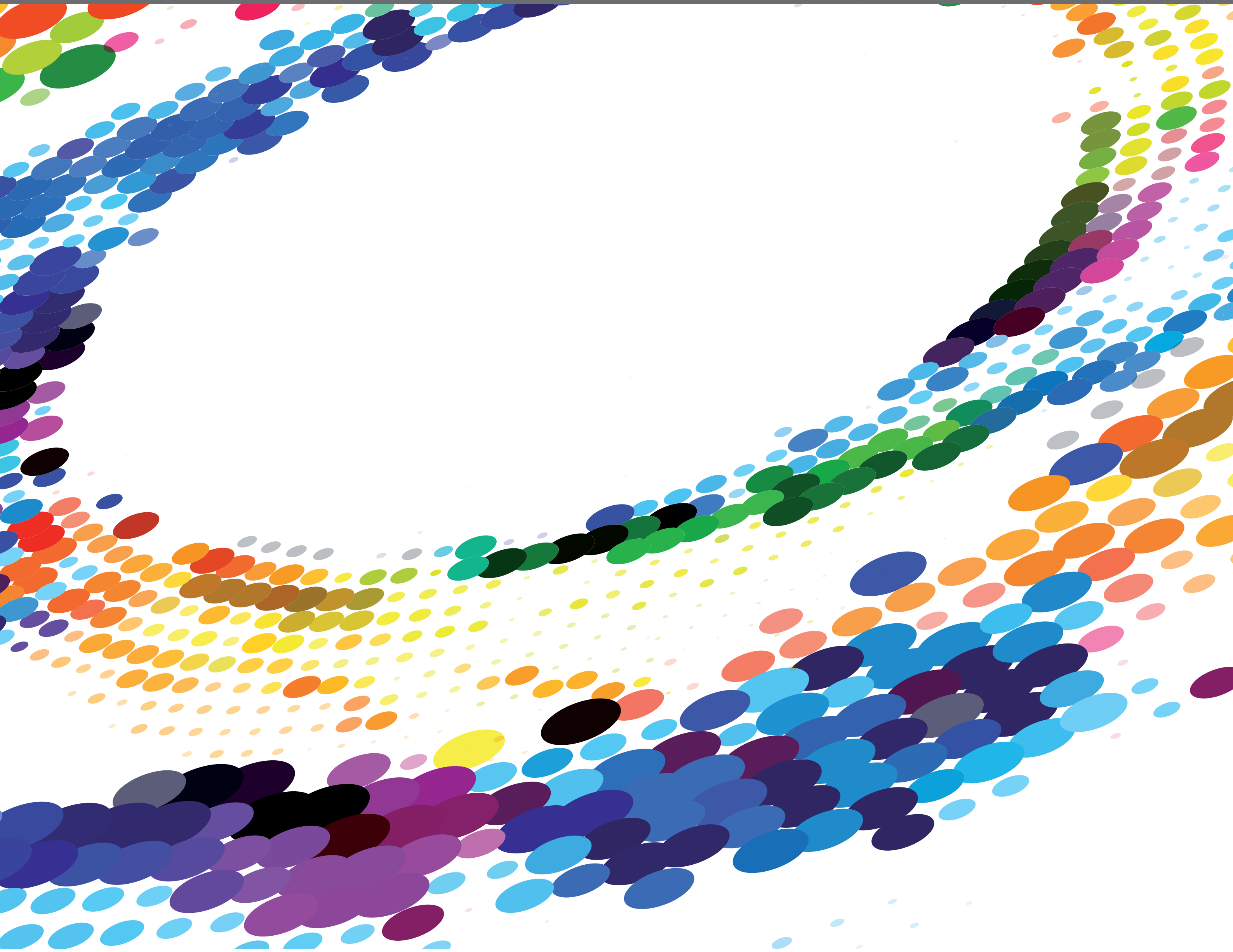
PRESENTATIONS AT THE 4th WORKSHOP OF THE GERMAN ASTROBIOLOGICAL SOCIETY (DABG) ON ASTROBIOLOGY, 26–27 SEPTEMBER 2019, VIENNA, AUSTRIA

EDITED BY: Tetyana Milojevic, Rosa De La Torre Noetzel and Oliver Strbak

PUBLISHED IN: Frontiers in Astronomy and Space Sciences



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88966-678-2
DOI 10.3389/978-2-88966-678-2

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Participants of the 4th Workshop of the German Astrobiological Society (DAbG) on Astrobiology, 26–27 September 2019, Vienna, Austria.
Image. Regina Prossinagg.

Citation: Milojevic, T., De La Torre Noetzel, R., Strbak, O., eds. (2021). Presentations at the 4th Workshop of the German Astrobiological Society (DAbG) on Astrobiology, 26–27 September 2019, Vienna, Austria.
Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-678-2

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frontiers
in Astronomy and Space Sciences

EDITORIAL
published: 18 January 2021
doi: 10.3389/fspas.2020.634989

Editorial: Presentations at the 4th Workshop of the German Astrobiological Society (DAbG) on Astrobiology, 26–27 September 2019, Vienna, Austria

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Keywords: biosignatures, habitability, planets, extremophiles, exobiology, astrobiology, space missions and technologies

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Specialty section:
This article was submitted to Astrobiology,
a section of the journal Frontiers in Astronomy and Space Sciences

Received: *29 November 2020*
Accepted: *08 December 2020*
Published: *18 January 2021*

Citation:
Milojevic T, De La Torre Noetzel R, Strbak O and Schulze-Makuch D (2021) Editorial: Presentations at the 4th Workshop of the German Astrobiological Society (DAbG) on Astrobiology, 26–27 September 2019, Vienna, Austria.
Front. Astron. Space Sci. 7:634989.
doi: 10.3389/fspas.2020.634989

Editorial on the Research Topic

Presentations at the 4th Workshop of the German Astrobiological Society (DAbG) on Astrobiology, 26–27 September 2019, Vienna, Austria

Astrobiology investigates the origin, evolution, occurrence of past, present and future life in the Universe. The questions on how and when life originated on Earth are therefore linked to any astrobiological reasoning (Cottin et al., 2017a; Cottin et al., 2017b). It is a multidisciplinary science that embraces a variety of scientific disciplines including but not limited to physics, chemistry, biology, paleontology, geology, space exploration, planetary science, astronomy, and astrophysics (Martins et al., 2017; Cottin et al., 2017a; Cottin et al., 2017b). There are numerous universities and research institutions in the German-speaking world where both astrobiology research and teaching are being conducted. As a common umbrella organization for the astrobiology researchers working in those institutions, the German Astrobiological Society Deutsche Astrobiologische Gesellschaft, 2020, which started its activities in 2016, seeks to increase the international visibility of astrobiology in Germany and in other German speaking countries by bringing together astrobiology researchers to stimulate their collaboration across the borders. The DAbG considers itself as a part of the European Astrobiology Network Association (EANA) (Rettberg et al., 2015). According to its statutes, the DAbG is committed to serve to both scientific community and to the general public.

The goals and objectives of the DAbG are, in particular (Deutsche Astrobiologische Gesellschaft, 2020):

(1) Promoting research, teaching and education activities in the field of astrobiology.
(2) Promoting and maintaining the scientific exchange of information in the field of astrobiology between people from German-speaking countries and German-speaking persons living abroad.
(3) Facilitating collaboration with international organizations active in the field of astrobiology, in particular with the European Astrobiology Network Association (EANA) (Rettberg et al., 2015).
(4) Advocating scientific cooperation in the field of astrobiology, in particular at universities and other research institutions.

(5) Promoting university teaching in the field of astrobiology.
(6) Promoting early career scientists.
(7) Promoting the astrobiology publications and information systems in the German-speaking areas.
(8) Popularizing astrobiology for the German-speaking public.

The fourth DAbG workshop was organized under the overarching theme "Life in the Universe". The sessions of the workshop were dedicated to space biology, origin of life, prebiotic chemistry, habitability, biosignatures, extremophiles, exoplanets, and space missions and technologies. In this context, this Research Topic aims to bring together contributions from scientists working in various disciplines who have a common interest in astrobiology at diverse scales and in a variety of extreme environments. The scientific disciplines involved include extreme microbiology, geomicrobiology, biogeochemistry, astrochemistry, space engineering, philosophy in science, and these articles demonstrate the diversity and the breadth of astrobiology topics. In this research collection we present two mini reviews, two original research papers, two brief research reports, one Hypothesis and Theory article, and one perspective article.

The topic opens with a hypothesis and theory article by Witzany on a definition of life for astrobiological research. Witzany proposes a novel twenty-first century definition of life based on communicative interactions that are inherent to all processes of life. He formulates life as primarily communicative interaction between cells, viruses, and RNA networks. Witzany's life definition emphasizes biocommunicative structure of life, which was rather ignored by the mechanistic understanding of life that prevailed in the 20th century. Several further papers in this Research Topic are focused on the question of life search and detection of traces of life. The perspective article by Groemer and Ozdemir delivered a robust "Exploration Cascade" of planetary analog field operations which helps to optimize the search for extinct and/or extant traces of life, the scientific output of planetary surface missions and also helps to characterize the geological context of the exploration sites. Albu et al. presented in their research article a novel correlative microscopic method to structurally characterize extraterrestrial minerals. This method is a promising next generation technique which should be included in a toolbox for the astrobiological assessments of various astromaterials, e.g., enabling a detailed investigation of meteorites down to the nano- and atomic range and a characterization of primordial conditions in the Universe. Bredehöft comprehensively reviewed the most recent astrochemical research on abiotic CO_2 formation and radiation induced reactions between carbon monoxide and water on grain surfaces of interstellar dust. This work discusses the role of the HOCO radical in the abiotic oxidation of carbon monoxide. Skladnev et al. proposed a nanobiotechnological approach for life detection in extraterrestrial low-temperature environments.

Microbial life under multiple extremes was a topic of several other papers in this Research Topic. Microbial preservation under low temperature was investigated by Milojevic et al., their work showed that the optimized nutrient fitness favors microbial preservation in extreme stressful conditions. Verseux reviewed extensively bacterial behavior under low pressure conditions and provided insights on physiological adaptation of bacteria to hypobaria. Kölbl et al. investigated the influence of long-term desiccation on the mineral-grown polyextremophilic archaeon *Metallosphaera sedula* and described several specific metal-bearing substrates which were particularly beneficial to preserve a viability and cellular integrity of dehydrated metal-encrusted microbial cells.

In summary, this Research Topic of Frontiers in Astronomy and Space Science shows that astrobiology is an actively developing research field supported by vibrant and dynamic contributions of DAbG scientific community.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

We sincerely thank all authors for submitting articles to the research topic. The authors would like to thank the reviewers for the time spent on the manuscripts and for valuable suggestions.

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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.

frontiers
in Astronomy and Space Sciences

HYPOTHESIS AND THEORY
published: 18 March 2020
doi: 10.3389/fspas.2020.00007

What is Life?

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In searching for life in extraterrestrial space, it is essential to act based on an unequivocal definition of life. In the twentieth century, life was defined as cells that self-replicate, metabolize, and are open for mutations, without which genetic information would remain unchangeable, and evolution would be impossible. Current definitions of life derive from statistical mechanics, physics, and chemistry of the twentieth century in which life is considered to function machine like, ignoring a central role of communication. Recent observations show that context-dependent meaningful communication and network formation (and control) are central to all life forms. Evolutionary relevant new nucleotide sequences now appear to have originated from social agents such as viruses, their parasitic relatives, and related RNA networks, not from errors. By applying the known features of natural languages and communication, a new twenty-first century definition of life can be reached in which communicative interactions are central to all processes of life. A new definition of life must integrate the current empirical knowledge about interactions between cells, viruses, and RNA networks to provide a better explanatory power than the twentieth century narrative.

Keywords: sign mediated interactions, communication, cellular life, viruses, RNAs, evolution, essential agents of life, biocommunication

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Specialty section:
This article was submitted to Astrobiology, a section of the journal Frontiers in Astronomy and Space Sciences

Received: *22 January 2020*
Accepted: *17 February 2020*
Published: *18 March 2020*

Citation:
Witzany G (2020) What is Life? Front. Astron. Space Sci. 7:7. doi: 10.3389/fspas.2020.00007

INTRODUCTION

Scientifically, the first half of the twentieth century was the most successful period for empirically based sciences. Basically, explorations in physics and chemistry paved a path on which science could delimitate validity claims against all other concepts of thoughts such as the broad range of philosophical disciplines, theology, and poetry. Philosophers and physicists such as Wittgenstein, Carnap, Goedel, Russell, and Tarski suggested that exact sciences are strictly based on exact scientific sentences describing empirical facts coherent with observations and measurements in experimental setups (Wittgenstein, 1922; Carnap, 1931, 1939; Gödel, 1931).

The formal language to describe this was mathematical equations that would depict material reality. Information theory and cybernetic systems theory encouraged this progress (Bertalanffy, 1940; Wiener, 1948; Shannon and Weaver, 1949; Turing, 1950; Neumann, 1966). Milestone publication, "Principia Mathematica," outlined by Bertrand Russel and Alfred North Whitehead was further developed by David Hilberts axiomatic system with error-free logical sentences (Whitehead and Russell, 1910/1912/1913; Hilbert and Bernays, 1934/1939). This exact scientific language was applied to nearly all disciplines of scientific investigations in natural sciences as well as social sciences.

Molecular biology, genetics, and biochemistry started their success stories, which have lasted until today. The role of physicalism in biology was so dominant that biology became a subdiscipline of physics and chemistry (Brenner, 2012). Because biological organisms, cells, tissues, and organs consist of molecules, constructed out of atoms, empirical and measurable features may be described by physics and chemistry. The genetic information storing molecules represent "aperiodic crystal structures" as assumed by Erwin Schroedinger.

THE PHYSICALISTIC PARADIGM IN THE BIOLOGY OF THE TWENTIETH CENTURY

"We shall assume the structure of a gene to be that of a huge molecule, capable only of discontinuous change, which consists of a rearrangement of the atoms and leads to an isomeric molecule. The rearrangement may affect only a small region of the gene, and a vast number of different rearrangements may be possible. The energy thresholds, separating the actual configuration from any possible isomeric ones, have to be high enough (compared with the average heat energy of an atom) to make the change-over a rare event. These rare events we shall identify with spontaneous mutations" (Schroedinger, 1944).

These spontaneous mutations occur strictly by chance and are statistically determined, according to natural laws. Evolutionarily relevant changes in molecular structures occur if, in replication processes. The replicated molecular structure is not an identical copy of the former sequence structures but a variation that is not identical with the master blueprint. This variation is a result of a replication error. Erwin Schroedinger introduced the "code-script" to denote that genetic information is a natural code. With the rise of molecular biology, and genetics, he adapted the code-metaphor to describe various features of the genetic code as the result of a molecular ensemble of nucleotides that underlies statistical fluctuations. These are the consequences out of thermodynamics of living systems. Schroedinger's book, "What is Life?" and his suggestion therein, "Life is physics and chemistry," became one of the most influential works of the twentieth century.

A big step forward in this direction occurred, when Manfred Eigen introduced information theory to molecular biology and adapted information as a molecular property of matter, which may reproduce itself (Eigen, 1971). Eigen's quasispecies theory and its core assumption that mutation caused the diversity of RNA populations dominated paradigmatically nearly half a century (Biebricher and Eigen, 2006). It represents the "exploitation of the formal mathematical analogy of quasispecies dynamics and the statistical mechanics" of quantum theory (Domingo and Schuster, 2016). "Biological selection can be viewed as condensation or localization of sequence distribution in a limited area in sequence space" (Biebricher et al., 1985). That finally should lead to a theory of evolution based upon "biochemical kinetics" (Schuster, 2011).

Eigen follows the basics of information theory as a mathematical theory of communication that is quantifiable and underlies natural laws strictly. And he insisted that the genetic code is a natural language and not a metaphor (Eigen and Winkler, 1983).

All these assumptions are driven by the general agreements of natural scientists at that time: how to discuss matter, natural laws, and biological affairs. To summarize, the physicalistic paradigm in biology that dominated the language of observation as well as the language of theory in biology until today are as follows:

- life is physics and chemistry (and information)
- information is a characteristic of matter
- difference between abiotic matter and biology is gradual
- natural languages and codes are determined by their syntax structure
- syntax structure of natural codes represent the logical structure of matter
- mathematics is the only language that can exactly depict the logical structure of matter
- evolution is variation (mutation) and selection
- mutations are error replications that result out of elementary physical processes and indeterminate due to their quantum-mechanical nature.

WHAT WE KNOW TODAY ABOUT BIOLOGICAL PROCESSES

We may analyze the available parts of a machine to get a functional blueprint for its construction. Then, we may reproduce it trying to optimize the construction. This is an important motif in genetic engineering also. However, if we observe living organisms and their interactional patterns, beginning from single cells to tissues, organs, and complex organisms, we find a variety of non-mechanistic circumstances, features, and capabilities that cannot be part of a mechanistic explanation. These features include common coordination to adapt to new and unexpected environmental circumstances. Machines cannot create new programs out of functional blueprints (Witzany, 1995).

The study of living organisms as machines presupposes biological information as a result of coded content according to principles of biochemical kinetics. Biolinguistics, bioinformatics, information theory, systems theory of (context-free) languages, or similar mathematical theories of language and communication cannot explain the essential features of natural languages and the codes used in communication processes. They investigate quantifiable sets of signs by mathematical procedures as statistical mechanics and, therefore, completely forget the essential agents in real life world being necessary to use languages and codes (Witzany, 1995, 2000, 2010). The formalizable sender–receiver model that was used to describe natural communication processes cannot identify the context dependence of meaning and its deep grammar that may represent different, and even contradictory, meanings to the superficial grammar that is present in identical sign sequences (Austin, 1975; Searle, 1976; Habermas, 1987). The social character of real-life organisms is not within their expertise because social-interacting organisms do not behave like formalizable abiotic elements (Witzany and Baluska, 2012a). Social-interacting living organisms may generate new sign sequences, behavior, and interactional motifs, for which no algorithm is available in principle.

In biology, in contrast to physics, entanglement with language and communication is double-sided. Biological disciplines in the twentieth century were convinced that, if they use the language of physics, they would be accepted as part of the exact science community of natural sciences (Chomsky, 1965). On the other side, the explanatory models to coherently describe genetic information used physics and mathematics to explain the features of DNA sequences as physical properties (Stadler and Schuster, 1992).

Let us remember dominant terms in molecular biology and genetics in the second half of the last century; they include "genetic code," "code without commas," "genetic information," "gene expression," "transcription," "translation," "nucleotide sequences," "messenger RNA," "protein-coding sequences," "cell-to-cell communication," "open reading frame," "immune response," "recognition sites," and so on. From a philosophy of science perspective, these terms could not be substantiated by the biological disciplines themselves, because they all were defined by physicochemical features, which clearly failed to coherently justify original linguistic terms (Witzany, 1995, 2005).

The search is, thus, for how to combine linguistic terms used in biology with current knowledge about natural languages/codes and communication without entering the "mouse-trap" of physicalism, which leads back to a view that living organisms are mechanistic cause and reaction machines (Witzany, 2017a). If we want to use linguistic terms without their physicalistic paradigm, we must be aware of what natural languages/codes used in communication processes mean, if we study biological processes.

WHAT DO WE KNOW ABOUT NATURAL LANGUAGES/CODES AND COMMUNICATION?

If we now consider the basic foundations in the language science and communication theory, we may also find the basic functions and processes in which these terms are appropriate descriptions (Morris, 1946; Wittgenstein, 1953; Austin, 1975; Searle, 1976; Habermas, 1994).

Current empirical facts about communication indicate that communication involves interaction processes between living agents mediated by signs in contrast to interactions in abiotic matter where no signs are present (Witzany, 2011a; Baluška and Witzany, 2014). No signs are present if water freezes to ice.

All living agents that communicate share real-life histories and traditions, and environmental conditions. Experiences and the organization and coordination of everyday life practice dominate communication patterns primarily (Witzany, 2014a, 2015). This everyday life practice is the original source of natural languages.

Communicative interactions need some natural language or code that consists of signs. We may differentiate three kinds of signs used in communicative interactions by competent sign users. Signs may be indices, icons, or symbols. These three kinds of signs found in natural languages or codes are used according to three levels of rules (syntax, semantics, pragmatics). Since Charles Morris, we know that if one level is missing, one cannot speak seriously about a real natural language or code (Morris, 1946). Syntactic rules guarantee the correct generation and combination of signs to sign sequences; semantic rules guarantee the correct combination of signs and signified objects, and pragmatic rules are relevant for the correct combination of signs and the concrete context in which signs are used by a real sign-using agent. Rule-following agents may also fail to follow these rules.

The meaning of sign sequences is dependent on shared rules of the population members: how to reach a common agreement upon what the signs designate and, most importantly, what the sign user tries to transport or trigger. Without common agreement, no coordination of common behavior can be reached (McCarthy, 1984). Even here, meaning (semantics) is a social function (Mead, 1934). Slight differences in experiences of real-world environments may lead to slightly different sign interpretation, as documented in the variety of dialects, for example, in bee languages as proved by Karl von Frisch (1971). That is important in optimizing energy costs because language/code-using agents do not need new signs for every circumstance but can use limited characters/signs and limited rules to generate unlimited sign sequences. To summarize that:

- Communication depends on natural languages or codes, i.e., signs that can be combined to sign sequences
- No natural language or code speaks itself or codes itself. There must be living agents that use such natural languages or codes
- Inherently, communication is a social interaction
- Correct use of natural languages or codes underlies syntactic (combinatorial), pragmatic (contextual), and semantic (content-specific) rules
- The meaning of information is a social function.

WHAT REMAINS TODAY FROM THESE TWENTIETH-CENTURY NARRATIVES?

We now examine the start of the third decade in the twenty-first century: which assumptions of the former narratives are still valid, and which ones must be refuted or revised to better integrate empirical data than the previous ones? The debate in the philosophy of sciences on how to generate correct scientific sentences in observation and theory lasted from 1920 to the 1980's (Witzany, 2010). Some results of these debates were:

1. The concept of an axiomatic system with error-free logical sentences is impossible in principle. The project of exact science and exact scientific language that depicts reality in a 1:1 fashion is a pipe dream. Gödel proved that in every complex system, there is at least one formula or utterance, which can neither be proved nor refuted. If someone thinks about such an undecideable formula in a non-formalizable language, he has the opportunity to determine whether this formula is true or false. For a machine, this is impossible.
2. Universal Turing and von Neuman machines that could reproduce itself have been proposed for more than half a century. No single self-reproducing machine has been built or seen until today because the theoretical construction depends on wrong assumptions.
3. No natural language speaks itself, as no natural code codes itself. Natural languages or codes depend on usage by living

agents that generate, arrange, and rearrange sign sequences that are syntactically, pragmatically, and semantically correct. The semiotic features of natural languages or codes exclude randomly derived sequences. No natural language or code emerges as a randomly derived mixture of an alphabet or characters without sign-using agents.

4. In real life, no context-free language exists. The meaning (semantics) of information in natural languages depends on the real-life context (pragmatics) within which linguistic signs are used and not on its syntax.
5. Every natural code or language must embody syntactic (combination), pragmatic (context), and semantic (content) rules, which cannot be reduced to one other. Such semiotic rules are rather conservative but—in contrast to natural laws—under circumstances may change and lead to rearrangements of sequences or their generation *de novo*.
6. The (1) central dogma of molecular biology (DNA–RNA–protein), (2) "one gene–one protein" thesis, and (3) "non-coding DNA is junk" has been falsified. There are several ways that environmental influences on proteins affect RNAs to react accordingly, which, itself, may be coded into DNA. RNA editing, splicing, and epigenetic imprintings demonstrate that one gene can be transcribed and translated into different proteins with different functions. Non-coding DNA, which is transcribed into non-coding RNAs, leads to an abundance of regulatory agents and networks essential for gene expression.

If we want to use the usual linguistic terms in biology also in the future and cannot use it according to the physicalism of the twentieth century, we should apply current knowledge about natural languages/codes and communication to biological processes (Mattick, 2009; Shapiro, 2009; Witzany, 2014a, 2019).

LIFE: THE COMMUNICATIVE STRUCTURE

To investigate communication processes in the living world, we have to identify the various levels of communicative (sign-mediated) interactions. The different levels are intertwined but share level-specific interaction patterns (Witzany, 1993, 2000, 2015).

- Every cell or organism is constantly confronted with abiotic influences such as light, gravity, wind, water, dryness, heat, cold, etc., and has to interpret the relevance for itself and react accordingly to survive. In most cases, these abiotic influences result in experiences that have to be memorized. That facilitates a faster and more appropriate reaction if the same circumstances return.
- Every organism is confronted with organisms that do not relate to the same or similar organisms of its population. These may be hunters and predators and may also be symbiotic partners essential for survival. Sign-mediated interactions between non-related organisms are of a certain quality and help us to understand the rich symbiotic life on this planet.
- The most popular communication processes we can observe and investigate are interorganismic interactions, which mean coordination and organization of common behavior in populations of the same or related organisms.
- Last but not the least, we may observe and investigate communication processes within an organism, i.e., between intercellular and intracellular parts.

All these levels of sign-mediated interactions, i.e., communication processes can be investigated throughout all domains of life as demonstrated within the last decade (Witzany, 2010, 2011b, 2012a, 2014b, 2017b; Witzany and Baluska, 2012b; Witzany and Nowacki, 2016).

I developed biocommunication theory to investigate communication processes within and among cells, tissues, organs, and organisms as sign-mediated interactions. Additionally, biocommunication theory investigates nucleotide sequences as a natural code, that is, language-like text, which follows in parallel three kinds of rules: combinatorial (syntactic), context sensitive (pragmatic), and content specific (semantic) (Witzany, 2015). Natural genome editing from a biocommunicative perspective means competent agent-driven generation and integration of meaningful nucleotide sequences into pre-existing genomic content arrangements and the ability to (re-) combine and re(regulate) them according to context-dependent (i.e., adaptational) purposes of the host organism (Witzany, 2000, 2016a).

Cellular Organisms

Cellular organisms of all domains of life actively compete for environmental resources. They assess their surroundings, estimate how much energy they need for particular goals, and then realize the optimum variant. They take measures to control certain environmental resources. They perceive themselves and can distinguish between self and non-self. These organisms process and evaluate information and then modify their behavior accordingly. All these coordinated activities are the result of communication processes in the four levels mentioned above in **Figure 1** (Witzany, 2016a).

Akaryotes

Akaryotes, such as bacteria and archaea, have been considered the most primitive organisms. They are also the oldest ones, with which cellular life started approximately 4 billion years ago. For a long time, they have been viewed as determined by a strict input–output reaction pattern. In the last three decades, this picture has changed dramatically. Their capability to organize, e.g., biofilm communities, using several messenger molecules, has been investigated in numerous studies (Witzany, 2011b, 2017b). They can coordinate common behavior like a multicellular organism (interorganismic) (Shapiro, 1998). Quorum sensing is a well-investigated process in akaryotic populations (Bassler, 1999). It is the start of common decision processes important for biofilm formation, bioluminescence, sporulation, or virulence (Kaiser and Losick, 1993; Ben Jacob et al., 2004). Their coordination and organization of common behavior by sign-mediated interactions made them the oldest biological agents that can colonize each ecological niche. Although they may colonize all kingdoms of life

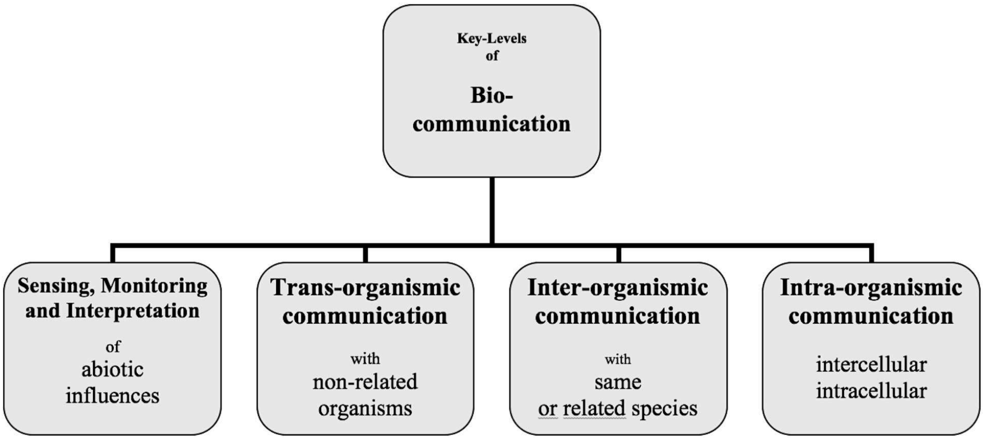

FIGURE 1 | The biocommunication approach identified four levels into which cellular organisms are involved since the start of their life until death.

(protozoa, animals, fungi, plants) in a parasitic or even neutral way, additionally, they serve as essential symbionts to all higher eukaryotes, without which they could not survive (transorganismic) (Schauder and Bassler, 2001).

They are in a constant struggle with their predators and co-evolutionary partners, that is, bacteriophages, which determine their gene word order (intraorganismic) and provide them with toxin/antitoxin or restriction/modification modules (Villarreal, 2005; Harms et al., 2018). Half of all bacteria in the oceans are killed every day by phages, but they survive because the other half reproduces so fast (Rohwer et al., 2014).

The signaling molecules used in akaryotic communication processes are, for example, acyl-homoserine lactones (AHLs), linear oligopeptides, cyclized oligopeptides, g-butyrolactone, furanosyl diester, cis-11-Methyl-2-dodecanoic acid, 4-hydroxy-2-alkyl quinelines, bacteriocins, cyanobactin, terpene, and palmitic acid methyl ester. Each of these signaling molecules is used for different interactions for coordination purposes (Visick and Fuqua, 2005; Witzany, 2011c; Caetano-Anolles et al., 2017; Wang and Lu, 2017).

Eukaryotes

The emergence of single-celled eukaryotes was surely a key step in biological evolution. What was formerly assumed to be a result of small steps of mutations and their selection was later explained by Lynn Margulis and her serial endosymbiotic theory in a completely contrary way (Bermudes and Margulis, 1989; Margulis, 2004). She proved that the membrane-bound fixation, rather than mutation, of a community of former free-living akaryotes was responsible. The coordination of such a community foundation depends on complex communication processes within the cellular membrane, that are fixed genetically. The crucial difference to akaryotes is the nucleus that assembles chromosomes. There are several indicators that the eukaryotic nucleus stems from a large double-stranded DNA virus that became an essential part of the group identity of the eukaryotic cell (Bell, 2006).

The natural signs in the communication processes of protozoa are hormones and secondary metabolites with which these organisms coordinate their behavioral motifs.

If we focus on protozoa, single-celled eukaryotes like ciliates, we can find various signaling molecules used to coordinate interactions, such as reproduction, mating, feeding, attack, and defense (Luporini et al., 1995, 2006; Jacob et al., 2015). Several classes of hormones and other secretions, such as secondary metabolites, have been identified (Plattner, 2016). Ciliates synthesize and secrete cell-type-specific proteins into their extracellular medium. The proteins are then taken up by species-specific receptors and interpreted by population members to generate appropriate reactions (Witzany, 2016b).

Most probably, the kingdom of fungi emerged from single-celled eukaryotes, as we can find single-celled as well as multicellular fungi, which are the evolutionary forerunners of animals and plants (Villarreal, 2005; Bonneville et al., 2020). The communication of fungi is rather complex and diverse, with a variety of substances that serve as semiochemicals (Regnier, 1971). Fungi feed on biotic surfaces, degrading them to soluble nutrients especially for plants. Fungi produce, release, and uptake a rich variety of semiochemicals for reproduction purposes, attack and defense activities, as well as developmental processes coordination and virulence (Witzany, 2012a). In single-celled fungi, we find quorum sensing, a similar communication pattern as in akaryotes. Fungi are communicating by semiochemicals such as mitogen-activated protein kinase, cAMPs, RAS, rapamycin, or calcium–calmodulin–calcineurin, to list the most prominent ones, each one used in different contextual needs (Hogan, 2006; Leeder et al., 2011; Potapova, 2012; Soll, 2012).

The evolution of animals started with the emergence of early metazoans and neuronal tissues in jelly fish (Yin et al., 2019). That was the start of electrical signaling in cell-to-cell communication and was unarguably an evolutionary key innovation. The uptake and release of chemical signaling molecules within the whole body such as hormones and secondary metabolites were enriched by the much faster electrical signaling of neuronal networks. The central nervous system enabled electrical communication over far distances within the body (Witzany, 2014b).

