential commodity is poly(3-hydroxybutyrate) (PHB), a common biopolyester targeted for additive manufacturing of durable goods. Here, we present an integrated two-module process for the production of PHB from $CO_2$. An autotrophic *Sporomusa ovata (S. ovata)* process converts $CO_2$ to acetate which is then directly used as the primary carbon source for aerobic PHB production by *Cupriavidus basilensis (C. basilensis)*. The *S. ovata* uses $H_2$ as a reducing equivalent to be generated through electrocatalytic solar-driven $H_2O$ reduction. Conserving and recycling media components is critical, therefore we have designed and optimized our process to require no purification or filtering of the cell culture media between microbial production steps which could result in up to 98% weight savings. By inspecting cell population dynamics during culturing we determined that *C. basilensis* suitably proliferates in the presence of inactive *S. ovata*. During the bioprocess 10.4 mmol acetate $L^{-1}$ $day^{-1}$ were generated from $CO_2$ by *S. ovata* in the optimized media. Subsequently, 12.54 mg PHB $L^{-1}$ $hour^{-1}$ were produced by *C. basilensis* in the unprocessed media with an overall carbon yield of 11.06% from acetate. In order to illustrate a pathway to increase overall productivity and enable scaling of our bench-top process, we developed a model indicating key process parameters to optimize.

**Keywords: biomanufacturing, $CO_2$ reduction, biopolymer, acetogen biocatalyst, *in situ* resource utilization**

## INTRODUCTION

Space exploration remains a key aspect of technical and scientific programs of multiple nations (ISECG, 2018). For instance, the National Aeronautics and Space Administration (NASA) has received increased federal funding in the United States as strategic investments call for expansion of crewed space exploration capabilities (National Research Council, 2012, 2014). These efforts are highlighted by the newly established Artemis Program which aims to land women and men on the Moon and Mars, as well as various directorates to fund research into systems enabling human-led deep space exploration (Northon, 2017; NASA, 2018, 2019).

However, the exorbitant costs to transport goods into space represent a major roadblock for space exploration (Wertz and Larson, 1996; Linck et al., 2019). Notably, as human-based mission lengths increase so does the demand for consumables (Moore, 2010). Additionally, tenuous re-supply lines to faraway locations like Mars could be easily disrupted (Tanner et al., 2006). Therefore, it is pivotal to transition from missions that solely rely on re-supplied or pre-deployed stores of single-use consumables to those that sustainably produce and recycle consumables.

*In situ* resource utilization (ISRU) is the practice to generate products in space from local materials and chemicals. The purpose of ISRU is mainly to support astronauts on long expeditions and could provide products and materials for life support, construction, propellants, and energy harvesting (Linne et al., 2017). This would also allow for the transport of more goods that would be prohibitively difficult to generate in space (e.g., photovoltaics and experimental equipment) and allow for production flexibility given a change of mission demand. Successful implementation of ISRU will require the harmonious integration of new technological platforms interfacing biotechnology, systems engineering, solar energy harvesting, agriculture, remediation, and manufacturing (Berliner et al., 2020).

There has been heightened interest and research into microorganisms as the core platform to generate consumables and life-support materials in space (Menezes, 2018; Nangle et al., 2020). Autotrophic and diazotrophic microorganisms fix $CO_2$ and $N_2$, both of which are present in the Martian atmosphere (Owen et al., 1977; Ragsdale and Pierce, 2008). Single-celled organisms replicate and self-repair; thus, only dried inocula would need to be transported. Bacteria have been reported to produce value-added products including bioplastics, biofuels and pharmaceuticals from $CO_2$, fix $N_2$ to fertilizer and aid in waste remediation (Kobayashi and Kobayashi, 2006; Liu et al., 2015, 2016, 2017; Soundararajan et al., 2019; Su et al., 2020). Many strains may be powered directly by solar-generated electricity or by electrochemically produced reducing equivalents such as $H_2$ (Sakimoto et al., 2018; Cestellos-Blanco et al., 2020). Here, we present a bacteria-based bioprocess to renewably produce a biopolymer poly(3-hydroxybutyrate) (PHB) from $CO_2$ while optimizing for ISRU specific requirements.

Poly(3-hydroxybutyrate) is a type of polyhydroxyalkanoate (PHA) belonging to a class of bio-polyesters (Sudesh et al., 2000). It accumulates in different bacterial cells and archaea intracellularly triggered by physiological stress when lacking certain nutrients (Koller, 2019; Müller-Santos et al., 2020). Bacteria employ the biopolymer resulting from carbon assimilation of glucose, starch and organic acids as a form of carbon and energy storage as well as a protectant against stressors (Sheu et al., 2009; Obruca et al., 2018; Dalsasso et al., 2019). PHB is synthesized by the reduction of two condensed acetyl-CoA molecules to produce the hydroxybutyryl-CoA monomer. PHB is biodegradable and has material properties resembling those of polyethylene (McAdam et al., 2020; Meereboer et al., 2020). Additive manufacturing techniques such as 3D-printing can employ PHB that has been extracted from cells and pelletized (Alarfaj et al., 2015; Vigil Fuentes et al., 2020; Kovalcik, 2021). Altogether, PHB represents a viable pathway for biomanufacturing of material products in space.

Acetogenic bacteria fix $CO_2$ to acetate and biomass through anaerobic respiration. The Wood–Ljungdhal pathway in acetogens undertakes the reduction of $CO_2$ to acetyl-CoA which is then used in biosynthesis or converted to acetate gaining ATP. Other minor products of the pathway may include ethanol, 2,3-butanediol, and hexanoic acid depending on growth conditions (Fernández-Naveira et al., 2017). Acetogens obtain electrons directly from the reduction of minerals, or through the oxidation of electron shuttles or reducing equivalents. Certain acetogens like *Sporomusa ovata* (*S. ovata*) have been found to directly obtain electrons from a poised cathode or from electrochemically generated reducing equivalents like $H_2$ (Nevin et al., 2011; Rodrigues et al., 2019). This enables $CO_2$ bioelectrosynthesis to acetate with high selectivity and long-term stability.

We have employed *S. ovata* as a self-replicating and self-regenerating biocatalyst to fix $CO_2$ to acetate. We then selected *Cupriavidus basilensis* (*C. basilensis*) for its ability to consume acetate as a primary carbon feedstock (**Figure 1**; Wierckx et al., 2010; Friman et al., 2013). In addition, *C. basilensis* produces PHB with high carbon efficiency, and as a significant proportion of biomass. Few accounts have been reported of PHB/PHA production starting from $CO_2$ as a carbon source (Liu et al., 2015; Pepè Sciarria et al., 2018). And several components of these accounts do not translate well to ISRU-based space production including the low rate of $CO_2$ fixation and the requirement to purify and concentrate the intermediate carbon molecules before their use in PHA bioproduction. *S. ovata* can directly take up electrons from a cathode for $CO_2$ to acetate conversion but this approach is limited by the alkaline pH change at the cathode and the bacteria/electrode interface (Lovley and Nevin, 2013). Therefore, we employ $H_2$ generated efficiently in a separate module to circumvent these challenges. Direct $CO_2$ bioelectrosynthesis to PHB has also been reported (Liu et al., 2016). However, the segmented approach ($CO_2$ to acetate, acetate to PHB) prevents incompatibilities that arise from combining electrochemistry and biological catalysts such as the requirement to maintain neutral pH. Additionally, a segmented process flow allows for individual unit optimization and overall biomanufacturing modularity (Fast and Papoutsakis,

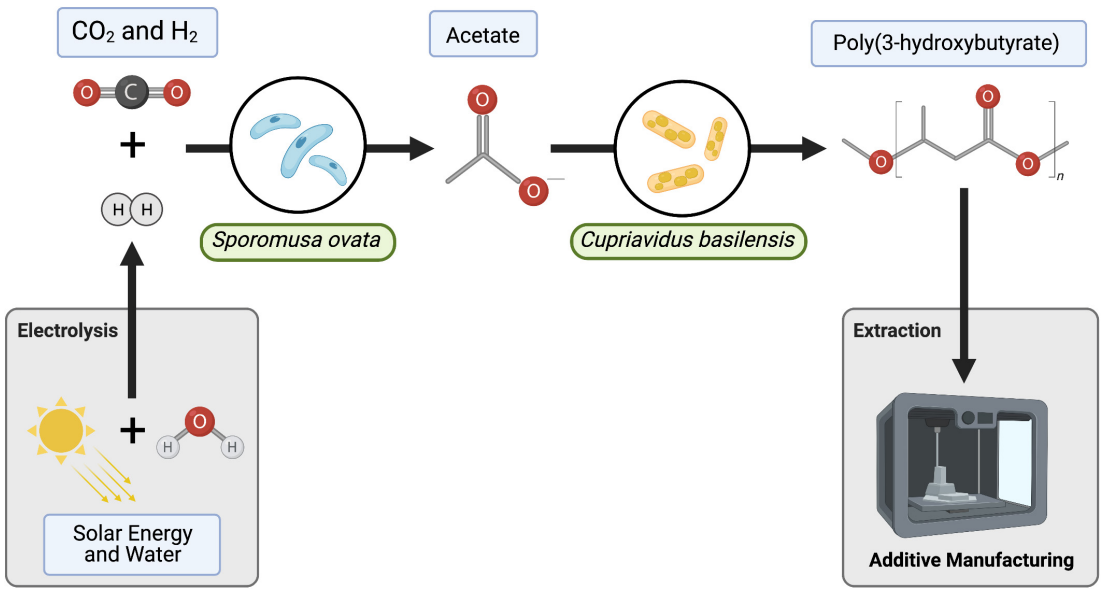


**FIGURE 1 |** Process diagram for two-stage bioprocess with media recycle. *Sporomusa ovata* oxidizes $H_2$ to fix $CO_2$ into acetate. *C. basilensis* grown in the unprocessed spent medium converts acetate to Poly(3-hydroxybutyrate) (PHB). Modules to be tested for end-to-end biomanufacturing including $H_2O$ reduction and PHB extraction to 3D printing in gray.

2012; Kiefer et al., 2021). For these reasons, we have designed our bioprocess to include a base medium used for both bioreactions–acetate and PHB generation. The ability to directly recycle media circumvents the need for acetate purification and concentration steps which require significant non-ISRU resources (**Supplementary Table 4**). Altogether, we demonstrate peak acetate production from $CO_2$ of 10.4 mmol acetate $L^{-1}$ $day^{-1}$ which translates to a titer of acetate of 25 mM in 2.5 days. This is sufficient for the generation of 12.54 mg PHB $L^{-1}$ $hour^{-1}$. Through modeling this process, we have determined that we can reduce the time to generate 25 mM of acetate by 75%. This can be accomplished by increasing the gas to liquid mass transfer of $H_2$ through improvements in bioreactor design.

# MATERIALS AND METHODS

## Cultivation of *Sporomusa ovata*

*Sporomusa ovata* was obtained from the American Type Culture Collection (ATCC 35899) and rehydrated as indicated. It was then grown in DSMZ 311 medium and aliquoted with DMSO as a cryoprotectant at −80°C. Frozen cells were inoculated in DSMZ 311 medium and cultured for two cycles before inoculating in yeast medium (DSMZ 311 medium, casitone, betaine, and resazurin omitted). *S. ovata* from yeast medium were inoculated at 5% (v/v) in autotrophic *S. ovata* medium (DSMZ 311 medium, casitone, betaine, resazurin, and yeast omitted) with $H_2/CO_2$ 80/20% headspace at 30 PSI. *S. ovata* was consistently incubated at 35 °C in starting pH 7.2 and an orbital shaker was employed for autotrophic growths. Acetate concentration was monitored by 1H-qNMR spectroscopy with sodium 3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionate as the internal standard. *S. ovata* from yeast cultures were also inoculated in deoxygenated DM9 (**Supplementary Table 1**) at 10% (v/v) with $H_2/CO_2$ 80/20% headspace at 30 PSI. Balch-type anaerobic culture tubes with butyl stoppers were employed to maintain anaerobicity throughout.

## Cultivation of *Cupriavidus basilensis* 4G11

*Cupriavidus basilensis* 4G11 was retrieved from a previously isolated culture from the Oak Ridge Field Research Center Site by Ray et al. (2015). To recover the strain from frozen glycerol stocks, it was initially plated on R2A medium agar plates and incubated overnight until colonies formed. A single colony was used to inoculate a starter culture (5 mL) of DM9 (**Supplementary Table 1**) medium containing 25 mM sodium acetate at pH 7.2 incubated at 30 °C. After overnight incubation, this culture was centrifuged and washed twice with sterile water to remove residual media before a final resuspension in PBS. Washed cells were used to inoculate experimental conditions at 1% v/v. Optical density of *C. basilensis* cultures was measured at a wavelength of 600 nm using a spectrophotometer (Genesys 30 visible spectrophotometer, thermo fisher scientific part number 76308-728).

## PHB Cell Biomass Preparation and HPLC Analysis

Bacterial culture samples were centrifuged and dried before digestion in 99.99% sulfuric acid (Sigma) for 30 min at 90°C; vortexing briefly at 10 min intervals, according to the protocol

of Tyo et al. with variations mentioned herein (Tyo et al., 2006). The acid-digested samples were then allowed to cool to room temperature for 30 min prior to filtering the samples through a 0.2 μm PVDF syringe filter (Pall part number 4406). PHB standards were prepared by quantitatively dissolving PHB powder (Sigma–Aldrich part number 363502-10G) in chloroform and aliquoting requisite amounts, after which the chloroform was allowed to evaporate overnight. The dried PHB standards were then processed alongside the dried cell biomass samples and analyzed as described below.

Poly(3-hydroxybutyrate) content was measured in processed samples using a Shimadzu Prominence HPLC system equipped with a Reactive Index Detector. Processed samples were eluted and separated using an Aminex HPX-87H column (BioRad part number 1250140) equipped with a micro-guard cation H guard column (BioRad part number 1250129) heated to 40°C and using 5 mM $H_2SO_4$ as the mobile phase flowing at 0.6 mL/min. PHB was enumerated as crotonic acid monomers (products of the concentrated acid digestion protocol mentioned above) at 206 nm. Pure crotonic acid (Sigma Aldrich part number 113018), diluted in 5 mM H2SO4, was also analyzed to verify accurate crotonic acid retention time.

## Dilution and Aeration of Media

The spent and acetate-containing *S. ovata* medium was depressurized and exposed to air prior to *C. basilensis* inoculation. The bottle was loosely covered with a sterile cap and shaken gently for 12 h to aerate. If necessary, the medium was diluted with sterile water to a final acetate concentration of 25 mM. Cultures of the aerated medium (400 mL) were inoculated at 1% v/v with washed *C. basilensis* culture.

## Calculation of Carbon Yield and PHB Productivity

Poly(3-hydroxybutyrate) productivity was calculated by considering the peak rate of PHB synthesis during the 32-h growth period. The net mass of PHB was divided by the window of time within which it was produced to give units of g PHB $L^{-1}$ $hr^{-1}$. Carbon yield from acetate to PHB was derived from the ratio of acetate-carbon consumed to PHB-carbon produced within the window of peak PHB production rate. The units for this metric are reported as % and reflect the fraction of carbon atoms from acetate that were used for PHB synthesis.

## SEM Imaging

Culture aliquots were taken from the *S. ovata*/*C. basilensis* mixed culture at specific time points. These aliquots were supplemented with glutaraldehyde at 2.5% (v/v) and kept at room temperature overnight. The samples were washed and consecutively dehydrated with 10, 25, 50, 75, 90, and 100% ethanol each for 10 min. 50 μL of dehydrated samples were dropcasted on a 1 × 1 cm silicon substrate, allowed to dry and imaged with a Quanta 3D field-emission gun scanning electron microscopy (SEM) (FEI) operated at 10 kV accelerating voltage after gold sputtering.

## Modeling of *S. ovata* Growth and Acetate Production With Periodic Replenishment of $H_2/CO_2$

### Liquid Phase Mass/Mole Balances

Growth of cells can be modeled according to the standard design equation for a well-mixed batch bioreactor (Harvey and Blanch, 1997).

$$\frac{d\left(c_X V_L\right)}{dt} = \mu V_L c_X \tag{1}$$

where, $c_X$ is the concentration of cells, $V_L$ is the liquid volume of the reactor, and μ is the specific growth rate ($hr^{-1}$). We assume that acetate is a purely growth-associated product, resulting in:

$$\frac{d\left(c_{Ac} V_L\right)}{dt} = \alpha\mu V_L c_X \tag{2}$$

where, α is the molar ratio of growth-associated product formed to cells produced. Solubilized (in the liquid phase) $H_2$ and $CO_2$ are consumed during the production of biomass and acetate, and mass transfer from the gas phase occurs simultaneously. For $H_2$ in the liquid phase, the resulting balance is:

$$\frac{d\left(c_{H_2} V_L\right)}{dt} = V_L\left(-\frac{1}{Y'_{X/H_2}}\mu c_X - \frac{1}{Y'_{Ac/H_2}}\alpha\mu c_X\right) + V_L k_L a(c_{sat,H_2} - c_{H_2}) \tag{3}$$

where, $Y'_{X/H_2}$ is the molar biomass yield on $H_2$, $Y'_{Ac/H_2}$ is the acetate yield on $H_2$, $k_L a$ is the gas-liquid mass transfer rate ($hr^{-1}$) and $c_{sat,H_2}$ is the saturation concentration of $H_2$. The saturation concentration is given, to a reasonable approximation, by:

$$c_{sat,H_2} = H_{H_2} p_{H_2} \tag{4}$$

where, $H_{H_2}$ is Henry's constant for $H_2$ in water and $p_{H_2}$ is the partial pressure of $H_2$ in the gas phase.

Equations (3, 4) are also valid for $CO_2$:

$$\frac{d\left(c_{CO_2} V_L\right)}{dt} = V_L\left(-\frac{1}{Y'_{X/CO_2}}\mu c_X - \frac{1}{Y'_{Ac/CO_2}}\alpha\mu c_X\right) + V_L k_L a(c_{sat,CO_2} - c_{CO_2}) \tag{5}$$

$$c_{sat,CO_2} = H_{CO_2} p_{CO_2} \tag{6}$$

### Gas Phase Mole Balances

In the gas phase, $H_2$ and $CO_2$ are consumed by mass transfer into the liquid phase according to:

$$\frac{d\left(p_{H_2} V_G\right)}{dt} = -V_L k_L a(c_{sat,H_2} - c_{H_2})RT \tag{7}$$

and

$$\frac{d\left(p_{CO_2} V_G\right)}{dt} = -V_L k_L a(c_{sat,CO_2} - c_{CO_2})RT \tag{8}$$

Note that these equations assume the gas follows ideal behavior.

### Equation Coupling and Monod Growth Kinetics

We assume that the specific growth rate follows Monod kinetics. In this case, $\mu$ can be written as:

$$\mu = \mu_{max}\left(\frac{c_{H_2}}{K_{S,H_2}+c_{H_2}}\right)\left(\frac{c_{CO_2}}{K_{S,CO_2}+c_{CO_2}}\right) \quad (9)$$

Because both $V_L$ and $V_G$ are constant, the liquid phase mass/mole balances are:

$$\frac{dc_X}{dt} = \mu_{max}c_X\left(\frac{c_{H_2}}{K_{S,H_2}+c_{H_2}}\right)\left(\frac{c_{CO_2}}{K_{S,CO_2}+c_{CO_2}}\right) \quad (10)$$

$$\frac{dc_{Ac}}{dt} = \alpha\mu_{max}c_X\left(\frac{c_{H_2}}{K_{S,H_2}+c_{H_2}}\right)\left(\frac{c_{CO_2}}{K_{S,CO_2}+c_{CO_2}}\right) \quad (11)$$

$$\frac{dc_{H_2}}{dt} = \left(-\frac{1}{Y_{X/H_2}}\mu c_X - \frac{1}{Y_{Ac/H_2}}\alpha\mu c_X\right) + k_L a(c_{sat,H_2} - c_{H_2}) \quad (12)$$

$$\frac{dc_{CO_2}}{dt} = \left(-\frac{1}{Y_{X/CO_2}}\mu c_X - \frac{1}{Y_{Ac/CO_2}}\alpha\mu c_X\right) + k_L a(c_{sat,CO_2} - c_{CO_2}) \quad (13)$$

Mole balances in the gas phase result in:

$$\frac{dp_{H_2}}{dt} = -\frac{V_L}{V_G}k_L a(c_{sat,H_2} - c_{H_2})RT \quad (14)$$

and

$$\frac{dp_{CO_2}}{dt} = -\frac{V_L}{V_G}k_L a(c_{sat,CO_2} - c_{CO_2})RT \quad (15)$$

### Periodic Gas Phase Replenishment

To describe the periodic (24-h) replacement of the gas phase, we generalize the initial conditions for $H_2$ and $CO_2$ pressure as:

$$p_{H_2}(t = 24n) = p_{H_2,0}$$
$$p_{CO_2}(t = 24n) = p_{CO_2,0} \quad (16)$$

where, $n \in \mathbb{N}$ and $t$ has units of hrs.

## Modeling of *C. basiliensis* Growth and PHB Accumulation Fed by *S. ovata* Spent Medium

### Liquid Phase Mole Balances

We again use the standard bioreactor design equation:

$$\frac{d(c_X V_L)}{dt} = \mu_X V_L c_X \quad (17)$$

To describe the accumulation of *C. basiliensis* cells in the liquid volume. Following Mozumder et al. (2014) the accumulation of PHB is given as:

$$\frac{d(c_{PHB}V_L)}{dt} = \mu_{PHB}V_L c_X \quad (18)$$

Acetate is consumed both by biomass and PHB accumulation, resulting in:

$$\frac{d(c_{Ac}V_L)}{dt} = -\frac{1}{Y'_{X/Ac}}\mu_X V_L c_X - \frac{1}{Y'_{PHB/Ac}}\mu_{PHB}V_L c_X \quad (19)$$

Nitrogen is also consumed by biomass accumulation, described as:

$$\frac{d(c_N V_L)}{dt} = -\frac{1}{Y'_{X/N}}\mu_X V_L c_X \quad (20)$$

As above, the liquid volume is approximately constant throughout the duration of the experiment.

### Growth and PHB Accumulation Kinetics

We use acetate and nitrogen inhibition-modified Monod (Andrews–Haldane) kinetics to describe the kinetics of cell growth:

$$\mu_X = \mu_{max,X}\left(\frac{c_{Ac}}{c_{Ac}+K_{S,Ac}+\frac{c_{Ac}^2}{K_{I,Ac}}}\right)\left(\frac{c_N}{c_N+K_{S,N}+\frac{c_N^2}{K_{I,N}}}\right) \quad (21)$$

where, $K_{I,i}$ are inhibition constants. We modify Mozumder et al. (2014) to describe PHB accumulation:

$$\mu_{PHB} = \mu_{max,PHB}\left(\frac{c_{Ac}}{c_{Ac}+K_{S,Ac}+\frac{c_{Ac}^2}{K_{I,Ac}}}\right)\left[1-\left(\frac{f_{PHB}}{f_{PHB,max}}\right)^{\beta}\right]\left(\frac{K_{S,N}}{c_N+K_{S,N}}\right) \quad (22)$$

where, $f_{PHB,max}$ is the maximum PHB-to-biomass ratio. In this model we neglect $O_2$ because $O_2$ was fully saturated throughout the duration of the experiment. We also neglect the potential PHB consumption due to cell growth once the acetate source is exhausted because PHB is the intended product, so careful process design, e.g., whereby cells are harvested before PHB consumption occurs, can avoid this parasitic impact on PHB accumulation. All model parameters are compiled in **Supplementary Tables 2**, **3**.

# RESULTS

## Optimization of Base Medium for Bioprocess

We firstly cultured *S. ovata* autotrophically in balch-type 25 mL culture tubes with an 80/20% $H_2/CO_2$ headspace. In these conditions acetate generation rate amounts to 10.4 mmol $L^{-1}day^{-}$, a rate modestly higher than previous reports (Blanchet et al., 2015). We selected 25 mM acetate as the feedstock concentration for *C. basilensis*. This titer of acetate was achieved

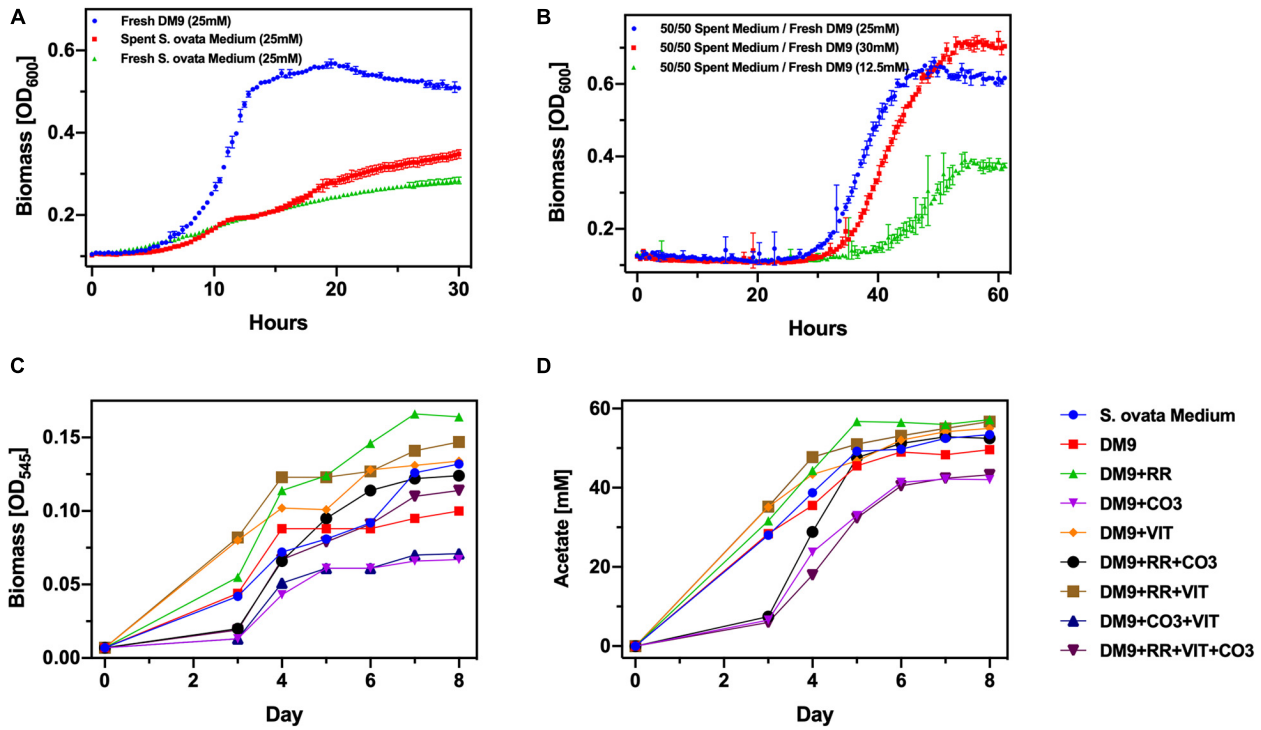


**FIGURE 2 |** Optimization of media compatibility **(A)** Biomass yield of *Cupriavidus basilensis* in DM9, and in spent and fresh *Sporomusa ovata* medium. **(B)** Biomass yield of *C. basilensis* in spent *S. ovata* medium diluted with fresh DM9 to a range of acetate concentrations. **(C)** Biomass and **(D)** acetate yield of *S. ovata* in DM9 with additional components (RR, reducing reagent; VIT, vitamins; $CO_3$, sodium bicarbonate) compared to those in *S. ovata* medium.

within the first 48–72 h of the autotrophic *S. ovata* culture. However, the *S. ovata* culture could reach a concentration of 50–60 mM acetate in 5–7 days without adjusting for pH which decreases due to acetate accumulation. The *S. ovata* cultures were stopped at 25 mM acetate and aerated which rendered the *S. ovata* inactive. *C. basilensis* was then inoculated in the spent *S. ovata* cultures (**Figure 2A**). The increase in biomass as detected by $OD_{600}$ was used to monitor the ability of *C. basilensis* to grow and use the $CO_2$-derived acetate. The baseline OD resulting from the presence of *S. ovata* biomass in the spent medium was deducted. As compared to *C. basilensis* grown in fresh DM9, which is the recommended *C. basilensis* culture medium, with synthetic acetate the biomass yield in the spent *S. ovata* medium was only 50% within the timeframe of the experiment. We employed a second control with cultured *C. basilensis* in fresh *S. ovata* medium (no prior *S. ovata*) with synthetic acetate. This culture achieved the same biomass yield as the one in the spent *S. ovata* medium indicating that acetate is not incompatible with *C. basilensis* but rather that a component in the *S. ovata* medium inhibits *C. basilensis* growth. In a second medium optimization experiment, we again cultured *S. ovata* autotrophically until the acetate concentration reached 25 mM. The spent *S. ovata* medium was diluted twofold with fresh DM9 containing synthetic acetate at 35, 25, and 0 mM. Thus the mixed media which were inoculated with *C. basilensis* contained total acetate concentrations of 30, 25, and 12.5 mM, respectively (**Figure 2B**). The biomass yield in the 25 mM culture equaled that of *C. basilensis* in fresh DM9 in the prior experiments. The biomass yields in the 30 and 12.5 mM were congruent with the available acetate. While adding fresh DM9 clearly enhanced *C. basilensis* growth, the cultures containing spent *S. ovata* medium had a 20 h longer lag phase. The peak optical densities were only reached in twice the length of time. Furthermore, it is burdensome to formulate two sets of media. Therefore, we attempted to grow *S. ovata* directly in deoxygenated DM9. We found that a 10% (v/v) inoculum is necessary when culturing *S. ovata* in DM9 whereas normally only a 5% (v/v) would be required. Next, we employed culturing controls in which we added the recommended *S. ovata* medium components to a DM9 base including reducing reagent, vitamins and carbonate (**Figures 2C,D**). Favorably *S. ovata* not only grows well in DM9 but also generates a similar amount of acetate as in the recommended *S. ovata* medium. Significant decreases in growth and acetate generation are only detected in the carbonate containing DM9 which could be a result of the increased osmotic pressure of the saline medium (Wood, 2015).

## Process Integration for PHB Production

Based on the results from the previous experiments we concluded to use DM9 as the base medium for our bioprocess. Before inoculating with *S. ovata*, we deoxygenated the DM9 medium. To gauge PHB productivity, we scaled up our process from 25 mL tubes to 1L balch-type bottles each with 270 mL of culture medium (**Figure 3A**). There was a significant scale-up associated

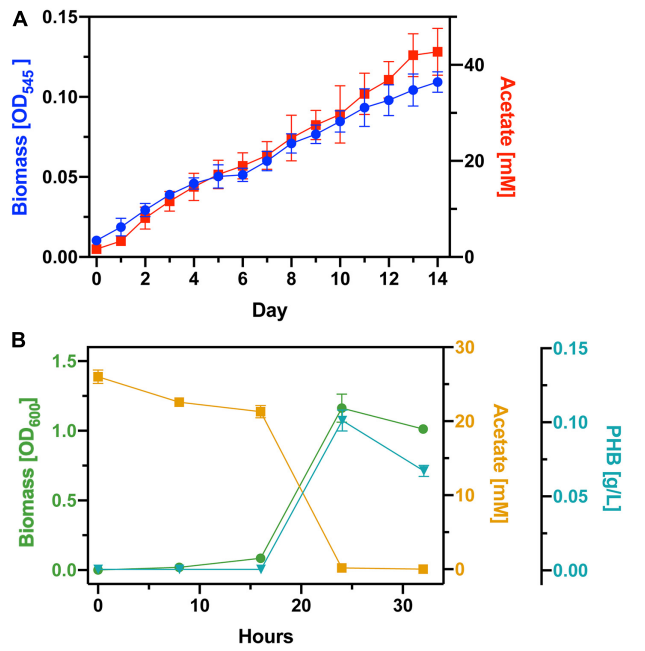


**FIGURE 3 |** Full bioprocess integration. **(A)** Cell growth and acetate generation by *S. ovata* in DM9 **(B)** Biomass yield, acetate consumption, and PHB accumulation by *C. basilensis* acetate rich spent DM9.

loss in acetate productivity with the cultures reaching 25 mM only after 8 days. The *S. ovata* cultures were grown for 14 days and the acetate concentration reached 42 mM. We proceeded to culture *C. basilensis* in the aerated spent DM9 which was diluted to 25 mM (**Figure 3B**). As expected, the acetate concentration decreases as biomass accumulates. The biomass yield achieved in this experiment was decidedly greater than that obtained in fresh DM9 in **Figure 2A**. Additionally, there is no protracted lag phase with the use of 100% DM9 as the base medium. Next, we determined that PHB correlates well with biomass production and we calculated a PHB generation rate of 12.6 mg $L^{-1}$ $hour^{-1}$ with an overall 11.06% carbon yield from acetate. After 24 h there is a decrease in the PHB concentration which correlates with the complete consumption of acetate. This could be due to the cells depleting PHB reserves to acquire carbon and energy.

## Qualitative Cell Population Analysis

Scanning electron microscopy has been established as a standard method to elucidate bacterial cell morphology. *S. ovata* is rod-shaped circa 4 μm long and 1 μm wide whereas *C. basilensis* is spherical to oval shaped and circa 2 μm in diameter. These differences in morphology allowed us to qualitatively determine the changing cell populations and establish that *C. basilensis* can grow in spent medium containing inactive *S. ovata*. We fixed culture samples at the 0 h time point just after inoculating the spent DM9 with *C. basilensis*. As *S. ovata* had achieved approximately 0.12 $OD_{545}$, we expected to mostly detect *S. ovata*. On the SEM micrographs rod-shaped *S. ovata* is abundantly visible with very few *C. basilensis* cells (**Figures 4A,B**). Additionally, we fixed culture samples at the 24 h time point after the exponential growth of *C. basilensis*. At this stage, *C. basilensis* cells clearly dominate the landscape (**Figures 4C,D**). *C. basilensis* closely co-exist with inactive *S. ovata* cells, evidently pointing to the fact that *S. ovata* cells are at least innocuous to *C. basilensis* and underscoring the ability to use spent *S. ovata* medium directly with minimal processing.

## Bioprocess Modeling

To determine process parameters that limit the production rate of PHB, we developed simple models of each step in the bioprocess: $H_2$-driven acetate production with *S. ovata* and acetate-driven PHB production with *C. basiliensis*. Experimental data of acetogenesis with *S. ovata* was fit well by adjusting the ratio of carbon diverted to acetate or biomass (α) and the gas-liquid mass transfer coefficient ($k_La$) (**Figures 5A,B**). Because $CO_2$ solubility (∼33 mM/bar) is much greater than $H_2$ solubility (∼0.78 mM/bar), this indicates that $H_2$ transfer from the gas phase to the liquid phase limits the acetate production rate. Modeled partial pressures in the gas phase (**Figure 5C**) and concentrations in the liquid phase (**Figure 5D**) support this conclusion: daily replenishment of the headspace maintains partial pressures of both substrate gasses at > 85% of their initial value. Liquid-phase $CO_2$ is similarly maintained at > ∼80% of its initial value. However, $H_2$ is nearly completely consumed in the liquid phase within ∼24 h and the concentration does not recover as the gas headspace is replenished, indicating that microbial growth and acetate production consumes $H_2$ nearly immediately as it is transferred into the liquid phase.

Experimental PHB production is similarly fit well to a simple Monod-like model of biomass growth and PHB accumulation (**Figures 6A–C**). Because specific biomass and PHB accumulation rates are determinant of the PHB production and acetate consumption rates, and acetate consumption is complete within ∼24 h, this portion of the integrated bioprocess does not limit overall productivity. Hence, $H_2$ gas-liquid mass transfer is the central bottleneck in the full process.

# DISCUSSION

Previous works have described the production of acetate from $CO_2$ by *S. ovata* and the production of PHB by closely related *Cupriavidus* strains from acetate (Kedia et al., 2014; Aryal et al., 2017). The traditional manufacturing approach to linking two such processes would be to purify the acetate intermediate between synthesis steps. This process is costly in the terrestrial industry and that cost would be prohibitively magnified in the context of space travel. Here, we have demonstrated the viability of serial biocatalyst culturing in a single media batch without processing of intermediate compounds.

Toward this goal, we sought to formulate a minimal medium that would support growth of both bacterial strains. It was determined that DM9 medium was suited for this task as it was already the preferred medium for *C. basilensis* growth and we observed only a minimal reduction of productivity for *S. ovata* when grown in DM9 compared to the traditional media for

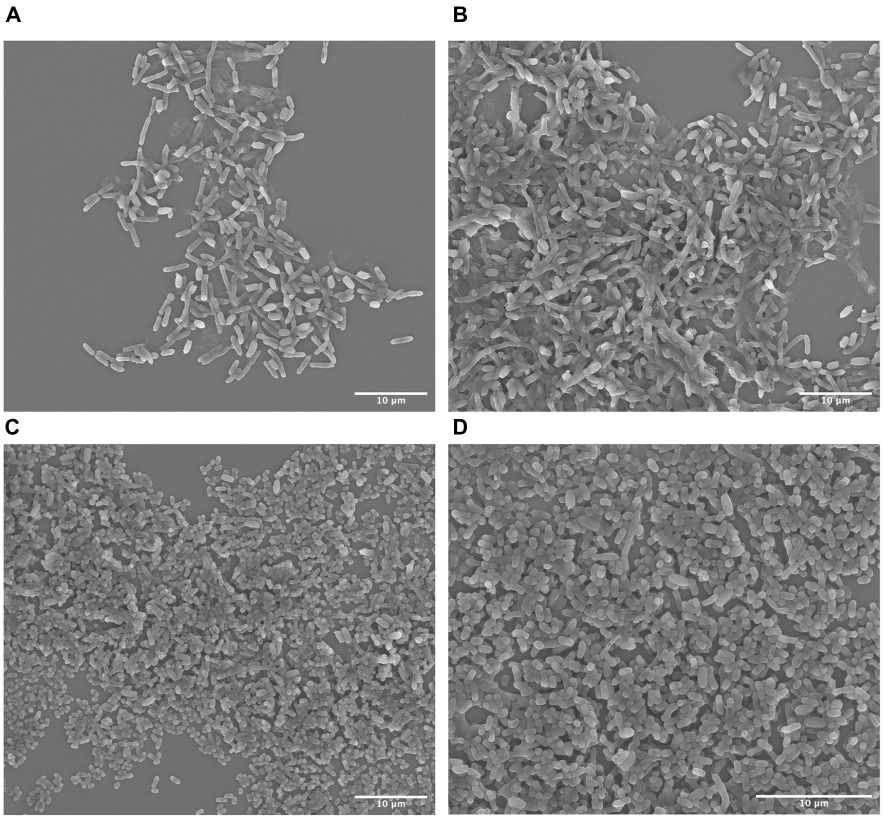


**FIGURE 4 |** Scanning electron micrographs of bioprocess. **(A,B)** 0 h time point post *Cupriavidus basilensis* inoculation in *S. ovata* containing spent DM9. **(D,C)** 24 h time point after *C. basilensis* exponential growth.

this strain. DM9 is an excellent media for space applications due to its extremely simple composition. The media as used here is primarily composed of phosphate buffer which serves to maintain a stable pH throughout bacterial growth. While pH regulation through buffering is common practice for bench-scale, batch reactors, it is highly uncommon and inefficient for large scale industrial settings. The alternative is continuous pH monitoring of the reaction and automated adjustment as needed with concentrated acids and/or bases (Jeevarajan et al., 2002). Therefore, our process in an industrial or space setting would require even fewer components.

Another component of the DM9 medium is the ammonium salt used as a nitrogen source for both strains. Fixed nitrogen could potentially be sourced from the Martian atmosphere as a nitrogen source is likely still required for *S. ovata* growth (Liu et al., 2017). However, it could also likely be favorable to reduce the nitrogen available to *C. basilensis*. It has been shown elsewhere that nutrient starvation, and especially nitrogen, is key to triggering the PHB accumulation mechanisms in *C. basilensis* strains (Mozumder et al., 2014). Therefore, sequestering a high density of active, PHB-poor cells into a nitrogen limited environment might improve the productivity and carbon-flux from acetate to PHB substantially.

We produced bio-acetate to a final concentration of ∼42 mM over the course of 14 days. By modeling this process, we can predict that the rate limiting step of acetogenesis is the gas to liquid transfer rate of $H_2$. As this experiment was only performed in shaken balch-type cell cultures bottles with pressurized headspace, there is significant opportunity for operational optimization to increase the gas-to-liquid transfer of $H_2$ from the headspace and thus the $k_La$ value representing this process. Such improvements may include agitation of the gas-liquid interface by stirring or bubbling. If the $k_La$ was increased even modestly to a value of 10, the time required to produce the equivalent mass of acetate would decrease by 75% (Supplementary Material section "Discussion"). Productivity in this stage of the process could be at least doubled in a flow-through, chemostat reactor setting, as opposed to batch, in which bio-acetate laden media is removed continually as it is diluted with fresh media. This approach increases efficiency by keeping the active cell number high and decreasing the need for a time-consuming lag phase in cell growth (Supplementary Material section "Discussion").

Next, the spent medium containing 42 mM acetate was diluted down to a final concentration of 25 mM acetate. The aeration of the medium at this step was done as a precaution against oxygen limiting conditions but optimization at this phase could prove this unnecessary, especially with the nearly two-fold dilution of the medium in presumably oxygenated fresh liquid. This minimal processing is the key operational cost reduction uncovered by this study.

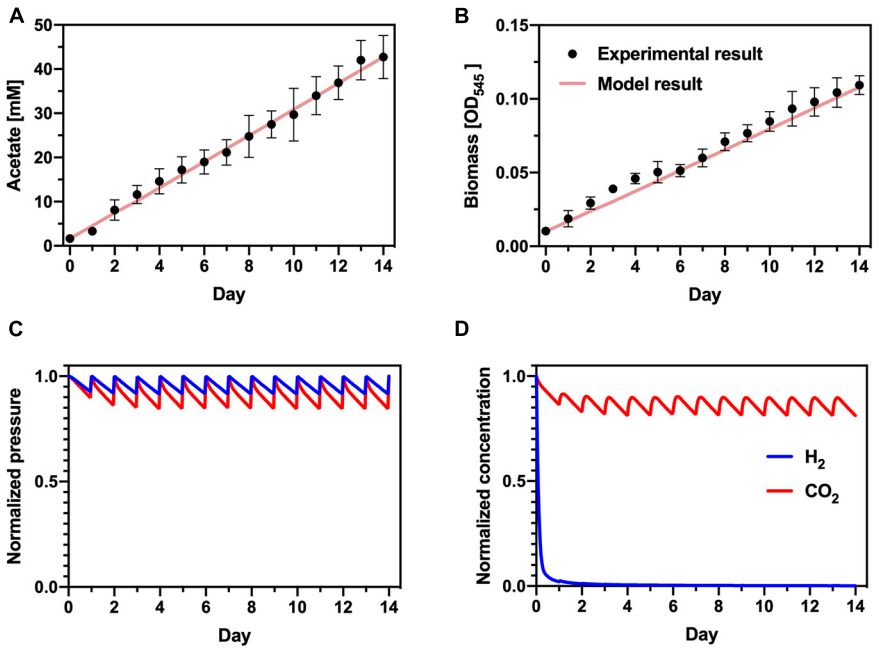


**FIGURE 5 |** Acetogenesis model. Comparison of experimental data and modeling results for **(A)** acetate and **(B)** biomass accumulation. **(C)** Calculated normalized $H_2$ and $CO_2$ partial pressures in the headspace of the bioreactor. **(D)** Calculated normalized dissolved concentrations of $H_2$ and $CO_2$ in the liquid phase of the acetogenesis bioreactor.

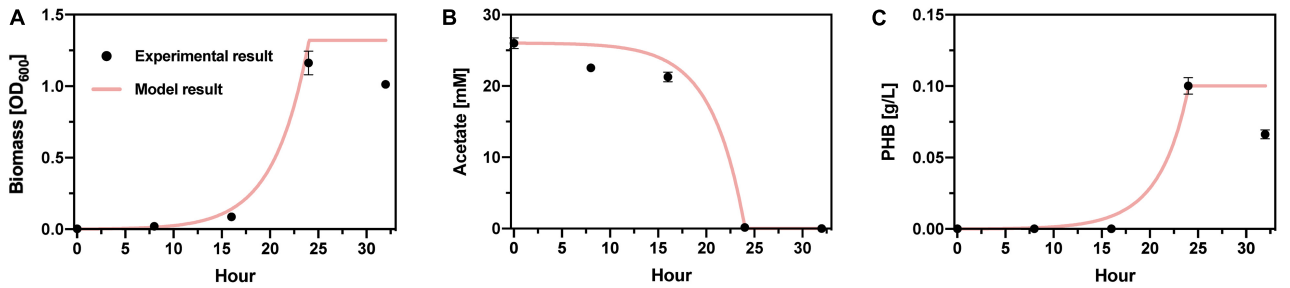


**FIGURE 6 |** *Cupriavidus basilensis* bioreactor model. Comparison of experimental and model results for **(A)** biomass yield, **(B)** acetate consumption, and **(C)** PHB accumulation.

It was demonstrated clearly that acetate is consumed proportionally to the PHB produced as it is presumed to be the only available carbon source for *C. basilensis*. This assumption is further enforced by the degradation of PHB which occurs after the acetate has been exhausted. In a biomanufacturing setting, acetate levels would need to be monitored so that the PHB could be harvested before it was repurposed as a fuel for biomass accumulation.

The productivity of *C. basilensis* in this study of 12.54 mg PHB $L^{-1}$ $hr^{-1}$ falls below demand of 1.55 g PHB $L^{-1}$ $hr^{-1}$ required for 3D printing an extraterrestrial habitat structure of 12 $m^3$ within a reasonable time frame (Menezes et al., 2015). In this demonstration experiment, optimization was not undertaken to improve this metric. The maximal PHB production rate of *Cupriavidus* species when grown in optimized conditions has been reported as high as 1.85 g $L^{-1}$ $hr^{-1}$ (Atlić et al., 2011). We hypothesize that, with additional strain and process optimization, productivity values for our media recycling approach should far exceed its present performance (Kedia et al., 2014; Li et al., 2019; Li and Wilkins, 2020).

Removal of the intermediate purification step between acetate production and PHB production offers both decreased system mass requirements and increased operational simplicity. Purification of acetate from aqueous solution is a delicate and energy intensive task most often performed either by expensive column separation or liquid-liquid extraction followed by distillation. These conventional industrial approaches would prove inefficient and inflexible for continued operation in the context of space travel and may increase the system mass of biological PHB production above the threshold mass of simply bringing pre-made PHB. The method presented here allows

for nearly "straight-pipe" integration of the bioprocesses and considerably decreases the complexity of this system.

Even with an increased productivity to 0.04 g PHB $L^{-1}$ $hr^{-1}$, the mass of media components required for generation of 1 kg of PHB would exceed that of simply bringing 1 kg of PHB from Earth. It was estimated that with our current media formulation, 12 kg of media components (primarily phosphate buffer) would be required to produce 1 kg PHB in 1 day (**Supplementary Table 1**). However, without the straight spent media utilization as presented, the process would require either a distillation column or nearly one ton of ethyl acetate for liquid extraction, assuming 1:1 solvent to media extraction. Altogether, our process affords nearly 98% weight savings as compared to a process involving liquid extraction of acetate. An attractive and thematically aligned approach would be subsequent reuse of media batches for further rounds of acetate and PHB production. While this method was not tested here, studies which determine the rate of nutrient depletion of the media so that it may be supplemented or assigned a maximum life cycle could decrease mass requirements for media significantly. An additional benefit of ISRU as it pertains to producing biopolymer for additive manufacturing is that astronauts would be able to alter the material on demand. A PHA with different material properties could be produced instead of relying on further deployments of goods to fulfill a pressing need.

In all, we have presented an approach for serial biomanufacturing with limited intermediate purification and processing steps. This streamlined approach reduces the need for costly materials that would need to be transported into space in order to support a biomanufacturing facility. Further optimizations of each biocatalyst step should be undertaken to maximize the cost-savings benefit of this process for production of PHB from $CO_2$. Lastly, there are additional considerations for microbial production of chemicals and materials in space that are currently under investigation including the effect of cosmic radiation on single-cell organisms, the effect of low gravity environments on $CO_2$ conversion and *in situ* resource utilization of Martian water for electrochemical processes.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

SC-B, SF, APA, and PY designed the experiments. SC-B and JMK carried out *S. ovata* cell work. SF and KBS performed *C. basilensis* culturing. AJA designed the models with experimental inputs. SC-B and SF co-wrote the manuscript with inputs from AJA and KBS. All authors discussed the results and revised the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.700010/full#supplementary-material

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# Influence of Martian Radiation-like Conditions on the Growth of *Secale cereale* and *Lepidium sativum*

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The Martian surface is constantly exposed to a high dose of cosmic radiation consisting of highly energetic particles and multiple types of ionizing radiation. The dose can increase temporarily by a factor of 50 through the occurrence of highly energetic solar flares. This may affect crop growth in greenhouses on the Martian surface possibly making settlement of humans more complicated. Shielding crops from radiation might be done at the expense of lighting efficiency. However, the most energy-efficient cultivation may be achieved through the use of natural daylight with the addition of LED lights. The goal of our research was to investigate whether Martian radiation, both the constant and the solar flares events, affects plant growth of two crop species, rye and garden cress. The levels of radiation received on the surface of Mars, simulated with an equivalent dose of $^{60}$Co $\gamma$-photons, had a significant negative effect on the growth of the two crop species. Although germination percentages were not affected by radiation, biomass growth was significantly decreased by 32% for cress and 48% for rye during the first 4 weeks after germination. Part of the biomass differences may be due to differences in temperature between radiation and control treatment, however it cannot explain the whole difference between the treatment and control. Coloring of leaves, necrosis and brown parts, was observed as well. Temporary increases in ionizing radiation dose at different development stages of the plants did not significantly influence the final dry weight of the crops.

**Keywords: radiation, Mars, *Secale cereale*, rye, *Lepidium sativum*, garden cress, regolith, food**

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## INTRODUCTION

The intention of the National Aeronautics and Space Administration (NASA) to send the first humans to Mars in the 2030's means that (semi-) permanent space communities will be set up at the Martian surface. An important task will be crop production, which is needed to provide the astronauts with sufficient fresh food (Horneck et al., 2006; Wamelink et al., 2014). There are several options to grow food on Mars. The first option is hydroponics. A second option is aeroponics (Maggi & Pallud, 2010). A third option is to use the regolith on Mars or the Moon for crop growth, the option used in this research. Wamelink et al. (2014) showed that different crop species are able to germinate and grow on Mars regolith simulant JSC 1A. However, crop

production on Mars will be more difficult compared to Earth due to the many adverse conditions present. One of those adverse conditions is the permanent higher ionizing radiation (IoR) on the Martian surface (Guo et al., 2017).

The source of IOR on the Martian surface is bipartite. The permanent part consists of galactic cosmic rays (GCRs) at a relatively low, constant flux (ICRP, 2013). Their composition varies slightly over time but in general 85–90% consist of protons (p), 10–13% of He-ions (α), 1% of electrons (e) and 1% of heavier nuclei (Hassler et al., 2013). Measurements done by the Mars Curiosity Rover in 2012 showed that the total amount of radiation received at the surface is 233 ± 12 μGy/d (Matthiä et al., 2017). This is approximately 17 times higher than the highest natural absorbed dose measured on the Earth's surface (UNSCEAR, 2008). Measurements in Skylab showed mean dose rates up to 860 μGy/day and Apollo 14 even up to 1,270 μGy/day (Benton & Benton, 2001). This absorbed dose (further referred to as IoR dose) is easily increased with a factor of 50 (Zeitlin et al., 2004) by the second, variable source of IoR, the solar energetic particles (SEPs). SEPs are primarily protons which are accelerated up to hundreds of MeV by sun flares and coronal mass ejections and their corresponding chocks (Hassler et al., 2013). SEP fluxes are relatively sporadic in nature and depend on the reversal of the Sun's magnetic field each one-half of the 22 years Hall cycle. SEPs have a mean duration of 4.13 days (Jun et al., 2007), but the duration of an individual SEP can vary from a few hours up to a week.

The SEPs and the GCRs together form a complex radiation environment which differ significantly from the radiation environment present at the Earth's surface. On Earth, radiation is a less influential factor for crop growth since the Earth's thick atmosphere and its strong magnetic field protects crops against high doses and UV-radiation (Jakosky & Phillips, 2001; Nicholson et al.,2005). Nevertheless, crops generally possess a high degree of resistance to single, short-term high IoR doses which are not present under natural circumstances; in the case of barley (*Hordeum vulgare*) seeds, a dose of X-rays of 500 Gy was lethal (Caldecott, 1955). Curiosity measurements on Mars showed a dose of 233 ± 12 μGy/d (Matthiä et al., 2017), which is magnitudes lower than the experiment by Caldecott.

Other experiments have shown that a single exposure to 500 Gy of $\gamma$-photons still resulted in 20% seedling survival by different Poaceae species (Kianian et al., 2016). Experiments with *Arabidopsis thaliana* showed that seeds displayed 100% survival under normal radiation conditions after exposure to $\gamma$-photons with a total dose of ±1800 Gy (Hase et al., 2017). Survival rates for doses obtained by radiation with ionizing particles was much lower. Single exposures to a dose of ±100 Gy obtained by using 15.8 MeV $u^{-1}$ neon ions resulted in only 40% survival. The survival of seeds exposed to 17.3 MeV $u^{-1}$ carbon ions with a total dose of 300 Gy was 17% (Hase et al., 2017).

Experiments assessing the effects of long-term IoR exposure are scarce and show that plants are able to deal with chronic doses which are factors higher than the Martian radiation dose of 233 μGy $day^{-1}$ (Van Hoeck et al., 2017). Therefore, it is assumed that the Martian IoR doses will not influence plant growth and development. However, as far as we know, this has never been tested. In particular, sudden dose increases, comparable to SEP events, have not been investigated yet.

In this study we investigated how non-ionizing radiations on the surface of Mars may affect the growth of rye (*Secale cereale*) and garden cress (*Lepidium sativum*). The goal of the experiment was to investigate whether or not germination and subsequent growth and development was influenced by the presence of a simulated Martian radiation environment. Both crops were sown in Mars regolith simulant and exposed to a constant Martian radiation dose during the course of the experiment. SEP-events were simulated on plants during different development stages to determine whether radiation changes over time affect plants.

# MATERIALS AND METHODS

## Plant Material

The experiment was carried out with two crop species *Secale cereale* and *Lepidium sativum*. Both species were used in earlier experiments on growing crops on Mars (and Moon) regolith simulants JSC 1A and MMS (Wamelink et al., 2014, 2019). Both plant species germinate quickly (normally within 24 h) have no seed dormancy, rapid growth and grew well on Mars regolith simulant JSC 1A (enriched with organic material) if nutrient solution was supplied (Wamelink et al., 2019). Seeds of both species were sown in pots located in six rectangular trays of 52 by 32 cm (**Figure 1A**). Each tray consisted of 84, 4 × 4 × 6 cm (l x w x d), pots located in seven columns and twelve rows. On the bottom of a pot a piece of paper was put to prevent leaking of soil/simulant. Trays with pots were placed in trays to collect leakage water.

Rye seeds were sown in the center of the 84 pots filled with potting soil (composition: Welkoop, 2017; www.welkoop.nl; Control Earth; CE rye) and in 84 pots filled with Mars regolith simulant (Mars regolith simulant JSC-1A; Control Mars; CM rye). Cress seeds were sown in two other trays in the same way (CE Cress & CM Cress), with two seeds per pot in opposing corners of each pot. All seeds were pushed gently into the soil/simulant without covering the seeds with the substrate. The CE and CM trays were placed in two similar fume hoods (90 × 70 × 95 cm) without radiation and functioned as the control experiments. Note that each of the two fume hoods only contained one plant species.

In the remaining two trays, consisting of 72 pots filled with Mars regolith simulant and 12 pots filled with potting soil, seeds were sown in the same way as in the control treatments, with each species in its own tray (**Figure 1B**). Each pot was randomly allocated to one Solar Energetic Particle (SEP) treatment. SEP treatment was applied to part of the seeds or germinated plants (see 2.3). Seeds sown in the pots with potting soil were marked with Earth (E) and were not imposed to a SEP event. Subsequently, the two trays were put in a fume hood containing a radiation field.

To sum up, we had per plant species:

- Control: 84 pots in potting soil and 84 pots in Mars regolith simulant.

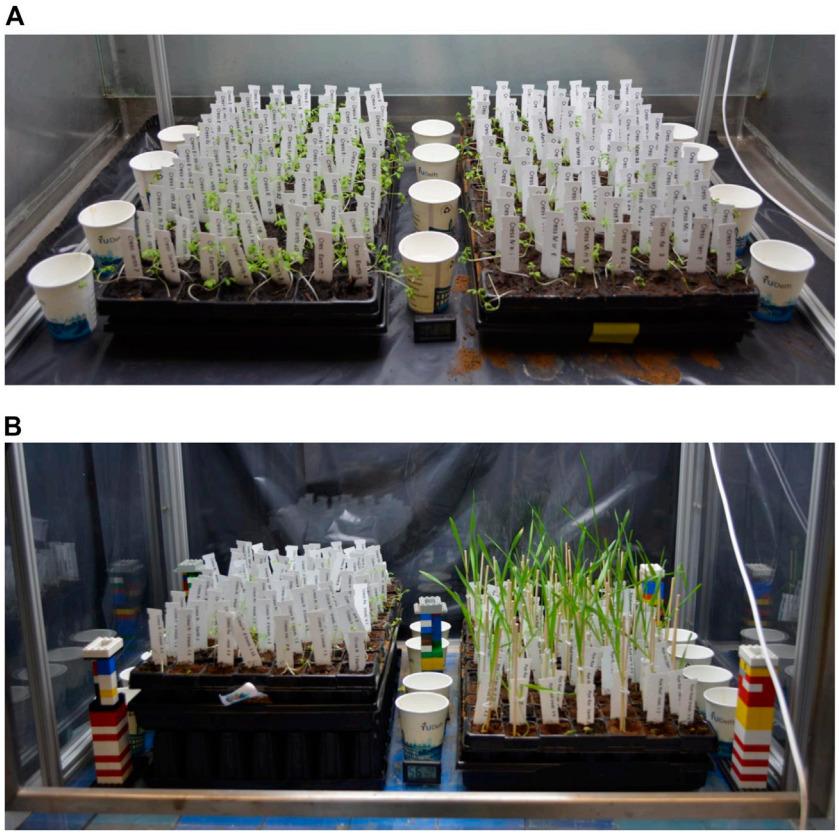


**FIGURE 1 | (A)** sowing the cress control Earth (CE, left) and control Mars (CM, right). Shown are the pots with trays, the water cups and the temperature and humidity meter located in between the two trays. **(B)** is showing the radiation treatments in the radiation fume hood surrounded by lead walls. Treatments of cress (left) and rye (right) are indicated with white labels. Lego towers hold thermoluminescent dosimetry (TLD) cups at the top. Pictures were taken on the morning of harvest.

- Radiation field: 72 pots in Mars regolith simulant treated with a SEP event (12 per treatment) and 12 pots in potting soil.

## Radiation Environment

A radiation environment was created in a Perspex fume hood (110 × 65 × 90 cm) surrounded by a 4.5 cm thick lead wall at three sides in order to protect the surrounding environment against the IoR used. An aluminum frame was placed in the fume hood. At 80 cm from the bottom surface of the fume hood wires were connected to the framework. To the wires five plastic capsules were attached in a five-point dice formation. The four capsules in the corners were attached at a distance of 5 cm in width and 7 cm in length from the corner edge. The fifth capsule was located exactly in the middle of the aluminum framework (**Supplementary Appendix 1**).

In 17 plastic High-Density Polyethylene (HDPE) type E cups, 25 mg of $^{59}$Co powder was placed. The cups were piled and put into a plastic irradiation capsule. The capsule was irradiated with a thermal neutron flux of 3.7*10$^{12}$ neutrons cm$^{-2}$ s$^{-1}$ for 5 h in the Befa-08 irradiation facility of the Reactor Institute Delft (RID): Delft, Netherlands (www.rid.tudelft.nl). This resulted in $^{60}$Co, and some other radionuclides as by-products. The yield of these radionuclides is very limited (<< 0.1%) and/or their half-life is much shorter than that of the $^{60}$Co and therefore did not influence our experiment. After one day of cooling, individual cups were transferred to five source holders. The four source holders in the corner received three irradiated cups each and the remaining five cups were put in the central source holder to create an even field. The total dose rate of the radiation environment at the growing tips of the plants was equal to 270.50 Gy d$^{-1}$ as measured by thermoluminescence dosimetry at five different locations in the radiation fume hood (**Supplementary Appendix 2**). The meters were placed in the fume hood below the five $^{60}$Co emitters and raised during the experiment to match the top of the plants (**Figure 1B**, the small 'Lego' towers). The exposure to the roots was not measured. The dose rate of 270.50 ± 25.31 μGy d$^{-1}$ was higher compared to the dose rate measured at the Martian surface of 233 ± 12 μGy/d (Matthiä et al., 2017), but close enough for our purpose. All irradiated plants were placed in the led walled fume hood.

## Solar Energetic Particle Events

SEP-events were simulated by imposing seeds, seedlings and plants to the beam of the $^{60}$Co source 8,290 of the RID with an equivalent calculated dose rate of on average 1,333 μGy h$^{-1}$. The time plants were exposed to the $^{60}$Co source slightly increased over the experiment due to decreasing activity of the $^{60}$Co source but equaled on average 22.51 h. The duration of each

**TABLE 1 |** Overview of imposed simulated Solar Energetic Particle (SEP) events per plant species ($n$ = 12 per treatment). A SEP-event was simulated by exposing the plant material 22.51 h to a $^{60}$Co-source with a dose rate of 1,333 μGy h$^{-1}$. Days are counted from the start of the experiment.

| Treatment code | Plant material | Day | Species | Day | Species |
|---|---|---|---|---|---|
| T1 | Seeds | 1 and 2 | *S. cereale* | 1 and 2 | *L. sativum* |
| T2 | Seedlings | 8 and 9 | *S. cereale* | 9 and 10 | *L. sativum* |
| T3 | Plants | 13 and 14 | *S. cereale* | 14 and 15 | *L. sativum* |
| T4 | Plants | 20 and 21 | *S. cereale* | 21 and 22 | *L. sativum* |
| T5 | Plants | 27 and 28 | *S. cereale* | 29 and 30 | *L. sativum* |

SEP-treatment was calculated by dividing the intended total dose of 30 mGy by the absorbed dose rate of the $^{60}$Co source at the moment of radiation. The total dose of 30 mGy was chosen based on the records of a real SEP-event on Mars (Zeitlin et al., 2004).

In our experiment one SEP-event consisted of a dose of on average 27.29 ± 1.17 mGy (**Supplementary Appendix 3**). The distance between the $^{60}$Co source and the plant material was 300 cm during each treatment to get the correct absorbed dose ($D$). 5 days before sowing, twelve seeds of both species (T1) were exposed to a simulated SEP-event. Subsequently, every week a new set of twelve plants (whole plants including the roots in the soil/simulant) of each species grown in the radiation environment was exposed to a simulated SEP-event, treatments T2-T5 (**Table 1**). Another twelve pots were marked with No-SEP (NSEP) and similar to the Earth (E) treatments they were never exposed to a simulated SEP-event. So, for every treatment we had twelve plants giving twelve independent responses per treatment.

During each SEP-event the total absorbed dose was measured by thermoluminescence dosimetry in quadruple. At the end of each SEP-event TLD cups were collected and stored till the end of the experiment. The final radiation dose was calculated by multiplying the amount of counts with a $^{60}$Co-conversion factor of 2.29 μGy count$^{-1}$. After exposure to a SEP-event, all plants were put back in the fume hood with the constant radiation environment at places similar to the places they stood before.

## Growth Conditions

All treatments were placed in fume hoods. This was not ideal for plant growth, but a necessity because of safety precautions related to the radiation treatment. In the two fume hoods used for the control treatments ventilation was shut down to improve environmental conditions regarding temperature and moisture content, which would be too low with the ventilation on. In the radiation fume hood ventilation was kept on, with an air speed of 0.25–0.45 m/s at the opening (according to NEN standard), because of radiation safety regulations.

The glass or Perspex windows of all fume hoods were covered with light-impermeable black garbage bags, to prevent light coming from the outside to influence the experiment. Lighting was done by PAN-KW-60–60 LED-lights (40W, white, 5000 K, 5630 SMD Samsung) with dimensions of 60 × 60 × 11 cm. The lights were adjusted to the aluminum frame at 85 cm above the pots, yielding around 2,200 Lux (or around 42 μmol/m$^2$/s) at pot level. A time switch was used to switch the lights on at seven AM and off at seven PM resulting in a 12 h photoperiod.

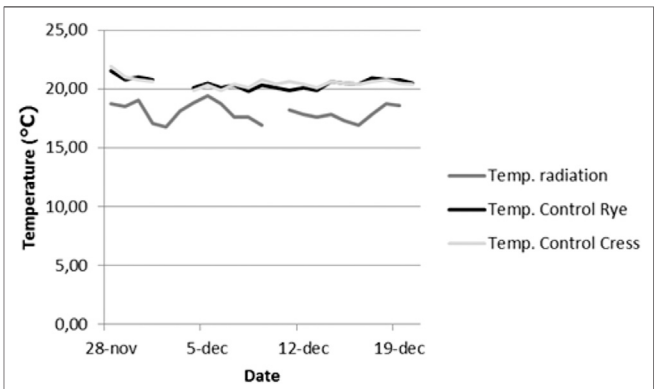


**FIGURE 2 |** Temperature changes over the course of the experiment for control and radiation fume hoods. Gaps in lines indicate missing data. Temperatures were obtained with a 2-in-1 humidity- and temperature measurement device (see also **Supplementary Appendix 4**).

Water was supplied as normal tap water. Once a week instead of water a standard nutrient solution was supplied (with EC 1.45 mS, pH 5.7). The solution was made by adding 25.2 L Zwakal, 44.2 L BFK, 14.4 L Baskal, 13.8 L Amnitra, 10.4 L Magnitra and 64 L Calsal to 100,000 L of water (standard nutrient solution at WUR, see also Wamelink et al., 2019). Plants were watered daily with paper cups to keep the soil/simulant moist, excessive water was stored in a second tray underneath the tray with the pots. Spraying was used to increase humidity. Average air humidity was 50 ± 8% for control experiments and 47 ± 3% for the radiation experiment and do not differ significantly overall (**Supplementary Appendix 4**). Once a week instead of water a standard nutrient solution was supplied (with EC 1.45 mS, pH 5.7). The solution was made by adding 25.2 L Zwakal, 44.2 L BFK, 14.4 L Baskal, 13.8 L Amnitra, 10.4 L Magnitra and 64 L Calsal to 100,000 L of water (standard nutrient solution at WUR, see also Wamelink et al., 2019). Average air temperature was 20.5 ± 0.4 C for the control experiments and 18.0 ± 0.8 C for the radiation experiment (**Figure 2**). Air temperature and humidity were measured daily in all fume hoods with a 2-in-1 humidity- and thermometer (www.woodandtools.com, ref. number WT1485393632).

## Data

The germination rate was determined daily during the experiment and final germination percentages were calculated. Differences between germination rates for the various treatments

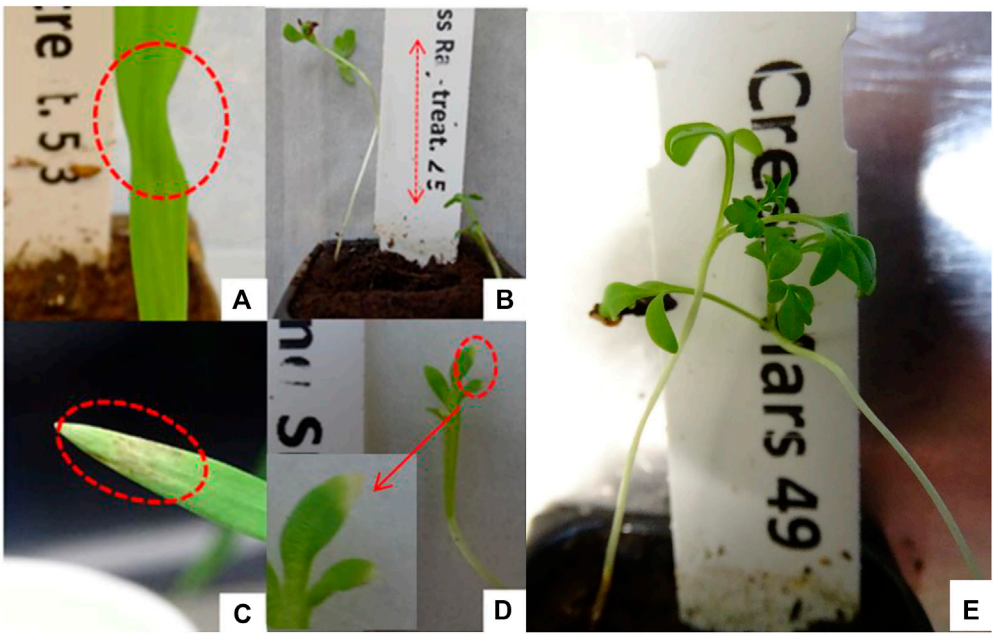


**FIGURE 3 |** Pictures of individual plants showing the observed malformations in rye **(A)** and cress **(B)** and the abnormal coloring of leaves for both rye **(C)** and cress **(D)**. **(E)** gives 'normal' non-irradiated cress plants on Mars regolith simulant.

were pairwise tested by means of *t*-test. After 28 days, plants were harvested and dry weight (DW) was determined after two days of oven (Marius Nieuwegein. 380 v, 8 KW) drying at 70 C. DW was statistically analyzed by means of regression from which *p*-values for pairwise differences were obtained using a Fisher exact test by calculations in Genstat (VSN International, 2020). Differences were assumed significant when $p < 0.01$ in all tests performed.

# RESULTS

## Germination

For both rye and cress, the highest germination percentage (98.8%) was observed in the CE treatments. Lowest germination percentage for rye was found for the T5 treatment (**Table 1**, 75.0%). For cress, the lowest germination percentage (91.7%) was found for T1, T3, T4 and E (**Supplementary Appendix 5**). Pairwise comparisons of germination percentages proved no significant differences among treatments ($n = 12$ per treatment; 12 Earth control and six SEP events on Mars regolith simulant of 12 replicas, $p < 0.01$; **Supplementary Appendix 6**). There is only an indication for a difference in rye between T5 and CE ($p = 0.011$; **Supplementary Appendix 6**).

Germination under a Martian radiation dose did not significantly differ from germination in a non-radiation environment for both rye and cress. The Control and Martian germination percentages were all above 96%. Only for some specific SEP radiation treatments germination of rye dropped below 90%. These were not statistically different from other treatments, also due to the small number of seeds ($n = 12$, $p = 0.01$) used for the SEP treatments. Experiments with more seeds are required to detect germination differences.

## Visible Coloring and Malformation of Leaves

For both rye and cress, remarkable malformations and coloring of leaves were observed for irradiated plants. Malformation included divergent leaf form (**Figure 3A**) and lagging of length growth without malformation of leaves (**Figure 3B**). Coloring was mainly observed at leaf tips for both crops (**Figures 3C,D**). Colors varied from light green to yellow or brown and necrosis varied from 1 mm to approximately 2 cm. Coloring and malformation occurred for all radiated plants on both Earth potting soil and Mars regolith simulant. No malformations or colorings were observed in the non-irradiated pots.

## Dry Weight

For rye, the highest mean DW was found for the Control Earth (CE) treatment, 0.0414 g ($n = 12$, **Figure 4A** and **Supplementary Appendix 7A**). This mean differed significantly ($p = 0.000$) from the Control Mars (CM) treatment mean of 0.0215 g. DW of both CE and CM differed significantly with treatments T1-T5 and NSEP (**Supplementary Appendix 7A**). Treatment Earth (CE) differed significantly with all other treatments except CM. The treatments T1-T5 and NSEP did not differ significantly among each other.

For cress, the mean DWs of CE (0.00767 g) and CM (0.00523 g) differed significantly ($n = 12$ per treatment, $p < 0.01$, **Figure 4B** and **Supplementary Appendix 7B**). All radiation treatments differed significantly from the CE treatment, but only T3, T4 and T5 did differ significantly from the CM treatment. The lowest treatment mean, 0.00264 g, was

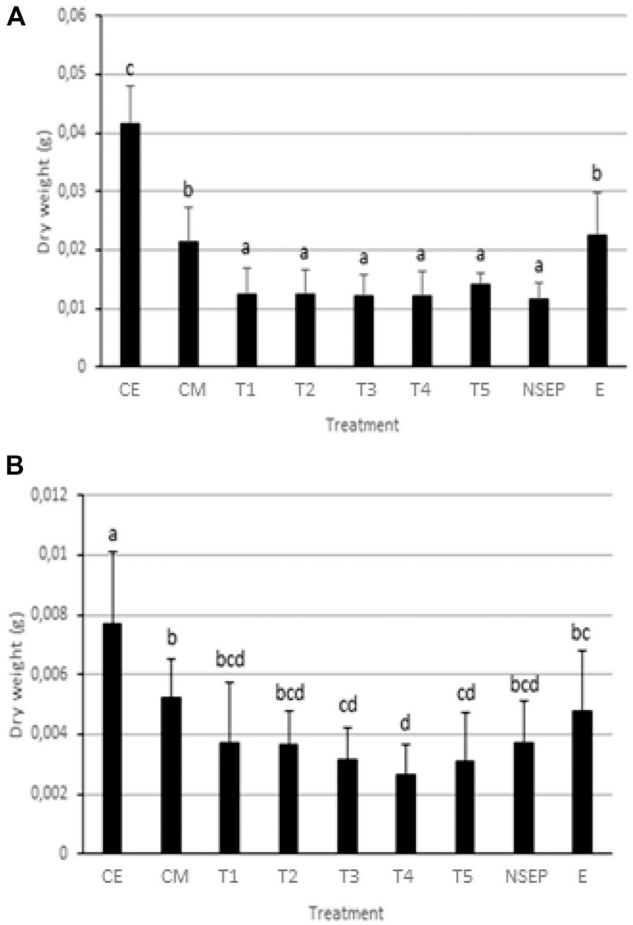


**FIGURE 4 |** Mean dry weight (DW) of Secale cereale (rye) **(A)** and Lepidium sativum (cress) **(B)** in different treatments, with $n = 12$ per treatment. CE = Control Earth (Potting soil), CM = Control Mars regolith simulant, T1 = SEP 5 days before sowing, T2 = 4 days after sowing (a.s.), T3 = 7 days a. s., T4 = 14 days a. s., T5 = 21 days a. s., NS = No SEP, E = Earth (Potting soil), No SEP. Significant differences ($p < 0.01$) between treatments are indicated by different letters plotted above the bars. Error bars represent positive standard errors of the treatment mean.

found in the T4 treatment and differed significantly from CE, CM and E. No significant difference was found among E, CM and the radiation treatments (**Supplementary Appendices 7B, 8**).

# DISCUSSION

## Germination

The results we obtained are in agreement with germination experiments of Hui et al. (2017). They showed that wheat seeds (*Triticum aestivum* L.) irradiated with doses varying from 646 to 1,060 μGy $s^{-1}$, displayed germination percentages varying from 96 to 99%. An explanation for this high germination percentage can be found in the upregulation of the reactive oxygen species (ROS) scavenging processes. Under natural conditions, these processes are needed to compensate for the negative effects of the increased ROS production, inherent to the reactivation of the metabolic system during germination (Bailly, 2004). The ROS formed due to IoR can be scavenged by these processes as well, limiting the negative effects of IoR on plants. For *Arabidopsis thaliana* and *Raphanus sativus* seeds it was shown that for IoR up to 1 kGy almost all seeds germinated. Above this dose scavenging of ROS was not efficient enough to protect the seeds against the high concentrations of ROS (Kumagai et al., 2000). Since the maximum total dose obtained by seeds in our experiment was ±35 mGy, our results are in agreement with these observations.

## Physiological and Morphological Reactions

The continues low dose radiation showed leaf malformation, leaf coloring and dwarf growth for irradiated individual plants. Tobacco plants exposed to relative low doses showed that malformation was more severe for the lowest doses compared to higher doses (Koeppe et al., 1970). At higher doses cessation of growth was observed, explaining the lack of new malformed leaves. Low chronic doses of gamma-photons also influenced leaf shape and coloring in *Curcuma alismatifolia*. A possible explanation may be that the accumulation of ROS induces more double strand breakage, leading to genetic chimerism in the plant (Taheri et al., 2016). In future experiments, chronic low dose radiation experiments should therefore include ROS-concentration measurements.

Leaf coloring in both rye and cress may have been caused by IoR damaging the photosynthetic system. Rice leaves exposed to a very low dose of 5.34 μGy $d^{-1}$ for 3 or 4 days already showed a decrease of greenness of the leaves (Rakwal et al., 2009). Moreover, stress-related accumulation of two major rice phytoalexins, sakuranetin and momilactone A, was shown by Rakwal et al. (2009). Those phytoalexins are involved in the regulation of the ROS concentration in leaves and may protect the plant against the negative consequences of ROS. Related phytoalexins are, as well, involved in the initiation process of apoptosis (Pervaiz, 2004), which may explain the brown necrosis observed in our experiment. ROS mostly occurs at higher doses of radiation, in our case only for the SEP events. A low dose of ionizing radiation normally produces too few reactive oxygen particles to directly affect antioxidant concentrations in cells (Smith et al., 2012).

## Dry Weight

The significant decrease of DW for both crops under IoR conditions is unexpected since earlier experiments did not show detrimental effects on plant growth and development with doses even higher than the doses in our experiment (part of the difference could be due to the set-up, giving differences in temperature, see below). Sheppard et al. (1982) showed that chronic exposure of *Pinus sylvestris L.* did not influence total DW of seedlings at doses till 0.7 mGy $h^{-1}$. Severe impairment of growth and total biomass was only observed at a dose rate of 7.0 mGy $h^{-1}$, which is more than 600 times higher compared to the dose rate of 0.011 mGy $h^{-1}$ in our experiment. However, results comparable with our experiment were reported by Chandorkar and Clark, (1986). They showed that

photosynthesis of *Pinus strobus L.* and *Pinus sylvestris L.* seedlings exposed for 150 days to gamma dose rate of 0.1015 mGy $h^{-1}$ was reduced by 16–19%. Also, the respiration rate was lowered by about 14–23% and the amount of 80%-ethanol soluble sugar content decreased with 14–25%. Overall results included smaller seedlings with a decreased weight and a more compact phenotype (i.e., plants were more compact). Both were also observed in our experiment.

## Temperature, Humidity and Light

Due to radiation safety regulations the control was placed in a separate room. This resulted in temperature differences between the control experiments and the radiation experiment. Overall differences in humidity were not significant (50 ± 8% and 47 ± 3%). Differences for temperature were significant (20.5 ± 0.4°C and 18.0 ± 0.8°C, $p < 0.001$). The temperature differences may significantly influence the total DW accumulated by plants due to the increased metabolic speed at higher temperatures (Berry & Björkman, 1980). Optimum growth temperature for rye, as given by the FAO (Acevedo et al., 2002), is between 12 and 25 C. According to experiments carried out by Yamori et al. (2006) a deviation of 2.5 C causes a decrease of photosynthesis of 11.6% at most.

There may be an effect of temperature on the growth in our experiment resulting in lower DW. But it is uncertain if it can explain the differences found in this experiment. The effect of temperature and air flow in the fume hoods may also influence moisture content of the soil/simulant and thus the growth. In a later conducted experiment under the same circumstances, it showed that there was almost no difference in moist content of the soil and simulant for both fume hoods (see further Pouwels, 2019). Air flow may also significantly influence the photosynthesis rate (Kitaya et al., 2012), the effects are not as such monitored in this project.

The used LED-lights yielded 42 μmol/$m^2$/s at the pot level. This is rather low for the standards in 2021. The panels were chosen also because they had to be fit in the lead walls to contain the radiation. This was at the moment of the experiment (2017) the optimal LED panel. Nowadays smaller and more powerful LED lights are available and that would be our choice. Nevertheless, the less powerful lights may have influenced the growth of the plants and looks to be visible in the elongated growth of the garden cress. All treatments had the same light conditions and most likely the results of the experiment will not be influenced. But, an interaction between treatment and light intensity can not be excluded.

## Radiation Conditions

The intended daily radiation was to 233 ± 12 μGy $d^{-1}$, as measured by the Mars Curiosity Rover between 15 November and 15 January in the Gale crater (Matthiä et al., 2017). The thermoluminescence dosimeters (TLDs) showed that the average dose in our experiment was 270.5 μGy $d^{-1}$. This implies that during the 26 experimental days plants received ±975 μGy more than on average on Mars.

Moreover, the dose to which our plants were exposed was obtained by employing high-energetic $^{60}$Co $\gamma$-photons. This neglects the composition of the Martian radiation spectrum, consisting of 85–90% protons, 10–13% of He-ions, 1% of electrons and 1% of heavier nuclei (Hassler et al., 2013). The lack of high energetic particle radiation may have caused the damage observed in the plants to be less severe than can be expected at the Martian surface. Especially, since plants appear to be less resistant to particle radiation compared to photon radiation, due to local energy disposure (Hase et al., 2017). Plants may partly be protected against those particles by using polymers rich in low Z-materials, such as hydrogen (Nambiar & Yeow, 2012). However, such materials are not sufficient to protect against the highly energetic $\gamma$-photon rays present, which may cause a significant decrease in DW, as shown in our experiment.

The DW decrease we showed as a result of the Martian radiation dose is a major concern for future civilization on Mars, since this indicates that crop growth at the Martian surface, indoors in a greenhouse, may not be very successful. Yield will be limited resulting in the need for large-area cultivation, which is economically and technically difficult, due to the likely scarce resources, e.g., construction materials and essential plant resources. Therefore, crop cultivation is most likely to succeed at locations under the Martian surface where the Martian regolith will provide protection against the galactic cosmic rays (GCR) and the SEP-events.

# CONCLUSION

Crops grown at the Martian surface will constantly be exposed to a radiation dose 17 times higher compared to Earth. This dose of Martian radiation, simulated with $^{60}$Co $\gamma$-photons, could have a significant negative effect on two crop species, rye and garden cress. During the first 4 weeks after germination biomass production was almost halved for both cress and rye and visible coloring of the leaves for both crops was observed.

Germination was not significantly influenced by long-term exposure to ionizing radiation. A temporary increase in the dose of radiation, in order to simulate Martian SEP-events, at different developmental stages of the young plants did not significantly influence the final dry weight of the crops.

# DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

# AUTHOR CONTRIBUTIONS

NT did day to day work and wrote the MS. GW, AD, MS, HH and HW overviewed the experiment and reviewed the MS. GW and MS were direct supervisors. GW designed the original set up and came up with the idea. PG carried out the statistics.

## ACKNOWLEDGMENTS

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fspas.2021.665649/full#supplementary-material

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# Ground Demonstration of the Use of *Limnospira indica* for Air Revitalization in a Bioregenerative Life-Support System Setup: Effect of Non-Nitrified Urine–Derived Nitrogen Sources

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Long-duration human space missions require considerable amounts of water, oxygen, and nutritious biomass. Additionally, the space vehicles must be well equipped to deal with metabolic human waste. It is therefore important to develop life-support systems which make these missions self-sufficient in terms of water, food, and oxygen production as well as waste management. One such solution is the employment of regenerative life-support systems that use biological and chemical/physical processes to recycle crew waste, revitalize air, and produce water and food. Photosynthetic cyanobacteria *Limnospira* could play a significant role in meeting these objectives. *Limnospira* can metabolize $CO_2$ and nitrogen-rich human waste to produce oxygen and edible biomass. So far, life-support system studies have mainly focused on using chemical/physical methods to recycle water, degrade human waste, and recycle $CO_2$ into oxygen. Nowadays, additional microbial processes are considered, such as nitrification of urea–ammonium–rich human waste and then using the nitrate for cyanobacterial cultivation and air vitalization. This cascade of multiple processes tends to increase the complexity of the life-support systems. The possibility of using non-nitrified urine for *Limnospira* cultivation can partially solve these issues. Our previous studies have shown that it is possible to cultivate *Limnospira* with urea and ammonium, the prominent nitrogen forms present in non-nitrified urine. In this study, we investigated the possibility of cultivating *Limnospira* with the different nitrogen forms present in non-nitrified urine and also evaluated their effect on the oxygen production capacity of *Limnospira*. For this 35-day-long study, we worked on a simplified version of the European Space Agency's MELiSSA. During this ground demonstration study, we monitored the effect of urea and ammonium (vs. nitrate) on the oxygen production capacity of *Limnospira*. A deterministic control law, developed and validated on the basis of a stochastic light-transfer model, modulated (increase/decrease) the incident light on the photobioreactor (with *Limnospira*) to control oxygen levels in the closed loop. The $CO_2$ from the mouse compartment was recycled as a carbon source for *Limnospira*. We observed that while the system could meet the desired oxygen levels of 20.3% under the

nitrate and urea regime, it could only reach a maximum $O_2$ level of 19.5% under the ammonium regime.

**Keywords: regenerative life-support system, crewed space exploration, *Limnospira indica*, oxygen production, nitrogen and carbon waste recycle, MELiSSA loop**

## INTRODUCTION

Current space missions mainly rely on physical/chemical processes for water recycling and air revitalization. Food and other consumables are periodically resupplied from Earth. However, constraints associated with the payload, launch technologies, and distance would drastically restrict such resupplies for future space missions to distant planets like Mars. Depending on the orbital distance and the synodic period, a one-way trip to Mars can take around 300 days (Portee, 2001). An average person needs 15–20 kg of essential supplies per day in terms of $O_2$, food, drinking water, and hygiene water (Farges et al., 2008; Anderson et al., 2018). This effectively means that around 90–120 ton of essential supplies would be needed for a crew of 6.0 people for a 1,000-day return mission to Mars. Lowering this payload will be an asset for the long-duration human space missions. Although certain chemical methods are currently used to recycle water and $O_2$ on board space flights, food and other essential supplies have to be periodically resupplied from Earth. Furthermore, provisions must be made to safely deal with human metabolic waste, and efforts must be made to reduce the dependence on physical and chemical waste treatment processes. It is therefore important to look for practical alternatives to increase the self-sustainability of long-term human space missions in terms of food production, air revitalization, waste management, and water recycling.

Several international space agencies and research organizations have been investigating the possibility of cultivating photosynthetic species (microalgae, cyanobacteria, and plants) on board space flights for food production, air revitalization, waste management, and water recycling (Karel, 1982; MacElroy and Bredt, 1984; MacElroy et al., 1987; Mergaey et al., 1988; Gitelson et al., 1989; Lasseur et al., 1996; Kibe et al., 1997; Perchonok et al., 2001; Farges et al., 2008; Lasseur et al., 2010; Hader, 2019; Helisch et al., 2020; Zhang et al., 2020).

The erstwhile USSR (now Russia) was one of the pioneers that worked on microalgae-based regenerative life-support systems (LSSs) (Gitelson et al., 1989). Their LSS mainly had four compartments: one for algae (*Chlorella* sp.), one for the crew, and two for plants (wheat and vegetables). The first test on this system (1972) mainly focused on water and $CO_2$ recycling and lasted for 180 days. For this study, food (for human consumption) was sourced externally, and no provision was made for metabolic waste treatment within the LSS.

The National Aeronautics and Space Administration (NASA), United States, started to work on their microalgae-based regenerative LSS under the project name Controlled Ecological Life Support System (CELSS) (MacElroy and Bredt, 1984; Averner, 1990). The CELSS was conceived with the aim of producing edible biomass from plants and microalgae (*Chlorella* sp.). The system also aimed to purify water, revitalize air, and recycle waste under closed-loop conditions (MacElroy et al., 1987). However, the CELSS project was soon discarded, and NASA decided to focus their life-support research on the plant compartment under the project name VEGGIE (NASA Techport, 2017; Massa et al., 2018; Monje et al., 2020). Nowadays, NASA is exploring the possibility of using their VEGGIE compartment for the cocultivation of plants and the microalgae *C. reinhardtii*. NASA researchers have recently been working on the development of bioprocesses and cultivation systems that can protect the microalgae from the inhibitory effects of microgravity and harmful radiations (Monje et al., 2020). Zhang et al. (2020) used molecular techniques to design tissue bag–based cultivation systems for the cultivation of UV–resistant mutants of the microalgae *C. reinhardtii* in the VEGGIE growth chamber.

The Japanese Aerospace Exploration Agency (JAXA) is also investigating the possibility of including photosynthetic microalgae (*Chlamydomonas* sp.) for waste recycling and production of food and $O_2$ under the umbrella of their regenerative LSS project, the Closed Ecology Experiment Facility (CEEF) (Kibe et al., 1997; Kage et al., 2011; Kage et al., 2013).

The European Space Agency (ESA), through its MELiSSA (Micro Ecological Life Support System Alternative) project, is currently involved in the development of a self-sustainable regenerative LSS project. Structurally, MELiSSA is inspired by the function of a natural lake ecosystem. The five compartments of the MELiSSA loop (see **Figure 1A**, De Meur, 2017) mimic the various functions of the lake ecosystem. Briefly, the first compartment (CI), which is inhabited by consortia of thermophilic bacteria, degrades the complex human waste and inedible plant biomass to volatile fatty acids (VFAs), carbon dioxide ($CO_2$), and ammonium ($NH_4^+$). These VFAs are then further oxidized to $CO_2$ and $NH_4^+$ in the second compartment (CII) inhabited by the purple photosynthetic bacteria *Rhodospirillum rubrum*. The nitrifying bacteria in the third compartment (CIII) then oxidize $NH_4^+$ to nitrate ($NO_3^-$), which is then used as the nitrogen (N) source along with $CO_2$ for cultivation of the cyanobacteria *Limnospira indica* (formerly *Arthrospira* sp. PCC 8005) and plants in the producer compartments CIVa and CIVb, respectively. The $O_2$, water, and edible biomass produced in the producer compartments are consumed by the crew in the consumer compartment.

MELiSSA scientists and engineers have conducted several studies in the past to investigate the efficiency and performance of the interconnectivity of two or more compartments under closed-loop conditions (Demey et al., 2000; Alemany et al., 2019). These terrestrial studies, also known as ground demonstration (GD) studies, were based on

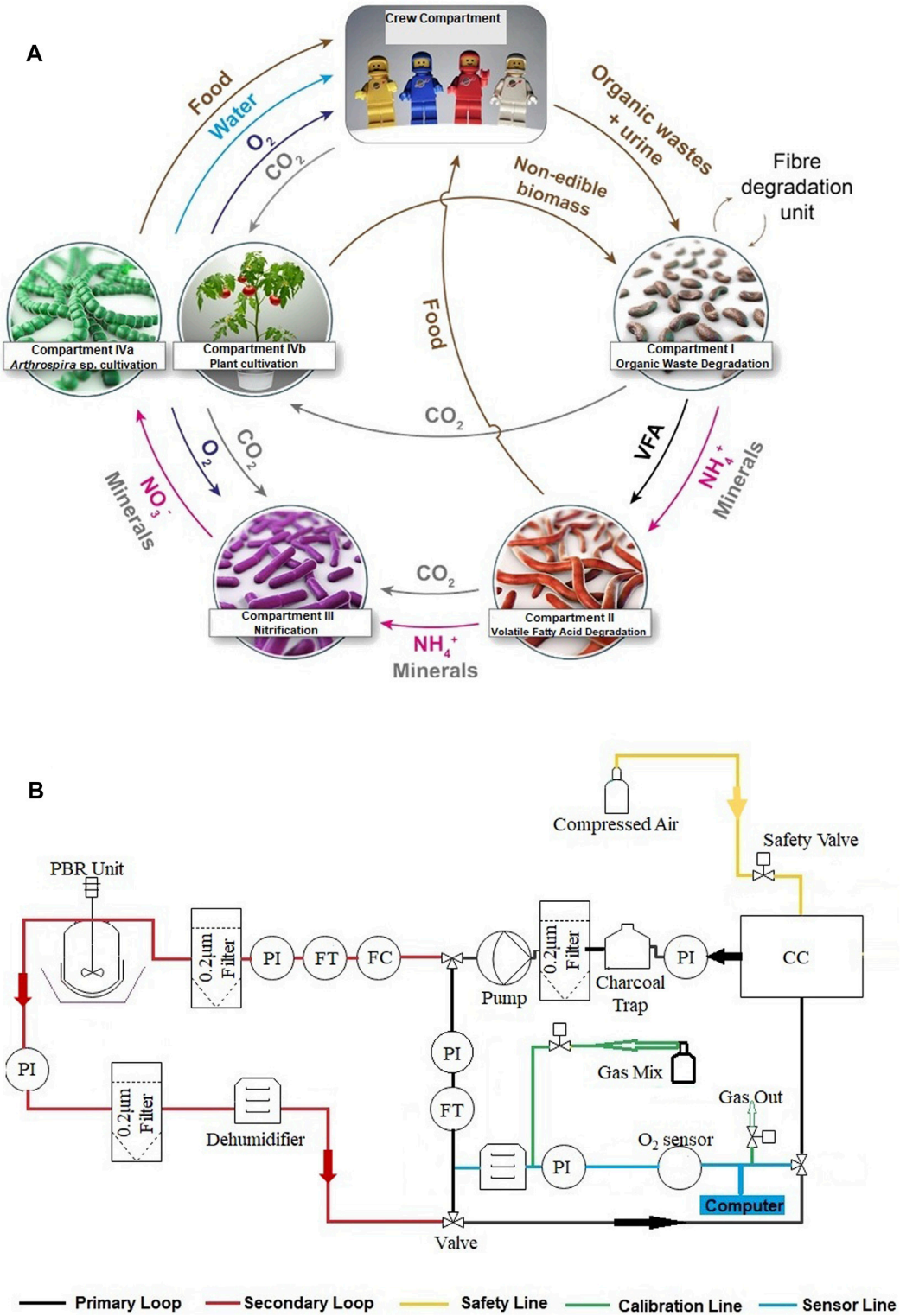


**FIGURE 1 | (A)** Schematic of the Micro Ecological Life Support System Alternative (MELiSSA) loop. **(B)** Schematic of the breadboard layout used for the ground demonstration study. PBR unit, photobioreactor plus the lightening unit; PI, pressure monitor; FT, flow transmitter; FC, flow controller; CC, consumer compartment; $O_2$, oxygen sensor.

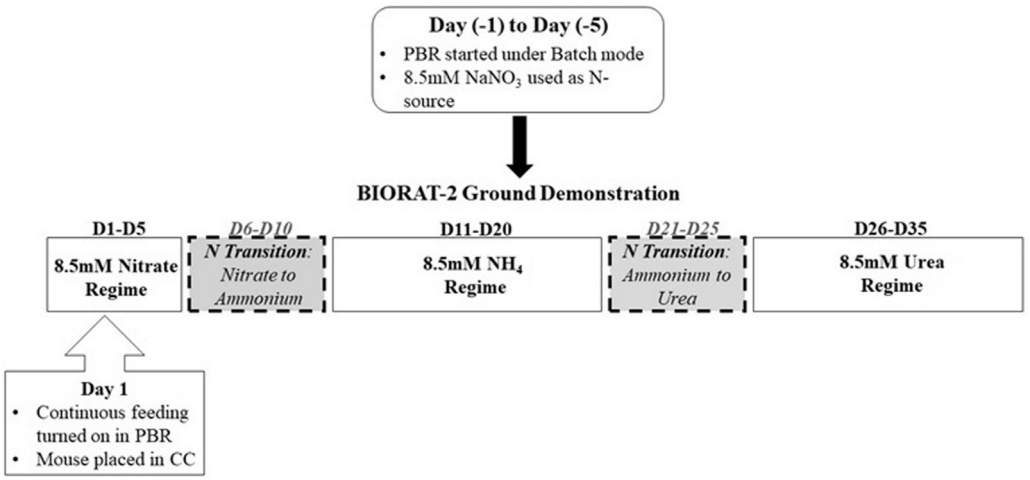


**FIGURE 2 |** Schematic of the timelines and study protocol adopted for the ground demonstration study.

the premise that the nitrification compartment (CIII) will convert urea and $NH_4^+$ to $NO_3^-$, which will then be used for cyanobacterial cultivation in the producer compartment. However, the suboptimal operation of the nitrification compartment could result in incomplete nitrification. This, in turn, would result in the transfer of urea- and $NH_4^+$-rich waste streams into compartment CIVa. Therefore, it is necessary to evaluate the possibility of using partially nitrified or even non-nitrified urine (urea and $NH_4^+$ rich) for cyanobacterial cultivation and understand their effect on the biomass yield, biomass composition/quality (biochemistry), and $O_2$ productivity.

Several studies have been conducted in the past to evaluate the effect of different N-sources ($NH_4^+$, $NO_3^-$, $NO_2^-$, and urea), their feeding mode (batch, fed batch, or continuous), and concentrations on the overall yield and quality/composition of *Limnospira* biomass (Sassano et al., 2004; Avila-Leon et al., 2012; Markou, 2015; Deschoenmaeker et al., 2017; Sachdeva et al., 2018a; Sachdeva et al., 2018b). These studies have shown that compared to $NO_3^-$, urea enhances the protein and pigment content in the *Limnospira* biomass (Sassano et al., 2004; Avila-Leon et al., 2012 and Sachdeva et al., 2018b). In contrast, $NH_4^+$ feeding has been reported to increase the lipid and exopolysaccharide content in the *Limnospira* biomass while decreasing biomass yield (Sassano et al., 2010; Markou et al., 2014; Deschoenmaeker et al., 2017; Sachdeva et al., 2018a; Sachdeva et al., 2018b). These previous studies have hence established the following: 1) it is possible to cultivate *Limnospira* with alternative N-sources and 2) changing the N-concentration, N–source type, and feeding mode could modify the biochemical composition and biomass yield of *Limnospira*.

However, none of these studies had evaluated the effect of the alternative N-sources on the $O_2$ production and air revitalization potential of *Limnospira*. In order to fill this gap, we conducted the present GD study, wherein we evaluated the $O_2$ production capacity and air revitalization characteristics of *Limnospira* under closed-loop conditions of the MELiSSA LSS. For this study, *Limnospira* was cultivated in a photobioreactor (PBR)/producer compartment, and the mouse, housed in a closed animal cage, was used as the consumer. The producer compartment and the consumer compartment were connected through a closed and airtight gas loop. The complete setup (breadboard) is described under **Figure 1B**.

The $O_2$ produced in the producer compartment was used for air revitalization in the consumer compartment, and the $CO_2$ from the consumer compartment was used as the carbon source for cyanobacterial cultivation in the producer compartment. A stochastic light-transfer–based model developed in our previous study (Sachdeva et al., 2018a) was used to define a deterministic control law which controlled the $O_2$ levels in the producer compartment by modulating the incident light flux on the PBR. As part of study protocol (**Figure 2**), the N-sources in the cyanobacterial feeding medium were changed between $NO_3^-$, $NH_4^+$, and urea.

The preliminary data obtained through this 35-day-long GD study clearly demonstrated the following: 1) it is possible to couple the producer compartment and the consumer compartment under the closed-loop conditions for air revitalization and 2) it is possible to use different N-forms (present in untreated urine) for cyanobacterial cultivation and $O_2$ production.

## MATERIALS AND METHODS

### System Setup: Connections Between the Compartments and Data Monitoring

The basic layout of the GD breadboard is represented in **Figure 1B**. The producer compartment and the consumer compartment were connected *via* a closed gas loop. The EU directive 2010/63/EU mandates a minimum of 10 air exchanges per hour for an animal compartment. Accordingly, a minimum air flow rate of 55.0 L/h was required for this 11.5-L cage. On the other hand, the gas flow rate in the PBR had to be maintained

below 3.0 L/h to avoid cell shearing and culture foaming. This PBR flow rate was decided as per the results of our preliminary tests (data not shown). To accommodate two separate gas flow rates, the gas loop was bifurcated into the primary and secondary loops.

The primary loop mainly consisted of the consumer compartment, pressure monitor (PI), flow transmitter (FT), and flow controller (FC). The gas flow rate in the primary loop was maintained at 55 L/h using an external pump. The presence of the activated charcoal trap and 0.2-micron filter prevented the transmission of microbes and ammonia from the consumer compartment to the gas loop (in the primary loop). The consumer compartment outlet was connected to a sensor line, which continuously monitored the $O_2$ levels in molar percentage. This sensor line consisted of a dehumidifying bottle, a flow controller, and an $O_2$ sensor. The $O_2$ sensor (9212-5AD, Analox Sensor Technology, United States) used for this study was found to be sensitive to high gas flow rates. Therefore, the gas flow rate in the sensor line was controlled at 100 ml/min.

A calibration line was also provided along the sensor line to ensure periodic calibration of the $O_2$ sensor without opening the gas loop. Two gas mixes were used: 1) 99.99% $N_2$ (for zero) and 2) 21% $O_2$, 1.0% $CO_2$, and 78% $N_2$ (similar to the atmospheric $O_2$ composition). A safety line (safety valve) was also connected to the consumer compartment outlet. This safety valve ensured the opening of the closed gas loop in case the $O_2$ levels in the consumer compartment increased or decreased beyond the safety range (17.5–24% $O_2$). The other end of this safety line was connected to a compressed air cylinder that ensured air supply to the consumer compartment in the case of system failure.

The secondary loop consisted of the producer compartment (the PBR and lightening unit). The manual valves, flow transmitter, and low controller were provided throughout the secondary loop to control and monitor the gas flow. The control law (see below) modulated the incident light flux of this lightening system that is attached to the PBR. The outlet of the PBR was connected to a dehumidifying bottle, PI, and 0.2-micron filter.

## Cyanobacterial Culture and Photobioreactor Setup

The stock culture of the *Limnospira indica* (previously *Arthrospira* sp. PCC 8005) strain was maintained under axenic conditions in Cogne-Modified Zarrouk Medium (Cogne et al., 2003) with 8.5 mM $NaNO_3$ as the N-source (Sachdeva et al., 2018b). For the GD test, *Limnospira indica* was cultivated in a 2.0-L double-jacked cylindrical PBR (Biostat®, Sartorius AG, Germany). The PBR was radially illuminated using 14 cool-white light bulbs (4000 K, 12 V, Philips), and the culture was mixed at 150 rpm using a Rushton turbine. The PBR working volume, dilution rate, and incident light flux were selected on the basis of the respiratory requirements of the 21.4-gm mouse used as the consumer for this study (see the following section). The details of the criteria used to define these working parameters have been defined in the study by Demey et al. (2000) and will not be discussed here. This setup (the PBR culture vessel and lightening unit) was termed as the producer compartment. *Limnospira indica* was cultivated at a controlled temperature (36°C) and pH (8.5) in carbonate- and bicarbonate-free Zarrouk medium. $CO_2$ from the consumer compartment served as the carbon source for *Limnospira*. pH was controlled using 0.5 N HCl and 1.0 N NaOH. Based on the study protocol (**Figure 2**), the N-source (8.5 mM each) in the feeding medium was changed between $NaNO_3$, $NH_4Cl$, and urea. A pH value of 8.5 was chosen to avoid loss of $NH_4^+$ as $NH_3$ (Sachdeva et al., 2018a). Under the current cultivation conditions (pH, dilution rate, and biomass concentrations), $CO_2$/carbon limitation was not foreseen. This simulation is based on the results of a previous MELiSSA LSS study (Demey et al., 2000) and hence is not discussed here.

Prior to the start of the GD study, the PBR was maintained under the batch mode (with 8.5 mM $NaNO_3$) for five days [Day (−1) to Day (−5)]. Once the culture reached an optical density (at 750 nm) of 1.5 ± 0.2, the continuous feeding was started, and this day was termed as Day (D) 1 of the GD study. The dilution/feeding rate for the continuous feeding was fixed at 0.2 (volume) per day to maintain the culture OD at 1.5 ± 0.2. This dilution rate and reference OD were adopted on the basis of the results of our previous study (Sachdeva et al., 2018a), which was used to define the control law used for this GD study (see following sections). Based on this dilution rate, one residence time was defined at 5 days. The mouse was put in the consumer compartment on D1 of the GD study.

The nitrate regime was used as the control for this study, and accordingly, the PBR was maintained under the continuous feeding of 8.5 mM $NaNO_3$ for five days (D1–D5). This period is termed as the $NO_3^-$ regime.

The first transition to 8.5 mM $NH_4Cl$ was performed on D6. The first five days (D6–D10) of the $NO_3^-$-to-$NH_4^+$ transition were excluded from the $NH_4^+$ regime (**Supplementary Figure 1**) to avoid quantitative bias (see the discussion section for more details). Consequently, D11–D20 were designated under the $NH_4^+$ regime. Similarly, for the urea regime, the first five days of the $NH_4^+$-to-urea transition (D21–D25) were excluded from quantitative analysis, and D26–D35 were considered under the urea regime.

## Test Animal and Consumer Compartment

An eight-week-old C57BL/6J male mouse was used as the consumer for this GD study. The research work (on the mouse) for this study was conducted in accordance with the ethical committee agreement LA 1500024 (Project Code: RI-10-01) dated August 28, 2018.

The mouse weighed 21.2 gm at the beginning of the study. The mouse was housed in a stainless steel cage (the consumer compartment), which had a total volume of 11.5 L. The design of the cage was compliant with the EU Directive 2010/63/EU.

Internally, the cage was separated into two parts by a perforated slide. While the mouse occupied the upper compartment, the lower compartment was layered with dried wooden shavings. The perforated slide between the upper and lower compartments enabled the mouse urine and feces to fall into the lower compartment (containing the wooden shavings), to

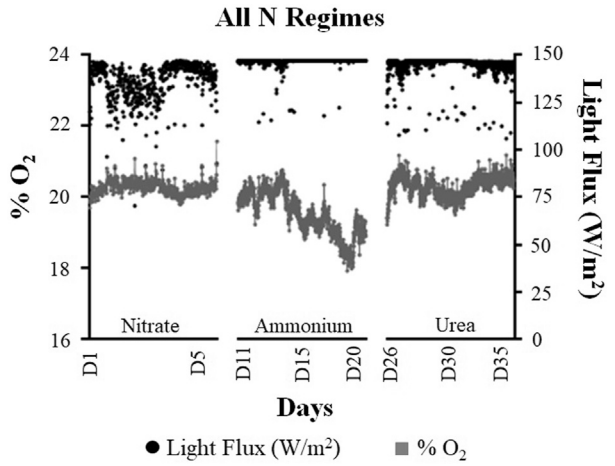


**FIGURE 3 |** Comparison of the average oxygen (% $O_2$) and light flux ($W/m^2$) values quantified during the three nitrogen regimes of the ground demonstration study. For the graphical representation of data, the values have been reported as the 15-min average of each analyte.

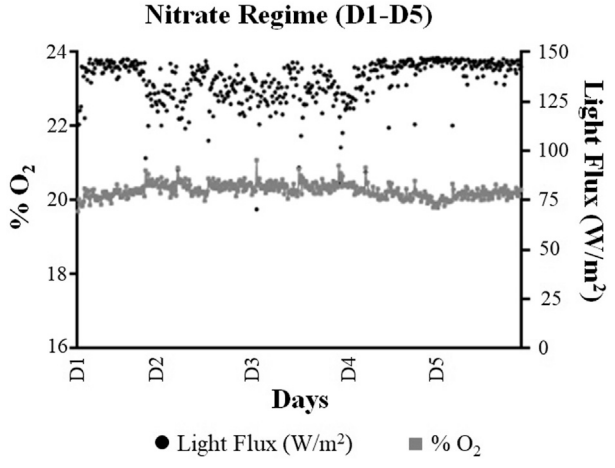


**FIGURE 4 |** Comparison of the average oxygen (% $O_2$) and light flux ($W/m^2$) values quantified using the monitoring system during the nitrate regime (Day 1–Day 5). For the graphical representation of data, the values have been reported as the 15-min average of each analyte.

facilitate moisture absorption and maintain proper sanitation in the upper compartment occupied by the mouse. This enabled a smooth, nonstop running of the GD study for 35 days without the need to open the cage for cleaning and sanitization. The exterior of the cage had an inlet and an outlet for gas exchange.

The food and water requirements of the mouse were calculated as per its weight before the start of the experiment. An eight-week-old C57BL/6J male mouse normally requires 8–10 ml water and 3.5–4.5 gm of food per day (Bachmanov et al., 2002). A mouse requires 3.3 gm $O_2$/kg.h (vs. 5,144 gm $O_2$/kg.h for an average human (Demey et al., 2000). Food and water were provided *ad libitum*. A 500-ml drinking water bottle was connected to the cage *via* a drinking nipple. Pre-weighed food pellets were provided through the food chute, located on the side of the cage. An automated light switch in the cage ensured that the mouse was exposed to a 12:12 h dark–light cycle. One cool fluorescent light bulb (3000 K, 36 V, GE) was used to illuminate the consumer compartment. This selection was made on the basis of animal study protocol directives. The CFL light bulb used in animal housing had no direct contact with or exposure to the PBR unit used for cyanobacterial cultivation.

Prior to the start of the GD study, the mouse was allowed to acclimatize to its new environment. For this acclimatization period, the mouse was housed in the cage for 8 h per day for a total period of 5.0 days. During this acclimatization period, we monitored the mouse for behavioral changes and response to the pump noise, temperature, new feeding and drinking mode, etc. No data were collected during this period.

The gender of the mouse was not foreseen to affect the study outcome. Only one mouse was used for this study, mainly as per the EU Directive 2010/63/EU and clauses of the ethical committee agreement used to conduct this animal study.

## Control Law and Safety System

The control law used for this study was trained and validated in accordance with the updated light-transfer model (Photosim 2.0 model hereafter) reported by Sachdeva et al. (2018a). The Photosim 2.0 model and its prediction parameters had already been tested and validated in the context of the cultivation parameters used for this GD study and thus would not be discussed here.

The control law also ensured safety within the closed loop in case of system failure. It controlled the opening/closing of the safety valve in accordance with the defined safety parameters. The safety parameters were defined at < 17.5% and ˃ 24% for $O_2$. In a scenario where the $O_2$ levels in the loop were higher or lower than these safety limits, the control law automatically opened the safety valve attached to the consumer compartment and ensured the supply of compressed air to the animal compartment to avoid animal distress.

## Data Collection and Analysis

The Arduino program recorded $O_2$ in molar percentage (%$O_2$) and incident light flux ($W/m^2$) once every 5.0 s. For the graphical representation, each data point on the graph is reported as the average of 15 min. The paired *t*-test was used to calculate the *p*-value.

# RESULTS

## Nitrate Regime

A total of 553 readings (for the light flux and $O_2$ level) were recorded under the $NO_3^-$ regime (D1–D5). Overall, the system applied an average light flux of 136 $W/m^2$ to maintain the average $O_2$ levels of 20.3% over the five days (D1–D5) of the $NO_3^-$ regime (**Figures 3**, **4**). Out of these 553 recorded readings, only 34 readings had $O_2$ levels less than 20%. The minimum $O_2$ level of 19.9% was observed for only 3 (recorded) readings during the

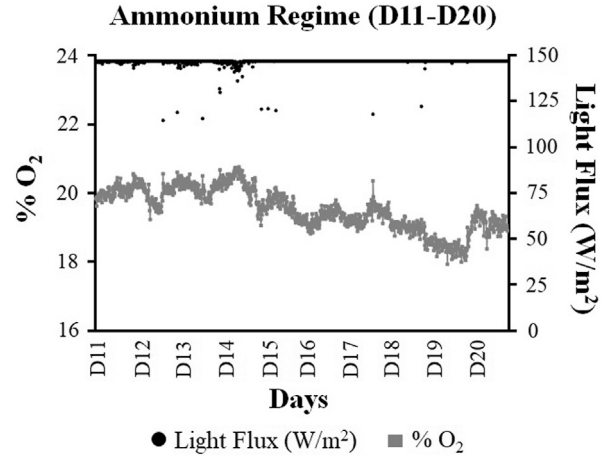


**FIGURE 5 |** Comparison of the average oxygen (% $O_2$) and light flux ($W/m^2$) values quantified using the monitoring system during the ammonium regime of the ground demonstration (Day 11–Day 20). For the graphical representation of data, the values have been reported as the 15-min average of each analyte.

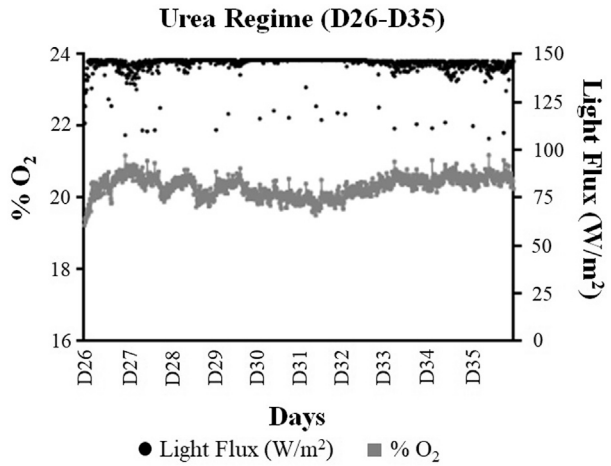


**FIGURE 6 |** Comparison of the average oxygen (% $O_2$) and light flux ($W/m^2$) values obtained during the urea regime of the ground demonstration (Day 26–Day 33). For the graphical representation of data, the values have been reported as the 15-min average of each analyte.

five days of the $NO_3^-$ regime, where the system was seen to reach the illumination limit (in terms of light flux) of 147.1 $W/m^2$. The system reached the highest $O_2$ level of 21.1% under the $NO_3^-$ regime.

## Ammonium Regime

The first transition to $NH_4^+$ was performed on D6 of the GD (**Figure 2**). Since the system was replacing residual $NO_3^-$ with $NH_4^+$, the first five days (D6–D10) of the $NO_3^-$-to-$NH_4^+$ transition were not considered under the $NH_4^+$ regime. Consequently, D11–D20 were considered under the $NH_4^+$ regime (**Figures 3**, **5**).

A total of 1,155 readings (for the light flux and $O_2$ level) were recorded under the $NH_4^+$ regime. The system was found to work under the illumination limit (147.1 $W/m^2$ light flux) on 82.9% of the recordings (958 out of 1,155 recordings) and could reach the $O_2$ set point (20.3% or higher) for only 9% of these recorded values. Overall, the system maintained an average $O_2$ level of 19.5% during the ten days of the $NH_4^+$ regime, under an average light flux of 146.6 $W/m^2$. The highest and lowest $O_2$ levels observed under the $NH_4^+$ regime were, respectively, 20.5% (a light flux of 114.7 $W/m^2$) and 17.9% (a light flux 147.1 $W/m^2$).

Interestingly, the $O_2$ levels were found to decrease toward the latter half of the $NH_4^+$ regime (**Figure 5**). The $O_2$ decreased significantly ($p$-value < 0.05) from an average of 20% (D11–D15) to 19% over the last five days (D16–D20) of the $NH_4^+$ regime.

## Urea Regime

The transition to urea was performed on D21 of the GD. As was done for the $NH_4^+$ transition, the first five days (D21–D25) of the $NH_4^+$-to-urea transition were not considered for analytical purposes. Consequently, D26–D35 were considered under the urea regime.

A total of 1,190 readings were recorded under the urea regime. The system maintained an average $O_2$ level of 20.3% over the 10 days of the urea regime and applied an average light flux of 146 $W/m^2$ (**Figures 3**, **6**). The system applied the highest possible light flux (147.1 $W/m^2$) on 34.6% of the observed timepoints (412 out of 1,190). The highest and lowest reached $O_2$ levels observed under the urea regime were 21.2% (light flux 106 $W/m^2$) and 19.2% (light flux 123.21 $W/m^2$), respectively.

In contrast to the $NH_4^+$ regime, where the $O_2$ levels decreased toward the latter half of the $NH_4^+$ regime, the $O_2$ levels in fact increased toward the end of the urea regime (**Figures 3**, **6**). While the system reached the average $O_2$ levels of 20% during the first five days (D26–D30) of the urea regime, this value increased to 20.4% ($p$-value < 0.05) toward the last five days (D31–D35) of the urea regime.

## Impact on Mouse

At the end of the GD study, the mouse was taken out of the consumer compartment, and its health and activities were observed for one week. The mouse weighed 21.2 gm before the start of the study, and it weighed 21.4 gm at the end of the study. There were no visible signs of stress or injury on the body of the mouse, and it responded well to stimuli. In general, no behavioral changes were observed in the mouse after the GD test.

# DISCUSSIONS

The BIORAT-2 GD lasted for a total of 35 days (in the continuous feeding mode). During these 35 days, we evaluated the following: 1) the effect of alternative N-sources ($NO_3^-$, $NH_4^+$, and urea) on $O_2$ levels in the gas loop, 2) the possibility of using $CO_2$ (not monitored) from the consumer compartment as the C-source for *Limnospira indica*, and 3) the efficiency of the control law with regard to simulating $O_2$ production in the producer compartment by modulating the incident light flux (reaching the PBR) in

accordance with the changing of $O_2$ requirements in the consumer compartment.

