



# Differential Effects of Varying Concentrations of Phosphorus, Iron, and Nitrogen in N<sub>2</sub>-Fixing Cyanobacteria

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Diazotrophs or N<sub>2</sub>-fixers are one of the most ecologically significant groups in marine ecosystems (pelagic and benthic). Inorganic phosphorus (PO<sub>4</sub><sup>3-</sup>) and iron (Fe) can limit the growth and N<sub>2</sub>-fixing capacities of cyanobacteria. However, studies investigating co-limitation of these factors are lacking. Here, we added different concentrations of PO<sub>4</sub><sup>3-</sup> and Fe in two cyanobacterial species whose relatives can be found in seagrass habitats: the unicellular *Halotheca* sp. (PCC 7418) and the filamentous *Fischerella muscicola* (PCC 73103), grown under different nitrate (NO<sub>3</sub><sup>-</sup>) concentrations and under N<sub>2</sub> as sole N source, respectively. Their growth, pigment content, N<sub>2</sub>-fixation rates, oxidative stress responses, and morphological and cellular changes were investigated. Our results show a serial limitation of NO<sub>3</sub><sup>-</sup> and PO<sub>4</sub><sup>3-</sup> (with NO<sub>3</sub><sup>-</sup> as the primary limiting nutrient) for *Halotheca* sp. Simultaneous co-limitation of PO<sub>4</sub><sup>3-</sup> and Fe was found for both species tested, and high levels of Fe (especially when added with high PO<sub>4</sub><sup>3-</sup> levels) inhibited the growth of *Halotheca* sp. Nutrient limitation (PO<sub>4</sub><sup>3-</sup>, Fe, and/or NO<sub>3</sub><sup>-</sup>) enhanced oxidative stress responses, morphological changes, and apoptosis. Furthermore, an extensive bio-informatic analysis describing the predicted Pho, Fur, and NtcA regulons (involved in the survival of cells to P, Fe, and N limitation) was made using the complete genome of *Halotheca* sp. as a model, showing the potential of this strain to adapt to different nutrient regimes (P, Fe, or N).

**Keywords:** *Halotheca* sp. PCC 7418, *Fischerella muscicola* PCC 73103, phosphorus, iron, nitrogen, N<sub>2</sub>-fixation, reactive oxygen species production, Pho-Fur-NtcA regulon

## INTRODUCTION

Diazotrophs or N<sub>2</sub>-fixers, one of the most important ecological groups, are microorganisms capable of converting atmospheric dinitrogen gas (N<sub>2</sub>) into a readily usable form of dissolved inorganic nitrogen, i.e., ammonia (NH<sub>3</sub>). This process is carried out through the nitrogenase protein complex (encoded by *nifH*, *nifD*, and *nifK*) by nitrogenase-containing autotrophic and heterotrophic bacteria (Hoffman et al., 2014). Biological N<sub>2</sub>-fixation is an essential process in the marine N-cycle, providing new N to the oceans and compensating ocean denitrification (Sohm et al., 2011). In coastal benthic communities (e.g., Mediterranean seagrass

*Posidonia oceanica* communities), N<sub>2</sub>-fixers are also important sources of new N, potentially supplying the entire demand of N of the associated plants (Agawin et al., 2016, 2017). These benthic communities can harbor a high diversity of N<sub>2</sub>-fixing cyanobacteria among which are different groups of cyanobacteria that can be classified as unicellular, filamentous heterocyst forming, and filamentous non-heterocyst forming (Zehr, 2011).

Factors affecting the growth and abundance of cyanobacteria include both abiotic [e.g., macronutrients (e.g., phosphorus, P, and nitrogen, N), micronutrients (e.g., iron, Fe), light, and temperature] and biotic factors (e.g., viral cell lysis or grazing) (Sohm et al., 2011; Zehr, 2011). The nutrients P, N, and Fe are suggested to be the most important limiting nutrients for cyanobacterial growth (Mills et al., 2004; Browning et al., 2017). Phosphorus (P) has key structural (i.e., for nucleic acids and for the cell membranes of most bacteria) and functional roles in various metabolic processes and limiting concentrations of P affect photosynthesis, respiration, and activities of ATP-dependent enzymes (Tripathi et al., 2013; Santos-Beneit, 2015). Nitrogen is essential for protein synthesis, and limiting concentrations of N can reduce cellular growth rates and contribute to the phenomenon called chlorosis, which causes degradation of phycobiliproteins and can lead to posterior downregulation of photosynthesis (Richaud et al., 2001; Klotz et al., 2015). Iron (Fe) is also important for photosynthesis and growth (Sunda and Huntsman, 2015); however, cells must maintain a state of homeostasis since elevated Fe-intracellular concentrations can enhance oxidative stress via Fenton and Haber–Weiss reactions (Kranzler et al., 2013). Under Fe limitation, cyanobacteria can import Fe into the cells through various high-affinity Fe transporters (Kranzler et al., 2014) or through the liberation of low-molecular-weight Fe chelators (e.g., siderophores) (Kranzler et al., 2013). In order to survive and adapt in limiting nutrient conditions, cyanobacteria must trigger molecular mechanisms by transcription factors (TFs). The most well-studied TFs in marine bacteria are PhoB, ferric uptake (Fur), and global nitrogen (NtcA) regulators involved in the acquisition of P, Fe, and N, respectively (Baichoo and Helmann, 2002; Yuan et al., 2006; Llacer et al., 2010). The PhoB, together with PhoR, an inner-membrane histidine kinase, forms a two-component regulatory system (Santos-Beneit, 2015). These TFs are activated or repressed to adapt to a changing environment (e.g., changes in nutrient concentrations) and recognize consensus sequences called transcriptional binding sites (TBS), namely, the PHO box, Fur, and NtcA boxes (Baichoo and Helmann, 2002; Yuan et al., 2006; Llacer et al., 2010), which control the expression of specific genes. The genes controlled by these TFs (PhoB, Fur, or NtcA) are part of what is known as the Pho, Fur, and NtcA regulons, and their prediction can help us understand how cells may adapt and survive under limiting concentrations of P, Fe, and N, respectively.

The demand for P, Fe, and N for N<sub>2</sub>-fixing cyanobacteria may be different from that for non-N<sub>2</sub>-fixing bacteria. Generally, cyanobacteria have elevated ratio of nitrogen (N):phosphorus (P) (a molar ratio above 25 compared with the general Redfield ratio of 16 in marine phytoplankton) (Redfield, 1934; Geider and La Roche, 2002; Quigg et al., 2011). For cyanobacteria,

Fe requirements are 10-fold higher than in non-photosynthetic bacteria (Kranzler et al., 2013). For cyanobacterial N<sub>2</sub>-fixers, Fe is even more important, as Fe is an important structural component of the nitrogenase complex (i.e., *nifH*) (Hoffman et al., 2014). N<sub>2</sub>-fixation also requires a large amount of photosynthetically derived energy, which in turn is P-dependent (Sañudo-Wilhelmy et al., 2001). While non-N<sub>2</sub>-fixing cyanobacteria are usually limited by N, N<sub>2</sub>-fixing cyanobacteria can use ubiquitous atmospheric N<sub>2</sub>, conferring them a competitive advantage over non-N<sub>2</sub>-fixing cyanobacteria in N-limited waters (Sohm et al., 2011). Usually, under limiting concentrations of inorganic N (e.g., NH<sub>3</sub> and NO<sub>3</sub><sup>-</sup>), N<sub>2</sub>-fixation is stimulated, while high concentrations of inorganic N inhibit N<sub>2</sub>-fixation (Manhart and Wong, 1980; Nelson et al., 1982; Knapp, 2012). In a previous study, the P-requirements of *Halothece* sp. PCC 7418 increased in N-limited conditions (i.e., when N<sub>2</sub>-fixation was stimulated), consequently activating their P-acquisition mechanisms [i.e., alkaline phosphatase activity (APA) and P-uptake], which were in turn controlled by Fe availability (Fernández-Juárez et al., 2019). How the concentrations of P and Fe (and their interacting effects) affect the different groups of N<sub>2</sub>-fixing cyanobacteria grown in differing N conditions remains to be investigated.

Here, we investigated the response to different concentrations of PO<sub>4</sub><sup>3-</sup> and Fe, in a multi-factorial design, of two species of N<sub>2</sub>-fixing cyanobacteria: the unicellular *Halothece* sp. PCC 7418 and the filamentous heterocyst-forming *Fischerella muscicola* PCC 73103, grown under different nitrate (NO<sub>3</sub><sup>-</sup>) concentrations and under N<sub>2</sub> as sole N source, respectively. Relatives of these two cyanobacteria have been previously found in association with the seagrass *P. oceanica* (**Supplementary Material, Supplementary Figure S1**, Agawin et al., 2017). The responses were measured in terms of physiological and molecular parameters (growth, pigment content, N<sub>2</sub>-fixation rates, oxidative stress, morphology, and apoptosis). To understand how *Halothece* sp. (PCC 7418) adapts in nutrient limiting environments, an extensive *in silico* analysis was done, taking as a model the complete genome of *Halothece* sp. to predict the Pho, Fur, and NtcA boxes, and the Pho, Fur, and NtcA regulons, respectively.

## MATERIALS AND METHODS

### Strains and Growth Conditions

N<sub>2</sub>-fixing bacterial strains (*Halothece* sp. PCC 7418 and *F. muscicola* sp. PCC 73103) were obtained from Pasteur Culture Collection of Cyanobacteria (PCC). Experiments were conducted in 250-ml acid-clean quartz Erlenmeyer flasks filled with 150 ml of their corresponding culture media. We used ASN-III + Turks Island salts 4× medium and BG11<sub>0</sub> culture medium for *Halothece* sp. and *F. muscicola*, respectively (Stanier et al., 1979). These media were supplemented with 0.1–0.3% (p/v) glucose, and cells were cultivated under aerobic condition in 12:12-h light–dark cycle at 25°C and under continuous low-intensity fluorescent light ( $\cong 30 \mu\text{E m}^{-2} \text{s}^{-1}$ ) in a rotatory shaker (120 rpm). The batch cultures were maintained for over 10–14 days for each experiment, and the initial inoculum of cells was added

at the exponential phase [O.D.<sub>750 nm/975 nm</sub>  $\cong$  0.2 (*Halotheca* sp.)/1.0 (*F. muscicola*)] from their original culture medium. All samples were manipulated in a class-100 clean hood to avoid Fe contamination. The subcultures for seeding were centrifuged and washed with their corresponding medium without PO<sub>4</sub><sup>3-</sup>, Fe, and NO<sub>3</sub><sup>-</sup>.

For *Halotheca* sp., three conditions were established for PO<sub>4</sub><sup>3-</sup> concentrations: [Low PO<sub>4</sub><sup>3-</sup>] (0.1 μM), [Medium PO<sub>4</sub><sup>3-</sup>] (1 μM), and [High PO<sub>4</sub><sup>3-</sup>] (45 μM). Furthermore, three conditions for Fe were also established: [Low Fe] (2 nM), [Medium Fe] (20 nM), and [High Fe] (7.5 μM). These PO<sub>4</sub><sup>3-</sup> and Fe concentrations were combined in nine experimental conditions ([Low PO<sub>4</sub><sup>3-</sup>-Low Fe], [Low PO<sub>4</sub><sup>3-</sup>-Medium Fe], [Low PO<sub>4</sub><sup>3-</sup>-High Fe], [Medium PO<sub>4</sub><sup>3-</sup>-Low Fe], [Medium PO<sub>4</sub><sup>3-</sup>-Medium Fe], [Medium PO<sub>4</sub><sup>3-</sup>-High Fe], [High PO<sub>4</sub><sup>3-</sup>-Low Fe], [High PO<sub>4</sub><sup>3-</sup>-Medium Fe], and [High PO<sub>4</sub><sup>3-</sup>-High Fe]) (**Supplementary Table S1**). The solutions of PO<sub>4</sub><sup>3-</sup>, Fe, and NO<sub>3</sub><sup>-</sup> were prepared from dipotassium phosphate (K<sub>2</sub>HPO<sub>4</sub>), ferric citrate (C<sub>6</sub>H<sub>5</sub>FeO<sub>7</sub>), and sodium nitrate (NaNO<sub>3</sub>), respectively. As reference for the nutrient concentrations for [Low PO<sub>4</sub><sup>3-</sup>] and [Low Fe], we followed the surface water concentrations of the Mediterranean Sea (Statham and Hart, 2005; Powley et al., 2017), while for [High PO<sub>4</sub><sup>3-</sup>] and [High Fe], we followed the optimal concentrations of ASN-III + Turks Island salts 4× medium. We performed two sets of experiments following Fernández-Juárez et al. (2019): under 4.4 mM of NO<sub>3</sub><sup>-</sup> (optimal concentration) and 0.15 mM of NO<sub>3</sub><sup>-</sup> (referred to from now on as [Low NO<sub>3</sub><sup>-</sup>] and represents the minimum amount of NO<sub>3</sub><sup>-</sup> in which *Halotheca* sp. grew with maximal N<sub>2</sub>-fixation rates). The cells were incubated for 10 days in these experiments (**Supplementary Table S1**). After these experiments, we selected the experimental conditions – [Low PO<sub>4</sub><sup>3-</sup>-Low Fe], [Low PO<sub>4</sub><sup>3-</sup>-High Fe], [High PO<sub>4</sub><sup>3-</sup>-Low Fe], and [High PO<sub>4</sub><sup>3-</sup>-High Fe] – in which *Halotheca* sp. cells were grown under extremely limiting NO<sub>3</sub><sup>-</sup> concentrations (6.66 nM), following NO<sub>3</sub><sup>-</sup> concentrations in the Mediterranean Sea as reference (Powley et al., 2017; **Supplementary Table S1**). The cultures were maintained over 12 days. To check the reversibility of the phenotypic characteristics of the cells, we re-inoculated with PO<sub>4</sub><sup>3-</sup>, Fe, and/or NO<sub>3</sub><sup>-</sup> in optimal concentrations (PO<sub>4</sub><sup>3-</sup>: 45 μM, Fe: 7.5 μM, and NO<sub>3</sub><sup>-</sup>: 4.4 mM) at day 12. Inorganic phosphorus (PO<sub>4</sub><sup>3-</sup>) was added to [Low PO<sub>4</sub><sup>3-</sup>-Low Fe], and NO<sub>3</sub><sup>-</sup> was added to [High PO<sub>4</sub><sup>3-</sup>-High Fe] in extremely limiting NO<sub>3</sub><sup>-</sup> treatments; and PO<sub>4</sub><sup>3-</sup> and Fe were added to [Low PO<sub>4</sub><sup>3-</sup>-Low Fe] in optimal NO<sub>3</sub><sup>-</sup> treatments. These cultures were maintained for over 4 days more (**Supplementary Table S1**).

