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Brief Report

Two co-dominant nitrogen-fixing cyanobacteria demonstrate distinct acclimation and adaptation responses to cope with ocean warming

Ping-Ping Qu,[1*] Fei-Xue Fu,[1] Xin-Wei Wang,[2] Joshua D. Kling,[1] Mariam Elghazzawy,[1] Megan Huh,[3] Qian-Qian Zhou,[4] Chunguang Wang,[4] Esther Wing Kwan Mak,[5] Michael D. Lee,[6,7] Nina Yang[1] and David A. Hutchins[1*]

[1]*Department of Biological Sciences, University of Southern California, Los Angeles, CA, 90089.*
[2]*School of Marine Sciences, Ningbo University, Ningbo, Zhejiang, 315211, China.*
[3]*Department of Preventative Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA, 90089.*
[4]*Third Institute of Oceanography, Ministry of Natural Resources, Xiamen, Fujian, 361005, China.*
[5]*Department of Ocean Sciences and Institute of Marine Sciences, University of California, Santa Cruz, CA, 95064.*
[6]*Exobiology Branch, NASA Ames Research Center, Mountain View, CA, 94035.*
[7]*Blue Marble Space Institute of Science, Seattle, WA, 98154.*

Summary

The globally dominant N_2-fixing cyanobacteria *Trichodesmium* and *Crocosphaera* provide vital nitrogen supplies to subtropical and tropical oceans, but little is known about how they will be affected by long-term ocean warming. We tested their thermal responses using experimental evolution methods during 2 years of selection at optimal (28°C), supra-optimal (32°C) and suboptimal (22°C) temperatures. After several hundred generations under thermal selection, changes in growth parameters, as well as N and C fixation rates, suggested that *Trichodesmium* did not adapt to the three selection temperature regimes during the 2-year evolution experiment, but could instead rapidly and reversibly acclimate to temperature shifts from 20°C to 34°C. In contrast, over the same timeframe apparent thermal adaptation was observed in *Crocosphaera*, as evidenced by irreversible phenotypic changes as well as whole-genome sequencing and variant analysis. Especially under stressful warming conditions (34°C), 32°C-selected *Crocosphaera* cells had an advantage in survival and nitrogen fixation over cell lines selected at 22°C and 28°C. The distinct strategies of phenotypic plasticity versus irreversible adaptation in these two sympatric diazotrophs are both viable ways to maintain fitness despite long-term temperature changes, and so could help to stabilize key ocean nitrogen cycle functions under future warming scenarios.

Received 28 March, 2021; accepted 18 December, 2021. *For correspondence. E-mail dahutch@usc.edu (D. A. H) and pqu2@stanford.edu (P. P. Q); Tel. 213-740-5616 (D. A. H) and 213-675-7391 (P. P. Q). [†]Present address: Department of Psychiatry and Behavioral Sciences, Department of Genetics, Stanford University School of Medicine, Stanford, CA, 94305, USA.

Introduction

Diazotrophic cyanobacteria fix new nitrogen that supports food webs and carbon export in the subtropical and tropical oceans (Sohm *et al*., 2011; Zehr, 2011; Tang *et al*., 2019). Among the most important diazotrophs is *Trichodesmium*, a globally distributed, colonial cyanobacterium that contributes a major fraction of marine new nitrogen inputs (Capone *et al*., 1997; Hood *et al*., 2004; Bergman *et al*., 2013). *Crocosphaera* is a sympatric unicellular N_2-fixer with comparable biomass-normalized nitrogen fixation rates to *Trichodesmium*, and also supports a large proportion of total marine nitrogen fixation (Moisander *et al*., 2010; Knapp *et al*., 2012; Berthelot *et al*., 2016).

The short-term (weeks) responses of *Trichodesmium* and *Crocosphaera* to climate change factors such as CO_2, temperature and nutrients have been used to infer potential impacts on biogeochemical cycles in the future ocean (Fu *et al*., 2008, 2014; Garcia *et al*., 2013; Hutchins *et al*., 2013; Boatman *et al*., 2017; Li *et al*., 2018; Qu *et al*., 2019). However, climate change occurs on

timescales of years to decades. Thus, long-term evolutionary experiments spanning hundreds of generations may provide more realistic predictions of the responses of important marine microbial groups.

Past experimental evolution studies have examined adaptation of several eukaryotic algae to changing temperature, CO_2, light and salinity (Schlüter *et al*., 2014; Padfield *et al*., 2016; Jin and Agustí, 2018; Kremer *et al*., 2018; O'Donnell *et al*., 2018), and of diazotrophic cyanobacteria to elevated CO_2 (Hutchins *et al*., 2015). Surprisingly, long-term thermal adaptation has not been examined in *Trichodesmium* and *Crocosphaera*, even though the low latitude regions where they live are among the fastest-warming parts of the global ocean (Liu *et al*., 2005; Collins *et al*., 2010). Tropical phytoplankton species are likely to be especially vulnerable to warming, since their thermal optima are close to the current mean temperatures of their surroundings (Thomas *et al*., 2012). Currently, the maximal sea surface temperatures (SSTs) of the low-latitude Atlantic and Pacific Oceans are approximately 26°C–28°C and 25°C–30°C respectively (Keenlyside and Latif, 2007; Hurrell *et al*., 2008; Muñoz *et al*., 2012; Dunstan *et al*., 2018), and the mean SST of the ocean has increased ∼0.44°C from 1971 to 2010 (IPCC, 2013). In comparison, the upper threshold for growth of both *Trichodesmium* and *Crocosphaera* is ∼30°C (Breitbarth *et al*., 2007; Sohm *et al*., 2011; Fu *et al*., 2014). With projected increases in both mean temperatures and extreme heat wave events caused by climate change (IPCC, 2012; Thornton *et al*., 2014; Boyd *et al*., 2016; Smale *et al*., 2019), the maximal SST of the tropical ocean is likely to exceed the upper thermal limit of these two diazotrophs. It is thus imperative to understand whether these two key diazotrophs have the capacity to acclimate or adapt to the future warming ocean, or whether they will be excluded from the warmest parts of their present low-latitude habitat (Breitbarth *et al*., 2007; Boatman *et al*., 2017; Li *et al*., 2018).

Here, we compare the potential for short-term acclimation and long-term adaptation of *Trichodesmium erythraeum* IMS101 and *Crocosphaera watsonii* WH0005 to optimal (28°C), supra-optimal (32°C) and suboptimal (22°C) temperatures by investigating both transient plastic capacity and lasting evolutionary fitness changes of these two diazotrophs. In particular, 32°C represents likely future warmer conditions in the tropical oceans (Bopp *et al*., 2013). In our study, acclimation and adaptation capacity was characterized using short-term temperature performance curve (TPC) determinations for both diazotrophs before and after 2 years of thermal selection. Adaptive responses were indicated by irreversible phenotypic changes as determined using reciprocal transfer experiments following the TPC determinations. Moreover, genomic sequence variations in temperature-selected *Crocosphaera* cell lines were analyzed to explore thermal evolution at the genetic level. Our results reveal fundamental differences in the acclimation and adaptation strategies that these two sympatric nitrogen-fixing cyanobacteria groups may employ to respond to the challenges of living in a rapidly warming ocean.

Results

Growth rates and TPCs

The growth rates of ancestral cell lines of both *Trichodesmium* (Fig. 1A) and *Crocosphaera* (Fig. 1B) were ∼0.3 day^{-1} at the optimal growth temperature of 28°C before the long-term evolution experiments. Immediately following the initial transfer from 28°C to the other two selection temperatures, growth rates decreased significantly at suboptimal (22°C) and supra-optimal (32°C) temperatures by ∼30%–50% (*Crocosphaera*: adjusted *p*-values <0.01 for 28°C vs. 22°C and 28°C vs. 32°C; *Trichodesmium*: adjusted *p*-value <0.001 for 28°C vs. 22°C and 28°C vs. 32°C; Fig. 1).

After ∼2 years at the three selection temperatures, *Trichodesmium* IMS101 specific growth rates remained ∼0.3 day^{-1} at 28°C and were still significantly lower at 22°C (∼0.2 day^{-1}, adjusted *p*-value <0.01, 28°C vs. 22°C). In contrast, final *Trichodesmium* growth rates at 32°C had increased to ∼0.28 day^{-1}, not significantly different from those at 28°C (Fig. 1A). Growth rate phenotype showed very similar trends in long-term thermally selected *Crocosphaera* WH0005. Final specific growth rates were still ∼0.3 day^{-1} at 28°C and had increased to identical levels at 32°C (∼0.3 day^{-1}), while they remained low and unchanged at 22°C (∼0.2 day^{-1}, adjusted *p*-value <0.001, 28°C vs. 22°C; Fig. 1B).

TPCs of *Trichodesmium* IMS101 and *Crocosphaera* WH0005 cell lines maintained at the three temperatures were determined at the end of the long-term selection period, in the 21st (*Crocosphaera*) and 24th (*Trichodesmium*) month after the initial transfer. The generation time for both species was 2–3 days at 28°C and 32°C, and 3–4 days at 22°C. Thus, long-term *Crocosphaera* cell lines were selected for ∼315 generations at 28°C and 32°C, and ∼160 generations at 22°C, while *Trichodesmium* IMS101 cell lines underwent selection for ∼360 generations at 28°C and 32°C, and ∼180 generations at 22°C.

