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REVIEW

Crocosphaera watsonii – A widespread nitrogen-fixing unicellular marine cyanobacterium

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Abstract

Crocosphaera watsonii is a unicellular N_2-fixing (diazotrophic) cyanobacterium observed in tropical and subtropical oligotrophic oceans. As a diazotroph, it can be a source of bioavailable nitrogen (N) to the microbial community in N-limited environments, and this may fuel primary production in the regions where it occurs. *Crocosphaera watsonii* has been the subject of intense study, both in culture and in field populations. Here, we summarize the current understanding of the phylogenetic and physiological diversity of *C. watsonii*, its distribution, and its ecological niche. Analysis of the relationships among the individual *Crocosphaera* species and related free-living and symbiotic lineages of diazotrophs based on the *nif*H gene have shown that the *C. watsonii* group holds a basal position and that its sequence is more similar to *Rippkaea* and *Zehria* than to other *Crocosphaera* species. This finding warrants further scrutiny to determine if the placement is related to a horizontal gene transfer event. Here, the *nif*H UCYN-B gene copy number from a recent synthesis effort was used as a proxy for relative *C. watsonii* abundance to examine patterns of *C. watsonii* distribution as a function of environmental factors, like iron and phosphorus concentration, and complimented with a synthesis of *C. watsonii* physiology. Furthermore, we have summarized the current knowledge of *C. watsonii* with regards to N_2 fixation, photosynthesis, and quantitative modeling of physiology. Because N availability can limit primary production, *C. watsonii* is widely recognized for its importance to carbon and N cycling in ocean ecosystems, and we conclude this review by highlighting important topics for further research on this important species.

KEYWORDS
Chroococcales, *Crocosphaera watsonii*, diazotroph, ecological niche, phylogenetic clustering, physiological diversity

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Abbreviations: C, carbon; DIN, dissolved inorganic nitrogen; DIP, dissolved inorganic phosphorus; DOP, dissolved organic phosphorus; EPS, extracellular polysaccharide; Fe, iron; HGT, horizontal gene transfer; LD, light–dark; N, nitrogen; NH_4^+, ammonium; NO_3^-, nitrate; OCP, orange carotenoid protein; P, phosphorus; PIT, phosphate inorganic transport; PSI, photosystem I; PSII, photosystem II; PST, phosphate-specific transport; rD1, rogue D1; TEP, transparent exopolymeric particles; UCYN, unicellular cyanobacteria.

INTRODUCTION

Crocosphaera watsonii, a unicellular cyanobacterium, is one of the key N_2-fixing planktonic microorganisms (diazotrophs) in tropical and subtropical oceans. *C. watsonii* WH8501 (Figure 1a) and WH8502 strains were initially isolated as *Erythrosphaera marina* gen. nov. sp. nov. in 1984 (Waterbury et al., 1988; Waterbury & Rippka, 1989), but the importance of *C. watsonii* as a diazotroph was only realized a decade later. The discovery of *nif*H genes from pico- and nano-plankton (0.22–20 μm fraction; Zehr et al., 1998) and the observation of phycoerythrin containing 2–3 μm unicellular cells (Figure 1b,c) in low-nitrogen waters that had measurable phosphorus (Neveux et al., 1999) pointed to the importance of N_2 fixation from unicellular cyanobacteria (Zehr et al., 2001). The significance of unicellular cyanobacteria as diazotrophs was later evidenced by high rates of N_2 fixation from in situ <10 μm fraction samples (Montoya et al., 2004). The unicellular diazotrophic cyanobacteria are phylogenetically divided into three groups based on *nif*H gene and 16S rRNA gene sequences: (i) symbiotic *Candidatus Atelocyanobacterium thalassae* (Oren et al., 2020) corresponding to UCYN-A as recovered in *nif*H field studies (Zehr et al., 2008), (ii) free-living autotrophic *C. watsonii* corresponding to UCYN-B in *nif*H studies (Mareš et al., 2019; Zehr et al., 2001), and (iii) other *Crocosphaera* (formerly "*Cyanothece*") species, such as *C. subtropica* (corresponding to UCYN-C *nif*H cluster) and *C. chwakensis* (Foster et al., 2007; Mareš et al., 2019; Turk-Kubo et al., 2017; Zehr et al., 2001). In this review, we use UCYN-B when we talk about *nif*H amplicon distribution, *C. watsonii* for culture studies, and *Crocosphaera* if the data have not been identified at the species level.

