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Dissolved organic matter offsets the detrimental effects of climate change in the nitrogen-fixing cyanobacterium *Crocosphaera*

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**Scientific Significance Statement**
Diazotrophs fix dinitrogen into bioavailable nitrogen, sustaining ocean primary productivity. Increasing anthropogenic CO2 emissions decrease seawater pH and induce warming enhancing water column stratification. This will reduce inorganic nutrient availability, forcing microbial communities to obtain resources from dissolved organic matter instead. While the source of nitrogen for diazotrophs is inexhaustible, their ability to use dissolved organic matter (DOM) may influence their performance in the future warm and acidified ocean. Here, we examine the response of *Crocosphaera* to DOM additions under three climate change scenarios. Our findings show that the availability of dissolved organic matter helps diazotrophs facing energetic stress under high temperature and low pH, allowing a more balanced metabolism and a winning strategy to survive in the future warm and acidified ocean.

**Abstract**
Diazotrophs provide a significant reactive nitrogen source in the ocean. Increased warming and stratification may decrease nutrient availability in the future, forcing microbial communities toward using dissolved organic matter (DOM). Not depending on reactive nitrogen availability, diazotrophs may be “winners” in a nutrient-depleted ocean. However, their ability to exploit DOM may influence this success. We exposed cultures of the widespread *Crocosphaera* to low (26°C, pH 8.1), moderate (28°C, pH 8.0), and extreme (30°C, pH 7.9) climate change conditions. Our results show that the availability of dissolved organic matter helps diazotrophs facing energetic stress under high temperature and low pH, allowing a more balanced metabolism and a winning strategy to survive in the future warm and acidified ocean.
Increasing anthropogenic CO₂ emissions induce ocean warming and acidification, affecting ecosystems and biogeochemical cycling (Gruber 2011; Cheng et al. 2019; Rossi 2023). Stratification inhibits the vertical supply of nutrients impacting biological productivity and consequently biomass transfer and carbon export (Passow and Carlson 2012). In the future ocean, reduced nutrient availability may force microbial communities to depend on regenerated compounds such as dissolved organic matter (DOM; Thornton 2014; Lønborg et al. 2020). Increased CO₂ also alters the seawater carbonate system, weakening the ocean’s buffering ability and reducing pH (Orr et al. 2005; Doney et al. 2009). While low pH has been generally observed to decrease phytoplankton growth (Burkhardt et al. 1999; Hinga 2002), increased CO₂ concentrations can improve the affinity of the RuBisCO enzyme enhancing primary productivity (Riebesell et al. 2007; Wu et al. 2010). In contrast, heterotrophic bacteria are more resistant to low pH and their metabolic rates tend to increase with temperature (Grossart et al. 2006; Kirchman et al. 2009; Morán et al. 2017). Thus, ecosystem functioning in the future ocean will be significantly driven by the sensitivity of different planktonic groups to changes in CO₂, pH, and temperature (Riebesell and Tortell 2011; Dutkiewicz et al. 2015; Sunagawa et al. 2015).

Prokaryotic cells called “diazotrophs” replenish the nitrogen oceanic reservoir by fixing dinitrogen (N₂), sustaining primary productivity (Zehr 2011). As the main suppliers of reactive nitrogen, the response of diazotrophs to future ocean conditions can have profound implications in productivity and the ocean’s role as a climate buffer (Hutchins and Capone 2022). Diazotrophic cyanobacteria, including Trichodesmium and Crocosphaera, predominate in (sub)tropical waters where temperatures range between ~20°C and 27°C (Breitbarth et al. 2007; Sohm et al. 2011), while the symbiotic UCYN-A and noncyanobacterial diazotrophs (NCDs) can be found across wider latitudinal ranges (polar to tropical waters; 4 to > 25°C; Martínez-Pérez et al. 2016; Harding et al. 2018; Turk-Kubo et al. 2022). Despite the diversity of diazotrophs, most climate change research has focused on Trichodesmium and Crocosphaera (e.g., Kranz et al. 2009; Garcia et al. 2013a; Hutchins et al. 2013). The different temperature optima of Trichodesmium and Crocosphaera, 24–28°C and 26–31°C, respectively (Webb et al. 2009; Sohm et al. 2011; Fu et al. 2014) will likely influence their biogeography and N₂ fixation activity as the ocean becomes warmer (Breitbarth et al. 2007; Hutchins et al. 2007).