The control law used for this study was formulated on the basis of the Photosim 2.0 model described in our previous study (Sachdeva et al., 2018a). The Photosim 2.0 model is a light-transfer–based stochastic model that takes into account the stoichiometric characteristics of the cyanobacterial biomass, the cultivation parameters of the bioprocess, and the geometry of the PBR to predict the volumetric biomass and $O_2$ productivities. The original Photosim model (Cornet and Dussap, 2009) was developed and validated for the *Limnospira indica* PCC 8005 strain (used for this study) cultivated at a pH value of 9.5 with 28 mM $NO_3^-$ (as the N-source). Since the nutrient source, its concentration, and other cultivation parameters are known to impact the biochemical and stoichiometric content of the photosynthetic species (Sachdeva et al., 2016a; Sachdeva et al., 2016b), we updated the original Photosim model in the context of the revised cultivation parameters used for this GD study. Therefore, all the cultivation parameters (dilution rate, residence time, N–feeding concentration, etc.) used for this GD have also been adapted from our previous study, which was used to validate the Photosim 2.0.

The Photosim 2.0 model was only updated to be used for $NO_3^-$ and $NH_4^+$ (as N-sources) feeding conditions. Accordingly, the control law used for this GD study was optimized for $NO_3^-$ and $NH_4^+$ feeding conditions and not for urea. Regardless, we decided to include urea as one of the N-sources for this study, mainly because of two reasons. First, urea is one of the main components of non-nitrified (untreated) urine (Chang et al., 2013), and if untreated/partially treated urine is to be used as the N-source for cyanobacterial cultivation, it is important to test the effect of urea on the $O_2$ production capacity of *Limnospira indica*. Second, our previous studies (Deschoenmaeker et al., 2017) have indicated that *Limnospira* sp. have better tolerance for urea (vs. $NH_4^+$), so it was important to compare the $O_2$ production capacity of *Limnospira indica* under all three N-sources in the same experiment. Interestingly, the control law performed exceptionally well for the urea regime.

It is also important to note here that attaining the "gas steady state" was used for defining the steady-state criteria for this GD study. This was in contrast to most bioprocess studies, which normally focus on attaining the use of "liquid steady state." The gas steady-state criteria were mainly based on the objective of this GD study, which aimed to evaluate the effect of alternative N-sources on the $O_2$ production capacity of *Limnospira indica*. In order to attain the "liquid steady state," the system must be maintained under continuous feeding of a particular N-source for a minimum of three residence times (15 days), which would have considerably increased the overall duration of the GD study. On the other hand, the constraints linked with maintenance of the mouse in the consumer compartment for longer than 40 days (cage hygiene and animal stress) and ethical guidelines for the animal experimentation did not permit us to conduct such long-duration experiments. Therefore, we focused on attaining the "gas steady state" (two residence times) for the purpose of this GD study.

Another important consideration for this GD study was the exclusion of the first five days of N-source transition ($NO_3^-$ to $NH_4^+$ and $NH_4^+$ to urea) from quantitative analysis. This criterion was also defined on the basis of our previous study (Sachdeva et al., 2018a), wherein we observed that the Photosim 2.0 model worked best for the timepoints wherein the PBR was under a single N-source (with 80% fitting between experimental and simulated values). The prediction efficiency of the model was reduced (to 50% fitting between experimental and simulated values) when the PBR was undergoing a transition between two N-sources. In other words, the Photosim 2.0 model prediction was found to be less efficient when two N-sources were simultaneously present in the PBR. Based on these results, we decided to exclude the first five days of $NO_3^-$-to-$NH_4^+$ transition (D6–D10) and $NH_4^+$-to-urea transition (D21–D25) from our quantitative analysis (**Supplementary Figure 1**). The effect of the simultaneous presence of multiple N-sources on the volumetric productivity of the PBR is currently being studied by our group in order to be able to more accurately describe the effect of N-transition.

Overall, the system maintained the average $O_2$ levels of 20.3% during the $NO_3^-$ regime and applied an average light flux of 136W/m$^2$ (**Figure 3**). Overall, the $O_2$ levels were found to be quite stable under the $NO_3^-$ regime compared to the $NH_4^+$ and urea regimes (**Figure 3** and **Supplementary Figure 1**). We did observe some fluctuations in the individual $O_2$ readings (after averaging). But these variations were due to the perturbation of the electrochemical $O_2$ sensor (used for this study) due to the high gas flow rates in the sensor line. For more accurate measurements, highly accurate sensors like the ones that work on the principle of paramagnetic susceptibility of $O_2$ should be used for future studies.

The system was constantly working under the maximum light flux of 147.1 W/m$^2$ (possible with the light unit used for this GD study) for most parts of the $NH_4^+$ regime (83% of observed timepoints). The system reached average $O_2$ levels of 19.5% under the $NH_4^+$ regime, by applying an average light flux of 146.6 W/m$^2$ (**Figure 3**). Interestingly, the average $O_2$ levels further decreased during the latter half of the $NH_4^+$ regime. The lower $O_2$ levels observed under the $NH_4^+$ regime (vs. the $NO_3^-$ regime) were in accordance with the results of our previous study (Sachdeva et al., 2018a), wherein we had reported a 21.5% decrease in $O_2$ yields under the $NH_4^+$ regime (vs. the $NO_3^-$ regime). A brief simulation summary comparing the $RO_2$ (oxygen production rate, mM$O_2$/L/hr) of *Limnospira indica* cultivated at different light fluxes (q0) under the $NO_3^-$ and $NH_4^+$ regimes has been described under **Supplementary Appendix 1**. **Supplementary Appendix 1** summarizes the $RO_2$ for both the $NO_3^-$ and $NH_4^+$ regimes as the function of light flux ($q_0$; W/m$^2$) and biomass concentration (g/L) (**Supplementary Appendix 1**). Finally, a comparison has been made between the (difference of) $RO_2$ for the $NO_3^-$ and $NH_4^+$ regimes at different biomass concentrations (g/L) at the applied light flux of 146 W/m$^2$ (**Supplementary Appendix 1**). These simulation data also suggest that at the desired biomass concentration of 2.0 g/L (used for validation of the Photosim 2.0 model) and the applied light flux of 146 W/m$^2$, 20% lower $RO_2$ is expected under the $NH_4^+$ regime (vs. the $NO_3^-$ regime).

Evidently, the system would have to produce 121% higher $O_2$ under the $NH_4^+$ regime to compensate for the 21.5% lower $O_2$ yields (vs. the $NO_3^-$ regime). This theoretically means that a higher light flux of 155.8 $W/m^2$ would be needed to reach the desired set point of 20.3% $O_2$ under the $NH_4^+$ regime. This could only be achieved by increasing the photosynthetic efficiency in the producer compartment, since the nutrient concentration, feeding rate, and other cultivation parameters could not be altered (as per study protocol). Moreover, the rate of photosynthesis was not foreseen to be limited by $CO_2$ levels under the present working conditions. This is mainly because under the cultivation parameters (biomass concentration, dilution rate, $O_2$ yield, and light flux) used for this GD study, the product of the respiration quotient and the photosynthetic quotient was approximately 1.2 (see Demey et al., 2000 for details). This means that the rate of production of $O_2$ is more than the rate of consumption of $CO_2$. Hence, the lower $O_2$ levels observed under the $NH_4^+$ regime could only be improved by increasing the incident light flux. However, due to the limitation of the hardware used in this GD, it was practically impossible to attain this level of light flux. For the future LSS studies (especially where $NH_4^+$ is used as the N-source), it would be important to evaluate the possibility of using higher light flux to enhance the photosynthetic efficiency (and hence the $O_2$ production capacity) of *Limnospira* without subjecting the cells to photo-inhibition. Such an investigation becomes even more important in the context of LSS studies wherein the cyanobacterial biomass is intended to be used as a nutrition source for the crew. Since light stress is known to change the biochemical composition of the cyanobacterial biomass and reduce its nutrient content (Chentir et al., 2018), extensive research needs to be undertaken to derive the ideal working light intensities that can increase the $O_2$ yield of *Limnospira* culture fed with $NH_4^+$ without subjecting it to photo-inhibition or compromising on the nutrient content of the biomass.

We also observed a considerable amount of biofilm deposition in the PBR and cyanobacterial cell bleaching (from bright green to pale green) under $NH_4^+$ feeding (data not shown), which increased toward the latter half of the $NH_4^+$ regime. The decreased $O_2$ levels and increased biofilm deposition in the PBR clearly indicated that the cyanobacterial cells were under nutrient stress (Rossi and De Philippis, 2015), indicating that $NH_4^+$ would not be able to meet the metabolic needs of *Limnospira*. Based on these results, we can say that in order to avoid these inhibitions (in terms of lower $O_2$ levels and nutrient stress), either the $NH_4^+$-only N-regimes would need to be limited to shorter durations or $NH_4^+$ would have to be combined with other N-sources to overcome this inhibition. Consequently, it would be interesting to analyze the effect of the mixture/combination of $NH_4^+$ with other N-sources on the metabolic characteristics and oxygen production capacity of *Limnospira indica* through future studies.

In contrast to the $NH_4^+$ regime, the system could effectively reach the desired (set point) $O_2$ level of 20.3% under the urea regime (**Figure 6**). Although comparable (average) $O_2$ levels were observed under the urea and $NO_3^-$ regimes, the system had to apply under a higher light flux to reach these levels under the urea regime (average light flux 146 $W/m^2$). But it is important to reiterate here that the control law and the Photosim 2.0 model had not been previously validated under urea feeding conditions. The light-transfer parameters used to validate the Photosim 2.0 model (and hence the control law) are influenced by the nutrient source, its effect on the stoichiometry of the cyanobacterial biomass, and the resulting light-transfer/absorption coefficients (Cornet et al., 1992; Cornet et al., 1995; Cornet and Dussap, 2009). Consequently, these prediction parameters are expected to change under urea feeding conditions.

Even though the system worked under light saturation for both the $NH_4^+$ and urea regimes, it could maintain an $O_2$ level of 20.3% under the urea regime. This indicated that urea is a better N-source (vs. $NH_4^+$) for supporting the photosynthetic needs of *Limnospira indica*. Moreover, contrary to the $NH_4^+$ regime, where the $O_2$ levels decreased during the last five days of $NH_4^+$ feeding, the $O_2$ levels slightly increased during the latter half of the urea regime (**Figure 3**). The higher $O_2$ levels under the urea regime could be attributed to its stoichiometry. Urea metabolism produces 2-$NH_4^+$ and $CO_2$. The influx of additional $CO_2$ (from urea metabolism) might have supported photosynthesis (and hence $O_2$ production). Higher N and carbon availability under the urea regime could be the reason for the revival of cells (from nutrient stress). This was also evident from the decreased biofilm deposition in the PBR once the urea feeding was started. The biofilm formation and cell bleaching observed during the $NH_4^+$ regime started to decrease with urea feeding and disappeared by the second half of the urea regime (data not shown). These physical indicators further showed that urea is a better N-source than $NH_4^+$ (both stoichiometrically and bioenergetically) for meeting the metabolic needs of *Limnospira indica* (Sassano et al., 2004).

## CONCLUSION AND FUTURE PERSPECTIVES

This GD study broadened our knowledge and understanding about the possibility of using different N-sources present in non-nitrified urine for cyanobacterial cultivation and air revitalization. In our previous batch and continuous feeding studies, we have already evaluated the effect of the different N-sources and their varying concentrations on the yield and biochemical content (carbohydrates, lipids, proteins, phycobiliproteins, and chlorophyll) of the *Limnospira* biomass (Deschoenmaeker et al., 2017; Sachdeva et al., 2018a; Sachdeva et al., 2018b). Since $NH_4^+$ and urea are the main N-sources present in non-nitrified urine (Chang et al., 2013), it was important to test the effect of these N-sources on the $O_2$ production capacity of *Limnospira indica* compared to $NO_3^-$ (the main component of nitrified urine).

In summary, we found that the system could easily maintain the ambient $O_2$ levels of 20.3% under the $NO_3^-$ and urea regimes, indicating that *Limnospira indica* PCC 8005 adapted better to urea than to ammonium under comparable cultivation conditions.

However, it would be necessary to validate the Photosim model for urea feeding conditions. This could also help to

improve the control law for urea feeding conditions. This understanding would be even more important when the tests/experiments would be conducted in the actual setup of the MELiSSA LSS. For the present study, we used a modified version of the MELiSSA loop, wherein we had a closed loop for the "gas phase" of the system, but the system was open (not fully closed) on the liquid side, and thus, $CO_2$ accumulation was not anticipated. However, under the actual MELiSSA loop setup, attaining $O_2/CO_2$ equilibria would be even more complex due to the involvement of several other compartments (see **Figure 1A**). In such a scenario, we will have to focus on attaining carbon balance and not just $CO_2$ balance.

Furthermore, it would be important to investigate the effect of a mix of N-sources on cyanobacterial cultivation and air revitalization, mainly for future LSS studies, wherein fluctuating streams of partially treated urine are planned to be used as the N-source for cyanobacterial cultivation.

More importantly, it would also be interesting to investigate the cumulative effect of different N-sources, high urine salinity, different organic compounds (metabolites, hormones, amino acids, etc.), and other personal hygiene products present in human urine on the overall metabolism, stoichiometry, and $O_2$ production capacity of *Limnospira indica*. These experiments become even more important for regenerative LSS studies, mainly because an influx of external carbon sources from organic compounds (present in urine) will not only change the cultivation mode from photoautotrophic to mixotrophic but would also have an effect on the biochemical/nutrient content of the biomass. In this regard, we are conducting further studies to evaluate the effect of the simultaneous presence of different N-sources, organic compounds, and high urine salinity mixtures of different N-sources on the biomass and oxygen productivities of *Limnospira indica*. The biochemical data obtained from these ongoing studies will be used to optimize the prediction parameters of the Photosim model, which will then be used to perform future GD studies.

In conclusion, this GD study provided preliminary indications toward the possibility of using $NH_4^+$- and urea-rich medium and $CO_2$ from the consumer compartment for *Limnospira indica* cultivation and air revitalization. The cumulative conclusions from our previous, present, and upcoming studies would not only aid in designing more efficient and robust regenerative life-support systems for future space missions but would also help in gaining a better understanding of the design of economically viable microalgae-based wastewater remediation systems and photosynthetic biorefineries for terrestrial purposes.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the ethical committee agreement LA 1500024 (Project Code: RI-10-01) dated 28th August, 2018.

## AUTHOR CONTRIBUTIONS

NS conducted all the experiments and collected the data. NS and LP worked on the Photosim 2.0 model. BL and OG worked on the control law. CD, CL, BL, and RW supervised the study. NS wrote the manuscript. All authors contributed equally to data analysis, interpretation, and manuscript review.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fspas.2021.700270/full#supplementary-material

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# Supporting Simultaneous Air Revitalization and Thermal Control in a Crewed Habitat With Temperate *Chlorella vulgaris* and Eurythermic Antarctic Chlorophyta

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Including a multifunctional, bioregenerative algal photobioreactor for simultaneous air revitalization and thermal control may aid in carbon loop closure for long-duration surface habitats. However, using water-based algal media as a cabin heat sink may expose the contained culture to a dynamic, low temperature environment. Including psychrotolerant microalgae, native to these temperature regimes, in the photobioreactor may contribute to system stability. This paper assesses the impact of a cycled temperature environment, reflective of spacecraft thermal loops, to the oxygen provision capability of temperate *Chlorella vulgaris* and eurythermic Antarctic Chlorophyta. The tested 28-min temperature cycles reflected the internal thermal control loops of the International Space Station (*C. vulgaris*, 9–27°C; Chlorophyta-Ant, 4–14°C) and included a constant temperature control (10°C). Both sample types of the cycled temperature condition concluded with increased oxygen production rates (*C. vulgaris*; initial: 0.013 $mgO_2$ $L^{-1}$, final: 3.15 $mgO_2$ $L^{-1}$ and Chlorophyta-Ant; initial: 0.653 $mgO_2$ $L^{-1}$, final: 1.03 $mgO_2$ $L^{-1}$) and culture growth, suggesting environmental acclimation. Antarctic sample conditions exhibited increases or sustainment of oxygen production rates normalized by biomass dry weight, while both *C. vulgaris* sample conditions decreased oxygen production per biomass. However, even with the temperature-induced reduction, cycled temperature *C. vulgaris* had a significantly higher normalized oxygen production rate than Antarctic Chlorophyta. Chlorophyll fluorometry measurements showed that the cycled temperature conditions did not overly stress both sample types ($F_V/F_M$: 0.6–0.75), but the Antarctic Chlorophyta sample had significantly higher fluorometry readings than its *C. vulgaris* counterpart ($F = 6.26$, $P < 0.05$). The steady state *C. vulgaris* condition had significantly lower fluorometry readings than all other conditions ($F_V/F_M$: 0.34), suggesting a stressed culture. This study compares the results to similar experiments conducted in steady state or diurnally cycled temperature

conditions. Recommendations for surface system implementation are based off the presented results. The preliminary findings imply that both *C. vulgaris* and Antarctic Chlorophyta can withstand the dynamic temperature environment reflective of a thermal control loop and these data can be used for future design models.

**Keywords: bioregenerative life support systems, Antarctica, McMurdo Dry Valleys, thermal control, air revitalization, Chlorophyta**

## INTRODUCTION

Engineers have developed technologies to provide air revitalization [carbon dioxide ($CO_2$) scrubbing/oxygen ($O_2$) provision], waste removal and processing, food, and thermal control for crewed spaceflight. While these systems have been proven on the International Space Station (ISS) and in Low-Earth Orbit (LEO), many require regular and frequent resupply missions (NASA, 2015). With the current launch technologies, this approach is unsustainable for longer duration missions traveling farther from Earth, as resupply missions can become mass prohibitive (NASA, 2015). Therefore, exploring closed-loop, robust, and adaptable environmental control and life support (ECLS) systems for longer duration and surface missions (i.e., Martian and Lunar missions) is imperative. Using multifunctional approaches that address multiple life support roles concurrently may further reduce system mass, power, and volume (Matula and Nabity, 2016).

Bioregenerative ECLS technologies, specifically algal photobioreactors, can simultaneously fix atmospheric carbon dioxide and produce oxygen through photosynthesis. Additional species-dependent functionalities include waste remediation (through nitrogen and phosphorous assimilation) and producing edible biomass (thereby providing carbon and nutrient loop closure) (Chinnasamy et al., 2009; Kumar et al., 2010; Jaatinen et al., 2015; Tuantet et al., 2019). Preliminary studies using photobioreactors for the support of human spaceflight were conducted both terrestrially and in LEO (Popova et al., 1989; Gilles et al., 2008; Lasseur et al., 2011; Helisch et al., 2020; Poughon et al., 2020). Typically, these systems focused on food production and air revitalization (Javanmardian and Palsson, 1991; Li et al., 2013).

Including the water-based growth media in the culture utilization design space can offer added benefit to a multifunctional system. The current ISS thermal control system and proposed sustained surface missions use water loops snaked throughout the cabin walls to absorb cabin waste heat before radiating it off to space (Reysa and Thurman, 1997; Westheimer, 2019; NASA, 2020). Taking advantage of the already allocated cabin volume and plumbing, using algal media in place of water for thermal control may further reduce ECLS mass, power, and volume (Matula and Nabity, 2016). However, using media for thermal control could expose the algal culture to a very dynamic thermal environment (**Figure 1**). This temperature profile depends on the commanded flow rate and cabin heat load. **Table 1** presents the operating characteristics of each of the ISS internal thermal loops and spacecraft cabin (The Boeing Company, 2001; Valenzano et al., 2003; Anderson et al., 2018). Mass production of microalgae in raceway ponds may sustain diel temperature cycles from +10 to +45°C (Ras et al., 2013). However, many studies have shown that, within a species-dependent range, there is a positive correlation of photosynthetic rate to temperature (Öquist, 1983; Dauta et al., 1990; Davidson, 1991; Ras et al., 2013). Therefore, a reduction in environmental temperature may lead to a decrease in the air revitalization capacity of these photobioreactors (Maxwell et al., 1994; Morales et al., 2018).

Maxwell et al. successfully sustained *Chlorella vulgaris* at a constant +5 and +27°C for 10 days. They noted the +5°C culture reduced cellular chlorophyll (Chl) content; thereby preserving cell sustainment through reduced irradiance absorption and excitation pressure on photosystem II (PSII) (Maxwell et al., 1994; Morgan-Kiss et al., 2006; Teoh et al., 2013). Limited studies investigating the effects of diurnal cycles in irradiance and temperature (+3 to +28°C) suggest that microalgae are adaptable and viable beyond optimal culturing conditions (+26 to +36°C) (Chisti, 2007; Ugwu et al., 2007; Costache et al., 2013). Additionally, there was no significant difference in specific growth rate between cultures experiencing diurnal cycling and time-averaged constant temperature controls (Tamiya et al., 1955; Tamburic et al., 2014; Yang et al., 2016). Davidson suggested that microalgae easily acclimate to temperature change over the course of weeks to days. He stated, however, that little is known about the rate of acclimation, especially within 24-h time scales; which a brief literature survey showed is still true today (Davidson, 1991). Spacecraft thermal loops can have a turnover rate of a few minutes. References for this study reported the optimal temperature range for *Chlorella* taxa was just within the spacecraft cabin temperature range (**Table 1**; Dauta et al., 1990; Maxwell et al., 1994; Serra-Maia et al., 2016; Anderson et al., 2018).

The referenced studies were conducted with lab-sustained *Chlorella* taxa, a readily available freshwater genus that easily grows in a range of temperature and pH conditions (Mayo, 1997; Ras et al., 2013; Zhao et al., 2013). Non-toxic to humans, and after minimal processing, it is fit for consumption as nutritional

**Abbreviations:** Chl-a,b, chlorophyll-a,b; $CO_2$, carbon dioxide; ECLS, environmental control and life support; $F_V/F_M$, maximum fluorescence; IATC, internal active thermal control; ISS, International Space Station; $k_{stir}a$, volumetric mass transfer coefficient ($d^{-1}$); LHC, light harvesting complex; LTL, lower temperature loop; LEO, low earth orbit; MDV, McMurdo Dry Valleys; MTL, moderate temperature loop; $N_{0,t}$, biomass dry weight at time $t$; $O_2$, oxygen; $O^*_{2sat}$, dissolved oxygen saturation in water at measured temperature (g $L^{-1}$); $O_{2t}$, dissolved oxygen at time $t$; $\Delta O_2$, excess dissolved oxygen (g $L^{-1}$); PAM, pulse amplitude modulation; PS I,II, photosystem I, II; Y, biomass yield rate (gDW $L^{-1}d^{-1}$).

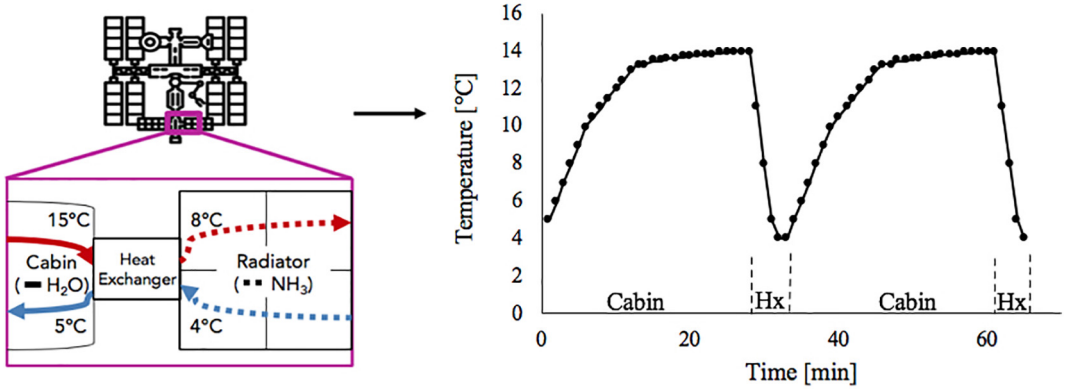


**FIGURE 1 |** Absorption of cabin heat and heat exchange between low temperature loop and ammonia radiator (*left*) results in temperature cycles in low temperature loop water as it flows between cabin and heat exchanger (*right*).

supplementation, closing the carbon loop for spaceflight (Powell et al., 1961; Hedenskog et al., 1969; Gitelson et al., 2003; Morris et al., 2008; Carlson, 2011; Solter and Beasley, 2013; Tibbetts et al., 2015). Therefore, this genus is widely accepted by the spaceflight community and has extensively published terrestrial data with limited spaceflight studies (Myers and Johnston, 1948, 1949; Myers, 1964; Lasseur et al., 2011; Drexler and Yeh, 2014; Serra-Maia et al., 2016; Rabbow et al., 2017; Niederwieser et al., 2018; de Vera et al., 2019; Helisch et al., 2020; Pickett et al., 2020).

Ras et al. (2013) denoted that microalgae generally adapt to a new environment over the course of a season, acclimating to the environment after enough generations. They added, "Optimal temperature should therefore be associated to the environmental conditions for which they (microalgae) have been obtained." This implies that psychrotolerant or cryophilic microalgae may be the most appropriate for use in a thermal control system, as they may be tolerant of the temperature extremes found in a cabin cooling loop. Spaceflight designs may benefit from bioprospecting by using extremophiles, taking advantage of their robustness and adapted metabolic processes (Beattie et al., 2011; Malavasi et al., 2020).

The McMurdo Dry Valleys (MDV), located on the west coast of McMurdo Sound, is the largest polar desert in Antarctica and exceptional planetary (Martian) analog (Doran et al., 2010). The MDV is home to ephemeral, glacial-fed streams where diverse types of perennial algal mats are common. These extremophilic microalgae grow as dense mats consisting of mixtures of cyanobacteria and eukaryotic microalgae (including taxa of *Prasiola*, *Chlorella*, and *Chlamydomonas*) (Kohler et al., 2016; Van Horn et al., 2016). Desiccated and cryopreserved in 24-h darkness for the winter, these mats photosynthesize within 10–20 min of rewetting in the summer, when the first glacial melt water saturates the stream bed and surface flow begins (McKnight et al., 1999; Darling et al., 2017). Through the summer, these species experience daily thermal swings between +2 and +15°C. This temperature range is driven by changing solar positions, where higher temperatures correlate to greater irradiance over the course of the summer. In the MDV, the 24-h irradiance in the summer imparts four times more UV-radiation than occurs in the United States (Cozzetto et al., 2006; Darling et al., 2017; Obryk et al., 2018; Sengupta et al., 2018). The irradiance conditions, along with the nutrients mobilized from weathering reactions in the underlying sediment of the streambed, supports this cold-environment photosynthesis. The freshwater algal mats from this location thrive in an environment analogous to conditions within a planetary habitat (Gooseff et al., 2010).

This study compares the air revitalization capabilities of commercially grown and Antarctic-sampled Chlorophyta, as potential microalgae for use in a multifunctional bioregenerative life support system. The thermal cycles imparted on these microalgae during the experiment are reflective of a spacecraft or surface habitat thermal loop. The work presented in this paper is the first comparative study investigating the impact of rapid, dynamic thermal environment on psychrotolerant and temperate-grown microalgae.

**TABLE 1 |** Internal active thermal control system and crewed cabin operational characteristics (The Boeing Company, 2001; Valenzano et al., 2003; Anderson et al., 2018).

| Internal system | Operational temperature range (°C) | Loop volume (L) | Heat transferred (kW) |
|---|---|---|---|
| Lower temperature loop (LTL) | 3.0–15 | 63 | 7 |
| Moderate temperature loop (MTL) | 10–21 | 200 | 12.5 |
| Spacecraft cabin | 18–27 | – | – |

# MATERIALS AND METHODS

## Site Description and Sample Methods

This study included *Chlorella vulgaris* (Bacteria-free agar slant, item #152075 isolated from UTEX 398, Carolina Biological) and green (Chlorophyta-Ant) mat gathered from the McMurdo Dry Valley, Antarctica.

Green algal mat was collected from Von Guerard stream in the Taylor Valley (77°26′38.717″S, 163°9′33.321″E) of the McMurdo Dry Valley, South Victoria Land, Antarctica (**Figure 2**). Sampling was conducted in January 2019 from the main thalweg of the stream ensuring, peak stream flow. Von Guerard Stream drains off of Von Guerard Glacier in the Kukri Hills, east of the Crescent Glacier (Cozzetto et al., 2006). Preliminary analysis of these green mats by Van Horn et al. (2016) suggested they are comprised of taxa of the genera *Prasiola*, *Chlorococcum*, and *Chlorella*. The availability of these species was confirmed through both light microscope (Olympus IMT, 40× dry objective) and fluid imagery microcopy (FlowCAM VS-IV, 10×).

Forceps and a spatula, triple-rinsed in stream water, were used to gather 3 cm × 6 cm sections of mat. Mat sections were each placed in a sterile 200 mL Nalgene screw-top sample bottles, triple-rinsed in stream water before sample deposit, and filled with approximately 100 mL of associated stream water. At the University of Colorado, the lids to the bottles were loosened to allow for ventilation and the foil-wrapped bottles were placed on a dark shelf of a +7°C (±1°C) incubator.

## Culturing Conditions

Subsamples of mat were transferred from the sample bottles to 250 mL Erlenmeyer flasks of 125 mL sterile Bold's (Bristol's) Modified Media (50× Bold Modified Basal Freshwater Nutrient Solution, Sigma Aldrich) and lightly capped with foil. After final dilution with ultrapure water (Milli-Q Direct, Millipore-Sigma), the freshwater media contained 2.94 mM $NaNO_3$, 0.17 mM $CaCl_2$–$H_2O$, 0.3 mM $mgSO_2$–$7H_2O$, 0.43 mM $K_2HPO_4$, 1.29 mM $KH_2PO_4$, and 0.43 mM NaCl (pH 6.4). Over the incubation period, sterile Bold's media was added to the flasks to return the total volume to 125 mL. The flasks were placed on an orbital shaker table, in the +7°C incubator, under a cool fluorescent lamp providing an irradiance of 50 μmol $m^{-1}$ $s^{-1}$ with a 12:12-h light:dark cycle. The incubator temperature was reflective of Von Guerard's average summer temperature (+7°C) (Cozzetto et al., 2006). While the Antarctic austral summer has 24-h sunlight, the light:dark cycles reflect the relative motion of the sun across the MDV, which produces diurnal cycles of direct solar radiation on the microbial mats (Darling et al., 2017).

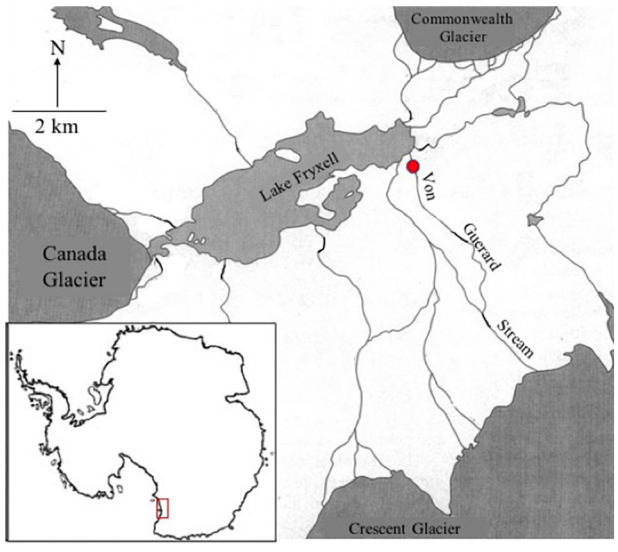


**FIGURE 2 |** Reference map of the sampled McMurdo Dry Valleys (MDV), Antarctica (red box, *lower left*). Sample site a Von Guerard Stream is marked with a red circle.

A continuous, xenic seed culture of *C. vulgaris* was sustained at a constant 20°C in Bold's media. The 1 L Pyrex beaker of maintained mother culture was placed on a magnetic stir plate, under a bank of fluorescent bulbs (F40PL/AQ/ECO 49893 Bulb, GE Lighting) providing 24-h irradiance. The top of the beaker was covered lightly with clear cling wrap to allow 120 μmol $m^{-1}$ $s^{-1}$ irradiance at the culture surface. Free gas exchange with the clean, but not sterile, lab environment more closely replicated direct exposure to spacecraft or surface habitat conditions.

## Temperature Treatment

A Peltier cooler system was developed to control the temperature of the 12-well plates (229111, CellTreat) used in these experiments. The thermal loop profile was replicated using an Arduino-based system (Mega, Arduino). The Arduino received temperature input through a K-type thermocouple (K-type braided, Omega and K-type Thermocouple Amplifier, AdaFruit), and controlled the Peltier cooler (Peltier Thermo-Electric Cooler, Adafruit), in conjunction with a resistive heating pad (Heating Pad, Sparkfun). Those wells in direct contact with the cooler (B2, B3, and B4) were filled with 3.5 mL of culture. The initial culture density of *C. vulgaris* was 5 × $10^5$ cell $mL^{-1}$ and Chlorophyta-Ant was approximately 0.34 mg $mL^{-1}$. The plates had free gas exchange through a clear, gas-permeable membrane (Sealing membrane, Breathe-Easy) that sealed the tops of the plates to reduce evaporation. The membrane created a seal around the inserted measurement probes (pH, dissolved oxygen, temperature). An orbital shaker table (7744-01000, Bellco Orbital Shaker) continuously agitated the plates at 100 RPM, to reduce media temperature gradients within the well and promote gas exchange between the environment and media. Overhead lighting (F40PL/AQ/ECO 49893 Bulb, GE Lighting) provided 120 μmol $m^{-1}$ $s^{-1}$ at the plate surface, under 24-h irradiance. Dauta et al. (1990) suggests that this irradiance intensity reduced the risk of photoinhibition in *C. vulgaris* for the tested temperatures (Morales et al., 2018). This system was at pressure equilibrium with the surrounding environment in Boulder, CO (approximately 84.1 kPa). Triplicate experiments were executed for 7 days to capture the exponential growth phase.

The range and period of the tested sinusoidal temperature cycles were modeled from the ISS's thermal control system and cabin environment (**Figure 3**). Temperature ranges for each experiment were adjusted to span both the operational conditions of the thermal loop but also the in-situ sample temperatures of each sample. The *C. vulgaris* experiment used a +9 to +27°C (±2°C) temperature profile, which included the LTL and cabin environment (Anderson et al., 2018). The Chlorophyta-Ant experiment used +4 to +14°C (±2°C), which replicated

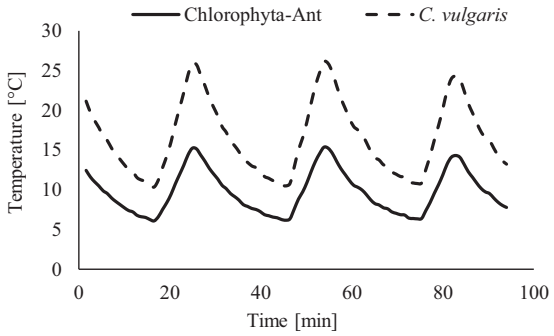


**FIGURE 3 |** Temperature profile used for Chlorophyta-Ant and *C. vulgaris*, representative of spacecraft thermal loops.

the operational range of the LTL [personal communication, Michael Holt, NASA Crew Thermal Systems, May 18, 2017]. The lower temperature bounds were dictated by the cooling capacity of the Peltier cooler and environmental chamber. The 28 min cycle period reflected the flow rate of the ISS Internal Active Thermal Control System (IATCS) (Hanford and Ewert, 1996; Patel et al., 2001; The Boeing Company, 2001). A constant temperature control was executed for both sample types at 10°C, which was the time-averaged temperature of the Chlorophyta-Ant experiment but also within the *Chlorella* experiment temperature range.

## Real-Time Measurements

PLX-DAQ software continuously recorded well temperature and pH (pH kit, Atlas-Scientific) measurements. Dissolved oxygen (Robust Oxygen Probe, PyroScience), with associated temperature compensation, was measured and recorded with the associated PyroScience software. All measurements had a sampling rate of 0.2 Hz. Before inoculating with algal culture, an operational baseline for dissolved oxygen and pH was established with sterile Bold's media using the tested temperature profiles for 3 days.

## Non-continuous Measurements

Invasive sampling methods are required for calculating specific growth rate, and a minimal amount of biomass was available for each sample type. Therefore, the biomass measurement frequency was restricted to the start of end of each experimental run. *C. vulgaris* experiments used optical density measurements to measure biomass growth via spectrophotometer (Thermo Scientific Multiskan FC, Fisher Scientific) at 690 nm (Raso et al., 2011; Santos-Ballardo et al., 2015). The equivalent biomass dry weight was calculated using a conversion constant calculated from optical density (OD) measurements of standard dilutions of inoculate and corresponding dry weight biomass density measurements.

$$Dry\ weight\ [g\ L^{-1}] = 1.46 \times OD_{690} \quad (1)$$

Both Chlorophyta-Ant experiments and the optical density conversion (Eq. 1) used a vacuum filtration method for preparing samples for biomass dry weight measurements. Well samples were deposited on quartz fiber filters (25 mm QE200, Advantec) and triple rinsed with ultrapure water before drying in a 60°C oven for >24-h.

Daily measurements of chlorophyll fluorometry provided a qualitative assessment of the cell's photosystem II (PSII) response efficiency (measured on a scale of 0–1, 1 being the highest efficiency). This non-invasive method determined relative culture health, referred to as photosynthetic quantum yield [Y(II)]. Measuring a well containing 3.5 mL of sterile Bristol's media before each culture measurement calibrated the pulse amplitude modulation system (Junior-PAM, Walz). Cultures were allowed to come to a steady state temperature (19°C for *C. vulgaris*, 10°C for Chlorophyta-Ant) after turning off the overhead light panel for at least 15 min to measure maximum fluorescence ($F_V/F_M$) (Schreiber, 2004). The PAM probe was held to the bottom of the culturing plate, in consistent locations, and the saturation pulse was initiated and recorded with associated PAM-Walz software (saturation: 1,500 μmol $m^{-1}$ $s^{-1}$, 0.4 s; actinic light: 420 μmol $m^{-1}$ $s^{-1}$).

## Analytical Methods

Dissolved oxygen, pH, and PAM measurements were recorded in 24-h increments and were post-processed with the corresponding Bold's baseline.

$$\Delta O_2 = O_{2\,t} - O^*_{2\,sat} \quad (2)$$

Where $\Delta O_2$ is the excess dissolved oxygen concentration, g $L^{-1}$, $O_{2t}$ is the measured dissolved oxygen concentration, g $L^{-1}$, and $O^*_{2sat}$ is expected oxygen saturation for the media at the measured temperature, g $L^{-1}$. Real-time data (pH and dissolved oxygen) were averaged over the 24-h period for both the control and cycled temperature conditions.

While it was understood that both sample types would experience a lag phase, the onset of exponential growth using biomass was not identified due to the restricted biomass sampling schedule. Therefore, the specific growth rate could not be calculated, and the biomass yield rate was used instead. The yield rate was calculated with the biomass dry weight and the following equation

$$Y = (N_t - N_{t0})/(t - t_0) \quad (3)$$

Where $Y$ is the biomass yield rate, gDW $L^{-1}$ $d^{-1}$, $N(t$ or $t_0)$ is the biomass dry weight at time $t$ and $t_0$, gDW $L^{-1}$, and $t$ is time, $d$. The yield rate was used to compare biomass accumulation rates over the course of the experiment (Wood et al., 2005). Resulting media pH, oxygen production, yield rates, and photosynthetic yield measurements were averaged across the triplicate experiments.

Two-way ANOVA with replication determined statistical significance of real-time measurements between sample types with subsequent Tukey *post hoc* tests. One-way ANOVA calculated the significance of data associated with biomass dry weight. All statistical calculations were conducted with Microsoft Excel 2016 with the Data Analysis package with a critical value of $P < 0.05$ for all statistical tests. All presented data represents averaged triplicate results, and the associated standard deviation

as the error bars calculated by Microsoft Excel 2016, unless specifically noted.

# RESULTS

## Biomass Yield Rate

The clumping nature of Chlorophyta-Ant made it difficult to prepare consistent, homogeneous samples for daily measurements. Therefore, biomass dry weight was measured at the beginning and end of each experiment. **Table 2** presents the calculated (Eq. 3) yield rate for each experiment. The temperature-cycled *C. vulgaris* condition had a significantly higher yield rate than the Chlorophyta-Ant sample ($F = 9.44$, $P < 0.05$); while the yield rate under steady-state temperatures (10°C) were similar magnitude for both sample types ($F = 0.02$, $P > 0.05$). The growth rate of Chlorophyta-Ant cultured at a steady state temperature was comparable to the cycled temperature condition ($F = 0.01$, $P > 0.05$). However, the yield rates for this sample type were less than those reported in literature culturing within similar temperature environments (0.11–0.15 g $L^{-1}$ $d^{-1}$) (Yang et al., 2016). The yield rate of the *C. vulgaris* sample type at a constant temperature was also less than reported values (0.14–0.17 g $L^{-1}$ $d^{-1}$). Yang et al. (2016) subjected isolated, temperate *Chlorella* to diurnal temperature fluctuations between 10°C/28°C, this condition resulted in growth rates significantly less than that presented here (0.21 g $L^{-1}$ $d^{-1}$).

## Oxygen Production

Oxygen production increased over the course of the experiment for each treatment and sample type, except for *C. vulgaris* at the steady state 10°C condition (**Figure 4**). The dissolved oxygen probe was available for only one of the Chlorophyta-Ant at 10°C trials. Therefore, these preliminary data do not have associated standard deviation and they are presented for the comparisons of trends. The isolated trial for Chlorophyta-Ant under constant temperature conditions resulted in an 160% increase in dissolved oxygen. Both temperature-cycled conditions (Chlorophyta-Ant and *C. vulgaris*) had significantly higher dissolved oxygen than the constant 10°C *C. vulgaris* trials ($F = 205.9$, $P < 0.001$; $F = 14.7$, $P < 0.001$, respectively). While the temperature-cycled *Chlorella* condition experiments concluded with an excess dissolved oxygen concentration three times greater than that of the Chlorophyta-Ant sample type (3.15 versus 1.03 $mgO_2$ $L^{-1}$), the difference was insignificant ($F = 2.28$, $P > 0.05$).

Excess dissolved oxygen measurements were normalized with corresponding biomass dry weight measurements, as it is indicative of algal response to the culturing environment (**Table 3**). The *C. vulgaris* sample types decreased in normalized dissolved oxygen (9–27°C: decreased by 37%; 10°C: decreased by 22%). The normalized dissolved oxygen for the cycled temperature *C. vulgaris* was significantly reduced over the course of the experiment ($t = 4.3$, $P < 0.05$). However, the cycled temperature *C. vulgaris* results were significantly greater than both the cycled Chlorophyta-Ant and the constant temperature *C. vulgaris* condition (Chlorophyta Ant: $F = 2.91$, $P < 0.05$; constant *C. vulgaris*: $F = 7.85$, $P < 0.05$). The Chlorophyta-Ant (9–27°C) sample type rate increased by 33%. The constant temperature Chlorophyta-Ant resulted in a slight reduction in normalized dissolved oxygen concentration. Only one constant temperature Chlorophyta-Ant experiment had dissolved oxygen readings, therefore additional trial runs are needed to confirm the precision of this observation.

**TABLE 2 |** Biomass yield rates calculated from biomass dry weight measurements (Chlorophyta-Ant) and $OD_{690}$ conversion (*Chlorella vulgaris*).

| Experimental condition | Y (gDW $L^{-1}$ $day^{-1}$) |
|---|---|
| Chlorophyta-Ant (4–14°C) | $0.02 \pm 0.03^{A}$ |
| *C. vulgaris* (9–27°C) | $0.75 \pm 0.41^{B}$ |
| Chlorophyta-Ant (10°C) | $0.02 \pm 0.04^{A}$ |
| *C. vulgaris* (10°C) | $0.03 \pm 0.02^{A}$ |

*Different letters in the superscript next to each value indicate significant differences ($P < 0.05$) between temperature conditions.*

## Media pH

The increase in media pH through the execution of this experiment was expected for both sample types across all test conditions, as cultures fixed dissolved carbon species (**Figure 5**). The cycled temperature condition of the *C. vulgaris* sample type was the only case to result in a significant increase in media pH (Initial pH: 6.4, Final pH: 10.0; $F = 5.14$, $P < 0.01$). While both *C. vulgaris* temperature conditions started at approximately the same media pH, the cycled temperature condition had a significantly greater increase in pH than the constant temperature condition ($F = 24.31$, $P < 0.001$).

## Chlorophyll Fluorescence

Daily maximum chlorophyll fluorescence measurements were used as both a proxy for culture stress levels and estimating the onset of exponential growth. Both *C. vulgaris* conditions experienced at least a 2-day lag phase. The significant decrease in $F_V/F_M$ values, in conjunction with a decrease in pH and oxygen production, suggested the lag phase duration. As cultures acclimated to the culturing environment, a subsequent rise in values followed (Maxwell and Giles, 2000; Kula et al., 2017). The $F_V/F_M$ values for all conditions peaked around day six before declining again on day seven. It was hypothesized this was the onset of the stationary phase. As the Chlorophyta-Ant mats were partially comprised of *Chlorella*, it was assumed that the Chlorophyta-Ant sample type exhibited the same $F_V/F_M$ patterns as *C. vulgaris* while transitioning into the various growth regimes. The constant temperature *C. vulgaris* condition had significantly lower fluorescence values than all other tested conditions ($F = 47.55$, $P < 0.001$). Although all tested conditions resulted in reduced maximum fluorescence readings through the duration of the experiment, the constant temperature *C. vulgaris* condition was the only condition to have a significantly impeded PSII response ($F = 87.09$, $P < 0.001$). Between the two cycled temperature conditions, Chlorophyta-Ant had a significantly

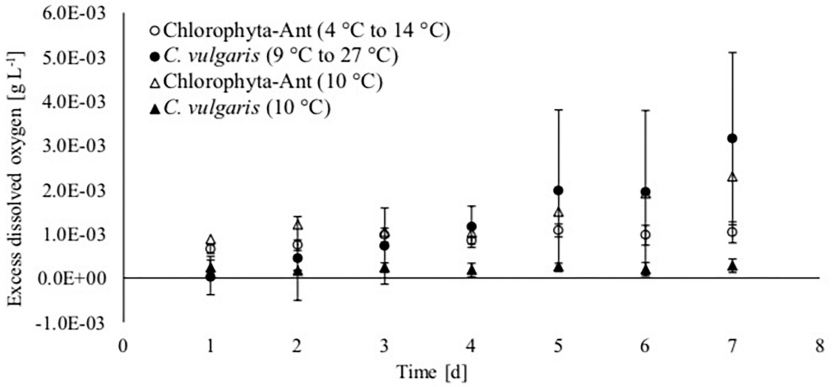


**FIGURE 4 |** Excess dissolved oxygen based on saturated dissolved oxygen measurements of Antarctic Chlorophyta and temperate *Chlorella vulgaris* grown in thermal environments of a spacecraft.

more responsive PSII than *C. vulgaris* ($F = 6.26$, $P < 0.05$). Carotenoid content was not directly measured but there was no significant yellowing observed for all experiments, indicative of significant carotenoid production, nor was there photobleaching.

## DISCUSSION

The tested temperature environments reflecting spacecraft thermal loops had a measurable impact on the performance of both *C. vulgaris* and Chlorophyta-Ant sample types. Those trials with cycled media temperature had higher metabolic metrics than their constant-temperature counterparts. This suggests if using sub-optimal environmental temperatures, incorporation of a temperature respite, regardless of cycle period, may improve culture stability and metabolic function.

While both sample types increased in oxygen production over the course of the experiment, the cycled temperature *C. vulgaris* condition concluded with production close to three times greater than Chlorophyta-Ant. Two factors may have contributed to the extreme production rate increase for *C. vulgaris*, initial excess dissolved oxygen concentration and rapid generational acclimation. The initial dissolved oxygen concentration for *C. vulgaris* (0.013 $mgO_2\ L^{-1}$) was almost two orders of magnitude less than Chlorophyta-Ant (0.653 $mgO_2\ L^{-1}$). Inoculated *C. vulgaris* was sustained in and acclimated to a temperate lab environment (20°C). The time-averaged temperature of cycled *C. vulgaris* was 16°C, which could have elicited a low temperature response from the culture within the first day. Standard deviation of both *C. vulgaris* conditions suggest that there may have been some active oxygen consumption during the first few days of the trial. Öquist and Huner observed immediate reduction in photosynthetic activity through diversion of energy from the PSII to PSI when *C. vulgaris* cultures were moved from 27 to 5°C environment. They hypothesized this reduced PSII excitation pressure and minimized production of damaging reactive oxygen species (Öquist, 1983; Hüner et al., 1998; Morgan-Kiss et al., 2006; Sydney et al., 2013). Literature suggested that physiological acclimation to lower steady state temperatures may take a few hours to days including reduction in chlorophyll-a per cell, increased RuBisCO content, and eventual alterations to lipid components of thylakoid membrane (Davidson, 1991; Maxwell et al., 1994; Cao et al., 2016).

**TABLE 3 |** Excess dissolved oxygen normalized with corresponding measured biomass dry weight.

| Experimental condition | Initial ($mgO_2\ gDW^{-1}$) | Final ($mgO_2\ gDW^{-1}$) |
|---|---|---|
| Chlorophyta-Ant (4–14°C) | $2.24 \pm 0.42^{A}$ | $3.0 \pm 1.71^{A}$ |
| *C. vulgaris* (9–27°C) | $8.84 \pm 4.40^{B}$ | $6.51 \pm 1.88^{C}$ |
| Chlorophyta-Ant (10°C) | 2.57 | 2.34 |
| *C. vulgaris* (10°C) | $4.45 \pm 3.89^{A}$ | $3.47 \pm 1.26^{A}$ |

*Superscripts indicate significant difference between initial and final values and within test conditions ($P < 0.05$).*

The cycled *C. vulgaris* had multiple generational turnovers over the course of the experiment, with a final biomass concentration 350 times greater than the initial concentration. Morgan-Kiss et al. explained that genetic adaptation occurs over a time scale of many generations while exposed to transitory changes in environmental conditions. Therefore, it is unlikely that the culture completed genetic adaptation within these experiments, as the temperature fluctuations were within the lifetime of these cells (Morgan-Kiss et al., 2006). However, with multiple generations across the experiment duration, *C. vulgaris* cells were exposed to this dynamic temperature environment at infancy. This may have expedited the acclimation reflected in increased photosynthetic yield and oxygen production (**Figures 4**, **6**). Large variations in dissolved oxygen measurements were observed during days 5, 6, and 7 for the cycled temperature *C. vulgaris* condition. During one experimental run, the Peltier cooler stopped working on day 4 and allowed the culture temperature to increase to 19°C for 6 h before reinitiating the temperature cycle. The experimental run continued using the same temperature cycle for the rest of day 4 and 5 through 7. The last 3 days of that isolated run resulted in substantial increases in oxygen production. The 6-h

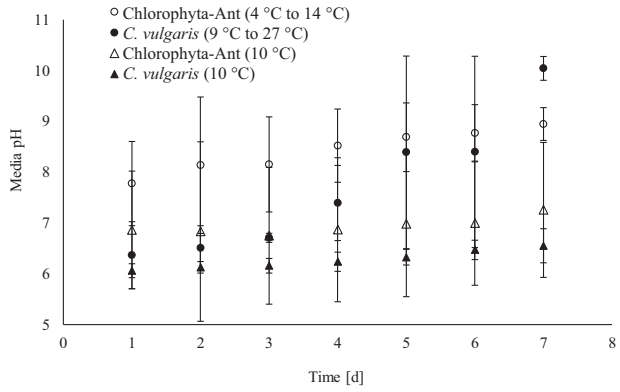


**FIGURE 5 |** Real-time media pH measurements averaged across 24 h for qualitative insight of culture growth.

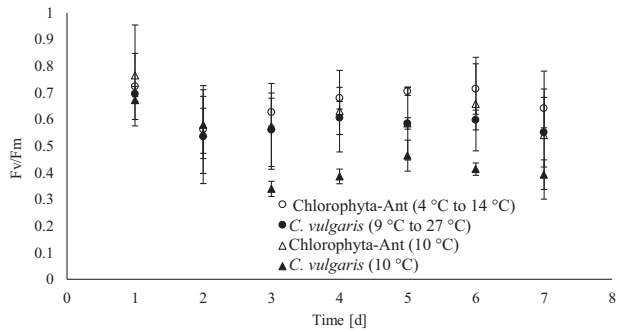


**FIGURE 6 |** Daily maximum chlorophyll fluorescence for each tested temperature condition, measured after allowing cultures to come to a steady state temperature (19°C for *C. vulgaris*, 10°C for Chlorophyta-Ant).

temperature respite may have increased cellular reproduction rate for a brief period, increasing biomass density and oxygen production in general.

Oxygen production normalized by the biomass dry weight elucidated any changes to oxygen production capability at the biomass level. **Figure 4** illustrated increased excess dissolved oxygen for both cycled *C. vulgaris* and Chlorophyta-Ant. However, both *C. vulgaris* conditions resulted in a decrease in oxygen production per biomass over the duration of the experiment (**Table 3**). This suggests that the population accrued biomass, as storage products, at a disproportionate rate to oxygen production and that the culture may have been acclimating to the lower temperature regime of the tested cycle. Reallocation of energy from PSII to photosystem I (PSI) in the cold-stressed cells reduces light-dependent production of oxygen (Hüner et al., 1998, 2020; Melis, 2009). In the case of the cycled temperature *C. vulgaris*, the higher temperature respite periods allowed cells to increase cellular reproduction, diverting energy from protein and lipid accumulation (Yang et al., 2016). No to minimal decrease in normalized oxygen production was recorded in either Chlorophyta-Ant condition (**Table 3**). This suggests complete genetic adaptation of these sample types to the tested temperature environment. Sustained or increased normalized oxygen production may indicate pre-established levels of regulatory enzymes and chlorophyll for intercellular energy balance (Hüner et al., 1998, 2020). While the two different cycled temperature profiles were reflective of the LTL and MTL on the ISS and considered the optimal growing regimes of each sample type, in the future, a more balanced comparison could be made by executing the same temperature profile for both sample types.

Due to long-duration exposure to the polar environment, it was assumed Chlorophyta-Ant adaptations were well-established. These included increased polyunsaturated fatty acid concentrations within the thylakoid to sustain membrane fluidity (Morgan-Kiss et al., 2006, 2008). Cao et al. also observed that Antarctic *Chlorella* reduced the light harvesting complex (LHC) through decreased Chl-b content at low temperatures <15°C, causing reduced light absorption to prevent photoinhibition. Sustained and increased oxygen production rates in both Chlorophyta-Ant conditions suggested adaptation to the tested conditions. The equipment schedule limited the test duration to 7 days, however, increasing the duration to 14 days or a few months would have been a better reflection of long duration surface operations, and may have given more insight to the long-term acclimation of these cultures.

Microalgae will preferentially absorb carbonic acid typically available at a lower media pH (Girard et al., 2008). If negligible changes in alkalinity are assumed, increases in media pH can be associated with depletion of carbonic acid and the transition to bicarbonate consumption (Wolf-Gladrow et al., 2007). As dissolved carbon dioxide (carbonic acid in pH $\leq$ 6) was fixed by the culture, the pH started to increase (**Figure 6**). Additionally, the microalgae released hydroxide ions corresponding with photosynthetic activity, which further increased the media pH. Mayo (1997) suggested that the optimal pH for *C. vulgaris* and other freshwater microalgae was between 4.0 and 8.5 (Scherholz and Curtis, 2013). Both cycled temperature conditions were beyond the optimal media pH by day six of the trials. Long-term operation of photobioreactors may require scheduled sparged carbon dioxide impulses with bicarbonate dosing and biomass removal to keep the system within a favorable pH range. The experiments for this study were conducted at 84.1 kPa with approximately 0.04% $CO_2$ (0.034 kPa), which is approximately a fifteenth of the carbon dioxide concentration on the ISS (0.507 kPa) (Anderson et al., 2018). However, terrestrial studies with temperate *Chlorella* have demonstrated positive correlation of photosynthetic rate with influent carbon dioxide concentration, up to 6% (v/v) (Chinnasamy et al., 2009). The variability in initial biomass concentration and culture productivity contributed to the wide standard deviation in pH measurements. It was difficult to homogenize the Chlorophyta-Ant samples for precise initial biomass densities, which led to some variability in initial biomass densities. However, the standard deviation in the pH measurements is constant across these tests. The standard deviation for pH measurements of the cycled temperature *C. vulgaris* condition increased on day four, in conjunction with the culture temperature coming to a constant 19°C for 6 h. This increased carbon dioxide fixation by the

culture, which dramatically increased the pH measurements for this test and increased the condition's standard deviation. On day seven, the standard deviation decreased for this condition, as the other tests increased their pH, as expected.

## Culture Volume for Surface Habitat Support

The launch mass of supply missions may constrain surface habitat infrastructure, thereby directing systems engineers to design for habitat system efficiency. If using the culture density prepared for these experiments, three times as much volume of Chlorophyta-Ant than *C. vulgaris* (but only two times as much biomass) is needed for sufficient oxygen supply. These estimated magnitudes in volume and dry weights are reflective of the tested environment (irradiance and $CO_2$ concentration). Photobioreactors designed and optimized for oxygen production in spaceflight applications estimate needing approximately 20–200 L of culture per crewmember (Javanmardian and Palsson, 1992). Testing the temperature profiles presented here with greater biomass density cultures, to investigate density limitations, may help reduce the required culture volume for oxygen provision. However, increasing the culture density may reduce irradiance penetration length, change mixing patterns and nutrient availability, thereby possibly changing the oxygen production rate.

## Modifying Irradiance for Culture Stress Reduction

Culture health and stress levels were estimated by chlorophyll fluorescence measurements using maximum quantum yield ($F_v/F_M$). Ritchie (2006) explains that nominal measurements for higher plants is approximately 0.7–0.83, however, microalgae may have lower values closer to 0.65–0.8, with values <0.6 associated with stress (Masojídek et al., 2004; Schreiber, 2004; Ritchie, 2006). *C. vulgaris* at a constant 10°C was the only series that was <0.6 for more than two consecutive days, signifying suboptimal PSII response. These measurements detected photoinhibition and loss of function of the reaction centers through reduction in $F_v/F_M$ (Guidi et al., 2019). Maxwell et al. (1994) also observed reduced $F_v/F_m$ measurements (approximately 0.55) for *C. vulgaris*, attributed this to excess excitation pressure on PSII at the tested temperature and irradiance (constant 5°C and 150 $m^{-1}$ $s^{-1}$) (Vonshak et al., 2001; Yang et al., 2016).

The experiments in this study used 24-h low-level irradiance (120 μmol $m^{-1}$ $s^{-1}$), which may have incited photoinhibition of *C. vulgaris*. Increasing the environmental temperature or reducing the irradiance promotes cellular equilibrium and increases maximum quantum yield. Öquist (1983) compared the inhibition of electron transport for higher plants and noted that there was less photoinhibition when plants chilled in darkness than in high irradiance. They also stated this chilling stress was observed in thermophilic cyanobacteria but was recoverable (hours to days) after returning to optimal temperatures (Öquist, 1983; Ensminger et al., 2004; Hüner et al., 2020). While the experiments presented here tested culture performance under 24-h irradiance, it may not be reflective of potential system operation. Depending on the heat exchanger interface or photobioreactor installation in the crewed habitat, these cultures may experience periods of darkness. Recognizing that cooling periods would typically be associated with darkness (culture flowing into a metal heat exchanger or night of a Martian sol) may relieve some of the PSII excitation pressure. Conversely, increasing irradiance would nominally be tied to warming periods (habitat-installed photobioreactor exposed to environmental lighting or day of a Martian sol). Coupling environmental temperature and photoperiods may result in enhance culture performance in the cooling loop thermal regime. In the MDV, the algal mats from which the Chlorophyta-Ant culture is derived, are growing under a daily cycle of cooler stream temperatures (4°C) when the sun is low on the horizon and warmer temperatures (up to 15°C) when the sun is directly overhead, and the light intensity is greatest. Adaptation to these conditions may be a factor contributing to the Chlorophyta-Ant performing better in normalized oxygen production and photosynthetic yield.

## Impact of Growth Rate on System Stability

Cycled temperature Chlorophyta-Ant had a significantly lower biomass yield rate than its *C. vulgaris* counterpart (**Table 2**). Field studies have documented gradual growth, as these green mats are slow to reestablish after scouring by major flood events (Kohler et al., 2015). Increasing time between harvest by using slow-growing microalgae may be beneficial for specific types of system operation. Biomass harvesting and processing will require added energy with filters and pumps or crew time. Proposed Martian mission plans include dormant periods between crew occupations (Williams-Byrd et al., 2016; Badger et al., 2018). Reducing harvest frequency resulting from slower growth (Chlorophyta-Ant) may reduce overall energy consumption, and the potential for wasted biomass between occupied periods. Conversely, the resulting higher yield rate for cycled temperature *C. vulgaris* may support habitat operations that require rapid reestablishment or growth (e.g., post culture crash, oxygen or nutritional supplementation, or increase in habitat population). Biomass yield rate was calculated here due to the reduced sampling schedule. More frequent (daily) sampling could capture the onset of the exponential phase and allowing specific growth rate calculations. Thereby, enabling doubling rate calculations for harvesting rate estimation and comparison of metabolic response to environmental conditions.

Closing the carbon loop has been a focus of NASA as missions extend farther from Earth and are unable to rely on frequent resupply missions (NASA, 2015). Using harvested algal biomass as a nutritional supplement or feedstock is one way to provide loop closure. Algal biomass is touted as a superfood with a high edible biomass ratio, and high in protein and fatty acids (Kovač et al., 2013). While a majority of algal biomass is safe for human consumption, literature does not provide a clear consensus on the recommended daily amount of biomass (Hedenskog et al., 1969; Gitelson, 1992; Salisbury et al., 1997; Carlson, 2011;

Caporgno and Mathys, 2018). If the biomass is not consumed or used, it will become waste and reduce the closure efficiency of the system. Preliminary investigation into the composition of the selected Antarctic algal mat identified *Prasiola*, *Chlorococcum*, and *Chlorella* (Van Horn et al., 2016). However, Van Horn et al. suggest conducting a targeted quantitative estimation of each of the identified genera for abundance mapping. This may also benefit spaceflight applications, as various dominant genera may require different preparation for human consumption (Sathasivam et al., 2019).

## Establishing Sustained Operations and Forward Work

The study presented here focused on the metabolic response of microalgal cultures to a dynamic thermal environment reflective of a spacecraft thermal loop. This study did not take into consideration the concept of operations for transporting, establishing, or sustaining these cultures. The following sections are initial suggestions for these operations based on terrestrial findings but should be further tested to establish their suitability.

A surface habitat could integrate a multifunctional bioregenerative life support system into the cabin in a multitude of ways. This study hypothesized that an algal culture would be inoculated into a water-based thermal control system mounted onto the cabin walls. The thermal control system would use opaque tubing to allow the transfer of cabin illumination and cabin heat. Pumps would keep the media constantly moving, lifting the culture, and reducing temperature and nutrient gradients. Gas transfer would be accomplished by nonporous membranes, allowing for simultaneous carbon dioxide, oxygen, and heat transfer as described in Matula and Nabity (2021). Interface heat exchangers incorporated into the loops would transfer collected heat from the media to external ammonia loops or radiator systems. Sample ports throughout the system would allow for harvesting of biomass and nutrient dosing.

It may not be prudent to transport liters of media due to volume and mass constraints, but microalgae may be transported by streaking concentrated cells on agar slants for short durations (approximately 6 months) at room temperature (Hu et al., 2020). However, this approach may require refrigeration to reduce extraneous bacterial growth. Antarctic algal mats are naturally cryodesiccated between austral summers, also reducing the amount of water for transport (Howard-Williams et al., 1986; Hawes et al., 1992). Currently, there is no consensus on proper technique for laboratory cryodesiccation of Antarctic green algal mat with high post-wetting viability percentages (Davey, 1989; Day et al., 1997; Šabacká and Elster, 2006). The Martian surface may provide water from ice harvested from polar icecaps or subglacial brine lakes for rehydration or inoculation of these cultures (O'Callaghan, 2020, 2021; Lauro et al., 2021; Scheller et al., 2021).

A slip stream of 95% $CO_2$ Martian atmosphere could be collected, diluted with $N_2$ or any other inert gas, and sparged into the photobioreactor system (Jha et al., 2006). Lunar surface habitats may use combustion or decomposition of harvested biomass as an additional supply of carbon dioxide. Planetary surface habitats with some amount of gravity may use the buoyancy effects of sparging system to supply carbon dioxide, release oxygen, and mix the system. Conducting experiments with Antarctic Chlorophyta exposed to elevated concentrations of carbon dioxide could help bioregenerative ECLS designers understand operational conditions but also help polar phycologists understand potential future impacts of variations in alkalinity due to accelerated weathering and increasing carbon dioxide concentrations on Antarctic algal mats.

Nutrient dosing was not studied here but it is understood that long-term operation of these photobioreactors, depending on the selected species, may need supplementation of nitrogen, phosphorous, and certain micronutrients for cell sustainment. Human urine has been considered for nitrogen and phosphorous supplementation in spaceflight applications (total nitrogen: approximately 5,600 mg $L^{-1}$, total phosphorous: 310 mg $L^{-1}$) (Blüm et al., 1994; Tikhomirov et al., 2007; Tuantet et al., 2014a; Pickett et al., 2020). Terrestrially, wastewater remediation has successfully cultivated microalgae (specifically *Chlorella* sp.) on wastewater (Wang et al., 2010; Jaatinen et al., 2015; Tuantet et al., 2019). Typically, these studies use diluted urine (1:2–1:200). Urea hydrolysis can rapidly occur in undiluted urine, increasing pH, and precipitating necessary nutrients, thereby reducing growth (Zhang et al., 2014).

Irradiance may be a deciding factor for efficiency of the overall air revitalization and thermal control system. Using in-situ irradiance at the planetary surface may be the most power, mass, and thermally effective way to illuminate the cultures. The Martian surface receives a photosynthetic photon flux density (PPFD) of approximately 80–350 $\mu mol\ m^{-1}\ s^{-1}$ with a yearly average of 230 $\mu mol\ m^{-1}\ s^{-1}$, based upon the Viking Lander 1 location (Clawson and Hoehn, 2005). The Lunar surface receives a nearly continuous 62.1 $\mu mol\ m^{-1}\ s^{-1}$ at the south pole and a maximum of 2,300 $\mu mol\ m^{-1}\ s^{-1}$ during its 29-day lunar day cycle (Badescu, 2012). Lighting systems using LEDs tuned to the photosynthetically active radiation (PAR) spectrum may supplement this *in-situ* resource. However, resulting heat loads require investigation of several types of irradiance sources to benefit the overall efficiency of the photobioreactor system.

Various spaceflight experiments have experienced biofilm formation, and for microalgae, this occurred in areas of concentrated irradiation (Kim et al., 2013; Zea et al., 2018; Helisch et al., 2020). Reduction in irradiance penetration length can result in systems unable to accommodate biofilm accumulation on surfaces, thereby reducing oxygen production capacity of the system (Lee and Palsson, 1994; Tuantet et al., 2014b; Helisch et al., 2020). A cleaning or flow rate regimen in conjunction with material selection may mitigate buildup. Fortunately, wall cleanliness may have minimal impact on the heat transfer capabilities of the system. Algal cells are approximately 99% water by weight (Mata et al., 2012). Gryta (2008) observed that biological deposits on heat transfer surfaces with high water content had significantly less influence on heat transfer when compared to inorganic aggregation. Therefore, if biofilming

progressed, air revitalization capabilities may be affected before heat transfer capabilities.

## CONCLUSION

In this study, the response of *C. vulgaris* and Antarctic Chlorophyta sampled from the MDV to rapid temperature cycles reflective of a spacecraft thermal loop was compared. The measured oxygen production and yield rates were used to estimate the feasibility of using these microalgae for simultaneous closed-loop air revitalization and thermal control in a surface habitat. Due to the intrinsic psychrotolerant properties of Antarctic Chlorophyta, it was hypothesized that these microalgae would outperform their temperate *C. vulgaris* counterparts in the tested temperature regimes. Holding the environmental temperature of *C. vulgaris* at a constant 10°C significantly reduced culture growth and oxygen production, and stressed culture PSII. However, cycling temperature between +9 and +27°C provided environmental respite, reduced cellular stress, and promoted environmental acclimation, observed through increasing media pH, chlorophyll fluorescence, and excess dissolved oxygen concentration. The tested temperature regimes elicited minimal stress from both Antarctic Chlorophyta conditions, as normalized oxygen production either increased or had a minimal decrease and chlorophyll fluorescence was greater in this sample type than in *C. vulgaris*. However, even with the temperature-induced reduction, cycled temperature *C. vulgaris* had a significantly higher normalized oxygen dissolved oxygen concentration than Antarctic Chlorophyta. This preliminary investigation suggests that both *C. vulgaris* and Antarctic Chlorophyta can withstand the dynamic temperature environment reflective of a thermal control loop. Using a bioregenerative approach to simultaneous air revitalization and thermal control may provide carbon loop closure for long-duration surface habitats. However, mission design requirements including radiation environment, habitat thermal loads, and air revitalization demands may favor one tested sample type over the other. Therefore, future research should include longer-duration trial runs with both sample types using the same temperature profile for a balanced comparison. Including elevated carbon dioxide environments and variation in irradiance regimes may also be a better reflection of the surface habitat environment.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

EM developed the concept and design of the study, executed the experiments, produced the statistical analysis, and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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# Photobioreactor *Limnospira indica* Growth Model: Application From the MELiSSA Plant Pilot Scale to ISS Flight Experiment

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Bioregenerative life support systems (BLSS) are the foundation stone to self-sustainable manned space missions. The MELiSSA is a BLSS concept that has evolved through a mechanistic engineering approach designed to acquire both theoretical and technical knowledge on each subsystem independently and, therefore, produces the necessary knowledge and experience needed to co-integrate all the subsystems together with a high level of control. One of the subsystems is a photobioreactor colonized by an axenic culture of the cyanobacterium *Limnospira indica* PCC8005 for revitalizing the air for the crew. This subsystem was extensively studied, and a mass balanced mechanistic model was developed to describe, predict, and control the photobioreactor. The model was based on a light transfer limitation model coupled to a kinetic model for the cyanobacteria growth through a Linear Thermodynamics of Irreversible Processes (LTIP) approach, including substrate limitation. The model was integrated into several hydrodynamic models adapted to several photobioreactors design and experiments, from a 100 L airlift pilot scale ground photobioreactor to a 50 ml membrane photobioreactor for ISS flight. Through this article we detail the principles of this mechanistic model and their application to different photobioreactor scales for predictive and descriptive simulations.

**Keywords: radiative transfer model, Growth model, bioregenerative life support system, Limnospira indica, MELiSSA loop**

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## INTRODUCTION

MELiSSA (Micro Ecological Life Support System Alternative) is an international effort developing technology for regenerative life support to enable long-term human exploration missions in space. Inspired by an aquatic ecosystem, it is conceived as a loop of interconnected bioreactors providing the basic functions of life support (Lasseur et al., 2010; Lasseur and Mergeay, 2021). MELiSSA has six major compartments (C1-C5) or subsystems, coupled together in a closed circulatory loop. Respectively, it involves an anaerobic digestion compartment (C1) followed by an anaerobic mineralization compartment to completely mineralize the organic wastes (C2), an aerobic nitrification compartment for ammonia and urine oxidation (C3), a photobioreactor (C4a), and a higher plants chamber (C4b) for air revitalization and to supply the crew compartment (C5) with water and food. Compared with their Earth counterpart, the drastic reduction in size of artificial LSS, the few degrees of freedom of BLSS, the presence of multiple producers and

consumers, tight technical and operational restrictions, and the different dynamics interacting within and between different compartments result in a complex control problem that requires the replacement of the stochastic "natural" control by a brain-level (intelligent) control. So, it was obvious that an engineering approach for MELiSSA development was mandatory. On one hand, all unit operations in charge of the elementary functions constitutive of the entire Life Support System (LSS) are studied, up to a thorough understanding. On the other hand, the systemic approach of complex, highly branched systems with feed-back loops is performed. Intelligence of the system is based on the adequacy of the knowledge models for representing each unit operation and their interrelations in a suitable degree of accuracy and adequate range of validity of the models for implementing a hierarchical strategy of control. Such a mechanistic modeling is a key stone for supporting the understanding and the maturity of the knowledge of a compartment required, to integrate it in a complex recycling system.

The present work concerns the fourth MELiSSA compartment (C4a), which is the photosynthetic microalgae compartment in charge of producing part of oxygen for the LSS and converts part of waste nitrogen into consumable biomass material. A detailed mechanistic understanding of a light energy transfer mechanism in dense and absorbing media has permitted linking the metabolic activity of the microbial photosynthetic cells to the mixing properties of the reactor and to the light energy supply by external light sources. This has offered the possibility to control the productivity of C4a by modulating the external light supply, to satisfy a fluctuating oxygen demand. It has been shown that it was absolutely mandatory to develop an as complete as possible knowledge model for associating metabolism determinism with physical transfer limitation and mixing properties of the bioreactor. This modeling level has been achieved by integrating both radiative transfer mechanisms and thermodynamic constraints imposed on cell metabolism, including detailed light energy conversion mechanisms at the photosystems level. This level of understanding has been progressively developed during the past few decades by the MELiSSA team, leading to engineering different types of photobioreactors of different sizes.

The present article presents the progressive steps for developing such advanced knowledge models of microalgae photobioreactors and the elemental bricks of science that are necessary to assemble to achieve the suitable level of understanding for including photobioreactors in an LSS such as MELiSSA. Two examples of applications of the photobioreactor model are presented, the first one being related to the operation and control of a pilot scale 80 L airlift photobioreactor operated for years at the MELiSSA Pilot Plant at the University de Barcelona in real life support conditions, the second one being a miniaturized 50 ml membrane photobioreactor that has been operated during 4 weeks in microgravity aboard the ISS in space.

## GROWTH MODEL FOR CYANOBACTERIUM *LIMNOSPIRA* (AKA *ARTHROSPIRA* OR SPIRULINA)

The mechanistic growth model proposed was developed for *Limnospira*, previously called *Arthrospira* and also commonly known as "Spirulina," which is the cyanobacteria grown in the

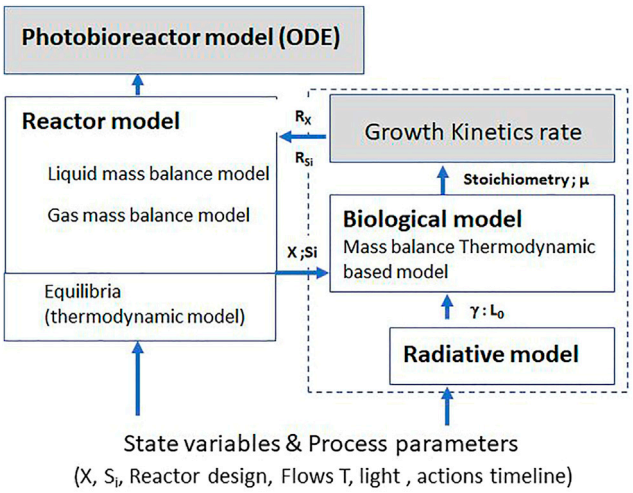


**FIGURE 1 |** Principle of the modeling of photobioreactors using the LTIP growth kinetic model (dashed selection) as an elementary brick.

C4a photoautotrophic compartment of the MELiSSA loop (Lasseur et al., 2010). The strain used in MELiSSA is *Limnospira indica* PCC 8005, a Gram-negative photosynthetic cyanobacterium, from the "Pasteur Culture Collection" (PCC), which was cultivated and maintained in axenic conditions. Recently, taxonomic classification of cyanobacteria has been revised and a new genus, *Limnospira*, and species *indica* has been established in place of the former *Arthrospira sp.* PCC 8005 (Nowicka-Krawczyk et al., 2019).

The growth model detailed here was first proposed by Cornet et al. (1998) as "PhotoSim." This model has been extended and improved since 1998 up to a complete modeling approach for photobioreactors and photoreactors for synthetic photosynthesis (Dauchet et al., 2016). This mechanistic growth model is one brick in a complete photobioreactor model (**Figure 1**). It predicts the growth and the oxygen production rates, and the biomass composition. It includes the mass balance constraints of *L. indica* as a function of reactor design and operating conditions (incident light), so that it can be used for various purposes and reactors. It is composed of two sub-models. The first one is a radiative transfer model, predicting the light distribution in the reactor. The second one is the biological model itself, predicting the biomass composition, the stoichiometric equation representative of cell growth, and the rates as a function of the previously predicted light distribution profile. The growth model presented and discussed hereafter is the essential part of the PBR model and is associated with a second brick, the reactor model, describing flow dynamics and mass transfer. The simplest approach used to describe the dynamic of a reactor is to consider a perfectly mixed reactor for both liquid and gas. The validity of this assumption is checked by either experimental methods (Residence Time Distribution) or numerical methods (Computed Fluid Dynamics). The results presented in the present article consider only perfectly mixed reactors (at least for the liquid phase), but depending on the design and operation of the reactor, the perfectly mixed assumption may not be satisfied, impacting mass and energy

transfer. It must be outlined that the growth model proposed can be used for a non-perfectly mixed reactor by using a more complex reactor model with a discretized approach for example.

## Radiative Transfer (Light) Model

A cell culture in photobioreactor (PBR) is heterogeneous in terms of light availability. The first approximation consists in considering that the system is uniformly distributed according to the direction of the light illumination. This direction is the depth (z) for the flat reactor with one or two sides lighted and the radius of the reactor (r) for cylindrical reactors (**Supplementary Figure S1**). The attenuation of light inside a photosynthetic culture can be calculated by determining the value of irradiance, G (z) ($W.m^{-2}$ or μmol photons $m^{-2}.s^{-1}$), for any depth (z) or radius (r) of the culture, following the two-flux model analytical solution of one of the dimensionless equations (**Eq. 1**, **Eq. 2**, or **Eq. 3**) (Cornet and Dussap, 2009), respectively, for a flat reactor geometry with one side lighted, or flat two sides lighted and/or a cylindrical reactor radially lighted. Even if these analytical solutions result from a simplified approach of the problem of light diffusion, these equations have already been proved efficient in several studies (Cogne et al., 2005; Ifrim et al., 2014; Pruvost et al., 2016):

$$\frac{G_z}{q_0} = 2\left(\frac{n+2}{n+1}\right)\frac{(1+\alpha)e^{\delta(L-z)} - (1-\alpha)e^{-\delta(L-z)}}{(1+\alpha)^2 e^{\delta L} - (1-\alpha)^2 e^{-\delta L}} \quad (1)$$

$$\frac{G_z}{q_0} = 2\left(\frac{n+2}{n+1}\right)\frac{e^{\delta z}\left((1+\alpha)+(1-\alpha)e^{-\delta L}\right) + e^{-\delta z}\left((\alpha-1)+(1+\alpha)e^{\delta L}\right)}{(1+\alpha)^2 e^{\delta L} - (1-\alpha)^2 e^{-\delta L}} \quad (2)$$

$$\frac{G_r}{q_0} = 2\left(\frac{n+2}{n+1}\right)\frac{I_0(\delta r)}{I_0(\delta L) + \alpha . I_1(\delta L)} \quad (3)$$

Here, $n$ is the degree of collimation for the radiation field: $n = 0$ for isotropic intensities and $n = \infty$ for collimated intensity. For *L. indica*, collimated intensity is assumed, so $(\frac{n+2}{n+1}) = 1$.

$I_n$ is the modified Bessel function of the first kind.

$\delta = \frac{(n+2)}{(n+1)X\sqrt{E_a(E_a+2bE_S)}}$ is the two-flux extinction coefficient and is a function of the time-dependent biomass concentration;

$\alpha = \sqrt{\frac{E_a}{(E_a+2bE_S)}}$ is the linear scattering modulus.

$E_a$ and $E_s$ are the mass absorption and the mass scattering coefficients and b is the backward scattering fraction (dimensionless). These optical and radiative properties are determined experimentally by spectrophotometric tools and for *Limnospira indica PCC 8005* specifically. They are important variables describing the so-called shadow effect and how the absorption of light intensity is occurring in the dense media. For *Limnospira indica PCC 8005,* the Ea, Es, and b values are respectively 300, 1,100 $m^2.kg\ X^{-1}$, and 0.03 (Rochatte, 2016). $q_0$ represents the hemispherical incident light flux or photons flux density (PFD). X is the biomass concentration inside the photobioreactor and L is the depth or radius of the photobioreactor. From a biological point of view, photosynthesis is driven by photon flow (μmol.$s^{-1}$) and wavelength. The presented expressions of the radiative model are used for a single-photon wavelength and integrated over the spectrum of the light source. The radiative model used for the growth model (**Figure 1**) is an integrated expression over the light spectrum. Therefore, the radiative properties and the irradiance are averaged values that should be calibrated for the light spectrum used. The parameters used in this article are for a white lamp spectrum.

The radiative transfer model allows calculating the light availability that is defined by the liquid volume fraction: $\gamma = min(1, \frac{L_0}{L})$, where $L_0$ is the depth for which light intensity is equal to the light compensation point. Thus, $\gamma$ represents the illuminated fraction of the reactor and depends on the incident light intensity and the biomass concentration. To control the productivity of a photobioreactor via light availability, one should keep at all times $\gamma < 1$, *i.e.* the cell culture is maintained and grown in light-limiting conditions.

## Biological Model

The biological model is composed of the growth kinetic model, accounting for the photosynthetic activity of the cells, and for the stoichiometry of the photosynthetic growth leading to the relationships between the compounds consumption (nutrients) and production (biomass) rates.

A single mass and elemental (C, H, O, N, S, P) balance supported by the stoichiometric equation (**Eq. 4**) was obtained from a structured approach of the metabolism of *Limnospira indica* PCC 8005 including anabolic reactions rates coupled to the rate of chemical energy carriers ($J_{ATP}$) and of reduced power cofactors (Jcov) driven by photosynthesis (Z scheme) (Cornet et al., 1998). In this equation, $x_{EPS}$ is the molar fraction of exopolysaccharide in the total biomass, so that the stoichiometric equation accounts for variable coefficients and a variable biomass composition, depending on the light energy transducing process.

$$\begin{aligned} &CO_2 + (0.673 + 0.137\,x_{EPS})\,H_2O + 1.192\,(1 - x_{EPS})\,HNO_3 \\ &+ (0.005 + 0.01x_{EPS})\,H_2SO_4 + 0.06\,(1 - x_{EPS})H_3PO_4 \\ &\rightarrow CH_{1.566+0.084x_{EPS}}O_{0.405+0.545x_{EPS}}N_{0.192\,(1-x_{EPS})}S_{0.005+0.010x_{EPS}}P_{0.060\,(1-x_{EPS})} \\ &+ (1.444 - 0.484x_{EPS})\,O_2 \end{aligned} \quad (4)$$

It was demonstrated (Dauchet et al., 2016) that the energy transducing process can be described by the ratio ($J_{ATP}$)/(Jcov), which is termed the $P/2e^-$ ratio, and thus can be handled with the theory of the linear energy converter in terms of Linear Thermodynamics of Irreversible Processes (LTIP) as:

$$x_{EPS} = 1.33\left(\frac{P}{2e^-} - 1.23\right) with\ \frac{P}{2e^-} = \frac{(1+0.91x)}{0.91\,(0.91+x)} \quad (5)$$

where x is obtained as the solution of the following second-order equation:

$$0.91\,(1 + 1.82x + x) - (0.91 + x)\,(1 + 0.91x)\left(1 + \overline{\beta}\right) = 0 \quad (6)$$

$$\overline{\beta} = \frac{1}{\gamma}\left(\frac{1}{L}\int_0^{L0}\frac{Gz}{K+Gz}dz\right) \quad (7)$$

for a flat reactor geometry with one side lighted

$$\overline{\beta} = \frac{1}{\gamma}\left(\frac{1}{L}\left(\int_0^{L0}\frac{Gz}{K+Gz}dz + \int_{L-L0}^{L}\frac{Gz}{K+Gz}dz\right)\right) \quad (8)$$

for a flat reactor with two sides lighted

$$\overline{\beta} = \frac{1}{\gamma}\left(\frac{1}{\pi R^2}\int_{L0}^{R}\frac{Gz}{K+Gz}dz\right) \quad (9)$$

for a cylindrical reactor radially lighted The kinetic of growth associated with **Eq. 4** is equal to the average photosynthetic growth rate, $<r_x>$, calculated for the full liquid volume of the reactor by integrating the local light flux G(z) along the culture depth (**Eq. 10**) (Cornet et al., 1998; Cornet and Dussap, 2009). The respiration of *Limnospira indica PCC 8005* in the dark was assumed to be negligible for a perfectly mixed illuminated bioreactor with a low residence time of the liquid medium in the dark zone, so that only the illuminated fraction of the reactor was really active and contributed to the growth:

$$r_x = (1-f_d)\,\rho_M\,\Phi\,K\,Ea\,\gamma\,\overline{\beta}\,X \quad (10)$$

$f_d$ is the design dark fraction of the reactor, $\rho_M$ the maximum energetic yield for photon conversion, Φ the mass quantum yield for the Z-scheme of photosynthesis, $K$ the half saturation constant for photosynthesis, and $\overline{\beta}$ calculated depending on the reactor design (**Eq. 7**, **Eq. 8**, and **Eq. 9**).

Therefore, this average photosynthetic growth rate considers the light limitation, but not other limitations such as substrates concentrations, effect of temperature and of pH. For substrates, a Monod limiting term was multiplied to $r_x$ for each substrate. In real culture conditions, the inorganic N, S, and P sources used in **Eq. 4** are under their dissociated ionic form. The proportion of dissociated forms for $CO_2$ and $H_3PO_4$ are dependent on pH. Even if the carbon source is written in the form of $CO_2$ in **Eq. 4**, in practice both dissolved $CO_2$ and $HCO_3^-$ support photosynthesis. But as pH 9 ± 1 is optimal pH for the growth of *L. indica*, the carbon source is mainly present in the liquid phase in the bicarbonate, $HCO_3^-$ form. For the same reason, the P source is mainly in the $H_2PO_4^-$ form. The Monod half saturation constants for $NO_3^-$, $SO_4^{2-}$, and $H_2PO_4^-$ are respectively 8.5 $10^{-5}$, 2.6 $10^{-5}$, and 2.8 $10^{-5}$ mol.$L^{-1}$ (Cornet et al., 1998). For the carbon source, because of the intracellular bicarbonate accumulation mechanism and the influence of both light limitation and pH, it was difficult to get reliable values for half saturation constant from the literature and depending on the reactor model developed (accounting or not with pH and substrate dissociated forms) it was chosen for the model that the limiting term can be calculated either for both $CO_2$ and $HCO_3^-$, or only $HCO_3^-$, or TIC (Total Inorganic Carbon), with respectively the following Monod half saturation constant, 1 $10^{-6}$, 5 $10^{-3}$, and 1 $10^{-3}$ molC.$L^{-1}$.

According to Cornet et al. (1998) temperature can be also taken into account for the calculation of $r_x$, using the corrective factor for temperature (optimal temperature = 34.7°C):

$$f(T) = \frac{1.95\,10^{14}\,e^{\left(\frac{-8.3\,10^4}{R\,T}\right)}}{1+2.71\,10^{36}\left(\frac{-2.16\,10^5}{R\,T}\right)} \quad (11)$$

with R the perfect gas constant (8.314 J.mol$^{-1}$.K$^{-1}$) and T the temperature (in K).

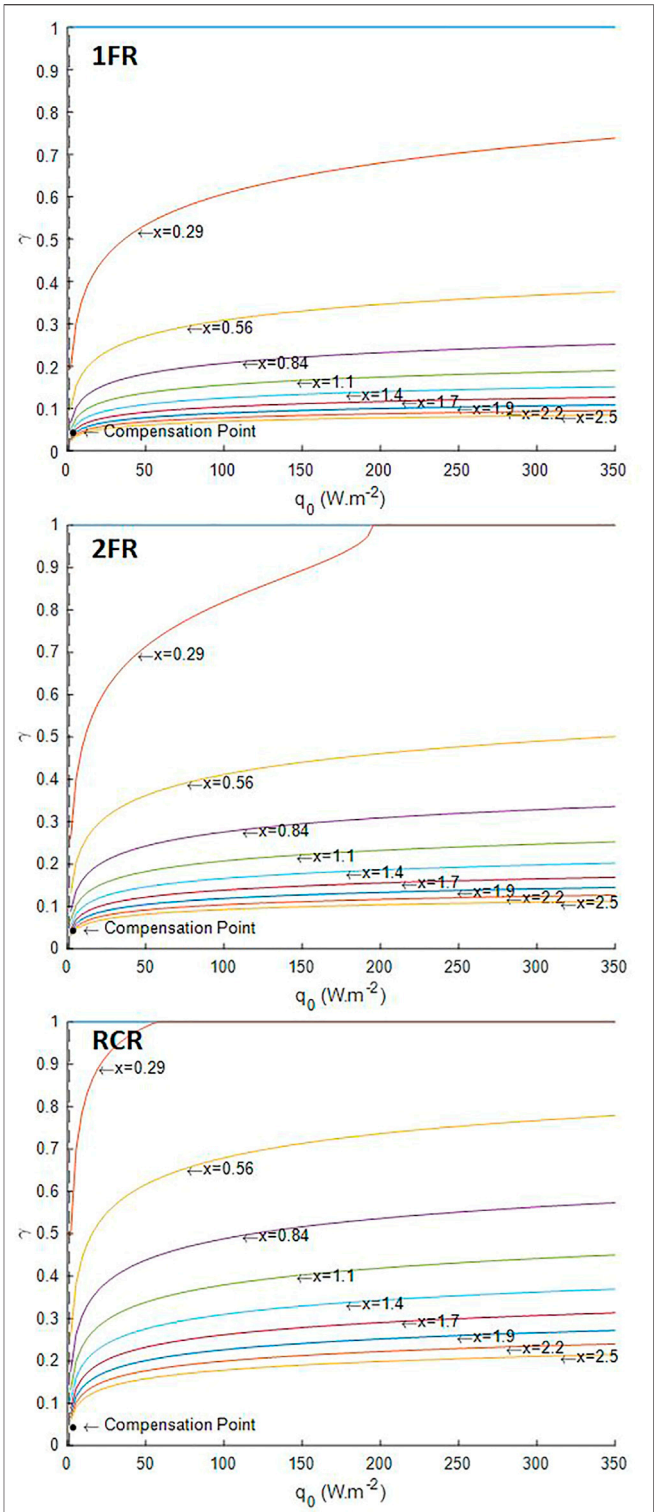


**FIGURE 2 |** Illuminated fraction predicted by the radiative transfer model. 1FR—one-side lighted flat reactor; 2FR—two-side lighted flat reactor; RCR—radially lighted cylindrical reactor.

The kinetic rate of each compound (i) involved in the mass balanced growth equation **Eq. 4** is calculated as:

$$\langle r_i \rangle = Y_{i/x} \langle r_x \rangle \quad (12)$$

where $Y_{i/x}$ is the mass yield in g(i). $gX^{-1}$ calculated according to the stoichiometric coefficients of **Eq. 4** and $\langle r_x \rangle$ the biomass growth rate calculated with **Eq. 10**.

## MODEL FOR THE PREDICTION OF LIGHT DISTRIBUTION, BIOMASS COMPOSITION, OXYGEN PRODUCTIVITY, AND CRITICAL SIZE FOR THREE PHOTOBIOREACTORS DESIGN

The first usage of the growth model of *L. indica* is its application with the three designs of reactor considered in the radiative transfer model, namely the one-side lighted flat reactor (1FR), the two-side lighted flat reactor (2FR), and the radially lighted cylindrical reactor (RCR). The simulation results presented here were obtained assuming no compound limitation, which also implies no pH-related equilibria for substrates and perfectly mixed reactor without other physical limitation than the light, and a white lamp spectrum for the light source. In such conditions, the four parameters required for the model are the characteristic dimension (L) of the reactor (the depth for flat reactor and the radius for the cylindrical reactor), the design dark fraction of the reactor ($f_d$), the biomass concentration (X), and the light incident flux ($q_0$). $f_d$ was taken equal to 0, meaning that all volume of the reactor is lighted. The dimensions, L, are 0.1 m for 1FR, 0.15 m for 2FR, and 0.075 m for RCR that are classical dimensions for laboratory scale pilot reactors (5–100 L). Simulations were done over a range of 0.01–2.5 $gX.L^{-1}$ and 2–350 $W.m^{-2}$.

The light availability defined by the lighted liquid volume fraction $\gamma$ as predicted by the radiative transfer model is presented in **Figure 2**. For the one-side and two-side flat reactors, as normally attempted by their respective design ($0.5L_{2FR}/L_{1FR}$ = 0.75), the asymptotic value of $\gamma$ for the same biomass has a relative ratio $\gamma_{1FR}/\gamma_{2FR}$ of 0.75. It can be noted that for the three designs, the value of $\gamma$ quickly decreases, even with a relatively low concentration of 0.56 $gX.L^{-1}$ and for an incident light of 150 W $m^{-2}$, which is an average value used at laboratory scale artificially lighted photobioreactors, $\gamma$ is respectively equal to 0.33, 0.44, and 0.72 for 1FR, 2FR, and RCR. For 2FR, the shape for 0.29 $gX.L^{-1}$ is classical for two-side lighted reactors (**Supplementary Figure S2**). It is important to recall that the prediction of the depth of the reactor illuminated, represented by the $\gamma$ value, is a key parameter for the prediction of the metabolic $P/2e^-$ (**Supplementary Figure S3**) and therefore for the calculation of the biomass composition and growth rate.

The mass fraction of exopolysaccharide (EPS) predicted for different incident light fluxes conditions using the LTIP model (**Figure 3**) is consistent with experimental observations and metabolic analysis of *Limnospira indica* PCC 8005 growth in the photobioreactor (Cogne et al., 2003). For $P/2e^-$ values greater than a thermodynamic threshold of 1.5, the light energy absorbed by the microorganisms is above their metabolic capacities and leading to physiological limitations. If from a numerical point of view, it is possible to solve the mass balance equation (**Eq. 4**, **Eq. 5**), such solutions are not possible from a biological point of view so that they were highlighted as black dotted area in **Figure 3** and **Figure 4**. Cogne et al. (2003) have indeed demonstrated by analyzing the metabolic network of *L. indica* (formerly called *Arthrospira platensis*) that the metabolic constraint of converting $NADH,H^+$ into $NADPH,H^+$ (which is the only form regulated by photosynthesis) can only be achieved *via* a shunt of phosphoenolpyruvate (PEP) to pyruvate through PEP carboxylase. Calculating the metabolic fluxes for various $P/2e^-$values has led us to demonstrate that an upper limit near 1.47 exists for the $P/2e^-$. This threshold limit corresponds to a metabolic flux through the shunt tending to 0. This upper limit is close to the thermodynamic threshold of the $P/2e^-$ of 1.5. It can be observed in simulations, especially for RCR (**Figure 2**, **Supplementary Figure S3**), that the prediction of the metabolically unfeasible domain is very sensitive to the value of this threshold. For example taking a threshold at 1.482 (~99% of 1.5) predicts that at 350 $W.m^{-2}$, even at 2.5 $gX.L^{-1}$ *L. indica* is above its metabolic capacities (**Supplementary Figure S4**). As a consequence, this permits drawing the conclusion that the behavior of the cyanobacteria is more difficult to predict at high light flux in radially lighted cylindrical, where the high-intensity radiative energy is more homogeneous than in flat reactors where the presence of a dark zone buffers the average high light energy availability. In the same way, this also permits deriving that the lighting conditions need to be reduced at low biomass concentrations, *i.e.* at the beginning of a batch culture after the inoculation, to start the culture without inhibition by a surplus of light energy (also called photoinhibition).

For Continuous Stirred Tank Reactor (CSTR), in steady state, the dilution rate (D) is expressed as $D = \frac{\langle r_x \rangle}{X}$, so that the biological model is used to predict the oxygen productivity and the feasible operating domain (D, $q_0$) for CSTR (**Figure 4**), assuming no other limitation than the light in the reactor (especially no substrate limitation for the growth). The presented results were obtained considering a maximum biomass concentration of 2.5 $g.L^{-1}$. For higher biomass concentrations, it is possible to operate CSTR at lower D than the ones presented in **Figure 4**. As previously discussed, the metabolically unfeasible area (for $P/2e^-$>1.5) is indicated and reduces significatively the operating domain of CSTR. Considering the area/volume ratios 10, 13, and 27 for 1FR, 2FR, and RCR respectively, it is obvious that $O_2$ volumetric productivity ($g.L^{-1}.h^{-1}$) is much higher in the radially lighted cylindrical reactor. It is also observed that when the cylindrical PBR is operated at the highest $O_2$ productivity, the range of possible dilution rates is reduced compared to that for the flat reactors (**Figure 4**). The simulations also highlight that it is difficult to operate RCR as CSTR at steady-state and high light incident flux. This is consistent with the assumption that when $P/2e^-$ is close (*i.e.,* 99%) to the threshold of 1.5, which is the theoretical thermodynamic limit for the continuing metabolic activity, the operation of the CSTR is predicted impossible for incident intensities greater than 350 $W.m^{-2}$ for a biomass lower

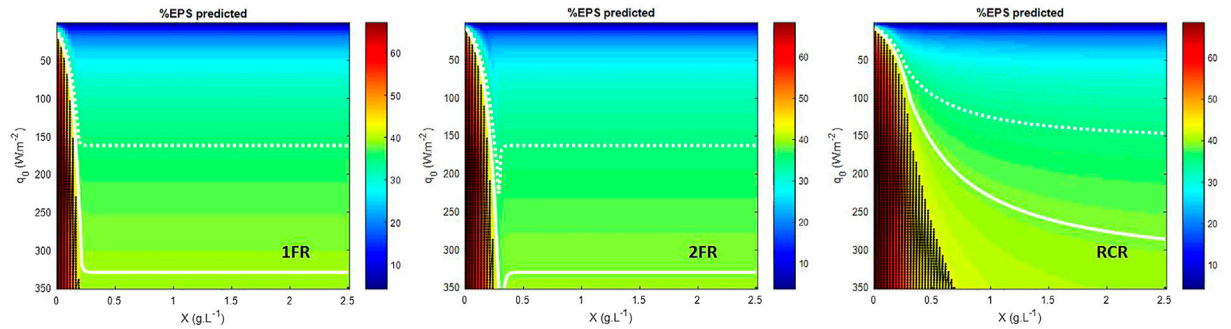


**FIGURE 3 |** EPS biomass fraction predicted. 1FR—one-side lighted flat reactor; 2FR—two-side lighted flat reactor; RCR—radially lighted cylindrical reactor. Dark dot area is the metabolically unfeasible domain predicted for a P/2e$^-$ > 1.5. White dashed line gives the 35% EPS limit and plain white line gives the 39% EPS limit.

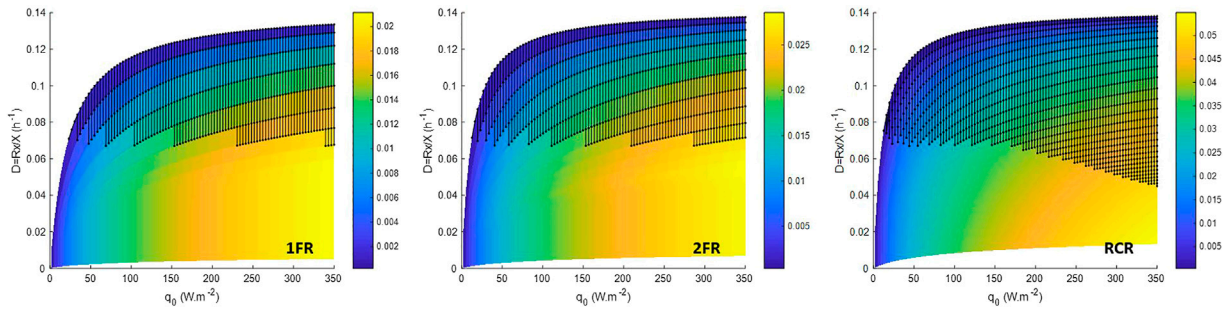


**FIGURE 4 |** Volumetric oxygen productivity (in g.L$^{-1}$ h$^{-1}$) in steady-state CSTR for an operating domain for biomass ranging from 0.01 to 2.5 g.L$^{-1}$. 1FR—one side lighted flat reactor; 2FR—two sides lighted flat reactor; RCR—radially lighted cylindrical reactor. Dark dot area is the metabolically unfeasible domain predicted for a P/2e$^-$ > 1.5.

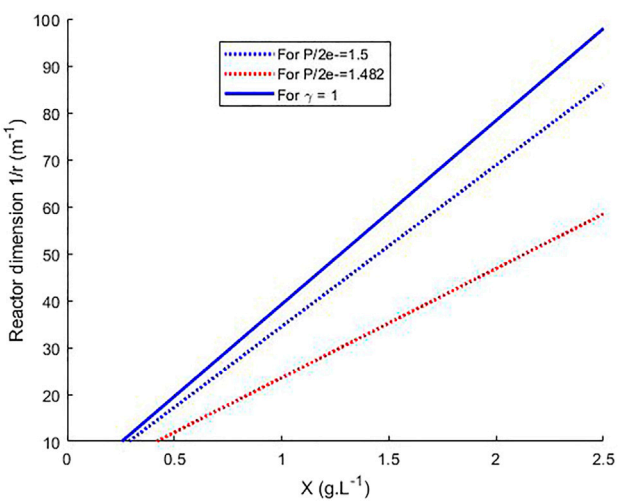


**FIGURE 5 |** Calculation of the radius for a cylindrical PBR for the growth of *L. indica* without other limitations than light in continuous steady-state and for light incident flux of 200 W m$^{-2}$, as a function of biomass concentration and for three criteria, $\gamma$ = 1, P/2e$^-$ = 1.5 and P/2e$^-$ = 1.482.

than 2.5 g L$^{-1}$ or D > 0.012 h$^{-1}$ (**Supplementary Figure S4**). Finally, it is possible to use the model to estimate the critical characteristic dimension of the PBR for example for a given light flux in function of the biomass concentration attempted in steady state (**Figure 5**).

## MODEL FOR OPERATION OF THE C4A IN MELISSA PILOT PLANT

The MELiSSA Pilot Plant was developed with the main goal of demonstrating long-term continuous operation feasibility of the MELiSSA loop under the supervision of a control system at a pilot-plant scale. In the MPP, the different compartments have been scaled up to achieve the oxygen production equivalent to the respiration needs of one human, with 20–40% concomitant production of food (Godia et al. 2014). The photosynthetic bioreactor (compartment 4a in the MELiSSA loop) is an 80 L cylindrical radially lighted external-loop gas lift photobioreactor providing between 5 and 10% of oxygen requirements for one human. Alemany et al. (2019) have demonstrated the validity of the radiative transfer model with a series of experimental tests of

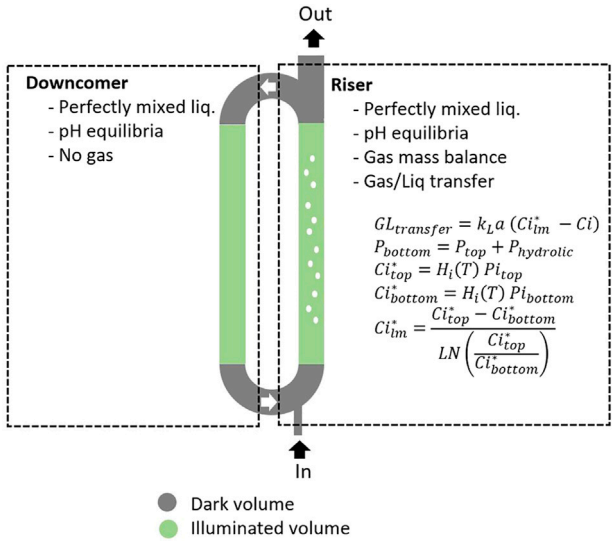


**FIGURE 6 |** Principle of the hydrodynamic model for MELiSSA Pilot Plant PBR.

gas-connected C4a and mock-up crew compartment (C5) that has been conceived and operated as a 3-rats' isolator. The photobioreactor model used by Alemany et al. (2019) is a "N-tank in series" model to characterize the nonideal mixing status of both liquid and gas phases into the 80 L air-lift PBR that is used. The biological model relies on a former approach of *L. indica* growth with a model based on separate kinetics for active biomass synthesis (proteins and main cell constituents) and exopolysaccharides (EPS).

The results obtained for a newer version of the MPP-PBR, which has been refurbished with an efficient LED-based lightening system, are presented hereafter. This equipment allows reaching up to 364 $W.m^{-2}$ for incident light energy flux for the C4a. The LTIP model used for the growth of *L. indica* was associated (**Figure 1**) with a reactor model for the air lift PBR considering riser/downcomer sections. The reactor is sketched as a series of two perfectly mixed reactors for the liquid phase, one for the riser and one for the downcomer. The gas phase flow is represented by a plug flow model in the riser, which means the mass transfer driving force for gas/liquid exchange has to be considered a Log average between the top and the bottom of the column for the gaseous compounds (**Figure 6**). The TIC (i.e., all dissociated forms of $CO_2$) are calculated considering the pH equilibria of $CO_2$<-> $HCO_3^-$<-> $CO_3^{2-}$, coupled to the $CO_2$ gas/liquid mass transfer and equilibrium. The mass balance equations of the PBR are written and solved for each section. For simulations, the following parameters were used: liquid volume of 83 L, gas volume of 1 L, design dark fraction ($f_d$) of 0.2, column radius of 0.075 m, gas flow rate of 2.8 $NL.min^{-1}$ (NL is the gas volume in L measured at 1 atm and 0°C), pressure in the headspace of the reactor 1 atm, temperature of 36°C TIC are taken as carbon source in the model instead of only $HCO_3^-$.

The accuracy of model prediction is compared for four steady-state experiments (SS1, SS2, SS3, SS4, with four different dilution rates) obtained at MELiSSA Pilot Plant (**Table 1**). At D = 0.01 $h^{-1}$, the TIC limitation is predicted, indicating a fair consistency between observations and simulation. It is also to outline that for D = 0.025 $h^{-1}$ the experiment has failed for an incident light of 364 $W.m^{-2}$, which is consistent with the former predictions on the effect of light energy inhibition at high intensities and the maximum value of $P/2e^-$ and the threshold that is reached at the metabolic level when the metabolic flux of the phosphoenolpyruvate (PEP) shunt was decreased to 0. In engineering terms and bioreactor control, it was necessary to decrease light energy flux down to 250 $W.m^{-2}$. The C4a MPP column, with a radius of 0.075 m, can be compared to the ideal RCR reactor (**Figure 3**, **Figure 4**). Interestingly, the previous conclusions obtained for bench scale reactors, when the operating conditions are too close to the metabolic $P/2e^-$ threshold, remain fully valid for the real pilot-scale reactor, having a much more complex liquid and gas phases behaviors.

Therefore, the model suitably describes and predicts the dynamic growth of the microalgae and the productivities ($O_2$ production and $CO_2$ uptake rates per unit of time and of PBR volume) for a large set of PBR operating conditions. This has been described in literature for various reactor designs (Cogne et al., 2005; Cornet and Dussap, 2009; Ifrim et al., 2014; Rochatte, 2016) and also for the MELiSSA C4a (Alemany et al., 2019).

From the above, the model can be used to simulate the PBR over a large range of conditions producing a large amount of data that could not be obtained experimentally without spending time and resources. From these simulations, abacuses are created, supporting a complete understanding of the process over various combinations of operating conditions. An example of abacuses is given in **Figure 7** for the MPP C4a operated at steady

**TABLE 1 |** Comparison between steady-state results (SS1—SS4) obtained with the C4a MPP model (simulation) and experiments.

| | SS1 | SS2 | SS3 | SS4 |
|---|---|---|---|---|
| D ($h^{-1}$) | 0.010 | 0.015 | 0.020 | 0.025 |
| Light ($W.m^{-2}$) | 364 | 364 | 364 | 250 |
| pH | 8.4 | 8.3 | 8.54 | 8.54 |
| Gas in $0_2$% | 20.47 | 20.47 | 20.47 | 20.47 |
| Gas in $CO_2$% | 2 | 3 | 3 | 3 |
| TIC in (mg $C.L^{-1}$) | 0 | 0 | 0 | 0 |
| **TIC out (mg $C.L^{-1}$)** | | | | |
| Simulation | 282 | 445 | 510 | 484 |
| Experiment | 230 ± 5 | 425 ± 4 | 423 ± 3 | 403 ± 2 |
| **Biomass ($g.L^{-1}$)** | | | | |
| Simulation | 2.29 | 1.68 | 1.26 | 0.88 |
| Experiment | 2.25 ± 0.07 | 1.74 ± 0.05 | 1.26 ± 0.03 | 0.96 ± 0.01 |
| **Gas out $O_2$%** | | | | |
| Simulation | 21.8 | 21.8 | 21.8 | 21.7 |
| Experiment | 21.9 | 21.8 | 21.9 | 21.8 |
| **Gas out $CO_2$%** | | | | |
| Simulation | 0.68 | 1.23 | 0.91 | 0.88 |
| Experiment | 0.81 | 1.22 | 1.18 | 1.10 |
| **$rO_2$ (g $O_2.h^{-1}$)** | | | | |
| Simulation | 3.2 | 3.5 | 3.4 | 3.0 |
| Experiment | 3.3 ± 0.1 | 3.8 ± 0.1 | 3.9 ± 0.1 | 3.5 ± 0.1 |
| Monod C limitation | 0.86 | 0.95 | 0.96 | 0.96 |

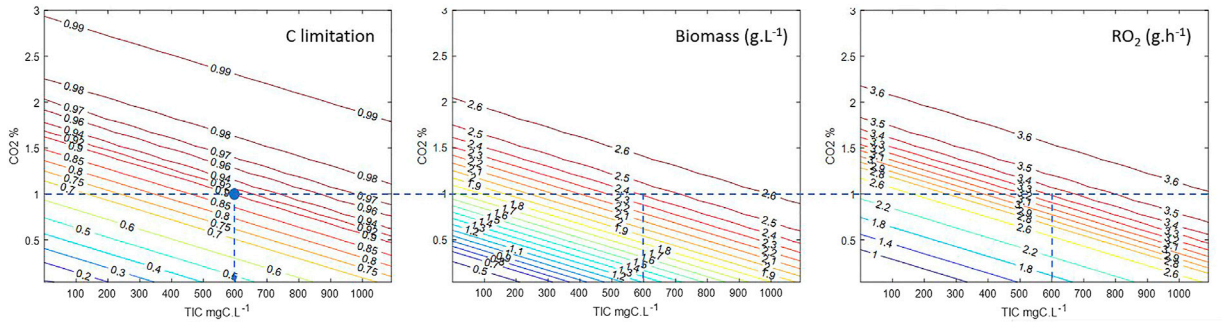


**FIGURE 7 |** Abacuses calculated for simulation of MPP C4a at D = 0.01 $h^{-1}$, pH = 9.5, q0 = 364 $W.m^{-2}$ over a range of TIC inputs of 0–1,100 mg $C.L^{-1}$ and $y_{CO2}$ input of 0–3%. Dotted line gives a lecture of the figures for a criteria $y_{CO2}$ = 1% and C limitation = 0.9.

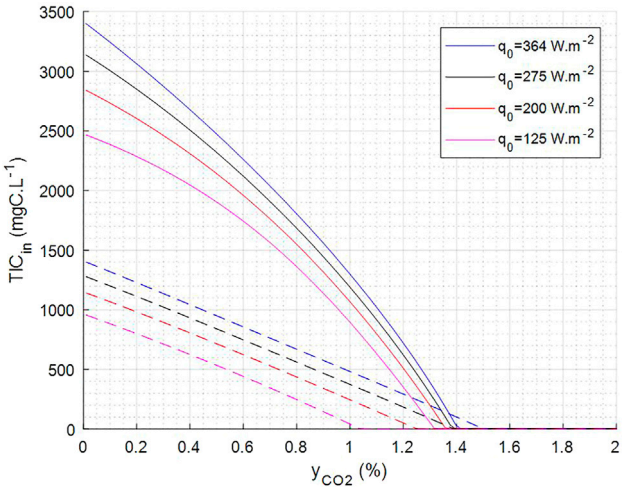


**FIGURE 8 |** Predicted operating condition to keep C limitation = 0.85 (dashed lines) and yCO2 out = 0.2% (plain line). Operation shall be above dotted line to have >0.85 C limiting and below plain line to have <0.2% yCO2 at output.

state with a hydraulic residence time (HRT) of 100 h, at the maximum light flux allowed by the LED lighting system of the reactor and pH 9.5. With these abacuses the risk of C limitation is directly evaluated as a function of the two input variables TIC and $y_{CO2}$, displaying the corresponding PBR performances, biomass concentration, and oxygen productivity. In terms of LSS control, namely in the MELiSSA loop, these inputs are not necessarily fixed, considering that TIC depends on the other MELiSSA compartments, as liquid output from C3 is the liquid input flow of C4a, and $y_{CO2}$ depends on C5 (crew compartment). Conceptually, the MELiSSA LSS loop is a highly branched and interconnected system, which is a common situation for any LSS, calling for a strictly controlled system. By example, the interpretation of these abacuses indicates that if $y_{CO2}$ is controlled at 1%, the minimal TIC input to provide is 600 mg $C.L^{-1}$, leading to a steady-state biomass concentration of 2.4 $g.L^{-1}$ and an oxygen production rate of 3.3 $O_2$ $h^{-1}$ defining directly the expected performances of the system.

Moreover, these abacuses can be fitted by quadratic functions to prevent an online integration of the model and therefore to obtain simpler expressions (**Supplementary Figure S5**) that are further used to define operating domain and operating set points. For example, the fit of $y_{CO2}$ at the output and of Monod C limiting factor calculated by the model (average over the riser part and the downcomer part) for TIC input and $y_{CO2}$ input varying respectively in a range of (0–1,100) $mgC.L^{-1}$ and (0–2)% was done for a hydraulic retention time (HRT) of 100 h, for pH 9.5 and four different light incident fluxes. The correlations obtained were used to define the operating domain (**Figure 8**—domain between dashed lines and plain lines) for which C limitation is above 0.85 and $y_{CO2}$ at output is below 0.2%. This indirect use of the mechanistic model of the PBR can be used for the control strategy of the complete MELiSSA loop, as it allows defining set points for the control of the complete loop under selected constraints. With the simple example presented here, it gives the set point domain for the TIC output of the C3, *i.e.,* TIC input of the C4a, and for the $y_{CO2}$ output of the C5, i.e., $y_{CO2}$ input of the C4a as the selected constraints on C4a.

