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**The response of N₂-fixing bacteria to pH changes, temperature, and elevated levels
of CO₂ can depend on their nutrient status**

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Keywords: ocean acidification and warming, *Posidonia oceanica*, CO₂, phosphorus and
iron, diazotrophs, alkaline phosphatase activity (APA), and reactive oxygen species
(ROS).

ABSTRACT

Ocean acidification and warming are current global challenges that marine diazotrophs, i.e., bacteria capable to reduce gas N₂ to ammonium, must cope with. Barely anything is known about the effects of changes in pH and temperature with increased CO₂ levels in combination with different nutrient regimes in N₂-fixers, especially in heterotrophic bacteria, and those found in association with seagrasses. Here, we selected four cultivable diazotrophs, i.e., cyanobacteria and heterotrophic bacteria, found in association with the endemic Mediterranean seagrass *Posidonia oceanica*. We tested different pH (from pH 4 to 8) and temperature levels (from 12 to 30 °C), both under different nutrient levels of phosphorus, P (0.1 µM and 1.5 mM) and iron, Fe (2 nM and 1 µM). We also tested different CO₂ concentrations [410 and 1000 particles per million (ppm)] under different P/Fe and temperature levels (12, 18 and 24 °C). We reveal the sensitivity of the diazotrophs tested to lower pH (pH < 7-8) and warmer temperatures (°C > 12-24). Growth responses were dependent on their nutrient status, as was evidenced for the heterotrophic bacteria, which displayed a higher susceptibility to changes in pH, temperature, and CO₂ than the cyanobacterial species. High CO₂ levels (1000 ppm) decreased heterotrophic growth only when cultures were nutrient-limited, regardless of the temperature. On the other hand, cyanobacteria were insensitive to CO₂ increased levels, independently of the nutrient and temperature levels. Changes in N₂-fixation were mainly controlled by changes in growth. Our findings show that diazotrophic responses against climate change factors can be dependent on their nutrient status and their mode of life.

INTRODUCTION

Carbon dioxide (CO₂) emissions have been increasing since the start of the industrial revolution in 1750 [1]. Anthropogenic activities will continuously emit CO₂ into the atmosphere, and CO₂ concentration is expected to reach between 750 and 1000 particles per million (ppm) by the end of this century from the current atmospheric levels of 418 ppm [National Oceanic and Atmospheric Administration (NOAA), <https://www.esrl.noaa.gov/gmd/ccgg/trends/weekly.html>, retrieved February, 2022) [2]. Increasing CO₂ levels have clear consequences on the chemistry of marine environments, as one-third of the anthropogenic emissions are accumulated in the oceans [3]. Therefore, the accumulation of CO₂, which leads to the formation of carbonic acid (H₂CO₃) and then bicarbonate (HCO₃⁻), releasing protons (H⁺), causes ocean acidification. By the end of this century, it is predicted that pH will reach values of 7.7 from current pH values of 8-8.25 [4]. Increasing CO₂ emissions also cause changes in the Earth's energy heat balance, leading to an increase in air and seawater temperature. As a result, it is predicted an increase from 2 to 6 °C in the ocean temperature before the turn of the century [5].

The effect of increased CO₂ levels and concomitant changes in pH and temperature have been studied in phytoplankton or microorganisms in general, but often with contradictory results. Some reports speculate that marine phytoplankton can be resistant to ocean acidification [6–8], and can even stimulate cell abundance [9]. However, others claim that small changes in the pH may change the microbial structure [10], while warmer temperatures can enhance phytoplanktonic abundance and microbial activity [5]. It is extremely important to understand how microbial communities will respond to global climate change, especially those involved in nutrient cycles, e.g., N₂-fixing microorganisms, also so-called diazotrophs, which provide new inorganic nitrogen (N) to

the oceans, reducing the atmospheric nitrogen (N_2) to inorganic nitrogen [11]. Several studies described the role of CO_2 and temperature in diazotrophs, although these reports are mainly focused on cyanobacteria, e.g., the filamentous *Trichodesmium*, or in the unicellular *Cyanothece* and *Crocospaera* [12–15]. In general, they show that the responses are dependent on nutrient and light availability. Cyanobacteria can fix CO_2 , and thus, buffering the negative effect of CO_2 in ocean acidification. In some cyanobacterial species, increasing CO_2 levels can decrease the activity of the carbon concentration mechanisms (CCMs) in the carboxysomes, saving energy which can be used to enhance the N_2 -fixation and diazotrophic growth [14]. However, different cyanobacterial species may have different CCMs, which causes different CO_2 sensitivities, suggesting that the effect of increased CO_2 levels in cyanobacteria is species-specific [16].

In the last years, the potential of the heterotrophic bacteria as a source of new nitrogen has drawn attention. Various studies report the wide distribution and expression of nitrogenase genes (e.g., *nifH*, *nifK* and *nifD*) in heterotrophic bacteria, suggesting that they may play a very important role in global N_2 -fixation rates [17, 18]. Heterotrophic bacteria may be associated with marine particles, wherein their N_2 -fixation activities can take place under low-oxygen conditions [19]. However, very little is known about the responses of heterotrophic N_2 -fixers in the context of CO_2 , acidification, and warming. Some heterotrophic bacteria can fix and assimilate CO_2 through different carboxylation reactions via phosphoenolpyruvate carboxylase and pyruvate carboxylase, producing oxaloacetate that can be incorporated into the Krebs cycle [20]. Given that cyanobacteria and heterotrophic bacteria have different use and pathways of assimilation of CO_2 , they may respond differently to global climate change, and their impact in the N cycles is unknown.

It is predicted that N₂-fixation rates will increase to 22 -27% due to an increase in water temperature by the end of this century [21–23]. These general predictions should be viewed with caution since the N₂-fixing community is composed of different groups which can differ with their optimal pH, temperature, and nutrient requirements for their growth and microbial activities [6, 24–26]. These factors can regulate their responses in future ocean acidification and warming scenarios [27]. It is well described that diazotrophs need a particularly higher nutrient demand, especially for phosphorus (P) and iron (Fe), than other microorganisms [28, 29] since P (as ATP source) and Fe (as a cofactor of the nitrogenase complex) are required to catalyze the N₂-fixation reaction [30].

Despite the importance of the diazotrophs found in association with seagrasses, and the ecological functions that they offer, very little is known about the individual responses of these diazotrophs to abiotic (e.g., changes in nutrient availability, plastic pollution, or anthropogenic CO₂) and biotic factors (e.g., grazers or virus). The endemic Mediterranean seagrass *Posidonia oceanica* is a key component of the Mediterranean ecosystems and harbors a high diversity of diazotrophs associated, which can provide the entire demand of nitrogen[31] . From our early investigation, we described how global climate change (i.e., CO₂ and temperature) can change the N₂-fixation rates and the diazotrophic community structure on *P. oceanica* [32, 33]. Considering the sensitivity of their cyanobacteria and heterotrophic bacteria associated to different abiotic factors (e.g., nutrients or anthropogenic pollution) [34–37], it is of utmost importance to understand and predict how diazotrophs associated with *P. oceanica* respond individually to global climate change.

Here, we studied the individual responses of four cultivable diazotrophs (two cyanobacteria and two heterotrophic bacteria) found in association with *Posidonia oceanica* against changes in (I) pH (from pH 4 to 8) and (II) different temperature levels

(12-30 °C), both under different nutrient concentrations of inorganic phosphorus (i.e., PO_4^{3-}) and Fe. Due to the complexity of this study, we focus on the unicellular cyanobacteria *Halotheca* sp. and the heterotrophic bacterium *Cobetia* sp. for testing the effects of (III) different levels of CO_2 (atmospheric CO_2 , aCO_2 : 410 ppm and elevated CO_2 as predicted at the end of the century, eCO_2 : 1000 ppm) under different nutrients (P and Fe) and temperature (18-24 °C) levels. We evaluated physiological responses in terms of growth and N_2 -fixation activities. We also measured the P-acquisition mechanisms (alkaline phosphatase activity [APA]), and oxidative stress (through the measurement of the reactive oxygen species [ROS]), which can be used as biomarkers for evaluating the plasticity of these communities (i.e., the ability to persist when they are subject to an environmental stressor).

MATERIALS AND METHODS

Culture strains tested

We selected four N₂-fixers found in association with *P. oceanica*, based on our previous sequencing works with Illumina and Oxford Nanopore [38], represented by their most related cultivable strains: two cyanobacteria, i.e., a unicellular *Halotheca* sp. PCC 7418 and a filamentous heterocyst-forming *Fischerella muscicola* PCC 73103, and two heterotrophic bacteria, i.e., *Pseudomonas azotifigens* DSM 17556^T and *Cobetia* sp. UIB 001, which was isolated as a N₂-fixer from the roots of *P. oceanica* [37] (Supplementary Table S1). Prior to the experiments, the stock cultures were maintained in their optimal culture media: ASNIII + Turks Island Salts 4X for *Halotheca* sp., BG11₀ for *F. muscicola* and marine broth for the heterotrophic bacteria. They were incubated at 24 °C and 120 rpm in a rotatory shaker with a photoperiod of 12 h dark:12 h light under low-intensity fluorescent light (30 $\mu\text{E m}^{-2} \text{s}^{-1}$).

Experimental culture conditions

All the experiments were performed in triplicates ($n = 3$) and carried out in 2 mL microtiter plates (1.8 mL) or 50 mL falcon tubes (30 mL) in batch cultures, using modified artificial seawater medium [36]. Inorganic phosphorus (P, in the form of K₂HPO₄), iron (Fe, in the form of ferric citrate, [C₆H₅FeO₇]) and inorganic nitrogen (N, in the form of NH₃Cl) were added depending on the nutrient regimen and the response variable selected (Supplementary Table S1). Cells were precipitated and washed with artificial seawater without P, Fe and N, inoculating in each treatment $\sim 20\text{-}60 \times 10^4$ cells mL⁻¹. Cells were then maintained again at the initial conditions of 120 rpm, 12 h dark:12 h light and under low-intensity fluorescent light (30 $\mu\text{E m}^{-2} \text{s}^{-1}$) for 72 h under different experimental conditions (Supplementary Table S1). To avoid Fe contamination, all the samples and

materials (acid-cleaned) were manipulated in a class-100 clean hood. Cell abundance, N₂-fixation rates, alkaline phosphatase activity (APA), and reactive oxygen species (ROS) were measured as response variables (Supplementary Table S1).

Effects of variation in pH and temperature levels

For the pH experiment, the pH of the cultures was adjusted to different levels- pH 4, 5, 6, 7 and 8- using Tris-HCl or Trizma base (Sigma-Aldrich) (0.1 mM final concentration) in a calibrated pH-Meter BASIC 20 (CRISON) (Supplementary Table S1). These levels were chosen based on the microenvironments that can be found in the seagrasses, in which the pH can be below 6 [39]. Cells were incubated at a constant temperature (24 °C), that is the temperature at which all the bacterial species tested have high growth rates. For the temperature experiment, the cultures were incubated at different temperatures levels - 12, 18, 24 and 30 °C - with coolers and thermostats (Supplementary Table S1). The initial pH was adjusted to 8, and the cultures were incubated at different nutrient concentrations in both pH and temperature experiments: at optimal nutrient concentrations (1.5 mM PO₄³⁻ and 1 µM Fe), based on the optimal P and Fe concentration of the artificial seawater medium, and under nutrient limitation (0.1 µM PO₄³⁻ and 2 nM Fe), based on our previous nutrient limitation studies in these bacteria [34–37], both under low levels of NH₃ (0.15 mM) to avoid N₂-fixation inhibition [40].

Effects of increased CO₂ levels

To investigate the effects of increased CO₂ levels, we selected the unicellular cyanobacterium, *Halotheca* sp., and the heterotrophic bacterium, *Cobetia* sp. Because of the complexity of the experimental set-up due to the large number of combined variables we focused only on these bacteria as our model, which were already selected in our

previous studies [34–37]. We tested two different levels of CO₂: atmospheric, aCO₂: 410 ppm and elevated, eCO₂: 1000 ppm (as predicted at the end of the century, IPCC, 2014), and included a control with no CO₂ influx. All the experiments were performed under low levels of NH₃ (0.15 mM). Combinations with two variables (Fe and temperature) and different CO₂ levels were conducted: (i) CO₂ and Fe (1 μM and 2 nM) at constant temperature (24 °C) and (ii) CO₂ and temperature (18 °C and 30 °C) with 1 μM of Fe (Supplementary Table S1). Both types of experiments were subject to different concentrations of PO₄³⁻ (optimal, 1.5 mM; limited, 0.1 μM), and the initial pH was adjusted to pH 8 in each treatment.

The microplates and Falcon tubes with the cultures were placed inside hermetic tanks, without bubbling (Supplementary Figure S1). Mass flow controllers (MFCs, Aalborg) were set-up to control the air mixture. The gas mixture was introduced inside the hermetic tanks, with an outlet hole to allow the flow-through of the gas mixture. To achieve these mixes, air from an air-pump was connected to a filter with soda lime to remove CO₂ and mixed with pure CO₂ from a bottle to reach the desired concentration. Mixing was achieved in a tube with marbles to assure the homogenization of gases. After splitting the resulting treatment air gas mixture, the volume that entered each tank was regulated by a flow meter with a volume of 2.5 L min⁻¹ (LPM). The pH was continuously measured and monitored (ENV-40-pH) every 30 min, and the resulting data was stored in two control boxes (IKS-AQUASTAR) (Supplementary Figure S1).

Flow cytometry and growth measurement

Fresh unfixed cells at the initial (T_0) and the final time point (T_f) of the experiments were counted with a Becton Dickinson FACS-Verse cytometer (Beckton and Dickinson, Franklin Lakes, New Jersey, USA). Fluorescent beads, BD FACSuite™ CS&T research beads (Beckton and Dickinson and Company BD Biosciences, San Jose, USA), were used as an internal standard to calibrate the instrument. Cells were separated by combinations of the flow cytometer parameters: forward scatter (FSC, reflecting cell size), side scatter (SSC, reflecting internal complexity of the cells) and/or fluorescein isothiocyanate (FITC, 488 nm excitation, 530/30 nm emission) parameters, recording for each sample a total of 1×10^4 cells.

Nitrogen fixation activity

N_2 -fixation rates were measured through the acetylene reduction assay (ARA) at the end of the experiments (i.e., after 72 h), following the general method described in [41]. Only cultures cultivated under P-optimal conditions were measured. Briefly, a known volume of the culture media (8 mL) was transferred into a 10 mL vial. Liquid saturated acetylene was injected in these vials achieving a final concentration of 20% (v/v) and incubated for 3 h. Ethylene and acetylene were determined using a gas chromatograph (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 μ m film thickness), using the set up described in [34–37]. Ethylene produced was calculated using the equations in [42]. The acetylene reduction rates were converted to N_2 -fixation rates ($\text{nmol mL}^{-1} \text{ h}^{-1}$) using a factor of 4:1 [43].