Research on neurobiology demonstrated that neuronal communication in animals is the most complex and specialized form of intraorganismic biocommunication on earth (Kandel, 1976). In addition to hormonal and neuronal communication processes, animals communicate interorganismically and transorganismically via auditory, visual, and tactile signs, which lead to an abundance of expression patterns of animal communication. Animal communication is very diverse through genera, families, and species and reaches a complexity peak in human communication that enables human species to act with collective intentionality as a driving force of coordinated and organized division of labor (Tomasello, 2008). The variety of animal communication has been investigated extensively more recently in genera such as chimpanzees, elephants, wolfs, dogs, rodents, mice, rats, spiders, ants, termites, crows, parrots, birds, salamanders, chelonians, cetacean, fish, cephalopods, corals, and nematodes (Witzany, 2014b). Karl von Frisch received a Nobel Prize for deciphering bee languages and their dialects by investigating how moving patterns serve as signals to communicate nutrition sites.

Plants represent the evolutionary youngest organismic kingdom. Up until now, they are regarded as mechanistic growth automatons, maybe because of their sessile lifestyle and rather slow moving patterns in growth and developmental processes in comparism to animals. However, research within the last two decades has changed this perspective dramatically (Baluska et al., 2006; Baluska and Ninkovic, 2010; Perotto and Baluska, 2012; Blande and Glinwood, 2016). The complex communication profiles of plants with non-plants, other plant species, and within plant bodies on the intracellular and intercellular level indicate that plants can communicate at all levels in parallel, in contrast to animals with their centralized nervous system. The interwoven transorganismic communication with bacteria and fungi in the plant root zone and their intraorganismic communication within the plant body has been highly investigated (Bais et al., 2003). Intraorganismic communication in plants coordinates cellular growth, development, shape, and dynamics on the local level and in rather separated parts. Semiochemical communication happens by vesicular trafficking or via plasmodesmata.

Additionally, we can find physical communication by airborne, electric-like, hydraulic, and mechanical signs (Callaway, 2002; Braam, 2005). We may find nucleic acids, oligonucleotides, proteins and peptides, minerals, oxidative signals, gases, mechanical signals, electrical signals, fatty acids, oligosaccharides, growth factors, several amino acids, various secondary metabolite products, and simple sugars as signaling molecules. Today, we know 100,000 different metabolites used in plants (Dunn and Handelsman, 2002; Fleming, 2005).

Virus Communication

Traditionally, viruses have been seen as infective, disease-causing entities with often epidemic consequences that infect all kinds of organisms. Evolutionary, they have been viewed as parasites that escaped out of cells because they cannot reproduce by themselves but need cellular hosts (Villarreal and Witzany, 2010). More recently, this perspective has been corrected (Villarreal, 2005, 2009a; Forterre and Prangishvili, 2013; Moelling and Broecker, 2019). The disease-causing agents are the minority. Most viruses are integrated into the cytoplasm or nucleoplasm of host cells, without harming the host.

The persistent lifestyle is the predominant one, and the symbiotic and coevolutionary lifestyle is omnipresent because, since the start of life, every cell, tissue, organ, and organism on this planet has been persistently infected by multiple viruses (Ryan, 2009; Broecker and Moelling, 2019a; Koonin et al., 2019). Their persistence, in most cases, does not occur as fully functional but as parts of infectious agents that remain as useful tools for cellular needs because such viral parts can be exapted and co-opted by cellular organisms and which we can identify as non-coding RNAs with repetitive sequence syntax (Jurka et al., 2007; Witzany, 2009, 2017c). Defective parts of infectious agents may serve as signals for immune functions against related genetic parasites (Ariza-Mateos and Gómez, 2017).

Viruses and their defective parts play essential roles in genetic content composition and arrangements that help organisms rearrange genetic content for adaptational purposes, such as in immunity systems or in the evolution of new organs, e.g., placenta and the role of syncytin genes (Villarreal, 2009a, 2016a; Perot et al., 2012; Koonin and Krupovic, 2017; Broecker and Moelling, 2019b). We know them as endogenous viruses and defectives, transposons, retrotransposons, long terminal repeats, non-long terminal repeats, long interspersed nuclear elements, short interspersed nuclear elements, alu's, group I introns, group II introns, phages, and plasmids. Because nearly all the remnants of former infectious genetic parasites share a repeat nucleotide syntax, in contrast to the protein-coding non-repeated nucleotide syntax, we now know that, in most cases, they remain as non-coding RNAs (see below) (Witzany, 2011d). In this respect, we may study cellular DNA as the preferred living habitat of an abundance of RNA inhabitants (Brookfield, 2005; Le Rouzic et al., 2007; Vennera et al., 2009; Villarreal and Witzany, 2013a).

We should be reminded that the human genetic content that codes for proteins is 1.5% only, whereas the non-coding but regulation-relevant genetic content is about 98.5% (Boland, 2017). Additionally, we must not forget that viruses represent the most abundant genetic elements on this planet, outnumbering cellular genetic content by 10 times. In this respect, cellular genomes seem like rare islands in an ocean of the global virosphere (Forterre and Prangishvili, 2009; Koonin, 2009; Rohwer et al., 2014).

Many infectious agents have been identified during the last decades as inhabitants of all prokaryotic as well as eukaryotic genomes (Koonin and Dolja, 2014; Koonin et al., 2015). They infect, insert and delete, cut and paste, or copy and paste. Many of them spread within the genome. They can change host genetic identity by insertion, recombination, or epigenetic regulation or re-regulation of genetic content, and co-evolve with the host (Witzany, 2006, 2014c; Catania et al., 2020).

Most interestingly and unknown, the formerly termed RNA virus populations, quasispecies, are now recognized as highly interactive and cooperative agents (Villarreal and Witzany, 2013b). Virus communication demonstrates that quasispecies populations and subpopulations may cooperate and compete in parallel, dependent on the circumstantial context of host life (Villarreal and Witzany, 2019). Social interacting persistent viruses play important roles as host gene regulatory elements—in most cases represented by repetitive sequences—that may react to nearly every unexpected circumstance (Díaz-Muñoz et al., 2017; Sanjuán, 2018).

Viruses are the only biotic agents that can generate code sequences *de novo*, identify sequence-specific target sites, integrate into pre-existing genetic content, integrate without damage of previous coding regions, recombine according to adaptational purposes, and mark sequence sites to epigenetically fix identity content (Villarreal, 2005, 2009b). The whole range of epigenetic marking, which is so essential for cellular-based organisms to coordinate the variety of developmental stages stems from these infectious agents and has been adapted to cellular needs (Witzany, 2009). Viruses may divide into multipartite genome segments, spread their parts non-randomly throughout host genomes, and reassemble into full functional viral genomes again (Sicard et al., 2016, 2019; Lucía-Sanz and Manrubia, 2017).

Current research demonstrated that viruses communicate to coordinate their behavior: whether it should be lytic or remain in a lysogenic style. The semiochemicals used to communicate at interorganismic levels are peptides (AimP), which reduce the expression of the negative regulator of lysogeny (AimX) by binding to the transcription factor (AimR) promoting lysogeny (Erez et al., 2017; Stokar-Avihail et al., 2019). Interactional motifs in virus communication range from conflict to cooperation in various forms and mimicry, dependent on situational context (Mei and Zhang, 2019; Seligmann, 2019).

Viruses are the only living entities that may exchange genetic sequences as module-like tools between double-stranded DNA, single-stranded DNA, single-stranded RNA, double-stranded RNA, and retroviruses. Most interestingly, they may cooperate and compete as viral clouds in parallel (Koonin et al., 2015; Stedman, 2015, 2018; Berliner et al., 2018).

The most important behavioral motif from both an evolutionary and functional perspective is that viruses can integrate a persistent lifestyle into cellular host organisms by "addiction" modules (Villarreal, 2012, 2016b). This means that former competing viral groups counterbalance each other, together with the host immune system (Koonin et al., 2019). One can find such counter-regulating paired genes of the addiction modules in the restriction/modulation (RM) systems, as well as the toxin/antitoxin (TA) systems (Mruk and Kobayashi, 2014). Insertion/deletion functions represent similar modules as do the RM systems. This infectious technique to colonize host genomes is the key process in generating a new sequence space without error replication (Villarreal, 2009b; Villarreal and Witzany, 2015).

To consider the genetic information in cellular genomes of all domains of life without the remnants of persistent viral infections would be as curious as to consider a language text consisting of various characters as molecular bricks without agents that can write coherent linguistic texts and are competent in generating it according to syntactic, pragmatic, and semantic rules (Witzany, 2012b, 2017c).

RNA Communication

The steps from viruses to pure RNA stem-loops can be much easily understood if we consider viroids, that is, short strands of circular, single-stranded RNA virus without a protein coat (Catalán et al., 2019). However, in evolutionary periods, interacting RNA networks most propably predated viruses and cellular life (Root-Bernstein and Dillon, 1997; Witzany, 2011b; Root-Bernstein and Root-Bernstein, 2015; Demongeot and Seligmann, 2019) Here, we focus on the RNA strand level, which clearly shows infective and host-manipulating properties (Diener, 1989; Flores et al., 2012, 2014). This is coherent with the RNA world of RNA stem-loop groups and RNA group identities as result of biotic behavior represented by biological selection processes (Petrov et al., 2014; Ariza-Mateos et al., 2019; Villarreal and Witzany, 2019; Demongeot and Seligmann, 2020).

To understand the social interactions between the proponents of the RNA world means to understand a fascinating sphere of what was formerly assumed to be impossible: that mere sequences of molecules do not behave like physical/chemical entities in an abiotic world but as competent agents on genetic code syntax that cooperate and organize, constitute, and generate sequence structures and groups that depend on group selection (Higgs and Lehman, 2015). As was shown, a single RNA stem-loop behaves like a random assembly of nucleotides without selective forces governed strictly by physical laws (Smit et al., 2006; Vaidya et al., 2012, 2013). Biological selection starts only if they are assembled to groups, consortia (Hayden and Lehman, 2006).

RNA world agents can serve as a template or be active as a catalyst (Gwiazda et al., 2012). This double function of genotype and phenotype is unique in biology, where we can find most organisms divided into genetic coding and protein-based bodies within their real-life interactions. In former times, it was thought that information transfer is a one-way road from genotype to phenotype, but with epigenetics and RNA biology, it became obvious that the reverse direction of information flow—indicating complex sign-mediated interactions (i.e., communication)—plays an important role in evolution, development, and adaptational flexibility (Shapiro, 2009, 2014, 2016; Spadafora, 2016).

RNA stem-loop groups interact with other DNA, RNA, or proteins forming the most important ribo–nucleo–protein complexes (RNP), such as the subgroups of ribosome, spliceosome, and editosome (Mercer and Mattick, 2013). Their active site that leads to group behavior is the single-stranded loops or bulges being essential for self/non-self recognition and group identity.

Additionally, these single-stranded loops are actively prone to integration or rejection of foreign RNA stem-loops. Their highly interaction-prone nucleotide "surface" serves as signs (indices) for competing or cooperating RNA stem-loops, based on complementary base-pairing rules (Schudoma, 2011). This is relevant also in RNA mimicry as demonstrated recently (Ariza-Mateos and Gómez, 2017; Grüll and Massé, 2019).

We should consider such ensembles of RNA stem-loops as RNA populations, which are investigated as varieties of small non-coding RNAs such as snRNA, snoRNAs, piwi RNAs, tRNAs, rRNAs, mRNA, siRNAs, and microRNAs (Bartel, 2004; Carthew and Sontheimer, 2009; Malone and Hannon, 2009; Gebetsberger et al., 2017).

Ensembles of RNA fragments that self-ligate into self-replicating ribozymes may form cooperative networks unexpectedly (Briones et al., 2009; Cheng and Unrau, 2010). It has been shown that three-member networks represent cooperative growth dynamics. When such cooperative networks compete against selfish RNA stem-loop groups, they grow faster. This indicates that RNA populations can evolve higher complexity by cooperative interactions. This also demonstrated that cooperation outcompetes selfishness (Hayden and Lehman, 2006; Vaidya et al., 2012). The primacy of the selfish gene hypothesis, therefore, is outdated on the RNA level.

Non-coding RNAs interact with all relevant key players in the biological world: DNA, RNA, and proteins. They play important roles in nuclear organization, transcription, post-transcriptional, and epigenetic processes and are transcribed in the sense and/or antisense directions (Cech and Steitz, 2014; Long et al., 2017). They may be expressed in different cell types, subcellular compartments, and developmental stages, in every case in a context-dependent manner (Mattick and Gagen, 2001; Mattick, 2003; Clark et al., 2013). Non-coding RNAs can interact cooperatively in a module-like manner (Manrubia and Briones, 2007; Higgs and Lehman, 2015).

Non-coding RNAs may regulate coordinated or independently, autonomously, or functionally interrelated. Such RNAs regulate single genes or bigger genetic networks. They also may control the spatiotemporal coordination of gene expression rather precisely (Witzany, 2009). Additionally, non-coding RNAs can target each other for post-transcriptional regulation, alternative splicing, polyadenylation, and non-templated modifications (Doudna et al., 1989). Another ribonucleoprotein complex, the editosome, plays important roles in transmitting environmental (contextual) information to the epigenome (Cech, 2012). In addition, other ribonucleoprotein complexes may undergo nuclear–cytoplasmic, nuclear–mitochondrial, and axodendritic trafficking. They serve as appropriate RNA–protein consortia that spatiotemporally distribute combinations of ncRNAs, messenger RNAs, and RNA-binding proteins (Atkins et al., 2010; Noller, 2012; Petrov et al., 2015; Villarreal, 2015; Tartaglia, 2016).

Basic tools of such RNA consortia are their complementary composition of base pairing stems and non-base-pairing (single-stranded) loops, which are results of an inherent property of RNA to fold back building stem-loop structures (Villarreal and Witzany, 2013b). The variety of regulations on protein-coding genes, as well as the processing of these regulatory RNAs by a number of defined steps in RNA splicing and RNA editing of RNA transcripts, makes an understanding nearly impossible because of the coordinated complexity (Witzany, 2016c). It is now very clear that non-coding RNAs build those agents that determine the regulation of all steps and substeps of gene regulation in cellular organisms (Mattick, 2009). The interactive connection between the RNA-world agents and cellular organisms is based on the abundance of infectious genetic parasites, viruses, and related agents that transfer and insert all relevant RNA features to the cellular world or, as Frantisek Baluska noted, "without infection, no evolution" (Baluska, 2009; Baluška and Witzany, 2014).

In contrast to DNA viruses, RNA viruses have much smaller genomes on RNA bases without proofreading and repair. "Error-prone" RNA viruses are a key narrative in physicalistic biology. However, it has been formerly assumed as deficit because the "error rate" is very high. The contrary perspective is more effective: to regard it as "innovation rate" because it assembles the property of invention of new sequence contents, *de novo*, that is, that have not been existent before. This is important for variation as well as infection, immunity, and identity, for both diversified viral and cellular populations and continued interaction between cellular immune systems and infectious genetic parasites throughout the whole history of life (Villarreal, 2015). "Error-threshold" was used to designate the critical state of too many error replications to stabilize newly derived variations (Shah et al., 2019). Replication must be faster than the breakdown rate of the newly derived strands. Because "errors" are not appropriate to describe RNA strand innovation we now may consider these events as "innovation overload."

SUMMARIZING CURRENT EMPIRICAL DATA

We have seen that the use of linguistic terms, such as genetic code, code without commas, transcription, translation, reading frame, immune response, etc., in biology, is still possible. However, the physicalistic paradigm that introduced these terms to biology is no longer valid. Physicalism, as well as other mathematical theories of language, cannot substantiate these terms because this is outside their expertise: Natural languages and codes are the result of social interacting agents, according to competent and commonly shared rules of sign use and allow living agents not only to coordinate and organize their behavioral features and capabilities but also to generate completely new sign sequences and behavioral adaptations that cannot be predicted or computed by algorithm-based procedures. Nucleotide sequences of the genetic code are not the result of the self-organization of matter. The genetic code is not the result of statistical mechanics (**Table 1**).

Today, we know that life is constituted of three levels of interactions: cells that are genetically regulated by RNA networks, which are remnants of former genome-invading agents such as viruses and their relatives. Quantitatively, cellular genes represent rare islands in an ocean of viruses and virus-related infectious genetic parasites (virosphere). The invasion strategy of genomic parasites resulted in persistence within host genomes that now represent evolutionary novel genetic identities than before the invasion. Immune systems in cellular organisms represent communicating networks of persistent genetic parasites that serve as immune function against related parasites. Former competing genetic parasites, together with host immune function, generate a variety of regulatory tools that are counter regulated. The three interaction levels (a) RNA-groups, (b) viruses, and (c) cell-based organisms not only constitute and regulate but also inherently are basically open for generating new,

TABLE 1 | Different paradigms investigating living agents and defining life: the molecular biological paradigm explains all life processes primarily by the physical–chemical properties and statistical mechanics.

Different Paradigms: Explaining and Understanding "Life"		
Concept of	**Molecular biology**	**Biocommunication**
"Dead"	Pre-biotic chemical reactions	No sign-mediated interactions
"Living"	Replication/biological selection (molecular reactions)	Sign-mediated interactions (social events)
Determinants	Natural laws (thermodynamics)	Semiotic rules
RNA-ensembles	Molecular assembly	Agent-groups integrate or preclude non-self agents
Viruses	Escaped selfish parasites	Essential agents of life
Genetic variation	Error replication	RNA interaction based innovation generation
Genetic novelty	Random mutations	Viruses and subviral RNA- networks edit code
Biological selection	Fittest type	Fittest consortium
Genetic code	Genetic material	Semiotic text (according syntax, pragmatics, semantics)
Biological information	Shanon entropy; content depending on molecular syntax	Content depending on context of use by competent agent-groups
Communication	Information transfer via coding/decoding mechanisms	Agent-based social interactions mediated by signs according semiotic rules
Defintion of "Life"	Machine-like statistical mechanics	Social event realized by communicative interactions

The biocommunication approach explains life as a social event realized by communicative interactions of cells, viruses, and RNA consortia.

unexpectable, and non-computable interaction profiles. This means genetic *de novo* sequences, new cooperation pathways, exaptation and new traits of former generated module like parts that evolved for different purposes, re-use of former degraded modules (viral defective minorities), and new behavioral motifs of cellular life forms.

CONCLUSION

Primarily, life is a process. The main characteristic of this process is the coordinated organization of complex interactions that we see as protein-based organisms of three domains of life, their reproduction, and metabolism all mediated by complex interwoven gene regulation as a result of communication. Living nature is structured and organized by language and communication within and among organisms, viruses, and RNA networks. If communication is damaged or disturbed, coordination and organization may be incomplete, and normal function becomes disregulated, leading to the broad variety of diseases. Without RNA world agents, no cellular gene regulation could take place. Without viruses and related infectious agents, these capabilities of RNA stem-loop group behavior as gene inventors and regulators would not have been integrated into cellular host genomes.

Therefore, we must ask whether mutation ("error replication") is the correct term to desígnate genetic variation in the future. Error replications, which in most cases means DNA damage without successful repair, is an empirical fact but does not play important roles in genetic innovation. Evolutionary relevant genetic variations are the result of natural genome editing by competent agents such as viruses and RNA networks with their inherent competence to generate and modify nucleotide sequences. This is competent nucleotide sequence editing. In contrast to former convictions, this is an agent-based interaction process, which is far from statistical mechanics and biochemical kinetics. Instead of error replication, we should use now "genetic innovation," which much better fits to the empirically documented events. Darwinian evolution then could be revised to "innovation and selection."

If we want a new definition of life for astrobiological research, we must integrate the complementarity of cells, viruses, and RNA networks into a communicative life world. Life as a process depends on these interactional agents. Yes, all living agents are constituted by elements that underlie physics and chemistry. However, in contrast to abiotic planets, life on this planet depends on communication processes involved in all sign-mediated interactions of cells, viruses, and subviral RNA networks. Therefore, we can formulate a new definition of life: life is communicative interaction, which means life is primarily a social event.

Life is a social event. Social events are realized by communicative interactions on three complementary levels in parallel: cell communication, RNA communication, and virus communication.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

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

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer LV declared a past co-authorship with the author to the handling editor.

frontiers
in Astronomy and Space Sciences

MINI REVIEW
published: 23 June 2020
doi: 10.3389/fspas.2020.00030

Bacterial Growth at Low Pressure: A Short Review

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Biological life-support systems could greatly increase the sustainability of crewed missions to the Moon or Mars. Understanding how bacteria react to hypobaria is critical to their optimization: if enclosed within crewed compartments, microbial modules may be exposed to the lower-than-Earth atmospheric pressure considered for future space vehicles and habitats and, if deployed outside, they would best rely on a low pressure to minimize both engineering constraints and risks of leakage. Bacterial behavior at low pressures is of relevance to other fields as well, both within astrobiology (e.g., habitability and planetary protection) and outside of it (e.g., aerobiology and food preservation). Unfortunately, while microbial survival under vacuum has been largely investigated, little work has focused on metabolism at low but growth-permissive pressures. Nonetheless, recent studies brought some insights. Limits were outlined: a few bacterial species can grow just above water's triple point, more can multiply down to around 25 mbar, and shifting pressure within 100 mbar to 1 bar seems not to largely affect growth of most species when the partial pressures of metabolizable gases are not limiting. Some mediating mechanisms have been proposed: hypobaria can affect bacteria by desiccation, via a reduced availability of specific gases, and through various other physico-chemical effects, interdependent and dependent on other environmental factors. A limited number of studies also gave insights into how bacteria cope with low pressure, and how much they can adapt to it. But, overall, much remains to be discovered on bacterial growth under hypobaric conditions.

Keywords: low pressure, hypobaria, life-support systems, hypobarophiles, hypopiezotolerance, space exploration

Edited by:
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Specialty section:
This article was submitted to Astrobiology, a section of the journal Frontiers in Astronomy and Space Sciences

Received: *23 February 2020*
Accepted: *11 May 2020*
Published: *23 June 2020*

Citation:
Verseux C (2020) Bacterial Growth at Low Pressure: A Short Review. Front. Astron. Space Sci. 7:30. doi: 10.3389/fspas.2020.00030

INTRODUCTION

Data is scarce on how microorganisms cope with low pressure. As space microbiology progresses and practical applications are looming, this area should no longer be neglected.

Among those applications is the development of biological life-support systems (BLSS), of which microorganisms may be critical components (Godia et al., 2002; Hendrickx and Mergeay, 2007; Verseux et al., 2016a). First, if enclosed within crewed compartments, for instance to contribute to air revitalization, they may be exposed to atmospheric conditions differing from Earth's: future space vehicles, and Moon and Mars habitats, may rely on a lower total pressure and increased O_2 concentration (e.g., 0.55 bar, 32% O_2; NASA, 2006) for engineering considerations (notably, to reduce the mass of structural components), for reducing the amounts of necessary gas consumables, and to facilitate extravehicular activities while maintaining low risks of decompression sickness (e.g., NASA, 2006; Norcross et al., 2013). Second, if BLSS modules are deployed outside to spread over larger surfaces, to rely on local resources, or both, they would best rely on low pressures to increase cost-efficiency, to reduce engineering constraints, and to minimize risks of outward contamination (e.g., Boston, 1981; Richards et al., 2006). On Mars, relying on a gas composition close to ambient could allow for more efficient use of atmospheric carbon and nitrogen (Verseux et al., 2016b).

Knowledge on bacterial behavior under hypobaric conditions could also help assess Mars's habitability, both to identify where indigenous life may exist and to develop appropriate planetary protection strategies. Measured values of Mars's surface pressure have varied between ∼6 and 11 hPa (sol average), with large seasonal and diurnal variations (see Harri et al., 2014; Martínez et al., 2017). Aside from their limiting the stability of liquid water at Mars's temperatures (see **Figure 1**; whether liquid water could persist at the surface or in the subsurface is largely discussed elsewhere, for instance in Haberle et al., 2001; Orosei et al., 2018; Hecht, 2002; Sori and Bramson, 2019), low pressures may limit microbial metabolism in potential Martian habitats.

The rationale for studying bacteria's lower pressure limits extends outside of astrobiology. To aerobiology, for instance: viable microbes (especially bacteria) abound in the troposphere (DeLeon-Rodriguez et al., 2013), and some were isolated from the stratosphere and mesosphere (Smith et al., 2010; Smith, 2013). Airborne dissemination may be an essential part of the life cycle of many microorganisms (Morris et al., 2011) and, in turn, those may have a significant impact on atmosphere chemistry and hydrological cycles (DeLeon-Rodriguez et al., 2013). It is unclear whether airborne microorganisms are metabolically active. A better understanding of microbial abilities to cope with high atmospheric conditions, including low pressures, is desirable.

Various other applications on Earth or beyond, from food packaging (Arashisar et al., 2004; Burg, 2004) to ecopoiesis (McKay et al., 1991; Thomas et al., 2006), would benefit from research on microbial metabolism at low pressures.

Unfortunately, little work has focused on this area. Elements of answers can nonetheless be drawn from various studies; they are summarized below, with the intention to provide researchers venturing into hypobare microbiology with an overview of the field and, perhaps, some research directions. It is noteworthy that, when this short article was in its final writing phase, Schwendner and Schuerger (2020) published a review on microbial activity under low pressure, which the reader is invited to consult for some further information (notably on experimental methods). Here the focus is on bacteria, with occasional comparisons with fungi or eukaryotic microalgae. The reader interested in plants under hypobaria is referred to a review by Paul and Ferl (2006).

BACTERIAL GROWTH AS A FUNCTION OF ATMOSPHERIC PRESSURE

Vacuum

Microorganisms have numerous times been exposed to pressures below water's triple point (6.1 mbar, 0.01°C; Haynes, 2017), both in space and on the ground (e.g., Cottin et al., 2017; Martins et al., 2017; de Vera et al., 2019). At those pressures, water is either solid or gaseous (depending on temperature), and in most microbiology experiments such vacuum led to desiccation. While some bacteria can survive desiccation (see for instance Billi and Potts, 2002; Cortesão et al., 2019; Beblo-Vranesevic et al., 2020) and rare microbial species are capable of metabolism at a water activity below 0.6 (Stevenson et al., 2017; Steinle et al., 2018), most are inactive below 0.9 a_w (Stevenson et al., 2015).

Mars-Like Pressure

Above its triple point, water remains stable with respect to boiling and freezing within a range of temperatures. Growth on solid medium was reported at 7 mbar (in a hypobaric desiccator flushed with CO_2 and maintained at 0°C) for bacterial species from various genera: *Carnobacterium* (Nicholson et al., 2013; Schuerger and Nicholson, 2016), *Serratia* (Schuerger et al., 2013; Schuerger and Nicholson, 2016), *Bacillus*, *Clostridium*, *Cryobacterium*, *Paenibacillus*, *Rhodococccus*, *Streptomyces*, *Carnobacterium*, *Exiguobacterium*, and *Trichococcus* (Schuerger and Nicholson, 2016).

From the environmental samples where those organisms were found, no fungi or archaea were isolated that could grow at 7 mbar, suggesting that this ability is limited to bacteria (Schuerger and Nicholson, 2016). However, reports were made of metabolic activity (albeit no growth) in lichens under similarly low pressures. *Xanthoria elegans* seemed to maintain its photosynthetic activity (as assessed by PAM fluorometry) in both ambient and 95%-CO_2 atmosphere down to close to 6 mbar (de Vera et al., 2010). The authors hypothesized that, under ambient air composition, the fungal symbiont may have been the source of CO_2 for photosynthesis. Another lichen, *Pleopsidium chlorophanum*, showed similar abilities under 95% CO_2 at 800 mbar total. Photosynthetic activity even seemed to increase over time, suggesting a physiological adaptation (de Vera et al., 2014). This capacity is most likely uncommon among photosynthetic microorganisms, as illustrated by the failure of the cyanobacterium *Plectonema boryanum* (not part of a lichen) to produce measurable O_2 under similar atmospheric conditions (Kleina et al., 2019).

25 to 100 mbar

More and more species can grow as pressure increases but, among a high number of tested bacterial strains (excluding those mentioned above), only a few could grow at 25 mbar under either high CO_2 or ambient air composition (Schuerger et al., 2013). For those that could (belonging to the genera *Escherichia*, *Bacillus*, *Enterococcus*, *Proteus*, *Staphylococcus*, and *Paenibacillus*), 25 mbar was close to the lower limit (Schuerger and Nicholson, 2006; Schuerger et al., 2006, 2013; Nicholson et al., 2010). In one experiment where *Escherichia coli* and *Serratia liquefaciens* were grown at 20°C in a CO_2 atmosphere, cell densities after 7 days were similar under 1,013, 100, and 25 mbar (Berry et al., 2010). However, no intermediate cell counts were performed, and growth rates could consequently not be compared.

Between 25 and 100 mbar, growth inhibition in most bacteria seems to decrease with increasing pressure. As an example, growth rates and cell counts after 24 h of *Bacillus subtilis* 168 decreased semilogarithmically when lowering pressure from 100 to 75 and 50 mbar (Nicholson et al., 2010).

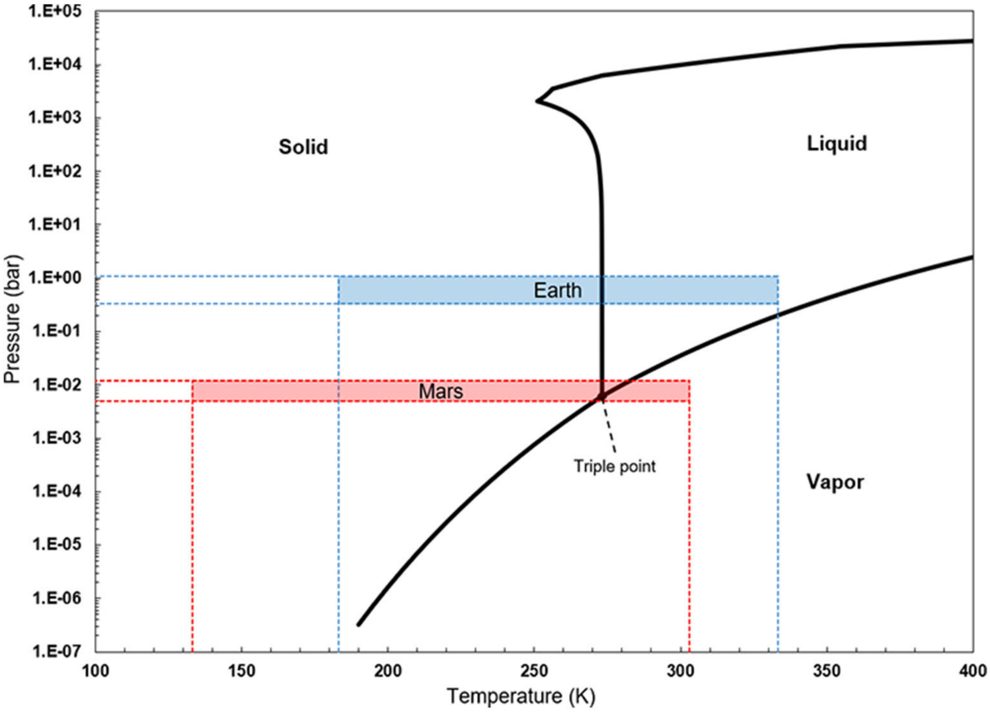


FIGURE 1 | Water's phase diagram, with approximate pressure and temperature ranges found on Earth's and Mars's surfaces. Modified from Verseux (2018).

100 mbar to 1 bar

Between 100 mbar and 1 bar, bacterial growth inhibition by hypobaria seems weak at most, as illustrated by the following examples. (i) In a study by Schuerger et al. (2013) involving 26 bacterial strains from 22 species, all showed vigorous growth at 100 mbar. (ii) Final cell counts of *E. coli* and *B. subtilis* strains after 19 days under 1 bar, 670 mbar, and 330 mbar showed no distinct trend (but growth rates were not assessed) (Pokorny et al., 2005). (iii) Growth rates of *B. subtilis* 168 were similar at 100 mbar and 1 bar of ambient air (Nicholson et al., 2010). (iv) Total bacterial cell counts in plants' hydroponic solution were unaffected by reducing pressure to 100 mbar, though community-level physiological profiles (the abilities of the microbial community to metabolize individual carbon sources) were altered (MacIntyre, 2013). (v) Under non-limiting partial pressures of CO_2, the cyanobacteria *Synechocystis* sp., *Arthrospira platensis*, and *Anabaena cylindrica* could grow at least as efficiently under 100 mbar as under 1 bar (Kanervo et al., 2005; Murukesan et al., 2015).

Consistently, metabolism in *S. liquefaciens* was constant between 1 bar and 100 mbar of ambient air but largely affected below (Schwendner and Schuerger, 2018). *B. subtilis* was also shown to induce the SigB-mediated global stress response at 100 mbar and below, but not at 250 mbar or above (Waters et al., 2014).