## MODEL FOR DESCRIPTION/ANALYSIS OF ARTHROSPIRA-B PHOTOBIOREACTOR SPACE FLIGHT EXPERIMENT

Another application of the growth model of *L. indica* in PBR was also successfully used to describe the Arthrospira-B experiment, which was the first experiment in space with online measurements of both oxygen production rate and growth rate in four batch photobioreactors running under microgravity on-board ISS (Poughon et al., 2020). This application concerns a membrane PBR with a liquid volume of about 50 ml, which is a one-side lighted flat PBR, in a size 1,400 times smaller than the 80 L MPP C4a gas lift PBR. The ability to use a growth model with such different reactors in their design and scale demonstrates the robustness and the importance of the mechanistic approach that was followed for PBR modeling, including light energy transfer, mixing, and metabolism determinisms. As for the MPP C4a, the reactor model that was associated with the growth model (**Figure 1**) was developed specifically for the Arthrospira-B

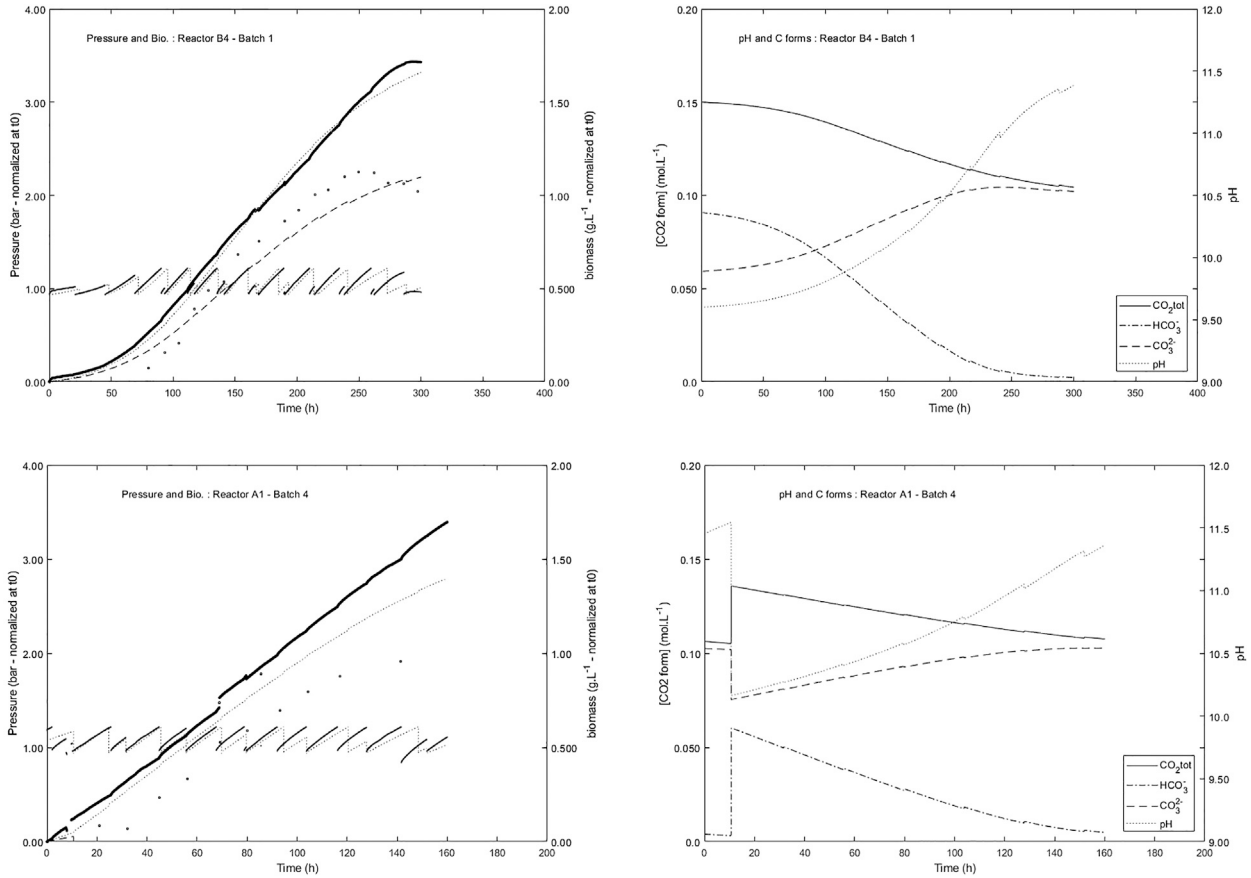


**FIGURE 9 |** Comparison of experimental results (pressure and biomass) with simulation for two batches of reactors on board ISS during Arthospira-B experiment (dot and plain line are experimental results. Dashed lines are simulations). pH and ionic forms of inorganic carbon are predicted. The evolution of the pressure is the result of the release of the pressure relief valve either automatically (set point 1.15 bar) or daily scheduled for the biomass sampling.

reactors. The liquid phase is described by a perfectly mixed model for implementing the mass balances for a batch reactor; the gas phase is described by a gas pressure/overpressure model. The micro-size reactor was only controlled for overpressure release and there was neither pH control nor pH measurement, due to the unavailability of a miniaturized space compatible pH probe stable operating in the high-pH medium. The growth model was therefore completed by a model for pH prediction in the liquid phase allowing the assessment of pH increase associated with the bicarbonate consumption for the biomass growth (Poughon et al., 2020). With a nominal incident light flux of 7.6 $W.m^{-2}$ (maximum 9.8 $W.m^{-2}$) and a depth of 0.015 m, the growth was never over the $P/2e^{-}$ threshold, even at start-up with low biomass concentrations (ca. 0.120 $g.L^{-1}$), which guaranties the light inhibiting conditions were never reached. For this experiment the pH increase is a key factor for the prediction of the growth, the carbon source being bicarbonate ions $HCO_3^{-}$. Therefore, the important variable was not the TIC but the actual concentration of $HCO_3^{-}$. This allows considering the potential pH limitation due to the fact that pH was increasing as bicarbonate was exhausted in the liquid medium. When pH is modified, the equilibria of the dissolved carbon forms (carbonate, bicarbonate, and $CO_2$) are displaced so that molecular dissolved $CO_2$, which concentration is linked to the $CO_2$ partial pressure, is decreased. In the Arthrospira-B experiment, the pressure increase is directly linked to the oxygen production, and the online pressure measurement and the cumulative pressure calculation were the main variables used to compare the experiments and the model simulations. Over the 16 batches produced on-board ISS (four batches for each of the four reactors integrated in the ISS Biolab facility) two were presented here (**Figure 9**): one is the first batch of reactor called B4 and one is for the fourth batch of the reactor called A1. A quite satisfactory fit was achieved between the experimental and simulation results, especially at the beginning of the batches. At the end of the batches, deviations are observed in the pressure profiles between simulation and measurements. A lower $O_2$ productivity (lower pressure increase) observed is not fully explained in the model by the $HCO_3^{-}$ limitation associated with the pH increase. The pH predicted by the model at the end of the batch is greater than 11 and limits for predictivity by the *L. indica* growth model were obviously reached considering that it was never experimentally verified and validated for such a high pH value. Nevertheless, when a suitable mixing of the liquid phase was maintained in microgravity

conditions the satisfactory prediction of the model that was built from ground experiments has demonstrated that microgravity had no first-order effect on the oxygen production rate of *Limnospira indica* PCC8005 in a photobioreactor operating in space in zero gravity conditions.

## CONCLUSION

Mechanistic modeling is a prerequisite for intensive applications of any system, whatever this system uses living microorganisms or not. The previous results clearly prove that a mechanistic modeling of a photobioreactor must associate physical understanding of light energy transfer in dense and absorbing media, physicochemical equilibria of dissociated electrolyte such as carbonate, bicarbonate and pH influence, liquid and gas phase mixing properties in defined volumetric characteristics and finally a thorough understanding of the metabolic level for describing the functioning and regulation of both photosynthetic organelles and metabolic fluxes, including thermodynamic constraints. This robust modeling strategy, which has been illustrated here, has permitted us to suitably understand and control a pilot-scale PBR (air-lift technology) functioning in actual LSS conditions and to interpret the results obtained from a micro-sized membrane reactor that has been operated in microgravity on ISS. Besides the fact that this modeling approach has been used in completely different and challenging conditions, including the design phases of the two PBR, it has permitted us to complete the unavailable non-measured variables, such as the pH and the production rates in microgravity, and to anticipate the control variables for supporting the respiration needs of a completely closed system, associating the constraints set related to a closed recirculating system functioning with minimal mass buffers.

Therefore, the modeling issues not only provide a clue for interpreting the metabolic functioning of living organisms in real environment of bioreactors but they also become a corner stone for designing efficient devices and intelligent multilayer control of complex systems. The price to pay is definitively to be capable of developing a totally multidisciplinary approach associating physical, chemical and biochemical sciences, in a common simulation platform, supporting the view of the phenomena at various scales, from molecular and metabolism to physical transfers and mixing properties and accounting for the coupling effects between different potentially limiting rates.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

LP worked on the PhotoSim model (v20.1.1) implementing and compiling past results obtained by the GEPEB team of Institut Pascal under supervision of C-GD and CC. FG and NL are supervisors of MELiSSA related activities respectively at the MPP in UAB and for ArtEMISS project at SCK CEN, including the Arthrospira-B flight experiment. LP wrote the manuscript. All authors contributed equally to manuscript review.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fspas.2021.700277/full#supplementary-material

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# Understanding Reduced Gravity Effects on Early Plant Development Before Attempting Life-Support Farming in the Moon and Mars

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Plants are a necessary component of any system of bioregenerative life-support for human space exploration. For this purpose, plants must be capable of surviving and adapting to gravity levels different from the Earth gravity, namely microgravity, as it exists on board of spacecrafts orbiting the Earth, and partial-*g*, as it exists on the surface of the Moon or Mars. Gravity is a fundamental environmental factor for driving plant growth and development through gravitropism. Exposure to real or simulated microgravity produces a stress response in plants, which show cellular alterations and gene expression reprogramming. Partial-*g* studies have been performed in the ISS using centrifuges and in ground based facilities, by implementing adaptations in them. Seedlings and cell cultures were used in these studies. The Mars gravity level is capable of stimulating the gravitropic response of the roots and preserving the auxin polar transport. Furthermore, whereas Moon gravity produces alterations comparable, or even stronger than microgravity, the intensity of the alterations found at Mars gravity was milder. An adaptive response has been found in these experiments, showing upregulation of WRKY transcription factors involved in acclimation. This knowledge must be improved by incorporating plants to the coming projects of Moon exploration.

**Keywords: root meristem, cell proliferation, ribosome biogenesis, auxin, transcriptomics, random positioning machine, International Space Station, space farming**

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## INTRODUCTION

The achievement of plant cultivation in space, also called "space farming" is an important step in the development of bioregenerative life-support systems (BLSS) to enable long-term human space exploration. Plants are fundamental elements of these BLSS, since they can provide unique and essential factors and components for the support of the human life in extra-terrestrial environments. They are a source of oxygen and supply a variety of valuable nutrients of different types, including vitamins; they contribute to the regulation of the atmospheric humidity and are capable of removing and recycling carbon dioxide, one of the major waste elements of life whose accumulation destabilizes environmental balances (Ferl et al., 2002; Wheeler, 2017). Last, but not least, plant culture is an activity recognized by astronauts and cosmonauts as highly rewarding in terms of psychological well-being, a non-negligible factor to be taken into account for the success of space exploration enterprises (Haeuplik-Meusburger et al., 2014; Zhang et al., 2020).

The first step of space exploration consists of leaving the Earth, stepping into the outer reaches of atmosphere. Space explorers, and their accompanying plants, are exposed to microgravity

conditions, as they exist on spacecrafts and stations orbiting the Earth, such as the International Space Station (ISS). Moreover, to enable human settlements on nearby planets, the influence on the plant physiology of partial or reduced gravity levels, such as those existing on the surface of the Moon (1/6 = 0.17 *g*) or Mars (3/8 = 0.38 *g*) should be investigated (Kiss, 2014). Plant response to these partial gravity levels, is of utmost importance taking into account the plans of the space agencies to travel back to the Moon (Artemis and Gateway) in 2024 (Chavers et al., 2019; Von Ehrenfried, 2020) and to Mars in the near future. In this respect, it is worth mentioning that a terrestrial cotton seed was reported to have germinated on the lunar surface on board of the Chinese Chang'e 4 probe, which landed on the Moon in January 2019. Seed germination took place within the "life-regeneration ecosystem" facility carried by the craft. Details of the experiment, following the life trajectory of cotton seed germination, development, and final fate after long term exposure to super cold temperature, are publicly available, although only in the form of a preprint, and not of a peer-reviewed publication (Xie et al., 2021).

## PLANTS AND GRAVITY. PLANT RESPONSE AND ACCLIMATION TO MICROGRAVITY

Plants of different species have been successfully grown in space, under microgravity conditions, on numerous occasions, reaching different developmental stages, including the completion of the full seed-to-seed life cycle (Merkys et al., 1984; Musgrave et al., 2000; Karahara et al., 2020). The earliest plant growth experiment was performed within the Oasis 1 hardware aboard Salyut 1 in 1971 (Harvey and Zakutnyaya, 2011; reviewed by Zabel et al., 2016). Then, experiments were performed on board of spacecrafts such as the NASA Space Shuttle or the Soviet MIR Station, but the assembly and operation of the International Space Station (ISS) was a giant step forward in the achievement of this objective (Vandenbrink and Kiss, 2016). In parallel, similar plant growth experiments on Earth, using ground based facilities for microgravity simulation, such as clinostats and random positioning machines have been successfully run (Kiss et al., 2019). They have provided a reliable replica of the biological results obtained in space experiments under real microgravity, even though the gravity vector is not possible to be avoided or removed on the Earth surface (Herranz et al., 2013; Medina et al., 2015; Van Loon, 2016).

These works using plants should be considered in the context of studies of microgravity effects extensively carried out in a wide range of living organisms, including the commonly recognized biological model species of most taxonomical categories, from mammals to bacteria. In general, microgravity is perceived by living beings as a stress-generating environmental factor, although the intensity of the physiological alterations caused is rather variable between the different organisms (Bizzarri et al., 2015; Van Loon et al., 2020). Two effects of the altered or null gravity signal, one direct and another indirect, overlap in the generation of a biological response: the direct effect is the modification of physiological processes (e.g., the changes in the mechanisms of biochemical reactions); the indirect effect is the physical change of collateral elements or factors which are not intrinsic part of the physiological processes, but may have an influence on them. Examples of these side-effects are the diffusion of water in the soil, that may alter the correct hydration of roots, or the convective processes, that may influence the correct gas exchange. It is rather difficult to separate these two types of effects when analyzing experimental results, although some attempts have been done using simulated microgravity (Herranz et al., 2013). These indirect effects can be mitigated with adequate ventilation and adequate watering systems, as is currently being done in Veggie and APH facilities on the ISS (Massa et al., 2016; Monje et al., 2019; Monje et al., 2020).

Moreover, these effects are difficult to overcome since terrestrial organisms have evolved under a constant gravity vector (Bizzarri et al., 2015). Although, in principle, terrestrial organisms do not seem prepared to be confronted with an environment devoid of the gravity force, and they indeed lack of a genetic equipment specialized in the response to this condition, signs of acclimation and adaptation processes have been found in some species, as a result of their genetic plasticity, a feature greatly varying between different taxa (Paul et al., 2012;

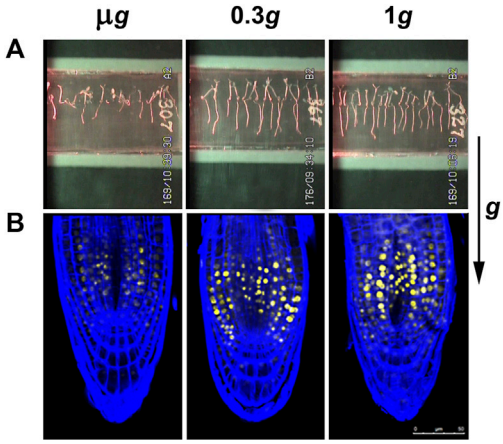


**FIGURE 1 |** Images obtained from the spaceflight experiment "Seedling Growth" at different levels of gravity. Seedlings of *Arabidopsis thaliana* were grown in the ISS for a period of 6 days from seed hydration, within the EMCS facility. Seedlings grew under a white light photoperiod for 4 days and under lateral red light photoactivation for the last 2 days. The gravity conditions were constant throughout the entire growth period. The levels 0.3 *g* (approx. the Mars gravity) and 1 *g* (Earth gravity) were obtained with a built-in centrifuge. (**A**): Images of seedlings in growth chambers (cassettes) at the end of the growth period. Seedlings grown in microgravity (μ*g*) appear disoriented, whereas under 0.3 and 1 *g* seedlings show a conspicuous gravitropism (the direction of the *g* vector is indicated at the right). (**B**): Confocal microscopic images of the root tips of seedlings of the DII-Venus reporter line of *A. thaliana* grown in the same conditions. Yellow fluorescence corresponds to a negative pattern of the distribution of the phytohormone auxin. Roots were counterstained with the SCRI 2200 dye (Renaissance Chemicals, North Duffield, UK) for the visualization of the cell wall in blue. Roots grown in microgravity show an altered pattern of auxin distribution with respect to the 1 *g* control, indicative of some inhibition of the auxin polar transport. On the contrary, the pattern of roots grown at 0.3 *g* is basically similar to the control pattern, indicating that auxin polar transport is not altered at these partial-*g* levels.

Medina et al., 2021). Actually, comprehensive studies on these processes are still insufficient and it appears that, similar as the intensity of the gravitational stress, different taxonomic categories display a different adaptability.

In the particular case of higher plants, gravity is an environmental factor decisively affecting plant growth, by means of the process called gravitropism, which modulates the growth orientation according to the gravity vector, with positive root gravitropism and negative shoot gravitropism. Nevertheless, in microgravity, the cue for this tropism (i.e., the gravity vector) is not present. The consequence is the substitution of gravitropism by automorphogenesis, a process producing in roots spontaneous curvatures followed by straight root elongations in random directions (Hoson and Soga, 2003; Driss-Ecole et al., 2008). This is accompanied by an intense reorganization of the transport and distribution of phytohormones, mostly affecting auxin, but also cytokinin, according to the available data, still incomplete, obtained in real and simulated microgravity (Manzano et al., 2013; Ferl and Paul, 2016; Yamazaki et al., 2016) (**Figure 1**). This hormonal perturbation is transduced to meristem, producing the alteration of cell proliferation rate and ribosome biogenesis during seedling development (Matía et al., 2010), which could have consequences at the level of the developmental pattern of the plant, ultimately relying on meristematic activity. In addition, an observed effect of microgravity is the induction in the plant of a higher sensitivity to other environmental cues, such as moisture or light, that are usually masked by dominant gravitropism (Manzano et al., 2021).

Physiological and cellular alterations are related to changes in gene expression, which have been identified by transcriptomic studies from space and ground experiments (Paul et al., 2012; Kwon et al., 2015; Johnson et al., 2017; Paul et al., 2017; Choi et al., 2019; Herranz et al., 2019; Kamal et al., 2019; Vandenbrink et al., 2019; Kruse et al., 2020; Villacampa et al., 2021a). In some cases, complementary proteomic studies were also performed (Kruse et al., 2020). Other than real or simulated microgravity, the environmental conditions of these experiments were far from homogeneous, which complicated direct comparisons. To overcome this inconvenience, an important effort of data sharing and harmonizing has been undertaken in the NASA-led initiative called GeneLab (Ray et al., 2018). This effort has allowed the identification of some common responses, often related to general mechanisms of defense against stress, such as the system of heat shock genes producing Heat Shock Proteins (HSP), which are molecular chaperones acting to protect and refold proteins in response to cellular damage. In addition, the oxidative stress pathways, involving the production of Reactive Oxygen Species (ROS), the cell wall remodeling system, cytoskeleton alterations and plastid genes, including those regulating photosynthesis, were included in the core set of functions appearing affected by spaceflight environment (Correll et al., 2013; Zupanska et al., 2013; Kwon et al., 2015; Johnson et al., 2017; Choi et al., 2019; Kruse et al., 2020). In none of these transcriptomic studies, specific genes for the response of plants to microgravity, or to the spaceflight environment, were found. For some authors these differentially expressed genes (DEGs) represent alterations caused by the gravitational stress (Johnson et al., 2017; Choi et al., 2019), whereas other interpretations highlight the fact that DEGs were found from viable samples, indicating that they reflect the mechanism of physiological adaptation of plants to spaceflight (Paul et al., 2012; Paul et al., 2017).

In any case, whether due to or despite genetic and physiological alterations, plants are capable of acclimating to the environmental change, since they survive and fulfill their entire life cycle in the microgravity environment of spaceflight (De Micco et al., 2014; Karahara et al., 2020; Khodadad et al., 2020). Most likely, plants could trigger an early and primary acclimation response to the environmental change from Earth to space, to overcome the early alterations that have been repeatedly detected in seedlings. In more advanced developmental stages, more stable adaptive responses could be produced, including genetic and epigenetic changes capable of being transmitted to the offspring, allowing the survival of plants in the space environment throughout successive generations. Many uncertainties on these mechanisms still remain and they pose a fundamental challenge for space plant biology, now and for the coming years.

## GROWTH OF PLANTS IN FRACTIONAL GRAVITY. STUDIES IN SPACE AND IN GROUND BASED FACILITIES

In contrast with the body of research acquired dealing with plant growth and development in microgravity, the number of studies investigating plant response to fractional or partial levels of *g* is considerably reduced. Prior to the assembly and operation of ISS only a few studies were carried out using partial-*g* levels, with the specific interest of the determination of thresholds for gravity detection (Kiss, 2014). On board of the American shuttle Columbia, using a centrifuge microscope, the orientation of the unicellular motile algae *Euglena* in water at different levels of gravity (gravitaxis) was followed. The threshold capable of triggering a gravitactic response was determined to be around 0.16 *g* (Häder et al., 1996). In other experiment, the gravitropic statolith system of the rhizoids of the green alga *Chara* was investigated in sounding rockets and also in parabolic flights using an on-board centrifuge. Acceleration of 0.14 *g* resulted in a displacement of statoliths toward the cell wall in the periphery of the cells, while acceleration of 0.05 *g* did not result in statolith movement (Limbach et al., 2005).

Once the ISS was assembled, the European Modular Cultivation System (EMCS), which was installed and actively operated from 2008 to 2018, made possible a significant progress in our knowledge of the plant response to spaceflight conditions by offering a reliable and versatile control of different environmental parameters related to plant growth, by overcoming some of the aforementioned unwanted side-effects of the microgravity environment, such as those affecting convection and watering. In addition, for the specific case of studies at fractional gravity levels, it provided the ability to apply different *g*-forces in space by means of a built-in centrifuge

(Brinckmann, 2005; Kittang et al., 2014). An intelligent and effective policy of cooperative agreements between agencies regarding the use of this facility multiplied the outcomes of the experiments performed in it. As an outstanding example, the NASA-ESA "Seedling Growth" series of experiments was executed in this hardware to test the influence of blue and red light photostimulation in the plant response to the reduced gravity stimuli (Kiss et al., 2014). In a first experiment, different gravity levels were applied (microgravity; 0.1 *g*; Moon; Mars; near-Earth *g*-level; 1 *g*) to blue-light stimulated *Arabidopsis thaliana* seedlings grown for 6 days and demonstrated a replacement of gravitropism by blue-light-based phototropism signaling at microgravity level (Vandenbrink et al., 2019), but a striking stress response was found at 0.1 *g*. Different components of the transcriptional response to the lack of gravity were determined as the *g*-gradient was progressively reduced (Herranz et al., 2019). In a successive experiment, three *g*-levels (microgravity, Mars gravity level and 1 *g* ground reference run) were applied and photostimulation was with red light. In this case, the analysis was carried out by combining morphological and molecular approaches (confocal and electron microscopy, and transcriptomics). Fundamental novel findings were obtained from this experiment. Firstly, seedlings grown under Mars gravity exhibited a conspicuous root gravitropic response, and, consequently, a balanced distribution of the phytohormone auxin throughout the root, indicative of a regular auxin polar transport (**Figure 1**). Furthermore, microgravity and partial gravity were found to trigger differential responses. The microgravity environment activates hormonal routes responsible for cell proliferation and cell growth and upregulates plastid/mitochondrial-encoded transcript expression, even in the dark. In contrast, the Mars gravity level inhibits these routes and activates responses to stress factors to restore cell growth parameters, only when red photostimulation is provided. This response is accompanied by upregulation of several transcription factors (TFs), such as the environmental acclimation-related WRKY family (Villacampa et al., 2021a). WRKYs are a family of numerous TFs, many of them involved in both abiotic and biotic stress responses, related to hormonal signaling and in acclimation processes (Phukan et al., 2016). This is a strong indication of the triggering of a transcriptional adaptive response to the new environment. Further validation of all transcriptomic results was obtained by comparison to other transcriptomic data obtained from *A. thaliana* seedlings grown during spaceflights and available in the GeneLab database (Ray et al., 2018).

Other studies in ISS have looked further into the determination of the threshold of gravity perceived by roots. A *g*-value of $2.0 \times 10^{-3}$ *g*, estimated punctually at $1.4 \times 10^{-5}$ *g*, was found enough to trigger some motion in statoliths, although the participation of the actomyosin system in this motion was considered essential (Driss-Ecole et al., 2008).

In parallel to the works carried out in space facilities, some studies have used terrestrial devices to reproduce Moon or Mars gravity levels and study their biological effects in experiments performed on Earth. An outstanding case of such these experiments consisted of the use of the Random Positioning Machine (RPM) and the incubation of *A. thaliana* seedlings in this device for the biological validation of the terrestrial analog of the partial-*g* conditions (Manzano et al., 2018). The RPM was originally designed to simulate microgravity, but an adaptation of this device was developed to expose living samples to simulated fractional levels of gravity. For this purpose, two different paradigms were used, one by implementing a centrifuge on a nominally operating RPM ($RPM^{HW}$), and the other by applying specific software protocols to driving the RPM motors, thus changing the nominal mode of operation of the facility ($RPM^{SW}$). Both approaches produced similar results, but the RPM software version showed an ideal working range at 0.05–0.4 *g*, while the RPM hardware version may work better at gravity levels above 0.3 *g*. The effects of the simulated partial gravity were tested in plant root meristematic cells, a system with known response to real and simulated microgravity. Cell proliferation appeared increased and cell growth was reduced under Moon gravity, compared with the 1 *g* control. The difference with control was even higher at the Moon *g* level than in simulated microgravity, indicating that meristematic competence (balance between cell growth and proliferation) was also affected at this gravity level. However, the results at the simulated Mars *g*-level were close to the 1 *g* static control. This suggests that we can separate the levels of gravity capable of being sensed at a molecular level by different intracellular mechanisms, from the gravity threshold capable of triggering a response in the root, visually detectable in the form of a defined growth direction according to the gravity vector. This threshold requires a level intermediate between Moon and Mars gravity (Manzano et al., 2018).

The $RPM^{HW}$ system was also used to incubate *in vitro* cell cultures of *A. thaliana* for a detailed study of the effects of simulated Mars gravity on the regulation of cell cycle progression and cell growth. Different times of exposure were tested. The effects observed at the Mars gravity levels were qualitatively similar as those caused by microgravity, but quantitatively less intense. Alteration in cell cycle regulator mechanisms associated to changes in the levels of some selected factors was detected, leading to changes in the duration of cell cycle phases, according to flow cytometry data. Nucleolar activity for ribosome biogenesis was reduced progressively with the exposure time and epigenetic mechanisms involved in chromatin remodeling were also altered (Kamal et al., 2018). Cell cultures were also exposed to partial-*g* levels by using parabolic flights and a clinostat (Fengler et al., 2016). It is worth noting that the reliability of the results obtained with these procedures is questionable. In the case of the parabolic flight, the alternation of the exposure of the biological samples to different levels of gravity in the same parabola complicates the attribution of specific effects to defined causes; in the case of the clinostat, fast clinorotation has been shown to produce unwanted side effects and additional forces that complicate the determination of the actual g-force sensed by the biological sample (Villacampa et al., 2021b; Herranz et al., 2021). Transcriptomic data obtained from the exposed cell cultures show a lower number of DEGs than in the case of microgravity and an

enrichment of DEGs in the cell wall metabolism and remodeling category (Fengler et al., 2016).

An interesting alternative for the study of the response of living beings, and particularly plants, to hypogravity is to make use of the Reduced Gravity Paradigm (RGP). It is based on the premise that adaptations expected or seen going from a higher to a lower gravity level are similar as changes seen going from 1 *g* to microgravity or partial gravity (Van Loon, 2016). The paradigm is not focused on the absolute acceleration values, but rather on the responses generated due to the delta between two gravity levels and it can be put in practice by using centrifuges for incubation of plant samples. In a typical experiment, the plant sample is incubated at the 2 *g* level until it shows a stable, steady state physiology. Then the acceleration of gravity is reduced and the plant will respond to this reduction. The hypothesis is that the adaptation e.g., from 2 to 1 *g* is similar to the adaptation from 1 *g* to microgravity. The magnitudes of the responses might be different, but the directions of the response would be the same (Van Loon, 2016).

## FUTURE PROSPECTS

In the long term, studies on plant growth and development at fractional *g*-levels will pave the way to understand the molecular mechanisms to improve the cultivation conditions of plants on different planets. These discoveries can be applied in the design of bioregenerative life-support systems and space farming. Regarding the use of ground based facilities, further research is needed, especially around and beyond Mars *g*-levels, to better understanding the differences and constraints in the use of these facilities for the space biology community. However, the most important effort should be invested in incorporating plants to the coming initiatives of deep space exploration. The cis-lunar space considered for the Gateway project should be efficiently used for incorporating plants (both model and crop species) to a research platform that will be a fundamental tool to fill this gap in knowledge (Paul and Ferl, 2018). This will be crucial for further exploration initiatives, such as an extended mission to Mars.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

FJM received the invitation to contribute to the Research Topic, proposed the topic to the rest of authors and wrote the first draft of the manuscript. AM, AV, MC, and RH carried out the experimental works reported in the manuscript, actively discussed the topics, ideas, concepts and hypothesis contained in the manuscript, provided corrections to the draft manuscript and approved the final version.

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# Evaluating the Cost of Pharmaceutical Purification for a Long-Duration Space Exploration Medical Foundry

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There are medical treatment vulnerabilities in longer-duration space missions present in the current International Space Station crew health care system with risks, arising from spaceflight-accelerated pharmaceutical degradation and resupply lag times. Bioregenerative life support systems may be a way to close this risk gap by leveraging *in situ* resource utilization (ISRU) to perform pharmaceutical synthesis and purification. Recent literature has begun to consider biological ISRU using microbes and plants as the basis for pharmaceutical life support technologies. However, there has not yet been a rigorous analysis of the processing and quality systems required to implement biologically produced pharmaceuticals for human medical treatment. In this work, we use the equivalent system mass (ESM) metric to evaluate pharmaceutical purification processing strategies for longer-duration space exploration missions. Monoclonal antibodies, representing a diverse therapeutic platform capable of treating multiple space-relevant disease states, were selected as the target products for this analysis. We investigate the ESM resource costs (mass, volume, power, cooling, and crew time) of an affinity-based capture step for monoclonal antibody purification as a test case within a manned Mars mission architecture. We compare six technologies (three biotic capture methods and three abiotic capture methods), optimize scheduling to minimize ESM for each technology, and perform scenario analysis to consider a range of input stream compositions and pharmaceutical demand. We also compare the base case ESM to scenarios of alternative mission configuration, equipment models, and technology reusability. Throughout the analyses, we identify key areas for development of pharmaceutical life support technology and improvement of the ESM framework for assessment of bioregenerative life support technologies.

**Keywords: techno-economic analysis, equivalent system mass, space exploration medical foundry, *in situ* resource utilization, pharmaceutical foundry, monoclonal antibody purification, human exploration mission, space systems bioengineering**

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# INTRODUCTION

## The Need for a Pharmaceutical Foundry in Space

Surveying missions to Mars, like the InSight lander[1] (Overview | Mission–NASA's InSight Mars Lander) launched in 2018 and Perseverance rover[2] in 2020, directly support the objectives of NASA's long-term Mars Exploration Program[3]: an effort to explore the potential for life on Mars and prepare for human exploration of Mars. The maturation of the program requires redefining the risks to human health as mission architectures transition from the current "Earth Reliant" paradigm used on the International Space Station (ISS) to the cislunar space "Proving Grounds" and finally to deep-space "Earth Independent" mission architectures, as defined in NASA's report titled, "Journey to Mars: Pioneering Next Steps in Space Exploration."[4]

Human missions to Mars will be "Earth Independent," meaning there will be very limited emergency evacuation and re-supply capabilities along with substantially delayed communications with the Earth-based mission team. The NASA Human Research Roadmap[5] currently rates most human health risks, which include "risk of adverse health outcomes and decrements in performance due to inflight medical conditions" and "risk of ineffective or toxic medications during long-duration exploration spaceflight," as either medium or high risk for a Mars planetary visit/habitat mission. Risk ratings are based on failure mode and effects analysis and on hazard analysis using dimensions of severity, occurrence, and detectability. A recent review highlights the current understanding of the primary hazards and health risks posed by deep space exploration as well as the six types of countermeasures: protective shielding, biological and environmental temporal monitoring, specialized workout equipment, cognition and psychological evaluations, autonomous health support, and personalized medicine (Afshinnekoo et al., 2020).

Of these countermeasures, it could be argued that medicine is the most crucial and least advanced toward mitigating space health hazards. There is very limited information on, and few direct studies of, pharmaceutical usage, stability, and therapeutic efficacy (i.e., pharmacokinetics, pharmacodynamics) in spaceflight or in a Mars surface environment (Blue et al., 2019). Furthermore, flown stores of pharmaceuticals face two additional barriers: (1) radiation-accelerated degradation (Du et al., 2011), and (2) addressing a myriad of low occurrence and high impact health hazards without the ability to fly and maintain potency of therapeutics for all of them. In these circumstances, it is often more beneficial to build robustness to these low occurrence health hazards rather than to try to predict them. It is therefore imperative that on-planet and/or in-flight pharmaceutical production be developed to bridge this risk gap. These pharmaceutical foundry technologies will supplement, not replace, the flown pharmaceutical formulary designed to treat anticipated medical threats during space missions.

Pharmaceuticals are produced either chemically or biologically. A recent review of pharmaceutical production for human life support in space compares these two methods, highlighting the need for biological production in order to address many low occurrence and high impact health hazards (e.g., sepsis, ear infection, and glaucoma) and further comparing different biological production systems (McNulty et al., 2021). One major advantage of biological production is the efficiency in transporting and synthesizing genetic information as the set of instructions, or sometimes the product itself, to meet the therapeutic needs for a variety of disease states. The emerging field of Space Systems Bioengineering (Berliner et al., 2020) encapsulates this need for biological production, of which pharmaceuticals is identified as an important subset.

## The Bottleneck of Space Foundries: Purification

Biopharmaceuticals must be purified after accumulation with the biological host organism, or cell-free transcription-translation reaction, in order to meet requirements for drug delivery and therapeutic effect (Harrison et al., 2015). The majority of commercial biopharmaceutical products are administered via intravenous and subcutaneous injection (Škalko-Basnet, 2014). Biopharmaceutical formulations for injection requires high purity (>95%) product, as impurities introduced directly into the bloodstream can trigger significant immune responses and reduce efficacy (Haile et al., 2015).

Downstream processing of biopharmaceuticals is therefore usually a resource-intensive section of overall processing, being cited as high as 80% of production costs (and contributions of input mass) for monoclonal antibody (mAb) therapeutics produced using mammalian cell cultures (Rathore et al., 2015; Budzinski et al., 2019). In addition to the processing burden for biopharmaceutical injectables, there are also often substantial storage costs involving complex supply chain and storage management with stability requirements for factors including temperature, time, humidity, light, and vibration (Sykes, 2018). There are several approaches being pursued to overcome the challenges and costs associated with downstream processing and formulation.

First are the tremendous efforts in process intensification (Strube et al., 2018). While the highly sensitive nature of biopharmaceuticals to minor process changes has introduced barriers and complexities to innovation through process intensification that have not been realized in non-healthcare biotechnological industries, there have been significant strides made in the past decade in the areas of process integration (Steinebach et al., 2017), automation (Pollard et al., 2017), and miniaturization (Adiga et al., 2018; Crowell et al., 2018).

**Abbreviations:** CHM, pre-packed chromatography; EHS, environmental, health, and safety; ELP, elastin-like polypeptide; ESM, equivalent system mass; Fc, fragment crystallizable; ISRU, *in situ* resource utilization; ISS, International Space Station; mAb, monoclonal antibody; MAG, magnetic bead; PMI, process mass intensity; OLE, oilbody-oleosin; RMA, reference mission architecture; SPN, spin column; VIN, plant virus-based nanoparticle.

[1]https://mars.nasa.gov/insight/mission/overview/

[2]https://mars.nasa.gov/mars2020/mission/overview/

[3]https://mars.nasa.gov/

[4]http://go.nasa.gov/1VHDXxg

[5]https://humanresearchroadmap.nasa.gov/Risks/

Another route that researchers are pursuing to reduce downstream processing costs and resources is a biological solution to processing technology. In the same vein that the biopharmaceutical industry sprung out of researchers leveraging the power of biology to produce therapeutically relevant molecules that were inaccessible or excessively costly by means of chemical synthesis, researchers are now also trying to apply that same principle to purifying therapeutically relevant molecules. The simplicity of production, reagents that can be produced using self-replicating organisms, and potential recyclability of spent consumables are significant advantages of biological purification technology for space or other limited resource applications. Examples of primary biological technologies include fusion tags (Bell et al., 2013), stimuli-responsive biopolymers (Sheth et al., 2014), hydrophobic nanoparticles (Jugler et al., 2020), and plant virus nanoparticles (Werner et al., 2006; Uhde-Holzem et al., 2016).

Lastly, there are vast efforts to establish alternative drug delivery modalities (Anselmo et al., 2018). Other modalities that do not require injection and which might be more compatible to administration in limited resource environments, such as oral consumption, nasal spray, inhalation, and topical application, have long presented challenges in biopharmaceutical stability (e.g., denaturation in stomach acid) and delivery to the active site (e.g., passing the gut-blood barrier) that minimize product efficacy and necessitate costly advanced formulations and chemistries (Mitragotri et al., 2014).

A particularly promising drug delivery technique to circumvent downstream processing burdens is to sequester the active pharmaceutical ingredient in the host cells of the upstream production system as a protective encapsulation in order to facilitate bioavailability through oral delivery (Kwon and Daniell, 2015). It represents an opportunity to greatly lower the cost of *in situ* production of human medicine for a space mission. This technique presumes that the host system is safe for human consumption, and so naturally lends itself to utility in systems such as yeast and plant production hosts. Oral delivery via host cell encapsulation has been recently established as commercial drug delivery modality with the US Food and Drug Administration approval of Palforzia as an oral peanut-protein immunotherapy (Vickery et al., 2018). However, this solution is not necessarily amenable to the diversity of pharmaceutical countermeasures that may be required, especially for unanticipated needs in which the product may not have been evaluated for oral bioavailability.

## Space Economics

In 2011, the space shuttle program was retired due to increasing costs, demonstrating that reduction of economic cost is critical for sustaining any campaign of human exploration (Wall, 2011). Although recent efforts in reducing the launch cost to low earth orbit by commercial space companies have aided in the redefinition of the space economy (Whealan George, 2019), the barrier to longer term missions, such as a journey to Mars, is still limited by the extreme financial cost in transporting resources. Additionally, it has been shown that as the mission duration and complexity increases–as expected for a human mission to Mars–the quantity of supplies required to maintain crew health also increases (Anderson et al., 2018). In the case of meeting the demand for medication, biopharmaceutical synthesis has been proposed as an alternative to packaging a growing number of different medications (Menezes et al., 2015; McNulty et al., 2021). Assuming that both technologies can meet mission demand, selection of the production-based biotechnology platform will be dependent on its cost impact. It is therefore critical that the cost model of biopharmaceutical synthesis accounts for and minimizes the cost of any and all subprocesses, including those for purification.

The current terrestrial biopharmaceutical synthesis cost model does not align with the needs for space exploration environments. For example, the literature highlights the high cost of Protein A affinity chromatography resin ($8,000–$15,000/L) and the need to reduce the price (Ramos-de-la-Peña et al., 2019). However, the purchase cost of chromatography resin is not nearly as critical in space environment applications where the major costs are more closely tied to the physical properties of the object (mass, volume, refrigeration requirements, etc.), as a result of fuel and payload limitations and the crew time required for operation (Jones, 2001). The distinct cost models of space and terrestrial biopharmaceutical production may increase the burden of identifying space-relevant processing technologies and may also limit direct transferability of terrestrial technologies without attention given to these areas.

On the other hand, changing incentives structures relating to sustainability and the advent of new platform technologies are rapidly increasing alignment and the potential for technology crossover. For example, companies like On Demand Pharmaceuticals[6], EQRx[7], and the kenUP Foundation[8], initiatives leading to industry adoption of environmental footprint metrics such as E-factor (Sheldon, 2007) and process mass intensity (PMI) (Budzinski et al., 2019), and diffusion from the adjacencies of green and white biotechnology (Tylecote, 2019) all promote development of accessible and sustainable technologies. As these trends pertain to space-relevant processes, these examples can also be viewed as driving more closed loop systems composed of simpler components.

### Reference Mission Architecture

The evaluation of biopharmaceutical system cost for space applications requires the establishment of a reference mission architecture (RMA) as a means for describing the envelope of the mission scenario and distilling initial technology specifications which relate to the proposed subsystem in question (Drake and Watts, 2014). This RMA can be used to orient and define the specific mission elements that meet the mission requirements and factor into the calculations of cost for deploying biopharmaceutical technologies. Ultimately, the RMA provides the means to determine and compare cost given specification of mission scenarios that utilize the technology in question. We envision developing and integrating

[6]ondemandpharma.com

[7]eqrx.com

[8]kenup.eu

biotechnological capabilities back-ended by purification and quality systems into standard methods composed of a series of unit procedures that maintain astronaut health via the Environmental Control and Life Support Systems (ECLSS) (Hendrickx et al., 2006). In this study, we begin to build toward this vision by proposing a high-level RMA that specifies a biopharmaceutical demand partially fulfilled through biomanufacturing over the course of a defined production window.

### Equivalent System Mass

In planning for future human exploration missions, technology choices and life-support systems specifications are often evaluated through the metric of the equivalent system mass (ESM) (Levri et al., 2003). Driven by the economic factor of cost in dollars required to transport mass into orbit, the ESM framework accounts for non-mass factors such as power, volume, and crew-time by relating them to mass through predetermined equivalency factors. ESM has been used to evaluate the mass of all of the resources of a larger system including water, shielding materials, agriculture and recycle loop closure. Currently, ESM remains the standard metric for evaluating advanced life support technology platforms (Hogan et al., 2000; Zabel, 2020). In the Space Systems Bioengineering context of realizing a biomanufactory on the surface of Mars (Berliner et al., 2020), recent advances in extending this metric have been proposed in the form of extended ESM which attempts to address complexities stemming from multiple transit and operations stages, as would be required to support a crewed mission to Mars (Berliner et al., 2021). It also accounts for uncertainties inherent in mission planning such as technology failures and their downstream effects as propagated through a mission such as refrigeration failures in systems housing medicine that requires specific cooling. Such advances in the ESM framework aid in the assessment of biopharmaceutical technologies as elements in the context of proposed ECLSS given the inherent stochastic nature of human health, especially in a space environment (Bizzarri et al., 2017). Here, we calculate ESM at multiple mission segments across which biopharmaceutical purification is deployed.

## MATERIALS AND METHODS

## Unit Procedure Selection

### Protein A-Based Affinity Capture Step

The medical significance of mAb therapies and the highly developed and specialized purification technology provide a fertile ground for techno-economic feasibility analysis of an *in situ* resource utilization (ISRU)-based pharmaceutical foundry for space. The first reason is that there are mAb therapies commercially approved or in development for multiple important disease states of spaceflight including osteoporosis (Faienza et al., 2018), migraines/headaches (Schuster and Rapoport, 2016), seizure (Zhao et al., 2017), pneumonia (Hua et al., 2014), ocular herpes (Krawczyk et al., 2015), otitis media (Iino et al., 2019), various oncological indications (Zahavi and Weiner, 2020), and fungal infections (Ulrich and Ebel, 2020). A second reason is that degradation products of mAb therapies are known to result in, not just reduced efficacy, but also deleterious effects (e.g., harmful immune reactions in patients) that further compound concerns of pharmaceutical stability over a long-duration mission (Laptoš and Omersel, 2018). Thirdly is that a common manufacturing system can be used to produce treatments for a variety of indications which is highly advantageous in mass and volume savings for spaceflight. And fourthly, the economic incentive of research into mAb purification technology has resulted in a plethora of technologies, enabling this analysis to include head-to-head comparisons between multiple mAb capture steps of different origins (e.g., biotic, abiotic) and different processing mechanisms (e.g., bind-and-elute mode liquid chromatography, precipitation). It is in comparing the differences between these technologies that we can uncover general insights into the desired components of a pharmaceutical foundry for space.

Monoclonal antibody therapy is a platform technology that supports human health across a diversity of medical indications with a generally maintained molecular structure, in large part due to the coupling of high target selectivity in the two small and highly variable complementarity-determining regions located in the antigen-binding fragments (Goding, 1996) and control of the biological action on that target (i.e., effector function) through the generally conserved fragment crystallizable (Fc) region (Kang and Jung, 2019). This otherwise high structural fidelity conserved across mAb therapy products (which are primarily of the immunoglobulin G class) spans a wide variety of therapeutic indications and creates an opportunity for generic mAb production process flows, which include technologies devised specifically for mAb production (Sommerfeld and Strube, 2005). This specialized manufacturing, which is most notable in the use of the affinity capture step targeting the Fc region of an antibody with the use of the protein-based ligands derived from the *Staphylococcus aureus* Protein A molecule, can be tuned for highly efficient purification of mAb and antibody-derived (e.g., Fc-fusion protein) class molecules (Ramos-de-la-Peña et al., 2019). Therefore, we have decided to investigate the Protein A-based affinity capture step in isolation as a starting point for understanding the costs of a potential pharmaceutical foundry in space.

It is worth noting that other similar protein ligands, such as Protein G and Protein L, are also widely used for their ability to capture different types of immunoglobulin classes and subclasses more efficiently (Choe et al., 2016).

### Abiotic and Biotic Protein A-Based Unit Procedures

We chose to analyze six Protein A-based capture step procedures: three commercially available abiotic technologies [pre-packed chromatography (CHM), spin column (SPN), and magnetic bead (MAG)] and three development-stage biotic technologies [plant virus-based nanoparticle (VIN), elastin-like polypeptide (ELP), and oilbody-oleosin (OLE)] (**Figure 1**). Commercial technology procedures are based on product handbooks while the procedures of developing technologies, which we would classify as Technology Readiness Level 2 per NASA's guidelines, are based on reports in literature. This set of procedures was selected

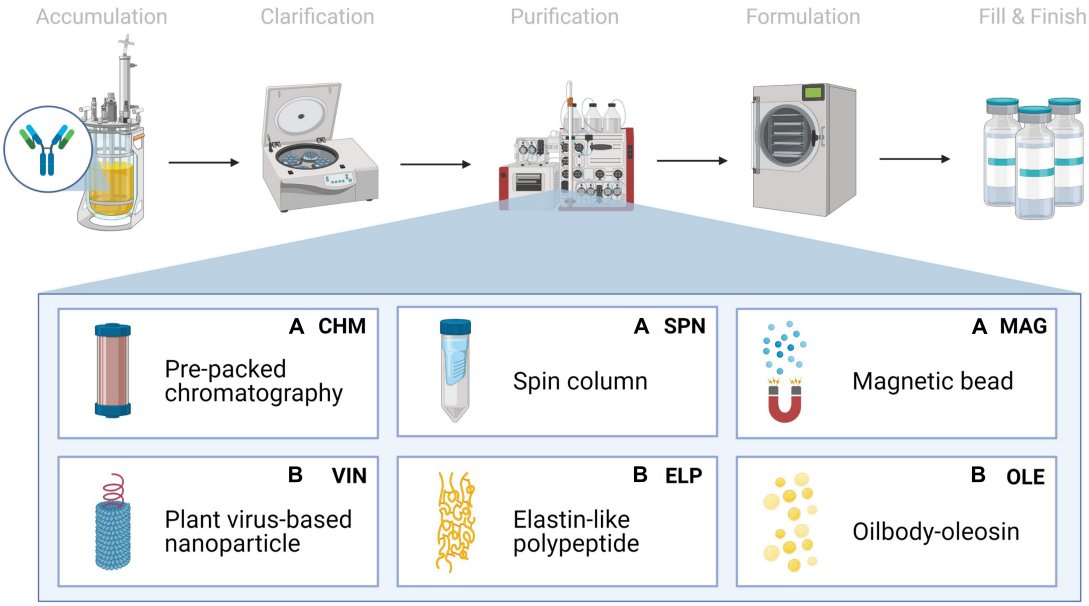


**FIGURE 1 |** Monoclonal antibody production consists generically of product accumulation, clarification, initial purification, formulation, and fill & finish. Here we investigate six technologies for the capture step within the first purification step in a space mission context using extended equivalent system mass. The manufacturing origin of the capture reagent is denoted as either **(A)** abiotic or **(B)** biotic.

**TABLE 1 |** List of Protein A-based monoclonal antibody capture step unit procedures included for analysis.

| Unit procedure ID | Method | Technology used | References |
|---|---|---|---|
| Pre-packed chromatography[(A)] | Liquid chromatography | Pre-packed HiTrap MabSelect SuRe column of novel alkali-tolerant recombinant Protein A-based ligand coupled with an agarose matrix | Vendor handbooks (Cytiva, 2006, 2020, 2021) |
| Spin column[(A)] | Centrifuge-assisted liquid chromatography | Pre-packed Protein A HP SpinTrap spin column containing Protein A Sepharose High Performance | |
| Magnetic bead[(A)] | Magnetic separation | Protein A Mag Sepharose superparamagnetic beads coupled with native Protein A ligands | |
| Plant virus-based nanoparticle[(B)] | Sedimentation complex | Plant virion, *Turnip vein clearing virus*, presenting a C-terminal coat protein fusion display of Protein A (domains D and E) | Werner et al., 2006 |
| Elastin-like polypeptide[(B)] | Inverse transition cycle | Elastin-like polypeptides [78 pentapeptide (VPGVG) repeats] fused with Z domain, an engineered B domain of Protein A | Sheth et al., 2014 |
| Oilbody-oleosin[(B)] | Liquid-liquid partition | *Arabidopsis* oleosin fused at the N-terminal with an engineered Protein A(5) | McLean et al., 2012 |

[A] *Abiotic technology.*
[B] *Biotic technology.*

to survey a wide range of operational modalities, technological chassis, and perceived advantages and disadvantages (**Table 1**).

All six of the unit procedures are operated in bind-and-elute mode, in which a clarified mAb-containing liquid stream is fed into a capture step containing Protein A-based ligand, which selectively binds the mAb and separates the mAb from the bulk feed stream. The mAb is eluted from the Protein A-based ligand and recovered using a low pH buffer to dissociate the mAb from the ligand. Finally, the low pH environment of the recovered mAb is pH neutralized for future processing or storage. The analysis does not consider differences in mAb processing upstream or downstream of the affinity capture step that may arise from differences in the unit procedure operations.

Pre-packed chromatography is a chromatography system consisting of a liquid sample mobile phase which is pumped through a pre-packed bed of Protein A-fused resin beads housed in a column. SPN is a similar system, in which a Protein A-fused resin bead bed has been pre-packed into a plastic tube housing and the mobile phase flow is controlled via centrifugation of the plastic tube. MAG is a slurry-based magnetic separation system that uses superparamagnetic particles coated with Protein A-fused resin mixed as a slurry with the feed mAb stream for capture and elution of the mAb by magnet. VIN is a sedimentation-based system that uses plant virion-based chassis fused with Protein A-based ligands in suspension for capture of the mAb and centrifugation, assisted by the sedimentation velocity contribution of the chassis, to isolate and elute the mAb. ELP is a precipitation-based system that uses stimuli-responsive biopolymers fused with Protein A-based ligands in suspension for capture of the mAb and external stimuli (e.g., temperature,

salt) to precipitate the bound complex and elute the mAb. OLE is a liquid-liquid partitioning system that uses oil phase segregating oleosin proteins fused with Protein A-based ligands to capture mAb in the oil phase and then elute the mAb into a clean aqueous phase.

## Techno-Economic Evaluation

Techno-economic evaluations are performed using the recently proposed equations for ESM that include calculation of costs at each mission segment (Berliner et al., 2021). ESM for the mission $ESM_0$ is defined as

$$\begin{aligned} \mathrm{ESM}_0 = \sum_{k}^{\mathcal{M}} L_{eq,k} \sum_{i}^{A_k} \big[ \big(M_{k_i} \cdot M_{eq,k}\big) + \big(V_{k_i} \cdot V_{eq,k}\big) \\ + \big(P_{k_i} \cdot P_{eq,k}\big) + \big(C_{k_i} \cdot C_{eq,k}\big) + \big(T_i \cdot D_k \cdot T_{eq,k}\big) \big] \\ = \mathrm{ESM}_{0,pd} + \mathrm{ESM}_{0,tr1} + \mathrm{ESM}_{0,sf} + \mathrm{ESM}_{0,tr2} \end{aligned}$$

where $M_i$, $V_i$, $P_i$, $C_i$, $T_i$ are the initial mass [kg], volume [$m^3$], power requirement [kW], cooling requirement [kg/kW], and crew-time requirement [CM-h/h], $M_{eq}$, $V_{eq}$, $P_{eq}$, $C_{eq}$, $T_{eq}$ are the equivalency factors for mass [kg/kg] (which is set to 1 in this study), volume [kg/$m^3$], power [kg/kW], cooling [kg/kW], and crew time [kg/CM-h], respectively, $L_{eq}$ is the location equivalency factor [kg/kg] that accounts for costs associated with mass transport occurring at a particular mission segment (e.g., orbital maneuvers required for the return transit), and $D$ is the duration of the mission segment [day] over a set of subsystems $i \in A$ and set of mission segments $k \in \mathcal{M}$. The mission ESM in this study is specifically defined as the sum of subtotal ESM for each mission segment within the scope of the RMA defined in this study (pre-deployment $ESM_{0,pd}$, crewed transit to Mars $ESM_{0,tr1}$, Mars surface operations $ESM_{0,sf}$, and return crewed transit to Earth $ESM_{0,tr2}$).

Key mission and pharmaceutical assumptions are summarized in **Table 2**. The mission timeline depicted in **Figure 2** provides insight into the proposed RMA and downstream crew needs and mAb production horizon. Here we assume a total mission duration of 910 days. First, a crew of 6 will travel from Earth to low Earth orbit, then board an interplanetary craft for a 210-day journey to Martian orbit, where the crew will descend to the surface in a separate craft, allowing the large transit vehicle to remain in orbit. Once on Mars, the crew will perform surface operations for 600 days. Following surface operations, the crew will leave Mars in a fueled ascent craft, board the interplanetary vehicle, and return to Earth orbit in 200 days. The mission timeline, crew size, and ESM equivalency factors are consistent with the recent RMA presented for inclusion of biomanufacturing elements (Berliner et al., 2021).

The mission demand for mAb therapies is assumed to be 30,000 mg over the entirety of the mission (supporting logic detailed in **Supplementary Table 1**). Pharmaceutical stores and production resources are assumed to be flown with the crew transit (no pre-deployment in order to maximize shelf-life). We assume that the production resources are stable throughout the mission duration. We conservatively assume (in the face of insufficient spaceflight stability data for biologics for a more refined estimate) that the first 600 days of pharmaceutical demand will be met through flown stores (20,000 mg), at which point pharmaceutical ISRU manufacturing is needed (10,000 mg) to alleviate the impact of accelerated pharmaceutical degradation and provide supplementary medication. The pharmaceutical production window opens prior to the ISRU demand timeframe and persists through a portion of the return transit (up to mission day 810) to reflect the expected life support advantage of maintaining capabilities to counter unanticipated needs or threats. We assume that the Protein A-based unit procedures consistently yield 98% recovery of mAb from the input stream.

**TABLE 2 |** Key mission and pharmaceutical reference mission architecture details and assumptions.

| **Mission scope** | |
|---|---|
| Pre-deployment | N/A |
| Transit to Mars | 210 days |
| Surface operations | 500 days |
| Return transit | 200 days |
| Total mission duration | 910 days |
| Crew size | 6 crew members |
| **Pharmaceutical scope** | |
| Mission demand, mAb | 30,000 mg |
| Biomanufacturing, mAb | 10,000 mg |
| Capture step recovery | 98% |
| Production window | 600 days |
| Feed mAb concentration | 1 mg/mL |
| Molecular weight, mAb | 150 kDa |

*mAb, monoclonal antibody.*

## Unit Procedure Simulation

Deterministic models for each unit procedure were developed in Microsoft Excel (**Supplementary Data Sheet 2**) using reference protocols cited in **Table 1** as a series of executable operations, each containing a set of inputs defined by cost categories (labor, equipment, raw materials, and consumables) that are correspondingly populated with characteristic ESM constituent (mass, volume, power, cooling, and labor time) values (model composition illustrated in **Supplementary Figure 1**). Unit procedures have been defined as the smallest single execution (i.e., unit) of the secondary purification capture step procedure according to the reference protocol. We define the unit capacity by volume according to the equipment and consumables used (e.g., 2 mL maximum working volume in a 2 mL tube) and by mAb quantity according to the binding capacity for the given method (e.g., 1 mg mAb/mL resin) (**Supplementary Table 2**). Unit procedures with no explicit working volume constraints (i.e., the liquid solution volume for biotic technologies) have been defined with a maximum unit volume of 2 mL. ESM-relevant characteristics of individual inputs (e.g., equilibration buffer, 2 mL tube) are defined based on publicly available

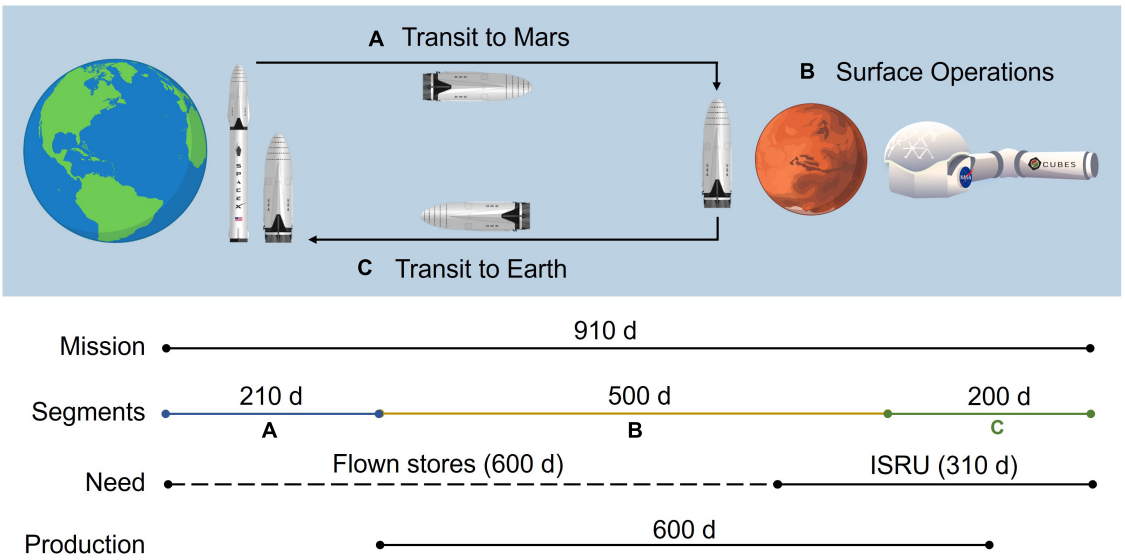


**FIGURE 2 |** An illustration of the reference mission architecture in which **(A)** a crewed ship is launched from the surface of Earth and lands on Mars and **(B)** assembles a pre-deployed habitat on the Martian surface to perform operations before **(C)** a return transit to Earth on the same ship. Pharmaceutical needs are supported by flown stores until partway through surface operations, at which point needs are met by pharmaceuticals produced using *in situ* resource utilization. Production is initiated prior to the need window to ensure adequate stocks are generated by the time it is needed. Rocket artwork adapted from Musk (2017). Habitat artwork by Davian Ho.

values, direct measurements taken, and assumptions (which are explicitly identified in the **Supplementary Data Sheet 2**).

There are several model features that we have considered and decided not to include within the scope of analysis. Packing and containers for the inputs are not included for three reasons: (1) the contributions of the container are considered negligible as compared to the input itself (e.g., container holding 1 L buffer as compared to the 1 L of liquid buffer); (2) materials flown to space are often re-packaged with special considerations (Wotring, 2018); and (3) the selection of optimal container size is non-trivial and may risk obscuring more relevant ESM findings if not chosen carefully. We do not consider buffer preparation and assume the use of flown ready-to-use buffers and solutions. Furthermore, refrigeration costs of the input materials and costs that may be associated with establishing and maintaining a sterile operating environment (e.g., biosafety cabinet, 70% ethanol in spray bottles) are expected to be comparable between unit procedures and not considered. Impacts of microgravity on unit procedure execution are not considered for the return transit production. Refrigeration costs associated with low temperature equipment operation (e.g., centrifugation at 4°C) are included in the equipment power costs.

Inputs common across unit procedures are standardized (**Supplementary Table 3**). One operational standardization is the inclusion of pH neutralization of the product stream following the low pH elution mechanism, which was explicitly stated in some procedures while not in others. Input quantities are scaled from a single unit to determine the number of units required to meet the RMA specifications. The ESM constituent inputs (mass, volume, power, cooling, and labor time) are converted into equivalent mass values using RMA equivalency factors (**Supplementary Table 4**).

# RESULTS AND DISCUSSION

## Standardization of Manufacturing Efficiency

Given the limited granularity of the presented RMA, which was scoped as such to reflect the lack of literature presenting an overarching and validated Concept of Operations for a Transit to Mars (Antonsen et al., 2017), we do not define strict manufacturing scheduling criteria for pharmaceutical production. Construction of a detailed pharmaceutical production RMA is hindered by uncertainty in the number and identity of mAb therapy products that would be included within mission scope, the decay rate of mAb therapy stores in the mission environments, and a reasonable basis for building robustness to unanticipated disease states. Rather, we choose to establish an objective comparison between unit procedures by normalizing for scheduling-associated manufacturing efficiencies. We accomplish this by first identifying the number of batches per mission (and thus batch size) needed to meet the mAb demand (base case of 10,204 mg mAb feed assuming 98% recovery) that minimizes the ESM output for a given unit procedure, and then running the simulation of pharmaceutical production at that number of mission batches, as shown in **Figure 3A** and tabulated in **Supplementary Table 5**.

In **Figures 3B–E**, we visualize a deconstruction of ESM output, using the VIN unit procedure as an example, by key performance metrics that vary with a scheduling dependence in order to

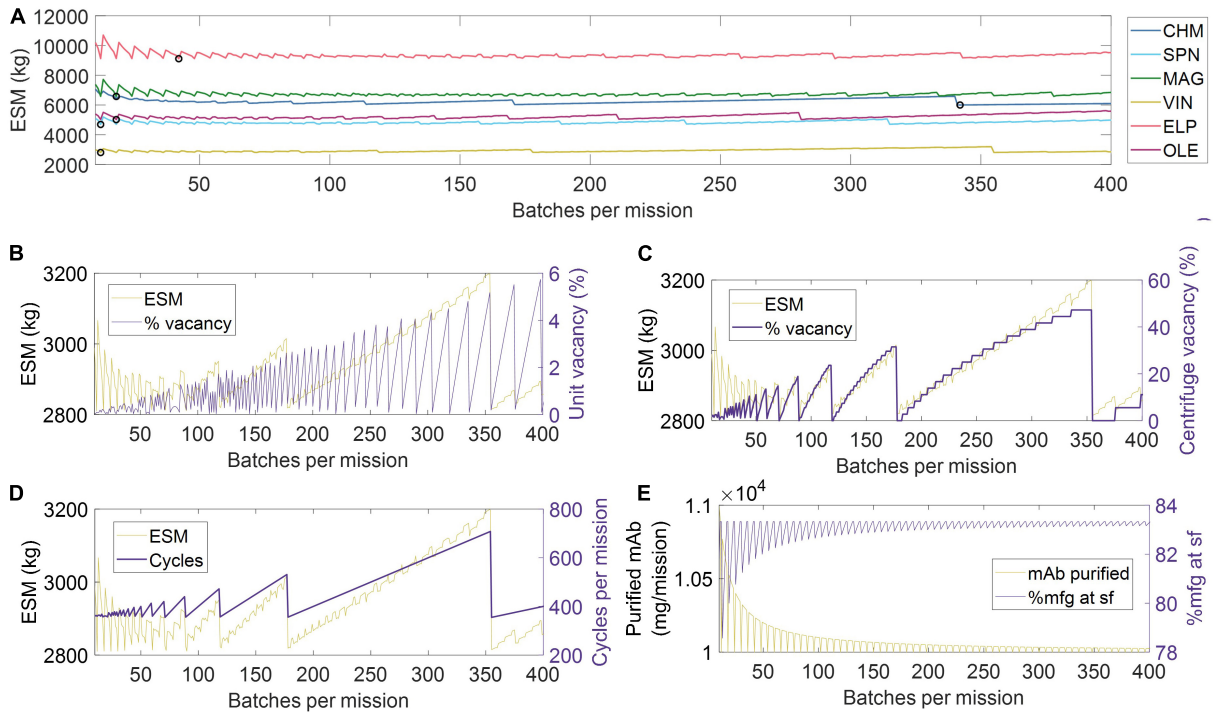


**FIGURE 3 | (A)** Scheduling optimization for the establishment of base case scenarios for each unit procedure. The value for number of batches corresponding to the minimum equivalent system mass for each unit procedure, as indicated by black circle (○) markers. Key operational parameters impacted by mission scheduling (shown using the VIN procedure) include **(B)** unit underutilization or vacancy, **(C)** equipment underutilization or vacancy, in this case represented by the centrifuge as the bottleneck, **(D)** the number of use cycles, and **(E)** the total quantity of monoclonal antibody (mAb) per mission and per surface operation (sf). CHM, pre-packed chromatography; SPN, spin column; MAG, magnetic bead; VIN, plant virus-based nanoparticle; ELP, elastin-like polypeptide; OLE, oilbody-oleosin.

illustrate the significance of batch optimization in unit procedure comparison. The processing of a given batch volume and mAb quantity is allocated into a number of units, as determined by the volume and mAb quantity constraints of a given unit procedure, and a number of use cycles per batch, as determined by the capacity of the equipment specified in the given unit procedure. We show how the variation in ESM output over the number of mission batches maps to extent of unit vacancy or underutilization (**Figure 3B**), extent of operational equipment (e.g., centrifuge) vacancy or underutilization (**Figure 3C**), and number of required use cycles (**Figure 3D**). We also show an oscillatory behavior in the scheduling (i.e., total mAb purified per mission, % purified at surface operations) that quickly dampens as number of mission batches increases (**Figure 3E**). This behavior is a result of the assumption that the mAb feed stream is coming from a discrete upstream production batch (e.g., batch-mode bioreactor) that does not output partial batch quantities, as opposed to a continuous upstream production for which there are no defined batches. Accordingly, partial batch needs are met by the processing of a full batch.

## Base Case Scenario

The ESM and output metrics of the base case scenario (10,000 mg mAb demand, 1 mg mAb/mL feed concentration, 98% recovery) for each of the six unit procedures are shown in **Figures 4A–F**. From this viewpoint of an ESM output for an isolated unit procedure outside the context of a full purification scheme, the ESM ranked from lowest to highest are VIN < SPN < OLE < CHM < MAG < OLE. However, we reason that it is more important to understand the model inputs that influence the ESM output rankings than to use the rankings in this isolated subsystem analysis to make technology selection choices, which requires the context of a full pharmaceutical foundry and of linkages to other mission elements.

We observe that mass costs are generally the primary contributor to ESM output, except for the MAG and ELP procedures in which labor time costs are larger. The mass costs are not closely associated to any given cost category across unit procedures, but rather the breakdown of mass costs varies widely by unit procedure.

Power costs (kW) are disproportionately high given that the static nature of ESM assumes constant usage, and thus energy (kW·h) in this context (i.e., the power supply to the equipment is not turned off in this analysis). These costs represent an upper bound assuming that the power supply system capacity is sized to support a maximal power consumption in which all power-drawing elements are simultaneously in operation. Time of power usage as a fraction of duration are as follows: CHM (99%) > MAG (78%) > ELP (48%) > SPN (45%) > OLE (42%) > VIN (30%). The lower use fraction unit procedures are therefore paying a relatively higher cost per unit power demand in this current method. The electrical needs of the equipment

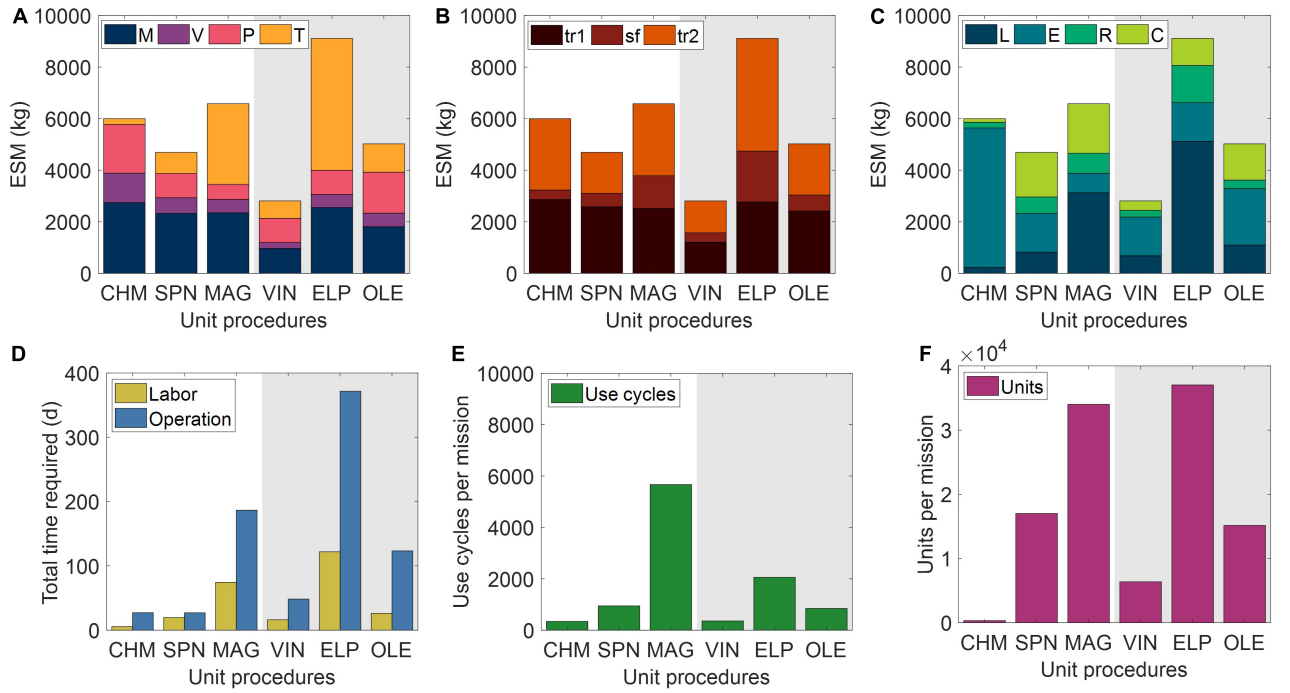


**FIGURE 4 |** Base case equivalent system mass results broken down by **(A)** mass (M), volume (V), power (P), and labor time (T) constituents, **(B)** transit to Mars ($tr_1$), surface operations (sf), and return transit ($tr_2$) mission segments, and **(C)** labor (L), equipment (E), raw materials (R), and consumables (C) cost category for the six tested Protein A-based monoclonal antibody affinity capture step unit procedures segregated by abiotic (white background) and biotic (gray background) technologies. Also shown are the **(D)** labor and operation times, **(E)** number of use cycles, and **(F)** number of units required for each unit procedure to meet the reference mission demand. CHM, pre-packed chromatography; SPN, spin column; MAG, magnetic bead; VIN, plant virus-based nanoparticle; ELP, elastin-like polypeptide; OLE, oilbody-oleosin.

used by the unit procedures are within NASA-proposed Mars mission RMA bounds, with energy use across all unit procedures would peak at ∼1% of a proposed Mars transfer vehicle electric capacity (50 kWe) or ∼5% of the habitat capacity (12 kWe) of a reference stationary surface nuclear fission power reactor (Drake et al., 2010).

The mission segment breakdown of ESM illustrates the relatively high costs of pharmaceutical manufacturing capabilities for transit, even for the transit to Mars ($tr_1$) in which there is no actual production taking place. There is a strong economic incentive to limit the amount of supplies flown on $tr_1$. Alternatives such as the pre-deployment of reagents and consumables and limiting of production to surface operations on Mars (which has lower RMA equivalency factors for mass and volume than transit operations) must be balanced against the risk to human health posed by removing pharmaceutical production capabilities from a mission segment and potentially exposing the supplies to longer storage times that could challenge shelf lives.

Labor and operation times are important parameters in the broader mission and pharmaceutical foundry context. These unit procedures represent a single step of pharmaceutical production, which if realized in a space mission context, would, in turn, need to be a small portion of a crew member's time allocation. Assuming 40-h work weeks for crew members, the labor time spans a range of ∼1% (CHM) to ∼14% (ELP) of the available crew time over the 600-day production window. It is not feasible to operationalize with such high labor and operation times at this scale of production, particularly as they stand for MAG and ELP. While strategies such as batch staggering and concurrency can be used to reduce durations, advanced automation will almost certainly need to be built into the core of a pharmaceutical foundry.

A prevailing trend throughout the unit procedures is that the number of unit executions and use cycles required by a given unit procedure are positive correlated with the ESM output value, except for the equipment cost-dominant and higher unit capacity CHM procedure. The equipment modeled in the analysis for CHM and the other unit procedures are almost certainly not space-ready and could be further designed to reduce mass and volume and increase automation to reduce crew labor time. The increased equipment costs in the CHM procedure are primarily due to automation and monitoring hardware for running liquid chromatography, which is reflected in the minimal labor costs of the CHM procedure. Miniaturization efforts, such as those focusing on microfluidic systems (Millet et al., 2015; Rodríguez-Ruiz et al., 2018; Murphy et al., 2019), are emerging as a potential path toward mitigating the high equipment costs associated with highly automated and tightly controlled manufacturing, which are crucial for freeing up valuable crew time.

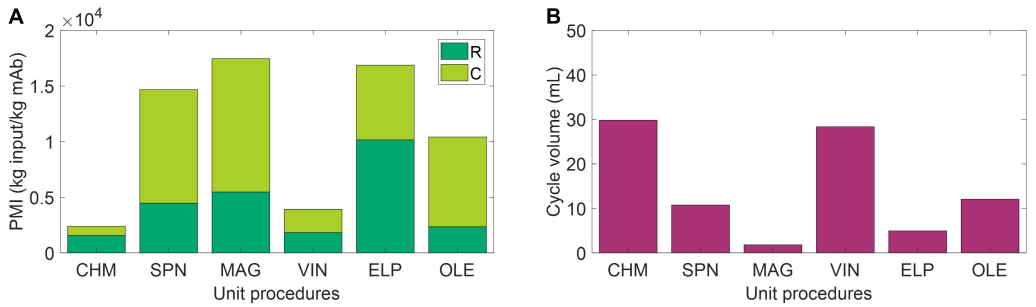


**FIGURE 5 | (A)** Process mass intensity (PMI) evaluation of the unit procedures broken down by raw materials (R) and consumables (C) contributions. **(B)** Cycle volume for each unit procedure. CHM, pre-packed chromatography; SPN, spin column; MAG, magnetic bead; VIN, plant virus-based nanoparticle; ELP, elastin-like polypeptide; OLE, oilbody-oleosin.

The number of unit executions is determined by the binding capacity of the technology and the nominal unit size. This indicates that the unit capacity for purification is an important consideration and influential factor. Unit sizing is an important consideration that is valuable to assess more holistically within the broader pharmaceutical production and mission context.

The number of use cycles is determined by the number of unit executions required and by the maximal unit capacity of the equipment items (e.g., if you presume that an 18-slot centrifuge is the equipment bottleneck then the effective number of batches is the number of units required divided by 18). Therefore, it can be understood that the equipment unit capacity is a critical parameter in tuning the number of use cycles and, by extension, the labor costs. For processes with lower labor costs, due to the intrinsic nature of the procedure or through automation of labor, equipment unit capacity will still influence the total duration and production throughout. The MAG and ELP procedures yield both high labor and duration times and are thus particularly sensitive to the equipment capacity.

### Contextualizing Equivalent System Mass With Supporting Evaluations

Having acknowledged shortcomings of ESM as a decision-making tool for comparison of alternative approaches in isolated subsystems, we propose that supplementary evaluations can assist in contextualization. A primary gap of an isolated subsystem ESM analysis is a lack of information on the holistic usefulness or cost of a given employed resource, which could include its synergy with other mission subsystems and its extent of recyclability, or waste loop closure, within the mission context. For example, the isolated subsystem analysis does not capture information on the broad applicability that a centrifuge might have for use in other scientific endeavors, nor do the ESM outputs reflect the >93% recyclability of water achieved by the recycler on the ISS (Steven Siceloff, 2008) that may be generalizable to future missions.

The use of environmental footprint metrics, such as PMI, may be one valuable step toward capturing missed information on recyclability. PMI is a simple metric of material efficiency defined as the mass of raw materials and consumables required to produce 1 kg of active pharmaceutical ingredient. The study by Budzinski et al. (2019) introducing PMI for biopharmaceuticals presents data from 6 firms using small-scale (2,000–5,000 L reactor) and large-scale (12,000–20,000 L reactor) mAb manufacturing operations, finding an average 7,700 kg of input is required to produce 1 kg of mAb. **Figure 5A** presents PMI evaluation for the six capture steps included in analysis, which result in PMI outputs as low as 2,390 kg of input (CHM) and as high as 17,450 kg of input (MAG) per 1 kg of mAb. A comparison of these outputs to those of Budzinski et al. (2019) indicates that we may be observing roughly similar values after accounting for the high cost of initial purification in the study, representing ∼60% of the total PMI reported, the elevated feed mAb concentration (i.e., cell culture titer) of 1–5.5 g mAb/L, and adjustments for economies of scale when operating at such low cycle volumes (**Figure 5B**). Consumable costs appear to be the most sensitive to scale, which represents ∼1% total PMI on average in the values reported by Budzinski et al. (2019) and ranges from 35% (CHM) to 77% (OLE) here. Budzinski et al. (2019) also go one step further to distinguish water as a separate category from raw materials and report that >90% of the mass is due to water use. Here we assume pre-made buffers and do not directly add water in this study, so we refrain from a similar calculation, but it is worth noting that the extent of water use may also serve as a reasonable starting surrogate for extent of achievable recyclability in a space mission context.

## Scenario Analysis

We analyzed the specific ESM output broken down by cost category for the six unit procedures over a range of input stream mAb concentrations (**Figure 6**) and mission demand for mAb (**Figure 7**). Specific ESM, termed cost of goods sold in traditional manufacturing analyses, is the ESM output required to produce 1 mg mAb. This is used in the scenario analyses to normalize ESM output across variation in mission demand for mAb. The optimal number of batches per mission was found and used for each unit procedure and scenario tested (**Supplementary Tables 7, 8**).

We observe the general and expected trends that specific ESM decreases with an increasing feed stream mAb concentration and mission demand. The CHM procedure exhibits notably limited sensitivity to feed stream mAb concentration, which can be attributed to the equipment-dominated cost profile, fixed column size, and nature of the governing reference protocol that does not

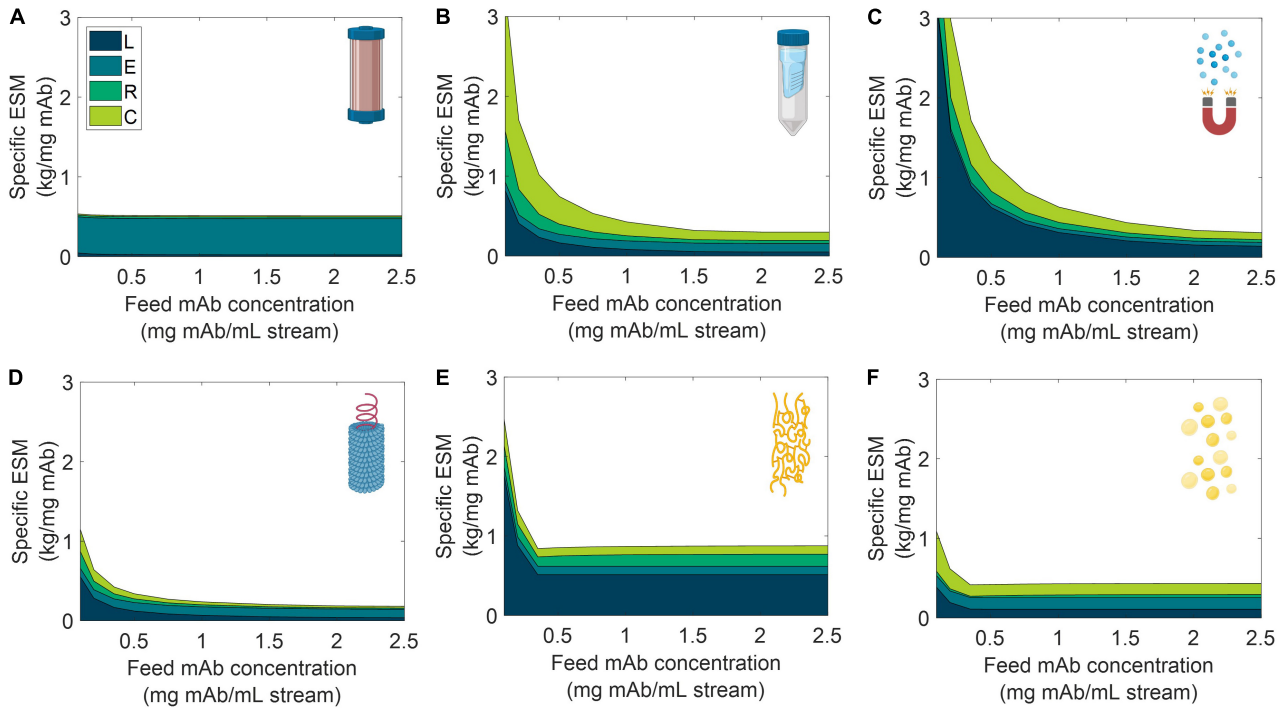


**FIGURE 6 |** Specific equivalent system mass (per unit mass monoclonal antibody produced) broken down by labor (L), equipment (E), raw materials (R), and consumables (C) cost categories as a function of feed monoclonal antibody (mAb) concentration for **(A)** CHM, **(B)** SPN, **(C)** MAG, **(D)** VIN, **(E)** ELP, and **(F)** OLE. CHM, pre-packed chromatography; SPN, spin column; MAG, magnetic bead; VIN, plant virus-based nanoparticle; ELP, elastin-like polypeptide; OLE, oilbody-oleosin.

specify restrictions on sample load volume. Depending on the pre-treatment of the feed stream, it may be more reasonable to impose constraints on the sample load volume. In contrast, the specific ESM output of the CHM procedure is the most sensitive to mission mAb demand with higher demand increasingly offsetting the fixed capital costs. The CHM procedure is also the largest capacity unit modeled in the analysis (i.e., CHM capacity is 30 mg mAb/unit as compared to 2.7 mg mAb/unit for MAG, the next highest capacity unit) and is accordingly expected to scale well with demand.

The SPN, ELP, OLE procedures exhibit behaviors in which the specific ESM output abruptly plateaus with an increasing feed stream mAb concentration. This observation can be attributed to the unit procedure operating in a mAb binding capacity-limited regime (as opposed to volume-limited for more dilute feeds) which also then controls and maintains unit procedure throughput (e.g., the ELP number of units, 37,044, and use cycles per mission, 2,058, is constant at and above 0.35 mg mAb/mL input stream concentration). This can be de-bottlenecked via technology (e.g., improved chemistry of the capture step unit leading to higher binding capacity) or methodology (e.g., increased concentration of the capture step unit leading to higher binding capacity) improvements.

Low demand scenarios are particularly relevant for examination in a space health context, as small capacity redundant and emergency utility is a likely proving ground for inclusion of a space pharmaceutical foundry. At the lower boundary of the tested range (1,000 mg mAb/mission), we see the ESM outputs from lowest to highest are re-ordered as MAG < VIN < SPN < OLE < ELP < CHM. Minimization of equipment costs are particularly important in this regime, and it is observed that, indeed, the ESM output near completely aligned with the ranking of equipment cost (MAG < VIN < SPN < ELP < OLE < CHM). It is likely that other non-ESM factors such as integration with other flown elements will understandably influence the design and composition of early and low capacity flown pharmaceutical foundries.

## Alternate Scenarios

### Mission Configurations

We explored variations to the base case RMA for all six unit procedures including scenarios in which the pharmaceutical manufacturing resources are shipped prior to the crew in pre-deployment, (+)pd, the production window has been truncated to close with the end of surface operations, $(-)tr_2$, and a combination of the two prior modifications, (+)pd $(-)tr_2$ (**Figure 8**). Costs of pre-deployment are included in the analyses and mission demand is kept constant regardless of the production window.

In all cases the ESM totals were reduced from the base case. Additionally, the general trend held that $(-)tr_2$ scenario resulted in lower ESM totals than (+)pd scenario except for

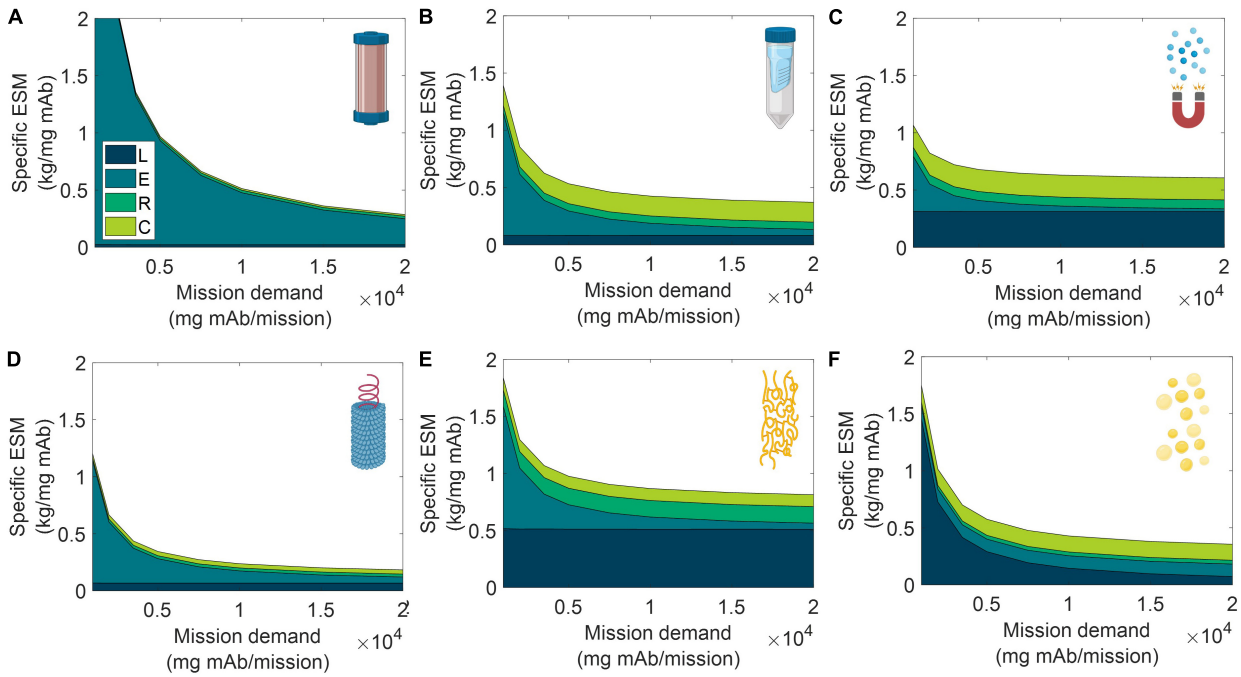


**FIGURE 7 |** Specific equivalent system mass (per unit mass monoclonal antibody produced) broken down by labor (L), equipment (E), raw materials (R), and consumables (C) cost categories as a function of mission production demand for monoclonal antibody for **(A)** CHM, **(B)** SPN, **(C)** MAG, **(D)** VIN, **(E)** ELP, and **(F)** OLE. CHM, pre-packed chromatography; SPN, spin column; MAG, magnetic bead; VIN, plant virus-based nanoparticle; ELP, elastin-like polypeptide; OLE, oilbody-oleosin.

SPN, in which the increased raw material and consumable costs of (−)$tr_2$ were sufficiently large to outweigh the reduction in equipment and labor costs of (+)pd. The combination (+)pd (−)$tr_2$ scenario resulted in the lowest ESM totals at a fraction of the base case (as high as 39% reduction in SPN and as low as 21% reduction in ELP).

## Equipment and Unit Throughput

Acknowledging the significance of the equipment capacity on ESM output, we further explored this contribution by comparing the base case ESM output of the centrifuge-utilizing procedures (SPN, VIN, ELP, and OLE) to that resulting from the use of alternative centrifuge models (**Supplementary Table 9**). This effectively results in a trade of equipment costs and batch throughput. The optimal number of batches per mission was found and used for each unit procedure and interval tested (**Supplementary Table 10**).

We observe in **Figure 9** that the ESM values increased with the size of the centrifuge model, 12-slot < 18-slot (base) < 48-slot. The labor and consumables savings of higher batch throughput were outweighed by the higher equipment costs (including higher power costs). Operation duration is an important metric relevant to a pharmaceutical foundry that is not well reflected in ESM that is also impacted by this alternative scenario. The exception to this trend is the 48-slot condition for the ELP procedure, in which a lower consumable cost related to the number of use cycles per mission (i.e., pipette tips, tubes, and gloves) sufficiently lowered the total ESM below the 18-slot condition.

## Technology Reusability

The number of use cycles for liquid chromatography resins is an important economic parameter in commercial pharmaceutical manufacturing (Pathak and Rathore, 2016). Here we explore the impact of use cycles on the CHM and ELP procedures in a space mission context, looking at no reuse nor regeneration operation of the purification technology, (−)Reuse, and at an increased number of use cycles, (+)Reuse (**Figure 10**).

We observe that the terrestrial importance of use cycles does not prevail in this isolated ESM evaluation in a space context. The high purchase costs of resin are not considered in ESM and the impact of the reuse cycles is reduced to the mass and volume savings of the pre-packed column consumable. There is a minor decrease in ESM of the (+)Reuse over the base case scenario, but both of these result in substantially higher ESM than the (−)Reuse scenario, particularly for the ELP procedure, in which the regeneration operation has been removed in addition to the reusability of the technology.

These results echo the trend of single-use technology in commercial biotechnology in which manufacturers look to disposable plastic bioreactor and buffer bags as a means to reduce cleaning and validation costs (Shukla and Gottschalk, 2013). It would be valuable to further consider the utilization of single-use technology in a space pharmaceutical foundry, and in other space systems bioengineering applications, but it is important to point out the limited scope of this ESM analysis. Here we reiterate that the single unit procedure scope establishes a modular basis for pharmaceutical foundry ESM evaluation but does not realize

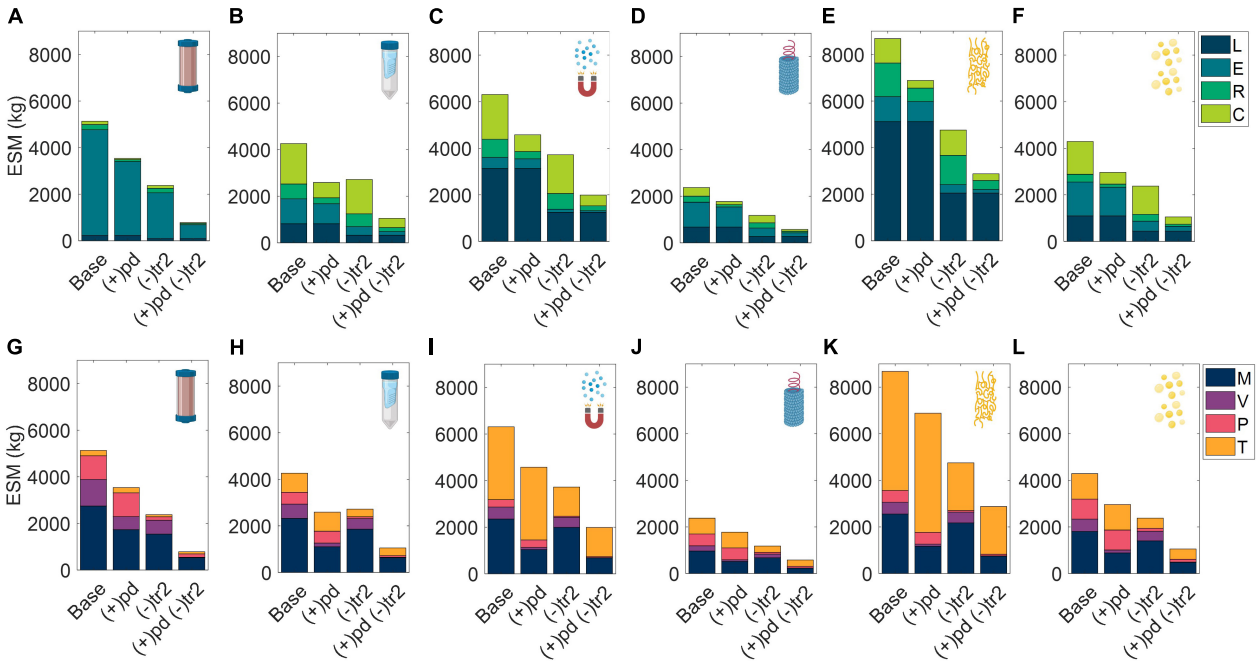


**FIGURE 8 |** Evaluation of extended equivalent system mass values in various mission configurations broken down by labor (L), equipment (E), raw materials (R), and consumables (C) cost categories cost category and mass (M), volume (V), power (P), and labor time (T) constituents for CHM, **(A,G)**, SPN, **(B,H)**, MAG, **(C,I)**, VIN, **(D,J)**, ELP **(E,K)**, and OLE, **(F,L)**. Configurations include the base case scenario of manufacturing resources flown with the crew for pharmaceutical production on the surface and return transit (Base), and alternatives in which the manufacturing resources are flown prior to the crew in pre-deployment, (+)pd, the production window is limited to surface operations, (–)$tr_2$, and a combination of the two previously stated alternatives, (+)pd (–)$tr_2$. CHM, pre-packed chromatography; SPN, spin column; MAG, magnetic bead; VIN, plant virus-based nanoparticle; ELP, elastin-like polypeptide; OLE, oilbody-oleosin.

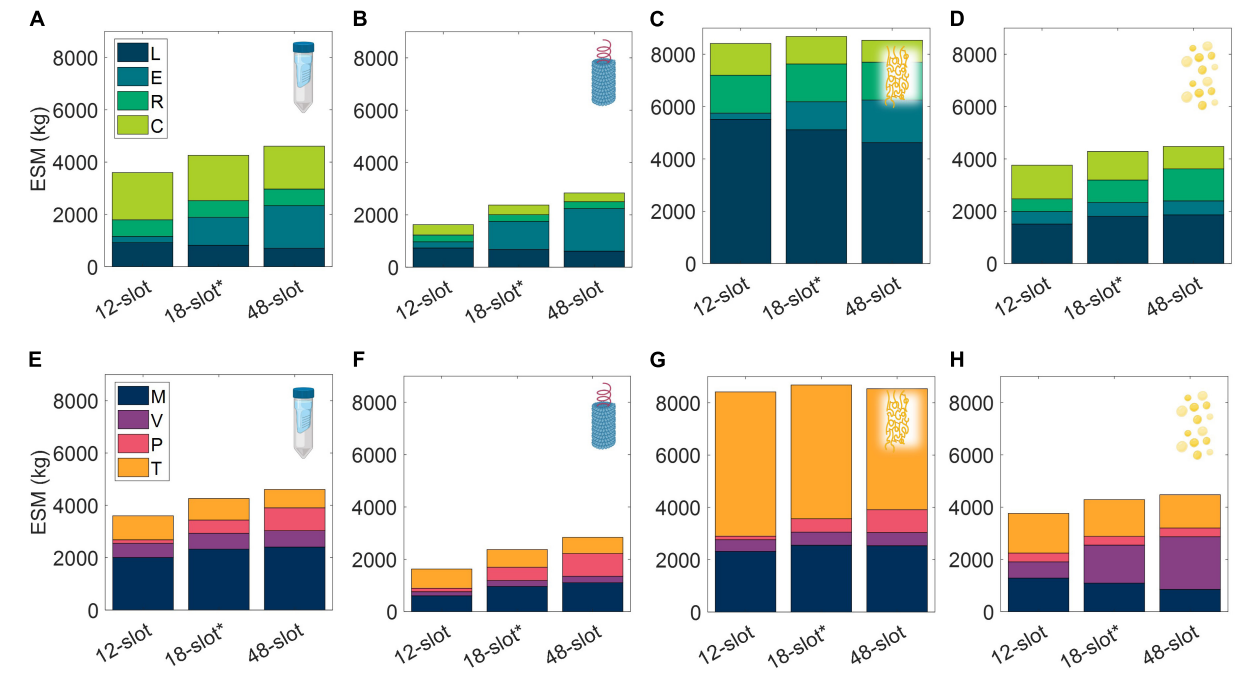


**FIGURE 9 |** Changes in extended equivalent system mass values with different capacity centrifuge models broken down by labor (L), equipment (E), raw materials (R), and consumables (C) cost categories and mass (M), volume (V), power (P), and labor time (T) constituents for SPN **(A,E)**, VIN **(B,F)**, ELP **(C,G)**, and OLE **(D,H)**. SPN, spin column; VIN, plant virus-based nanoparticle; ELP, elastin-like polypeptide; OLE, oilbody-oleosin.

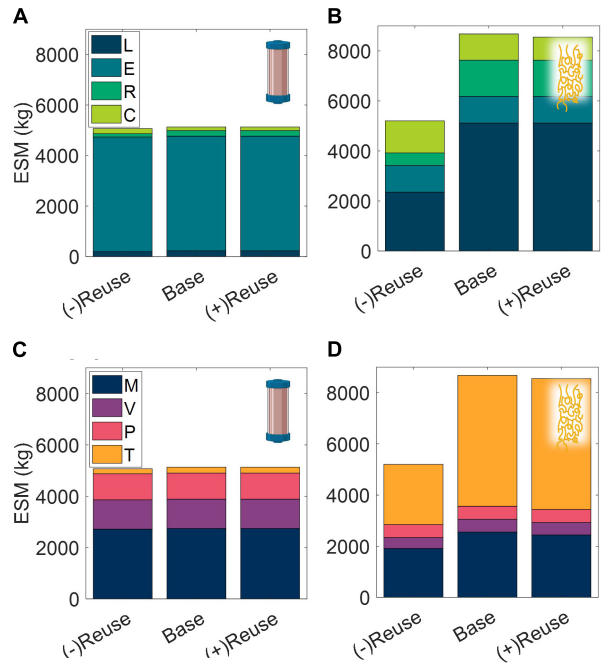


**FIGURE 10 |** Changes in extended equivalent system mass values with reusability of purification technology broken down by labor (L), equipment (E), raw materials (R), and consumables (C) cost categories and mass (M), volume (V), power (P), and labor time (T) constituents for CHM **(A,C)**, and ELP **(B,D)**. (–)Reuse considers the technology as single-use and accordingly discards the unit procedure cleaning operations; (+) Reuse considers additional reuse cycles of the technology. CHM, pre-packed chromatography; ELP, elastin-like polypeptide.

the true circular economy advantages of reuse, which may be considerable for the regeneration step, and of biological systems for production of the purification reagent in general.

## CONCLUSION AND FUTURE DIRECTIONS

In this study, we have introduced and applied the ESM framework to biopharmaceutical processing as a first step toward modeling and understanding the costs of Space Systems Bioengineering and, more specifically, of a long-duration space exploration medical foundry, which we believe may 1 day constitute a critical bioregenerative component of ECLSS for humans to be able to explore the surface of Mars. We have observed that the static behavior of ESM, while certainly maintaining usefulness in early stage analyses, may stymie later-stage analyses of bioregenerative life support technologies, which tend to behavior more dynamically than traditional abiotic counterparts. In the future, higher fidelity analyses may be performed using tools such as HabNet (Do et al., 2015), although the use of such dynamic mission design and modeling tools will require additional software engineering efforts. As it stands now, our techno-economic calculations both satisfy the three fundamental aspects for life support modeling (Jones, 2017) and provide helpful directions for future efforts to incorporate purification processes in space systems bioengineering.

The mAb affinity capture step represented an ideal starting point for biopharmaceutical purification cost analysis given the breadth of the mAb treatments for space-important health indications, the fact that mAb purification is considered a platform technology, and the diversity of affinity capture technologies. However, there are additional processing categories, such as size exclusion, ion exchange, and hydrophobic interaction unit procedures, which could be similarly studied in isolation for their general relevance in biopharmaceutical manufacturing. Establishing a unit procedure knowledge base for space-relevant economics of biopharmaceutical purification would provide additional benefit to the community.

We acknowledge that the ESM analysis performed in this study utilizes current Earth-based technologies, not Mars-designed processes, and that as technologies evolve and expand the analysis will need to be updated. The need to revisit and update ESM analyses periodically as technology develops is standard practice. This is well illustrated in a recent ESM analysis of plant lighting systems that compares solar fiber optics to photovoltaic-powered light emitting diode hybrid systems (Hardy et al., 2020). The study results reversed decade-old trade study outcomes in which solar fiber optics scored more favorably, citing rapid advances in solar photovoltaics and light emitting diode technologies.

Furthermore, the analysis presented does not encapsulate potentially significant characteristics of the unit procedures at the interfaces of the upstream and downstream biomanufacturing elements. For example, at the upstream interface the biotic unit procedures (VIN, ELP, and OLE) have been reported in literature to be effective capture mechanisms in "dirtier" feed solutions, perhaps absolving the need for more complex pre-capture clarification steps by virtue of process integration. At the downstream end, the eluate of the CHM unit procedure can be directly fed to the subsequent processing step, which would be particularly amenable for other column-based unit procedures, resulting in lower labor time and manufacturing duration. We also do not account for the uncertainty in performance associated with the developmental state of the technology. There have been substantially lower research and development investments in the biotic technologies than in the commercially available abiotic technologies; one may reasonably assume that there is more potential for improvements through biotic unit procedure optimization, while also considering that a larger driving force in abiotic unit procedure optimization for commercial terrestrial operations may balance or outweigh this. Forecasting on the technology development dynamics in the context of these, and other, forces could provide significant additional insights.

Several overarching lessons on the development required for deployment of pharmaceutical purification technology to support human health in space can be gleaned from the cost breakdown of the ESM framework employed in this study. The high mass costs for the mAb capture technologies investigated suggest strong incentives to pursue efforts in miniaturization to reduce not only equipment mass, but also reagent mass, as preparation for pharmaceutical foundries in space. The high labor costs and

duration of some of the technologies studied likewise suggests that automatization of biopharmaceutical purification would be impactful. Automatization could also conceivably be valuable in reducing mass costs associated with manual manipulation, such as pipette tips and gloves, and those associated with ensuring sterile operation. We also underline the importance of scheduling and equipment sizing optimization; for example, the ESM penalty for capturing the mission demand of mAb with the VIN unit procedure yielded up to 40% higher total ESM for non-optimal scheduled manufacturing batches. Given the advantage of *in situ* manufacturing to respond to uncertainty in mission medicine demand, further research to explore scheduling and equipment sizing under uncertainty would provide valuable insight.

There are a series of challenges facing pharmaceutical foundries in space beyond processing. Perhaps the most daunting of these is the incompatibility of existing pharmaceutical regulatory compliance frameworks with the design constraints of *in situ* manufacturing. There are currently dozens to hundreds of analytical tests required to confirm process and product quality prior to release of the pharmaceutical for administration to human patients (Morrow and Felcone, 2004), which translates into a highly burdensome cost for *in situ* manufacturing of pharmaceuticals in space. Fortunately, there is a strong and parallel terrestrial need to reduce the burden of regulatory compliance while maintaining standards of quality assurance and control for personalized medicine, an individualized and patient-specific approach to medical care with widespread support. As mentioned earlier, trends of distributed and sustainable biomanufacturing on Earth provide additional support for reducing ESM-relevant costs.

The analyses presented in this study motivate future investigation into the ESM output of a complete pharmaceutical foundry for a more complete comparison to other ECLSS needs and subsequent formal evaluations of medical risk (i.e., loss of crew life, medical evacuation, crew health index, risk of radiation exposure-induced death from cancer) mitigation as a balance to the ESM costs. The Integrated Scalable Cyto-Technology system (Crowell et al., 2018), reported in literature as capable of "end-to-end production of hundreds to thousands of doses of clinical-quality protein biologics in about 3 d[ays]," is an automated and multiproduct pharmaceutical manufacturing system that may serve well as a starting point for a complete pharmaceutical foundry evaluation. While downstream costs are typically a large proportion of terrestrial biopharmaceutical production costs, they may represent an even higher proportion of the overall ESM costs. ESM is more closely aligned to PMI as a metric than to cost of goods sold in dollars, suggesting that downstream contributions to ESM may similarly dominate. Budzinski and team found that downstream operations contributed 82% of the total PMI for commercial mAb production (Budzinski et al., 2019).

Assembly of a complete pharmaceutical foundry ESM model would also enable investigation of more nuanced RMA design considerations, such as those relating to the influence of a fixed set, or anticipated probability distribution, of pharmaceutical product diversity and batch size on optimal system composition to meet given medical risk thresholds.

As stated in the original presentation of ESM theory and application, comparison of multiple approaches for a given subsystem with ESM, such as we are studying with the capture step of a mAb pharmaceutical foundry, should satisfy the same product quantity, product quality, reliability, and safety requirements (Levri et al., 2000). Of these assumptions, the product quality and safety requirements prove challenging for implementation in pharmaceutical foundry comparisons. It is worth noting that reliability is not considered in the scope of this preliminary study, given the varying technology readiness levels of the unit procedures, but that it should be included in future analyses of full purification schemes. By extension, the impact of microgravity and reduced gravity on reliability and unit operation performance, while not investigated in this study, is an important and complex consideration, that requires significant research to address. Similarly, stability of the production resources over the course of a mission duration should be further considered in future works. High product sensitivity to process changes, and the large battery of testing sometimes required to observe them (the extent of which will also change with the processes employed), creates a situation where ESM comparisons of pharmaceutical foundries that serve as technology decision making tools will absolutely need to meet this requirement, albeit at a considerable cost and/or complexity of execution.

The assessment of equivalent safety requirements, to the best of the knowledge of the authors, has been approached thus far in an *ad hoc* and qualitative manner, relying on extensive subject manner expertise and working process knowledge. One promising route to strengthening these critically important safety assessments would be to implement a formal assessment framework based on the environmental, health, and safety (EHS) assessment proposed by Biwer and Heinzle (2004), in which process inputs/outputs are ranked based on a series of hazard impact categories (e.g., acute toxicity, raw material availability, global warming potential) and impact groups (e.g., resources, organism). The key to a systematic space health-centric safety assessment like this is to establish space-relevant EHS impact categories (e.g., planetary protection, crew and ship safety). An improvement of the EHS underpinnings has the potential to provide significant benefits to future ESM analyses in the increasingly complex mission architecture of longer-duration missions.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

MM, AB, SN, and KM designed the study. MM, AB, PN, LM, OH, and KY built the model used in the study. MM analyzed the data and wrote the first draft of the manuscript. All authors were

involved in project discussion and interpretation and contributed to manuscript revision.

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The illustrative figures in part were created with BioRender.com.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.700863/full#supplementary-material

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REVIEW
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# To Other Planets With Upgraded Millennial Kombucha in Rhythms of Sustainability and Health Support

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Humankind has entered a new era of space exploration: settlements on other planetary bodies are foreseen in the near future. Advanced technologies are being developed to support the adaptation to extraterrestrial environments and, with a view on the longer term, to support the viability of an independent economy. Biological processes will likely play a key role and lead to the production of life-support consumables, and other commodities, in a way that is cheaper and more sustainable than exclusively abiotic processes. Microbial communities could be used to sustain the crews' health as well as for the production of consumables, for waste recycling, and for biomining. They can self-renew with little resources from Earth, be highly productive on a per-volume basis, and be highly versatile—all of which will be critical in planetary outposts. Well-defined, semi-open, and stress-resistant microecosystems are particularly promising. An instance of it is kombucha, known worldwide as a microbial association that produces an eponymous, widespread soft drink that could be valuable for sustaining crews' health or as a synbiotic (*i.e.*, probiotic and prebiotic) after a rational assemblage of defined probiotic bacteria and yeasts with endemic or engineered cellulose producers. Bacterial cellulose products offer a wide spectrum of possible functions, from leather-like to innovative smart materials during long-term missions and future activities in extraterrestrial settlements. Cellulose production by kombucha is zero-waste and could be linked to bioregenerative life support system (BLSS) loops. Another advantage of kombucha lies in its ability to mobilize inorganic ions from rocks, which may help feed BLSS from local resources. Besides outlining those applications and others, we discuss needs for knowledge and other obstacles, among which is the biosafety of microbial producers.

**Keywords: kombucha, space exploration, postbiotic, extraterrestrial outposts, microbial technologies, bioregenerative life support system, in situ resource utilization, cellulose biofabrication**

## INTRODUCTION

Long-duration stays on the Moon are scheduled to start this decade (Foing et al., 2018, 2021; Heinicke and Foing, 2021; Silk et al., 2021). One may realistically expect the first steps on Mars to take place within the subsequent few years and to be followed, on a longer term, by settlements on the red planet (Ehrenfreund et al., 2012; Musk, 2017). Advanced biotechnologies could be central to the fulfilment of such plans: they lend themselves to highly efficient use of resources and tend to minimize both waste generation and environmental impact (Camere and Karana, 2018), which will be (or, we argue, should be) priorities of settlements on other celestial bodies. Bioregenerative life-support systems (BLSS; confined self-sustained artificial ecosystems for the generation and recycling of life-support consumables), in particular, may be key to meeting those constraints (Mitchell, 1994). Besides basic BLSS functions, the dependency on Earth could be decreased by the biofabrication of goods such as food, fabrics, construction materials, and medicines. The use of local resources (*in situ* resource utilization, ISRU) could decrease the reliance of bioprocesses on Earth-imported materials and, thus, improve their cost-effectiveness. Researchers and engineers are, for instance, designing ways of growing plants on lunar or Martian analogue substrates (Lytvynenko et al., 2006; Zaets et al., 2011; Duri et al., 2020), possibly after processing them using microorganisms to release nutritive elements (Auerbach et al., 2019; Castelein et al., 2021).

Here, we argue that the kombucha microbial community (KMC) is worth considering as a multipurpose component of crewed space missions. Kombucha has been consumed for millennia as a health-promoting drink. Its use beyond Earth has been considered for over a decade (Kozyrovska and Foing, 2010; Kozyrovska et al., 2012), and the idea gained momentum in the frame of the BIOMEX project (de Vera et al., 2019; Reva et al., 2015; Zaets et al., 2016; Podolich et al., 2017a,b; 2019; 2020; Góes-Neto et al., 2021; Orlovska et al., 2021; Lee et al., 2021). The applications of KMC we envision can be gathered in two broad categories: 1) prophylaxis of spaceflight-related health disorders, based on both live nutraceuticals and cell-free postbiotics (as well as, perhaps more anecdotally, the psychological benefits of tending cultures), and 2) resource production, which encompasses functions in BLSS/ISRU processes as well as, especially in advanced settlements, the waste-free fabrication of consumer goods, such as comfort drinks and products derived from bacterial cellulose.

The aim of this review is to provide insights into how KMC could be integrated into BLSS for the Moon and Mars, draw attention to obstacles on which research efforts should be focused, and give an overview of the foreseen benefits. Some applications we consider are well-proven and could be implemented at little cost; others may be more speculative and harder to deploy in the foreseeable future. If we elected to include the latter as well, it is with the goal of stimulating constructive discussions, which may help evidence which among them are worth pursuing.

Defining the relevance and feasibility of using KMC beyond Earth will require significant research efforts. We suggest that such efforts are justified, as the benefits may be vast.

## KOMBUCHA *PER SE:* ECHOES OF THE PAST OR BACK TO THE FUTURE

### Kombucha as a Multimicrobial Community

KMC is an example of advanced microbial social interactions between representatives of two domains of living beings, based on metabolic cooperation and competition (May et al., 2019). More specifically, it is a mutualistic multiculture of acetic acid bacteria (AAB) and osmophilic yeasts, which produces an acidic medium at the surface of which floats a cellulose-based pellicle biofilm. This community is not found in nature; when it was formed is not precisely known, but it is thought of having existed in anthropic environments (human habitations) for over two millennia. The core bacterial community within KMC is dominated by AAB: representatives of the *Komagataeibacter, Acetobacter,* and *Gluconobacter* genera (Jayabalan et al., 2014). The relative dominance of yeast genera, such as *Zygosaccharomyces, Brettanomyces/Dekkera, Schizosaccharomyces, Saccharomyces*, and *Pichia,* varies in accordance with the geographical variants of KMC (Jayabalan et al., 2014). Lactic acid bacteria are involved in the fermentation process in some KMC ecotypes (Chakravorty et al., 2016; Coton et al., 2017). The most beneficial genera detected in KMC samples were *Bacteroides* and *Prevotella*, which are known as dominant human gut microbiota members (Lavefve et al., 2021). Metagenomics studies also predicted opportunistic bacteria like *Bacillus, Pseudomonas,* etc. (Arikan et al., 2020; Villarreal-Soto et al., 2020; Barbosa et al., 2021; Harrison and Curtin, 2021), as well as bacteriophages and yeast viruses (Góes-Neto et al., 2021). There is a metabolic interplay between yeasts that convert sugar into monosaccharides and ethanol, in a liquid phase, and AAB that use glucose, fructose, and ethanol to produce organic acids and bacterial cellulose (BC), in a pellicle film phase (Villarreal-Soto et al., 2018). The biofilm, as a three-dimensional microbial hub, supports an evolutionary stable social cooperation between its inhabitants, in a way that is analogous to tissues in multicellular organisms. The biofilm may also optimize oxygen concentrations for the microbial microcolonies, which are stratified in the cellulosic matrix, as well as protect the community from environmental stressors such as UV radiation (Williams and Cannon, 1989). The capability of KMC to generate and tolerate acidic conditions, metabolize ethanol, and produce organic acids, protects the system from invasion by competitor microbes. The bacterially produced cellulose-based pellicle biofilm may also provide protection from contaminants by inhibiting the diffusion of their extracellular metabolites. The KMC as a multipurpose ecosystem has the potential for usage in wide sectors of human activity. The areas of application of kombucha microbial culture products are represented in **Figure 1**.

### Kombucha as Health-Promoting Products

Home-made drinks and pellicle films have been used for prophylaxis, as well as for the treatment of various ailments, before kombucha was widely commercialized (Danielian, 2005). Cultivation was typically initiated by placing a piece of a kombucha pellicle biofilm in a sweetened tea infusion. Owing

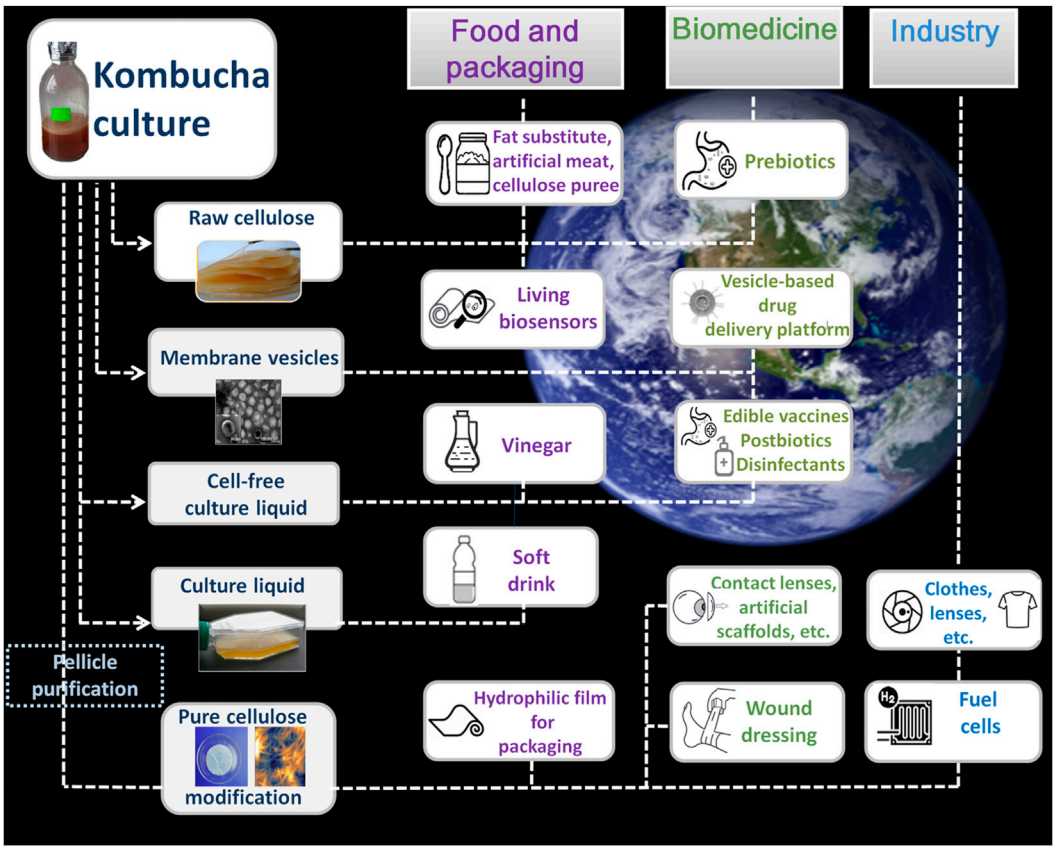


**FIGURE 1 |** Kombucha's cellulose-based pellicle as a three-dimensional hub for microbial community-member cells and raw material for fermented food and consumer goods fabrication. Background image credits: ESA.

to its antimicrobial properties, which were later evidenced (Sreeramulu et al., 2000), and its presumed detoxifying and energizing properties (Jayabalan et al., 2016), kombucha's products served as medicines for the lack of synthetic drugs. In clinical settings of the former U.S.S.R., planktonic cultures were used against severe alterations of the arterial pressure, various forms of acute tonsillitis, chronic enterocolitis, and bacterial dysentery. The cellulose-based pellicle, which resembles mammal skin, has high biocompatibility and water holding capacity, and contains valuable metabolites, was used as a matrix for skin regeneration and healing (Barbanchik, 1954). Highly acidic solutions obtained by long fermentation times were used to disinfect wounds or household items during epidemics (Danielian, 2005). Such uses were largely abandoned with the rise of the pharmaceutical industry.