Models predict altered nutrient availability changing interspecies competition, favoring diazotrophs (Dutkiewicz et al. 2021). Under increased CO₂ levels, the energy saved in intracellular carbon storage allows diazotrophs to invest more energy in fixing N₂ (Levitan et al. 2007; Kranz et al. 2011). Previous work examined the response of Trichodesmium and Crocosphaera cultures to partial pressure of CO₂ (pCO₂) levels between 150 and 1500 ppm (e.g., Hutchins et al. 2007, 2015; Fu et al. 2008; Kranz et al. 2011). Some studies observed increased N₂ fixation from 380 to 750 ppm pCO₂ (Hutchins et al. 2007; Levitan et al. 2007; Boatman et al. 2017), but others only observed enhanced N₂ fixation when comparing much larger pCO₂ ranges (e.g., > 190 vs. > 350 ppm; Kranz et al. 2009; Garcia et al. 2013a). These positive responses only seem to hold under high iron or light conditions (Fu et al. 2008; Garcia et al. 2013b). However, culture-based studies do not reflect field studies, where diazotroph assemblages show a high resilience to short-term pCO₂ perturbations (Böttjer et al. 2014; Gradoville et al. 2014a). In contrast, when temperature and pCO₂ increase are combined, Trichodesmium growth decreases if temperature exceeds the strain optimum (Boatman et al. 2017), suggesting that the interlinked climate change stressors will affect diazotrophic performance in the future ocean.

Although CO₂ and photosynthesis provide the main sources of carbon and energy for marine cyanobacteria—including diazotrophs—DOM may supply an alternative source of carbon, energy, and nutrients when facing stress (e.g., Muñoz-Marín et al. 2013). For example, the unicellular cyanobacterium Cyanothece can grow on glycerol (Feng et al. 2010), the filamentous Nodularia can assimilate leucine (Hietanen et al. 2002), and UCYN-A and Crocosphaera may use sugars when photosynthesis is energy-limited (Moisander et al. 2012). Trichodesmium and Crocosphaera are able to use phosphoesters (Dyhrman et al. 2002; Orchard et al. 2003), but only Trichodesmium obtains phosphor from phosphonates (Dyhrman et al. 2006). Crocosphaera can obtain both phosphorus and energy from phosphoanhydrides (Filella et al. 2022). These studies point toward an overlooked role of osmotrophy, the uptake of DOM, for cyanobacteria survival in the future ocean. In this study, we sought to understand the potential of DOM as an alternative carbon and/or energy source for Crocosphaera when subjected to future temperature and pH conditions (IPCC 2014; Pachauri et al. 2014). Combining molecular, physiological, and biogeochemical techniques, we find that DOM is an important carbon source for Crocosphaera, compensating for the energy and stress imbalances faced under future climate change conditions. These
results call for a reassessment of the role of DOM for diazotrophic cyanobacteria, as nitrogen inputs in the future stratified ocean may be closely linked to these interactions.