After brief acclimation for 2 weeks, a typical 'increase-peak-decline' pattern was found in all TPCs, but *Trichodesmium's* temperature range was 3°C–4°C wider than that of *Crocosphaera* (Fig. 1C and D; Table 1). Thermal response modelling based on Eppley–Norberg curves (Thomas *et al*., 2012) yielded a calculated optimal

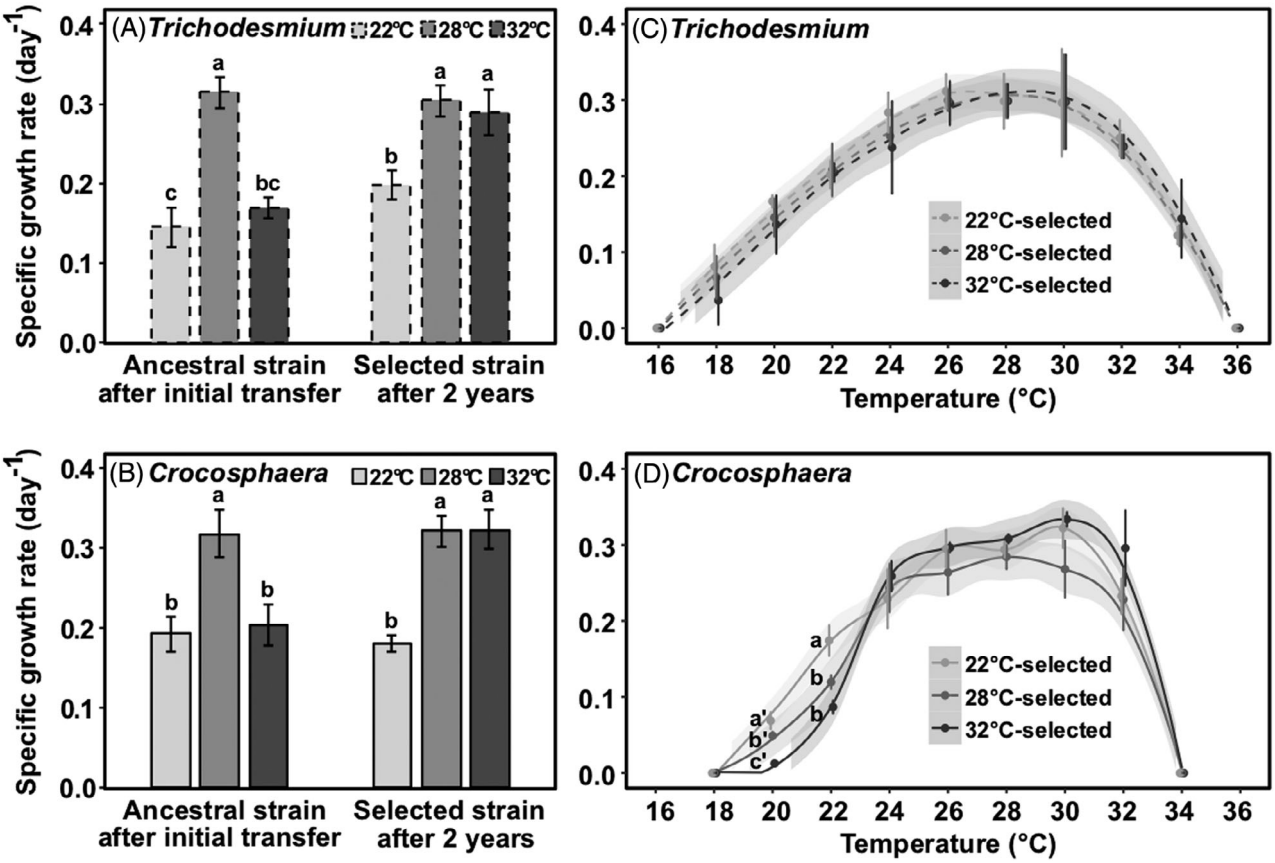

Fig. 1. Growth rates of ancestral and thermally selected *Trichodesmium* and *Crocosphaera* cell lines versus temperature. Growth rates of ancestral cell line of (A)*Trichodesmium erythraeum* IMS101 and (B) *Crocosphaera watsonii* WH0005 when initially transferred to three selection temperatures (22°C, 28°C and 32°C), and of the same cell lines (six *Trichodesmium* replicates and three *Crocosphaera* replicates at each temperature) at the end of the 2-year selection period. The letters a–c on each bar in (A) and (B) show significance grouping results based on TukeyHSD multiple comparisons for growth rates of the ancestor and thermally selected cell lines for each species. Values and error bars represent the means and standard deviations of triplicate cell cultures in each treatment. Also shown are measured growth rate-based thermal response curves of 2-year thermally selected (C) *Trichodesmium* (dashed lines) and (D) *Crocosphaera* (solid lines), two weeks after transfer from the selection treatments to the TPC. The lines and shading show the smoothing results using the loess method and the 95% confidence interval respectively. The letters a–b and a′–c′ in (D) represent the significance grouping results based on TukeyHSD multiple comparisons for the growth rates of selected *Crocosphaera* cell lines at 22°C and 20°C respectively.

temperature niche of 27°C–30°C for both diazotrophs, and maximum growth rates of all *Trichodesmium* and *Crocosphaera* cell lines were comparable at 0.31–0.36 day^{-1} (Table 1).

By the end of the 2-week acclimation period, growth rates of *Trichodesmium* cell lines selected under three different temperatures were congruent at each temperature across the entire TPC (Fig. 1C; multiple comparisons among treatments for each temperature from 16°C to 36°C individually). In contrast, long-term thermally selected *Crocosphaera* only achieved similar growth rates at each temperature within its optimal temperature range (spanning the middle of the TPC, 24°C–30°C), but responded quite differently at the two ends of the thermal response curve (Fig. 1D). At the cold end of the response curves, 22°C-selected *Crocosphaera* cell lines had significantly higher growth rates at 20°C and 22°C than the 32°C and/or 28°C-selected cell lines (multiple comparisons, at 20°C: adjusted p-value <0.001, 22°C- vs. 32°C-selected and <0.05, 22°C- vs. 28°C-selected cells; at 22°C: adjusted p-value <0.05, 22°C- vs. 32°C-selected and 22°C- vs. 28°C-selected cells). Besides the growth difference between 22°C- vs. 32°C-selected *Crocosphaera* cell lines at 20°C, 32°C-selected cells grew significantly more slowly than 28°C-selected cells at this lowest temperature (adjusted p-value <0.01; Fig. 1D).

At the warm end of the response curves, the growth rates of the 32°C-selected *Crocosphaera* cell lines were ~25% higher at 32°C than those of the 22°C- and 28°C-selected *Crocosphaera*, although the difference was not significant. At a stressful high temperature of 34°C, none of the selected *Crocosphaera* cultures was able to grow actively (Fig. 1D). However, the 32°C-selected cell lines

Table 1. Thermal performance curve (TPC) parameters of *Trichodesmium* and *Crocosphaera* cell lines selected for 2 years at 22°C, 28°C and 32°C.

Species	Selection condition	Maximal growth rate (day^{-1})	Optimum temperature (°C)	Thermal niche width (°C)	Correlation factor (R^2, 0–1)	Maximal survival temperature (°C)
Trichodesmium	22°C-selected	0.30	27.89	19.79	0.96	34
	28°C-selected	0.31	28.52	19.64	0.92	34
	32°C-selected	0.31	27.64	19.91	0.95	34
Crocosphaera	22°C-selected	0.36	29.35	15.33	0.95	32
	28°C-selected	0.31	28.78	15.93	0.94	32
	32°C-selected	0.33	28.64	16.94	0.96	34[a]

Values in columns 3–5 were calculated by incorporating growth rates measured over 2 weeks across the temperature range 16°C–36°C into the thermal response model. The maximal growth rate is the predicted rate at the corresponding optimum temperature, as calculated by the model. Maximal survival temperature in the last column was the highest observed survival temperature in the TPC experiments.
[a]At this temperature, 32°C-selected *Crocosphaera* survived 2 weeks (the length of TPC experiment) by maintaining cell numbers and nitrogen fixation, but was not able to grow.

maintained a stable level of living biomass for 16 days and continued to fix nitrogen at 34°C (see below), while 22 and 28°C-selected cells died within a week at this temperature (Table 1).

Nitrogen and carbon fixation rates

After thermal selection for 2 years, long-term 28°C-selected *Trichodesmium* cells had the highest nitrogen (Fig. 2A) and carbon fixation (Fig. 2B) rates, followed by 32°C-selected cells, while the rates of 22°C-selected cells were the lowest. In particular, carbon and nitrogen fixation rates between 28°C-selected and 22°C-selected *Trichodesmium* cell lines were significantly different (Fig. 2A and B; multiple comparisons, both carbon and nitrogen fixation: adjusted p-value <0.05). During the 2-week period of TPC experiments, thermally selected *Trichodesmium* cells demonstrated a rapid acclimation. No significant difference was observed in the nitrogen and carbon fixation rates of temperature-selected cell lines at each of the five temperatures from 20°C to 34°C (Fig. 2A and B).

Like *Trichodesmium*, long-term 28°C and 32°C-selected *Crocosphaera* cell lines had identical nitrogen and carbon fixation rates at their selection temperatures, while 22°C-selected cells had significantly lower rates (multiple comparisons, carbon fixation: adjusted p-value <0.001, 22°C- vs. 28°C-selected and 22°C- vs. 32°C-selected cells; nitrogen fixation: adjusted p-value <0.05, 22°C- vs. 28°C-selected cells; Fig. 2C–F). After the 2-week incubations at 22°C and 28°C, all three thermally selected *Crocosphaera* cell lines matched the carbon and nitrogen fixation rates of long-term 22°C-selected and 28°C-selected cells respectively (Fig. 2C and D). In contrast, at the 32°C assay temperature, long-term 22°C and 28°C-selected *Crocosphaera* cell lines had much lower nitrogen and carbon fixation rates than 32°C-selected cells (Fig. 2C–F). In particular, the carbon fixation rates of 28°C-selected and the nitrogen fixation rates of 22°C-selected cell lines transferred to 32°C were significantly lower than those of 32°C-selected cells after the 2-week TPC determinations (carbon fixation: adjusted p-value <0.001, 32°C- vs. 28°C-selected cells; nitrogen fixation: adjusted p-value <0.01, 32°C- vs. 22°C-selected cells; Fig. 2C and D). After a 1-month transfer to 32°C in the switch experiments, 32°C-selected *Crocosphaera* cells had significantly higher carbon fixation rates compared to 22°C-selected cells (adjusted p-value <0.05; Fig. 2E and F).

It was worth noting that only 32°C-selected *Crocosphaera* cells survived and had the ability to fix a considerable amount of carbon and nitrogen at 34°C (Fig. 2C and D). At this temperature, the 28°C and 22°C-selected cell lines stopped growing and nitrogen and carbon fixation rates were mostly below detection limits (multiple comparisons, both nitrogen and carbon fixation: adjusted p-value <0.05, 32°C- vs. 22°C-selected and 32°C- vs. 28°C-selected cells). Clearly, the 32°C-selected cell lines were more tolerant of elevated temperature than 22°C and 28°C-selected cell lines.