In the 90s, kombucha grew as a commercial product and rapidly regained popularity (Kim and Adhikari, 2020), which drew scrutiny on its putative health properties. Reconstructions from kombucha metagenomes (Arıkan et al., 2020; Villarreal-Soto et al., 2020; Góes-Neto et al., 2021) predicted the production of various health-supporting components, such as short-chain fatty acids (SCFA), which influence the gut-brain communication (Silva et al., 2020); polyunsaturated fatty acids, which are essential for brain activity and reduce the risk of heart disease (Freitas et al., 2018); glutamate, a neurotransmitter known for its role in mental health (Zhou and Danbolt, 2014); glucosamine, an amino sugar known to support the function of healthy joints (Leal et al., 2018); and a variety of vitamins ($B_1$, $B_2$, $B_3$, $B_6$, $B_{12}$, C, $K_2$). Several organic acids, including detoxifying glucuronic acid (Martínez-Leal et al., 2020), sugars, polyols, kombucha-specific theobromine, rutin, quercetin and chlorogenic acid, were detected experimentally (Tran et al., 2020; Villarreal-Soto et al., 2020; Barbosa et al., 2021). *In vitro* assays and model experiments on animals demonstrated antioxidant, anticancer, antiviral, and anti-inflammatory activities (Mousavi et al., 2020). Nonetheless, *in vivo* assays remain scarce, and clinical trials are lacking to ascertain the health benefits of drinking kombucha (Morales, 2020). Given the fast-increasing interest in this beverage, one may expect more conclusive evidence in the near future.

## Kombucha Multimicrobial Community Are Highly Resilient Under Martian Conditions Simulated Beyond Earth

A common pitfall in BLSS modules or other microbial cultures foreseen for biotechnologies in space is an expected lack of stability under the unusual culture conditions found (or

anticipated) in facilities beyond Earth. The resilience of KMC was put to an extreme test in the Biology and Mars Experiment (BIOMEX): biofilm samples of the KMC ecotype IMBG-1 (Ukraine) were exposed at the outer surface of the International Space Station (ISS) to simulated Mars-like conditions for 18 months (de Vera et al., 2019; Podolich et al., 2019). KMC IMBG-1 tolerated those conditions; however, shotgun metagenomic analyses showed that the community was altered with an overall decrease in diversity and richness (the number of species in a community) of microorganisms (Góes-Neto et al., 2021). Among others, the relative diversity of dominant *Komagataeibacter* spp. increased, suggesting that stress exposure had fostered the development of bacterial species usually found below detection levels. The most striking changes were found in the *Komagataeibacter* representatives after exposure to the Mars-like UV spectrum: the relative abundance of *K. saccharivorans* (which is normally dominant) decreased while that of *K. hansenii* increased. Viral species in KMCs also increased after the exposure experiment. On the other hand, the diversity of the yeast component was not affected significantly by space exposure; however, the dominant genus *Schizosaccharomyces* was replaced with other yeast genera (Góes-Neto et al., 2021). Results of this metagenomic study will be used for the design of robust, rationally assembled mini-kombuchas composed of both core community members and purposely selected microbial partners. The composition of extracellular membrane vesicles (EMVs), which are thought to mediate communication between cells within and across domains, was altered in the post-flight KMC community members (Podolich et al., 2020). The changed composition of membranes correlated with changes in EMV fitness: the activity of membrane-associated enzymes seemed to be increased by UV radiation.

In spite of those alterations, exposure in space did not lead to significant differences in the community functions, suggesting that kombucha samples were ecologically resilient (Góes-Neto et al., 2021). It should also be noted that conditions outside the ISS were far more challenging than those foreseen for KMC when used in support of crewed missions, where it would be grown mostly in crewed modules and, if cultivated outside, would be contained in a bioreactor (and, thus, would not be exposed to vacuum, UV, or extreme temperatures).

# BIOSAFETY OF THE KOMBUCHA MICROBIAL COMMUNITY

## Biosafety Risks Associated With Microbial Communities

Any crewed mission will be accompanied by microorganisms: they are both necessary and inevitable (see, for instance, Guerrero and Berlanga, 2009; Lloyd-Price et al., 2016). Around half of the microorganisms on inner surfaces of crewed compartments is expected to be represented by the crew's microbiota (Singh et al., 2018; Checinska et al., 2019; Avila-Herrera et al., 2020), the rest coming from a variety of sources (*e.g.*, imported consumables, various environmental sources, BLSS). The resulting indoor ecosystem should be well-understood: its mismanagement may lead to the emergence of pathogens (or opportunistic pathogens) which would threaten the crew's health, as well as that of technophiles which might damage equipment.

In order to avoid such a situation in the new indoor environments that will be created by settlers, the philosophy of the interdependence of humans and living microbial beings in the context of co-becoming fates has to go beyond anthropocentrism. The more diverse the microbiota, the better the balance of useful and opportunistic/detrimental counterparts, and the healthier the ecosystem from the standpoint of human health. A reasonable approach to the maintenance of microbiome health is a reconstruction of an Earth-like biosphere and interrelationships with the surrounding biota through a BLSS, as described by Hao et al. (2018) and Chen et al. (2020). The contact with plants for several hours in a day and the consumption of plant fiber diet would increase the relative abundance of friendly microbiota and would reduce proportions of potential pathogens in the crew.

More pragmatically, extensive efforts should be deployed to characterize and, to some extent, design the microbial communities that will accompany crews; in other words, to diversify the microbiota in the indoor ecosystem to reduce the risk of acquired overcompetitiveness. KMC, as a microecosystem organized on metabolic symbiosis and which evolved alongside humans, could contribute to the establishment of an environment free of human pathogens.

## Kombucha Microbial Community Biosafety: What Is Known From Our Shared History on Earth

The biosafety of KMC is known from its use over millennia as well as from recent studies (Dutta and Paul, 2019; Martínez-Leal et al., 2020), including some based on metagenomics (Arikan et al., 2020; Villarreal-Soto et al., 2020; Góes-Neto et al., 2021). While adverse health effects have been attributed to the consumption of home-brewed kombucha (Murphy et al., 2018), such cases have been reported very rarely and were probably related to inappropriate cultivation processes, which led to contamination of the product with pathogenic bacteria. Nevertheless, the United States Food and Drug Administration (FDA, 1995) states that kombucha is safe for human consumption when properly prepared, and kombucha, therefore, has been commercialized in a large number of countries (*e.g.*, Kim and Adhikari, 2020).

*Komagataeibacter xylinus,* a keystone species of KMC, is recognized as a safe microorganism for human consumption. The safety status of *K. xylinus* was determined by feeding rats with doses of $10^{16}$ bacterial cells (Lavasani et al., 2019). Moreover, the toxicological and dietetic aspects of BC have been assessed in a rat model, which led to the conclusion that BC is safe for applications in food technology (Dourado et al., 2017). From a nutritional point of view, BC can be considered a promising low-calorie fiber-rich ingredient or fat substitute. Cellulose generated by *Komagataeibacter* spp. can be a valuable resource for the food industry in compliance with the current legislation of some countries, *e.g.*, the FDA considers BC as GRAS (Vigentini

et al., 2019). Nevertheless, the EFSA (European Food Safety Authority) scientific panel has not decided yet on this issue, in spite of the fact that the chemical structure of BC is well known and identical to that of the vegetable cellulose. Furthermore, AAB that synthesize it are recognized as GRAS, belong to the risk group 1, and are components of fermented foods with a long history of safe human consumption.

## Kombucha Microbial Community Biosafety: What Is Known From a Space Experiment

A recent shotgun metagenomic study on the microbial community structure in kombucha exposed to open space and Mars-like conditions on the ISS demonstrated a disturbing effect of abiotic stressors on the KMC relative abundance and taxonomic composition (Góes-Neto et al., 2021). Nonetheless, KMC core microbial community members were preserved, providing the community with needed gene sets for the formation of a cellulose-based pellicle, which allowed for the survival of the community. After 2 years of subculturing in lab conditions on Earth, the returned KMCs displayed vulnerability for contaminations that should be considered as a possible safety risk. It is worth noting that the KMC was exposed to extremely harsh conditions of real space and simulated Martian conditions that never would occur at human habitation where KMC would be cultured.

In addition to the risk of contamination, undesirable changes in the fermentation process and activation of probable pathogenic factors present in genomes of kombucha community inhabitants must be investigated in detail in future studies. In our recent study, despite alterations in membranes, kombucha's EMVs did not acquire endotoxicity, cytotoxicity, or neurotoxicity (Podolich et al., 2020). Metagenome-assembled genomes did not show the presence of pathogenic microorganisms in KMC IMBG-1, neither before nor after the exposure experiment (Lee et al., 2021). We were also interested in deciphering genomes of core community members in order to analyze them on the presence of genes for toxins and toxin transport systems, extracellular enzymes, genetic mobile elements, and so on. Recently, two dominant bacterial strains of *K. oboediens* isolated from the dehydrated KMC biofilms (ESA BIOMEX), one maintained on Earth and another sent to space/Mars-like conditions on the ISS, had their genomes sequenced and extensively compared. Despite some differences in phenotypic features (Podolich et al., 2020), the genomic analysis revealed that their genomes were quite similar (de Carvalho et al., 2021; personal communication), which made us consider epigenetic events on the space-flown strain.

It is time to examine both the accumulated experience on human coexistence with the KMC microecosystem and the scientific evidence on its unique properties, and to rigorously assess its value as a potential functional food/edible vaccine and environmentally-friendly materials in the context of crewed missions to other planets. The known advantages of KMC as a companion to astronauts and settlers come from the fact that it is: a self-organized system that can be purposively modified and is resistant to contamination; tolerant to space/Mars-like stressors; simple to cultivate; easy to transport; a multimicrobial multifunctional community; ban immortal bank of probiotic and cellulose-synthesizing bacteria and yeasts of biotechnological importance; a multivariate functional food (as a drink, smoothies, candies, vinegar, etc.); a prophylaxis and healing agent; an indicator of local biosafety; a means of psycho-emotional relaxation (taking care of mother culture, handicrafts, and installations using bacterial cellulose, etc.).

The putative strategies for using KMCs, including purposively upgraded variants, for space exploration are summarized in **Figure 2** and outlined below.

# RATIONALLY ASSEMBLED KOMBUCHA MICROBIAL COMMUNITY COULD SUPPORT ASTRONAUT HEALTH LONG-TERM SPACE MISSIONS

## During Missions, the Improvement of Human Health Will Be Profitable Under the Assistance of Health-Promoting Microorganisms

Among the physiological changes undergone by crewmembers in space is a modification of the composition and functionality of the gut microbiome (*e.g.,* Voorhies et al., 2019). The highly comprehensive analyses of the human microbiome using high-throughput "omics"-based technologies evidenced a clear link between gut microorganisms and health problems (Gilbert et al., 2018; Parker et al., 2020). The human intestinal microbiota is essential for microbial homeostasis, regulation of metabolism and immune tolerance (Ahlawat et al., 2020), and a deficit in bacterial products can deregulate brain function, leading to neurobehavioral problems (Cryan et al., 2019). A decrease in the richness of probiotic bacteria in the gut was observed in spaceflight, notably in NASA's Twins Study (Garrett-Bakelman et al., 2019), alongside a reduction in the number of anti-inflammatory bacteria (Voorhies et al., 2019). Altogether, these results indicated that gut microbiome alterations predispose astronauts to illnesses (reviewed in Turroni et al., 2020). Thus, the maintenance of healthy gut microbiota during interplanetary journeys to Mars or other celestial bodies will require means of mitigation.

There are several countermeasures that might be investigated for mitigating dysbiosis in crews: 1) modification in nutrient supply (David et al., 2014; Fetissov, 2017); 2) non-selective modification of the gut microbiome using fecal microbiota transplantation (Cheng et al., 2019; Guo et al., 2020); 3) semi-selective modification of the gut microbiome using antibiotics (Bajaj et al., 2018); and 4) biological modification of the intestinal barrier. All the aforementioned approaches have *pros* and *contra.* Prophylaxis and correction of dysbiosis with microbiome-targeted therapeutics, such as probiotics/synbiotics/postbiotics (**Box 1**), can reasonably be expected to be beneficial but have not been used so far.

In spite of the interest in probiotics for astronauts' diets (Voorhies et al., 2019; Turroni et al., 2020), the in-flight safety of probiotic microorganisms remains unclear. Currently,

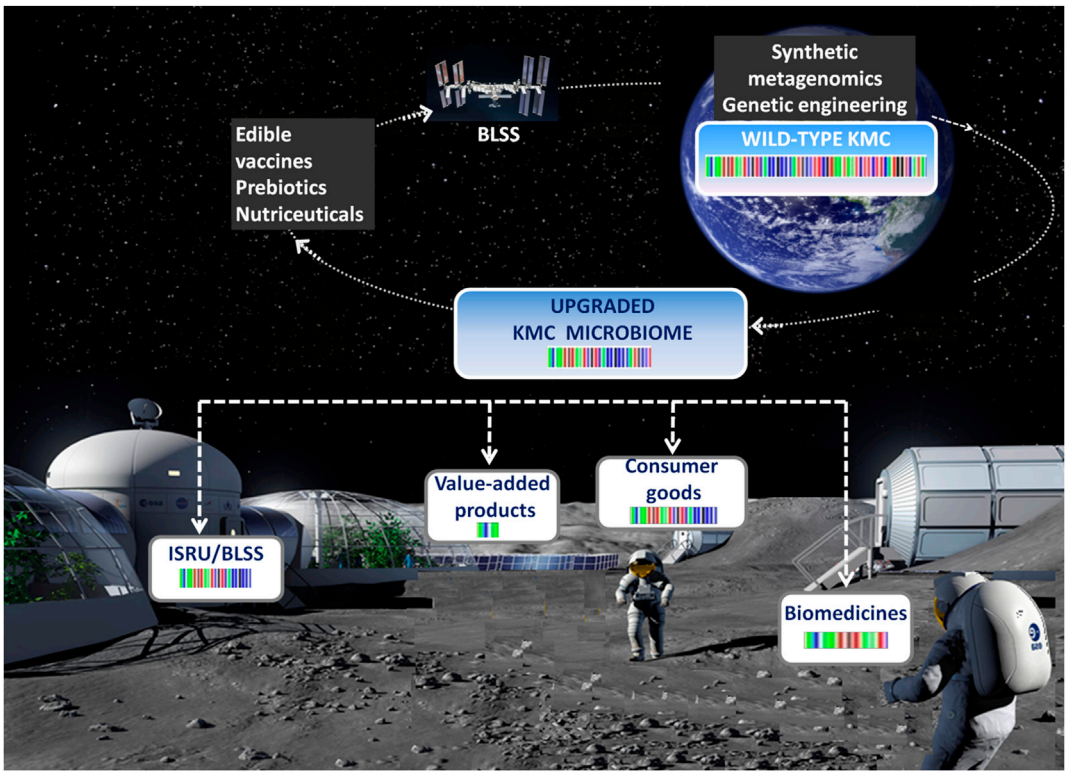


**FIGURE 2 |** A model scheme of the kombucha's travels from the laboratory to the International Space Station and extraterrestrial settlements.

**BOX 1 | Probiotics, prebiotics, synbiotics, and postbiotics**

*Probiotics* are viable microorganisms whose health effects are independent of the site of action and route of administration. The potential benefits of probiotics were first proposed over a century ago by the 1908 Nobel Prize winner Elie Metchnikov (Metchnikoff, 1908), and because of him, fermented milk products entered the daily diet. The term "probiotic" was introduced by Lilly and Stillwell (1965). Later, probiotics were officially defined by the FAO and WHO as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002; Hill et al., 2014). Probiotics are efficient in treating gut-associated disorders, including allergic disorders and metabolic syndrome (Molina-Tijeras et al., 2019), and improving local immunity. *Prebiotics* are non-digestible food ingredients resistant to destruction by mammalian body enzymes but metabolized by intestinal microbiota and that stimulate, therefore, the growth and/or activity of intestinal bacteria associated with health (Gibson et al., 2017). This definition will continue to evolve in line with expanding knowledge about dietary fiber. *Synbiotics* are health-promoting products, containing a combination of prebiotic and probiotic products. *Postbiotics* are overall cell-free metabolites or separate components, *e.g.*, extracellular membrane vesicles of probiotic bacteria.

neither probiotic/synbiotic products nor its structural components were evaluated on the potential risk for astronauts, except for the first exposure of lactic acid probiotic bacteria preparations during a 1-month trial on the ISS (Sakai et al., 2018), which showed that this probiotic had not lost basic therapeutic properties. Further profound systemic studies will be required to determine the safety and stability of probiotic microorganisms during space missions. Criteria for the scientific substantiation of health claims on foods and issues on probiotics, synbiotics, postbiotics and prebiotics remain to be addressed (Vargas et al., 2021).

Kombucha is a functional food (Dimidi et al., 2019) with features of a probiotic product as it contains lactic acid bacteria (LAB) and probably probiotic yeasts (*Shizosaccharomyces, Saccharomyces,* and *Lachancea*), as mentioned above. The main LAB strains isolated from kombucha are the well-known *Lactobacillus bulgagricus, Streptococcus thermophilus,* and *Lactobacillus plantarum* (Pei et al., 2020), as well as *Pediococcus pentosaceus* (Matei et al., 2018). Marsh et al. (2014) reported that *Lactobacillus* and *Lactococcus* are dominant in kombucha pellicles, and the content of LAB depends on the kombucha's origin. LAB isolated from kombucha are considered potential probiotics since they meet most of the probiotics criteria, *e.g.*, bacteriocin production and bile salt tolerance, in according to the FAO/WHO Guidelines for the Evaluation of Probiotics in Food (2002) (Matei et al., 2018; Pei et al., 2020; Vargas et al., 2021). Incoming LAB easily become community members (Zaets et al., 2016; Cvetković et al., 2019; Bueno et al., 2021) and improve kombucha's biological properties, *e.g.,* antibacterial and antioxidant activities (Nguyen et al., 2015). Another approach is to design rationally assembled

(upgraded) kombucha composed of the defined health-promoting bacterial and yeast species capable of kombucha fermentation (Savar et al., 2021).

## Kombucha Dietary and Prophylaxis Projects During Spaceflights

Whether *Komagataeibacter* spp. (and other dominant members of the kombucha community) behavior as probiotics has not been ascertained, these bacteria possess several characteristics which suggest that research on their dietary properties could be fruitful. First of all, their product, cellulose polymer, which is non-digestible in the human gut but can be consumed by cellulolytic bacteria in the distal gut, can be considered a prebiotic (Holscher, 2017; Nsor-Atindana et al., 2020). Low-fiber diets lead to slow gastro-intestinal transit and a microbiome starving in crewmembers. Cellulose is useful for digestion because it stimulates the work of intestines that increases stool bulk and water absorption, and, thereby, decreases the gastrointestinal transit time. Earlier, we proposed KMC as a source of prebiotic for astronauts (Kozyrovska et al., 2012), and most recently, kombucha fermentation was reported to produce SCFAs (Utoiu et al., 2018; Annunziata et al., 2020) or support a significant potential prebiotic effect (Lavefve et al., 2021). The AAB, producing cellulose, are food-grade (Alexandraki et al., 2013) or GRAS bacteria (Gorgieva and Trček, 2019), which gives *Komagataeibacter* species potential for pro/pre/postbiotic use. Among them, *K. xylinus,* one of the dominant core kombucha bacteria, exhibited some probiotic features: it was shown to survive in the conditions of a rat gut tract, being resistant to acid, bile, low oxygen pressure, and rat body temperature (Lavasani et al., 2019). Komagataeibacter cells contain valuable antioxidants and lipids which can improve cognitive functions (Fukami et al., 2009) and prevent stratum corneum dryness (Tsuchiya et al., 2020).

During space missions, crewmembers experience nutrient deficiency because of a reduced appetite compared to that on Earth, for many reasons (Turroni et al., 2020). Correction of nutrient and energy deficiency by live kombucha-related products is a reasonable and facile way to enhance energy level and crew's appetite under specific conditions. Both beverage and pellicle film possess inherently a great collection of various essential and valuable dietary compounds highlighted above, always available fresh and organic, which probably could aid in prophylaxis of health problems in parallel with pharmacological interventions, lowering doses of medicines. Special attention should be given to kombucha's energizing capability: the formation of the iron and gluconic acid chelating complex increases the level of hemoglobin and stimulates ATP synthesis under the support of energy regeneration by the vitamin B group that promotes enzymatic activation of lipid and protein metabolism (Dufresne and Farnworth, 2000).

In addition to both nutrient and energy deficiency, various health risks (zero- and reduced-gravity effects, ionizing radiation, etc.) affect several crewmembers' organs and systems during missions. Beyond Earth's protective magnetosphere, the exposure of astronauts to galactic cosmic radiation could result in degenerative tissue diseases (Thirsk, 2020). A high oxidant and low antioxidant level in the human body is associated with the development of chronic inflammatory diseases, such as cardiovascular and neurologic diseases (Barcellos-Hoff et al., 2015; Vernice et al., 2020) and, thus, lead to deregulation of the immune and metabolic systems (Garrett-Bakelman et al., 2019). Intake of dietary antioxidants may inhibit the formation of peroxides and their absorption in the gastrointestinal tract (Xia et al., 2019). Kombucha-derived products, such as beverage and pellicle-based products, exhibit antioxidant capacity, which has been associated with the presence of polyphenols and derived phenolic compounds, such as gallic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, and ferulic acid among many others (Villarreal-Soto et al., 2020). Furthermore, kombucha modulates the immune system in animals, inducing the production of anti-inflammatory cytokines (*e.g.,* IL-4 and TGF-β) and inhibiting the production of pro-inflammatory cytokines, such as IFN-$\gamma$ and IL-17 (Haghmorad et al., 2020). The microbiome shift is one of the six fundamental changes in human bodies during spaceflights (Afshinnekoo et al., 2020), and it is already known that kombucha brew intake corrects changes in intestinal microbiota promoted by non-alcoholic fatty liver disease (Jung et al., 2019).

## An Alternative Approach to the Mitigation of Crew's Health Dysfunctions: *In Situ* Biofabricated Acellular Postbiotics

Microorganisms may not be the only players in probiotic products that affect the recipient's biology and safety; their extracellular membrane vesicles (**Box 2**) could contribute to health effects under the changed conditions.

Several members of the KMC produce EMVs, which could serve a range of health-related purposes. In this case, microbial EMVs could act as a surrogate of probiotics, carrying the same bioactive molecules but limiting the risks associated with the administration of live bacteria (Kozyrovska et al., 2018; Podolich et al., 2020), or act as living edible vaccines.

Hereafter, we report examples of such applications. Some microbial EMVs carry surface immunomodulatory proteins that could be used for protective mucosal immunization through different routes (oral, nasal, rectal, vaginal), to facilitate the restoration of normal immune responses (Gorreja, 2019; Kuhn et al., 2020). The probiotic *Propionibacterium freudenreichii* produces EMVs that export proteins (*e.g.*, the surface-layer protein B), which interact with the host proteins and attenuate inflammation (Rodovalho et al., 2020). EMVs from various bacteria regulate brain functions by interacting with the peripheral nervous system and delivering neurotransmitters to the central nervous system, which may lead to novel therapeutic approaches to neurodevelopmental and mood disorders (Haas-Neill and Forsythe, 2020). Furthermore, EMVs may also be used to alleviate metabolic dysfunctions (Ashrafian et al., 2019). As the last example, bacterial EMVs are emerging as nanodrugs (Jahromi and Fuhrmann, 2021) and platforms for drug delivery (Li et al., 2020).

**BOX 2 | Extracellular membrane vesicles**

EMVs are spherical, nanosized (20–400 nm) bilayered proteolipids that contain different molecular cargo from the cytosol, periplasm, and cellular membranes. EMVs are released by cells of all the three domains of life represented in the KMC, however, depending on their origin, vesicles differ in unicellular and multicellular organisms, but all the EMVs facilitate intercellular communication by shuffling molecular cargo between cells. Proteomic and biochemical analyses have shown that EMVs of Gram-negative bacteria contain a variety of bacterial components from the outer membrane (like lipopolysaccharide and lipoproteins), periplasmic proteins, DNA, and RNA species, which have been suggested to modulate biological processes, such as biofilm formation, survival, competition, defense, etc. Moreover, EMVs of probiotic bacteria mediate communications with the host via molecular pattern molecules, interacting with innate immune receptors (reviewed in Nagakubo et al., 2020). Nano-sized bacterial EMVs withstand long-time circulation through body organs and fluids and are able to penetrate the blood-brain barrier. Eventually, their effect on living systems is comparable to that of the gut microbiota (Muraca et al., 2015; Tulkens et al., 2018).

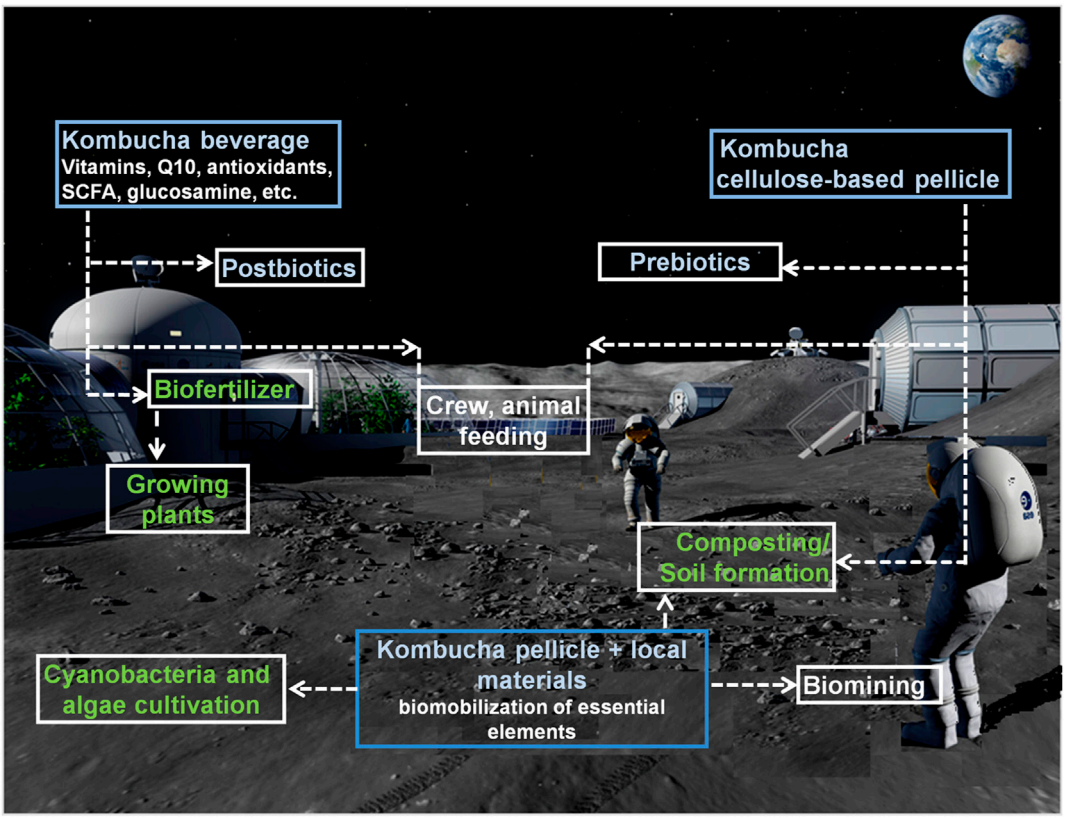


**FIGURE 3 |** A schematic presentation of different approaches for manipulation of the kombucha microbial community to enhance life-support efficacy in extraterrestrial settlements.

Rather than being sent from Earth, EMVs could be produced on-site, which would circumvent issues related to long-term conservation. This could be facilitated by the fact that bacterial EMV production seems to be intensified in space (Zea et al., 2017; Ott et al., 2020). EMVs from ground control and space-flown KMC samples did not exhibit signs of cyto-, endo- or neurotoxicity (Podolich et al., 2019), and neither did EMVs of the dominant kombucha community member *Komagataeibacter oboediens* (Podolich et al., 2020): membrane alterations occurred but did not lead to toxicity for eukaryotic cells *in vitro*. This experience suggests that EMVs, originating from carefully selected, non-pathogenic Gram-negative bacteria such as *K. oboediens*, can be considered as candidates for postbiotics or edible mucosal vaccines produced *in situ*, beyond Earth.

## Kombucha Microbial Community Could Be a Practical Element of the ISRU-Based Bioregenerative Life Support System on Mars and the Moon

The KMC could find application in ISRU-based BLSS, either as a primary goal or via satellite products from other programs (health support, cellulose production, etc.) (**Figure 3**).

## Kombucha for Growing Plants in BLSS

The lunar and Martian regoliths are expected to contain all the mineral nutrients required for plant growth (Helmke and Corey, 1989). However, whether either could support plant growth has not been fully demonstrated. The biological tests performed with

Moon regolith during and shortly after the Apollo years (reviewed in Ferl and Paul, 2010) mostly aimed at ensuring that the regolith did not present any risk to the Earth's biosphere. As for Mars, no regolith sample has been brought back so far. Plants were successfully germinated and grown in Mars regolith simulants (Wamelink et al., 2014; Duri et al., 2020), but those differ from the regolith they mimick in ways that are critical to biological experiments. Generally speaking, it is thought that regolith from both bodies would require some physical, chemical, and/or biological modifications (*e.g.*, Ming and Henninger 1994; Maggi and Pallud, 2010; Fackrell et al., 2021) before use as a substrate for plant growth, due to, for instance, poor water retention, low availability (or release up to toxic concentrations) of mineral nutrients, toxins, root damage by abrasive particles, and/or lack of metabolizable nitrogen. These limitations may be addressed by using microorganisms (*e.g.*, Ehrlich, 1989; Ming and Henninger, 1994; Zaets et al., 2011): some may coat the sharp edges of grains, thereby mitigating damage to plant roots (Slenzka and Kempf, 2010); their biomass would enrich the soil in organic compounds, improving both fertility and water retention; and microorganisms capable of bioleaching could foster the release of essential elements (Wamelink et al., 2014). Associated to plant roots in anorthosite, microbial communities were shown to help balance levels of mineral nutrients, favoring release while preventing accumulation to toxic levels (Zaets et al., 2011). Thus, microbial communities, possibly planktonic KMC, could have a positive impact on plant growth, either as an inoculant or by pre-processing the regolith. For the case where such a treatment would not suffice for growing plants of high interest, an approach has been proposed where "first-generation plants" (*i.e.*, plants with higher fitness in such an environment, such as *Tagetes patula* or *Kalanhoe daigremontiana*) would be grown first, supported by microbial communities, and composted to enrich a protosoil itself used for plants of direct interest such as calorie-dense crops (*e.g.*, Lytvynenko et al., 2006; Zaets et al., 2011). In line with this, Duri et al. (2020) suggested planting leafy vegetables on Martian regolith ameliorated with organic materials produced *in situ*. A composted kombucha mat could be an additional source of nutrients and other plant-supporting biologicals.

Another approach may lie in using such communities to mobilize ions from regolith beforehand, for the production of fertilizers. It was shown that in a tea infusion, supplementation with anorthosite led to an accumulation of inorganic ions in a KMC cellulose biofilm matrix: metals concentrations exceeded that in the anorthosite-free matrix by, for instance, 10 times for Ca, and 43–51 times for Mn and Mg (Zaets et al., 2014). Inorganic ions accumulated in the film can readily be extracted. The presence of KMC also increased the concentration of ions in the anorthosite-supplemented tea: after 1.5 months, for instance, the concentrations of Mn, Cu, and Zn increased 5–7 times, and Mg and Fe 12–22 times, compared to a medium with anorthosite but without KMC. The extensive growth of the $SiO_2$ group of secondary minerals on the bottom surface of the pellicle evidenced the primary dissolution of the silica-rich minerals in the anorthosite rock, and its accumulation on and around the pellicle (Podolich et al., 2017a). Kombucha's leaching activity presumably stems from metabolites (organic acids, siderophores, enzymes) and a proton excess (Hopfe et al., 2017). Increased levels of EMVs production were also noted in the KMC grown with anorthosite, which correlated with the alteration of the ionome in the pellicle. Furthermore, Al and Fe were found in the pure preparations of EMVs isolated from KMC grown in the presence of anorthosite (Podolich et al., 2017a), consistently with observations by Matlakowska et al. (2012), suggesting a role for EMVs in biomineralization reactions during bioweathering of aluminosilicate rocks.

The use of cyanobacteria for the development of ISRU-based BLSS has been under investigation for several years (e.g., Brown et al., 2008; Olsson-Francis and Cockell, 2010; Rothschild, 2016; Verseux et al., 2016). Extremophilic desert strains of *Chroococcidiopsis* spp. that have been exposed in space and were tested for resistance to high doses of ionizing and ultraviolet radiation (see Billi et al., 2020), as well as nitrogen-fixing, rock-weathering cyanobacteria of the genus *Anabaena* (or related genera) were shown to be suitable as a source of nutrients for heterotrophic microorganisms (Verseux, 2018; Billi et al., 2021; Verseux et al., 2021). As a proof-of-concept, a desert strain of the cyanobacterium *Chroococcidiopsis* was grown in a Mars-relevant perchlorate concentration, *i.e.*, 2.4 mM (Billi et al., 2021); perchlorate ions and the cyanobacterial lysate successfully utilized to feed *Escherichia coli.* Dual cyanobacterium—kombucha cultures are under consideration for processing locally available regolith to make rock-extracted nutrients available for plants of interest. The cyanobacteria-kombucha mat will form a protosoil, also making rock-extracted nutrients available for growing plants.

A challenge for growing KMC in space is the carbon and energy sources. The traditional substrate for KMC, *Camellia sinensis* or Chinese tea, is a limited resource for space travels, therefore, tea must be replaced by alternative components, considering a self-sustainable production based on green ecology (Kołodziejczyk, 2018). One such possibility is the use of photosynthetic microorganisms, for example, common *Chlorella* algae, which can grow in bioreactors onboard habitats or spaceships (Suchan, 2020). KMC cultures set on naturally basic *Chlorella* extract must be reduced to pH = 6. Bacterial nanocellulose produced in green solutions is white, delicate, and glossy (**Figure 4A**). Other types of algae tested in order to optimize KMC growth in space are extremophilic microorganisms such as endolythic *Galdieria sulphuraria* and *Cyanidioschyzon merolae*. These algae are easy to cultivate, and they do not create protective biofilms. Instead of sucrose, there are few options such as using starch or cellulose digested by bacteria and fungi within the BLSS loop. It is important to note that adding a new organism to the consortium takes time and sometimes it is not possible for KMC to accept a new partner and alternatively, yeasts will be modified with genetic engineering tools.

## Kombucha for Biomining

Biomining describes a process of dissolving ingredients and removing them out by biological agent, *e.g.*, metals of

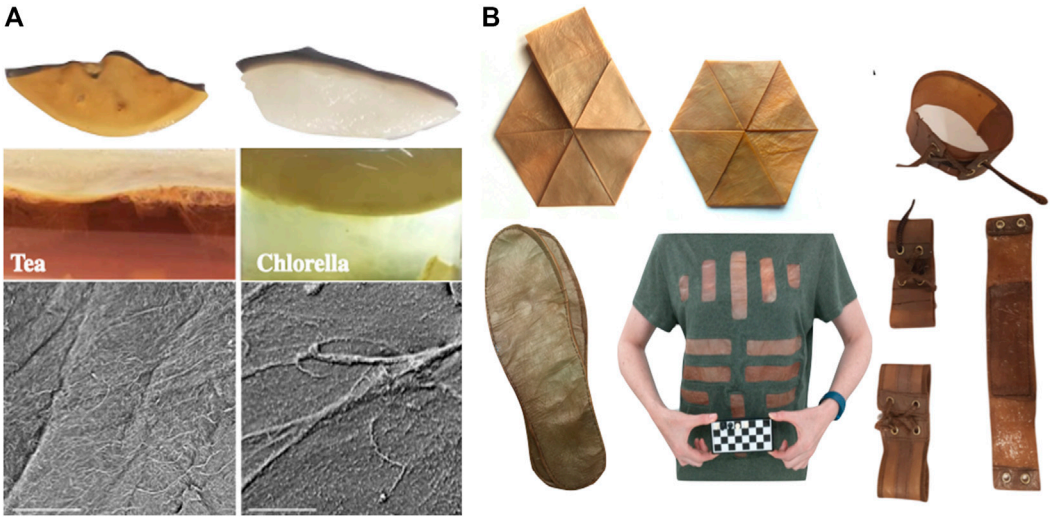


**FIGURE 4 |** Bacterial nanocellulose can be used for clothes in space. **(A)** The traditional substrate for KMC, a tea, must be replaced by alternative components, e.g., photosynthetic algae, for biofabrication of cellulose. The comparison between two types of KMC cultures: (left) grown on tea, and (right) grown on algae. Hydrogel bacterial cellulose is similar in chemical composition but physical properties are slightly different. Nanocellulose grown on algae extract is much softer and more prone for damage. Scanning electron microscope images reveal the ultrastructure of nanocellulose filaments. Scale bar 50 microns (modified after Suchan, 2020). **(B)** KMC's material absorbs odours. It can be easily cleaned and sterilised. It folds gently to very compact volumes. Folding does not implicate changes in structure of the material. Kombucha's cellulose does not generate allergic responses in direct contact with human skin. It makes skin softer and visibly healthier (Kołodziejczyk, 2018; Kamiński et al., 2020).

economic interest from rock ores or mine waste (Rawlings and Silver, 1995; Johnson, 2014). When the metal of interest is directly dissolved, the biomining process is called "bioleaching". The bioleaching of elements occurs directly by electron supply based on the pH difference between the environment and the microbial cytoplasm, which generates a natural proton motive force across the cellular membrane. Another type is indirect leaching by metabolic products of the microorganisms (organic acids, siderophores, enzymes, etc.) (Potysz et al., 2018). Biological technologies offer obvious advantages over the traditional mining procedures due to their low cost and eco-friendliness, and they do not require high amounts of energy. A biomining is also of great advantage since microbes can discard low-grade ores. Moreover, in some cases, bioleaching is significantly more efficient using bacteria when compared to abiotic technology (Auerbach et al., 2019). Microorganisms are increasingly used for metal extraction worldwide, primarily for copper and gold but for other metals as well (see Schippers et al., 2013).

The use of biomining for missions beyond Earth has been considered for over a decade (Dalton and Roberto, 2008; Cockell, 2010; Cockell et al., 2020). Although estimates of the effectiveness and efficiency of such processes on Mars or the Moon can only be tentative, a few assays based on basalts, anorthosite, or slightly more sophisticated simulants were performed for such purposes (*e.g.,* Volger et al., 2020; Olsson-Francis and Cockell, 2010; Navarrete et al., 2012; Castelein et al., 2020), giving insights into the rates of metal release in the liquid phase, metal assimilation in the biomass, and bacterial growth. Besides, an experiment on the ISS showed that microgravity or Martian gravity did not impair biomining (Cockell et al., 2020). Kombucha's bioleaching abilities could be used for the mining of metals for applications beyond BLSS. KMC has shown to foster the release of the rare earth elements (REEs) from fluorescent tubes (Hopfe et al., 2017) and, as described above, that of various metals from anorthosite. Their use on the Moon on Mars may, thus, be worth considering for leaching small amounts of iron, REEs, and other elements from regolith or waste (Zaets et al., 2011).

## BACTERIAL CELLULOSE IS A MULTIPURPOSE BIODEGRADABLE ROBUST NANOMATERIAL FOR EXTRATERRESTRIAL BASES

Since economic and environmental issues will be critical in the early beginning of extraterrestrial settlements, sustainable bio-based products will be desired. BC is an example of biofabricated multipurpose products, valuable for sustainable eco-friendly extraterrestrial economies.

### Bacterial Cellulose Fibrils Are Synthesized by Acetic Acid Bacteria

BC is secreted out of cells as linear polysaccharide polymer, where D-glucose units are linked by β-1,4-glycosidic linkages, similar to plant-derived cellulose (Ross et al., 1991). Nonetheless, in contrast, BC generates structural hydrogel with interconnected ribbons of around 100 μm in length and 100 nm diameter composed of a three-dimensional nanofibrous network (Wang

et al., 2020), which is free of both lignin and pectin. Such a peculiar supramolecular structure engineered by nature makes BC stable and robust. The crystallinity of BC is higher than cellulose produced by plants, and this results in higher thermal stability. Being one hundred times thinner than cellulose fibrils obtained from plants, BC possesses high tensile strength. The highly porous structure of the cellulose nanonetwork and controlled shape are attractive for the manufacture of new ultra-light and stable nanomaterials, and various possibilities of the BC-polymer modifications open up the unrestricted development of new cellulose-based nanocomposites.

## Bacterial Cellulose has the Potential to Be Used in the Extraterrestrial Economy

The robust cellulose properties, such as high crystallinity, water holding capacity, thermo- and radiation resistance, mechanical properties, biocompatibility with the human body offer a wide range of applications in different fields, including optics, electronics, biomedical and food industry (Blanco Parte et al., 2020; Choi and Shin, 2020; Sriplai and Pinitsoontorn, 2021). The worldwide market for bacterial cellulose is valued at 324.5 million USD in 2020 and is expected to reach 785.1 million USD by the end of 2026 (Global microbial and bacterial cellulose market research report, 2020). Therefore, BC has a high sustainable value and a great potential to be used in the extraterrestrial economy for providing biodegradable and reusable goods and materials. BC's exceptional bioaffinity will promote the development of BC-based biomedical products for outposts, such as tissue-engineered scaffolds, wound-dressing materials, dental implants, artificial blood vessels and nerves, surgical mesh, bone fillings, heart valve, meniscus, artificial cartilage, etc. (Swingler et al., 2021). When bacterial cellulose is modified with conductive compounds, such as metal nanoparticles or graphite, it naturally becomes conductive of electricity and, therefore, promising in developing materials for electrical applications (Andriani et al., 2020). KMC living cellulose-based materials can sense and respond to their environment. Synthetic biology methods provide tools to create hybrid cellulose materials with predictable novel or enhanced characteristics matching the special purposes of the developing settlements, which cannot be achieved by conventional tools. For example, engineered to secrete enzymes into bacterial cellulose, yeast strains can be taught to sense and respond to chemical and optical stimuli. This means that the modified KMC can be biosensors for many purposes (Mustafa and Andreescu, 2020; Gilbert et al., 2021). Moreover, another especially potential use will be intelligent packaging for foods with short shelf-life, as well as biosensors of microbial contamination and mycotoxins.

## Bacterial Cellulose for Clothes in Space

Astronauts must take all clothes for the time of their mission because the supply of water on board is too limited to permit clothes being washed by hand, let alone by a washing machine. The price for each lifted kilogram into space ranges from $10 000 to $25 000, which means that clothes in space are an expensive issue regarding long-term missions (https://www.nasa.gov/vision/space/livinginspace/Astronaut_Laundry.html). There were many discussions about solving the clothing problem in space. One option was proposed to clean dirty clothes using physical methods such as radiation, UV light, or vacuum. Other options suggested using smell-free clothes, for example, Wakata's clothes developed by Yoshiko Taya designed to be flame-resistant, anti-static, anti-bacterial, to absorb water, insulate the body, and dry quickly, not to mention comfortable and stylish. The last option was to reuse dirty clothing for nourishing plants and bacteria cultivation (Space.com, 2021). Interestingly, KMC-derived bacterial nanocellulose may be easily and ecologically processed to obtain material similar to animal leather (Kołodziejczyk, 2018; Kamiński et al., 2020). It has similar properties to Wakata's clothes and it is fully biodegradable. The whole process from setting the KMC culture to obtaining material of the desired shape takes around 3 weeks. Transformation of a raw kombucha hydrogel cellulose into a dry and strong anti-flammable material takes another 3 days (Kołodziejczyk, 2018) (**Figure 4B**). In addition to indoor clothing, *e.g.*, T-shirts shown in **Figure 4**, the potential use of kombucha nanocellulose as a component of spacesuits is currently being investigated in the Analog Astronaut Training Center (Poland) in collaboration with Jagiellonian University (Krakow, Poland). In particular, post-processed kombucha material reveals new features such as resistance to damage, friction or fire (Kamiński et al., 2020). In support of the fact of cellulose robustness under extreme conditions, Orlovska et al. (2021) provided the first data on the cellulose polymer resilience after exposure of live cellulose-based pellicle films to the simulated Martian environment on the ISS. The ATR-FTIR absorption spectra analysis showed the preservation of its characteristic vibrations. In addition, the characteristic hydroxyl bonding in cellulose structure due to preservation of an Iα crystalline phase may serve as the evidence of structural integrity of cellulose polymer in the space-exposed samples. Notably, after a long-term exposure experiment, the mechanical properties of the *de novo* synthesized cellulose were slightly changed in the BC-producers, especially, in unprotected KMCs, which exhibited reduced cellulose yield, compared to ground control KMC pellicles. After a long period of permanent culturing, the mechanical properties of cellulose produced by KMCs improved.

## Kombucha Microbial Community Is a Hub for the Cellulose-Synthesizing Bacteria

Increasing attention is paid to the cellulose-producing organisms and their appropriate genetic modification as candidates for cellulose biofabrication in earthly and extraterrestrial conditions. For example, the reprogrammed bacterial strains that are capable to produce a high yield of BC in low-cost media with precise control of heterologous cellulose biosynthesis gene expression or metabolic pathway modulation have been designed (Florea et al., 2016; Teh et al., 2019; Ryngajłło et al., 2020; Singh et al., 2020). Our results show that cellulose-producing bacteria of the *Komagataeibacter* genus are tolerant to spaceflight and Mars-like stressors (Podolich et al., 2019). Nonetheless, the rate of tolerance may depend on the

genotype of the species. Among the species of *K. saccharivorans, K. hansenii, K. rhaeticus, K. oboediens, K. intermedius* and *K. europaeus* detected in the IMBG-1-ecotype, the relative abundance of *K. hansenii* was substantially increased in the KMC exposed to Mars-like UV compared to the initial KMC, where *K. saccharivorans* was dominant. Similarly, *K. oboediens, K. intermedius* and *K. europaeus* were enriched in the exposed KMCs. We observed that the Mars-like stressors did not alter the topology or induced mutations in any of the bacterial cellulose synthesis (*bcs*) genes of *K. oboediens,* reisolated from the returned KMC (Góes-Neto et al., 2021). Nevertheless, in the revived *K. oboediens*, the cellulose synthase gene *bscA* was down-regulated, in spite of the full homology/topology of the *bcsABCD* to wild type gene cluster, supporting the observation of the lower cellulose output by revived bacteria compared to the ground reference (Orlovska et al., 2021). Since komagataeibacters show a significant potential of survival in extraterrestrial conditions, these bacteria have a chance to be safely delivered within a cellulose-based pellicle matrix to far destinations.

In addition to *Komagataeibacter* spp., KMC pellicle is a matrix for safe delivery of a miniature collection of microbial organisms, which could later be developed into a real microbial collection hosted by a local research unit. KMC members are co-existed on a principle of cooperation and competition to produce public goods with the endless biotechnological value and myriads of applications. For example, *Gluconobacter oxydans* could be a candidate for biofabrication of goods for application in pharmaceutical and food industries (vitamin C, a sweetener, co-enzyme Q10, biosensors, etc.) (Moghadami et al., 2019). In extraterrestrial bases, *Saccharomyces cerevisiae* is of great importance for various biotechnological applications, especially, in such vitally important industries as bread and biofuel production (Parapouli et al., 2020). *Schizosaccharomyces pombe* is a promising yeast species for the laccase production needed for degrading and detoxifying various synthetic compounds, which inevitably will be accumulated in crewed bases, or CoQ10 as an antioxidant food supplement (Nishida et al., 2019). Moreover, lactic acid bacteria could be an inexhaustible source of probiotics from the kombucha ecosystem (Zaets et al., 2016).

## Kombucha and the Zero-Waste Production Philosophy

KMC culturing for the biofabrication of cellulose for industrial purposes lies in the context of eco-friendly, zero-waste production. Importantly, the kombucha-related industry is to operate without wastes and side products. For example, residues of cellulose pellicle in the manufacture of clothes can be used in formation of protosoil in BLSS gardens (Camargo et al., 2020) or as animal feed (Afsharmanesh and Sadaghi, 2014). A sour kombucha drink left could be transformed into vinegar and balms, as well as used in the production of disinfectants (Ryssel et al., 2009). Notably, acetic acid is a cheap and effective measure to eradicate biofilms (Halstead et al., 2015), which would be of great importance in fighting against biofilm-forming bacteria usually competitive in confined systems of crew habitations. *Komagataeibacter* cells, as a by-product of the BC production, may be reasonably used as a biologically active additive, containing valuable antioxidants and lipids for improving cognitive function (Fukami et al., 2009) and as a product preventing skin dryness (Tsuchiya et al., 2020).

## Perspectives

Kombucha culture has evolved over significant periods of time in various artificial environments. An upgraded, rationally assembled, well-defined version could benefit crews, living in the confined space, in long-term missions. It is known as a health-promoting functional food and could provide a wide range of nutrients and other metabolites (including nutraceuticals for prophylaxis, either synbiotic or postbiotic) for crewmembers, animals, and plants. It is also a producer of cellulosic materials, which could find a wide range of applications in human communities beyond Earth, be they small habitats or outposts possessing their own economic systems. A significant advantage of KMC is the zero-waste production in systems ranging from small-scale, ISRU-based BLSS to large-scale cellulose biofabrication. Its organization as a microecosystem provides strong advantages over most microorganisms of biotech value: resistance to contamination, ease of cultivation, and high versatility. As kombucha is a complex microecosystem, it probably complicates operation and its control, especially in alien conditions. The improvement of an advanced toolbox of genetic metabarcoding and shotgun metagenomic technologies (to monitor accurately the microbial populations), would be highly valuable. On the other hand, alternative low-resource tools and novel biological sensing platforms for pathogen monitoring should be designed for easy use by crews. Relying on a combination of systems biology and *Meta*-Omics methodologies, it is suggested to compose and study different ecotypes of the kombucha microbiota (or related communities) to select an operational, minimal rationally assembled (upgraded) community with defined essential community members for different purposes (health promotion, biosensing, biomining, etc.) (Savar et al., 2021). Our results showed that the diversity within a KMC was not affected significantly by space exposure, suggesting that keystone tolerant species will be maintained and controlled. Minor metagenomes can be customized by including specific transcripts or sequence variants serving specific purposes. Synthetic metagenomics could be used to gather metabolic networks from various members into a single yeast species, then used as a chassis (Belda et al., 2021). Systems and synthetic biology could be used to enhance KMC's biotechnological features. For example, metabolic engineering could lead to more efficient pathways of carbohydrate utilization from waste plant material produced on-site (rather than imported sugars) by some KMC members (*e.g.*, yeasts).

Even though KMC is quite resistant to contaminating biota, some of the remaining challenges pertain to safety, most importantly with regards to the consumption of fermented products. Studies of the safety of a well-defined yeast-bacterial community, under normal and stressed conditions, as well as the improvement of an advanced toolbox of genetic metabarcoding and shotgun metagenomic technologies (to monitor accurately the microbial populations), would be highly valuable. On the other hand, alternative low-resource tools and novel biological

sensing platforms for pathogen monitoring should be designed for easy use by crews. A set of criteria for the scientific substantiation of health claims on foods for consumption beyond Earth are being elaborated, but issues on fermented food, probiotics, synbiotics, postbiotics remain to be addressed (Vargas et al., 2021).

Significant research and development efforts are required to fulfil the potential of KMC beyond Earth. However, those can be provided progressively: from the simplest applications in orbital stations to industrial manufacturing in planetary settlements, incremental developments will reap incremental benefits. Humankind seems to be in the process of expanding its presence beyond Earth: after 2 decades of permanent presence in low Earth orbit, a permanent presence on the Moon is expected to start this decade, followed by crewed missions to Mars. In a more distant future we may, as many have claimed, become a multiplanetary species. KMC-based processes could develop alongside human activities in the Solar System. In any case, the idea of fermentation in space is gaining popularity among scientists from academia, industry, research institutes, and public interest groups, creating—it seems—the required momentum. The evolution of space-related regulations, which may be relaxed as new safety-related knowledge is obtained, could support this initiative.

In short, we argue that KMC applications beyond Earth should be investigated: biotechnological processes may result which could largely benefit human activities, from orbital stations to planetary settlements. On a less tangible side (but nonetheless worthy of consideration), we suggest that the acceptance of microorganisms—such as upgraded KMC—as partners, and of the principle of co-existence, would greatly benefit the creation of a healthy biosphere in alien worlds.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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# Development of Nitrogen Recycling Strategies for Bioregenerative Life Support Systems in Space

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To enable long-distance space travel, the development of a highly efficient and robust system to recover nutrients from waste streams is imperative. The inability of the current physicochemical-based environmental control and life support system (ECLSS) on the ISS to produce food *in situ* and to recover water and oxygen at high enough efficiencies results in the need for frequent resupply missions from Earth. Therefore, alternative strategies like biologically-based technologies called bioregenerative life support systems (BLSSs) are in development. These systems aim to combine biological and physicochemical processes, which enable *in situ* water, oxygen, and food production (through the highly efficient recovery of minerals from waste streams). Hence, minimalizing the need for external consumables. One of the BLSS initiatives is the European Space Agency's (ESA) Micro-Ecological Life Support System Alternative (MELiSSA). It has been designed as a five-compartment bioengineered system able to produce fresh food and oxygen and to recycle water. As such, it could sustain the needs of a human crew for long-term space exploration missions. A prerequisite for the self-sufficient nature of MELiSSA is the highly efficient recovery of valuable minerals from waste streams. The produced nutrients can be used as a fertilizer for food production. In this review, we discuss the need to shift from the ECLSS to a BLSS, provide a summary of past and current BLSS programs and their unique approaches to nitrogen recovery and processing of urine waste streams. In addition, compartment III of the MELiSSA loop, which is responsible for nitrogen recovery, is reviewed in-depth. Finally, past, current, and future related ground and space demonstration and the space-related challenges for this technology are considered.

**Keywords: space exploration, bioregenerative life support systems, MELiSSA, urine recycling, nitrogen recovery, nitrification, ureolysis**

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## INTRODUCTION

Space missions are primarily driven by the desire to acquire new knowledge in a wide variety of scientific fields, such as life sciences, physics, material science, and planetary science. Currently, all long-distance space missions to Mars, for example, are conducted by robots. While cheaper than a crewed mission, more accurate and reliable, these expeditions mainly serve as precursor

missions. Humans can handle tasks far more complicated than those which can be performed with robotic automation. They bring versatility, adaptability, a hands-on approach, and problem solving skills to the table. All of which cannot be underestimated in distant and high-risk environments. However, due to the complicated nature of crewed missions over extended time periods, many new technologies and engineering processes still need to be developed (Crawford, 2004; Rovetto, 2016).

One of the greatest challenges of long-distance space travel and the establishment of bases beyond Earth's orbit is the ability to provide food, water, and a breathable atmosphere for the crew in a stable and secure manner with a high reliability over time. It is estimated that the life support of a single crew member demands 1.83 kg of food and 2.50 kg of water per day (Anderson et al., 2018). Assuming a 3-year mission to Mars with a crew of four, a total payload of 25,287 kg would be needed for food and consumable water alone. From a logistics perspective, carrying out such a mission by relying on periodic resupply missions with spacecrafts is challenging due to the large payloads and great distances from Earth. Also, prices of cargo to space are costly, currently exceeding $10,000 per kg which makes such an approach cost-prohibitive (Clauwaert et al., 2017; Pickett et al., 2020). Hence, minimization of transport costs is another incentive to reduce the payload size of any space mission.

*In situ,* production of vital resources tackles some of the aforementioned challenges and has already been implemented in space stations, such as Mir and the international space station (ISS) in the form of life support systems. In those technologies, water and air managements play an essential role in recovery of oxygen and potable water. The remaining waste is stored and destroyed upon re-entry in Earth's atmosphere. On the ISS, the environmental control and life support systems (ECLSSs) are entirely based on physicochemical processes and are responsible for the production of potable water and oxygen with the help of waste stream recycling (Bagdigian et al., 2015; Volpin et al., 2020). As a result, the transport payload of water can be reduced by as much as 96.5% (Hendrickx et al., 2006; Clauwaert et al., 2017). However, the operation of the ECLSS requires a steady supply of consumables. Furthermore, nutrition has to be provided from terrestrial sources since it is not considered in current operative systems.

Bioregenerative life support systems (BLSS) are being developed as alternatives to existing fully physicochemical technologies. A BLSS aims at covering the metabolic needs of the crew by recovery of nutrients from waste streams in a closed-loop system through the combination of biological and physicochemical processes. For these systems, a minimal amount of consumables is needed, generated waste can be recycled, and food, water, and oxygen are produced. In most of the BLSSs, urine recycling plays an essential role in the recovery of water, but also in providing a nitrogen source for higher plant and/or edible bacteria biomass growth. With an average daily excretion of 7–16 g N per crew member, urine accounts for 85% of the total potentially recoverable nitrogen in a BLSS, mostly under the form of urea. This makes urine the main source of nitrogen in these systems. Different strategies are applied to provide a suitable form of nitrogen for plants and micro-algae to assimilate, either by directly combining urine with a nutrient stream or indirectly through production of an appropriate fertilizer. Plants and micro-algae are cultivated with these nutrient streams and serve as an important source of the required dietary protein intake for crew members (Paradiso et al., 2014; Ilgrande et al., 2019a). Although biological systems that recover nitrogen are already operative in terrestrial settings, the space environment subjects organisms to microgravity and increased ionizing radiation intensities. These factors may affect the way microorganisms may behave, ultimately affecting the performance and stability of a BLSS. This should be taken into consideration when putting forward the nitrogen recovery space technologies development roadmap.

In this review, we describe the current ECLSS on the ISS and its limitations and summarize different BLSS systems and their urine treatment strategy. We also provide an in-depth report on the nitrogen recovery strategy of the Micro-Ecological Life Support System Alternative (MELiSSA) and its challenges for space travel. MELiSSA is the BLSS strategy of the European Space Agency (ESA) and is based on the ecosystem of a lake. It is subdivided in five compartments, each representing a subsection of a (closed-loop) lake ecosystem. The program has been under development for over 30 years, making it the longest-running program out of all BLSS initiatives. Moreover, it was the first of its kind, approaching BLSS development from an engineering point of view. These two factors combined to make it one of the most advanced and promising concepts to support future exploration. Recent research on both improvements and developments of the MELiSSA nitrogen recovery technology as well as studies on the biological effects of real and simulated space conditions on nitrifying bacteria is highlighted here.

## CURRENT ECLSS ON THE INTERNATIONAL SPACE STATION

ISS's ECLSS is comprised of the water recovery system (WRS) and the oxygen generation system (OGS; **Figure 1**). Operational since 2007 and 2008, respectively, it integrates the different life support systems in different modules, whereas before this, each space agency provided its own life support system. The current ECLSSs have helped to meet the water and oxygen demands of astronauts through physicochemical processes (Bagdigian et al., 2015).

Oxygen is produced in two interconnected processes: (1) in the oxygen generation assembly (OGA), the water ($H_2O_{liquid}$) obtained from Earth supplies and (2) from the WRS, where $H_2O$ is electrolyzed to $H_2$ and $O_2$. The produced $H_2$ reacts with metabolic $CO_2$, originating from crew respiration and collected from cabin atmosphere, *via* the Sabatier reaction (eq. 1) in the $CO_2$ reduction system (CRS). In this process, $CH_4$ and $H_2O$ are generated (Greenwood et al., 2018).

$$CO_2 + 4\,H_2 \rightarrow CH_4 + 2\,H_2O \quad (1)$$

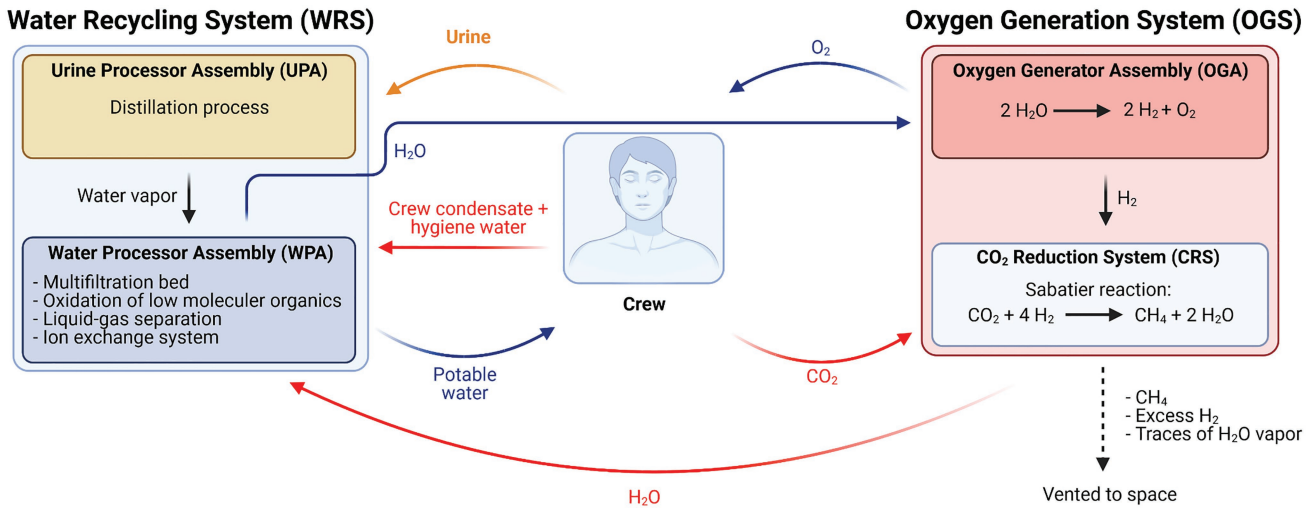


**FIGURE 1** | Simplified schematic overview of the Environmental Control and Life Support System (ECLSS) currently in use on the International Space Station (ISS; Adapted from Volpin et al., 2020).

Produced $H_2O$ is purified in the WRS, together with a water stream originating from processed urine from the Urine Processor Assembly (UPA), and returned to the OGA. $CH_4$ is vented to space (Greenwood et al., 2018).

## Water Recovery System

The WRS can be subdivided into two assemblies operating in concert with each other. The Water Processor Assembly (WPA) collects condensate from the cabin atmosphere originating from crew perspiration and respiration, water from the OGS produced during the Sabatier reaction, and distillate from the second module, the UPA. The UPA's position in the ECLSS is shown in **Figure 1**. In **Figure 2**, a schematic overview of the UPA module is provided (Bagdigian et al., 2015; Volpin et al., 2020).

In total, an average of 1.80 l of urine (4–9 g N/L $d^{-1}$) and urine flush water per crew member per day is collected in the Wastewater Storage Tank Assembly (WSTA). Here, the urine load is kept sterile and is chemically stabilized to avoid scaling (precipitation of solid minerals in urine collection systems) by mixing it with an $H_3PO_4$ and $Cr^{6+}$ solution. Originally, $H_2SO_4$ played the role of $H_3PO_4$ in the system, acidifying urine and converting the volatile ammonia ($NH_3$) originating from the hydrolysis of urea to non-volatile ammonium ($NH_4^+$). However, astronaut's urine contains higher amounts of $Ca^{2+}$, which can precipitate as $CaSO_4$ and cause scaling and pipeline clogging which eventually leads to system failure. This issue was initially addressed by reducing UPA water recovery from 85 to 75%, which prevented the $CaSO_4$ concentration from reaching its solubility limit in water. The introduction of $H_3PO_4$ to acidify stored urine significantly reduced SO42-content and hence scaling potential. This allowed the increase of water recovery efficiency up to 85% again. $Cr^{6+}$ acts as an oxidizing agent, preventing urea hydrolysis and thus avoiding $NH_4^+$ formation (Muirhead et al., 2018; Volpin et al., 2020). Chemically stabilized urine is pumped from the WSTA to the distillation assembly (DA) after enough urine has been collected in the storage tank (Bagdigian et al., 2015; Anderson et al., 2018; Volpin et al., 2020).

In the DA, water is separated from waste products through evaporation, leaving a concentrated waste mixture behind. This waste stream passes through the Advanced Recycle Filter Tank Assembly (ARFTA) where concentrated brine is collected with a series of membrane filters (Link et al., 2010; Bagdigian et al., 2015; Volpin et al., 2020). A downstream brine filter removes leftover solid precipitates formed during the process (Link et al., 2010). After every cycle, the ARFTA becomes saturated and is emptied and the removed concentrated brine is stored until eventual disposal. The resulting filtered waste stream is then returned to the DA for a new distillation cycle. Finally, the produced water vapor from the distillation process is separated from purge gases before passing to the WPA (Bagdigian et al., 2015; Volpin et al., 2020). In the WPA, water undergoes the final purification steps. Here, contaminants are removed in a four-step process: (1) removal of inorganics and non-volatile organic compounds in an ion-exchange resin multifiltration bed, (2) oxidation of low molecular organics with oxygen at 130°C, (3) liquid–gas separation of oxygen and other leftover gaseous by-products, and (4) removal of carbonate and bicarbonate ions through an ion-exchange system (Volpin et al., 2020).

## Limitations of the Current ECLSS

Although the current ECLSS is suitable for space missions in Low Earth Orbit (LEO), there are several limitations that need to be addressed to enable its use in long-term space missions. In order to make long-distance space exploration feasible, a life support system should recover more than 98% of water and nutrients from waste streams and produce food to meet the human metabolic needs, with minimal use of additional resources (Pickett et al., 2020). The UPA operating efficiency

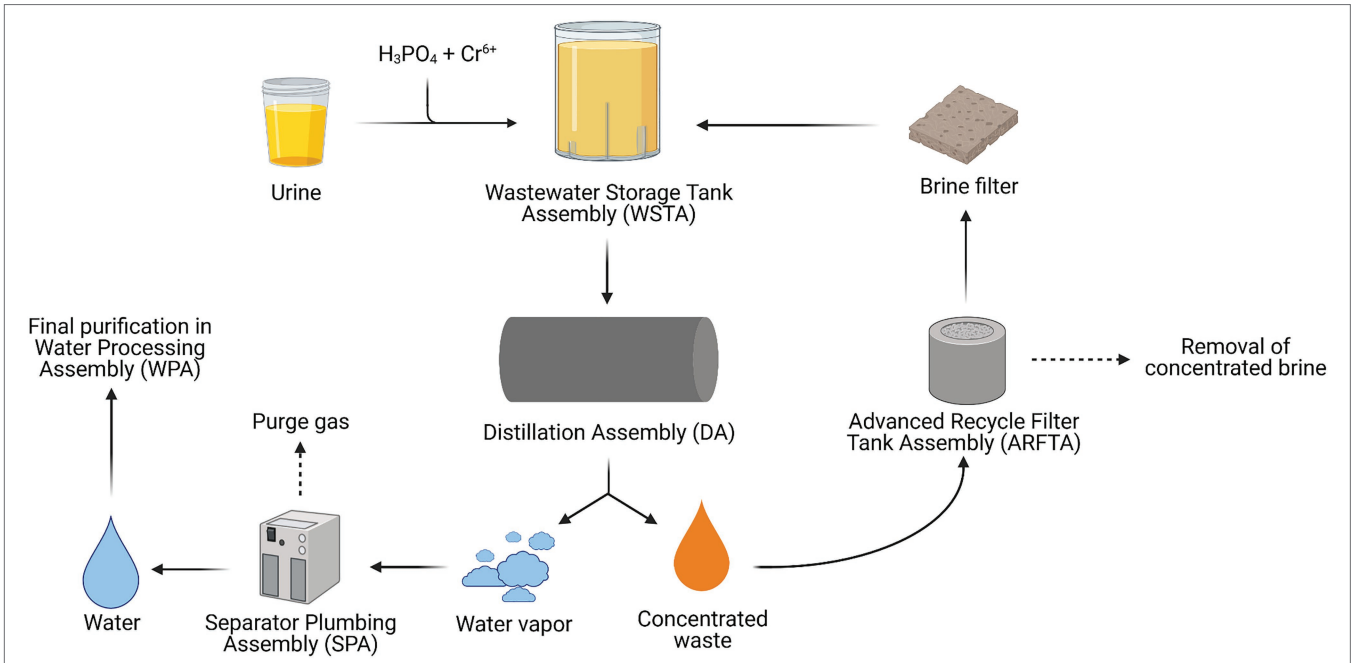


**FIGURE 2 |** Schematic overview of the Urine Processor Assembly (UPA) module of the Water Recycling System (WRS) operating on the International Space Station (ISS; Adapted from Bagdigian et al., 2015).

of only 85% results in a significant loss of available $H_2O$. In addition, not all water vapor that can be used for oxygen production is condensed out of the $CH_4$ waste stream in the OGS, thereby venting valuable water to space. The combination of the $H_2$ lost under the form of $CH_4$ (as H-atoms) and the loss of uncondensed $H_2O$ produced in the Sabatier reaction results in only 50% of $O_2$ recovery produced with the Sabatier reaction from metabolic $CO_2$ (Greenwood et al., 2018). Consequently, though designed for high reliability, the current ECLSS does not meet the necessary requirements for long-distance space travel. Water resupply from Earth is necessary to enable system operation. Moreover, 0.21 kg of disposable hardware (saturated filters, malfunctioning hardware, etc.) is consumed per 1 l of potable water produced, which need to be replaced during resupply (Volpin et al., 2020). In conclusion, while payload size and storage requirements on the ISS have been vastly reduced by the use of the ECLSS, they are still considerable and should be minimized further. Moreover, energy requirements to employ ECLSS modules are substantial (Jones et al., 2014). Hence, technological improvements (e.g., higher water recovery efficiencies, nutrient recovery, and higher system reliability) or a switch to new innovations, such as a BLSS, is required (Jones, 2016). The latter would allow tapping on waste streams rich in valuable compounds (N, P, and K), such as brine produced from urine during water purification and solid wastes, both of which are currently stored to be incinerated upon re-entry into Earth's atmosphere. These compounds could be alternatively used for the production of fertilizer for food production (Volpin et al., 2020). It would help to (partially) solve the inability of the current system to produce food and reduce the need for frequent supply missions to space.

## BIOREGENERATIVE LIFE SUPPORT SYSTEMS

Bioregenerative life support systems are able to convert inorganic and organic waste into food, water, and oxygen through the combination of biological and physicochemical processes. *In situ,* production of a balanced diet for crew members during a long-distance space mission is currently only possible when incorporating biotechnological aspects into life support systems. Several projects were developed or are still in development at different major space organizations, and an overview of these projects is provided in the following paragraph. In the scope of this review, the unique urine processing systems of each of these technologies are emphasized (**Table 1**).

### Bioregenerative Life Support Systems in History

Russia spearheaded the development of BLSSs with the development of the Hybrid Biosphere System (BIOS) program, which was a large scale confined analogue environment manned by humans. It used microalgae and higher plants to meet a person's oxygen demand (BIOS-1 and BIOS-2) and later expanded to also include plant-based food production (BIOS-3). Here, unprocessed human urine (containing urea) was mixed directly with nutrient solutions to successfully cultivate higher plants hydroponically (Salisbury et al., 1997; Guo et al., 2017). Other similar projects are the Biosphere 2 project of NASA, the Closed Ecological Experiment Facility (CEEF) of the Japan Aerospace Exploration Agency (JAXA), and the Lunar Palace 1 program of the National Natural Science Foundation of China. These

**TABLE 1** | Overview of different BLSS systems.

| BLSS | Organization | Urine and nitrogen processing system | Bacterial consortium | Advantages | Disadvantages | Space experiments | Nitrogen recovery efficiency | References |
|---|---|---|---|---|---|---|---|---|
| BIOS-3 | Institute of Biophysics of Siberian Branch of the Russian Academy of Sciences | Unprocessed urine mixed with a nutrient solution fed to higher plants | None | Simplicity of technology | Accumulation of NaCl in the inedible biomass of plants; nitrogen demands of cultivated wheat plants are not entirely met with urine alone | None | N/A | Lisovsky et al. (1997) and Salisbury et al. (1997) |
| Biosphere 2 | Biosphere 2 | Marsh biome system | Natural bacteria in Marsh biome | N/A | N/A | None | N/A | Guo et al. (2017) |
| Closed Ecological Experiment Facility (CEEF) | JAXA | Waste incineration | None | $CO_2$ production for crop growth | High energy consumption; high temperatures for incineration; highly oxygen consuming process | None | N/A | Ashida and Nitta (1995) and Tako et al. (2017) |
| Lunar Palace 1 | National Natural Science Foundation of China | Reduced-pressure distillation followed by treatment in a membrane aerated, activated carbon bioreactor | Populated naturally by microorganisms from Lunar Palace environment & wastewater | Potentially high degree of adaptability to space conditions | Loss of urea-nitrogen during distillation process | None | 20.5 % | Xie et al. (2017) |
| Lunar Palace: In development | | Aerobic membrane bioreactor and anaerobic membrane bioreactor | Sludge from municipal wastewater treatment plants | Potentially high degree of adaptability to space conditions; ability to recover nitrogen from urea | High energy costs | None | 80 - 99 % | Cheng et al. (2019) |
| Closed Equilibrated Biological Aquatic System (C.E.B.A.S.) | DLR | Ammonia oxidizing biofilter | Bacteria of the *Nitrosomonas* and *Nitrobacter* genera | N/A | N/A | C.E.B.A.S MINI MODULE | N/A | Blüm et al. (2003) |
| Combined Regenerative Organic food Production (C.R.O.P.) | DLR | Biofiltration process (biological trickle filter) | Natural community of soil microorganisms including *Nitrosomonas* and *Nitrobacter* | Low energy consumption; easy handling; low maintenance; high degree of adaptability to space conditions; low space occupancy requirements | Depends on convection and sedimentation forces; adaption required to membrane aerated flow filters/artificial gravity system for space | Eu:CROPIS (malfunctioned) | 66 - 87 % | Bornemann et al. (2015, 2018) and Hauslage et al. (2018) |
| Micro-Ecological Life Support System Alternative (MELiSSA) | ESA | Fixed-bed bioreactor | Defined nitrifying community of *N. europaea* and *N. winogradskyi* | Easier to characterize and model; acceptable degree of robustness to space conditions | Less robust than more diverse bacterial communities; requires pretreatment of organic urine compounds | BISTRO; NITRIMEL | 50 - 100 % | Perez et al. (2004, 2005) and Cruvellier et al. (2017) |
| MELiSSA: In development | | To be determined, currently tested in CSTR | Synthetic community with a urease-positive heterotrophic strain, *N. europaea*, and *N. winogradskyi* | Easier to characterize and model; ability to recover nitrogen from urea in synthetic and fresh real urine; acceptable degree of robustness to space conditions | Less robust than more diverse bacterial communities; Oxygen competitiveness between constituents remains challenging | URINIS A1 and A2 (to fly) | 35 - 94 % | Christiaens et al. (2019a) and Ilgrande et al. (2019a) |

facilities were built with a focus on *in situ* cultivation of higher plants and/or animals for future Lunar or Martian bases (Ashida and Nitta, 1995; Guo et al., 2017; Xie et al., 2017). The Biosphere 2 consists of a 1.27 ha structure of different biomes (marshland, rainforest, savannah, etc.). In the 1990s, it was used to study artificial closed ecology systems with a human crew. Human urine was deposited in the marshland biotope, where it was digested through natural processes and bioavailable nitrogen could be redirected toward the remaining biomes (Nelson et al., 1999; Guo et al., 2017). In the CEEF, JAXA chose to incinerate human fecal material, urine, and inedible crop waste in an effort to recover $CO_2$ for crop production. No nitrogen recovery system was put in place (Tako et al., 2017). More recently, China completed a 106-day terrestrial manned mission in the Lunar Palace 1 facility. A BLSS was successfully used to support a crew of four with minimal external input of food, water, and oxygen for the duration of the trial (Xie et al., 2017; Cheng et al., 2019). During the experiment, nitrogen was recovered from urine with an efficiency of 20.5% through reduced-pressure distillation, where ammonia was distilled together with the water vapor. The distillate from this process was combined with kitchen and sanitary wastewater and processed in a membrane bioreactor followed by activated carbon treatment. The former is populated by bacteria (including nitrifying strains) originating from the Lunar Palace environment and wastewater stream (Xie et al., 2017). It is worth mentioning that this system design was mainly focused on pollutant removal rather than optimization of nutrient recovery. In the second phase of Lunar Palace, an anaerobic and aerobic membrane bioreactor will replace the current setup. In preliminary experiments preceding a new manned mission in Lunar Palace, successful ureolysis has been observed in the anaerobic reactor, while the ammonium removal efficiency in the aerobic reactor sits between 80 and 99% during stable operation (Cheng et al., 2019).

The German Aerospace Center (DLR) initiated two smaller scale BLSS research projects. In the 1990s and the 2000s, the Closed Equilibrated Biological Aquatic System (CEBAS) combined aquatic vertebrates and invertebrates with microalgae, aquatic plants and nitrifying bacteria in a closed-loop ecosystem. An ammonia oxidizing biofilter, which contains bacteria from the *Nitrosomonas* and *Nitrobacter* genera, processed the excreted ammonium from organisms in the aquatic animal tank (Blüm et al., 2003). The second, more recent Combined Regenerative Organic-food Production (C.R.O.P) project utilizes a biofiltration process with a natural bacterial community (including *Nitrosomonas* and *Nitrobacter*) that populates a trickle filter. In this setup, nitrogen can be recovered from synthetic human urine that carries ammonium as N-source. The microalgae species *Euglena gracilis* provides oxygen necessary for nitrification activity in this setup. Tomato plants are cultivated using the resulting nutrient stream from the trickle filter. Depending on the dilution of a synthetic urine matrix, a 66–87% nitrogen recovery efficiency was realized. Here, an undiluted feed negatively impacted the nitrate production rates, while 60 and 80% diluted urine seemed to be within the optimal range for nitrogen recovery (Bornemann et al., 2015, 2018; Hauslage et al., 2018).

## The Micro-Ecological Life Support System Alternative

European Space Agency initiated the ambitious MELiSSA program in the late 1980s. It is currently the longest-running BLSS program to date. Based on a lake ecosystem, MELiSSA aims to develop a closed-loop bioregenerative system using an engineering-based approach. Five interdependent compartments utilize a variety of specific microorganisms or higher plants to produce oxygen, water, and food from human metabolic waste (Hendrickx et al., 2006; Lasseur et al., 2010). In contrast to the previous described projects and facilities that focused on the development of a large analogue human test facility, the MELiSSA Pilot Plant, a testbed facility of the MELiSSA loop, is designed to sustain the respiration needs of a single person while also providing 20–40% of necessary nutrition (Alemany et al., 2019). Hence, it is not designed to be a closed full-scale testing facility that can fully support a complete crew. The plant has been operative since 2009. The MELiSSA program is the oldest and most advanced circular BLSS currently in development. At the same time, it serves as a pioneer for the terrestrial circular economy as it provides state-of-the-art technology that can also be applied on Earth (Paladini et al., 2021).

In the MELiSSA context, astronauts act as consumers, microorganisms are the recyclers, and plants and cyanobacteria close the loop as the producers (**Figure 3**; Lasseur et al., 1996; Hendrickx et al., 2006). In compartment I (CI), a consortium of bacteria digest/liquefy fecal and solid wastes and urine mainly into $CO_2$, volatile fatty acids (VFAs), and minerals, including free $NH_4^+$. Bioreactor conditions are thermophilic and anaerobic to prevent possible pathogen proliferation and methanogenesis, respectively. Methane is a potentially hazardous and flammable biogas and hence it should be avoided on space vessels. Moreover, its potential use as a biogas fuel results in loss of carbon from the loop while the biological recovery of carbon from methane implies the introduction of two more bioreactors in the already highly complicated loop (Hendrickx et al., 2006; Hendrickx and Mergeay, 2007). Furthermore, carbon sources will also be lost as methane and recovering carbon from methane implicates two additional compartments, overcomplicating the MELiSSA design (Hendrickx et al., 2006). VFAs and minerals are further processed in Compartment II (CII) by the photoheterotrophic bacterium *Rhodospirillum rubrum* to produce biomass and additional $NH_4^+$ (Hendrickx et al., 2006; Mastroleo et al., 2009). Produced biomass could potentially serve as an additional nutrition source, while $NH_4^+$ is diverted to the third, nitrifying compartment (CIII; Hendrickx et al., 2006; Mastroleo et al., 2013). Moreover, removal of VFAs from the waste stream is necessary to ensure proper functioning of CIII, since its presence negatively affects nitrite oxidation in the dedicated compartment (Oguz et al., 2006; Mastroleo et al., 2013). Nitrogen availability is crucial for cyanobacteria (*Limnospira indica*) and higher plant growth in compartment CIVa and CIVb, respectively (Paradiso et al., 2014; Clauwaert et al., 2017; Alemany et al., 2019; Poughon et al., 2020). As such, to ensure optimal food production, an efficient nitrogen recovery system is indispensable. Although nitrogen fixation from urea by plants is possible, studies have

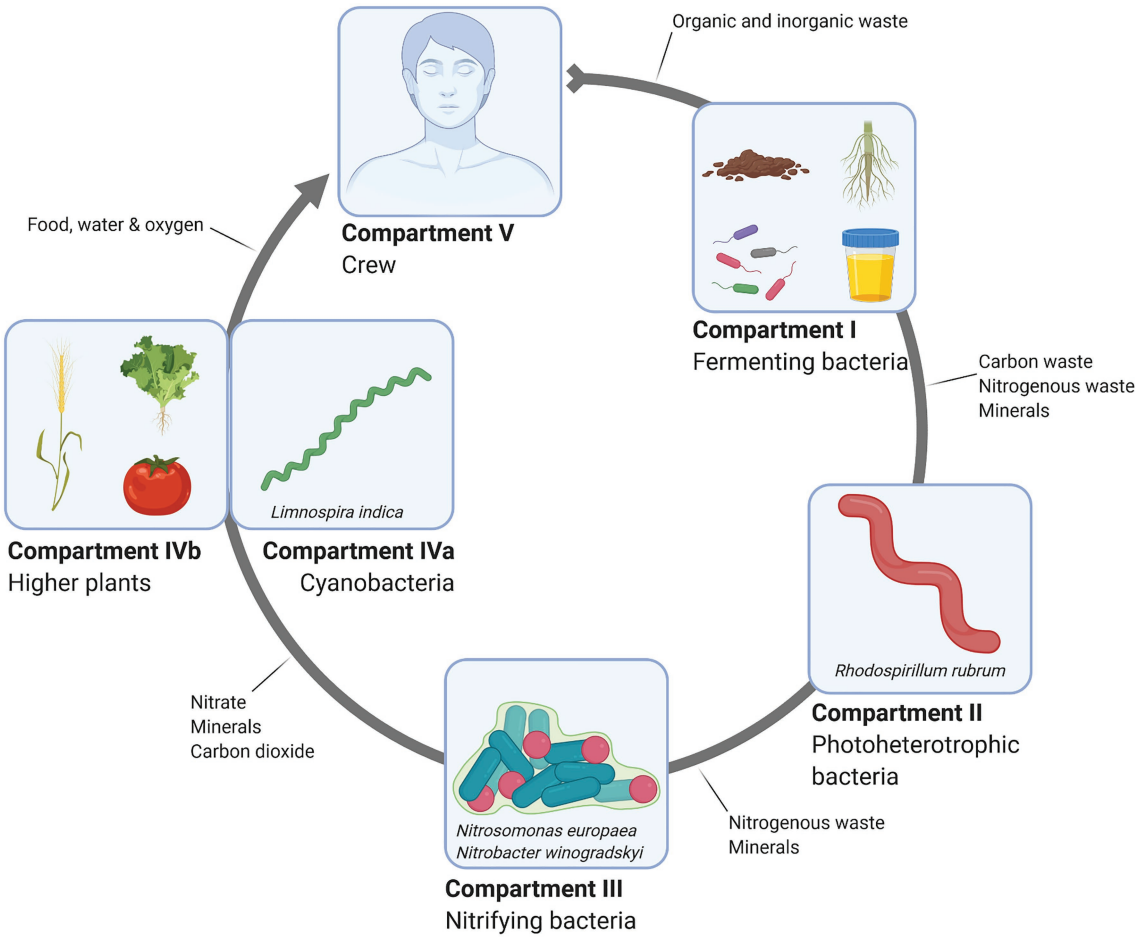


**FIGURE 3 |** Schematic overview of the MELiSSA loop.

shown that it is ineffective as plant fertilizer (Ilgrande et al., 2019a). Plants prefer to assimilate nitrogen from inorganic $NH_4^+$ and NO3-molecules while *L. indica* prefers NO3-over $NH_4^+$ and urea (Clauwaert et al., 2017; Ilgrande et al., 2019a). Therefore, in MELiSSA, urea is biologically converted to $NH_4^+$ and $NO_3^-$. In the context of a BLSS for space applications, NO3-is especially preferred due to the fact that $NH_4^+$ can easily convert to the volatile $NH_3$, which is toxic at high concentrations in atmosphere. It can be harmful to the astronaut's health if leaked into a closed environment, such as the ISS or any other spacecraft (Ilgrande et al., 2019a). Finally, $NH_4^+$ is also detrimental for plant growth at high concentrations (Paradiso et al., 2014; Ilgrande et al., 2019a). In CIII, $NH_4^+$ is oxidized to nitrate in a two-step process using $O_2$ as electron acceptor. *Nitrosomonas europaea* is an ammonium-oxidizing bacterium that converts ammonium to nitrite, which is subsequently consumed by the nitrite oxidizing bacterium (NOB) *Nitrobacter winogradskyi*. Here, nitrogen is effectively converted to the form preferred by plants or cyanobacteria. It serves as a fertilizer in the subsequent biomass-and oxygen-producing and $CO_2$-consuming compartment IV (Farges et al., 2012; Clauwaert et al., 2017; Ilgrande et al., 2019b). The final compartment V is the crew, who is consuming produced biomass and $O_2$ on the one hand, and producing $CO_2$, urine and fecal waste on the other hand. These waste products enter CI, closing the MELiSSA-loop.

In the MPP, bioreactors are operating and being characterized both individually and in an interconnected setup (Albiol et al., 2000; Gòdia et al., 2004). An important objective is to design associated technology that facilitates a continuous process that is robust and that runs as optimally as possible, as well as to develop mathematical models based on experimental data obtained from real operating conditions (Albiol et al., 2000).

## NITROGEN RECOVERY IN CIII OF MELISSA

Traditionally, nitrifiers are responsible for ammonia nitrogen removal in wastewater treatment plants (Poughon et al., 2001; Farges et al., 2012). Autotrophic nitrifying bacteria have been proven to nitrify ammonium to nitrate in wastewater at rates which are orders of magnitude higher than those of heterotrophic bacteria (Perez et al., 2004). For nitrification of ammonium to nitrate, the MELiSSA system uses a synthetic community

of two axenic autotrophic strains, i.e., *Nitrosomonas europaea* ATCC 19718 and *Nitrobacter winogradskyi* ATCC 25391, instead of a mixed undefined community from wastewater sludge (Cruvellier et al., 2016). An axenic co-culture does not perform significantly better than its open culture counterparts in a bioreactor (Zeghal et al., 1994), but it is easier to study, characterize, and model (Christiaens et al., 2019a). *N. winogradskyi* is the NOB considered in the MELISSA CIII because out of all NOB strain candidates studied, it proved to be the least affected by high concentrations of both ammonium and nitrite. This is a critical point in a system like CIII where ammonium load and, consequently, nitrite load can temporarily fluctuate to higher levels (Cruvellier et al., 2016). Biological activity at high efficiencies in highly saline environments is another prerequisite for nitrogen recovery from urine, where salinity can rise to 45–75 mS $cm^{-1}$ in nitrified undiluted urine from 20 mS $cm^{-1}$ in fresh urine (Christiaens et al., 2019a). A co-culture of *N. europaea* and *N. winogradskyi* was proven to tolerate salinities of up to 45 mS $cm^{-1}$ in a synthetic urine matrix, working at nitrification efficiencies of 90–94% in a continuously stirred tank reactor (CSTR). The use of this co-culture could allow urine nitrification with limited dilution (Christiaens et al., 2019a).

## Nitrate Production With Nitrifying Bacteria

The metabolic pathway of nitrification in *N. europaea* and *N. winogradskyi* is shown in **Figure 4**. Full nitrification is achieved by the nitrifiers in the following reactions (Cruvellier et al., 2016; Caranto and Lancaster, 2018):

$$NH_4^+ + 1.5\,O_2 \rightarrow NO_2^- + 2\,H^+ + H_2O \quad (2)$$

$$NO_2^- + 0.5\,O_2 \rightarrow NO_3^- \quad (3)$$

The formation of nitrite from ammonium in *N. europaea* is called nitritation (1), and it is achieved in several enzymatic reactions. In the first step, ammonia is converted into hydroxylamine ($NH_2OH$) by the membrane-bound ammonia monooxygenase (AMO). The next step involves hydroxylamine oxidoreductase (HAO), which was originally hypothesized to catalyze the oxidation of $NH_2OH$ to $NO_2$- with $O_2$ acting as the electron acceptor (Chain et al., 2003). Recently, however, nitric oxide (NO) has been demonstrated to be the enzymatic product of HAO instead of $NO_2^-$. Meanwhile, the production of $NO_2$-from NO is probably catalyzed by a third, unidentified enzyme in the nitritation (Coleman and Lancaster, 2020). The presence of such an enzyme is necessary to outcompete the side reactions that can produce by-products, such as NO3-and/or $N_2O$, but also to prevent the spontaneous formation of $NO_2^-$. The latter is required since non-enzymatic oxidation implies a loss of electrons, which are captured during the enzymatic reaction (Caranto and Lancaster, 2018). Finally, $NO_2$-is oxidized to NO3-by the membrane-bound nitrite oxidoreductase (NXR) of *N. winogradskyi* during the so-called nitratation (2), also using $O_2$ as an electron acceptor (Starkenburg et al., 2006; Cruvellier et al., 2016; Caranto and Lancaster, 2018; Coleman and Lancaster, 2020).

These nitritation and nitratation processes only take place in aerobic conditions since they are highly dependent on the oxidative power of $O_2$. An anaerobic environment imposes a lack of oxygen on the cells for the nitrification reaction. In those situations, NO3-can be reduced to $NO_2$-by *N. winogradskyi* using the NXR enzyme, which now acts as a reductase instead of an oxygenase under these circumstances (Freitag et al., 1987). *N. europaea* can further convert $NO_2$-into gaseous $N_2O$, NO, and $N_2$ (Schmidt et al., 2004). This so-called nitrifier denitrification leads to nitrogen loss and production of undesired gasses. Following the requirements of an efficient and safe BLSS, denitrification should be strongly avoided.

## Characterization of the Fixed-Bed Bioreactor of the MELiSSA CIII

The nitrification reactor in the MPP is a fixed-bed bioreactor of 8.1 l that houses a nitrifying co-culture of *N. europaea* and *N. winogradskyi*. It operates at a pH of 8.1 at 28 ± 0.1°C and is magnetically stirred at the inlet to enable mixing of the bioreactor content (Montras et al., 2008). Due to the autotrophs' characteristic low biomass growth and high conversion yield, the co-culture is grown as a biofilm immobilized on 4 mm diameter polystyrene beads to avoid washout (Zeghal et al., 1994; Perez et al., 2004; Montras et al., 2008). Growth on biofilms allows decoupling of the liquid residence time in the reactor from the biomass retention time (critical for slow-growing organisms, such as nitrifiers), although substrate diffusion through biofilms can result in lower maximal conversion rates (Perez et al., 2004).

During an extended period of continuous operation (4.8 years), nitrogen conversions in the order of 95–100% efficiency were reported in the MPP CIII, at ammonium loading rates of up to 1.35 kg N $m^{-3}$ $d^{-1}$ (Gòdia et al., 2002; Perez et al., 2004; Montras et al., 2008). Despite the observed long-term stable operation of the bioreactor, it remains important to characterize the effects of certain perturbations on the process stability in the reactor. As mentioned, a critical parameter that influences nitrification efficiency is the dissolved oxygen (DO) availability in the reactor. Depending on how extensive oxygen limitation is, different steps in the nitrification process are affected (Gòdia et al., 2002). Hence, experiments were performed to quantify the bulk DO level needed in the system to ensure full nitrification (Zeghal et al., 1994; Gòdia et al., 2002; Perez et al., 2004). In a two-step decrease of DO concentration from 80 to 40% and 40 to 20%, ammonium was not completely converted to nitrite but all nitrite was oxidized to nitrate in the first step DO concentration decrease (80 to 40%). This indicates that DO concentration in this range is limiting for *N. europaea*, but not for *N. winogradskyi* (Gòdia et al., 2002). In the second DO decrease step (40 to 20%), however, ammonium was fully oxidized after an adaption period but nitrite conversion was incomplete. Here, *N. europaea* regained the upper hand in competition for oxygen with its co-culture counterpart and dominated the co-culture. These results showed that at 80%

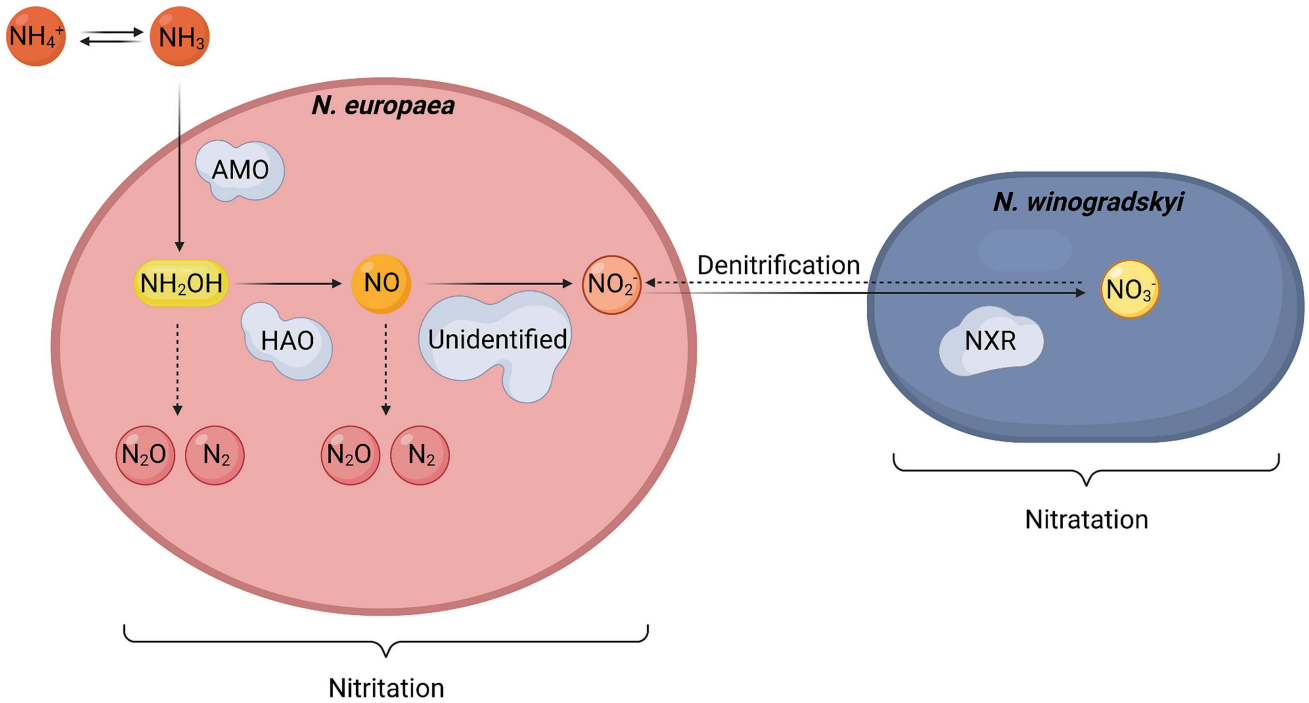


**FIGURE 4 |** Simplified schematic representation of the nitrification pathway in an axenic co-culture of *Nitrosomonas europaea* and *Nitrobacter winogradskyi*. Full lines represent nitrification in aerobic conditions. Dashed lines represent the denitrification pathway in anaerobic conditions. Abbreviations: AMO: ammonia monooxygenase, HAO: hydroxylamine oxidoreductase, NXR: nitrite oxidoreductase.

DO concentration, full nitrification was achieved while lower DO concentrations caused partial nitrification. It is thus essential to monitor DO in the bioreactor in order to control the nitrification process both in steady state and in dynamic situations (Gòdia et al., 2002).

Aside from high nitrification efficiency, another requirement for MELiSSA is stringent controllability of the compartments. MELISSA considers three control levels to ensure controllability (and thus stability) of the complex biological system: (1) real-time monitoring and control of predetermined parameters in each compartment (such as pH, temperature, and dissolved oxygen (DO) in the CIII), (2) implementation of a control law for each bioreactor chamber, and (3) an intercompartment coordination system for the loop (Gòdia et al., 2004). Specifically for the CIII compartment, several mathematical models have been constructed and validated to simulate and control the MPP CIII bioreactor in real-time. For instance, a hydrodynamic model able to predict the N-species response to ammonium load disturbances was developed, hence enabling the user to keep nitrite accumulation to a minimum (Perez et al., 2005). This is required due to the latter compound's toxic properties for both plants and humans. Using the model, one can simulate the N-species ($NH_4^+$, $NO_2^-$, and $NO_3^-$) concentrations and biomass concentration in the fixed-bed bioreactor. The model serves as a valuable tool to maintain stable operation in CIII by identifying boundary conditions and operating the bioreactor within these boundaries (Perez et al., 2005). Beyond that, the heterogeneous distribution of the co-culture species was characterized along the length of the bioreactor after 4.8 years of operation and this information was used to expand on the previous model to predict population dynamics and nitrification efficiency, while also considering diffusion of nutrients into the biofilm (Montras et al., 2008). Finally, Cruvellier et al. was able to determine that online measurements of base addition to counteracting nitritation-driven acidification (1) and online measurements of oxygen consumption can be used as predictive variables for partial nitrification (Cruvellier et al., 2016).

## FUTURE PERSPECTIVES FOR NITROGEN RECOVERY

### Urea Hydrolysis: The Addition of Ureolytic Bacteria to the Nitrification Bioreactor

Nitrogen recovery from ammonium oxidation has been well characterized in ammonium-containing medium in the MELISSA CIII compartment at the MPP (Gòdia et al., 2004, 2004; Perez et al., 2015; Cruvellier et al., 2016, 2017). However, CIII cannot be used to directly process urine at present. Urine currently enters CI together with other waste products (Ilgrande et al., 2019b). Urea can be converted to ammonium by ureolytic bacteria through the action of the hydrolytic urease enzyme (Defoirdt et al., 2017; Ilgrande et al., 2019a). Urease catalyzes the conversion of urea into ammonium and carbamic acid. The latter then spontaneously hydrolyzes into a second ammonium molecule and carbonic acid, resulting in two

ammonium molecules from one urea molecule and an increase in pH due to bicarbonate ($HCO_3^-$) formation (Defoirdt et al., 2017; De Paepe et al., 2020b).

$$CO(NH_2)_2 + 3\,H_2O \rightarrow 2\,NH_4^+ + HCO_3^- + OH^- \quad (4)$$

Full urine hydrolysis can take more than a month when relying on the spontaneous action of urease-positive bacteria populating urine pipelines and storage equipment. Storing urine for these extended periods of time is impractical for space applications (Defoirdt et al., 2017). By including an ureolytic heterotrophic bacterium directly in the nitrifying community in CIII, urea can be converted into ammonium (Defoirdt et al., 2017; Christiaens et al., 2019a; Ilgrande et al., 2019a). Low concentration organic compounds (~10 g COD (Chemical Oxygen Demand) $L^{-1}$) present in urine should also be removed from urine by the heterotroph to provide a fertilizer free of CODs, reducing the risk of biofouling of the nutrient solution for CIV (Christiaens et al., 2019a; Ilgrande et al., 2019a; De Paepe et al., 2020a).

Currently, research on adding one (or multiple) ureolytic strain(s) to the nitrifying co-culture in the CIII compartment is ongoing. The combination of a heterotroph with nitrifying bacteria in a defined community has not been demonstrated before, as opposed to wastewater treatment with mixed microbial communities. In the same study that demonstrated halotolerance of the nitrifying co-culture by Christiaens and co-workers, the co-culture was combined with three heterotrophic ureolytic strains (*Pseudomonas fluorescens* DSMZ 50090, *Acidovorax delafieldii* DSMZ 64, and *Delftia acidovorans* DSMZ 14801). Complete ureolysis and nitrification were achieved at similar efficiencies as a nitrifying co-culture (107 ± 8% and 94 ± 8%, respectively) when treating a 5% fresh real urine feed. When using a 10% urine influent, ureolysis and nitrification efficiencies significantly dropped to 66 ± 9% and 35 ± 3%, respectively. In another study by Ilgrande and coworkers, five ureolytic heterotrophic strains (*A. delafieldii, Comamonas testosteroni* I2, *Cupriavidus necator* DSMZ 13513, *D. acidovorans, P. fluorescens, Vibrio campbellii* LMG 22895) were also combined individually with the nitrifying co-culture. *C. necator* and *V. campbellii* containing communities did not show active nitrification of $NH_3$. Meanwhile, ammonia oxidation rates in consortia with *P. fluorescens* and *C. testosteroni* were twice as high as those observed in *A. delafieldii* or *D. acidovorans* communities, indicating a beneficial interaction between the heterotroph and *N. europaea* (Ilgrande et al., 2019a). Moreover, in axenic conditions, *C. testosteroni, V. campbellii, and P. fluorescens* were proven to hydrolyze urea present in low concentrations, while the other strains (*A. delafieldii, C. necator,* and *D. acidovorans*) did not exhibit ureolytic activity here (Ilgrande et al., 2019a). Hence, the three former strains are favored over the latter three strains for possible inclusion in the nitrifying community since urea consumption will be more complete. Halotolerance of those ureolytic heterotrophs was also assessed and no ureolytic activity was observed for the selected species in high salinity (> 30 mS $cm^{-1}$) conditions in an artificial urine matrix. However, ureolytic activity at lower salinities was not inhibited, implying the need for dilution of urine before feeding it to the bioreactor (Ilgrande et al., 2019a). Finally, organics removal was limited with all influent urine concentrations and has to be improved before space applications are possible (Christiaens et al., 2019a). Nonetheless, the inclusion of an ureolytic step in a nitrifying co-culture already shows successful ureolysis and nitrification, which is encouraging to the implementation of direct urine treatment in CIII.

## Stabilization of Fresh Urine for Long-Term Storage

Due to storage limitations in space, urine accumulation in a tank for extended periods of time is discouraged. However, storage cannot be avoided because urine is produced discontinuously over the course of a day. Unfortunately, untreated urine is highly unstable. As show in eq. 4, urea hydrolysis by urease-positive bacteria in the urine collection and processing system results in ammonium formation and a pH increase, causing ammonia ($NH_3$) volatilization. This impacts nitrogen recovery and can have detrimental effects on crew members health. Moreover, salt precipitation due to high pH levels causing scale formation and clogging of the urine processing system (Coppens et al., 2016; De Paepe et al., 2020a,b). The toxic compounds ($H_3PO_4$ and $Cr^{6+}$) that are used currently in the UPA system in ISS to stabilize stored urine can be replaced by a safer, electrochemical method (De Paepe et al., 2020b). Recirculation over a cathode in a three-compartment electrochemical cell is a potential alternative. Here, hydroxyl ($OH^-$) ions are created from $H_2O$ and the pH is increased to 10–11 (due to hydroxyl-ion production), inhibiting enzymatic urease activity in a non-toxic manner (De Paepe et al., 2020b). Meanwhile, calcium and magnesium concentrations are reduced due to precipitation caused by supersaturation. This mineral removal process in the electrochemical cell also minimizes downstream clogging of the system (De Paepe et al., 2020b).

## Ureolysis and Nitrification in Space Conditions

A key hurdle for nitrification in space applications is the diffusion of oxygen into the nitrifying culture due to reduced gravity conditions on Mars and microgravity conditions in LEO and deep space. Fluid dynamics are restricted to diffusion due to the absence of convection forces in these scenarios, causing limited oxygen distribution (Monti, 2001; Ilgrande et al., 2019a; De Paepe et al., 2020a; Acres et al., 2021). One possible way to circumvent this problem is the use of a membrane-aerated biofilm reactor (MABR) in which aeration takes place *via* gas-permeable membrane tubes populated by a biofilm of nitrifiers on the outer surface of the membranes. Since microorganisms directly take up the oxygen that diffuses through the membrane, no air bubbles are formed. However, bacteria closest to the gas-permeable membrane will consume most oxygen. This could be an issue if fast-growing ureolytic heterotrophs are present in the system, potentially limiting oxygen availability for nitrifiers on the outer layers of the

biofilm. Additionally, anoxic areas may lead to denitrification by the autotrophic nitrifiers or by heterotrophic denitrifiers (using available COD) if an open community is used in the MABR. These denitrification reactions result in the loss of nitrogen as $N_2$. COD removal with an ureolytic heterotroph in the reactor to minimize denitrification is too low. One option to tackle that issue while also preventing oxygen limitation for autotrophic nitrifiers is the addition of an upstream microbial electrolysis cell (MEC) populated by a ureolytic strain. The bacteria in the MEC remove organics efficiently using an anode as electron acceptor (**Figure 5**). Here, energy from COD is also partially converted to hydrogen gas. Ultimately, a low COD, nitrate-rich effluent suitable for CIV cyanobacteria and plant growth is obtained after treatment in a MEC-MABR setup (De Paepe et al., 2020a). The combination of physicochemical and biological treatment of urine optimizes nitrogen recovery at minimal energy input and high conversion efficiency while avoiding the use of toxic compounds for urine storage (De Paepe et al., 2020a,b).

Potential application of an electrochemical urine pretreatment module and a MEC-MABR module in the MELiSSA CIII shows promises, but still require modifications for operation in LEO. For example, hydrogen gas formed in the liquid in the MEC in space cannot escape due to the microgravity conditions. A possible solution is the use of a gas diffusion air cathode that prevents $H_2$ formation in the MEC (De Paepe et al., 2020a).

While the former technologies are promising for improving the overall process of urine nitrification, the use of an anaerobic bioreactor followed by an aerobic bioreactor as will be applied in the Lunar Palace also show promise (Cheng et al., 2019). Thus, in recent years, exciting concepts have been demonstrated for the combination of physicochemical and biological elements for urine processing in space.

## Terrestrial Applications of Nitrogen Recovery Technologies

Several terrestrial applications based on space research for nitrogen treatment have been developed or are currently in development. For example, Biostyr™ is an innovative process that can be implemented to remove, among others, nitrogenous ($NH_4^+$, $NO_3^-$) compounds using both nitrification and denitrification in a compact structure, thereby presenting a low environmental footprint. Another technology based on a self-sustaining groundwater filtration system that removes nitrates with denitrification from the ground water to produce potable water has been installed and is currently in used in Morocco (MELiSSA Foundation, 2021). Finally, the Antarctica research facility Concordia has been fitted with a grey water processing system that includes biological components to remove nitrogen (MELiSSA Foundation, 2021).

As mentioned, untreated urine causes scaling and biofouling in downstream processes during urine recycling in CIII. This also holds true for pipelines in sewer and sewage plants. Stabilization of urine by controlled anaerobic ureolysis at the source rather than in a centralized manner can reduce divalent cation salt precipitation ($Ca^{2+}$ and $Mg^{2+}$) and release $NH_3$ in a controlled manner. The latter is important to avoid nitrogen loss and thus improve downstream nutrient recovery, yielding environmental benefits as a consequence (Christiaens et al., 2019b). In a second potential system, an electrochemical cell drives precipitation of the divalent cation minerals at the toilet and, unlike the aforementioned technology, inhibits urea hydrolysis through alkalization of the urine. Source-separated urine can be stored and, when needed, discharged to a wastewater treatment plant that can recover nitrogen, reducing nitrogen loss (De Paepe et al., 2020b).

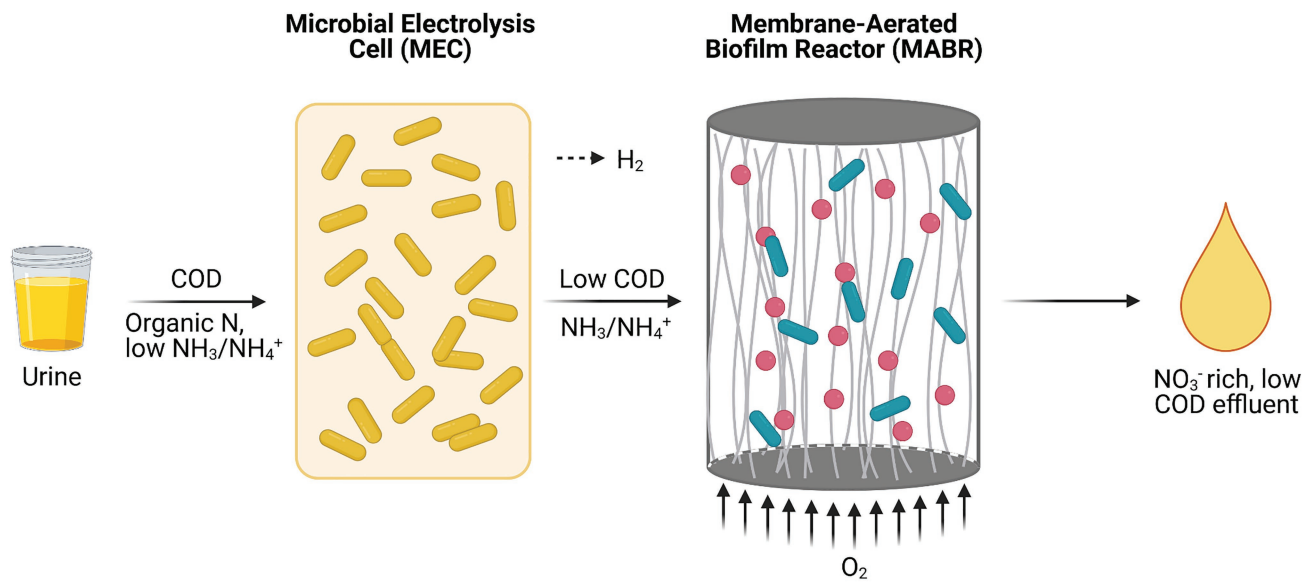


**FIGURE 5 |** Schematic representation of a microbial electrolysis cell (MEC) and a membrane-aerated biofilm reactor (MABR) in a serial setup (Adapted from De Paepe et al., 2020a).

Hydrogen-oxidizing Bacteria (HOB) have been under the spotlight in recent years due to their high growth rate, low resource requirements, and a high protein content (> 70% of their cell dry weight). Hence, they have great potential to be used as microbial protein (MP) source (Christiaens et al., 2017; Yang et al., 2021). In recent studies, the growth of HOB on recycled nitrogen has been investigated (Christiaens et al., 2017; Yang et al., 2021). HOBs typically rely on ammonium as their nitrogen source and use $H_2$ and $O_2$ as electron donor and an electron acceptor, respectively, to fixate $CO_2$ as carbon source (Yang et al., 2021). However, conventional ammonia removal and production with the Haber-Bosch process (an artificial industrial process to fixate nitrogen by converting atmospheric $N_2$ to $NH_3$ with hydrogen under high pressure and temperature) has a high energy cost. As a potential alternative, a proof-of-concept study validated the use of an electrochemical cell to extract $NH_4^+$ from hydrolyzed urine produced in, for example, a MEC. In the system, the urine is alkalified in a cathode compartment, which enables gas stripping of $H_2$ and $NH_3$. Then, the effluent is redirected to an anode compartment where $NH_4^+$ is pulled to the cathode compartment over an ion-exchange membrane. The $O_2$ and $H_2$ needed are generated at the anode and cathode, respectively, whereas the $CO_2$ used as carbon source is a by-product of ureolysis. The use of this technology greatly reduces energy costs compared to $NH_4^+$ production with Haber-Bosch. A study on HOB species *C. necator* 335 confirmed its ability to grow at high $NH_4^+$ loads. Hence, it is possible to grow MP from HOB *via* $NH_4^+$ recovered from high $NH_4^+$ load waste streams such as urine (Yang et al., 2021). By supplementing human food or feed for cattle with MP from HOB produced from recovered nitrogen, nitrogen losses, energy costs, and surface area per unit of protein mass produced can be significantly reduced. This offers a strategy to tackle to challenges of future food production for an ever-growing human population (Christiaens et al., 2017; Yang et al., 2021).

# IMPACT OF SPACE CONDITIONS ON UREOLYTIC AND NITRIFICATION BACTERIA

In LEO, the metabolism of the ureolytic and nitrifying bacteria could be affected by increased ionizing radiation dose rates and changes in gravity conditions (Senatore et al., 2018; Milojevic and Weckwerth, 2020). Both spaceflight experiments and simulations on Earth have been conducted to observe and gain a broad understanding of the bacterial responses and, more importantly, to assess the feasibility of a BLSS for space exploration (Leys et al., 2009; Mastroleo et al., 2009, 2013; Lindeboom et al., 2018; Ilgrande et al., 2019b; Poughon et al., 2020; Senatore et al., 2020). It is, however, extremely challenging to perform flight experiments to assess the biological effects of a real space environment due to the high costs and great interest in flying an experiment in combination with limited crew time and space aboard the ISS and other space stations. Hence, terrestrial methods have been favored to simulate microgravity and increased ionizing radiation intensities to assess their effects in a more accessible manner.

## Bacterial Response to Simulated Microgravity

Depending on the location in spaceflight, microgravity decreases from $10^{-3}$ to $10^{-6}$ times the terrestrial gravity (Huang et al., 2018). These conditions indirectly impact bacterial life through the alteration of fluid mechanics. Buoyancy due to density differences, convection and hydrostatic forces are eliminated, causing a low-shear fluid environment for bacteria, in which nutrients and metabolites only spread by diffusion along a concentration gradient. This results in the formation of nutrient-depleted and metabolite-enriched zones around bacteria (Monti, 2001; Huang et al., 2018; Senatore et al., 2018; Acres et al., 2021). Microbial behavior in real microgravity conditions has been analyzed on the orbital stations Mir and ISS. Most experiments, however, have been conducted on Earth, using different variations of devices called clinostats, which simulate microgravity. The rotating wall vessel (RWV) is a type of 2D clinostat that rotates perpendicular to the gravity vector, causing low-shear fluid conditions and preventing the microbe from adapting to a specific gravitational orientation. These factors result in a low shear-modelled microgravity (LSMMG) environment (Orsini et al., 2017; Huang et al., 2018; Senatore et al., 2018; Acres et al., 2021). Alternatively, the random positioning machine (RPM), a type of 3D clinostat, changes the position of an experiment randomly at arbitrary speeds and directions in the 3D space. In doing so, the object in suspension experiences a net gravity vector close to zero over a certain time interval. For microorganisms, the RWV is the most commonly used device for experiments in simulated microgravity, while the RPM is generally applied for experiments with higher organisms, but several applications for bacterial experiments are also known (Mastroleo et al., 2009, 2013; Huang et al., 2018; Senatore et al., 2018, 2020; Acres et al., 2021). The effects of an LSMMG environment have been assessed for a wide variety of microbial species and have been observed to impact cell growth, cell morphology, cell metabolism, cell–cell communication, cell pigmentation, biofilm formation, stress response, gene expression, virulence, genetic transfer, and even cause genomic changes (Mastroleo et al., 2009, 2013; Orsini et al., 2017; Tirumalai et al., 2017; Senatore et al., 2020; Acres et al., 2021).

Very limited work has been performed on ureolytic and nitrifying bacteria or microbial consortia in simulated microgravity. Ilgrande and co-workers exposed a synthetic community of *C. testosteroni, N. europaea,* and *N. winogradskyi* to LSMMG in a 2D clinorotation experiment, showing similar ureolytic and nitritation activity in regular cultures compared to those subjected to microgravity (Ilgrande et al., 2019a). However, nitrite accumulated in the LSMMG-exposed samples, indicating inactive nitratation by *N. winogradskyi*. This observation has been attributed to the experiment setup, which was performed in a fluid processing apparatus used in former

spaceflight experiments (Ilgrande et al., 2019a). It is hypothesized that this device could limit oxygen availability. The resulting competition for oxygen probably inhibited *N. winogradskyi* in the nitrifying community, likely causing nitrite accumulation in this scenario (Laanbroek and Gerards, 1993; Ilgrande et al., 2019a). Further work should ensure that the setup allows adequate oxygen availability for all members of the synthetic community. Moreover, an omics approach will aid in unraveling the underlying molecular mechanisms and constructing a thorough understanding of the behavior of nitrifying consortium in simulated microgravity conditions.