**Methods**

To provide a comprehensive assessment of the behavior of *Crocosphaera* under future ocean conditions, we combined temperature and pH levels simulating representative concentration pathways (RCP) 2.6 (26°C and pH 8.1), 4.5 (28°C and pH 8.0), and 6.0 (30°C and pH 7.9) (IPCC 2014), here called “low,” “intermediate,” and “extreme” treatments (10.6084/m9.figshare.22773503). Cultures of *Crocosphaera* were acclimated to the different treatments for 1 month in 500-mL bottles before the start of the experiment in larger volumes (40 liters; duplicate low and triplicate intermediate and extreme treatments) with a starting concentration of $\sim 1.5 \times 10^4$ cells mL$^{-1}$ (Fig. 1A). The experiment was run for 8 d (here days −2 to 5) under a 12-h light : dark cycle with a light intensity of $\sim 250 \mu$mol photons s$^{-1}$. The temperature and pH levels of each treatment were maintained constant along the experiment using an IKS aquastar system (Supporting Information). Temperature and salinity were measured daily with a YSI Pro30 probe, while pH was measured spectrophotometrically (Supporting Information). Cell abundance and nutrient concentrations were measured daily using standard microscopy and colorimetry methods (Supporting Information). On alternate days (days 0, 2 and 4), subvolumes were sampled from each treatment and incubated to measure single-cell $N_2$ and dissolved inorganic carbon (DIC) fixation using dissolved $^{15}N_2$ and $^{13}C$-HCO$_3^-$, respectively. Similarly, a mix of $^{13}C$-labeled organic compounds representative of marine DOM including the amino acids alanine, leucine, and arginine, and the carbohydrates glucose and N-acetylglucosamine was used to measure DOM uptake rates and RNA expression responses (see Supporting Information). RNA was sequenced using an Illumina NovaSeq 6000 platform generating more than 30 million paired end reads per sample (2 $\times$ 50 bp). RNA reads were annotated using Diamond and the databases Kofam UniProt’s SwissProt, trEMBL, and NCBI’s RefSeq (see Supporting Information).

**Results and discussion**

**Responses to combined temperature and pH treatments**

Temperature and pH remained close to the target values in all treatments (10.6084/m9.figshare.22773503). Nutrient conditions were similar between treatments and favorable for diazotrophic growth, indicating that any responses in growth, DIC and $N_2$ fixation can be confidently attributed to the different treatments (Supporting Information). Cells grown in batch incubations without continued nutrient additions, slowly consume limiting elements contained in the culture medium (YBC-II), including vitamins and iron which cause cultures to end collapsing.

*Crocosphaera* grew exponentially from day −2 until day 0 in all treatments, increasing from $\sim 1.5$ to $>2.7 \times 10^4$ cell mL$^{-1}$ (Fig. 1A). Growth in the low and extreme treatments reached a plateau between days 0 and 2, then decreased until day 5 (Fig. 1A). Conversely, cells in the intermediate treatment continued growing until day 4 and only started decaying after. The intermediate treatment thus was the most beneficial for *Crocosphaera*, in agreement with previous studies where the thermal range of *Crocosphaera* oscillates between 26°C and 31°C and optimal growth is $\sim 28$°C (Waterbury and Willey 1988; Falcion et al. 2005; Webb et al. 2009; Fu et al. 2014).

DIC and $N_2$ fixation rates differed among treatments (ANOVA; $p < 0.001$; Supporting Information Fig. S1), ranging between 0 and 34.4 fmol C cell$^{-1}$ d$^{-1}$ and between 0 and 10.3 fmol N cell$^{-1}$ d$^{-1}$, respectively (Fig. 1B), in the range of previous field measurements at similar temperatures (i.e., $\sim 26$–30°C; Foster et al. 2013; Bonnet et al. 2018; Benavides et al. 2022). While neither DIC nor $N_2$ fixation rates varied significantly with time in the low treatment, rates increased