Fungi may have a similar threshold: inhibition (reduced mycelial growth and delay in germination) of various species increased with decreasing pressure from 133 mbar of ambient air, but behavior was constant between 200 mbar and 1 bar (Apelbaum and Barkai-Golan, 1977; Romanazzi et al., 2001). No growth or germination occurred at 33 mbar, though it did at 67 mbar (Apelbaum and Barkai-Golan, 1977).

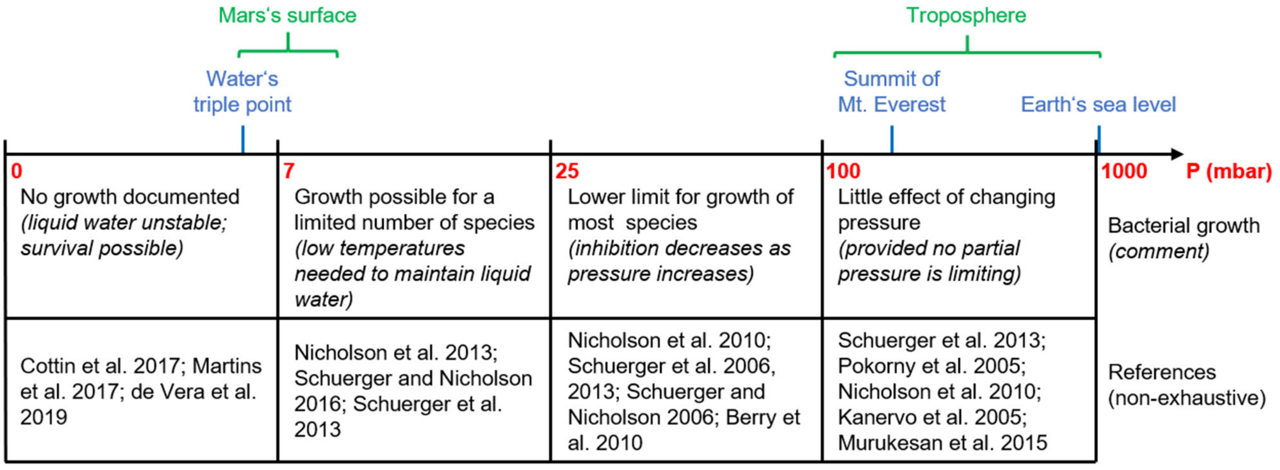
Interestingly, 100 mbar is roughly the lowest pressure of the troposphere (Holton et al., 1995), where bacteria abound (DeLeon-Rodriguez et al., 2013) and above which the density of microorganisms is expected to be low (as a temperature inversion strongly limits the ascension of aerosols through the tropopause). It may also be worth noting here that, at the onset of the Viking Labeled Release experiment (following first nutrient injection), shown in ground-based tests to support metabolism of numerous terrestrial microorganisms, pressure was about 92 mbar (Levin and Straat, 1976, 1979).

A rough summary of bacterial growth as a function of pressure, as described above, is given in **Figure 2**.

MECHANISMS BY WHICH LOW PRESSURE AFFECTS BACTERIAL GROWTH

Desiccation

The most obvious mechanisms for the effects of low pressure may be those of desiccation. When water does not boil, it can still evaporate if the gas phase above is not saturated with water, at rates increasing with decreasing pressure. It is, however, not the only cause for bacterial inhibition at low pressures: growth of *E. coli* K12 and *B. subtilis* 168 in liquid medium was impaired below



0	7	25	100	1000
No growth documented *(liquid water unstable; survival possible)*	Growth possible for a limited number of species *(low temperatures needed to maintain liquid water)*	Lower limit for growth of most species *(inhibition decreases as pressure increases)*	Little effect of changing pressure *(provided no partial pressure is limiting)*	Bacterial growth *(comment)*
Cottin et al. 2017; Martins et al. 2017; de Vera et al. 2019	Nicholson et al. 2013; Schuerger and Nicholson 2016; Schuerger et al. 2013	Nicholson et al. 2010; Schuerger et al. 2006, 2013; Schuerger and Nicholson 2006; Berry et al. 2010	Schuerger et al. 2013; Pokorny et al. 2005; Nicholson et al. 2010; Kanervo et al. 2005; Murukesan et al. 2015	References (non-exhaustive)

FIGURE 2 | Simplified overview of bacterial growth as a function of pressure. This information is to be taken with caution, given today's paucity of data in this area. See text for details.

100 mbar, and that of the latter was close to detection limit at 25 mbar (Schuerger et al., 2006, 2013; Nicholson et al., 2010).

Partial Pressures

Part of the effects of low pressure can be attributed to the low partial pressures of non-inert gases, which go down, roughly proportionally, with total pressure (Dalton's law), leading to (again, roughly proportionally) reduced concentrations of dissolved gases in liquid phases (Henry's law).

Unfortunately, low-pressure studies often do not single out the effects of the involved gases' partial pressures. Qin et al. (2014), for instance, showed that the growth of cyanobacteria (*Microcystis aeruginosa*, *Merismopedia* sp., and *Anabaena* spp.) in nitrate-rich medium was reduced at 0.5 bar of ambient air compared to 1 bar. However, the atmospheric composition was unchanged, leading to, notably, a halved pCO_2—and pCO_2 is limiting even under ambient atmosphere, below about 4 mbar (Murukesan et al., 2015). As another example, an experiment demonstrating the lower rates of methanogenesis at 50 mbar compared to 400 mbar of 3 hydrogenotrophic methanogens, *Methanothermobacter wolfeii*, *Methanosarcina barkeri*, and *Methanobacterium formicicum* (Kral et al., 2011), relied on a 50:50 mixture of H_2:CO_2 in both cases. While studies suggest that the partial pressure thresholds for use of H_2 and CO_2 by hydrogenotrophic methanogens are, respectively, within 0.1–50 mbar—depending on species and culture conditions (Lovley, 1985; Lee and Zinder, 1988; Conrad and Wetter, 1990; Kral et al., 1998; Sprenger et al., 2007)—and below 6 mbar (Chen et al., 2019), both gases become limiting from much higher partial pressures (e.g., Agneessens et al., 2017; Chen et al., 2019).

A few studies, however, have investigated the role played by the partial pressure of specific gases in mediating the effects on microorganisms of a low total pressure.

Some of the most clear-cut evidence comes from microalgae. By contrast with results from Qin et al. (2014), experiments with *Chlorella* spp. where pCO_2 was kept constant (total pressures were adjusted using a non-reactive gas, the nature of which seems not to make a difference with *Chlorella* spp.; Ammann and Lynch, 1966; Orcutt et al., 1970) suggested a positive impact of lowering the pressure to 250 mbar (Orcutt et al., 1970), and showed no negative impact of lowering it to 565 mbar when pO_2 was also kept constant (Niederwieser et al., 2019). *Chlorella* spp. are eukaryotes, but similar results were observed with cyanobacteria: lowering pressure down to 100 mbar inhibited the growth of *Synechocystis* sp. under ambient air composition but did not reduce its growth, or that of other cyanobacterial species, when CO_2 was not limiting (Kanervo et al., 2005; Murukesan et al., 2015).

Though one of those cyanobacteria, *A. cylindrica*, is diazotrophic, nitrates were provided in the media; one may wonder how reducing the total pressure with non-limiting CO_2 would affect the diazotrophic growth of nitrogen fixers. At 1 bar of total pressure, pN_2 became limiting for growth of *A. cylindrica* and *A. variabilis* below 500 mbar, but growth was still vigorous at 100 mbar (Silverman et al., 2018). Those results are consistent with values obtained by others with non-cyanobacterial nitrogen fixers: Klingler et al. (1989) showed that growth rates of *Azotobacter vinelandii* and *Azomonas agilis* decreased with decreasing pN_2 down from about 400 mbar, but that nitrogen fixation was still possible at 5 mbar (though not at 1 mbar), and MacRae (1977) found an increase in nitrogen fixation in *Beijerinckia indica* and *B. lacticogenes* with pN_2 increasing between 5 and 400 mbar. Consistently, nitrogen fixation by *Bradyrhizobium japonicum* was reported under a pN_2 of 190 mbar, under a total pressure of 250 mbar (MacIntyre, 2013), though quantitative results were not presented.

Further evidence on the role of partial pressures comes from the fact that growth inhibition of *E. coli* K12 in LB at 50 and 25 mbar was attenuated by the addition of substrates for anaerobic metabolism (Schuerger et al., 2013), suggesting a role of decreased oxygen availability in the effect of reduced total pressure.

One may be tempted to conclude that the effects of hypobaria on bacteria come from changes in partial pressures of non-inert gases and, in solid media, from desiccation. However, evidence suggests effects independent of both. The addition of substrates for anaerobic metabolism did not, or barely, relieve the low-pressure-induced growth inhibition in *Bacillus* spp. as it did for *E. coli* K12, though the former also are capable of anaerobic metabolism (Schuerger and Nicholson, 2006; Schuerger et al., 2013). Besides, endospores of 2 facultatively anaerobic *Bacillus* spp., *B. nealsonii* and *B. licheniformis*, could germinate at 1 bar of a high-CO_2 atmosphere but not at 25 mbar of either ambient composition or high CO_2 (Schuerger and Nicholson, 2006), further pointing toward pO_2-independent effects of low pressure. Consistently, a strain of *B. subtilis* evolved toward higher fitness at 5 kPa had no advantage at 1 bar in oxygen-limited conditions (Nicholson et al., 2010), and the *des* gene it upregulated at low pressure (and whose deactivation decreased its fitness at 5 kPa) was not upregulated by low oxygen levels (Fajardo-Cavazos et al., 2012).

Evidence that is less conclusive, but nonetheless worth reporting here, was obtained with other organisms. Various *Serratia* spp. and *Carnobacterium* spp. were shown to grow better in 7 mbar of a high-CO_2 atmosphere than under 1 bar at the same composition and, in the case of *Carnobacterium* spp., better than under ambient atmosphere (Nicholson et al., 2013; Schuerger et al., 2013; Schuerger and Nicholson, 2016). However, one cannot rule out, on the basis of the reported data, that those observations are due to CO_2's toxicity at high partial pressures (e.g., Dixon and Kell, 1989; Kimura et al., 1999; Thomas et al., 2005) and, for *Carnobacterium* spp., from an advantage of reducing pO_2 (compensated for by CO_2 toxicity in the 1 bar, high-CO_2 control). Increasing O_2 concentrations attenuated low-pressure growth inhibition in several fungal species, but not fully: inhibition under 67 mbar at a pO_2 of 8 mbar was higher than at 1 bar with the same pO_2 (Apelbaum and Barkai-Golan, 1977). In a different study, fungal growth was further delayed by a reduced pressure (135 mbar) of ambient air than under ambient pressure at similar pO_2 (Wu and Salunkhe, 1972). However, those results may stem from the reduction in pCO_2 rather that in total pressure (see, e.g., Bahn and Mühlschlegel, 2006).

pCO_2-independent effects of total pressure were reported in microalgae. First, growth rates of *Synechocystis* sp. were about 5 times higher within 60–150 mbar of a 100%-CO_2 atmosphere than under ambient air, an increase higher than can be explained by pCO_2 alone: at the presumably non-limiting value of 4 mbar, growth increased only 3.5-fold (Murukesan et al., 2015). However, pO_2 was not constant, and the lack of photorespiration (which would reduce carbon fixation) might explain such results. Second, growth of *Chlamydomonas reinhardtii* decreased with pressure between 1 bar and 700 mbar under high CO_2 concentrations (Wagner and Posten, 2017). It should however be noted that, although CO_2 was presumably non-limiting even at the lowest tested pressures, its concentration (4.8% v/v) in the sparging gas—not its partial pressure—was constant between samples.

Physico-Chemical Effects of Low Total Pressure

The effects of low pressure itself, not accounted for by desiccation and the partial pressures of the component gases, can only be described tentatively: data is scarce.

As suggested by others (Nicholson et al., 2010; Waters et al., 2014; Schwendner and Schuerger, 2018, 2020), one can make tentative inferences from studies on the effects of high pressure on microorganisms. Volume reduction caused by high pressure leads to structural alterations in biomolecules, thereby impacting numerous cellular processes and threatening cell integrity. Membranes are particularly sensitive—their rigidity tends to increase with pressure (Macdonald, 1984; Winter and Jeworrek, 2009)—, but various biomolecules, notably proteins and nucleic acids, may be impacted as well (see Bartlett, 2002; Oger and Jebbar, 2010; Mota et al., 2013). Pressure also affects chemical equilibria and reaction rates: following Le Chatelier's principle, higher pressures stabilize the state corresponding to the lowest volume (see for instance Smeller, 2002). One may expect opposite influences when pressure is reduced, such as a tendency toward volume increase of molecular and cellular structures (consistently, Wagner and Posten, 2017 observed a swelling of *C. reinhardtii* cells when pressure was abruptly lowered), leading, notably, to a fluidification of plasma membranes.

Further effects may be mediated by changes in gas diffusion and solubility. A beneficial impact of reducing pressure while maintaining the partial pressure of metabolism-supporting gases (e.g., Orcutt et al., 1970; Murukesan et al., 2015) may be due to the fact that diffusion coefficients increase proportionally to decreasing pressure (e.g., Chen and Othmer, 1962). Gas exchanges may thus be enhanced and the microenvironment around cells become more supportive of metabolism. This positive impact has not been systematically observed, possibly because diffusion is not always limiting (e.g., Niederwieser et al., 2019) or because other effects can counterbalance it. For similar reasons, one could expect temperature (with increasing temperatures, diffusion rates increase but solubility decreases) and salts (increasing salt concentrations generally decrease gas solubility; Schumpe, 1993) to affect, when the availability of some gases is near the threshold at which they become limiting, the response of bacteria to low total pressures.

The interplay between pressure, temperature, and salinity is more complex than the above could suggest. Schuerger and Nicholson (2006) noted interactive effects of low pressure, temperature, and gas compositions that could hardly be explained by diffusion and solubility alone. Berry et al. (2010) showed that growth in CO_2 atmospheres of a strain of *E. coli* was inhibited by 5% $MgCl_2$, or 5% NaCl, at 1 bar, but not at 100 or 25 mbar. While one can hypothesize that growth inhibition came from the combined toxicity of high CO_2 and high salt concentrations, the former being relieved at lower pressures, the presented data do not allow for a conclusion. More broadly, much remains to be defined, such as the role played by salts' chaotropicity (see Hallsworth et al., 2003, and Ball and Hallsworth, 2015 for an overview of how chaotropic compounds affect bacteria, and Rummel et al., 2014 for insights in the context of Martian habitability), or the joint outcome of temperature and low pressure's influences on membrane fluidity.

Finally, bacterial isolates including *Streptomyces* spp. grew at 7 mbar (high-CO_2, 0°C) in presence of soil from their original environment but failed to grow in such conditions after being streak-purified, suggesting that geochemical or biological components from their original surroundings are needed to cope with low pressure (Schuerger and Nicholson, 2016).

Hypobaria thus seems to affect cells even when desiccation is prevented and when partial pressures of non-inert gases are constant, in ways that are dependent on various other physico-chemical factors. Owing to their complexity and to the paucity of related data, those ways remain poorly understood.

Bacteriostatic or Bactericidal?

Desiccation set aside, the effects of low pressure seem bacteriostatic rather than bactericidal: growth inhibition at low pressures tends to be relieved when pressure is brought back to normal (Kanervo et al., 2005; Schuerger and Nicholson, 2006; Nicholson et al., 2010, 2013; Schuerger et al., 2013). A similar observation was made on fungi (Apelbaum and Barkai-Golan, 1977). Exceptions were, however, reported: *S. liquefaciens* incubated for 49 days under 7 mbar of high-CO_2 atmosphere, at 0°C, did not return to fully normal metabolic activity when back to 1 bar of ambient air, at 30°C (Schwendner and Schuerger, 2018), and *B. subtilis* 168 pre-incubated at 50 mbar grew more slowly when brought back to ambient air than cells not previously exposed to low pressure (Nicholson et al., 2010). In the latter case, growth (which had stopped at a low density at 50 mbar) resumed without a lag phase when returned to ambient air, suggesting that low pressures reversibly inactivate some biomolecules. On the other hand, the slower growth rate suggested a lasting physiological alteration, possibly mediated by damage to cell components that require recovery time (Nicholson et al., 2010).

BACTERIAL ADAPTATION TO HYPOBARIA

All bacteria are not equal in the face of low pressures. Out of nearly 10^4 colonies from permafrost soil samples, only 6, all *Carnobacterium* spp., grew under 7 mbar of CO_2 (Nicholson et al., 2013). Numerous species seem unable to grow at 25 mbar (Schuerger et al., 2013). Wide differences can be found even within a genus: a strain of *B. subtilis* could germinate and grow under 35 mbar of Earth-normal air but not of a high-CO_2 atmosphere, while the reverse was true for *B. nealsonii* and *B. licheniformis* (Schuerger and Nicholson, 2006). Two out of 8 tested *Serratia* spp. strains could not grow under 7 mbar of CO_2 (Schuerger and Nicholson, 2016). Cell differentiation matters, too: under 25 mbar of Earth-normal air composition, vegetative cells of 7 *Bacillus* spp. grew but endospores of the same strains failed to germinate (Schuerger and Nicholson, 2006).

What enables some bacteria to cope better than others is unclear. Below about 25 mbar, temperatures must be lowered below mesophiles' optimal values to reduce evaporation (whose rates decrease with pressure and increase with temperature) and prevent boiling. At Mars-like pressures, temperatures must be so low—the boiling point of pure water is, for instance, around 2.4°C at 7 mbar—that psychrophilic or psychrotrophic properties are required for growth. Equally obvious, a microorganism depending on a given gas cannot grow if the total pressure is below the partial pressure threshold for use of that gas. It is, for instance, safe to assume that the low-pressure threshold for diazotrophic or photosynthetic growth is limited by pN_2 or pCO_2. Consistent with both considerations, all bacteria grown below 10 mbar were obligate or facultative anaerobes, and many came from cold environments (Nicholson et al., 2013; Schuerger and Nicholson, 2016).

Beyond this, describing coping strategies is tentative. Earth's surface is mostly devoid of environments where abilities to grow at low pressures give an advantage: its lowest pressure, at the top of Mount Everest, is above 0.3 bar (West, 1999). Some microorganisms may evolve mechanisms favoring tropospheric endeavors (Morris et al., 2011; DeLeon-Rodriguez et al., 2013)—supporting this is the threshold for effect at 100 mbar described above, which roughly corresponds to the lowest pressure in the troposphere—but selective pressure may be more toward survival and transportation efficiency (e.g., suitability for carriage by air masses, or ability to foster precipitations for faster fallout; Smith, 2013) than multiplication. Even so, selection would only happen down to the vicinity of 100 mbar.

One may thus expect responses to very low pressures to be maladaptive. Transcription analyses are consistent with this. Growing *B. subtilis* at 50 mbar rather than 1 bar altered the levels of 363 transcripts from several global regulons. Most notable was the strong induction at low pressure of the SigB-mediated general stress response regulon, which seemed non-optimal: inactivation of *sigB* did not significantly change fitness at either pressure (Waters et al., 2014). Under a high-CO_2 atmosphere at 0°C, the expression of 184 genes in *S. liquefaciens* differed significantly between 7 mbar and 1 bar (Fajardo-Cavazos et al., 2018). No genes were identified that could be reasoned to facilitate growth at low pressure. Some of the most up-regulated were involved in transport and utilization of various sugars (none of which was present in the medium), and the most strongly downregulated ones were involved in transport of sulfate, or the sulfur-containing amino acid cysteine, which here again was presumed to come from a maladaptive answer.

More broadly, no optimized low-pressure answer has been reported. Schuerger et al. (2013) suggested that organisms likely to cope best at low pressure are, rather than specialized extremophiles, those able to adapt to a broad range of environmental conditions.

One may wonder whether this only comes from the lack of a role for hypobaria in natural selection and whether, if exposed to low pressure over multiple generations, microorganism could evolve toward higher tolerance. Studies suggest a positive answer: the fitness of *B. subtilis* at 50 mbar of ambient air had increased after 1,000 generations (Nicholson et al., 2010), and in large part within the first 200 generations (Waters et al., 2015).

Unfortunately, the molecular basis for this adaptation is unclear. Microarray analyses revealed a higher transcription of the *des*, *desK*, and *desR* genes, encoding, respectively, the Des membrane fatty acid desaturase, the DesK sensor kinase, and the DesR response regulator. Consistently, lowered pressure caused an up-regulation of *des* mRNA levels in the evolved strain only, and deactivating the *des* gene slightly reduced its fitness at 50 mbar (Fajardo-Cavazos et al., 2012). Such a result is somewhat surprising: reducing pressure tends to increase membrane fluidity (Macdonald, 1984), and the *des-desKR* system mediates an acute response that fluidifies membranes when temperatures go down, counterbalancing cold's tendency to rigidify them (Aguilar et al., 2001). In the ancestral strain, reducing the pressure led to seemingly conflicting responses: an increase in the proportion of saturated fatty acids (which would tend to increase rigidity) accompanied by an increase in the proportion of anteiso-fatty acids (which would tend to increase fluidity). At 50 mbar, fatty acid membrane compositions were similar in the ancestral and evolved strains, suggesting that adaptation did not come from there (though the evolved strain had a lower proportion of anteiso-fatty acids at 1 bar, which may give an initial advantage following a drop in pressure).

Whole-genome sequencing revealed that the adapted strain had amino acid-altering mutations in the coding sequences of 7 genes, 2 of which are involved in the maintenance of cell wall integrity, and a 9-nucleotide in-frame deletion in the *rnjB* gene that encodes a component of the RNA degradosome—and whose knockout increased competitive fitness of *B. subtilis* at both low pressure and 1 bar (Waters et al., 2015). However, whether and how those different mutations may enhance growth specifically at low pressure remains to be elucidated, and most of the increase in fitness occurred before those mutations spread in the evolving population.

CONCLUDING REMARKS

Implications for potential metabolism on Mars and in the troposphere of bacteria's capabilities to grow at low pressure were discussed elsewhere (Nicholson et al., 2013; Schuerger et al., 2013; Waters et al., 2014; Schuerger and Nicholson, 2016; Schwendner and Schuerger, 2020), but less so the opportunities for BLSS. Results presented above suggest that microbial modules in crewed compartments of future space vehicle and habitats would be little affected by the envisioned reduced pressure (NASA, 2006) itself; more important would be the partial pressures of the component gases.

Lower pressures would offer advantages for BLSS deployed outside, such as cyanobacterium-based ones (CyBLiSS): it has been argued that some species of diazotrophic, rock-weathering cyanobacteria could be used as a basis for life-support systems on Mars that would rely on local resources—atmospheric gases, water mined on site, and mineral nutrients from the regolith—, thereby greatly reducing the mass of consumables to be sent from Earth (Verseux et al., 2016b). One of the factors that will determine the efficiency of CyBLiSS is the behavior of cyanobacteria under non-Earth atmospheres. On the one hand, growing them under atmospheric conditions close to Mars's (low total pressure, high pCO_2, low pN_2) would simplify the system, minimize the mass of structural materials and consumables, and lower the risk of organic matter leakage (Lehto et al., 2006; Verseux et al., 2016b). On the other hand, changes in gas composition and pressure affect cyanobacterial behavior. Opening cultures directly to the Martian atmosphere is, of course, excluded: both total pressure and pN_2 are too low. However, changing the CO_2/N_2 ratio and pressurizing slightly, both of which could be done with technologies routinely used on Earth, could suffice. A total pressure of 100 mbar or below, with a few percent CO_2, is not expected to be limiting *per se*; growth would presumably depend largely, atmosphere-wise, on pN_2. What atmospheric conditions offer the most relevant compromise between engineering and biology is currently being investigated.

Overall, while the impacts of extreme conditions on bacteria have been quite intensely studied (e.g., Rothschild and Mancinelli, 2001; Harrison et al., 2013), high hypobaria has been largely neglected, owing in large part to its absence from Earth's surface and to the need for specific experimental hardware. Only a limited number of organisms, isolated from a small set of environments, have been measured against it. The mechanisms mediating the effects of low pressure, and their interactions, are poorly understood. How far the low-pressure limits of bacteria could be pushed, by genetic engineering or directed evolution, is unknown. Much remains to be discovered in the field of low-pressure microbiology. Here is a call for interested researchers to join this investigation.

AUTHOR CONTRIBUTIONS

CV conceived and wrote the manuscript.

ACKNOWLEDGMENTS

CV acknowledges funding from the Alexander von Humboldt Foundation, and thanks both reviewers for their valuable comments.

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Conflict of Interest: The author declares 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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MINI REVIEW
published: 24 June 2020
doi: 10.3389/fspas.2020.00033

CO_2: A Small Ubiquitous Molecule With a Lot of Astrochemical Debate Attached

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OPEN ACCESS

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Specialty section:
This article was submitted to Astrobiology, a section of the journal Frontiers in Astronomy and Space Sciences

Received: *19 February 2020*
Accepted: *25 May 2020*
Published: *24 June 2020*

Citation:
Bredehöft JH (2020) CO_2: A Small Ubiquitous Molecule With a Lot of Astrochemical Debate Attached. Front. Astron. Space Sci. 7:33. doi: 10.3389/fspas.2020.00033

Water, carbon monoxide and carbon dioxide are the most abundant molecules in the condensed phase of interstellar dust grains. Water is formed by hydrogenation of oxygen species adsorbed on the surface of dust grains, while carbon monoxide is formed by reactions between ionized carbon and hydroxyl species in the gas phase. It is the second most abundant molecule in the gas phase after molecular hydrogen. The formation of carbon dioxide, however, cannot proceed in the gas phase as the addition of an oxygen to carbon monoxide is so exothermic that the new-formed molecule quickly breaks a C-O bond without a third body to transfer the excess energy to. It is commonly accepted that carbon dioxide is formed by energetic processing of carbon monoxide and water on grain surfaces. The exact mechanism of the oxidation of carbon monoxide and the intermediates formed in the process, however, are under some dispute. The role of the $HOCO^{\cdot}$ radical in the oxidation of carbon monoxide is especially contested, as theory predicts it should not be able to dissociate the H-O bond to yield carbon dioxide, but it is experimentally observed in some studies of the reaction. The author presents here a short overview of established and recent experimental results in an attempt to reconcile seemingly contradictory results and give a comprehensive and consistent picture of the radiation induced reactions between carbon monoxide and water on grain surfaces.

Keywords: astrochemistry, comets, ISM, icy grains, secondary electrons

INTRODUCTION

Carbon dioxide (CO_2) is found everywhere in the Universe. It has been determined the second or third most abundant condensable[1] molecule after water (H_2O) and carbon monoxide (CO) (Hama and Watanabe, 2013). It has been identified in dense clouds, young stellar objects (Ehrenfreund and Charnley, 2000), comet Hale-Bopp (Irvine et al., 2000), and its abundance has even been measured *in situ* on the nucleus of comet 67P/Churyumov-Gerasimenko (Goesmann et al., 2015). The only exception to this is the high-mass protostellar object W33A, in which the abundance of methanol (CH_3OH) exceeds that of both CO and CO_2 (Gibb et al., 2000). It is generally understood that CO_2 forms by oxidation of CO in the ice mantles surrounding interstellar dust grains. This is in agreement with the very low observed gas phase abundances of CO_2 (about a factor of 100

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[1] Note that the two non-condensable substances hydrogen (H_2) and Helium are disregarded throughout this entire review, because they can by their nature not contribute to condensed-phase chemistry.

less than in condensed phase) (Boonman et al., 2003) and the fact that gas-phase synthesis of CO_2 from CO and O atoms is very inefficient without a third body to transfer excess energy to. In fact, the electronically excited $O(^1D)$ is very efficiently quenched to the $O(^3P)$ ground state by interaction with CO. The intermediate CO_2 rapidly decays to vibrationally excited CO and ground state $O(^3P)$ (Shortridge and Lin, 1976). The exact mechanism by which oxidation of CO occurs, however, is less well agreed upon. While it is conceivable that CO undergoes radiolysis to $C^{\cdot}$ and $O^{\cdot}$ atoms, the latter of which can react with CO, the most abundant molecule in interstellar ices is water, which would hinder this reaction by dilution of CO and by rapid reaction with O atoms. It is thus reasonable to expect H_2O to play the role as oxygen donor in the net reaction $CO + H_2O \rightarrow CO_2 + H_2$. But since condensed phase chemistry rarely ever proceeds by such simple routes, the question of the exact mechanism of the oxidation of CO by H_2O needs to be answered by experiment.

EXPERIMENTAL DATA ON CO + H_2O

There have been numerous studies of the radiation-induced chemistry of CO and H_2O, as should be expected for the two most abundant molecules in the Universe (disregarding H_2). The means of irradiation span UV light (Milligan and Jacox, 1971; Allamandola et al., 1988; Watanabe and Kouchi, 2002; Watanabe et al., 2007), slow electrons (Yamamoto et al., 2004; Schmidt et al., 2019), fast electrons (Bennett et al., 2011; Petrik et al., 2014a,b), X-ray (Laffon et al., 2010) as well as wide range of ion beams. Since ion beams introduce another potential reaction partner, complicating the reaction routes further, they will not be discussed here in depth. The proton beam experiments alone would warrant a full review paper for their extremely rich and interesting chemistry.

In all of the above studies, CO and H_2O are condensed at cryogenic temperatures (10–35 K) and are then be subjected to irradiation. Along with CO_2, formaldehyde (H_2CO), formic acid (HCOOH), and CH_3OH were all identified as products of energetic processing. In all but the Schmidt et al. study, reaction progress was monitored by infrared (IR) spectroscopy (and sometimes complementary techniques as well). This allowed the authors to monitor stable products as well as reactive intermediates, as long as their abundance was high enough. The downside of IR spectroscopy in condensed phase is that bands tend to be very broad and overlap due to the manifold chemical surroundings experienced by individual molecules. This makes definite band assignment difficult or downright impossible. Further complicating the issue is the fact that IR spectra of intermediate species are often not well-known, or intermediates are species that are not IR active at all, such as atomic O. The two key intermediate radical species $HCO^{\cdot}$ and $HOCO^{\cdot}$ were, however, observed.

In experiments with isotopic labeling, Yamamoto could show that the formation of CO_2 predominantly proceeds by a reaction between CO and H_2O rather than from CO alone. Experiments by Petrik et al. showed that CO_2 yields are highest, when CO and H_2O are well-mixed, while in diffusion-limited scenarios the hydrogenation products H_2CO and CH_3OH are favored because of the high mobility of $H^{\cdot}$ radicals even at cryogenic temperatures. These observations led to the rationalization that the reaction is triggered by radiolysis of H_2O, forming $H^{\cdot}$ and $OH^{\cdot}$ radicals. These react with CO to form $HCO^{\cdot}$ or $HOCO^{\cdot}$, respectively. Subsequent additions of further $H^{\cdot}$ and $OH^{\cdot}$ radicals then yield H_2CO, CH_3OH, and HCOOH. CO_2 formation was explained by the loss of an $H^{\cdot}$ from the $HOCO^{\cdot}$ intermediate.

The problem with this interpretation is that for every cleavage of H_2O, equal numbers of $H^{\cdot}$ and $OH^{\cdot}$ radicals are formed. This means that the ratios between the different products should be predictable and, above all, fixed. Which they weren't. In their 1988 study, Allamandola et al. found a much higher abundance of CO_2 than Milligan and Jacox did in 1971. Moreover, while Milligan and Jacox saw a significant IR signal, which was later assigned to $HOCO^{\cdot}$, the later study couldn't find a trace of the same intermediate. Watanabe and Kouchi did observe that the rate of decrease in CO was faster than the rate of increase in CO_2 abundance, which hinted at some intermediate, but could not identify it in their IR measurements. In their later 2007 study Watanabe et al. did observe some small traces of $HCO^{\cdot}$ but no $HOCO^{\cdot}$ which led them to propose a reaction scheme based solely around the $HCO^{\cdot}$ intermediate. And all this was just for the UV irradiation.

In the 2011 electron irradiation experiments by Bennett et al. $HOCO^{\cdot}$ was unambiguously identified as an intermediate. By that time, however, quantum-chemical calculations had shown that the $HOCO^{\cdot}$ radical should be stabilized in a water matrix, quickly losing all its excess energy and making the reaction to CO_2 impossible (Goumans et al., 2008), a concept that would later also be shown by molecular-dynamics simulations (Arasa et al., 2013). One huge benefit that the Bennett study had over the previous studies was, however, that it looked at more than one product. The authors monitored CO_2, H_2CO, and HCOOH at the same time. The difficulties in identifying all products and intermediates from an IR spectrum, led most authors to focus on one product of the reaction and observing its formation with increasing dose of radiation. Bennet et al. circumvented this in part by also looking at the stable reaction products by mass spectrometry. By simultaneously looking at several products, some additional insight into the messy situation around the $HOCO^{\cdot}$ radical could be gained. The authors proposed for the first time that $HOCO^{\cdot}$ was the precursor to HCOOH. But ultimately, they also couldn't explain the formation of CO_2 comprehensively.