Marine N_2-fixation studies historically focused on *Trichodesmium*, and this genus has been the subject of a number of intensive reviews of its physiological features, distribution, and physiology (Capone et al., 1997; Kranz et al., 2011; McKinna, 2015). The symbiotic unicellular diazotroph *Candidatus Atelocyanobacterium thalassae* (UCYN-A) was discovered relatively recently (Zehr et al., 1998) and has been the subject of interest and extensive research efforts (Farnelid et al., 2016; Zehr et al., 2016). The function and the distribution of marine diazotrophs, including those of *Crocosphaera watsonii* (UCYN-B), have been summarized previously (Luo et al., 2012; Masuda et al., 2022; Shao et al., 2023; Tang & Cassar, 2019; Thompson & Zehr, 2013; Zehr & Capone, 2020). In this review, we have focused on *C. watsonii*, summarizing the phylogenetic clustering, physiological diversity, ecological niche, and regulation of photosynthesis and N_2 fixation of *C. watsonii*. We conclude by summarizing the knowledge obtained by quantitative modeling of *C. watsonii* physiology.

PHYLOGENETIC CLUSTERING

The ability to fix N_2 occurs across cyanobacterial lineages, and the complete *nif* gene operon has been detected in more than one-third of sequenced cyanobacterial genomes (Latysheva et al., 2012; Watanabe & Horiike, 2021). Among marine plankton, diazotrophs are represented by several phylogenetically separated lineages (see Appendix S1 in the Supporting Information; Figure 2a). *Crocosphaera* spp. (UCYN-B and -C) and *Candidatus Atelocyanobacterium thalassae* (UCYN-A) form a unique lineage of unicellular representatives of cyanobacterial diazotrophic marine plankton (Figure 2a) that belongs to a broader cluster of single-celled N_2-fixing cyanobacteria inhabiting various ecosystems, including terrestrial, epilithic, soil, and freshwater habitats (genera *Rippkaea*, *Aphanothece*, *Gloeothece*; Mareš et al., 2019). The systematics of unicellular cyanobacteria has been a challenge due to morphological convergence and researchers using diverse taxonomic concepts over time, including traditional botanical (Geitler, 1932), bacteriological (Rippka et al., 1979), and more recently phylogenetic (Johansen & Casamatta, 2005) approaches. Recently, the taxonomy of unicellular diazotrophs was revised based on modern comprehensive techniques, which involved

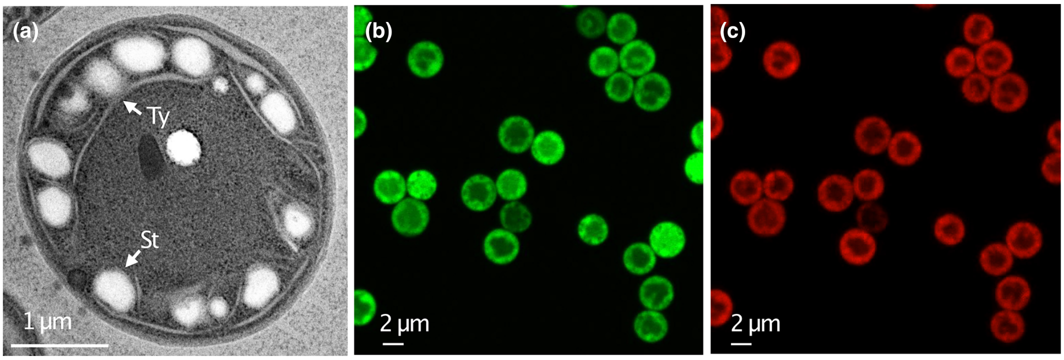

FIGURE 1 (a) TEM image of *Crocosphaera watsonii* WH8501, (b) phycoerythrin, and (c) chlorophyll autofluorescence images of *C. watsonii* PS0609. St, starch granule; Ty, thylakoid membrane. [Color figure can be viewed at wileyonlinelibrary.com]