For *F. muscicola*, PO<sub>4</sub><sup>3-</sup> and Fe were added in nine combination treatments as previously described for *Halotheca* sp. but using 180 μM for [High PO<sub>4</sub><sup>3-</sup>] and 30 μM for [High Fe]. These optimal concentrations for PO<sub>4</sub><sup>3-</sup> and Fe are recommended for BG11<sub>0</sub> medium (Stanier et al., 1979). These PO<sub>4</sub><sup>3-</sup> and Fe combinations were performed without any combined N source, and cultures were maintained over 14 days.

All cultures with their respective treatment conditions were performed in duplicate, and growth, pigment content, N<sub>2</sub>-fixation rates, reactive oxygen species (ROS) production and morphological and/or cellular response (e.g., apoptosis) were

measured. For *Halotheca* sp., subsamples of the culture (1.5 ml) were taken from the culture flasks at the final stage of the experiment and were counted through flow cytometric analysis to normalize the results per cell and for the phenotype recovery experiments. For *F. muscicola*, results were normalized per total chlorophyll (Chl).

## Growth Measurement

The growth of *Halotheca* sp. and *F. muscicola* was measured by optical density (O.D.) using a quartz cuvette and read in Cary Eclipse spectrometer. Growth measurements were measured in *Halotheca* sp. every 2 days at 750 nm. In *F. muscicola*, we measured O.D. at 975 nm (maximum O.D. of the cells), and this was measured every 2–4 days. Growth rate (μ, day<sup>-1</sup>) and generation time (T<sub>g</sub>, days) were calculated following the formula described in Widdel (2010) and Ng et al. (2015):

$$\mu = \frac{\ln(O.D.f) - \ln(O.D.i)}{Tf - Ti} \quad (1)$$

$$T_g = \frac{\ln(2)}{\mu} \quad (2)$$

where O.D.<sub>f</sub> is the O.D. at the final time (T<sub>f</sub>) and O.D.<sub>i</sub> is the O.D. at the initial time (T<sub>i</sub>) of the exponential phase of the growth curve.

To evaluate the type of co-limitation of the growth response of N and P (simultaneous co-limitation, independent co-limitation, or serial limitation), we followed the log ratio effect-size criteria based on the mean treatment and control response in Harpole et al. (2011) and Hedges et al. (1999). We calculated the following log growth response ratios as follows:

$$\text{Nitrogen (N) response} : \ln(N_1P_0/N_0P_0) \quad (3)$$

$$\text{Phosphorus (P) response} : \ln(N_0P_1/N_0P_0) \quad (4)$$

$$\text{N + P response} : \ln(N_1P_1/N_0P_0) \quad (5)$$

For *Halotheca* sp., N<sub>0</sub>P<sub>0</sub> represents the growth rate under extremely limiting NO<sub>3</sub><sup>-</sup> conditions at [Low PO<sub>4</sub><sup>3-</sup>], N<sub>1</sub>P<sub>0</sub> is the growth rate under [Low NO<sub>3</sub><sup>-</sup>] or optimal NO<sub>3</sub><sup>-</sup> at [Low PO<sub>4</sub><sup>3-</sup>] conditions, N<sub>0</sub>P<sub>1</sub> is the growth rate under extremely limiting NO<sub>3</sub><sup>-</sup> conditions at [High PO<sub>4</sub><sup>3-</sup>], and N<sub>1</sub>P<sub>1</sub> is the growth rate under combinations of under [Low NO<sub>3</sub><sup>-</sup>] or optimal NO<sub>3</sub><sup>-</sup> at [High PO<sub>4</sub><sup>3-</sup>] conditions. Responses = 0 are responses identical to control values (no response), >0 are positive responses, and <0 are negative responses. The critical threshold value, representing the minimum significant treatment responses at p = 0.05 relative to N<sub>0</sub>P<sub>0</sub>, was calculated to graphically determine the nature of co-limitation. To choose this critical threshold value, we used the T-score criteria, in which those values that surpass the critical T-score were considered statistically significant (**Supplementary Material**, Harpole et al., 2011). We also evaluated the type of co-limitation growth response of PO<sub>4</sub><sup>3-</sup> and Fe under NO<sub>3</sub><sup>-</sup> optimal and [Low NO<sub>3</sub><sup>-</sup>]

conditions, substituting Fe for N in the eqs. 3–5 above, with  $\text{Fe}_0\text{P}_0$  representing the growth rate under [Low Fe] at [Low  $\text{PO}_4^{3-}$ ] conditions. For *F. muscicola*, we could only evaluate the nature of P and Fe co-limitation because all the treatments were incubated with no added combined N source.

Monod kinetic models were performed with data of the initial concentrations of  $\text{PO}_4^{3-}$ , Fe, and  $\text{NO}_3^-$  (Monod, 1949). The Monod growth curves for *Halotheca* sp. were plotted against different concentrations of  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  incubated at different  $\text{PO}_4^{3-}$  ([Low  $\text{PO}_4^{3-}$ ], [Medium  $\text{PO}_4^{3-}$ ], and [High  $\text{PO}_4^{3-}$ ]) and Fe ([Low–Medium Fe] and [High Fe]) concentrations, respectively. The Monod growth curves for *F. muscicola* incubated at different Fe concentrations ([Low–Medium Fe] and [High Fe]) were also plotted against different concentrations of  $\text{PO}_4^{3-}$ . Monod kinetic parameters were calculated from plotted hyperbolic functions of the Monod equation:

$$\mu = \frac{\mu'_{\max} (Q - Q_{\min})}{Q} \quad (6)$$

where  $Q$  is the concentration of  $\text{NO}_3^-$  or  $\text{PO}_4^{3-}$ ,  $Q_{\min}$  is the minimal concentration of  $\text{NO}_3^-$  or  $\text{PO}_4^{3-}$  for growth, and  $\mu'_{\max}$  is the maximum specific growth rate. Half-velocity constant ( $K_{\mu}$ ) was calculated as the concentration at half of the  $\mu'_{\max}$  (Monod, 1949).

## Abundance Measurement of *Halotheca* sp. Using Flow Cytometry

*Halotheca* sp. cells at the end of the different experiments were fixed with glutaraldehyde 25% (v/v) in  $\text{H}_2\text{O}$  (Sigma-Aldrich) [final concentration 0.05% (v/v)] and were counted in a Becton Dickinson FACSVerse cytometer (Becton and Dickinson, Franklin Lakes, NJ, United States). Fluorescent beads, BD FACSuite™ CS&T research beads (Becton and Dickinson and Company BD Biosciences, San Jose, CA, United States), were used as internal standard to calibrate the instrument. To count *Halotheca* sp. cells, we selected fluorescein isothiocyanate (FITC) (488-nm excitation, 530/30-nm emission) and phycoerythrin (PE) (488-nm excitation, 576/26-nm emission) combination fluorescence signals, which show clearly the population of the cells, recording for each sample a total of  $10 \times 10^3$  cells.

## Pigment Measurement in *Fischerella muscicola*

Chlorophyll (Chla, Chlb, and total Chl) and phycobiliprotein [R-PE and R-phycoyanin (R-PC)] content for *F. muscicola* were measured and calculated following Porra et al. (1989) and Sobiechowska-Sasim et al. (2014). Briefly, for chlorophyll measurements, 5-ml samples of the cultures were filtered through MFV5-025 glass microfiber filters (MFV5-025, FilterLab), grounded, and extracted with 4 ml of acetone 80% for 2 h. After extraction, cells and filter debris were discarded by centrifugation, and the supernatant was read at 647 and 664 nm in quartz cuvette in a Cary Eclipse spectrometer. For phycobiliproteins, 5-ml samples of the culture were filtered as described above, grounded, and extracted in a buffer medium composed of 0.25 M of Trizma

base, 10 mM of disodium EDTA, and 2 mg  $\text{ml}^{-1}$  of lysozyme. After extraction, cells and filter debris were centrifuged, and supernatants were read at 564, 618, and 750 nm in quartz cuvette in a Cary Eclipse spectrometer.

## $\text{N}_2$ -Fixation Rate Measurement by Acetylene Reduction Assay

$\text{N}_2$ -fixation rates of *Halotheca* sp. ( $\text{pmol N}_2\text{-fixed cell}^{-1} \text{ h}^{-1}$ ) and *F. muscicola* ( $\text{nmol N}_2\text{-fixed nmol total Chl}^{-1} \text{ h}^{-1}$ ) were measured through acetylene reduction assay (ARA) at the end of the experiments, following the method of Agawin et al. (2014). *Halotheca* sp.  $\text{N}_2$ -fixing activities were measured under known  $\text{N}_2$ -fixing conditions (i.e., anaerobic condition and during the dark photoperiod and in [Low  $\text{NO}_3^-$ ]) (Reddy et al., 1993). For *F. muscicola*, measurements of  $\text{N}_2$ -fixation rates were performed under aerobic conditions during both light and dark photoperiods. Samples of known volume of the cultures (10 ml) were filtered through 0.45- $\mu\text{m}$  GF/F filters (MFV5-025, FilterLab), these filters were incubated with acetylene at 20% (v/v) final concentration injected in each vial using gas-tight Hamilton syringes, and ethylene production was measured (after 3 h of incubation) by gas chromatographic (GC) methods. Ethylene and acetylene were determined using a GC (model HP-5890, Agilent Technologies) equipped with a flame ionization detector (FID). The column was a Varian wide-bore column (ref. CP7584) packed with CP-PoraPLOT U (27.5-m length, 0.53-mm inside diameter, 0.70-mm outside diameter, 20- $\mu\text{m}$  film thickness). Helium was used as carrier gas at a flow rate of 30  $\text{ml min}^{-1}$ . Hydrogen and airflow rates were set at 30 and 365  $\text{ml min}^{-1}$ , respectively. The split flow was used so that the carrier gas flow through the column was 4  $\text{ml min}^{-1}$  at a pressure of 5 psi. Oven, injection, and detector temperatures were set at 52, 120, and 170°C, respectively. Before passing the samples in the GC, standard ethylene and acetylene were ran, and their retention times noted. Ethylene produced was calculated using the equations in Stal (1988). The acetylene reduction rates were converted to  $\text{N}_2$ -fixation rates ( $\text{nmol ml}^{-1} \text{ h}^{-1}$ ) using a factor of 4:1 (Jensen and Cox, 1983).

## Measurement of Reactive Oxygen Species Production

The ROS production was measured using the molecular probe 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma) in culture media (ASN-III + Turks Island salts 4 $\times$  or BG11<sub>0</sub>), which was added to a 96-well microplate (Thermo Scientific) containing cyanobacterial samples (final concentration of probe at 15  $\mu\text{g ml}^{-1}$ ). Within the cells,  $\text{H}_2\text{O}_2$  production oxidizes DCFH-DA to 2',7'-dichlorofluorescein (DCF) whose green fluorescence can be measured at 25°C in a FLx800 Microplate Fluorescence Reader (BioTek Instruments, Inc.) for 1 h with an excitation of 480 nm and emission of 530 nm. The measurements (the slope of the linear regression obtained) were expressed as arbitrary units (AU) per  $\text{cell}^{-1}$ . DCFH-DA was added in ASN-III + Turks Island salts 4 $\times$  or BG11<sub>0</sub> without cells as blanks under the same conditions as above.