Crocosphaera genomic analysis

Based on the growth and nitrogen/carbon fixation rate measured during the 2-week period of TPC experiments, thermally selected *Trichodesmium* cells showed a quick acclimation to new temperatures (Figs 1C and 2A and B). These rapidly reversible shifts in *Trichodesmium* TPCs were characteristic of non-genetic phenotypic plasticity (Reusch and Boyd, 2013). In contrast, the persistent changes in 32°C-selected *Crocosphaera* thermal phenotypes observed in TPC determination and switch experiments were suggestive of true adaptation (Figs 1D and 2C–F).

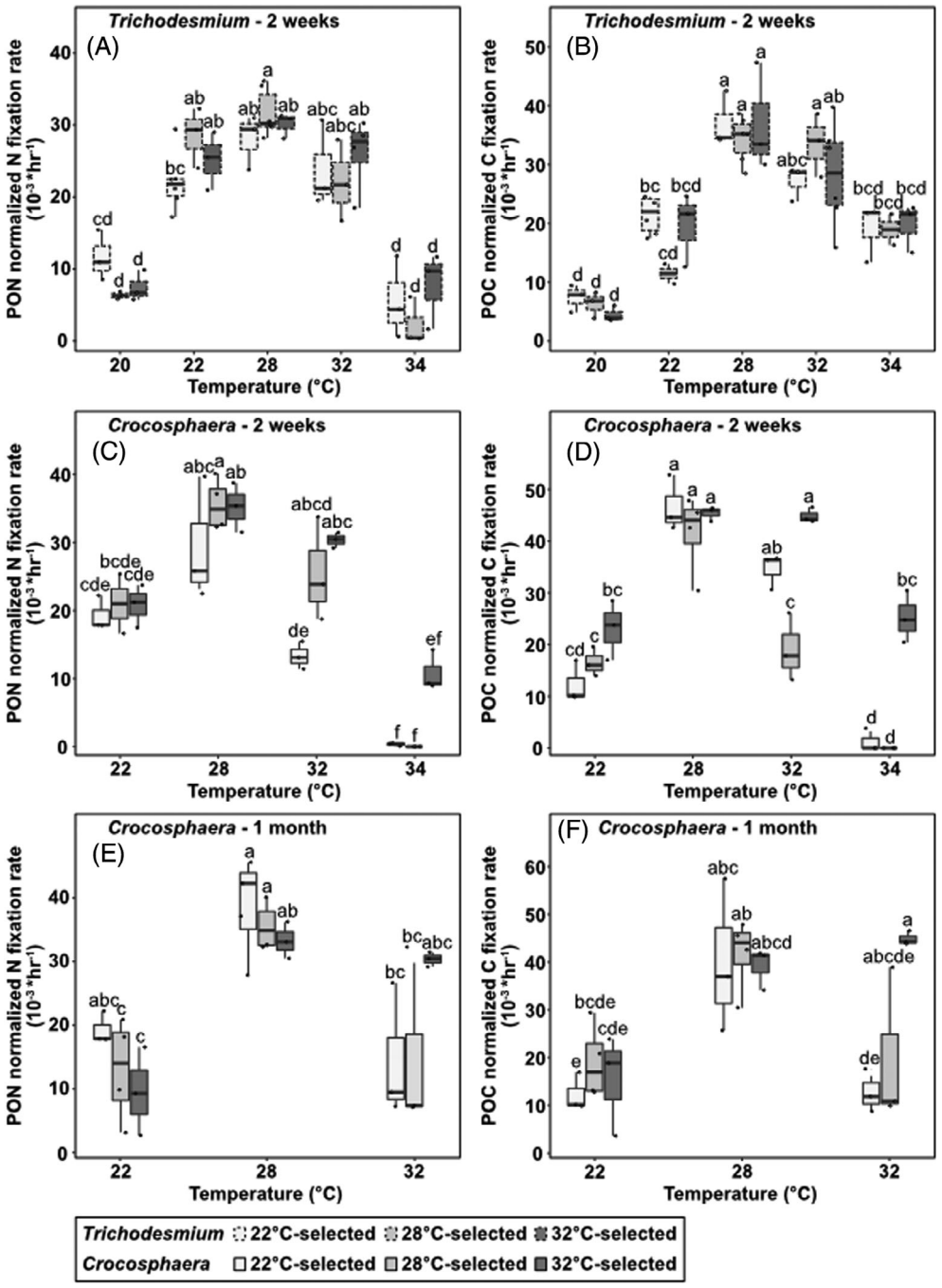

Fig. 2. Nitrogen and carbon fixation rates of 2-year thermally selected *Trichodesmium* and *Crocosphaera* cell lines. Dashed line boxes in (A) and (B) show the (A) N fixation and (B) C fixation rates of *Trichodesmium* at different temperatures after the 2-week TPC incubation. Solid line boxes in (C–F) show *Crocosphaera* (C) N fixation and (D) C fixation rates after a 2-week TPC experiment and (E) N fixation and (F) C fixation rates at the end of the 1-month switch experiment. N and C fixation rates are respectively normalized to PON and POC for both species. Open circles show the values of replicates in each treatment (six *Trichodesmium* replicates and three *Crocosphaera* replicates in each long-term selection treatment, and three replicates of each species in each short-term temperature treatment). The letters a–f above boxes represent the significance grouping results after the TukeyHSD multiple comparisons among all treatments in each panel. Take panel (A) as an example, letters a–d indicate the significant difference among 15 treatments (three temperature-selected groups × five assay temperatures) instead of among three groups at each temperature.

Accordingly, we tested for genetic changes by performing whole-genome Illumina sequencing on *Crocosphaera* cell lines. Based on the principal component analysis (PCA) of 10558 single nucleotide variants (SNVs) and 740 insertion–deletion variants (Indel) among nine samples (including one ancestral

strain sample, two 22°C-selected, three 28°C-selected and three 32°C-selected cell lines), three 32°C-selected replicates clustered together, apart from the other samples (Fig. 3). With another pipeline that subsampled sequence reads before calling variants, the influence of varying sequencing depth on these findings was eliminated and PCA using uniformly subsampled reads resulted in a similar pattern (Fig. S1).

Moreover, more than 400 nonsynonymous SNVs and Indels were detected in the *Crocosphaera* genome among the three temperature-selected treatments. Thirteen of these passed the cutoff for significance (one-way ANOVA for each variant, *p*-values listed in Table 2), and nine of these 13 occurred in the three 32°C-selected replicates. The ratios of non-synonymous to synonymous substitutions (Ka/Ks, as known as d_N/d_S) for genes with nonsynonymous SNVs of statistical significance among treatments were all below the hallmark of 1, indicating functional roles in the *Crocosphaera* genome (Table 2). Annotation results also showed that genes influenced by nonsynonymous variants were involved in numerous important cellular functions and biological processes (discussed below).

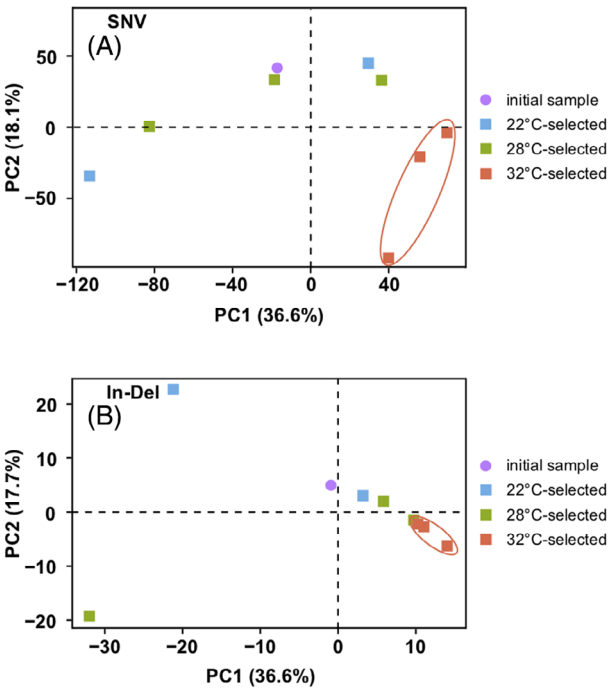

Fig. 3. PCA of *Crocosphaera* genomic mutations in ancestral and 2-year thermally selected cell lines. (A) SNVs and (B) insertions and deletions (Indels). Principal components 1 (PC1) and 2 (PC2) are shown on the *x*-axis and *y*-axis respectively. In both panels, the initial DNA sample of the ancestral *Crocosphaera* strain is represented by the purple solid circle. DNA samples of the long-term thermally selected *Crocosphaera* cell lines are shown for 22°C (blue squares, two replicates), 28°C (green squares, three replicates) and 32°C (orange squares, three replicates).

Discussion

Plasticity versus adaptation in thermally selected Trichodesmium and Crocosphaera

Our results suggest that *Trichodesmium* IMS101 possesses a considerable amount of inherent thermal plasticity, allowing it to quickly acclimate to temperature shifts within the range of 18°C–34°C. This species showed no sign of irreversible thermal adaptation, even after 2 years of thermal selection. Like *Trichodesmium* in our study, the dinoflagellate *Alexandrium ostenfeldii* also demonstrates plastic responses to warming instead of adaptation (Jerney *et al*., 2019). In contrast, >4.5 years of selection by elevated CO_2 results in irreversible adaptation of *Trichodesmium* IMS101, following an initial plastic acclimation period (Hutchins *et al*., 2015). The selection time in our thermal study was ∼44% shorter than that in the CO_2 selection experiment, offering a possible explanation for the differing responses to selection by two global change drivers in the same *Trichodesmium* strain. In other words, longer selection time may produce true thermal adaptation in temperature-selected *Trichodesmium* cell lines. Alternatively, CO_2 enrichment and warming may very well elicit distinct coping responses in *Trichodesmium*.

Unlike *Trichodesmium*, true thermal adaptation is likely to have occurred in *Crocosphaera* WH0005 during long-term selection, indicated by the stable phenotypic changes in TPC parameters in 32°C and 22°C-selected cell lines. Although *Crocosphaera* cell lines selected at three temperatures demonstrated a similar acclimation to the temperatures in the middle, optimum region of their TPC (24°C–30°C), 32°C-selected and 22°C-selected cell lines responded quite differently to cold and warm temperature extremes in both TPC determinations and switch experiments. Another key piece of physiological evidence suggesting true adaptation is that only 32°C-selected *Crocosphaera* could survive and fix nitrogen at the most intense warming temperature of 34°C, while cell lines selected at lower temperatures died quickly there. In this way, the thermal niche width of these 32°C-selected *Crocosphaera* cell lines for survival was actually broadened by at least 2°C. This pattern seems to support the 'hotter is broader' model, which suggests that adaptation to elevated temperatures can generate a broader growth reaction norm and wider temperature range (Knies *et al*., 2009; Jin and Agustí, 2018).