Determination of reactive oxygen species

The molecular probe 2'-7'-dichlorofluorescein diacetate (DCFH) (Sigma) was used to measured reactive oxygen species (ROS) production after 72 h [34]. Only cultures cultivated under P-optimal conditions were measured. The DCFH diluted in artificial seawater (previously stabilized in acetone at 1 mg mL⁻¹) was added in a 96-well microplate (Thermo Scientific) containing the bacterial samples, achieving a final concentration of 15 µg mL⁻¹. The emission of the green fluorescence of the resulting 2'-7'-dichlorofluorescein was measured by a Cary Eclipse spectrofluorometer (FL0902M009, Agilent Technologies). Fluorescence was monitored for 1 h with an excitation of 480 nm and an emission of 530 nm. The slope of the linear regression between the fluorescence and time elapsed was reported as the ROS production expressed in arbitrary units (A.U.) and normalized by the cell number. The DCFH was added in artificial seawater without cells under the same conditions as above and served as blanks.

Determination of the alkaline phosphatase activity

Alkaline phosphatase activity (APA) was evaluated after 72 h through a fluorometric assay measuring the hydrolysis of the substrate 4-methylumbelliferyl phosphate (MUF-P, Sigma-Aldrich) to 4-methylumbelliferyl (MUF) [35]. An endpoint enzymatic assay was conducted at the end of the experiment with 8 µM of MUF-P (Supplementary Table S1). For the pH experiments, only *Halothece* sp. and *Cobetia* sp. were chosen as the main model cultivated under P-limiting conditions to detect the APA. After 1 h incubation in darkness, APA was measured in a microtiter plate that contained borate pH buffer 10 (3:1 of sample: buffer). The MUF production was measured at 359 nm (excitation) and 449 nm (emission), using a calibration standard curve with commercial MUF (Sigma-

Aldrich), with the Cary Eclipse spectrofluorometer (FL0902M009, Agilent Technologies).

Statistical analysis

Normality and homogeneity of variances were checked, and the statistical significance level was set at $p < 0.05$. Parametric analysis was used to examine normally distributed data using ANOVAs with Bonferroni post-hoc test. Spearman correlation analysis was used to determine the relationships between ROS vs. cell abundance, and APA vs. cell abundance. All analyses were done in R-Studio, R version 3.6.3 (2020-02-29).

RESULTS

Effect of varying levels of pH and temperature in N₂-fixing bacteria

Growth responses

The growth responses of the diazotrophs tested to varying pH and temperature levels were dependent on the nutrient status of the cells (ANOVA, $p < 0.05$, Figures 1 and 2). Across the pH and temperature treatments, cells under replete nutrient conditions (P and Fe) grew 4-fold more than those grown under nutrient-limited conditions (Figures 1A and 2). The magnitude of the difference between the growth under nutrient replete and nutrient-limited cells varied among the species tested and the pH (Figures 1A and 1B). Under nutrient replete conditions, a clear regression was found, in which decreasing pH levels negatively affected the growth of all species tested (Spearman's correlation, $p < 0.05$, $n = 20$, $r^2 = 0.6$, Figure 1A). Under this condition, we did not detect any differences between cyanobacteria or heterotrophic bacteria, since the cyanobacterium, *F. muscicola*, and the heterotrophic bacterium, *Cobetia* sp. were the most affected species to low pH levels, both lowering the growth 30-fold (Bonferroni test, $p < 0.05$, Figure 1B). Under nutrient-limiting conditions, we found the opposite response, reporting a negative regression, in which increasing pH levels decreased growth (Spearman's correlation, $p < 0.05$, $n = 20$, $r^2 = -0.2$, Figure 1B). Under this condition, differential responses between cyanobacteria and heterotrophic bacteria were found. The optimal pH for growing in the cyanobacteria species tested shifted towards an acidic pH (i.e., pH 5), with 9-fold higher growth compared with the rest of the pH treatments (Bonferroni test, $p < 0.05$, Figure 1B). A slight change from pH 8 towards the acidic-neutral pH (at pH 6-7) was also observed for the heterotrophic species tested under nutrient limitation (Figure 1B).

The response of the N₂-fixers tested at different temperatures was also dependent on the nutrient status of the cells and the species (ANOVA, $p < 0.05$, Figure 2). For heterotrophic bacteria, the nutrient replete cells, *Cobetia* sp. and *P. azotifigens*, grew better in warmer temperatures (24-30 °C) than the cyanobacterial strains, *Halothece* sp. and *F. muscicola* (12-18 °C) (Figure 2). However, under nutrient limiting conditions, the optimal growth temperature of *Cobetia* sp. was 12 °C (Bonferroni test, $p < 0.05$, Figure 2), while for *P. azotifigens*, we did not detect significant changes under nutrient limitation (Bonferroni test, $p > 0.05$, Figure 2). Under the same nutrient condition for the cyanobacterial strains, their optimal temperature range increased up to 24 °C (Bonferroni test, $p < 0.05$, Figure 2).

Changes in N₂-fixation rates

Although both groups have higher N₂-fixation rates at alkaline pH (Figure 3A), heterotrophic bacteria maintained active N₂-fixation rates at pH 6-8 (Figure 3A), while cyanobacteria have active N₂-fixation rates under acidic conditions (< pH 7) (Figure 3A). Regarding temperature, cyanobacteria (i.e., *Halothece* sp. and *F. muscicola*) reached the maximum N₂-fixation rates at 24 °C, while in the heterotrophic bacteria, it was at 18 °C (Figure 3B). Moreover, the results indicate that temperatures over 30 °C may have an inhibitory effect on the N₂-fixation processes (Figure 3B). However, these results were not statistically significant.

Changes in P-acquisition mechanisms

For *Halothece* sp. and *Cobetia* sp., the maximum APA was achieved at alkaline pH (i.e., above pH 7) (Figure 4A), and the rates dropped significantly from pH 8 to 6.5 for both bacteria (ANOVA, $p < 0.05$, $n = 3$, Figure 4A). Different temperature levels also

affected the APA in the diazotrophs tested (ANOVA, $p < 0.05$, Figure 4B). The optimal temperature for the APA varied by species (i.e., for cyanobacteria at 24 °C and heterotrophic bacteria at 12-18 °C). However, for all the species we tested, the APA had lower rates at 30 °C (Bonferroni test, $p < 0.05$, Figure 4B).

Changes in the ROS production

Significantly high ROS production was measured for *Halotheca* sp., *F. muscicola* and *P. azotifigens* at the pH of normal ocean water (i.e., pH 8) compared to other pH treatments. However, we found species-specific responses since for *Cobetia* sp. pH 8 decreased ROS (Bonferroni test, $p < 0.05$, Figure 5A). Temperature also affected ROS production, but their response was also dependent on the species tested (Figure 5B). For *Halotheca* sp. and *P. azotifigens*, ROS production was lower at intermediate temperatures than at the lowest (i.e., 12 °C) and highest (i.e., 30 °C) temperature treatments (Bonferroni test, $p < 0.05$, Figure 5B). For the other species, i.e., *F. muscicola* and *Cobetia* sp., no significant differences were detected (Bonferroni test, $p > 0.05$, Figure 5B).

Varying levels of CO₂ (aCO₂: 410 ppm and eCO₂: 1000 ppm) combined with different Fe and temperature levels

Growth responses

Elevated CO₂ (eCO₂, 1000 ppm) resulted in lower pH than at ambient levels (aCO₂, 410 ppm) after 72 h (Supplementary Figure S2). The differences of pH between the two CO₂ treatments were lower for the cyanobacterium, *Halotheca* sp. (an average of 0.21 units of pH), than for the heterotrophic bacterium, *Cobetia* sp. (an average of 0.55 units of pH). The growth of the unicellular cyanobacterium tested (*Halotheca* sp.) did not vary significantly at the different CO₂ levels applied in our study under different nutrient

regimes (PO_4^{3-} and/or Fe) nor temperature levels (i.e., 18 °C and 30 °C) (ANOVA, $p > 0.05$, Figures 6A-D).

On the contrary, for the heterotrophic bacterium, *Cobetia* sp., different CO_2 concentrations combined with varying nutrients had a significant effect on their growth (ANOVA $p < 0.05$, Figures 6A-D). In both, CO_2 -Fe and CO_2 -temperature experiments, growth at eCO_2 was lower compared with aCO_2 only when cultures were P-limited (Bonferroni test, $p < 0.05$, Figures 6B and 6D). In the CO_2 -Fe experiments, growth was significantly lower at eCO_2 compared to the control (i.e., no CO_2 influx) without Fe at optimal P concentration (Bonferroni test, $p < 0.05$, Figure 6A). Under P-limitation, growth was drastically reduced at eCO_2 compared to the control, independently of the Fe level (Bonferroni test, $p < 0.05$, Figure 6A). In the CO_2 -temperature experiments, under P-optimal concentrations, growth was higher at aCO_2 and eCO_2 compared to the control, irrespective of the temperature level (Bonferroni test, $p < 0.05$, Figures 6C), while under P-limited conditions, growth at eCO_2 was lower compared to the control, irrespective of the temperature level (Bonferroni test, $p < 0.05$, Figures 6C and 6D).

Changes in N_2 -fixation rates

No significant changes in the N_2 -fixation rates, irrespective of the CO_2 , Fe and/or temperature levels (ANOVA, $p > 0.05$, Figures 3C and 3D) were found for *Halothece* sp. For the heterotrophic species tested, *Cobetia* sp., continuous influx of CO_2 (i.e., both aCO_2 and eCO_2) significantly enhanced the N_2 -fixation rates compared to the control in the CO_2 -Fe experiments at 24 °C, irrespective of the Fe level (Bonferroni test, $p < 0.05$, Figure 3C).

Changes in the P-acquisition mechanisms

Generally, for *Halotheca* sp., under both, aCO₂ and eCO₂ levels, APA decreased significantly compared to the control treatment (Bonferroni test, $p < 0.05$, Figures 4C and 4D). However, we did not find any significant differences between aCO₂ and eCO₂ (Bonferroni test, $p < 0.05$, Figures 4C and 4D). Moreover, APA was negatively affected with increasing temperatures (i.e., 30 °C) (ANOVA, $p < 0.05$, Figure 4C), as we previously reported in the temperature experiments (Figure 4B). For the heterotrophic species, *Cobetia* sp., the temperature was the main factor controlling APA responses against CO₂. For this bacteria, elevated CO₂ levels enhanced the APA irrespective of the Fe levels at 24 °C compared with the control and compared with aCO₂ levels in the Fe-CO₂ experiments (Bonferroni test, $p < 0.05$, Figure 4C). Yet, elevated CO₂ levels significantly reduced APA at 18 °C, compared to the control and aCO₂ levels in the CO₂-temperature experiments (Bonferroni test, $p < 0.05$, Figure 4D). At 30 °C, we did not find any significant differences among treatments (ANOVA, $p > 0.05$, Figure 4D).

Changes in ROS production

ROS production was not significantly affected in response to the addition of CO₂ combined with different Fe and temperature levels for *Halotheca* sp. (ANOVA, $p < 0.05$, Figure 5C). For the heterotrophic bacteria *Cobetia* sp., eCO₂ levels with added Fe, ROS production increased 5-fold compared with the control and aCO₂ (Bonferroni test, $p < 0.05$, Figure 5C). However, no significant ROS production was observed with the addition of CO₂ at different temperature levels (ANOVA, $p > 0.05$, Figure 5D).

Reactive oxygen species (ROS) and APA as molecular biomarkers

Cell abundance of all the species tested and treatment tested (pH, temperature, CO₂ and nutrient level) were negatively correlated with the cell specific production of ROS and APA (Spearman's correlation, $p < 0.05$, $n = 144$, $r^2 = -0.41$ and $r^2 = -0.34$, respectively, Figures 7A and 7B).

Discussion

Dependence on nutrient status of N₂-fixers in their growth responses to global climate change factors

Here we provide evidence that P and Fe concentration can control the growth responses of N₂-fixers to global climate change factors (pH, temperature, and CO₂) but their response was also dependent on their nature (cyanobacteria or heterotrophic bacteria) (Figures 1, 2 and 6). Diazotrophs are primarily controlled by the availability of P and Fe [11]. Therefore, diazotrophs require large amounts of P to fuel N₂-fixation processes (i.e., requiring up to 16 ATP) [44]. Iron (Fe), is also a primary factor limiting N₂-fixation since it is a co-factor of the nitrogenase complex (i.e., contains 38 Fe atoms per holoenzyme) [45], and it is especially critical for cyanobacteria since Fe can limit photosynthetic processes [46]. Nutrient-limited conditions can promote higher alkaline phosphatase activity (APA), oxidative stress, cell break, and apoptotic processes, as reported in our previous studies [34, 35, 37]. This explains the higher growth in nutrient-replete cells compared with a nutrient-limited situation (Figures 1 and 2). Although cyanobacteria and heterotrophic bacteria can have different nutrient requirements (i.e., cyanobacteria have higher nutrient requirements than other phytoplanktonic taxa, as suggested by their Redfield N:P ratio of 25, which is significantly higher than other marine microbial groups with a ratio of 16), here, we did not find any difference between these microbial groups (Figures 1 and 2). According to these data, P and Fe can be the main abiotic factors regulating the responses of these N₂-fixers.

Although several reports focus on the effect of pH in N₂-fixers from the soil [48, 71–73], to our knowledge, this is the first study that considers the independent effect of ocean acidification for marine diazotrophs regardless of the CO₂ level. Previous studies suggest that marine microorganisms will not be affected by small changes in the pH [7]. Here we

also found that the species tested were not affected by pH changes dropping to 6.5 (data did not show). Therefore, future pH changes in the water column, dropping the pH up to 7.6-7.7, should not affect the diazotrophs, considering that it is improbable that the oceans will reach an acidic pH (i.e., below pH 7). However, seagrasses are complex systems. The epiphytic population in the rhizosphere can be subjected to pH microenvironments, finding very low pH values [39]. Ocean acidification can exacerbate this process, decreasing the pH level. Interestingly, growth responses below pH 6 are dependent on nutritional status and bacterial nature (i.e., cyanobacteria or heterotrophic bacteria) (Figures 1A and 1B). Under a nutrient replete situation, it is already described in phytoplankton that decreasing pH levels negatively affects cell growth [6] (Figures 1A and 1B). Lower pH levels reduce Fe-uptake since Fe (III) is less available for organic chelators (e.g., siderophores), and Fe (II) can be oxidized in non-bioassimilable forms. The reduction of Fe-uptake along with changes in cell membranes and gene expression can limit microbial activity with low pH levels under a replete nutrient condition [12, 50–52]. Surprisingly, we detected that under nutrient-limiting conditions, the optimal pH changed toward more towards acidic pH (Figure 1A), especially for the cyanobacterial species (*Halotheca* sp. and *F. muscicola*) since these species grew better below pH 6 (Figure 1B). These results show that cyanobacteria can be more resistant to lower pH than heterotrophic bacteria (*Cobetia* sp.) (Figure 1B). How these species take profit from an acidic pH (i.e., high $[H^+]$) under a nutrient limitation situation is unknown, although some cyanobacteria have a high affinity to Na^+/H^+ antiporters, through which intracellular Na^+ is exported to maintain cell homeostasis [53]. This data highlights the differential survival strategies that phototrophic and heterotrophic bacteria exhibit against pH changes in marine environments.