The most recent study of the problem is by Schmidt et al. (2019). The authors build on the Bennett experiments in the sense that they too used mass spectrometry and they too looked at all known products of the reaction. To overcome the limitation of the previous study, however, they also implemented another experimental technique that Yamamoto et al. tried in 2004: Looking at product yields not in dependence of irradiation time, but in dependence of electron energy E_0. Yamamoto et al. looked at the CO_2 yield after 10 min of electron irradiation at 5, 10, 15, 20, 25, 30, 40, and 50 eV of E_0. The energy-dependence of the process yields some interesting information about the primary interaction of radiation with H_2O molecules. In order to understand why product yields at different electron energies E_0

are useful in understanding the underlying chemistry, a very brief explanation of some basic concepts of electron-driven chemistry might be needed.

A BRIEF INTRODUCTION TO ELECTRON-MOLECULE INTERACTIONS

The reason why UV light, electron beams and X-rays should produce the same chemical products from condensed H_2O:CO mixtures might at first be surprising. The modes of primary interaction between the different types of radiation and a molecule are quite different. UV light typically has energies (3–10 eV) that can excite valence shell electrons of a molecule $M \rightarrow M^*$, where the asterisk denotes an (electronically) excited state, while X-rays with their energies in the 100s of eV have enough energy to knock a core electron out of a molecule $M \rightarrow M^+$. Electrons on the other hand can have energies from near 0 eV all the way up to GeV, as seen in cosmic rays. Therefore, they can trigger a huge range of different processes. This is why the study of condensed phase astrochemistry is so often conducted using electron beams. They can trigger a huge variety of processes and at the same time are much easier to operate, tune and quantify than sources for X-Ray or extreme UV radiation.

But why do UV, electrons and X-ray cause the same types of chemical reactions to occur? This has to do with the processes that happen after the primary interaction. Any type of radiation that has an energy above the ionization threshold of a substance can knock an electron out of a molecule[2]. The electron that leaves the molecule, however, does not simply disappear. It can interact with surrounding molecules, of which there are many in the condensed phase, just as an electron from an electron beam would. These so-called "secondary electrons" typically have energies in the range between 2 and maybe 10–20 eV. The cross-section for electron-molecule interactions in this energy range is very large (Böhler et al., 2013), and they are produced in vast numbers (Boyer et al., 2016). This makes them responsible for the majority of chemical processes that are observed in energetic processing of ices. There are three principal ways of interaction of an electron e^- and a molecule M. The electron can excite the molecule, transferring some of its energy. This can happen when E_0 is above the excitation threshold of the molecule, from which energy the cross section steadily rises:

$$M + e^- \rightarrow M^* + e^-(\text{slower})$$

The electron can knock an additional electron from the molecule, if its E_0 is above the ionization threshold of the molecule, again with rising cross section for higher energies

$$M + e^- \rightarrow M^{+\cdot} + 2\,e^-,$$

and finally the electron can attach to the molecule, which can happen in narrow, well-defined energy ranges of E_0, called resonances:

$$M + e^- \rightarrow M^{-\cdot}.$$

Any of these three forms of the molecule M^*, M^+, M^- can go on to dissociate by breaking a bond. In the case of neutral excitation, the dissociation of the molecule is called neutral dissociation (ND) and it typically yields two radicals

$$M^* \rightarrow A^{\cdot} + B^{\cdot}.$$

The case of the molecule losing an electron is called electron impact ionization (EI), in case the energy of the impinging electron is high enough, this will lead to dissociative ionization (DI),

$$M^{+\cdot} \rightarrow A^+ + B^{\cdot},$$

and finally electron attachment can also lead to something called dissociative electron attachment (DEA):

$$M^{-\cdot} \rightarrow A^- + B^{\cdot}.$$

In all of the cases, a radical species ($B^{\cdot}$) is formed. These radicals are responsible for the formation of new bonds and thus chemical change. Since the energy dependence of these processes is different (resonant vs. steadily rising from different onsets), the processes can be distinguished by looking at the energy dependence of the formation of a product.

RESOLVING THE ISSUE OF CO_2 FORMATION WITH SLOW ELECTRONS

The 2019 Schmidt et al. study made use of slow electrons with an energy resolution of 0.5 eV in the range between 2 and 20 eV, which is the energy range for secondary electrons. By looking at the energy dependence of the formation of the known products, CO_2, H_2CO, and HCOOH by post-irradiation mass spectrometry, they could finally untangle the reaction sequence and shed some light on the formation pathways not only for CO_2, but also for H_2CO and HCOOH. It was observed that all three products had a common energy dependence with a steady rise in product yield starting from around 6–7 eV. This clearly was an ND process, as it was not resonant and started at an energy far below the ionization threshold of either H_2O or CO. This indicated that there must be one common or at least similar reaction pathway leading to either of the three products. Superimposed on the energy dependencies of H_2CO and HCOOH, but not CO_2, there were two resonant structures, one at around 4 eV in H_2CO formation and one at around 10 eV for HCOOH formation. These resonances coincide with known electron attachment resonances. The lower energy channel at 4 eV leads to formation of $CO^{\cdot-}$ which is very unstable and immediately detaches the electron in pure CO. In a water matrix, however, it can react to form OH^- and $HCO^{\cdot}$. The higher

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[2] Hence the name "ionizing radiation."

energy channel is an electron attachment to H_2O, which decays into H^- and $OH^\cdot$. The $OH^\cdot$ radical then reacts with CO in a barrierless addition to form $HOCO^\cdot$. While the intermediates themselves could not be observed, their reaction products H_2CO and HCOOH could. It would indeed seem that $HOCO^\cdot$ is an important intermediate of the reaction between CO and H_2O, it just is not an intermediate to CO_2 formation. This reconciles a lot of the previous work in which $HOCO^\cdot$ was experimentally observed with the theoretical predictions that the reaction of $HOCO^\cdot$ to CO_2 would be energetically infeasible.

But if neither $HCO^\cdot$ nor $HOCO^\cdot$ are intermediates on the route to CO_2, what is? The energy dependence of CO_2 formation strongly suggests an ND process, but ND to water yielding $H^\cdot$ or $OH^\cdot$ is ruled out for significant CO_2 production, as there is no enhanced production of CO_2 at the resonance energies where $HCO^\cdot$ and $HOCO^\cdot$ are known to exist. There is another known ND process in CO yielding $C^\cdot$ and $O^\cdot$, but since it was experimentally observed that most CO_2 is formed by involving H_2O (Yamamoto et al., 2004; Laffon et al., 2010; Schmidt et al., 2019) this seems very unlikely. Also, the energy at which this process starts is much higher than the observed onset (McConkey et al., 2008). At the energies observed here, there is however, another ND process in H_2O. Starting from around 7 eV, H_2O can dissociate into H_2 and $O(^1D/^3P)$. This would seem counter-intuitive at first, since dissociation of both H-O bonds in H_2O requires significantly more energy than 7 eV, but the energy yield from the recombination of 2 $H^\cdot$ to H_2 is enough to offset the deficit. The authors thus present their finding that the formation of CO_2 is one of the extremely rare cases where a net reaction equation like

$$CO + H_2O \rightarrow CO_2 + H_2$$

is indeed indicative of the actual reaction mechanism. By carefully looking at all products of the reaction between H_2O and CO, and by doing so with an energy resolution that allowed the authors to distinguish different reaction channels, they could work out that, in the end, everybody was right: $HOCO^\cdot$ is indeed an important intermediate, just not on the path to CO_2, $HOCO^\cdot$ is indeed stabilized by the matrix, which is why it could be observed in some cases, and the net stoichiometric equation for oxidation of CO is truly describing the reaction mechanism.

AUTHOR CONTRIBUTIONS

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

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Conflict of Interest: The author declares 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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BRIEF RESEARCH REPORT
published: 08 July 2020
doi: 10.3389/fspas.2020.00037

Cultivation With Powdered Meteorite (NWA 1172) as the Substrate Enhances Low-Temperature Preservation of the Extreme Thermoacidophile *Metallosphaera sedula*

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Specialty section:
This article was submitted to
Astrobiology,
a section of the journal
Frontiers in Astronomy and Space
Sciences

Received: 18 April 2020
Accepted: 28 May 2020
Published: 08 July 2020

Citation:
Milojevic T, Zebec Z and Schimak MP
(2020) Cultivation With Powdered
Meteorite (NWA 1172) as the
Substrate Enhances
Low-Temperature Preservation of the
Extreme Thermoacidophile
Metallosphaera sedula.
Front. Astron. Space Sci. 7:37.
doi: 10.3389/fspas.2020.00037

Recent studies have uncovered a vast number of thermophilic species in icy environments, permanently cold ocean sediments, cold sea waters, and cool soils. The survival of thermophiles in psychrobiotic habitats requires thorough investigation of the physiological and molecular mechanisms behind their natural cryopreservation. Such investigations are mainly impeded due to a restricted cultivation of thermophiles at low temperatures under the laboratory conditions. Artificial culture media used under the laboratory conditions usually fail to support cultivation of thermophiles at low-temperature range. In this study we cultivated the extreme thermoacidophilic archaeon *Metallosphaera sedula* with the preliminary powdered and sterilized multimetallic extraterrestrial mineral material (the meteorite NWA 1172) under a low temperature regime in laboratory conditions. Our data indicate that *M. sedula* withstands cold stress and can be maintained at low temperatures, when supplemented with the meteorite NWA 1172 as the sole energy source. Cultivation with the meteorite NWA 1172 opens up new, previously unknown psychrotolerant characteristics of *M. sedula*, emphasizing that culture conditions (i.e., the "nutritional environment") may affect the microbial survival potential in stress related situations. These observations facilitate further investigation of strategies and underlying molecular mechanisms of the survival of thermophilic species in permanently cold habitats.

Keywords: ***Metallosphaera sedula*, meteorite, low temperature, cold environments, cryopreservation, thermophiles, archaea**

INTRODUCTION

Diverse extremophilic microorganisms have been discovered in habitats characterized by parameters that go beyond the range of their physiological activity. For example, a variety of heat-loving microbial species have been isolated from low temperature environments across the globe. Recent independent investigations have uncovered a vast number of thermophilic species in icy habitats (Bulat et al., 2004; Lavire et al., 2006; Bulat, 2016; Papale et al., 2019; Gura and Rogers, 2020); permafrost environments (Gilichinsky et al., 2007; Demidov and Gilichinsky, 2009; Mironov et al., 2010; Shcherbakova et al., 2011); cool soils with temperatures constantly below 25°C (Marchant et al., 2002, 2008, 2011; Rahman et al., 2004; Zeigler, 2014); "arctic thermophiles" in permanently cold (−2° to 4°C) ocean sediments (Hubert et al., 2009, 2010; de Rezende et al., 2013; Müller et al., 2014; Robador et al., 2016; Bell et al., 2018, 2020; Chakraborty et al., 2018); and non-spore-forming hyperthermophiles in cold (2–4°C) seawater (Huber et al., 1990; Wirth, 2017). How these thermophilic species, abundantly represented in permanently cold habitats, can tolerate low temperatures significantly below their minimum requirement for growth is an intriguing subject of current investigations. Thermophilic spore-forming bacteria in the marine sediments of Svalbard have been constantly reported (Vandieken et al., 2006; Hubert et al., 2009, 2010; Cramm et al., 2019). Their endospores with specialized cellular features that protect cells from extreme harsh environmental factors contribute to the survival of these thermophilic bacteria in Arctic sediments. Black smoker-associated hyperthermophiles utilize both adherence to suitable surfaces and fast motility as the driving forces for survivability in cold seawater for prolonged periods of time (Mora et al., 2014; Wirth, 2017). Wiegel (2002) proposed the hypothesis of temporary nanoniches in the mesobiotic environments. Such temporary nanoniches provide short-termed limited conditions for alkaliphilic thermophilic bacteria and therefore dictate fastened growth rate in order to cope with this limitation (Wiegel, 2002). Icelandic basaltic and rhyolitic glass and minerals in a sub-Arctic environment with the temperature range below required for thermophile activity were proposed to support transient growth of thermophiles in summer months (Cockell et al., 2015). The low albedo of these rocks has been suggested to facilitate their thermal conductivity and support the function of these igneous materials as microclimatic environments to harbor thermophilic microbial communities (Kelly et al., 2010, 2011; Cockell et al., 2015). Apparently, these nanoniches inside the rock pores provide a certain potential for their temporary "re-awakening" of such communities during the warm periods. On the molecular level, it has been suggested that microorganisms capable of growth in temperature range of both thermobiotic and mesobiotic environments may contain two different sets of key enzymes whose synthesis are regulated by temperature (Wiegel, 1990).

Understanding freezing tolerance and survival limits of thermophiles in low temperature habitats is also crucial for studies of microbial transfer through space and between celestial bodies, e.g., in the context of lithopanspermia. One of the scientific concepts for the origin and distribution of life is a long-distance shielded interplanetary viable transfer of the most ancient microbial forms of life entombed in lithic habitats (Mileikowsky et al., 2000; Fajardo-Cavazos et al., 2007; Horneck et al., 2008; Nicholson, 2009; Onofri et al., 2012; Kawaguchi et al., 2016). Extreme temperature fluctuations affect microbial "space travelers" during their interstellar transfer. Knowledge of the thermal limits of microbial life embedded into extraterrestrial mineral materials is crucial for envisaging the microbial survival during all lithopanspermia's stages (microbial launching due to the impact ejection from the planet of origin, long-term interplanetary traveling of microbes inside of rocks, and the capture of new life by the recipient planet). Furthermore, microbial cold stress experiments are helpful in terms of interpreting possible spatial and temporal environmental micro(nano)niches suitable for microbial life on Mars. Such near-surface microenvironments of Mars can provide potential habitable niches protected not only from UV, but also from extreme temperature fluctuations. Chin et al. (2010) indicated that chaotropic metabolites and chaotropic environments can increase tolerance to subzero temperatures, extending growth windows in cold ecosystems. Chaotropic ions in the Mars regolith might form microenvironments that support potential Martian biosphere, favoring the growth and preservation of a microbiota at low temperature. Comprehensive laboratory investigations on the survival limits of thermophiles in low temperature habitats can help to address the question if there are habitable niches on Mars today and if they harbor life.

However, our understanding of the physiology of how non-spore-forming thermophiles adapt to the cold is far from being explicit. The major limitation in the field comes from a restricted cultivation of thermophiles at low temperature regiment under laboratory conditions. Artificial culture media used under laboratory conditions usually fail to support cultivation of thermophiles at low-temperature range. One of the rare exceptions is *Geobacillus thermoleovorans* strain T80 which was cultivated at 4°C during a long-term period (9 month) and supported in soil microcosms at low temperatures during short-term experiments (1 week; Marchant et al., 2008). Wiegel (1990) described that extreme thermophile *Methanobacterium thermoautotrophicum* can grow between 22 and 78°C and the addition of sterile anaerobic sediments permitted its incubation at lower temperatures. The studies obtained under laboratory conditions show that hyperthermophiles can survive at least 9 months in cold surroundings when stored in low-temperature seawater (Mora et al., 2014). In this data report, we show that the extreme thermoacidophile *Metallosphaera sedula* is among a very few thermophiles supported at cold temperatures under laboratory conditions. Supplementation with the stony meteorite NWA 1172 permits the preservation of the thermoacidophile *M. sedula* under low temperature regime and provides further possibilities to study the molecular machinery and mechanisms implicated in survivability of non-spore-forming thermophiles at deep subfreezing temperatures.

RESULTS AND DISCUSSION

The heat-, acid-, and heavy metal-resistant *M. sedula* represents a robust microbiological subject for stress related investigations, with a number of studies published (Peeples and Kelly, 1995; Beblo et al., 2009, 2011; Maezato et al., 2012; Mukherjee et al., 2012; McCarthy et al., 2014; Milojevic et al., 2019a). At the same time, there is an evident gap of knowledge regarding cold stress reactions of this extreme thermophilic archaeon. *M. sedula* has been described as a well-defined, obligate thermophile which requires a temperature range from 50 to 80°C for growth, with an optimum of 73°C (Huber et al., 1989; Auernik et al., 2008). However, the sediments from Pisciarelli solfatara, a volcanic field near Naples, Italy, where *M. sedula* was first isolated, are much cooler, between 25 and 52°C (Huber et al., 1989). Employing standard culture techniques and evaluating microbial growth, it is obvious that none of the validated terrestrial energy sources (chalcopyrite, pyrite, and other inorganic electron donors) support the cultivation of *M. sedula* in this lower temperature range (Huber et al., 1989; Auernik et al., 2008). However, chemolithoautotrophic cultures of *M. sedula* in presence of preliminary sterilized meteorite material (the stony chondrite H5 type NWA 1172 Russell et al., 2002; Milojevic et al., 2019b) as the sole energy source permitted the maintenance of *M. sedula* during 2 months at the average temperature of 12°C (**Table S1**). Ramping down the cultivation temperature of *M. sedula* to this cold temperature regime was achieved by providing a regularly exchanged ice-supplemented environment for the glass bioreactors (**Figure 1**). Similar incubation on sulfide ores did not yield in detectable cells after 2 months (**Figure S1**). Examination by multi-labeled fluorescence *in situ* hybridization (MiL-FISH) with a *M. sedula*-specific 16S rRNA-targeted probe, confirmed the identification of cells from the cultures supplemented with the meteorite at cold regime as *M. sedula* cells (**Figure 2** and **Figure S2**). Furthermore, sequencing of the functional and phylogenetic *M. sedula* marker gene *msed0966* (putative rusticyanin gene; Auernik and Kelly, 2008, 2010a,b) as well as its 16S rRNA gene confirmed *M. sedula* presence in low temperature cultures (**Figure S3**). Additionally, the content of the cultures was analyzed by scanning electron microscopy (SEM) (**Figures 3A–C** and **Figure S4**). Interestingly, our SEM observations of cold-maintained *M. sedula* indicated a presence of an extracellular matrix evenly spread over the cell surface, appearing as a layer of cellular appendages wrapping around the colonies of *M. sedula* similar to a biofilm layer (**Figure 3** and **Figure S4**). In conjunction with these observations, when analyzing the cold-maintained cultures by fluorescence microscopy, slightly autofluorescing, dense, and opaque formations were observed (**Figures 2A–C**), which can be inferred to be the same aforementioned extracellular matrix. A branched extracellular matrix has not been detected in *M. sedula* cultures grown at 73°C on terrestrial minerals (Blazevic et al., 2019; Milojevic et al., 2019a) and NWA 1172 (Milojevic et al., 2019b), and might represent an adaptive feature of this thermophilic archaeon for withstanding stressful cold conditions. Another property of cold-maintained cells of *M. sedula* is their tendency to clump and condense into cellular aggregates (**Figure 3** and **Figure S4**), which is an additional possible strategy for coping with low temperatures.

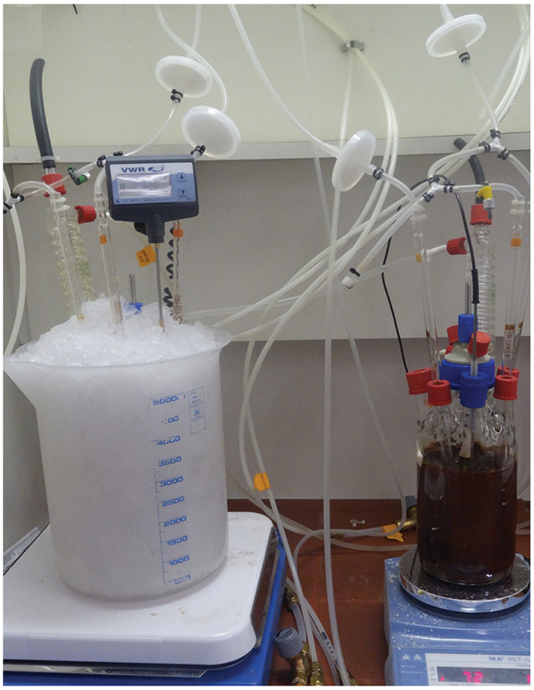

FIGURE 1 | Cultures of *M. sedula* at cold temperature regime. Fermentation set up for cultivation of *M. sedula* in 1L glassblower modified Schott-bottle bioreactors at 73°C (right) and at cold temperature regime in ice-supplemented environment with a tap water heat exchange system (left).

Our study indicates that the utilization of a specific mineral source and/or nutrients can influence the resistance to cold stress, contributing to the preservation of microbial cells in the cold. The characteristic inability of thermophilic *M. sedula* to be preserved in common laboratory media at temperatures below 50°C can be attributed to deficiencies in these media, e.g., in certain essential metabolites/metals which are indispensable for the cultivation of this organism at low temperatures, but that are not required at 73°C. Our previous studies depicted the beneficial contribution of NWA 1172 as the sole electron donor with a superior growth rate of *M. sedula* over chalcopyrite, suggesting preferential nature of this multimetalic material as energy source for *M. sedula* (Milojevic et al., 2019b). The NWA1172 stony meteorite is a non-carbonaceous H type ordinary chondrite, with high iron abundance (largely present in metallic form; Russell et al., 2002) and a wide range of other metal elements (Milojevic et al., 2019b). These metals might be alternatively used by *M. sedula* as specific metabolic cofactors offering more optimal structural and/or constitutive elements for enzyme activities, e.g., altering protein structural flexibility and shaping protein biophysical and biochemical properties.

A plausible explanation of the observed resistance of NWA 1172-grown *M. sedula* to cold stress can be inferred from heavy metals chaotropicity that extends the life window at low temperatures (Chin et al., 2010). Heavy metals may chaotropically enhance macromolecular flexibility (Chin et al., 2010; Cray et al., 2012) and so enable cell function/help maintain cellular structure at low temperatures. By exerting chaotropic activity heavy metals can cause specific toxic effects, inhibiting growth already at low concentrations in susceptible microorganisms. However, the studied archaeon *M. sedula* is an extreme metallophilic microorganism that tolerates elevated heavy metal concentrations (Maezato et al., 2012; Mukherjee et al., 2012; Blazevic et al., 2019; Milojevic et al., 2019a). Cultivation on multimetalic NWA 1172 meteorite

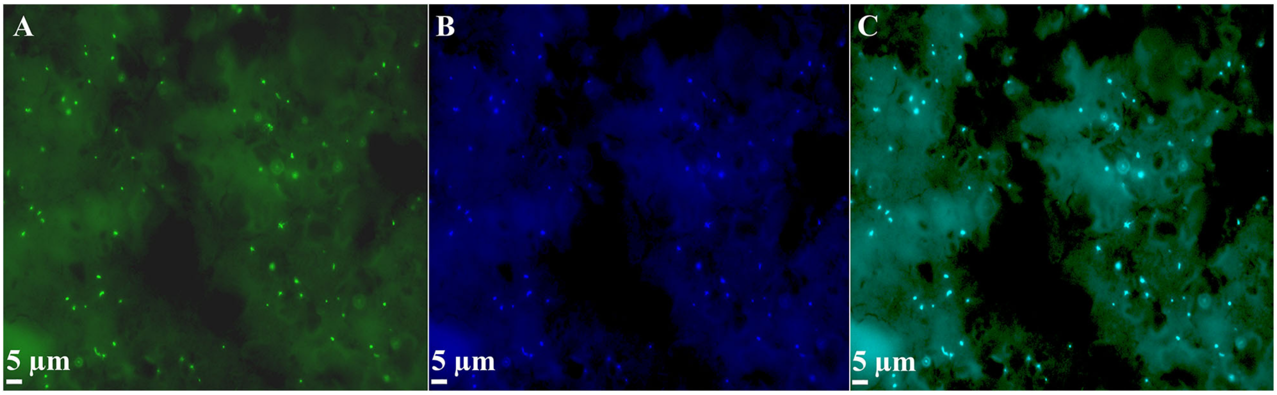

FIGURE 2 | MiL-FISH (multi-labeled fluorescence *in situ* hybridization) of *M. sedula* cells supplemented with the stony meteorite NWA 1172 as the sole energy source at cold temperature regime. **(A)** MiL-FISH image of cells (green) after hybridization with the specific oligonucleotide probe targeting *M. sedula.* **(B)** DAPI staining of the same field (blue). **(C)** Corresponding overlaid epifluorescence image, showing 100% overlap of specific probe with DAPI signals. Cultures of *M. sedula* were examined with MiL-FISH conducted as described in Kölbl et al. (2017) and Milojevic et al. (2019a) after 2 months of cultivation at the average temperature of 12°C.

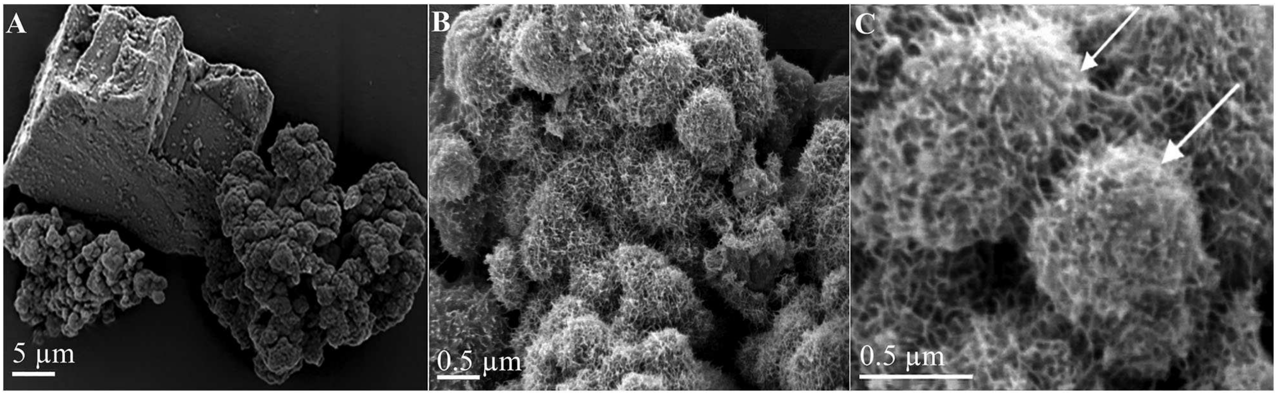

FIGURE 3 | Scanning electron microscopy images of *M. sedula* supplemented with the stony meteorite NWA 1172 as the sole energy source at cold temperature regime**. (A)** Scanning electron image, showing an angular mineral fragment of NWA 1172 clustered by *M. sedula* aggregated cells at the cold temperature regime. **(B,C)** Higher magnification scanning electron images displaying single cells of *M. sedula* covered with extracellular matrix. Cells of *M. sedula* are indicated with white arrows. Cultures of *M. sedula* were examined with SEM after 2 months of cultivation at the average temperature of 12°C.

(see Milojevic et al., 2019b for heavy metal composition of NWA 1172) exposes *M. sedula* to higher heavy metal concentrations under physiological conditions, thus helping to increase psychrotolerance. It is important to note that the effect of chaotropes that enhance microbial activity and growth at low temperatures appears to be initiated at around 10–12°C (Chin et al., 2010), which is the temperature range in our study. Thus, heavy metal chaotropicity may provide a mechanistic understanding of the increased psychrotolerance of *M. sedula* grown on NWA 1172.

CONCLUSION

In this study we report that when cultivated with meteorite, but not with chalcopyrite, cells of *M. sedula* can be preserved at cold temperature regime, denoting that the optimized nutrient fitness favor microbial preservation in extreme stressful conditions. Cultures of *M. sedula* supported at low temperature may serve as a laboratory model to explore metabolic potential of non-spore-forming thermophiles in psychrobiotic environments and the molecular mechanisms behind natural cryopreservation. It is important to note that future follow-up investigations should deliver a systematic study to determine which constituents of the meteorite are the crucial ingredients to ensure *M. sedula* culture preservation at low temperatures. In this regard, the survivability of *M. sedula* at low temperatures is a topic that deserves more attention and thorough analysis in the future. Moreover, the fact that an ancient inhabitant of terrestrial thermal springs and extreme thermophilic chemolithotroph *M. sedula* can be maintained at cold temperatures may complement the on-going scientific debates concerning psychrophilicity or thermophilicity of a last universal common ancestor (Wächtershäuser, 1992; Bada et al., 1994; Bada and Lazcano, 2002; Stetter, 2006; Akanuma et al., 2013), adding new circumstances in terms of survivability of ancestral thermophiles in cool primordial environments.

MATERIALS AND METHODS

Strain and Media Composition

Metallosphaera sedula (DSMZ 5348) cultures were grown aerobically as described previously (Kölbl et al., 2017; Blazevic et al., 2019; Milojevic et al., 2019a,b) in DSMZ88 *Sulfolobus* medium containing 1.3 g $(NH_4)_2SO_4$, 0.28 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7\ H_2O$, 0.07 g $CaCl_2 \cdot 2\ H_2O$, and 0.02 g $FeCl_3 \cdot 6\ H_2O$ dissolved in 1 L of water. After autoclaving, Allen's trace elements solution was added to 1 L media resulting in 1.80 mg $MnCl_2 \cdot 4\ H_2O$, 4.50 mg $Na_2B_4O_7 \cdot 10\ H_2O$, 0.22 mg $ZnSO_4 \cdot 7\ H_2O$, 0.05 mg $CuCl_2 \cdot 2\ H_2O$, 0.03 mg $Na_2MoO_4 \cdot 2\ H_2O$, 0.03 mg $VSO_4 \cdot 2\ H_2O$, and 0.01 mg $CoSO_4$ final concentration. The pH was adjusted to 2.0 with 10 N H_2SO_4. Chemicals of high purity grade were used for media preparation.

Cultivation Setup

Chemolithoautotrophic cultivation of *M. sedula* was performed as described before (Blazevic et al., 2019; Milojevic et al., 2019a,b) in DSMZ88 *Sulfolobus* medium defined above in 1L glassblower modified Schott-bottle bioreactors (Duran DWK Life Sciences GmbH, Wertheim/Main, Germany), operated with a thermocouple linked to a heating and magnetic stirring plate (IKA RCT Standard/IKA C-MAG HS10, Lab Logistics Group GmbH, Meckenheim, Germany) for agitation and temperature control. The cultivation of *M. sedula* under cold temperature regime was achieved by providing a regularly exchanged ice-supplemented environment of fermentation glass bioreactors (**Figure 1**). For this the bioreactors were placed in plastic vessels filled with crushed ice and additionally supplemented with a tap water heat exchange system, consisting of 10 mm inner diameter silicon tubes, which were connected to a tap water supply. Melting of ice due to the room temperature fluctuations resulted in an established dynamic temperature profile of *M. sedula* cultures with the average temperature of 12°C, which was monitored with an electronic thermocouple inside the bioreactor and with a thermometer outside the bioreactor. For fermentation at cold temperature regime, the media was maintained at room temperature for 2 h prior inoculation. Directly after inoculation the glass bioreactors were provided with ice-supplemented environment and water heat exchange system in order to maintain cold temperature regime. Inocula used were exponentially growing H_2-oxidizing autotrophic cultures of *M. sedula*. For chemolithoautotrophic growth cultures were supplemented with 10 g/liter either chalcopyrite (provided by E. Libowitzky from the mineral collection of the Department of Mineralogy and Crystallography, University of Vienna) or NWA 1172 (provided by the NHM, Vienna). The minerals were ground and temperature sterilized at 180°C in a heating oven for a minimum of 24 h prior to autoclaving (121°C, 20 min). Cells were monitored by phase contrast/epifluorescence microscopy. For the visualization of cells wiggling on solid particles they were stained by a modified "DAPI" (4′-6′- Diamidino-2-phenylindole) procedure (Huber et al., 1985), observed and recorded with ProgRes® MF cool camera (Jenoptik) attached to Nikon eclipse 50i microscope, operated with F36-500 Bandpass Filterset (ex, 377/50 nm; em, 447/60 nm).

PCR Assays for Marker Genes

DNA extracted by two different DNA isolation methods (Bead beating with Phenol-Chloroform extraction; MoBio PowerSoil DNA Isolation Kit) was used as template for PCR (Polymerase Chain Reaction) amplification. The PCR was targeting the 16S rRNA gene and the *msed0966* gene (*M. sedula* putative rusticyanin) with the archaeal-specific 16S rRNA primers (109f 5′- ACKGCTCAGTAACACGT-3′; 1492r 5′-GGYTACCTTGTTACGACTT-3′), the universal bacterial primers (27f 5′-AGAGTTTGATYMTGGCTCAG-3′; 1492r 5′-GGTTACCTTGTTACGACTT-3′) and newly designed *M. sedula msed0966* gene-specific primers msed0966f (5′-TACCTTCCCAATTACTACTCTCAGCAATCTGTCG-3′), and msed0966r (5′-CGTCACCACGATCTCCCC-3′), respectively. The obtained PCR products (**Figure S3**) were cloned and sequenced, retrieving *M. sedula* 16S rRNA and *msed0966* gene sequences.