## Microscopy and Morphological Studies

Autofluorescence, morphological changes, and DCF oxidized by ROS were observed with a confocal microscope (Leica TCS SPE, Leica Microsystems). Samples were placed onto a clean glass microscope slide, and 40× and 100× objective lens were used to visualize the cells. Images were processed using the software Leica application suite (Leica Microsystems). Autofluorescence was detected with an excitation of 532 nm and an emission of 555- to 619-nm wavelengths. For visualization of DCF oxidized by ROS, we used excitation of 488 nm and emission of 493- to 562-nm wavelengths. Cell apoptosis for *F. muscicola* was observed with Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit (Thermo Fisher), in which apoptotic cells were detected when the exposed phosphatidylserine of these cells conjugates with annexin. Annexin was detected and visualized at excitation of 530–570 nm and emission of 488-nm wavelengths.

## Bio-Informatic *in silico* Analysis

The TBSs (PHO box, Fur, and NtcA boxes) for SphR (i.e., PhoB), ferric uptake regulator (Fur), and global nitrogen regulator (NtcA) already described in cyanobacteria were explored and downloaded from RegPrecise (May, 2020), a web resource for collection, visualization, and analysis of transcriptional regulons reconstructed by comparative genomics (Novichkov et al., 2013). From this database, multifasta files were generated for each TBS, including several genera of cyanobacteria (*Cyanothece*, *Gloeobacter*, *Microcystis*, *Nostoc*, *Prochlorococcus*, *Synechococcus*, *Synechocystis*, *Thermosynechococcus*, and *Trichodesmium*). We obtained three separated multifasta files (one for each TBS type) that contained 77 sequences for PHO box, 167 sequences for Fur boxes, and 228 sequences for NtcA boxes (**Supplementary Material**). For the prediction of the TBS (PHO box, Fur, and NtcA boxes) for *Halotheca* sp., we used a user-friendly web interface for prediction of prokaryote promoter elements and regulons, PePPER, using the DNA motif build and search platform<sup>1</sup> (de Jong et al., 2012). Position frequency matrices (PFMs; using MEME and subsequently transposed to a MOODS compatible PWM format) were made from each multifasta file (**Supplementary Material**). The PFMs generated were searched in the whole genome of *Halotheca* sp. PCC 7418 (GenBank: NC\_019779.1), obtaining 804 hits for SphR (PhoB), 822 hits for Fur, and 238 hits for NtcA. The corresponding hits were manually explored with UGENE (Golosova et al., 2014). We selected the potential TBS on the basis of their relative positions to the genes (a maximum of –800 bp upstream or +40 bp downstream). DNA sequence logos were generated with WebLogo (Crooks et al., 2004) to graphically indicate the relative frequency of each amino at each position, i.e., predicting the consensus sequences for Pho, Fur, and NtcA boxes. Finally, the genes detected as associated to PHO box, Fur, and NtcA boxes were annotated and submitted to EggNOG v5.0 for further identification of orthology relationships and for functional analysis to describe the Pho, Fur, and NtcA regulons, respectively (Huerta-Cepas et al., 2018).

<sup>1</sup>[http://genome2d.molgenrug.nl/g2d\\_pepper\\_motifs.php](http://genome2d.molgenrug.nl/g2d_pepper_motifs.php)

## Statistical Analysis

Non-parametric test, Kruskal–Wallis analysis followed by an unpaired two-samples Wilcoxon test was used to study the effect of the nutrient treatment conditions to growth, pigment concentrations, N<sub>2</sub>-fixation rates, and ROS production. Spearman's correlation was used to determine the relationships between growth and N<sub>2</sub>-fixation rates. The statistical analyses were performed using the SPSS program version 21 (IBM Corp year 2012).

## RESULTS

### Effect of Varying Nutrient Concentrations (P, Fe, and/or N) on *Halotheca* sp. and *Fischerella muscicola*

#### Growth of *Halotheca* sp. Under Different Concentrations of NO<sub>3</sub><sup>–</sup>

The decreasing concentrations of inorganic nitrogen (i.e., NO<sub>3</sub><sup>–</sup>) negatively affected the growth of *Halotheca* sp. PCC 7418 (Kruskal–Wallis,  $H = 24.55$ , 2 d.f.,  $p = 0.045$ , **Table 1**). Average growth rates ( $\mu$ , of all the combinations of different PO<sub>4</sub><sup>3–</sup> and Fe concentrations) under optimal, low, and extremely limiting NO<sub>3</sub><sup>–</sup> conditions were  $0.33 \pm 0.017 \text{ day}^{-1}$  ( $T_g$ , 2.18 days),  $0.24 \pm 0.023 \text{ day}^{-1}$  ( $T_g$ , 3.37 days), and  $0.046 \pm 0.0075 \text{ day}^{-1}$  ( $T_g$ , 16.52 days), respectively. Under extremely limiting NO<sub>3</sub><sup>–</sup> conditions, the growth of the unicellular cyanobacteria dramatically decreased with rates eight times lower than in optimal NO<sub>3</sub><sup>–</sup> conditions, and cells were not able to overcome the latent growth phase in the cultures (**Table 1**).

#### Growth of *Halotheca* sp. Under Different PO<sub>4</sub><sup>3–</sup> and Fe Concentrations

No significant differences in growth of *Halotheca* sp. were observed among different combination conditions of PO<sub>4</sub><sup>3–</sup> and Fe under optimal NO<sub>3</sub><sup>–</sup> (Kruskal–Wallis,  $H = 7.21$ , 8 d.f.,  $p = 0.51$ , **Table 1**; and  $H = 9.02$ , 8 d.f.,  $p = 0.34$ , **Figure 1A**). On the contrary, under [Low NO<sub>3</sub><sup>–</sup>] conditions, significant differences were found (Kruskal–Wallis,  $H = 15.83$ , 8 d.f.,  $p = 0.045$ , **Figure 1B**). Under [Low NO<sub>3</sub><sup>–</sup>], at high concentrations of PO<sub>4</sub><sup>3–</sup>,  $\mu$  was generally higher than at medium and low concentrations (**Table 1** and **Figure 1B**) except when it was combined with high Fe levels (**Table 1** and **Figure 1B**), showing that high Fe levels had a negative impact on their growth at [High PO<sub>4</sub><sup>3–</sup>] (**Figure 1B**).

#### Recovery of *Halotheca* sp. After Nutrient Re-inoculation

The recovery of *Halotheca* sp. cells from low PO<sub>4</sub><sup>3–</sup> and low Fe (and/or under extremely limiting NO<sub>3</sub><sup>–</sup>) to high PO<sub>4</sub><sup>3–</sup> and high Fe concentrations with re-inoculation of the nutrients (PO<sub>4</sub><sup>3–</sup>, Fe, and/or NO<sub>3</sub><sup>–</sup>) in the medium was dependent on the initial conditions of PO<sub>4</sub><sup>3–</sup>, Fe, and NO<sub>3</sub><sup>–</sup> concentrations in the culture medium (**Figure 1C**). In the [Low PO<sub>4</sub><sup>3–</sup>–Low Fe] condition under extremely limiting NO<sub>3</sub><sup>–</sup> conditions, the cells exhibited total chlorosis, and the addition of PO<sub>4</sub><sup>3–</sup>, Fe,

**TABLE 1** | Growth rate ( $\mu$ ) and generation time ( $T_g$ ) for *Halotheca* sp. (at different  $\text{NO}_3^-$  concentrations) and *Fischerella muscicola* at different combinations of  $\text{PO}_4^{3-}$  and Fe concentrations.

Treatments/ parameters	<i>Halotheca</i> sp.				<i>Fischerella muscicola</i>			
	Optimal $\text{NO}_3^-$ (4.4 mM)		Low $\text{NO}_3^-$ (0.15 mM)		Under extremely limiting $\text{NO}_3^-$ (6.66 nM)		$\text{N}_2$ as only source of N	
	$\mu \pm \text{SR}$ ( $\text{d}^{-1}$ )	$T_g \pm \text{SR}$ (d)	$\mu \pm \text{SR}$ ( $\text{d}^{-1}$ )	$T_g \pm \text{SR}$ (d)	$\mu \pm \text{SR}$ ( $\text{d}^{-1}$ )	$T_g \pm \text{SR}$ (d)	$\mu \pm \text{SR}$ ( $\text{d}^{-1}$ )	$T_g \pm \text{SR}$ (d)
[Low $\text{PO}_4^{3-}$ - Low Fe]	0.31 $\pm$ 0.06	2.33 $\pm$ 0.01	0.19 $\pm$ 0.01	3.74 $\pm$ 0.26	0.05 $\pm$ 0.00	12.91 $\pm$ 0.77	0.30 $\pm$ 0.03	2.32 $\pm$ 0.22
[Low $\text{PO}_4^{3-}$ - Medium Fe]	0.31 $\pm$ 0.06	2.36 $\pm$ 0.02	0.13 $\pm$ 0.02	5.29 $\pm$ 0.65	*	*	0.26 $\pm$ 0.06	2.81 $\pm$ 0.62
[Low $\text{PO}_4^{3-}$ - High Fe]	0.35 $\pm$ 0.08	2.08 $\pm$ 0.01	0.13 $\pm$ 0.01	5.56 $\pm$ 0.65	0.03 $\pm$ 0.00	20.87 $\pm$ 0.00	0.43 $\pm$ 0.26	2.53 $\pm$ 0.88
[Medium $\text{PO}_4^{3-}$ - Low Fe]	0.33 $\pm$ 0.09	2.24 $\pm$ 0.03	0.25 $\pm$ 0.03	2.82 $\pm$ 0.30	*	*	0.42 $\pm$ 0.12	1.79 $\pm$ 0.52
[Medium $\text{PO}_4^{3-}$ - Medium Fe]	0.27 $\pm$ 0.01	2.60 $\pm$ 0.05	0.29 $\pm$ 0.05	2.49 $\pm$ 0.41	*	*	0.22 $\pm$ 0.04	3.28 $\pm$ 0.58
[Medium $\text{PO}_4^{3-}$ - High Fe]	0.42 $\pm$ 0.00	1.63 $\pm$ 0.03	0.20 $\pm$ 0.03	3.47 $\pm$ 0.53	*	*	0.25 $\pm$ 0.10	3.25 $\pm$ 1.29
[High $\text{PO}_4^{3-}$ - Low Fe]	0.28 $\pm$ 0.05	2.56 $\pm$ 0.02	0.40 $\pm$ 0.02	1.75 $\pm$ 0.10	0.07 $\pm$ 0.02	10.84 $\pm$ 2.84	0.45 $\pm$ 0.23	2.06 $\pm$ 1.05
[High $\text{PO}_4^{3-}$ - Medium Fe]	0.35 $\pm$ 0.04	2.04 $\pm$ 0.01	0.39 $\pm$ 0.01	1.77 $\pm$ 0.04	*	*	0.50 $\pm$ 0.09	1.44 $\pm$ 0.25
[High $\text{PO}_4^{3-}$ - High Fe]	0.34 $\pm$ 0.03	2.04 $\pm$ 0.03	0.20 $\pm$ 0.03	3.54 $\pm$ 0.54	0.03 $\pm$ 0.01	21.36 $\pm$ 3.49	0.74 $\pm$ 0.00	0.94 $\pm$ 0.00

Values are the mean with their spanning range (SR) between the duplicate measurements. Asterisks (\*) are conditions in which  $\mu$  and  $T_g$  were not measured.

and  $\text{NO}_3^-$  (at final concentrations of 45  $\mu\text{M}$ , 7.5  $\mu\text{M}$ , and 4.4 mM, respectively) was not sufficient to restore their original phenotype. However, in [High  $\text{PO}_4^{3-}$ -High Fe] treatments under extremely limiting  $\text{NO}_3^-$  conditions, re-inoculation of  $\text{NO}_3^-$  exhibited slight recovery (recovering their greenish color; data not shown). In addition, in [Low  $\text{PO}_4^{3-}$ -Low Fe] treatment under optimal  $\text{NO}_3^-$ , which was re-inoculated with  $\text{PO}_4^{3-}$  and Fe, the cells responded with higher growth and recovered their greenish color (Figure 1C).