Temperature is a key factor controlling microbial activity in the oceans, playing a critical role in bacterial metabolism [54]. Under a nutrient replete situation, heterotrophic bacteria preferred warmer temperatures (i.e., $\geq 24\text{ }^{\circ}\text{C}$) than cyanobacteria (i.e., $\leq 18\text{ }^{\circ}\text{C}$) (Figure 2B). Higher temperatures can stimulate heterotrophic bacteria respiration and growth [54], while cyanobacteria prefer cooler temperatures to achieve maximum growth rates since higher temperatures can decrease photosynthetic processes [55]. However, for some bacteria, i.e., *Cobetia* sp., we found the opposite growth responses under a limiting nutrient condition, in which increasing temperature decreased growth (Figure 2). This shows that nutrient availability could be one of the main factors controlling the bacterial optimal temperature [54] (Figure 2). Early investigations [56, 57] report changes of 3-6 $^{\circ}\text{C}$ in the optimal temperature according to the nutritional status in several phytoplankton groups (e.g., *Synechococcus* sp., *Skeletonema costatum*, *Emiliana huxleyi* or *Thalassiosira pseudonana*), probably by alterations in enzymatic kinetics. For heterotrophic bacteria, ocean warming can also increase nutrient consumption rates, nutrient limiting the media and limiting growth [58]. These results highlight again the species-specific responses that can be found between cyanobacteria and heterotrophic bacteria.

Cyanobacteria, as phototrophs, perform photosynthesis, removing CO_2 from the media through efficient carbon concentration mechanisms (CCMs), which transport and concentrate CO_2 into the carboxysomes (i.e., structures in which the CO_2 -fixing enzyme, RuBisCO, is encapsulated) [59]. Thus, cyanobacteria can buffer the negative effect of CO_2 , decreasing the pH level (Supplementary Figure S2). This explains why the cyanobacterium tested did not respond to CO_2 increased levels irrespective of the nutrient and temperature levels (Figures 6A-D). Our results are consistent with other studies on known cultures of N_2 -fixing cyanobacteria (i.e., *Cyanothece* sp. ATCC51142,

Crocospaera watsonii WH850/WH0401/WH0402 and *Trichodesmium erythraeum* IMS101) [16, 60–64]. However, in other studies about N₂-fixing cyanobacteria (e.g., *Crocospaera*, *Cyanothece*, *Trichodesmium* and *Nodularia*), the authors report a growth increase or decrease due to elevated CO₂ levels depending on the nutritional status [63, 65–67]. This data suggests different CCMs efficiencies, and thus, the responses to global climate change in cyanobacteria can be species-specific.

On the contrary, we showed higher sensitivity against ocean acidification in the heterotrophic bacteria, as they do not have efficient mechanisms to remove CO₂ from the media, and thus, they are more exposed to the acidification of the media (Supplementary Figure S2). Studies about CO₂ and heterotrophic N₂-fixing bacteria are scarce, and only some reports evaluate the role of CO₂ in marine non-diazotrophic heterotrophic bacteria [68–70]. In agreement with these studies, elevated CO₂ levels in heterotrophic bacteria cultured under enriched nutrient media (i.e., P and Fe) do not affect microbial growth (Figures 6A and 6C), yet higher CO₂ concentrations could increase bacterial production, triggering changes in the metabolism [68, 69]. However, we report the negative effect of increasing CO₂ levels on the heterotrophic growth only when cells were under low P-concentration, irrespective of the temperature level (Figures 6A-D). We hypothesized that since low nutrient availability can limit growth and protein synthesis and activity [71], a combined effect with decreasing pH levels, as a consequence of increasing CO₂ values, may exacerbate the negative growth responses in heterotrophic bacteria. These results show the role of nutrients controlling CO₂ responses in heterotrophic bacteria.

Global climate change effect on N₂-fixation

Higher pH levels may enhance N₂-fixation rates, which is similar to what is reported in previous studies carried out on soil samples. It is suggested that the decrease in the

cytosolic pH can change the structure and catalysis of the nitrogenase complex [47–49]. Changes in temperature levels affect differently according to the cyanobacteria and heterotrophic bacteria, suggesting species-specific responses, but in both groups, a temperature of 30 °C had an inhibitory effect in N₂-fixation rates (Figure 3B). Studies conducted in marine bacteria show that N₂-fixation rates are active within temperature ranges from 24 to 30 °C, but at temperatures > 30 °C the activity decrease, suggesting that temperature can control nitrogenase activity and expression [13, 23]. It is important to mention that considering the effect of pH, temperature, and CO₂ on the growth of the diazotrophs tested (Figures 1 and 2), changes in their abundance can affect the bulk N₂-fixation rates.

Contrary to the previous reports conducted in other cyanobacterial species, in which CO₂ can enhance or reduce N₂-fixation rates depending on the nutrient and temperature level [12], we did not find any significant change in the cyanobacterium tested (*Halothece* sp.) (Figures 3C and 3D). In some cyanobacteria, high CO₂ levels can downregulate carboxysome genes, decreasing the energy used for concentrating CO₂ in these organelles [72]. It is hereby suggested that cyanobacteria can save energy by concentrating CO₂ and use this energy instead for N₂-fixation [14, 73]. However, the absence of effect due to an increase in CO₂, in the cyanobacterium tested, suggests that regulatory processes of carbon concentrating or uptake mechanisms can be species-specific, and thus, N₂-fixing responses in diazotrophic cyanobacteria to global climate change factors cannot be generalized. On the other hand, some of the results obtained for *Cobetia* sp. suggest that CO₂ may regulate N₂-fixation processes in heterotrophic bacteria (Figure 3C). Some heterotrophic bacteria can fix CO₂ through different carboxylation reactions catalyzed by phosphoenolpyruvate (PEP) carboxylase and pyruvate carboxylase. These enzymes can convert HCO₃⁻ in oxalacetate, which can be included in the Krebs cycles [20],

contributing to 2-8% of the cell's biomass carbon abundance [74]. Therefore, heterotrophic bacteria can take advantage of increased CO₂ levels, raising the ATP pool, which can fuel the N₂-fixation processes (Figure 3C). In the genome of *Cobetia* sp. (UIB 001) (accession number: CP058244-CP058245), we found that this bacterium contains a PEP carboxylase (locus tag: HA399_04700) and two SulP family inorganic anion transporters (locus_tag: HA399_00940 and HA399_13445), responsible for bicarbonate transport into the cells. These findings suggest its potential as CO₂-fixing bacteria.

P-acquisition mechanisms and oxidative stress as biomarkers for the prediction of microbial disturbances against global climate change

The increase of APA is a survival mechanism active under low P concentrations and/or when cells need higher P-requirements (e.g., under a N₂-fixing situation), releasing inorganic P from dissolved organic phosphorous [75]. N₂-fixation is tightly linked to APA since this process can fuel the P-requirements of the nitrogenase complex [33]. Thus, APA can be activated when cells are subjected to different environmental stressors, increasing the P-demand, which can be followed by the APA measurement. Our results show a clear negative correlation between the increase in APA per cell and the decrease in cell abundance (Figure 7A). This suggests that APA can be used as a biomarker to study changes in bacterial communities. Generally, for both groups, i.e., cyanobacteria and heterotrophic bacteria, an acidic pH decreased the APA rates (Figures 4A, 4C and 4D). Studies in cyanobacteria (e.g., *Nostoc flagelliforme*) and heterotrophic bacteria (e.g., *Cobetia amphilecti* KMM 296) show the dramatical inhibition of APA with acidification, possibly due to loss of the stability of the enzyme [76–78]. Interestingly, APA was more sensitive for cyanobacterial strains, being highly inhibited below pH 7, while heterotrophic bacteria were able to maintain higher rates of APA in a range from 6.5-8

(Figure 4A). Considering the concomitant water acidification from increased CO₂ levels, future pH values may affect the P-acquisition mechanisms in cyanobacteria over heterotrophic bacteria. Nevertheless, low pH can cause fewer free phosphate ions to be transported into the cells [79], and consequently increasing APA, as evidenced by the enhancement APA for *Cobetia* sp. at eCO₂ levels only at 24 °C (Figure 4C). This indicates the role of the temperature modulating the APA responses. Indeed, we found an interesting trend in which APA can be inhibited over 24 °C (Figure 4A). Contrary to our *in vivo* results, *in vitro* studies with the purified alkaline phosphatase (e.g., PhoD) show that for cyanobacteria (e.g., *N. flagelliforme*) and heterotrophic bacteria (e.g., *C. amphilecti* KMM 296) the optimal APA is within a range of 40 to 45 °C [76, 78, 81]. To date, this is the first study reporting the *in vivo* temperature effect of the APA in marine N₂-fixing bacteria (Figure 4B), considering that the stability of the enzyme, the velocity of organic phosphate breakdown, and enzyme-substrate affinity are controlled by temperature [82].

Reactive oxygen species (ROS) are biochemical biomarkers used for marine plants and animals [83, 84], and they can be used to evaluate the plasticity of the bacterial community against different environmental factors [85, 86]. ROS are reactive molecules that, if not removed, their accumulation can end in oxidative stress and, therefore, affect cell viability [86]. Here, we found that cell abundance was negatively correlated with ROS production (Figure 7B). This implies, as with APA, that ROS production can be used as a biomarker to assess the health of diazotrophic microorganisms exposed to global climate change factors. In agreement with our results, in other studies in phytoplankton cells, e.g., *Chattonella marina* and *Heterosigma akashiwo*, alkaline pH increases ROS production [87, 88]. Yet, our results indicate species-specific ROS production responses to alkaline pH since for *Cobetia* sp. higher pH increased ROS production (Figure 5A).

We also observed the same trend for the temperature experiments, i.e., species-specific responses, and cooler or warmer temperatures can promote adaptive responses to survive under suboptimal conditions, which can trigger higher ROS production [88, 89] (Figure 5B). Our results showed an interactive effect between CO₂ and Fe, increasing the ROS levels for *Cobetia* sp. High Fe-levels can enhance Fenton and Haber-Weiss reactions, which generate free radicals such as hydroxyl radicals ($\cdot\text{OH}$), being extremely toxic for cells [90]. On the other hand, CO₂ can downregulate the catalase expression, which is responsible for the detoxification of the H₂O₂, causing a 6-fold decrease in ROS removal [91]. Therefore, both CO₂ and Fe can increase ROS production in such a way that N₂-fixing heterotrophic bacteria cannot compensate with their antioxidant defenses.

CONCLUSIONS

We show the susceptibility of the N₂-fixers found in association with the Mediterranean seagrass *Posidonia oceanica* against ocean acidification and warming due to increased CO₂. However, the impact may depend on whether they are phototrophic or heterotrophic bacteria, and the nutrient status, suggesting that the community structure of the N₂-fixing community may also change. Our results reveal the susceptibility of the heterotrophic bacteria. Here, we suggest the utilization of the P-acquisitions mechanisms, i.e., APA, and biochemical parameters, i.e., ROS, as molecular biomarkers to assess the responses of diazotrophic cells to climate change factors. Yet, these results should be taken with caution since we only tested a few N₂-fixers found in association with *P. oceanica*, and, hence, further physiological, and molecular studies must be conducted in the natural communities found in association seagrass (e.g., *P. oceanica*) to figure out which are the responses of these N₂-fixers against the global climate change and how this can impact in the health of the plant.

Author contributions

VFJ conducted all experiments with the help of EHZ, EPP and NSRA in the various parameters measured in the study. VFJ and NSRA led the writing of the MS, and NSRA is the supervisor of the laboratory.

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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- 671 1. Etheridge DM, Steele LP, Langenfelds RL, et al (1996) Natural and anthropogenic
672 changes in atmospheric CO₂ over the last 1000 years from air in Antarctic ice and
673 firm. *Journal of Geophysical Research Atmospheres*.
674 <https://doi.org/10.1029/95JD03410>
- 675 2. Raupach MR, Marland G, Ciais P, et al (2007) Global and regional drivers of
676 accelerating CO₂ emissions. *Proceedings of the National Academy of Sciences of*
677 *the United States of America* 104:10288–10293.
678 <https://doi.org/10.1073/pnas.0700609104>
- 679 3. Friedlingstein P, O’Sullivan M, Jones MW, et al (2020) Global Carbon Budget
680 2020. *Earth System Science Data* 12:3269–3340. [https://doi.org/10.5194/essd-12-](https://doi.org/10.5194/essd-12-3269-2020)
681 [3269-2020](https://doi.org/10.5194/essd-12-3269-2020)
- 682 4. Jiang LQ, Carter BR, Feely RA, et al (2019) Surface ocean pH and buffer capacity:
683 past, present and future. *Scientific Reports* 9:1–11.
684 <https://doi.org/10.1038/s41598-019-55039-4>
- 685 5. Sarmiento H, Montoya JM, Vázquez-Domínguez E, et al (2010) Warming effects
686 on marine microbial food web processes: How far can we go when it comes to
687 predictions? *Philosophical Transactions of the Royal Society B: Biological*
688 *Sciences* 365:2137–2149. <https://doi.org/10.1098/rstb.2010.0045>
- 689 6. Berge T, Daugbjerg N, Andersen BB, Hansen PJ (2010) Effect of lowered pH on
690 marine phytoplankton growth rates. *Marine Ecology Progress Series* 416:79–91.
691 <https://doi.org/10.3354/meps08780>
- 692 7. Nielsen LT, Hallegraeff GM, Wright SW, Hansen PJ (2012) Effects of
693 experimental seawater acidification on an estuarine plankton community. *Aquatic*
694 *Microbial Ecology* 65:271–285. <https://doi.org/10.3354/ame01554>
- 695 8. Oliver AE, Newbold LK, Whiteley AS, van der Gast CJ (2014) Marine bacterial
696 communities are resistant to elevated carbon dioxide levels. *Environmental*
697 *Microbiology Reports* 6:574–582. <https://doi.org/10.1111/1758-2229.12159>
- 698 9. Kim JM, Lee K, Shin K, et al (2006) The effect of seawater CO₂ concentration on
699 growth of a natural phytoplankton assemblage in a controlled mesocosm
700 experiment. *Limnology and Oceanography* 51:1629–1636.
701 <https://doi.org/10.4319/lo.2006.51.4.1629>
- 702 10. Krause E, Wichels A, Giménez L, et al (2012) Small changes in pH have direct
703 effects on marine bacterial community composition: a microcosm approach. *PLoS*
704 *ONE* 7:. <https://doi.org/10.1371/journal.pone.0047035>
- 705 11. Zehr JP, Capone DG (2020) Changing perspectives in marine nitrogen fixation.
706 *Science* 368:. <https://doi.org/10.1126/science.aay9514>
- 707 12. Shi D, Kranz SA, Kim JM, Morel FMM (2012) Ocean acidification slows nitrogen
708 fixation and growth in the dominant diazotroph *Trichodesmium* under low-iron
709 conditions. *Proceedings of the National Academy of Sciences of the United States*
710 *of America* 109:. <https://doi.org/10.1073/pnas.1216012109>
- 711 13. Breitbarth E, Oschlies A, LaRoche J (2007) Physiological constraints on the global
712 distribution of *Trichodesmium* - Effect of temperature on diazotrophy.
713 *Biogeosciences* 4:53–61. <https://doi.org/10.5194/bg-4-53-2007>
- 714 14. Eichner M, Kranz SA, Rost B (2014) Combined effects of different CO₂ levels
715 and N sources on the diazotrophic cyanobacterium *Trichodesmium*. *Physiologia*
716 *Plantarum* 152:316–330. <https://doi.org/10.1111/ppl.12172>
- 717 15. Boatman TG, Lawson T, Geider RJ (2017) A key marine diazotroph in a changing
718 ocean: The interacting effects of temperature, CO₂ and light on the growth of