Scanning Electron Microscopy

Cells of *M. sedula* harvested at stationary phase were prepared for electron microscopy by fixing in a solution of 1% (v/v) glutaraldehyde in Na-Cacodylate buffer. Samples were dehydrated in a graded series of ethanol solutions and dried chemically using Hexamethyldisilazan (HMDS). Fixed samples were mounted on aluminum stubs, sputter-coated with Au, and examined with a Hitachi S-4100 SEM (Hitachi, Tokyo, Japan).

Multi-Labeled-Fluorescence *in situ* Hybridization (MiL-FISH)

Metallosphaera sedula cells were fixed in 2% (v/v) paraformaldehyde (PFA) at room temperature for 1 h, washed three times in distilled water, centrifuged at 10,000 rpm and

TABLE 1 | Oligonucleotide probe used in this study.

Probe	Sequence 5′-3′ (reverse complementary)	Target gene	Label	Synthesis	Taxon	Target species	FA (%)	Color
M.sedula_174_ 17mer	AGA UUC CCU UGC CCG CU	16S rRNA	Atto488	Click chemistry	Archaea	*Metallosphaera sedula*	30	Green
EUB338	GCT GCC TCC CGT AGG AGT	16S rRNA	Atto488	Click chemistry	Bacteria	Most bacteria	30	Green

Probe name, nucleotide sequence—underscore indicates nucleotide: fluorochrome conjugates, target gene, label type, label synthesis, target taxon or higher, target species and probe color during imaging.

stored in 50:50, ethanol:PBS (phosphate buffer saline). A 16 S rRNA phylotype specific probe for *M. sedula* was designed with the software package ARB31 and labeled with 4x Atto488 via Click chemistry (biomers.net GmbH, Ulm, Germany; **Table 1**). Fixed cells were mounted on 10 well Diagnostica glass slides (Thermo Fisher Scientific Inc. Waltham, USA) and MiL-FISH conducted directly on them as described previously (Schimak et al., 2015; Kölbl et al., 2017; Milojevic et al., 2019b). Briefly, cells were hybridized with 30% (v/v) formamide for 16 h. For experimental positive controls *Gramella forsetii* strain KT0803 was hybridized with a 4x labeled general bacterial probe EUB338. Positive control for the specificity of the phylotype specific probe M.sedula_174 was given by including *M. sedula* DSM5348 in all experiments. After hybridization slides were washed for 15 min at 48°C [14–900 mM NaCl, 20 mM Tris-HCl (pH 8), 5 mM EDTA (pH 8), and 0.01% SDS (v/v)] at a stringency adjusted to the formamide concentration used. Cells were counterstained by incubation for 10 min with 10 mg ml^{-1} DAPI followed by rinsing in distilled water 3 times before CitiFluor (CitiFluor Ltd., London, England) mounting medium was applied to slides with a coverslip. Fluorescence images were taken with an AxioCam Mrm camera mounted on an Axioscope2 epifluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with F36-525 Alexa 488 (ex, 472/30 nm; em, 520/35 nm) filter cube. Images were recorded with the PC-based AxioVision (release 4.6.3 SP1) imaging software.

DATA AVAILABILITY STATEMENT

All data generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

ZZ, MS, and TM performed experiments and provided editorial input. All authors made extensive contributions to the analysis, acquisition, and interpretation of data provided in this report. All authors reviewed the report and accepted the final version of it.

FUNDING

This study was supported by the Austrian Science Fund (FWF) within an Elise-Richter Research Program V333 Iron- and Sulfur-oxidizing Machinery of the bioleaching Archaeon *Metallosphaera sedula*.

ACKNOWLEDGMENTS

We warmly thank Andreas Klingl and Michaela Stieglmeier (Biocenter LMU Munich, Germany) for the support of with electron microscopy investigations. Christian Koeberl, Ludovic Ferrière, and Brandstätter Franz (Natural History Museum of Vienna, Austria) are deeply acknowledged for providing the NWA 1172 meteorite sample and fruitful discussions. Simon K.-M. R. Rittmann, Christa Schleper, and the other team members of Archaea Biology and Ecogenomics Division (University of Vienna) are greatly appreciated for fruitful discussions and help with low temperature cultivation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fspas.2020.00037/full#supplementary-material

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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.

frontiers
in Astronomy and Space Sciences

BRIEF RESEARCH REPORT
published: 11 August 2020
doi: 10.3389/fspas.2020.00041

Desiccation of the Extreme Thermoacidophile *Metallosphaera sedula* Grown on Terrestrial and Extraterrestrial Materials

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Prokaryotes are among the most versatile organisms on Earth and their ability to adsorb metals for nutrient, energy, or protection purposes can be noted in many different environments on our planet. The extreme thermoacidophilic archaeon *Metallosphaera sedula* is a metal-mobilizing archaeon capable of redox transformations during chemolithoautotrophic growth on diverse metal-bearing compounds. Examining the interfaces of this extreme metallophilic archaeon with various metal-bearing substrates of terrestrial and extraterrestrial origin, we have detected its selective preservation after desiccation. Cultivated on specific metal-bearing materials, e.g., tungsten-bearing scheelite, tungsten-bearing polyoxometalate, multimetallic waste products, and the NWA 1172 meteorite, cells of *M. sedula* can be preserved after dehydration, and therefore can potentially serve as a microbial fingerprint of the presence and/or activity of metal-transforming microorganisms. Preservation of desiccated *M. sedula* cells reported in this study has a discriminatory character, depending on the content and nature of the metal-containing compound used for cultivation of this metallophilic microorganism. The achieved preservation of dehydrated *M. sedula* cells facilitates our survivability studies with this desiccated microorganism during future space exposure experiments and under simulated space environmental conditions.

Keywords: desiccation, meteorite, *Metallosphaera sedula*, metals, SEM

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Specialty section:
This article was submitted to Astrobiology, a section of the journal Frontiers in Astronomy and Space Sciences

***Received:** 22 April 2020*
***Accepted:** 16 June 2020*
***Published:** 11 August 2020*

Citation:
Kölbl D, Blazevic A, Albu M, Fasching C and Milojevic T (2020) Desiccation of the Extreme Thermoacidophile Metallosphaera sedula Grown on Terrestrial and Extraterrestrial Materials. Front. Astron. Space Sci. 7:41. doi: 10.3389/fspas.2020.00041

INTRODUCTION

Liquid water is a vital requirement for life on our planet and is the main research focus for scientists looking for life beyond Earth. Nonetheless, various organisms, including unicellular and multicellular life forms, can be preserved in dehydrated settings for long periods. However, dehydration remains a severe stress that leads to cellular perturbation interfering with survival. It has been widely accepted that intracellular compatible solutes impact microbial tolerance to desiccation. A potential positive influence of compatible solutes, e.g., trehalose or sucrose, on cell membranes during dehydration has been shown by Hincha and Hagemann (2004). The efficient ability of trehalose to preserve the function of dried biological macromolecules is widely used for protecting cell membranes and proteins from extreme temperatures, desiccation, and osmotic shock in a wide range of microorganisms. Trehalose biosynthesis genes were described in the genomes of thermophilic members of the *Sulfolobaceae* family, including *Metallosphaera* spp. (Seo et al., 2008; Okazaki et al., 2012; Moon et al., 2016). In addition to metabolic defense with non-reducing sugars, key molecular elements for the repair of dehydration effects (e.g., oxidative stress and DNA damage) might also be engaged by microorganisms. Apart from intracellular molecular protectants, production of stress-induced biofilms can have a substantial influence on the dehydration tolerance of thermophilic archaea and bacteria, physically preventing water loss in the cells (La Paglia and Hartzell, 1997; Mortel and Halverson, 2004; Chang et al., 2007; Beblo et al., 2011). Another strategy, an inorganic protection by sulfidic ore particles, has been described for the extreme thermophile *Hydrogenothermus marinus* exposed to a combination of desiccation and irradiation (Beblo et al., 2011), asserting a "shielding by ore" effect.

A multitude of multifunctional biogenic structures emerged repeatedly and independently over the course of Earth's history. Armor as a protective mechanism against harsh environmental conditions has been particularly successful. Multicellular organisms, e.g., chitons, have taken advantage of metal-encrusted armor for more than 200 million years (Li et al., 2015). Metal-bearing cell walls of single-celled microorganisms can serve as a protective armor against phytoplankton predators. Microbial survival in extreme environments with high metal concentrations is usually connected with cell surface absorption and/or extracellular polymeric substance (EPS) complexation of various surrounding metals (Schultze-Lam et al., 1996; Loaëc et al., 1998; Gupta and Diwan, 2017; Hickman-Lewis et al., 2019). Moreover, for endolithic and metal-respiring microorganisms, metal accumulation on the cell surface can serve as a mechanism for nutrient and energy source. Certain cell wall structures serve as a nucleation site and might enhance the metal accumulation process by providing a surface that promotes adsorption or precipitation of metals. Prokaryotes (e.g., cyanobacteria, proteobacteria, archaea) are able to adsorb metals, and even radionuclides on their surfaces (proteinaceous S-layer, sheaths, capsules, cell walls), which can serve as an extracellular nucleation site (van Gemerden, 1986; Beveridge, 1989; Ehrlich, 1999). The potential to withstand and promote metal accumulation followed by subsequent cell preservation has been examined using different microbial strains (archaea and bacteria); however, only limited reports exist revealing archaeal–metal interactions. To gain deeper insight into what drives microbial metal accumulation, precipitation, and subsequent cellular preservation, microbes from the *Bacteria* and *Archaea* kingdoms have been artificially encrusted (impregnated) with metals since the 1970s. Cyanobacteria have been successfully silicified, which leads to microbial structures that are well-preserved and thoroughly embedded in a crystalline silica matrix (Oehler and Schopf, 1971). The first experimental silicification of archaeal strains was performed with two strictly anaerobic and hyperthermophilic microorganisms, *Methanocaldococcus jannaschii* and *Pyrococcus abyssi* that could have inhabited hydrothermal environments on the early Earth and probably the early Mars as well. The obtained observations demonstrated that not all (micro)organisms are susceptible to metal precipitation and preservation; they behave differently even though they are closely related phylogenetically. Although both strains possess similar cell wall structures, most *M. janaschii* cells could not withstand silicification and lysed quickly, whereas silica precipitated on the cell wall of *P. abyssi* and its cells were well-preserved (Orange et al., 2009). Experimental silicification of the Gram-positive bacterial species *Geobacillus* SP7A over a period of 5 years led the authors to the conclusion that this species impregnates with silica faster than other thermophilic or mesophilic Gram-negative bacteria and archaea, due to a Gram-positive characteristic thick peptidoglycan layer, containing abundant anionic functional groups as primary silica binding sites (carbonyl, hydroxyl, phosphoryl groups). Furthermore, fast and efficient metal accumulation processes seem to be crucial to prevent degradation of organic material and to preserve the structural integrity of cellular material throughout a long period of time (Li et al., 2014; Orange et al., 2014). Rapid and high-precision preservation of microorganisms in the geological record is of utmost importance for the successful fossilization process (Orange et al., 2014). Experimental fossilization as a tool to study the preservation potential of different Bacterial and Archaeal communities could improve our understanding of the nature of Earth's earliest fossils. Importantly, the experimental fossilization approach enables thorough investigation of the influence of geological/mineralogical settings on microbial preservation potential. To extend the search of preserved biomarkers from Earth to Mars, the polyextremotolerant bacterium *Yersinia intermedia* was artificially encrusted by silica and gypsum under cold and anoxic settings, similar to current Martian conditions. *Yersinia* cells interacted immediately with the metal-bearing materials, which favors the preservation of cells during aging due to early entombment in a metal-bearing matrix (Gaboyer et al., 2017). The capacity of biosorption depending on a specific cell wall structure has been examined in two halophilic archaeal strains of *Halobacterium noricense*, which sequesters uranium in cell agglomerates and structures (Bader et al., 2017).

The extreme thermoacidophilic archaeon *Metallosphaera sedula* is a metal-mobilizing organism capable of redox transformations of a variety of metal-bearing substrates (Huber et al., 1989; Peeples and Kelly, 1995; Auernik and Kelly, 2008, 2010a; Maezato et al., 2012; Mukherjee et al., 2012; Wheaton et al., 2016; Kölbl et al., 2017; Blazevic et al., 2019; Milojevic et al., 2019a,b). Our recent studies indicate that this metallophilic archaeon can form a metal-bearing crust encasing its S-layer (Blazevic et al., 2019; Milojevic et al., 2019a,b). Examining the physiology of this extreme metallophilic archaeon cultivated with diverse metal-bearing substrates of terrestrial (Blazevic et al., 2019; Milojevic et al., 2019a) and extraterrestrial origin (Kölbl et al., 2017; Milojevic et al., 2019b), we have observed its selective preservation under the conditions of desiccation. Here, we report on several cases of the metal-grown polyextremophilic archaeon *M. sedula*, which were particularly beneficial to preserve its viability and cellular integrity after long-term desiccation.

MATERIALS AND METHODS

Strain and Media Composition

Metallosphaera sedula (DSMZ 5348) cultures were grown aerobically as described before (Kölbl et al., 2017) in DSMZ88

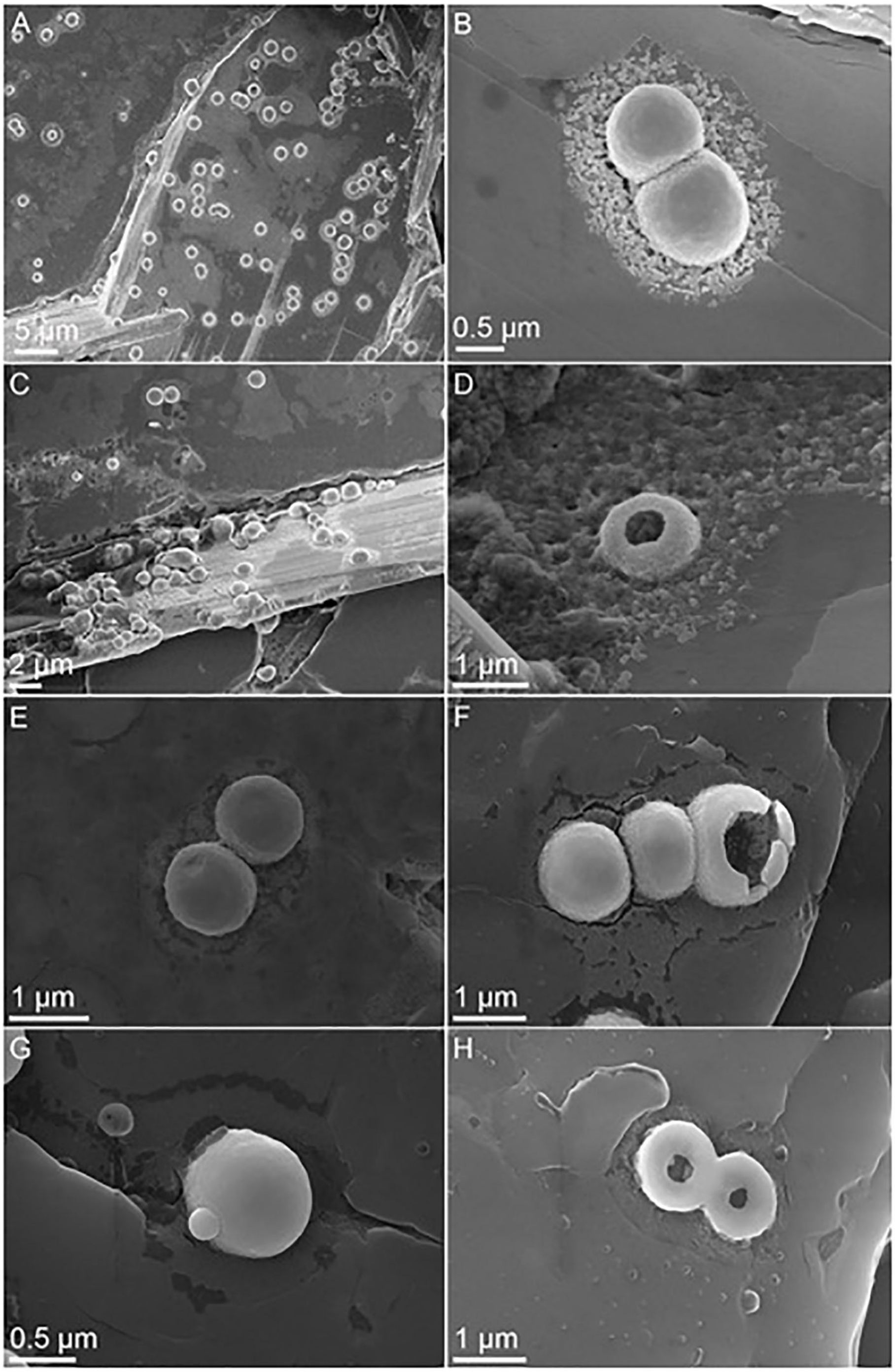

FIGURE 1 | Scanning electron microscopy (SEM) images of desiccated *M. sedula* cells grown on tungsten (W) ore scheelite and preserved after 2 months of dehydration. **(A,C)** SEM images of dehydrated colonies of *M. sedula* cells grown on scheelite. **(B)** Magnified SEM image showing single scheelite-grown cells of *M. sedula* preserved after dehydration. **(D–H)** SEM images of intact cells of *M. sedula* maintaining the integrity after dehydration and broken dried cells of *M. sedula* revealing their cellular interior.

Sulfolobus medium containing 0.28 g KH_2PO_4, 1.3 g $(NH_4)_2SO_4$, 0.07 g $CaCl_2 \cdot 2\ H_2O$, 0.25 g $MgSO_4 \cdot 7\ H_2O$, and 0.02 g $FeCl_3 \cdot 6\ H_2O$ dissolved in 1 L of water. After autoclaving, Allen's trace elements solution was added to 1 L of media resulting in 4.50 mg $Na_2B_4O_7 \cdot 10\ H_2O$, 1.80 mg $MnCl_2 \cdot 4\ H_2O$, 0.05 mg $CuCl_2 \cdot 2\ H_2O$, 0.22 mg $ZnSO_4 \cdot 7\ H_2O$, 0.03 mg $VSO_4 \cdot 2\ H_2O$, 0.03 mg $Na_2MoO_4 \cdot 2\ H_2O$, and 0.01 mg $CoSO_4$ (final concentration). The pH was adjusted with 10 N H_2SO_4 to 2.0.

Cultivation Setup

Chemolithoautotrophic cultivation of *M. sedula* was performed in DSMZ88 *Sulfolobus* medium defined above as described earlier (Milojevic et al., 2019a,b). Chemolithoautotrophic cultures were supplemented with 10 g/L of (i) a tungsten-bearing scheelite ore containing impurities of Mn and Fe oxides (Blazevic et al., 2019; see **Supplementary Table 1** for chemical composition); (ii) the Dawson-type tungsten-bearing polyoxometalate W-POM ($K_6[\alpha\text{-}P_2W_{18}O_{62}]\cdot nH_2O$) (Contant et al., 2007; Milojevic et al., 2019a); (iii) the stony meteorite NWA 1172 (H5 ordinary chondrite Northwest Africa 1172) (Russell et al., 2002; Milojevic et al., 2019b; see **Supplementary Tables 1, 2** for chemical and mineralogical composition); (iv) multimetallic waste products (voestalpine BÖHLER Edelstahl GmbH & Co KG); and (v) multimetallic Martian regolith simulants (MRSs; Kölbl et al., 2017; see **Supplementary Tables 1, 2** for chemical and mineralogical composition). The metal-containing compounds were temperature sterilized in a heating chamber (180°C) for a minimum of 24 h prior to autoclaving (121°C for 20 min). Abiotic controls containing uninoculated culture media with all aforementioned metal-bearing sources were included through all the experiments. Growth of cells was examined by phase contrast/epifluorescence microscopy and metal release (Kölbl et al., 2017; Milojevic et al., 2019a,b).

Dehydration Experiments

Dehydration of *M. sedula* cultures grown on various metal-bearing materials was performed under oxic laboratory conditions and under atmospheric pressure. For the dehydration experiments, cultures of *M. sedula* autotrophically cultivated on metal-bearing materials were harvested at middle stationary phase (**Supplementary Table 3**) omitting centrifugation and concentration, deposited by spreading evenly on glass plates (VWR International, Ø7 cm), and desiccated at room temperature within 60 days. Abiotic controls containing uninoculated culture media with the corresponding metal-bearing sources were included throughout the experiments. The morphology of the desiccated cells of *M. sedula* and crystalline and amorphous precipitates were examined by means of scanning electron microscopy (SEM). The contents of glass plates with desiccated cells were transferred into the culture media supplemented with 1% tryptone extract and incubated heterotrophically in a shaking orbital bath at 73°C. Growth of the cells was monitored during 1-week post-inoculation by phase-contrast/epifluorescence microscopy with 60× and 100× magnification (**Supplementary Figure 1**).

Scanning Electron Microscopy

The precipitates obtained after dehydration experiments were examined with a Zeiss Supra 55 VP scanning electron microscope, operated with a field emission gun (Schottky-FE, DENKA). Prior to SEM, the dehydrated samples were coated with a 3 nm Au/Pd layer (spincoater Laurell WS-650-23).

Statistical Analysis

The Excel 2016 (version 7.0) and Sigma plot (version 13.0) software packages were used to perform statistical analysis and graphical representation of the obtained data.

RESULTS

Dehydration of *M. sedula* Cells

The cultures of *M. sedula* chemolithoautotrophically grown on various metal-bearing materials (**Supplementary Table 2**, Kölbl et al., 2017; Blazevic et al., 2019; Milojevic et al., 2019a,b) were dehydrated for 60 days at room temperature under atmospheric laboratory conditions. The surface of the precipitates obtained after dehydration was examined using SEM. The cell cultures were deposited and dehydrated in their respective glassware as monolayers composed of single cells (**Figures 1–3**), thus avoiding protective "shielding" effect of cellular multilayers during dehydration. In the case of *M. sedula* grown on MRSs, no preserved cells were detected after the 60-day period of dehydration (**Figures 3G–J**).

Preservation of Desiccated *M. sedula* Cells Grown on Tungsten (W) Ore Scheelite

SEM analysis revealed that after a long-term treatment under dehydration conditions (up to 2 months), most *M. sedula* cells grown on scheelite tungsten ore as the sole energy source (Blazevic et al., 2019) remained intact, did not show significant alteration in the overall morphology, and preserved their cellular integrity, maintaining the structural stability and exposing a fine-scale irregular surface (**Figures 1A–C**). The cells of *M. sedula* with budding vesicles attached to the cell surface occurred in this case as well (**Figure 1G**). To a minor extent, lysed and broken cells were clearly recognizable too, revealing their empty interior content (evidence of cell lysis, **Figures 1D,F,H**).

Preservation of Desiccated *M. sedula* Cells Grown on Tungsten Polyoxometalate (W-POM)

Dehydration by evaporation (up to 2 months) has been applied toward microbial cultures harvested after cultivation of *M. sedula* with W-POM (Milojevic et al., 2019a). The crystalline material obtained after dehydration was examined by SEM. Colonies of *M. sedula* cells were found attached to the surface of the obtained material (**Figures 2A–D**), revealing that after long-term treatment under dehydrating conditions, cells of *M. sedula* visually remained undamaged, did not show SEM-detectable alterations in the overall morphology, and preserved their cellular integrity in frames of SEM detection, exposing a fine-scale irregular surface (**Figures 2C,D**). Particularly in regard with W-POM grown cells, no broken cells or cells with a damaged cell surface were detectable after a long-term dehydration. Physical reorganization of cellular structures after dehydration did not occur, nor was structural evolution of the cell surface after the dehydration process observed in this case (**Figure 2**). In addition, the desiccated cells forming budding vesicles were recognizable too (**Figure 2**).

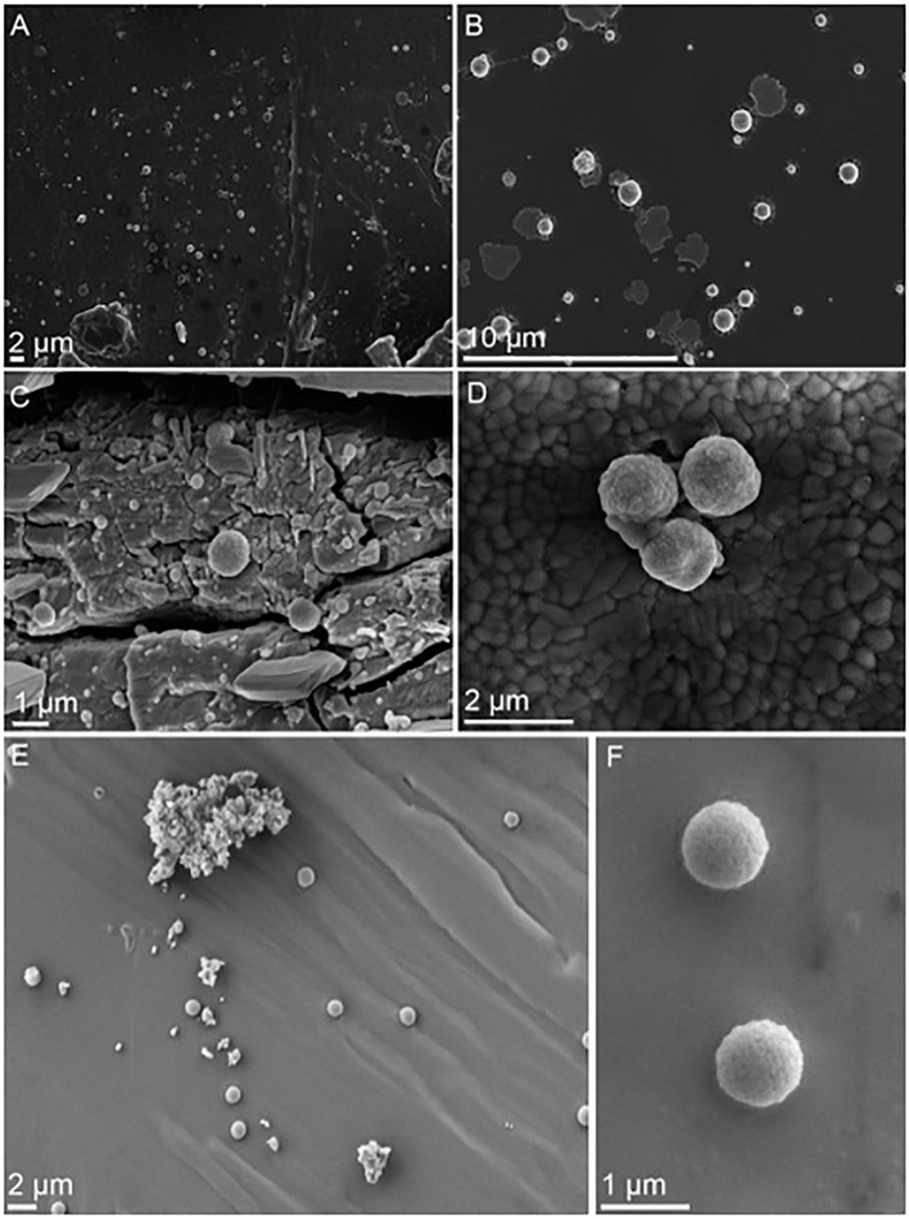

FIGURE 2 | SEM images showing desiccated cells of *M. sedula* cultivated with W-POM and multimetallic waste material after 2 months of dehydration. **(A–D)** SEM images of dehydrated *M. sedula* cells cultivated with W-POM. **(E,F)** SEM images of dehydrated *M. sedula* cells cultivated with multimetallic waste material.

Preservation of Desiccated *M. sedula* Cells Grown on Multimetallic Waste Material

Cells were harvested after cultivation with multimetallic waste material as the sole energy source and subjected to dehydration by slow evaporation for up to 2 months. Extensive post-dehydration monitoring of these cultures was performed. SEM-assisted evaluation of the post-dehydration structural integrity revealed intact colonies of dried *M. sedula* cells attached to the surface of the obtained dehydrated material (**Figures 2E,F**). Single undamaged dried cells were inspected after a long-term treatment with dehydrating conditions. The cells did not show alterations in their overall morphology and preserved their cellular integrity, exposing a fine-scale irregular surface (**Figures 2E,F**).

Preservation of Desiccated *M. sedula* Cells Grown on the NWA 1172 Meteorite

Cultures of *M. sedula* grown on the stony meteorite Northwest Africa 1172 (NWA 1172; an H5 ordinary chondrite; Milojevic et al., 2019b) were subjected to a dehydration procedure for 60 days. Dehydration of *M. sedula* cultures by evaporation resulted in the formation of amorphous and crystalline materials.

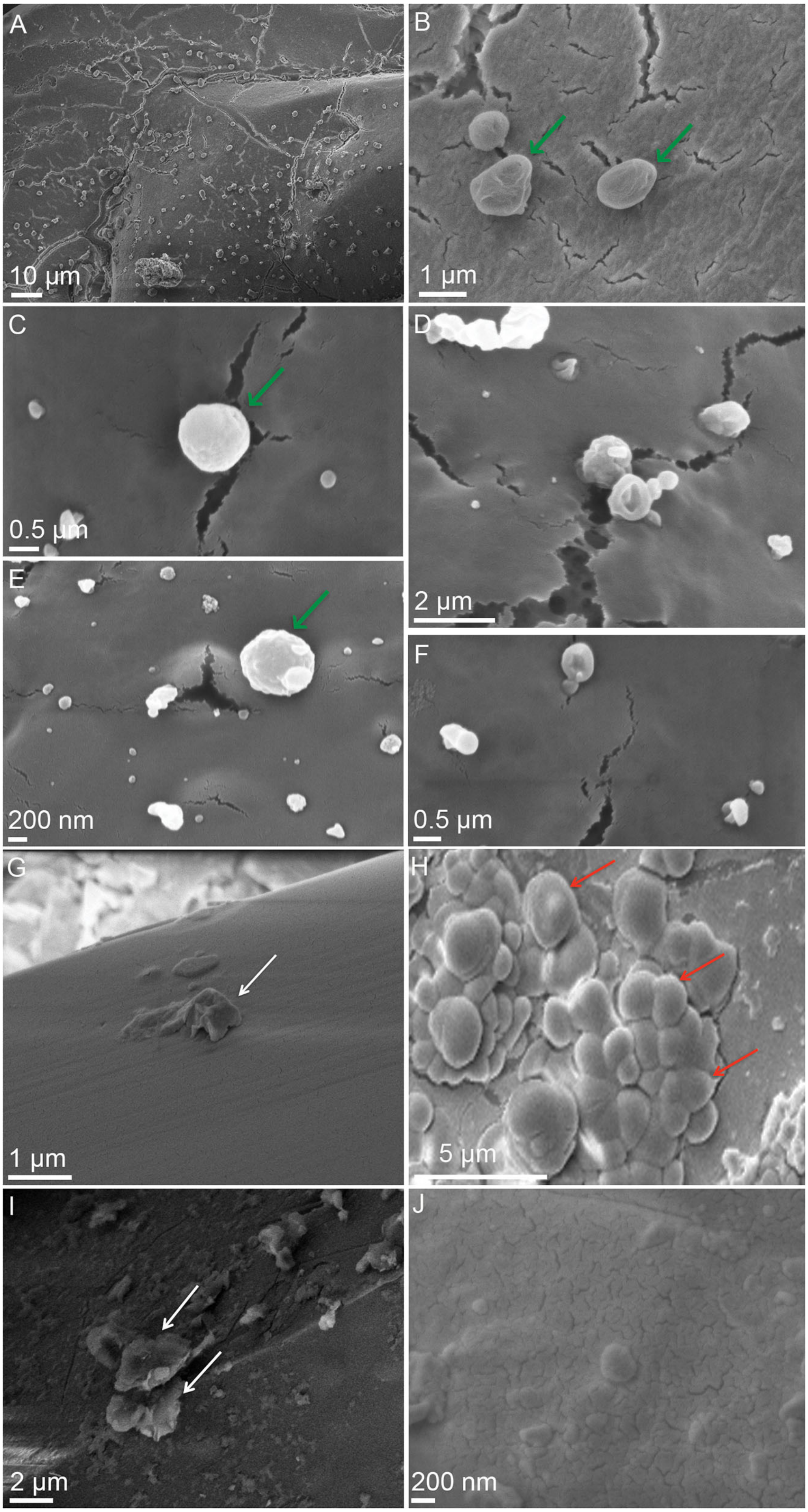

FIGURE 3 | SEM images showing desiccated cells of *M. sedula* cultivated with the stony meteorite NWA 1172 and surfaces of Martian regolith simulants (MRSs) dehydrated for 2 months after cultivation with *M. sedula*. **(A–F)** SEM images of dehydrated *M. sedula* cells cultivated with NWA 1172. **(B,C,E)** Magnified SEM images *(Continued)*

FIGURE 3 | showing single cells of *M. sedula* preserved after dehydration. **(D,F)** Magnified SEM images of broken dried cells of *M. sedula* revealing their cellular interior. **(G–J)** SEM images of MRSs dehydrated for 2 months after cultivation with *M. sedula*. **(G)** Scanning electron image showing a surface of precipitate obtained after the cultivation of *M. sedula* on JSC 1A. **(H)** Scanning electron image showing a surface of precipitate obtained after the cultivation of *M. sedula* on P-MRS. **(I)** Scanning electron image showing a surface of precipitate obtained after the cultivation of *M. sedula* on S-MRS. **(J)** Scanning electron image showing a surface of precipitate obtained after the cultivation of *M. sedula* on MRS07/52. *M. sedula* cells are shown with green arrows. White arrows indicate cell debris (e.g., cells and EPS remnants). Al-rich microspheroids are depicted with red arrows. Representative SEM-EDS analysis of carbon-rich cells, cell debris, and Al-rich microspheroids is provided in **Supplementary Figure 2**.