### Growth of *Fischerella muscicola* Under Different $\text{PO}_4^{3-}$ and Fe Concentrations

Unlike what was observed for *Halotheca* sp., decreasing concentration of  $\text{NO}_3^-$  did not affect the  $\mu$  of *F. muscicola* PCC 73103 (data not shown). With  $\text{N}_2$  as sole N source, *F. muscicola* reached the maximum  $\mu$  of 0.74  $\text{day}^{-1}$  ( $T_g$ , 0.94 days) at the highest concentrations of  $\text{PO}_4^{3-}$  and Fe, and it was higher than the rest of the treatments (Kruskal-Wallis,  $H = 16.03$ , 8 d.f.,  $p = 0.042$ , Figure 2A). The cells under [High  $\text{PO}_4^{3-}$ -High Fe] conditions also showed the highest average content of Chla, Chlb, and total chlorophyll (total Chl) (Figure 2B) and phycobiliprotein (R-PC and R-PE) content (Figure 2C).

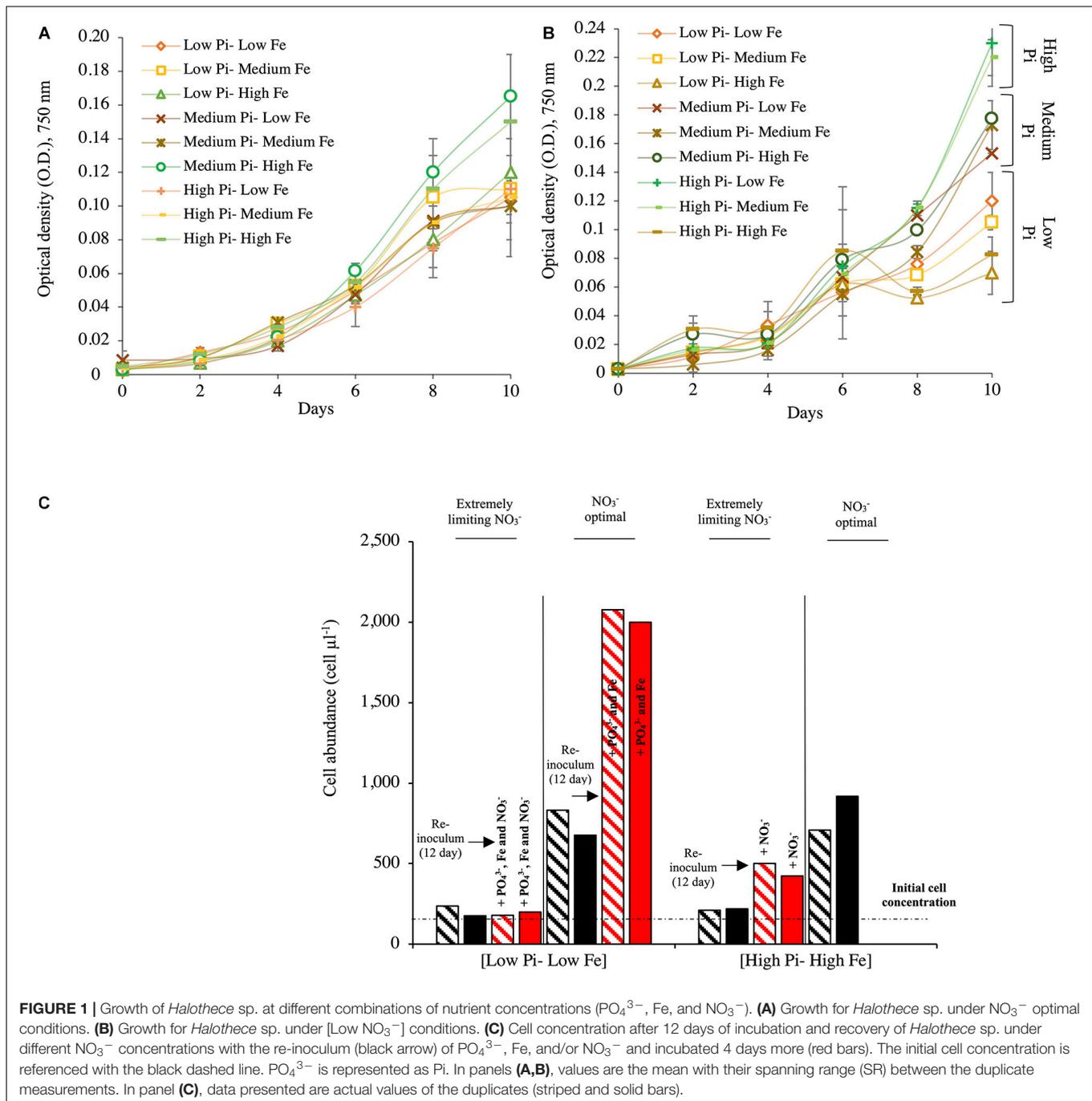
### Nature of Limitation of $\text{PO}_4^{3-}$ , Fe, and/or $\text{NO}_3^-$ in *Halotheca* sp. and/or *Fischerella muscicola*

The evaluation of the limitation of  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  in *Halotheca* sp. under extremely limiting  $\text{NO}_3^-$  conditions concentrations revealed that cells were serially limited, i.e., first with  $\text{NO}_3^-$

and then with  $\text{PO}_4^{3-}$  (N response > 0, P response = 0 and N + P response > 0) (Figure 3A).  $\text{PO}_4^{3-}$  did not have any effect on the growth of cells when they were extremely N limited. Evaluating the effects of the addition of  $\text{PO}_4^{3-}$  and Fe with increased N supply, we showed that under optimal  $\text{NO}_3^-$ , *Halotheca* sp. did not respond significantly to any addition of  $\text{PO}_4^{3-}$  and Fe ( $p < 0.05$ , Figure 3B). Under sufficient N supply (i.e., at [Low  $\text{NO}_3^-$ ] when maximum rates of  $\text{N}_2$ -fixation were measured),  $\text{PO}_4^{3-}$  was the main limiting nutrient and Fe had a negative effect on the growth of *Halotheca* sp. at high Fe levels (Fe response < 0, P response > 0, and P + Fe response > 0) (Figure 3C). However, we also found a simultaneous co-limitation of  $\text{PO}_4^{3-}$  and Fe (Fe response = 0, P response = 0, and P + Fe response > 0) when both elements were added at medium  $\text{PO}_4^{3-}$  and medium-high Fe concentrations for *Halotheca* sp. (Figure 3C). For *F. muscicola*, a simultaneous co-limitation of  $\text{PO}_4^{3-}$  and Fe was also observed (Fe response = 0, P response = 0, and P + Fe response > 0) when both elements were added at high  $\text{PO}_4^{3-}$  and Fe concentrations (Figure 3C).

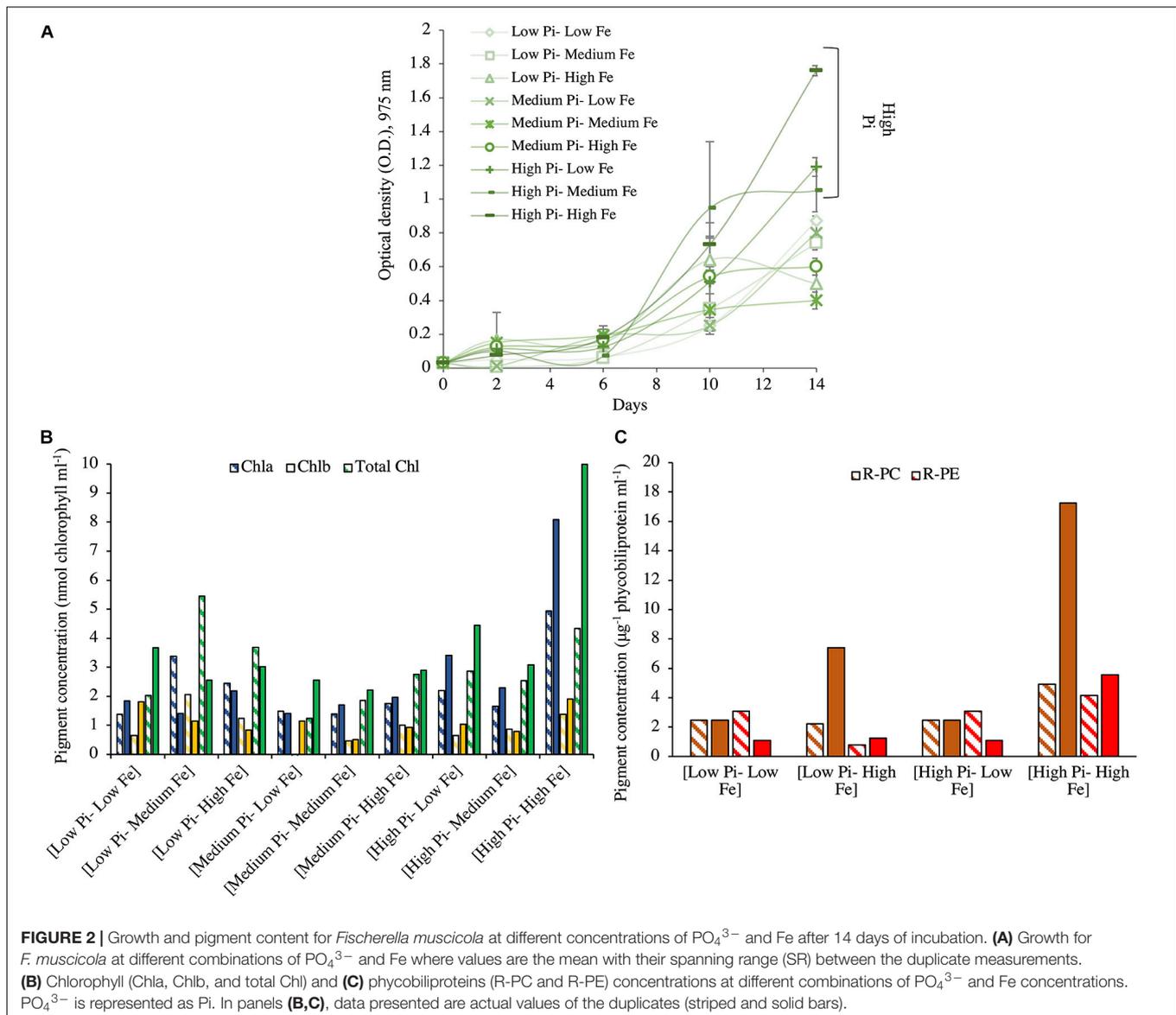
### Monod Growth Kinetics of *Halotheca* sp. and *Fischerella muscicola*

The N-dependent Monod growth kinetics of *Halotheca* sp. incubated at different  $\text{PO}_4^{3-}$  concentrations showed P dependence (Figure 4A). *Halotheca* sp. had higher maximum growth at high and medium  $\text{PO}_4^{3-}$  levels ( $\mu'_{\text{max}} = 0.50 \text{ day}^{-1}$ ) compared with low  $\text{PO}_4^{3-}$  levels



( $\mu'_{\max} = 0.44 \text{ day}^{-1}$ ). However, the half-velocity constant at [High  $\text{PO}_4^{3-}$ ] ( $K_{\mu} = 0.217 \text{ mM}$ , with a  $Q_{\min} = 1.55 \text{ mM}$ ) was the lowest compared with that at [Medium  $\text{PO}_4^{3-}$ ] ( $K_{\mu} = 0.45 \text{ mM}$ , with a  $Q_{\min} = 1.19 \text{ mM}$ ) and [Low  $\text{PO}_4^{3-}$ ] ( $K_{\mu} = 0.53 \text{ mM}$ , with a  $Q_{\min} = 1.22 \text{ mM}$ ) (**Figure 4A**). The P-dependent growth kinetics of *Halotheca* sp. incubated at different Fe concentrations showed  $\mu'_{\max}$  ( $0.25 \text{ day}^{-1}$ ) and  $Q_{\min}$  ( $5.37 \mu\text{M}$ ) at high Fe levels that were lower and higher, respectively, than at low to medium Fe levels ( $\mu'_{\max} = 0.43 \text{ day}^{-1}$ ,  $Q_{\min} = 3.13 \mu\text{M}$ ); but  $K_{\mu}$  was lower

at [High Fe] ( $K_{\mu} = 0.01 \mu\text{M}$ ) than in [Low-Medium Fe] ( $K_{\mu} = 0.35 \mu\text{M}$ ) (**Figure 4B**). On the other hand, *F. muscicola* generally had higher values of the P-dependent kinetic parameters than *Halotheca* sp. when incubated at different Fe concentrations (**Figure 4B**). *F. muscicola* had the higher maximum growth ( $\mu'_{\max} = 0.83 \text{ day}^{-1}$ ), half-velocity constant ( $K_{\mu} = 1.85 \mu\text{M}$ ), and higher minimal cell quota ( $Q_{\min} = 12.58 \mu\text{M}$ ) at high Fe treatments as compared with cells at low to medium levels of Fe ( $\mu'_{\max} = 0.62 \text{ day}^{-1}$ ,  $K_{\mu} = 0.03 \mu\text{M}$ ,  $Q_{\min} = 12.33 \mu\text{M}$ ) (**Figure 4B**).