- Trichodesmium erythraeum* IMS101. PLoS ONE 12:1–20.
<https://doi.org/10.1371/journal.pone.0168796>
16. Eichner M, Rost B, Kranz SA (2014) Diversity of ocean acidification effects on marine N₂ fixers. *Journal of Experimental Marine Biology and Ecology* 457:199–207. <https://doi.org/10.1016/j.jembe.2014.04.015>
 17. Riemann L, Farnelid H, Steward GF (2010) Nitrogenase genes in non-cyanobacterial plankton: Prevalence, diversity and regulation in marine waters. *Aquatic Microbial Ecology* 61:235–247. <https://doi.org/10.3354/ame01431>
 18. Moisaner PH, Benavides M, Bonnet S, et al (2017) Chasing after non-cyanobacterial nitrogen fixation in marine pelagic environments. *Frontiers in Microbiology* 8:. <https://doi.org/10.3389/fmicb.2017.01736>
 19. Chakraborty S, Andersen KH, Follows MJ, Riemann L (2021) Quantifying nitrogen fixation by heterotrophic bacteria in sinking marine particles. *Nature Communications*. <https://doi.org/10.1038/s41467-021-23875-6>
 20. Santruckova H, Bird MI, Elhottová D, et al (2005) Heterotrophic fixation of CO₂ in soil. *Microbial Ecology* 49:218–225. <https://doi.org/10.1007/s00248-004-0164-x>
 21. Jiang HB, Fu FX, Rivero-Calle S, et al (2018) Ocean warming alleviates iron limitation of marine nitrogen fixation. *Nature Climate Change* 8:709–712
 22. Boyd PW, Doney SC (2002) Modelling regional responses by marine pelagic ecosystems to global climate change. *Geophysical Research Letters* 29:53-1-53–4. <https://doi.org/10.1029/2001gl014130>
 23. Fu FX, Yu E, Garcia NS, et al (2014) Differing responses of marine N₂ fixers to warming and consequences for future diazotroph community structure. *Aquatic Microbial Ecology* 72:33–46. <https://doi.org/10.3354/ame01683>
 24. Lagus A, Suomela J, Weithoff G, et al (2004) Species-specific differences in phytoplankton responses to N and P enrichments and the N:P ratio in the Archipelago Sea, northern Baltic Sea. *Journal of Plankton Research* 26:779–798. <https://doi.org/10.1093/plankt/fbh070>
 25. Striebel M, Schabhüttl S, Hodapp D, et al (2016) Phytoplankton responses to temperature increases are constrained by abiotic conditions and community composition. *Oecologia* 182:815–827. <https://doi.org/10.1007/s00442-016-3693-3>
 26. Thackeray SJ, Jones ID, Maberly SC (2008) Long-term change in the phenology of spring phytoplankton: Species-specific responses to nutrient enrichment and climatic change. *Journal of Ecology* 96:523–535. <https://doi.org/10.1111/j.1365-2745.2008.01355.x>
 27. Wrightson L, Tagliabue A (2020) Quantifying the impact of climate change on marine diazotrophy: insights from earth system models. *Frontiers in Marine Science* 7:1–9. <https://doi.org/10.3389/fmars.2020.00635>
 28. Ward BA, Dutkiewicz S, Moore CM, Follows MJ (2013) Iron, phosphorus, and nitrogen supply ratios define the biogeography of nitrogen fixation. *Limnology and Oceanography* 58:2059–2075. <https://doi.org/10.4319/lo.2013.58.6.2059>
 29. Falkowski PG (1997) Evolution of the nitrogen cycle and its influence on the biological sequestration of CO₂ in the ocean. *Nature* 387:272–275. <https://doi.org/10.1038/387272a0>
 30. Hoffman BM, Lukoyanov D, Yang Z-Y, et al (2014) Mechanism of nitrogen fixation by nitrogenase: The next Stage. *Chemical Reviews* 114:4041–4062. <https://doi.org/10.1021/cr400641x>
 31. Agawin NSR, Ferriol P, Cryer C, et al (2016) Significant nitrogen fixation activity associated with the phyllosphere of Mediterranean seagrass *Posidonia oceanica*:

- 769 First report. Marine Ecology Progress Series 551:53–62.
 770 <https://doi.org/10.3354/meps11755>
- 771 32. García-Márquez MG, Fernández-Juárez V, Rodríguez-Castañeda JC, Agawin
 772 NSR (2022) Response of *Posidonia oceanica* (L.) Delile and Its Associated N2
 773 Fixers to Different Combinations of Temperature and Light Levels. Frontiers in
 774 Marine Science 8: <https://doi.org/10.3389/fmars.2021.757572>
- 775 33. Agawin NSR, Gil Atorrasagasti MG, Frank Comas A, et al (2021) Response of the
 776 seagrass *Posidonia oceanica* and its associated N2 fixers to high business-as-usual
 777 climate change scenario in winter. Limnology and Oceanography 66:2346–2359.
 778 <https://doi.org/10.1002/lno.11758>
- 779 34. Fernández-Juárez V, Bennasar-Figueras A, Sureda-Gomila A, et al (2020)
 780 Differential Effects of Varying Concentrations of Phosphorus, Iron, and Nitrogen
 781 in N2-Fixing Cyanobacteria. Frontiers in Microbiology 11:1–19.
 782 <https://doi.org/10.3389/fmicb.2020.541558>
- 783 35. Fernández-Juárez V, Bennasar-figueras A, Tovar-sanchez A, R. Agawin NS
 784 (2019) The role of iron in the P-acquisition mechanisms of the unicellular N2 -
 785 fixing cyanobacteria *Halotheca* sp., found in association with the Mediterranean
 786 seagrass *Posidonia oceanica*. Frontiers in Microbiology 10:1–22.
 787 <https://doi.org/10.3389/fmicb.2019.01903>
- 788 36. Fernández-Juárez V, López-alforja X, Frank-comas A, Echeveste P (2021) “ The
 789 Good , the Bad and the Double-Sword ” Effects of Microplastics and Their Organic
 790 Additives in Marine Bacteria. Frontiers in Microbiology 11:1–12.
 791 <https://doi.org/10.3389/fmicb.2020.581118>
- 792 37. Fernández-Juárez V, Jaén-Luchoro D, Brito-Echeverría J, et al (2021) Everything
 793 Is Everywhere: Physiological Responses of the Mediterranean Sea and Eastern
 794 Pacific Ocean Epiphyte *Cobetia* Sp. to Varying Nutrient Concentration. Microbial
 795 Ecology. <https://doi.org/10.1007/s00248-021-01766-z>
- 796 38. Agawin NSR, Ferriol P, Sintés E, Moyà G (2017) Temporal and spatial variability
 797 of in situ nitrogen fixation activities associated with the Mediterranean seagrass
 798 *Posidonia oceanica* meadows. Limnology and Oceanography 62:2575–2592.
 799 <https://doi.org/10.1002/lno.10591>
- 800 39. Moßhammer M, Brodersen KE, Köhl M, Koren K (2019) Nanoparticle- and
 801 microparticle-based luminescence imaging of chemical species and temperature in
 802 aquatic systems: a review. Microchimica Acta 186:20–23.
 803 <https://doi.org/10.1007/s00604-018-3202-y>
- 804 40. Knapp AN (2012) The sensitivity of marine N2 fixation to dissolved inorganic
 805 nitrogen. Frontiers in Microbiology 3:1–14.
 806 <https://doi.org/10.3389/fmicb.2012.00374>
- 807 41. Agawin NSR, Benavides M, Busquets A, et al (2014) Dominance of unicellular
 808 cyanobacteria in the diazotrophic community in the Atlantic Ocean. Limnology
 809 and Oceanography 59:623–637. <https://doi.org/10.4319/lo.2014.59.2.0623>
- 810 42. Stal LJ (1988) Acetylene reduction technique for assay of nitrogenase. 167:474–
 811 484
- 812 43. Jensen BB, Cox RP (1983) Direct measurements of steady-state kinetics of
 813 cyanobacterial N2uptake by membrane-leak mass spectrometry and comparisons
 814 between nitrogen fixation and acetylene reduction. Applied and Environmental
 815 Microbiology 45:1331–1337
- 816 44. Sanudo-Wilhelmy SA et al (2001) Phosphorus limitation of nitrogen fixation by
 817 *Trichodesmium* in the central Atlantic Ocean. Nature 411:66–69

45. Berman-Frank I, Cullen JT, Shaked Y, et al (2001) Iron availability, cellular iron quotas, and nitrogen fixation in *Trichodesmium*. *Limnology and Oceanography* 46:1249–1260. <https://doi.org/10.4319/lo.2001.46.6.1249>
46. Haramaty L, Quigg A, Berman-Frank I, et al (2007) Nitrogen-fixation strategies and Fe requirements in cyanobacteria. *Limnology and Oceanography* 52:2260–2269. <https://doi.org/10.4319/lo.2007.52.5.2260>
47. Pham DN, Burgess BK (1993) Nitrogenase reactivity: Effects of pH on substrate reduction and CO Inhibition. *Biochemistry* 32:13725–13731. <https://doi.org/10.1021/bi00212a043>
48. Schubert E, Mengel K, Schubert S (1990) Soil pH and calcium effect on nitrogen fixation and growth of broad bean. *Agronomy Journal* 82:969–972. <https://doi.org/10.2134/agronj1990.00021962008200050026x>
49. Fernandez Valiente E, Leganes F (1990) Regulatory effect of pH and incident irradiance on the levels of nitrogenase activity in the cyanobacterium *Nostoc UAM 205*. *Journal of Plant Physiology* 135:623–627. [https://doi.org/10.1016/S0176-1617\(11\)80647-4](https://doi.org/10.1016/S0176-1617(11)80647-4)
50. F.J. Millero, R. Woosey, B. Ditrolio, J. Water (2009) effect of Ocean acidification on the Speciation of Metals in Seawater. *Oceanography* 22:72–85
51. Shi D, Xu Y, Hopkinson BM, Morel FMM (2010) Effect of ocean acidification on iron availability to marine phytoplankton. *Science* 327:676–679. <https://doi.org/10.1126/science.1183517>
52. Yu T, Chen Y (2019) Effects of elevated carbon dioxide on environmental microbes and its mechanisms: A review. *Science of the Total Environment* 655:865–879. <https://doi.org/10.1016/j.scitotenv.2018.11.301>
53. Rungaroon Waditee, Takashi Hibino, Yoshito Tanaka, Tatsunosuke Nakamura AI and TT (2001) Halotolerant Cyanobacterium *Aphanothece halophytica* Contains an Na⁺/H⁺ Antiporter, Homologous to Eukaryotic Ones, with Novel Ion Specificity Affected by C-terminal Tail. *Journal of Biological Chemistry* 276:36931–36838. <https://doi.org/10.1074/jbc>
54. Pomeroy LR, Wiebe WJ (2001) Temperature and substrates as interactive limiting factors for marine heterotrophic bacteria. *Aquatic Microbial Ecology* 23:187–204. <https://doi.org/10.3354/ame023187>
55. Schabhüttl S, Hingsamer P, Weigelhofer G, et al (2013) Temperature and species richness effects in phytoplankton communities. *Oecologia* 171:527–536. <https://doi.org/10.1007/s00442-012-2419-4>
56. Marañón E, Lorenzo MP, Cermeño P, Mouriño-Carballido B (2018) Nutrient limitation suppresses the temperature dependence of phytoplankton metabolic rates. *ISME Journal* 12:1836–1845. <https://doi.org/10.1038/s41396-018-0105-1>
57. Thomas MK, Aranguren-Gassis M, Kremer CT, et al (2017) Temperature–nutrient interactions exacerbate sensitivity to warming in phytoplankton. *Global Change Biology* 23:3269–3280. <https://doi.org/10.1111/gcb.13641>
58. Akpor O, Olaolu TD, Okolie EC (2014) The effect of temperature on nitrate and phosphate uptake from synthetic wastewater by selected bacteria species. *British Microbiology Research Journal* 4:328–342. <https://doi.org/10.9734/bmrj/2014/6407>
59. Zhang A, Carroll AL, Atsumi S (2017) Carbon recycling by cyanobacteria: Improving CO₂ fixation through chemical production. *FEMS Microbiology Letters* 364:1–7. <https://doi.org/10.1093/femsle/fnx165>

- 866 60. Barcelos e Ramos J, Biswas H, Schulz KG, et al (2007) Effect of rising
867 atmospheric carbon dioxide on the marine nitrogen fixer *Trichodesmium*. Global
868 Biogeochemical Cycles 21:1–6. <https://doi.org/10.1029/2006GB002898>
- 869 61. Fu FX, Mulholland MR, Garcia NS, et al (2008) Interactions between changing
870 pCO₂, N₂ fixation, and Fe limitation in the marine unicellular cyanobacterium
871 *Crocospaera*. Limnology and Oceanography 53:2472–2484.
872 <https://doi.org/10.4319/lo.2008.53.6.2472>
- 873 62. Kranz SA, Sültemeyer D, Richter KU, Rost B (2009) Carbon acquisition by
874 *Trichodesmium*: The effect of pCO₂ and diurnal changes. Limnology and
875 Oceanography 54:548–559. <https://doi.org/10.4319/lo.2009.54.2.0548>
- 876 63. Garcia NS, Fu FX, Breene CL, et al (2011) Interactive effects of irradiance and
877 CO₂ on CO₂ fixation and N₂ fixation in the diazotroph *Trichodesmium*
878 *erythraeum* (cyanobacteria). Journal of Phycology 47:1292–1303.
879 <https://doi.org/10.1111/j.1529-8817.2011.01078.x>
- 880 64. Garcia NS, Fu FX, Breene CL, et al (2013) Combined effects of CO₂ and light on
881 large and small isolates of the unicellular N₂-fixing cyanobacterium *Crocospaera*
882 *watsonii* from the western tropical Atlantic Ocean. European Journal of Phycology
883 48:128–139. <https://doi.org/10.1080/09670262.2013.773383>
- 884 65. Garcia NS, Fu FX, Hutchins DA (2013) Colimitation of the unicellular
885 photosynthetic diazotroph *Crocospaera watsonii* by phosphorus, light, and
886 carbon dioxide. Limnology and Oceanography 58:1501–1512.
887 <https://doi.org/10.4319/lo.2013.58.4.1501>
- 888 66. Levitan O, Rosenberg G, Setlik I, et al (2007) Elevated CO₂ enhances nitrogen
889 fixation and growth in the marine cyanobacterium *Trichodesmium*. Global Change
890 Biology 13:531–538. <https://doi.org/10.1111/j.1365-2486.2006.01314.x>
- 891 67. Wannicke N, Endres S, Engel A, et al (2012) Response of *Nodularia spumigena*
892 to pCO₂ - Part 1: Growth, production and nitrogen cycling. Biogeosciences
893 9:2973–2988. <https://doi.org/10.5194/bg-9-2973-2012>
- 894 68. Teira E, Fernández A, Álvarez-Salgado XA, et al (2012) Response of two marine
895 bacterial isolates to high CO₂ concentration. Marine Ecology Progress Series
896 453:27–36. <https://doi.org/10.3354/meps09644>
- 897 69. James AK, Kelly LW, Nelson CE, et al (2019) Elevated pCO₂ alters marine
898 heterotrophic bacterial community composition and metabolic potential in
899 response to a pulse of phytoplankton organic matter. Environmental Microbiology
900 21:541–556. <https://doi.org/10.1111/1462-2920.14484>
- 901 70. Grossart HP, Allgaier M, Passow U, Riebesell U (2006) Testing the effect of CO₂
902 concentration on the dynamics of marine heterotrophic bacterioplankton.
903 Limnology and Oceanography 51:1–11. <https://doi.org/10.4319/lo.2006.51.1.0001>
- 904 71. Bristow LA, Mohr W, Ahmerkamp S, Kuypers MMM (2017) Nutrients that limit
905 growth in the ocean. Current Biology 27:R474–R478.
906 <https://doi.org/10.1016/j.cub.2017.03.030>
- 907 72. Morris JJ, Kirkegaard R, Szul MJ, et al (2008) Facilitation of robust growth of
908 *Prochlorococcus* colonies and dilute liquid cultures by “helper” heterotrophic
909 bacteria. Applied and Environmental Microbiology.
910 <https://doi.org/10.1128/AEM.02479-07>
- 911 73. Kranz SA, Eichner M, Rost B (2011) Interactions between CCM and N₂ fixation
912 in *Trichodesmium*. Photosynthesis Research 109:73–84.
913 <https://doi.org/10.1007/s11120-010-9611-3>