Our SEM analysis, performed after the dehydration period, displayed *M. sedula* cells deposited on the surface of the obtained amorphous and crystalline material (**Figures 3A–F**; see **Supplementary Figure 2C** for SEM-EDS analysis). Most of these desiccated cells remained intact on the level of SEM detection and visually preserved their cellular and structural integrity, exposing a fine-scale irregular surface (**Figures 3B,C,E**). Flourishing and dividing cells occurred as well; however, lysed and broken cells were clearly recognized too, revealing their empty interior content (evidence of cell lysis) (**Figures 3D,F**). Precipitated nanoglobules were detected attached to the surface of *M. sedula* cells in this case as well (**Figures 3D,E**).

Dehydration of *M. sedula* Grown on MRSs

Cultures of *M. sedula* grown on multimetallic MRSs as the sole energy sources (Kölbl et al., 2017) were subjected to dehydration by slow evaporation for 2 months at room temperature under atmospheric laboratory conditions. Neither preserved cells, nor cellular-like morphologies with broken, damaged, or lysed exterior were detected on the surface of dehydrated MRSs precipitates (**Figures 3G–J**). In the case of MRSs, we solely observed the formation of the aluminum/chlorine containing microspheroids published previously (Kölbl et al., 2017; **Figure 3H** and **Supplementary Figure 2A**). These hemispheroid formations were mostly composed of oxygen, chlorine, and aluminum and were characterized as nearly carbon-free metal inclusions, thus excluding the cellular nature of these morphologies (Kölbl et al., 2017; **Figure 3H** and **Supplementary Figure 2A**). Apart from aluminum/chlorine containing microspheroids, the deposition of cell debris materials was frequently detected in dehydrated cultures of *M. sedula* grown on MRSs (**Figures 3G,I** and **Supplementary Figure 2B**).

Survivability of *M. sedula* After Desiccation

The cells of *M. sedula* grown on various metal-bearing substrates after desiccation treatment for 2 months were transferred into the culture medium and were allowed to recover heterotrophically for 120 h. The cells of *M. sedula* grown and desiccated on NWA 1172, multimetallic waste, and tungsten-bearing materials (scheelite and W-POM) possess the ability to survive desiccation (**Figure 4**). The cells of *M. sedula* grown on four MRSs (JSC-1A, P-MRS, S-MRS, and MRS 07/52) showed impeded recovery after desiccation treatment when compared to NWA 1172, multimetallic waste, and tungsten-bearing materials scheelite and W-POM (**Figure 4**). Overall, heterotrophic re-cultivation of desiccated *M. sedula* cells on NWA 1172 yielded almost 2-fold higher cell number after 120 h compared to multimetallic waste, scheelite, and W-POM, showing a comparable re-cultivation capacity of cells grown on these substrates. The reduction of cell number after the desiccation treatment (“0” time point) and the subsequent impeded recovery of cells (120 h) was observed with all four types of tested MRSs (**Figure 4**). The cells grown and desiccated on MRS-07/52 exhibited the highest recovery among all four types of tested MRSs, which was however about two orders of magnitude lower than the recovery of the cells grown and desiccated on NWA 1172 and again, a 70- to 100-fold lower when compared to multimetallic waste and both tested tungsten-bearing materials (**Figure 4**). Among all examined metal-bearing substrates, the highest number of recovered cells after 120 h of post-desiccation incubation was observed in the case of NWA 1172 (**Figure 4**).

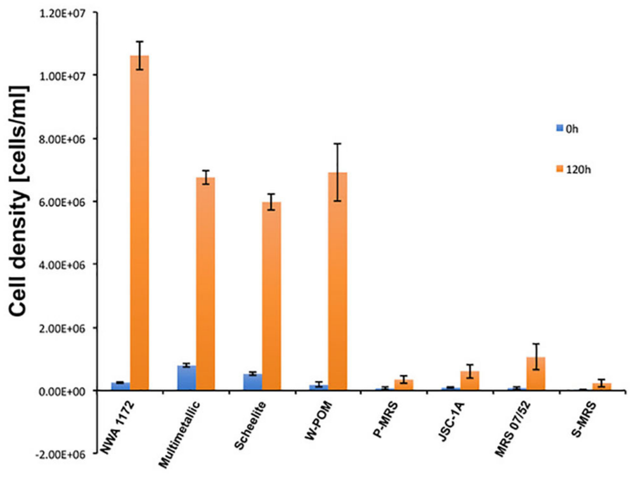

FIGURE 4 | Cell densities of *M. sedula* cells recovered after desiccation for 2 months. Blue bars represent cell counts after immediate re-cultivation in heterotrophic medium (“0”), orange bars represent cell counts after 120 h of growth after re-culturing. Columns and error bars show the mean and error-represented standard deviation, respectively, of $n = 3$ biological replicates. If not visible, error bars are smaller than symbols.

DISCUSSION

Our results showed that cells of *M. sedula* cultivated with certain metal-bearing materials (tungsten-bearing substrates, the stony chondrite meteorite NWA 1172, and multimetallic waste product) can be well-preserved (**Figures 1–3**) and recovered after dehydration up to 60 days under atmospheric conditions (**Figure 4**). Dehydrated cells of *M. sedula* cultivated on the solid metal-bearing materials were dried in a single cell layer to avoid microbial cell-aggregates, such that a "cell-by-cell shielding effect" expected in cellular multilayers is minimized. We have previously shown that metal incorporation by the cell surface of *M. sedula* leads to the formation of a metal crust around a cell (Blazevic et al., 2019; Milojevic et al., 2019a,b). Such metal-bearing crust might protect cell integrity, providing an additional barrier against complete water loss by the cell and thus retains a minimal level of the water phase, with implication for microbial preservation in severe dehydrated conditions. Various metal-containing precipitates and accumulations were reported previously on the S-layer of other extremophilic bacteria and archaea (Orange et al., 2011, 2014; Oggerin et al., 2013; Sanchez-Roman et al., 2015; Kish et al., 2016). Interestingly, cell surface deposition of metals and subsequent cellular encrustation does not necessarily lead to the cell entombment and death. Microorganisms capable of metal sorption and encrustation have developed strategies to deal with such micro-barriers. Recent work conducted with the archaeon *S. acidocaldarius* (closely related to *M. sedula*) demonstrated that this microorganism forms encrusted outer membrane vesicles when heavily encrusted with Fe-containing accumulations (Kish et al., 2016). This strategy is likely attributed to the removal of impaired S-layer proteins to enable substitution with new, precipitate-free proteins. Elimination of damaged encrusted S-layer portions may serve as an important strategy for *Sulfolobales* members to regenerate their cell wall structure and survive in extreme environments.

Moreover, differences in preservation of dried *M. sedula* cells were observed depending on the nature of metal-bearing materials used for cultivation of this chemolithotroph. In contrast to cultures grown on tungsten-bearing substrates, the NWA 1172 meteorite, and multimetallic waste products, no cells after desiccation could be recovered or detected when *M. sedula* was cultivated on sulfide ores, elemental sulfur, molecular hydrogen (Beblo et al., 2009, 2011), volcanic glass (**Supplementary Figure 3**), and synthetic extraterrestrial materials such as MRSs (**Figures 3G–J**). The chemolithotrophic growth of *M. sedula* on all these metal-bearing matrixes has been well documented (Huber et al., 1989; Auernik and Kelly, 2008, 2010a,b; Kölbl et al., 2017; Blazevic et al., 2019; Milojevic et al., 2019a,b). Similar to our observations, Beblo et al. (2009) also reported no survival after long-term desiccation of *M. sedula* grown on elemental sulfur, sulfide ores, or on solfatara sand. In these cases, it is possible that even when cells of *M. sedula* were subjected to metal sorption, the composition of these metal-containing substrates did not provide a sufficient protection layer to resist dehydration. The observed selective preservation of *M. sedula* cells after desiccation in these cases can be explained by a discriminating "shielding by metal nano-precipitates" effect. Apparently, only certain cell wall–metal interactions can strengthen prokaryotic cell envelopes and augment their structural stability implicating in cell preservation during harsh long-term dehydration. In this regard, the tungsten incorporation by the cell envelope of *M. sedula* (Blazevic et al., 2019; Milojevic et al., 2019a) may certainly serve as an efficient strengthening strategy, as this is a hard element with the highest melting point and extraordinary properties among all metals. Being suitable for high-temperature applications in energy and lighting technology, and in the space industry, it is also used as alloys, superalloys, and radiation-shielding. We have previously reported that *M. sedula* (cultivated on scheelite and W-POM) mineralizes its S-layer via encrusting with crystalline nanoparticles containing tungsten carbide-like structures (Blazevic et al., 2019; Milojevic et al., 2019a). Tungsten exhibits a hardness of ∼9–9.5 on the Mohs hardness scale (Tabor, 1954) and can potentially provide an efficient barrier against water loss, warranting preservation of cell integrity after desiccation. *M. sedula* cells grown on the NWA 1172 meteorite are also heavily mineralized with the amorphous crust containing a $Cu_xFe_yO_z$(SPNiAl)–SiO_2 product (Milojevic et al., 2019b). This multimetallic crust of mixed Ni/Al/Si content might have implications for the preservation of desiccated cells. Growth on elemental sulfur and related sulfur-bearing substrates (Beblo et al., 2009, 2011) as energy sources would imply sulfur incorporation by S-layer of *M. sedula*. Sulfur is a soft and very brittle material [2 on the Mohs scale of mineral hardness Tabor, 1954]. Therefore, sulfur-bearing crust might not provide sufficient mechanic protection against desiccation and sulfur-supplemented growth represents a contrasting case in which the substrate does not provide the required raw materials for defense against desiccation stress.

In environments of low organic carbon content, high temperature, acidic pH, and iron-rich surroundings, microorganisms face multiple challenges maintaining their cell population and growth. The four MRSs used in this study were modeled to represent global Martian regolith chemistry and are therefore limiting possible microbial–mineral interactions compared to natural minerals (e.g., NWA 1172, scheelite ore). In addition, these synthetic mixtures do not take Mars' actual Fe^{2+}/Fe^{3+} ratios into account (Ramkissoon et al., 2019), generating regolith simulants high in Fe^{3+} rather than abundantly detected and metabolically feeding Fe^{2+} on the planet's surface (Boynton et al., 2008; Nixon et al., 2013). Elevated cultivation temperatures for *M. sedula*, the high ferric iron content, and the synthetic and therefore trace metal-depleted nature of the used MRSs (**Supplementary Table 1**) could have played a major role in the degradation of organic matter, since organic carbon is thermodynamically unstable in the presence of abundant Fe^{3+} (Sumner, 2004; Hays et al., 2017), even though there is evidence for organic matter-Fe coprecipitation and conservation over extended timescales (Lalonde et al., 2012). Our SEM observations (**Figures 3G–J**) and subsequent re-culturing under heterotrophic conditions show that thermoacidophilic *M. sedula* cells were not entombed on the surface of the MRSs mineral mixtures under the given experimental circumstances.

Apart from abundant Fe^{3+} content, additional reasons may contribute to the observed low efficiency of MRSs in the preservation of dehydrated cells. Elemental and mineralogical comparison of MRSs with genuine extraterrestrial and terrestrial materials shows that natural minerals (e.g., NWA 1172 and scheelite) exhibit a rich and complex mixture of elements in bulk as well as in trace concentrations (**Supplementary Table 1**). After cultivation of *M. sedula* on the ordinary chondrite

NWA 1172, HAADF-STEM analysis detected complex mixed mineral phases (e.g., Cu, Fe, and Al) encasing individual cells (Milojevic et al., 2019b). Such complexity of encapsulated cell wall may contribute to cellular preservation under dehydrating conditions in the case of NWA 1172. Furthermore, NWA 1172 is characterized by much pronounced Mg and Ni elemental content (**Supplementary Table 1**) and its microbially mediated biotransformation leads to the formation of magnesium and nickel sulfates $MgSO_4x7H_2O$ and $NiSO_4x6H_2O$ (Milojevic et al., 2019b). These sulfate salts may assert their kosmotropic effect, bringing order to the surrounding solution by accumulating several water layers around them and acting as potential protein (organic matter) stabilizers under desiccation stress conditions (Okur et al., 2017; Kang et al., 2020). On the contrary, each regolith simulant lacks an authentic composition and subsequently diminishes the possibilities of more complex mineral–microbe interactions. Since growth of *M. sedula* on simulated Martian regolith was successful and the hydrogeological and iron-rich makeup of Mars could have supported iron and/or sulfur-transforming, chemolithoautotrophic microorganisms at one point, the identification of their putative (iron-) relevant fingerprints is of general interest (Amils et al., 2011; Nixon et al., 2012; Kölbl et al., 2017). Despite not being able to withstand dehydrating conditions in this study, we suggest that further investigations with alternative MRSs/analogs varying in overall and iron chemistry [reviewed by Ramkissoon et al. (2019)] should be performed. Since *M. sedula* cells were preserved after growth and desiccation on the meteorite NWA 1172, which exhibits a similar chemical composition to synthetic MRSs, most valuable would be the investigation of chemolithotrophs grown on real Martian meteorite materials.

Metal-organic associations in the fossil record are key indicators of past life and can be crucial in assessing whether microstructures found in rocks are of biological origin. Metabolic activity of certain microorganisms leads to mineral precipitation extracellularly; however, in metal-laden environments, e.g., associated with contamination or natural enrichments of S, Fe, Mn, and other metals, cells are subjected to metal sorption and mineral nucleation followed by metal encrustation of the cell surface (Schultze-Lam et al., 1996). Cell surface biomineralization can serve as a powerful microbial fingerprint and potential biosignature for the presence and/or activity of metal-transforming microorganisms. Assigning biogenicity to a certain structure or signature in ancient rocks remains very challenging due to probable degradation of microbial remains during diagenesis or microbial-like morphologies being produced abiotically (Gaboyer et al., 2017). The heavily encrusted desiccated cells depicted in our study may serve as relevant biosignatures to be looked for in the geological record, if they are not destroyed during diagenetic or metamorphic processes and are intact during different stages of fossilization. With regard to the subject of the preservation potential of desiccated microbes, many fascinating questions remain to be answered, including the influence of complex environmental parameters (e.g., the environmental chemistry, the rapidity of mineral encapsulation, and various post-diagenetic factors) on biomineralized cell wall. Such investigations may serve in establishing mineralogical and morphological criteria for the identification of metal-containing microfossils. The results of our study also suggest that desiccation-resistant and heavily encrusted cell walls might be identified in other representatives of archaeal order *Sulfolobales*, including fossil species where the S-layer could be preserved due to biomineralization, and remain intact.

CONCLUSION

Communities of archaea grown on tungsten-bearing materials, the NWA 1172 meteorite, and a multimetallic waste product provide well-preserved and recoverable cells of *M. sedula* under dehydrating conditions with NWA 1172 grown cells exposing the highest number of recovered cells after the desiccation treatment. Preservation of desiccated *M. sedula* cells described in the present study appears to be a discriminatory process, which depends on the nature and content of metal-bearing source used for growth of this metal-oxidizing archaeon. Our research report emphasizes the importance of considering microorganisms in their geological/mineralogical setting during investigations of geobiological environmental constrains. More importantly, the preservation of dehydrated *M. sedula* cells on metal-bearing substrates described herein suggests that this polyextremophilic archaeon is an ideal candidate for further survivability studies during future space exposure experiments and under the simulated space environmental conditions, including the testing of *M. sedula* persistence in a vacuum and after a combination of multiple stressors, for example, vacuum conditions combined with UV and gamma irradiation.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DK, AB, MA, and TM performed experiments. DK and TM performed, planned, and interpreted experiments described in this article. All authors provided editorial contribution to the manuscript, critically revised the report, and accepted the final version.

FUNDING

This study was supported by the Austrian Science Fund (FWF) with an Elise-Richter Research fellowship V333 Iron- and Sulfur-oxidizing Machinery of the bioleaching Archaeon *Metallosphaera sedula*.

ACKNOWLEDGMENTS

We warmly thank Stephan Puchegger (University of Vienna, Physics Faculty Center for Nano Structure Research) for the support of electron microscopy investigations. Christian Koeberl, Ludovic Ferrière, and Franz Brandstätter (Natural History Museum of Vienna, Austria) are deeply acknowledged for providing the NWA 1172 meteorite sample and fruitful discussions. The authors would also like to thank Steffen Schmidt (Wolfram Bergbau und Hütten AG) for providing scheelite and Felix W. von Aulock (School of Environmental Sciences, University of Liverpool) for providing volcanic glass.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fspas.2020.00041/full#supplementary-material

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Conflict of Interest: CF was employed by company Voestalpine BÖHLER Edelstahl GmbH & Co KG, Austria.

The remaining 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.

frontiers
in Astronomy and Space Sciences

ORIGINAL RESEARCH
published: 19 November 2020
doi: 10.3389/fspas.2020.544331

Multiscale and Correlative Analytical Electron Microscopy of Extraterrestrial Minerals

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This paper presents a unique correlative microscopic method for the structural characterization of extraterrestrial minerals. A fragment from the pallasite Seymchan meteorite that consists of olivine grains mixed into a metallic iron matrix with variable nickel content was studied from mm-down to nm-size by using the Raman Imaging and Scanning Electron Microscopy and analytical scanning transmission electron microscopy. Hyperspectral fast acquisition for energy-dispersive X-ray spectroscopy mapping of a couple of mm2 large area correlated with additional hyperspectral Raman analysis of smaller regions in the same area on one hand, and hyperspectral analytic STEM investigations at the atomic resolution, on the other hand, provided valuable information about the chemical composition, bonding, and crystallography. The analysis revealed particles of troilite, schreibersite, and forsterite but also regions of mixed iron oxides, carbonates, and amorphous carbon as well as plessite regions with nanometre-sized taenite needles dispersed in the kamacite matrix.

Keywords: Seymchan meteorite, correlative microscopy, scanning electron microscopy, Raman, scanning transmission electron microscopy, energy dispersive X-ray spectroscopy, electron energy loss spectroscopy

Edited by:
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Specialty section:
This article was submitted to
Astrobiology,
a section of the journal
Frontiers in Astronomy and Space
Sciences

Received: *20 March 2020*
Accepted: *27 October 2020*
Published: *19 November 2020*

Citation:
Albu M, Fitzek H, Moser D, Kothleitner
G and Hofer F (2020) Multiscale and
Correlative Analytical Electron
Microscopy of Extraterrestrial Minerals.
Front. Astron. Space Sci. 7:544331.
doi: 10.3389/fspas.2020.544331

INTRODUCTION

Pallasites are exquisite meteorites made of millimeter- to centimeter-sized olivine grains and FeNi metal alloys. The pallasites comprising the main group (PMG), about 90% of all pallasite meteorites found on Earth, have similar composition and are thought to originate from a single asteroid body whereas the other 10% are probably derived from five or six other asteroids. The classification of meteorites, that provides information on the geological past, is historically based on the Fe content extracted via wet chemistry analysis and electron microprobe. The analysis of oxygen isotope or the isotope ratios of other noble gases (He, Ne, Ar, Kr) that have been trapped into the stone, the analysis of the rare Earth elements content and crystal pattern information are other methods used to classify a meteorite. Unlike chondritic meteorites that are fragments of asteroids, which avoided melting and differentiation, the pallasites undergone thermal metamorphism that produced metal separation.

The primitive (unaltered) chondrites contain complex mixtures of micrometer sized magnesian olivine and low-Ca pyroxene, amorphous ferromagnesian silicates, Fe, Ni-metals, sulphides and organic phases. These meteorites also contain fractions of material that was probably vaporized and recondensed during high-temperature transient heating events associated with the formation of chondrules and refractory inclusions (Kereszturi et al., 2015; Bizzaro et al., 2017). The differentiated meteorites, on the other hand, are fragments of asteroids that experienced chemical and physical changes that led to a separation of the metallic part from silicates leading to metallic core and silicate mantle. They are considered core-mantle mixtures, and could come from many different asteroid bodies with different cooling and shock histories (Fritz et al., 2017; Hunt et al., 2018). Microstructural, chemical, and isotopic analyzes of meteorites provide unique information on their origin, thermochemical and shock history (Hunt et al., 2018; Gyollai et al., 2019); The Meteorite Exchange, (2014). Furthermore, their specific chemical composition, which contain elements throughout the entire periodic table (albeit in ppm concentration), is very important for the astrobiological assessments of the necessary conditions for primordial life in the Universe. It has been shown that interstellar medium, asteroids, comets, and meteorites host the organic matter necessary for life. It is also assumed that the impact of such objects (asteroids) brought volatile organic matter to the ancient Earth. For example, meteorites are considered major sources of carbon and reactive phosphorus (pyrophosphate, triphosphate)—a likely requirement for the emergence of life (Domagal-Goldman and Wright 2016). For the progress in astrophysics and astrobiology, a detailed investigation of meteorites down to the nano- and atomic range became therefore necessary.

Seymchan, a differentiated meteorite classified as heterogeneous pallasite consists of regions of pure metal—plessite (FeNi alloy-estimated to ~34.6 vol%) and pure silicate (~60.4 vol%) but also mesosiderite regions (Kichanov et al., 2018). Moreover, chromite phosphate and troilite phases (FeS) are present with a fraction of about 5 vol%. This meteorite is the only pallasite that contains all three morphologies of olivine grains, olivine clusters and large metal veins (Bösenberg et al., 2012; Kichanov et al., 2018). With a Ni content of 9.51 wt%, the Seymchan was first classified as an IEE-related iron meteorite (Scott and Watson 1976) but was later, after a new expedition in 2004 where additional fragments were found, identified as a main-group anomalous pallasite (Van Niekerk et al., 2007; Wilson, 2012). Its Widmanstätten pattern-cooling rate was estimated to be 7.1 + 1.2 K/Myr (Yang et al., 2010). Besides Fe and Ni other elements with trace concentrations (ppm) such as: Co (0.48 wt%), Cr, Cu, Ga, As, W, Ir, and Au were identified by neutron-activation analysis (Wasson and Wang, 1986).

Regular bulk and microstructure investigations of meteorites involve X-ray and infrared spectroscopy (Skulteti et al., 2020a; Skulteti et al., 2020b), optical microscopy and Raman analysis (Kereszturi et al., 2017; Tulej et al., 2017), metallographic methods, X-ray tomography and other synchrotron radiation techniques. Furthermore, bulk analyzes performed using both X-ray methods and infrared spectroscopy help to link the meteorites to their parent asteroids and therefore also support the next space missions. However, each method has advantages and disadvantages. For example, the metallographic methods are destructive but offer microstructure information down to µm range, on the other hand X-ray tomography and the synchrotron radiation relied methods are nondestructive but most of them only offer bulk-like information (Kichanov et al., 2018; Blukis et al., 2020). Few methods based on neutron scattering can reach a higher spatial resolution (Lehmann et al., 2017). Nano-SIMS (secondary ion mass spectrometry) and RIMS (resonance ionization mass spectrometry), however, deliver exact chemical information down to nanometer scale but no information about the crystallography (Ott and Hope, 2006). In the last decade, the analytical scanning and transmission electron microscopy (SEM and TEM) investigations have proven to be an important tool for both microstructure and crystallographic investigations of extraterrestrial minerals (Lin et al., 1977; Vollmer et al., 2014; Lo et al., 2019; Vollmer et al., 2019).

In this work in addition to SEM and TEM, we apply a new correlative microstructure analysis of the Seymchan meteorite by using the Raman Imaging and Scanning Electron Microscopy RISE (Jiruše et al., 2014; Schmidt et al., 2019). The RISE method is a seamless combination of two techniques, that offers the possibility of imaging by SEM and chemical analysis with the attached Raman microscope. In addition, a modern X-ray silicon drift detector that enables hyperspectral analysis in a comparatively short time rounds up the correlative investigations. Furthermore, transmission electron microscopy in scanning mode along with analytical capabilities such as electron energy loss (EELS) and energy dispersive X-ray spectroscopy (EDXS), provided information about both crystal structure and chemical composition down to atomic resolution and with the best detection limit for trace elements measured with EELS that exist at this moment.

MATERIALS AND METHODS

A small fragment of the Seymchan meteorite metallic part was cut and prepared for SEM and Raman investigations. The surface was first polished with standard procedure and etched in order to identify the plessite regions. Usually, to avoid charging effects, a coating (C or Au) needs to be applied prior to SEM investigations. However, in this particular case, the preparation of the sample surface needs to be coating-free for additional Raman spectroscopy investigation of the same area. Focused ion beam (FIB) lamellas were additionally prepared from plessite regions for the analytical STEM analysis.

The SEM imaging was performed using the scanning electron microscope Sigma 300 VP (Zeiss, Oberkochen, Germany) and the chemical analysis with the attached Raman microscope from WITec (Ulm, Germany). The electron microscope is also equipped with a silicon drift detector from Oxford (UK) for EDXS that allows for fast acquisition of spectra and maps (Schmidt et al., 2019). The energy resolution of the Oxford EDS detector as specified by the provider is: at C Ka −50 eV, F Ka −57 eV, Mn Ka −127 eV. The spatial resolution—as simulated with the Monte Carlo software (Drouin et al., 2007)—is between 100 nm (for Fe and Ni) and 300 nm (for O, Mg, P, and Si). The Raman measurements were performed using a 532 nm laser with the laser power reduced to 2.5 mW in order to minimize sample damage. The integration time was adjusted for every spectrum to give a reasonable signal to noise ratio and standard spectrum correction, such as cosmic ray removal and background correction were performed directly in the instrument software (Project FIVE, Witec).

TEM investigations such as: Energy filtered transmission electron microscopy (EFTEM), high resolution high angular annular dark field imaging (HAADF), EELS and EDXS spectroscopy, were performed on FIB lamellas. The lamellas were investigated in TEM and STEM mode by HAADF imaging and hyperspectral X-ray mapping. For TEM investigations a Tecnai F20 microscope was used whereas the STEM investigations were performed with an aberration corrected FEI Titan G3 60-300 microscope operated at 300 kV. This microscope is equipped with a Schotky emitter, a monochromator, a Super X detector (Chemi-STEM technology with four silicon drift detectors surrounding the sample) for EDXS (Schlossmacher et al., 2010), and with a Dual EELS Quantum Gatan Imaging Filter (GIF) for EELS (Gubbens et al., 2010). A direct electron detection camera (Gatan-K2) allows for detection of EELS spectra with even higher energy resolution and detection limits. We used the Gatan's Digital Micrograph Software to process the micrographs, EELS and EDXS spectra, and the VELOX software by Thermo Fisher Scientific to acquire and process the hyperspectral EDXS mapping. The k-method was used for the quantification of the EDXS spectra (Goldstein et al., 1986).

RESULTS

Previous neutron tomography investigation showed that the metal component of the Seymchan meteorite consist of a dendrite-like complex network of metals veins (Kichanov et al., 2018). We performed our microscopic investigation in a large metallic region as depicted on the optical micrograph in **Figure 1A**, with focus on plessite areas (FeNi-rich) and areas with mixed Fe–Ni–P, Fe-sulfate, -oxide, -hydroxide, and -carbonate.

The inset on the SEM image in **Figure 1C** depicts one plessite area from which a lamella was prepared by FIB. STEM micrographs and EDXS maps from plessite areas are presented in **Figures 2B**,D,E. These areas contain needle-like Ni-rich—taenite particles embedded into a kamacite matrix. The taenite needles of about 50–100 nm in diameter and 100–700 nm in length were studied by EDXS. The needles contain Fe (43.7 ± 7 wt%), Ni (54.4 ± 8.8 wt%), and other elements with very low concentrations C: 0.72 ± 0.09 wt%, Si 0.81 ± 0.2 wt%, P: 0.20 ± 0.04 wt%, Co: 0.2–0.55 ± 0.09 wt% and Cu: 0.5 ± 0.06. The Ni and P contents in the kamacite grains however, vary from 4.00 to 7.50 ± 1.5 wt% and 0.09 to 0.23 ± 0.02 wt%, respectively. Low concentrations of C: < 0.69 ± 0.1 wt%, Si < 0.43 ± 0.15 wt%, and Co: ~1.2 ± 0.25 wt% were also found. The Si concentration is higher in the taenite needles while Co is dispersed in the kamacite phase. However, the Co–Kα peak in the EDXS spectra is overlapping with the Fe–Kβ peak and due to the fact that the energy resolution in EDX is not sufficient to separate the peaks, it is difficult to extract the exact intensity without multiple fitting. The P–K peak in the EDXS spectra could only be clearly visible after long acquisition time of the spectrum image and addition of the spectra over large regions of kamacite- and taenite phase. It has to be noted that the SuperX detector also offers a better detection of all elements since we have four detectors surrounding the sample, thus detection of trace elements is possible.

The electron energy loss spectroscopy (EELS) investigations definitively revealed the low concentration of Co (<1 wt%) dispersed in the kamacite matrix (**Figure 2C**) and of Si mainly in the taenite needles. An energy shift of about 1.0 eV toward lower energy was measured for the Ni-L_3 ionization edge (ionization energy of the subshell L3 of the Ni atoms at 855 eV) in the taenite phase, thus confirming the different bonding of Ni in the two phases. It has to be noted that the low concentrations of Co could only be demonstrated by using the direct electron detection camera since its sensitivity is definitely higher than ordinary cameras used in electron microscopy.

The high-resolution STEM image from taenite needles and matrix is shown in **Figure 2G**. The fast Fourier transform (FFT) image calculated from an image at higher magnification, enables the measurement of the distance between the atomic columns in a certain orientation relative to the electron beam. The kamacite matrix was described in the literature as α-Fe with a disordered cubic structure (4/m −3 2/m, $a = 2.86$ Å). We indeed observed the strained structure on the high-resolution images due to the collision history of the meteorite, but also due to the presence of impurities. The FFT image (**Figure 2H**) from a region of **Figure 2G** at higher magnification matches the mentioned body centered cubic structure in the [111] zone axis. The element profile in **Figure 2J** over one needle (**Figure 2I**) confirms the relationship between Fe and Ni in taenite.

A region containing μm large particles that have been revealed by etching the surface, was analyzed in SEM by EDXS mapping (**Figure 3**; **Supplementary Figure S1**). We identified the particles to be schreibersite $(Ni,Fe)_3P$ and one forsterite (Mg_2SiO_4) (or ringwoodite—high pressure modification of olivine—cubic crystal structure). The schreibersite particles have dimension between 200 nm and 2 μm and show elliptical, spherical and needle-like shapes while the forsterite particle is about 2 μm large and has a triangle morphology. Their location into the metallic kamacite region and the chemical composition suggest them to be presolar grains. However, we cannot measure their isotopic signature in SEM or STEM.