A similar positive role of selection history was seen in the responses of the CO_2-selected picoplankter *Ostreococcus* to a new CO_2 concentration (Schaum *et al*., 2016). *Ostreococcus* lineages selected at high CO_2 levels (∼1000 µatm) had higher growth rates than lineages selected at ambient CO_2 levels (∼400 µatm) when both were moved to a further elevated CO_2 concentration (∼2000 µatm). Thus, prior exposure to

Table 2. Nonsynonymous mutations detected in the genome of temperature-selected *Crocosphaera* cell lines.

Contig	Position[a]	KEGG identifier	Temperature of frequency change (°C)	Cutoff	Variation type	Protein production encoded	Ka/Ks ratio
130	938, 943, 952	K00986	32	FDR 1.71E-05, 1.43E-06, 8.20E-04	SNV	RNA-directed DNA polymerase	0.16 ± 0.03
19	69328	K07496	32	FDR 1.45E-04	SNV	Putative transposase	0.05 ± 0.01
61	16663	K13069	28	FDR 1.23E-04	SNV	Diguanylate cyclase	0.34 ± 0.02
26	44451	K21348	32	FDR 4.07E-05	Indel	Calcium-binding and coiled-coil domain-containing protein 2	N/A
64	4687	K18640	32	FDR 1.47E-03	SNV	Plasmid segregation protein ParM	0.30 ± 0.01
28	3523	K16150	22	FDR 1.07E-02	Indel	Glycogen synthase	N/A
17	44132	K01369	32	Raw *p*-value 6.65E-03	Indel	Legumain-like sequence, asparaginyl endopeptidase	N/A
102	2119	N/A	32	FDR 2.46E-04	SNV	Not annotated	0.20 ± 0.07
4	136478	N/A	32	FDR 3.14E-77	Indel	Not annotated	N/A
6	114320	N/A	32	FDR 3.14E-77	Indel	Not annotated	N/A
7	114729	N/A	22	FDR 7.47E-04	Indel	Not annotated	N/A

Shown are results of cultures selected at 22°C, 28°C, or 32°C at different cutoffs by SNV and Indel analysis and annotation. The *p*-values were calculated by one-way ANOVA and adjusted using the FDR method. The contig (column 1) and position information (column 2) were defined according to the *Crocosphaera watsonii* WH0005 reference genome assembled in this study. KEGG identifier (column 3) and protein production encoded by genes affected (column 7) are based on the information from the KEGG Orthology Database (Kanehisa *et al*., 2016). The ratios of non-synonymous to synonymous substitutions (Ka/Ks) for mutated genes are listed in column 8.
N/A: not available.
[a]Gene positions are located on the positive (+) strand.

projected future conditions such as mild warming or CO_2 enrichment could help marine microbes maintain fitness under more intense subsequent environmental changes.

The results of genomic analysis also provide robust evidence supporting the potential genetic adaptation of *Crocosphaera* cell lines with a long-term selection history. The SNV PCA results indicate substantial genetic divergence between 32°C-selected *Crocosphaera* cell lines and the others (Fig. 3A), although this separation is less clear based on the Indel variants (Fig. 3B). This could be biased by the limited number of Indel variants detected (740) relative to SNVs (10,558). Another possible interpretation for the closeness between 32°C-selected cell lines and one 28°C-selected replicate (labelled as '28-3') (Fig. 3B) is that the genomic differences between the replicate 28-3 and three 32°C-selected replicates are still relatively small, which is consistent with their similar phenotypic performance. For instance, the growth rate of the replicate 28-3 in the TPC determination and its nitrogen and carbon fixation rates in the switch experiment were closer to those of the 32°C-selected cell lines, instead of the other two 28°C-selected replicates.

Potential functions of the rapidly evolving genes in the Crocosphaera genome

The most rapidly evolving genes detected in the genome of thermally selected *Crocosphaera* in our study are associated with numerous fundamental biological functions. Mutations in these genes may help improve fitness of *Crocosphaera* under thermal selection and contribute to the observed phenotypic changes. For instance, genes that encode RNA-directed DNA polymerase (also known as reverse transcriptase, RT) and plasmid segregation protein ParM play an important role in DNA replication (Lennarz and Lane, 2013) and plasmid partitioning processes (Møller-Jensen *et al*., 2002) during cell division.

Cyanobacterial RTs include three substantially characterized classes [retrons, group II introns and diversity-generating retroelements (DGRs)] and many uncharacterized types (Simon and Zimmerly, 2008; Toro and Nisa-Martínez, 2014). Retrons synthesize multicopy single-stranded DNAs in cells (Lampson *et al*., 1989; González-Delgado *et al*., 2021) and play a role in antiphage defence (Millman *et al*., 2020). Bacterial group II introns exhibit autonomous mobility and function in RNA splicing (Stamos *et al*., 2017). DGRs, which exist in the *Trichodesmium* and *Crocosphaera* genomes, can drive mutations that optimize signal response and regulatory networks and promote adaptation to stressful conditions (Vallota-Eastman *et al*., 2020).

As self-replicating, extra-chromosomal genetic materials, plasmids can also facilitate the adaptation of bacteria to environmental stress (Leplae *et al*., 2006; Heuer and Smalla, 2012). To ensure equal inheritance of plasmids by daughter cells during cell division, bacterial

plasmids encode efficient plasmid partitioning machineries, such as the ParMRC system (Gayathri and Harne, 2017). These actin-like proteins are responsible for segregating large DNA plasmids by forming filaments and pushing two sister plasmids towards two opposing poles, together with ParRC complexes (Salje *et al*., 2010; Gayathri *et al*., 2012). In this way, ParMs enhance the stability of plasmid transmission and inheritance during cell division and have been reported to diverge under additional positive selection (Gunning *et al*., 2015).

In our study, the mutation in the RT genes only occurred in the three 32°C-selected *Crocosphaera* replicates, and the mutation frequency of the ParM coding genes in 32°C-selected *Crocosphaera* showed a significant difference compared to 22°C- and 28°C-selected cell lines. The enhanced growth and physiological rates in 32°C-selected *Crocosphaera* coincided with the potential roles of the mutated genes encoding for RTs and ParMs in helping bacteria cope with environmental challenges.

Furthermore, mutations in transposase genes were correlated with the phenotypic changes in the 32°C-selected *Crocosphaera* replicates (Table 2), implying a potential role of transposable elements in *Crocosphaera* responses to environmental changes, as in other organisms (Casacuberta and González, 2013). The genome of this cyanobacterium has large numbers of mobile genetic elements and hundreds of transposase genes (Bench *et al*., 2013). Most transposase genes in *Crocosphaera* genomes fall into the insertion sequence (IS) families, especially IS200/605 (Hewson *et al*., 2009; Bench *et al*., 2013). Some transposases interrupt reading frames of genes (e.g. RT genes discussed above) and genomic rearrangements have been reported between different *Crocosphaera* strains and the alignment of environmental bacterial artificial chromosome-cloned sequences to the draft genome of *Crocosphaera* WH8501. Such genetic mobility likely contributes to the maintenance of phenotypic and genetic diversity in *Crocosphaera* populations (Zehr *et al*., 2007; Bench *et al*., 2011, 2013). Moreover, positive selection has also been observed in the transposase genes of *Crocosphaera* (Mes and Doeleman, 2006), indicating the importance and sensitivity of these mobile elements in evolutionary adaptation processes.

The rest of the rapidly evolving genes are also involved in universal cellular functions, which may also contribute to adaptation. The gene encoding glycogen synthase (*glg*A) controls bulk synthesis of glycogen (Wilson *et al*., 2010), the major carbon and energy storage compound in cyanobacteria (Xu *et al*., 2013). The mutation in the gene *glg*A paralleled a depressed growth status in 22°C-selected *Crocosphaera* cells, implying its potential contribution to adaptation to the possibly changed energy balancing at the sub-optimal temperature (22°C). The genes encoding diguanylate cyclase play a vital role in second messenger signalling and biofilm formation (Boehm *et al*., 2009; Pfiffer *et al*., 2019). Calcium-binding and coiled-coil domain-containing protein 2 (also known as NDP52) is a well-known autophagy receptor which can mediate intracellular bacterial degradation and modulate antiviral responses (Verlhac *et al*., 2015a, 2015b; Mohamud *et al*., 2019; Fan *et al*., 2020). Legumain-like gene products are mainly responsible for hydrolysis of proteins (Rawlings and Salvesen, 2013). More detailed descriptions of the biological functions of these mutated genes are in the Supplementary Materials.

Considering the ecological significance of *Crocosphaera* as an N_2-fixer, genes encoding nitrogen fixation functions are always of great interest. In this study, however, no mutations were detected in nitrogen fixation-related genes (e.g. *nif* genes, Table S2). For mutations observed in genes related to other crucial functions, such as energy generation, ATP transport and nutrient assimilation (Table S3), the difference in variant ratios among three temperature-selected treatments did not pass the false discovery rate (FDR) cut-off of 0.05, and thus are not discussed.

The link between physiological plasticity and evolutionary adaptation

Previous studies show that adaptation can protect phytoplankton from extinction in new, stressful environments (Collins *et al*., 2014), but plasticity can also move the population closer to a new phenotypic optimum (Ghalambor *et al*., 2007). Not surprisingly, many studies have found that diatoms (Anning *et al*., 2001), cyanobacteria (Stomp *et al*., 2008), green algae (Kremer *et al*., 2018) and dinoflagellates (Jerney *et al*., 2019) can respond to changing environmental factors (temperature, light and salinity) using inherent plasticity instead of evolutionary adaptation. In recent experimental evolution studies, adaptive responses to CO_2 or temperature changes have also been reported in phytoplankton taxa including *Trichodesmium* (Hutchins *et al*., 2015), diatoms (Jin and Agustí, 2018), green algae (Padfield *et al*., 2016) and coccolithophores (Schlüter *et al*., 2014).