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**Fig. 1.** (A) *Crocosphaera* cell abundance in the low, intermediate, and extreme treatments along the experiment, where each dot represents the average, and the error bars the standard deviation of analytical duplicates in each treatment replicate. (B) Single-cell DIC and (C) $N_2$ fixation rates in the different treatments on days 0, 2, and 4, where each dot represents the uptake rate for single cells and boxplots show the 25th to 75th percentile, error bars the minimum and maximum percentile and the black dot and crossbar the median and mean values, respectively. Negative day values (days −2 to 0) correspond to the post-acclimation period, when *Crocosphaera* cells are transferred from concentrated cultures to the larger tanks, while positive day values (days 0–5) correspond to the experimental period.
on day 2 in the other two treatments (Fig. 1B,C). However, on day 4 both rates increased in the intermediate treatment where cells were actively growing but decreased in the other two treatments (Fig. 1A,B). Our results agree with previous reports of enhanced DIC and N₂ fixation with increased temperature or pCO₂ during exponential growth (Fu et al. 2014; Gradoville et al. 2014a,b). The drop in DIC and N₂ fixation rates observed in the extreme treatment on day 4 suggests that cells cannot withstand these harsh conditions for experimental periods of time. Similarly, natural assemblages of picocyanobacteria increase their photosynthetic rates with elevated pCO₂ initially but are unable to maintain elevated rates after 3 d of incubation (Lomas et al. 2012). A closer look at the intrapopulation variability confirms that while extreme conditions may enhance DIC and N₂ fixation momentarily, they became detrimental over time as suggested by the decrease of metabolically active cells between days 2 and 4 (Supporting Information Fig. S4). Similar intra-population variability has been observed in wild and cultured Crocosphaera (Foster et al. 2013, Masuda et al. 2020), which questions previous interpretations of increased pCO₂ having a positive effect on Crocosphaera.

Comparative transcriptomics captures changes in gene expression identifying regions involved in the regulation of gene expression (Ozsolak and Milos 2011, Wilhelm and Landry 2009). Although gene expression is a complex process regulated at many cellular levels (e.g., Buccitelli and Selbach 2020), it is generally defined as the production of an observable phenotype by a gene, ultimately directing the synthesis of a protein, thus showing how cells attempt to regulate their metabolism (Alberts et al. 2002). Here, we use transcriptomics to investigate differential gene expression of Crocosphaera under three future temperature and pH treatments in control or DOM-amended conditions. We screened for the most differentially expressed genes among treatments and experimental time points, covering many important metabolic pathways of this species (Supporting Information Table S1).

Connecting physiological responses to gene expression, we observed that the DIC fixing gene rbcL, encoding for part of the RuBisCO enzyme, was least expressed in the extreme treatment and most expressed in the low treatment on day 4 (Supporting Information Fig. S3), as previously observed in Synechocystis (Price et al. 2008). This could be due to the thermal damage of RuBisCO, as seen in Trichodesmium (Boatman et al. 2017). Increased pCO₂ is thought to alleviate the energy investment that concentrating carbon involves, in turn promoting both DIC and N₂ fixation (Hutchins et al. 2007; Levitan et al. 2007). N₂ fixation (nif) genes expression were not mirrored with N₂ fixation rates at any time point of the experiment (Supporting Information Fig. S3; Fig. 1B), which may be due to post-translational modifications and/or differing energy availability for nitrogenase activity (Kranz et al. 2011; Levitan et al. 2010). Alternatively, the time of RNA sampling at sunrise might not reflect the effect of treatments on N₂ fixation genes, as nif gene expression in Crocosphaera peaks at sunset (Wilson et al. 2017).

Through 1st steps of photosynthesis, light is harvested and transferred to generate energy and reductants that keep most metabolic processes active including DIC and N₂ fixation (Steward 1980; Kranz et al. 2011). The lower expression of the light-harvesting machinery coincided with lower DIC and N₂ fixation rates in the low and extreme treatments (Fig. 1B; Supporting Information Fig. S3), reflecting a close link between the expression of energy-producing genes and Crocosphaera’s main metabolic activity. Stress-induced protein genes such as heat shock chaperones were highly expressed in the low and extreme treatments (Supporting Information Fig. S3). Molecular chaperones allow cyanobacteria to maintain protein conformation homeostasis, which is key for temperature stress adaptability (Blondin et al. 1993). Synechocystis and Synechococcus upregulate about 90 different heat shock proteins after 1 h exposure to heat stress (Slabas et al. 2006) and similar responses are observed when exposed to cold temperatures (Porankiewicz and Clarke 1997). Moreover, osmotic and oxidative stresses promote heat shock expression in cyanobacteria (Apte et al. 1989), suggesting that these genes regulate essential cellular functions (Rajaman et al. 2014). Collectively, our comparative transcriptomics analysis confirms that cells in the low and extreme treatment were more stressed than in the intermediate treatment where the higher expression of light-harvesting genes allowed cells to keep balanced DIC and N₂ fixation activity (Supporting Information Fig. S3). Moreover, the high expression of stress-induced proteins maintained over time only in the extreme treatment suggests a strong effect of low pH and high temperature, negatively affecting all cellular functions despite potential energetic benefits of higher pCO₂.