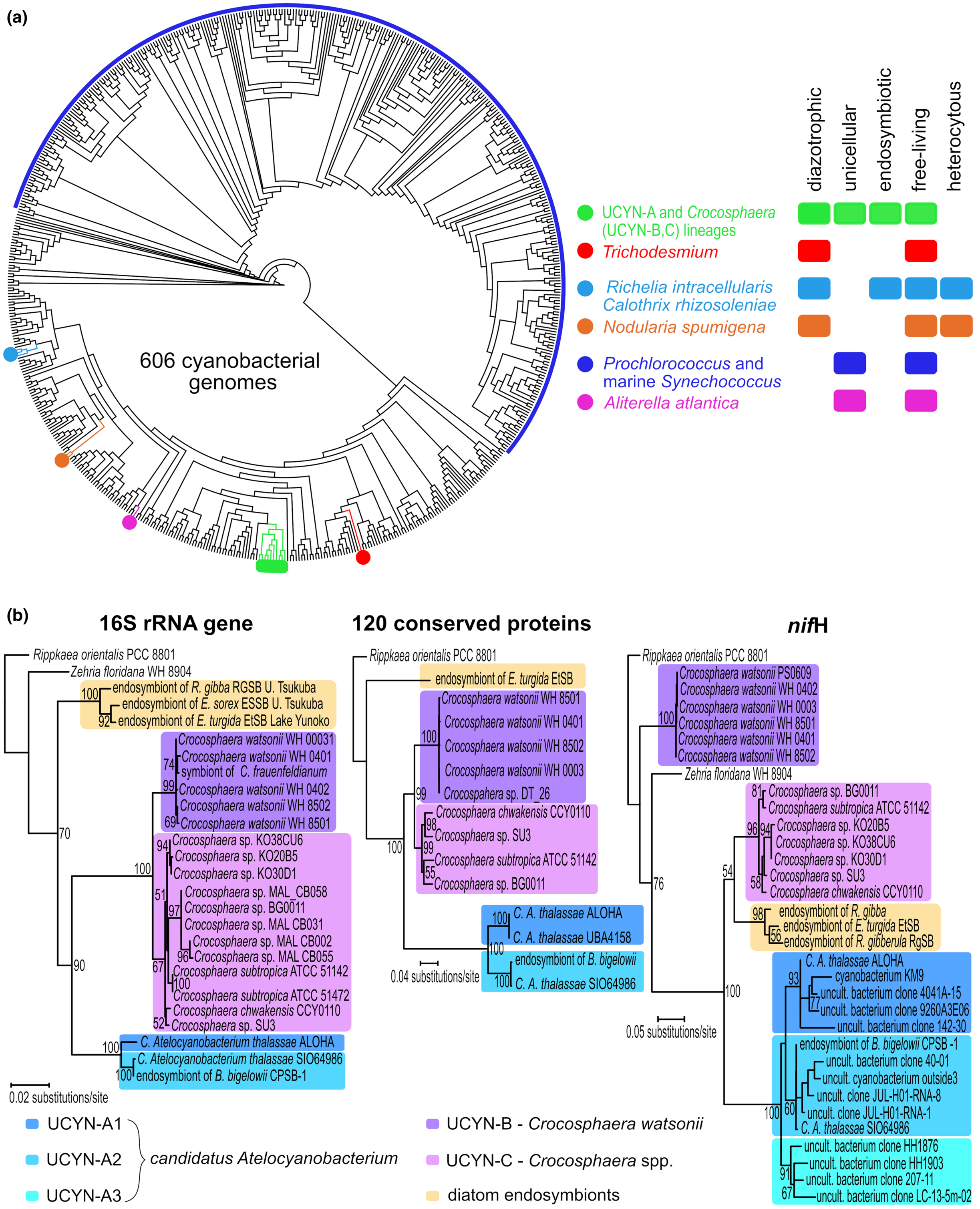

FIGURE 2 Phylogeny of *Crocosphaera* and related organisms. (a) Phylogenomic tree (120 conserved proteins) of cyanobacteria, showing the clustering of planktonic marine diazotrophs in multiple separated derived lineages. The *Crocosphaera* (UCYN-B and C) and UCYN-A lineages host unicellular diazotrophic cyanobacteria, while other planktonic marine cyanobacteria are either filamentous diazotrophs or unicellular non-diazotrophs. (b) Comparison of phylogenetic clustering within the UCYN-A, B, and C lineages based on 16S rRNA gene, multilocus (120 proteins), and *nif*H trees. The UCYN A–C clades and the clade of related diatom endosymbionts are detectable in all three trees; however, the topology of the *nif*H tree is incongruent with the conserved loci, indicating possible horizontal gene transfer among related lineages. The methods for phylogenetic inference are provided as Appendix S1. [Color figure can be viewed at wileyonlinelibrary.com]