Although it remains unclear why particular microbes employ distinct strategies (acclimation, adaptation, or both) in response to environmental changes, the relationship between phenotypic plasticity and evolutionary adaptation has been extensively debated (Price *et al*., 2003; Ghalambor *et al*., 2007; Chevin *et al*., 2010; Collins, 2011). On the one hand, plasticity may facilitate evolution by maintaining a large population size, with

greater genetic and mutational variance (Lande, 2009; Draghi and Whitlock, 2012; Collins *et al*., 2014). On the other hand, if acclimation alone increases fitness without a large trade-off or cost, plastic genotypes may be shielded from selection pressure. Thus, further evolutionary adaptation may not occur (Price *et al*., 2003; Ghalambor *et al*., 2007).

The latter situation may be why no apparent adaptation occurred for our *Trichodesmium* after 2 years of thermal selection. The considerable capacity of *Trichodesmium* to quickly acclimate to new temperatures may alleviate thermal selection, thus retarding evolution. In contrast, *Crocosphaera* responded to strong selective pressure with irreversible phenotypic changes and apparent genotypic shifts, especially at the challenging temperature of 32°C. This demonstrates its ability to adapt more rapidly, but also implies a potential lack of thermal plasticity compared to *Trichodesmium*. However, it is unknown whether adaptation was halted for *Trichodesmium* or might occur later with longer selection time. Despite this open question, our study provides insights into modes of correlation between plasticity and evolution, including positive, negative and timescale-dependent interactions.

Genetic backgrounds and lifestyles of the two diazotrophs

The genetic backgrounds and lifestyles of these two diazotrophs may provide clues about how to interpret their contrasting plastic and evolutionary responses under long-term thermal selection. Both *Trichodesmium* and *Crocosphaera* genomes contain widespread, highly expressed non-coding spacers with unclear functions; the coding percentage in *Trichodesmium* IMS101 is ∼60%, while in *Crocosphaera* it is ∼75%–80% (Walworth *et al*., 2015). Recently, epigenetic modifications underlying phenotypic adaptation have also been described in *Trichodesmium* IMS101 (Walworth *et al*., 2017, 2020). More work is needed to examine whether genomic and epigenetic differences might account for the differing thermal responses of the two diazotrophs.

The different lifestyles of *Trichodesmium* and *Crocosphaera* may also be involved in their divergent responses. For instance, interactions between colony-forming *Trichodesmium* and a diverse bacterial microbiome in both natural populations and laboratory cultures are thought to mediate numerous metabolic processes (Frischkorn *et al*., 2017; Lee *et al*., 2017; Basu *et al*., 2019). The responses of *Trichodesmium* to environmental change may be modulated to some extent by the other members of this complex epibiotic consortium, and thus could differ from those of largely free-living unicellular *Crocosphaera*.

Future marine nitrogen fixation and species competition between the two diazotrophs

Using differing but successful strategies, both diazotrophs thrive in the contemporary oligotrophic central gyres and contribute substantially to new nitrogen inputs in the tropical and subtropical ocean (Bonnet *et al*., 2009; Moisander *et al*., 2010; Zehr and Capone, 2020). How future warming will influence the survival and competitive success of these two crucial diazotrophs is hence of paramount interest. In our study, changes in growth, nitrogen- and carbon-fixation rates with temperature had generally parallel trends for the two diazotrophs. At a fairly moderate level of warming (e.g. 32°C), rates of the two diazotrophs were still relatively equally matched, but all decreased compared to optimal temperatures, suggesting the possibility of reduced nitrogen fixation and carbon sequestration in the future ocean.

However, extreme warming beyond the thermal range of these two diazotrophs may change their relative competitive fitness. When temperature further increased to 34°C, nitrogen fixation of *Trichodesmium* and surviving *Crocosphaera* first declined substantially, to one third or less of amounts at optimal temperatures. All our *Trichodesmium* cell lines, however, acclimated rapidly to 34°C, while only 32°C-selected *Crocosphaera* cell lines could survive and fix nitrogen at this temperature. This finding indicates that non-adapted *Crocosphaera* may fail to survive or be outcompeted by *Trichodesmium* during more frequent and intense marine heat waves in the future ocean (Scannell *et al*., 2016; Frölicher and Laufkötter, 2018; Oliver *et al*., 2018; Smale *et al*., 2019). Nevertheless, if given the chance to adapt to the gradual temperature increases which constitute the mean rate of climate change, *Crocosphaera* may be able to expand their upper thermal limit to 34°C. This differs from predictions based on short-term acclimated experiments that lasted weeks (Fu *et al*., 2014). Overall, our study suggests that thermal acclimation of *Trichodesmium* and adaptation of *Crocosphaera* may together help to sustain the biodiversity of diazotrophs, and thus help to mitigate declines in new nitrogen supplies to the warming tropical and subtropical oceans of the near future.

Experimental procedures

Cultures and experimental evolution

Trichodesmium erythraeum IMS101 and *Crocosphaera watsonii* WH0005 were respectively isolated from the Atlantic Ocean (Prufert-Bebout *et al*., 1993; Chen *et al*., 1996) and from the North Pacific Ocean (Webb *et al*., 2009). All cultures were grown in 250 ml sterile polystyrene flasks using autoclaved artificial seawater,

with replete phosphorous, iron, trace metals and vitamins added according to the modified AQUIL recipe (Price *et al*., 1989; Sunda and Morel, 2005). An identical 12 h dark:12 h light cycle at 150 μmol photons m^{-2} s^{-1} was provided with cool white fluorescent bulbs for all cultures.

Ancestral cell lines of both strains maintained long-term at 26°C were acclimated to 28°C for 3 months before transferring to the three selection temperatures, which covered the optimal (28°C), suboptimal (22°C) and supra-optimal (32°C) temperatures for both strains (Fu *et al*., 2014). Six replicate cell lines of *Trichodesmium* IMS101 and three of *Crocosphaera* WH0005 were maintained until the end of the ~2-year experiment using semi-continuous incubation methods as described in Supplementary Materials and Qu *et al*. (2018).

Temperature performance curves determination

After ~2 years' selection at the three temperatures, TPCs of selected *Trichodesmium* and *Crocosphaera* cell lines were determined from 16°C to 36°C at intervals of 2°C. Traits such as optimal growth temperature, maximal growth rate and thermal niche (the full temperature range for survival) of selected cell lines were investigated under experimental conditions identical to those in the long-term incubations.

For the 16 day short-term incubations, cultures were diluted every 4 days. The initial biomass of each replicate for each cycle was carefully controlled at ~80–100 μmol L^{-1} of POC to make sure the cells remained in exponential growth stage throughout the entire dilution cycle. Specific growth rates were used as a proxy of relative fitness (Hutchins *et al*., 2015). TPC parameters (i.e. predicted maximal growth rate, optimum growth temperature and thermal niche width) were calculated using the thermal response model of Norberg (2004) and Thomas *et al*. (2012). Detailed statistical methods and model formulas can be found in the supplement materials of Thomas *et al*. (2012).

Nitrogen and carbon fixation rates were measured at three selection temperatures 22°C, 28°C and 32°C, as well as at an extension temperature of 34°C at the warm end. Since the temperature range of *Trichodesmium* was found to be wider than that of *Crocosphaera*, physiological rates were also determined at 20°C for *Trichodesmium* cell lines. Detailed descriptions of all these measurements are presented in the *Physiological measurements* section of Supplementary Materials.

Switch experiments

After TPC determination experiments showed potential adaptive responses in long-term temperature-selected *Crocosphaera* cells, reciprocal transfer or 'switch' experiments were conducted with these cell lines among the three selection temperatures (Hutchins *et al*., 2015), as shown in Fig. S2. The experimental conditions and dilution frequencies in the switch experiments were identical to those used in the long-term incubations. Following the switches, physiological sampling of short-term switch and long-term selected cell lines at three temperatures took place after 1 month to document the potential changing phenotypes of each set of cell lines at the other two assay temperatures. In the switch experiment, the parameters measured were identical with those examined in the TPC determination experiments.

Statistics of physiological measurements

R 3.3.1 software was used for the statistical analysis. One-way ANOVA was used to analyze the physiological differences among long-term cell lines, a series of temperature treatments of TPC determination experiments for both diazotrophs, and the switch experiments with *Crocosphaera*. When significance was found, the Tukey's Honest Significant Difference (hereafter called TukeyHSD) test was applied for multiple comparisons between treatments. For data that failed the Shapiro–Wilk test or Fligner-Killeen test for normality and homogeneity of variances, the Kruskal–Wallis test followed by the Dunn test as a *post hoc* was used to compare among multiple groups instead. All significance testing was done at the adjusted p-value <0.05 level and p-values 0.01 and 0.001 were also used for reference.

Genomic analysis of Crocosphaera WH0005

Crocosphaera DNA sample collection and sequencing. Since the growth and physiology of long-term selected *Crocosphaera* cell lines showed potential thermal adaptation after 2 years' selection, they were sampled by centrifugation, followed by DNA extractions and genome sequencing (details in Supplementary Materials). These thermally selected genomes were then compared to the genomes of two ancestral *Crocosphaera* cell lines collected at the initial time point. For sequencing and subsequent analyses, the two initial samples were combined into one due to the limited DNA quantity.

Resequencing the Crocosphaera WH0005 reference genome. Prior to this study, the available genome for strain WH0005 (GCA_001050835.1, downloaded from NCBI November 2019) was split between 1266 contigs (N50 = 9619). In order to improve our ability to detect sequence variation as a result of long-term thermal selection, we generated additional sequencing data to reduce the gaps in the sequence. For this purpose, both paired-end 2 × 150 short reads (HiSeq, Illumina, San Diego, CA, USA) and long reads (Sequel II, Pacific Biosciences, CA, USA) were generated at Novogene from DNA extracted from cultures maintained under experimental conditions at 28°C. The

Crocosphaera WH0005 reference in this study was assembled with both short and long reads, as described in the Supplementary Materials.

The final reference genome assembled here achieved good completeness (97.18%) with only 126 contigs, ~1/10 of the number of contigs in the previous reference of this strain (Bench *et al*., 2013). The contamination estimation of our assembly is 2.81%. Coding regions were identified using Prodigal (v.2.6.3) (Hyatt *et al*., 2010) and annotated with kofamScan (v.1.1.0) (Aramaki *et al*., 2020).