74. Spona-Friedl M, Braun A, Huber C, et al (2020) Substrate-dependent CO₂ fixation in heterotrophic bacteria revealed by stable isotope labelling. *FEMS Microbiology Ecology*. <https://doi.org/10.1093/femsec/fiaa080>
75. Santos-Beneit F (2015) The Pho regulon: A huge regulatory network in bacteria. *Frontiers in Microbiology* 6:1–13
76. Li P, Liu W, Gao K (2013) Effects of temperature, pH, and UV radiation on alkaline phosphatase activity in the terrestrial cyanobacterium *Nostoc flagelliforme*. *Journal of Applied Phycology* 25:1031–1038. <https://doi.org/10.1007/s10811-012-9936-8>
77. Yu Plisova E, Balabanova LA, Ivanova EP, et al (2005) A highly active alkaline phosphatase from the marine bacterium *Cobetia*. *Marine Biotechnology* 7:173–178. <https://doi.org/10.1007/s10126-004-3022-4>
78. Noskova Y, Likhatskaya G, Terentieva N, et al (2019) A novel alkaline phosphatase/phosphodiesterase, CamPhoD, from marine bacterium *Cobetia amphilecti* KMM 296. *Marine Drugs* 17:1–20. <https://doi.org/10.3390/md17120657>
79. Cerozi B da S, Fitzsimmons K (2016) The effect of pH on phosphorus availability and speciation in an aquaponics nutrient solution. *Bioresource Technology* 219:778–781. <https://doi.org/10.1016/j.biortech.2016.08.079>
81. Golotin V, Balabanova L, Likhatskaya G, Rasskazov V (2015) Recombinant production and characterization of a highly active alkaline phosphatase from marine bacterium *Cobetia marina*. *Marine Biotechnology* 17:130–143. <https://doi.org/10.1007/s10126-014-9601-0>
82. Hernández I, Niell FX, Whitton BA (2002) Phosphatase activity of benthic marine algae. An overview. *Journal of Applied Phycology* 14:475–487. <https://doi.org/10.1023/A:1022370526665>
83. Ferrat L, Pergent-Martini C, Roméo M (2003) Assessment of the use of biomarkers in aquatic plants for the evaluation of environmental quality: Application to seagrasses. *Aquatic Toxicology* 65:187–204. [https://doi.org/10.1016/S0166-445X\(03\)00133-4](https://doi.org/10.1016/S0166-445X(03)00133-4)
84. Benedetti M, Giuliani ME, Regoli F (2015) Oxidative metabolism of chemical pollutants in marine organisms: Molecular and biochemical biomarkers in environmental toxicology. *Annals of the New York Academy of Sciences* 1340:8–19. <https://doi.org/10.1111/nyas.12698>
85. Litchman E, Edwards KF, Klausmeier CA, Thomas MK (2012) Phytoplankton niches, traits and eco-evolutionary responses to global environmental change. *Marine Ecology Progress Series* 470:235–248. <https://doi.org/10.3354/meps09912>
86. Lesser MP (2006) Oxidative stress in marine environments: Biochemistry and Physiological Ecology. *Annual Review of Physiology* 68:253–278. <https://doi.org/10.1146/annurev.physiol.68.040104.110001>
87. Liu W, Au DWT, Anderson DM, et al (2007) Effects of nutrients, salinity, pH and light:dark cycle on the production of reactive oxygen species in the alga *Chattonella marina*. *Journal of Experimental Marine Biology and Ecology* 346:76–86. <https://doi.org/10.1016/j.jembe.2007.03.007>
88. Twiner MJ (2000) Possible physiological mechanisms for production of hydrogen peroxide by the ichthyotoxic flagellate *Heterosigma akashiwo*. *Journal of Plankton Research* 22:1961–1975. <https://doi.org/10.1093/plankt/22.10.1961>
89. Chattopadhyay M (2006) Mechanism of bacterial adaptation to low temperature. *Journal of Biosciences* 31:157–65.

- 963 90. Julia M Diaz, Sydney Plummer (2018) Production of extracellular reactive oxygen
964 species by phytoplankton: past and future directions, Journal of Plankton Research,
965 Volume 40, Issue 6, Pages 655–666, <https://doi.org/10.1093/plankt/fby039>
966 91. Hennon GM, Morris JJ, Haley ST, et al (2017) The impact of elevated CO2 on
967 *Prochlorococcus* and microbial interactions with a ‘helper’ bacterium *Alteromonas*.
968 ISME Journal 12:520–531. <https://doi.org/10.1038/ismej.2017.189>
969
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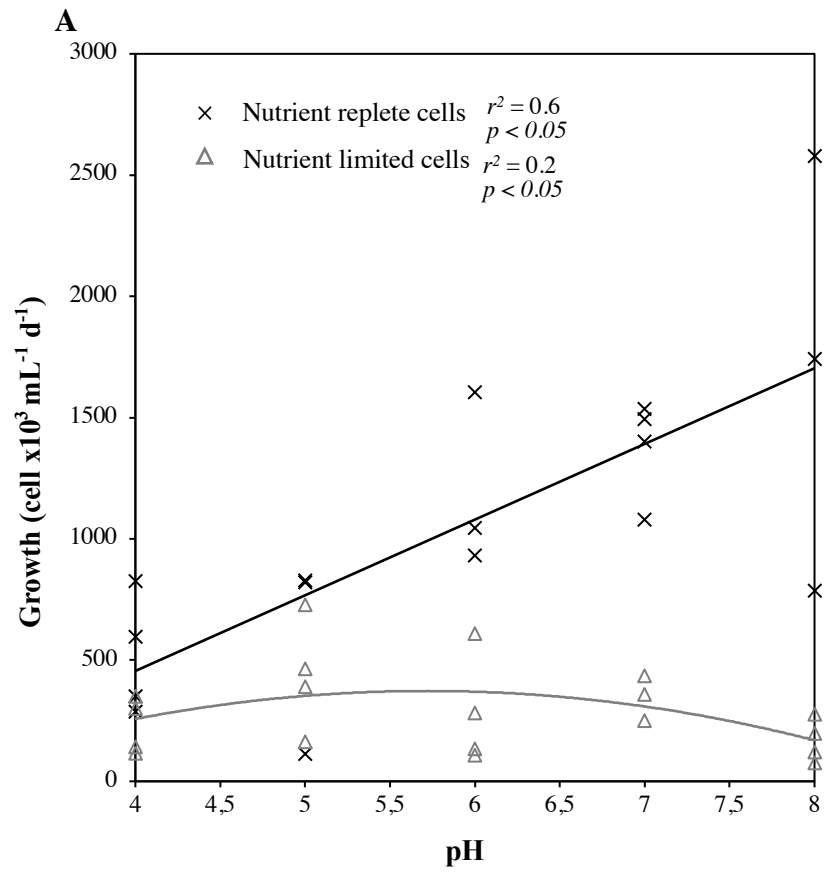
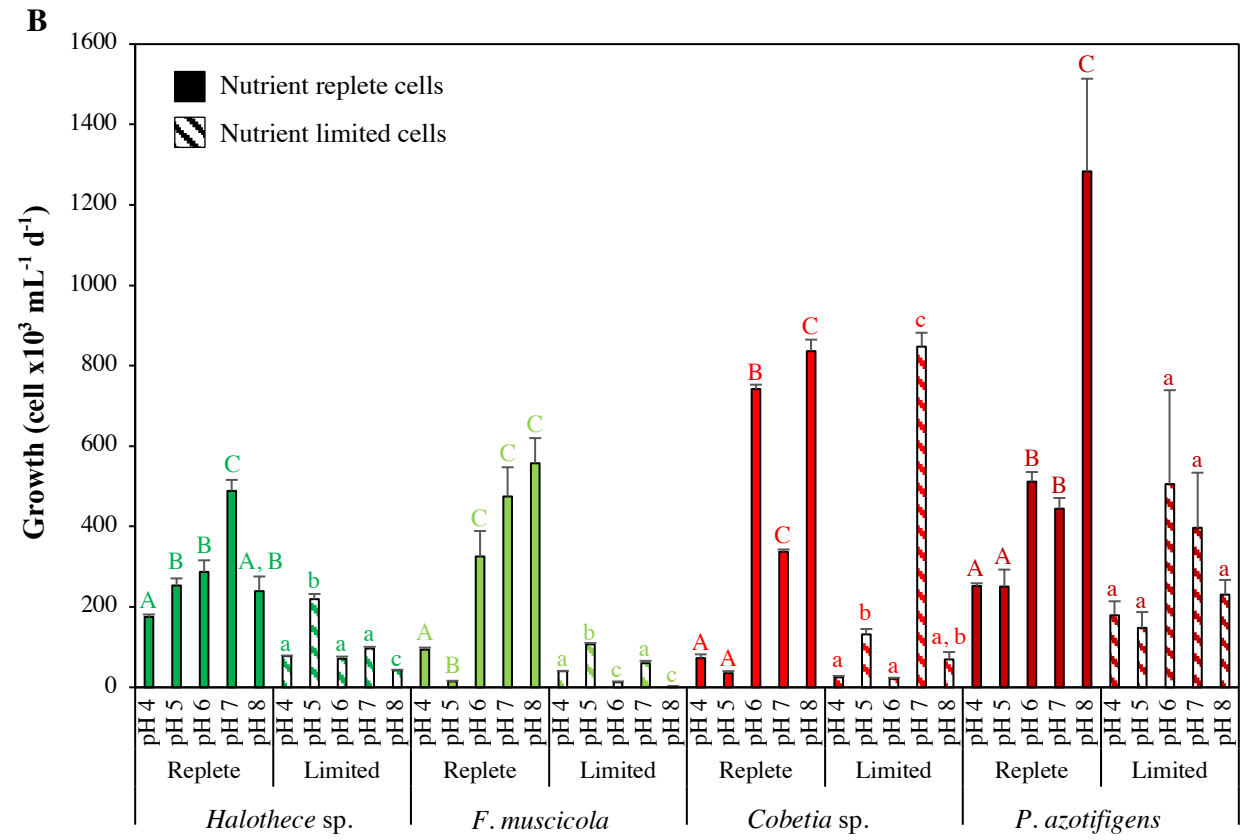


Figure 1. Effect of different levels of pH (4-6) on growth after 72 h under different nutrient concentrations at 24 °C. **A)** Correlation between growth and pH level according to the nutritional level in all the strains and triplicate measured. **B)** Effect of pH according to the species tested and nutritional level. In B, values are the mean $\pm$ SE ($n = 3$). Letters (i.e., capital letters for the nutrient replete condition or lower case for nutrient limiting condition) indicate significant differences ($p < 0.05$) with the rest of the treatments for each strain and nutrient level, using a posthoc test (Bonferroni test) after ANOVA over the whole dataset.

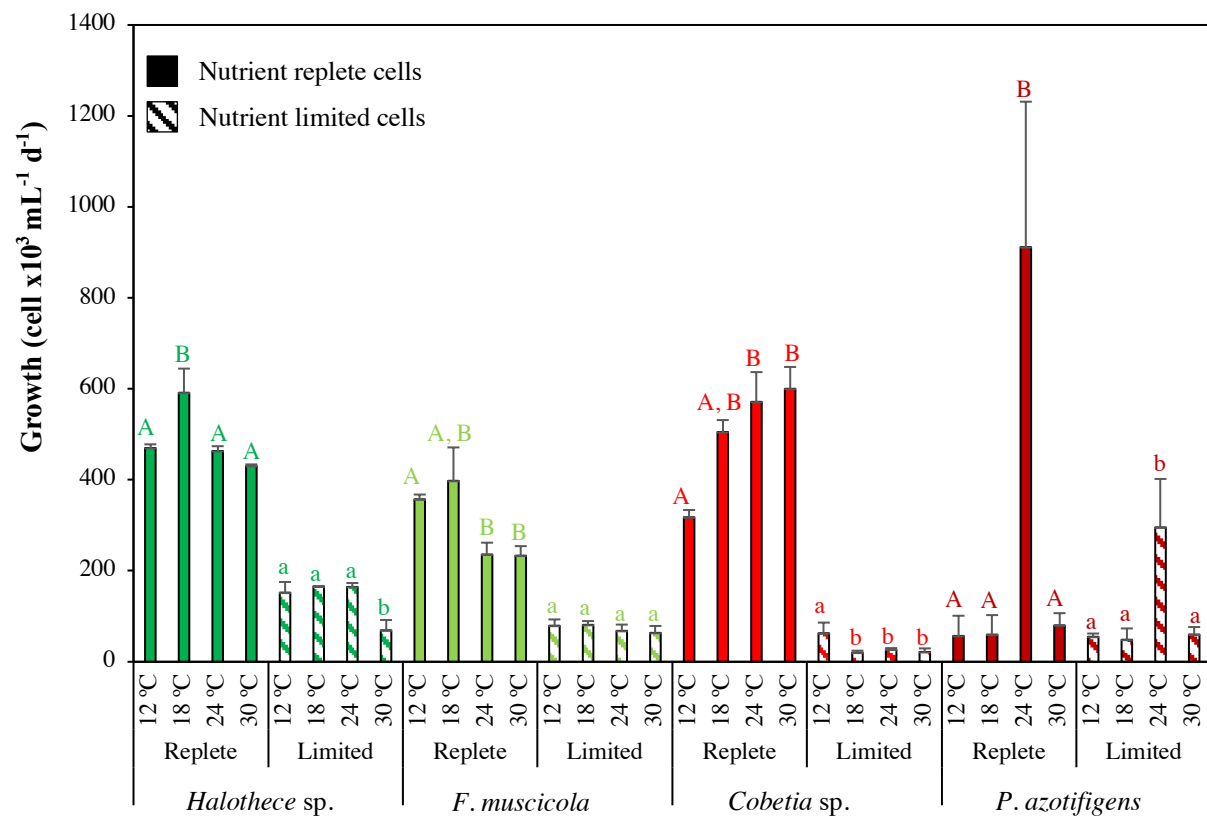


Figure 2. Effect of different levels of temperature on growth after 72 h under different nutrient concentrations. Values are the mean $\pm$ SE ($n = 3$). Letters (i.e., capital letters or lower case) indicate significant differences ($p < 0.05$) with the rest of the treatments for each strain and nutrient level, using a posthoc test (Bonferroni test) after ANOVA over the whole dataset.