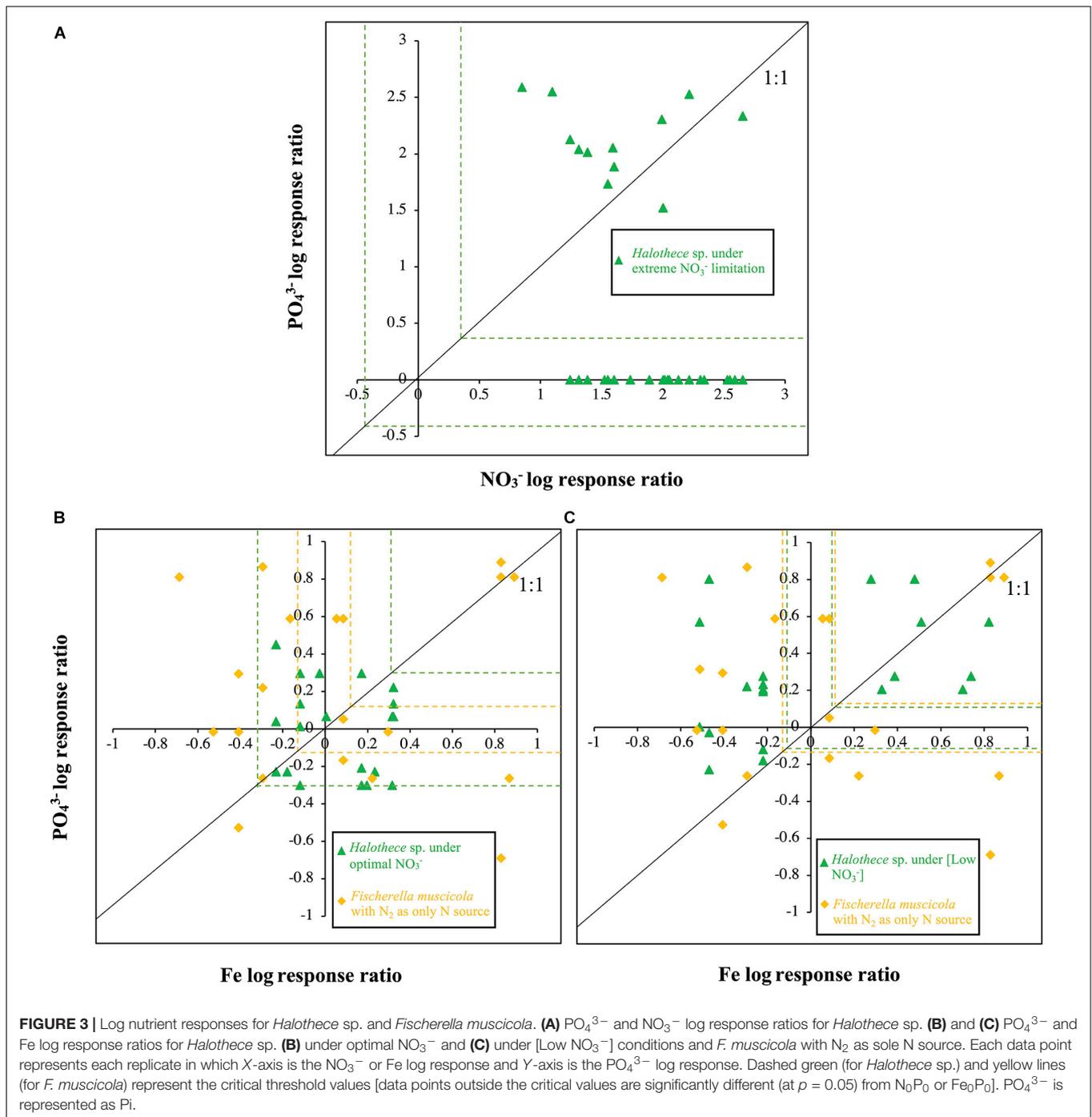
## Effect of Varying Concentrations of P and Fe on $\text{N}_2$ -Fixation Rates of *Halotheca* sp. and *Fischerella muscicola*

*Halotheca* sp.-specific  $\text{N}_2$ -fixation rates under [Low  $\text{NO}_3^-$ ] at different treatment combinations of  $\text{PO}_4^{3-}$  and Fe were significantly linearly correlated with cell concentrations (Spearman's correlation,  $r = 0.7$ ,  $p < 0.05$ ,  $n = 18$ ).  $\text{N}_2$ -fixation rates at [Low  $\text{PO}_4^{3-}$ ] conditions were undetectable and generally increased at medium  $\text{PO}_4^{3-}$  and at increasing Fe concentrations (Figure 5A). However, at the highest  $\text{PO}_4^{3-}$  and Fe concentrations,  $\text{N}_2$ -fixation was again undetectable (Figure 5A). For *F. muscicola*, where specific  $\text{N}_2$ -fixation rates were measured during the light and dark photoperiods, there were no significant differences among the treatments during the light and dark phase (Kruskal-Wallis,  $H = 15.45$ , 8 d.f.,  $p = 0.051$ ; data not shown). Higher  $\text{N}_2$ -fixation rates under high  $\text{PO}_4^{3-}$

levels were detected, especially when combined with high Fe concentrations (Figure 5B).

## Effect of Varying Concentrations of P and Fe on the Production of Reactive Oxygen Species of *Halotheca* sp. and *Fischerella muscicola*

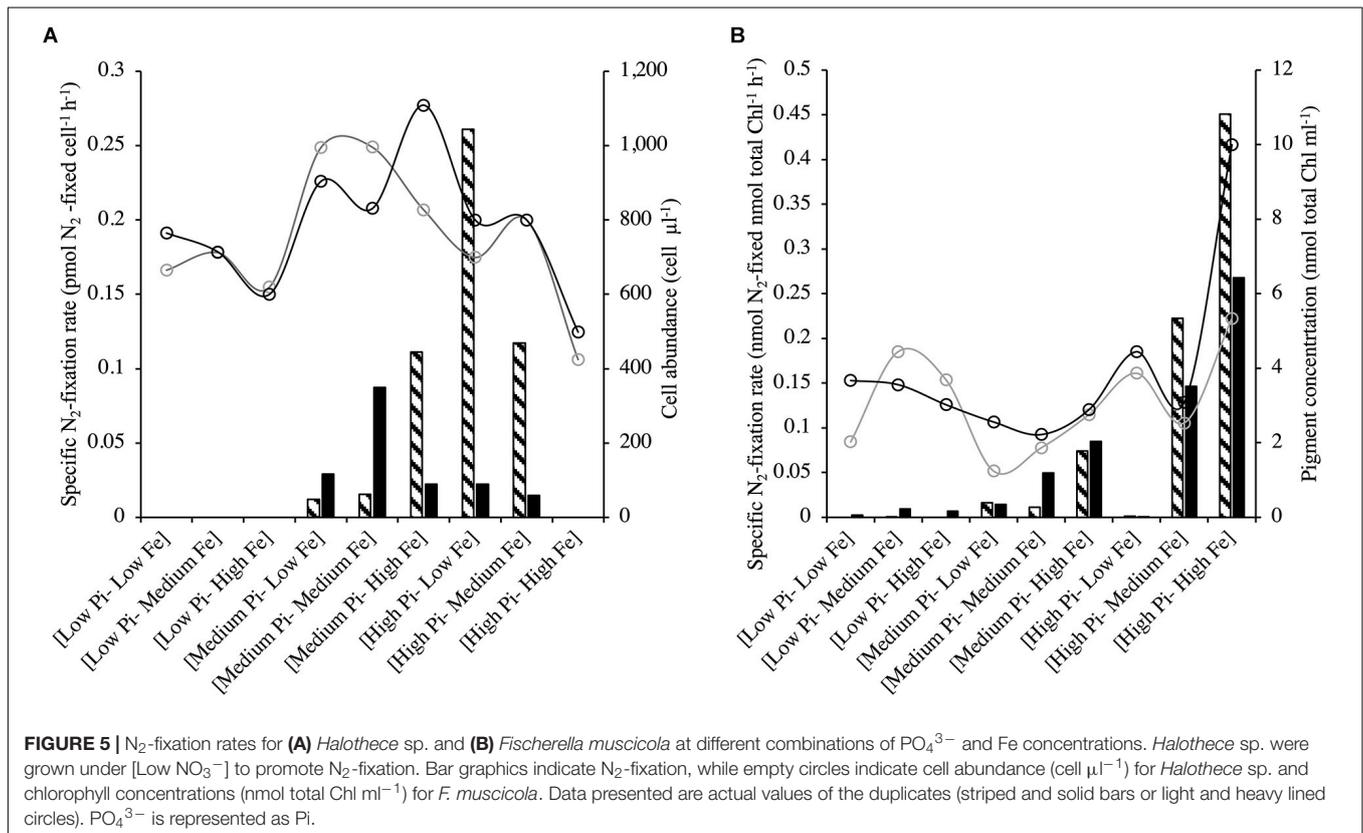
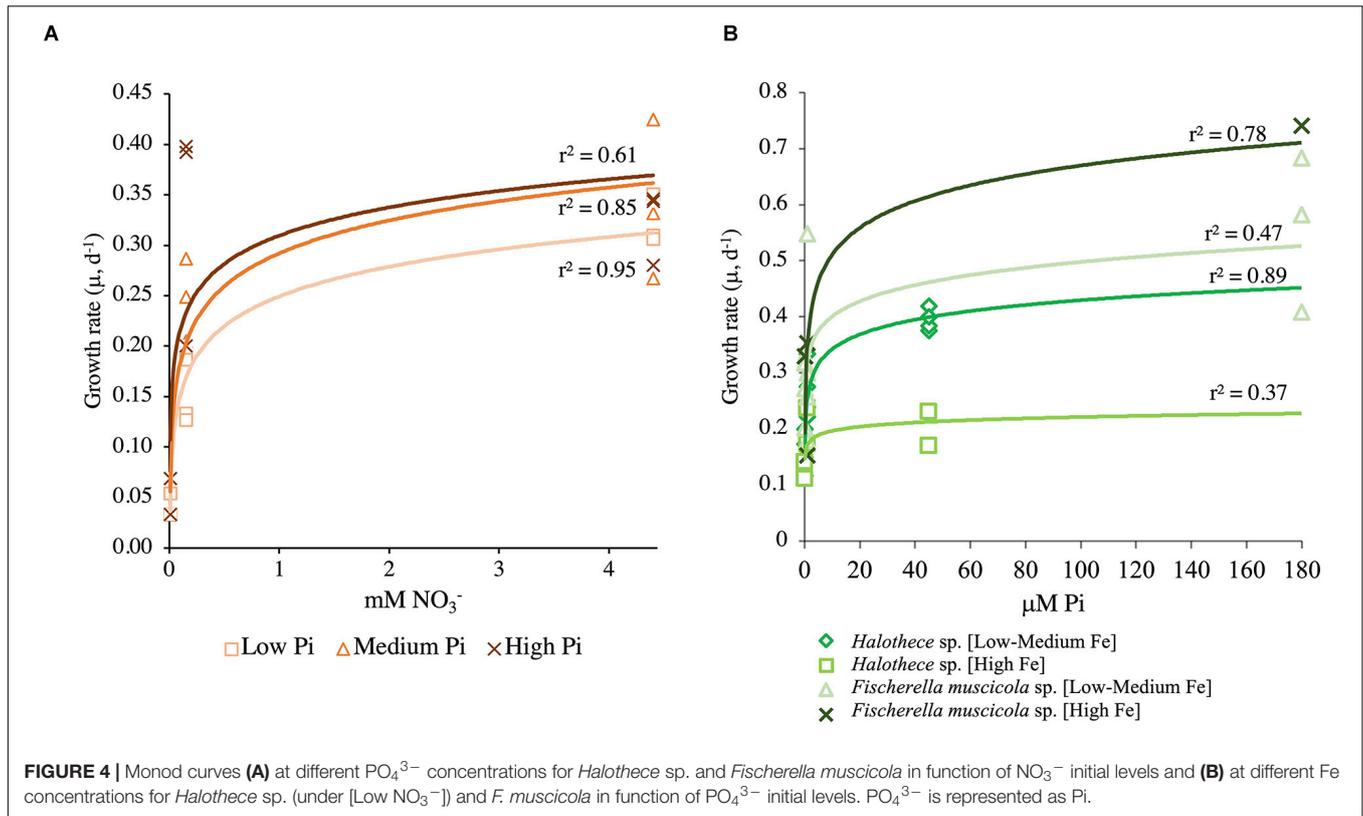
Reactive oxygen species production for *Halotheca* sp. was measured under optimal  $\text{NO}_3^-$  and [Low  $\text{NO}_3^-$ ] conditions (Figures 6A,B). Under optimal  $\text{NO}_3^-$ , there were significant differences among the treatments (Kruskal-Wallis,  $H = 15.67$ , 8 d.f.,  $p = 0.047$ , Figure 6A). ROS production was higher at [Low  $\text{PO}_4^{3-}$ -Low Fe] conditions compared with increasing concentrations of  $\text{PO}_4^{3-}$  at the same [Low Fe] conditions (Figure 6A). At [Low  $\text{PO}_4^{3-}$ ] conditions, increasing Fe concentration reduced the ROS production (Figure 6A). On the