Variant calling and principal component analysis. For all long-term selected *Crocosphaera* samples, sequencing reads were aligned to the *Crocosphaera* WH0005 reference genome assembled above after quality check and adapter trimming (Supplementary Materials). One replicate of the 22°C-selected culture was excluded from the following analyses due to inadequate sequencing depth. Samtools mpileup and BCFtools (v.0.1.19) were used to call genomic variants, including SNVs and insertion–deletion mutations (Indels). Low-quality variants were eliminated with bcftools filter utility, and only the SNVs and Indels with coverage >10 were included in the analysis. For each site, the variant ratio was calculated by dividing the counts of variants by the counts of all reads at that site. PCA used package factoextra (Kassambara and Mundt, 2017) and ade4 (Bougeard and Dray, 2018) in R to detect the classification of nine genome samples based on the variant ratios of SNVs and Indels respectively.

Detection of nonsynonymous mutations and gene functions. One-way ANOVA analysis was conducted at each mutation position to detect significant differences among three temperature treatments. An FDR corrected p-value <0.05 was considered significant. Nonsynonymous SNVs were detected with SNPGenie (Nelson *et al*., 2015) and nonsynonymous Indels manually with the ExPASy tool (https://web.expasy.org/translate/). For nonsynonymous mutations displaying a significant difference among three selection treatments, the potential functions of genes affected by the temperature treatments were identified based on the annotation result and literature search. The ratios of nonsynonymous to synonymous substitutions (Ka/Ks, as known as d_N/d_S) were calculated with SNPGenie for genes with nonsynonymous SNVs of statistical significance.

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Data Availability

Sequence data (raw Illumina reads) of *Crocosphaera watsonii* WH0005 have been deposited on the NCBI Sequence Read Archive under the Bioproject ID PRJNA714247. SRA accession numbers and associated metadata can be found in Table S1. The genome assembly and annotation data and codes are publicly available at https://figshare.com/account/home#/projects/99914.

References

Anning, T., Harris, G., and Geider, R. (2001) Thermal acclimation in the marine diatom *Chaetoceros calcitrans* (Bacillariophyceae). *Eur J Phycol* **36**: 233–241. https://doi.org/10.1080/0967026011000173538 8.

Aramaki, T., Blanc-Mathieu, R., Endo, H., Ohkubo, K., Kanehisa, M., Goto, S., and Ogata, H. (2020) KofamKOALA: KEGG Ortholog assignment based on profile HMM and adaptive score threshold. *Bioinformatics* **36**: 2251–2252. https://doi.org/10.1093/bioinformatics/btz859.

Basu, S., Gledhill, M., de Beer, D., Matondkar, S.P., and Shaked, Y. (2019) Colonies of marine cyanobacteria *Trichodesmium* interact with associated bacteria to acquire iron from dust. *Commun Biol* **2**: 1–8. https://doi.org/10.1038/s42003-019-0534-z.

Bench, S.R., Heller, P., Frank, I., Arciniega, M., Shilova, I.N., and Zehr, J.P. (2013) Whole genome comparison of six *Crocosphaera watsonii* strains with differing phenotypes. *J Phycol* **49**: 786–801. https://doi.org/10.1111/jpy.12090.

Bench, S.R., Ilikchyan, I.N., Tripp, H.J., and Zehr, J.P. (2011) Two strains of *Crocosphaera watsonii* with highly conserved genomes are distinguished by strain-specific features. *Front Microbiol* **2**: 261. https://doi.org/10.3389/fmicb.2011.00261.

Bergman, B., Sandh, G., Lin, S., Larsson, J., and Carpenter, E.J. (2013) *Trichodesmium* – a widespread marine cyanobacterium with unusual nitrogen fixation properties. *FEMS Microbiol Rev* **37**: 286–302. https://doi.org/10.1111/j.1574-6976.2012.00352.x.

Berthelot, H., Bonnet, S., Grosso, O., Cornet, V., and Barani, A. (2016) Transfer of diazotroph-derived nitrogen towards non-diazotrophic planktonic communities: a comparative study between *Trichodesmium erythraeum*, *Crocosphaera watsonii* and *Cyanothece* sp. *Biogeosciences* **13**: 4005–4021. https://doi.org/10.5194/bg-13-4005-2016.

Boatman, T.G., Lawson, T., Geider, R.J., and Cockshutt, A. M. (2017) A key marine diazotroph in a changing ocean: the interacting effects of temperature, CO_2 and light on the growth of *Trichodesmium erythraeum* IMS101. *PLoS One* **12**: e0168796. https://doi.org/10.1371/journal.pone.0168796.

Boehm, A., Steiner, S., Zaehringer, F., Casanova, A., Hamburger, F., Ritz, D., *et al*. (2009) Second messenger signalling governs *Escherichia coli b*iofilm induction upon ribosomal stress. *Mol Microbiol* **72**: 1500–1516. https://doi.org/10.1111/j.1365-2958.2009.06739.x.

Bonnet, S., Biegala, I.C., Dutrieux, P., Slemons, L.O., and Capone, D.G. (2009) Nitrogen fixation in the western

equatorial Pacific: rates, diazotrophic cyanobacterial size class distribution, and biogeochemical significance. *Global Biogeochem Cy* **23**: GB3012. https://doi.org/10.1029/2008GB003439.

Bopp, L., Resplandy, L., Orr, J., Doney, S., Dunne, J., Gehlen, M., *et al*. (2013) Multiple stressors of ocean ecosystems in the 21st century: projections with CMIP5 models. *Biogeosciences* **10**: 6225–6245. https://doi.org/10.5194/bg-10-6225-2013.

Bougeard, S., and Dray, S. (2018) Supervised multiblock analysis in R with the ade4 package. *J Stat Softw* **86**: 1–17. https://doi.org/10.18637/jss.v086.i01.

Boyd, P.W., Cornwall, C.E., Davison, A., Doney, S.C., Fourquez, M., Hurd, C.L., *et al*. (2016) Biological responses to environmental heterogeneity under future ocean conditions. *Glob Chang Biol* **22**: 2633–2650. https://doi.org/10.1111/gcb.13287.

Breitbarth, E., Oschlies, A., and LaRoche, J. (2007) Physiological constraints on the global distribution of *Trichodesmium* – effect of temperature on diazotrophy. *Biogeosciences* **4**: 53–61. https://doi.org/10.5194/bg-4-53-2007.

Capone, D.G., Zehr, J.P., Paerl, H.W., Bergman, B., and Carpenter, E.J. (1997) *Trichodesmium*, a globally significant marine cyanobacterium. *Science* **276**: 1221–1229. https://doi.org/10.1126/science.276.5316.1221.

Casacuberta, E., and González, J. (2013) The impact of transposable elements in environmental adaptation. *Mol Ecol* **22**: 1503–1517. https://doi.org/10.1111/mec.12170.

Chen, Y.B., Zehr, J.P., and Mellon, M. (1996) Growth and nitrogen fixation of the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium s*p. IMS 101 in defined media: evidence for a circadian rhythm. *J Phycol* **32**: 916–923. https://doi.org/10.1111/j.0022-3646.1996.00916.x.

Chevin, L.-M., Lande, R., and Mace, G.M. (2010) Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. *PLoS Biol* **8**: e1000357. https://doi.org/10.1371/journal.pbio.1000357.

Collins, M., An, S.-I., Cai, W., Ganachaud, A., Guilyardi, E., Jin, F.-F., *et al*. (2010) The impact of global warming on the tropical Pacific Ocean and El Niño. *Nat Geosci* **3**: 391. https://doi.org/10.1038/ngeo868.

Collins, S. (2011) Many possible worlds: expanding the ecological scenarios in experimental evolution. *Evol Biol* **38**: 3–14. https://doi.org/10.1007/s11692-010-9106-3.

Collins, S., Rost, B., and Rynearson, T.A. (2014) Evolutionary potential of marine phytoplankton under ocean acidification. *Evol Appl* **7**: 140–155. https://doi.org/10.1111/eva.12120.

Draghi, J., and Whitlock, M.C. (2012) Phenotypic plasticity facilitates mutational variance, genetic variance, and evolvability along the major axis of environmental variation. *Evolution* **66**: 2891–2902. https://doi.org/10.1111/j.1558-5646.2012.01649.x.

Dunstan, P.K., Foster, S.D., King, E., Risbey, J., O'Kane, T. J., Monselesan, D., *et al*. (2018) Global patterns of change and variation in sea surface temperature and chlorophyll *a*. *Sci Rep* **8**: 1–9. https://doi.org/10.1038/s41598-018-33057-y.

Fan, S., Wu, K., Luo, C., Li, X., Zhao, M., Song, D., *et al*. (2020) Dual NDP52 function in persistent CSFV infection. *Front Microbiol* **10**: 2962. https://doi.org/10.3389/fmicb.2019.02962.

Frischkorn, K.R., Rouco, M., Van Mooy, B.A., and Dyhrman, S.T. (2017) Epibionts dominate metabolic functional potential of *Trichodesmium* colonies from the oligotrophic ocean. *ISME J* **11**: 2090–2101. https://doi.org/10.1038/ismej.2017.74.

Frölicher, T., and Laufkötter, C. (2018) Emerging risks from marine heat waves. *Nat Commun* **9**: 650–650. https://doi.org/10.1038/s41467-018-03163-6.

Fu, F.X., Mulholland, M.R., Garcia, N.S., Beck, A., Bernhardt, P.W., Warner, M.E., *et al*. (2008) Interactions between changing pCO_2, N_2 fixation, and Fe limitation in the marine unicellular cyanobacterium *Crocosphaera*. *Limnol Oceanogr* **53**: 2472–2484. https://doi.org/10.4319/lo.2008.53.6.2472.

Fu, F.-X., Yu, E., Garcia, N., Gale, J., Luo, Y., Webb, E., and Hutchins, D. (2014) Differing responses of marine N_2 fixers to warming and consequences for future diazotroph community structure. *Aquat Microb Ecol* **72**: 33–46. https://doi.org/10.3354/ame01683.