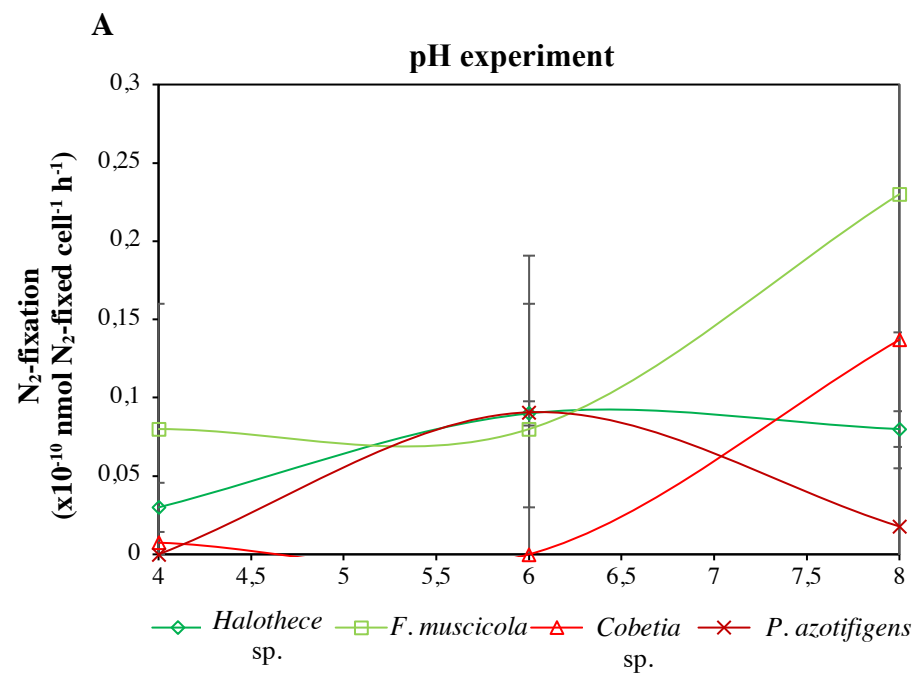
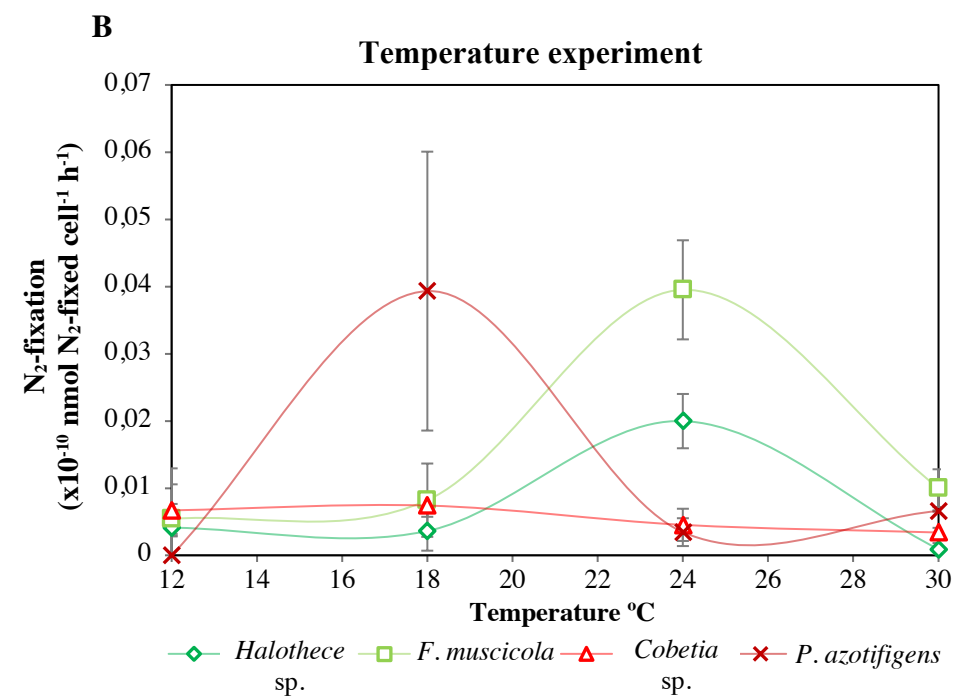
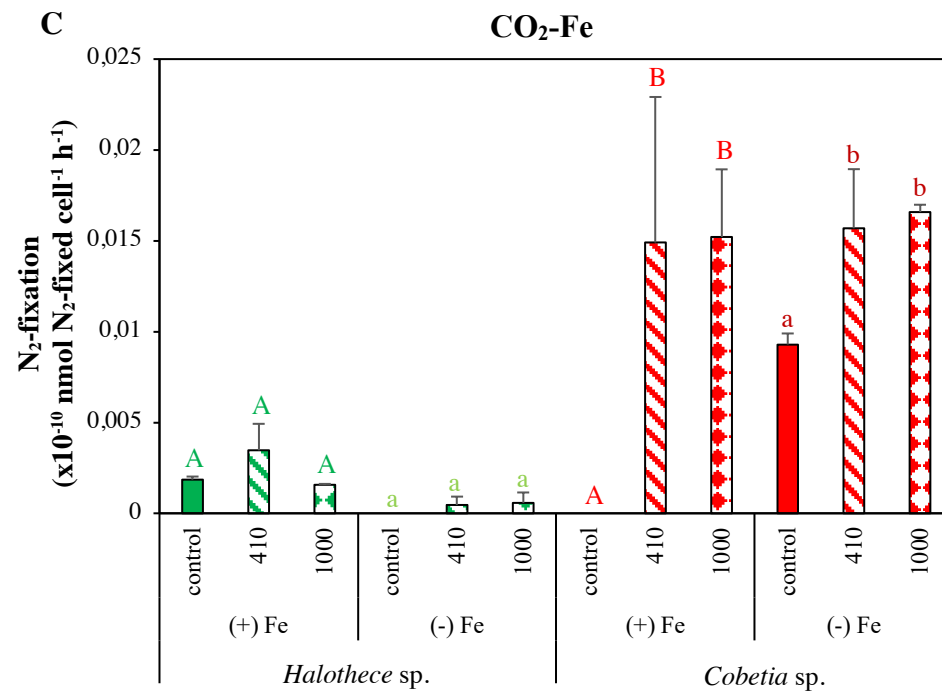
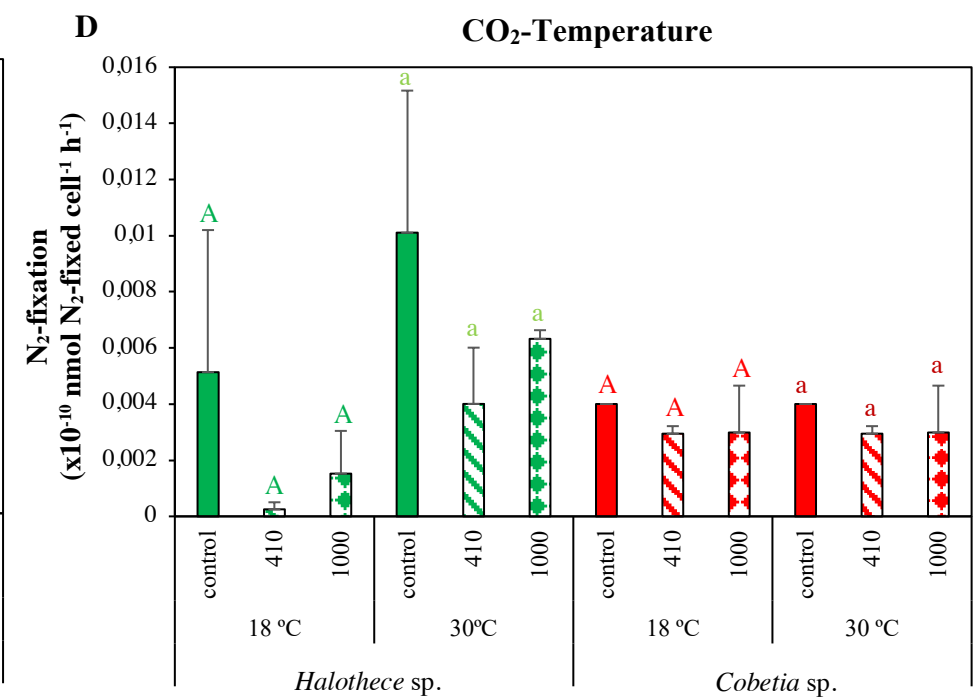


Figure 3. Effect on N₂-fixation rates after 72 h, under different **A)** pH and **B)** temperature levels, and **C-D)** different concentrations of CO₂ [control, atmospheric CO₂ (aCO₂): 410 ppm, and elevated CO₂ (eCO₂): 1000 ppm] in combination with different **C)** Fe (+Fe, 1 µM and -Fe, 2 nM) and **D)** temperature levels (18 °C and 30 °C). All the treatments were incubated under optimal P-concentration to detect N₂-fixation rates. Values are the mean ± SE (n = 3). Letters indicate significant differences ($p < 0.05$) with the rest of the treatments for each strain and experimental set-up, using a posthoc test (Bonferroni test) after ANOVA over the whole dataset.

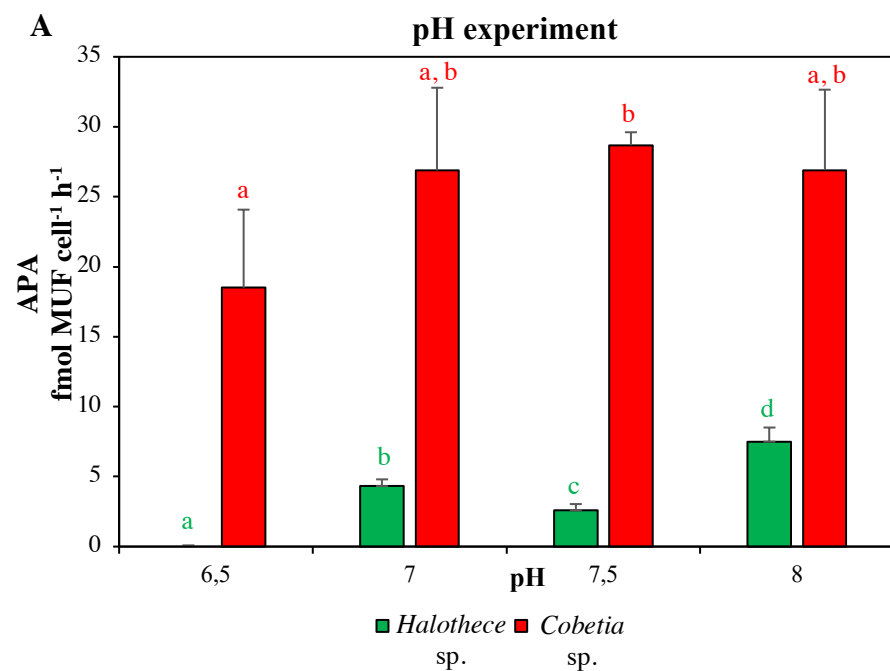
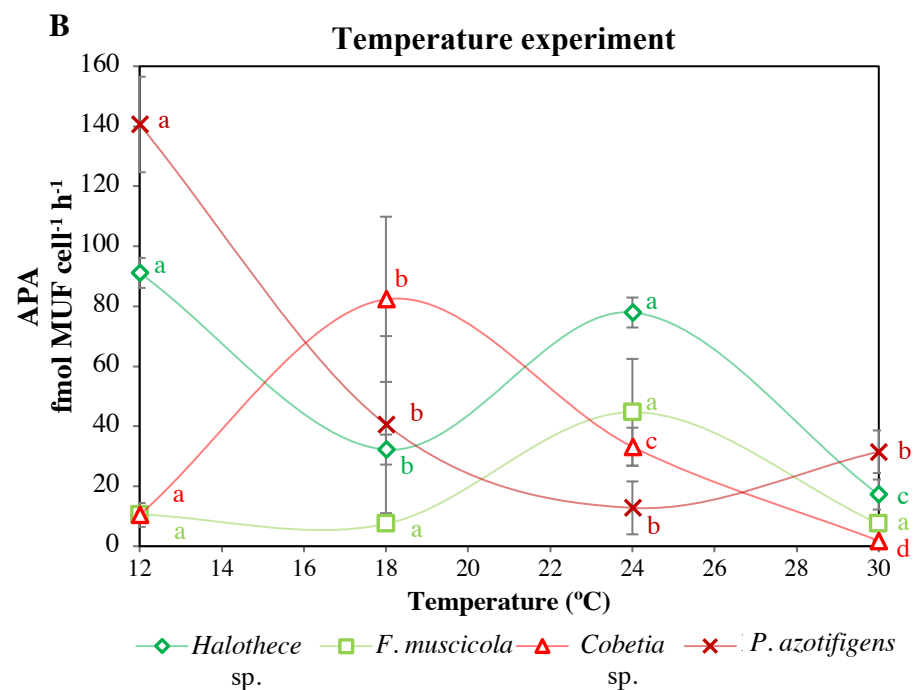
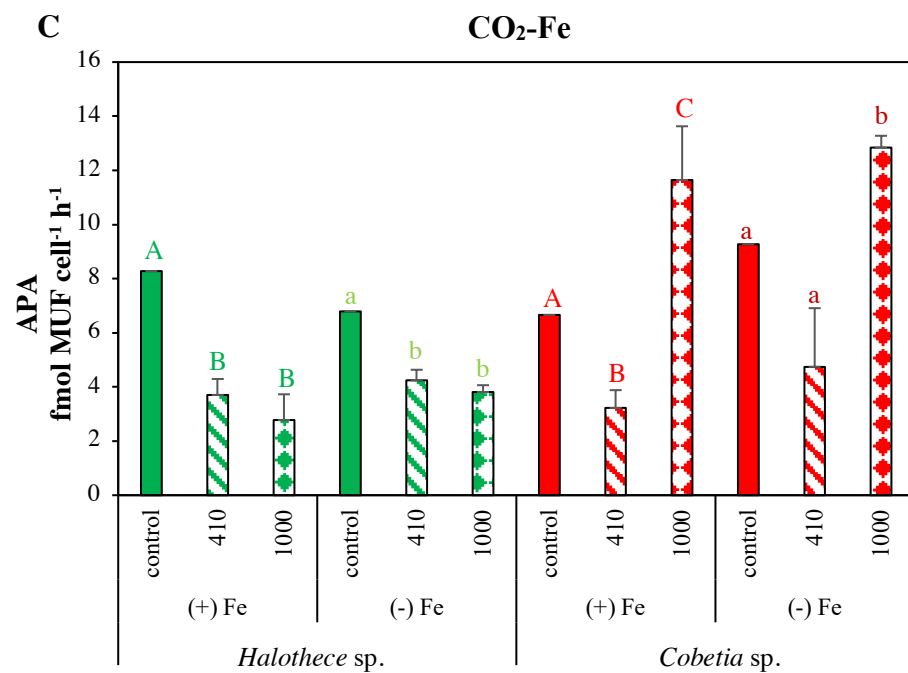
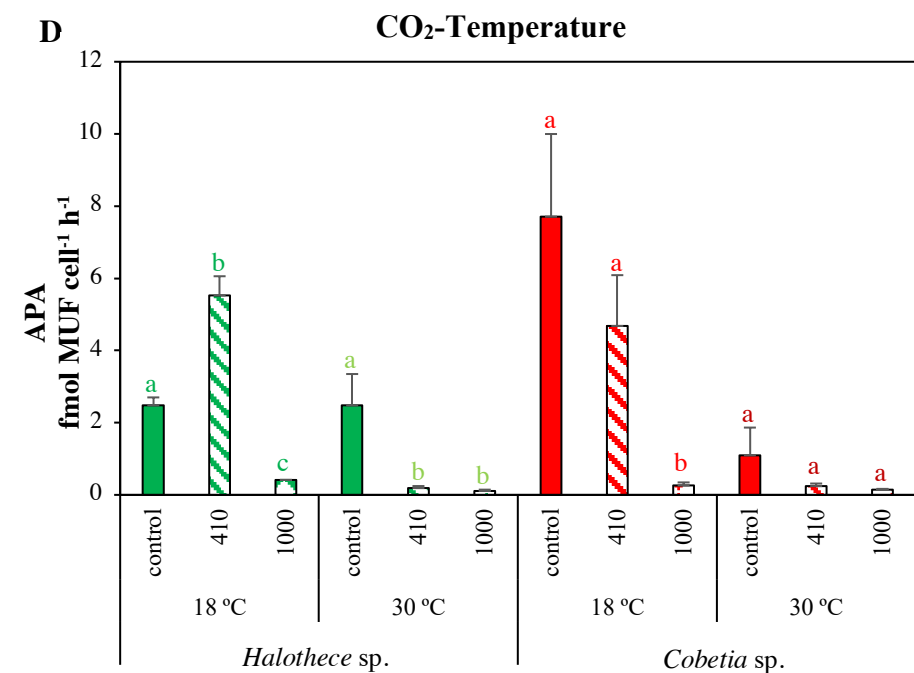