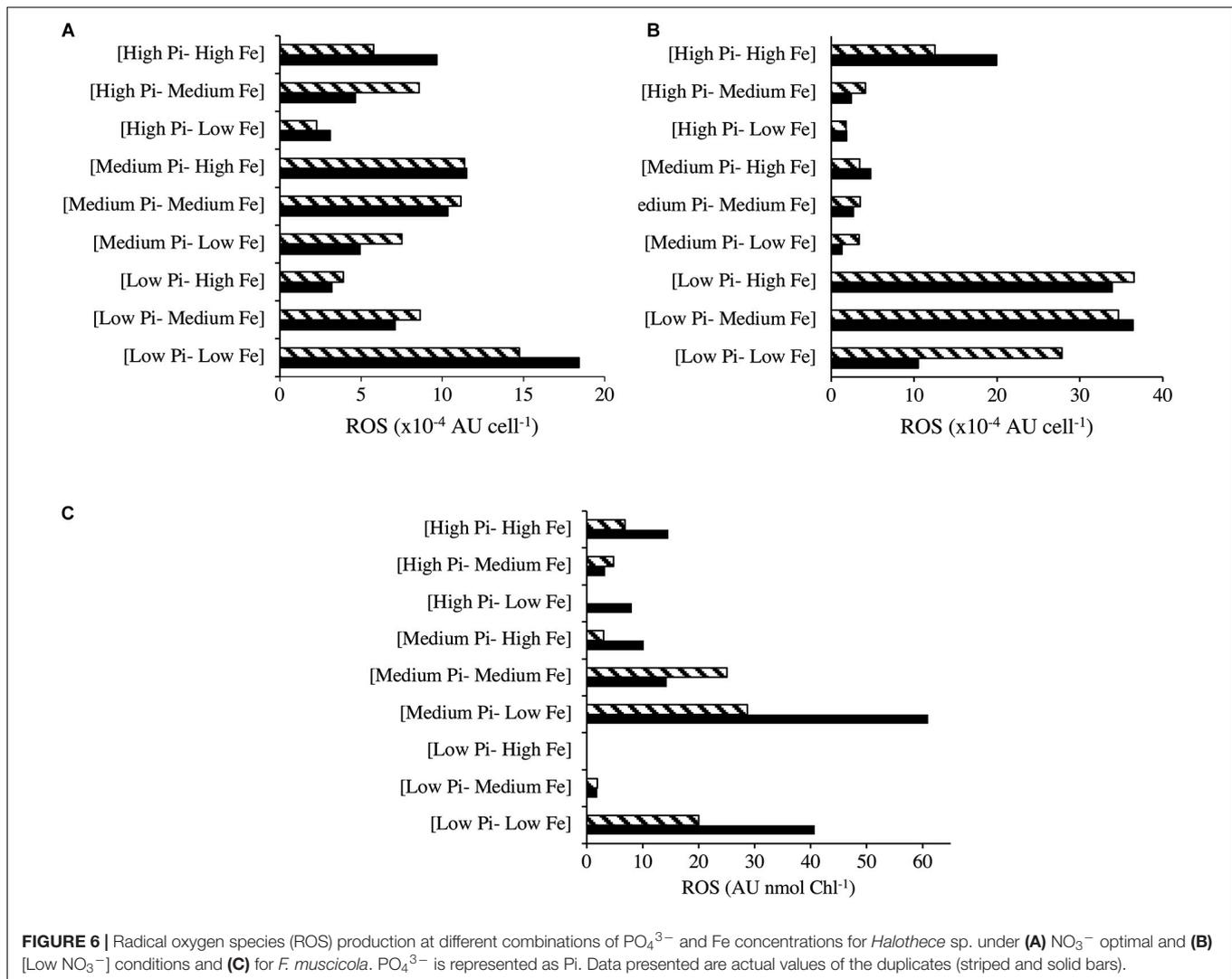


other hand, cells under  $\text{PO}_4^{3-}$  and Fe limitation ([Low  $\text{PO}_4^{3-}$ –Low Fe]) and optimal  $\text{NO}_3^-$  showed higher ROS production, as observed with the greener color (oxidized DCF) of cells, compared with the red color of cells that were not producing ROS in [High  $\text{PO}_4^{3-}$ –High Fe] (Figures 7A,B). On the other hand, under [Low  $\text{NO}_3^-$ ] conditions, we did not detect significant differences in ROS production (Kruskal–Wallis,  $H = 13.80$ , 8 d.f.,  $p = 0.087$ , Figure 6B). Nonetheless, we observed ROS production under [Low  $\text{NO}_3^-$ ] conditions to be higher at

[Low  $\text{PO}_4^{3-}$ –Medium/High Fe] than the rest of the treatments except at [High  $\text{PO}_4^{3-}$ –High Fe] and [Low  $\text{PO}_4^{3-}$ –Low Fe] (Figure 6B). At [Medium Fe] conditions, increasing  $\text{PO}_4^{3-}$  concentrations reduced ROS production (Figure 6B).

The ROS production of *F. muscicola* did not vary among the different treatment combinations of  $\text{PO}_4^{3-}$  and Fe (Kruskal–Wallis,  $H = 12.30$ , 8 d.f.,  $p = 0.14$ , Figure 6C). Nonetheless, there was a tendency of higher ROS production at [Low  $\text{PO}_4^{3-}$ –Low Fe] compared with [High  $\text{PO}_4^{3-}$ –High Fe] conditions, consistent





with the confocal images showing oxidative stress, observing more green-yellowish color cells (indicative of ROS production) in [Low PO<sub>4</sub><sup>3-</sup>-Low Fe] treatments (Figure 7C) than in [High PO<sub>4</sub><sup>3-</sup>-High Fe] conditions (Figure 7D).

### Effect of Varying Concentrations of P and Fe on the Morphology and Physiology of *Halotheca sp.* and *Fischerella muscicola*

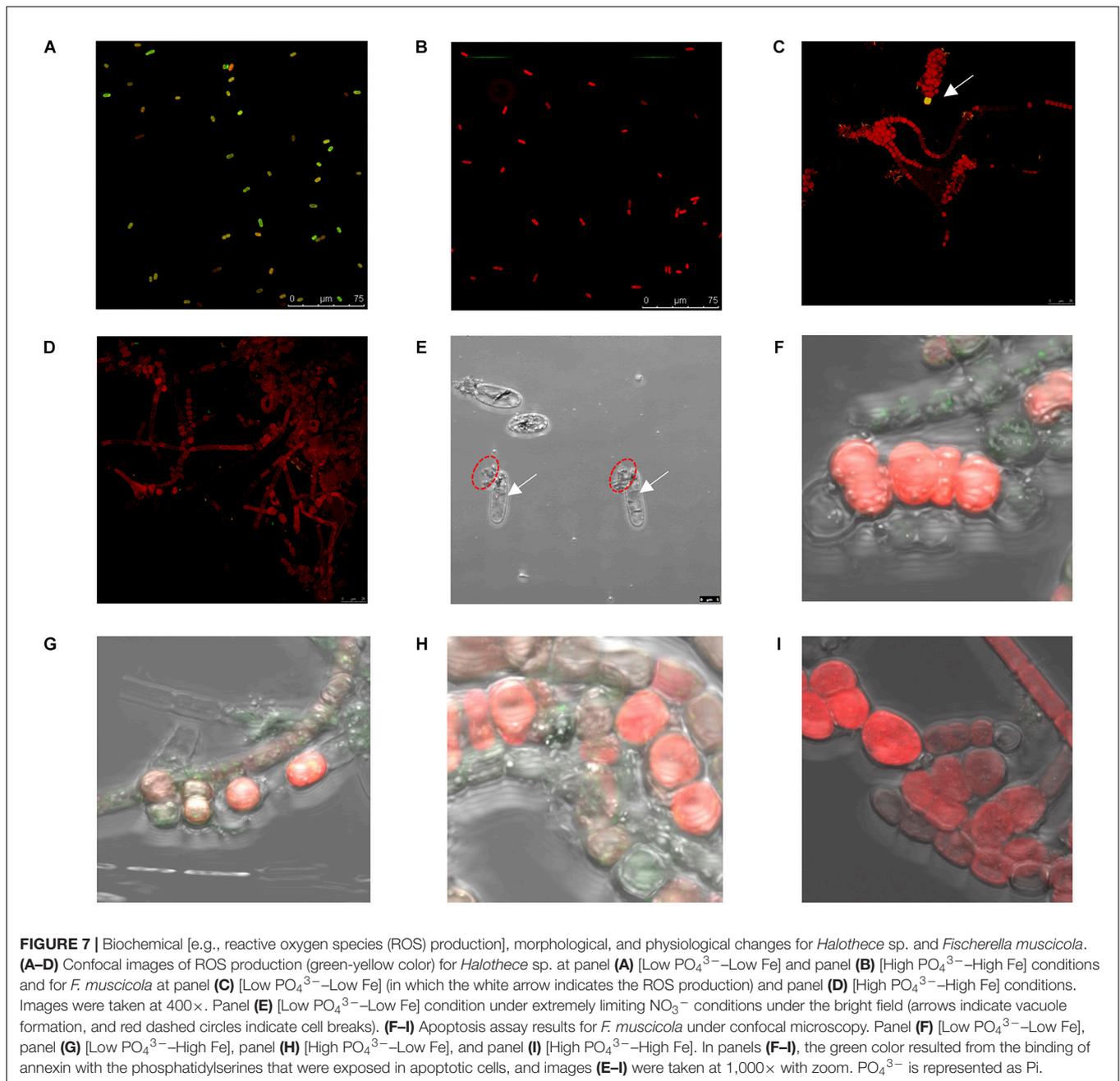
*Halotheca sp.* cells under optimal nutrient conditions (PO<sub>4</sub><sup>3-</sup>, Fe, and/or NO<sub>3</sub><sup>-</sup>) exhibited sizes between 4 and 7 μm. Limitation of PO<sub>4</sub><sup>3-</sup> and Fe under NO<sub>3</sub><sup>-</sup> optimal and [Low NO<sub>3</sub><sup>-</sup>] conditions did not cause changes in sizes. However, extremely limiting NO<sub>3</sub><sup>-</sup> conditions caused an increase in size of the cells (up to two-fold with respect to the normal size), reaching an average size of 12.7 ± 0.74 μm. Under extremely limiting NO<sub>3</sub><sup>-</sup> conditions together with PO<sub>4</sub><sup>3-</sup> and Fe limitation, cells experience vacuole production together with modification of shape and breakage of the cells (Figure 7E). In addition, we also observed that under these same conditions, cells experience total chlorosis

(dramatic loss of phycobiliproteins), and cells were barely visible under confocal microscopy compared with cells under optimal nutrient conditions.

Programmed cell death for *F. muscicola* was studied through apoptosis assay to detect mortality in four treatments: [Low PO<sub>4</sub><sup>3-</sup>-Low Fe] (Figure 7F), [Low PO<sub>4</sub><sup>3-</sup>-High Fe] (Figure 7G), [High PO<sub>4</sub><sup>3-</sup>-Low Fe] (Figure 7H), and [High PO<sub>4</sub><sup>3-</sup>-High Fe] (Figure 7I). Results showed that PO<sub>4</sub><sup>3-</sup> and Fe limitation caused increased mortality. At [High PO<sub>4</sub><sup>3-</sup>-High Fe], there was no signal of annexin V in conjugation with phosphatidylserine detected, indicating the good status of the cells in this treatment.

### Prediction of Genes Involved in P, Fe, and N Adaptation and Survival in *Halotheca sp.*

We predicted the consensus sequences for TBSs for SphR-PhoB (PHO box), Fur regulator (Fur Box), and NtcA protein (NtcA box) for *Halotheca sp.* For PHO box, we



established that consensus sequence was formed by three tandem repeats of 8 bp ( $[\text{ATTTAAAT}]_3$ ) separated by 3 bp (**Figure 8A**). Fur Box was formed by 19 bp inverted repeats ( $\text{ATTGAAAATTATTTT}[\text{T/C}]\text{AAT}$ ) (**Figure 8B**). Finally, NtcA box was constituted by  $\text{TGTAN}_8\text{TACA}$  in which GTA at positions 2–4 and TAC at positions 13–15 were well conserved (**Figure 8C**). From the prediction of these boxes, we described what potential genes are implicated in Pho, Fur, and NtcA regulons (**Supplementary Tables S2–S4**).