## Bacterial Response to Ionizing Radiation

A second major difference in the space environment compared to the terrestrial environment is the chronic low-dose ionizing radiation exposure. In LEO, radiation dose rates (400–600 μGy $d^{-1}$) can be 150–200 times higher than those experienced on Earth (2–4 μGy $d^{-1}$; Vanhavere et al., 2008; Mastroleo et al., 2009; Dachev et al., 2015). The average ionizing radiation exposure in lunar orbit was measured at 200–300 μGy $d^{-1}$ and recent lunar surface measurements determined an average absorbed dose rate of 316.8 μGy $d^{-1}$ (Reitz et al., 2012; Zhang et al., 2020). Finally, radiation doses in Mars transit are estimated to average 460 μGy $d^{-1}$ while the Mars surface dose rate is approximated at an average of 210 μGy $d^{-1}$ (Durante, 2014). Biological effects of ionizing radiation result from both direct and indirect damage to biomolecules. First, molecular bonds in biomolecules can be broken directly by radiation, which interacts with molecules by excitation and ionization. High energy, high density (HZE) particles are the main contributors to these effects. However, ionizing radiation mainly causes indirect damage by interacting with $H_2O$ molecules, creating reactive oxygen species that attack biomolecules and cause oxidative stress (Dartnell et al., 2008; Moeller et al., 2010; Hassler et al., 2014; Siasou et al., 2017).

There have been many studies on the effects of ionizing radiation on bacterial species. Cell survival to acute high-dose exposure is suggested to not rely on just a single mechanism, such as the ability to repair DNA, but rather a complex set of cellular responses. A review on studies of the effects on bacterial populations exposed to long-term chronic low doses in the Chernobyl restricted zone describes that radioresistance seems to improve (Mollert and Mousseau, 2016). This implies adaption of bacteria to long-term chronic low-dose radiation exposure. However, more rigorous experimentation is required to solidify this claim (Mollert and Mousseau, 2016). In experiments with *E. coli*, selective pressure caused by high-dose acute ionizing radiation (1,000 Gy) led to highly radioresistant phenotypes (Byrne et al., 2014). The findings in the discussed experiments do imply the presence of selective pressure when bacteria are exposed to either chronic low-dose irradiation or acute high-dose irradiation.

Effects of space relevant doses of ionizing radiation mimicking LEO conditions have also been studied. It is impossible to precisely imitate the complex radiation environment on board the ISS due to the wide array of radiation types stemming from secondary particles generated from interactions of primary particles with the spacecraft's hull. HZE particles are the main protagonists in radiobiological damage in space. Hence, irradiation with these types of particles in terrestrial simulation can give a close approximation of the biological effects of cosmic radiation (Moeller et al., 2010). Moreover, to more closely mimic the radiation environment in space, one could irradiate biological samples with both low-and high-energy particles. An experiment on *R. rubrum* (a MELiSSA CII strain) comparing the impact of simulated LEO ionizing radiation and a spaceflight experiment in ISS, showed a pronounced transcriptomic response to the radiation exposure. *R. rubrum* is thus able to respond and cope with conditions linked to spaceflight in LEO (Mastroleo et al., 2009).

To our knowledge, no research has been done on the effects of ionizing radiation on ureolytic and nitrifying strains or consortia. Only one study indicates that members of the ammonium-oxidizing *Nitrosomonadacaea* family, of which *N. europaea* is a member, are more sensitive to γ-irradiation than other soil microorganisms, and post-exposure recovery proceeds slowly (Shah et al., 2013). To provide an idea of the performance of the axenic ureolytic heterotrophs and nitrifying autotrophs in space, it is necessary to evaluate the biological effects of chronic low-dose ionizing radiation exposure on these strains. Moreover, valuable information can be gathered on radioresistance of these bacteria with an acute high-dose irradiation survival experiment.

## Bacterial Response to Spaceflight

Several studies have already provided researchers with insight into the effects of the LEO space environment on bacteria. Since it is hard to mimic the exact conditions of real space ionizing radiation and microgravity conditions and the combination of both, space experiments provide very valuable information of bacterial response to real space conditions. Such effects include variations in cellular metabolism, microbial proliferation rate, cell division, biofilm formation, cell morphology, cell motility, and genetic transfer between cells. Limited information is available on molecular responses of bacteria in space (Senatore et al., 2018; Milojevic and Weckwerth, 2020).

Nitrification in space has already been demonstrated in biofilters of the CEBAS experiment during the SPS-89 mission of the Columbia space shuttle (Blüm et al., 2003). A second biofilter experiment called C.R.O.P. failed due to hardware malfunctioning during spaceflight (Hauslage et al., 2018). Furthermore, two spaceflight experiments (NITRIMEL and BISTRO) have been conducted to specifically study the effects of space conditions on nitrifying bacteria and consortia. Both focused on the storage and survival of inactivated cultures of nitrifiers, rather than assessing the effects of space conditions on active cultures and the active nitrogen conversion process (Lindeboom et al., 2018; Ilgrande et al., 2019b). The impact of space conditions on reactivation ability of the nitrogen cycle cultures is important in case of system failure during

spaceflight (Lindeboom et al., 2018). During the NITRIMEL experiment, reactivation of pure cultures of ureolytic heterotroph *Cupriavidus pinatubonensis, N. europaea,* and *N. winogradskyi*, a bipartite culture of *N. europaea* and *N. winogradskyi,* a synthetic consortium of all 3 strains, and a nitrifying bioreactor culture were assessed after a 44-day mission in LEO on board the FOTON-M4 research satellite. Samples were exposed to 547–827 μGy $d^{-1}$ and microgravity ($10^{-3}$ - $10^{-4}$ g) conditions. No impact on ureolysis, nitritation or nitratation rates was observed compared to control cultures stored on the ground (Lindeboom et al., 2018). These results were substantiated by the BISTRO fight experiment where *C. pinatubonensis,* autotrophic nitrifying *Nitrosomonas ureae, N. europaea, N. winogradskyi*, the bipartite community of *N. europaea* and *N. winogradskyi*, and a synthetic consortium of the aforementioned strains with or without *N. ureae* added to the community, were sent to the ISS for 7 days. Here, the radiation exposure was 400 μGy $d^{-1}$. A general biomass decay was observed for all ISS cultures as opposed to control samples, but reactivation of nitrifying processes was successful in all populations (Ilgrande et al., 2019b). The observations in both studies conclude that ureolytic and nitrifying strains can cope with exposure to space conditions for a duration of missions that can take us to at least the Moon and encourage further experimentation toward their application in a BLSS for space travel (Lindeboom et al., 2018; Ilgrande et al., 2019b).

In the current URINIS (Urine Nitrification in Space) project, the impact of spaceflight conditions on actively growing nitrification cultures in space will be assessed for the first time. A batch experiment followed by a bioreactor experiment will be flown to ISS to assess the *in situ* effect of space conditions on the nitrifying activity of pure strains and the synthetic consortium of *C. testosteroni, N. europaea,* and *N. winogradskyi*. Activity tests, viability tests, transcriptome, metabolome, and proteome analysis, phenotypical characterization and biofilm analyses will aid in understanding microbial responses to that environment and provide more answers for the feasibility of CIII in space travel.

## CONCLUSION

Bioregenerative life support systems will play an important role for both terrestrial and space applications in the future. In space, regenerative systems will be required for long-term space expeditions and colonization missions of celestial bodies. A closed loop system can meet the metabolic needs of the crew (food, water, and oxygen) where nitrogen recovery plays a vital part. Extensive research on urine recycling systems has enabled the development of several experimental technologies for which proof-of-concept experiments have validated their potential. However, the harsh space conditions and the stringent requirements for space applications require more research and development to further minimize energy costs, crew time, and nitrogen losses and to optimize efficiencies and continuous runtimes of the bioreactor process and robustness of the biological systems. Resource exploitation without adequate regeneration and/or recycling of waste results in overconsumption, pollution and resource scarcity. As such, recovery of valuable compounds from waste products could partly solve many environmental as well as societal issues (Pearson, 2007). Technologies obtained in the development of BLSSs for space travel show high potential for transfer to the resource recovery industry on Earth.

## AUTHOR CONTRIBUTIONS

TV and FM collected literature and wrote the original draft. All authors critically reviewed the manuscript and figures and approved the final manuscript.

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# Identification of Plant Growth Promoting Bacteria Within Space Crop Production Systems

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As we establish colonies beyond Earth, resupply missions will become increasingly difficult, logistically speaking, and less frequent. As a result, the on-site production of plants will be mission critical for both food production as well as complementing life support systems. Previous research on space crop production aboard the International Space Station (ISS) has determined that the spaceflight environment, though capable of supporting plant growth, is inherently stressful to plants. The combined stressors of this environment limits yield by inhibiting growth, as well as increasing susceptibility to infection by plant pathogens such as *Fusarium* spp. We propose that a consortium of space-viable, plant growth-promoting bacteria (PGPB) could assist in mitigating challenges to plant growth in a sustainable fashion. Here, we utilize biochemical and phenotypic assessments to identify potential PGPB derived from previously acquired isolates from the VEGGIE crop production system aboard the ISS. These assays confirmed the presence of bacteria capable of producing and/or interfering with plant hormones, facilitating plant uptake of high-value target nutrients for plants such as iron and phosphorus, and able to inhibit the growth of problematic fungal species. We discuss our findings with regards to their potential to support plant growth aboard spaceflight platforms as well as the Moon and Mars.

**Keywords: regenerative life support, plant growth promoting (PGP) bacteria, space agriculture, astrobiology, space crop production**

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## INTRODUCTION

To ensure the longevity of human space colonization it is imperative to develop safe and sustainable methods of life support on the surface of a planetary body, as well as on deep space transports. Bioregenerative life support systems (BLSS) use biological processes to support a human crew by recycling air and water, and in more advanced systems provide food. Food production is an extremely important aspect of BLSS for deep space missions such as establishing a Mars colony. As humanity establishes colonies further from Earth the logistics of resupply missions will become increasingly complicated, limiting their frequency. Relying on resupply missions to provide food for an off-world colony will not only be infeasible, but failed or delayed resupplies would be detrimental, potentially lethal, to colonists.

Current food production systems in space are limited to modified hydroponic systems in microgravity such as the Veggie growth system or the Advanced Plant Habitat aboard the International Space Station (ISS). Crop selection for these systems focuses on relatively fast-growing, pick-and-eat foods (*i.e.,* selections that require no processing/cooking to be eaten) that can supplement the astronaut diet. These selections provide nutrition lacking in prepackaged food such as potassium, or may not be stable over long duration missions, such as vitamin C (Cooper, 2013), but are not a significant source of caloric intake. Food production systems for the Moon and Mars will likely have the same considerations for early phase crop selection, retaining these nutritious accessory crops, while also moving toward the production of staple crops with higher caloric density.

The other benefit of these systems that has been noted is a psychological benefit, both to eating fresh food, and tending to the plants (Odeh and Guy, 2017). These benefits to the emotional and psychological health of crew members have been reported not only in space, but in the Neumayer Station in Antarctica (Mauerer et al., 2016). These findings underscore the importance that plant growth systems will play in overall crew health in deep space missions, as well as on colonies on the Moon and Mars.

The Veggie system is a modified hydroponic plant growth system that uses a "root pillow" containing an arcillite growth substrate which helps to carry water and suspended nutrients to the roots in microgravity (Massa et al., 2017a). Initial flight tests of the Veggie system, supported that the average fresh mass of surviving plants was higher in space flight samples compared to ground controls, albeit with large variability. However, the total number of ground control plants which survived over the duration of the study was significantly higher than those in microgravity (Massa et al., 2017a). Many of the plants in the flight system exhibited guttation, stunted growth, leaf curling, and chlorosis, all hallmarks of different stress responses, and ultimately resulted in the death of many of these plants. One of the major sources of stress in these systems is the behavior of water in the microgravity environment which can limit nutrient uptake, suffocate roots, and allow potentially pathogenic microorganisms, such as fungi, better access to host tissues as water builds up on leaves and around roots (Massa et al., 2017a). Strategies to improve plant growth, relieve stress, and improve disease resistance are in high demand for these environments.

On Earth, plants are assisted by a multitude of microorganisms, generically classified as plant growth-promoting bacteria (PGPB) which provide a variety of functions to their hosts, typically in exchange for a variety of carbon sources contained within root exudates. These include functions such as: 1) the production and manipulation of plant growth hormones (Ludwig-Müller, 2015), 2) interference of ethylene production by deamination of 1-aminocyclopropane-1-carboxylate (ACC) (Gamalero and Glick, 2015), 3) fixation of atmospheric nitrogen (Franche et al., 2009), 4) scavenging nutrients from the environment (Rodríguez et al., 2006; Scavino and Pedraza, 2013), or 5) provide resistance to potential pathogens (Compant et al., 2005). We propose that PGPB could perform a similar function aboard the ISS and other off-world sites to ameliorate the stressors associated with these environments.

In order to be sustainable, colonies on the Moon and other planets will need to practice *in situ* resource utilization (ISRU), or using resources available at the site of the colony to ensure sustainable operations. ISRU efforts toward food production often look at using the regolith available on site as a plant growth substrate, though studies using regolith simulants have shown the need for applied fertilizers (Bugbee and Salisbury, 2015; Eichler et al., 2021). Bacteria capable of scavenging nutrients from regolith, or simply facilitating nutrient uptake from fertilizer solutions would reduce the demand for those nutrients from Earth. Many PGPB can solubilize the key nutrient, phosphate, which may be present in regolith minerals, or may precipitate out of solution when interacting with other geologic material such as calcium, iron, or aluminum (Rodríguez et al., 2006; Ambrosini and Passaglia, 2017).

The general potential for PGPB to limit the growth of potential pathogens, either by producing antimicrobials or through effective competition, is of great interest for future BLSS (Compant et al., 2005), as it has been proven difficult to completely eradicate potential plant pathogens on the ISS via sterilization and sanitization. Some PGPB bacteria can produce organic compounds known as siderophores as biocontrol agents for potential pathogens. These compounds frequently work by chelating iron and there is wide diversity among siderophores produced by different organisms, along with a variety of transport proteins. This allows an organism to compete for resources by making the nutrient unavailable to competitors (Scavino and Pedraza, 2013). Such siderophores may also have the potential to scavenge iron from the rich iron-oxide deposits in Martian regolith making this crucial nutrient more available to plants in this environment (Blake et al., 2013).

While the biological control of potential pathogens is an important role for PGPB, the most common functions of PGPB are those that improve overall plant growth. One common mechanism for improving plant growth is through manipulation of plant hormones, such as the production of indole-3-acetic acid (IAA), or the interference of ethylene production. IAA is the most common form of auxin, the plant growth hormone. Stimulating auxin responses can encourage root and shoot elongation in most plants, while facilitating nodulation in legumes (Ludwig-Müller, 2015). Ethylene is a volatile hormone commonly used by plants in abiotic stress responses, as well as in defense against pathogens, and is associated with a reduction in overall growth. Some PGPB, as well as some pathogens, can inhibit ethylene production by metabolizing the ethylene precursor 1-Aminocyclopropane-1-carboxylic acid (ACC) as a nitrogen source. This allows the plant to become more resistant to abiotic stress, by ignoring these effects at low concentrations. Reduced ethylene production is frequently associated with increased plant growth and nodulation given the inhibitory effect this hormone can have on auxin responses (Gamalero and Glick, 2015). In summary, there are a variety of ways in which PGPB may ameliorate the challenges plants face in both the spaceflight environment as well as on an off-world colony.

However, for the protection of the crews who work with the plants, just like all the other items sent to space, everything in these systems are either sterilized or sanitized in the case of the seeds used (Massa et al., 2017b), and these practices are likely to continue. Yet despite these efforts to remove microbes from these systems, the ISS has a fairly diverse microbiome both in the plant growth systems (Khodadad et al., 2020), and on the general space station surfaces (Checinska Sielaff et al., 2019). The microbiome on the ISS is predominantly derived from the human microbiome (Avila-Herrera et al., 2020), which is then spread to the plants via astronauts during watering, pruning, and multi-round harvesting.

Seed-borne endophytic microbes which were unaffected by the surface sanitization of the seeds are likely to contribute to the plant microbiome as well (Ravel et al., 1997; Pitzschke, 2016). It is from this group of seed-borne endophytes that there may be a subset of plant growth promoting bacteria (PGPB) growing in the plant growth systems of the ISS. Yet, bacterial populations in Veggie are fairly heterogenous between various flight experiments, and while certain genera are consistently present, there are seemingly random differences in their distributions (Khodadad et al., 2020). It is therefore prudent to consider the development of a curated microbiome enriched with specific PGPB with which seeds could be inoculated.

The routine use of such a PGPB "probiotic" can improve current and future space food production systems, enhancing the sustainability of these systems on the Moon and Mars. We hypothesized that previous isolates from the Veggie system, were an ideal starting point for the identification of potential PGPB as they already have spaceflight history, indicating their ability to survive the launch process as well as grow in the spaceflight environment. (Khodadad et al., 2020). These bacterial isolates from the Veggie production system aboard the ISS were provided by NASA Kennedy Space Center (KSC) for study and a subset were screened for the presence of PGPB. Specifically, we have investigated these microorganisms for potential PGP functions including siderophore production, phosphate solubilization, indole production, ACC deaminase activity, and fungal growth inhibition. Our efforts confirm the existence of multiple PGPB strains as candidates for further study for their ability to improve yield and reduce stress on plants aboard the ISS. We discuss our findings in the context of their short-term benefits aboard the ISS along with the long-term potential to the development of bioregenerative life support systems in off-world sites.

# MATERIALS AND METHODS

## Maintenance of International Space Station Isolates

Parameters of the Veggie system are described in (Massa et al., 2017a). The isolation and identification of the bacterial and fungal species used in this study were previously reported as well (Khodadad et al., 2020). From this study a subset of bacteria and fungi were selected for their evaluation as PGPB. Bacterial species used in the present study include: *Acinetobacter genomospecies 3, Bacillus altitudinis, Bacillus amyloliquefaciens, Bacillus pseudomycoides, Bacillus pumilus, Bacillus subtilis, Burkholderia pyrocinnia, Cupriavidus pauculus, Curtobacterium flaccumfaciens, Curtobacterium pusillum, Leifsonia aquatica, Methylobacterium rhodinium, Microbacterium marytipicum, Paenibacillus macerans, Paenibacillus pabuli, Pantoea agglomerans, Paracoccus yeeii, Pseudomonas fulva, Ralstonia picketii, Sphingobacterium multivorum,* and *Stenotrophomonas rhizophila*. Fungal species used in this study include: *Fusarium anthophilum, Aspergillus sydowii, Aspergillus ustus,* and *Emericella parvathecia*.

Bacterial samples were taken from freezer stocks made using 30% glycerol media maintained at −80°C. Fresh cultures were maintained on 1.5% solid agar King B media, containing 1.5% (v/v) glycerol, 20 g/L peptone, 1.15 g/L $K_2HPO_4$, and 1.50 g/L $MgSO_4.7H_2O$. Fungal samples were maintained on Inhibitory Mold Agar (IMA) (BBL, Difco, Beckton Dickenson, Franklin Lakes, NJ). All isolates used were cultured from microbial screens of Veggie system flight samples. Stocks were streaked every 2 weeks to maintain fresh cultures.

## Siderophore Production

Methods for detection of bacterial siderophores were adapted from Ambrosini and Passaglia (2017). Chrome azurol S dye solution (CAS) was made by combining, an iron (III) chloride solution, a chrome azurol S solution, and cetrimonium bromide solution were combined with a piperazine/HCl buffer, filter sterilized, then added to 0.2X King B molten agar in a 100 ml/L ratio.

21 Bacterial isolates were cultured in King B liquid media for 48–72 h at 30°C, then pipetted onto four spots on solid assay plates containing 0.2X King B with CAS. Assay plates were incubated for 7 days at 30°C, then the orange halos were measured. Isolates that produced particularly large halos were redone with one central spot per plate in triplicate.

## Phosphate Solubilization

Assessment methods for phosphate solubilization were adapted from Ambrosini and Passaglia (2017) using GY/tricalcium phosphate solid medium. GY solid growth medium was prepared using glucose, yeast extract, and 1.5% agar, then while molten sterile solutions of $K_2HPO_4$ and $CaCl_2$ were added just before pouring. The addition of these two solutions causes insoluble calcium phosphate to precipitate, making the medium cloudy.

21 Bacterial isolates were cultured in King B liquid media for 48–72 h 30°C, then pipetted onto four spots on GY/tricalcium phosphate solid medium. Plates were incubated for 7 days at 30°C then observed for growth and zones of clearing.

## Fungal Biocontrol

Fungal biocontrol assays were performed on tryptic soy agar (TSA) (BBL, Difco, Beckton Dickenson, Franklin Lakes, NJ) plates. Bacterial isolates were cultured in King B liquid media for 48–72 h before inoculating assay plates. Fungal samples were prepared by suspending stock isolates into sterile water until an optical density at 600 nm ($OD_{600}$) of 0.1 was reached. A sterile

swab was used to streak lawns of fungal suspension on TSA plates, then bacterial samples were pipetted onto 4 spots on the plate. Plates were allowed to grow for 7 days then observed for interactions. 15 bacterial isolates were assayed for biocontrol capability against 4 fungal isolates.

## Indole Production

Twenty-one bacterial isolates were grown in King B liquid media supplemented with 2.5 mM tryptophan. IAA production is determined by a spectrophotometric described in Ambrosini and Passaglia (2017), which uses Salkowski's reagent, containing 0.012 g/ml $FeCL_3$ and 7.75 M $H_2SO_4$ to react with indoles present in the culture solution.

Liquid cultures were incubated for 48–72 h at 30°C. Aliquots were taken to measure the $OD_{600}$ of the culture, then centrifuged for 10 min at 5,400 × *g*. The supernatant was pipetted off, mixed with Salkowski's reagent, then allowed to incubate at room temperature in the dark for 30 min. Samples were run in triplicate through a spectrophotometer at 550 nm with a set of IAA standards ranging from 0 to 250 µg/ml. Sample IAA concentrations calculated from the standard curve were normalized to the measured $OD_{600}$ of the sample to express the amount of indole produced relative to the final concentration of bacteria.

## ACC Deaminase Activity

The ACC deaminase activity assay uses an indirect method described in Ambrosini and Passaglia (2017). Bacteria are grown on Dworkin and Foster (DF) salts solid media, containing 2% agar, 2 g/L glucose, 2 g/L gluconic acid, 4 g/L $K_2HPO_4$, 6 g/L $Na_2HPO_4$, 0.2 g/L $MgSO_4.7H_2O$, 2 g/L citric acid, 1 mg/L $FeSO_4.7H_2O$, 0.01 mg/L $H_3BO_4$, 0.011 mg/L $MnSO_4{\cdot}H_2O$, 0.12 mg/L $ZnSO_4.7H_2O$, and 0.008 mg/L $CuSO_4.5H_2O$. Each isolate is grown on two plates side by side, on one, an ACC solution is spread over the plate providing a 1 mM concentration of ACC, providing the only source of nitrogen. The other plate is left as a nitrogen free control.

21 Bacterial isolates were cultured in King B liquid media for 48–72 h 30°C. Aliquots were centrifuged at 5,400 × *g* for 10 min, the spent media pipetted off, and the bacteria resuspended in 0.85% saline. This rinse step was repeated 3 times, then the final resuspension pipetted onto three spots on each plate containing DF salts solid medium with or without ACC. Plates were placed in an incubator at 30°C for 7 days.

# RESULTS

From the previously isolated microbiome samples from the Veggie crop production system (Khodadad et al., 2020, 21 bacteria were identified (*materials and methods* for full list) that were readily culturable under general conditions (30°C on King B solid media) and remained viable in freezer stocks. Also selected from these previous microbiome studies were 4 fungal species to be used in the antifungal assessment. These samples were isolated from leaf tissue, root tissue, or from the root substrate of the spaceflight Veggie system and are therefore likely candidates to have derived from seed-borne endophytic populations.

## Siderophore Production

Siderophores are heavily involved in iron uptake and in doing so can provide effective competition for nutrient resources. Excess iron taken up by PGPB can then be passed off to host plants to improve plant performance. CAS was used to determine the presence of siderphores in culture. This dye solution forms a blue dye complex consisting of chrome azurol S, iron (III), and cetrimonium bromide, which turns orange when a strong chelator, such as a siderophore, removes the iron (Ambrosini and Passaglia, 2017). 9 out of 20 tested isolates were able to grow on the media and produce orange halos including; *Acinetobacter genomospecies* 3, *Burkholderia pyrocinnia*, *Cupriavidus pauculus*, *Curtobacterium flaccumfaciens*, *Paenibacillus macerans*, *Paenibacillus pabuli*, *Pantoea agglomerans*, *Pseudomonas fulva*, and *Ralstonia picketii* (**Table 1**). Of these, the 5 isolates *B. pyrocinnia*, *C. flaccumfaciens*, *P. macerans*, *P. agglomerans*, and *R. picketii* exhibit strong siderophore production, producing large halos with diameters ranging from 4.47 to 5.87 cm (**Figure 1A**), compared to the other 4 isolates whose diameters ranged from 0.88 to 1.20 cm (**Figure 1B**) (**Table 1**).

## Phosphate Solubilization

Phosphate is a crucial nutrient for plants and bacteria that can easily precipitate out of solution depending on the environmental pH and geochemical interactions. Certain PGPB are able to solubilize phosphate to assist with uptake by both the bacteria and host plant. In this assay bacteria incapable of solubilizing the calcium phosphate precipitate will be unable to grow, bacteria that are particularly efficient will develop clear halos around the colony (Ambrosini and Passaglia, 2017). Out of 20 isolates, 5 were able to grow including; *Bacillus pumilus*, *B. pyrocinnia*, *C. flaccumfaciens*, *P. macerans*, and *P. agglomerans* (**Table 1**). Clear halos developed around colonies of *B. pyrocinnia*, *P. macerans*, and *P. agglomerans* (**Figure 2A**) while the media around *B. pumilus* and *C. flaccumfaciens* remained opaque with no halo (**Figure 2B**) (**Table 1**).

## Fungal Biocontrol

PGPB can play a crucial role in plant pathogen defense by providing competition or producing antimicrobial compounds (Compant et al., 2005). Assessments were made using a co-culturing assay examining simultaneous bacterial and fungal growth. Bacterial antifungal properties were classified on a rating scale from 1 to 4, with 1 being ineffective and 4 being most effective (**Figure 3**). Bacteria rating a 4 developed clear zones around the colony, i.e., no fungal growth was observed (**Figure 3A**) while those with a rating of 3 developed with a zone of reduced fungal growth around the colony (**Figure 3B**). In samples with a rating of 2, bacteria developed with no fungal growth on the colony but with no effects on the surrounding fungal growth (**Figure 3C**). Finally, bacteria with a rating of 1 showed no

**TABLE 1 |** Siderophore production; growth and resulting halo diameter on 0.2X King B/CAS media after 1 week of growth. *N* = 4 for halos under 1.5 cm, *N* = 3 for halos over 1.5 cm. Phosphate Solubilization; growth and resulting halo diameter on GY/tricalcium phosphate media after 1 week of growth. *N* = 4 for all samples. Indole production; results of spectrophotometric assay for indole production. Concentration of IAA is calculated based on a set of standards, expressed in μg/mL. IAA concentration was normalized to the final $OD_{600}$ of the culture to account for slow growing bacteria producing low but significant amounts of indole. ACC deaminase; ACC deaminase indirect assay rankings. 1 indicates no ACC deaminase activity, 2 indicates mild ACC deaminase activity, 3 indicates strong ACC deaminase activity.

| Species | Siderophore production | Phosphate solubilization | | | Indole production | | ACC deaminase |
|---|---|---|---|---|---|---|---|
| | Growth (+/–) Halo diameter (cm) | Growth (+/–) | Halo (+/–) | Halo diameter (cm) | (IAA) (μg/ml) | (IAA)/$OD_{600}$ | Ranking |
| *A. genomospecies 3* | +; 1.20 ± 0.07 | – | – | — | 18.46 ± 0.13 | 36.55 ± 0.25 | 3 |
| *B. altitudinis* | – | – | – | — | 13.08 ± 0.42 | 27.47 ± 0.88 | 3 |
| *B. amyloliquefaciens* | – | – | – | — | 15.85 ± 0.58 | 53.92 ± 1.98 | 3 |
| *B. pseudomycoides* | – | + | – | 0.30 ± 0.00 | 25.09 ± 0.13 | 53.85 ± 0.27 | X |
| *B. pumilus* | – | – | – | — | 11.10 ± 0.25 | 31.71 ± 0.72 | X |
| *B. subtilis* | – | – | – | — | 26.66 ± 0.75 | 84.64 ± 2.39 | 3 |
| *B. pyrocinnia* | +; 5.87 ± 0.45 | + | + | 1.08 ± 0.19 | 10.74 ± 0.38 | 12.82 ± 0.45 | 2 |
| *C. pauculus* | +; 0.88 ± 0.08 | – | – | — | 15.62 ± 0.56 | 20.68 ± 0.75 | 3 |
| *C. flaccumfaciens* | +; 4.47 ± 0.24 | + | – | 0.38 ± 0.04 | 64.61 ± 0.56 | 203.82 ± 1.78 | 1 |
| *C. pusillum* | – | – | – | — | 17.65 ± 0.63 | 52.84 ± 1.90 | 1 |
| *L. aquatica* | – | – | – | — | 15.50 ± 0.55 | 22.96 ± 0.82 | 1 |
| *M. rhodinium* | – | – | – | — | 10.65 ± 0.13 | 64.55 ± 0.77 | 1 |
| *M. marytipicum* | – | – | – | — | 21.86 ± 0.13 | 31.97 ± 0.19 | 1 |
| *P. macerans* | +; 4.60 ± 0.47 | + | + | 0.53 ± 0.08 | 22.40 ± 0.46 | 263.56 ± 5.38 | 1 |
| *P. pabuli* | +; 0.90 ± 0.07 | – | – | — | 2.58 ± 0.25 | 2.98 ± 0.29 | 3 |
| *P. agglomerans* | +; 4.83 ± 0.48 | + | + | 0.85 ± 0.09 | 118.13 ± 14.85 | 309.23 ± 38.96 | 3 |
| *P. yeeii* | – | – | – | — | 9.75 ± 0.46 | 19.31 ± 0.91 | X |
| *P. fulva* | +; 1.05 ± 0.18 | – | – | — | 35.41 ± 0.91 | 319.02 ± 8.24 | 1 |
| *R. picketii* | +; 5.80 ± 0.08 | – | – | — | 11.64 ± 0.34 | 12.47 ± 0.36 | 3 |
| *S. multivorum* | – | – | – | — | 45.92 ± 0.56 | 110.64 ± 1.36 | 3 |
| *S. rhizophila* | – | – | – | — | 17.83 ± 0.83 | 44.13 ± 2.06 | X |

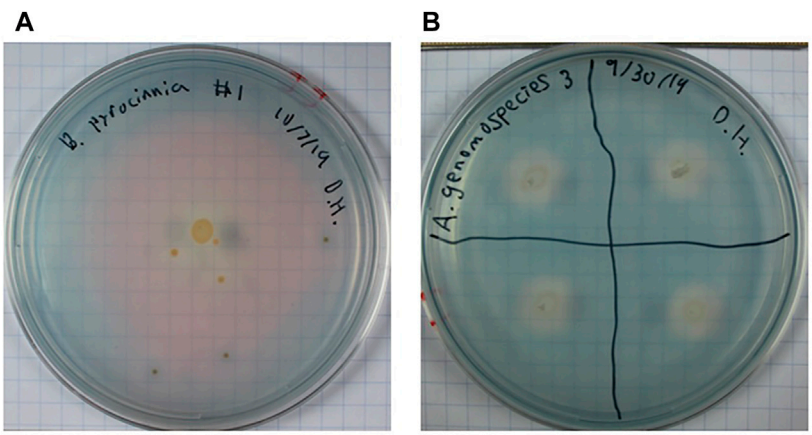


**FIGURE 1 |** Growth of bacterial isolates on 0.2X King B/CAS assay plates showing examples of **(A)** a "strong" siderophore producer, *Burkholderia pyrrocinia*, and **(B)** a "weak" siderophore producer, *Acinetobacter genomospecies* 3. Orange/yellow zones indicate removal of iron from the CAS dye complex.

observable impacts on fungal growth on the plate or the colony itself (**Figure 3D**).

The assessment for each combination of bacteria and fungi is described in **Table 2**. More than half of the combinations (38/60) were given a rating of 2 against the fungal species *Fusarium anthophilum*, *Aspergillus sydowii*, *Aspergillus ustus*, and *Emericella parvathecia*. Only 5/60 combinations were given rating 1 against the fungi, three of them paired against *F. anthophilum*; *Leifsonia aquatica*, *Methylobacterium rhodinum*, and *P. agglomerans*, as well as *M. rhodinum* paired against *A. sydowii* and *E. parvathecia*. 13/60 combinations were given rating 3, most notable being *Bacillus amyloliquefaciens* causing

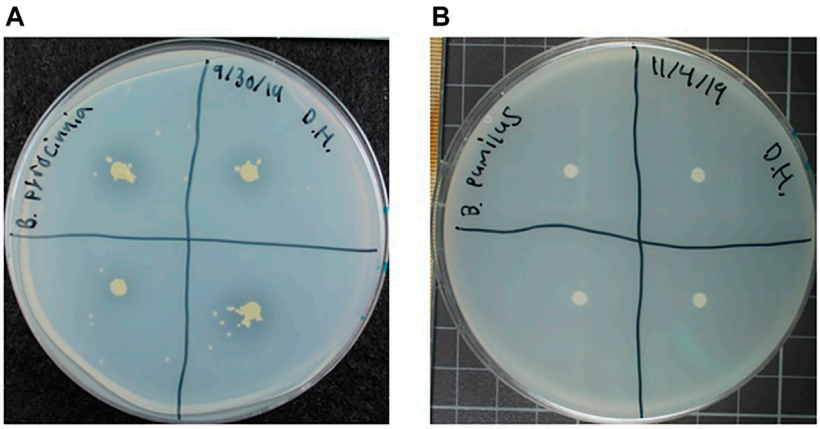


**FIGURE 2 |** Growth of bacterial isolates on GY/tricalcium phosphate assay plates showing **(A)** *Burkholderia pyrrocinia*, capable of developing clear zones around colonies, and **(B)** *Bacillus pumilus*, capable of growing on insoluble phosphate but unable to develop clear zones. Growth and/or development of clear zones indicate ability to solubilize calcium phosphate.

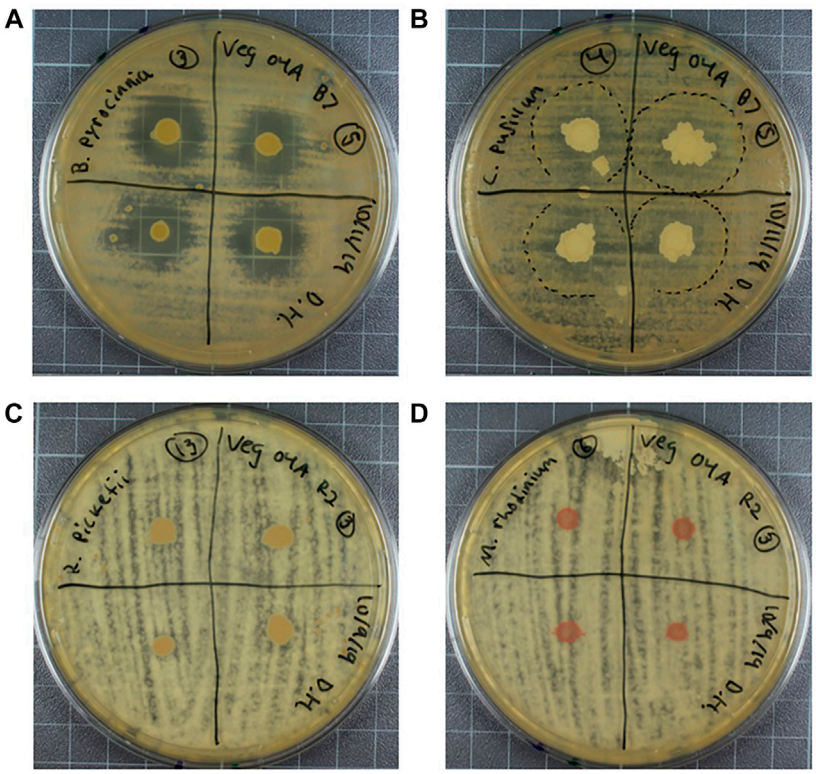


**FIGURE 3 |** Antifungal assay plates showing examples of **(A)** Rating 4 growth of *Burkholderia pyrrocinia* on *Emericella parvathecia*, **(B)** Rating 3 growth of *Curtobacterium pusillum* on *Emericella parvathecia*, **(C)** Rating 2 growth of *Ralstonia picketii* on *Aspergillus sydowii* and **(D)** Rating 1 growth of *Methylobacterium rhodinum* on *Aspergillus sydowii*.

decreased growth effects on all 4 fungal species, and *B. pumilus* causing decreased growth effects on all except *F. anthophilum*. Other rating 3 combinations include: *Curtobacterium pusillum* against *A. sydowii* and *E. parvathecia*; *B. pyrocinnia*, *P. fulva,* and *Stenotrophomonas rhizophila* against *A. ustus*; and *Bacillus pseudomycoides* against *A. sydowii.* The 4 remaining combinations were given a rating of 4; *B. pyrocinnia* exhibited cleared zones with *A. ustus* and *E. parvathecia*, and *B.*

**TABLE 2 |** Antifungal assessment results of bacterial isolates on four fungal isolates. Rating 4 exhibits zones of no fungal growth around the bacterial colony. Rating 3 bacteria have zones of reduced fungal growth around the colony. Rating 2 bacteria only prevent fungal growth within the bacterial colony. Rating 1 bacteria are unable to prevent fungal growth in or around the bacterial colony.

| Species | *F. anthophilum* | *A. sydowii* | *A. ustus* | *E. paravathecia* |
|---|---|---|---|---|
| *A. genomospecies 3* | 2 | 2 | 3 | 2 |
| *B. amyloliquefaciens* | 3 | 3 | 3 | 3 |
| *B. Pseudomycoides* | 2 | 3 | 4 | 4 |
| *B. pimilis* | 2 | 3 | 3 | 3 |
| *B. pyrocinnia* | 2 | 4 | 3 | 4 |
| *C. pusillum* | 2 | 3 | 2 | 3 |
| *L. aquatica* | 1 | 2 | 2 | 2 |
| *M. rhodinum* | 1 | 1 | 2 | 1 |
| *M. marytipicum* | 2 | 2 | 2 | 2 |
| *P. pabuli* | 2 | 2 | 2 | 2 |
| *P. agglomerans* | 1 | 2 | 2 | 2 |
| *P. yeeii* | 2 | 2 | 2 | 2 |
| *P. fulva* | 2 | 2 | 3 | 2 |
| *R. picketii* | 2 | 2 | 2 | 2 |
| *S. rhizophila* | 2 | 2 | 3 | 2 |

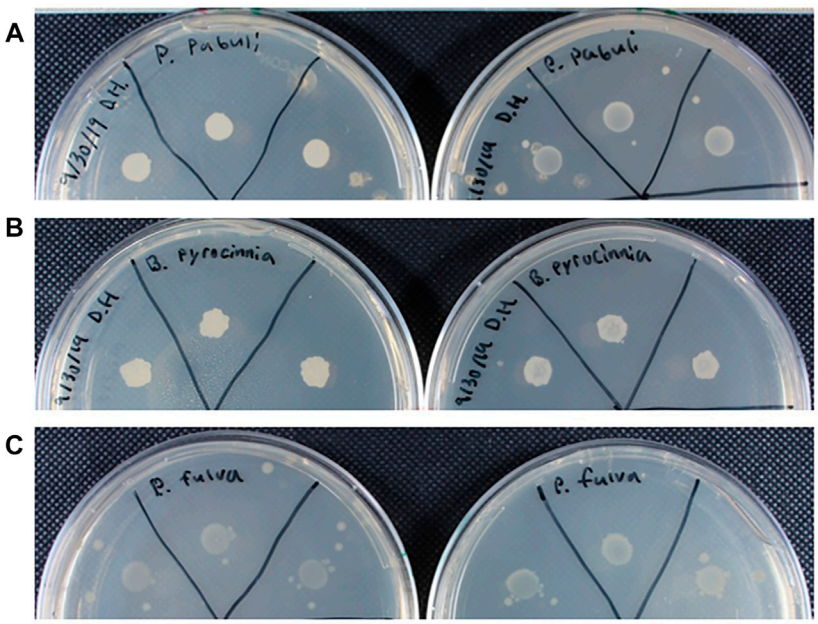


**FIGURE 4 |** Growth of bacteria on DF salts medium with 1 mM ACC (left) and nitrogen free (right). **(A)** Example of rating 3 growth, *P. pabuli*, showing improved growth in the presence of ACC. **(B)** Example of rating 2 growth, *B. pyrocinnia*, showing mildly improved growth in the presence of ACC. **(C)** example of rating 1 growth, *P. fulva*, showing no improved growth in the presence of ACC.

*pseudomycoides* exhibited cleared zones on *A. sydowii* and *E. parvathecia.*

## Indole Production

Bacterial production of IAA, the most common auxin, can improve plant growth by stimulating root and stem elongation. A spectrophotometric assay (Ambrosini and Passaglia, 2017) determined that most bacterial isolates produced some amount of indole, typically in the 10–25 μg/ml range, though *P. pabuli* produced a particularly low amount of indole that may be false due to instrument sensitivity (**Table 1**). Others produced a particularly high amount of indole, such as *P. agglomaerans*. To account for cultures with low cell density at time of analysis, calculated indole measurements were normalized to the $OD_{600}$ of the culture, creating ratio of indole concentration to relative cell abundance. This revealed that *C. flaccumfaciens*, *P. fulva*, *Sphingobacterium multivorum*, and *P. macerans* produce more significant amounts of indole than the raw data would indicate. This

normalization also confirms that other bacteria, such as *P. pabuli and R. picketii,* do not produce notable amounts of indole.

## ACC Deaminase Activity

The plant hormone ethylene is heavily involved in plant stress response and is responsible for many of the resulting phenotypes of stress conditions. The precursor molecule to ethylene, ACC, can be used as a nitrogen source by PGPB, and in doing so, the bacteria limits the amount of ethylene produced. An indirect assay was used to detect ACC deaminase based on growth patterns (Ambrosini and Passaglia, 2017). Isolates were rated based on differential growth between plates with and without ACC on a scale of 1-3, with 3 indicating improved growth on plates with ACC (**Figure 4A**), 2 indicating mildly improved growth (**Figure 4B**), and a 1 indicating poor growth on both plates (**Figure 4C**) Bacteria that produce ACC deaminase were able to grow on the plate with ACC and poorly on the plate without ACC, whereas bacteria that do not produce ACC deaminase grew poorly on both. Bacteria that showed growth unlike these, such as growing well on both, or better on the ACC free plate, are described as indeterminant. These bacteria are likely capable of nitrogen fixation and therefore do not depend on nitrogen in the growth media. Of the 21 isolates examined, 9 species were given a rating of 3, 1 species was given a rating of 2, 4 species a rating of 1, and the remaining isolates were classified and indeterminant (**Table 1**).

# DISCUSSION

The spaceflight environment has proven to be stressful to plants, yet long-term operations in deep space depend on successful plant growth for supplemental life support, food production, and psychological benefits. The isolation and detection of PGPB with spaceflight history provides an opportunity to improve plant growth through the development of a curated microbiome for the growth of plants aboard the ISS, on the Moon, or another planet. Using several biochemical and phenotypic assessments of bacteria isolated from the Veggie crop production system, we have confirmed the potential for several PGPB to exist in conjunction with plants grown on the ISS. However it remains to be seen if these bacteria are indeed capable of encouraging plant growth within the spaceflight environment. Of the 21 bacterial isolates, 17 were shown to be capable of at least one plant beneficial function, though most performed 2 to 3 of these functions. Of those appeared to generate indole, further studies will need to confirm the presence of IAA, especially those that did not exhibit other plant growth promoting functions, due to the assay used being general for all indoles.

These assessments provide a collection of culturable, space viable PGPB for the selective application to space food production systems. The selection of microorganisms for our planned community could be made smaller and more manageable by the selection of "generalist" PGPB, *i.e.* those that perform two or more of the plant-growth promoting functions evaluated herein. A potential minimal microbiome from this set of isolates would consist of just *P. agglomerans*, and *B. pyrocinnia*, both capable of phosphate solubilization, siderophore production, and ACC deamination. *P. agglomerans* also produces indoles while *B. pyrocinnia* provides antifungal capabilities.

Both species have been previously studied for plant growth promotion. Studies have shown that certain strains of *P. agglomerans* have nitrogen fixing capabilities, produce not only IAA, but also the phytohormones abscisic acid, gibberellic acid, and cytokinins (Feng et al., 2006). Inoculations of rice seedlings with the species increases overall growth rate and improved nutrient uptake preventing malnutrition stress (Feng et al., 2006; Jiang et al., 2015) other studies show that *P. agglomerans* also has the ability to reduce heavy metal toxicity (Luziatelli et al., 2020) and provide biocontrol against the plant pathogen *Ralstonia solanacearum* (Singh et al., 2020). *B. pyrocinnia* is a member of the *Burkholderia cepacia* complex and has been investigated for its biocontrol potential against plant pathogen *Rhizoctonia solani* via production of the antibiotic pyrrolnitrin (Schmidt et al., 2009) and studies on its siderophores have shown the ability to improve seed germination, plant size and fruit size (Min et al., 2019).

An inoculated microbiome can also be tailored for specific functions. ISRU approaches using regolith as a plant growth substrate on Mars will need to prioritize the acquisition of mineral nutrients, so the selection of microbes may be expanded to include *C. flaccumfaciens* and *P. macerans*, both of which solubilize phosphate and produce siderophores. Hydroponic systems would not require the highly active nutrient scavenging bacteria, but inoculation with indole producing, ethylene reducing, and pathogen mediating bacteria can help increase biomass and yield in hydroponic systems (Lee and Lee, 2015; Paradiso et al., 2017). Thus, including indole producing bacteria such as *P. fulva* and *P. macerans*, ACC deaminating bacteria such as *P. pabuli* and *R. picketii*, and antifungal bacteria such as *B. pseudomycoides* and *B. amyloliquefaciens* would improve the robustness and efficiency of the system.

Ultimately, the selection of the most appropriate PGPB will be determined by the location of Lunar and Martian colony sites, as well as the method of plant growth. ISRU approaches will have to be tailored not just to the planetary body, but also the specific colony site as locations will vary in mineralogy, which will also require the development of site specific regolith simulants important for research in these systems (Ramkissoon et al., 2019; Fackrell et al., 2021). The use of siderophore producing or phosphate solubilizing bacteria will be dependent on the amount of insoluble nutrients available in the on-site substrate, as well as what interactions applied fertilizers may have with the geologic material.

Compounds contained in on-site regolith may also create a need for plant growth promoting functions not investigated here, such as remediation of perchlorate compounds found on the Martian surface (Hecht et al., 2009; Navarro-González et al., 2010). These compounds have the potential to bioaccumulate in plant tissues (Sanchez et al., 2005) and

be toxic to Martian colonists (Lawrence et al., 2000), but can also be a source of breathable oxygen when degraded by perchlorate reducing bacteria (Carlström et al., 2015).

Before implementing these bacteria into food production systems, each species will need to be validated for use. Further testing should include *in planta* studies to confirm that the strains investigated are plant growth promoting. Certain species investigated, such as *C. flaccumfaciens* have variants that are plant growth promoters (Bulgari et al., 2014) and others that cause disease such as bacterial wilt (Osdaghi et al., 2020). Species of interest for food production systems will also need to be screened for their potential as human pathogens, whether opportunistic or otherwise to ensure crew safety. However, it should be noted that the strains analyzed here are already present in the plant growth systems being tended to by astronauts on the ISS.

As research into BLSS continues, further investigation of PGPB will be crucial. The ISS has already shown that current sterilization and sanitization practices have been shown to be ineffective at preventing opportunistic microorganisms from infecting plants, and the stress effects caused by the space environment can be mitigated to improve plant growth and yield. As crops are selected for space food production systems, and sites are selected for colonization on the Moon and Mars, this work will go towards providing the foundation for developing robust and sustainable agricultural systems.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

DH contributed the bulk of experimental design, performed experiments and data analysis and was primary writer. MH, AD and AR contributed equally to determining the scope, experimental design, and were secondary writers. GM is the point of contact for Veggie and helped provide access to key isolates and consulted on experimental design. AP consulted on experimental design, determining the scope of the manuscript, and was a secondary writer.

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# Integration of Nitrifying, Photosynthetic and Animal Compartments at the MELiSSA Pilot Plant

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MELiSSA (Micro Ecological Life Support System Alternative) is developing bioregenerative Life Support technologies for long-term Space missions. The MELiSSA concept is conceived as a loop with several compartments, each one performing a specific function, providing all together edible material production, atmosphere regeneration and water recovery with a concomitant use of wastes, i.e., $CO_2$ and organic wastes. Each one of the compartments is colonized with specific bacteria or higher plants depending on its specific function. The MELiSSA Pilot Plant is a facility designed for the terrestrial demonstration of this concept, hosting laboratory rats as a crew mock-up mimicking the respiration of humans. Currently, the MELiSSA Pilot Plant focus on the integration of three compartments: Compartment 3 (nitrifying packed-bed bioreactor based on the co-culture of immobilized *Nitrosomonas europaea* and *Nitrobacter winogradsky*), compartment 4a (an air-lift photobioreactor for the culture of the edible cyanobacteria *Limnospira indica* with concomitant oxygen production) and Compartment 5 (an animal isolator with rats as mock-up crew). The output from these tests shows a high robustness and reliability and the performance of oxygen producing and oxygen consuming compartments is successfully demonstrated under transitory and steady-state conditions. This contribution reports on the current state of development of the MELiSSA Pilot Plant Facility and the most recent results of the integration work.

**Keywords: Continuous operation, bioreactors, *Limnospira indica*, *Nitrosomonas europaea*, *Nitrobacter winogradsky*, Micro Ecological Life Support System Alternative, Environmental Control and Life Support System**

## INTRODUCTION

The MELiSSA (Micro-Ecological Life Support System Alternative) project focusses on the development and integration of a system providing life support for long-term human missions in space (Mergeay et al., 1988), such as a base on Moon or Mars, and is led by the European Space Agency, in the context of an international consortium with 15 partners (Lasseur et al., 2010). The project approach is inspired in an ecological system by reproducing its main functions in specific compartments (Hendrickx et al., 2006). In the proposed closed loop structure, first, Compartments 1

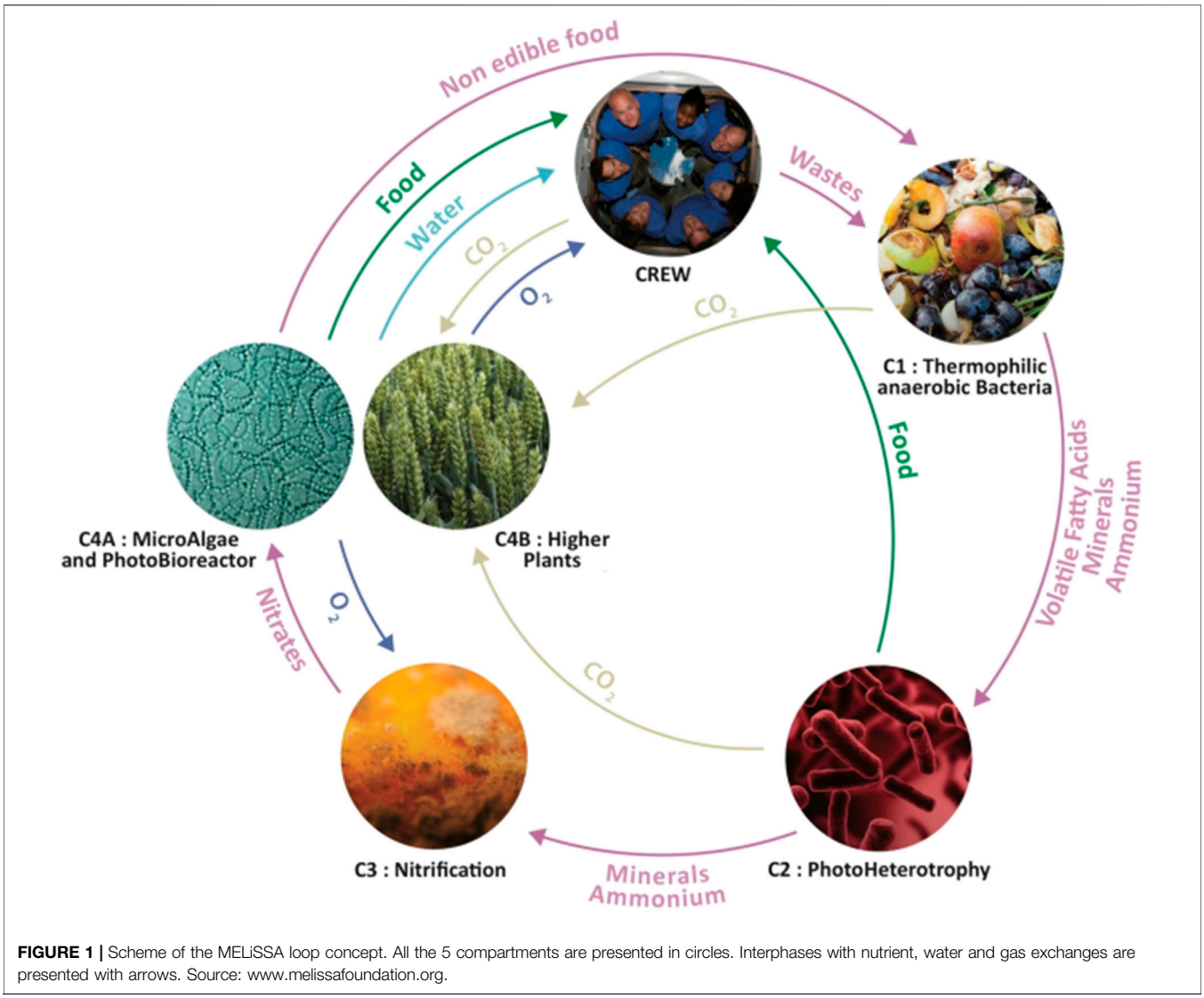


**FIGURE 1 |** Scheme of the MELiSSA loop concept. All the 5 compartments are presented in circles. Interphases with nutrient, water and gas exchanges are presented with arrows. Source: www.melissafoundation.org.

and 2 are dedicated to the degradation of wastes, with a first step based on thermophilic bacteria and a second complementary step of biotransformation of the wastes. Compartment 3 performs nitrification and it is an aerobic bioreactor working with an axenic pure co-culture of *Nitrosomonas* and *Nitrobacter* autotrophic bacteria. This co-culture transforms ammonium ($NH_4^+$) to nitrite ($NO_2^-$) and then to nitrate ($NO_3^-$), the last one being a more assimilable form of nitrogen for the photosynthetic elements of the loop. Some *Nitrospira* related microorganisms have been discovered over the past years that perform complete ammonium oxidation to nitrate in one organism, instead of the two-stage nitrification, such as *Nitrospira inopinata* (Daims et al., 2015). It is considered a promising discovery, but so far their application in a scenario of intensive bioprocessing has not yet been achieved to fulfill the MELiSSA and urine nitrification requirements. Hence, the canonical two-stage nitrification as selected originally for MELiSSA has been maintained. Compartment 4a and 4b are dedicated to oxygen, water and edible biomass production from the consumption of $CO_2$, evapotranspiration and using light as energy source, and they are a photobioreactor (PBR) working with an axenic pure culture of the cyanobacteria *Limnospira indica* and a higher plant compartment for the culture of three representative plants [*Lactuca sativa* (lettuce), *Triticum aestivum* (wheat) and *Beta vulgaris* (red beet)], respectively. Finally, Compartment 5 corresponds to the animal isolator, in which a group of rats is used to mimic the crew. The animals are basically connected to the rest of the loop in the gas phase to demonstrate the atmosphere regeneration to support breathing. A complete illustration of the loop is shown in **Figure 1** Those compartments are operated in continuous mode for long-term periods under controlled conditions, based on the use of mathematical models developed for each compartment, and are connected one to each other through various interfaces (gas, liquid and solid). Overall, the system should provide the essential functions of life support: food production, air regeneration, water reclamation and waste treatment when fully developed, and represents a unique effort in the development of life

support systems enhancing the self-sustainability of human activities in Space.

Taking into consideration the complexity of these proposed system, the MELiSSA Consortium has developed the MELiSSA Pilot Plant (MPP) with the main goal of demonstrating long-term continuous operation feasibility under the supervision of a control system (Gòdia et al., 2004; Poughon et al., 2009) with knowledge-based models that reproduce each compartment's individual characterization and intercompartment dynamics. Thus, in this pilot plant, the different compartments have been scaled-up to achieve the oxygen production equivalent to the respiration needs of one human ($0.84\ kg{\cdot}d^{-1}$) (Wieland, 2005), with 20–40% concomitant production of edible material. The MPP is developed using terrestrial conditions, with the main objective to demonstrate the feasibility of the MELiSSA loop concept, using an industrial approach based on systems engineering, operating under high quality standards, including clean room operation for the area hosting axenic compartments (Compartments 3 and 4a). This dedicated facility to MELiSSA loop demonstration is located at Universitat Autònoma de Barcelona and it is a European Space Agency external laboratory. To note, MELiSSA Consortium is performing in parallel studies to test MELiSSA compartments at Space conditions as well as studies on the application of the MELiSSA technology to Earth in order to foster circular systems.

The MELiSSA Pilot Plant is developed in a stepwise approach. First, the individual compartments have been designed according to the final scenario of the MPP based on the research work done previously to characterize them and develop their mathematical models. Once manufactured and installed in the MPP site, the compartments, associated instrumentation and auxiliary equipment have been operated and fully characterized for a wide range of operational conditions, while testing simultaneously the monitoring and control elements developed for each one of them. Second, the so called integration phase has followed. This phase implies the progressive connection of the compartments, involving gas, liquid and solid phases. Today, the integration work is at the level of connecting Compartment 3 (nitrification), Compartment 4a (photosynthesis) and Compartment 5 (crew compartment), both in gas and liquid phase. The results of the integration are reported in this contribution and have been obtained in three consecutive steps. First, compartments 4a and 5 have been connected in close loop in the gas phase. The results from this first connection have been reported previously (Alemany et al., 2019). Second, compartments 3 and 4a have been connected in the liquid phase. Third, the two previous connections have been combined, so, compartments 5 and 4a connected in gas phase and 3 and 4a connected in liquid phase. Each integration step has been operated for long-term periods (several months of continuous operation) under different operational conditions, including several steady-state conditions and the corresponding transitory phases. In the following, the results obtained in the last two steps of integration are discussed, representing a step forward in the integration of the building blocks that should lead to the demonstration of the MELiSSA loop concept and envisage how it would contribute to human exploration missions in Space.

# MATERIALS AND METHODS

## Nitrifying Reactor (Compartment 3) Description

The nitrification compartment (Compartment 3) is a cylindrical packed-bed bioreactor of 7 L operation volume (SNC-Lavalin, Brussels, Belgium and Bioprocess Technologies, Madrid, Spain). It is packed with polystyrene beads and colonized by a co-culture of *Nitrosomonas europaea* and *Nitrobacter winogradsky* that grow forming a biofilm. *N. europaea* is a Ammonia Oxidizing Bacteria (AOB) capable to oxidize ammonia into nitrite. Then, complete nitrification is achieved by the oxidation of nitrite into nitrate by the Nitrite Oxidizing Bacteria (NOB) *N. winogradsky*. 2 mol of oxygen are needed for the complete oxidation of 1 mol of $N\text{-}NH_4^+$ making it an aerobic process. On top of the central packed-bed section, the reactor has a bottom section, mechanically stirred, where fresh liquid feeding, liquid recirculation inlet and gas sparging take place. There is as well a top section after the packed-bed for gas-liquid separation, and liquid and recirculation outlets. Both top and bottom section hold the instrumentation for on-line monitoring of the compartment (pH, T, $pO_2$, conductivity). It is operated in a recirculation closed gas-loop mode regulated by means of a mass flow-meter (Bronkhorst, F-202D-FA, Bronkhorst, Ruurlo, Netherlands). pH is measured by means of two sterilizable glass pH probe connected to a pH amplifier (Mettler Toledo, Inpro 3253, Greifensee Switzerland). $pO_2$ is measured by means of two Clark amperometric sensor (Mettler Toledo, InPro6950i/12/320, Greifensee, Switzerland) (see **Figure 2**).

## Photobioreactor (Compartment 4a) Description

The photobioreactor compartment (Compartment 4a) is an 83L external-loop gas lift photobioreactor (Bioengineering Ag, Wald, Switzerland). It is used for *Limnospira indica* cultivation. The reactor consists of two glass cylindrical tubes with 15 cm diameter and 1.5 m height with an illuminated volume of 55 L. Illumination is provided by 350 halogen lamps (MR16HM, (12 V, 20 W), Sylvania, Mississuaga, Ontario, Canada) around the glass tubes and can be adjusted by the control system of the loop in order to regulate oxygen production. The upper and lower parts of the tubes are connected by U-shaped stainless steel sections that allow improved liquid circulation and heat exchange through an external jacket. The gas phase is injected through the bottom of the right column (riser) and exits from the gas separation section situated at the top of that same column, which creates a difference of density that allows the fluid to return through the left column (downcomer) (see **Figure 2**).

For continuous operation of the bioreactor during the experiment, the inlet feed medium is pumped by a variable speed gear pump (Lewa, EEC0002S11, Hispania S.L., Leonberg, Germany) followed by a 0.2 μm filtration

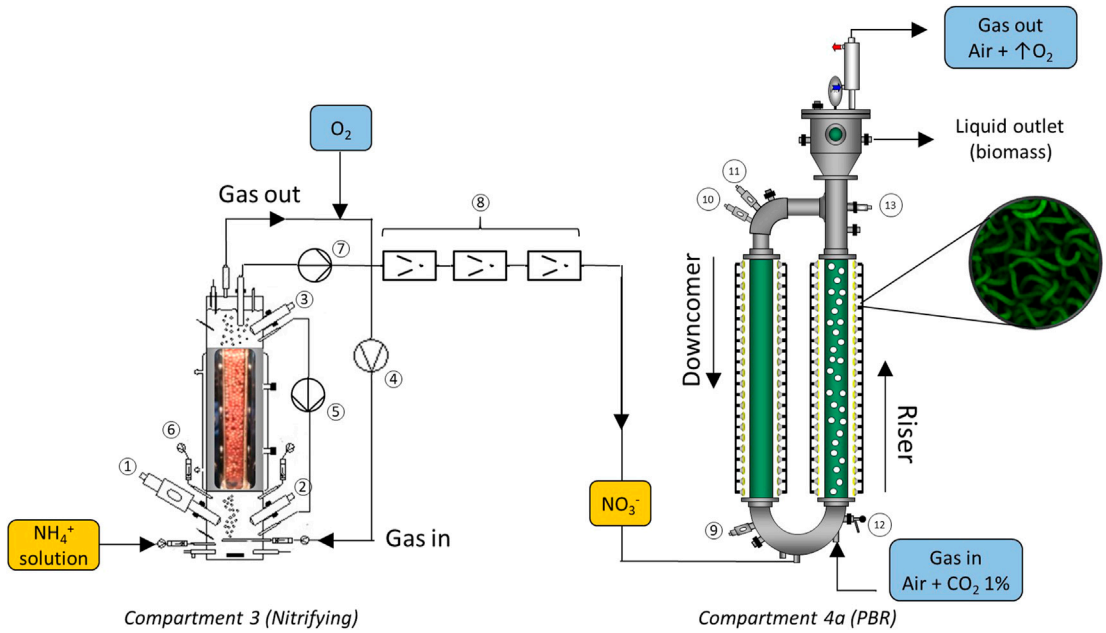


**FIGURE 2 |** Diagram for liquid connection between nitrifying and photosynthetic compartments (3-C4a). Nutrient solution containing ammonium is fed into Compartment 3 where nitrification takes place. The output containing nitrate is pumped into Compartment 4a for photosynthesis. **Nitrifying bioreactor elements:** *1*-pH sensor, 2,3 $pO_2$ sensors, 4-recirculating gas closed-loop, 5-liquid recirculation loop, 6-acid and base additions, 7-outlet pumping system, 8-filtration system to avoid nitrifying bacteria reaching the photobioreactor. **Photobioreactor elements:** 9,10-pH sensors, 11-pO2 sensor, 12-sampling valve, 13-biomass sensor.

(MCY4440DFLPH4, Pall Corporation, New York, United States). Outlet flow is regulated with a second variable speed gear pump (Lewa Hispania S.L., EEC0002S11, Leonberg, Germany) Inlet and outlet air supply is regulated and measured by means of three flow-meters and controllers (Bronkhorst, F-202D-FA, Bronkhorst, Ruurlo, Netherlands). pH is measured by means of a conventional sterilizable glass pH probe connected to a pH amplifier (Mettler Toledo, Inpro 3253, Switzerland). Control of pH is performed by addition of NaOH (2 M) or $H_2SO_4$ (2 M) as required. The composition of the outlet gas is continuously monitored on-line by a IR analyser for $CO_2$ and a paramagnetic analyser for $O_2$ (600 series, CAI, United States).

## Animal Compartment Description

The crew mock-up compartment (Compartment 5) is an animal isolator of 1550 L volume (Hosokawa Micron Ltd., Runcorn, UK). It is divided in three zones: main chamber, transfer air-lock and recirculation loop. It is designed to host the animal crew with artificial light at periods of 12–12 h day-night cycles. It includes six 250 mm round glove ports at the front of the isolator to perform the animal support operations.

In the main chamber, an overpressure of 200 Pa is maintained by pressure control through two pressure transmitters (Rosemount, 2051 CG1,Shakopee, Minnesota, United States). An external 250 L buffer tank is connected to the isolator to compensate the effects of atmospheric pressure changes. Temperature and humidity in the isolator are monitored by the use of two transmitters (Vaisala, HMT337, Helsinki, Finland) and controlled at 22°C and 55%, respectively. A gas recirculation loop ensures a homogenous distribution of air in the isolator using a recirculation flow of 90 $m^3 \cdot h^{-1}$. An active carbon filter is installed (Bi-On® ACPA, Bioconservación, Barcelona, Spain) in order to avoid accumulation of contaminants during long operational periods. $CO_2$ and $O_2$ are measured on-line through an IR and paramagnetic analyzers respectively (SICK-Maihak, GSM810, Hamburg, Germany). Finally, the transfer airlock operates with an independent pressure control to allow the entry and exit of materials (cages, food, bedding, etc.) without affecting the main isolator variables.

Three Wistar rats (Charles River Laboratories, Domaine des Oncins, France) with an age of 12 weeks were selected as the suitable crew model for inhabiting the isolator and running the experiments. The rational for the animals selection has been described previously (Alemany et al., 2019). A different group of rats is used for each experimental condition where different liquid flow rates of the liquid loop are tested. The rats are fed with an *ab libitum* diet (Teckad 2914, Envigo, United States). Weekly monitoring of the rats weight is performed.

## Cell Strains and Culture Media

*Nitrobacter winogradsky* and *Nitrosomonas europaea* were provided by DSMZ, (Germany) for nitrifying bioreactor culture. *Limnospira indica* PCC 8005 was provided by SCK CEN (Mol, Belgium) for PBR cultivation. The strains were grown axenically during the experiment in their corresponding bioreactor. Culture media was a combination of a modified Zarrouk medium (Cogne et al., 2003) and the medium defined in the literature for nitrifying cultures (Pérez et al., 2004):

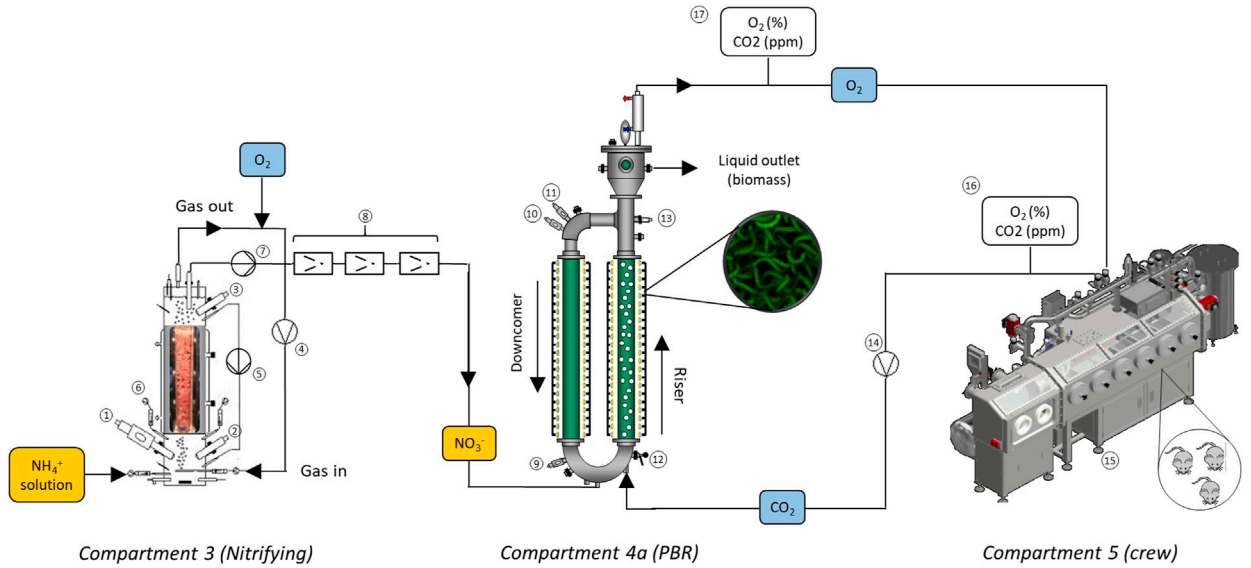


**FIGURE 3 |** Diagram for liquid connection between intriguing and photosynthetic compartments (C3-C4a), and gas connection between compartment 5 (animal isolator) and compartment 4a. The gas phase from the PBR (rich in O2) is send to the isolator. Then, the gas rich in CO2 is pumped back to the PBR. Compartment elements: see **Panel 2** for elements from 1 to 13, 14-gas connection line between C4a and C5, 15-animal isolator, 16-gas analyser in C5, 17-gas analyser in C4a outlet.

1.358 g·L$^{-1}$ of $(NH_4)_2SO_4$, 1 g·L$^{-1}$ of $K_2SO_4$, 1 g·L$^{-1}$ of NaCl, 0.816 g·L$^{-1}$ of $NaHCO_3$, 0.71 of Na2HPO4, 0.68 of KH2PO4, 0.5 of K2HPO4, 0.1 g·L$^{-1}$ of $MgSO_4{\cdot}7H_2O$, 0.18 g·L$^{-1}$ of $(NH_4)_6Mo_7O_2{\cdot}4H_2O$, 0.08 g·L$^{-1}$ EDTA-2Na·2$H_2O$, 0.01 g·L$^{-1}$ of $FeSO_4{\cdot}7H_2O$, 0.04 g·L$^{-1}$ of $CaCl_2{\cdot}2H_2O$, 1.81 mg·L$^{-1}$ of $MnCl_2{\cdot}4H_2O$, 0.22 mg·L$^{-1}$ of $ZnSO_4{\cdot}7H_2O$, and 0.079 mg·L$^{-}$1 of $CuSO_4{\cdot}5H_2O$.

## Liquid Connection Between Compartment 3 and 4a

A liquid stream was constantly supplied from a reservoir tank to the inlet of the nitrifying bioreactor. Liquid effluent from nitrifying bioreactor was constantly processed by a three-step filtration to maintain axenicity in both bioreactors. This was a critical operation to avoid any cells detached from Compartment 3 biofilm reaching Compartment 4a, with the associated contamination effects. The effluent from nitrifying bioreactor was pumped to the inlet of the PBR with a gear pump (Lewa, EEC0002S11, Hispania S.L., Leonberg, Germany). A variable peristaltic pump (Watson-Marlow 323Du, Watson Marlow Fluid Technology Group, Wilmington, United States) at the nitrifying bioreactor outlet was used to keep an overpressure in the liquid connection line at 200 mbarg. Finally, the PBR effluent was continuously collected in a harvesting tank (see **Figure 2**).

A set of different liquid flow rates corresponding to three $NH_4^+$ loads (ppm·d$^{-1}$) into compartment 3 were initially tested: 10, 20 and 30 L·d$^{-1}$. A combination of testing conditions was defined by setting two illumination levels ($q_o$): 120 and 285 W·m$^{-2}$. So, a total of six conditions were tested. Operational conditions in the PBR were stablished as follows: pH was set at nominal value of 8.5, $CO_2$ was constantly injected at 1% composition in the air inlet at a total gas flow of 2.8 L·min$^{-1}$ and temperature was set at 36°C. Conditions in Compartment 3 were as follows: pH was controlled at 8.1 by the addition of $H_2SO_4$ (0.1 M) and KOH (1.9 M), pO2 was maintained at 80% thanks to the injection of external pure $O_2$ into the recirculation closed gas-loop, a recirculation liquid flow between the top and bottom section of the bioreactor was set at 4.5 L·h$^{-1}$ to improve the homogeneity and temperature was set at 30°C.

## Gas Connection Between Compartments 4a and 5

The animal isolator was connected to the PBR at the entrance and output of the compartment so that closed gas loop was established by using a diaphragm vacuum pump (GAST, 22D1180-202-1005, Benton Harbor, Michigan, United States).

After completion of previous tests, involving Compartments 3 and 4a in the liquid phase, a new series of experiments was carried out. Now, the animal isolator was connected in the gas phase to the PBR, and liquid connection between nitrifying bioreactor and PBR was maintained (see **Figure 3**). Therefore, the three compartments were connected. In this scenario, the liquid flow-rate was increased to 20, 30 and 40 L·d$^{-1}$ and the N-$NH_4^+$ concentration was maintained at 300 ppm at 20 and 30 L·d$^{-1}$, while it was decreased to 240 ppm at 40 L·d$^{-1}$. The main control variable of these experiments was the light intensity of the PBR. The control system adjusted the light intensity to maintain an $O_2$ concentration set-point in the animal isolator and compensating the animals oxygen needs along the day/night cycles. For each liquid loop flow rate condition, the $O_2$ set-point in the isolator was sequentially changed following this

sequence: 21–19-20–21%. The rationale between this operation schedule was to test the capacity of the control system to adjust the illumination conditions in the PBR each time a new $O_2$ set-point was fixed in the animal isolator. Additionally, the experimental period at a given flow rate between the nitrifying bioreactor and PBR coincided with a new group of rats in the isolator.

## Biomass Concentration Determination

Off-line monitoring of biomass in the PBR was carried out by cell dry weight (CDW) determination and OD measurement. For CDW, 25–50 ml of culture broth were filtered through a 47 mm-glass microfiber (GMFC-52047, Scharlab, Barcelona, Spain), dried at 105°C until constant weight. OD measurement was performed at 750 nm in an optical spectrophotometer (UV-VIS DR6000, Hach, Düsseldorf, Germany).

## TIC Total Inorganic Carbon and Nitrogen Determination

Filtered fractions for biomass determination in the PBR were used for $NO_3^-$ and TIC analysis. Samples were diluted 1:100 with milli-Q $H_2O$ and $NO_3^-$ was analysed by spectrophotometry with LCK 339 kits (Hach, Weinheim, Germany). For TIC determination a 1:5 dilution with milli-Q H2O was required. 1.5 ml of sample were loaded to a multi N/C2100S equipment (Analytik Jena, Jena Germany). 200 µL from the sample were injected into the acidic TIC reactor and 0.5 ml of $H_3PO_4$ at 10% were added to the chamber. $CO_2$ generated from the acidification was detected by the NDIR detector and quantified by triplicate.

In Compartment 3, $NO_3^-$ at the bioreactor outlet was analysed following the same method as in Compartment 4a. $NH_4^+$ and $NO_2^-$ were analysed with LCK 303/304 and LCK 342 (Hach, Weinheim, Germany).

## Gas Analysis, $O_2$ Production and $CO_2$ Consumption

Outlet gas composition from the PBR was measured on-line by an IR analyser for $CO_2$ coupled to a paramagnetic analyser for $O_2$ (600 series CAI, Orange, California, United States). In the case of Compartment 4a and 3 liquid connection, oxygen volumetric production rate ($g{\cdot}L^{-1}{\cdot}h^{-1}$) was calculated on-line based on continuous monitoring of outlet gas composition in the PBR:

$$r_{O2} = ((F_{in} - F_{CO2}) \cdot 0.2089 - (F_{out} \cdot O_{2out}/100))/(22.4 \cdot MW_{O2} \cdot VR) \quad (1)$$

where $F_{in}$ is the inlet air flow rate, $F_{CO2}$ is the inlet $CO_2$ flow rate, $F_{out}$ is the total outlet gas flow rate, $O_{2out}$ is the outlet mole fraction of $O_2$ in %, $MW_{O2}$ is the molecular weight of $O_2$ ($32\ g{\cdot}mol^{-1}$). The flow rates are expressed at normal conditions (T = 273.15 K; $p$ = 1 atm). In the case of the second integration step (Compartments 5 and 4a connected in the gas phase and 3 and 4a connected in the liquid phase), the inlet gas composition of PBR is analysed by means of the gas analyser in compartment 5. Then, oxygen volumetric production rate in PBR is calculated as follows:

$$r_{O2} = ((F_{in} \cdot O_{2in} - (F_{out} \cdot O_{2out}/100))/(22.4 \cdot MW_{O2} \cdot VR) \quad (2)$$

where $O_{2in}$ is the inlet mole fraction of $O_2$ (%) in the PBR.

PBR $CO_2$ consumption was calculated for each condition considering on-line gas analysis and off-line TIC concentration in the steady state. In such situation, no carbon accumulation takes place and $CO_2$ can be calculated in $g{\cdot}L^{-1}{\cdot}h^{-1}$ by means of the following mass balance:

$$r_{CO_2} = CO_{2Loutlet} - (CO_2 \text{gas transfer}) \quad (3)$$

$$r_{CO_2} = C_{Lout} \cdot D + F_{in}(X_{COUT} - X_{CIN})/22.4 \cdot MW_{CO_2} \quad (4)$$

where $C_{LOUT}$ is the carbon concentration ($g{\cdot}L^{-1}$) in the liquid outlet converted into $CO_2$ by means of molecular weight, $D$ is the dilution rate ($h^{-1}$), $F_{in}$ ($L{\cdot}h^{-1}$) is the bioreactor gas flow rate, $X_{COUT}$ is the molar fraction of $CO_2$ in the outlet gas, $X_{CIN}$ is the molar fraction of $CO_2$ in the inlet gas and $MW_{CO2}$ is the molecular weight of $O_2$ ($44\ g{\cdot}mol^{-1}$). When PBR and animal compartment where connected in the gas phase, $X_{CIN}$ was the corresponding $CO_2$ concentration in the animal compartment.

Considering that $O_2$ was the controlled variable in the animal compartment, $O_2$ consumption in animal compartment was assumed to be the same as $O_2$ production in the PBR. In the case of $CO_2$ consumption ($g{\cdot}h^{-1}$) of the rats, calculation was as follows:

$$r_{CO_2} = F_{in}(X_{COUT5} - X_{CIN5})/22.4 \cdot MW_{CO_2} \quad (5)$$

where $X_{COUT5}$ and $X_{CIN5}$ are the molar fractions of $CO_2$ at the outlet and inlet of animal compartment respectively.

## Statistical Analysis

One-way ANOVA analysis assuming differences in variances was performed to assess variability of $O_2$ generation of the system. This statistical approach was also used to study the differences in body weight of the animals. A significance value of $p < 0.05$ was considered in all cases.

# RESULTS

## Compartment 3 and 4a Liquid Connection

Nitrifying bioreactor and PBR are connected in the liquid phase in continuous operation mode for a total period of 276 days. The nitrification compartment provides the necessary nitrogen source for the growth of cyanobacteria (Clauwaert et al., 2017) A total of six different conditions were tested corresponding to three different ammonium loads: 435, 870, and 1300 $ppm{\cdot}d^{-1}$ into Compartment 3 at 10, 20, and 30 $L{\cdot}d^{-1}$ respectively. These flow rate conditions are combined with two levels for $q_o$ in the PBR: 120 and 285 $W{\cdot}m^{-2}$.

**Figures 4D–F** presents the operation profile of the PBR in terms of oxygen production and biomass cell density for the complete series of experimgents. In all the tested conditions steady state in the PBR is achieved as it is maintained with stable parameters for a period of at least 3 Hydraulic Residence

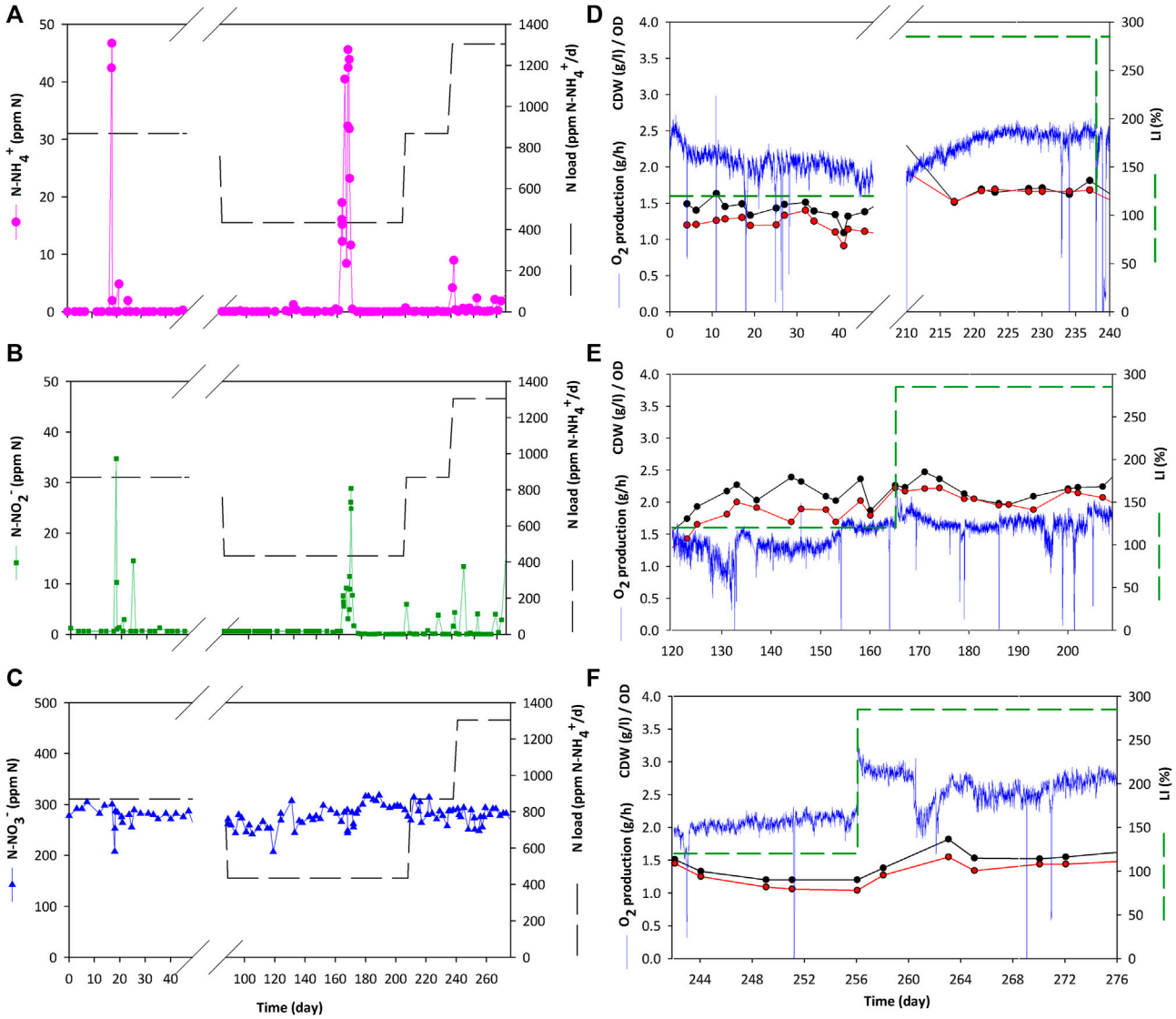


**FIGURE 4 |** Results for integration experiments between nitrifying and photosynthetic compartment. Panels **(A–C)** show the nitrogen species at the nitrification bioreactor outlet. N-NH4+ concentration is presented in dotted pink points. N-NO2- is presented in square green points. And N-NO3- is displayed in blue triangle points. Panels **(E–F)** illustrates the oxygen production and biomass content in the PBR for different combinations of light and liquid flow rate [**(D)** 20 $L{\cdot}d^{-1}$; **(E)** 10 $L{\cdot}d^{-1}$; **(F)** 30 $L{\cdot}d^{-1}$]. Light intensity is presented in dashed green line, O2 production is showed in solid blue lines, biomass related parameters are displayed in black and red dotted points for CDW and OD respectively.

Times (HRT) for each condition. The highest biomass concentration is obtained for 10 $L{\cdot}d^{-1}$ condition (**Figure 4E** with values between 2.1 and 2.2 $g{\cdot}L^{-1}$, while the lowest cell density is reached at 30 $L{\cdot}d^{-1}$ (**Figure 4F**) with values between 0.9 and 1.1 $g{\cdot}L^{-1}$. On the contrary, the highest biomass productivity results from 30 $L{\cdot}d^{-1}$ conditions, followed by 20 and 10 $L{\cdot}d^{-1}$ respectively. On-line oxygen production monitoring is also presented in **Figure 4**. The dynamics of the system can be observed for each $q_o$ increase followed by a stabilisation profile. The highest oxygen production is reached at 30 $L{\cdot}d^{-1}$ condition (between 2.1 and 2.7 $g{\cdot}h^{-1}$) while the lowest production occurs at 10 $L{\cdot}d^{-1}$ (1.4–1.7 $g{\cdot}h^{-1}$). The same pattern is also observed from specific production values ($q_{O2}$). Detailed data are presented in **Table 1**. **Figure 5** reflects the influence of light and flow in the performance of the PBR in terms of oxygen and biomass production. A Pareto's ANOVA was performed to study the influence of those factors. The liquid flow is the factor showing the greatest effect on biomass and oxygen production. The influence of flow on biomass concentration is 85%, while it is 69% for oxygen production. In contrast, light intensity shows little influence in biomass concentration (4%), while its contribution increases up to 21% regarding oxygen production. The interaction effect between factors in both cases is at 11 and 10% respectively.

Values from carbon dioxide consumption in the PBR are also presented in **Table 1**, showing that carbon dioxide consumption

**TABLE 1 |** Tested conditions and results in PBR for liquid connection between Compartment 3 (nitrification) and 4 (PBR).

| Condition | Inlet $NH_4^+$ (N-ppm) | N-$NH_4^+$ load ($ppm \cdot d^{-1}$) | $q_o$ ($W \cdot m^{-2}$) | Liquid flow ($L \cdot d^{-1}$) | CDW ($g \cdot l^{-1}$) | $Prod_X$ ($g \cdot h^{-1}$) | $Prod_{O2}$ ($g \cdot h^{-1}$) | $qO_2$ ($mmol \cdot g^{-1} \cdot h^{-1}$) | $Cons_{CO2}$ ($g \cdot h^{-1}$) | $qCO_2$ ($mmol \cdot g^{-1} \cdot h^{-1}$) | $Q_P$ ($mol_{O2} \cdot mol_{CO2}^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| #1 | 300 | 870 | 120 | 20 | 1.41 ± 0.12 | 1.18 ± 0.10 | 2.03 ± 0.15 | 0.54 | −1.55 | −0.30 | 1.80 |
| #2 | | 435 | 120 | 10 | 2.24 ± 0.15 | 0.93 ± 0.06 | 1.43 ± 0.18 | 0.24 | −1.51 | −0.18 | 1.30 |
| #3 | | | 285 | 10 | 2.15 ± 0.15 | 0.89 ± 0.06 | 1.6 ± 0.14 | 0.30 | −1.61 | −0.21 | 1.44 |
| #4 | | 870 | 285 | 20 | 1.70 ± 0.07 | 1.42 ± 0.06 | 2.44 ± 0.13 | 0.54 | −1.95 | −0.31 | 1.72 |
| #5 | | 1300 | 120 | 30 | 1.20 ± 0.02 | 1.50 ± 0.02 | 2.11 ± 0.08 | 0.66 | −1.70 | −0.39 | 1.71 |
| #6 | | | 285 | 30 | 1.50 ± 0.08 | 1.87 ± 0.10 | 2.76 ± 0.10 | 0.69 | −2.33 | −0.43 | 1.63 |

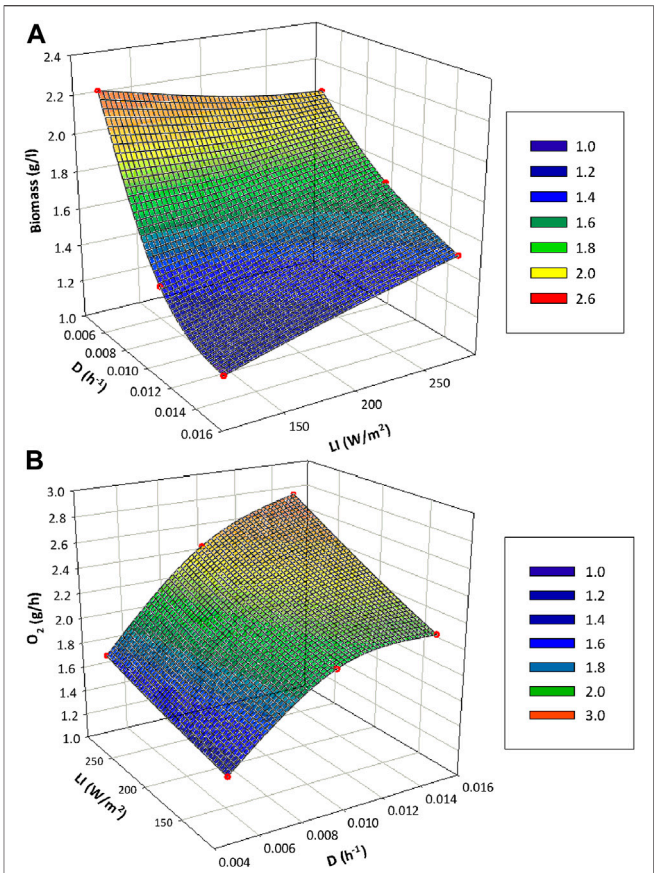


**FIGURE 5 |** Influence of light and liquid flow rate in the performance of the PBR in terms of biomass content **(A)** and oxygen production **(B)**. The individual point data is interpolated to reach the mesh representation.

by *L. indica* increases in correspondence with oxygen production. 2.3 g $CO_2 \cdot h^{-1}$ are consumed when the cells are exposed to the maximum $q_o$ of 285 $W \cdot m^{-2}$ at a continuous flow of 30 $L \cdot d^{-1}$. The minimum carbon dioxide consumption is found at the lowest flow rate of 10 $L \cdot d^{-1}$ and minimum light intensity (120 $W \cdot m^{-2}$). Photosynthetic quotient ranges between 1.3 and 1.8 $molO_2 \cdot molCO_2^{-1}$, which is in agreement with theoretical values between 1.2 and 1.4 when $NO_3^-$ is used as nitrogen source according to the stoichiometry (Cornet, 2007).

In terms of N balance, all the nitrogen supplied to compartment 4 is in form of $NO_3^-$ previously converted from $NH_4^+$ in compartment 3. The ammonium conversion is maintained at 99% during most of the experiment (**Figures 4A–C**). Only minor transitory peaks occur because of operation activities in the bioreactor focused on biomass removal with an external circulation system coupled with filtration for biomass retention. During these short periods the maximum N-$NH_4^+$ and N-$NO_2^-$ concentration in the PBR is 0.1 and 1 ppm respectively (data not shown) and therefore can be considered negligible. The outlet N-$NO_3^-$ concentration from the nitrifying bioreactor is between 275 and 300 N-ppm (**Table 2**). Yield values are higher than 90%, whilst conversion of ammonium is at 99% during all steady states (transitory peaks

**TABLE 2 |** Nitrogen balances observed in nitrifying bioreactor for every experimental condition during liquid connection experiments between nitrifying and photosynthetic compartment. The outlet of $NO_3^-$ from nitrifying bioreactor is considered as the inlet concentration in the PBR.

| | Nitrifying compartment | | | | | | PBR | | |
|---|---|---|---|---|---|---|---|---|---|
| **Cond** | **Inlet $NH_4^+$ (N-ppm)** | **Load (N-ppm·d$^{-1}$)** | **$NO_3^-$ outlet (N-ppm)** | **$NH_4^+$ outlet (N-ppm)** | **$NH_4^+$ removal (%)** | **Conversion (%)** | **NO3- outlet (N-ppm)** | **$Y_{N/X}$ (g·g$^{-1}$)** | **$Y_{N/O2}$ (g·g$^{-1}$)** |
| #1 | 300 N-$NH_4^+$ | 870 | 274.8 | 0.01 | 100.00 | 91.6 | 116.06 | 0.11 | 0.065 |
| #2 | | 435 | 288.5 | 0.05 | 99.98 | 96.2 | 66.35 | 0.10 | 0.064 |
| #3 | | | 297.7 | 0.02 | 99.99 | 99.2 | 65.71 | 0.11 | 0.058 |
| #4 | | 870 | 279.1 | 0.10 | 99.97 | 93.0 | 95.15 | 0.11 | 0.063 |
| #5 | | 1300 | 274.7 | 0.20 | 99.93 | 91.6 | 152.80 | 0.10 | 0.072 |
| #6 | | | 276.8 | 1.00 | 99.67 | 92.3 | 121.60 | 0.10 | 0.070 |

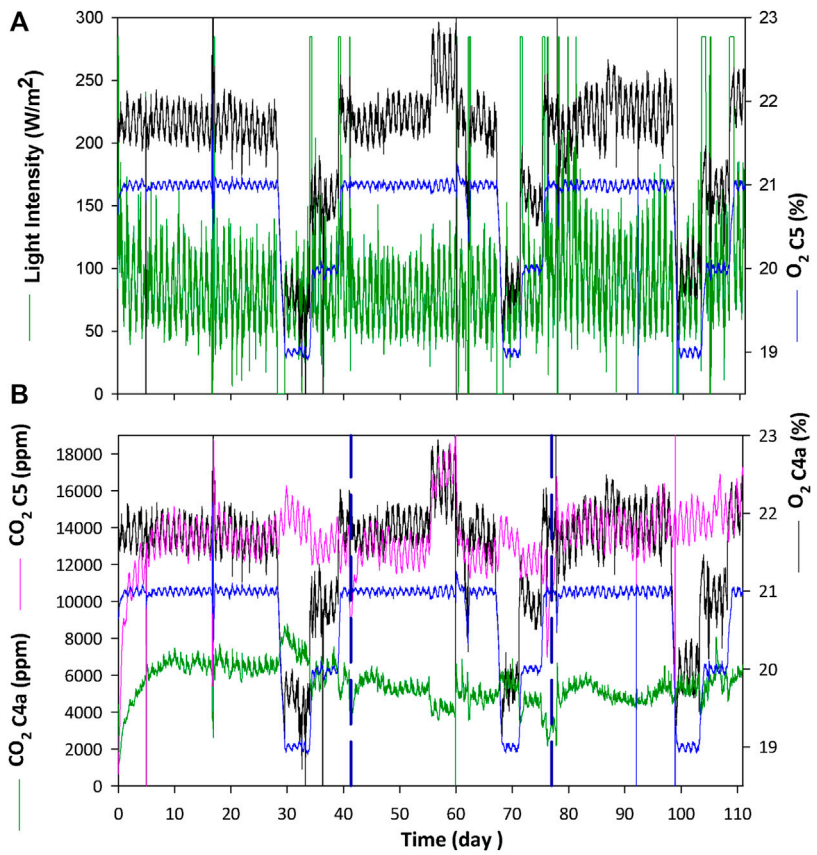


**FIGURE 6 |** Gas phase results for the gas interface connection between the PBR and the crew compartment during the experimental period of 111 days. The same oxygen set-point conditions in the crew compartment are repeated for each liquid flow rate tested in the PBR, which are indicated by vertical dashed lines in graph **(B)**. Graph **(A)** focuses on the oxygen profile between compartments and light intensity in the PBR (**Green:** light intensity in PBR; **Blue:** $O_2$% in animal isolator; **Black:** $O_2$% at the PBR outlet). Graph **(B)** shows the behaviour of carbon dioxide composition (**Green:** CO2 at PBR outlet; **Pink:** CO2 in Animal isolator; **Blue:** $O_2$% in animal isolator; **Black:** $O_2$% at the PBR outlet).

are not considered). The yield is higher (96–99%) with the lowest ammonium load of 435 N-ppm·d$^{-1}$, but the yield decreases (91–93%) when the load increases to 870 and 1300 ppm·d$^{-1}$. Inlet N-$NH_4^+$ into nitrifying bioreactor was maintained constant at 300 N-ppm for all conditions, resulting in different ammonium loads depending on the liquid flow rate. The output from nitrifying bioreactor was the inlet of the PBR, so $Y_{N/X}$ was calculated considering that input. Results show that $Y_{N/X}$ is maintained constant no matter the experimental condition used. 0.10–0.11 g of N-$NO_3^-$ are needed to generate 1 g of biomass. In terms of oxygen as a product, $Y_{N/O2}$ is between 0.06 and 0.07 g·g$^{-1}$ in all tested conditions. The yields obtained fit

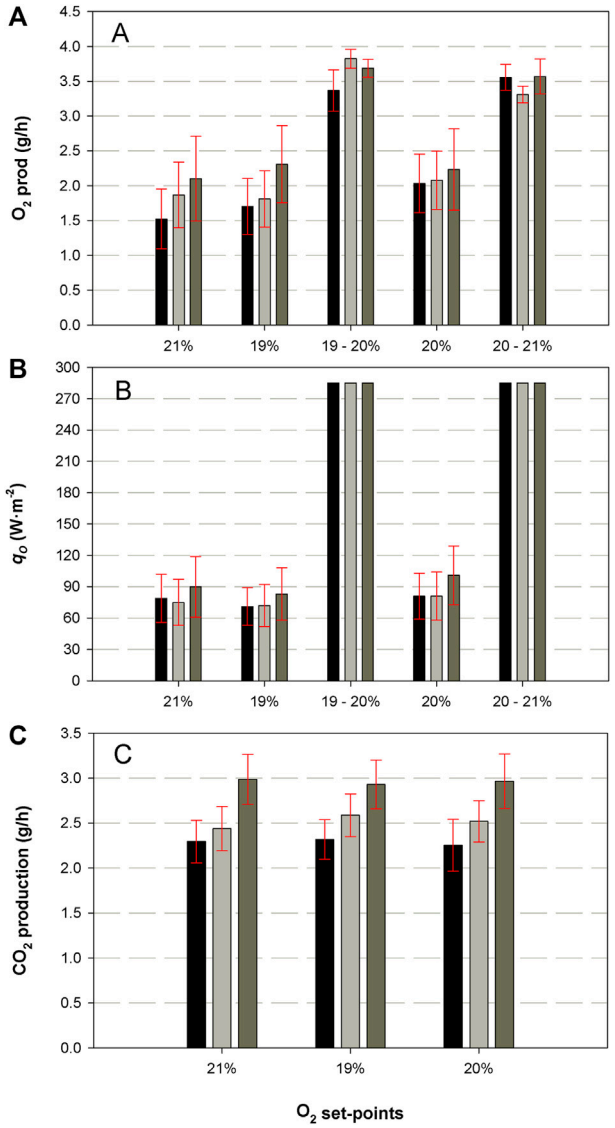


**FIGURE 7 |** Results for oxygen production **(A)** and light irradiance **(B)** in the PBR during the integration with the crew compartment. Carbon dioxide production by the crew compartment are presented in **(C)**. These values are presented for each $O_2$% set-point concentration in the animal isolator. Black, grey and brown columns correspond to the first, second and third group of rats respectively.

with the reaction stoichiometry described in previous works (Cornet et al., 1998).

## Compartment 3 and 4 Liquid Connection Combined with Compartment 4a and 5 Gas Connection

### Gas Phase Performance

The connection between the PBR and the animal isolator was conducted during 110 days Three female Wistar rats were connected in the gas phase with the PBR, which provided the oxygen needed in the animal isolator to maintain the oxygen atmospheric concentration at the target value (21–19 to 20–21%). On the other hand, the carbon dioxide produced by the rats was supplied to the PBR to cover the carbon needs for photosynthetic activity. In parallel, the nitrogen needs of the PBR were supplied by the nitrifying bioreactor after complete removal of ammonium and conversion into nitrate. Different liquid flows between nitrifying bioreactor and the PBR (20-30-40 L·d$^{-1}$, each one with a different group of rats in the animal isolator for age reasons) were tested in order to assess the effect of hydraulic changes in the PBR.

**Figure 6A** provides the gas phase evolution of the animal isolator and the PBR. The oxygen concentration of the isolator is controlled first at the set-point of 21% following the sequential changes (21–19 to 20–21%) for each animal group. A repetitive cyclic pattern in the oxygen concentration profile is observed, caused by the circadian activity (day and night cycles) of the rats. Light irradiance in C4a changes likewise to adapt the oxygen production to the animals needs. Changes in oxygen set-point concentration are made when 6 HRT are reached in the PBR. During oxygen set-point decrease (from 21 to 19%), the control system turns off the lights and it takes 30, 26 and 20 h for the first, second and third group of rats respectively to reach the target value. In transition periods where the oxygen set-point is increased from 19 to 20%, the lights are set by the control system to the maximum intensity (285 W·m$^{-2}$) and it takes 8, 7.8 and 19.5 h for each group respectively to reach the target set-point. Finally, when oxygen set-point is increased from 20 to 21% the transition takes 8.5, 8.3 and 19.8 h respectively. **Figure 6B** focuses in the carbon dioxide concentration of the animal isolator and the PBR. Carbon dioxide in the animal isolator is kept between 12,000 and 16,000 ppm (1.2–1.6%) during all the test, except for the period between day 55–60, when it reaches 18,000 ppm because of a perturbation in the gas flow exchange between the PBR and animal isolator. However, normal values are recovered afterwards. The cyclic biological activity of the rats during day and night is also observed in carbon dioxide dynamics.

Results for oxygen production in the PBR, carbon dioxide production by the crew and light intensity for each group of animals and for each oxygen concentration in the isolator are presented in **Figures 7A–C**. The average oxygen needs of the rats are different for each group and consequently the oxygen production. The average oxygen production by the PBR is 1.5, 1.9 and 2.1 g·h$^{-1}$ for the first, second and third group respectively during the 21% set-point. The same pattern is observed for 19 and 20% concentration. Regarding carbon dioxide generation of the rats. 2.3, 2.4 and 3.0 g·h$^{-1}$ are produced by each group at 21% $O_2$ concentration. RQ of the rats at 21% is 1.0, 0.9 and 0.97 $mol_{O2}{\cdot}mol_{CO2}{}^{-1}$ for each group (data not shown). Average light intensity ($\overline{q_o}$) provided by the control system to the PBR does not correlate with oxygen production as $\overline{q_o}$ in the second group (75 W·m$^{-2}$) is lower than in the first group (79 W·m$^{-2}$). During the third group $\overline{q_o}$ increases to 90 W·m$^{-2}$. Focusing on transition periods (19–20% and 20–21%) where the $\overline{q_o}$ is at maximum, the oxygen generation in the PBR are statistically different in both transitions ($p < 0.05$). During the

**TABLE 3 |** PBR performance results in terms of $O_2$ and $CO_2$ generation and biomass concentration reached for every condition. $CO_2$ uptake is only expressed at 21% $O_2$ set-point as liquid phase is not stable at 19 and 20% leading to variability in TIC concentration.

| Rat's group – Liquid flow | $O_2$ SP (%) | $q_o$ ($W \cdot m^{-2}$) | X ($g \cdot L^{-1}$) | $O_2$ prod ($g \cdot h^{-1}$) | $CO_2$ uptake ($g \cdot h^{-1}$) | $qO_2$ ($mmol \cdot g^{-1} \cdot h^{-1}$) | $qCO_2$ ($mmol \cdot g^{-1} \cdot h^{-1}$) | PQ ($rO_2 \cdot rCO_2^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| #1 | 21 | 79 | 1.24 | 1.52 | 2.18 | 0.46 | 0.48 | 0.96 |
| — | 19 | 71 | 1.07 | 1.70 | — | 0.60 | — | — |
| 20 L/d | 20 | 78 | 1.22 | 1.81 | — | 0.63 | — | — |
| #2 | 21 | 75 | 0.98 | 1.87 | 1.92 | 0.72 | 0.54 | 1.33 |
| — | 19 | 72 | 0.77 | 1.81 | — | 0.89 | — | — |
| 30 L/d | 20 | 81 | 0.89 | 2.08 | — | 0.88 | — | — |
| #3 | 21 | 90 | 0.81 | 2.10 | 2.08 | 0.98 | 0.71 | 1.39 |
| — | 19 | 83 | 0.72 | 2.31 | — | 1.21 | — | — |
| 40 L/d | 20 | 101 | 0.83 | 2.23 | — | 1.01 | — | — |

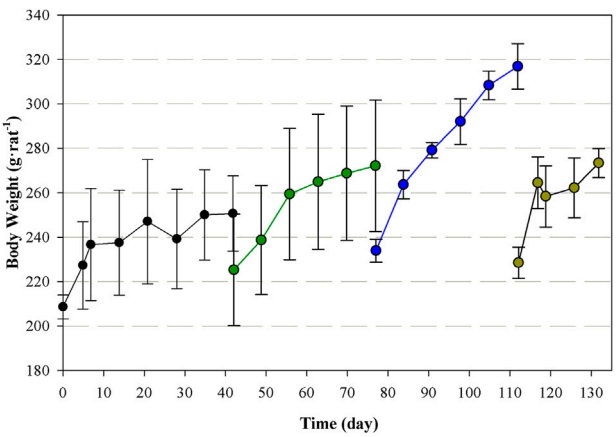


**FIGURE 8 |** Body weight for each group of rats (**first:** black; **second:** green; **third:** blue) during their corresponding experimental period. The last group of rats **(brown)** corresponds to the control group.

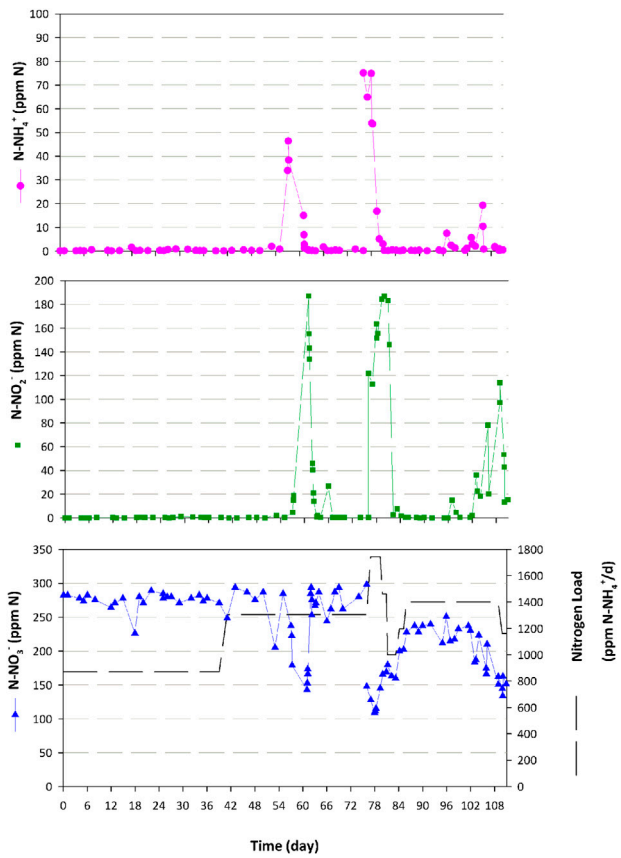


**FIGURE 9 |** Nitrogen species at the nitrification compartment outlet during the integration experiments where Compartment 3 and 4a are connected in the liquid phase and Compartment 4a and 5 in the gas phase. [**Dotted pink:** $N\text{-}NH_4^+$ (ppm); **Square green:** $N\text{-}NO_2^-$ (ppm); **Triangle blue:** $N\text{-}NO_3^-$ (ppm)].

transition from 19 to 20%, the oxygen production is 3.4, 3.8 and 3.7 g·h-1 for the first, second and third groups respectively. In the second transition step the oxygen generation is 3.6, 3.3 and 3.6 $g \cdot h^{-1}$.

**Table 3** shows the results obtained in the PBR. Biomass concentration is different depending on the group (and flow) tested. In the steady state, which takes place at 21% oxygen set-point, the highest concentration is 1.24 $g \cdot L^{-1}$ when the system works at 20 $L \cdot d^{-1}$. The lowest concentration, 0.81 $g \cdot L^{-1}$ is observed in the last group of rats when inlet liquid flow rate in the PBR is 40 $L \cdot d^{-1}$. Changes within the same group are also observed for each oxygen set-point. Carbon dioxide uptake is only presented at 21% set-point condition as stability of TIC concentration in the liquid phase of the PBR is only achieved at 21% after 6 HRT. Maximum carbon dioxide demand is 2.18 $g \cdot h^{-1}$ at 20$L \cdot d^{-1}$ (first group). Similar uptake is observed for the other tested groups (1.92 and 2.08 $g \cdot h^{-1}$). Specific oxygen production rates and carbon dioxide consumption rates ($q_{O2}$ and $q_{CO2}$) increases for each group of rats sequentially, indicating a higher efficiency in oxygen production, but also a higher carbon dioxide demand. PQ ranges between 1 and 1.4.

**TABLE 4 |** Nitrogen balances observed in nitrifying bioreactor and photobioreactor for every experimental condition during liquid connection between nitrifying and photosynthetic compartments and gas connection between photosynthetic and crew compartment. The outlet of $NO_3^-$ from nitrifying bioreactor is considered as the inlet concentration in the PBR.

| | Nitrifying compartment | | | | | PBR | | |
|---|---|---|---|---|---|---|---|---|
| Liquid flow ($L \cdot d^{-1}$) | Load ($N\text{-}ppm \cdot d^{-1}$) | $NO_3^-$ intlet (N-ppm) | $NO_3^-$ outlet (N-ppm) | $NH_4^+$ removal (%) | Conversion (%) | $NO_3^-$ outlet (N-ppm) | $Y_{N/X}$ ($g \cdot g^{-1}$) | $Y_{N/O2}$ ($g \cdot g^{-1}$) |
| 20 | 870 | 300 | 274.8 | 99 | 91.6 | 160 | 0.09 | 0.064 |
| 30 | 1,304 | 300 | 289 | 99 | 96.3 | 202 | 0.09 | 0.059 |
| 40 | 1,400 | 241 | 227 | 99 | 94.2 | 155 | 0.09 | 0.057 |

Finally, the daily monitoring of the rats (**Figure 8**) shows that the weight gain differs between groups. Average weight gain of the first group is 0.77 $g \cdot d^{-1} \cdot rat^{-1}$, followed by the second group (1.35 $g \cdot d^{-1} \cdot rat^{-1}$) and the third group (1.30 $g \cdot d^{-1} \cdot rat^{-1}$). The fourth group of rats, which corresponds to the control group, showed a weight gain of 1.75 $g \cdot d^{-1} \cdot rat^{-1}$. Pearson's correlation shows a positive correlation between the starting weight of the rats and the weight gain rate ($r = 0.95$; $p < 0.05$). All the animal groups did not show any relevant alteration in their physiological parameters, behaviour or clinical evaluation. Food and water intake are normal in all the groups (data not shown).

### Liquid Phase Results

As mentioned, nitrogen in form of $NO_3^-$ was provided to the PBR through the liquid phase, which was connected to the outlet of the nitrifying bioreactor. Nitrification profile during the whole experiment is presented in **Figure 9**. Ammonium conversion is maintained at 100% and no accumulation of nitrite nor ammonium takes place during the first hydrodynamic condition (20 $L \cdot d^{-1}$) at 870 $ppm \cdot d^{-1}$. In the second experimental period (30 $L \cdot d^{-1}$) a peak of nitrite (187 N-ppm) and ammonium (46 N-ppm) is measured at the outlet of nitrifying bioreactor on day 60, probably caused by the biomass removal in the packed-bed as a nominal operation (excess biomass accumulation is removed periodically from the packed-bed to avoid clogging). Recovery of the complete nitrification takes place within 6 days. In the last hydraulic condition (40 $L \cdot d^{-1}$) the tested nitrogen load of 1740 results in ammonium (78 N-ppm) and nitrite (186 N-ppm) accumulation at the bioreactor outlet, therefore showing that the maximum load capacity was achieved. Then, the system is recovered after decreasing the nitrogen load to 1400 $ppm \cdot d^{-1}$, reaching total nitrification as in previous conditions. Finally, at the end of the same hydraulic condition, a third and last nitrite pick (113 N-ppm) occurs due to a second operation to control biomass in the bioreactor to avoid clogging. Those peaks are translated in a slight presence of nitrite in the PBR: maximum concentration of 78, 58 and 36 N-ppm respectively. No presence of ammonium is detected in the PBR. In the first and second cases, the presence of nitrite in the PBR is translated into a light intensity increase reaching maximum $q_o$ during night periods (data not shown), except in the last group.

Nitrogen balances during steady state of the PBR (corresponding to $O_2$ concentration in the animal isolator at 21%) are presented in **Table 4**. Ammonium is not detected in the liquid outlet of the nitrifying bioreactor, so ammonium removal is at 99% for all conditions. Conversion of ammonium into nitrate is at 91.6, 96.3 and 94.2% for each condition respectively, which is in line with the performance of the bioreactor in previous integration experiment (*Compartment 3 and 4a Lliquid Connection*). The outlet nitrate concentration in the PBR depends on the hydraulic condition. Higher concentration is measured at 30 $L \cdot d^{-1}$ (202 N-ppm) than at 20 $L \cdot d^{-1}$ (160 N-ppm). It is not the same behaviour as at 40 $L \cdot d^{-1}$ (155 N-ppm) because of the inlet concentration reduction to diminish the ammonium$^+$ load into the system. Results show that the same $Y_{N/X}$ is obtained in all conditions. 0.09 g of $N\text{-}NO_3^-$ are needed to generate 1 g of biomass. In contrast, around 0.06 g of $N\text{-}NO_3^-$ are needed to generate 1 g of oxygen. These values are in the same range as those obtained in the first series of experiments.

## DISCUSSION

### Compartment 3 Ad 4a Liquid Connection

The obtained results prove that liquid connection between nitrifying and photosynthetic bioreactors was successful. Nitrogen requirements of *L. indica* were met thanks to the nitrate supplied by the nitrifying bioreactor after a complete nitrification process performed by the co-culture of *N. europaea* and *N. winogradsky*. Indeed the operation of the photobioreactor was very stable and robust in the range of 10–30 $L \cdot d^{-1}$ and $q_o$ of 120 $W \cdot m^{-2}$ and 285 $W \cdot m^{-2}$. Stability for each condition was maintained at steady-state to obtain reliable data. In terms of nitrification activity, the system has been proved to be robust enough to maintain the ammonium conversion at 100% for 200 days no matter the load. There were minor accumulation peaks that were rapidly recovered and had no impact on photosynthetic activity of *L. indica*. These peaks were most likely caused after biomass removal from the packed-bed reducing the amount of active biomass. Then, once the previous active levels of biomass were reached, the nitrification activity recovered to 100%. Additionally, residual $N\text{-}NO_2^-$ concentration in the PBR supernatant was below the limit for drinking water (World Health Ogranization, 2017).