Garcia, N.S., Fu, F., Breene, C.L., Yu, E.K., Bernhardt, P.W., Mulholland, M.R., and Hutchins, D.A. (2013) Combined effects of CO_2 and light on large and small isolates of the unicellular N_2-fixing cyanobacterium *Crocosphaera watsonii* from the western tropical Atlantic Ocean. *Eur J Phycol* **48**: 128–139. https://doi.org/10.1594/PANGAEA.835431.

Gayathri, P., Fujii, T., Møller-Jensen, J., Van Den Ent, F., Namba, K., and Löwe, J. (2012) A bipolar spindle of antiparallel ParM filaments drives bacterial plasmid segregation. *Science* **338**: 1334–1337. https://doi.org/10.1126/science.1229091.

Gayathri, P., and Harne, S. (2017) Structure and dynamics of actin-like cytomotive filaments in plasmid segregation. *Prokaryotic Cytoskeletons* **84**: 299–321. https://doi.org/10.1007/978-3-319-53047-5_10.

Ghalambor, C.K., McKay, J.K., Carroll, S.P., and Reznick, D. N. (2007) Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Funct Ecol* **21**: 394–407. https://doi.org/10.1111/j.1365-2435.2007.01283.x.

González-Delgado, A., Mestre, M.R., Martínez-Abarca, F., and Toro, N. (2021) Prokaryotic reverse transcriptases: from retroelements to specialized defense systems. *FEMS Microbiol Rev* **fuab025**: 1–19. https://doi.org/10.1093/femsre/fuab025.

Gunning, P.W., Ghoshdastider, U., Whitaker, S., Popp, D., and Robinson, R.C. (2015) The evolution of compositionally and functionally distinct actin filaments. *J Cell Sci* **128**: 2009–2019. https://doi.org/10.1242/jcs.165563.

Heuer, H., and Smalla, K. (2012) Plasmids foster diversification and adaptation of bacterial populations in soil. *FEMS Microbiol Rev* **36**: 1083–1104. https://doi.org/10.1111/j.1574-6976.2012.00337.x.

Hewson, I., Poretsky, R.S., Beinart, R.A., White, A.E., Shi, T., Bench, S.R., *et al*. (2009) *In situ* transcriptomic analysis of the globally important keystone N_2-fixing taxon

Crocosphaera watsonii. *ISME J* **3**: 618–631. https://doi.org/10.1038/ismej.2009.8.

Hood, R.R., Coles, V.J., and Capone, D.G. (2004) Modeling the distribution of *Trichodesmium* and nitrogen fixation in the Atlantic Ocean. *J Geophys Res Oceans* **109**: 1–25. https://doi.org/10.1029/2002JC001754.

Hurrell, J., Hack, J., Shea, D., Caron, J., and Rosinski, J. (2008) A new sea surface temperature and sea ice boundary dataset for the community atmosphere model. *J Climate* **21**: 5145–5153. https://doi.org/10.1175/2008JCLI2292.1.

Hutchins, D.A., Fu, F.-X., Webb, E.A., Walworth, N.G., and Tagliabue, A. (2013) Taxon-specific response of marine nitrogen fixers to elevated carbon dioxide concentrations. *Nat Geosci* **6**: 790–795. https://doi.org/10.1038/ngeo1858.

Hutchins, D.A., Walworth, N., Webb, E., Saito, M., Moran, D., McIlvin, M., *et al*. (2015) Irreversibly increased nitrogen fixation in *Trichodesmium* experimentally adapted to elevated carbon dioxide. *Nat Commun* **6**: 8155. https://doi.org/10.1038/ncomms9155.

Hyatt, D., Chen, G.L., Locascio, P.F., Land, M.L., Larimer, F. W., and Hauser, L.J. (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinform* **11**: 119. https://doi.org/10.1186/1471-2105-11-119.

IPCC. (2012) *Managing the Risks of Extreme Events and Disasters to Advance Climate Change Adaptation: Special Report of the Intergovernmental Panel on Climate Change*. Cambridge, UK: Cambridge University Press. https://doi.org/10.5860/choice.50-4454.

IPCC. (2013) *Summary for Policymakers. In Climate Change 2013 – The Physical Science Basis: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, UK: Cambridge University Press. https://doi.org/10.1017/cbo9781107415324.004.

Jerney, J., Suikkanen, S., Lindehoff, E., and Kremp, A. (2019) Future temperature and salinity do not exert selection pressure on cyst germination of a toxic phytoplankton species. *Ecol Evol* **9**: 4443–4451. https://doi.org/10.1002/ece3.5009.

Jin, P., and Agustí, S. (2018) Fast adaptation of tropical diatoms to increased warming with trade-offs. *Sci Rep* **8**: 1–10. https://doi.org/10.1038/s41598-018-36091-y.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M. (2016) KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res* **44**: D457–D462. https://doi.org/10.1093/nar/gkv1070.

Kassambara, A., and Mundt, F. (2017) Package 'factoextra': extract and visualize the results of multivariate data analyses. *R Package Version* **1**: 337–354.

Keenlyside, N.S., and Latif, M. (2007) Understanding equatorial Atlantic interannual variability. *J Climate* **20**: 131–142. https://doi.org/10.1175/JCLI3992.1.

Knapp, A.N., Dekaezemacker, J., Bonnet, S., Sohm, J.A., and Capone, D.G. (2012) Sensitivity of *Trichodesmium erythraeum* and *Crocosphaera watsonii* abundance and N_2 fixation rates to varying NO_3^- and PO_4^{3-} concentrations in batch cultures. *Aquat Microb Ecol* **66**: 223–236. https://doi.org/10.3354/ame01577.

Knies, J.L., Kingsolver, J.G., and Burch, C.L. (2009) Hotter is better and broader: thermal sensitivity of fitness in a population of bacteriophages. *Am Nat* **173**: 419–430. https://doi.org/10.1086/597224.

Kremer, C.T., Fey, S.B., Arellano, A.A., and Vasseur, D.A. (2018) Gradual plasticity alters population dynamics in variable environments: thermal acclimation in the green alga. *Proc R Soc B* **285**: 20171942. https://doi.org/10.1098/rspb.2017.1942.

Lampson, B.C., Sun, J., Hsu, M.Y., Vallejo-Ramirez, J., Inouye, S., and Inouye, M. (1989) Reverse transcriptase in a clinical strain of *Escherichia coli*: production of branched RNA-linked msDNA. *Science* **243**: 1033–1038. https://doi.org/10.1126/science.2466332.

Lande, R. (2009) Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. *J Evol Biol* **22**: 1435–1446. https://doi.org/10.1111/j.1420-9101.2009.01754.x.

Lee, M.D., Walworth, N.G., Mcparland, E.L., Fu, F.-X., Mincer, T.J., Levine, N.M., *et al*. (2017) The *Trichodesmium* consortium: conserved heterotrophic co-occurrence and genomic signatures of potential interactions. *ISME J* **11**: 1813–1824. https://doi.org/10.1038/ismej.2017.49.

Lennarz, W.J., and Lane, M.D. (2013) *Encyclopedia of Biological Chemistry*. Netherlands: Elsevier Science.

Leplae, R., Lima-Mendez, G., and Toussaint, A. (2006) A first global analysis of plasmid encoded proteins in the ACLAME database. *FEMS Microbiol Rev* **30**: 980–994. https://doi.org/10.1111/j.1574-6976.2006.00044.x.

Li, X., Fonseca-Batista, D., Roevros, N., Dehairs, F., and Chou, L. (2018) Environmental and nutrient controls of marine nitrogen fixation. *Prog Oceanogr* **167**: 125–137. https://doi.org/10.1016/j.pocean.2018.08.001.

Liu, Z., Vavrus, S., Wen, N., and Zhong, Y. (2005) Rethinking tropical ocean response to global warming: the enhanced equatorial warming. *J Climate* **18**: 4684–4700. https://doi.org/10.1175/JCLI3579.1.

Mes, T.H.M., and Doeleman, M. (2006) Positive selection on transposase genes of insertion sequences in the *Crocosphaera watsonii* genome. *J Bacteriol* **188**: 7176. https://doi.org/10.1128/JB.01021-06.

Millman, A., Bernheim, A., Stokar-Avihail, A., Fedorenko, T., Voichek, M., Leavitt, A., *et al*. (2020) Bacterial retrons function in anti-phage defense. *Cell* **183**: 1551–1561. https://doi.org/10.1016/j.cell.2020.09.065.

Mohamud, Y., Qu, J., Xue, Y.C., Liu, H., Deng, H., and Luo, H. (2019) CALCOCO2/NDP52 and SQSTM1/p62 differentially regulate coxsackievirus B3 propagation. *Cell Death Differ* **26**: 1062–1076. https://doi.org/10.1038/s41418-018-0185-5.

Moisander, P.H., Beinart, R.A., Hewson, I., White, A.E., Johnson, K.S., Carlson, C.A., *et al*. (2010) Unicellular cyanobacterial distributions broaden the oceanic N_2 fixation domain. *Science* **327**: 1512–1514. https://doi.org/10.1126/science.1185468.

Møller-Jensen, J., Jensen, R.B., Löwe, J., and Gerdes, K. (2002) Prokaryotic DNA segregation by an actin-like filament. *EMBO J* **21**: 3119–3127. https://doi.org/10.1093/emboj/cdf320.

Muñoz, E., Weijer, W., Grodsky, S.A., Bates, S.C., and Wainer, I. (2012) Mean and variability of the tropical Atlantic Ocean in the CCSM4. *J Climate* **25**: 4860–4882. https://doi.org/10.1175/JCLI-D-11-00294.1.

Nelson, C.W., Moncla, L.H., and Hughes, A.L. (2015) SNPGenie: estimating evolutionary parameters to detect natural selection using pooled next-generation sequencing data. *Bioinformatics* **31**: 3709–3711. https://doi.org/10.1093/bioinformatics/btv449.

Norberg, J. (2004) Biodiversity and ecosystem functioning: a complex adaptive systems approach. *Limnol Oceanogr* **49**: 1269–1277. https://doi.org/10.4319/lo.2004.49.4_part_2.1269.

O'Donnell, D.R., Hamman, C.R., Johnson, E.C., Kremer, C. T., Klausmeier, C.A., and Litchman, E. (2018) Rapid thermal adaptation in a marine diatom reveals constraints and trade-offs. *Glob Chang Biol* **24**: 4554–4565. https://doi.org/10.1111/gcb.14360.