Figure 4. Effect in P-acquisition mechanisms (alkaline phosphatase activity [APA]), after 72 h, under different **A)** pH (6.5-8) and **B)** temperature levels (12-30 °C), and **C-D)** different concentration of CO₂ [control, atmospheric CO₂ (aCO₂): 410 ppm, and elevated CO₂ (eCO₂): 1000 ppm] in combination with different **C)** Fe (+Fe, 1 µM and -Fe, 2 nM) and **D)** temperature levels (18 °C and 30 °C). The measurements were taken under P limitation (0.1 µM) to induce APA. Note that for the pH experiment APA was only measured in *Halothece* sp. and *Cobetia* sp. in a pH range between 6.5-8. Values are the mean ± SE (n = 3). Letters indicate significant differences ($p < 0.05$) with the rest of the treatments for each strain and experimental set-up, using a posthoc test (Bonferroni test) after ANOVA over the whole dataset.

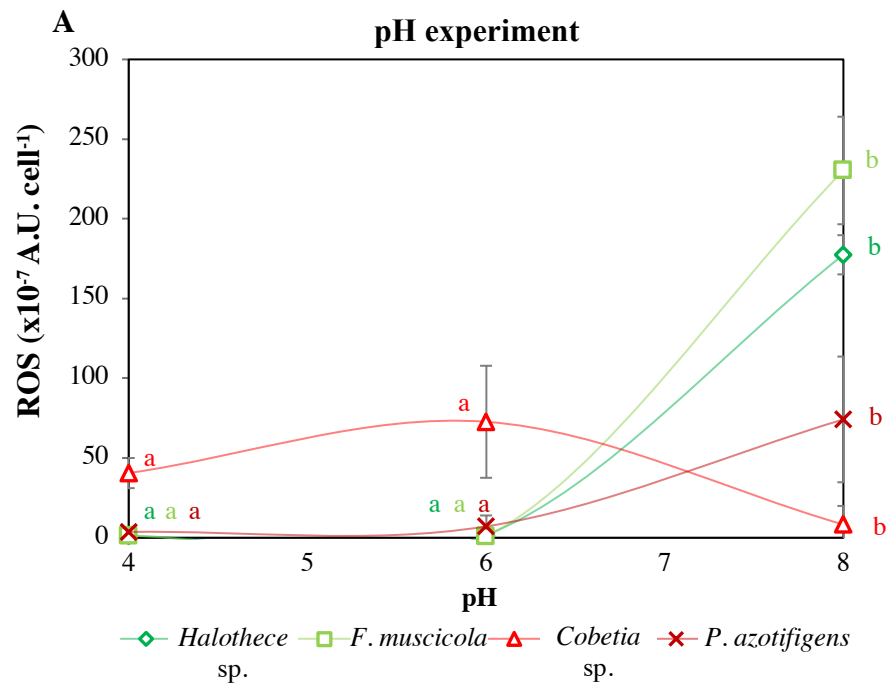
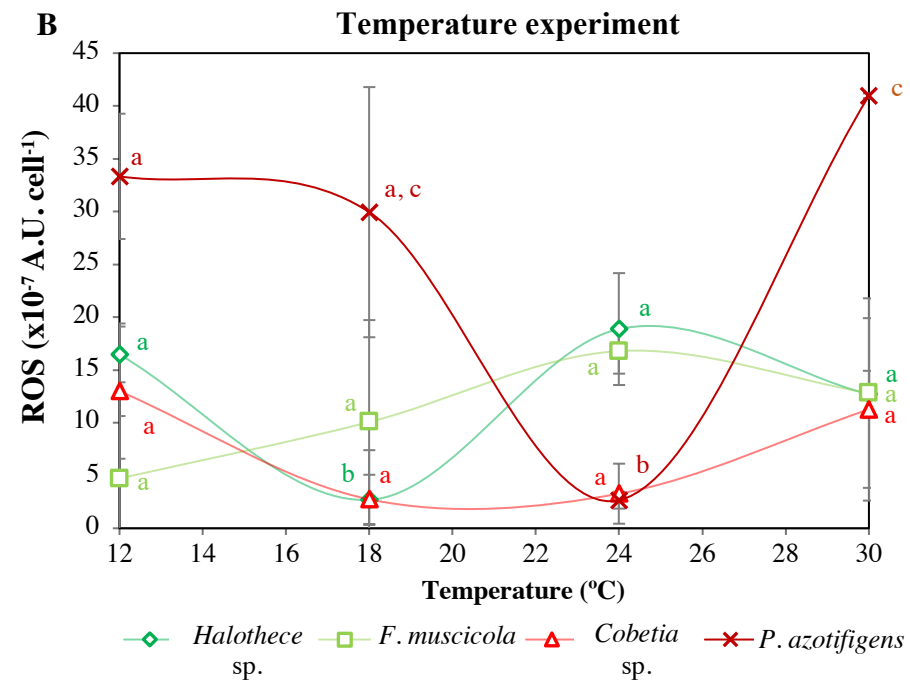
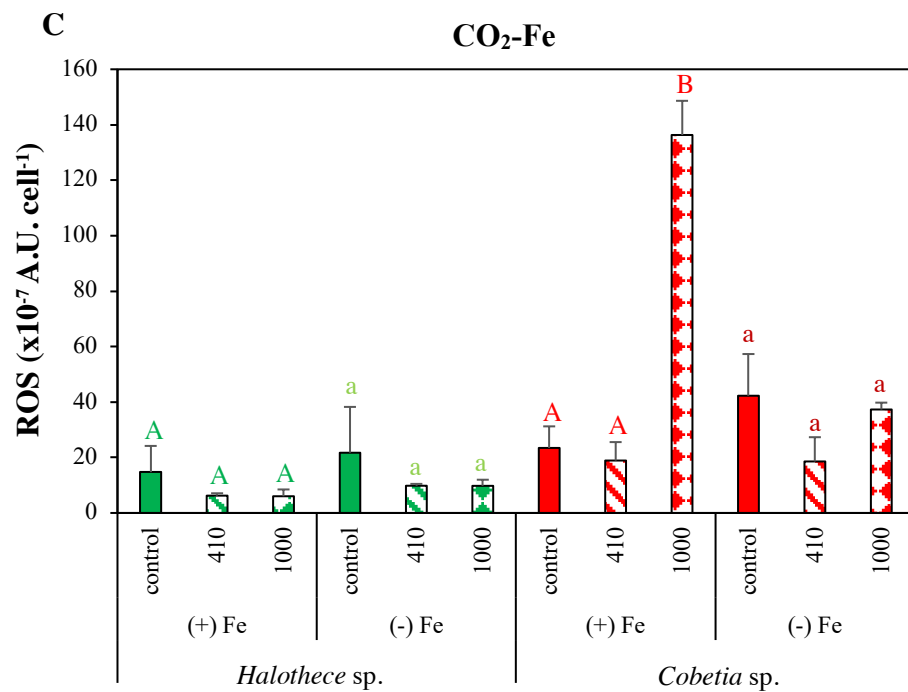
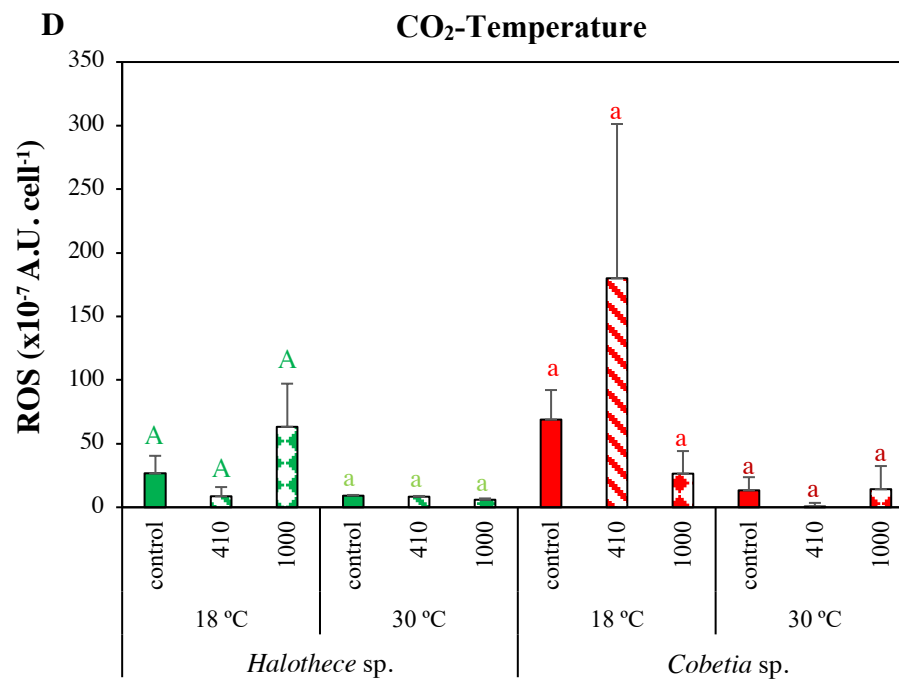


Figure 5. Effect at the oxidative stress level (i.e., reactive oxygen species, ROS), after 72 h, under different **A)** pH and **B)** temperature levels, and **C-D)** different concentration of CO₂ [control, atmospheric CO₂ (aCO₂): 410 ppm, and elevated CO₂ (eCO₂): 1000 ppm] in combination with different **C)** Fe (+Fe, 1 µM and -Fe, 2 nM) and **D)** temperature levels (18 °C and 30 °C). All the treatments were incubated under optimal P-condition. Values are the mean ± SE (n = 3). Letters indicate significant differences ($p < 0.05$) with the rest of the treatments for each strain and experimental set-up, using a posthoc test (Bonferroni test) after ANOVA over the whole dataset.