Si and Mg condensate as forsterite and enstatite and are thought to be present in O-rich circumstellar shell of giant stars and in planetary nebulae (Molster et al., 2002a; Molster et al. 2002b; Molster et al. 2002c; Suh 2002). The silicates can be crystalline and amorphous; thicker and cooler circumstellar shells would favor crystalline silicates. Presolar silicates are not often observed in meteorites because the silicates are more susceptible to metamorphism and chemical changes than presolar carbonaceous and oxide grains (Lodders and Amari, 2005). Aqueous alteration for example erases the presolar isotopic signatures in the silicates if the parent meteorite body experienced much aqueous exposure. It is also difficult to locate the presolar silicate among abundant solar system silicate that are major constituents of meteorites, except when the particles are embed into metallic matrix as in the case of Seymchan meteorite. Forsterite particles were only measured by O-isotope ion imaging with the NanoSIMS so far (Messenger et al., 2003). Iron, on the other hand, form FeNi alloy or condensates as troilite and schreibersite but these phases have not yet been directly identified in the dusty circumstellar shells of M-giants because of the very low infrared signal in spectra with a low resolution. However, for spectra acquired with the Infrared Space

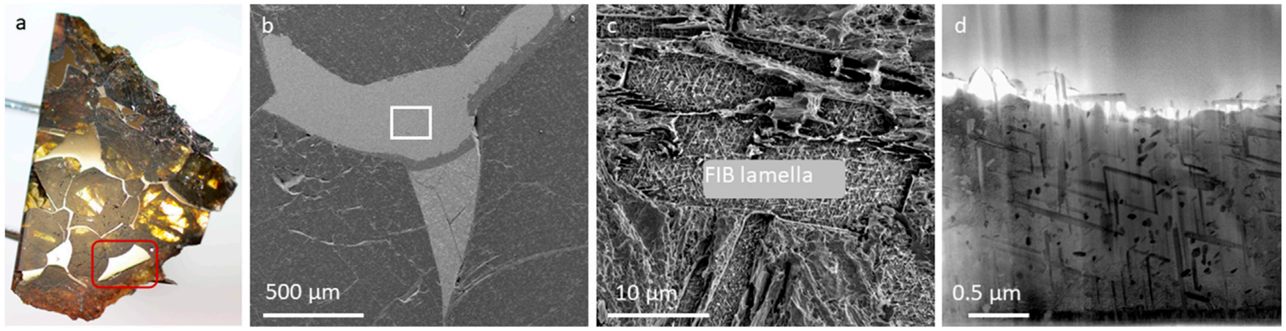

FIGURE 1 | (A) Optical microscopy image of the Seymchan meteorite fragment of approximatively 2 cm^2, **(B)** SEM image at low magnification, **(C)** SEM image of the etched surface with the inset indicating the plessite region from where a FIB lamella was cut, and **(D)** STEM image of the FIB lamella.

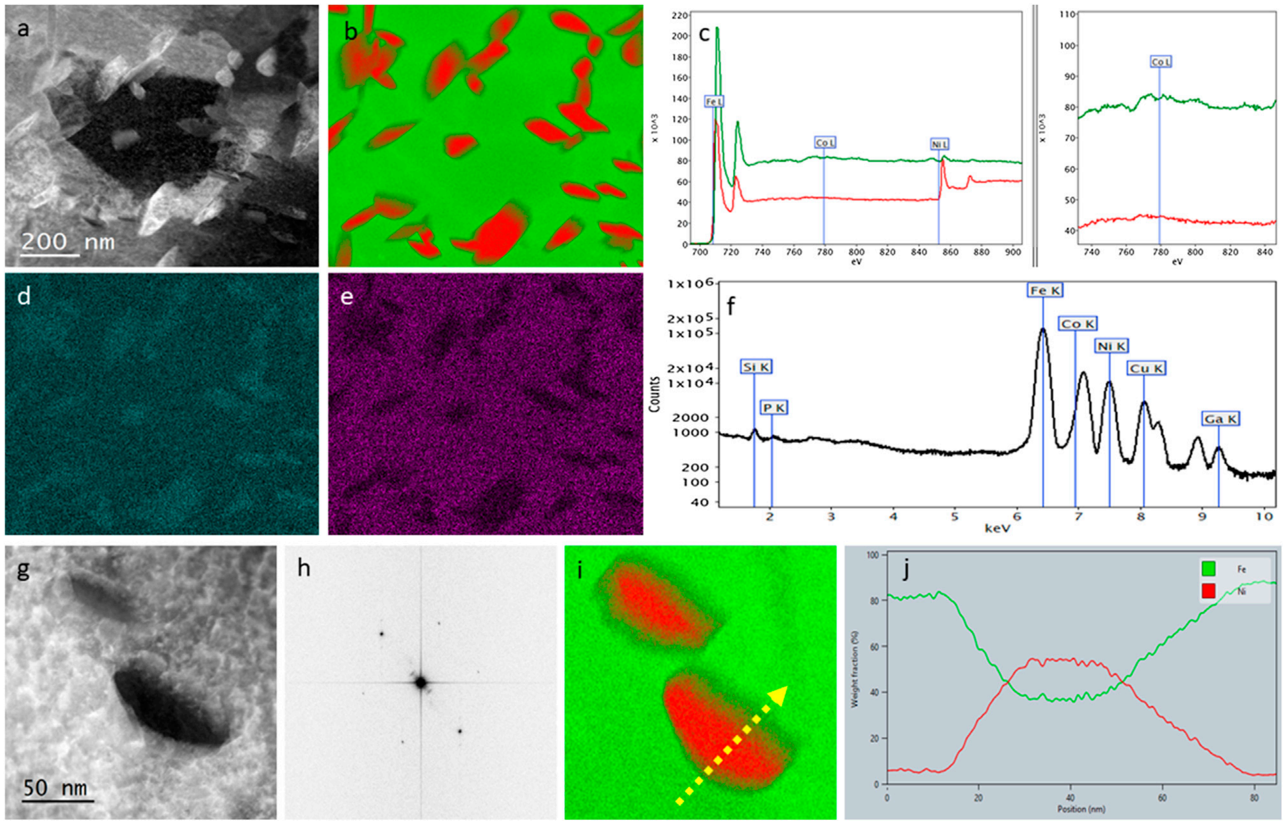

FIGURE 2 | (A) HAADF image of taenite needles in kamacite matrix; **(B)** composite image of EDXS maps: red-Ni and green-Fe, **(C)** EELS spectrum from the matrix (note the low Co signal), **(D)** Si EDXS maps, **(E)** Co SI EDXS map, **(F)** EDXS spectrum sum over the whole area (note the low P peak), **(G)** HAADF image of taenite needles in kamacite matrix, **(H)** Fast Fourier Transform of an area at higher magnification in **(G)** that indicates the [111] zone axis of cubic kamacite; **(I)** composite image of EDXS maps: red-Ni and green-Fe, **(J)** element profile over taenite needle and kamacite phase (concentrations in wt%).

Observatory at intermediate resolution, Kemper et al. estimated the contribution of non-spherical metallic iron to the near-infrared spectrum from a giant branch star (Kemper et al., 2002). Amorphous and/or graphitic carbon, is also believed to be present around many C-stars (Swart et al., 1983; Blanco et al., 1994).

A multiscale analysis using SEM-EDXS and Raman microscopy is shown in **Figure 4**. Starting from the large scale EDXS-mapping (**Figure 4A**) an interesting P-containing region was chosen for further analysis. In the small scale EDXS-mapping a predominantly Fe-containing phase, a mixed Fe–Ni–P phase and some oxide-phases are apparent. Focusing in further the Raman analysis (**Figure 4B**), note that on the right-hand side spectra with similar composition have been averaged according to the color of their position

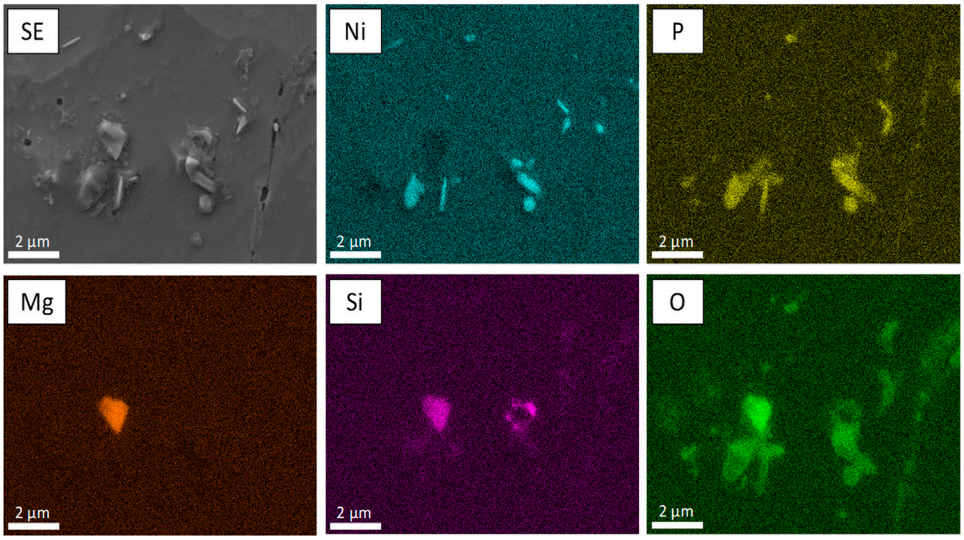

FIGURE 3 | SEM, EDXS Maps of a particle group containing a Mg_2SiO_4 particle (forsterite phase) and schreibersite $(Ni,Fe)_3P$ – 2 µm sized particles.

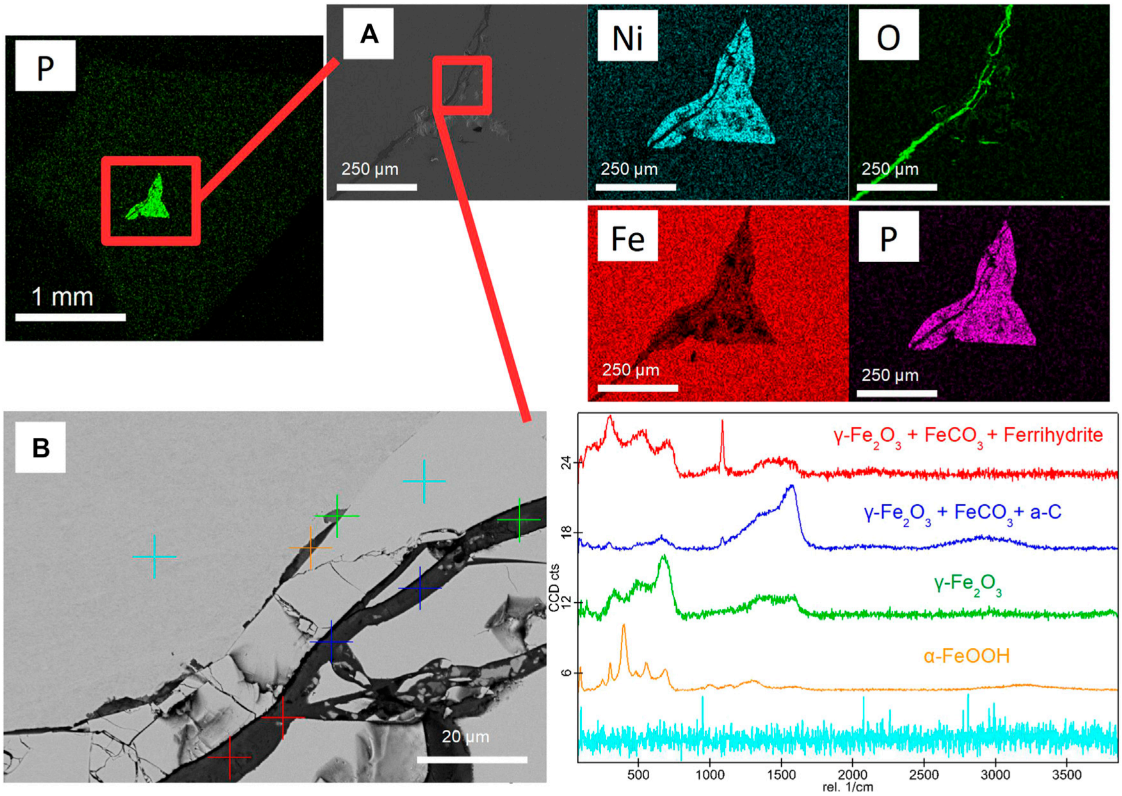

FIGURE 4 | Correlative Raman-SEM-EDX analysis of a Fe–Ni–P inclusion. **(A)** SE-image and EDXS-mapping of the whole inclusion. **(B)** Raman point measurements of the matrix materials and the oxygen rich veins.

indicator on the left, both the Fe- and Fe–Ni–P phase show no Raman signal indicating predominantly metallic phases. In the oxide-phase on the other hand a complex composition of multiple different materials is revealed by Raman. The phase contains iron oxide (γ-Fe_2O_3 maghemite), iron hydroxides (ferrihydrite and goethite), iron carbonate and amorphous carbon. The spectral interpretation is based on reference spectra from (Hanesch, 2009).

DISCUSSION

The goal of the measurement was actually to identify and localize presumable elements with trace concentrations (Ca, Cr, Ti, Al, Co, Si, P, Ga, As, W, Ir, Au) that have been confirmed by bulk measurements using the neutron-activation technique. Our microscopes are well suited with some of the best equipments for this type of analytical investigations: SuperX detector for EDXS detection and a DualEELS spectrometer and a K2 electron direct detection camera for EELS. Therefore, we were able to measure the Co content (<1.00 wt%) and Si (<1.00 wt%) with EELS, and Co, Si, and P (<0.25 wt%) with EDXS. Also, we measured with the EDXS a broad triangle-like feature centered around Ar–K (3 keV) peak. This feature appeared in the measurements performed for this meteorite at longer acquisition times and is slightly pronounced for the kamacite region. Our findings are in excellent agreement with the research on kamacite formation proposed by Goldstein and Doan (1972).

They established that below 7 wt% Ni kamacite is formed by only one reaction path, γ (taenite) $\rightarrow$ α (kamacite) + γ. The phases with low concentration of Ni (5–3–5.9 wt% or 5.8–6.5 wt%) known as hexahedrites and hexaoctahedrites are described by this reaction. However, the equilibrium nucleation temperature for the kamacite was shown to be greatly increased by phosphorus presence. Above 7 wt % Ni, kamacite is formed by either of two reaction paths depending on the P content. The first reaction path is the same as for lower concentrations of Ni: $\gamma \rightarrow \alpha + \gamma$ and is preferred at low P contents. At higher P contents (0.2 wt%) the reaction path: $\gamma \rightarrow \gamma$ + Ph (phosphide, $(FeNi)_3P$) $\rightarrow \alpha + \gamma$ + Ph controls the formation of kamacite.

The plessite regions we investigated in STEM showed an average concentration of Ni lower than 7 wt%, however some small kamacite grains contain slightly higher concentrations than 7 wt%. The crystal structure of these small kamacite grains differs from the cubic bcc α-Fe. The *hkl* values we measured on the FFT, however, do not correspond to any known allomorph phase of Fe on Earth, but correspond to the metastable tetragonal iron found in other meteorites and it is considered to be a transitional state in the transformation of γ-phase alloy into α-phase alloy (Ramsden and Cameron, 1966). Furthermore, the schreibersite particles we found by etching the pure kamacite regions, and the concentration of 0.2 wt% of P indicated that the formation of some kamacite phase also take place by the second reaction path γ + Ph $\rightarrow \alpha + \gamma$ + Ph.

The RISE investigations of metallic parts from the Semchan meteorite, revealed particles of troilite, schreibersite and forsterite but also regions of mixed iron oxides, carbonates and amorphous carbon.

Assessing the morphology and composition of those particles, and the fact that were incorporated into the metallic kamacite matrix we assume them to have a presolar origin. The presence of presolar grains in the Seymchan meteorite was not indicated so far. Given that is the only meteorite that contains all three morphology types of olivine and the presolar grains we suspect, further detailed investigations by NanoSIMS should be considered.

CONCLUSION

A correlative investigation by using the method RISE and scanning transmission electron microscopy was applied to investigate the Seymchan meteorite. Valuable information about the morphology, chemistry and crystallography from millimeter down to nanometer sizes was acquired.

The analyses revealed nanometer sized taenite needles dispersed in the kamacite matrix. The taenite needles showed a very low content of silicon and the kamacite a very low content of cobalt and phosphorus. Moreover, due to the collision history of the meteorite and the presence of trace elements in the matrix, the crystal structure of the kamacite is highly defective and stressed. Additionally, presolar grains in the Seymchan meteorite and regions of iron- oxide, carbonate, and hydroxides as well as amorphous carbon were observed.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MA performed high resolution STEM and wrote the manuscript, HF and DM performed SEM and Raman investigations, FH designed the experiment and all co-authors corrected and improved the manuscript.

FUNDING

RISE was enabled by the project “HRSM-Projekt ELMINet Graz—Korrelative Elektronenmikroskopie in den Biowissenschaften” (i.e., a cooperation within “BioTechMed-Graz,” a research alliance of the University of Graz, the Medical University of Graz, and Graz University of Technology), which was financed by the Austrian Federal Ministry of Education, Science and Research (BMBWF). The combination with the EDX detector could be realized by the project “Innovative Materialcharakterisierung” (SP2016-002-006), which is part of “ACR Strategisches Projektprogramm 2016” of the Austrian Cooperative Research (ACR). The STEM investigations received financial support by the Austrian Science Fund (FFG): SP2018-003-006 (Microstructure of 3D printed metallic parts), the “Zukunftfonds Steiermark” for the purchase of the K2-camera through the “ASTEM upgrade” project #9016, and from the European Union’s Horizon 2020 research and innovation program under grant agreement No. 823717—ESTEEM3.

ACKNOWLEDGMENTS

The authors would like to thank Martina Dienstleder and Sebastian Rauch for sample preparation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fspas.2020.544331/full#supplementary-material

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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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ORIGINAL RESEARCH
published: 23 November 2020
doi: 10.3389/fspas.2020.00059

Detection of Microorganisms in Low-Temperature Water Environments by *in situ* Generation of Biogenic Nanoparticles

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Specialty section:
This article was submitted to Astrobiology, a section of the journal Frontiers in Astronomy and Space Sciences

Received: *01 January 2020*
Accepted: *31 July 2020*
Published: *23 November 2020*

Citation:
Skladnev DA, Vasilyeva LV, Berestovskaya YY, Kotsyurbenko OR, Kalenov SV and Sorokin VV (2020) Detection of Microorganisms in Low-Temperature Water Environments by in situ Generation of Biogenic Nanoparticles. Front. Astron. Space Sci. 7:59. doi: 10.3389/fspas.2020.00059

A new nanobiotechnological approach for the detection of extraterrestrial Earth-like biological forms is proposed. The approach is based on the ability of microbial cells to reduce artificially added cations with the generation of crystalline nanoparticles (NPs) from zero-valent atoms. The method is named DBNG (Detection of Biogenic Nanoparticles Generation). The subglacial low-temperature oligotrophic Lake Untersee in Antarctica was used as a model of putative extraterrestrial water environments inhabited by Earth-like type microorganisms. The DBNG protocol for the comparative study of microbial communities of low-temperature oligotrophic environments was optimized on the base of experiments with the pure culture of psychroactive bacterium *Cryobacterium* sp. 1639 isolated earlier from Lake Untersee. The formation of silver nanoparticles (Ag°NPs) has been conducted in natural water samples of three horizons at low temperature (+5°C), which was in the temperature range registered in the Lake Untersee. The generation of biogenic Ag°NPs was detected only at the presence of indigenous microorganisms in all studied samples. No Ag°NPs generation was observed in the lake water samples artificially free of cells or exposed to pasteurization (two types of controls). The miniature microfluidic chip for an automated version of the device, based on using different analytical methods for recording *in situ*-formed biogenic nanoparticles, is proposed. The device allows the detection of the biological objects directly at the sampling site.

Keywords: astrobiology, extraterrestrial life, nanoparticles, psychrophiles, DBNG method

INTRODUCTION

Nowadays, the topical challenge of astrobiology is the development and testing of new methods for searching for Earth-like biological forms in Space. It is assumed that metabolically active microorganisms are present mainly in a state of aqueous suspensions in terrestrial environments. Many cells of such suspensions can remain viable for a very long time, especially in oligotrophic environments (Gordon et al., 2000; Vishnivetskaya et al., 2000; Ponder et al., 2008). Accordingly, searching for extraterrestrial biosignatures or biological Earth-like objects in most studies is focused on cosmic bodies containing water and ice (Andersen et al., 1995; Priscu et al., 1998; Storrie-Lombardi and Sattler, 2009; Haendel et al., 2011; Filippova et al., 2013; Westall et al., 2013; Koo et al., 2017, 2018). Various approaches to detect biosignatures have been suggested (Rivkina et al., 2000; Gilichinsky et al., 2007; Rogers et al., 2010; Andersen et al., 2011; Zhang et al., 2014; Bedrossian et al., 2017; Managadze et al., 2017). Nevertheless, the development of new effective techniques for the detection of putative life beyond Earth is still an important problem to be solved (Nadeau et al., 2008; Garcia-Descalzo et al., 2012; Judge, 2017; Nelis et al., 2018). The basic astrobiological methods are mostly related to search for biosignatures of a geological, chemical, and biomorphological nature (Cockell, 2010; Westall et al., 2015; Hays et al., 2017; Mickol et al., 2018). Here, we propose a novel nanobiotechnological approach for the detection of microbiological objects in water or melted ice samples from extraterrestrial low-temperature environments. The approach is named the Detection of the Biogenic Nanoparticles Generation (DBNG), and it based on the ability of microorganisms, in particular bacteria, to produce biogenic crystalline nanoparticles of reduced Ag° atoms of the artificially added silver salt solution.

It has already been shown that microorganisms are capable of reducing cations and generating nanoparticles from reduced zero-valent atoms of metals (Wang et al., 2012; Wu et al., 2013). This is one of the natural non-specific mechanisms that are probably involved in the detoxification of metals, including the protection from cation entry into the cell (Merroun, 2007; Gadd, 2010). All tested microorganisms had this ability to reduce cations with different valences (Velusamy et al., 2016; Patil and Kim, 2017; Sorokin et al., 2019; Singh et al., 2020). It was also demonstrated that a much greater variety of nanoparticle assemblies could be produced by cells along the "bottom-up" approach (Zhang et al., 2011; Woehl et al., 2014; Sánchez-López et al., 2020) (**Figure 1**). Observations clearly show that nanocrystals can nucleate from an aqueous solution *via* a three-step mechanism: spinodal decomposition, clusterization, and nanocrystallization (Luo et al., 2017; Tan et al., 2017; Xie et al., 2017; Liao et al., 2019; Mikhailov and Mikhailova, 2019; Wang et al., 2019). The amorphous clusters of zero-valent atoms (up to 1–2 nm) are direct precursors of the bigger crystalline nanoparticles (Malis et al., 2011; Wan et al., 2013; Loh et al., 2017; Singh et al., 2020). Several studies have reported natural polymers (such as chitosan, proteins and amino acids, sugars, starch, and tannic acid) as reducing agents for the synthesis of metallic nanoparticles (Prakash and Sharma, 2010; Kharissova et al., 2013; Velusamy et al., 2016; Masse et al., 2019; Navarro Gallón et al., 2019). Differences in generated biogenic nanoparticles depend on the physiology of microorganisms as well as on the medium composition and conditions at their formation (Narayanan and Sakthivel, 2010; Zhang et al., 2011; Wang et al., 2012; Sorokin et al., 2013; Wu et al., 2013; Tyupa et al., 2016; Muller, 2018; Siddiqi et al., 2018). However, only metabolically active cells can rapidly (in minutes) generate metal nanoparticles from metal ions of the added salt due to their ability to constantly "efflux" electrons and electron donors into the medium (Zhou et al., 2013; Skladnev et al., 2017a; Wang et al., 2019). Inactive cells or cells with destroyed and non-active biostructures have a much weaker ability to reduce cations and form only the amorphous nanoclusters (up to 1–2 nm) as precursors of nanoparticles (Woehl et al., 2014; Liao et al., 2019). In solution, the organic components of the cytoplasms of destroyed cells can only act as low concentrated and "slow" chemical reducing agents. The generation of nanoparticles, when a source of metal cations is added into a sample, can be considered as an indicator of the presence of biological objects.

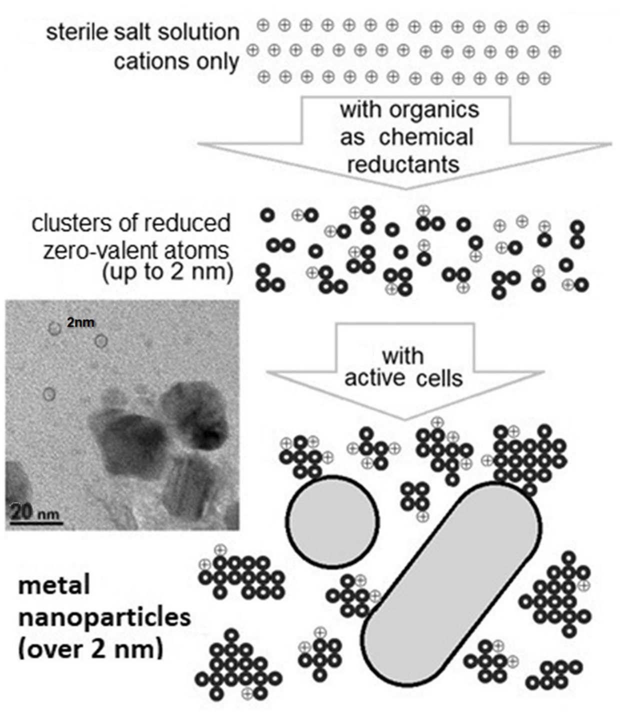

FIGURE 1 | The scheme of stages of biogenic nanoparticles generation.

To confirm the efficiency of the proposed DBNG method, we applied it to natural water samples taken from the Antarctic Lake Untersee—an analog terrestrial system for putative cold extraterrestrial ecosystems (Andersen et al., 1995, 2011; Filippova et al., 2013; Heinz et al., 2018). The lake is an ice-covered reservoir, oligotrophic, and isolated from the Earth's atmosphere for millions of years. In previous studies, microbial communities present in the Lake Untersee have been shown to survive in extreme conditions, such as near-zero temperatures and low content of organic compounds (Fomenkov et al., 2017; Pikuta et al., 2017). The water reservoir with likely similar characteristics, was found under a layer of Martian ice in the southern polar region (Jones et al., 2018; Orosei et al., 2018; Post et al., 2019). The goal of the present work was to demonstrate the efficiency of the proposed nanobiotechnological DBNG method for astrobiology applications. The aim of the investigation was to study the biogenic Ag°NPs formation *in situ* in water samples from different horizons of subglacial Antarctic Lake Untersee containing indigenous microorganisms under low-temperature conditions.

TABLE 1 | Some characteristics of water sampling horizons from the Lake Untersee (Andersen et al., 2011).

Horizon depth, m	T, °C	pH	Dissolved oxygen, mg L^{-1}	Features of lake water horizons
20	0.3	10.1	20	Aerobic layers with very low organic content
40		10.3	18	
72	4.9	7.9	0.2	Microaerobic conditions in chemocline

MATERIALS AND METHODS

Sampling of Different Horizons of the Lake Untersee, East Antarctica

Three water samples were taken aseptically from the subglacial low-temperature Lake Untersee, in East Antarctica (71°20′ S, 13°45′ E) located in the interior of the Gruber Mountains of central Queen Maud Land. This extremely oligotrophic lake is situated 563 m above the sea level and is the largest by surface area (11.4 km^2) lake in East Antarctica. Samples from the lake were taken using 1-liter bathometers at the station of the ice camp, as described in the NASA 2015 Annual Science Report[1] The water samples were collected by the depth profile of the column at 20, 40, and 72 m (starting from the smallest depth). Some environmental parameters are shown in **Table 1**. Water temperature in the horizons was from +0.3 to +4.9°C. The samples were aseptically transferred to sterile laboratory flasks and transported to the laboratory in heat-insulating containers and subsequently stored at +5°C. For the current study, about 4 ml of those water samples were kindly provided by Acad. Gal'chenko. The experiments for the generation of nanoparticles were started in 4 months of sample collection. The aliquots for analysis were also taken aseptically.

The Pure Culture of Psychroactive Bacterium

The psychroactive bacterium *Cryobacterium* sp. 1639 was isolated in pure culture from the Lake Untersee water sample taken from a depth 72 m with registered *in situ* temperature of +4.9°C. The 16S rRNA sequence of the isolate was deposited in the NCBI GenBank database (MT364260).

Formation of Biogenic Silver Nanoparticles at DBNG Method

The principal scheme of the DBNG method is presented in **Figure 2**. The formation of biogenic Ag°NPs was carried out in 50-μL aliquots. The source of silver cations for generation of Ag°NPs was an aqueous solution of $Ag(NH_3)_2NO_3$ synthesized from $AgNO_3$ and ammonia according to a modified Tollens protocol (Anh-Tuan et al., 2010). Sterile 2 mM $Ag(NH_3)_2NO_3$ solution was added directly to samples to achieve a final concentration of 0.1 mM. The generation of silver nanoparticles in native water samples was carried out at +5°C. The generation of silver nanoparticles by cells of strain *Cryobacterium* sp. 1639 was carried out at the exponential growth phase at two cultivation temperatures: +5°C (close to the Lake Untersee horizon temperature) and at +24°C (room temperature). The duration of exposure to silver salt in all experiments was 20 min (Sorokin et al., 2013). The experiments were conducted in triplicate. Sterility of $Ag(NH_3)_2NO_3$ solution was proved by the absence of microbial growth in the LB medium. The absence of NPs in native water samples and $Ag(NH_3)_2NO_3$ solution was confirmed by transmission electron microscopy. As cell-free control aliquots, 1.0 mL of the native water samples and the cultural fluid of *Cryobacterium* sp. 1639 free of microbial cells previously removed by centrifugation (10,000 g, 15 min) were used. In all cell-free controls formation of silver nanoparticles was carried out at two temperatures +5 and +24°C.

Electron Microscopy and X-Ray Microanalysis

Specimens were studied using a JEM-1400 microscope (JEOL, Japan) equipped with an X-ray microanalyzer (Oxford Instruments, United Kingdom) at an accelerating voltage of 80 keV. The samples were prepared using standard copper grids with Formvar film reinforced with carbon. Five microliters aliquots of each variant were applied to the copper grids and dried for 15 h.

A direct cell counting was conducted for the samples taken from different water horizons by the TEM examination of grids with native variants.

Analysis of the linear dimensions of Ag°NPs and their classification was carried out using the "Compass 3D-V14" software and a specially developed algorithm for calculating linear dimensions based on electron microscope images of at least 300 nanoparticles.

Inactivation of Cells of *Cryobacterium* sp. 1639 Culture and Water Samples From Horizons of the Lake Untersee

Four samples were pasteurized at 70°C for 30 min and used as negative controls (Cebrián et al., 2017).

RESULTS

Formation of Silver Nanoparticles by Cells of Psychroactive Bacterium *Cryobacterium* sp. 1639

The psychroactive bacterium *Cryobacterium* sp. 1639 was isolated from horizon depth 72 m of the Lake Untersee. The culture *Cryobacterium* sp. 1639 grew well at +5°C and was able to reduce silver cations and forming Ag°NPs at +5°C during 20 min (**Figure 3A**). The size of 50% of these particles did not exceed 20 nm, while larger size appeared rare (**Figure 3B**). When the temperature was higher than 20°C, the growth of psychroactive bacterium *Cryobacterium* sp. 1639 was very slow, and the formation of Ag°NPs was not detected (**Figure 3C**). Cells of *Cryobacterium* sp. 1639 grown at +5°C lost their ability

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[1] https://astrobiology.nasa.gov/nai/annual-reports/2015/seti/lake-sediment-habitats-lake-habitability-and-sediment-biosignatures/

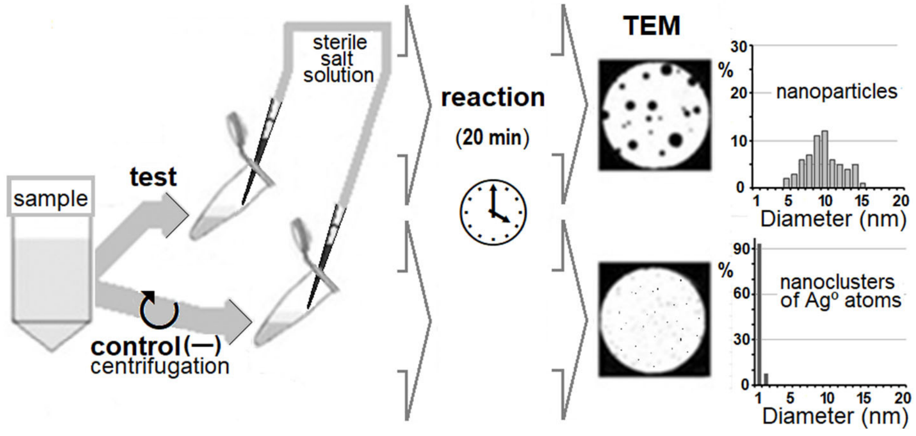

FIGURE 2 | The principal scheme of the DBNG method.