**DOM as a source of carbon when facing environmental stress**

Crocosphaera obtained at least twice more carbon from DOM as from DIC, independently of the treatment (ANOVA; p = 0.12; Fig. 2). DOM thus provided a preferred source of carbon under all conditions tested here, suggesting that osmotrophy is an important trait of Crocosphaera. Given the lability of the amended 13C-DOM, these results should be carefully extrapolated to natural environments where most DOM is generally refractory. Considering that 1% of the background DOM in our cultures was labile, DOM uptake rates decreased by 94% and were two times lower than DIC fixation rates in all treatments on day 0, and remained lower (t-test, p < 0.01) in the intermediate but similar (t-test, p > 0.1) in the low and extreme treatments for the rest of the experiment. In nature, DOM uptake rates by Crocosphaera may be in the above ranges. Furthermore, despite the competitiveness of Crocosphaera against other community components for these labile substrates is unknown, this diazotroph shows
significant uptake of organic carbon derived from a mixture of representative natural carbohydrates and amino acids (see Supporting Information). Diazotrophic cyanobacteria may use DOM as an alternative source of carbon, nutrients and/or energy (Dhryman et al. 2006; Filella et al. 2022), for example Trichodesmium can obtain as much carbon from amino acids as from DIC (Benavides et al. 2017). In contrast, the non-diazotrophic cyanobacterium Prochlorococcus obtains more carbon from DIC than from DOM uptake but can assimilate glucose in culture and field conditions (Moisander et al. 2012; Muñoz-Marín et al. 2013; Duhamel et al. 2018). Direct glucose assimilation is more energetically favorable than producing it through the Calvin Cycle (Muñoz-Marín et al. 2013). Moreover, while DIC is tightly coupled to the reactive carbonate system and hence susceptible to pH changes, the chemical composition of DOM does not change significantly with varying pH, providing a more stable carbon source under changing environmental conditions (Zark et al. 2015). The cellular machinery to assimilate DOM may also be more resistant to pH changes than that required to fix DIC, as observed by similar gene expression of the main carbohydrate degradation pathways regardless of pH changes (Supporting Information Fig. S3). In all, DOM seems a more stable source of carbon for cyanobacteria under low pH conditions.

Diazotrophic cyanobacteria are able to obtain carbon and phosphorus from DOM, which can enhance their N₂ fixation activity (Benavides et al. 2017, 2020; Dyhrman et al. 2007). However, organic nitrogen molecules contained in DOM may instead inhibit N₂ fixation (Mulholland and Capone 1999; Walworth et al. 2018). The DOM mix used here contained alanine, arginine and leucine, but average N₂ fixation rates were similar under DOM-amended or unamended conditions with few exceptions (Fig. 3). Again, single-cell analyses showed beneficial responses to DOM additions, masked if treating the population as whole (Fig. 3; Supporting Information Fig. S2). On day 4, N₂ fixation rates were close to zero in the low and extreme treatments (Fig. 3), where only ~18% and 21% of the cells were actively fixing N₂, respectively (Supporting Information Fig. S2), while upon DOM amendments ~30% and 54% of the measured cells actively fixed N₂ (Supporting Information Fig. S2; Fig. 3). In contrast, the percentage of cells actively fixing N₂ remained similar over time in the intermediate treatment where cells were healthier (Fig. 1A; Supporting Information Fig. S2). Our results support previous field experiments arguing that DOM aids cyanobacterial diazotrophs fixing N₂ when facing environmental stress (Benavides et al. 2018; Bonnet et al. 2013).