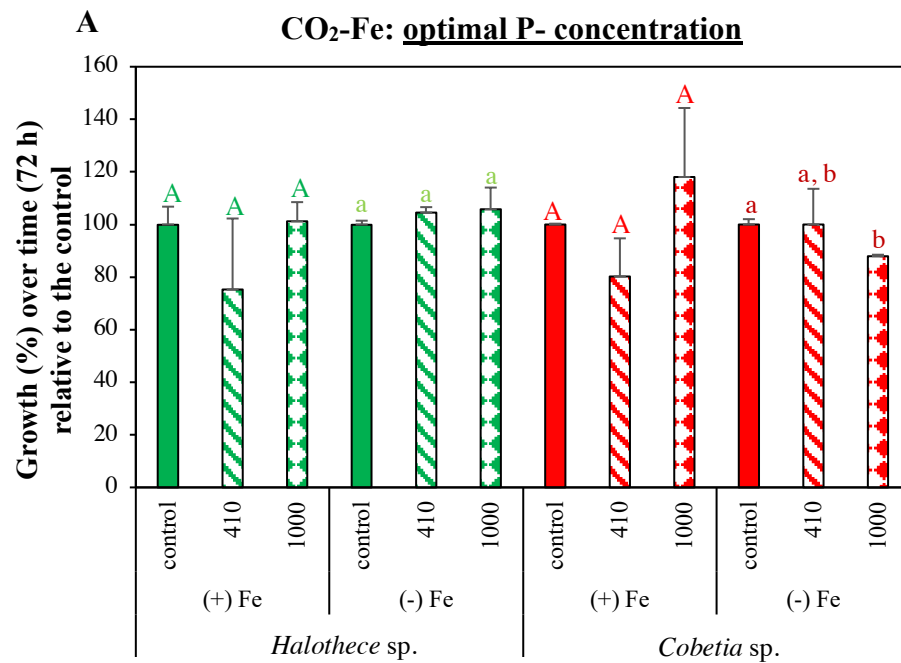
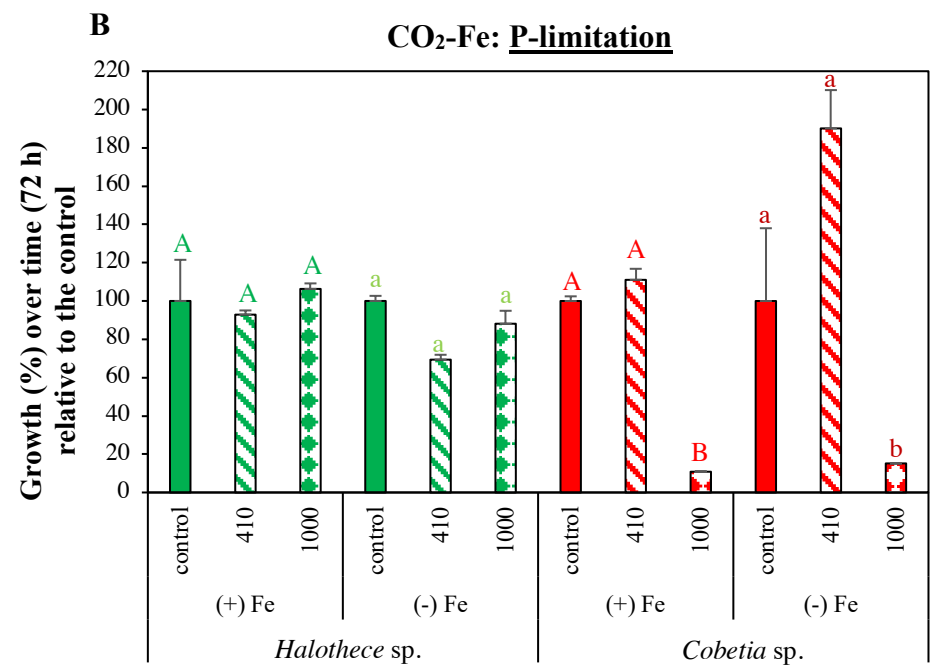
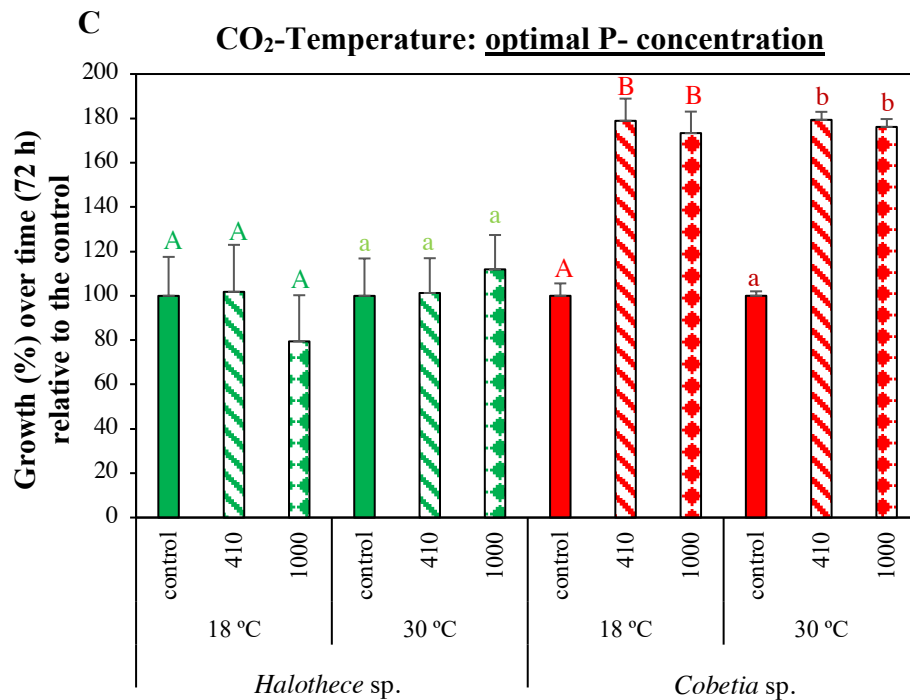
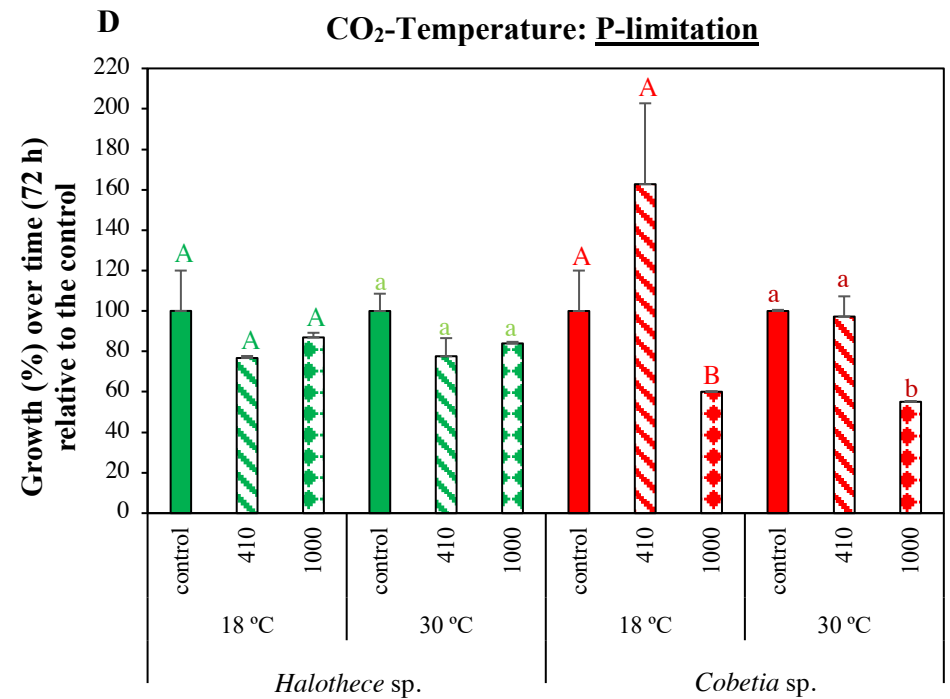


Figure 6. Growth (calculated as % difference relative to the control over the incubation time of 72 h) after the exposure to atmospheric CO₂ (aCO₂, 410 ppm) and elevated CO₂ (eCO₂, 1000 ppm). **A-B)** Exposure with CO₂ at different concentrations of Fe (+Fe, 1 µM and -Fe, 2 nM) under **A)** P-optimal conditions (1.5 mM) and **B)** P-limitation (0.1 µM). **C-D)** Exposure with CO₂ at different temperature levels (18 °C and 30 °C), under **C)** P-optimal conditions and **D)** P- limitation. Values are the mean ± SE (n = 3). Letters indicate significant differences ($p < 0.05$) with the rest of the treatments for each strain and experimental set-up, using a posthoc test (Bonferroni test) after ANOVA over the whole dataset.

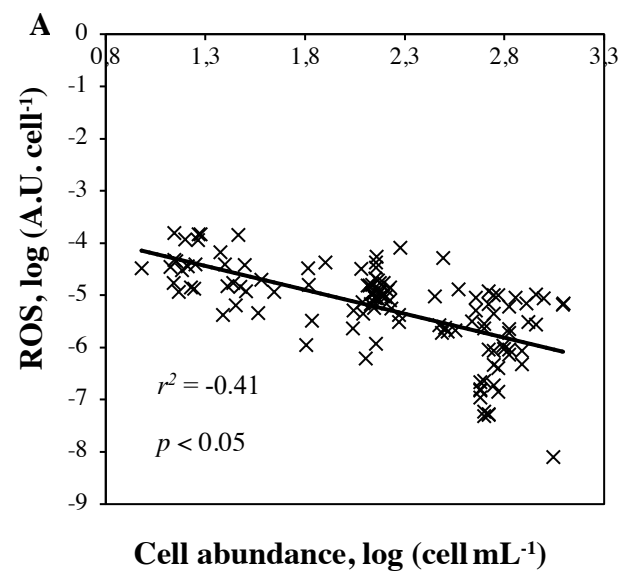
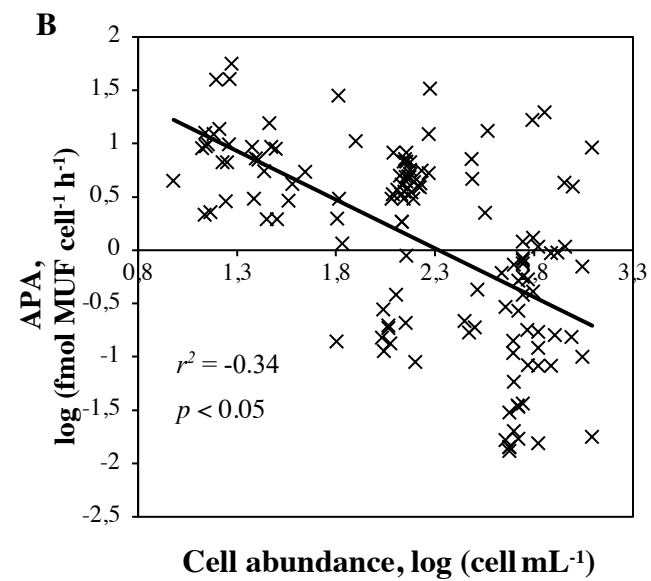
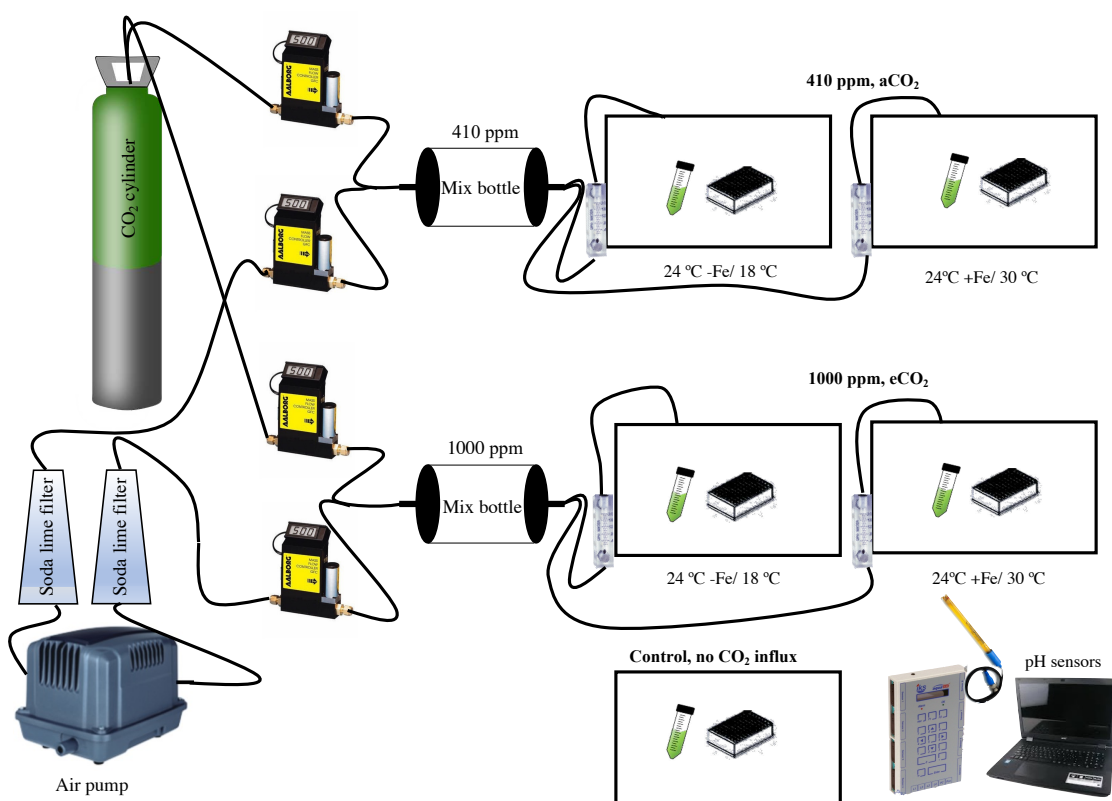


Figure 7. Linear regression analyses between **A)** cell abundance and ROS per cell and **B)** cell abundance and APA per cell. All treatments and species from the pH, temperature and CO₂ experiments were combined.

Supplementary Table S1. List of all treatments, strains and variables studied in this report. * Only tested for *Halotheca* sp. and *Cobetia* sp.

(I, II) Without the infusion of CO ₂							
Strains	(I) pH experiment (at 24° C)			(II) Temperature experiment (adjusted to pH 8)			Description
<i>Halotheca</i> sp. PCC 7418, Pasteur Culture Collection	pH 4			12 °C			<u>Under replete nutrients conditions:</u> 1.5 mM PO ₄ ³⁻ , 1 µM Fe and 0.15 mM NH ₃
<i>Fischerella muscicola</i> PCC 73103, Pasteur Culture Collection	pH 5			18 °C			
<i>Cobetia</i> sp. UIB 001, obtained: isolated directly from <i>P. oceanica</i> roots (Fernández-Juárez et al., unpublished data). GenBank: CP058244-CP058245	pH 6			24 °C			<u>Under P and Fe limitation.:</u> 0.1 µM PO ₄ ³⁻ , 2 nM Fe and 0.15 mM NH ₃
	pH 6.5*	pH 7*		30 °C			
<i>Pseudomonas azotifigens</i> DSM 17556 ^T , obtained from German Collection of Microorganisms and Cell Cultures GmbH (DSMZ)	pH 7.5*	pH 8*					
(III) With the infusion of CO ₂ (410 and 1000 ppm)							
Strains	(i) CO ₂ -Fe (at 24°C, adjusted to pH 8)			(ii) CO ₂ -temperature (adjusted to pH 8)			Description
<i>Halotheca</i> sp. PCC 7418	(+) Fe, 1 µM (+) PO ₄ ³⁻ , 1.5 mM	(-) Fe, 2 nM (+) PO ₄ ³⁻ , 1.5 mM	(+) or (-) Fe, (-) PO ₄ ³⁻ , 0.1 µM	18 °C (+) Fe, 1000 nM (+) PO ₄ ³⁻ , 1.5 mM	30 °C (+) Fe, 1000 nM (+) PO ₄ ³⁻ , 1.5 mM	18 °C or 30°C (-) PO ₄ ³⁻ ,0.1 µM	Control, no CO ₂ influx:
							aCO ₂ : 410 ppm
							eCO ₂ : 1000 ppm
<i>Cobetia</i> sp. UIB 001							At 120 r.p.m 12 h dark:12 h light photoperiod for 72 h, performed under 0.15 mM NH ₃)
Response variables							
(1) Growth		(2) N ₂ -fixation rates		(3) ROS production		(4) P-mechanisms (APA)*	

Supplementary Figure S1. Experimental set-up of the CO₂ experiments, considering two different levels of CO₂, aCO₂: 410 ppm and elevated, eCO₂: 1000 ppm, and included a control with no CO₂ influx. The pH level was monitored for aCO₂ and eCO₂.



Supplementary Figure S2. pH monitoring under a continuous influx of atmospheric CO₂ 410 ppm (atmospheric CO₂, aCO₂) and elevated CO₂ 1000 ppm (elevated CO₂, eCO₂) for *Halothece* sp. and *Cobetia* sp.