The effect of light and dilution rate have been proved to contribute in a different degree to the performance of the PBR. Liquid flow, and consequently dilution rate, is the factor having the greatest effect on biomass and oxygen productivity. It is an indication that what really influences the behaviour of cyanobacteria is light availability rather than the amount of light. **Figure 5** shows that light availability was directly proportional to the amount of light, but inversely proportional to the amount of cells. Results in terms of oxygen production were very relevant for the definition of the next integration step including the gas connection of the animal compartment to the PBR. Animal compartment in the MELiSSA loop aims to mimic the human activity. In this case the rats are the mock crew. As reported previously (Alemany et al., 2019), the oxygen needs for 1 rat is 0.48–0.7 $g{\cdot}h^{-1}$. The analysis of the obtained results for Compartment 3 and 4a liquid connection shows that depending on the liquid flow between bioreactors, different number of rats could be used in the experimental connection with the animal compartment. When working at 10, 20 or 30 $L{\cdot}d^{-1}$, the maximum oxygen productions achieved in the steady state were 1.6, 2.44 and 2.76 $g{\cdot}h^{-1}$ respectively. Under this scenario, the maximum number of rats that could be selected in the animal compartment is 4 with a theoretical minimum and maximum oxygen demand of 1.92 and 2.8 $g{\cdot}h^{-1}$ respectively. Nevertheless, under this scenario the system would be at the maximum capacity to meet the maximum oxygen requirements. It is for this reason that 3 rats were selected for the experiments where nitrifying bioreactor and the PBR were connected in the liquid phase, and the PBR was connected through the gas phase with animal compartment. This solution provides some margin to the system to react against potential perturbations or changes imposed by the user in the gas phase composition of the animal isolator. The selection of 3 rats imposes a theoretical oxygen demand into the system of 1.44–2.1 $g{\cdot}h^{-1}$ for minimum and maximum values. In order to meet this demand, the minimum and maximum carbon dioxide needs of the PBR would be 1.32 and 1.93 $g{\cdot}h^{-1}$ respectively assuming a middle range experimental $Q_P$ of 1.5 $molO_2{\cdot}molCO_2^{-1}$ (**Table 1**). The animal compartment would provide 1.78–2.60 $g{\cdot}h^{-1}$ of carbon dioxide if a theoretical RQ of 0.9 $molCO_2{\cdot}molO_2^{-1}$ is considered (McGregor and Lee, 1998). So, the carbon needs of the PBR would be met. In terms of N needs, no lack of nitrate is expected in the PBR to meet the $O_2$ requirements if the same $N\text{-}NH_4^+$ loads in nitrifying bioreactor are maintained. It has been demonstrated that according to yields displayed in **Table 2**, the amount of $N\text{-}NO_3^{-1}$ needed in the PBR would be between 0.09 and 0.12 $g{\cdot}h^{-1}$. Hence, any of the 3 tested loads conditions in the nitrifying bioreactor are acceptable.

According to previous rationale, the best hydrodynamic conditions for the next integration phase would be selecting a liquid flow of 20 and 30 $L{\cdot}d^{-1}$ between nitrifying bioreactor and the PBR. The liquid flow of 10 $L{\cdot}d^{-1}$ should be discarded as the system could be in the limit in terms of oxygen production, especially during high activity periods of the rats. Additionally, the exploration of a wider experimental liquid flow domain could provide of relevant information for further optimisation of the MELiSSA loop. This is why a liquid flow of 40 $L{\cdot}d^{-1}$ was also selected as an experimental condition for the second phase tests.

## Compartment 3 and 4 Liquid Connection Combined With Compartment 4a and 5 Gas Connection

After a 110 days experiment, where the PBR and the animal isolator were connected in the gas phase, it has been demonstrated that the system is able to cover the oxygen needs of the rats and carbon needs of *L. indica* while operating in a closed gas loop. This condition is in agreement with previous results (Alemany et al., 2019). The added value of the current work is that nitrogen requirements of *L. indica* to cover the oxygen demand, have been covered by the continuous operation of a nitrifying bioreactor. This is particularly relevant since it represents a further step in the completion of the overall closed-life support system.

Focusing on the gas phase dynamics, the system could maintain a constant oxygen concentration in the animal isolator. Oscillations (±0.04%) in the measured value are the direct cause of circadian activity of the rats. In all oxygen set-points (21,19, 20%), the activity of the rats was higher during night periods because of its photoperiod (Antle and Mistlberger, 2005). In all cases the RQ of the rats ranged between 0.9 and 1.0 molO2·molCO2$^{-1}$, which is consistent with the experimental data reported in the literature (McGregor and Lee, 1998; Jørgensen et al., 2010).

Transition periods, where oxygen set point was increased, took less than 10 h in two groups of rats and less than 20 h in the last group. This proves the fast response of the system to adjust to a change in the parameters of the crew compartment. It should be reminded that the aim of Compartment 4a (PBR) in the loop is to respond to oxygen demand variations thanks to its fast dynamics. The explanation of a higher time period for the transition in the last group of rats is the higher oxygen demand of the rats. It was demonstrated by the average oxygen produced in the PBR for each rat's group (**Figure 7**), which increased up to 2.1 $g{\cdot}h^{-1}$ in that case. Indeed, the body weight gain of the rats, which could be an indication of the physiological activity of the rats, has been correlated with the oxygen demand of the system. In contrast, the transition periods for the first group of rats was not lower than in the second group, even a lower oxygen demand (1.5 vs. 1.9 $g{\cdot}h^{-1}$). This effect could be explained by the differences in the PBR capabilities. In the first case, the biomass concentration in the PBR was higher than in the second case (1.24 g·L-1 vs. 0.98 $g{\cdot}L^{-1}$), potentially limiting the light availability to the cells and decreasing the photosynthetic activity at the same light irradiance. This hypothesis is plausible considering the average $\overline{q_o}$, which is higher in the first group (79 $W{\cdot}m^{-2}$) than in the second the group of rats (75 $W{\cdot}m^{-2}$) even the lower oxygen need. From this observation, it is confirmed that the cells were in a "physical limitation regime" where the light is the limitation parameter of the growth kinetics (Cornet, 2007). The higher light intensity observed in the last group could be explained by the high oxygen demand.

In terms of carbon dioxide concentration, the levels were always maintained below 15,000 ppm. This was below the acceptance limit of 3% for rats (Krohn and Hansen, 2002; Krohn et al., 2003). However, future experiments in the system should consider lowering this level and make it closer to the limits accepted for humans (<0.5%). This point will address in future integration steps of the MELiSSA loop.

Results from liquid connection experiments between Compartment 3 and 4a showed that maximum oxygen production in the PBR was around 2.4–2.8 $g{\cdot}h^{-1}$ with a flow rate of 20–30 $L{\cdot}d^{-1}$. However, during the second phase of the experiments (liquid connection between Compartment 3 and 4a and gas connection between Compartment 4a and 5) the system has proved to be over the previous determined limits. Light irradiance was maintained around 30% of its capacity with enough margin to meet the oxygen demand during transition periods. Indeed, the maximum oxygen production in those transition phases exceeded by 25% the reported values of the first experimental phase. This difference evidences that transition phases should be treated differently than steady state. Comparing biomass concentration at maximum light intensity condition in both experimental phases shows that cell density is lower in the second phase, leading to a higher light availability. From a biological perspective this effect might be explained by the molecular composition of the cells. The photosynthetic capacity of *L. indica* is influenced by the pigment content. When light availability is increased, it takes some hours (Tomaselli et al., 1997) to reduce the pigment content to protect the cells against potential photoinhibition (Muramatsu and Hihara, 2012; Weiwen Zhang, 2018). It is during that transition that the closed loop benefits from the still high pigment content to exploit the maximum photosynthetic activity of the cells. It can be concluded that the capacity of the system in transition steps cannot be extrapolated at long-term, when the system would evolve to reach a new steady state with lower oxygen generation capacity.

Regarding nitrogen needs of *L. indica* to meet the oxygen requirements of the animal crew, it has been demonstrated that nitrifying bioreactor is capable to fulfil them. Indeed, nitrate concentration in the PBR outlet was always kept higher than 20 N-ppm, which is considered a potentially limiting concentration (Depraetere et al., 2015). This scenario avoided any metabolic drift in *L. indica*, allowing an optimal performance in terms of air revitalisation. However, the limiting capacity of the nitrifying bioreactor was reached in the last condition (40 $L{\cdot}d^{-1}$), so ammonium load had to be reduced in order to avoid nitrogen species accumulation. One of the outputs from this work is that the maximum capacity of nitrifying bioreactor in the MELiSSA loop is 1400 $ppm{\cdot}d^{-1}$, which should be taken into consideration when it comes to introduce the urea in the loop. Once the ammonium load was decreased, the full nitrification capacity was fully recovered. Although complete nitrification was dominant during most of the test, three partial nitrification events occurred. Nitrite and ammonium reached the PBR resulting in different consequences. Whilst nitrite was present at detectable levels, no ammonium was detected at the PBR outlet. This result shows that *L. indica* can use ammonium as nitrogen source as previously reported in the literature (Sachdeva et al., 2018). Even nitrite concentration in the PBR reached up to 78 N-ppm during peaks periods, no irreversible effects were detected in *L. indica* culture. In the first and second accumulation events (78 and 58 N-ppm), the presence of nitrite had an effect on photosynthetic activity. The control system adjusted the lights up to the maximum $q_o$ of 285 $W{\cdot}m^{-2}$ during 2–3 h in order to cover the oxygen needs of the animal compartment. This behaviour was not observed in the last accumulation event (36 N-ppm) suggesting that a concentration higher than 40 N-ppm of nitrite have an inhibitory effect on *L. indica* photosynthetic activity. Nevertheless, this event shows the robustness of the system in such a wide range of conditions and in front of potential perturbations. The main function of air revitalisation in Compartment 4a was never at risk and oxygen needs of the rats were fulfilled during 110 days of continuous operation.

## CONCLUSION

The work presented here has demonstrated the feasibility of integrating three compartments of the MELiSSA loop: Compartment 4a (photosynthesis) and 5 (crew compartment) in the gas phase and Compartment 3 (nitrification) and 4a in the liquid phase. The step-wise approach has been proved to be a key factor in the progress to demonstrate the MELiSSA loop concept. Results from the first experimental period have been relevant to define the integration conditions for the following step. The system has succeeded to maintain its main functions to guarantee the survival of the animal crew for a long operational period, proving as well its robustness and reliability against perturbations, which is very relevant in the MELiSSA loop context. It can be concluded that this experimental work represents a step forward in the demonstration of the MELiSSA loop concept and its contribution to bioregenerative life support.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article material, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Committee on Ethics in Animal and Human Research (4017 CEEAH—UAB).

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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## NOMENCLATURE

$C_{LOUT}$ outlet liquid $CO_2$ concentration ($mmol \cdot L^{-1}$)

$D$ dilution rate ($h^{-1}$)

$F_{CO2}$ inlet $CO_2$ gas flow ($L \cdot h^{-1}$)

$F_{in}$ bioreactor inlet gas flow ($L \cdot h^{-1}$)

$F_{out}$ bioreactor total outlet gas flow ($L \cdot h^{-1}$)

**HRT** Hydraulic Residence Time (days)

$O_{2out}$ outlet $O_2$ fraction (%)

**PQ** Photosynthetic quotient ($mol_{O2} \cdot mol_{CO2}^{-1}$)

$q_O$ light irradiance in the PAR region ($W \cdot m^{-2}$)

$q_{O2}$ specific $O_2$ production ($mmol \cdot g^{-1} \cdot h^{-1}$)

$q_{CO2}$ specific $CO_2$ consumption ($mmol \cdot g^{-1} \cdot h^{-1}$)

$r_{CO2}$ $CO_2$ volumetric consumption rate ($g \cdot L^{-1} \cdot h^{-1}$)

**RQ** respiratory quotient ($mol_{CO2} \cdot mol_{O2}^{-1}$)

$r_{O2}$ $O_2$ volumetric production rate ($g \cdot L^{-1} \cdot h^{-1}$)

$r_X$ biomass volumetric production rate ($g \cdot L^{-1} \cdot h^{-1}$)

$V_R$ bioreactor volume (L)

$X$ biomass concentration ($g \cdot L^{-1}$)

$X_{COUT}$ molar fraction of $CO_2$ in the outlet gas ($mol \cdot mol^{-1}$)

$X_{CI}$ molar fraction of $CO_2$ in the inlet gas ($mol \cdot mol^{-1}$)

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# Supplemental Food Production With Plants: A Review of NASA Research

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Bioregenerative life-support systems for space have been investigated for 60 years, and plants and other photosynthetic organisms are central to this concept for their ability to produce food and $O_2$, remove $CO_2$, and help recycle wastewater. Many of the studies targeted larger scale systems that might be used for planetary surface missions, with estimates ranging from about 40 to 50 $m^2$ (or more) of crop growing area needed per person. But early space missions will not have these volumes available for crop growth. How can plants be used in the interim, where perhaps <5 $m^2$ of growing area might be available? One option is to grow plants as supplemental, fresh foods. This could improve the quality and diversity of the meals on the International Space Station or on the Lunar surface, and supply important nutrients to the astronauts for missions like Mars transit, and longer duration Martian surface missions. Although plant chambers for supplemental food production would be relatively small, they could provide the bioregenerative research community with platforms for testing different crops in a space environment and serve as a stepping stone to build larger bioregenerative systems for future missions. Here we review some of NASA's research and development (ground and spaceflight) targeting fresh food production systems for space. We encourage readers to also look into the extensive work by other space agencies and universities around the world on this same topic.

**Keywords: crop, nutrient, salad, veggie, greenhouse, sustainable, ECLSS, controlled ecological life-support systems**

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## INTRODUCTION

Bioregenerative life support systems (BLSS) have been one of the most enduring life science research themes since the beginning of the space era in the 1950's (Myers, 1954). The use of photosynthetic organisms for food and oxygen production, along with $CO_2$ removal and water processing is central to this concept (Miller and Ward, 1966; Salisbury et al., 1997) and in 1962 discussions began on what crop plants to consider for space missions (Pilgrim and Johnson, 1962). But opportunities to test BLSS at a relevant scale in space have been limited due to volume and mass constraints of the spacecraft. Modest efforts at space crop production on board NASA's Space Shuttle, the Russian Mir station, and the International Space Station (ISS) have been underway since the 1990's, but most have been short duration studies and all have been limited due to volume, mass, and power constraints. To date, life-support systems for spacecraft and space stations have been based on physico-chemical (PC) principles, some of them regenerative, others relying on resupply (Shaw et al., 2020). For

example, urine from the crew on the ISS is currently processed through a vapor compression distillation system and purified to recover potable water (Carter et al., 2018). Here we review only plant related testing in space for food production and eventual BLSS applications.

## Food Production in Bioregenerative Life Support Systems

To supply food on space missions, the only option to date has been stowage and resupply of packaged, stabilized foods (Perchonok et al., 2012). Currently, the ISS receives several resupply missions of food each year, but this approach will be more costly as mission durations and distances increase (Perchonok et al., 2012). About 40–50 $m^2$ of crops grown under high light intensities would be needed to produce enough dietary calories for one human, and when coupled with insects to degrade inedible biomass and provide supplemental protein, the area could be reduced to 35–40 $m^2$ per person (Salisbury et al., 1997; Wheeler et al., 2008; Fu et al., 2016). Plantings of this size would also supply all the $O_2$ production and $CO_2$ removal for one human. However, these BLSS studies have all been ground-based, and opportunities to test and implement them in space have been limited. A logical approach might be to sequentially develop smaller BLSS capabilities on space missions where PC life-support systems are already in place; then as durations and distances increase, expand BLSS components where applicable (Gitelson et al., 1995; Wheeler, 2002; Morrow et al., 2004). While earlier approaches focused on larger scale food production for more full life-support (carbohydrate, fat, protein), micronutrients were not given as high a priority (e.g., Mitchell et al., 1996; Salsibury and Clark, 1996). But supplying even smaller amounts of fresh produce could supplement micronutrients such as vitamins C and B1, which may degrade in the packaged diet, provide dietary anti-oxidants, and improve the overall acceptability of meals for the crew (Cooper et al., 2017). Extensive work has been done by other space agencies and universities around the world to address these same issues (Wheeler, 2017). For example, the 1970's Oasis tests on the Russian Salyut Space Station were the first attempts at a human-monitored plant BLSS research in space (Porterfield et al., 2003). Here we review some NASA sponsored ground-based and space research with plants that could be used as supplemental fresh foods on early missions.

## Preparing Crops for Space: The Concept of Crop Readiness Level

Space brings with it unique environmental constraints for crops. This inspired the concept of a Crop Readiness Level or CRL (Wheeler and Strayer, 1997), which is a maturation scale analogous to technology readiness levels (TRL) but for crops and BLSS. For example, short or dwarf growth, high harvest index, high yields, organoleptic acceptance, good nutrient content, and ability to control microbial contaminants are all desirable traits for the selection and maturation of CRL for space (Romeyn et al., 2019; Spencer et al., 2021). The current scale is focused on ISS and Mars transit needs, but surface settings with larger BLSS crop systems might consider different criteria or factors appropriate for those settings, such as higher macronutrient content, ability to grow in multispecies plantings, or radiation tolerance. Like applying TRL for aerospace hardware, a CRL approach provides a logical progression of testing for future space crops.

## Historical Context of Preparing Crops for Spaceflight: Ground-Based Research

Decades of ground and flight research have gone into our current supplemental crop growth systems on the ISS. Around 1980, NASA started its Controlled Ecological Life-Support Systems (CELSS) program (MacElroy and Bredt, 1984). The CELSS Program focused largely on BLSS research for surface missions and agronomic crops that might be grown in large plantings (MacElroy et al., 1990; Wheeler, 2017), but CELSS also proposed a smaller "rack" sized plant system for growing supplemental food crops for near term and Mars transit missions. The term "salad machine" or "vegetable production unit" was used for this concept (Kliss and MacElroy, 1990).

Unlike the staple crops tested by NASA in the 1980's and 1990's (e.g., wheat, soybean, potato, peanut, sweet potato), leafy greens and small fruit crops (e.g., tomato and pepper) can be grown on the ISS and early missions to supplement the crew's diet. These supplemental food crops have a short shelf life but can have a high impact on the diet (Cooper et al., 2012). NASA ground testing included species such as spinach, lettuce, chard, green onion, leafy mustards such as pak choi, mizuna, and Chinese cabbage, radish, beet, dwarf tomato, dwarf pepper, strawberry, and dwarf plum trees (Knight and Mitchell, 1983; Gilrain et al., 1999; Subbarao et al., 1999; Goins and Yorio, 2000; Richards et al., 2004; Massa et al., 2006; Hummerick et al., 2010; Graham et al., 2015; Massa et al., 2016; Graham and Wheeler, 2016). To date, many of the leafy greens and "Red Robin" tomato have performed very well in these studies (Spencer et al., 2019; Spencer et al., 2020).

# CROP PHYSIOLOGICAL CONCERNS

## Impact of Atmospheric $CO_2$ Concentrations

Significant vegetable crop testing by NASA focused on the effects of $CO_2$ on crop growth and development (McKeehen et al., 1996; Spencer et al., 2019; Burgner et al., 2020). Elevating the $CO_2$ from ambient levels ~400 ppm to 1,000–2000 ppm increased growth and yield for most crops, as expected. But yields of some crops like radish and lettuce dropped at super-elevated $CO_2$ concentrations, e.g., 5,000 and 10,000 ppm, compared to 1,000 ppm (Mackowiak et al., 1994). Thus for typical $CO_2$ levels on the ISS (~3,000 ppm), most of these crops should grow well. But for Chinese cabbage, cv. Tokyo Bekana, the combination of moderately elevated $CO_2$ (900 ppm) and LED lighting decreased growth compared to lower $CO_2$ levels (Burgner et al., 2020). This response highlights the importance of conducting thorough ground testing prior to spaceflight (Romeyn et al., 2019).

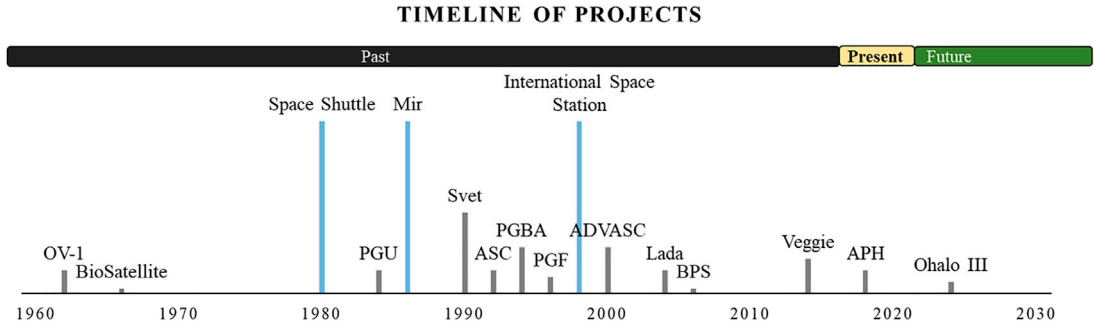


**FIGURE 1 |** Timeline of projects. Spaceflight hardware for bioregenerative crop production from 1960–2030. Past (black), present (yellow), and future (green) are indicated above the timeline. Platforms are indicated with a blue line while plant-specific hardware is indicated by a gray line. Length of the gray line represents the relative length of time that the hardware was/has been in operation. Orbital Vehicle 1 (OV-1; US Air Force), BioSatellite I and II (NASA), Plant Growth Unit (PGU; NASA), Svet (Roscosmos), Astroculture (ASC; Univ. Wisconsin/NASA), Plant Generic Bioprocessing Apparatus (PGBA; BioServe/NASA), Plant Growth Facility (PGF; NASA), Advanced Astroculture (ADVASC; Univ. Wisconsin/NASA), Lada (Roscosmos), Biomass Production System (BPS; NASA), Veggie (NASA), Advanced Plant Habitat (APH; NASA). Veggie and APH are currently in use on the International Space Station.

## Plant Lighting

Using light emitting diodes (LEDs) to grow plants was proposed and patented through a NASA Commercialization Center at the University of Wisconsin (Barta et al., 1992). LEDs were first used in the Astroculture (ASC) plant chamber aboard the Space Shuttle (Morrow et al., 1995) then the Advanced Astroculture Chamber (ADVASC) (Link et al., 2003), and later the Veggie and Advanced Plant Habitat for the ISS, both of which are currently flying aboard the ISS (Massa et al., 2016; Morrow et al., 2016). To support the development of LED lighting for space, NASA sponsored ground testing from the early 1990's through the mid 2000's with leafy greens and other crops (Goins et al., 1997; Kim et al., 2004; Massa et al., 2008). These studies showed that both red and blue light improved plant photosynthesis and growth (Bula et al., 1991; Dougher and Bugbee, 2001; Yorio et al., 2001; Douglas et al., 2016). Subsequent LED studies revealed important roles for green and far-red light as well (Spencer et al., 2020). Recent NASA research showed that supplementing with far-red LEDs can act like adding more photosynthetically active radiation--PAR (400–700 nm) (Zhen and Bugbee, 2020), and that LEDs can achieve remarkable efficiencies (>3 μmol/J), which could greatly reduce electrical power needs for BLSS (Kusuma et al., 2020). This has far-reaching implications for future missions. In addition to electric lighting systems, solar lighting techniques that use concentrators and fiber optics were also explored for growing crops (Cuello et al., 2000; Nakamura et al., 2009).

Regardless of the lighting approach, nearly all these studies showed greater yields in response to increased PAR (Knight and Mitchell, 1983; Knight and Mitchell, 1988; Richards et al., 2004). Although some leafy greens are prone to physiological disorders like leaf tip burn at higher PAR (Barta and Tibbitts, 1991; Frantz et al., 2004), this key relationship between PAR and yield becomes a driving factor for planning crop systems for space. For NASA's Veggie plant chamber on the ISS, the PAR is adjustable up to ~450 μmol $m^{-2}$ $s^{-1}$ depending on distance between the plants and lights, but has typically been operated between 200 and 300 μmol $m^{-2}$ $s^{-1}$ (Massa et al., 2016).

## NASA'S PAST, PRESENT, AND PLANNED PLANT CHAMBERS FOR SPACE

The first attempt to test BLSS concepts in space was with the Orbital Vehicle (OV-1) satellite mission in 1966, where NASA and the US Air Force monitored photosynthetic and respiratory gas exchange between duckweed (*Spirodella*) and *Chlorella* aglae (Ward et al., 1970). The subsequent BioSatellite 2 experiments tested wheat seedlings and pepper plants in microgravity (Conrad, 1968; Johnson and Tibbitts, 1968), but these were short flights with limited data recovery. NASA funded investigators F.B. Salisbury and G.E. Bingham also collaborated with Russian colleagues for a series of tests in the Svet plant chamber on Mir space station through much of the 1990's with research focusing on wheat production (Bingham et al., 1996, 2000). With the Shuttle program came more frequent trips to space and more chamber options for plant growth, such as the Plant Growth Unit (PGU) and subsequent Plant Growth Facility (PGF) (Halstead and Dutcher, 1987; Paul et al., 2001). These chambers were primarily for space and gravitational research. In comparison, the Astroculture (ASC) and Plant Generic Bioprocessing Apparatus (PGBA) were also used on the Shuttle but more focused on BLSS concepts (Bula et al., 1991; Morrow et al., 1995; Hoehn et al., 1997; Porterfield et al., 2003; Zabel et al., 2016) (**Figure 1**).

Compared to standard gravity, watering systems for μ-gravity must deal with water containment, the lack of natural drainage and impaired aeration of the rootzones (Bingham et al., 2000; Steinberg et al., 2002; Jones et al., 2011). This began ground testing of porous membranes, tubes, or plates to contain the water and allow capillary movement to the roots (Wright et al., 1988; Dreschel and Sager 1989; Koontz et al., 1990), as well as flight testing of hybrid approaches that use porous tubes to sub-irrigate a solid rooting matrix (Morrow et al., 1992; Bula et al., 1991). ASC and PGBA also used porous thermo-electric plates to cool and dehumidify the air, while recycling the condensate back to the plants (Morrow et al., 1995; Hoehn et al., 1997; Conrad, 1968; Dreschel and Sager 1989;Johnson and Tibbitts, 1968; Khodadad et al., 2020; Koontz et al., 1990; Morrow et al., 1992; Schuerger et al., 2021; Stutte et al., 2005; Wright et al., 1998).

**TABLE 1 |** NASA crops grown in spaceflight in Veggie and APH.

| Crop | Scientific Name | Experiments | Experiment Flight Grow Dates |
|---|---|---|---|
| Red Romaine Lettuce | *Lactuca sativa* cv. Outredgeous | VEG-01A | May 8, 2014–June 10, 2014 |
| | | VEG-01B | July 8, 2015–Aug. 10, 2015 |
| | | VEG-03A | Oct. 25, 2016–Dec. 28, 2016 |
| | | VEG-03D | Sept. 26, 2017–Nov. 23, 2017 |
| | | VEG-03E | Feb. 5, 2018–April 6, 2018 |
| | | VEG-03F | Feb. 9, 2018–April 9, 2018 |
| | | VEG-03I | Jan. 4, 2021–Feb. 2, 2021 |
| | | VEG-03J | Jan. 4, 2021–Feb. 2, 2021 |
| Green Leaf Lettuce | *Lactuca sativa* cv. Waldmann's Green | VEG-03D | Sept. 26, 2017–Nov. 23, 2017 |
| | | VEG-03E | Feb. 5, 2018–April 6, 2018 |
| | | VEG-03F | Feb. 9, 2018–April 9, 2018 |
| Dwarf Romaine | *Lactuca sativa* cv. Dragoon | VEG-03G | Oct. 25, 2018–Nov. 28, 2018 |
| | | VEG-03I | Jan. 4, 2021–Feb. 2, 2021 |
| Zinnia | *Zinnia hybrida* cv. Profusion | VEG-01C | Nov. 16, 2015–Feb 14, 2016 |
| Chinese Cabbage | *Brassica rapa* var. Chinensis cv. Tokyo Bekana | VEG-03B | Jan. 20, 2017–May 31, 2017 |
| | | VEG-03C | April 3, 2017–May 31, 2017 |
| Mizuna Mustard | *Brassica rapa* var. japonica | VEG-03D | Sept. 26, 2017–Nov. 23, 2017 |
| | | VEG-03E | Feb. 5, 2018–April 6, 2018 |
| | | VEG-04A | June 4, 2019–July 9, 2019 |
| | | VEG-04B | Oct. 1, 2019–Nov. 28, 2019 |
| Red Kale | *Brassica napus* cv. Red Russian Kale | VEG-03G | Oct. 25, 2018–Nov. 28, 2018 |
| | | VEG-03I | Jan. 4, 2021–Feb. 2, 2021 |
| Wasabi Mustard | *Brassica juncea* cv. Wasabi | VEG-03H | March 9, 2019 - April 6, 2019 |
| | | VEG-03I | Jan. 4, 2021 - Feb. 2, 2021 |
| Dwarf Pak Choi | *Brassica rapa* var. Chinensis cv. Extra Dwarf | VEG-03H | March 9, 2019–April 6, 2019 |
| | | VEG-03I | Jan. 4, 2021–Feb. 2, 2021 |
| | | VEG-03L | Feb. 8, 2021–Apr. 13, 2021 |
| Amara Mustard/Ethiopian Kale | *Brassica carinata* | VEG-03K | Feb. 8, 2021–Apr. 13, 2021 |
| Radish | *Raphanus sativus* | PH-02 | Nov. 3, 2020–Nov. 30, 2020 |
| Pepper | *Capsicum annuum* cv. Española Improved | PH-04 | July 12, 2021 |

By 2000, plant growth systems were launched to the ISS. First was the ADVASC (Link et al., 2003) and then the Biomass Production System (BPS) (Stutte et al., 2005), both of which were double, mid-deck, locker-sized chambers. In 2014 the Veggie chamber was added to the ISS, with a second unit added in 2017. The Advanced Plant Habitat (APH), a quad, locker-sized chamber that provides a wide range of environmental control, was based on Astroculture principles (Zhou et al., 1998) and installed on the ISS in 2017 (Massa et al., 2016; Morrow et al., 2016). Unlike the ADVASC, BPS, and APH, Veggie was intended to be collapsible for stowage, open to the cabin atmosphere, and easily accessible for the crew, much like the Russian Svet and Lada systems (Bingham et al., 1996; 2000) To date, Veggie has been used more than any other chamber in space to study fresh food production for astronauts.

## Recent NASA Spaceflight Studies of Supplemental Food Production

To date, the series of 12 leafy green tests in NASA's Veggie chamber on the ISS included lettuce (several cultivars), mizuna, Chinese cabbage, wasabi mustard, red Russian kale, amara mustard, and pak choi (**Table 1**). The first studies used "Outredgeous" red romaine lettuce and an ornamental crop, "Profusion" zinnia (Massa et al., 2015). Tests with Chinese cabbage showed leaf chlorosis on some of the plants (Burgner et al., 2020). Analyses of nutrient content of red romaine lettuce grown in Veggie found no significant difference between the ground control and spaceflight treatment of each experiment. Some growth differences were found across the three studies, each conducted a year apart, and are attributed to different environmental conditions (Khodadad et al., 2020). The crop microbiome did vary, with a considerably more diverse microbial community found on space ground produce, especially the leaves. Lettuce growth improved with better approaches to watering by astronauts. Throughout most of the Veggie tests, water management has been challenging, with lettuce having insufficient water in some early tests, while zinnia plants had too much, primarily due to a ventilation system failure (Schuerger et al., 2021). The baseline Veggie watering system is a passive wicking design, where water from a reservoir wicks to plant pillows, which are filled with arcillite media and controlled-released fertilizer (Massa et al., 2017a; Massa et al., 2017b). The capillary watering approach has thus

far been inconsistent due to materials and design issues. These issues have not been addressed to date so Veggie experiments have relied largely on direct manual watering. The watering system in APH consists of porous tubes surrounded by arcillite media (Morrow et al., 2016), and to date it has worked well. Recently the radish plants grown in APH were consumed by astronauts (John et al., 2021). A second crop, chile peppers (Spencer et al., 2019), began growing for the first time in APH in orbit on July 12, 2021 (**Table 1**).

### Ohalo III

NASA's next step toward space crop production will be the Ohalo III chamber, targeted for the ISS as early as 2024. This rack-sized crop production system will be atmospherically closed to recycle transpired humidity and will contain various automation and sensing capabilities. Ohalo III is designed to be evolvable and expandable and will initially test different water delivery and volume optimization concepts for growing plants in microgravity. As a permanent addition to the ISS, Ohalo III should be able to investigate operational challenges associated with the sustained production of crops in space and will hopefully achieve the long desired "vegetable production unit" for a Mars transit mission (Kliss and MacElroy, 1990; Kliss et al., 2000).

## DISCUSSION

NASA has sponsored extensive research on growing various species of leafy vegetables and small fruits in controlled environment chambers. This research revealed the importance of managing water and nutrient supplies to the plants, the effects of elevated and super-elevated $CO_2$ on plants, and the profound influence of light on crop growth and development. This in turn has driven the development and testing of LED lighting and other new technologies for space crop production. Other key gaps exist in our knowledge base, such as the effects of reduced gravity on the plants and their support systems, such as water delivery, and the effects of space radiation. Most of this testing occurred in "ground" settings, but small plant chambers have been built and tested in space. These chambers have become successively larger with better environmental control, but none have been used with the sole intent for providing fresh food for the astronauts. Exploratory tests with the Veggie plant chamber are beginning to do this, but a dedicated "vegetable production unit" with better environmental control is still needed.

### Concluding Remarks

BLSS using plants to generate food and oxygen while recycling $CO_2$ and water will help achieve more autonomous living on other planets; however, developing a large BLSS on surface settings could be decades in the future, and this will be dictated in part by mission architectures of the various space agencies. Nonetheless, continued ground research on these systems is needed to understand their integration with other environmental control technologies, their sustainability, and their costs in terms of mass, power, volume, and crew time. Part of the evolution toward these BLSS systems will be testing smaller components or subsystems in space settings like the ISS and early surface missions. A logical stepping stone in this progression will be using smaller plant chambers to provide supplemental, fresh foods to augment stored foods. These fresh foods could reduce diet fatigue and provide key nutrients that degrade in the packaged food supplies. Understanding the operation, cost, and sustainability of these smaller food-crop production systems will provide critical information for evolving toward a larger BLSS of the future.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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# Investigating the Growth of Algae Under Low Atmospheric Pressures for Potential Food and Oxygen Production on Mars

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With long-term missions to Mars and beyond that would not allow resupply, a self-sustaining Bioregenerative Life Support System (BLSS) is essential. Algae are promising candidates for BLSS due to their completely edible biomass, fast growth rates and ease of handling. Extremophilic algae such as snow algae and halophilic algae may also be especially suited for a BLSS because of their ability to grow under extreme conditions. However, as indicated from over 50 prior space studies examining algal growth, little is known about the growth of algae at close to Mars-relevant pressures. Here, we explored the potential for five algae species to produce oxygen and food under low-pressure conditions relevant to Mars. These included *Chloromonas brevispina*, *Kremastochrysopsis austriaca*, *Dunaliella salina*, *Chlorella vulgaris,* and *Spirulina plantensis*. The cultures were grown in duplicate in a low-pressure growth chamber at 670 ± 20 mbar, 330 ± 20 mbar, 160 ± 20 mbar, and 80 ± 2.5 mbar pressures under continuous light exposure (62–70 μmol $m^{-2}$ $s^{-1}$). The atmosphere was evacuated and purged with $CO_2$ after sampling each week. Growth experiments showed that *D. salina, C. brevispina*, and *C. vulgaris* were the best candidates to be used for BLSS at low pressure. The highest carrying capacities for each species under low pressure conditions were achieved by *D. salina* at 160 mbar (30.0 ± 4.6 × $10^5$ cells/ml), followed by *C. brevispina* at 330 mbar (19.8 ± 0.9 × $10^5$ cells/ml) and *C. vulgaris* at 160 mbar (13.0 ± 1.5 × $10^5$ cells/ml). *C. brevispina, D. salina*, and *C. vulgaris* all also displayed substantial growth at the lowest tested pressure of 80 mbar reaching concentrations of 43.4 ± 2.5 × $10^4$, 15.8 ± 1.3 × $10^4$, and 57.1 ± 4.5 × $10^4$ cells per ml, respectively. These results indicate that these species are promising candidates for the development of a Mars-based BLSS using low pressure (∼200–300 mbar) greenhouses and inflatable structures that have already been conceptualized and designed.

**Keywords: BLSS, life on mars, space biology, low pressure chamber, extremophilic algae**

## INTRODUCTION

Human exploration of Mars is one of the key scientific and technological undertakings of our time, providing critical information enabling the discovery and settlement of another world while also facilitating the development of technologies on Earth. Future human space exploration may include returning to the moon, as well as missions to Mars (Henn, 2013; Martinez et al., 2013; National Academies of Sciences, Engineering, and Medicine, 2016), with NASA aiming to send humans to Mars by the 2030s (Miranda, 2020). Current research and planning to send crewed missions to Mars for long term space exploration has underscored the critical need for advanced Bio-regenerative Life Support Systems (BLSS), which are complex mixtures of biological and engineering systems that include atmosphere revitalization, water recycling, food production, and organic waste recycling (Revellame et al., 2021). Algae, which produce much of the oxygen on Earth, can similarly be used to recycle $CO_2$ and provide $O_2$ and food to astronauts (Häder, 2020), and therefore, have previously been proposed for space life support systems (Averner et al., 1984).

Since the beginning of human spaceflight missions, algae have been considered promising candidates for space life support systems due to their rapid growth rates, the fact that they are straightforward to grow, and edible biomass (Powell et al., 1961; Rangel-Yagui et al., 2004; Soletto et al., 2005; Ganzer and Messerschmid, 2009; Wells et al., 2017). In the late 1960s, a bio-regenerative system utilizing the algae *Chlorella* was first studied in ground experiments for $CO_2$ cleansing and $O_2$ provision (Eckart, 1996). Since then, various experiments have been conducted to study the effects of radiation, microgravity, space vacuum, and temperature extremes on algae growth for space exploration missions (Horneck et al., 2003; Thirsk et al., 2009), and it has been shown that algae are capable of surviving exposure to spaceflight conditions (Niederwieser et al., 2018).

The atmospheric pressure at the surface of Mars ranges from 1 mbar to 14 mbar depending on the location and season, which is very low compared to the 1013 mbar on average at sea level on Earth (Forget, 2009). One of the goals in space exploratory missions and on Mars is to minimize the amount of mass and energy required to launch and maintain life support systems. Low-pressure is sought by the human spaceflight programs to decrease the engineering cost associated with space vehicles, as it allows a reduction in their size and in the quantity of accompanying consumable materials (Paul and Ferl, 2006). The ability to grow photosynthetic organisms under low pressure conditions is therefore an important step toward establishing advanced life support systems for long-term space missions.

Early studies of algae growth at low pressures ranging from 250 to 500 mbar suggested that low atmospheric pressures have no inhibitory effect and might slightly stimulate growth (Orcutt et al., 1970). Some algae have also developed anoxic metabolisms to adapt to low oxygen conditions (Yang et al., 2016). Limited studies have examined cyanobacterial growth under low atmospheric pressures relevant to potential growth on Mars (Kanervo et al., 2005; Murukesan et al., 2015; Verseux et al., 2021). These studies reported the successful growth of cyanobacteria at pressures as low as 100 mbar achieved with the continuous replenishment of $CO_2$ and nitrogen. However, the experiments were conducted for relatively short duration (7–10 days) and detailed observations of growth dynamics such as carrying capacities and growth trends at reduced pressures were not reported (Murukesan et al., 2015; Verseux et al., 2021). To the best of our knowledge from the existing literature, including a review of over 50 space studies examining algal growth (Niederwieser et al., 2018), few studies have examined the growth of extremophilic algae, with high nutritional potential, at low pressures relevant to Mars.

Algae are found in almost all ecosystems on Earth (Rajkumar and Yaakob, 2013; Malavasi et al., 2020). Algae are diverse organisms with specialized adaptations that enable them to survive under extreme environmental conditions including hot or cold deserts (Lewis and Lewis, 2005; Schmidt et al., 2011), hypersaline habitats (Vinogradova and Darienko, 2008; Oren, 2014a; Leena et al., 2018; Cycil et al., 2020), extreme concentrations of heavy metals (Garbayo et al., 2012; Malavasi et al., 2020), deep-sea hydrothermal vents (Edgcomb et al., 2002; Malavasi et al., 2020), and extreme elevations such as the highest volcanoes on Earth (Schmidt et al., 2018; Vimercati et al., 2019). Some preliminary studies also indicated the ability of cyanobacteria and algae to grow using Martian Regolith Simulant (MRS) demonstrating their ability for *in-situ* resource utilization (Arai et al., 2008; Cycil et al., 2021). In this study, we selected algae species that are ecologically diverse and may have adaptations to thrive under extreme environmental conditions that may help them to grow under conditions relevant to Mars. Snow algae, for example, are the primary oxygen producers in challenging high UV, low temperature, and low nutrient snow environments in lower atmospheric pressures up to 6,000 m above sea level (Painter et al., 2001; Schmidt et al., 2018; Solon et al., 2018; Vimercati et al., 2019; Hoham and Remias, 2020), and can reach concentrations of over one million cells/ml (Hoham, 2001). Halophilic algae are salt-loving algae that are the main or only primary producer in most light-exposed hypersaline environments approaching salt saturation (Banciu et al., 2020). The halophilic algae *Dunaliella salina,* similar to snow algae, are the primary oxygen producers in hypersaline environments such as The Great Salt Lake, Dead Sea, Lake Tyrell, solar salterns, and brine inclusions (Oren, 2014b).

In this study, we utilized five algae strains to study their growth under Mars-relevant low-pressure conditions: three extremophilic algae, the snow algae *Chloromonas brevispina* (Hoham et al., 1979) and *Kremastochrysopsis austriaca* (Remias et al., 2020), and the halophilic algae *Dunaliella salina* (Teodoresco, 1905), in addition to two well-studied edible algal strains *Chlorella vulgaris* and *Spirulina plantensis* that have been used in multiple spaceflights and ground-based studies (Lee et al., 2001; De Morais and Costa, 2007; Daliry et al., 2017; Niederwieser et al., 2018; Detrell et al., 2019; Häder, 2020). Edible microalgae are a source of potentially healthy and sustainable nutrients. *D. salina, C. vulgaris,* and *S. plantensis* have been reported to have commercial applications as food supplements due to their rich protein content, presence of vitamins A and B12 and the abundance of β-carotene which is an

antioxidant (Mokady et al., 1989; Panahi et al., 2015; Kumudha and Sarada, 2016; Lupatini et al., 2017; Canelli et al., 2020; Sui and Vlaeminck, 2020). Some reports also indicate potential applications of snow algae metabolites in the pharmaceutical industry (Sathasivam et al., 2019; Hans et al., 2021). Therefore, these algae species also have the potential to serve as healthy food sources.

# MATERIALS AND METHODS

## Algae Strains and Culturing

Xenic cultures of the snow algae *C. brevispina* (from the University of Texas Culture Collection of Algae UTEX B SNO96) were provided by James Raymond, and the snow algae *K. austriaca* was isolated and provided by Daniel Remias. The *C. brevispina* culture was first isolated from Lac Laflamme by Hoham et al. (1979), and the *K. austriaca* culture was first isolated from Tyrol, Austria by Remias et al. (2020). In these experiments, *C. brevispina* and *K. austriaca* cultures were maintained on the M1 growth medium described by Hoham et al. (1979). To prepare M1 medium, 1% v/v of trace metal solution was autoclaved and added to the M1 medium prior to adding 0.1% v/v of vitamin solution (1 mg/ml vitamin B12, 5 mg/ml biotin and 1 mg/ml thiamine-HCl), which was filter-sterilized separately using a 0.2 μm filter and then added to the autoclaved M1 medium (Harrold et al., 2018; Phillips-Lander et al., 2020).

Xenic cultures of the algae *C. vulgaris* (UTEX 2714), *S. plantensis* (UTEX LB 1926), and *D. salina* (UTEX LB 200) were purchased from the UTEX Culture Collection of Algae, University of Texas, Austin along with their recommended growth media (**Table 1**). *S. plantensis,* originally isolated by Lewin (1969) from Del Mar Slough, San Diego Co., California, United States was maintained on sterile Enriched Seawater Medium from UTEX (Lewin, 1979). *D. salina* (Teodoresco, 1905), originally isolated from a salt lake in Russia, was maintained on sterile 2X Erdschreiber's Medium (2X ERD UTEX) described by Foyn (1934). Xenic cultures of *C. vulgaris* (originally isolated by González et al., 1997, from a wastewater-treatment stabilization pond, Santa Fe de Bogota, Colombia) were cultivated using the sterile Proteose Medium (UTEX), where proteose is added to a Bristol medium (Bold, 1949).

## Mars-Relevant Low-Pressure Chamber Design

Except for the algae experiments at 670 mbar pressures, which were performed in a modified vacuum chamber (**Supplementary Figure 26**), all algae growth experiments were carried out in a 11.4-L (25.4 cm diameter by 23 cm tall) aluminum vacuum chamber (SlickVacSeal) (**Figure 1**). It is equipped with a –30–0 inch Hg (0–1014 mbar) gauge with ± 2% accuracy for routine pressure measurements. The clear tempered glass lid on the top of the chamber allowed exposure to light (**Figure 1**). The chamber was designed for a near full vacuum (–29.9 in Hg, ∼1 mbar) as per the manufacturer's description. The low-pressure environment inside the chamber was generated by a Labconco direct-drive rotary vane vacuum pump (Model 117, LABCONCO). The pump has 117 (LPM) free air capacity with a vacuum to single mbar levels (SlickVacSeal, 2020). For the lowest pressure experiments at 80 mbar, instead of using the SlickVacSeal gauge, the pressure was monitored using a high sensitivity vacuum gauge (Ashcroft) that can measure –30–0 Hg (0–1014 mbar) vacuum with ± 0.25% accuracy to ensure accurate measurement of the low-pressure environment (SlickVacSeal, 2020).

The outlet on the chamber was used to establish low pressure using the vacuum pump as described above and to administer $CO_2$ through a valve manifold (**Figure 1**). To provide a Mars-relevant atmosphere, the atmosphere within the low-pressure chamber was evacuated to the desired low-pressure and then replaced with $CO_2$ using 16-gram food-grade $CO_2$ threaded cartridges (ASURA). After filling the chamber with $CO_2$, the chamber was evacuated again to achieve the desired pressure. This process was repeated three times at the beginning of every experiment, which took approximately 5–8 min. The atmosphere was then evacuated, purged with $CO_2$, and evacuated again after each sampling to maintain Mars-relevant atmospheric conditions. Pressures could increase up to 1 inch Hg (34 mbar) in 1 week, after which the chamber was again evacuated to the required pressure. The cause of the increase was unclear, but media vaporization, degassing and leakage of the chamber are possible factors. Two Sun Blaster T5 high output fluorescent grow lamps were placed on the top of the tempered glass lid at a distance that allowed 62–70 μmol $m^{-2}$ $s^{-1}$ of continuous light exposure to the cultures. The range of 62–70 μmol $m^{-2}$ $s^{-1}$ was based on previous experiments by Harrold et al. (2018). The distance for proper light exposure was established by placing the handheld digital lux meter (URCERI) on the bottom of the chamber with the lid on. From the bottom of the chamber, all measurements were between 62 and 70 μmol $m^{-2}$ $s^{-1}$ with an accuracy of ±3% based on the meter manufacturer's instructions. Each culture was set up in duplicate with abiotic controls that contained only media without cultures. To prevent sedimentation of algae and to allow a homogenized distribution of gasses and nutrients within the medium, the chamber was shaken at a rate of 150 revolutions per minute (RPM) on a standard shaker plate (VWR).

## Experimental Setup

Prior to initiating the low-pressure growth experiments, each culture was first inoculated on a solid agar plate (2% agar in respective media) using the streak plate method. An individual colony for each species was then picked from the agar plates and grown in its respective liquid medium under optimum conditions. The liquid cultures were used for all further experiments.

The growth phase of each culture under optimum conditions was tracked to the mid-logarithmic phase by measuring the optical density (OD) of a 1 ml sample of each culture using a GENESYS 10S UV-VIS spectrophotometer (Thermo Scientific) at 750 nm ($OD_{750}$). The 750 nm wavelength is out of the absorbance range of algal pigments and hence is a preferred choice for OD measurement (Griffiths et al., 2011). The logarithmically growing

**TABLE 1 |** Selected algae species and their growth conditions.

| | *Chloromonas brevispina* | *Kremastochrysopsis austriaca* | *Dunaliella salina* | *Spirulina plantensis* | *Chlorella vulgaris* |
|---|---|---|---|---|---|
| Classification | Snow (Psychrophilic) | Snow (Psychrophilic) | Halophilic | Mesophilic | Mesophilic |
| Media | M1[b] | M1[b] | 2X Erd Medium[c] | Enriched Seawater Medium[d] | Proteose Medium[e] |
| Pressures (mbar)[f] | ($OD_{750}$, cell cts)[g] 670 ± 20, 330 ± 20, 160 ± 20, 80 ± 2.5 | ($OD_{750}$)[g] 670 ± 20, 330 ± 20, 160 ± 20, 80 ± 2.5 | ($OD_{750}$, cell cts)[g] 670 ± 20, 330 ± 20, 160 ± 20, 80 ± 2.5 | ($OD_{750}$)[g] 670 ± 20, 330 ± 20 | ($OD_{750}$, cell cts)[g] 670 ± 20, 330 ± 20, 160 ± 20, 80 ± 2.5 |
| Temperatures (°C) | 4.0 ± 0.1 | 4.0 ± 0.1 | 20.8 ± 2.6 (10.0 ± 0.1 at 80 ± 2.5 mbar) | 20.8 ± 2.6 | 20.8 ± 2.6 (10.0 ± 0.1 at 80 ± 2.5 mbar) |
| Light levels ($^a\mu mol\ m^{-2}s^{-1}$) | 62–70[a] | 62–70[a] | 62–70[a] | 62–70[a] | 62–70[a] |
| Incubation time | 33–54 days | 33–54 days | 33–62 days | 62 days | 33–62 days |

[a] *As measured using a handheld digital lux meter (URCERI) ± 3% accuracy, where the 62–70 μmol of photons $m^{-2}$ $s^{-1}$ range was chosen based on previous work by Harrold et al. (2018).*
[b] *M1 medium (Hoham et al., 1979).*
[c] *2X Erdschreiber's Medium (modified Erdschreiber's medium, Foyn, 1934).*
[d] *Enriched Seawater Medium (Lewin, 1979).*
[e] *Proteose Medium (Bold, 1949).*
[f] *Values of uncertainties are based on the accuracy of the gauges monitoring the pressure.*
[g] *Both cell counts (cell cts) and $OD_{750}$ readings were measured for C. brevispina, D. salina and C. vulgaris and for K. austriaca and S. plantensis only $OD_{750}$ readings were measured due to their minimal growth.*

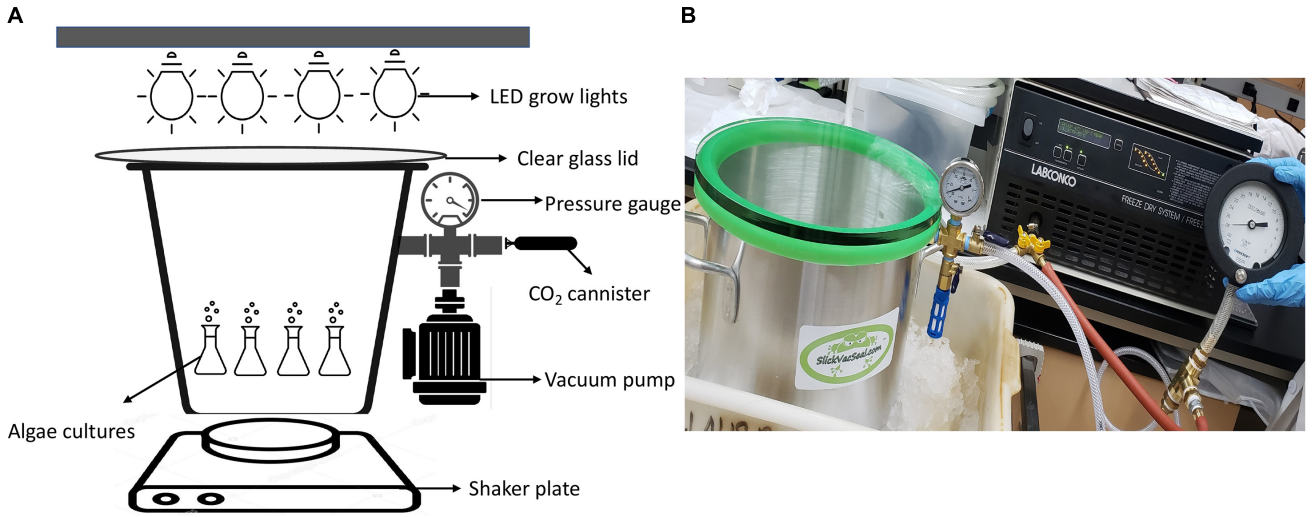


**FIGURE 1 | (A)** Design of low-pressure growth chamber used for pressures of 330 mbar and below, **(B)** Photograph of the experimental setup for low-pressure growth experiments of 330 mbar and below using the SlickVacSeal aluminum vacuum chamber.

cultures were then used to inoculate the algae growth experiments at the first set of low pressure conditions at 670 mbar.

For each experiment, 100 ml of autoclave-sterilized medium specific to each alga culture was used in 200 ml Erlenmeyer glass flasks. The flasks were first acid washed (10% nitric acid) and then rinsed three times with 18 MΩ $cm^{-1}$ $H_2O$. All equipment used for experimental setup and sampling was autoclaved before use. Cultures were inoculated with 10% inoculum in each case. The first sampling was performed immediately after inoculation to determine the initial optical density (OD) and cell counts of the inoculated culture. As soon as cultures were inoculated and sampled, they were placed in the low-pressure chamber, which was then evacuated and purged three times as described above. The temperature was maintained at 4.0 ± 0.1°C for the snow algae and room temperature (20.8 ± 2.6°C) for other algae. However, at the lowest pressure (80 mbar), algae other than snow algae were kept at 10.0 ± 0.1°C to reduce the vapor pressure under these low pressure conditions.

Algae growth was measured at four different pressures: 670, 330, 160, and 80 mbar. To allow cultures to potentially adapt to decreasing pressures, the inoculum for pressure conditions 330, 160, and 80 mbar was prepared so that half the volume of the inoculum was from cultures growing logarithmically outside the low-pressure growth chamber under normal atmospheric conditions, and the other half was an equal volume of the culture

growing at 670, 330, and 160 mbar, respectively, after steady state was achieved.

## Sampling

Sampling was performed once a week for the duration of the experiments (33–62 days) to allow the cultures to reach the stationary growth phase. Algae growth was qualitatively determined by taking the $OD_{750}$ measurements for each duplicate culture ($n = 2$) (Moheimani et al., 2013), whereas the quantitative growth measurements via cell counts were performed using two measurements each of duplicate cultures, making the total number of measurements $n = 4$. At all pressures, for growth experiments of the cultures for which cell counts were performed (*C. brevispina, D. salina* and *C. vulgaris*), cell concentrations for the last 2–4 time points were averaged and used to estimate the carrying capacity for all conditions except for *D. salina* at 670 mbar which was assumed to be at or near stationary growth at the end of the experiment (**Supplementary Tables 1–3**). For sampling, the low-pressure chamber was first brought to atmospheric pressure by opening the valve of the pressure outlet on the low-pressure chamber allowing air to enter the chamber. The cultures were then removed for sampling. Sampling was performed in a laminar flow hood (Horizon, LABCONCO) using aseptic techniques and took about 5–20 min. Samples were then returned to the low-pressure chamber, which was evacuated and purged as described above.

The laminar flow hood workspace was sterilized before and after sampling via UV lights (Pure UV) for 15 min and 70% ethanol to prevent contamination. Before sampling, cultures were first homogenized by gentle swirling to ensure uniform distribution of cells and then 1 ml of sample was extracted from each flask for growth measurement using a sterile pipet. Growth was measured immediately after sampling using $OD_{750}$ for all cultures and cell count measurements for *C. brevispina, D. salina,* and *C. vulgaris* (**Table 1**). $OD_{750}$ and cell counts measurements were used because both are a direct reflection of the biomass in each culture (Chioccioli et al., 2014).

## Growth Measurements

Algal cell counts were measured as previously described by Harrold et al. (2018) and Phillips-Lander et al. (2020). Briefly, samples were first vortexed to homogenize the cultures and 10 μL of the sample was used for each cell count reading using disposable Incyto C-chip hemocytometer chambers (Model #DHC-N01). For low-moderate cell concentrations, cells were counted within each of five large grid zones ($V_{grid,L} = 1 \times 10^{-4}$ ml), and for high cell concentrations, cells were counted within five to thirteen small grid zones ($V_{grid,sm} = 1 \times 10^{-6}$). For the lowest cell concentrations, an entire hemocytometer grid was counted. All cell counts were performed using an Olympus BH microscope under 400× magnification. Concentrations of algal cells were determined according to Eq. (1):

$$C_{algae} = \frac{N}{n \times V_{grid}} \quad (1)$$

where algae cell concentration ($C_{algae}$) (cells ml $^{-1}$) was determined by measuring the total number of cells ($N$) in the grid blocks ($n$), where $V_{grid}$ is the volume per grid used for enumeration. All cell counts were performed using two measurements of duplicate cultures, making the total number of measurements $n = 4$, and the standard deviation was calculated using the Excel Analysis ToolPak function (Excel, Microsoft Office 365, v. 16.43).

## Modeling Cell Growth

To determine whether culture growth was statistically significant, $P$-values and $R^2$ (correlation coefficients) were calculated, with values of $P < 0.05$ considered statistically significant. The goodness of fit determined by $R^2$ (Kuśnierz and Łomotowski, 2015) was measured for the regression analysis of each growth curve of the algae cultures plotted as a log scale of their exponential growth measured as average cell counts against time at each pressure condition (**Supplementary Tables 9**, **10**). The statistical analysis was performed in Microsoft Excel Analysis Tool Pak (v. 16.43) (**Supplementary Table 9**).

The primary, overarching goal of this work was to test growth under low pressures, and we therefore performed fewer sampling sessions to minimize the amount of time that cultures were returned to normal terrestrial atmospheric conditions during sampling. These growth curves therefore contain fewer time points than many microbial growth experiments, and we anticipated that fitting these data with the logistic growth curve (Eq. 2) would be less constrained than had we more data points. However, fitting these data with the logistic growth curve (Eq. 2) can help assess the potential of algae for production of oxygen and food for astronauts by helping constrain the doubling time and lag phase duration (LPD) of these cultures under low pressure conditions. Although the generated curves are less constrained, we did fit the data using the logistic growth curve using the Solver function in Microsoft Excel (v. 16.43). The logistic growth curve fittings are in **Supplementary Figures 1–24**.

The logistical growth equation (Eq. 2) was used to fit algal cell concentration data from 0–62 days of incubation, where the average of the two cell count measurements of each culture ($n = 2$) was fit separately (**Supplementary Figures 1–24**).

$$C_{algae}(t) = \frac{C_{algae,max}}{1 + e^{-r(t-t_{half})}} \quad (2)$$

where $C_{algae}$ $(t)$ is the concentration of algae at time $t$, $C_{algae,max}$ is the maximum concentration of algae or the carrying capacity of the culture, *t-half* is the time at the sigmoid midpoint, and $r$ is the slope at the sigmoid midpoint. $C_{algae,max}$ was estimated by averaging the last 2–4 cell concentration measurements except for *D. salina* at 670 mbar, for which 1 point was used (**Supplementary Tables 1–3** and **Table 2**), and was then used as an input in fitting the logistic growth curve to the data using the Excel Solver function in the Analysis Tool Pak (v. 16.43). Logistical growth curves were fit by minimizing the residual sum of squares and yielding best-fit *t-half* and $r$ values, where the mean of cell counts (Y), Standard error (SE) of (Y),

**TABLE 2 |** Table showing the carrying capacities ($C_{algae,max}$) computed at different pressures for *Chlorella vulgaris* (CV) *Dunaliella salina (DS),* and *Chloromonas.*

| Pressure (mbar) | Average carrying capacity of duplicates ($C_{algae,max}$)* | Uncertainty* | n (number of time points the average carrying capacity is based on) |
|---|---|---|---|
| ***Chloromonas brevispina*** | | | |
| 670 ± 20 | $161.1 \times 10^4$ | $5.2 \times 10^4$ | 2 |
| 330 ± 20 | $198.0 \times 10^4$ | $8.8 \times 10^4$ | 3 |
| 160 ± 20 | $86.8 \times 10^4$ | $6.2 \times 10^4$ | 3 |
| 80 ± 2.5 | $43.4 \times 10^4$ | $2.5 \times 10^4$ | 3 |
| ***Dunaliella salina*** | | | |
| 670 ± 20 | $2.3 \times 10^6$ | $1.5 \times 10^{5}$** | 1 |
| 330 ± 20 | $121.3 \times 10^4$ | $7.5 \times 10^4$ | 3 |
| 160 ± 20 | $30.0 \times 10^5$ | $4.6 \times 10^5$ | 4 |
| 80 ± 2.5 | $15.8 \times 10^4$ | $1.3 \times 10^4$ | 4 |
| ***Chlorella vulgaris*** | | | |
| 670 ± 20 | $32.8 \times 10^4$ | $1.1 \times 10^4$ | 3 |
| 330 ± 20 | $78.8 \times 10^4$ | $3.6 \times 10^4$ | 3 |
| 160 ± 20 | $13.0 \times 10^5$ | $1.5 \times 10^5$ | 4 |
| 80 ± 2.5 | $57.1 \times 10^4$ | $4.5 \times 10^4$ | 3 |

*The carrying capacities are the averages of the duplicate experiments reported in **Supplementary Table 11** with the uncertainties propagated for the average of the duplicate experiments.

**This uncertainty represents half the range between the duplicates.

Sum of Square of Residuals, Critical T, Degree of freedom and Confidence intervals were generated and were then used by the Solver function in the Excel for Best-fit logistic curve fitting (**Supplementary Tables 4–6**; Motulsky and Christopoulos, 2004). To examine differences in the time required by each species to acclimatize at different pressures, the length of the lag phase was estimated as the point at which the algae concentration calculated using the logistic growth curve was 15% that of the carrying capacity. This allowed a comparison of the duration of the estimated lag phase between different species, and between the same species at different pressures.

The exponential growth rate equation (Eq. 3) was used to fit algal concentration data spanning 0 days of incubation up to one time point beyond the best fit $T_{half}$ value as determined from the logistic curve, where the average of the duplicate measurements of each culture ($n = 2$) was fit separately (**Table 3**):

$$C_{algae}(t) = C_{algae,0}e^{rt} \quad (3)$$

where $C_{algae}$ *(t)* is the concentration of algae at time *t*, $C_{algae,0}$ is the initial algal concentration in the experiment resulting from inoculation before growth has occurred and is input as a fixed parameter based on measured values, and the equation was solved for the growth rate *(r)* using Microsoft Excel. The growth rate (*r*) was then used to determine the doubling time ($T_d$) using Eq. 4:

$$T_d = \frac{\ln(2)}{r} \quad (4)$$

The goodness of fit ($R^2$) for the exponential algal growth models of each culture condition is given in **Table 3**.

# RESULTS

## Algae Growth Dynamics Under Different Pressure Conditions

The statistical analysis on the OD measurements at 670 mbar indicated that *C. brevispina, C. vulgaris, K. austriaca,* and *D. salina* showed statistically significant ($p < 0.05$) growth at 670 mbar, whereas *S. plantensis* did not show statistically significant growth (**Figure 2** and **Supplementary Table 9**). However, despite showing statistically significant growth at 670 mbar, *K. austriaca* showed very minimal growth at this pressure (OD values slightly more than doubled over the course of the experiment). We therefore chose the strains *C. brevispina, D. salina,* and *C. vulgaris* as candidate strains for further detailed quantitative growth analysis using cell counts at lower pressures (**Supplementary Tables 1–3** and **Figure 2**).

*C. brevispina*, *D. salina,* and *C. vulgaris* exhibited exponential growth at 670 mbar as indicated by the goodness of fit ($R^2$) values obtained by the exponential growth models of their OD values which measured as 0.93, 0.90, and 0.88, respectively (**Supplementary Figure 25** and **Supplementary Table 9**).

### Carrying Capacity

The carrying capacity for the cultures, measured as the average of the last $n = 2$–4 time points except for *D. salina* at 670 mbar, for which 1 point was used, ranged from $16.0 \pm 1.3 \times 10^4$ cells/ml to $30.0 \pm 4.6 \times 10^5$ cells/ml. The highest carrying capacity for each species was observed at the pressures of 330 mbar for *C. brevispina* at $19.8 \pm 0.9 \times 10^5$ cells/ml, at 160 mbar for *D. salina* at $30.0 \pm 5.6 \times 10^5$ cells/ml and for *C. vulgaris* at $13.0 \pm 1.5 \times 10^5$ cells/ml (**Table 2**).

### Doubling Time

The doubling time for the cultures ranged from $3.7 \pm 0.7$ to $32.5 \pm 7.4$ days, with the fastest doubling time for each species being at the pressures of 330 mbar for *C. brevispina* ($5.4 \pm 1.1$ days) and *D. salina* ($3.7 \pm 0.7$ days) and at 160 mbar for *C. vulgaris* ($3.9 \pm 2.0$ days). Due to our experimental setup, designed to minimize changes in pressure required by sampling, and thus with measurements 1 week apart, the uncertainty on the doubling times is large (**Table 3**), but these fastest doubling times under low pressures are similar to those previously measured for *C. brevispina* under optimum conditions ($5.2 \pm 0.1$ days; Harrold et al., 2018).

### Lag Phase Duration

The estimated LPD for the cultures ranged from $0.20 \pm 0.05$ to $25.8 \pm 1.4$ days, with the shortest lag phase for each species being at the pressures of 670 mbar for *C. brevispina* ($6.8 \pm 0.2$ days), at 80 mbar for *D. salina* ($0.8 \pm 0.3$ days), and at 160 mbar for *C. vulgaris* ($0.20 \pm 0.05$ days) (**Table 3**).

**TABLE 3 |** Estimated Lag Phase Duration (LPD), growth rate (r), doubling time ($T_d$), and correlation coefficient ($R^2$) value for the candidate algae at different pressures.

| Pressure (mbar) | *Chloromonas brevispina* (CB) | | | | | *Dunaliella salina* (DS) | | | | | *Chlorella vulgaris* (CV) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | [a]LPD* | [b]r* | [c]$T_d$* | [d]$R^2$ | | [a]LPD | [b]r | [c]$T_d$ | [d]$R^2$ | | [a]LPD | [b]r | [c] $T_d$ | [d]$R^2$ | |
| | | | | CB1 | CB2 | | | | CB1 | CB2 | | | | CB1 | CB2 |
| 670 ± 20 | 6.8 ± 0.2 | 0.11 ± 0.02 | 6.4 ± 1.3 | 0.86 | 0.91 | 25.8 ± 1.4 | 0.09 ± 0.01 | 8.2 ± 1.4 | 0.73 | 0.87 | 8.61 ± 0.07 | 0.02 ± 0.005 | 32.5 ± 7.4 | 1 | 0.89 |
| 330 ± 20 | 10.7 ± 0.7 | 0.13 ± 0.02 | 5.4 ± 1.1 | 0.89 | 0.86 | 9.7 ± 0.7 | 0.19 ± 0.03 | 3.7 ± 0.7 | 0.92 | 0.99 | 2.6 ± 0.1 | 0.13 ± 0.03 | 5.7 ± 1.6 | 0.89 | 0.89 |
| 160 ± 20 | 9.0 ± 0.4 | 0.08 ± 0.01 | 9.0 ± 1.7 | 0.93 | 0.86 | 2.7 ± 0.8 | 0.07 ± 0.02 | 9.5 ± 3.6 | 0.81 | 0.91 | 0.20 ± 0.05 | 0.14 ± 0.06 | 3.9 ± 2.0 | 0.78 | 0.79 |
| 80 ± 2.5 | 13.6 ± 0.2 | 0.11 ± 0.02 | 6.1 ± 1.1 | 0.94 | 0.83 | 0.8 ± 0.3 | 0.08 ± 0.03 | 8.6 ± 3.3 | 0.84 | 0.69 | 6.6 ± 2.1 | 0.11 ± 0.04 | 6.4 ± 2.6 | 0.89 | 0.78 |

**Lag phase duration (LPD), growth rate (r), doubling time ($T_d$), for Chloromonas brevispina (CB), Dunaliella salina (DS), and Chlorella vulgaris (CV) were the averages of the duplicate experiments reported in* ***Supplementary Table 12*** *with the uncertainties on LPD, doubling time and growth rate are propagated for the average of the duplicate experiments.*

*[a]Lag phase duration (LPD) was estimated as 15% of the culture carrying capacity and is reported in days.*

*[b]Growth rate (r) as solved using the exponential growth equation (Eq. 3) and is reported as per day.*

*[c]Doubling time ($T_d$) is the time it takes for a population to double in size found using Eq. 4 and is reported in days.*

*[d]Correlation coefficient ($R^2$) is used to measure the goodness of fit for non-linear regression.*

### Trends With Decreasing Pressure

The strain *C. vulgaris* showed the clearest trends with decreasing pressure. With the exception of the 80 mbar pressure, *C. vulgaris* displayed increasing carrying capacities with decreasing pressure, with carrying capacities reaching $32.8 \pm 1.1 \times 10^4$ cells/ml at 670 mbar, $78.8 \pm 3.6 \times 10^4$ cells/ml at 330 mbar, and $13.0 \pm 1.5 \times 10^5$ cells/ml at 160 mbar (**Table 2**). Similarly, with the exception of the 80 mbar pressure condition, a decreasing trend was observed in the estimated LPD with decreasing pressure, with the length of the estimated lag phase decreasing from 8.61 ± 0.07 at 670 mbar to 2.6 ± 0.1 at 330 mbar to 0.20 ± 0.05 at 160 mbar (**Tables 2**, **3** and **Figure 3**).

Similarly, with decreasing pressure, *D. salina* showed a consistent decrease in the estimated LPD from 25.8 ± 1.4 days at 670 mbar, to 9.7 ± 0.7 days at 330 mbar, to 2.7 ± 0.8 days at 160 mbar, and 0.8 ± 0.3 days at 80 mbar. However, no clear trend in carrying capacity was observed for *D. salina,* with the highest carrying capacity of $30.0 \pm 4.6 \times 10^5$ cells/ml observed at 160 mbar pressure (**Tables 2**, **3** and **Figure 3**). For *C. brevispina,* LPD generally increased with decreasing pressure, and no clear trend was observed for carrying capacity with pressure (**Tables 2**, **3** and **Figure 3**).

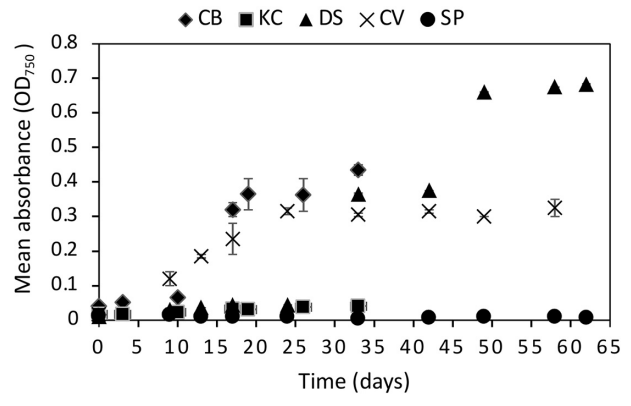


**FIGURE 2 |** Growth curves of all five algae *Chloromonas brevispina* (CB), *Kremastochrysopsis austriaca* (KC), *Chlorella vulgaris* (CV), Spirulina plantensis (SP) and *Dunaliella salina* (DS) grown at low pressures of 670 ± 20 mbar plotted as a mean of absorbance measured by optical density measurement at 750 nm. Error bars represent the range in $OD_{750}$ values between duplicate experiments. Where error bars are not visible, they fall within the symbol.

## DISCUSSION

Human exploration of Mars is one of the key scientific and technological undertakings of our time. Current research is ongoing to successfully support astronauts' food and oxygen needs for long-term space exploration journeys including to Mars (Massa et al., 2017). The results of this research underscore the critical need for advanced BLSS to support human life during extended space flight and on long planetary surface expeditions (Monje et al., 2003; Revellame et al., 2021). Algae are considered an excellent food source for astronauts because they (1) contain all the essential amino acids, (2) are more digestible than traditional plant protein and (3) grow faster than traditional crops (wheat, rice, corn, etc.) (Bleakley and Hayes, 2017; Koyande et al., 2019; Yang et al., 2019). Algal oil also contains substantial contents of poly-unsaturated fatty acid (PUFA) and algal-specific super-antioxidants, both of which may have beneficial effects for astronauts exposed to harsh space environments (Harwood, 2019; Yang et al., 2019). Recent results also signified the biomedical applications of astaxanthin, a pigment produced by algae, in preventing certain cancers, aging, macular degeneration, and inflammation (Grimmig et al., 2017). All these reports suggests that algae can be a competitive food option for food and oxygen production in long-term space exploratory missions (Yang et al., 2019).

However, optimization of algal growth for their use in self-sustaining BLSS is needed. Therefore, the goal of this study was to grow algae for potential oxygen and food production under low pressure conditions, such as might be possible in an enclosed low-pressure environment made with flexible

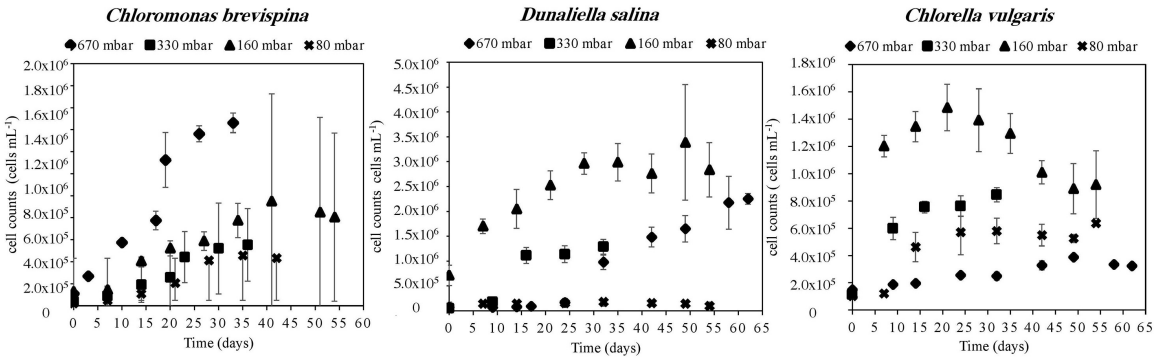


**FIGURE 3 |** Growth curves of *Chloromonas brevispina* (CB), *Chlorella vulgaris* (CV), and *Dunaliella salina* (DS) at pressures of 670 ± 20 mbar, 330 ± 20 mbar, 160 ± 20 mbar, and 80 ± 2.5 mbar plotted as a mean value ($n$ = 4) of duplicate cell count measurements of duplicate experiments. Error bars are 1 standard deviation of mean cell count values, and all data are shown in **Supplementary Tables 1–3**. Where error bars are not shown they lie within the points.

materials on Mars. Flexible materials, such as those used for the extravehicular mobility unit (EMU) spacesuit that enables pressurized oxygen, ventilation, as well as carbon dioxide, water vapor, and trace contaminant removal, maintains a pressure of 296 mbar (4.3 psi) (National Research Council, 1997). Low pressure (∼200–300 mbar) martian or lunar greenhouses and inflatable structures have already been conceptualized and designed (e.g., Cadogan et al., 1999; Wheeler and Martin-Brennan, 2000). The use of flexible materials to make inflatable structures would considerably reduce the mass and volume of any martian greenhouse structure making it more viable for deployment. Therefore, the ability to grow photosynthetic organisms under low pressures (<296 mbar) facilitates the use of BLSS that could be utilized on Mars. Furthermore, pressurizing such a structure initially with the martian atmosphere would reduce transported oxygen/air resource requirements.

In this study, the maximum growth for each species was observed at the pressures of 330 mbar for *C. brevispina* with $19.8 \pm 0.9 \times 10^5$ cells/ml and at 160 mbar for *D. salina* with $30.0 \pm 5.6 \times 10^5$ cells/ml and for *C. vulgaris* with $13.0 \pm 1.5 \times 10^5$ cells/ml. To put these cell concentrations into context, here we compare them with optimum growth conditions as reported in both natural and laboratory conditions. For *C. brevispina,* under optimum conditions, cell concentrations were observed reaching $10^6$ cells/ml (Hoham et al., 1979; Harrold et al., 2018), and for *D. salina,* the highest cell concentrations reached $10^6$ –$10^7$ cells/ml under laboratory conditions (García et al., 2006; Hadi et al., 2008; Ahmed et al., 2017). For *C. vulgaris*, the maximum growth under optimum conditions was also observed to range from $10^6$ to $10^7$ cells/ml under laboratory conditions (Mandalam and Palsson, 1995; Adamczyk et al., 2016; Sánchez-Saavedra et al., 2020). The ability of our candidate algae species to grow under low pressure conditions and reach cell concentrations close to maximum cell counts observed for these species under optimum conditions, makes them excellent candidates to be used for BLSS.

The results of our experiments also show that three species showed substantial growth at 80 mbar and 160 mbar (**Supplementary Figures 27**, **28**), well below the 200–300 mbar lower limit generally proposed for flexible materials on Mars, and well below the value thought to be the limit for vascular plant growth (Hublitz et al., 2004; Paul and Ferl, 2006; Richards et al., 2006). Under these conditions of very low pressure (80 mbar), the growth rates of the cultures *C. brevispina, D. salina,* and *C. vulgaris* were relatively slow (with doubling times of ∼5–9 days, although these are comparable to *C. brevispina* growth under optimum conditions (Harrold et al., 2018).

According to previous estimates, each astronaut performing 2 h of intense physical activity each day would consume approximately 1 kg of $O_2$ per day (Horneck et al., 2003), which can be photosynthetically produced by bio-fixation of 1.3 kg of $CO_2$ (Verseux et al., 2016). Previous work indicates that for C. *vulgaris* species, a maximal bio-fixation rate of 1.4 g $CO_2$ /L/d was observed at a cell concentration of $1.3 \times 10^7$ cells/ml under optimum conditions (Adamczyk et al., 2016) and for D. salina species, under optimum conditions, the $CO_2$ bio-fixation rate was observed to range from 0.71 g $CO_2$/L/d to 1.102 g $CO_2$/L/d at maximum biomass concentrations (Mortezaeikia et al., 2016). Based on these values of $CO_2$ bio-fixation rates, it can be estimated that the cell counts of *C. vulgaris* and *D. salina* measured in our experiments reaching $13.0 \pm 1.5 \times 10^5$ cells/ml and $30.0 \pm 4.6 \times 10^5$ cells/ml at 160 mbar, respectively, could potentially generate enough oxygen for astronaut consumption. Snow algae *C. brevispina* is also known to be an important $CO_2$ sink in snow environments (Williams et al., 2003) and their cell counts reaching $19.8 \pm 0.9 \times 10^5$ cells/ml at 330 mbar in our experiments indicate their potential to photosynthetically generate substantial oxygen via $CO_2$ bio-fixation. These calculations, however, are simply estimates as the photosynthesis machinery of algae can be influenced by various environmental factors [light exposure, pressure, activity of reactive oxygen species (ROS), pH fluctuations, etc.]. Therefore, even though the biomass data suggest that these strains could be pursued as food and oxygen producers on Mars, further research is needed to directly optimize and quantify $CO_2$ fixation and $O_2$ generation under these low-pressure settings.

The observed decrease in duration of the estimated lag phase with decreasing pressure for *D. salina* and *C. vulgaris,* and the increasing carrying capacities with decreasing

pressure observed for *C. vulgaris* (**Table 2**) suggest that the cultures may be acclimatizing to the decreasing pressure conditions. Few studies have explored the mechanisms of adaptation of microorganisms under low pressure conditions (Kanervo et al., 2005; Nicholson et al., 2013; Murukesan et al., 2016; Schuerger and Nicholson, 2016; Verseux, 2020). Previous research on prokaryotes growing at low pressures, including transcription analysis, revealed that owing to their specialized adaptations to thrive in extreme environments, extremophiles are most likely to be better suited to survive under low pressures conditions (Schuerger et al., 2013; Verseux, 2020). This could explain the successful growth at low pressure of the extremophilic candidate strain *D. salina* and *C. brevispina*, where their natural adaptations to cope with high salinity, cold temperatures, and high irradiance might be responsible for their tolerance to low pressures as well. Some reports also suggest that microorganisms could evolve toward higher tolerance if exposed to low pressure over multiple generations (Verseux, 2020). This may explain the trend observed in the *C. vulgaris* species where the increasing trend in carrying capacity was observed with decreasing pressure. Additionally, *C. vulgaris* is globally distributed in both aquatic and terrestrial habitats (Bock et al., 2011; Aigner et al., 2020) suggesting that the species has adaptations to survive in these contrasting habitats. The molecular mechanisms of such adaptations are not clearly understood (Aigner et al., 2020) but may also contribute to the tolerance to low pressures as well.

Further analysis of the molecular basis of low-pressure adaptations will be required to understand different growth dynamics of algae species under low pressure conditions and may reveal key genes or quantitative trait loci that are involved in growth at low pressure. Such key genes or quantitative trait loci could potentially be selected for use in breeding studies, resulting in more useful algal strains. In addition, long-term growth under low pressure on Earth should lead to the development of strains with elevated productivities under low pressure. Together, such studies would accelerate the development of an algal-based BLSS for Mars.

## CONCLUSION

Life support represents one of the most critical technologies needed for successful and safe long-term deep space human exploration missions and will require substantial amounts of both oxygen and food production. The results from this study contribute to the development of a BLSS by demonstrating the potential contributions of three candidate species *C. brevispina, D. salina,* and *C. vulgaris.* All three of the candidates showed exponential growth at low pressures of 80 mbar and 160 mbar, which indicates the possibility of using inflatable greenhouses to produce oxygen on the surface of Mars. If these cultures produce approximately similar $O_2$ yields per unit of dry biomass as recorded previously (Kirensky et al., 1968; Gitelson, 1992), the biomass of the algae used as food could also generate enough $O_2$ for the astronauts' use. In addition, the lag phases of *D. salina* and *C. vulgaris* decreased with decreasing pressure, and the carrying capacity of *C. vulgaris* increased with decreasing pressure, which suggests that the cultures may be acclimatizing to the decreasing pressure conditions and may be increasingly useful in BLSS. Together these results indicate that these species may be able to contribute to potential BLSS on Mars using low pressure (∼200–300 mbar) greenhouses and inflatable structures that have already been conceptualized and designed.

## DATA AVAILABILITY STATEMENT

The data supporting the conclusions of this paper are available in the **Supplementary Material**.

## AUTHOR CONTRIBUTIONS

LC, EH, DM, and WR contributed to the conception and design of the study. LC conducted the experiments. EH and LC analyzed the data and performed the statistical analysis. CA contributed to the experimental design and wrote a section of the manuscript. JR and DR contributed to sample collection and experimental setup. All authors contributed to manuscript revision, read, and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.733244/full#supplementary-material

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# Hydrogels Improve Plant Growth in Mars Analog Conditions

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Sustainable human settlement on Mars will require *in situ* resource utilization (ISRU), the collection and utilization of Mars-based resources, including notably water and a substrate for food production. Plants will be fundamental components of future human missions to Mars, and the question of whether Mars soils can support plant growth is still open. Moreover, plant cultivation may suffer from the lack of *in situ* liquid water, which might constitute one of the biggest challenges for ISRU-based food production on Mars. Enhancing the crop yield with less water input and improving water utilization by plants are thus chief concern for sustainable ISRU food production. Hydrogels are polymers able to absorb large quantity of water and to increase soil water retention, plant establishment and growth. This work reports on the short-term assessment of plant growth in Mars soil analogs supplemented with hydrogels. Soil analogs consisted of sand and clay-rich material, with low organic matter content and alkaline pH. Soils were supplemented with 10% (w/w) potting medium and were sampled in Utah desert, in the vicinity of the Mars Desert Research Station, surrounded by soils sharing similarities in mineralogical and chemical composition to Martian soils. Height and dry biomass of spearmint (*Mentha spicata*) were compared under various irrigation frequencies, and seed germination of radish (*Raphanus sativus*) were monitored. Under limited irrigation, results indicate that the soil analogs were less capable of supporting plant growth as a comparison to potting medium. The effects of hydrogel supplementation were significant under limited irrigation and led to spearmint heights increased by 3 and 6% in clay- and sand-containing soils, respectively. Similarly, hydrogel supplementation resulted in spearmint mass increased by 110% in clay-containing soils and 78% in sand-containing soils. Additionally, while radish seeds failed to germinate in soil analogs, hydrogel supplementation allows for the germination of 27% of seeds, indicating that hydrogels might help loosening dense media with low water retention. Collectively, the results suggest that supplementation with hydrogel and plant growth substrate could help plants cope with limited irrigation and poor alkaline Mars soil analogs, and are discussed in the context of strategies for ISRU-based off-world colonization.

**Keywords: *in situ* resource utilization (ISRU), Mars, life support systems, astrobiology, colonization, plant, hydrogel**

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## INTRODUCTION

Sustained human settlement on Mars will raise considerable number of challenges, among which the use of plant-based bioregenerative advanced life support systems (ALS), with the potential to provide sustainable food production, air and water recycling, and to allow the minimization of resupply missions (Ming 1989; Richards et al., 2006). Critically, such systems may likely depend on *in situ* resource utilization (ISRU), i.e., the use of existing materials at the settlement site, including notably water and a substrate for food production (Wheeler 2010). Together, ISRU and addition of ALS systems to exploration missions might save cargo volumes in spacecrafts, minimize safety issues, extend the length of planetary explorations and support mission success (Richards et al., 2006).

Beside the necessity of highly sealed spaces, different models for future ground-based life support systems have been studied for off-world food production, among which soilless (i.e., hydroponics or aeroponics) and soil-based systems. In comparison to soilless models, soil-based systems offer the distinct advantages to limit fertilizer and the reliance on Earth-supplied resources and to improve waste recycling (Nelson et al., 2008).

Due to the necessity of developing ISRU systems with limited supplementation, the question of whether plants can grow on Martian soils is of chief importance (Wamelink et al., 2014; Fox-Powell et al., 2016). In view of fleets of orbital and landed spacecraft, our understanding of Martian soils has improved considerably over the last few decades (Billi et al., 2019). Although Martian soils contain a variety of necessary micro- and macronutrients in accessible forms for plants (Ehrenfreund et al., 2011; Cannon et al., 2019), substantial soils properties argue against efficient plant growth, such as high concentrations of calcium perchlorate (Hecht et al., 2009), soils with low water retention capacity (Hecht et al., 2009; Wamelink et al., 2014; Fox-Powell et al., 2016), or soils with moderately to high alkaline content (Fairén 2008; Hecht et al., 2009), a barrier for many plant species (Wamelink et al., 2005). Although large stores of underground water ice at various depth has been evidenced (Wilson et al., 2018; Piqueux et al., 2019), its availability for *in situ* use is still uncertain (Bullock et al., 2004; Möhlmann 2004), water stress being one of the major factors limiting crop growth and plant biomass production (Shormin 2009). The study of Terrestrial analogs of Martian regolith on plant growth in the context of limited water resources are thus critically needed.

Solutions has been reported to improve water use efficiency by plants: based on the ability of organic matter to store water available for plant growth (Hudson 1994), the supplementation with compost has been reported to improve water use efficiency and lettuce growth (Duri et al., 2020; Caporale et al., 2020).

Hydrogels are polymers able to absorb large quantities of water and fixing on plant roots. By improving water availability, they have been shown to reduce water stress and improve plant growth and survival (Montesano et al., 2015). However, some studies report negative effects on different soil types (Del Campo et al., 2011), and no study has been conducted so far with Mars soil analogs, as far as is known.

The Mars Desert Research Station (MDRS), is surrounded by a landscape that is an actual geologic Mars analog, with a mineralogy comparable to Mars, consisting of deposits of sands, clay minerals, iron oxides and traces of carbonates (Kotler et al., 2011; Direito et al., 2011).

This study reports on the effect of hydrogel supplementation on spearmint (*Mentha spicata*) growth parameters (i.e., height and dry biomass) under full and limited irrigation regimes, in two Mars soil analogs collected in the vincinity of the MDRS station. These soils consisted of sand and clay-rich material, with low organic matter content and alkaline pH, and were supplemented with 10% (w/w) potting medium. Additionally, the effect of hydrogel supplementation on seed germination and emergence of radish (*Raphanus sativus*) was addressed in the two soils analogs.

This study is a proof of concept to help the development of Mars and Moon regoliths-based food production, where *in situ* use of space resources and minimal water input are needed.

## MATERIAL AND METHODS

### Soils Sampling

All experiments were conducted during the UCL to Mars 2018 campaign (Université catholique de Louvain) within the constraints of *in situ* operations in the MDRS station, a Mars analog facility operated by the Mars Society (Saint-Guillain 2019; Wuyckens et al., 2019). Soil samples were collected in the vincinity of the MDRS station, in Utah desert, at an average altitude of 1391 m, and consisted in a white sand layer and a brown-reddish clay-rich material (hereafter referred as "sand" and "clay", respectively) (**Table 1**). These soils were previously analyzed for their composition during the EuroGeoMars 2009 campaign (Ehrenfreund et al., 2011), including elemental composition of nitrate, potassium, phosphorous, organic matter and carbonates. Soils were selected for their similarities with Mars soils, based on their 1) mineralogy (i.e., sand and phylosilicates [clay minerals]) and 2) composition (i.e., low amount of organic matter, iron oxides, and traces of carbonates) (Poulet et al., 2005; Chevrier and Mathé 2007; Boynton et al., 2009). Additionally, soils were analyzed *in situ* for their pH in water, indicating pH of 9.2 and 9.06 for sand and clay soils, respectively (**Table 2**).

### Water Holding and Release Properties of Hydrogels

Hydrogels are polymers that are able to increase water retention, and enhance plant growth (Wang and Boogher 1987; Montesano et al., 2015; El-Asmar et al., 2017). The superabsorbent polymer used in the present study was the commercial hydrogel STOCKOSORB® 660 Medium (Evonik Industries; hereafter referred as "hydrogel"), a crosslinked potassium polyacrylic acid designed to remain active in the soil for 1–3 years. Soils were supplemented with 0.1% (w/w) hydrogel (according to the manufacturer's instructions) prior to plant transplantation. Hydrogel were first analyzed for their ability to regenerate after drying, to mimic absorption by plant roots. A sample of 2 g of dry hydrogel was saturated with water, and weighed after

**TABLE 1 |** Characteristics of soils used in this study. * Soils sampled at the Mars Desert Research Station location, adapted from (Ehrenfreund et al., 2011).

| Soil nature | Location | Organic matter (%) | N-element (ppm) | P (ppm) | K (ppm) | Carbonates (%) |
|---|---|---|---|---|---|---|
| Sand * | N38.40737°W110.79261° | 1 | >75 | 12 | 175 | <1 |
| Clay * | N38.42638°W110.78342° | 2 | >75 | 100 | 200 | 2 |
| Potting medium | — | 48 | <10 | 22 | 150 | — |

**TABLE 2 |** Determined pH of soils and mixed soils used in this study.

| Soil | pH |
|---|---|
| Sand | 9.2 |
| Clay | 9.06 |
| Potting medium | 7.05 |
| Mix sand | 8.5 |
| Mix clay | 8.4 |

10 min at room temperature. This sample was dried on absorbing paper at room temperature for 24 h, and subjected to three additional hydration/dehydration cycles. Water holding capacity was measured in the soils with or without 0.1% (w/w) hydrogel supplementation. Water holding capacity of the soil was defined as the gain in weight at saturation point divided by the dry weight of the soil, expressed in percent (Montesano et al., 2015).

## Experimental Design for Plant Growth

All experiments were conducted in the greenhouse facility of MDRS station during the UCL to Mars 2018 campaign, from March 12, 2018 (Day 1) to March 25, 2018 (Day 13), with an average temperature of 24 °C (ranging from 16.2°C to 30.5°C) and an average air humidity of 43% during the experimental period. During this period, mean length of visible light was 14 h (solar irradiance ≈5.5 kW/m$^2$/day (Sengupta et al., 2018).

## Effect of Hydrogels on Plant Growth

For a short-term assessment of plant growth, spearmint (*Mentha spicata*) plants were selected for their rapid growth and their robustness under nutrient-poor conditions. Experiments aimed at addressing the effect of hydrogel amendment in Mars soil analogs, in normal or reduced irrigation conditions. To that end, plants were manually overhead irrigated with 70 ml of water (without addition of nutrient solution), daily for normal irrigation conditions, and each 4 days for reduced irrigation conditions, below requirements for *Mentha* species (Clark R.J. 1980; McConkey et al., 2000; Shormin 2009). Experiments were divided into three groups of soils: potting medium ("pot. medium"), sand supplemented with 10% (w/w) potting medium ("mix sand"), and clay supplemented with 10% (w/w) potting medium ("mix clay"). Each soil group was tested under normal or reduced irrigation regimes, performed in three independent experimental units. For each condition, plants of *Mentha spicata* were grown with or without 0.1% (w/w) hydrogel supplementation. This experimental set-up resulted in three soils x two irrigation conditions x two supplementation conditions (with or without hydrogel supplementation) x three replicates.

Plants of *Mentha spicata* were transplanted at Day 1 in single pots of 9 cm length and 10 cm depth with drainage outlet. Shoot heights were recorded 13 days after transplantation (Day 13). Initial plants were 23 ± 3 cm height. To normalize these variations and allow statistical comparisons, the growth of each plant was represented as a percentage of growth in comparison to its initial height (Day 1) (see **Figure 2A**).

Dry shoots masses were recorded for all plants 13 days after transplantation. Plants were dried in a forced air oven at 60°C until reaching a constant mass (Valmorbida and Boaro 2007).

## Effect of Hydrogels on Seed Germination and Emergence

To monitor the first growth stages, seeds of radish (*Raphanus sativus* 'Scarlet Globe') were selected for their fast germination. Experiments were designed to address the effect of hydrogel-supplemented Mars soil analogs on seed germination. To that end, the following treatments were compared: potting medium ("pot. medium"), sand supplemented with 10% (w/w) potting medium ("mix sand"), clay supplemented with 10% (w/w) potting medium ("mix clay"), sand and clay. This experimental set-up resulted in five soil conditions x two supplementation conditions (with or without hydrogel supplementation) x three replicates. For each condition, five seeds were positioned per pot at the same burial depth. The percentage of seed germination and emergence (resulting from both germination and emergence above the surface) were recorded at Day 13.

## Statistical Analysis

Statistical analyses were performed with GraphPad Prism version 8.3.1, GraphPad InStat v3.10 (GraphPad Software) and SPSS v25.0 (IBM Statistics). For comparison between hydrogel and control conditions, statistical differences were determined using unpaired Student's t-tests, with a threshold of statistical significance set to 0.05. $p$ values strictly inferior to 0.05, 0.01 and 0.001 were used to show statistically significant differences and are represented with *, ** or *** respectively. For multiple comparison between soils, statistical differences were determined using one-way ANOVA with Tukey's post hoc tests, with a threshold of statistical significance set to 0.05. Different letters indicate statistically significant differences.

# RESULTS

## Water Holding and Release Properties of Hydrogels

Hydrogels are polymers that can absorb large quantity of water during expansion (**Figure 1A**). When submitted to consecutives cycles of hydration and dehydration in order to mimic absorption by plant roots and watering, respectively, data

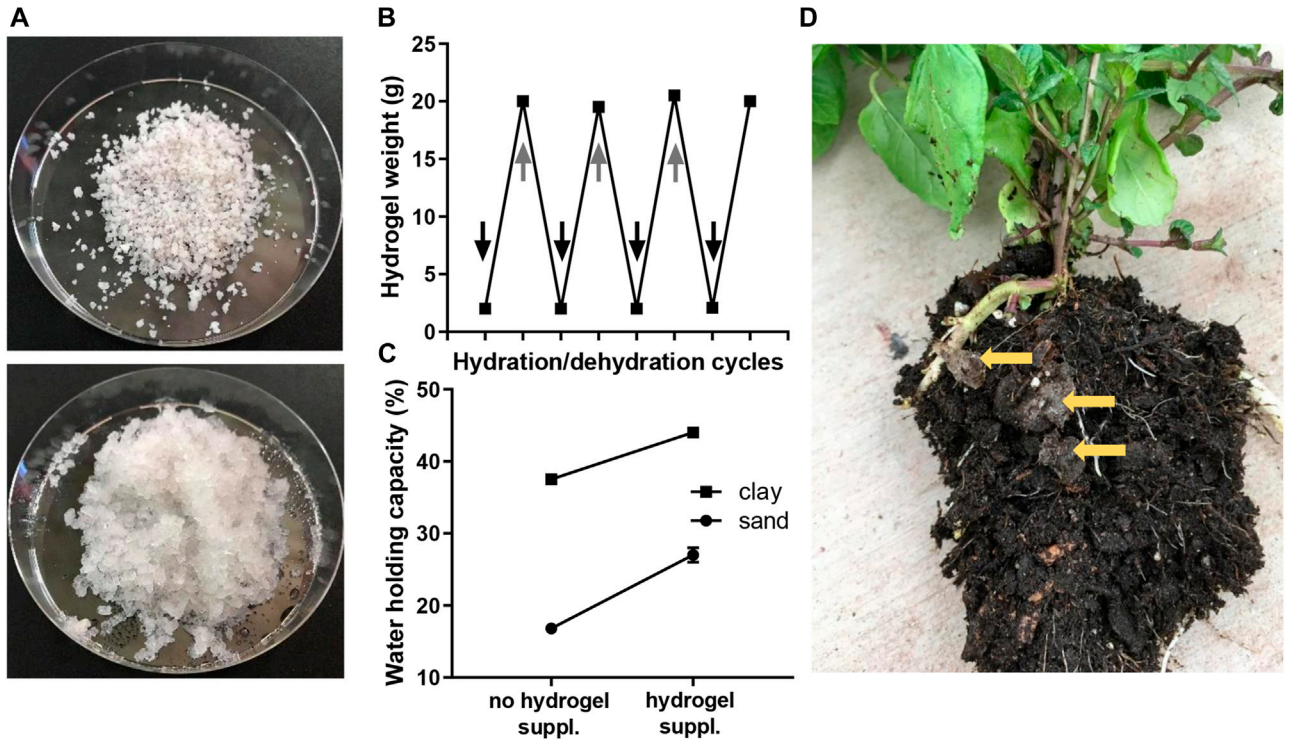


**FIGURE 1 |** Water holding and release properties of hydrogels **(A)** Sample of 1 g of dry hydrogel **(left)**, and saturated with water and incubated 10 min **(right) (B)** Hydrogel weight during hydration/dehydration cycles. A sample of 2 g of dry hydrogel was saturated with water (hydration; black arrows), incubated 10 min at room temperature prior to weighing, and dried on absorbing paper at room temperature for 24 h (dehydration; gray arrows) **(C)**) Water holding capacity of sand and clay soils with (hydrogel suppl.) or without (no hydrogel suppl.) 0.1% (w/w) hydrogel supplementation **(D)** Hydrogels (arrows) fixed on spearmint (*Mentha spicata*) roots.

indicated that they absorb on average 10 times their weight in water after 10 min, and that they efficiently regenerate for sustained periods of time (**Figure 1B**). The water holding capacity of sand increased from 16.8 to 27% with hydrogel supplementation (**Figure 1C**). The water holding capacity of clay was higher than in sand (37.5%), and hydrogel supplementation led to additional increase to 44%. Additionally, roots of *Mentha spicata* transplanted in potting medium amended with 0.1% (w/w) hydrogel were able to fix on hydrogels (**Figure 1D**).

## Effect of Hydrogels on Plant Growth

When grown in potting media, spearmint plants grew to 3% on average in 13 days, and the supplementation with hydrogels allowed for an 8% growth in comparison to their initial heights (**Figure 2A**), confirming the increased plant growth upon hydrogel amendment (Montesano et al., 2015). When compared to the control plants grown in potting medium without hydrogel supplementation, data indicated a significant reduction of plant growth in soil containing clay (101% their initial values; see uppercase letters in **Figure 2A**). By contrast, the growths recorded in clay- or sand-containing soils with hydrogel supplementation were statistically indistinguishable from those observed in potting medium (see lowercase letters in **Figure 2A**). The mixing of soils with potting medium decreased the initial pH from 9.2 to 8.5 and from 9.06 to 8.4, respectively (**Table 2**).

When compared with potting medium without hydrogel supplementation, the limited irrigation led to reduced growth during the course of experiments, with plant heights of 97 and 101% their initial values, respectively (see uppercase letters in **Figure 2A**).

Conversely, the growths recorded with hydrogel supplementation were not significantly affected among the different soils, except for clay-containing soil, albeit clay was able to support growth only in the presence of hydrogel. Under reduced irrigation frequencies, hydrogel amendment allowed for significantly improved growth in potting medium, clay- and sand-containing soils, leading to growth increased by 4, 3 and 6% respectively, in comparison with non-amended conditions.

Similar trends were observed when monitoring biomasses, and the effect resulting from hydrogel amendment were significative under reduced irrigation (**Figure 2B**). In comparison with non-amended conditions, hydrogel supplementation resulted in plant masses increased by 34% in potting medium, 110% in clay-containing soils and 78% in sand-containing soils. Consistently, the plant masses recorded with hydrogel supplementation were not significantly different among the different soils or irrigation regimes.

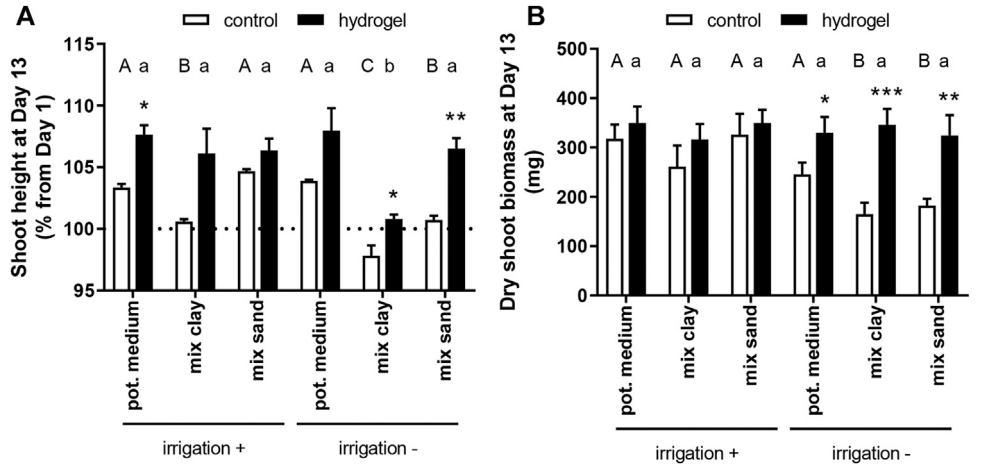


**FIGURE 2 |** Shoots heights **(A)** and dry shoots biomasses **(B)** of spearmint (*Mentha spicata*) 13 days after transplantation (Day 13), with (hydrogel) or without (control) 0.1% (w/w) hydrogel supplementation, under varying irrigation conditions. Pots were filled with soils from MDRS location (see **Table 1**) supplemented with 10% (w/w) potting medium or with potting medium alone (mix clay, mix sand and pot. medium, respectively). Plants were either daily watered (irrigation +) or each 4 days (irrigation –). All data are means of three replications. Statistical analysis for comparison between control and hydrogel conditions: unpaired Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. For comparison between soils: one-way ANOVA with Tukey's post hoc test. Different uppercase (control) or lowercase (hydrogel) letters indicate values significantly different from each other.

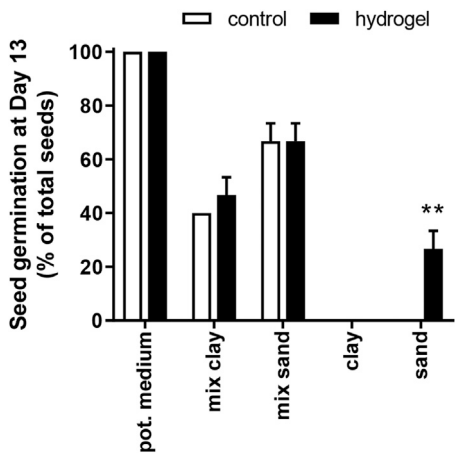


**FIGURE 3 |** Percentage of seed germination of radish (*Raphanus sativus*) after 13 days. For each condition, five seeds were positioned per pot, filled with potting medium, or soils from MDRS location (see **Table 1**) supplemented with 10% (w/w) potting medium, or soils alone, respectively, with (hydrogel) or without (control) 0.1% (w/w) hydrogel supplementation. Data are means of three replications. Statistical analysis for comparison between control and hydrogel conditions: unpaired Student's t-test. $^{**}p < 0.01$.

## Effect of Hydrogels on Seed Germination and Emergence

All the seeds germinated in pots filled with potting medium regardless of the presence or absence of hydrogel amendment, indicating the absence of toxicity of hydrogels on seed germination in these conditions (**Figure 3**). A reduced germination or emergence were observed in soils containing clay, and sand to a lesser extent, allowing for the germination and emergence of 40 and 66% of seeds respectively, without significative effect of hydrogel supplementation. In control conditions, seeds of radish failed to show any germination or emergence after 13 days in pots only filled with clay or sand, which both create dense media largely unfavorable for germination. Similarly, clay did not support germination or emergence of seeds of radish under hydrogel supplementation.

Additionally, hydrogel supplementation allows however for the germination and emergence of 27% of seeds in average in sand.

# DISCUSSION

This work takes place in body of studies that address the capacity of regolith simulants to support plant growth, in the context of ISRU for off-world colonization (Wamelink et al., 2014; Guinan 2018; Wamelink et al., 2019; Eichler et al., 2021). As a precursor to human visitations and future off-world agricultural systems, research regarding plant response to simulated Martian environment is of chief importance.

Our current knowledge of the Martian surface highly suggests that several mineral and chemical properties encountered will likely constitute barriers for plant growth. Firstly, Mars is covered with sand-sized particles with various cohesive strength (Bishop et al., 2002; Arvidson et al., 2004a; Arvidson et al., 2004b), as well as traces of clay minerals (Milliken et al., 2010). Low amounts of organic matter and carbon stored in carbonates have been found in specific regions or rock fractures (Boynton et al., 2009; Leshin et al., 2013; Moyano-Cambero et al., 2017). Secondly, the measurements so far indicate that soils are alkaline, with pH ranging from eight to nine (Fairén 2008), which may be problematic for many plant species, by decreasing nutrients

availability for plants (Wamelink et al., 2005). Finally, the availability of water for optimal plant cultivation might constitute one of the biggest issues raised by ISRU-based food production systems on Mars.

Several studies addressed the growth of plants in Martian regolith simulants, but some display critical differences with the chemical or mineralogical composition of Martian regolith such as the presence of organic matter (Seiferlin et al., 2008), or pH below the alkaline pH recorded at the Phoenix lander site (Fairén 2008; Hecht et al., 2009; Kounaves et al., 2010; McElhoney et al., 2014).

The proposed study tends to integrate the aforementioned parameters, and to assess the effect of hydrogel on plant growth in Mars soil analogs, consisting in sand and clay-rich material with low amount organic matter and alkaline pH.

Results indicate that hydrogels improved the water retention capacity of sand, and to a lesser extent, clay soil, the latter having a high intrinsic retention capacity (Montesano et al., 2015). This further confirms the interesting water holding and release properties of hydrogel in the tested conditions.

Although experiments were limited to the first growth stages of spearmint (*Mentha spicata*), hydrogel supplementation improved plant growth under full irrigation regime, albeit limited growth in the control conditions. The latter can be due either to the stress resulting from transplantation, the relatively low air humidity or the alkaline pH in the mixed soils. This mixing of soils with potting medium partially mitigate their alkalinity, leading to pH values in the range of pH of Martian soils (Fairén 2008).

Mint is a crop with a high water requirement during its active growth period (Shormin 2009). Due to the limited availability of liquid water for ISRU on Mars, the effects of hydrogel supplementation were then investigated under low irrigation conditions. The soil analogs, together with the limited irrigation frequency, were less capable of supporting plant growth as a comparison to potting medium. This indeed resulted in culture conditions well beyond *Mentha* species requirements i.e., slightly acidic pH media (Valmorbida and Boaro 2007; Shormin 2009; Mohammadi and Asadi-Gharneh 2018) and frequent irrigation (Clark R.J. 1980; McConkey et al., 2000; Shormin 2009). Under water stress, N-uptake as well as vegetative growth and biomass production of plants significantly decrease, leading to more pronounced effect of hydrogel supplementation under water deficit, both on plant heights and biomass. These observations are consistent with reports showing that water stress significantly decreases plant height (Shormin 2009) as well as findings on *Argania spinosa* in arid region (C Defaa 2015), and on cucumber and basil plants in sandy soils (Montesano et al., 2015). Moreover, a wilting of main branches of plants was observed in clay-containing soils, which appeared to be cohesive and unfavorable for plant growth, confirming that the deleterious effect of clay-containing soil was more pronounced under low irrigation frequencies. Despite the ability of organic matter to store water, the supplementation with potting media appeared insufficient to cope with water deficit and to support efficient plant growth in the two soil analogs.

Collectively, this suggests that supplementation with hydrogel and traditional plant growth substrate allows for enhanced plant growth in poor alkaline Mars soil analogs under water deficit.

Additionally, the soil analogs were unable to support seed germination and emergence of radish (*Raphanus sativus*), whereas hydrogel supplementation allowed for seed germination in sand, suggesting that hydrogels could help loosening dense soils. While preliminary, these results rise the hypothesis that hydrogels could facilitate seed germination in soils as the cohesive sand-like soils encountered in Mars (Arvidson et al., 2004a).

This study will require more replications to strengthen the findings highlighted in the present work, and to confirm that the beneficial effects of hydrogels are observed for the different stages of plant growth. The percentage of seed germination recorded in this study results from both germination and emergence above the surface, and additional experiments will be needed to delineate whether the effects of hydrogel are related to germination itself or to emergence of sprouts. Moreover, because benefits of hydrogel supplementation are generally related to the concentration applied to the soil, one can assume that higher concentrations could improve the plant growth further. Given the absolute necessity of sealed life support systems for future agricultural systems on Mars, further experiments will have to be reproduced in a full climate control environment to reach culture conditions relevant to future greenhouses on Mars (e.g., optimal temperature and air humidity), and to ensure the reproducibility of the findings highlighted in the present work.

More globally, this work further confirms the need of soil supplementation outlined by several studies to support the growth of plants. The use of fertilizer has been proposed as a source of nutrient to increase the crop yield (Li et al., 2016; Yamamoto et al., 2016). This notably comprises supplementation with nitrogen (through direct $NH_4/NO_3$ supplementation or nitrogen fixing bacteria), an essential nutrient for plant growth, which is absent in JSC-Mars-1A simulant (Wamelink et al., 2019), although nitrate in sedimentary and aeolian deposits has been detected in specific regions by Curiosity Mars Science Laboratory within Gale crater (Stern et al., 2015). Other reports highlight the importance of soil acidification to improve the plant viability, and the necessity of detoxifying substrates from perchlorates (Eichler et al., 2021), through e.g., perchlorate-reducing bacteria (Coates John et al., 1999).

This work is a short-term assessment conducted within the constraints of *in situ* operations and will require further studies on other plants, Mars soil analogs and hydrogels. Together, these results should be considered as a proof of concept that indicate the potential interest of hydrogels to limit water input in ISRU-based food production systems.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

FP conceived and designed the experiments, collected the data, performed the analysis and wrote the paper.

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# Integrated Pest Management Protocols for Space-Based Bioregenerative Life Support Systems

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Human missions to the Moon and Mars will necessarily increase in both duration and complexity over the coming decades. In the past, short-term missions to low-Earth orbit (LEO) or the Moon (e.g., Apollo) utilized physiochemical life support systems for the crews. However, as the spatial and temporal durations of crewed missions to other planetary bodies increase, physiochemical life support systems become burdened with the requirement of frequent resupply missions. Bioregenerative life support systems (BLSS) have been proposed to replace much of the resupply required of physiochemical systems with modules that can regenerate water, oxygen, and food stocks with plant-based biological production systems. In order to protect the stability and productivity of BLSS modules (i.e., small scale units) or habitats (i.e., large scale systems), an integrated pest management (IPM) program is required to prevent, mitigate, and eliminate both insect pests and disease outbreaks in space-based plant-growing systems. A first-order BLSS IPM program is outlined herein that summarizes a collection of protocols that are similar to those used in field, greenhouse, and vertical-farming agricultural systems. However, the space environment offers numerous unusual stresses to plants, and thus, unique space-based IPM protocols will have to be developed. In general, successful operation of space-based BLSS units will be guided by IPM protocols that (1) should be established early in the mission design phase to be effective, (2) will be dynamic in nature changing both spatially and temporally depending on the successional processes afoot within the crewed spacecraft, plant-growing systems, and through time; and (3) can prevent insect/phytopathology outbreaks at very high levels that can approach 100% if properly implemented.

**Keywords: Bioregenerative life support system, BLSS, Veggie, Advanced Plant Habitat, phytopathogens, space exploration, integrated pest management, IPM**

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## INTRODUCTION

Human crewed spacecraft since the Mercury and Gemini programs in the 1960's utilized physical and chemical (i.e., called *physiochemical* or *P/C*) life support systems to sustain their occupants. The P/C systems handled $CO_2$ absorption or recycling of the internal spacecraft atmospheres, $O_2$ generation, water purification, pressure control, humidity control, etc. (see reviews by Eckart, 1996; Seedhouse, 2020). Alternatives to P/C life support systems proposed using algae or higher plants in *bioregenerative life support systems* (BLSS) to recycle water and $O_2$ while producing food stocks for the crews (see reviews by Wheeler, 2004; Escobar and Nabity, 2017; Wheeler, 2017). As mission

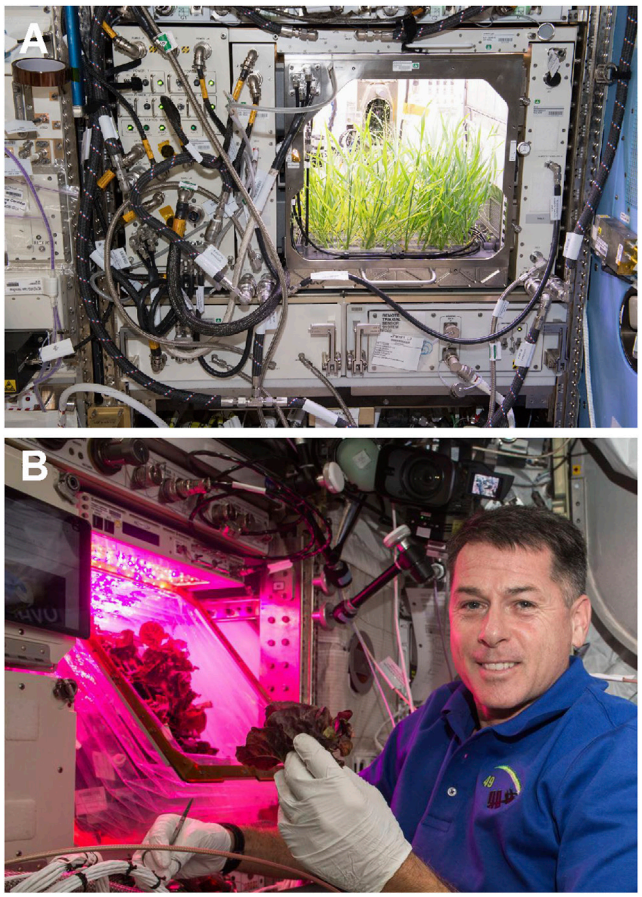


**FIGURE 1 |** Small plant-growth payloads currently onboard the International Space Station (ISS). **(A)** The Advanced Plant Habitat (*APH*; Monje et al., 2020) is a semi-closed research unit in which independent environmental controls (e.g., relative humidity, temperature, LED spectral balance) are available to alter the course of a disease outbreak. **(B)** The Vegetable Production System (*Veggie*; Massa et al., 2016; Massa et al., 2017) is an open crop production system used to grow fresh edibles for the ISS crew. Astronaut Shane Kimbrough can be seen harvesting lettuce from the Veggie (VEG-03A mission) system on the ISS. (Photos are courtesy of NASA; **(A)** image oo225510_web; **(B)** iss055e001010).

durations increase in time—or move away from low-earth orbit (LEO)—the energy, launch mass, resupply mass, and economic tradeoffs improve for BLSS compared to decreasing efficiencies of constant resupply for P/C systems. For example, Seedhouse (2020) compares the tradeoffs of 29 life-support factors between BLSS and P/C systems with BLSS approaches surpassing P/C systems when the mission duration exceeds 3 months.

*Equivalent system mass* (ESM) is a metric used by life-support engineers to contrast and compare mixtures of BLSS and P/C subsystems in order to select the best combination of hardware, materials, energy sources, and operational protocols to optimize life-support while minimizing the energy and overall launch mass of crewed spacecraft and habitats (Drysdale et al., 1999; Drysdale et al., 2001; Ewert et al., 2001; Escobar and Nabity, 2017). When applied to crewed missions in LEO (e.g., Space Shuttle, Skylab, Mir, and the International Space Station [ISS]), ESM modeling suggests that resupply missions for P/C systems are the most economical. Furthermore, in short-duration missions to the Moon (e.g., Apollo) the ESM tradeoffs also argued for a purely P/C approach to life support.