Oliver, E.C.J., Donat, M.G., Burrows, M.T., Moore, P.J., Smale, D.A., Alexander, L.V., *et al*. (2018) Longer and more frequent marine heatwaves over the past century. *Nat Commun* **9**: 1324. https://doi.org/10.1038/s41467-018-03732-9.

Padfield, D., Yvon-Durocher, G., Buckling, A., Jennings, S., and Yvon-Durocher, G. (2016) Rapid evolution of metabolic traits explains thermal adaptation in phytoplankton. *Ecol Lett* **19**: 133–142. https://doi.org/10.1111/ele.12545.

Pfiffer, V., Sarenko, O., Possling, A., and Hengge, R. (2019) Genetic dissection of *Escherichia coli*'s master diguanylate cyclase DgcE: role of the N-terminal MASE1 domain and direct signal input from a GTPase partner system. *PLoS Genet* **15**: e1008059. https://doi.org/10.1371/journal.pgen.1008059.

Price, N.M., Harrison, G.I., Hering, J.G., Hudson, R.J., Nirel, P.M., Palenik, B., and Morel, F.M. (1989) Preparation and chemistry of the artificial algal culture medium Aquil. *Biol Oceanogr* **6**: 443–461. https://doi.org/10.1080/01965581.1988.10749544.

Price, T.D., Qvarnstrm, A., and Irwin, D.E. (2003) The role of phenotypic plasticity in driving genetic evolution. *Proc R Soc B* **270**: 1433–1440. https://doi.org/10.1098/rspb.2003.2372.

Prufert-Bebout, L., Paerl, H.W., and Lassen, C. (1993) Growth, nitrogen fixation, and spectral attenuation in cultivated *Trichodesmium* species. *Appl Environ Microbiol* **59**: 1367. https://doi.org/10.1128/aem.59.5.1367-1375.1993.

Qu, P., Fu, F., and Hutchins, D.A. (2018) Responses of the large centric diatom *Coscinodiscus* sp. to interactions between warming, elevated CO_2, and nitrate availability. *Limnol Oceanogr* **63**: 1407–1424. https://doi.org/10.1002/lno.10781.

Qu, P., Fu, F.-X., Kling, J.D., Huh, M., Wang, X., and Hutchins, D.A. (2019) Distinct responses of the nitrogen-fixing marine cyanobacterium to a thermally variable environment as a function of phosphorus availability. *Front Microbiol* **10**: 1282. https://doi.org/10.3389/fmicb.2019.01282.

Rawlings, N.D., and Salvesen, G. (2013) *Handbook of Proteolytic Enzymes*. Cambridge, MA: Academic Press.

Reusch, T.B.H., and Boyd, P.W. (2013) Experimental evolution meets marine phytoplankton. *Evolution* **67**: 1849–1859. https://doi.org/10.1111/evo.12035.

Salje, J., Gayathri, P., and Löwe, J. (2010) The ParMRC system: molecular mechanisms of plasmid segregation by actin-like filaments. *Nat Rev Microbiol* **8**: 683–692. https://doi.org/10.1038/nrmicro2425.

Scannell, H.A., Pershing, A.J., Alexander, M.A., Thomas, A. C., and Mills, K.E. (2016) Frequency of marine heatwaves in the North Atlantic and North Pacific since 1950. *Geophys Res Lett* **43**: 2069–2076. https://doi.org/10.1002/2015GL067308.

Schaum, C.E., Rost, B., and Collins, S. (2016) Environmental stability affects phenotypic evolution in a globally distributed marine picoplankton. *ISME J* **10**: 75–84. https://doi.org/10.1038/ismej.2015.102.

Schlüter, L., Lohbeck, K.T., Gutowska, M.A., Gröger, J.P., Riebesell, U., and Reusch, T.B.H. (2014) Adaptation of a globally important coccolithophore to ocean warming and acidification. *Nat Clim Chang* **4**: 1024–1030. https://doi.org/10.1594/PANGAEA.837755.

Simon, D.M., and Zimmerly, S. (2008) A diversity of uncharacterized reverse transcriptases in bacteria. *Nucleic Acids Res* **36**: 7219–7229. https://doi.org/10.1093/nar/gkn867.

Smale, D., Wernberg, T., Oliver, E., Thomsen, M., Harvey, B., Straub, S., *et al*. (2019) Marine heatwaves threaten global biodiversity and the provision of ecosystem services. *Nat Clim Chang* **9**: 306–312. https://doi.org/10.1038/s41558-019-0412-1.

Sohm, J.A., Webb, E.A., and Capone, D.G. (2011) Emerging patterns of marine nitrogen fixation. *Nat Rev Microbiol* **9**: 499. https://doi.org/10.1038/nrmicro2594.

Stamos, J.L., Lentzsch, A.M., and Lambowitz, A.M. (2017) Structure of a thermostable group II intron reverse transcriptase with template-primer and its functional and evolutionary implications. *Mol Cell* **68**: 926–939. https://doi.org/10.1016/j.molcel.2017.10.024.

Stomp, M., van Dijk, M.A., van Overzee, H.M.J., Wortel, M. T., Sigon, C.A.M., Egas, M., *et al*. (2008) The timescale of phenotypic plasticity and its impact on competition in fluctuating environments. *Am Nat* **172**: E169–E185. https://doi.org/10.1086/591680.

Sunda, W.G.P.,.N.M., and Morel, F.M. (2005) Trace metal ion buffers and their use in culture studies. *Algal Culturing Tech* **4**: 35–63. https://doi.org/10.1016/b978-012088426-1/50005-6.

Tang, W., Wang, S., Fonseca-Batista, D., Dehairs, F., Gifford, S., Gonzalez, A.G., *et al*. (2019) Revisiting the distribution of oceanic N_2 fixation and estimating diazotrophic contribution to marine production. *Nat Commun* **10**: 1–10. https://doi.org/10.1038/s41467-019-08640-0.

Thomas, M.K., Kremer, C.T., Klausmeier, C.A., and Litchman, E. (2012) A global pattern of thermal adaptation in marine phytoplankton. *Science* **338**: 1085–1088. https://doi.org/10.1126/science.1224836.

Thornton, P.K., Ericksen, P.J., Herrero, M., and Challinor, A. J. (2014) Climate variability and vulnerability to climate change: a review. *Glob Chang Biol* **20**: 3313–3328. https://doi.org/10.1111/gcb.12581.

Toro, N., and Nisa-Martínez, R. (2014) Comprehensive phylogenetic analysis of bacterial reverse transcriptases. *PLoS One* **9**: e114083. https://doi.org/10.1371/journal.pone.0114083.

Vallota-Eastman, A., Arrington, E.C., Meeken, S., Roux, S., Dasari, K., Rosen, S., *et al*. (2020) Role of diversity-generating retroelements for regulatory pathway tuning in cyanobacteria. *BMC Genomics* **21**: 1–13. https://doi.org/10.1186/s12864-020-07052-5.

Verlhac, P., Grégoire, I.P., Azocar, O., Petkova, D.S., Baguet, J., Viret, C., and Faure, M. (2015a) Autophagy receptor NDP52 regulates pathogen-containing autophagosome maturation. *Cell Host Microbe* **17**: 515–525. https://doi.org/10.1016/j.chom.2015.02.008.

Verlhac, P., Viret, C., and Faure, M. (2015b) Dual function of CALCOCO2/NDP52 during xenophagy. *Autophagy* **11**: 965–966. https://doi.org/10.1080/15548627.2015.1046672.

Walworth, N., Pfreundt, U., Nelson, W.C., Mincer, T., Heidelberg, J.F., Fu, F., *et al*. (2015) *Trichodesmium* genome maintains abundant, widespread noncoding DNA in situ, despite oligotrophic lifestyle. *Proc Natl Acad Sci U S A* **112**: 4251–4256. https://doi.org/10.1073/pnas.1422332112.

Walworth, N.G., Hutchins, D.A., Dolzhenko, E., Lee, M.D., Fu, F.-X., Smith, A.D., and Webb, E.A. (2017) Biogeographic conservation of the cytosine epigenome in the globally important marine, nitrogen-fixing cyanobacterium *Trichodesmium*. *Environ Microbiol* **19**: 4700–4713. https://doi.org/10.1111/1462-2920.13934.

Walworth, N.G., Lee, M.D., Dolzhenko, E., Fu, F.X., Smith, A.D., Webb, E.A., and Hutchins, D.A. (2020) Long-term m5C methylome dynamics parallel phenotypic adaptation in the cyanobacterium *Trichodesmium*. *Mol Biol Evol* **38**: 927–939. https://doi.org/10.1093/molbev/msaa256.

Webb, E.A., Ehrenreich, I.M., Brown, S.L., Valois, F.W., and Waterbury, J.B. (2009) Phenotypic and genotypic characterization of multiple strains of the diazotrophic cyanobacterium, *Crocosphaera watsonii*, isolated from the open ocean. *Environ Microbiol* **11**: 338–348. https://doi.org/10.1111/j.1462-2920.2008.01771.x.

Wilson, W.A., Roach, P.J., Montero, M., Baroja-Fernández, E., Muñoz, F.J., Eydallin, G., *et al*. (2010) Regulation of glycogen metabolism in yeast and bacteria. *FEMS Microbiol Rev* **34**: 952–985. https://doi.org/10.1111/j.1574-6976.2010.00220.x.

Xu, Y., Guerra, L.T., Li, Z., Ludwig, M., Dismukes, G.C., and Bryant, D.A. (2013) Altered carbohydrate metabolism in glycogen synthase mutants of *Synechococcus* sp. strain PCC 7002: cell factories for soluble sugars. *Metab Eng* **16**: 56–67. https://doi.org/10.1016/j.ymben.2012.12.002.

Zehr, J.P. (2011) Nitrogen fixation by marine cyanobacteria. *Trends Microbiol* **19**: 162–173. https://doi.org/10.1016/j.tim.2010.12.004.

Zehr, J.P., Bench, S.R., Mondragon, E.A., McCarren, J., and DeLong, E.F. (2007) Low genomic diversity in tropical oceanic N_2-fixing cyanobacteria. *Proc Natl Acad Sci U S A* **104**: 17807–17812. https://doi.org/10.1073/pnas.0701017104.

Zehr, J.P., and Capone, D.G. (2020) Changing perspectives in marine nitrogen fixation. *Science* **368**: eaay9514. https://doi.org/10.1126/science.aay9514.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting information.