Pho regulon (**Figure 8D**) was mainly constituted by genes involved in inorganic ion transport and metabolism (P) (10%), replication, recombination and repair (L) (10%), and cell

wall/membrane/envelope biogenesis (M) (8%), detecting 218 genes (**Supplementary Table S2**). Among them, we detected genes that are part of the “classical” Pho regulon (e.g., some of them found in the same operon): PstS family phosphate ABC transporter substrate-binding protein-phosphate (*pstS*), ABC transporter permease subunit PstC-phosphate (*pstC*), ABC transporter permease PstA-phosphate (*pstA*), ABC transporter ATP-binding protein PstB (*pstB*), PhoQ sensor (*phoQ*), or alkaline phosphatase (*phoA*). In addition, we detected other genes under the control of PhoB such as *nbla* (involved in chlorosis processes); *gvpA*, *gvpN*, *gvpL*, and *gvpF* (involved in gas vesicles); *cas* genes (involved in CRISPR-Cas systems); and a



whole group of genes that codify for transferases, endonucleases, ABC transporters, or transposases (**Supplementary Table S2**).

Fur regulon (**Figure 8E**) mostly was distributed in P (13%), L (11%), coenzyme transport and metabolism (H) (9%), and signal transduction mechanisms (T) (9%), detecting 73 genes (**Supplementary Table S3**). We detected genes under the control of Fur box directly involved in Fe acquisition, such as energy transducer TonB (*tonB*), MotA/TolQ/ExbB proton channel family protein, and three operons of transporter permease subunit (*feoA-feoB*). Fur boxes were detected upstream of genes related in photosynthesis and respiration [e.g., photosystem II reaction center protein X (*psbX*)], in the pathway of chlorophyll biosynthesis [glutamate-1-semialdehyde-2,1-aminomutase (*gsaI*)], and in bacteriochlorophyll biosynthesis [magnesium-protoporphyrin IX monomethyl ester anaerobic oxidative cyclase/DNA-binding response regulator (*bchE*)].

Finally, NtcA regulon (**Figure 8F**) was constituted by genes mostly involved in P (17%), amino acid transport and metabolism (E) (14%), and energy production and conversion (C) (12%), detecting 40 genes (**Supplementary Table S4**). We detected genes involved in N<sub>2</sub>-fixation processes: FeMo cofactor biosynthesis protein (*nifB*) and molybdate ABC transporter substrate-binding protein (*modA*); in nitrogen assimilation: ferredoxin-nitrite reductase (*nirA*); in glutamine/glutamate assimilation: glutamate synthase [NADPH] large chain (*gltB*), type I glutamate-ammonia ligase (*glnN*) and sodium:glutamate symporter (*gltS*); and in ammonium assimilation: ammonium transporter (*amt*). In addition, Fur regulator itself potentially has a NtcA box.

## DISCUSSION

### Effect of Varying N Concentrations on *Halothece* sp. Compared With *Fischerella muscicola*

In this study, the dependence on the concentration of combined N sources such as NO<sub>3</sub><sup>-</sup> for growth of diazotrophic cyanobacterial cultures differed between the unicellular *Halothece* sp. and the filamentous heterocyst-forming *F. muscicola* (**Table 1** and **Figures 1A–C, 2A, 3A, 4A**). Theoretically, N<sub>2</sub>-fixers are independent of inorganic N source (e.g., *F. muscicola*) because of their capability of fixing N<sub>2</sub> (Knapp, 2012). However, at extremely limiting NO<sub>3</sub><sup>-</sup> conditions (i.e., 6.66 nM), almost having N<sub>2</sub> as sole N source, *Halothece* sp. were barely growing (**Table 1** and **Figures 1C, 4A**), and their morphology (increased size) and physiology (i.e., exhibiting extreme chlorosis) changed. Chlorosis can result from the deprivation of NO<sub>3</sub><sup>-</sup>, which promotes the degradation of phycobilisomes through the protein NblA (Klotz et al., 2015), which was detected here under the PhoB control (**Supplementary Table S2**). The observed increases in cell size, possibly, is a result of cell division cessation (Klotz et al., 2016). Moreover, vacuole formation was observed possibly due to the storage of compounds in response to severe N limitation (**Figure 7E**). Our results indicate that in some N<sub>2</sub>-fixing species, e.g., *Halothece* sp., a minimum amount of combined N sources is necessary for the proper functioning of the cells ( $Q_{\min}$  ranging

from 1.19 to 1.51 mM of NO<sub>3</sub><sup>-</sup>, **Figure 4A**). However, it remains to be investigated whether these cells in the natural environment, indeed, require a combined source of inorganic nitrogen and if this requirement can be supplied by other diazotrophs in N-limited waters.

Contrary with *Halothece* sp., *F. muscicola* cells were totally independent of combined N source and were able to grow with N<sub>2</sub> as the sole N source. The viability of unicellular N<sub>2</sub>-fixers to grow under N<sub>2</sub> as sole N source may be dependent on the presence and type of additional carbon (C) source to support their growth as shown with *Synechococcus* sp. strain SF1 (isolated from macroalgae, *Sargassum fluitans*), which was not able to grow with HCO<sub>3</sub><sup>-</sup> as sole C source (Spiller and Shanmugam, 1987). Here, *Halothece* sp. were cultured with glucose and citrate, and these may not be the optimal C source for their growth when grown at almost having N<sub>2</sub> as sole N source. Unicellular N<sub>2</sub>-fixers also have been shown to grow more when NO<sub>3</sub><sup>-</sup> is added compared when grown with only N<sub>2</sub> as sole N source (Agawin et al., 2007), since a larger energetic cost is associated with assimilating N<sub>2</sub> versus NO<sub>3</sub><sup>-</sup> assimilation. Since N<sub>2</sub>-fixation is sensitive to oxygen (O<sub>2</sub>), cyanobacteria must develop strategies to avoid N<sub>2</sub>-fixation inhibition by the O<sub>2</sub> liberated from photosynthesis. Unicellular cyanobacterial cells are able to separate temporarily two incompatible processes: N<sub>2</sub>-fixation (at night) and photosynthesis (at day) in the same cell (De Bruijn, 2015). These two processes have to be tightly controlled to avoid inhibition of N<sub>2</sub>-fixation through well-regulated circadian clocks (Toepel et al., 2008), and these strategies can be energy consuming. Aside therefore of the energy cost of assimilating N<sub>2</sub>, *Halothece* sp. may have an add-on cost of temporally separating N<sub>2</sub>-fixation and photosynthetic process, and thus they cannot be totally independent of combined sources of inorganic N. On the contrary, filamentous heterocyst-forming cells have specialized cells (heterocyst) in which N<sub>2</sub>-fixation takes place in anoxic conditions and may not have an add-on cost of temporally separating N<sub>2</sub>-fixation and photosynthetic processes and thus are better adapted to growing with N<sub>2</sub> as sole N source (De Bruijn, 2015; **Figure 5B**).

### Effect of Varying Concentrations of P and Fe on *Halothece* sp. and *Fischerella muscicola*

#### Concentration of P and Fe in *Halothece* sp. and *Fischerella muscicola*

Phosphorus (P) is needed for many cellular components, such as cellular membranes, nucleic acids, and ATP-dependent reactions such as N<sub>2</sub>-fixation, which is energetically costly, requiring 16 ATPs ( $N_2 + 8e^- + 16ATP + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16PO_4^{3-}$ ) (Hoffman et al., 2014). Under optimum conditions for N<sub>2</sub>-fixation (at low [Low NO<sub>3</sub><sup>-</sup>] for *Halothece* sp. and without any combined N source in the case of *F. muscicola*), increasing concentrations of PO<sub>4</sub><sup>3-</sup> generally increased the growth of both species, suggesting that PO<sub>4</sub><sup>3-</sup> was an important limiting factor for both species and affected their N<sub>2</sub>-fixation rates (**Figures 1–5**). These results are consistent with the previous study of Fernández-Juárez et al. (2019) reporting

the increased rates of P-acquisition mechanisms ( $\text{PO}_4^{3-}$ -uptake rates and APAs) at [Low  $\text{NO}_3^-$ ] for *Halotheca* sp. At high concentrations of  $\text{NO}_3^-$  where  $\text{N}_2$ -fixation rates were lower (and thus this P-requirement can be lower), growth of *Halotheca* sp. was independent of varying concentrations of  $\text{PO}_4^{3-}$ . It is well described that in some cyanobacteria, e.g., *Trichodesmium* sp., one of the major  $\text{N}_2$ -fixers in the Atlantic, P is the main element regulating  $\text{N}_2$ -fixation (Sañudo-Wilhelmy et al., 2001). Under P-limiting environments (e.g., the Mediterranean Sea),  $\text{N}_2$ -fixers must therefore depend on external P sources (such as Saharan dust deposition, Tanhua et al., 2013), and cells have to be well adapted to P limitation with mechanisms for phosphorus scavenging (Dyhrman and Haley, 2006; Fernández-Juárez et al., 2019).

Iron (Fe) had differing effects for *Halotheca* sp. (i.e., toxic effect under high Fe levels under [Low  $\text{NO}_3^-$ ]) and for *F. muscicola* (i.e., limiting growth under low Fe levels) (Table 1 and Figures 1B, 2A, 3B,C, 4, 5). The results for *F. muscicola* were consistent with studies on *Crocospaera watsonii* in which severe Fe limitation (3 nM) showed strong negative changes in growth and  $\text{N}_2$ -fixation, limiting both processes, while higher Fe levels (up to 400 nM) increased these two parameters (Jacq et al., 2014).  $\text{N}_2$ -fixation is an Fe-dependent process, since nitrogenase complex contains 38 Fe atoms per holoenzyme (Hoffman et al., 2014). This Fe dependence was observed for *F. muscicola* at high  $\text{PO}_4^{3-}$  levels (Figure 5B). However, in *Halotheca* sp., high amounts of Fe (7.5  $\mu\text{M}$ ) inhibited  $\text{N}_2$ -fixation but only under high  $\text{PO}_4^{3-}$  levels, suggesting toxicity at these levels of  $\text{PO}_4^{3-}$  and Fe for this species (Figures 3C, 4B, 5A). Nonetheless, Fe can control  $\text{N}_2$ -fixation in *Halotheca* sp. cells, considering the Fe dependence of their alkaline phosphatase D (PhoD), which releases  $\text{PO}_4^{3-}$  from organic sources to fuel  $\text{N}_2$ -fixation (Fernández-Juárez et al., 2019). Further studies must be conducted especially in *Halotheca* sp. to determine which threshold of Fe concentrations can Fe inhibit/enhance growth and  $\text{N}_2$ -fixation rates.