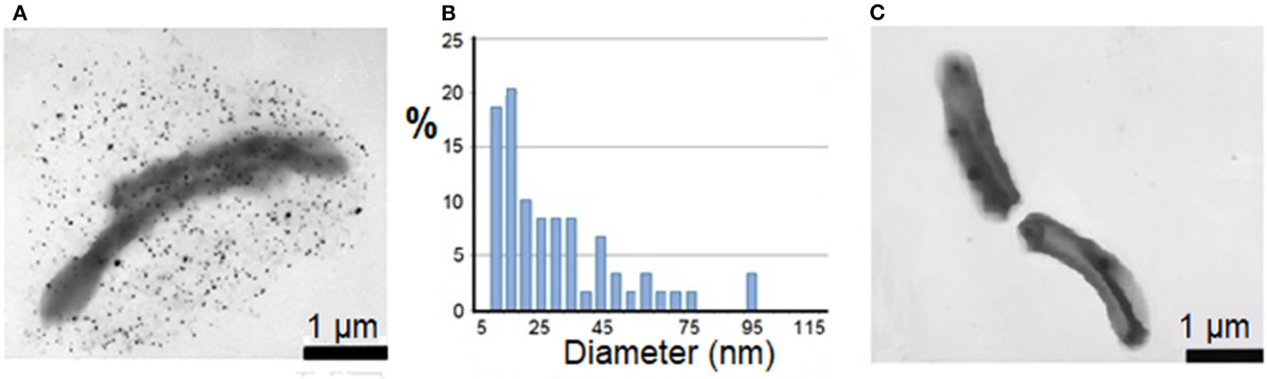

FIGURE 3 | Distribution in sizes **(B)** of biogenic Ag°NPs generated by psychroactive *Cryobacterium* sp. 1639 at +5°C in 20 min **(A)**. No Ag°NPs generated by cells *Cryobacterium* sp. 1639 at +24°C **(C)**.

to reduce Ag^+ cations fast and generate nanoparticles under non-optimal temperature conditions (at +24°C).

Formation of Silver Nanoparticles in Native Water Samples From the Lake Untersee

The direct cell counting in water horizons confirmed the presence of numerous indigenous microorganisms (from $3{\cdot}10^4$ to $2{\cdot}10^6$ mL^{-1}). In all three studied water samples, the formation of biogenic Ag°NPs of 15–150 nm in diameter at +5°C in 20 min has been observed (**Figure 4**). In cell-free controls no Ag°NPs have been detected.

The difference in the size distribution of Ag°NPs formed in water samples taken from three different horizons of the Lake Untersee was revealed. For these samples, distribution in size could be characterized by the normal law. Biogenic nanoparticles detected in the sample taken from 20 m depth had a maximum size of 40 nm. In the samples from depth 40 m and 72 m, the formed nanoparticles were twice as large. Nanoparticles from deeper samples had a larger size (35–120 nm for depth 40 m and 65–155 nm for depth 72 m), the predominant size was 80 nm.

Pools of biogenic nanoparticles formed in samples were different in both linear sizes and quantity. The same conditions for silver reduction in lake water samples made it possible to compare concentrations of biogenic Ag°NPs formed in these reactions. Amounts of biogenic Ag°NPs detected in water samples were normalized to those detected in the sample from the depth of 40 m (**Figure 5**).

Formation of Nanoparticles by Inactivated Cells of *Cryobacterium* sp. 1639 and Native Microflora of Water Horizons of the Lake Untersee

The standard protocol of the DBNG method was used for experiments with non-specific inactivation of cell suspensions of psychoactive bacterium *Cryobacterium* sp. 1639 and three studied water samples with indigenous microorganisms of the Lake Untersee. No silver nanoparticles were observed in these four samples with their previous pasteurization.

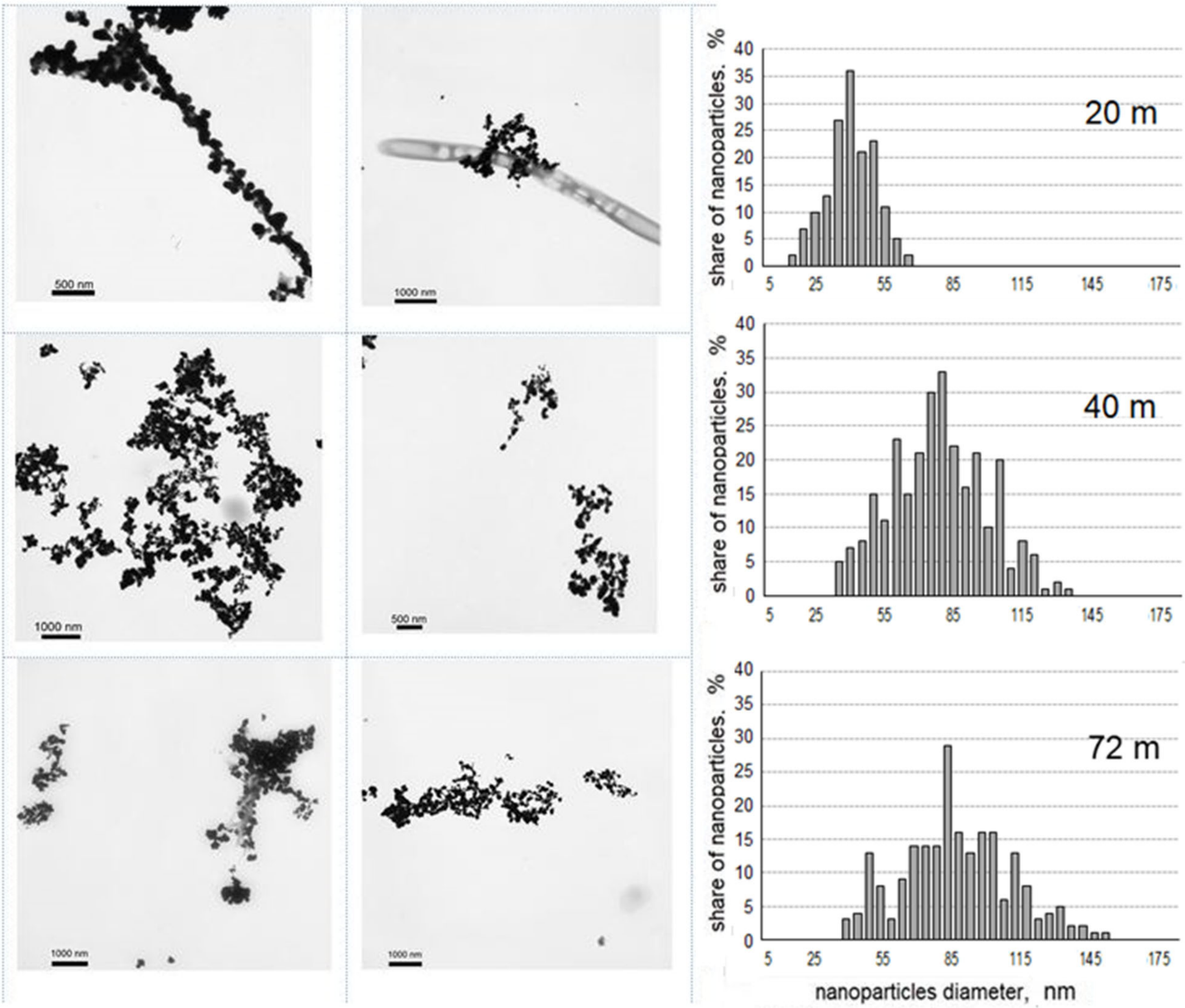

FIGURE 4 | Biogenic Ag°NPs, formed at +5°C in 20 min in water samples of the Lake Untersee and their distribution in size (in percentage to the total amount).

DISCUSSION

The Lake Untersee is highly oligotrophic, ice-covered, and has no anthropogenic impact. All these features make it a good terrestrial analog to putative cold extraterrestrial ecosystems and therefore an attractive subject for astrobiologists (Andersen et al., 1995, 2011; Cockell, 2010; Garcia-Lopez and Cid, 2017; Nelis et al., 2018; Post et al., 2019). All these characteristics make water samples from this lake acceptable for the approbation of the new method based on the ability of microbial forms to reduce Ag^+ cations followed by the formation of biogenic Ag°NPs from zero-valent silver atoms.

Adaptation of the DBNG Method for the Detection of Microorganisms in Low-Temperature Water Environments

To adapt the DBNG method for the detection of microorganisms in low-temperature water environments (by *in situ* formation of the biogenic nanoparticles), we used the results of experiments with a pure culture of psychroactive bacteria isolated from the 72 m horizon of the Lake Untersee. It has been demonstrated that the psychroactive bacterium *Cryobacterium* sp. 1639 has good growth at +5°C (Bajerski et al., 2011), within the temperature range characteristic of its native habitat in the horizon of the Lake Untersee (while at +25°C bacterium *Cryobacterium* sp. 1639 grew three times slower). For the first time, it has been shown that psychroactive bacteria are capable of biogenic Ag°NPs formation at +5°C during 20 min. It is important to note that silver nanoparticles generated at these conditions were relatively small in size (20 nm or smaller in average) in comparison to those formed by indigenous microorganisms in water samples of the Lake Untersee. The possible explanation of the difference in size is the biosynthesis of low-molecular extracellular bioactive compounds of *Cryobacterium* sp. 1639. These compounds can be absorbed by the "young" nanoparticles (nanoclusters) and prevent their further growth (Zhang et al., 2011; Zhou et al., 2013). There were no biogenic Ag°NPs generated at +24°C for 20 min. Thus, the temperature +5°C is optimal for the DBNG method for detecting microorganisms in a low-temperature water system.

The formation *in situ* biogenic Ag°NPs in two horizons of the Lake Untersee at +24°C were described earlier (Skladnev et al., 2017a). It was shown that: (i) for the depth of 40 m—the size distribution of nanoparticles had the normal distribution with a distinct peak at 12–13 nm; (ii) for the depth of 72 m—half of Ag°NPs had a very small diameter (about 5 nm), whereas other nanoparticles were larger (from 12 to 50 nm).

In our study, at +5°C Ag°NPs showed different size distribution (**Figure 4**). In this case, Ag°NPs size distributions for horizons 40 and 72 m had the pick at 80 nm. We emphasize that large nanoparticles were formed over 20 min, indicating the presence of metabolically active microorganisms in these horizons.

The comparison of average amounts of formed Ag°NPs demonstrate that in the water sample from 72 m, the number of biogenic Ag°NPs is larger than in the water sample of the 40 m depth. It was true for both temperature conditions: at +5°C (60: 1), and +24°C (60: 2.5) (**Figure 5**). The value of relative amount Ag°NPs at +5°C is higher, which means that cells at low temperature demonstrate higher reducing activity. The high sensitivity of the DBNG method directly for native water samples was shown even at low-concentrated cell suspensions (20 m—$3\cdot10^4$, 40 m—$4.1\cdot10^4$ cells per mL).

In general, based on the data obtained in our experiments, we can conclude that the reduction efficiency for artificially added silver cations to samples is higher at the temperature close to the native environment (+5°C) than at the room temperature (+24°C), and this indicates to the true psychrophilic physiology of inhabitants. Biogenic Ag°NPs with a diameter larger than 10 nm can be detected by a wide range of analytical methods (visible and fluorescent spectrometry, spectroscopy of surface-enhanced Raman scattering, etc.).

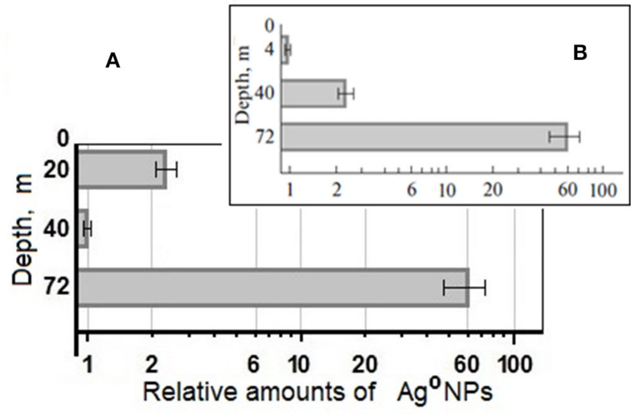

FIGURE 5 | Relative number of Ag°NPs, formed at +5°C in water samples of the Lake Untersee from different depths, normalized to the amount of biogenic nanoparticles in the sample from the depth 40 m **(A)**, **(B)** relative number of Ag°NPs, formed in the same water samples at +24°C (Skladnev et al., 2017a).

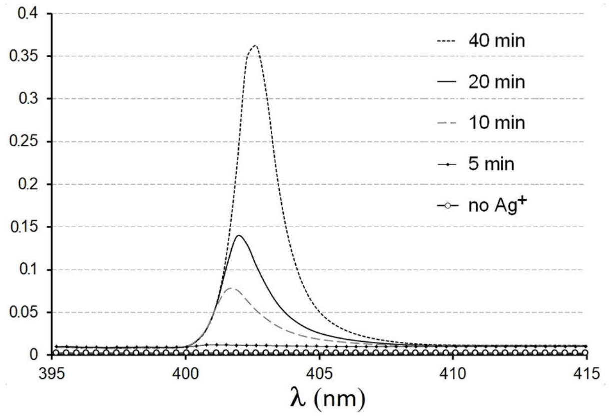

FIGURE 6 | Visible absorption spectra for different duration of biogenic Ag°NPs generation.

Technical Principles of Analytical Methodology of the Proposed DBNG Method for Perspective Astrobiology Missions

For reliable detection of life-forms using the DBNG method, two controls have to be used: (i) an aliquot of the test sample mechanically purified from microbial cells (for example, by centrifugation) and (ii) an aliquot with inactivated cells (for example by pasteurization). The formation of nanoparticles (from artificially added cations) in the native sample would prove the presence of Earth-type active living forms, if there are no nanoparticles (from artificially added cations) in the two controls.

UV-visible and fluorescence spectrometry are the most frequently used tools for the detection of metal nanoparticles (Gomez et al., 2014; Loh et al., 2017; Liao et al., 2019; Sánchez-López et al., 2020). In earlier work (Sorokin et al., 2013), it was shown that biogenic Ag°NPs generated under DBNG protocol were confidently registered by visible spectrometry (**Figure 6**). Several models of miniature spectrometers with high sensitivity are available now. The protocol of the DBNG method permits the use of microliter volumes of examined samples and salt solutions. Devices for the production of nanoparticles by chemical methods based on microfluidic lab-on-a-chip (LOC) technologies have already been demonstrated (Wu et al., 2012; McLeod et al., 2013; Gomez et al., 2014; Buja et al., 2017). So, similar chips for detecting biogenic nanoparticles formed by microorganisms in aqueous samples can be developed (Skladnev et al., 2017b). Such devices can certainly be useful for searching for Earth-like living forms directly on extraterrestrial objects.

CONCLUSION

The fast *in situ* formation of the biogenic Ag°NPs in water samples of the Lake Untersee at low temperature was demonstrated. The efficiency of the DBNG method to detect living biological forms directly in native water samples (from 10^4 cells per mL) was confirmed. For evidence of the biogenic character of formed nanoparticles, we used two controls: water samples after the indigenous cells were artificially removed by centrifugation and water samples exposed to pasteurization. Biogenic nanoparticles formed due to application of the DBNG protocol can be detected by spectrometry and/or other physical methods. A compact chip, based on principles of the DBNG method, can be constructed. In summary, the proposed and tested in this work method can be used for the detection of active microbial Life in native samples and can be considered as a new nanobiotechnological tool for searching for Earthlike living forms elsewhere in Space.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DS: general concept, nanoparticles forming experiments, literature review, and preparing the manuscript. LV and YB: isolation of microbial culture and incubation experiments. OK: general concept, correction of the manuscript, and organizational support. SK: literature review and correction of the manuscript. VS: general concept, electron microscopy investigations, and preparing the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors thank Acad. V. F. Gal'chenko, Dr. A. L. Mulyukin, and Jaime A. Cordova Jr. for help and support during our work. The work was carried out within the framework of a State Assignment of the Russian Ministry of Science and Higher Education. All microscopy studies were performed using the instruments in Core Facility Collection of Unique and Extremophilic Microorganisms (UNIQEM) in the Federal Research Center Fundamentals of Biotechnology of the Russian Academy of Sciences.

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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.

frontiers
in Astronomy and Space Sciences

PERSPECTIVE
published: 30 July 2020
doi: 10.3389/fspas.2020.00032

Planetary Analog Field Operations as a Learning Tool

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Mars and Moon analog field missions are established tools to investigate the potential of instruments, workflows, materials, and human factors for characterizing the astrobiological potential and geoscientific context of planetary surfaces. Historically, there is a broad spectrum on both the scientific focus and the performance parameters for analog missions. This applies specifically where performance parameters of coordinated deployment of mission assets (e.g., rovers, human crewmembers, or scientific instruments) are studied. We argue that scientific priorities and workflows shall be consolidated at an early planning stage of deep space missions such as during phase-0 or phase-A studies, while they can still impact the mission architecture design process. It is to be expected that a human-robotic mission to Mars or the Moon will include multiple field assets such as human explorers, robotic vehicles including aerial reconnaissance, mobility assets, habitat modules, stationary instruments, and engineering elements for power, communication, and *in-situ* resource utilization. These require more complex asset coordination compared to single-rover planetary missions. Therefore, we advocate an "Exploration Cascade," which helps to manage these multiple assets to optimize the scientific return of planetary surface missions, to search for extinct and/or extant traces of life, and to characterize the geoscientific context of the sites of interest.

Keywords: planetary exploration, astrobiology, spacesuit simulators, exploration cascade, tactical planning

Edited by:
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Specialty section:
This article was submitted to
Astrobiology,
a section of the journal
Frontiers in Astronomy and Space
Sciences

Received: *05 December 2019*
Accepted: *22 May 2020*
Published: *30 July 2020*

Citation:
Groemer G and Ozdemir S (2020)
Planetary Analog Field Operations as
a Learning Tool.
Front. Astron. Space Sci. 7:32.
doi: 10.3389/fspas.2020.00032

INTRODUCTION

Analog Missions as a Training Tool

Human-robotic Mars missions are likely to be launched within the next 2–3 decades. Some of these missions will include surface sojourns with at least 1 month in duration (Davila et al., 2010; Drake et al., 2010). Analog field campaigns have supported all previous planetary surface missions so far (Preston and Dartnell, 2014) and contributed to planetary missions such as NASA-MSL's Curiosity Rover (e.g., Grotzinger et al., 2015; Kah and MSL Science Team, 2015) and ESA's ExoMars (Vago et al., 2018). It is generally understood that astrobiology constitutes one of the primary scientific drivers for such missions (e.g., Belz et al., 2014; Domagal-Goldman et al., 2016; Fairén et al., 2019). Analog studies are generally considered an efficient tool to prepare for future Mars missions, complementing instrument validation campaigns. Various Mars and lunar analogs on Earth are used to train for scientific operations on planetary surfaces, or study extraterrestrial processes (e.g., Preston and Dartnell, 2014, also see **Figure 1**). Additionally, they help to enlighten the logistics and workflow-related aspects, focusing on human factors, engineering (e.g., human-robotic interaction) constraints, as well as safety considerations.

FIGURE 1 | Examples of analog missions. **(A)** NASA BASALT (USA), **(B)** D-MARS (Israel), **(C)** LUNARES (Poland), **(D)** ESA/PANGEA (Spain), **(E)** NASA/D-RATS (USA), **(F)** HI-SEAS (USA), **(G)** Mars Desert Research Station (USA), **(H)** NASA/NEEMO underwater station (USA), **(I)** NDU Habitat Demonstrator (USA), **(J)** OeWF/AMADEE-program (Austria, Oman), **(K)** Mars-500 (Russia), and **(L)** ESA/CAVES (Spain/Italy). Image credits: **(A)** photos with permission from Zara Mirmalek, **(B)** photos with permission from Hilel Rubinstein/D-MARS, **(C)** photos with permission from Anna B. Gregorczyk, **(D)** photos from permission from ESA/S. Sirios, **(E)** photos NASA, **(F)** photos with permission from Ross Lockwood, **(G)** photos with permissions from OeWF, **(H)** photos: NASA/Florida International University, **(I)** photos with permission from Pablo de Leon, University of North Dakota, **(J)** photos with permission from OeWF, **(K)** photos: Roskosmos, **(L)** photos: European Space Agency.

Notably, a representative metasearch using the Google-Scholar database on the number of publications focusing on the keyword "Mars analog research" yields an increase from 611 results in 1997, to 1,890 in 2007, to 3,150 in 2017 (similar increases were also observed on bibliographic databases NASA ADS and Pubmed), indicating the emergence of a new scientific field. Some examples of past analog campaigns include the NASA DESERT-RATS field campaigns conducted between 1997 and 2010 (Abercromby et al., 2013), the MOONWALK project (Imhof et al., 2015, 2017), the ESA CAVES missions (Bessone et al., 2015), the NASA HI-SEAS long-duration missions (Häuplik-Meusburger et al., 2017), an initiative by the UK Centre for Astrobiology: the subsurface analog research MINAR (Payler et al., 2017), ESA PANGEA (Bessone et al., 2018), the NASA BASALT campaigns (Lim et al., 2019), or numerous stand-alone expeditions studying highly specific astrobiological questions (e.g., Schulze-Makuch et al., 2018) and others. Notably, in 2011, the European Space Agency (ESA) created a topical team to investigate recent analog activities (Martins et al., 2017) by using the Earth as a tool for studying astrobiology, and to formulate inputs and scientific needs for the improvement of ground-based astrobiological research. The outcomes and lessons learned from these and other analog missions constitute the building blocks for OeWF's field campaigns.

Besides these professional programs, there are relevant studies performed at grassroots, mixed professional/citizen-science or outreach-focused analog sites, such as the Mars Desert Research Station MDRS in Utah (Kobrick et al., 2018), or NASA's Spaceward Bound Program (Allner et al., 2010; Rask et al., 2011).

Until now, the Austrian Space Forum (OeWF, German: Österreichisches Weltraum Forum) has conducted 12 Mars analog field campaigns, as part of the PolAres (2006–2017) (Groemer, 2009) and the subsequent AMADEE Program (since 2018). These missions included more than 750 h of simulated EVA (extravehicular activity) operations and the performance of more than 100 peer-review selected experiments. The aim of these initiatives is to identify research gaps in the exploration roadmaps such as the Global Exploration Roadmap (GER) (Crawford, 2014) of the International Space Exploration Coordination Group (ISECG), the NASA Mars Reference Architecture (DRM 5.0) (Drake, 2009), the Mars Exploration Robotic Program (MREP) of ESA (Geelen et al., 2013), as well as NASA's upcoming ARTEMIS program (Chavers et al., 2019). These programmatic roadmaps facilitated the AMADEE program assumptions in terms of projected crew composition, primary scientific objectives, and mission architectural considerations.

Human Element Controversies and Science as an Early Mission Design Driver

It could be argued that robotic exploration is a more efficient tool than human explorers to study planetary surfaces concerning the scientific aspect (as opposed to policy-driven programs like Apollo). However, by looking into the exploration efficiency of current and planned robotic missions, it becomes evident that a human component adds significantly to the throughput of, e.g., sample selection, *in-situ* analysis, and procurement workflows (Crawford, 2012). Glass and Briggs (2003) demonstrated in a field test at Devon Island that humans can be up to 25 times more scientifically productive than a rover under certain conditions. Another concern is the planetary protection aspect, as addressed by the respective COSPAR recommendations (Kminek et al., 2010), which requires a high level of cleanliness at the target site or even access denial to the restricted areas. However, this argument applies also to robotic elements, whereas the amount of contamination (despite proper cleaning efforts on Earth) increases with the mass of the rovers. Hence—besides ethical and societal arguments (e.g., Dunér et al., 2018; Szocik, 2019)—we argue that the expected benefits of including human explorers outweigh the risks from a primarily scientific perspective.

The bandwidth of analog missions ranges from non-scientific initiatives with a focus on education, like the Chinese Plan-C station in the northwestern Gobi desert, NASA's Spaceward Bound Program which aims to train the next generation of space explorers by hosting students and teachers within some analogs (see above for the references), and also highly focused projects like the NASA D-RATS (Abercromby et al., 2013) or BASALT missions (Lim et al., 2019), complemented by laboratory-type analog studies (e.g., the Russian MARS-500 study; Ushakov et al., 2014). In addition to that, analog missions vary in the complexity of their scope. On the one end of the spectrum there are direct instrument field validations and field data acquisitions pertinent to geoscientific workflows such as the AMASE expeditions verifying the performance of the ExoMars PanCam (Amundsen et al., 2010).

On the other side it features more complex surface-sojourn suites of experiments, including human-factors and science tactical decision making, such as the D-RATS studies (Litaker and Howard, 2013). Notably, one of the underground campaigns, MINAR 2017's main focus is to carry out not only geoscientific investigations and instrumental operations under planetary deep subsurface conditions but also to develop technologies for the mining industry (Payler et al., 2017). Therefore, due to the variety in campaign goals, analog missions do not always represent a realistic projection of planetary surface activities. For instance, only a few of them (such as D-RATS, BASALT, or AMADEE) include operational remote science support teams mimicking a major design driver for surface operations (e.g., Groemer et al., 2014, 2016): the modality of the decision-making process, including constraints such as time-delay, bandwidth-limitation, and segregated expertise. Hence, many operational lessons learned of analog missions might be challenging to implement in future flight missions.

We argue that the planetary surface operations—once mission safety criteria have been met—focusing on the astrobiological and geoscientific performance indicators, should be represented as an early-stage design driver for mission architectures. Current exploration frameworks, for instance, the NASA ARTEMIS program, at first define the engineering border conditions, including spacesuit designs, the Deep Space Gateway infrastructure, etc., and then the science objectives are identified. Long lead times in developing deep space infrastructure tend to be the first step in developing architecture and traditionally, science is involved at a later point. So, for industrial policy cycle considerations, capacity building needs to start prior to the surface mission science being consolidated.

However, we suggest that in contrast to the Apollo missions, a science-first principle will ultimately lead to a more effective mission architecture. For instance, the Mars Sample Return project IMOST exemplifies how significant it is to involve detailed science work combined with engineering (Beaty et al., 2018, 2019a,b). Hence, we favor a scientific consensus-building before committing to specific mission architectures. Having multiple disciplines represented over multiple missions, testing various instrumentation, facilitates the establishment of "common trunk infrastructures." Those provide the required technological and operational baselines that provide high flexibility for accommodating experiments and technologies.

RESEARCH GAPS IN TACTICAL DECISION MAKING: APPLICATIONS OF LESSONS LEARNED

Managing Multiple Field Assets During Analog Missions

Balancing the needs and cultures of operational and scientific teams during a mission can be crucial. For instance, during the D-RATS missions (Abercromby et al., 2013), a discrepancy between the space operations community (which had heritage from operational branches of NASA with marginal experience in geoscience field activities) and the scientific teams (stemming from the academic community with less spaceflight operational experience) was observed. This led to flight planning friction losses resulting in both communities feeling under-served. Following the D-RATS lessons learned (Eppler et al., 2013; Rader et al., 2013), as a best-practice example, the 2017 BASALT missions included a Science Traceability Matrix (STM) and operational concepts ("ConOps"; Lim et al., 2019) defining the missions' science objective. Additionally, they implemented a near-real-time interaction between field personnel and the science back rooms at Mission Control (Brady et al., 2019), as well as selected operational considerations (Beaton et al., 2019).

Although there was a lack of assets expected during an actual mission, such as rovers, drones, or a physiological load, and consumables modeling of astronauts, the BASALT field activities were supported by the Minerva software suite (Marquez et al., 2019), optimizing a traverse planning, timeline generation and display (via the PLAYBOOK software, Marquez et al., 2017), procedure management, execution monitoring, data archiving, and visualization (Deans et al., 2017) and included a set of codified flight rules, safety rules, and troubleshooting routines.

Similarly, the Austrian Space Forum has established codified standard operating procedures (SOPs), including workflows analogous to Minerva, but also including physiological modeling and monitoring and taking into account the limitations of operating in high-fidelity spacesuit simulators. These procedures are constantly trained and have evolved into an operational toolkit independent of the technical framework deployed (Groemer et al., 2016).

It is to be expected that a human-robotic mission to Mars will include multiple field assets ranging from human explorers, aerial and surface robotic vehicles, human mobility, habitat modules, stationary instruments and engineering elements for power, communication, and *in-situ* resource utilization. Notably, this variety will require a significantly more complex asset coordination compared to single-rover planetary missions, including the need for delay-tolerant networking and *in-situ* high-performance computing (Geist et al., 2019). The NASA MOSAIC initiative points out this challenge in hindsight of multiple robotic assets on the surface (Hook et al., 2018) but is not designed to address the scale and complexity of a human-robotic mission.

The Exploration Cascade

The "Exploration Cascade" (EC) is an OeWF-coined term for tactically optimizing the sequence of measurements for pursuing a pre-defined scientific question. Although the strategic aims may be set well prior to the flight mission architecture development, environmental dynamics (e.g., the Martian weather or solar activity), infrastructure limitations (e.g., communication ranges, safety rules), instrument anomalies (e.g., dust-induced degradation), human factors (e.g., reduced productivity due to isolation), and even (ground-based) data processing pipeline limitations will have a significant impact on the modalities of when and where to deploy which instruments. Building upon established workflows and SOPs, the Austrian Space Forum has devised the "Exploration Cascade" as an evolving algorithm, taking into account the aforementioned border conditions. Generally, science operations in multidisciplinary campaigns with a wide range of both requirements and expected data products may present challenges in the coordination workflows, but also offer synergistic effects, if properly applying the EC as an operational tool. To exemplify this, under certain circumstances, the high-res imagery obtained by an orbiting telescope may need certain orbital parameters to be met before the target of interest can be surveyed. Although aerial vehicles may need significantly longer time for surveying a site, they might still be the better choice as they would be readily available. This workflow defines when and where to deploy instruments, when their data are to be expected by the Mission Support Center on Earth and how fast the data processing can lead to knowledge influencing the decision making of the flight planners. The exploration cascade was first demonstrated in an early exploratory investigation during the AMADEE-18 field campaign in Oman in February 2018, bringing together 16 experiments (Garnitschnig, 2018), and will be furthered during the AMADEE-20 field campaign in Israel in late 2020.

The EC visualizes and optimizes instrument workflows and their required resources, environmental, and flight planning border conditions, as well as the ground segment data processing pipeline. In comparison to the established and well-tested PLAYBOOK software, the EC is also used as one of the selection criteria for experiment proposals at pre-mission phase, and it allows for an inclusion of the ground segments' remote science support. Initially, it can be considered an empty roadmap that is filled with mission aims, operational requirements (e.g., safety rules for astronauts), scientific priorities (e.g., prioritizing of biomarker detection over media activities), and finally with selected experiments during the mission preparation. Subsequently, after several Dress Rehearsals and map exercises, it evolves into a web of dependencies identifying critical pathways (aka sequence of experiment stages determining the minimum duration for an operation) and susceptibilities to external changes in the workflow as well as their potential alternatives by providing strong networking between experiment PIs, mission leaders, and team leaders, before and during the mission. Finally, it offers lessons learned for future missions, e.g., identifying the need for faster data processing pipelines or increasing instrument robustness for critical pathways.

Analog missions are tools to test permutations and the decision-making trees of the EC. We argue that if the field trials include representative scientific environments, such as a time-delayed remote science support teams and realistic data processing pipelines, multiple assets to be coordinated, and a plausible rule set for the field operations, then uncertainties and weaknesses in the flight mission planning can be substantially reduced. Especially given the cost-benefit ratio of analog missions, variants of the EC can be continuously field-tested with a moderate effort along the mission planning up to the landing of the actual flight missions.

CONCLUSIONS

The proper implementation of the Exploration Cascade is yet to be demonstrated, in particular, the robustness of the workflows needs to be tested in representative environments. As such, the AMADEE-20 mission in Israel in late 2020 will be a proving ground for applying the EC.

Mission teams have strived for a realistic projection of the workflows involving both flight crews on "Mars" and the remote science support teams on Earth. However, the multitude and peculiarities of instruments and technologies available to mission architects and researchers requires a plethora of planning decisions, as in contrast to, e.g., rover missions, the range of decision options rises exponentially with the number of field assets deployed. Also, the bandwidth of scientific priorities makes it challenging to identify patterns (e.g., perceived optimal sequence of workflows) and preferably strategies beyond anecdotal evidence. Therefore, we advocate for a deeper understanding of the scaling effects of an increasing number of field assets, considering long-duration surface sojourns with engineering constraints along with low bandwidth and time-delayed communication, as well as human factors.

The contribution of analog missions to flight mission architectures is strengthened by a clear definition of scientific priorities, awareness about mission architecture assumptions, and a well-structured workflow that allows for an in-depth analysis of the mission performance. Besides, a structured lessons-learned process and emerging well-maintained science data archives, that are open to the scientific community beyond individually funded missions, are key to optimizing the science return for future flight missions.

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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