However, we are now entering a new phase of human exploration of the solar system in which long-duration missions to the Moon and Mars begin to argue for ESM tradeoffs that will evolve away from small plant-growth payloads towards larger-scale BLSS habitats. For example, several space-based plant-growth modules are currently operational on the ISS for plant biology research and vegetable production for crews (Zabel et al., 2016). As of this writing, these systems include the *Advanced Plant Habitat* (APH; **Figure 1A**; Monje et al., 2020), *Vegetable Production System* (Veggie; **Figure 1B**; Massa et al., 2016; Massa et al., 2017), and the *Multi-Use Variable-Gravity Platform* (MVP; https://techshot.com/aerospace/technology/mvp/). The APH and MVP systems are semi-closed research modules while Veggie is an open-system that utilizes the ISS cabin air for dehumidification and temperature control. As space-based plant-growing systems get more complicated and transition from closed systems to open configurations, the ability to maintain the stability of internal microbiomes on the plants becomes more difficult to achieve. In general, open systems are more subject to microbial disease outbreaks because the crewed habitat microbiome can pass through the plant production modules.

Recently, such a scenario occurred on the ISS when cabin air containing infective propagules of the opportunistic phytopathogen, *Fusarium oxysporum*, caused a severe disease outbreak on *Zinnia hybrida* plants within a Veggie module (Schuerger et al., 2021a). Disease symptoms developed rapidly during a high-humidity event that created water-soaked leaves and stems within Veggie. The incident supports the conclusion that comprehensive management plans of the ISS and Veggie microbiomes are required to prevent or mitigate severe plant pathology issues in space-based plant growing systems. In essence, we are entering a phase of human exploration in space in which the same microbial and insect pest issues that occur in field, greenhouse, or vertical-farming agricultural operations may occur in future BLSS-supported missions to the Moon and Mars.

*Integrated Pest Management* (IPM) is a comprehensive approach to managing undesirable microbial and entomological issues that arise during nominal operations of agricultural systems. All aspects of plant production must be considered in an effective IPM program and should include considerations of plant nutrition, horticulture, hardware design, microbial and insect ecology, environmental controls, and operational constraints (to mention a few). First, IPM programs can be both preventive and curative in agricultural systems, are dictated by the pest and disease pressures that are encountered in agricultural systems, and are dynamic in nature. Insect or disease pressures can be defined as the interactions between the incidence (i.e., numerical occurrence) and severity (i.e., levels of damage at the endpoint of the occurrence) of pest/disease outbreaks. As insect or disease pressures increase in

incidence and severity, the earlier IPM programs need to be initiated.

An IPM program for space-based plant-growing systems will be proposed here that will be applicable to (1) small-scale plant-growth modules, (2) larger scale plant production systems that have multiple subunits of production but have not yet included waste management, and (3) full-scale BLSS habitats that include plant production and waste management. As the complexities of BLSS modules increase, the complexities of the IPM protocols required to maintain healthy crops will increase. Thus, it is beyond the scope here to propose any sort of comprehensive IPM program with precise specifications or protocols. Instead, the following discussions are intended as a first-order model of the types of IPM protocols that should be considered during currently planned crewed missions to the ISS, the Moon, and Mars over the next 25 years.

## INSECT AND DISEASE PRESSURES ON PLANTS IN SPACE

### Phytopathogens in Space

Historically, few outbreaks of phytopathogens have been observed in small-scale plant-growth modules, and no insect outbreaks have been noted. The primary reason for the previous success of keeping pests and diseases from developing in space-based plant habitats has been the utilization of closed or semi-closed production modules that were returned to Earth for post-flight processing and sanitation between missions (see reviews by Schuerger, 1998; Schuerger, 2004; Zabel et al., 2016; Schuerger et al., 2021b). Thus, the systems were in general cleaned regularly and rendered free of competing microbiomes separate from the intended research.

However, two naturally occurring disease outbreaks, and one directed plant-pathology experiment in space are noteworthy to discuss here. First, the fungus, *Neotyphodium chilense*, was identified as the causal agent of disease (i.e., a biological phytopathogen) on wheat seedlings grown in the now decommissioned *Plant Growth Unit* (PGU; Bishop et al., 1997). The disease occurred on surface-sterilized wheat seeds and was later identified as an endophytic phytopathogen within seeds. Although a heat-treatment of seeds to eliminate the fungus was later developed (Bishop et al., 1997), nearly 50% of the seedlings in the original Space Shuttle STS-63 mission (03-Feb-1995 to 11-Fe-1995) were lost. Second, an international team of scientists conducted the first directed space plant-pathology experiment on the Shuttle STS-87 flight (19-Nov-1997 to 05-Dec-1997) that involved flying infected roots of soybean plants previously inoculated with oospores of *Phytophthora sojae*; a common root-rotting mycoparasite of soybeans (Ryba-White et al., 2001). Root symptoms, colonization of infected root tissues, and numbers of new oospores observed in infected roots were all significantly higher in space-flown plants than ground controls. And third, Schuerger et al. (2021a) described an outbreak of *F. oxysporum* on zinnia plants grown in the Veggie VEG-01C mission (16-Nov-2015 to 14-Feb-2016) in which only one of six zinnia plants reached full maturity without being infected by the opportunistic fungus. In all three cases, low-light levels, elevated relative humidities, and the spaceflight environment are likely to have acted synergistically to enhance disease severity in microgravity ($\mu g$).

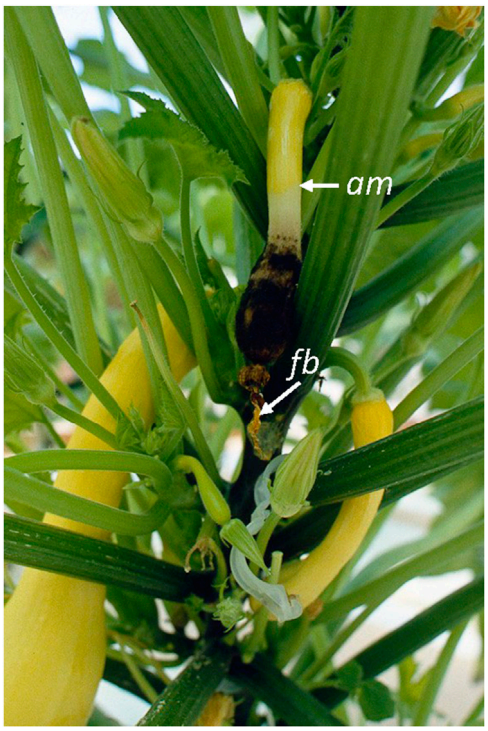


**FIGURE 2 |** The opportunistic fungus, *Choanephora* sp., infecting squash fruit via a senescent flower blossom (fb). The advancing margin (am) of infected tissue by *Choanephora* sp. can be observed 2–3 cm above the lawn of black conidia of the phytopathogen. (Photo credit: A.C. Schuerger and courtesy of The Land, Epcot®).

Recently, Schuerger et al. (2021b) described eight broad categories of plausible phytopathogens that should be considered for space-based BLSS modules, regardless of size, including the following (in priority order based on the likelihood of being introduced into space-based BLSS habitats): (1) microbial contaminants, toxins, and secondary metabolites whose presence alone subjects plants to stress without inciting an active disease outbreak; (2) volatile organic compounds—from either abiotic or biotic sources—that alter plant physiology in BLSS modules causing stress; (3) opportunistic phytopathogens that are generally not able to incite disease in healthy plants on Earth; (4) subclinical phytopathogens that might cause stunting and loss of yield without causing overt disease symptoms; (5) storage rots that develop on harvested or stored edible produce; (6) traditional phytopathogens that are accidentally introduced into spacecraft during prelaunch activities and cause the types of disease outbreaks typically encountered in field and greenhouse agricultural systems; and (7) obligate phytopathogens that have

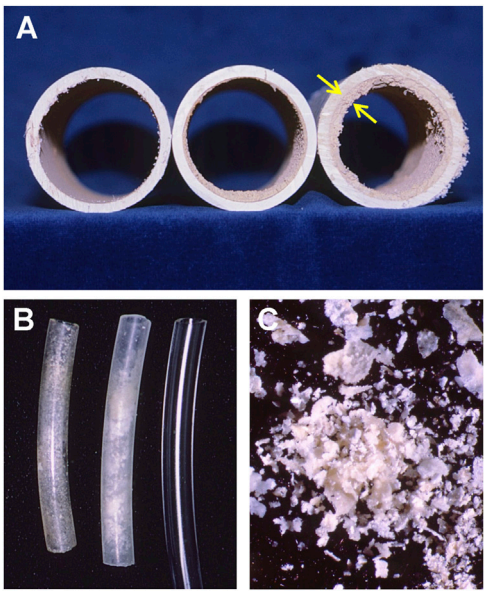


**FIGURE 3 |** Occluded hydroponic irrigation lines after 5.25 years of operation at The Land, Epcot greenhouse facility located in central Florida (circa 1988). **(A)** Precipitates and microbial biofilms adhered to the insides of nutrient delivery lines. The thickness of the precipitates/biofilms (arrows) was determined by the amount of total flow through the lines. **(B)** Smaller delivery tubes often had salt precipitates occluding the ends of the tubes where the nutrient/air transition occurred. **(C)** Shown here are white precipitates removed from 2 mm tubing used for aeration in small hydroponic systems (i.e., from **Figure 3B**). White precipitates in **Figure 3C** were rich in Fe (15.35 wt%), Ca (13.53 wt%), $PO_4$ (12.70 wt%), Mg (1.61 wt%), Al (1.16 wt%), K (0.54 wt%), Na (0.44 wt%), and lesser amounts of all ions present in the nutrient solutions (Schuerger, unpublished). (Photo credit: A.C. Schuerger and courtesy of The Land, Epcot®).

unique biotrophic relationships with specific crop cultivars (e.g., rust pathogens on their unique hosts).

To this list we can add several other factors that are likely to impact the health of crops in space-based BLSS. First, the spaceflight environment itself can alter plant and phytopathogen physiologies such that the combination could increase disease severity in space (Khodadad et al., 2020; Schuerger et al., 2021a; Schuerger et al., 2021b). For example, disease severity for the *N. chilense*/wheat, *P. sojae*/soybean, and *F. oxysporum*/zinnia events in LEO all exhibited increased symptoms and signs in the *μg* environment compared to Earth controls (Bishop, et al., 1997; Ryba-White et al., 2001; Schuerger et al., 2021a; respectively). Recently, Schuerger et al. (2021b) suggested that the increased severity of these three diseases in space may have been caused by up-regulation of phytopathogen virulence genes and down-regulation of plant resistance processes in *μg*. However, this remains only a hypothesis at this point and requires additional phytopathogen experiments in *μg*.

Second, the growth of saprophytic fungi on crop detritus during production or harvest cycles might increase the inoculum loads to levels that then overwhelm host resistance. For example, this problem has been observed in greenhouse-grown squash plants in which the typical saprophytic fungus, *Choanephora* sp., infects squash fruit through colonized senescent flower blossoms still attached to the distal ends of fruit (**Figure 2**; Schuerger, unpublished). Furthermore, the process of a microbial surge in population (i.e., often called *microbial flooding* or *microbial blooms*) can create physical problems to mechanical systems present in BLSS. For example, small or large irrigation tubing can become partially or fully occluded by biofilm formation concomitant to precipitation of nutrient salts (**Figure 3**; Schuerger, unpublished).

And lastly, closed ecosystems, root hypoxia, high humidity, altered gravity, and allelopathy may be concomitant factors that increase plant stress, and thus, potentially alter host resistance to microbial phytopathogens in space. Of these factors, root and canopy hypoxia are well established as plant stressors in *μg* due to low-shear forces in liquids and gases that result in stratified layers of stagnant water or air with very low gas diffusion rates (e.g., Stout et al., 2001; Kitaya et al., 2003; Maggi and Pallud, 2010).

In summary, a wide range of potential environmental or mechanical stressors—and indeed active phytopathogens—will likely impact the long-term stability of space-based BLSS modules in similar ways as these factors impact field, greenhouse, and vertical-farming agricultural systems on Earth. The spaceflight environment further adds a number of additional stressors that are not typically encountered on terrestrial surfaces due to the lack of a gravity-vector in space. In addition, the full range of physiological, anatomical, and genomic responses of both plants and their associated biological phytopathogens remains mostly unknown for space-based plant-growing systems and should be explored in the near-term with a wide range of directed plant host/phytopathogen experiments.

## Spacecraft Microbiomes and Sources of Potential Phytopathogens

Spacecraft microbiome studies reveal similar species diversity and bioloads among diverse missions (see reviews by Taylor, 1974; Schuerger, 1998; Schuerger, 2004; Schuerger et al., 2021b). For example, Apollo, Mir, Shuttle, and ISS spacecraft have exhibited similar species of bacteria and fungi because the common factor of these diverse missions are the human crews present for either short-durations or extended tenures in the spacecraft. Prior to circa 2005, the studies were exclusively based on the recovery and identification of culturable bacteria and fungi from spacecraft surfaces and human crews. However, since circa 2010, metagenomic studies of the ISS habitat modules and crews have expanded the known microbiomes in large complex spacecraft to include non-culturable bacteria, fungi, and some archaea (e.g., Mora et al., 2019; Avila-Herrera et al., 2020; Khodadad et al., 2020).

Potential fungal phytopathogens in these studies include species of *Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Penicillium*, and *Verticillium*. Potential bacterial phytopathogens recovered from spacecraft include species of *Agrobacterium*, *Burkholderia*, *Candida*, *Corynebacterium*, *Pseudomonas*, and *Xanthomonas*. More recently, a review of the genomic sequences in an ISS microbiome study (Avila-Herrera et al., 2020; as reviewed by Schuerger et al., 2021b) has suggested that phytopathogens like *Agrobacterium*

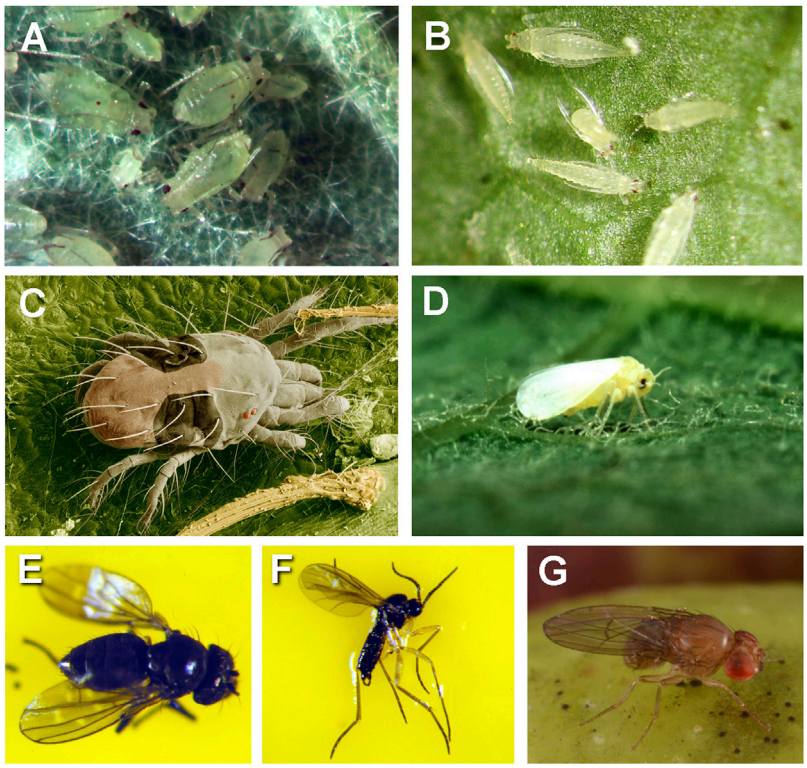


**FIGURE 4 |** Common airborne insect pests (except spider mites) of greenhouse hydroponic systems that are also possible as infestations in space-based BLSS habitats. **(A)** Green peach aphids (*Myzus persicae*), **(B)** thrips (*Echinothrips americanus*), **(C)** two-spotted spider mites (*Tetranychus urticae*), **(D)** white fly (*Bemisia tabaci*), **(E)** shore fly (*Scatella stagnalis*), **(F)** fungus gnat (*Bradysia* sp.), and **(G)** fruit fly (*Drosophila melanogaster*). All images were taken under 40× to 60× magnification using stereo-microscopes. (Photo credits: **(A, B, E, F)** courtesy of L. Osborne, Entomology & Nematology Department, University of Florida; **(C)** and **(D)** courtesy of E. Erbe, USDA and colorization of **(C)** by C. Pooley, USDA; and **(G)** courtesy of L. Bliss, Entomology & Nematology Dept., University of Florida).

*tumefaciens* (crown gall), *Alternaria brassicicola* (leaf black spot), *Erwinia amylovora* (soft rot), *Erwinia chrysanthemi* (wilts), *Fusarium oxysporum* (crown rot and wilts of vegetables), *Pectobacterium carotovorum* (soft rot), and *Puccinia striiformis* (leaf yellow rust of wheat) may already be present in the ISS microbiome. However, no ISS microbiome studies were found in which plant viruses, nematodes, mycoplasmas, or viroids were identified.

The brief discussion above is by no means exhaustive, but it does highlight the possibility that a range of phytopathogens may be introduced into space-based BLSS and crewed habitats. Then why have not more plants succumbed to disease outbreaks? Two factors are in play. First, the question is partially answered by the concept of the *disease triangle* (Agrios, 2005) in which disease only occurs if the following three factors occur simultaneously: (1) the host must be susceptible to the phytopathogen being considered, (2) the phytopathogen must be virulent and abundant in regards to the susceptible host, and (3) the environment for plant infection and disease development must remain in a conducive range for a long-enough period of time to permit disease development. If any of these factors are interrupted (e.g., resistant cultivars, alteration of the physical environment), then disease development is halted. Second, most plant-growth experiments in space prior to 2015 were conducted in payloads that were prepared prior to launch and maintained during the missions under aseptic conditions. In contrast, ISS plant growth experiments after 2015 included both open (Veggie) and semi-closed (APH) conditions. When under aseptic conditions, the Shuttle, Mir, or ISS microbiomes could not interact with the plants, and thus, disease outbreaks were limited to a single event when wheat seed were contaminated with an endophytic fungus (Bishop et al., 1997).

## Insect Pests in Spacecraft Plant-Growth Systems

In contrast to the development of phytopathogens on plants in space, the presence of phytophagus entomological species may be a serious issue because insects that feed on plants are typically generalists without a focused host-range. Although, the emphasis here is to propose IPM practices that are applicable to the development of plant diseases in space-based BLSS, insect pests should not be ignored. For example, the five most common insect pest in greenhouse crops are aphids, leaf miners, spider mites, thrips, and white flies of numerous species (**Figure 4**; Guillino et al., 2020). As of this writing, it is entirely unknown if these or other phytophagus insects have been introduced into the ISS environment. No reports were found in

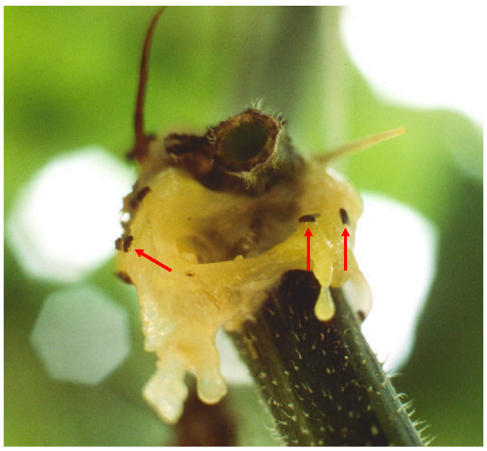

**FIGURE 5 |** *Drosophila* sp. fruit flies feeding on bacterial ooze from an active disease outbreak on squash plants. The fruit flies act as vectors of the bacterial soft-rot phytopathogen, *Pectobacterium carotovorum*, on greenhouse-grown plants. The image was taken with a 105 mm macrolens on a Nikon 2E single-reflex camera (approx. 7×). (Photo credit: A.C. Schuerger and courtesy of The Land, Epcot®).

the literature on the occurrence of these insect pests on the ISS or other spacecraft.

Second, the ability to vector phytopathogens needs to be considered for typically non-phytophagus insects. For example, fruit flies, fungus gnats, and shore flies are well known to act as vectors for a wide range of phytopathogens including *Pectobacterium carotovorum* (bacterial soft rot; **Figure 5**; Schuerger and Batzer, 1993), *F. oxysporum* f. sp. *radicis-lycopersici* (fungal crown rot of tomato; Gillespie and Menzies, 1993), *Pythium aphanidermatum* (a root rot eukaryotic mycoparasite of hydroponic crops; Goldberg and Stanghellini, 1990; Hyder et al., 2009; Braun et al., 2012), and *Thielaviopsis basicola* (fungal root rot of corn-salad, *Valerianella locusta*; Stanghellini et al., 1999). In addition, insects like aphids, thrips, and white flies are notorious for acting as vectors of plant viruses (e.g., Hogenhout et al., 2008; Whitefield et al., 2015).

In summary, searching for and identifying insects present in the ISS habitat should be a near-term research goal. It is likely that phytophagus insects have been periodically introduced into the Mir and ISS space stations but have not caused problems because they did not survive long enough to colonize plants grown in open plant-growth modules like the Veggie system. However, as crop production becomes a more permanent fixture in space-based crewed habitats, insects that can vector phytopathogens must be studied in order to evaluate the risks to BLSS plant production.

## INTEGRATED PEST MANAGEMENT FOR SPACE-BASED BIOREGENERATIVE LIFE SUPPORT SYSTEMS

*Integrated Pest Management* (IPM) is a thoroughly studied approach in field agriculture for insects, nematodes, rodents, and phytopathogens. Less work—but still substantial levels of research—have been developed for IPM programs in greenhouse and vertical-farming agricultural systems (e.g., Jarvis, 1992; Guillino et al., 2020). However, only a half-dozen or so papers have appeared in the literature on IPM programs for space-based BLSS habitats (e.g., Nelson, 1987; Gonzales et al., 1996; Schuerger, 1998; Schuerger, 2004; Schuerger et al., 2021a; Schuerger et al., 2021b).

The following is an outline for an IPM program for space-based plant-growth modules (e.g., APH, Veggie) and large-scale BLSS habitats for future crewed missions to the Moon and Mars. A comprehensive spaceflight IPM program is not specified here because modifications of the concepts below will be achieved through the implementation processes for future missions. Furthermore, aspects of the following IPM program will be adopted differentially for specific missions based on previous flight experience with plant growing systems and the risks of failure to the life-support systems for new missions. As higher plants are adopted for an increasing proportion of the crew's life-support requirements—and for longer-term missions—pest and disease management should become a priority.

### Universal Concepts for Space-Based Integrated Pest Management Programs

The following concepts will apply to almost all plant-growth systems in space regardless of size. However, as crop-production systems increase in size, complexity, and long-term use, these IPM protocols will become more complex and require significant preplanning for mission success. The following principles for BLSS/IPM protocols are adapted from Agrios (2005), Eckart (1996), Gonzales et al. (1996), Guillino et al. (2020), Jarvis (1992), Marschner (1995), Ming and Henninger (1989), Schuerger (1998), Schuerger (2004), Schuerger et al. (2021a), Schuerger et al. (2021b), and Seedhouse (2020).

Although traditional pesticides are an important component of field and greenhouse agricultural systems, it is proposed here that traditional pesticides should not be used in closed BLSS habitats in space due to the immediate effects (i.e., direct biotoxicity to astronauts) and biomagnification (i.e., increased concentrations through trophic levels) of toxic compounds in the closed ecosystems of spacecraft. However, some materials may be certified for use within closed ecosystems for the sanitation of hardware or plant surfaces. For example, during the *F. oxysporum* outbreak on zinnia plants on the ISS (Schuerger et al., 2021a), infected plant surfaces were sanitized with citric acid based wipes saturated with 1% Pro-San (Microcide, Inc., Sterling Heights, MI, United States); and hardware surfaces were sanitized with 0.4% benzalkonium chloride wipes (BZK Antiseptic Towelettes, Nice-Pak/PDI Inc., Orangeburg, NY, United States). In both cases, the wipes were precertified for safe use onboard the ISS prior to the disease outbreak; but their efficacy against *F. oxysporum* was not established prior to the disease event. Thus, the wipes were used because no other IPM protocols were in place when the fungal disease occurred.

Although plant parasitic nematodes, exotic phytopathogens (e.g., mycoplasma, spirochaetes), and many obligate biotrophic phytopathogens (e.g., corn smut, downy mildew, lettuce big vein virus) are likely not to be introduced into BLSS modules due to


**FIGURE 6 |** The Columbus module (launched on 11-Feb-2008) was assembled in a *closed-construction* cleanroom configuration inside the Operation & Checkout Facility at the Kennedy Space Center, FL. The assembly of most spacecraft and ISS modules are (or will be) assembled under such conditions and will reduce the diversity and bioburden of surface contamination. (Photo courtesy of NASA).

**FIGURE 7 |** The Biosphere 2 complex in Oracle, AZ was assembled in an *open-construction* configuration that permitted many more insect pests, plant diseases, and microbial communities than the closed-construction configurations of spacecraft, ISS modules (e.g., **Figure 6**), or future BLSS habitats. (Photo credit: A. C. Schuerger).

IPM sanitation and exclusion protocols, it remains plausible that they can be.

Allelopathy may be an issue in crops that share common nutrient loops and should be studied in pre-flight ground research of polycultures.

There are at least four key epidemiological strategies for disease control in BLSS habitats. First, prevent, eliminate, or reduce inoculum loads to tolerable levels. If infective propagules are not present within the crop production systems, then plants can avoid specific phytopathogens (i.e., called *escape*). Second, slow the rate of disease development by initiating pre-designed and available sanitation, therapeutics, or rogueing protocols. Third, alter the environmental conditions by manipulating the BLSS hardware to reduce the conducive conditions described above in the disease triangle concept. Fourth, IPM protocols must be in place to prevent disease. In most cases, once plants become infected by biological phytopathogens, the plants cannot be rendered free of the infective agents.

Space-based hardware, ISS modules, and full-scale Mars spacecraft are (or will be) assembled under controlled conditions within cleanrooms of increasing stringency of dust mitigation depending on mission criteria (e.g., **Figure 6**; Columbus module for the ISS). In contrast, greenhouse and vertical-farming agricultural systems are often constructed in an open configuration (e.g., **Figure 7**; Biosphere 2 complex). In open systems, all types of pests and microbial contamination are possible. For example, the open-construction approach in the Biosphere 2 complex led to serious insect and disease outbreaks (Silverstone and Nelson, 1996; Marino et al., 1999). Insect infestations in the Biosphere 2 complex included outbreaks of ants, aphids, cockroaches, broad mites, spider mites, pill bugs, mealybugs, scale insects, leaf hoppers, white flies, and psyllids. Furthermore, 20 parasitic nematodes were introduced into the agricultural systems with the most devastating being *Meloidogyne* spp. (root-knot nematodes). Plant diseases were also introduced and included numerous *Pythium* spp. (root rots of diverse crops). Thus, in the continuum from open-assembly configurations (Biosphere 2) to semi-closed or tightly closed spacecraft assembly facilities (e.g., APH, Columbus, ISS equipment), the more isolation that is possible, the more likely that insects and phytopathogens will be severely constrained in both diversity and abundance. Based on the perceived risks to the missions, tighter controls utilized during assembly will yield better chances that the launched hardware, equipment, and subsystems will not be carriers of the insects and disease phytopathogens outlined above.

Large-scale space BLSS habitats should be compartmentalized with separate nutrient and air recirculation systems in order to manage sets of crops under optimum conditions (e.g., warm- versus cool-temperature crops), and to isolate units if an insect outbreak or disease epidemic occurs.

The following are general principles for the ecology of phytopathogens that individually, or in combination, might be utilized for preventing or mitigating disease outbreaks in space-based BLSS.

i) Most fungi enter hosts tissues actively utilizing their endogenous energy reserves in spores to gain footholds in host tissues.
ii) Most bacteria and viruses enter hosts tissues via vectors or passively through mass flow of water into leaves, stems, or roots.
iii) Excluding vector-mediated infections, most phytopathogens require free moisture on susceptible tissues for infection, and thus, humidity control within a canopy is a key component of any IPM program for BLSS crops.
iv) Over fertilization (e.g., high N and P) will often promote disease development in crops.
v) High K and Ca fertilization will often suppress disease development by strengthening cell wall structures.
vi) Continual use of monocultures over time and space may increase inocula loads of phytopathogens in BLSS modules and could lead to the loss of host resistance. Thus, utilize crop rotation, genetic diversity in crop cultivars, and polycultures to prevent specific phytopathogens from developing into epidemics in BLSS crops. Crop rotation might include changing specific cultivars of one crop-type with divergent resistance genes or rotating to other crop species in time and location.
vii) Optimum temperatures for disease development are typically in the 25–30°C range. Thus, holding crops under slightly cooler conditions (i.e., <25°C) often can suppress the development of many diseases.
viii) Optimum relative humidity (RH) for disease development is >80%, with the presence of free-standing water on leaves and stems the most problematic. Thus, maintaining canopy humidities ≤70% RH will decrease both the incidence and severity of canopy infections.
ix) Phytopathogens are most likely to be introduced into BLSS habitats by the following routes (in priority): (1) airborne; (2) human-transmitted via equipment, food, clothing, or hardware; (3) insects missed by exclusion protocols, (4) seed (most can be sanitized prior to flight), and (5) vegetative transplants (if not properly processed through tissue-culture techniques).
x) There is generally a *grace-period* early in the operation of greenhouse and other closed agricultural systems in which few insect and phytopathology problems are encountered. However, as the operations of such systems—including space-based BLSS habitats—continue through time, the slow introductions of new materials and personnel will increase the risks of insect pest outbreaks and plant disease epidemics.
xi) Polygenic resistance is regarded as more durable than isogenic resistance (Agrios, 2005). Thus, it is harder for a phytopathogens to mutate multiple gene loci at once in order to attack a host that has a collection of polygenic resistance genes. When selecting crop cultivars for space-based BLSS, choose polygenic host resistance genes, if available.

## Integrated Pest Management Protocols for the Bioregenerative Life Support Systems Hardware Design Phase

There are several high-criticality phases for initiating a system-wide BLSS IPM program and include the following: (1) hardware design, (2) horticulture design phase, (3) payload and subsystem construction, (4) pre-launch protocols of assembled hardware, (5) crew training and experience in agricultural activities, (6) crop production, and (7) harvest and waste processing. The following are brief discussions of the IPM protocols that might be adopted during the hardware design phase of the BLSS subsystems.

Use anti-microbial tubing, films, and surfaces to keep microbial biofilms managed within the nutrient and water irrigation subsystems.

However, plan for the eventual occlusion of nutrient and water delivery subsystems, and thus, plant production hardware would


**FIGURE 8 |** Symptoms of calcium tip burn versus bacterial soft rot. **(A)** Lettuce plant (*Lactuca sativa*) affected by calcium-tip burn along leaf margins. **(B)** Chinese cabbage (*Brassica rapa*) infected with the bacterial soft-rot phytopathogen, *Pectobacterium carotovorum*. (Photo credits: **(A)** by R.M. Wheeler and courtesy of NASA; **(B)** by A.C. Schuerger and courtesy of The Land, Epcot®).

benefit from being modular, easily assembled/disassembled, and easily sanitized (i.e., not requiring sophisticated or complex sterilization equipment or protocols).

Excellent air flow through crop canopies would decrease internal canopy humidity levels. BLSS crop-production units would benefit from air flowing upward from the plant crowns; if such air flow dynamics can be easily designed into the systems.

Temperature (<25°C) and humidity (≤70% RH) control systems should be designed into long-term BLSS hardware whenever possible.

Irrigation of plant roots would benefit from minimal splashing or release of liquid nutrient solutions (even in a gravity-well like the Moon or Mars) in order to prevent algal or microbial blooms on hydrated surfaces.

Avoid the design of one large-scale BLSS habitat with common air, water, and nutrient loops. If BLSS modules can be designed with isolated loops, the chances of losing the entire BLSS habitat due to a mechanical or biological failure is significantly reduced. However, it is obvious that the ESM trade-offs between one large BLSS approach versus isolated modular subunits will factor into the eventual design of such systems.

Plant roots require dissolved oxygen in nutrient solutions. The saturated dissolved pO2 at 25°C on Earth at 1 g is approx. 7–8 ppm if nutrient solutions are aerated during recirculation. If a physical substrate is used for irrigation and plant support (e.g., Moon or Mars regolith (Ming and Henninger, 1989); Arcillite in plant pillows in the Veggie hardware (Massa et al., 2016; Massa et al., 2017), then the aeration of the roots is likely satisfied by the interstitial spaces within the materials. However, root hypoxia can still occur in *μg* due to low-shear forces (Stout et al., 2001; Kitaya et al., 2003; Maggi and Pallud, 2010), and thus, root aeration should be monitored in most systems.

All crops require frequent scouting to maintain optimum conditions for crop health free of insect infestations and disease outbreaks. It is beyond the scope of the current effort to go into detail on scouting protocols. Suffice it to note here that automatic remote sensing systems will likely be required to monitor crop health over time.

And lastly, rapid disease diagnostic tools are required for space-based diagnoses of phytopathogens such that the flight and ground crews can rapidly respond to specific issues (see Haveman and Schuerger, 2021). For example, foliar symptoms of tip-burn on cabbage (**Figure 8A**; i.e., usually caused by Ca imbalance in rapidly expanding leaves) and soft-rot bacterial disease (**Figure 8B**; e.g., caused by *Pectobacterium carotovorum*) appear very similar in the early stages of disease. Not knowing which is the causal agent of disease in **Figures 8A,B** will significantly hamper the disease control response.

## Horticultural Design Phase

In parallel to the physical design and construction of the BLSS hardware, horticultural activities will be developed to select a diversity of crops that will be compatible with the hardware. It cannot be over-emphasized that the hardware and horticultural design landscapes must be interwoven during the prelaunch phase to optimize all activities for mitigating disease outbreaks. The following suggestions are for the horticultural design phase of the BLSS habitat.

Select crop cultivars with resistance to known or anticipated phytopathogens. For example, to-date *Pythium* spp. (root rots) have not been recovered from spacecraft or the ISS (see Schuerger, 1998; Schuerger, 2004; Schuerger et al., 2021a; Schuerger et al., 2021b), and thus, efforts to develop crops resistant to this genus may not be fruitful for space-based BLSS modules. In contrast, *Fusarium* spp. (fungal wilts, rots, and head blights) are predicted to be major problems in space-based BLSS crops (see Schuerger and Mitchell, 1992; Schuerger et al., 2021a; Schuerger et al., 2021b), and thus, selecting crops resistant to known *Fusarium* spp. would be a logical decision. The lists of active phytopathogens in space-based BLSS habitats are likely to grow over time, and thus, plant breeders and horticulturalist should remain vigilant in testing

and improving crop resistance to the unique environmental and biological stressors experienced in spaceflight.

Avoid high-pollen producing crops because dispersed pollen can act as a rich organic food base for weak or opportunistic phytopathogens (see examples in Schuerger et al., 2021b).

Select crops for high harvest indices to reduce waste detritus at harvest. Senescent biomass is another easy source of proliferating saprophytic bacteria and fungi in closed ecosystems, and thus, should be either collected quickly and sterilized or avoided by developing crops with high harvest indices.

Avoid crops with succulent flower blossoms, fruit, or other structures because many phytopathogens (e.g., *Choanephora* sp.; **Figure 2**) can take advantage of succulent tissues, especially if high humidities and temperatures are present.

Avoid selecting crops with high levels of leaf guttation because the free water droplets along leaf margins may serve as sites for bacterial or fungal infections.

Crop rotation is a very effective IPM protocol to keep inoculum loads low in crop production systems due to the constantly changing genetic diversity of host tissues.

Select at least three genetically dissimilar cultivars of each crop to provide genetic diversity during the mission. The preferred cultivar could be grown until a phytopathological issue arises, upon which the crew could switch to other cultivars or crops.

Sanitize all seeds for both external microbial contamination and internal endophytic phytopathogens (e.g., Bishop et al., 1997). Seed sanitation protocols must be maintained through time among different seed lots.

Avoid the use of organic-rich rooting substrates (e.g., peat moss) because microbial blooms are more likely to occur in organic rich materials and such blooms can interfere with essential nutrient uptake for plants. Furthermore, peat moss can harbor pathogens and insect pests more easily than inorganic substrates (e.g., rockwool).

## Prelaunch Processing of Bioregenerative Life Support Systems Hardware

Once crop production hardware is constructed, and the crop cultivars selected, the next most crucial phase to mitigate downstream pathology issues is the pre-launch handling of payloads, spacecraft processing personnel, and astronauts. The following are brief discussions of a number of pre-launch IPM protocols that should be considered for both small-scale payloads (e.g., APH, Veggie) and complex BLSS modules. However, as mission criteria become aligned with utilizing BLSS habitats for significant amounts of regeneration of $O_2$, water, and food stocks, the risks of losing the BLSS functionality will increase, and thus, so too will the utilization of IPM protocols available to the ground and flight crews.

All flight hardware should be sterilized or sanitized prior to launch and kept in sealed bio-isolation wraps or containers to prevent recontamination. Such an approach will assure that the hardware itself is not the source of new phytopathogens.

All personnel handling flight hardware—even if they are not directly involved in handling BLSS crop production equipment—should be aware that they can act as vectors for both insects and phytopathogens into space launch systems, crewed habitats, and crop-production payloads. For example, individuals that handle tobacco products can frequently act as vectors of plant viruses like tomato mosaic virus (Ng and Perry, 2004; Whitefield et al., 2015). In addition, even the colors of clothing and flight suits can have repellant (white or silver) or attractant (yellow or light blue) effects to insects like thrips, white flies, fungus gnats, and aphids (Guillino et al., 2020). For example, the Columbus module engineers depicted in **Figure 6** are wearing light-blue cleanroom garments. Thus, flight support personnel would reduce the risks of accidentally introducing many insect pests or their vectored phytopathogens into flight systems by wearing non-attractant colors during handling of all flight hardware.

Insect barriers should be erected at the sites of launch vehicle ingress and egress to prevent flying insect pests from entering spacecraft prior to launch. A single air-curtain might not be adequate in all cases, and a double-exclusion barrier (i.e., a 2-stage cleanroom approach in which the spacecraft technicians and crew swap out street clothes for cleanroom garments in an anteroom before passing through an air-flushing and filtration room) might be considered if the threat from insect pests is documented. If a full-scale BLSS habitat gets a severe insect infestation (e.g., by spider mites or white flies), it would be very difficult to eliminate the infestation short of shutting the whole crop production system down and rebooting the BLSS habitat.

## Space-Based Integrated Pest Management/Bioregenerative Life Support Systems Activities

During each mission that utilizes a plant-growth module (e.g., APH and Veggie; **Figure 1**) or BLSS habitat (e.g., **Figure 9**), IPM practices will be utilized to (1) attempt to prevent the introduction of insects or phytopathogens, and then (2) mitigate all outbreak events that do occur. A comprehensive IPM program established during the design and construction of BLSS modules and habitats can reduce the incidence and severity of both insect pest and phytopathogen outbreaks by >90%. However, the unusual conditions encountered in microgravity—or the reduced gravity on the Moon (*1/6g*) and Mars (*1/3g*)—may create conditions that promote the development of opportunistic phytopathogens (e.g., *F. oxysporum* on zinnia plants on the ISS; Schuerger et al., 2021a). The following are suggested IPM protocols that should be considered for space-based BLSS modules.

Environmental manipulation is the best and easiest way to interfere in the disease triangle combination of factors that leads to disease development. Thus, (1) keep ambient RH ≤ 70% in crop canopies, (2) raise or lower temperatures (i.e., ideally with separate root and shoot temperature control subsystems), when feasible to move outside the conducive ranges of the phytopathogens being encountered, (3) avoid BLSS operations that create dew or standing water on crop canopies, and (4) keep root zone $pO_2$ near saturation to promote healthy root systems.

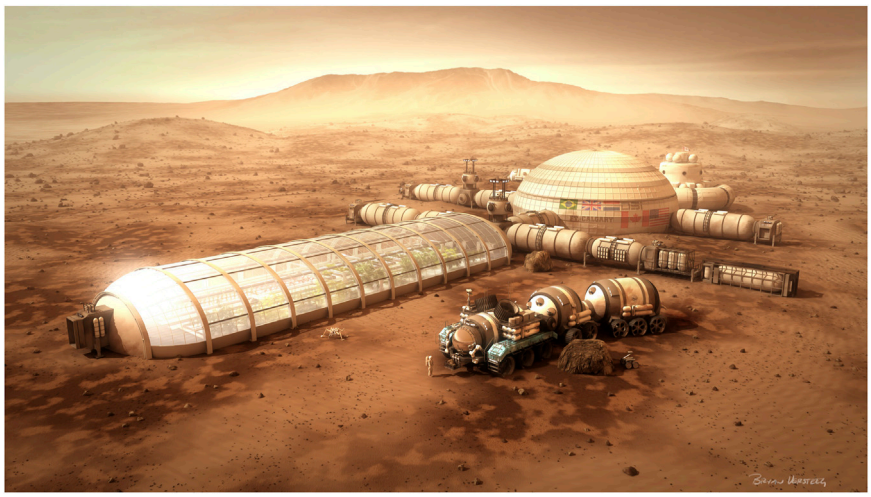

**FIGURE 9 |** A Mars-based BLSS module that uses transparent walls similar to a terrestrial greenhouse can provide significant amounts of water, $O_2$, and food recycling on other planetary bodies. Although humanity may be several decades away from such complex bases on Mars, activities on the ISS are paving the way for the design, flight, and operation of planetary BLSS habitats. (Photo credit: by B. Versteeg/Spacehabs.com).

Operate LED lighting systems, air-circulation, horticultural practices, and environmental controls to minimize open wounds on plants created during pruning activities. For example, crops should be pruned when canopies are dry, and leaf pruning should be directed at leaf abscission layers (when possible). If pruning cuts are made distal to the abscission layers of leaves, and leaf canopies are under high-humidity conditions, the cut bases of the petioles can act as sites for colonization by many fungi and bacteria. The microbial colonization of dead tissues that remain connected to stems is an easy route into the main stems of plants for aggressive phytopathogens (e.g., **Figures 2**, **5**).

Frequent sanitation of pruning equipment should be maintained during the missions to avoid the microbial contamination of the cutting surfaces. As the bioburden on the shears or scissors builds up, the chances of vectoring a traditional or opportunistic phytopathogen increases.

Senescent crop detritus should be avoided by selecting crops with high-harvest indices and by removing dead tissues as frequently as possible.

Edible portions of harvested crops should be produced on a *grow-and-eat cycle* to minimize the storage of edible biomass that might succumb to colonization of airborne storage rot fungi (e.g., *Aspergillus*, *Mucor*, *Penicillium*, and *Rhizopus* spp.) or bacteria (e.g., soft rot bacteria like *Erwinia* and *Pectobacterium* spp.).

Insects can be attracted by UV, blue, and yellow lights. Thus, small-scale and low-power insect traps should be developed that are strategically placed within BLSS habitats to draw flying insects away from crop production modules for capture.

Sanitize the crop production equipment and surfaces between crops. And where feasible, sanitize the internal nutrient flow plumbing to reduce or eliminate microbial biofilms.

Degrade, recycle, or remove crop detritus as quickly as possible after final harvests to prevent colonization of the detritus by saprophytic fungi and bacteria. If this process can be completed quickly, the removal of the detritus will act to suppress microbial blooms within BLSS equipment.

Crop detritus should also be heat-sterilized before reuse within nutrient recapture hardware, waste processing cycles, or incorporation into regolith growing media.

## Brief Bioregenerative Life Support Systems/ Integrated Pest Management Case Studies

The following brief case studies are given as first-order IPM programs for plant-growth modules (i.e., small scale) and BLSS habitats (i.e., large scale). Most of the IPM protocols or concepts given above are, in principle, applicable to all plant-growing systems currently on the ISS or to be built for future crewed missions to the Moon and Mars. In addition, small-scale modules will always be more easily managed for insect and disease problems than large-scale BLSS habitats. However, IPM programs must be (1) established early in the mission design phase to be effective, (2) will be dynamic in nature changing both spatially and temporally depending on the successional processes afoot within the crewed spacecraft through time; and (3) can prevent insect/phytopathogen outbreaks at very high levels of success that can approach 100% if properly implemented.

## Small-Payloads Plant-Growth Modules

A semi-closed plant-growth module like the APH unit (**Figure 1A**) is the conceptual endmember of IPM modeling that are the easiest to keep free of insect infestations and disease outbreaks. The fundamental approach should be to (1) sanitize or sterilize all components prior to launch, (2) screen seed or tissue-culture propagative materials for both external and endophytic insects and phytopathogens, (3) sanitize seed if such insects/microbes are present, (4) operate the horticultural activities in such a manner as to mitigate against the introduction or dispersal of phytopathogens (e.g., use the same set of dedicated crop-pruning tools that are frequently sanitized), (5) remove and

isolate all crop waste at harvest, and (6) sanitize and clean all equipment between cropping cycles. In the past, plant-growth modules were typically returned to the ground for reprocessing between flight experiments. However, the APH is a modular system in which some subsystems might be returned to the ground for reprocessing while other subunits may not. The subunits that remain on the ISS, will become more likely to be problematic over multiple cropping cycles and should be sanitized with the citric acid-based wipes saturated with 1% Pro-San or the 0.4% benzalkonium chloride wipes described in *Integrated Pest Management for Space-Based Bioregenerative Life Support Systems*. Such sanitation protocols will act to break insect reproductive and disease cycles and lower the overall risks to plants in these systems.

Although open-agricultural systems like Veggie will undergo a short grace-period when first launched—over time—the systems will begin to encounter more problems as the hardware begins to receive microbial contamination from the ISS microbiome. Open systems will require the same startup IPM protocols described above for the APH but will also require additional sanitation and sterilization scrutiny over time including: (1) replacement of uncleanable parts; (2) fallow periods of time to help break insect and pathogen life-cycles; (3) crop-rotation to avoid sequential monocultures of one crop in any specific Veggie unit; (4) multiple cultivars available to respond to disease outbreaks; and (5) remove individual plants that exhibit severe stress, insect infestations, or disease outbreaks as soon as possible after detection.

## Multi-Modules in Aggregate

Currently, there are two Veggie (**Figure 1B**) units operating on the ISS. Let us assume that eventually this builds up to 10 separate units to provide daily salad greens for an ISS crew of six astronauts and cosmonauts. A big advantage of multiple Veggie units that are sown with diverse crops is that the genetic diversity and staggered cropping cycles help protect the plant-growing area from outbreaks of a given insect pest or phytopathogen. For example, the genetic diversity of five crops sown into a polyculture (i.e., two Veggie units per crop) offers a significant improvement in ecological stability over monocultures. However, if all 10 Veggie units are side-by-side, insect infestations can spread rapidly. Thus, another IPM protocol in a multi-unit system would be to have one-half of all Veggie units located in one ISS module and the second-half in a different module. Such compartmentalization will increase the ecological stability of both growing regions with minimal increased hardware or crew time. Furthermore, if an insect infestation or a phytopathogen with a wide host-range occurs in one subsystem—but not in both—one half of the plant-growing units can be shut down, cleaned, sanitized and repropagated without losing all of the plant production in the ISS.

## Large-Scale Bioregenerative Life Support Systems Habitats

In the BLSS literature discussed above (e.g., Ming and Henninger, 1989; Eckart, 1996; Wheeler, 2017; Seedhouse, 2020), the authors outline multiple scenarios in which initially there are a few plant-growth modules in prototype spacecraft that evolve into larger systems that provide 50% of crew diets, and in-the-end will be full-scale habitats providing up to 90% of oxygen, water, and crops to support crews. Throughout this continuum, IPM protocols will start simple and develop into more complex programs through the design, construction, flight, operation, and recovery phases of any mission to the Moon and Mars. Most of the IPM protocols discussed above will be applicable to such complex BLSS habitats.

For example, in the BLSS module depicted in **Figure 9**, the plant production habitat is isolated from the main Mars base but will undoubtedly be connected to recycle water, $O_2$, and edible biomass. Every time materials and people pass between the BLSS habitats and the primary crew quarters, the microbiomes of each must be managed to mitigate against the introduction of microbial phytopathogens. However, if insect infestations do not arise in the first few cropping cycles, it is likely that the IPM prelaunch program was successful in preventing the introduction of eggs, larvae, or adults of the problematic insects. Once a BLSS module is isolated from Earth, insect infestations would be expected to be a non-issue until the next crew arrives. Thus, there will have to be IPM protocols associated with crew-rotations in which new crews do not bring with them fresh materials with new pests or phytopathogens.

In addition, a Mars mission can be broken down into three phases for spacecraft and crew operations: (1) outbound transit from Earth-to-Mars, (2) surface operations using one or more bases and rovers, and (3) an inbound Mars-to-Earth transit. The outbound transit to Mars is the most critical to assure that no insects are hitchhiking along as unwanted interlopers. If the 6–8 months outbound transit phase is free of insect infestations, then the crew rotation protocols will likely not be required. However, if outbound BLSS modules encounter either severe insect infestations and/or disease outbreaks, the crews and BLSS hardware within the outbound leg should be mothballed approx. 3–4 weeks prior to arrival in Mars orbit to break the insect and disease life cycles.

## Equivalent System Mass Considerations

In the *Introduction* section, the term equivalent system mass was introduced as a necessary modeling approach to examine the trade-offs among diverse spacecraft components, life-support systems, and mission scenarios. All of the IPM practices discussed above—including new protocols yet to be developed—will be evaluated on how they affect the ESM of the final configuration for human missions to the Moon and Mars. The closer the spacecraft and BLSS habitats are to Earth, the more relaxed such an IPM program can be because there are shorter resupply time-lags from Earth, and ground crews can be more responsive within the Earth/Moon system. In contrast, a crewed Mars trip will be a 3-year mission in which all life-support equipment and protocols must work effectively to maintain crew health. As soon as the BLSS approach is designed into bases and missions to provide for essential crew life support activities on Mars, an IPM program must be adopted to assure the long-term success of the systems.

Furthermore, any given set of IPM protocols might also have different levels of *intensity* within diverse scenarios depending on the risk to the mission and the distance from Earth. For example, the intensity of an inter-crop sanitization cycle might be a quick wipe-down of the Veggie hardware between crops when no problems have occurred; such an approach would be a low-intensity IPM program. In contrast, if an insect infestation occurs during transit between Earth and Mars, a system-wide shut-down, sanitation/sterilization process, and reboot procedure might be considered a very high-intensity IPM program. Thus, ESM modeling of different BLSS habitats with divergent mission goals should be a prelaunch priority and is likely to force different IPM programs to be adopted for the divergent missions. One IPM program will not fit all mission scenarios.

## CONCLUSION

Bioregenerative life support systems that utilize higher plants for the recycling of $O_2$, water, and food biomass have significant ESM advantages over pure P/C systems due to the reductions in resupply missions. Plant production science in the spaceflight environment has a long and storied history that began in the mid-1960's. However, a full-scale BLSS habitat with fully integrated food production, $O_2$ regeneration, and waste recycling subsystems has not yet been designed, built, or tested. As the science and technologies continue to be developed over the next decade, IPM programs for both insect pests and phytopathogens need to be developed in parallel with plant production technologies to assure crew safety for missions that leave the Earth/Moon system.

The IPM protocols and approaches discussed above represent a first-order IPM model for BLSS hardware and habitats. The individual IPM components can be used alone or in combination to help reduce the risks of losses from insects and phytopathogens. With a carefully integrated IPM program woven into BLSS technologies and operations, the risks of catastrophic failures can be dramatically reduced (i.e., possibly to near zero). Moreover, if an insect pest or phytopathogen outbreak does occur, preestablished IPM protocols on the outbound spacecraft (e.g., sanitation wipes, crop diversity, compartmentalization, etc.) will likely make it manageable without compromising the mission.

The future of space exploration is very exciting with both commercial and government organizations working toward missions to the Moon and Mars. However, as the space transportation systems begin to achieve fruition, the development of sustainable life support systems become imperative. Thus, the next decade will fit into space history as a key phase of human dispersal into the Solar system. We need to be prepared to support increasing populations of humans on multiple missions using BLSS approaches to reduce the Solar System wide ESM of all activities. Integrated Pest Management fits directly into the development of BLSS habitats and offers to significantly increase the ecological stability and reliability of plant-based life support systems.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

Written informed consent was not obtained from the individual in Figure 1 because it is standard NASA policy to name astronauts in public-domain photos. Thus, no consent was required.

## AUTHOR CONTRIBUTIONS

AS envisioned, wrote, and edited the entire paper as presented above.

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# Terraform Sustainability Assessment Framework for Bioregenerative Life Support Systems

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In this perspective paper, we raise attention to the lack of methods or data to measure claims of sustainability for bioregenerative life support system designs and propose a method for quantifying sustainability. Even though sustainability is used as a critical mission criterion for deep space exploration, there result is a lack of coherence in the literature with the use of the word sustainability and the application of the criterion. We review a Generalized Resilient Design Framework for quantifying the engineered resilience of any environmental control and life support system and explain how it carries assumptions that do not fit the assumptions of sustainability that come out of environmental science. We explain bioregenerative life support system sustainability in the context of seven theoretical frameworks: a planet with soil, biogeochemical cycles, and ecosystem services provided to humans; human consumption of natural resources as loads and disturbances; supply chains as extensions of natural resources engineering application of; forced and natural cycles; bioregenerative systems as fragmented ecosystems; ecosystems as a network of consumer-resource interactions with critical factors occurring at ecosystem control points; and stability of human consumer resources. We then explain the properties of environmental stability and propose a method of quantifying resistance and resilience that are impacted by disturbances, extend this method to quantifying consistence and persistence that are impacted by feedback from loads. Finally, we propose a Terraform Sustainability Assessment Framework for normalizing the quantified sustainability properties of a bioregenerative life support system using the Earth model to control for variance.

**Keywords: sustainability, resilience, bioregenerative life support system, ECLSS, space habitation, supply chain, fragmented ecosystem, terraform**

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## 1 INTRODUCTION

Across literature and popular science articles on bioregenerative life support systems (BLSSs), the word *sustainable* is commonly used as a critical mission criterion for deep space exploration (e.g., NASA 2012; Skibba 2018; Andrews 2019; NASA 2020; Kozyrovska et al., 2021; Maiwald et al., 2021). However, sustainability is not well-defined in the schema of BLSS. When the presentation and explanation of BLSS technologies that lay claim to sustainability are not accompanied by a discussion of parameters that would allow a measurement of sustainability, then such claims fall flat. The lack of supporting sustainability data is not surprising, considering that an understanding of sustainability is a relatively recent development among biological and environmental system researchers on Earth

(e.g., Primmer and Furman 2012; Nimmo et al., 2015; Donohue et al., 2016; Pimm et al., 2019). The lack of coherence also could be a result of the BLSS concept growing out of the preceding development of non-biological, non-regenerative schema of the environmental control and life support system (ECLSS). ECLSS designs involve a system life cycle that depends upon the ability to maintain and repair the ECLSS during its useful life and then replace it with a new system at the end of its limited, useful life. *Sustainable* and *limited* are opposite outcomes.

Within the context of a limited, useful life, ECLSS developers still needed a way to measure the capability of an ECLSS to operate in the adverse conditions of space. The concept of system resilience has been defined in the field of risk analysis by Haimes (2009) as "the ability of the system to withstand a major disruption within an acceptable degradation of parameters and to recover within an acceptable time and composite costs and risks." The noted challenges in quantifying a measure of resilience are that 1) one must identify the multitude of disruption scenarios specific to the design of the system in question and necessarily apply a probabilistic scoring system to a limited set of classes of disruptions, and 2) given the inherent limited life of engineered systems, resilience will degrade over time, thus further constraining the time frame of scoring (Haimes 2009). The two challenges have been addressed specifically for ECLSSs using a *Generalized Resilient Design Framework (GRDF)* that limits disturbances to the failure of parts within the system; approximates all part failures to happen with equal, low probabilities; and considers such resilience only within a maximum, useful lifetime period during which the probability of any given part failing does not change (Matelli and Goebel 2018). The generalized resilient design framework for quantifying resilience can be applied to any ECLSS. We will refer to this as *engineered resilience*.

There is one fundamental difference between a BLSS and an ECLSS that makes engineered resilience insufficient to the task of defining the sustainability of a BLSS: the BLSS has one or more living biological components, whereas the ECLSS is fundamentally non-regenerative. An ECLSS has no inherent ability in its nature to maintain or repair itself. Even with human intervention in maintenance and repair of an ECLSS, the cost of such maintenance or repair eventually exceeds the cost of replacing the ECLSS entirely, thus reaching the end of its useful life. The bioregenerative nature of the biological components of a BLSS are specifically utilized to give the BLSS the ability to theoretically function indefinitely without replacement. BLSS system developers use the word sustainability in the same way environmental scientists use it because the BLSS is literally intended to be a living environment. The flip side of this coin is the potential for a 100% die off scenario in which the bioregenerative portion of both main lines and redundant lines fail in quick succession. The big difference between non-regenerative parts and bioregenerative elements is that failed non-regenerative parts can be replaced and systems returned to full function. Bioregenerative elements that fail 100% are at risk of quickly failing at 100% again due to undetected and unknown environmental conditions, such as the presence of an alien biological vector or mutated-Earth pathogen, which could require years to figure out and resolve. Space explorers do not have years to fix a down life support system. Sustainable BLSS designs must take this into consideration.

According to the Oxford English Dictionary (2004), *environmentally sustainable* is the "degree to which a process or enterprise is able to be maintained or continued while avoiding the long-term depletion of natural resources." Similarly, the United Nations Educational, Scientific and Cultural Organization (2017) defines *sustainable engineering* as the "process of using resources in a way that does not compromise the environment or deplete the materials for future generations." Four contextual elements are implied in these definitions, the first being that sustainment is endless, the second being that humans are being sustained, the third being that a natural environment is involved, and the fourth being that the functions of the natural environment have a critical, natural-resource basis. Different from engineered resilience, sustainability is a term that has arisen out of environmental science and natural resource management in the context of excessive human activities impacting our Earth ecological systems and environment (Du Pisani 2006). Instead of a probability that excessive human activity will result in adverse impacts to the environment, both the excessive human activity and the adverse impacts have been shown to exist. Environmental scientists look at whether ecological systems and the environment can continue to function in the presence of the excessive human activity. Additionally, environmental sustainability is not defined by the useful life of Earth's ecological systems and environment. A sustainable ecological system continues to support humans indefinitely under human activity. Thus, the assumptions that have been developed for a *generalized resilient design framework* to calculate engineered resilience of an ECLSS do not fit the assumptions of sustainability that come out of environmental science. In the context of environmental science, the sustainability of a BLSS is its capability to continue functioning indefinitely under nominal and potentially abnormal human activity in the course of expected and unplanned events. Any quantitative method of assessing sustainability must apply these assumptions. This is vital, considering environmental failure endangering human life is a very real risk for a spaceship or off-Earth colony.

In this perspective paper, we start by explaining the basis of BLSS sustainability in the context of the following theoretical frameworks: (2.1) a planetary (e.g. Earth): basis, in which biogeochemical cycles based in the soil are driven by planetary and solar forces out of which emerge environmental processes; (2.2) human consumption and disruption of natural resources and environmental processes acting as loads and disturbances that impact the entire system; (2.3) the human use of supply chains to artificially extend natural resources to anywhere they are needed, including space; (2.4) engineered elements of the BLSS working on a spectrum of forced and natural cycles; (2.5) bioregenerative elements of the BLSS functioning as fragmented ecosystems; (2.6) stability of the overall system being governed as critical factors identified by consumer-resource interactions disproportionately influencing the ecosystem at control points; and (2.7) sustainability of the BLSS and its ecosystem services

network defined by stability of the human consumer resource(s) produced by the BLSS under human loads and impacted by disturbances. We then explain the properties of environmental stability, examine a method of quantifying *resistance* and *resilience* that are impacted by disturbances, extend this method to quantifying *consistence* and *persistence* that are impacted by feedback from loads, and propose a *Sustainability Assessment Framework* using the human consumer resources as the critical factors in these calculations. Finally, we propose a framework for normalizing the quantified sustainability properties of a BLSS using the Earth model to control for variance. The result is the *Terraform Sustainability Assessment Framework*, a method for quantifying the sustainability of a BLSS, and a framework that can be used to improve BLSS designs.

# 2 THE ENVIRONMENTAL BASIS OF SUSTAINABILITY

## 2.1 Soil, Biogeochemical Cycles, and Ecosystem Services

Earth's human-sustaining environment is broadly the result of biogeochemical cycles (interactions of living things, minerals, and chemicals in cycles) that provide the essential elements required for interactions between the environment's biotic and abiotic components. Biogeochemical cycles are driven in complex ways by interactions in the soil, as the soil is where water, air, geological minerals, organic matter, microbes, plants, and other organisms interact chemically and physically, driven by solar, gravitational, and geo-thermal energy to produce the systems that make up the natural environment. When we refer to sustainability, we are referring to the fundamental ability of a soil-based biosphere to sustain humans. The implication is that, for a BLSS that utilizes biological functions to be sustainable and thus provide the necessary services required for human survival, the sustainability of those biological functions can only be based on the natural environment and the "root" soil basis from which the biological elements of the BLSS derive.

The Millennium Ecosystem Assessment (2003) proposed a framework to assess how the natural environment enables human life through ecosystem services — a framework now used by scientists, policymakers and government agencies around the world to assess resource use and conservation (Millennium Ecosystem Assessment 2005; Daily and Matson 2008; Fisher et al., 2009; Primmer and Furman 2012; FAO 2021). The framework proposed four categories of ecosystem services, starting with supporting services (e.g., biogeochemical cycles, biodiversity, soil formation, photosynthesis) that then support regulating services (e.g., biological control, carbon storage sequestration, air quality, climate regulation) that then regulate provisioning services that relate to human needs (e.g., clean air to breathe) and cultural services that relate to the development of society (e.g., education, recreation, custom, commerce, governance). Without the basis of supporting services, in which soil is a critical part, ecosystems would not exist. With an estimated 95% of the food consumed by humans being produced from the soil (FAO 2015), humans are dependent on the sustainability of a soil-basis for survival. Furthermore, ecological system theory defines the sustainability of Earth's biosphere in terms of the stability of natural cycles and processes under varying load feedbacks and disturbances (Benedetti-Cecchi 2003; García Molinos and Donohue 2011; Moreno-Mateos et al., 2017). To assess the sustainability of BLSS, we need to explore ecosystem stability in the context of a system sustaining humans (providing provisioning services) while experiencing loads and disturbances.

## 2.2 Human Activity: Loads and Disturbances

Just as provisioning services provided by Earth's environment are described in terms of human needs, loads on provisioning services are specific to human activities. All other activities that naturally occur within the environment (e.g., animals feeding on plants and on other animals), are part of the environment and are not considered to be loads in the context of sustainability. Human activities include not just the loads of the humans themselves (e.g., eating food and breathing) but also the loads of the infrastructure and industry that humans build and operate (e.g., urbanization, intensive agriculture, and the resulting increase in greenhouse gas emissions that load the Earth biosphere and contribute to climate change). Infrastructure and industry are built up and expanded using supply chains. Supply chains act as artificial extensions of provisioning services to multiply the use of consumable resources and extend the use of the resources to humans in locations where the resources would not naturally be available. Thus, supply chains must then be considered as both part of the environmental processes and part of the loads in the determination of sustainability of the environment.

In ecology, a *disturbance* is any event "that disrupts the structure of an ecosystem, community, or population, and changes resource availability or the physical environment" (White and Pickett 1985). Disturbances can act on any parts of a system's natural cycles (e.g., Müller et al., 2002; Tylianakis et al., 2008; Jentsch and White 2019) and its supply chains (e.g., Davis 1993; Christopher and Lee 2004; Bhatnagar and Sohal 2005; Hobbs 2020), not just the points of the natural cycles and supply chains where human consumption normally occurs. Disruptions can greatly reduce or eliminate the availability of provisioning services and the resources they provide. As artificial extensions of provisioning services and resources, supply chains introduce additional potential types and points of disturbances.

## 2.3 Supply Chain Sustainability

Supply chain sustainability to load and disturbance is built upon performance factors affecting, for example, a supply chain's production flexibility, lead time, demand variability response, inventory, and overall product and service quality (Davis 1993; Levy 1995; Bhatnagar and Sohal 2005; Sreedevi and Saranga 2017). Location-specific factors include access to resources, cost, infrastructure, and geographic distance to the end-customer (Levy 1995; Bhatnagar and Sohal 2005). Relational factors are the various activities along the value chain of a product or service and the associated costs to link each into the supply chain (Levy 1995). Supply chain performance analysis has shown that the greater the quantity of performance factors, the more susceptible the supply chain is to natural disturbances such as weather events, and human disturbances such as distorted

information, border closings, strikes, and socioeconomic fluctuations (e.g., Lee et al., 1997; Kleindorfer and Saad 2005; FAO et al., 2020; Hobbs 2020). Thus, supply chains with long paths and the need to reduce lead times commonly have increased risk of disturbance to meeting supply, process, and demand. With a smaller margin of error, the cost to maintain or recover efficiency in the short term increases and sustainability degrades in the long term.

The 2020 COVID-19 Pandemic almost immediately revealed how unstable our long-but-efficient supply chains are to sudden disturbances. The uncharacteristic freezing temperatures in Texas in 2021 also revealed how even localized supply chains (e.g., Texas electric grid) with minimal relational factors and shorter geographical distances can be disrupted by disturbances. The Texas cold wave of 2021 also demonstrated the catastrophic levels of failure that result when risk mitigation steps and adaptive management plans are not in place to recover or create pre-disturbance sustainability in supply chains (Blackmon 2021; Calma 2021; Krauss et al., 2021).

Engineered life support systems derive their sustainability from long, artificial supply chains that extend across Earth and out into space as the systems rely on resupply from the provisioning services of Earth to maintain operation (e.g., the ISS). An alternative to using Earth as a basis for sustainability that we will mention here (but leave detailed discussion to another paper) is to use another planetary body, such as Mars, as a basis (e.g., Kading and Straub 2015; Irons 2018; Berliner et al., 2021). For example, Irons (2018) proposes a "quasi-closed agroecological system" that utilizes ecological buffer zones, *in situ* resources, and ecosystem service reservoirs to establish natural cycles independent of supply chains from Earth.

## 2.4 Engineered vs. Bioregenerative Resilience (Revisited From Introduction)

It is noteworthy to consider the assumption that BLSS is inherently more sustainable than ECLSS due to the presence of bioregenerative components. For any given functional objective and the best available ECLSS and BLSS technologies that can be selected to meet the objective, sustainability will be affected by the engineered resilience of the non-regenerative parts of the system (Haimes 2009; Matelli and Goebel 2018) and the particular supply chains needed for the given technology to provision maintenance materials, repair parts, and consumable materials. The expectation is that sustainability is heavily weighted by both ECLSS/BLSS design and supply chain performance under load, and the risk mitigations, and adaptive management plans prepared for the common and unique disturbance points.

A theoretical advantage in sustainability of a BLSS over an ECLSS emerges as internal, non-regenerative components of the design of a BLSS are replaced with bioregenerative components, eliminating potential non-regenerative part failure points and reducing the need for supply chains for replacement parts. A theoretical advantage in sustainability of a BLSS over an ECLSS also emerges when the bioregenerative function is applied in a natural cycle that is more adaptive to loads and disturbances, rather than a forced cycle that has fixed steady state modes that are non-adaptive (**Figure 1**). When operated closer to a natural cycle, the implication is that it becomes more dependent on local, naturally renewable resources and buffering capacities and less dependent on supply chains, making it more adaptable to recovery from unplanned disruptive events (Irons 2018). Based upon this implication, operating a BLSS within a natural cycle with few or no supply chains would make it inherently more sustainable.

## 2.5 Fragmented Ecosystems

An area of research and ecosystem management here on Earth that is highly relevant to the question of sustainability of a BLSS is that of fragmented ecosystems. Fragmented ecosystems, such as isolated forests resulting from clear-cutting practices and segmented agricultural fields, experience constant edge effects such as wind, temperature fluctuations, and pest disturbances, that negatively affect the populations and communities that are openly exposed to those elements. The stability and quality of such systems is constantly under threat of habitat degradation, loss of connectivity to biogeochemical cycle inputs and outputs, and the loss of biodiversity and genetic movement through the system (Fahrig 2003; Hanski 2011; Wilson et al., 2016). A BLSS operated with need for a supply chain from Earth is effectively a fragmented ecosystem, considering the biological elements within the engineered system will have a large "edge" comprised of the supply chain between it and the Earth-basis ecosystem from which is derived the resources needed by the BLSS.

One example of a BLSS that could require a supply chain is a hydroponics system. In addition to the edge effect of the supply chain, hydroponics systems create edge environments along plant roots that would have otherwise been protected and buffered by a natural soil environment microbiome on Earth. An edge effect the plant may experience is exposure to pathogens that would have otherwise been kept at bay by a soil's microbiome. A field-based agricultural system in an artificial habitation module, even if it uses natural Earth soil, will also have an edge where plantings end along the engineered boundary and would have a separate edge associated with a supply chain if fertilizer additions are required to support the load of crop production. Edge environments are more susceptible to disturbances and load feedbacks and can be a place to measure the most critical factors that will limit the sustainability of a BLSS.

## 2.6 Ecosystem Stability and Control Points

Maintaining a human presence in space over the long-term using BLSS implies the system must have stability under nominal and potentially abnormal load conditions, as well as both expected and unplanned disturbance events. Stability of forced cycle engineered systems has commonly been defined as an asymptotic measure of whether a system maintains an equilibrium state under load and how quickly it returns to its equilibrium state following a disturbance or shock event (Holling 1996). However, the goal of a BLSS is to support or produce a natural cycle. Stability of natural-cycle systems over a long term will not necessarily be restricted and asymptotic. In comparison, naturally cycling ecosystems are complex networks of individual

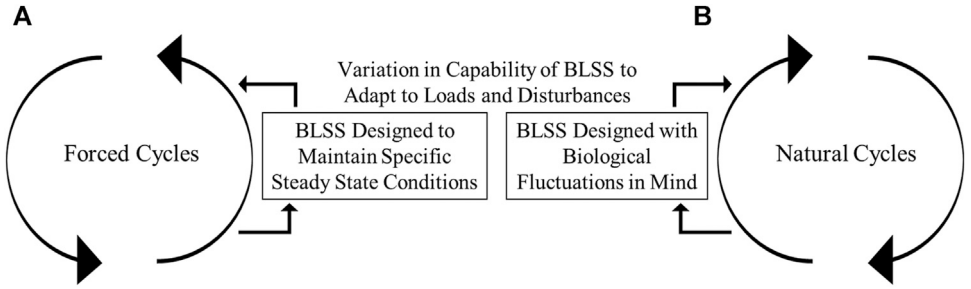


**FIGURE 1 |** Adaptability of bioregenerative life support systems (BLSS) in forced and natural cycles. **(A)** Approach in which a bioregenerative life support system is designed within engineered system processes and are expected to meet specific steady state parameters through forced cycle management. **(B)** Approach in which a bioregenerative life support system is designed within natural cycles with minimal to no forced cycle management.

species and groups of species that interact with the biotic and abiotic environment, creating unique and redundant functions and processes (Gray et al., 2021) made up of consumer-resource (i.e. producer) interactions that can vary over time and not necessarily around a mean (Benedetti-Cecchi 2003; Inouye 2005). Perturbations of varying magnitude, duration, and frequency over different spatial and temporal scales will elicit a range of responses that may affect the ecosystem immediately or may have long term effects that are not measurable on small time scales (Sousa 1984; Müller et al., 2002; Tylianakis et al., 2008; Pincebourde et al., 2012; Donohue et al., 2016; Jentsch and White 2019).

Considering the variance of the large number of consumer-resource interactions comprising the complex network of an ecosystem, quantifying stability as a binary measure of single dimension (i.e., output is nominal or off-nominal) as is done with forced-cycle engineered systems is inaccurate and non-predictive for measuring the sustainability of a natural cycle system and can lead to ineffective management (Donohue et al., 2016; Pimm et al., 2019). The marine resources industry is a key example of how management of marine ecosystems is shifting away from focusing on single species stability and single ecosystem service management to ecosystem-based management—a management strategy that incorporates all ecosystem interactions, including those of humans, into the research and adaptive management of the system (Pikitch et al., 2004; Tam et al., 2017). The multi-dimensional approach of such a management strategy should be applied to assessing the stability of a BLSS. However, the large number of potential factors represented by all the consumer-resource, send-receive, force-action interactions that make up the operation of a BLSS can be daunting. An approach is needed to select a subset of critical factors out of all interactions.

*Ecosystem control points* provide such an approach. In Bernhardt et al. (2017), e*cosystem control points* are defined as "areas of the landscape that exert disproportionate influence on the biogeochemical behavior of the ecosystem under study." For soil, these ecosystem control points are places where the movement of water and gases result in the transport, accumulation and export, and delivery of reactants to support permanent and activated biogeochemical activity. The concept of ecosystem control points can be applied to the process of an ecosystem service being delivered to humans by an Earth biogeochemical soil basis, a network of supply chains, and a BLSS comprised of non-regenerative parts combined with bioregenerative elements. One identifies the ecosystem control pointsto select a subset of all interactions and factors. This requires a careful analysis to identify the measurable, disproportionately influencing factors of the biogeochemical cycles and environmental processes, supply chains, non-regenerative subsystems of the BLSS, and bioregenerative subsystems of the BLSS. These disproportionately influencing factors are called *critical factors*.

Finally, the myriad possible ways that a system can be impacted by load feedbacks and disturbances necessitates limiting them to classes of load feedbacks and disturbances (Haimes 2009). For the BLSS schema, we recommend the following classes: non-regenerative part failure, biotic feed stock loss, pathogen and toxin introduction, mutation of biotic elements, unplanned load/leak, bioregenerative overgrowth. Classes of load feedbacks and disturbances for the ecosystem services on Earth and the associated supply chains that support the BLSS from Earth should also be utilized in the analysis. The BLSS development team should include biologists, environmental scientists, and supply chain experts to help with this analysis.

## 2.7 Sustainability of Life Support Systems in Space

To meet the mission criterion for a sustainable human presence in space, engineered life support systems tend to be defined by six functional objectives: 1) maintain closed-loop atmospheric pressure and composition within optimal parameters, 2) manage closed-loop water cycling, storage, and wastewater; 3) produce and store food to meet crew caloric and nutrition needs, 4) manage and recycle waste, 5) generate efficient energy for the system, and 6) ensure crew safety (e.g., Eckart 1996; NASA 2017). **Table 1** shows how these six functional objectives are supported by the ecosystem services network (i.e., Earth environment basis of natural cycles and the ecological services provided by such cycles, and the supply chains that extend the resulting provisioning services to the location of humans in space) and

**TABLE 1 |** Life support system functional objectives in the context of natural and forced cycle lead services and human loads.

| Natural cycle lead (Earth Basis) | | | Forced cycle lead | | Natural cycle lead | Typical functional objectives | Loads | |
|---|---|---|---|---|---|---|---|---|
| **Supporting services** | **Regulating services** | **Provisioning services** | **Supply chain** | **ECLSS or BLSS** | **BLSS** | | **Human** | **Infrastructure and industry** |
| Biogeochemical cycles, biodiversity, soil formation, photosynthesis | Decomposition, evapotranspiration, biological control, carbon storage/sequestration (soil), air quality regulation, climate regulation | Clean air to breath | → | Maintain atmospheric composition | | Maintain closed-loop atmospheric pressure and composition | Absorbs O2<br>Produces: CO2, VOCs, Airborne particulate | Absorbs O2<br>Produces: CO2, VOCs, Airborne particulate |
| Water cycle, soil creation, photosynthesis, biodiversity, habitat | Climate regulation, water regulation and purification, soil formation; primary productivity, air quality regulation | Clean water for drinking and personal hygiene | → | Manage water cycling and storage | | Manage closed-loop water cycling, storage, and wastewater | Uses water<br>Produces gray water<br>Produces black water | Uses water<br>Produces gray water |
| Biogeochemical cycles, nutrient cycling, photosynthesis, biodiversity, habitat | Pollination, climate regulation, water regulation and purification, biological control, erosion control, atmospheric regulation, disease regulation | Biodiversity to agricultural system | → | Produce and store food | | Produce and store food to meet crew caloric and nutrition needs | Eats food<br>Produces food waste | Uses plant material for spare parts |
| Nutrient cycling, biogeochemical cycles, soil formation, microbial primary productivity | Decomposition, carbon storage, climate regulation, water regulation, biological control, atmospherics | Waste recycling | → | Manage waste | | Manage and recycle waste | Produces trash | Produces industrial waste |
| Depending on energy source—water cycling, sunlight | Depending on energy source: water regulation, climate regulation | Sunlight, wind, flowing water, gravitational potential energy, biomass, other | → | Generate energy | | Generate efficient energy for the system | Consumes energy<br>Produces heat | Consumes energy<br>Produces heat |
| All | All | Raw materials and biodiversity for adaptation, human health and adaptability to known and unknown disturbances | → | Produce spare parts and consumables for engineered systems<br>Safeguard against known disturbances<br>— | | Ensure crew safety | Subject to unplanned events | Consumes material<br>Produces material waste<br>Produces additional hazards for humans |

the ECLSS or BLSS that convert the provisioned resources provided by the supply chain into the resources that meet human consumer need. **Table 1** also identifies the human loads and space-based infrastructure and industry loads that impact the sustainability of the whole system.

These load points are the final consumer-resource interactions in the complex network that make up the BLSS and its connection back to Earth and the soil basis. As such, the measures of resources directly consumed by humans are the dependent variable critical factors (DVCFs) of the independent variable critical factors (IVCFs) measured at the control points. The IVCFs can be impacted by feedbacks of the human loads (i.e., IVCFs pulled out of nominal by human load rates) and by disturbances (i.e. expected and unplanned events). Thus, the stability of the BLSS is dependent upon the stability of the human resource DVCF when the IVCFs are impacted. We define the sustainability of a BLSS to be the stability of the human resource DVCF produced by a BLSS in response to human load and disruption impacts on IVCFs at the environmental control points of the BLSS and its ecosystem services network. We apply this definition in our development of a Terraform Sustainability Assessment Framework.

# 3 TERRAFORM SUSTAINABILITY ASSESSMENT FRAMEWORK

## 3.1 Quantifying Sustainability

Studies to assess the stability of ecosystem critical factors have used variability, persistence, resistance, and resilience as properties of ecological stability (Pimm 1984). Where the challenge lies in quantifying these stability properties is a lack of clear definitions of the terms and a lack in understanding of how to capture the complexity and variety of ecosystem responses to disturbances (Donohue et al., 2016; Pimm et al., 2019).

Nimmo et al. (2015) proposes quantifying *resistance* and *resilience* as

$$Rs = X2/X1 \quad (1)$$

$$Rl = X3/X1 - Rs \quad (2)$$

*Resistance* (*Rs*) is the ratio of the minimum level to which a critical factor (*X*) drops due to a disturbance (*X*2) to its value prior to disturbance (*X*1), and defined as "the ability to maintain functional output immediately following a disturbance." *Resilience* (*Rl*) is the difference of the resistance ratio (*Rs*) and the ratio of the value to which the critical factor recovers following the drop (*X*3) to its value prior to disturbance (*X*1), defined as "the ability to return to functional output after a disturbance has passed." Resistance and resilience, as described here, are the stability properties associated with disturbances.

As resistance and resilience relate to disturbances, variability and persistence relate to load feedback processes. However, whereas *variability* is defined as how much a critical factor drops as a result of feedback from a load, we propose to use what we call *consistence* that we define as "the ability to maintain functional output immediately upon establishment of a load." *Persistence* is "the ability to return to functional output while loading is ongoing." Equations for consistence and persistence would then follow the same model as resistance and resilience

$$C = Y2/Y1 \quad (3)$$

$$P = Y3/Y1 - C \quad (4)$$

where consistence (*C*) is the ratio of the minimum level to which a critical factor (*Y*) drops under load feedback (*Y*2) to its value prior to loading (*Y*1), and persistence (*P*) is the difference of the consistence ratio (*C*) and the ratio of the value to which the critical factor recovers following the drop while still under load (*Y*3) to its value prior to loading (*Y*1). For clarification, symbols "X" and "Y" are used to differentiate disturbance critical factors (*X*) and load feedback critical factors (*Y*). It is worth noting that a given critical factor could be impacted by both disturbances and load feedbacks.

When *X* and *Y* are selected to be the same human resource DVCF of a BLSS, the stability properties represent measures of sustainability for the BLSS, as we defined in **subsection 2.7**. Ecosystem control points and the associated IVCFs for the given BLSS and its classes of disturbances and load feedbacks must be selected to meet five caveats identified by Nimmo et al. (2015) for use of these equations. The theories and methods discussed in **section 2** enable the BLSS developer to meet the caveats. With these theories and methods, BLSS developers can perform analyses to predict how feedbacks from human loads and impacts from disturbances uniquely and commonly affect IVCFs. BLSS developers can then perform computer simulations and physical tests to see how the classes of selected load feedbacks and disturbances affect any given IVCF (while controlling for the other IVCFs), and how the affected IVCF impacts the particular human resource DVCF produced by the BLSS. Values of the human resource DVCF prior to disturbance/loading (X1/Y1), immediately following disturbance/loading (X2/Y2), and at a later time following disturbance/loading (X3/Y3) are then used to calculate resistance, resilience, consistence, and persistence. Using this *Sustainability Assessment Framework*, values of these four properties of sustainability can be calculated for the worst-case scenario of each class of disturbance and load feedback for each BLSS.

## 3.2 Normalizing to Terraform Specific Stabilities

The Sustainability Assessment Framework we have provided thus far quantifies sustainability of a BLSS as a measure of four properties of stability of human resource DVCFs; however, we still have not accounted for the effect of variance of consumer-resource interactions on the measurements of critical factors. We next propose a *Terraform Sustainability Assessment Framework* to account for the effect of variance on an assessment of BLSS stability by comparing the four stability properties we can calculate for a BLSS to those of a functionally equivalent Earth environment model under proportional load and disturbance effects. We are controlling the effect of variance on the sustainability calculations of a BLSS by normalizing the stability properties to those of an equivalent (in bioregenerative aspects) Earth environment that would also be affected by variance. Any influence of variance on the *X3* and *Y3* critical factors of **Equations 2**, **4**, respectively, would be assumed to be in play in both the BLSS and Earth model environment(s), and thus, their influence on the calculations is controlled for in the normalization. The results are four new terms that we call *terraform specific stabilities*: *terraform specific resistance* ($Rs_T$), *terraform specific resilience* ($Rl_T$), *terraform specific consistence* ($C_T$), and *terraform specific persistence* ($P_T$).

$$Rs_T = Rs_{BLSS}/Rs_E \quad (5)$$

$$Rl_T = Rl_{BLSS}/Rl_E \quad (6)$$

$$C_T = C_{BLSS}/C_E \quad (7)$$

$$P_T = P_{BLSS}/P_E \quad (8)$$

where each normalization is the ratio of the value of a BLSS stability property (e.g., $Rs_{BLSS}$) to the stability property of the proportional Earth model (e.g., $Rs_E$). We use the word *terraform* for the normalized properties considering the calculations are developed on the basis of Earth sustainability; thus, any BLSS that achieves terraform specific stabilities of 1 has effectively terraformed the human habitation supported by the BLSS, making it as sustainable as an equivalent system on Earth.

## 3.3 Designing for Sustainability

Finally, we recommend a formalized and adaptable approach (**Figure 2**) to using the Terraform Sustainability Assessment Framework for BLSS system developers to assess and quantify sustainability. The assessment can be performed on a BLSS design at any phase of development as a tool for iterative improvement of the design and associated risk and adaptive management plans. Development teams should be staffed with engineers, supply chain experts, environmental scientists, ecologists, and science specialists (e.g., soil scientists) to ensure valid application of the theories we utilized to develop this framework.

By defining terraform specific stability properties of BLSSs on the basis of proportional Earth models, we provide a tool to assist

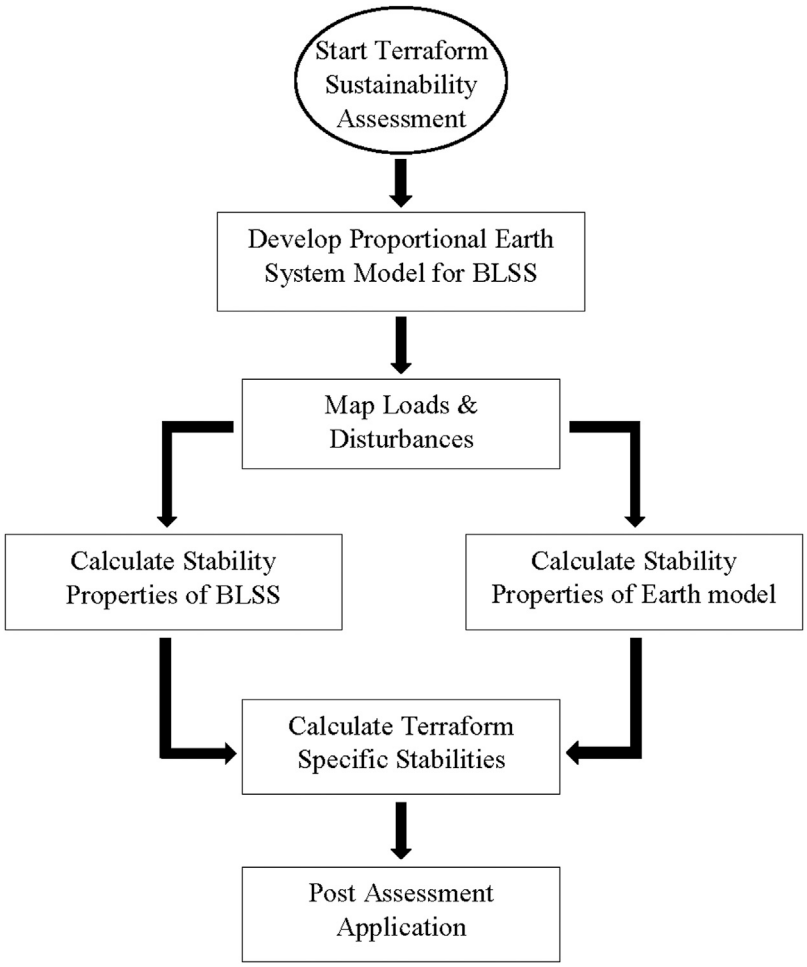


**FIGURE 2 |** Proposed Terraform Sustainability Assessment Framework process flow.

in the purposeful advancement of BLSS designs towards the ideal of no longer requiring Earth supply chains. Our hope is that previously unknown questions and development challenges will be discovered in the process of analyzing and managing the sustainability of any given BLSS using this framework. We encourage the development of case studies of BLSS designs using our proposed Terraform Sustainability Assessment Framework. Such case studies will not only help with the development of more sustainable life support for space but also the development of technologies that can be used for sustainable practices on Earth.

## 4 CONCLUSION

Terraforming has always been a concept in the realm of science fiction. However, it is an idea that has its roots in the science of Earth biogeochemistry, soil formation, and ecological system succession. By modeling the BLSS as an extension of the ecosystem services provided to humans on Earth and then applying theoretical developments of environmental science, we have provided a definition of sustainability of a BLSS and a framework for quantifying it, normalizing it, and applying it to design. Use of this framework will now provide research and development teams and program managers with a way to assess claims of sustainability of BLSS technologies. It also provides a tool for ongoing improvement of BLSS designs, making them more bioregenerative and more naturally cycling, as well as development of system complexes of BLSS systems (terraform life support systems—TFLSS) that will provide all human consumption resources for space explorers of the future. The ultimate objective is to move the science and engineering toward local, scalable terraforming on other planets. To support this endeavor, we see the need for new disciplines in astroecology, extraterrestrial biospherics, and terraform engineering. We encourage career researchers, early career scientists, and students to build on this perspective paper through multidisciplinary, collaborative research with the objective of building these new disciplines.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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# The Potential for Lunar and Martian Regolith Simulants to Sustain Plant Growth: A Multidisciplinary Overview

*Luigi Giuseppe Duri*[1†], *Antonio Giandonato Caporale*[1*†], *Youssef Rouphael*[1], *Simona Vingiani*[1,2], *Mario Palladino*[1], *Stefania De Pascale*[1] *and Paola Adamo*[1,2]

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Bioregenerative life support systems (BLSS) are conceived of and developed so as to provide food sources for crewed missions to the Moon or Mars. The *in situ* resource utilization (ISRU) approach aims to reduce terrestrial input into a BLSS by using native regoliths and recycled organic waste as primary resources. The combination of BLSS and ISRU may allow sustainable food production on Moon and Mars. This task poses several challenges, including the effects of partial gravity, the limited availability of oxygen and water, and the self-sustaining management of resources. Lunar and Martian regoliths are not available on Earth; therefore, space research studies are conducted on regolith simulants that replicate the physicochemical properties of extra-terrestrial regoliths (as assessed *in situ* by previous missions). This review provides an overview of the physicochemical properties and mineralogical composition of commercially available Lunar and Martian regolith simulants. Subsequently, it describes potential strategies and sustainable practices for creating regolith simulants akin to terrestrial soil, which is a highly dynamic environment where microbiota and humified organic matter interact with the mineral moiety. These strategies include the amendment of simulants with composted organic wastes, which can turn nutrient-poor and alkaline crushed rocks into efficient life-sustaining substrates equipped with enhanced physical, hydraulic, and chemical properties. In this regard, we provide a comprehensive analysis of recent scientific works focusing on the exploitation of regolith simulant-based substrates as plant growth media. The literature discussion helps identify the main critical aspects and future challenges related to sustainable space farming by the *in situ* use and enhancement of Lunar and Martian resources.

**Keywords: *in situ* resource utilization, regolith simulants, space exploration, Moon, Mars, extra-terrestrial farming, bioregenerative life support systems, food production**

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## 1 INTRODUCTION

Current scientific inventions and technological advancements may allow space travel and, in the far future, the development of bioregenerative life support systems (BLSS) on other celestial bodies (Zubrin and Wagner, 2011; NASA, 2018). From this perspective, the colonization of the Moon or Mars is closer to reality than it is to science fiction.

Studies focusing on various aspects of life on other celestial bodies have helped make it possible to contemplate extra-terrestrial colonization. These include studies on celestial bodies with a gravitational pull different from that on Earth (Hoson et al., 2000), the recycling of oxygen and water (Primm et al., 2018), and other issues related to sustainable food production in controlled environments or BLSS. The provision of terrestrial resources to permanent extra-terrestrial human settlements is not economically sustainable (Verseux et al., 2016), not only because of the high cost and resource/energy requirements, but also due to the difficulty and time needed to plan and execute launches (Llorente et al., 2018). Given the long-term nature of space missions and future space settlements, BLSS also need to be self-sustaining so as to reduce inputs from Earth and to deal with any challenges threatening the success of the missions. An efficient BLSS must be capable of purifying water, revitalizing the atmosphere, and producing food in a closed loop system (Menezes et al., 2015; Foing et al., 2018; Llorente et al., 2018). This can also be accomplished through *in situ* resource utilization (ISRU), which requires the use of native materials (Karl et al., 2018) and waste as primary resources (Menezes et al., 2015). Instead of relying on a closed loop, new materials found on site could be brought into the life support systems, thus making them sustainable and expandable.

Numerous studies have evaluated the feasibility of life on other planets. Some have conducted hydraulic and engineering tests to assess the practicality of building (Gertsch et al., 2008) and manufacturing (Chow et al., 2017; Karl et al., 2018), whereas others have experimented with microbial (Verseux et al., 2016; Kölbl et al., 2017) and plant growth (Gilrain et al., 1999; Kozyrovska et al., 2006; Wamelink et al., 2014) in an extra-terrestrial environment. Space farming based on local resource exploitation (Maggi and Pallud, 2010; Ramírez et al., 2019) is a promising strategy for food production (Ming and Henninger, 1989) on extra-terrestrial habitats, as it can allow water recycling, organic waste composting, and oxygen production or $CO_2$ consumption (Verseux et al., 2016; Llorente et al., 2018). This would also reduce the launch mass from Earth and the waste generated by human settlement (food cost-cutting). Another important aspect is the psychological comfort plants can provide for astronauts during their long period of isolation (Nechitailo and Mashinsky, 1993; Ivanova et al., 2005; Marquit et al., 2008; Bates et al., 2009).

An ISRU approach for fresh food production is crucial to guarantee sustainability in extra-terrestrial BLSS. Using the local regolith as "soil" for plant growth would be a viable way to grow food, even though "extra-terrestrial soil" is very different from vital and fertile "terrestrial soil" (Certini et al., 2009; Juilleret et al., 2016; Certini et al., 2020). The appropriate term for the surficial unconsolidated fine mineral material on other planetary bodies is regolith, as it lacks living matter and is still very similar to the underlying parent rock. Soil taxonomy defines soil as "*a natural body that comprised solids (minerals and organic matter), liquid, and gases that occur on the land surface, occupies space, and is characterized by one or both of the following: horizons, or layers, that are distinguishable from the initial material as a result of additions, losses, transfers, and transformations of energy and matter or the ability to support rooted plants in a natural environment*" (Soil Survey Staff, 2014). Regolith does not have associated organic matter or a microbiome. In theory, regolith can be classified as soil if it has undergone the same processes that the Earth-based regolith undergoes to become soil (e.g., the presence of oxygen, the influences of wind and water, and activity by living organisms). Likewise, regolith can be defined as soil if it has undergone organic processes. Due to the lack of a standardized method for evaluating regolith efficacy, many published studies claiming to have assessed regolith for plant growth have used microbiome-contaminated regolith, which is in effect a soil.

The Lunar science community uses the word "soil" in an engineering geology sense, identifying "Lunar soil" with the finer-grained fraction of the unconsolidated material (regolith) on the Lunar surface (Heiken et al., 1991). A more complex and informative definition of extra-terrestrial soils (along with attempts of classification) has been provided (Certini et al., 2009; Certini et al., 2020). These native soils can be defined as the (bio)geochemically/physically altered material on the surface of a planetary body that encompasses surficial extra-terrestrial telluric deposits. According to this definition, the soil is a material that retains information about its environmental history, and whose formation does not require the presence of life. In this context, and considering the known geochemical features of extra-terrestrial regoliths, the surface deposits on planetary and other celestial bodies—such as Venus, Mars and Earth-Moon—should be considered soils in a pedological sense (Certini et al., 2009). Moreover, the chemical diversity across *in situ* and regional soils on Mars suggests the existence of many different soil types and processes (Certini et al., 2020). In this review, we frequently use the term "soil" when referring to the surface of the Moon and Mars as a potential crop substrate, and adopt this term when discussing the literature.

The Lunar regolith has been studied on the Moon, and has also been analyzed on Earth using samples brought back by the Apollo missions. The regolith on Mars has been analyzed by rovers and robotic spacecrafts. These studies have elucidated the physical and chemical properties of Lunar and Martian native soils. Nevertheless, there is only a minimal quantity of Lunar material on Earth that is closely guarded, and no samples have been brought back from Mars to date. Therefore, most commercial regolith simulants have been produced by closely replicating the specific physicochemical properties of extra-terrestrial surfaces. Most existing simulants were developed to address specific application fields, and although their chemical interactions or properties related to mechanical abrasion have been assessed to mitigate potential risks (Rickman et al., 2013), their agricultural properties have rarely been evaluated. Naturally, plant growth, morphology, and physiology on the Moon or other planets are expected to be greatly affected by the sterile and nutrient-poor nature of extra-terrestrial soil and the different gravitational and climatic conditions. However, these regolith simulants, although not sterile, may play an essential role in improving our understanding of the environmental phenomena on the Moon and Mars. They may also help solve potential problems

**TABLE 1 |** List of the simulants considered in the present work, divided by reference category. In detail the availability on the market and the reference analysis for chemical and mineralogical characteristics.

| Category | Simulant name | Commercialised | Bulk chemistry | Mineralogy | References |
|---|---|---|---|---|---|
| Lunar Dust Simulants | BHLD20 | May Be Available | XRF | XRD and SEM | Sun et al. (2017) |
| | CLDS-i | May Be Available | XRF | XRD | Tang et al. (2017) |
| | DUST-Y | No | N/A | N/A | Britt and Cannon (2019), Cannon and Britt (2019) |
| | Kohyama Simulant | May Be Available | Not specified | Not specified | Sueyoshi et al. (2008) |
| Lunar Highlands Simulants | LHS-1 | Yes | XRF | XRD | https://sciences.ucf.edu/class/wp-content/uploads/sites/23/2019/02/Spec_LHS-1.pdf |
| | NAO-1 | May Be Available | XRF | Not specified | Li et al. (2009) |
| | NU-LHT/1M/2M/3M/1D/2C | May Be Available | Calculated | Not specified | Stoeser et al. (2010a), Zeng et al. (2010) |
| | OB-1 | May Be Available | QEMSCAN | EDS | Battler and Spray (2009) |
| | OPRH2N/H2W | Yes | Calculated | Use a mineral recipe | McKay et al. (1994) |
| | OPRH3N/H3W | Yes | Calculated | Use a mineral recipe | |
| Lunar Mare Simulants | BP-1 | May Be Available | XRF | XRD | Rahmatian and Metzger (2010), Stoeser et al. (2010b), Suescun-Florez et al. (2015) |
| | CAS-1 | May Be Available | XRF | CIPW normative | Zheng et al. (2009) |
| | CSM-CL | May Be Available | XRF | Not specified | van Susante and Dreyer (2010) |
| | CUG-1A | May Be Available | XRF | Not specified | He et al. (2010) |
| | FJS-1 | Yes | XRF | Not specified | Kanamori et al. (1998); Matsushima et al. (2009) |
| | FJS-2 | Yes | XRF | Not specified | |
| | FJS-3 | Yes | XRF | Not specified | |
| | JSC-1/1A/1AF/1AC/2A | May Be Available | XRF | XRD | McKay et al. (1994); Sibille et al. (2006) |
| | LMS-1 | Yes | XRF | XRD | https://sciences.ucf.edu/class/wp-content/uploads/sites/23/2019/02/Spec_LMS-1.pdf |
| | OPRL2N/L2W | Yes | Calculated | Use a mineral recipe | McKay et al. (1994) |
| | Oshima Simulant | May Be Available | Not specified | Not specified | Sueyoshi et al. (2008) |
| Mars Simulants | JEZ-1 | Yes | Calculated | XRD | Cannon et al. (2019) |
| | JMSS-1 | May Be Available | XRF | XRD and SEM-EDS | Zeng et al. (2015) |
| | JSC-Rocknest | May Be Available | XRF | XRD | Archer et al. (2018), Clark et al. (2020) |
| | KMS-1 | May Be Available | N/A | N/A | Lee, (2017) |
| | MGS-1/1S/1C | Yes | Calculated | XRD | Cannon et al. (2019) |
| | Y-Mars | May Be Available | XRF | XRD | Stevens et al. (2018) |
| | MMS-1 | Yes | WD- and ED-XRF | XRPD | Caporale et al. (2020) |

*All data are from the Planetary Simulant Database and are dated April 2019. May be available = simulant is not commercialised online, but, if requested for scientific purposes, may be available from the producers in small amounts; N/A = not available information; XRF = X-ray fluorescence; XRD = X-ray diffraction; SEM = scanning electron microscopy; QEMSCAN = scanning electron microscope, equipped with up to four light element energy-dispersive X-ray detectors and an electronic processing unit for automated quantitative evaluation of minerals; EDS = Energy dispersive X-ray spectrometry; WD- = wavelength dispersive; ED- = energy dispersive; XRPD = X-ray powder diffraction.*

related to the exploitation of Lunar and Martian regolith as plant growth substrates.

This review aims to provide a comprehensive overview of the potential for existing Martian and Lunar simulants to serve as substrates for growing crops in BLSS. First, we assess selected Lunar and Martian regolith simulants on the basis of their physicochemical and mineralogical properties. Second, we describe previously tested strategies and sustainable practices for using these simulants as plant growth media, with emphasis on the main critical aspects and challenges in deploying these systems. In this review, we consulted 74 scientific papers and 10 technical reports on Lunar and Martian regolith simulants published between 1970 and 2021. The main critical aspects of space agriculture are presumed to be related to nutrient availability, air and fluid movements in different gravitational conditions, and potentially toxic elements in the substrates. Potential future challenges include a lack of adequate knowledge about the extra-terrestrial environment and the development of best agronomic practices for the first space colony.

## 2 AN OVERVIEW OF THE PROPERTIES OF REGOLITH SIMULANTS

This review provides an overview of the petrographic/mineralogical compositions and bulk chemistry of Lunar and Martian regolith simulants developed over the last 3–4 decades, which are mostly available for research purposes. We included simulants listed in the Planetary Simulant Database (https://

simulantdb.com/) of the Colorado School of Mines. Of these, we selected 30 simulants whose petrographic/mineralogical and chemical characteristics had been described in 27 scientific papers and included in the Planetary Simulant Database (**Table 1**). According to the Planetary Simulant Database classification scheme, the included simulants were divided into four categories: 11 **Lunar Mare** simulants (McKay et al., 1994; Kanamori et al., 1998; Sibille et al., 2006; Sueyoshi et al., 2008; Matsushima et al., 2009; Zheng et al., 2009; Stoeser et al., 2010b; He et al., 2010; Rahmatian and Metzger, 2010; van Susante and Dreyer, 2010; Suescun-Florez et al., 2015); 6 **Lunar Highlands** simulants (McKay et al., 1994; Battler and Spray, 2009; Li et al., 2009; Stoeser et al., 2010a; Zeng et al., 2010); 4 **Lunar Dust** simulants (Sueyoshi et al., 2008; Sun et al., 2017; Tang et al., 2017; Britt and Cannon, 2019; Cannon and Britt, 2019); and 9 **Martian** simulants (Zeng et al., 2015; Lee, 2017; Archer et al., 2018; Stevens et al., 2018; Cannon et al., 2019; Caporale et al., 2020; Clark et al., 2020).

## 2.1 Mineralogy of the Lunar and Martian Simulants

There is no universal simulant that comprehensively represents the mineralogy of the Lunar and Martian surfaces. Similar to the Earth's crust, the surficial layers of the Moon and other planets show high heterogeneity and spatial variability. Therefore, it is difficult to create a simulant for every mineralogical combination or potential application. To develop a simulant, it is crucial to find terrestrial rocks with compositions and qualitative and quantitative mineralogical patterns similar to those of Lunar and Martian regoliths. Mineralogical assemblages can be modified to reproduce the general variability on the Moon and Mars. However, simulants comprised of the majority of minerals found in Lunar and Martian surficial regoliths often lack some minor or rare phases (including phosphates, sulfides, and phyllosilicates) that affect ISRU and plant growth. This limitation can be overcome by the exogenous addition of minerals that are deficient in the selected rocks. However, even with this addition, it is difficult to replicate all regolith characteristics in a single simulant (Seiferlin et al., 2008). As a result, many research teams have produced their own simulants over the years (Cannon et al., 2019). In any case, no existing simulants contain moisture or biological components (Gertsch et al., 2008).

The surface morphology of the Moon is dotted with meteorite and micrometeorite impact sites (Gertsch et al., 2008). Samples brought back to Earth by the Apollo missions at the end of the 1970s revealed that the Lunar regolith was a mixture of varying amounts of two primary rocks: 1) the Lunar Mare dark basalt and 2) the lighter-colored, feldspar-rich anorthosite of the Lunar Highlands. These are mixed with an approximately constant proportion of impact melt glass (McKay et al., 1994). Based on the mineralogical composition of this regolith, Earth & Space 2006 and the 2nd NASA/ARO/ASCE Workshop on Granular Materials in Lunar and Martian Exploration (Malla et al., 2006) proposed two compositional end-members of Lunar simulants to be used as an ideal set of root simulants: 1) low-Ti basalt for Lunar Mare and 2) high-Ca anorthosite for Lunar Highlands. Indeed, the Lunar Mare simulants FJS-3, Oshima simulant, FJS-2, OPRL2N, and FJS-1 are 81–100% basaltic (**Supplementary Table S1**), whereas the anorthositic rocks in the Lunar Highland simulants NU-LHT/1M/2M/3M/1D/2C, OPRH2N/H2W, and OPRH3N/H3W are 43–80% basaltic (**Supplementary Table S1**). As shown analytically (**Supplementary Table S1**) and synthetically (**Figure 1**; **Supplementary Table S2**), the mineralogical compositions of only 11 Lunar simulants have been characterized.

The mineralogy of the Lunar Mare and Highlands simulants primarily consists of plagioclases, mafic minerals (nesosilicates and inosilicates such as olivine and pyroxene), and glass plus opaque (with a prevalence of glass) (**Figure 1**; **Supplementary Table S2**). On average, compared to Lunar Highland simulants, Lunar Mare simulants are more enriched with mafic minerals (7 vs. 35%, respectively) and oxides (0.2 vs. 2.9%, respectively). However, Lunar Highland simulants contain higher levels of anorthite plagioclases than Lunar Mare simulants (59 vs. 34%, respectively).

During the period of the Apollo missions, exploration activities on the Lunar surface were seriously hampered by dust. Consequently, an additional type of simulant was proposed, called Lunar Dust. This simulant was created based on the data on Lunar dust collected by the Lunar Soil Characterization Consortium (Taylor et al., 2001; Wallace et al., 2009). Available data on the petrographic characteristics of this simulant indicate a wide range of rock types, ranging from gabbro to anorthosite (**Supplementary Table S1**). In accordance with the mineralogy of the Lunar dust regolith, the Lunar Dust simulant is enriched by glass and opaque than the Lunar Mare and Lunar Highland simulants (53 vs. 25% and 33%, respectively), and exhibits lower levels of nesosilicates, inosilicates, and tectosilicates (**Figure 1**; **Supplementary Table S2**).

The Martian surface was shaped by the combined action of the wind (physical erosion) and water (chemical weathering) and lava flows (Zeng et al., 2015; Cannon et al., 2019), all of which contributed to the formation of the Martian "soil" (Bandfield et al., 2011). The data collected by the Curiosity rover over the last decade have shed light on the composition and physical properties of the Martian regolith. Peters et al. (2008) reported that the Martian regolith is classified as a fine-grained and cohesionless rocky soil that is mixed with dust due to planet storms. The surface is covered by a basaltic sand that is mainly composed of plagioclases and mafic minerals (including nesosilicates and inosilicates such as olivine and pyroxene) (Peters et al., 2008; Zeng et al., 2015; Filiberto, 2017). The Martian regolith also contains relatively lower levels of phyllosilicates (smectite and saponite), sulphate salts (such as gypsum, anhydrite, and alunite-jarosite) (McSween and Keil, 2000; Gaillard et al., 2013; McCollom et al., 2013), and the iron oxides (such as magnetite, hematite, and ferrihydrite) (Benison et al., 2008; Peters et al., 2008; Zeng et al., 2015; Cannon et al., 2019) that

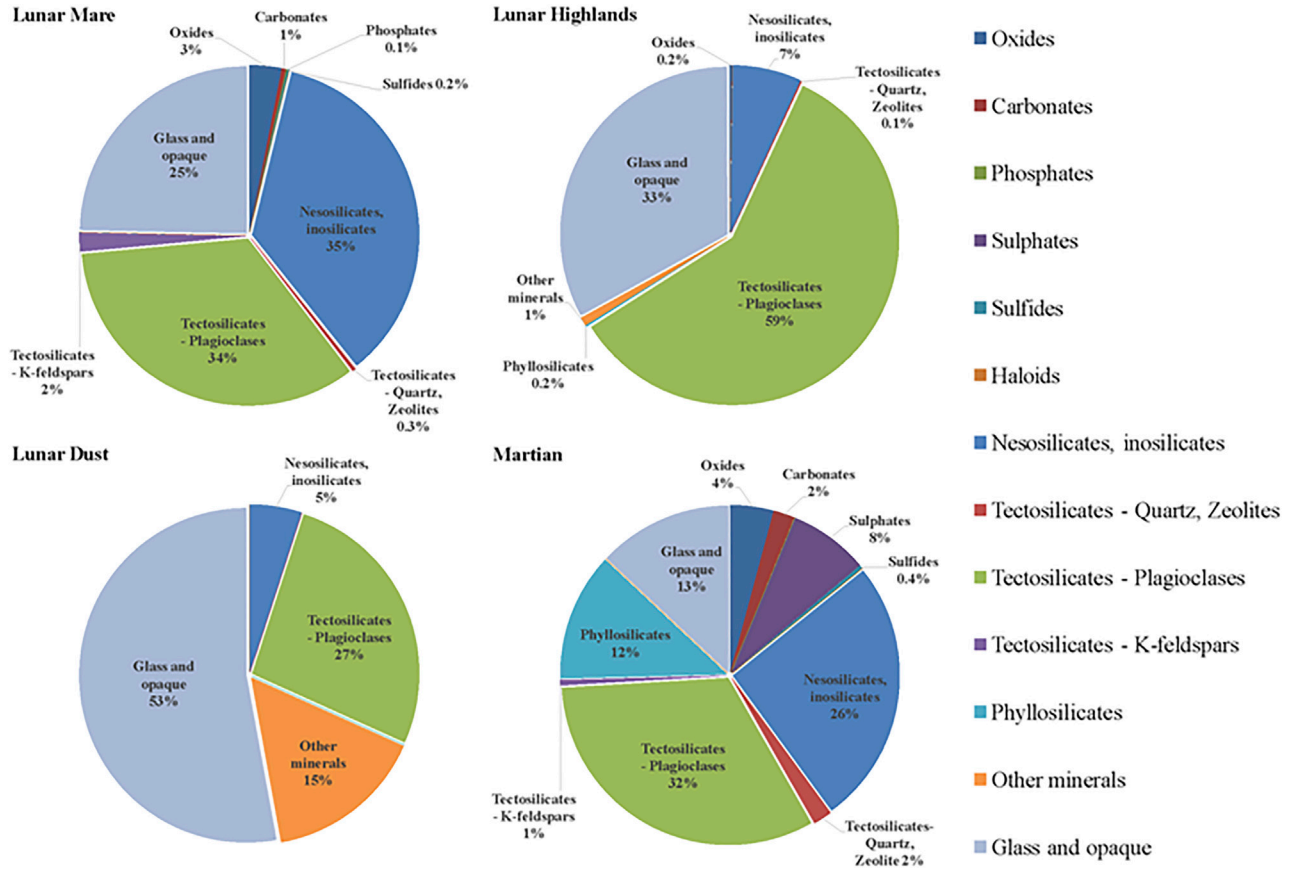


**FIGURE 1 |** Data of the figure are relative only to simulants (19/30) whose mineralogical composition is reported for more than 73% of the total mass in the literature (**Supplementary Table S1**). The mineralogical classes are split as follow: Oxides (Ilmenite; Magnetite; Ti-magnetite; Hematite; Ferrihydrite; Cr-spinel); Carbonates (Calcite; Fe-carbonate; Mg-carbonate); Phosphates (Apatite); Sulphates (Gypsum; Selenite; Mg-sulfate; Anhydrite); Sulfides (Pyrrhotite; Sulfide); Nesosilicates—inosilicates (Olivine + pyroxene + ilmenite; Olivine; Clinopyroxene; Augite; Enstatite; Pyroxene + Hornblende; Pyroxene; Diopside; Hypersthene); Tectosilicates—Quartz, Zeolite; Tectosilicates—Plagioclases (Plagioclase; Anorthite; Albite; Labradorite); Tectosilicates—K-feldspars (Sanidine; K-feldspar; Orthoclase); Phyllosilicates (Smectite; Saponite; Chlorite); Other minerals (undifferentiated). Glass + opaque (Plagioclase glass, basaltic glass, hydrated silica, Volcanic glass; Glass; Opaque + Glass).

make Mars "the red planet" (Grotzinger et al., 2014; Hurowitz et al., 2017).

## 2.2 Bulk Chemistry and Physicochemical Properties

The bulk chemistry and physicochemical properties of Lunar and Martian regolith simulants have been analyzed to assess their ability to support extra-terrestrial farming in the future. Studies are primarily focused on the essential nutrients required by plants to complete their life cycle. Plants can obtain large quantities of macronutrients (including N, P, K, S, Ca, and Mg) from the growth media. These, along with C, H, and O (derived from the atmosphere) contribute to over 95% of a plant's entire biomass (when measured as dry matter). Because micronutrients are required in lower quantities, their levels in plant tissues are measured in parts per million. These include Cl, B, Zn, Fe, Mo, Mn, Cu, and Ni.

In the absence of any organic matter, the main chemical compositions and mineralogy of Lunar and Martian simulants are very similar to those of their respective reference samples from the Lunar and Martian surfaces (Mortley et al., 2000; Beegle et al., 2007; Peters et al., 2008; Zeng et al., 2015; Kölbl et al., 2017). The data from rovers show low variability in bulk chemical composition of regolith in the areas where measurements have been acquired (Zeng et al., 2015). Nevertheless, we need to assume that the unexplored areas of the Lunar and Martian surfaces may exhibit different mineralogy and chemical compositions. NASA's Perseverance rover has successfully cored Martian rocks, and data received from this rover will certainly broaden our knowledge of Martian geochemistry. **Figure 2** shows the mean chemical compositions (as oxide percentages) of the 30 simulants listed in **Table 1**. The oxides of manganese (MnO), titanium (TiO), chromium ($Cr_2O_3$), phosphorus ($P_2O_5$), and sulfur ($SO_3$) occur at very low concentrations in each simulant category. Therefore, for the sake of clarity, these are all collated in the "others" category

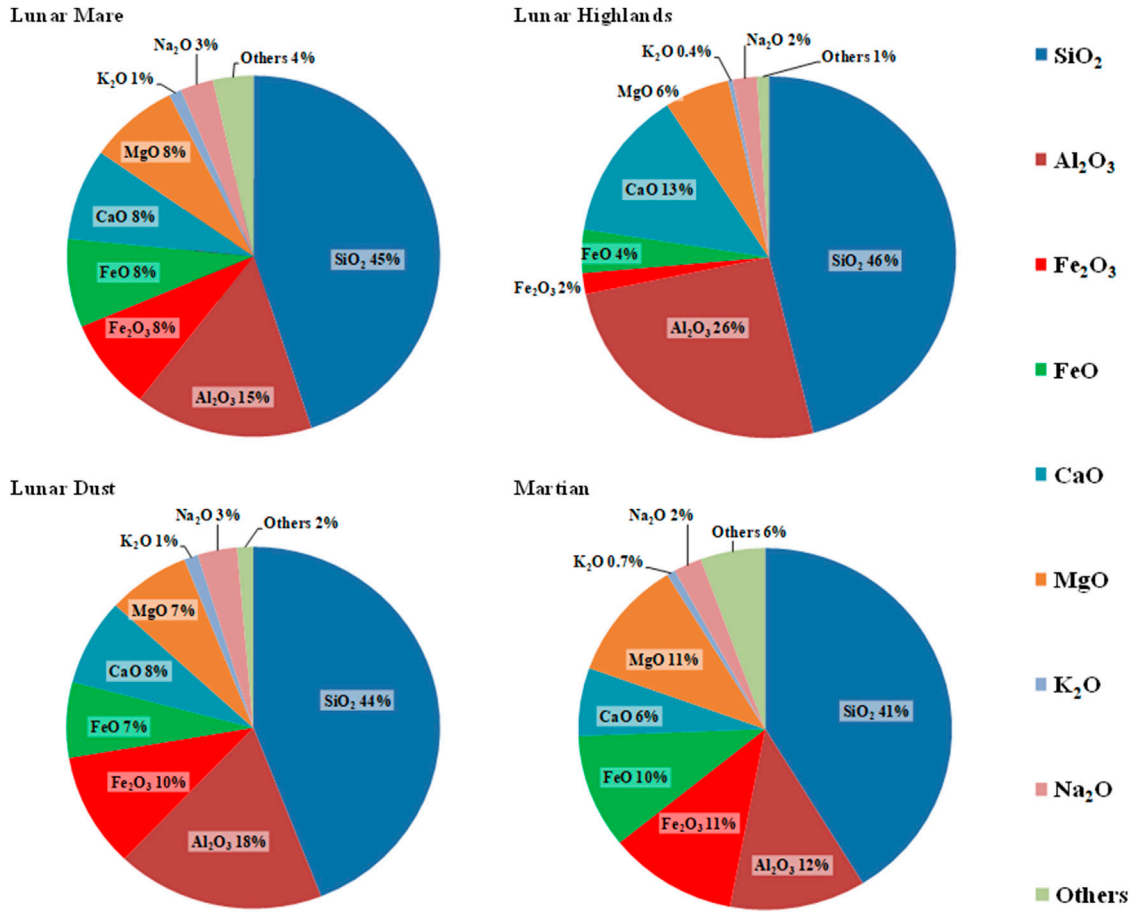


**FIGURE 2 |** Bulk chemistry of 30 lunar and martian simulants listed in **Table 1** (the source of the information used to build the graph and the analytical techniques used to produce chemical data are reported in **Table 1** and in the text).