## Interaction Between P and Fe Under Different Nitrogen Concentrations

### Cell Recovery Under P, Fe, and N Limitation in *Halotheca* sp.

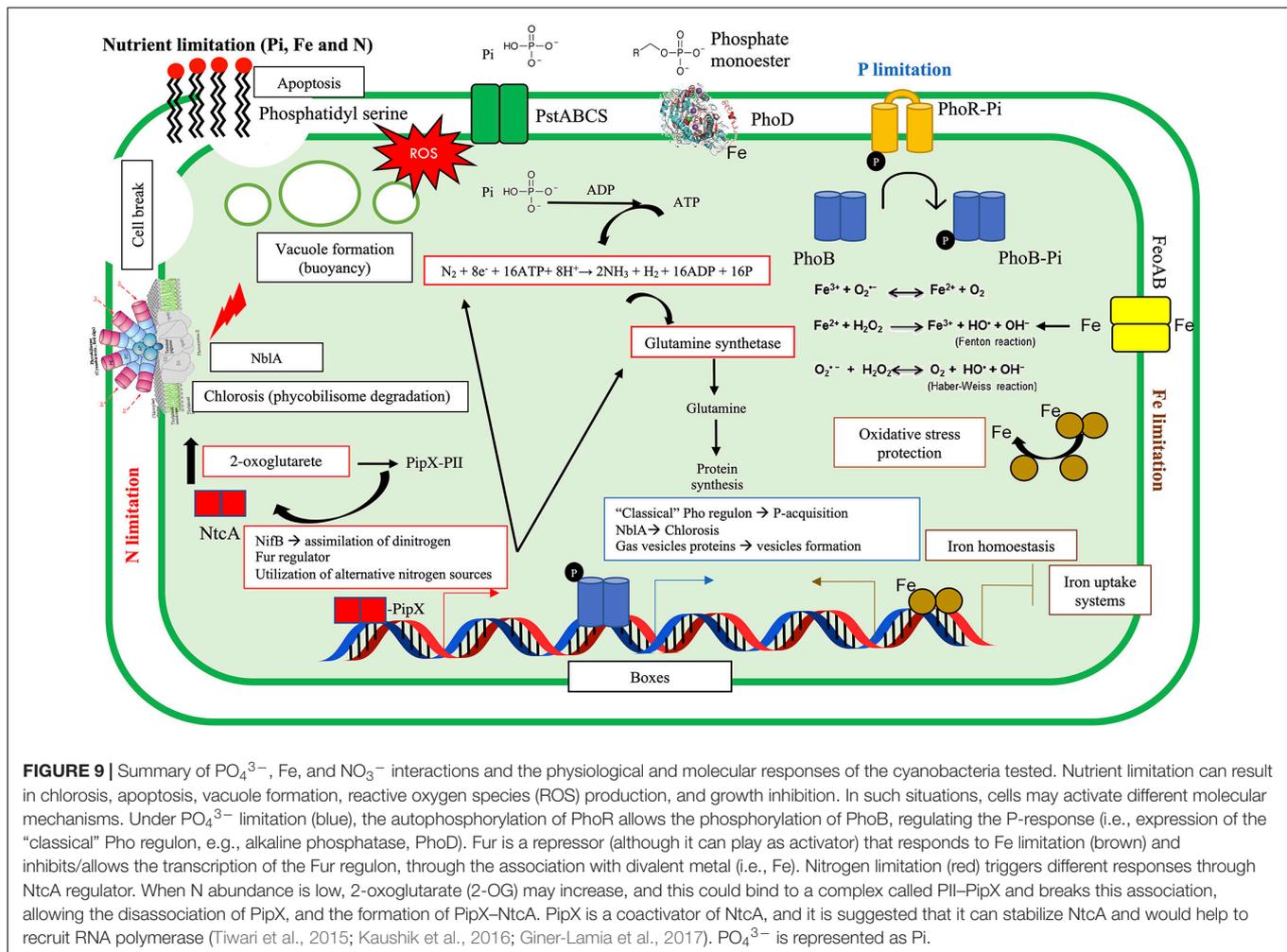
It is well documented how unicellular cyanobacteria cells are able to recover their phenotype by a genetically determined program (Klotz et al., 2016). Re-inoculum of  $\text{PO}_4^{3-}$  and Fe in [Low  $\text{PO}_4^{3-}$ -Low Fe] under  $\text{NO}_3^-$  optimal conditions and even in [High  $\text{PO}_4^{3-}$ -High Fe] under extremely limiting  $\text{NO}_3^-$  conditions with the addition of  $\text{NO}_3^-$  had as a consequence the partial or complete recovery of the green natural color and growth of the cells (Figure 1C). However, here, we observed that for *Halotheca* sp., this program is “canceled” under extreme  $\text{NO}_3^-$  limitation and co-limitation of  $\text{PO}_4^{3-}$  and Fe. Under extremely limiting  $\text{NO}_3^-$  and [Low  $\text{PO}_4^{3-}$ -Low Fe] conditions, addition of  $\text{PO}_4^{3-}$  and Fe did not result in growth recovery of the cells, suggesting that nutrient co-limitation ( $\text{PO}_4^{3-}$ , Fe, and  $\text{NO}_3^-$ ) could kill cells irreversibly by breakage of cells (Figures 1C, 7E).

## Nature of Nutrient Co-Limitation (P, Fe, and N) in *Halotheca* sp. and *Fischerella muscicola*

In oligotrophic areas, e.g., the tropical North Atlantic, bacterial productivity and biomass are usually co-limited with N, P, and/or Fe (Mills et al., 2004; Arrigo, 2005; Moore et al., 2008). The interactions between these limiting nutrients can trigger different types of limitation: simultaneous co-limitation (nutrient limitation of, e.g., N, P, and/or Fe have collective responses), independent co-limitation (nutrient limitation of, e.g., N, P, and/or Fe have different responses), and serial limitation wherein the response of a second limiting nutrient is appreciable after the previous addition of the primary limiting nutrient (Harpole et al., 2011). To our knowledge, this study is the first to show evidences of serial limitation of N ( $\text{NO}_3^-$ ) and P ( $\text{PO}_4^{3-}$ ) in diazotrophs with combined N as the primary limiting nutrient as suggested by the results for *Halotheca* sp. (Figure 3A). This suggests that in extremely N-limited waters, diazotrophs like *Halotheca* sp. are not competitive enough to compensate for N-deficits of the system through their  $\text{N}_2$ -fixation activities and cannot deplete the P concentration enough for the other co-occurring diazotrophic or non-diazotrophic species in the community to become P-limited. The reverse would follow for *F. muscicola*, which was independent of combined N source. The simultaneous co-limitation of  $\text{PO}_4^{3-}$  and Fe observed in Figure 3C for *Halotheca* sp. and *F. muscicola* may be due to the nature of the process of  $\text{N}_2$ -fixation, which is ATP dependent, and Fe is a structural component of the nitrogenase enzyme (Hoffman et al., 2014). Iron (Fe) is also a co-factor of alkaline phosphatases, which is one of the P-acquisition mechanisms of diazotrophs when P is in short supply (Santos-Beneit, 2015; Fernández-Juárez et al., 2019). However, the mechanisms behind the simultaneous limitation of  $\text{PO}_4^{3-}$  and Fe have to be further investigated as we also observed deleterious effects depending on the concentrations of these nutrients.

## Differences in Nutrient Kinetics in Diazotrophs at Different P and Fe Concentrations

Of the two species of cyanobacteria tested, the one that can most probably adapt to low levels of  $\text{PO}_4^{3-}$  is the unicellular cyanobacterium *Halotheca* sp. based on their nutrient kinetics (Figure 4B). Based on the Monod curves, the unicellular strain had the lowest  $K_\mu$  and  $Q_{\min}$ , indicating a better adaptation to  $\text{PO}_4^{3-}$  than the filamentous diazotroph (Figure 4B), albeit the  $K_\mu$  measured in *Halotheca* sp. under [High Fe] (i.e., 10 nM) shows lower affinity for  $\text{PO}_4^{3-}$  than that reported in the marine picophytoplankton *Synechococcus* (1 nM) (Kretz et al., 2015). In addition, its  $Q_{\min}$  (2.27  $\mu\text{M}$ ) was much higher than that of the marine *Synechococcus* (3 nM) (Kretz et al., 2015), indicating that *Halotheca* sp. is well adapted to P limitation but not at the level of *Synechococcus*. Unicellular cyanobacteria with smaller sizes are more efficient in acquiring nutrients in nutrient-limited waters because of their high surface: area ratios, while heterocyst-forming bacteria, which are generally bigger in size, need higher nutrient requirements (Haramaty et al., 2007). This is clearly shown in the Monod plot (Figure 4B), in which *F. muscicola* needs high amounts of Fe to growth, on the contrary of



*Halothece* sp. in which high Fe levels inhibited cell abundance (Figures 3C, 4B).

### Reactive Oxygen Species Production and Apoptotic Changes Derived From P, Fe, and N Limitation

Nutrient limitation (i.e.,  $\text{PO}_4^{3-}$ , Fe, and/or  $\text{NO}_3^-$ ) increased ROS in both species, *Halothece* sp. and *F. muscicola* (Figures 6A–C, 7A–D). Iron (Fe) had a key role in regulating ROS production. Increasing Fe levels can have different responses as shown in Figures 6A–C: (1) beneficial through increase activity of enzymatic antioxidant defenses or (2) extremely toxic through increase in Fenton and Haber–Weiss reaction (Latifi et al., 2009; Diaz and Plummer, 2018). The second case is suggested in Figure 6B, in which under [Low  $\text{NO}_3^-$ ] and [High  $\text{PO}_4^{3-}$ ], Fe increased ROS production and in turn can inhibit  $\text{N}_2$ -fixation (Alquéres et al., 2010). This could explain why *Halothece* sp. under [High  $\text{PO}_4^{3-}$ –High Fe] had lower growth and  $\text{N}_2$ -fixation rates than the other treatments with high levels of  $\text{PO}_4^{3-}$  (Figure 5A). On the contrary, in *F. muscicola*, Fe generally reduced ROS production (Figure 6C). The reduction of ROS production with Fe addition is consistent with a study on *Anabaena* PCC 7120, in which ROS increased up to 10-fold when

the cells were starved with Fe (Latifi et al., 2005). The results of our apoptosis tests (normally applied to eukaryotic cells) for *F. muscicola* when cells were limited by  $\text{PO}_4^{3-}$  and/or Fe are first evidences of programmed cell death in cyanobacteria by nutrient limitation (Figures 7F–I). Further investigations have to be performed to figure out the molecular mechanisms behind this.

### Prediction of Genes Involved in P, Fe, and N Limitation in *Halothece* sp.

We predicted the Pho, Fur, and NtcA boxes in the unicellular cyanobacterium *Halothece* sp. (PCC 7418), controlled by the master regulators in P, Fe, and N metabolism (PhoB, Fur, and NtcA, respectively), showing well-conserved sequences in this bacteria (Figures 8A–C), consistent with several studies (Tiwari et al., 2015; Kaushik et al., 2016; Giner-Lamia et al., 2017). A large majority of these genes associated to these boxes were involved in inorganic ion transport and metabolism (P) (Figures 8D–F and Supplementary Tables S2–S4), providing key information of how cyanobacteria respond to P, Fe, and N limitation (Figure 9). We detected genes involved in typical transporters in P, Fe, and N metabolism, in Fe storage, cell wall biosynthesis, amino acid metabolism,

photosynthesis, chlorosis, chlorophylls and PC biosynthesis, anti-phage systems (e.g., *cas* genes, implicated in the CRISPR-Cas systems), or even virulence (**Supplementary Tables S2–S4**). Through the created algorithms, we were able to detect elements from the “classical” Pho regulon (e.g., transporters, *pstABCS*, enzymes through which  $\text{PO}_4^{3-}$  is obtained from dissolved organic phosphorus, APases or stored phosphate, *ppK*), Fe transporters (e.g., *feoA* and *feoB*), and a whole group of genes involved in N metabolism (e.g., *gltBNS*, *amt*, and *nifB*) and thus being the most probable genes controlled by these TFs as is already described in *Cyanotheca*, *Gloeobacter*, *Microcystis*, *Nostoc*, *Prochlorococcus*, *Synechococcus*, *Synechocystis*, *Thermosynechococcus*, and *Trichodesmium* (**Supplementary Material** [Multifasta] and **Supplementary Tables S2–S4**). A  $\text{N}_2$ -fixation regulator, *nifB*, was predicted under the control of NtcA, whose product is crucial in iron-molybdenum biosynthesis (**Supplementary Table S4**). Downstream of this gene, we localized an entire cluster of genes related to  $\text{N}_2$ -fixation: Fd III 4Fe-4S, *nifS*, *nifU*, *nifH*, *nifD*, *nifK*, *nifZ*, *nifE*, *nifN*, *nifX*, DUF683, and *nifW*. In addition, some reports affirm that NtcA binds and controls Fur protein (López-Gomollón et al., 2007), as we predicted in this study, showing that N limitation can increase Fur protein levels and suggesting the narrow connection between N and Fe metabolism.

In summary, we show that two species of  $\text{N}_2$ -fixing cyanobacteria potentially associated with seagrasses have differing sensibilities to  $\text{PO}_4^{3-}$ , Fe, and  $\text{NO}_3^-$  concentrations, showing that these nutrients interact with each other based on our experimental and bio-informatic analysis. Despite the low number replications (being conscious that some results cannot be generalized), the growth responses reported here at varying concentrations of  $\text{PO}_4^{3-}$  and Fe at different  $\text{NO}_3^-$  conditions must be taken with precaution since the internal storage of these nutrients could affect these results considering that the cells were inoculated from optimal nutrients conditions. This is a pilot study in which insights on nutrient limitation in  $\text{N}_2$ -fixers are reported. Nonetheless, our study of a multifactorial design provides useful data and important findings of nutrient limitation in marine diazotrophs (at physiological, biochemical, and genetic levels). Deeper molecular assays (i.e., transcriptomic or proteomic assays) are recommended to investigate all the predicted genes involved in P, Fe, and N metabolism.

## DATA AVAILABILITY STATEMENT

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

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## AUTHOR CONTRIBUTIONS

VF-J and NA designed the experiments. VF-J conducted all experiments. All authors led the writing of the manuscript, reviewed, and supervised by NA.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.541558/full#supplementary-material>

**Supplementary Table 1** | List of all experimental treatments conducted in this study.

**Supplementary Table 2** | Pho regulon prediction. PHO boxes sequences, their position in the genome, orientation, the genes that are controlling (the Pho regulon), locus tag from these genes, the distance of the box from the gene and the gen function from single-letter of functional (COGs) are presented.

**Supplementary Table 3** | Fur regulon prediction. Fur boxes sequences, their position in the genome, orientation, the genes that are controlling (the Fur regulon), locus tag from these genes, the distance of the box from the gene and the gen function from single-letter of functional (COGs) are presented.

**Supplementary Table 4** | NtcA regulon prediction. NtcA boxes sequences, their position in the genome, orientation, the genes that are controlling (the NtcA regulon), locus tag from these genes, the distance of the box from the gene and the gen function from single-letter of functional (COGs) are presented.

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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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