Differential gene expression was observed in response to DOM additions, with changes among growth phases and treatment conditions (Fig. 4). Most genes increased their expression upon DOM additions on day 4, while only a few differences were measured in the intermediate treatment on day 2 (Fig. 4).

**Fig. 2.** (A) Single-cell DIC fixation and (B) DOC uptake rates in unamended and DOM-amended conditions, respectively, for each sampling time point. Each dot represents the uptake rate for single cells.
DOM amendments did not influence the expression of DIC fixation genes (Fig. 4). Expression of N₂ fixation genes increased slightly in all treatments on day 4, and in the extreme treatment on day 2 upon DOM additions (Fig. 4), providing inconclusive trends and uncoupled patterns with the measured rates as seen above (Supporting Information Fig. S3). Conversely, DOM additions enhanced the expression of light-harvesting genes in the low treatment and components of the photosystem II in all treatments on day 2 (Fig. 4).

In Crocosphaera, photosynthesis genes are expressed before sunrise (Wilson et al. 2017) and their expression depends on cellular resources produced on the previous day (Dron et al. 2012). Therefore, cells can easily face energy limitation if metabolic processes are not balanced throughout the diel cycle. For example, Crocosphaera shows a temporal delay in cell division of approximately 1 h after sunrise reflecting the need to recover cellular reserves with light-derived energy after night-time activity (Wilson et al. 2017). Hence, DOM could provide an additional and energetically cheaper source of carbon for restoring the light-harvesting components when cells are energetically deprived after the dark period. Overall, DOM enhanced the expression of the machinery required to produce cellular resources to fix DIC and N₂, improving cell performance specially under stress conditions including late growth stage, non-optimal temperature, and low pH.

**Crocosphaera in the future ocean**

In a future ocean, decreased nutrient supply may limit nondiazotrophic phytoplankton growth, benefitting diazotrophs as long as iron and phosphorus remain available (Weber and Deutsch 2010; Hutchins and Capone 2022). Phosphorus concentrations, unlike N₂ and carbon, will decrease considerably with intensified stratification, while the enhancement of desertification and dust deposition could increase iron and phosphorus inputs regionally, creating diazotroph niches (Jickells et al. 2005; Moulin and Chiapello 2006; Hamilton et al. 2020).

We show that DOM uptake was higher and more constant than DIC fixation regardless of the treatment, providing Crocosphaera with an extra and secure carbon source. DOM additions allowed cells in the low and extreme treatments to maintain a greater number of cells actively fixing N₂ than in unamended conditions, which coincided with increased expression of genes involved in the 1ˢᵗ steps of photosynthesis.
Overall, DOM became especially important in treatments or at growth stages where cells were more limited, presumably lacking stored resources to maintain a balanced metabolism over time.

Osmotrophic Crocosphaera may maintain balanced growth in a future stratified, warm, and acidic ocean, contributing to nitrogen inputs where they predominate. Warming may favor Crocosphaera relative to other coexisting diazotrophs in (sub)tropical waters like Trichodesmium (Fu et al. 2014; Breitbarth et al. 2007; Yang et al. 2021), while increasing pCO₂ may similarly affect both species (Hutchins et al. 2007; Fu et al. 2008). Our results indicate that warming and acidification are more adverse when combined, and that beyond thermal and pH limits, metabolic versatility to exploit DOM is likely to influence the expansion of diazotrophic niches, potentially dictating nitrogen inputs in the future. Therefore, investigating osmotrophy in other prominent diazotrophs such as Trichodesmium, UCYN-A and NCDs should be prioritized if we are to understand their role in the future ocean.

Fig. 4. Differentially expressed genes between DOM-amended and unamended samples (p < 0.01) for each treatment on days 2 and 4. Genes are grouped by common pathway or function (see Supporting Information Table S1 for gene full names and grouping). Positive fold changes (purple dots) indicate genes that were more expressed in DOM-amended samples than in unamended samples for each treatment. Negative fold changes and genes showing no differences between both conditions are colored in gray. The dot size increases with increasing fold change.
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