(**Figure 2**). $SiO_2$ is the principal constituent in all the simulants. $SiO_2$ levels are consistently ~45% in the Lunar simulants, and ~41% in the Martian ones. This difference is likely due to the higher occurrence of amorphous materials in Lunar simulants (**Figure 1**). Likewise, Lunar simulants show higher $Al_2O_3$ and CaO levels than Martian simulants, whereas the opposite is true for Fe (FeO and $Fe_2O_3$) and MgO (**Figure 2**). These trends are explained by higher levels of Ca-plagioclases in Lunar simulants and of Fe-(hydr)oxides (magnetite, hematite, ferrihydrite), nesosilicates (olivine), and inosilicates (pyroxene) in Martian ones. Lunar and Martian simulants also contain significant levels of other nutrients essential for plant growth. These include potassium (average, 0.9%; 0.7% of $K_2O$), phosphorus (0.3%; 0.3% of $P_2O_5$), sulfur (0.1%; 4.4% of $SO_3$), and manganese (0.1%; 0.1% of MnO), which are derived from K-feldspars, phosphates, sulfates, and Mn oxides, respectively (**Figure 1**). The simulants also contain inorganic carbon in the form of carbonates, and their levels are higher in Martian simulants than in Lunar ones. Simulants also contain non-negligible amounts of sodium (Lunar: 2.8% of $Na_2O$; Martian: 2.4% from Na-plagioclases) and potentially toxic elements (e.g., Cr as $Cr_2O_3$), which may induce salt or other abiotic stresses in rhizosphere-competent microorganisms and plants (Caporale et al., 2020). Simulants lack key nutrients for plants such as N, which is frequently absent in minerals and occurs in biomolecules. Micronutrients such as Mo, Ni, B, Cu, and Zn are generally occluded in accessory minerals in trace concentrations (i.e., in the order of parts per million).

The total amount of the most essential elements may be more than adequate to satisfy the requirements for plant growth in simulants. However, plants generally take up only the bioavailable forms of elements (such as the readily soluble and exchangeable forms), and not the elements occluded in mineral structures that are released only after mineral weathering. For plants, nutrient availability in soil is governed by the pseudo-equilibrium between aqueous and solid phases, rather than by the total nutrient content. Factors such as pH, redox potential, electrical conductivity (EC), texture, type and relative abundance of fine solid particles play a key role in regulating nutrient availability in a plant growth medium (Adamo et al., 2018). Thus, these factors should be assessed when growing plants in regolith simulant-based substrates. Unfortunately, the pH and EC of simulants are

not provided in the Planetary Simulant Database. Studies report that the Martian and Lunar simulants have a pH above 6, and have alkaline properties in some cases (Gilrain et al., 1999; Zaets et al., 2011; Wamelink et al., 2014; Caporale et al., 2020; Eichler et al., 2021), suggesting low rates of mineral weathering and cation release. The only data regarding EC in Martian simulants indicate low values of 0.2–0.3 dS $m^{-1}$ (Gilrain et al., 1999; Caporale et al., 2020), suggesting no adverse effect of salinity on plant growth.

Agronomic techniques and crop management also affects nutrient availability and dynamics in a growth substrate. Thus, the addition of sustainable amounts of organic amendments (e.g., compost or manure) or mineral fertilizers to Lunar and Martian simulants may enhance the bioavailability of essential nutrients and provide missing vital nutrients such as N and organic C. These practices can also aid in pH adjustment and have positive effects on microbial rhizosphere activity and nutrient biogeochemical cycles.

Potentially toxic elements such as Al and Cr usually precipitate and are poorly available in alkaline environments (Brautigan et al., 2012); therefore, their presence in non-negligible amounts should not hinder plant growth in simulants. However, analyzing the bioavailability of these elements in the substrates and their levels in plant tissues may help evaluate the potential risk they pose to space crews. Due to the phytochemistry of the cold and oxidizing environment, toxic perchlorate salts occur commonly on the Martian surface in concentrations of 0.5–1% (Oze et al., 2021); however, they were not present in any of the 30 simulants. Perchlorates can be taken up by plants and make their edible parts unsafe to eat. To remediate Martian soils rendered toxic by perchlorates, several papers have proposed a biochemical approach that involves transforming perchlorates into chloride and oxygen (Rikken et al., 1996; Davila et al., 2013).

## 2.3 Physical and Hydrological Properties

Reduced gravity (e.g., in a spacecraft in orbit) causes changes in crucial hydrological variables and alters some fundamental characteristics of water flow and liquid distribution inside a porous medium. The characteristic retention curve of a porous medium is of paramount importance for water movement and plant growth. In the terrestrial environment, water movement towards root hairs is determined by both gravity, which decreases with water depletion, and capillary forces, which increase as water content decreases. In microgravity, capillary forces exert complete control over liquid distribution in a plant growth medium. By simulating a wetting and drying cycle using conventional unsaturated flow models adapted to microgravity, Jones and Or (1999) showed that the retention curve has narrower pore-size distributions in microgravity. This may be due to particle rearrangement, increased air entrapment, and enhanced hysteresis. The authors also reported a decrease in unsaturated hydraulic conductivities, which can be explained by the modified hydrodynamics in microgravity. Chamindu Deepagoda et al. (2014) used equations to quantify the water retention of substrates in terrestrial conditions and under different gravity conditions. Their analysis concluded that the Lunar simulant was the worst in terms of water retention capacity, likely because of its large pore space (0.52 $cm^3$ $cm^{-3}$) and particle size (0.25–1.0 mm).

Maggi and Pallud (2010) evaluated the effect of Martian gravity on soil processes by using a highly mechanistic model. This model had been previously tested for terrestrial crops, and couples soil hydraulics and nutrient biogeochemistry. The net leaching of $NO_3^-$ solutes, gaseous fluxes of $NH_3$, $CO_2$, $N_2O$, NO, and $N_2$, the depth concentrations of $O_2$, $CO_2$, and dissolved organic carbon (DOC), and pH in the root zone were measured in two cropping units under a simulation of the gravitational conditions on Earth and Mars (9.81 and 0.38 *g*, respectively). These units were similarly fertilized and irrigated, but had different initial soil moisture content. The water and nutrient leaching of soil was reduced by 90% under Martian gravity. This enhanced the microbial metabolism, promoted faster decomposition of DOC, and much higher emission of NO, $N_2O$, $N_2$, and $CO_2$. The authors concluded that cultivation on Mars would require less water for irrigation and lower external nutrient supply than on Earth. Unless the soil bulk density is very high, it is expected to have small influences on plant growth. Based on data from the Pathfinder lander, the potential bulk density of Martian soil has been estimated at 1.07–1.64 g $cm^{-3}$ (Moore et al., 1999). The data from the Viking 1 lander suggests a bulk density of 1.15 g $cm^{-3}$ for Martian soil (Moore and Jakosky, 1989), with a volatile loss of 0.1–1.0% by weight at 500°C (Peters et al., 2008). The bulk density of Lunar soil varies between 1.5 and 1.7 g $cm^{-3}$ (Taylor, 2007).

Pore size distribution is potentially associated with the percolation process, such that an increase in particle size enhances the percolation process. The percolation threshold changes under reduced gravity, resulting in improved aeration conditions in the early stages of simulations. Although reduced gravity can influence gas and fluid movement, it does not necessarily lead to better rhizospheric conditions. This is because the root zone is primarily influenced by air in pore spaces (Chamindu Deepagoda et al., 2014). Therefore, if the small pore space is filled with air and the substrate is almost water-saturated, conditions could become critical for plant growth and would need to be managed carefully.

Particle size distribution is another critical aspect that influences the potential of a regolith to be a growth medium. On Earth, soils are made of particles (namely clay, silt, and sand particles) of different sizes. The optimal assemblage (loam texture) of certain proportions of sand, silt, and clay-sized particles can maximize the ability of the soil to sustain plant growth. The particle size distributions of Lunar and Martian simulants must be modified to create an optimal particle assemblage (Cannon et al., 2019) that promotes air permeability (to avoid anoxia stress) and geochemical and mechanical interactions to enhance element bioavailability (Beegle et al., 2007; Zeng et al., 2015; Cannon et al., 2019).

Caporale et al. (2020) conducted an interesting study on the mixing of organic compost with the MMS-1 Martian simulant. This study showed how the addition of green compost to the MMS-1 simulant affects the physical and hydrological properties of the mixture. As expected, the bulk density progressively decreased with increasing rates of compost in the mixture,

ranging from 1.39 g $cm^{-3}$ (pure simulant) to 0.60 g $cm^{-3}$ (pure compost). The addition of compost to pure simulant proportionally increased the maximum amount of water retained. The retention curves of the pure simulant and a 70:30 (v/v) simulant:compost mixture tended to converge when the matrix suction was approximately 60 cm. In contrast, the retention curve of a 30:70 simulant:compost mixture was always higher than those of the other two substrates. However, the suitability of a substrate for the cultivation of a candidate crop cannot be established solely through analysis of the retention curve. In other words, higher values of the saturated water content do not necessarily translate to better performance. The authors also showed that compost addition to the pure simulant exerted a more significant effect on the macropore region than on the micropore domain. Water held in macropores exceeding 120 μm in diameter was not directly beneficial for root water uptake in lettuce (the plant considered in the study), and even caused root asphyxia. Moreover, the three mixtures considered had a similar distribution of pore sizes with diameters below 50 μm. Based on its hydraulic properties, the 70:30 mixture was best substrate, as the percentage increase in large pores (diameter, 50–120 μm) was more significant in this mixture than in the 30:70 one. In terms of water and nutrient transport processes, all the mixtures were acceptable as growth media in a hydroponic cultivation system, where a timely water supply is guaranteed. However, if the objective is to manage lettuce irrigation to minimize irrigation frequency, the 70:30 mixture was the most promising substrate due to its hydraulic properties. Thus, the concerns change if the aim is to have an optimized collection system with more efficient energy consumption and system usage.

## 3 KEY STUDIES ON PLANT CULTIVATION ON REGOLITH SIMULANTS: CRITICAL ASPECTS OF - AND SOLUTIONS FOR - GROWING CROPS ON OTHER PLANETS

A good knowledge of the physicochemical and hydraulic properties of Lunar and Martian regolith simulants is of paramount importance in developing and building off-world BLSS based on an ISRU approach, in which native substrates are exploited as plant growth media. Evaluating how simulant properties can influence plant growth, physiology, and health can help overcome deficiencies and critical concerns in a sustainable and effective way. The macro- and micronutrient levels, porosity, and water availability are fundamental parameters in assessing the capability of a substrate to sustain plant growth. Many studies have evaluated substrate efficiency (Ming et al., 1993; Ming and Henninger, 1994; Aglan et al., 1998; Mortley et al., 2000) and water management (Ramírez et al., 2019; Wamelink et al., 2019) over the years. To be integrated with crop production, a good regolith simulant should have certain physical characteristics, including the following: 1) optimal water holding capacity to maintain an effective level of humidity after irrigation; and 2) optimal air circulation in the porous medium to allow efficient gas exchange and root and microbial respiration. Plants commonly take up nutrients from the soil solution, either in a dissolved form, through exchange, or through easy release at the solid-water interface. The fluxes of water, air, and nutrients in a growth substrate are closely linked to its physicochemical properties (McFee and VanScoyoc, 1975). Therefore, particle interactions or aggregation and the consequent formation of a structured substrate are critical for better plant growth. For extra-terrestrial farming, it is well-established that several simulant properties—such as an alkaline pH, a high availability of sodium, the predominance of macro-vs. micropores, and a scant water holding capacity—can influence plant growth, health, and vigor (Wamelink et al., 2014; Caporale et al., 2020). Thus, it is essential to evaluate whether these soil simulants can be exploited to support future colonies.

As reported in **Section 2.2**, plants need macro and micronutrients for optimal growth, and these can be found in both inorganic and organic forms in soil on Earth (Hopkins and Huner, 2008; Fageria, 2009). Terrestrial soil minerals (mainly feldspars and micas) are the main source of K. Calcium, Mg, S, and Fe, are usually abundant in soil, and microelements such as Zn, Cu, Mn, B, Mo, Cl, and Ni originate from both minerals and organic matter. Carbon is absorbed from the atmosphere through photosynthesis (Hopkins and Huner, 2008), and organic matter is the primary source of both N and P. Lunar and Martian soils lack organic matter and biotic activity (Seiferlin et al., 2008), although there is some evidence of a biofilm (Thomas-Keprta et al., 2014; Eigenbrode et al., 2018). It is also worth noting that the Curiosity rover investigations at Gale crater on Mars discovered indigenous N in sedimentary and aeolian deposits (Stern et al., 2015), although its exact concentration and potential use for plant growth is debatable. Thus, Lunar and Martian soils (and, therefore, regolith simulant-based substrates) are potentially deficient in all macro- and micronutrients, which are derived exclusively (N), mostly (P, S), or partly (K, Ca, Mg, Fe, Zn, Cu, Mn, B, Cl, and Ni) from the degradation of organic components. Thus, regolith simulants cannot support sustainable ISRU for crop production without exogenous inputs of inorganic fertilizers or organic matter.

To date, Lunar and Martian simulants have been primarily studied for applications other than agronomy (de Vera et al., 2004; Gertsch et al., 2008; Kölbl et al., 2017; Karl et al., 2018), although they have potential use as a substrate for crop growth (Mortley et al., 2000). Several strategies and treatments can be applied to ameliorate the nutrient deficiency of simulants and enhance their performance as plant growth substrates. The first solution involves the use of a stable organic amendment, not only as a supply of organic carbon, but also to improve the physical features of regolith (Gilrain et al., 1999). The use of this amendment and soil tillage can also help mitigate the effects of microgravity on water leaching (Maggi and Pallud, 2010). Furthermore, selected pioneer plants can be grown initially (Kozyrovska et al., 2006) to improve the root zone during cultivation and

**TABLE 2 |** Overview of soil-based space farming experiments.

| Ref. Soil | Simulant name | Treatments/ Sterilisation | Nutrient supply | Species | Propagation material | Crop cycle | Measurements | References |
|---|---|---|---|---|---|---|---|---|
| Moon | | Add lunar soil to support/ growth medium (a wood pulp product stabilized with acrylonitril resin)/ Sterilized (by steam autoclaving) | Yes | *Allium cepa* L.; *Anacystis nidulans* (Richt) Drouet; *Brassica oleracea* L.; *Capsicum frutescens* L.; *Chenopodium amaranticolor* Coste and Reyn.; *Chlorella pyrenoidosa* Chick; *Citrullus vulgaris* Schrad.; *Citrus limonia* L.; *Cucumis melo* L.; *Cucumis sativus* L.; *Glycine soja* L. Sieb and Zucc.; *Haplopappus gracilis* Nutt. Gray; *Helianthus annuus* L.; *Lactuca sativa* L.; *Lycopersicum esculentum* Mill.; *Lycopodium cernuum* L.; *Marchantia polymorpha* L.; 4 *Nicotiana tabacum* L. var.; *Onoclea sensibilis* L.; *Oryza sativa* L.; *Phaeodactylum tricornutum* Bohlin; *Phaseolus aureus* L.; *Phaseolus vulgaris* L.; *Pinus elliottii* Engelm.; *Pinus lambertiana* Dougl.; *Pinus palustris* Mill.; *Porphyridium cruentum* Ag. Naeg.; *Raphanus sativus* L.; *Saccharum officinarum* L.; *Solanum tuberosum* L.; *Sorghum vulgare* Pers.; *Spinacia oleracea* L.; *Todea barbara* L. Moore; *Triticum vulgare* Vill.; 2 *Zea mays* L. var. | (A); (S); (T); (TC) | Max 30 days | Seed germination; Biometric parameters; Chemical analyses; Histologic analysis; Plants color | Walkinshaw et al. (1970), Walkinshaw and Johnson (1971) |
| Moon | | Add lunar soil to growth medium/Sterilized (By washed with triple-distilled water and spectroquality chloroform and methanol) | Yes | *Nicotiana tabacum* L. | (TC) | 84 days | Pigment determinations; Lipid content; Growth rate; Total biomass | Weete and Walkinshaw (1972), Weete et al. (1972) |
| Moon | Analogous made by a mix of sand and rocks | A bacterial consortium was spayed onto seeds/ Sterilized (by heating at 170°C for 2 h and autoclaving at 112°C for 40 min) | No | *Tagetes patula* L. | (S) | 70 days | Elemental analyses; Fresh biomass | Zaets et al. (2011) |
| Moon | Analogous made by inert aggregates | No treatments/Not specified | Yes | *Ipomoea batatas* L. | (T) | 120 days | Biometric parameters | Aglan et al. (1998) |
| Moon and Mars | Not specified | No treatments/Not specified | Yes | *Ipomoea batatas* L. | (T) | 120 days | Biometric parameters | Mortley et al. (2000) |

(Continued on following page)

**TABLE 2 |** (*Continued*) Overview of soil-based space farming experiments.

| Ref. Soil | Simulant name | Treatments/ Sterilisation | Nutrient supply | Species | Propagation material | Crop cycle | Measurements | References |
|---|---|---|---|---|---|---|---|---|
| Mars | Analogous collected from desert | Salt stress/Not sterilized | Yes | *Solanum tuberosum* L. | (T) | 134 days | Biometric parameters; Stomatal conductance; Chl.phyll SPAD values | Ramírez et al. (2019) |
| Mars | JSC-1A Mars | Mix simulant with different ratios of leaf compost/Not sterilized | Yes | *Beta vulgaris* L. | (S) | 90 days | Plants weights; Substrate phisico-chemical analysis | Gilrain et al. (1999) |
| Moon and Mars | JSC Mars-1A and JSC1-1A Lunar | No treatments/Not sterilized | No | *Arnica montana* L.; *Sinapsis arvensis* L.; *Urtica dioica* L.; *Cirsium palustre* L.; *Sedum reflexum* L.; *Festuca rubra* L.; *Vicia sativa* L.; *Lupinus angustifolius* L.; *Melilotus officinalis* L.; *Lotus pedunculatus* Cav.; *Solanum lycopersicum* L.; *Secale cereale* L.; *Daucus carota* subsp. *sativus* Hoffm.; *Lepidium sativum* L. | (S) | 50 days | Seed germination; Biometric parameters | Wamelink et al. (2014) |
| Moon and Mars | JSC Mars-1A and JSC1-1A Lunar | Mix simulants with organic matter/Not sterilized | Yes | *Solanum lycopersicum* L.; *Secale cereale* L.; *Lepidium sativum* L.; *Allium ampeloprasum* L.; *Chenopodium quinoa* Willd.; *Pisum sativum* L.; *Raphanus raphanistrum* subsp. *Sativus* L.; *Diplotaxis tenuifolia* L.; *Allium tuberosum* Rottler ex Spreng | (S) | 159 days | New seeds germination; Total aboveground dry biomass | Wamelink et al. (2019) |
| Moon | Analogous made by anorthosite rocks | Seeds treatment with bacterial consortia and substrate inoculation with microorganisms/ Not specified | Not Specified | *Tagetes patula* L. | (S) | Not specified | Microbial activity | Kozyrovska et al. (2006) |
| Mars | JSC Mars-1A; MMS-1; MGS-1; MGS-1P | Add of perchlorate to the simulants/Not sterilized | Yes | *Lactuca sativa* L.; *Arabidopsis thaliana* L. | (S) | Max 28 days | Chlorophyll and carotenoid content | Eichler et al. (2021) |
| Mars | MMS-1 | Mix simulant with different percentage of compost/Not sterilized | Yes | *Lactuca sativa* L. | (T) | 19 days | Leaf Gas Exchange; Biometric parameters; Plant mineral composition; Chlorophyll and C vitamin content; Carotenoids and Polyphenols profile | Duri et al. (2020) |

*(A) algal cultures; (S) seed and/or spore; (T) transplanting; (TC) tissue cultures.*

provide plant residues for humification at the end of their life cycle.

The selection of candidate pioneer plant species is a key aspect to consider (Gilrain et al., 1999; Mortley et al., 2000; Kozyrovska et al., 2006; Wamelink et al., 2014; Ramírez et al., 2019). Plants produce $O_2$ and fix $CO_2$, serve as food for space crews, have a role in water recycling (Maggi and Pallud, 2010; Llorente et al., 2018), and are actively involved in soil structure formation. To improve ISRU, several researchers have proposed the development of microbial consortia (Zaets et al., 2011; Verseux et al., 2016; Llorente et al., 2018) that could improve the mineral uptake of plants.

## 3.1 Apollo-Era Plant Experiments With Lunar Samples

The first studies of plant growth on Lunar regolith were conducted in the early 70s in the Lunar Receiving Laboratory, where small amounts of real Lunar materials (brought from the Moon by the Apollo missions) were mixed with growth media

(such as wood pulp product) stabilized with acrylonitrile resin (Walkinshaw et al., 1970; Walkinshaw and Johnson, 1971; Weete et al., 1972; Weete and Walkinshaw, 1972). These studies were designed to determine whether Lunar materials contained any agents capable of generating an epiphytotic disease in representative species of the plant kingdom. The Lunar material was sterilized to avoid external contamination, and the entire laboratory was kept under quarantine conditions. The plant growth substrates containing extra-terrestrial materials were treated as inert media without considering their nutrient content and composition (**Table 2**). The main parameters monitored were seed germination capacity, growth alteration, phytotoxicity, and disease incidence. Walkinshaw et al. (1970) grew 35 representative plant species in aseptic conditions in different cultivation systems, including algae, seeds, spores, seedlings, gametophytes, and tissue cultures of higher plants. The authors noted the absence of disease agents in the plants tested under experimental conditions, and concluded that the Lunar material could potentially support the growth of a wide range of plant species. Specifically, ferns, liverworts, and tobacco were particularly effective in exploiting the Lunar material as a nutrient source. A year later, Walkinshaw and Johnson (1971) focused on the possible differences in chemical composition among plants grown on Lunar material. The results showed a direct interaction between plant species and Fe, Al, and Ti uptake from the Lunar substrate. Notably, cabbage and Brussels sprouts exhibited higher absorption of Mn.

Based on the findings of Walkinshaw et al. (1970), researchers further investigated the effect of the Lunar material on tobacco plants in terms of their constituent biomolecules and secondary metabolites. A tissue culture experiment over a period of 12 weeks used the Lunar material recovered from Walkinshaw's experiment, which was washed and sterilized before the trial. Weete et al. (1972) found that tissue grown in contact with Lunar material had a higher concentration of total sterols than in the control. They also found differences in absolute and relative fatty acid concentrations. Moreover, the chlorophyll and carotenoid concentrations were higher in treated plants, with chlorophyll *a* being the major pigment present (Weete and Walkinshaw, 1972). According to the review by Ferl and Paul (2010), the Apollo-era plant experiments with Lunar samples provided many insights into the biological impact of the Lunar environment on terrestrial life forms, which were useful for future research in support of Lunar exploration. The modern molecular approaches (-omic sciences) were not available during the Apollo-era plant experiments. However, those studies provided useful preliminary information on how Lunar samples and Earth biota interacted with and affected each other.

## 3.2 Plant Growth Experiments on Lunar and Martian Simulants

As a part of NASA's Advanced Life Support Program, Aglan et al. (1998) and Mortley et al. (2000), evaluated the response of sweet potato clones grown under microgravity in Lunar and Martian simulant media containing a buried microporous tube system for watering and fertigation. In these tests, the simulants mainly provided mechanical anchorage for the plant roots, and did not cause any adverse or toxic effects in the plants. Therefore, the authors concluded that both simulants showed potential for use as a substrate for crop production.

As a biotechnological approach to plant cultivation in an extreme environment (such as a Lunar base), Kozyrovska et al. (2006) proposed the growing of pioneer plants (*Tagetes patula* L.) in a Lunar rock anorthosite substrate. The simulants contained specific root-colonizing bacteria that could decompose the Lunar silicate rock and release the cations essential for plant growth. This strategy may prove to be a practical necessity in order to support plant growth in a substrate with low nutrient availability. The primary function of the pioneer plants with associated microorganisms is to form a soil with adequate fertility. This soil can then be used to grow plants of a second generation (such as wheat, rice, and soybean, among others) to provide Lunar explorers with fresh sources of vitamins, nutrients, and biomolecules. At the end of the growth cycle, the authors demonstrated that the first-generation plant residues could serve as a supply of green manure for humification and as a potential nutrient source.

The first large-scale controlled experiment evaluating potential plant growth (germination, growth, flowering, and seed formation) on the JSC1-1A Lunar and JSC-1A Martian regolith simulants was conducted by Wamelink et al. (2014). Fourteen different species of wild plants, crops, and nitrogen fixers (see **Table 2**) were grown for 50 days in isolation under Earth-like light and atmospheric conditions, while only using demineralized water and no fertilizers or substrate amendments. The results indicated that neither simulant was an adequate source of plant nutrients. Nevertheless, the Martian simulant outperformed the Lunar simulant in biomass production, as it had trace levels of ammonium nitrate and carbon and no stressors that could cause a higher pH or low water holding capacity. Tomato and wheat crops performed particularly well on the artificial substrates. Three species flowered, but only two produced seeds. In conclusion, the authors raised several open questions regarding the representativeness of the simulants, their water holding capacity, the availability of N and other nutrients on Mars and the Moon, and the influences of gravity, light, and other extra-terrestrial environmental conditions.

Waste management and efficient resource use are critical aspects of BLSS for both the Moon and Mars. A possible solution for the problem of waste management is composting, which can be incorporated into the agronomic treatment of regoliths as an amendment in line with the ISRU approach. By mixing compost with various regolith simulants, several studies have evaluated the role and potential utility of organic waste in plant cultivation and the management of extra-terrestrial settlements. These investigations not only provide a better approach to the management of residues, but elucidate the effects of organic matter amendment on mineral-based substrates (Gilrain et al., 1999; Wamelink et al., 2019; Duri et al., 2020). To help overcome the chemical constraints on plant growth in pure simulants, Gilrain et al. (1999)

conducted preliminary studies in an ALS plant growing system using a variety of proportional combinations of the JSC Mars-1 regolith simulant and a municipal leaf compost. The Swiss chard was used as a candidate crop, and half of the treatments received a modified half-strength Hoagland's solution. Plants grown in compost:simulant ratios of 1:0, 3:1, and 1:1 showed yields that were greater than those in the 1:3 and 0:1 ratios, and control plants irrigated with only water produced similar trends. However, overall plant growth was significantly lower, indicating that nutrient supply by both the compost and regolith simulant was not enough to sustain the entire plant growth cycle. The authors concluded that the compost mainly promoted plant growth by improving the physical features of the regolith that regulate water and/or nutrient availability.

A study reported the growth of 14 different plant species on a Martian soil simulant and, to a lesser extent, on a Lunar soil simulant (Wamelink et al., 2014). As a follow-up experiment, Wamelink et al. (2019) grew 10 different crop species (see **Table 2**) on the JSC1-1A Lunar and JSC-1A Martian regolith simulants (provided by NASA) containing organic residues from first harvests (fresh mown grass of *Lolium perennne* L.). A nutrient solution was also added to mimic the addition of human feces and urine. The main goal was the production of edible crops and their seeds for a next generation. The authors harvested the edible parts of nine out of ten crops. The biomass production was highest in the Earth control and the Martian soil simulant, but was significantly lower in the Lunar simulant. Only three species (radish, rye, and cress) produced seeds. Radish germination rates were lower in the Lunar simulant than in the Earth control soil and Marian simulant. The authors defined their study as a small step towards the implementation of a sustainable agricultural ecosystem for a Lunar or Martian colony. They further encouraged the search for the optimal organic matter content and physical characteristics of the simulants in future studies.

Recently, two butterhead lettuce (*Lactuca sativa* L. var. capitata) cultivars (green and red Salanova®) have been cultivated in the MMS-1 Mojave Mars simulant mixed with green compost at different rates (simulant:compost ratios, 0: 100, 30:70, 70:30, and 100:0; v:v) in a phytotron open gas exchange growth chamber (Caporale et al., 2020; Duri et al., 2020). A detailed characterization of the physicochemical, mineralogical, and hydrological properties of the simulant, compost, and their mixtures was provided. This was the first characterization of MMS-1 in terms of its mineralogical composition (x-ray diffraction) and spectroscopic features (by mid-infrared MIR spectroscopy). MMS-1 was found to be a coarse-textured alkaline substrate mostly composed of plagioclase, amorphous material, and (to a lesser degree) of zeolite, hematite, and smectites. Although it was a source of nutrients for lettuce, it did not supply organic matter, N, and S, and provided very scant amounts of P. As reported above in **Section 2.3**, organic amendment improved the physical properties of the simulant (such as bulk density and water holding capacity). It also lowered the pH of the alkaline simulant, enhanced its cation exchange capacity, organic C and N levels, and the availability of macro- and micronutrients. The red Salanova® lettuce grown in the 30:70 mixture showed the best crop performance, photosynthetic activity, intrinsic water use efficiency, and quality traits (mineral, carotenoid, and phenolic contents). The 70:30 mixture showed a slight decline in lettuce yield and quality; however, the authors concluded that it was a more sustainable choice for space farming, as it exhibited more efficient use of limited resources (e.g., compost). The study by Caporale et al. (2020) found discrepancies between the measured bulk chemistry of the MMS-1 simulant and that provided by the producer. This supported the observations by Cannon et al. (2019), who suggested that the MMS-1 simulant was derived from different source material than the original MMS. In the absence of rigorous documentation by producers of simulants, this shows the need for an adequate characterization of commercial simulants prior to the designing and planning of any scientific experiments.

Over the past 2 years, the new data collected by rovers has allowed the scientific community to broaden its knowledge of Martian environmental features. For instance, the Martian surface has been found to have a high salt concentration (Ramírez et al., 2019) and high levels of perchlorates (Eichler et al., 2021). To evaluate the impact of abiotic stressors on plant growth and health, Ramírez et al. (2019) tested the responses of 65 potato genotypes grown in Mars-like soil from the La Joya desert in Southern Peru (characterized by high EC, ranging from 19.3 to 52.6 dS $m^{-1}$). Only 40% of the genotypes survived and yielded crops (0.3–5.2 g tuber $plant^{-1}$). At the end of the study, the authors stated that the selection of tolerant genotypes, appropriate sowing methods, and soil management strategies were crucial for crops to withstand the extreme salinity and yield produce. More recently, Eichler et al. (2021) tested the growth rates of lettuce and *Arabidopsis* plants cultivated on three pure Martian regolith simulants—JSC-Mars-1A, Mars Mojave simulant (MMS), and Mars Global simulant (MGS-1)—enriched with calcium perchlorate (2% w/v). None of these simulants could support plant growth in the absence of nutrient supplementation. However, with the addition of nutrients, both plant species grew on JSC-Mars-1A and MMS, but did not grow on MGS-1. The authors linked this failure to the high alkalinity of MGS-1, and suggested acidifying the simulant to achieve plant growth. Calcium perchlorate-enriched simulants were unable to sustain plant growth, even with nutrient supply.

Fackrell et al. (2021) have developed and characterized five new Martian simulants—Global soil (MBas), Phyllosilicate-smectite (MPSmec), Phyllosilicate-illite/chlorite (MPChl), Sulfate-rich (MSul) and Carbonate-rich (MCarb)—for applications in space farming tests. These simulants have been found to be mineralogically, chemically, and spectrally comparable to Martian regolith and bedrock (according to available data), and are exploitable for plant growth in future studies on Martian surface analogues. In the conclusion to their work, the authors strongly advised that the fertility and feasibility of a simulant should be assessed not only on the basis of its mineralogical/chemical composition, but also on how physical

and (bio)chemical weathering of the substrate affects its nutrient bioavailability over time.

## 4 CONCLUSION

This paper was intended to be a comprehensive review of the potential for Lunar and Martian simulants being used as substrates for plant growth. Given the costs associated with shipping to either of these off-world sites, as well as the need to establish sustainable off-world operations, more research in this area is essential. This review analyzed more than 70 articles on the Lunar and Martian regolith simulants used as analogues in terrestrial experiments. We identified their main properties, critical aspects, currently available solutions to enable the growth of higher plants, and the potential challenges in deploying them in life support systems.

The literature review showed that pure regolith simulants may be suitable media for plant growth (at least for limited periods) and function as a source of essential nutrients such as K, Ca, Mg, and Fe. However, they lack organic matter and key macronutrients such as N, P, and S. Furthermore, these simulants generally exhibit numerous features harmful for plant health, such as an alkaline pH, high availability of Na, low cohesion of mineral components, the predominance of macro-vs. micropores, and low water holding capacity. In addition, the Martian regolith sometimes contains toxic perchlorates. Hence, the configuration of a mineral-rich and fertile biological substrate for edible plant growth based on regolith simulants still presents a challenge in space biology research.

In many studies, nutrient deficiency in simulants was overcome by fertigation with nutrient-rich solutions (e.g., Hoagland). However, this agronomic technique is not feasible and sustainable in space agriculture, as the nutritional resources must be carried from the Earth and cannot be produced in BLSS. A promising strategy is the adding of *in situ* recycled organic matter to enrich regolith simulants. This is a sustainable and effective technique to enhance the chemical and biological fertility and physical and hydraulic properties of regolith-based substrates (e.g., permeability and water retention). The organic waste produced by BLSS crews can help recover compounds and allow their use as fertilizers or compost to support plant growth. Thus, future studies should evaluate the efficacy of these treatments. Moreover, consecutive cycles of plant cultivation on the same regolith-based substrate can allow prolonged root exudation and the release of organic acid molecules and $CO_2$. This can lower the substrate pH, increase mineral weathering rates, enhance nutrient release/availability, promote the aggregation of particles of different sizes (to form a more efficient porous system), and overall contribute to soil improvement.

Many vital aspects of space farming have already been explored in the published experiments on plant growth in simulant-based substrates. Nevertheless, many other challenging factors still need to be considered to assess the true potential of extra-terrestrial farming based on the exploitation and development of *in situ* Lunar and Martian resources. For example, water management and recycling are paramount in sustainable BLSS modules developed and based on the ISRU strategy. Water is another limited resource in space, and we need a better understanding of water movement and fluxes in regolith-based substrate/plant systems under microgravity. In extra-terrestrial soil, water dynamics would regulate the extent of mineral weathering and the rate of organic matter decomposition, thus greatly affecting the biogeochemistry and bioavailability of nutrients and plant growth. Future studies simulating the potential environmental conditions of off-world bases are highly recommended.

Another aspect which needs further investigation is the effect of the space environment (such as different gravity and climatic conditions) on plant physiology (e.g., the biophysical limitations on gas exchange and transpiration), and how this affects plant growth and productivity and substrata properties. In sustainable scenarios of space farming, regolith-based substrates are required to sustain plant growth throughout the plant life cycle, including the complete seed maturation needed for reproduction. Although this may be feasible for microgreens or salad crops, it is more challenging for other candidate species such as potato (a source of carbohydrates) and soybean (a source of proteins), which require more nutrients and resources to produce a sufficiently edible yield than do salad crops.

The presence of—and interactions with—biota (including pathogens, cyanobacteria, plant growth-promoting bacteria, beneficial symbiotic fungi, and worms) and biostimulants will add further complexity to life support systems with extra-terrestrial soils. However, little is known about their effects on: 1) mineral weathering rates in the early stages of terraforming; 2) decomposition and recycling of organic plant residues and human excreta; 3) plant nutrition mediated by symbiotic fungi, compared with that mediated by entirely abiotic systems; and 4) protection of plants from environmental stresses. The occurrence of perchlorates in the Mars regolith provides a significant challenge to its use as an agricultural substrate. Thus, further steps—such as water rinses, phytoremediation, volatilization, and chemical reduction by using perchlorate-reducing bacteria—are necessary to make Mars regolith a viable growing substrate.

In conclusion, we encourage research that examines the effects of using *in situ* extra-terrestrial soils on several fundamental environmental functions (other than food security and biomass production) that constitute the primary functions of soil. On Earth, soils are the link between the air, water, rocks, and organisms. Soils are known to affect the climate, atmosphere composition, carbon and nutrient recycling, water quality and maintenance, biotic regulation, buffering, and the transformation of potentially harmful elements and compounds. In future, we should dedicate more attention to the development of pedogenesis on extra-terrestrial surface materials and explore the effects of regolith/simulant weathering on the soil-plant-atmosphere system. Providing ecosystem services by using a regolith-based substrate might be the key to a fruitful and sustainable Lunar or Martian BLSS.

## AUTHOR CONTRIBUTIONS

LD, SV, MP, and AGC, contributed to the original draft of the manuscript. YR, SDP, and PA guided the editing process and the literature search.

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# Large-Scale Crop Production for the Moon and Mars: Current Gaps and Future Perspectives

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In this perspectives paper, we identify major challenges for space crop production: altered convection in the microgravity environment, scheduling and logistics, crew time and the need for advanced automation, robotics, modeling, and machine learning. We provide an overview of the existing space crop production gaps identified by the Kennedy Space Center (KSC) space crop production team and discuss efforts in current development in NASA projects to address these gaps. We note that this list may not be exhaustive but aims to present the baseline needs for space crop production implementation and a subset of current solutions to the greater scientific community in order to foster further ingenuity.

**Keywords: automation, microgreens, plasma agriculture, microbiology, nutrient delivery system, reduced gravity, hydroponic, aeroponics**

## 1 INTRODUCTION

As humanity ventures into the Solar System and settles on other planetary bodies, movement towards independence from Earth to produce consumables such as food, oxygen, and water will be crucial. *In-situ* food production will depend on biological organisms such as plants and to a lesser extent, unicellular organisms. Large-scale crop growth systems included in bioregenerative life-support systems (BLSS) will allow for resource recycling and minimize resupplying from Earth. Several projects have aimed to identify the requirements for Lunar and Martian greenhouses, by assessing their feasibility, and developing concepts, theoretical designs, or prototypes at different scales and maturity levels (Wheeler and Martin-Brennan 2000; Stutte et al., 2009; Boscheri et al., 2016; Furfaro et al., 2016; Zeidler et al., 2017). KSC has a long history in controlled environment crop research. This began with the Biomass Production Chamber (BPC), extends to on-orbit platforms such as Veggie and the Advanced Plant Habitat (APH) and will continue with Ohalo III (a crop production chamber currently in development), with each platform deliberately selected and designed to lead to future crop production units destined for the Moon or Mars (**Figure 1**).


**FIGURE1 |** Examples of KSC's prior, current and future space crop production platforms selected and designed to lead to crop production units destined for the Moon or Mars.

Larger-scale platforms will bring new engineering challenges and needs. A set of logistical and physical obstacles have been identified as informed by the lessons learned from KSC's space crop production efforts on the International Space Station (ISS) using small scale platforms.

## 2 SPACE CROP PRODUCTION GAPS AND NEEDS

Space crop production efforts utilize the lessons learned from growing plants on the ground and in orbit. This knowledge, in combination with the expertise across NASA centers, academia, and industry serves to provide a framework for crop production systems for deep space travel (Johnson et al., 2021). Prior work conducted in plant chambers aboard the Shuttle and ISS has helped identify knowledge gaps and technology needs for space crop production (**Supplementary Table S1**). Throughout the remainder of this perspectives paper, we present a sampling of the current technologies being explored at NASA centers to address specific gaps, and we discuss their associated limitations and challenges.

### 2.1 Water and Nutrient Delivery: Challenges, Needs, and Current Research

Plant testing on the Shuttle and ISS revealed challenges with water and nutrient delivery. The lack (or reduction) of buoyancy-driven convection and the domination of surface-tension forces in reduced gravity leads to thick boundary layers of water (respectively air) forming around plant roots (respectively leaves) (Kitaya et al., 2003; Jones et al., 2012). Without appropriate water and nutrient delivery systems, roots suffer from hypoxia. Although substrate-based watering systems using capillary forces to guide water to the roots without suffocating them, are currently used on the ISS (e.g. Veggie and Advanced Plant Habitat (APH)) (Zabel et al., 2016), substrate-free or reduced-substrate water and nutrient delivery systems are being considered for future missions to reduce up-mass and waste (Morrow et al., 2017). We present recent work investigating spaceflight testing of a hydroponics system and an Earth-based analog demonstration of aeroponics.

#### 2.1.1 Substrate-Less Growth Systems

Space crop production efforts to date have focused on substrate-based approaches mainly using arcillite for plant growth, for proof of concept demonstration of crop growth (Massa et al., 2018). The approach is similar to terrestrial methods, yet as discussed above, watering proves a challenge in the microgravity. Among water solutions for substrate-based growth methods is a passive watering system named Passive Orbital Nutrient Delivery System (PONDS) where plants will draw from a free-standing reservoir of water (Levine et al., 2021). Recently, NASA has tested on-orbit a substrate-less hydroponics approach known as Plant Water Management (PWM). The PWM uses capillary geometry to contain poorly wetting aqueous solutions (e.g., sugary or nutrient-rich water), and provide sufficient aeration and hydration for low-g hydroponics (Mungin et al., 2019). Initial low-g data analysis showed that this could be a viable method, and further testing will include the use of space crops rather than the current simulated plants.

Furthermore, KSC's ongoing collaborative work with the German Aerospace Center (DLR) Institute of Space Systems in the analog extreme environmental setting of the EDEN ISS greenhouse facility at the Neumayer Station III site in Antarctica, has provided precedence for using an aeroponics approach (Zabel et al., 2017). Aeroponic nutrient delivery and a modified ebb/flow technique for plant growth systems in microgravity will also be evaluated using the eXposed Root On-Orbit Test System (XROOTS) (Morrow et al., 2017). In addition, the Porous Tube Nutrient Delivery System (PTNDS), which utilizes suction to hold plants seeded onto a porous ceramic water delivery tube, is in current development with additional watring approaches under development by university and industry researchers (Monje et al., 2019).

#### 2.1.2 Fertilizer/Nutrient Supply: Plasma Activation of Water

In the realm of nutrient delivery, a recent and interesting development in plasma agriculture (use of plasma, the fourth state of matter, to generate reactive species that interact chemically) is the plasma activation of water (PAW). For PAW, the introduction of plasma to water is able to change its properties (Foster et al., 2018). Plasma research is underway at KSC to explore the use of low temperature plasma to treat water, serve as a microbial sanitization method for hardware, and to explore plasma introduction of NOx groups and changes in pH for fertilizer-like applications. There is yet to be a complete understanding of plasma water chemistry, with prior and ongoing research at UC-Berkeley (Graves 2012) and recent modeling efforts at the University of Michigan (Kruszelnicki et al., 2019).

### 2.2 Crop Cultivation: Challenges, Needs, and Current Research at KSC

Volume is a limited resource in space, and future crop production systems will need to use available growth space effectively. In microgravity, plants can grow in all dimensions, thus making use of the entire available volume. Trade-off studies between monocrop and multicrop cultivation will help to determine if growth units will benefit from one or multiple crop species (Boscheri et al., 2016; Zeidler et al., 2017). To use volume efficiently, crop scheduling is also key. Depending on the growth cycles of individual species, crop planting could be staggered to meet dietary needs of the crew (Zeidler et al., 2017).

Together, NASA and the United States Department of Agriculture (USDA) are investigating the use of microgreens, plants that are small in size, nutrient-dense, and quick to grow with little crew time. Ready-to-eat plants like microgreens are easy to maintain and the rich nutrient and phytochemical composition of microgreens can help mitigate spaceflight-induced health risks (Anderson et al., 2017; Cooper et al., 2017). Microgreens have a high-volume optimization potential,

and efficiently use light, fertilizer, and water, all of which are important selection criteria for plants grown in space (Romeyn et al., 2019). These crops are harvested one to 3 weeks after planting, when the cotyledons are fully expanded, and the first true leaves have emerged (Xiao et al., 2012; Xiao et al., 2016). The short growth cycle of microgreens adds flexibility to crop systems, especially if disruptions occur. Microgreen production would require more seeds than full size crop production, but seeds are small and light, and the mass of seeds is largely offset by the reduced mass of fertilizer and their small volume requirements. An upcoming project at KSC will work to quantify this trade-off.

## 2.3 Environmental Monitoring Platform: Stress Detection and Food Safety Technologies

Studying plant growth in simulated and actual space flight environments, will enable the development of plant growth mechanistic and knowledge models. Such models can account for the combined physical, biochemical, and morphological phenomena involved; a necessary tool to accurately control and predict plant growth in life-support systems (Hézard et al., 2010; Poulet et al., 2020). In addition, we aim to achieve an advanced level of autonomy by implementing an integrated machine learning approach to monitor and coordinate space crop production systems, including the plant compartment atmospheric conditions, plant and microbiome genomic and metabolomic trends, water system microbiome and chemistry, and biomass recycling. Crew time for crop cultivation in future missions will be limited, as the primary activity of astronauts will be to accomplish mission tasks (Russell et al., 2006). Future space crop systems will need to be less crew time-intensive than current systems (Poulet et al., 2021; Zeidler et al., 2021) and utilize automation (e.g., watering or health monitoring). Small space crop production chambers on ISS, such as Veggie and the APH, can be used to validate and inform automation efforts, such as disease detection by imaging.

### 2.3.1 Hyperspectral Imaging

Monitoring plant growth and health during the entire life cycle of a crop is needed to ensure food safety of crops eaten by astronauts during cis-lunar, lunar and Martian missions (Anderson et al., 2017; Monje et al., 2019). Hyperspectral imaging can collect relevant spectral data for developing suitable vegetation indices, providing non-destructive and autonomous estimates of plant health with minimal crew time (Gowen et al., 2007; Araus and Cairns 2014; Huang et al., 2018; Zeidler et al., 2019). Early identification of plant responses to nutrient deficiencies, drought, flooding, or microbial/fungal infections, will provide the crew with enough time to mitigate these problems (Kim et al., 2001; Gowen et al., 2007). To this end, a prototype Plant Health Monitoring (PHM) system was developed by the USDA Environmental Microbial and Food Safety Laboratory (EMFSL, Beltsville, MD) and is in operation at KSC (Monje et al., 2021). The PHM is being used to develop a database of images from plants exposed to abiotic (e.g. drought) or biotic (e.g. fungal infection) stresses. This database will be used to develop suitable vegetation indices for autonomous early stress detection and for training future AI algorithms for plant health monitoring and to ensure food safety. In the future, miniature imaging systems will be deployed for monitoring plant health and microbial composition remotely in spacecraft such as ISS, Gateway, and Deep Space Transport.

### 2.3.2 Microbial Sequencing

Molecular methods have been essential for understanding plant-associated microbiomes (Bulgarelli et al., 2012; Chaparro et al., 2014; Knief 2014) and plant pathogens (Pecman et al., 2017; Díaz-Cruz et al., 2019; Piombo et al., 2021). Monitoring the microbes present in the spaceflight environment has been important since Apollo, but with short mission durations, not practical to perform during flight. For the ISS, requirements were generated to ensure that microbial contamination was periodically assessed in the ISS air, water, and on surfaces (Castro et al., 2004; Yamaguchi et al., 2014). From the beginning of plant growth on ISS, plants and plant growth hardware have been routinely sampled to understand the plant microbiome interaction and pathogen control in supplemental food production (Hummerick et al., 2010, Hummerick et al., 2011, Hummerick et al., 2012; Massa et al., 2017b; Khodadad et al., 2020). Veggie plant samples have been evaluated by culture-based isolation and identification, as well as microbiome analysis using Next Generation Sequencing on the Illumina MiSeq platform upon return to Earth (Khodadad et al., 2020). Such assessments of the microbiological food safety of crops grown on orbit has led to the development of a hazard analysis critical control point plan (HACCP) for space crop cultivation (Hummerick et al., 2011; 2012). The goal for spaceflight microbiology is to move beyond the need to culture potentially harmful microorganisms and to this end, culture-independent, swab-to-sequencer processes using the Oxford Nanopore MinION are now conducted onboard the ISS (Stahl-Rommel et al., 2021).

Future paths to assess plant-microbe interactions in the space crop production environment can benefit from the development of system level approaches where the power of automated sensing through imaging, omics (genomics, transcriptomics) and spectrometry (metabolomics, nutrients, volatiles) is realized. This surveillance, in combination with correlative and machine learning techniques, will allow for the detection of stress indicators, plant and human pathogens, reduced nutrient content, or the formation of health-hazardous volatiles, respectively.

## 2.4 Microbiome Ecosystem-Related Needs

### 2.4.1 Microbial Control Solutions for Watering Systems

Microbial biofilms have been a cause for concern in ISS biodeterioration, and as such, represent a risk to safe long-term human space exploration (Landry et al., 2020). Research into the effects of spaceflight on microbes has unveiled changes in virulence and biofilm formation under microgravity conditions (Kim et al., 2013), further underlining the need to develop robust biofilm management solutions (Landry et al., 2020). NASA continues to explore treatment methods such as biocides,

antimicrobial surfaces, nutrient filters, applied shockwaves, thermal and UV treatments (Velez-Justiniano et al., 2020). Biofilm management studies explore the use of other biological agents (such as bacteriophages or plasmids) to balance the ISS Water Processor Assembly (WPA) ecosystem (Sillankorva and Azeredo 2014) and even consider plasma-based approaches. The identification of the best solution for microbial control can be tailored to the relevant platform, be this a space crop production chamber, or the water processing system used for downstream irrigation.

#### 2.4.2 Plasma for Seed Sanitization

To avoid contamination by plant or human pathogens in space crop production platforms, seed surface sanitization is carried out before flight. Traditional methods of seed sanitization on the ground include alcohol soaking and chemical gas fumigation (Khamsen et al., 2016; Massa et al., 2017a), but these methods are not effective for all seeds. Plasma treatment of seeds, surfaces, or containers inoculated with fungi or bacteria have demonstrated significant log reductions after exposure (Filatova et al., 2009; Takemura et al., 2014; Puligundla et al., 2017a, Puligundla et al., 2017b; Zahoranová et al., 2018). The effectiveness of seed or surface sanitation may vary greatly based upon the plasma source and plasma gaseous medium being employed. Preliminary work is being conducted at KSC to test space crop production seed sanitization using different plasma sources and their effects on the associated seed microbiome.

## 3 DISCUSSION

### 3.1 Hyperspectral Imaging Current Challenges and Prospects

Although hyperspectral imaging enables precision plant health monitoring and early detection of diseases, it is currently limited by several factors. Data collection and analysis is dependent on the conditions in which measurements were performed, such as leaf orientation, illumination, or even leaf texture (Mahlein, 2016; Zeidler et al., 2019). Currently, spectral vegetation indices (SVI) are tied to specific wavelengths and obtained with specific environmental conditions and plant species, which means data to define SVI need to be collected in a high-fidelity environment. It is hard to apply SVIs to different conditions and plant types without risking data misinterpretation (Lowe et al., 2017; Thomas et al., 2018). The amount of data produced is large and therefore requires the development of efficient data handling and analysis methods (Mahlein, 2016; Thomas et al., 2018). For space applications, this raises the issue of telemetry limitations, which translates into the need for *in situ* processing. However, deep learning algorithms seem to be a promising solution for hyperspectral data analysis and interpretation (Thomas et al., 2018; Nagasubramanian et al., 2019), provided that they can be trained on a big enough dataset (Zeidler et al., 2019).

### 3.2 Substrate-Free vs. Substrate-Based

Alongside substrate-less systems presented in section 2.3.1, substrate-based systems using lunar or Martian regolith are being investigated (Wamelink et al., 2019; Eglin and Guinan, 2020; Eichler et al., 2021; Fackrell et al., 2021). Plant growth experiments have been conducted with returned Moon material (Ferl and Paul, 2010) and Wamelink et al. (2019) have successfully grown nine different species in JSC-1A regolith simulant mixed with organic material and further projects, such as the Mars Gardens, have compared this regolith simulant approach to hydroponic growth methods (Eglin and Guinan, 2020). Among the concerns raised when cultivating plants in regolith was the fact that it cannot be used as is; harmful compounds (e.g., perchlorates) need to be removed from Martian regolith and organic materials need to be added (Eichler et al., 2021). Furthermore, geomicrobiological methods have the potential to modify regolith to enable its utilization for plant growth. In this scenario, microorganisms are used to create soil from Lunar and Martian regolith (Verseux et al., 2016). Previous studies on plant growth in regolith simulants may have overlooked some important chemical composition aspects. However, the recent development of agriculturally relevant Martian regolith will enable more accurate studies (Fackrell et al., 2021). For the near term, hydroponic systems may prove a better option that is more amenable to a BLSS infrastructure. Hydroponic systems provide efficient absorption of nutrients as roots are directly in contact with the nutrient solution (Eglin and Guinan, 2020) and easy monitoring for subsequent control of the root zone microbiome.

### 3.3 Plasma Technology Development and Cost Reduction

Plasma applications in agriculture have largely focused on 1) plasma activated water, 2) seed germination, growth, and development, and 3) seed sanitization and biofilm sanitation. Uunderstanding of the chemistry and uses of plasma activated water is far from complete, but there are potential benefits in terrestrial and off-Earth controlled environment agriculture (Besten, 2019; Ranieri et al., 2020). As resupply is costly, plasma based applications and technologies provide an avenue of investigation worth researching to reduce the reliance on conventional methods of sanitation. The plasma source and gas requirements can be small, potentially even using breathable air. On Earth, use of plasma activated water is not widespread due to the problem of scaling for industrial use. Plasmas can be expensive outside of a laboratory setting and conventional methods are cheaper and within regulatory requirements. As advancements in electronics are rapidly decreasing costs, and regulations become stricter on particulate contaminants, plasma becomes a more favorable option. Additionally, new research is providing insight into plasma interactions with water and plasma-water chemistry that allow for system optimization of tasks. Although the application of plasma in various water systems is in its infancy, it should be considered as an aid or potential replacement to some current methodologies.

## 4 CONCLUDING REMARKS

As NASA and its commercial partners are getting closer to Gateway and robotic and crewed lunar surface missions, it is imperative to plan for large-scale crop production systems that will be deployed on the Moon and Mars. These present numerous challenges, and significant technology development and knowledge gaps need to be resolved prior to successful implementation. We have presented select considerations currently under investigation at NASA centers that can be integrated into large-scale crop production systems design to address these gaps. While not exhaustive, these state-of-the-art technologies include new methods for space crop production, environmental monitoring, water processing, and microbial containment approaches. Future crop production systems must be resilient, but also evolvable, so that new advances can be implemented as innovations occur. It will require contributions from numerous disparate disciplines to feed future exploration.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

LP, KE, TH, SS-R, Y-AV, SC-W, JB, OM, MH, CK, JS, MR, and GM contributed by writing the paper. All authors edited and revised the paper. AO coordinated the effort. GM and RW oversaw this coordination.

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# A Plant Characterization Unit for Closed Life Support: Hardware and Control Design for Atmospheric Systems

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Growth chambers are critical to the characterization of higher plant performance within BLSSs for long term crewed missions in Space. The Plant Characterization Unit (PCU) is a 2.16 $m^3$ environmentally controlled sealed chamber, realized in 2019 at the Laboratory of Crop research for Space of the University of Naples (Italy), within the European Space Agency Programme MELiSSA. The PCU enables terrestrial investigations of BLSS higher plant compartments that produce food, water, and oxygen for the crew. It accommodates two separate sub-systems, an atmospheric module and a hydroponic module. Such systems regulate autonomously temperature, relative humidity, light intensity and spectral composition, atmosphere gas composition, and air flow and pressure, as well as the composition, the temperature and the flow of the nutrient solution. This method paper describes the following phases of realization: 1) the definition of plant requirements; 2) the design of the two modules; 3) the development of the control system for the atmospheric sub-system.

**Keywords: controlled environment, Lactuca sativa L., air composition control, thermal control, air tightness, hydroponics, closed growth chamber, BLSS**

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## 1 INTRODUCTION

Human exploration beyond Low Earth Orbit (LEO) will require specific technologies to regenerate resources, while recycling the waste generated by the crew, and to exploit the *in situ* resources, to overcome the need of continuous resupplying from Earth, and to prevent pollution to extra-terrestrial bodies. Bioregenerative Life Support Systems (BLSSs) are artificial ecosystems in which appropriately selected organisms are assembled in consecutive steps of recycling, to reconvert the crew waste into oxygen, potable water, and edible biomass, developed to allow autonomous human long-term habitation in space (Hendrickx and Mergeay, 2007). Specifically, BLSSs consist of producers (higher plants and algae), consumers (humans and animals) and decomposers (microorganisms), as natural terrestrial ecosystems, able to recycle and regenerate air, water, food, and other essential substances needed for human survival, ultimately providing Earth-like comfortable environments (Liu et al., 2021). They are realized as modular systems including interconnecting compartments which host the above-mentioned organisms and enable different specific functions in a closed regenerative loop (Wheeler, 2010; Guo et al., 2017).

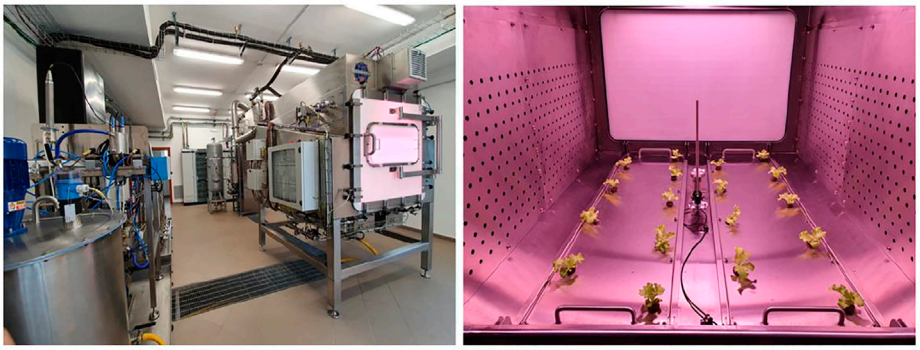


**FIGURE 1 |** Plant Characterization Unit (PCU) realized at the Laboratory of Crop research for Space of the University of Naples Federico II (Department of Agricultural Sciences, Portici Naples) within the ESA—MELiSSA project PlAnt Characterization unit for closed life support system—engineering, MANufacturing and testing (PaCMAN). Panoramic view of the laboratory and internal view of the chamber during a test on lettuce plants.

Higher plants are the most promising biological regenerators in BLSSs for space, as they regenerate air through the photosynthetic $CO_2$ assimilation and $O_2$ emission, to recover purified water through the transpiration, and to recycle waste products through the mineral nutrition, while providing fresh food for the crew diet (Hendrickx and Mergeay, 2007), and health and psychological benefit to the astronauts (Cahill and Hardiman, 2020; Heer et al., 2020). However, to perform these functions, plants need optimal environmental conditions for the growth. This requires the knowledge of their response to the different cultivation factors (e.g. controlled environment, hydroponic systems) and space factors (e.g., altered gravity, ionizing radiation) (De Pascale et al., 2021), as well as the design of specific agricultural systems (Wheeler, 2017). To this purpose, the creation of growth chambers that automatically control the technical and environmental parameters is crucial to study the plant performance. The goal is to define the conditions (e.g. light intensity and spectrum, $CO_2$ concentration, temperature, relative humidity) to optimize the growth and yield of candidate crops and the nutrient and nutraceutical value of plant food. Several review papers detail fully controlled plant chambers, developed for both ground demonstration and space flight experiments, and the needs for their improvement (Zabel et al., 2016; Wheeler, 2017; Liu et al., 2021).

Relevant efforts in developing modules for Earth and space applications are made by the European Space Agency (ESA), within the framework of the programme Micro-Ecological Life-Support System Alternative (MELiSSA) (MELiSSA foundation, 2022). The goal of the programme is to conceive an artificial ecosystem, inspired by the reconversion cycle of organic matter in natural lake ecosystems, founded on higher plants-, algae- and microorganisms-based technologies for resource regeneration for long-duration missions in space (Mergeay et al., 1988). The driving element of MELiSSA is the recovering of edible biomass, water and $O_2$ from organic waste (faeces, urine, $CO_2$ and minerals), using light as an energy source to promote photosynthesis (Hendrickx et al., 2006).

The MELiSSA cycle is a loop of five interconnected compartments, each with a specific biotransformation task, colonized by thermophilic anaerobic bacteria, photo-heterotrophic bacteria, nitrifying bacteria, photosynthetic organisms, and the crew as both the first producer (of waste) and the final user (of products) (Lasseur et al., 2010). The complete cycle is demonstrated in the MELiSSA Pilot Plant (MPP), a laboratory operating at the University Autònoma of Barcelona (Spain), dedicated to test the closed-loop at a pilot-scale in terrestrial conditions (Gòdia et al., 2004). In the MPP, compartment sizing provides the total oxygen and 20–40% of the food required by one human (Peiro et al., 2020). Within the loop, the Higher Plant Chamber (HPC) is a compartment designed to cultivate plants under fully controlled conditions. The HPC aims at regenerating air and water through photosynthesis and transpiration respectively, as well as produce fresh food for human consumption (Dixon et al., 2017). However, it does not allow the precise characterization of plant physiology in terms of rate of $O_2$, $CO_2$ and water absorption and production. Nonetheless it is not suited for computing separately the mass balances in the root and aerial zones, required for modelling each compartment (Favreau et al., 2005; Waters et al., 2005).

To address these limitations, the Plant Characterization Unit (PCU) was realized in 2019 at the Laboratory of Crop research for Space of the University of Naples Federico II (Department of Agricultural Sciences, Portici, Naples) (**Figure 1**), within the MELiSSA project PlAnt Characterization unit for closed life support system—engineering, MANufacturing and testing (PaCMAN). The PaCMAN project involves 5 partners: University of Naples Federico II (Naples, Italy), ODYS S.r.l. (Milan, Italy), EnginSoft S.p.A. (Bergamo, Italy), NTNU Centre for Interdisciplinary Research in Space—CIRiS (Trondheim, Norway), Hosokawa Micron Ltd. (England).

The PCU is conceived as a research facility for extensive scientific investigations on food production, air revitalization and water purification, in the view of the integration of the higher plant compartment in BLSSs. Specifically, it allows

**TABLE 1 |** Plant growth requirements in terms of environmental parameters for the different crop types (left panel), and Reference intervals for environmental parameters for plant cultivation in controlled environment at the NASA's Biomass Production Chamber (right panel) (Wheeler et al., 1996a).

| Leafy vegetables | Root and tuber vegetables | Fruit vegetables |
|---|---|---|
| Light intensity (μmol/m$^2$s) | >250–275 | >275–400 | >300–400 |
| Photoperiod (h) | 18–24 | 18 | 18 |
| Air Temperature (°C) | 22–28 | 15–25 | 20–28 |
| Relative air humidity (%) | 50–85 | 50–70 | 50–75 |

| Parameter | Reference interval |
|---|---|
| Air Temperature | 10–30°C |
| Relative Humidity | 40–90% |
| $P_{atm}$ | >30 kPa |
| $CO_2$ Partial pressure | 0.1–3 kPa |
| $O_2$ Partial pressure | >5 kPa |
| Inert gas composition | Optional |
| Ethylene | <50 ppb (at 100 kPa) |

testing of multiple crop growth scenarios under different conditions in terms of environmental and cultural variables (temperature, relative humidity, light intensity and spectral composition, air flow, pressure and gas composition, nutrient solution). The chamber is not intended for a large food production, while it offers the possibility to finely measure growth parameters (i.e. $O_2$ evolution, $CO_2$ absorption, edible biomass production, plant tissue nutritional and nutraceutical properties), and to determine the mass balance in both the rhizosphere (hydroponic module) and the aerial zone (atmospheric module), since the two zones are separate and leakage proof. This will allow data to be collected to develop mechanistic models for plant growth and also to validate theoretically developed models (Boscheri et al., 2012; Poulet et al., 2020).

This paper describes the design of the chamber, based on the plant requirements and the scientific purpose, and the development of the control system and algorithms of the PCU atmospheric sub-system, as well as the main results of environmental control in a life test on a lettuce crop. Data on growth (biomass accumulation and plant biometric parameters), plant physiology related results (gas exchange, mass balance), and food quantity and quality (yield and nutritional and nutraceutical composition) will be reported on a different article.

# 2 MATERIALS AND METHODS

## 2.1 Criteria for Plant Requirements Definition

In closed growth chambers for ground-based experiments, the control of environmental and cultivation parameters is essential to characterize the required inputs (energy, water, nutrients, $CO_2$, and $O_2$) and the subsequent outputs (water from transpiration, $O_2$ from photosynthesis, $CO_2$ from respiration, waste). Such parameters are required to model the cultivation system functioning. Accordingly, the following environmental parameters were considered for monitoring and control in the PCU atmospheric module: air temperature (T), relative air humidity (RH), light duration, intensity, and spectrum, and air composition.

Considering the diversity of plant needs, growth requirements were distinguished according to different crop types, hence different reference ranges were proposed for leafy vegetables (e.g., lettuce, kale, spinach), root and tuber vegetables (e.g., potato, carrot), and fruits vegetables (e.g., tomato, soybean), as shown in **Table 1**.

PCU validation required plant life tests and the species selection involved several crops, each with specific scientific interest for technical and/or nutritional and nutraceutical reasons., Plant requirements were developed accordingly to the following crops selection:

- lettuce (*Lactuca sativa* L.), as leafy vegetable;
- potato (*Solanum tuberosum* L.), as tuber plant;
- tomato (*Solanum lycopersicum* L.) and soybean (*Glycine* max L. Merr.), as fruit vegetables.

A literature review was performed to collect information on plant characteristics and optimal environmental conditions for these crops (**Table 2**) and possible additional crops to be included in future experiments.

## 2.2 PCU System Design

The PCU was designed by EnginSoft (Bergamo, Italy) in collaboration with Hosokawa Micron Ltd. (Runcorn, Cheshire, United Kingdom), and engineered as a combination of two sub-systems (**Figure 1**). The atmospheric module provides the air circulation in the growth chamber, while controlling and monitoring the environmental parameters and air quality. The hydroponic module recirculates the nutrient solution (NS), while controlling its pH and ions composition and concentration. Particular attention was paid to ensuring homogeneous environmental conditions, in both the root and the areal zones.

The PCU has a cultivation area of dimensions 1.5 × 1.2 × 1.2 m (L×W×H). The hydroponic system consists of 2 gullies, each of which can accommodate 9 plants (thus 18 plants in total) at the density of 10 plants m$^{-2}$.

Two centrifugal blowers provide air recirculation. An air velocity sensor measures the airflow in the heating, ventilation, and air conditioning (HVAC) vertical pipe. From the side plenums, the air is distributed in the chamber through perforated plates and is recovered again at the top through perforated plates (**Figure 2**).

The design of the PCU addressed several engineering challenges and specific techniques were implemented to meet the low leak rate required for the mass balance calculation. For instance, various phenomena produce pressure changes, such as

**TABLE 2 |** Main characteristics of the four crops proposed as candidates for PCU life tests (left panel; data elaborated from literature on plant cultivation in controlled environment for Bioregenerative Life Support Systems), and optimal ranges of air temperature, relative air humidity, and light intensity and photoperiod for the crops suggested to validate the PCU design (right panel).

| Crop variables | Lettuce | Soybean | Tomato | Potato |
|---|---|---|---|---|
| Plant height (cm) | 25 | 50 | 45 | 80 |
| Growing cycle duration (days) | 28 | 97 | 84 | 90 |
| Maximum photoperiod (hours) | 24 | 14 | 12 | 14 |
| Carbon dioxide accumulation (g/m$^2$ day) | 13.68 | 27.86 | 50.90 | 55.72 |
| Oxygen production (g/m$^2$ day) | 11.28 | 23.38 | 37.00 | 46.62 |
| Water production (g/m$^2$ day) | 1834 | 3,794 | 5,550 | 5,192 |
| Inedible biomass (g/m$^2$ day) | 0.72 | 11.48 | 9.80 | 15.12 |
| Edible biomass (g/m$^2$ day) | 8.16 | 6.58 | 9.80 | 21.00 |
| Crop variables | Lettuce | Soybean | Tomato | Potato |
| Temperature (°C) | 20–24 days<br>10–18 night | 18–30 | 22–28 days<br>13–16 night | 16–22 |
| Relative humidity (%) | 60–80 | 60–70 | 55–60 | 60–70 |
| Light intensity (μmol/m$^2$s) | 250–300 | 300–400 | 300–400 | 300–400 |
| Photoperiod (h) | 18–24 | 18 | 18 | 18 vegetative phase/12 tuberization phase |

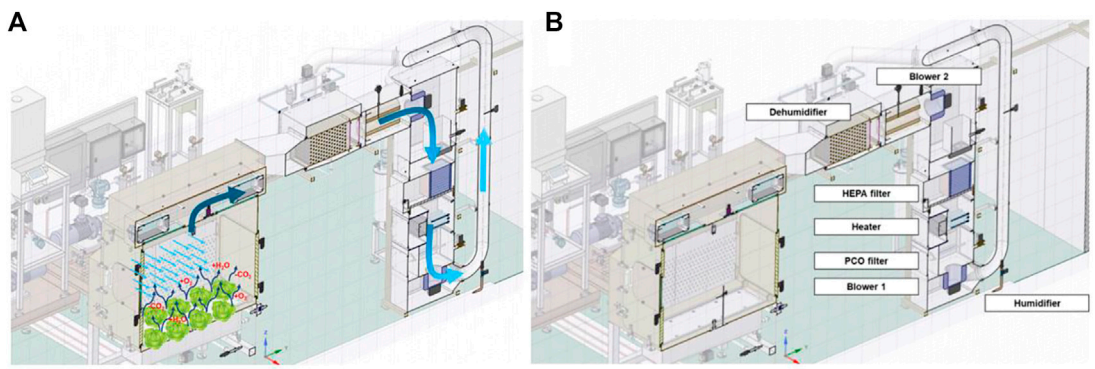



**FIGURE 2 |** Schematic view of the atmospheric system of the PCU: particular of the air flow from the plant chamber to the HVAC **(A)** and of the components position **(B)**.

external weather conditions, and temperature changes in the chamber simulating day and nights cycles. Therefore, the pressure inside the chamber must be continuously controlled, to limit the gradients between the internal and external environment. For this reason, an active pressure compensation system was developed. A compressor extracts air if pressure must be reduced and stores it in a tank. When pressure must be increased to the stored air is re-injected opening valve V125 (**Figure 3**). In addition, particular care was paid to the design of the connections among components. For example, inflatable seals are used to achieve airtight door closure.

Air relative humidity and air temperature were controlled based on RH and T sensor measurements in the chamber and a dedicated dehumidifier and heater (**Figure 2B**). The dehumidifier reduces excess air humidity, and the heater reaches proper T in the chamber. In addition, a nozzle sprays water when RH is low. Two additional RH/T sensors are placed in the chamber and in the air distribution pipe, and an additional thermistor is located downstream the dehumidifier. A stainless-steel tank collects the generated condensate.

Lighting is provided by two dimmerable light emitting diodes (LED) lamps (full spectrum blue, green, red and far red panel) and a dedicated fan removes generated heat.

An $O_2/CO_2$ gas analyzer measures the gas composition, and $CO_2$ concentration is controlled by the injection of pure $CO_2$ until reaching the desired level. The flow is measured by a mass flow transmitter allowing the calculation of the cumulated $CO_2$ volume injected. During standard activity of the chamber the $O_2$ concentration is monitored, and when a threshold value is reached, an aeration procedure is activated. This consists in opening two valves connecting the chamber with the external environment, until the $O_2$ falls below a user-defined threshold and the mass balance computation is reinitialized. In addition, $O_2$ injection can be foreseen in some advanced working mode. The flow is measured by a mass flow transmitter, allowing the calculation of the cumulated $O_2$ volume injected. A HEPA Filter and a PCO (Photo-Catalytic Oxidation) filter are used to trap contaminants.

The hydroponic loop includes a 300 L main stirred tank manufactured in stainless steel (316-L), and the distribution

piping composed of two lines that supply the NS flow to the two gullies through four branches each. Two pressure transmitters measure the liquid level in the gullies and a flow transmitter measures the NS total flow. The liquid level and the recirculating flow rate were controlled by means of three control valves one on each inlet line and one on the return line, an irrigation pump and a return pump.

The recirculating NS includes a system to remove contaminants, consisting in three membranes with different pore-size to remove microorganisms and suspended particles bigger than 0.2 microns.

An advanced nutrient delivery system gives the possibility to control the NS composition by dosing 8 stock solutions for a total of 10 side tanks, each equipped with a peristaltic pump and a scale to compute the dosed volume. This system gives a high flexibility in defining the NS recipe, and it is supported by state-of-the-art sensors. Electrical conductivity (EC), pH and temperature are controlled as standard parameters. In addition, dissolved $O_2$, dissolved $CO_2$ and $NO_3^-$, $NH_4^+$, $Ca^{2+}$, $K^+$, $Na^+$, $Cl^-$, $Mg^{2+}$, $HPO_4^{2-}$ concentrations are monitored to gain further understanding on root uptake phenomena. The pH and EC of the recirculating NS were measured by pH sensors and EC sensors, respectively. The pH control is performed according to the desired set point through the addition of acid (0.5 M $HNO_3$) or base (0.5 M KOH) that are stored in two dedicated 5-L polypropylene tanks. Two concentrated salt solutions (Stock A and B) are stored in dedicated 10-L polypropylene tanks and injected into the loop at a final concentration corresponding to the EC set point.

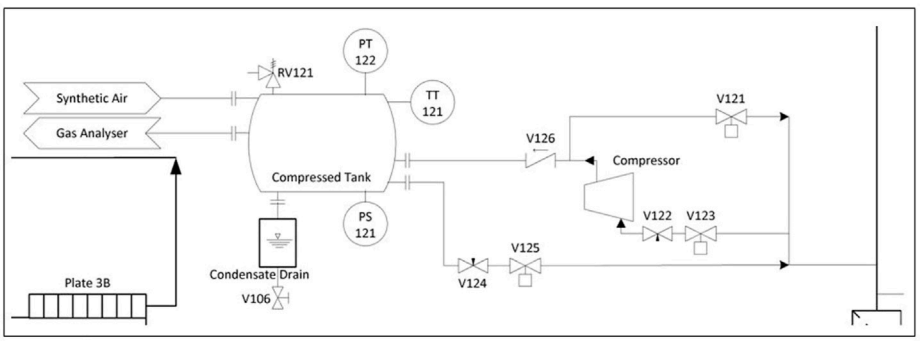
## 2.3 Atmospheric Control System

This section details the design of the control system for the PCU, carried out by ODYS Srl (Milan, Italy). To guarantee an optimal operation of the chamber here developed, the control software must manage all the aspects of the atmospheric and the hydroponic subunits. Nonetheless, their tight coordination must be guaranteed, to meet system-wide objectives involving aerial and hydroponic variables, such as pressure gradients. The controller must enable the operation of the system according to user-defined specifications, which are based on the requirements of the cultivated crops and the specific test plans.

The complexity of the above-mentioned tasks does not allow an exhaustive description of the whole control system, therefore in this paper we focus on the main atmospheric components, whose primary objective is to guarantee homogeneous and fully controlled environmental conditions in terms of air circulation, pressure, air composition, T, and RH.

### 2.3.1 Control System Overview

The control and monitoring functionalities are provided by two separate software products, a real-time controller, and a Graphical User Interface (GUI). Both applications have been



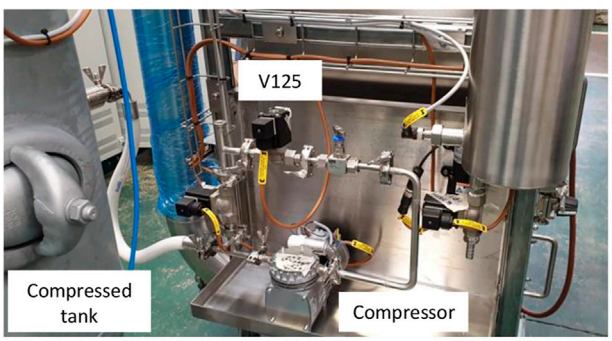




**FIGURE 3 |** Schematic view and picture of the pressure compensation system implemented in the PCU. The compressor extracts air from the chamber if the internal pressure must be reduced and stores it in the compressed tank, while when the pressure has to be increased the air stored in the compressed tank is re-injected by opening valve V125.

**TABLE 3 |** Requirements considered for the design of the atmospheric module of the PCU.

| Requirements description |
|---|
| The system shall allow the monitor and control of air temperature in the range 10–35°C, with the accuracy of 0.5°C |
| The system shall allow the monitor and control of air relative humidity in the range 40–90%, with the accuracy of 2% |
| The system will provide adjustable light intensity at floor level, in the range 0÷600 μmol $m^{-2}$ $s^{-1}$ |
| The system shall allow the regulation of the duration of lighting (photoperiod) until continuous lighting (24-h), with an increment time of 10 min |
| The system shall allow the manual adjustment of light intensity at the canopy level |
| The system shall provide homogeneous light intensity at the canopy level along the positions in the gullies (±40 μmol $m^{-2}$ $s^{-1}$ at different plant locations) |
| The system shall allow the change of light spectral composition (i.e. proportion of the selected wavelengths: blue 445–500 nm, red 620–700 nm and far red 700–775 nm and white light 400–700 nm) |
| The system shall allow the on-line monitoring and control of $CO_2$ air concentration from 400 ppm (ambient concentration) until at least 2000 ppm |
| The system shall allow the on-line monitoring of $O_2$ air concentration in the range between ambient concentration (around 21%) and 26%. Air renewal is needed above the threshold value of 26.5% |
| The system shall allow the control of ethylene ($C_2H_4$) air concentration below the threshold value of 50 ppb |
| The standard air flow velocity in the growth chamber shall be 0.3 m/s |
| The standard airflow velocity in the growth chamber shall be uniform at the level of the plant canopy |

developed in Python programming language, which guarantees code portability, faster turnarounds, and seamless functionality extensions, all being crucial features for prototypes development.

The real time controller runs on a Unix embedded platform. At the fastest rate, it executes the most critical tasks such as the monitoring of all the variables that are linked to a possible fault and/or alarm of the PCU, and the communication with the GUI. The atmospheric and hydroponic control loops run at slower sampling rates, the faster being 5 s. Given the different timescales of the variables at hand in the entire system, the sensors' reading as well as the actuations are implemented on several independent control rings. Data logging is provided as well by the real-time controller.

The GUI is a desktop application that can operate the system remotely. It enables real-time inspection of all the monitored values, as well as the current state of every manipulated variable. The configuration of each control task can be modified in real-time, such as set points or manual commands. The changes in the configurations can also be scheduled, enabling an effective planning of all the different phases of the experiment, according to the specific needs of the crop.

Research and scientific data acquisition are the main objectives of the PCU, therefore experiment flexibility, safety and easy extension of testing capabilities are of utmost concern. To this end almost every control loop, besides the most critical ones such as the pressure regulation system, allow for different operating regimes. The *OFF mode* disables any active controller of a specific task, as well as its alarm management. Only the measures from the sensors, if any, are collected while operating in this mode, and every actuator is in its default position. The *manual mode* consists in disabling the feedback routines of a specific control task, while allowing the operator to manually operate from the GUI all the actuators. Finally, the *automatic mode* is the one where the feedback algorithms of a control task are active. Given the complexity of the system, and the possibility of having the control loops in different combinations of operating modes, the PCU requires a very reliable handling of dangerous situations, as well as feedback controllers that are robust against unmatched disturbances, which are mainly caused by crops growing and operations in manual mode.

### 2.3.2 Model-free Controllers

The PCU is a medium-scale process, where many tasks consist in controlling Multi-Input Multi-Output (MIMO) systems, some of which are interacting with each other and must be coordinated for achieving process-wide goals. Moreover, different time-responses coexist especially in the coordination layer given the different nature of the variables. This challenge has been tackled with a hierarchical multilayer system, whose control actions are performed by several regulators working at different timescales. Advanced model-based control algorithms, among which we cite Model Predictive Control (MPC) (Rawlings et al., 2017), are a natural answer to the challenges posed by such control requirements and architecture. However, deriving control oriented mathematical models is time consuming and not flexible in a prototyping stage, making it preferable to exploit model-free controllers. Therefore, for the coordination layer we have opted for a state machine, whereas the low-level controllers rely on Proportional Integral Derivative (PID) regulators, bang-bang control, and the possible combinations of those.

PID controllers are implemented in *parallel form,* with anti-windup action for taking care of the nonlinearities introduced by actuators saturation, which is very frequent in valves control. Let $u(k) \in R$ be the manipulated input, $y(k) \in R$ the controlled output, $y_{\mathrm{ref}}(k) \in R$ the reference trajectory and $e(k) = y_{\mathrm{ref}}(k) - y(k)$ the tracking error, with $k$ the current timestep of a system with sampling time $T_s$. Let $\tilde{u}(k) = P(k) + I(k) + D(k)$ be the non-saturated input command, with $P(k)$ the proportional action, $I(k)$ the integral action and $D(k)$ the derivative action, then the saturated control command of a discrete time, parallel-form, PID controller is:

$$u(k) = \mu\left(y_{\mathrm{ref}}(k), y(k)\right) = max\left(min\left(\tilde{u}(k), u_{max}\right), u_{min}\right)$$

where $u_{min}, u_{max} \in R$ are the lower and upper bounds for the control command. We recall that P(k) is a term proportional to the current tracking error, $I(k)$ is proportional to the error integrated over a period of time, and $D(k)$ depends on the rate of change of the error with respect to time.

In the next we discuss the design of the crucial atmospheric controllers. It is worth noticing that many minor tasks are

involved in the atmospheric system even though they are not described in detail in this article. Among them, we cite the day/night cycle scheduling, with the regulation of light intensity and spectrum, the air filtering, and the photocatalytic oxidation for removing bio and non-biological contaminations.

### 2.3.3 Air Velocity Control

The automatic control of the air velocity is crucial in the PCU, because if an adequate flow rate of air is not guaranteed then it is not possible to effectively control T, RH, and air composition in the growth chamber. Nonetheless a correct air flow is crucial to guarantee heterogeneous conditions to all the plants. The objective of the control is to manipulate the rotational speed of two blowers, namely *blower 1* and *blower 2,* such that the air flow inside the chamber is regulated to a defined set-point, which can be dynamic during the experiment. *Blower 1* is located before the chamber, with the aim of overtaking pressure losses due to the chamber distribution unit and the dehumidification system, whereas *blower 2* is placed downstream the chamber, to overtake pressure losses in the remaining elements of the HVAC unit.

In order to coordinate the two actuators, *blower 1* is controlled in open-loop as a function of the flow rate set point. In the specific, let $u_{b1}$ be the rotational speed of *blower 1*, expressed in rpm, and $f_{a,\text{ref}}$ the air flowrate setpoint, expressed in *m/s*, we define $u_{b1}(k) = \eta(f_{a,ref}(k))$ the function computing the ideal rotational speed for $u_{b1}(k)$ such that the pressure upstream *blower 1* is identical to the pressure upstream *blower 2*. Such function has been experimentally derived. On the other hand, the rotational speed of *blower 2*, namely $u_{b2}$, is controlled in closed-loop by a PID ($\mu_1$) which computes the deviation from $u_{b1}(k)$ to effectively steer the air velocity $f_a$ to its set-point, despite any disturbance in the circuit. Additionally, given the wide operational range of the velocity and the abrupt impact that the change in the set-point has on the open-loop control, the reference trajectory is filtered by incremental constraints. In the specific the air velocity control can be summarized by the following equations:

$$
\begin{aligned}
f_{\text{a,ref}}(k) &= f_{\text{a,ref}}(k-1) + max\Big(min\Big(f_{\text{a,ref}}(k) - f_{\text{a,ref}}(k-1), \\
&\qquad d_{f,\max}\Big), d_{f,\min}\Big) \\
u_{b1}(k) &= \eta\Big(f_{a,\text{ref}}(k)\Big) \\
u_{b2}(k) &= u_{b1}(k) + \mu_1\Big(f_{a,ref}(k),\ f_a(k)\Big)
\end{aligned}
$$

where $d_{f,\min} < 0$ and $d_{f,\max} > 0$ are the minimum and maximum increments allowed for the set-point in a time step.

### 2.3.4 Pressure Control

The objective of this function is to limit the differential pressure between the chamber and the external environment, thus reducing potential gas leakage, while simultaneously maintaining an overpressure to prevent any air intake from outside.

The PCU is equipped with a synthetic air cylinder controlled by an on-off valve to increase the pressure in the system, and a compressor to extract air from the system and thus decreasing the pressure. The compressor is a binary actuator as well. The controller regulates the pressure upstream *blower 2*, indicated as $p_1$ ($barg$), which is the point of minimum pressure of the system. Let $p_{1,\text{ref}}$ be the target overpressure, a deviation $d_p$ from the setpoint is defined so to implement a bang-bang controller. Assuming that air injection or removal are mutually exclusive, $v_p$ is a virtual actuation which is mapped to air injection if $v_p = 1$, compressor operation if $v_p = -1$, and no actuation if $v_p = 0$. The pressure requirements can be then effectively handled with a hysteresis control as in the following:

$$
v_p(k) = \begin{cases}
-1 & \textbf{\textit{if}} \quad p_1(k) \geq p_{1,\text{ref}} + d_p \\
 & \textbf{\textit{or}} \quad v_p(k-1) = -1 \ \textbf{\textit{and}}\ p_1(k) > p_{1,\text{ref}} \\
1 & \textbf{\textit{if}} \quad p_1(k) \leq p_{1,\text{ref}} - d_p \\
 & \textbf{\textit{or}} \quad v_p(k-1) = 1 \ \textbf{\textit{and}}\ p_1(k) < p_{1,\text{ref}} \\
0 & \textbf{\textit{otherwise}}
\end{cases}
$$

### 2.3.5 Air Composition

In order to allow accurate measurement of plant gas-exchange, $O_2$ and $CO_2$ concentrations of the closed atmosphere in the chamber must be monitored and controlled. Under nominal conditions, the $O_2$ concentration is monitored continuously, and when the threshold value of 26% (maximum safety limit) is reached, an aeration procedure is activated. This consists into opening two valves connecting the chamber with the external environment, until the $O_2$ falls below a user-defined threshold. The PCU is however equipped with an $O_2$ cylinder, and $O_2$ injection can be activated to operate at oxygen concentrations higher than the atmospheric level.

The $O_2$ injection is therefore achieved by actuating a proportional valve $v_1$ for the $O_2$ cylinder. However, a prolonged injection can cause large violations on the chamber pressure limits. The correct coordination of pressure and oxygen control is guaranteed by a supervisor state-machine that can disable injection to avoid pressure violations. Let $o_2$ be the percentage of oxygen concentration of the chamber, $o_{2,\text{ref}}$ its setpoint to be reached at the beginning of the experiment or after an aeration, and $e_p(k) = p_{1,\text{ref}}(k) - p_1(k)$ the error in pressure regulation, the control operates at a sampling time of 5 s and such that:

$$
v_1(k) = \begin{cases}
\mu_2\Big(o_{2,ref}(k), o_2(k)\Big) & \textbf{\textit{if}} \quad e_p(k) \leq 0 \\
v_1(k-1) & \textbf{\textit{if}} \quad 0 < e_p(k) \leq o_r d_p \\
0 & \textbf{\textit{if}} \quad e_p(k) > o_r d_p
\end{cases}
$$

with $v_1(-1) = 0$ and $o_r \in [0, 1]$ a tuning coefficient. Please note that we indicate with $f_O$ the flow-rate in *l/h* of the injected oxygen when $v_1$ is actuated.

A single PID ($\mu_3$) is also the base for the $CO_2$ regulation in the closed atmosphere. A dynamic set-point ($c_{o,\text{ref}}$) can be imposed, and the controller actuates the valve ($v_2$) of a $CO_2$ cylinder to restore the $CO_2$ consumed by photosynthesis. We indicate by $f_C$ the flow-rate in *l/h* of the $CO_2$ injected in the chamber when $v_2(k)$ is actuated. There is however no control

action to remove the $CO_2$ produced during the respiration phase, as its net consumption is expected on a day/night cycle. Let $c_o$ be the $CO_2$ measure, the control operates such that:

$$v_2(k) = \begin{cases} \mu_3\left(c_{o,\mathrm{ref}}(k), c_o(k)\right) & \textit{if} \quad c_o(k) \le c_{o,\mathrm{ref}}(k) \\ 0 & \textit{otherwise} \end{cases}$$

Given the mono-directional control and the fact that it is continuously operating, a hysteresis method is built around $c_o(k)$ to improve the steady-state performance.

### 2.3.6 Air T and RH Control

The objective of this function is to regulate the temperature $t_1$ (in °C) and the relative humidity $h_1$ (in %) in the growth chamber, so to track time-varying references ( $t_{1,\mathrm{ref}}$ and $h_{1,\mathrm{ref}}$ respectively) that commonly change from day to night hours.

The system is equipped with a heating coil, a cooling coil, and a humidifier. The heater and the de-humidifier normalized proportional control actions are indicated by $g_1, g_2 \in [0, 1]$. The humidifier consists instead in a water spray nozzle and its on-off control action is indicated as $g_3 \in \{0, 1\}$.

Let $\omega_c(k)$ (in $g/Kg$) be the water content in the chamber at time step $k$, and $\omega_{c,ref}(k)$ the target water content given the setpoints of T and RH for the chamber, that are respectively $t_{1,ref}(k)$ and $h_{1,ref}(k)$. With these premises the combined air T and RH controller works as in the following:

$$\begin{aligned} b_h &= \mu\left(t_{1,ref}, t_1\right) \\ b_c &= \mu\left(-\omega_{c,ref}(k), -\omega_c(k)\right) \\ g_1 &= max\left(b_h, 0\right) \\ g_2 &= b_c - min\left(0, b_h\right) \end{aligned}$$

On the other hand, the humidifier $g_3$ is actuated by a hysteresis control, function of $h_{1,ref}$ and $h_1$. However, the humidifier is required only during the very early stages of plant growth, when the total transpiration rate is still low.

## 3 RESULTS AND DISCUSSION

### 3.1 Plant Requirements

The literature review performed to collect information on plant cultivation in a controlled environment provided useful data to define the optimal environmental parameters for the selected crops. **Table 1** shows the reference ranges adopted in NASA's plant cultivation experiments for BLSSs, performed in the Biomass Production Chamber (BPC) at Kennedy Space Center, consisting of a 20 $m^2$ growing area within a 113 $m^3$ closed growth chamber (Wheeler et al., 1996a). Extensive testing of crop responses to air T and RH, photoperiod, light intensity and spectral composition was conducted in the BPC as a part of the NASA CELSS (Controlled Ecological Life Support) program (Wheeler, 2017). The overall focus of these studies was to define the optimal environmental conditions able to boost the growth and yield of selected candidate species for space missions (Wheeler et al., 2003). The growth chamber was decommissioned in 1998 after nearly 10 years of continuous operation, and many of the results of the crop tests were reported in the literature, including biomass yield and gas exchange ($CO_2$ removal and $O_2$ production) (Wheeler 1992; 1996b), and radiation use efficiency (Wheeler et al., 2008). **Table 2** shows the reference values recorded in the BPC regarding some main biometric, physiological, and productive characteristics of four of the species selected for the PCU life tests (lettuce, soybean, tomato, potato).

The main plant requirements for the atmospheric module of the PCU involved monitoring and controlling air T, RH, and lighting conditions. In the specific, the required performance of the lighting system was analyzed in terms of photoperiod, light intensity and spectral composition, in order to provide different lighting durations, up to continuous lighting, to test the possibility of improving plant biomass yield as well as oxygen production in the PCU. The light intensity was dimmable, to adapt the radiation level to the needs of different crops, as well as to optimize the light use efficiency. In addition, the light radiation was homogeneously distributed to achieve a maximum fluctuation of 40 μmol $m^{-2}$ $s^{-1}$ at the canopy level for different plant positions. Finally, the spectral composition (red:blue ratio, red:far-red ratio, blue:green ratio, etc.) was customizable (**Table 3**), to influence the crop-specific response in terms of plant physiology (leaf gas exchange analysis) and morphogenesis (stem elongation, plant size) (Paradiso and Proietti, 2021). Based on the ranges of optimal values reported in the literature for the selected crops (Langhans and Tibbitts, 1997; Anderson et al., 2018, Favreau et al., 2005; Wheeler et al., 1996a, 1996b, 2003, 2008), and considering the possibility of including potential crops for future experiments, the ranges of each environmental parameter to be ensured in the PCU were determined (**Table 2**).

Finally, another key aspect of closed growth chamber is the control of airflow velocity and homogeneity. Previous ESA work (ACSA project) conducted in the HPC of the MELiSSA Pilot Plant showed that increased uniformity of airflow velocity significantly improved the homogeneity of biomass distribution throughout the growth chamber (Peiro et al., 2020). In the specific, under inhomogeneous air velocity conditions, the unbalanced airflow configuration negatively affected the homogeneity of plant growth, resulting in adverse environmental conditions in the different modules of the growth chamber, responsible for plant bolting, leaf rot and increased inedible biomass. Therefore, referring to the values found by Peiro et al. (2020), the airflow velocity in the PCU was set to an average of 0.3 m $s^{-1}$, trying to ensure optimal airflow uniformity.

The overview of the main requirements considered for the design of the PCU is summarized in **Table 3**.

Lettuce cultivar 'Grand Rapids' was the genotype selected for the first PCU life test. The test was conducted following the plan and protocol of a previuos ESA work (ACSA project) conducted at the MPP in 2017 (Peiro et al., 2020). The followed approach allowed to compare past data obtained during the ACSA project with the results of the present life test, thus having a reference PCU performance evaluation. Specifically, lettuce crop was grown in the PCU with conditions as close as possible to the ones of ACSA test. Those include genetic material, seedling production,

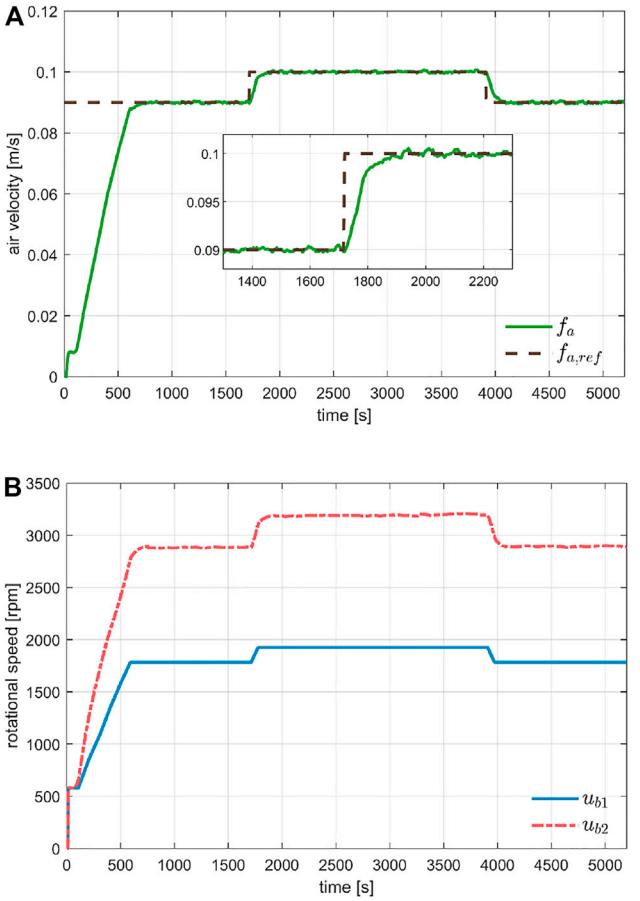



**FIGURE 4 |** Evaluation of air velocity control during system startup and sudden changes of the set-point. Plot **(A)** shows the tracking performance under different step changes, while Plot **(B)** the commanded rotational speed for the two blowers.

climatic conditions, nutrient solution composition and concentration, cycle length and phenological stages (Peiro et al., 2020). Lettuce plants in the PCU was grown according to a light/dark regime of 16/8 h with an intensity of 420 ± 40 µmol $m^{-2}$ $s^{-1}$, while air T and RH was set at 26/20°C and 50/70%, respectively. The experiment was carried out with a $CO_2$ concentration set at 1,000 ppm, while the harvesting of all the plants was performed at 28 days after transplanting.

## 3.2 Control System Validation

The performance of the atmospheric control system was evaluated on specific maneuvers designed to stress the challenge of the algorithms. For each controller, particular attention was devoted to tracking performance, limits satisfaction and actuation effort. Air T, RH, and $CO_2$ controls are common in other BLSS projects, at least in the most recent ones (Zabel et al., 2016), but discussions on the control strategies, and details about algorithm performance are an added value to the current state-of-the-art research.

**Figure 4** shows the air velocity control. The test scenario was a *cold start*, meaning a system startup from rest position, followed by a series of step changes in the air velocity set-point, commanded by the user. The transient of the cold start maneuver clearly shows the benefit of the incremental constraints on the velocity reference. The rise time for a step of $0.1m/s$ was about $500s$, and the steady state error amounts at just sensor and system noise. We recall that the total range for the set-point is $0.015m/s$ to $0.15m/s$. Smaller step changes were handled smoothly as well as shown in the picture.

**Figure 5** shows the pressure upstream blower 1, which was controlled by the bang-bang controller with hysteresis described in **Section 2.3.4**. The reference value for the overpressure was set to $50Pa$ while the maximum allowed deviation was $40Pa$. The figure shows the correct activation of the air injection system when the pressure falls below the lower limit, and the activation of the compressor when the pressure exceeds the upper limit. The hysteresis makes sure the pressure was close to the reference when the activation was turned off. It is important to consider that this data shows few seconds of the life test, therefore many sources of disturbance in the pressure were present. These include gullies level, air flow rate and temperature. The control strategy could keep the target pressure inside the prescribed limits with reasonable accuracy.

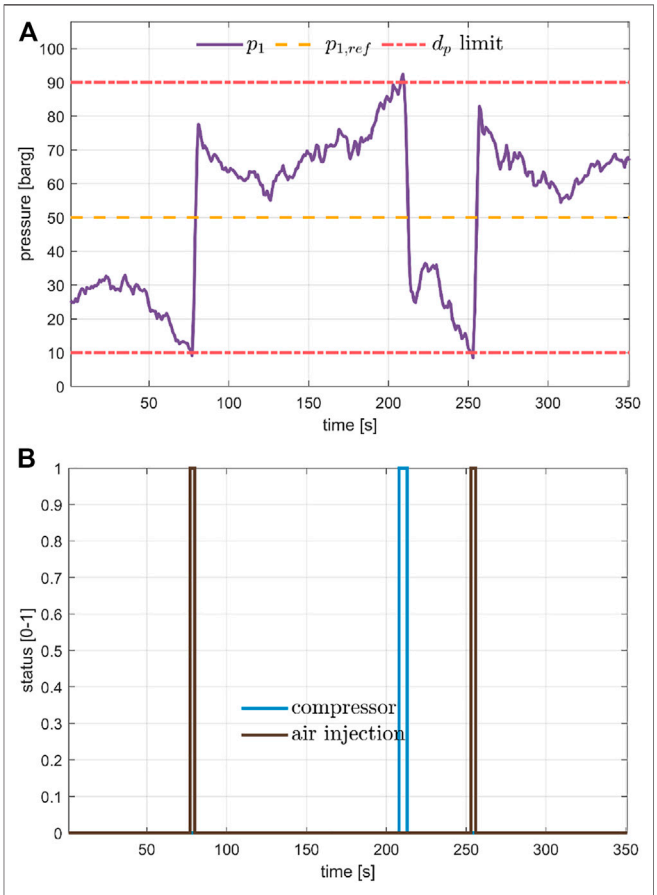



**FIGURE 5 |** Pressure control keeping the pressure upstream blower 1 inside the design constraint, plot **(A)** by switching ON and OFF the air injection and the compressor for air removal, shown in plot **(B)**.

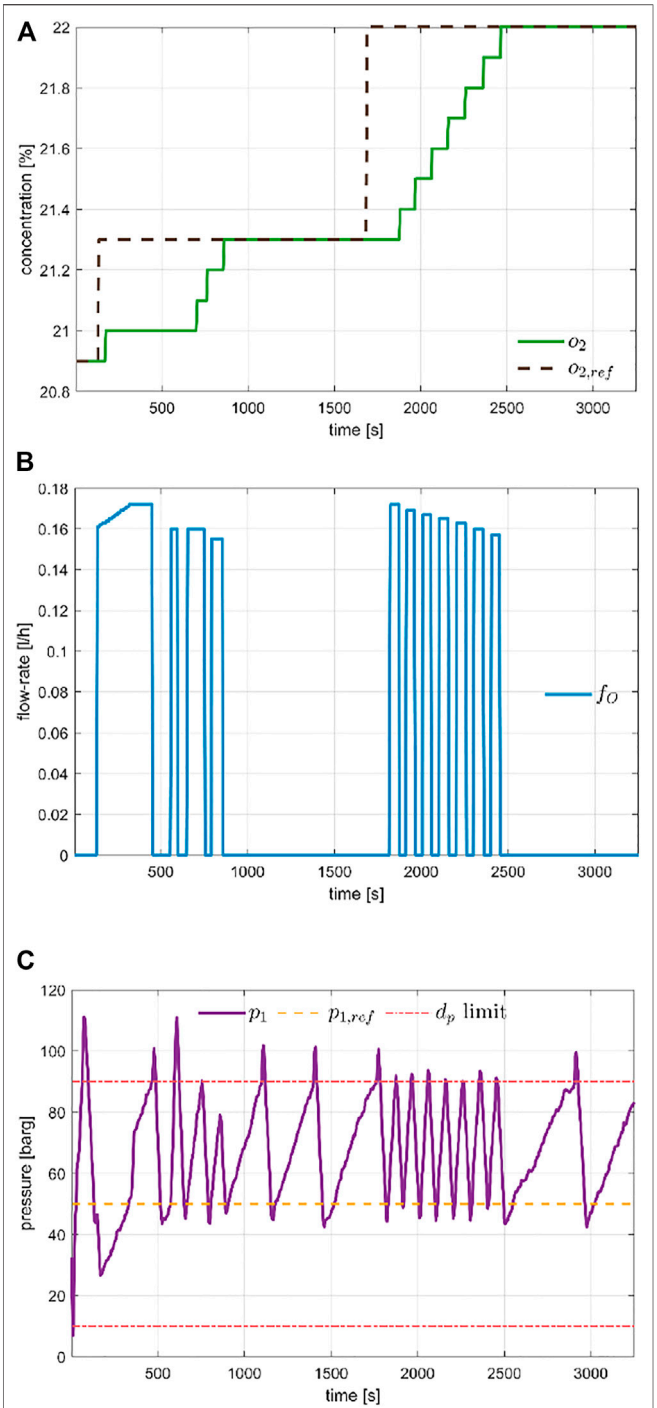



**FIGURE 6 |** Evaluation of $O_2$ recovery control, for different set-point values. Plot **(A)** shows the tracking performance, Plot **(B)** the actuation valve position and Plot **(C)** the simultaneous control of the chamber pressure that limits the $O_2$ injection.

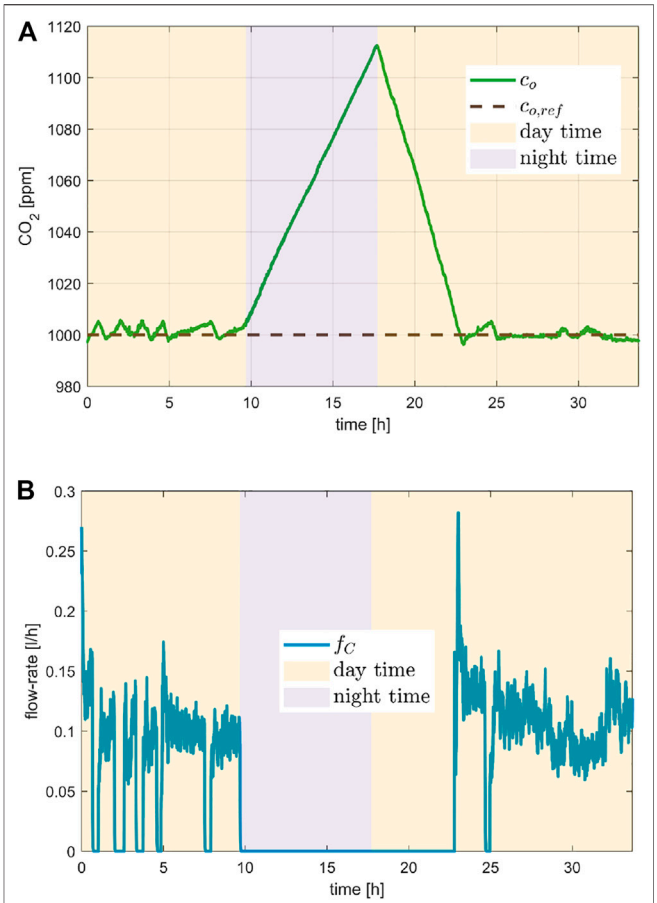



**FIGURE 7 |** $CO_2$ control performance evaluated in a day/night cycle at the second day of the experiment. Plot **(A)** shows the tracking of the $CO_2$ set-point and plot **(B)** the flow rate of the injected $CO_2$.

For the air composition we evaluated both $O_2$ and $CO_2$ concentrations. The $O_2$ control is mostly passive, as oxygen enrichment was not provided in this first life test. The control behaviour for an $O_2$ injection event was collected in **Figure 6**. The figure details the tracking performance for different concentration set-points. It was worth noticing that, as described in **Section 2.3.5**, the injection of $O_2$ is limited by the pressure limits, given the high impact on its dynamics. Indeed, the bottom plot shows the measure of $p_1$, which is the point of minimum pressure, to better understand the behaviour of the $O_2$ actuation. $O_2$ concentration set-point is reached exactly, and with the minimum rise-time allowed by the limits on the pressure control, which were correctly handled during the injection.

The regulation of the $CO_2$ concentration is depicted in **Figure 7** which collects the detail of a day-night cycle, specifically for the second day of the experiment. The objective was to show the controlled flow rate of the $CO_2$ injection while the $CO_2$ concentration must be maintained around the $1000 ppm$ set-point. During the night, the $CO_2$ produced by the plants contributes to steer the concentration above the target reference, and the nominal value was then reached again in a few hours after the photosynthesis has begun. When the controller was active, namely when the concentration is close to its reference, the steady state error was limited to $5 ppm$ and the controller correctly compensates the consumption of the plants. We also stress the fact that there was no means to remove $CO_2$ from the

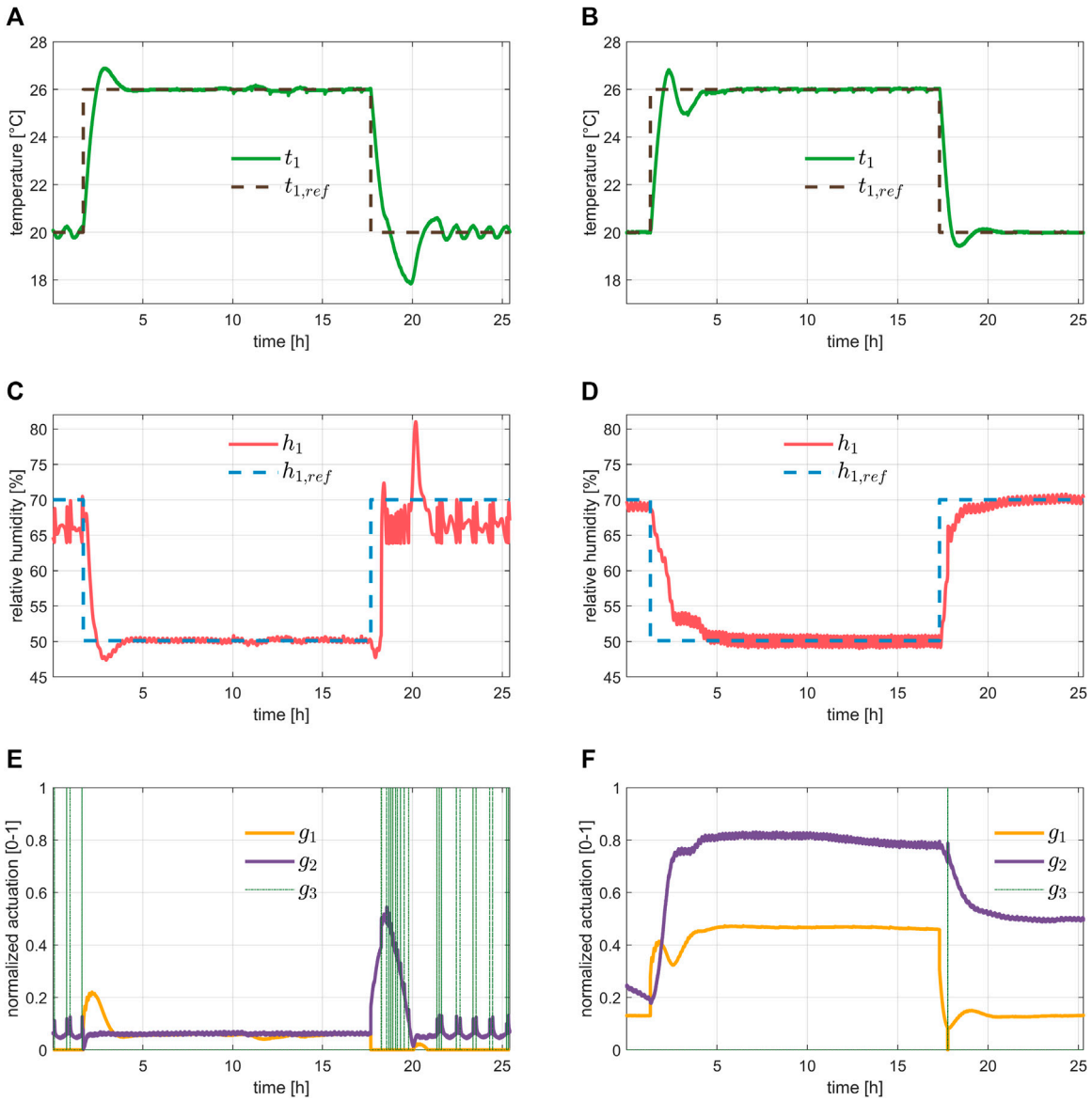



**FIGURE 8 |** Evaluation of temperature and relative humidity control for different set-points, typical of a day/night cycle. The left column shows the results during the *first day* of the experiment described in **Section 3.1**, while the second column shows the results during the *last day*. From top to bottom we have: the tracking performance of the temperature in plots **(A,B)**, the tracking performance of the relative humidity in plots **(C,D)**, and the input commands, namely the heater, dehumidifier, and humidifier in plots **(E,F)**.

atmosphere in this hardware setup, thus the typical saw-tooth profile of the $CO_2$ concentration.

Lastly, **Figure 8** shows the regulation of air T and RH in the growth chamber. The plots in the left column detail the 1st day of the experiment, the ones on the right the 27th day. The aim was to compare the performance at the beginning and at the end of the experiment, thus showing the impact of the plants on the tracking performance. Both air T and RH set-points underwent the typical step changes on the switch between day and night. As expected, the humidifier was mostly used at the beginning of the experiment. It must be pointed out that T and RH dynamics are a MIMO system that was inherently hard to control with coupled PIDs. As it was evident, the performance when plant transpiration was very low was much worse than that obtained at the end of the experiment, suggesting the need to develop more advanced control algorithms. Indeed, the control achievements were found to be sufficient to conduct a first complete life test, but the wide range of operating conditions for air T, RH, and air flow rate, as well as the impact of the lights and crops could be handled optimally only with a model-based controller.

Based on these results, MPC is a potential candidate for developing a robust and accurate predictive controller, and we

**TABLE 4 |** Total mass balance computation in terms of moles and % of volume during the 27 days life test on lettuce in the PCU.

| | Time interval | Hours | P system [*Pa*] | P tank [*Pa*] | T system [*K*] | T tank [*K*] | TOT [*mol*] | Leak [*mol/h*] | Volume gradient [*l/h*] | Absolute [*%V/h*] |
|---|---|---|---|---|---|---|---|---|---|---|
| Days 1–20 | 16/11/2020 22:30 | 480.00 | 99,955 | 341,715 | 298.0 | 297.4 | 222.9 | –0.149 | –3.71 | –0.081% |
| | 06/12/2020 22:30 | | 98,790 | 347,585 | 295.7 | 296.2 | 223.0 | | | |
| Days 22–27 | 07/12/2020 22:30 | 144.00 | 99,371 | 277,145 | 295.9 | 295.9 | 216.2 | –0.139 | –3.44 | –0.075% |
| | 13/12/2020 22:30 | | 99,536 | 292,289 | 295.6 | 295.1 | 218.5 | | | |

**TABLE 5 |** Daily mass balance computation in terms of % of volume per hour in the 27 days life test on lettuce in the PCU.

| | Leak [*mol/h*] | Volume gradient [*l/h*] | Absolute [*%V/h*] |
|---|---|---|---|
| Day 1 | –0.140 | –3.45 | –0.075 |
| Day 2 | –0.143 | –3.52 | –0.076 |
| Day 3 | –0.142 | –3.50 | –0.076 |
| Day 4 | –0.233 | –5.77 | –0.125 |
| Day 5 | –0.127 | –3.15 | –0.068 |
| Day 6 | –0.125 | –3.06 | –0.066 |
| Day 7 | –0.129 | –3.15 | –0.069 |
| Day 8 | –0.135 | –3.31 | –0.072 |
| Day 9 | –0.124 | –3.03 | –0.066 |
| Day 10 | –0.210 | –5.14 | –0.112 |
| Day 11 | –0.131 | –3.21 | –0.070 |
| Day 12 | –0.159 | –3.91 | –0.085 |
| Day 13 | –0.129 | –3.18 | –0.069 |
| Day 14 | –0.137 | –3.38 | –0.074 |
| Day 15 | –0.133 | –3.29 | –0.071 |
| Day 16 | –0.132 | –3.26 | –0.071 |
| Day 17 | –0.138 | –3.44 | –0.075 |
| Day 18 | –0.132 | –3.28 | –0.071 |
| Day 19 | –0.230 | –5.72 | –0.124 |
| Day 20 | –0.162 | –4.03 | –0.088 |
| Day 21 | — | — | — |
| Day 22 | –0.122 | –3.05 | –0.066 |
| Day 23 | –0.142 | –3.53 | –0.077 |
| Day 24 | –0.169 | –4.19 | –0.091 |
| Day 25 | –0.153 | –3.81 | –0.083 |
| Day 26 | –0.133 | –3.31 | –0.072 |
| Day 27 | –0.117 | –2.89 | –0.063 |

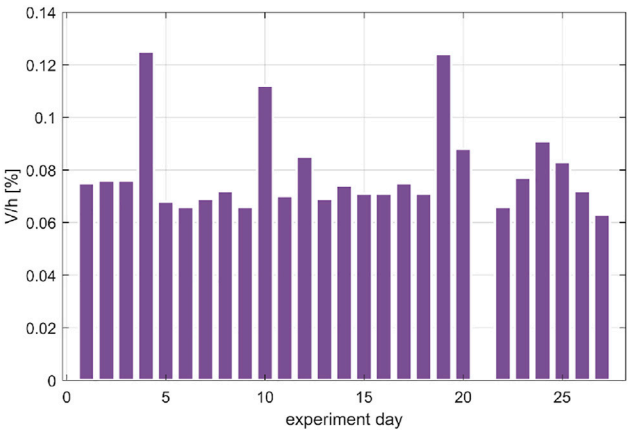



**FIGURE 9 |** Air leakage evaluation in terms of percentage of volume per hour in the 27 days life test on lettuce in the PCU (during day 21 an aeration was performed, thus the leakage is not reported).

foresee a future review of some control strategies to have more consistent dynamics over the entire operating range and possible disturbances. A modelling activity is foreseen to derive control-oriented models of plants' behaviour, necessary for accurate predictive control systems (Hu et al., 2008; Poulet et al., 2020).

## 3.3 Leak Rate Computation

The life test reported in this paper proved the capability of the system to collect the data required for mass balances computation. At the 21st day, the system was aerated due to the high $O_2$ concentration accumulated which could pose a safety risk. The air was renewed and mass balance computation reinitialized. Consequently, the total mass balance was computed for the first 20 days and for the following 6 days (**Table 4**). In addition, mass balance was computed daily in order to estimate the leak rate (**Table 5**). The calculation considers corrective factors due to atmospheric pressure, system pressure and temperature variations. To minimize the temperature corrective factor, the daily mass balances were computed when the temperature was in controlled and in stable conditions. For simplicity, the 22:00 of each day has been selected.

The results show a good system tightness, comparable with the preliminary tests and, more importantly, stable along the life test (**Figure 9**). The average leak rate for the first 20 days is 0.081 and 0.075% for the following 6 days. In addition, mass balance was computed also daily (**Figure 9**).

In conclusion, the research scope of the PCU implies the need to measure variables of interest for the mechanistic modeling of the higher plant compartment. Functional and life tests performed on the atmospheric module demonstrated the

efficiency of both the design and the realization of this subsystem. The controller was proven to finely regulate environmental parameters and to collect precisely data, in a efficiently sealed growth chamber., The performance assessment shows that the PCU is suitable for investigations aiming at modelling the higher plant compartment of BLSSs.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

SP and LB conceived the project. GC, AP, RP and CQ contributed to the realization of the project activities, performed the plant life test and wrote the first draft of the paper. LB, SP and YR revised the final version. All the authors contributed to writing the manuscript in equal part.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fspas.2022.820752/full#supplementary-material

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