morphological observations, transmission electron microscopy (TEM), phylogenetic analysis (of the 16S rRNA gene and two additional housekeeping loci), ITS rRNA region secondary structure prediction, and assessment of the source habitat type (Mareš et al., 2019). The most striking result from the analysis of rRNA operon data by Mareš et al. (2019) was that several strains previously referred to as *Cyanothece* sp. or *Gloeocapsa* sp. turned out to be members of the genus *Crocosphaera*. Consequently, the genus *Crocosphaera* currently contains not only *C. watsonii* (UCYN-B) but also species corresponding to the UCYN-C clade: *C. subtropica* (previously *Cyanothece* sp. ATCC51142), *C. chwakensis* (previously *Cyanothece* sp. CCY0110), and other species still waiting to be described (Figure 2b). Recently, a novel *Crocosphaera* species, *Crocosphaera waterburyi*, has been proposed based on metagenomic analysis from the North Pacific Ocean (Cleveland et al., 2023), and so the genus continues to expand. Furthermore, a morphologically and ecologically convergent group of diazotrophic *Crocosphaera*-like isolates has formed a separate genus-level lineage described as *Zehria* (Mareš et al., 2019).

A closer look at the whole lineage (Figure 2b) reveals that widespread non-photosynthetic diazotrophic unicellular endosymbionts of eukaryotic algae have formed sister clades to *Crocosphaera*. One of them, UCYN-A or *Candidatus Atelocyanobacterium thalassae*, contained endosymbionts of marine Prymnesiophytes (Hagino et al., 2013; Thompson et al., 2012); another more distantly related lineage contained the endosymbionts (also called "spheroid bodies") of rhopalodiacean diatoms (Nakayama & Inagaki, 2017). In both cases, the symbionts had abandoned oxygenic photosynthesis; they function almost as organelles, providing fixed N_2 to their hosts in exchange for fixed carbon (Prechtl et al., 2004; Thompson et al., 2012). Intriguingly, the marine diatom *Climacodium frauenfeldianum* has been shown to be capable of hosting *Crocosphaera* as a photosynthetic diazotrophic endosymbiont (Caputo et al., 2019; Carpenter & Janson, 2000).

The clustering and relationships among the individual *Crocosphaera*-like free-living and symbiotic lineages seem to be identical based on the 16S rRNA gene and a robust set of 120 universal bacterial housekeeping proteins (Parks et al., 2021) (Figure 2b), although the whole genome is missing for *Zehria*. Although the corresponding clades can be identified in the *nif*H tree (Figure 2b), the *C. watsonii* (UCYN-B) group holds a basal position, and its *nif*H gene sequences are more similar to *Rippkaea* and *Zehria* (89.2%–90.1% pairwise identity) than to other *Crocosphaera* species (80.5%–85.0%). Such a phylogenetic pattern possibly indicates a horizontal gene transfer (HGT) event leading to the replacement of the *C. watsonii nif*H gene by the gene from an unknown but related marine unicellular diazotrophic cyanobacterium. Alternatively, the 16S rRNA gene of *Zehria* could have been subject to HGT. Based on previous reports, HGT in the N_2 fixation gene cluster is possible, although uncommon, in cyanobacteria (Bolhuis et al., 2010; Latysheva et al., 2012). The *nif*H gene itself seems to exhibit HGT more frequently than other *nif* genes, and its phylogeny is often incongruent with the 16S rRNA gene phylogeny in N_2-fixing bacteria in general (Gaby & Buckley, 2014). Given the scarcity of reported HGT events in cyanobacterial rRNA genes (Johansen et al., 2017), we suggest that an HGT event in the *nif*H gene of *C. watsonii* might be more likely than in the 16S rRNA gene of *Zehria*. Future studies that include whole genome sequencing of *Zehria* are needed to validate this hypothesis.

Studies suggest low genomic diversity of *Crocosphaera watsonii* (Webb et al., 2009; Zehr et al., 2007). For example, analysis of a set of five functional genes showed greater than 99% nucleotide identity among seven *C. watsonii* strains (Zehr et al., 2007). In addition, sequences of the internal transcribed spacer region between the 16S and 23S rRNA genes (ITS rRNA region) from phenotypically distant cultures were highly similar (Webb et al., 2009). Cultivated and in situ *C. watsonii* genomes have shown an unusually high number of transposases (Bench et al., 2011, 2013; Hewson et al., 2009; Mes & Doeleman, 2006; Zehr et al., 2007), suggesting a mechanism for adaptation to changing environmental conditions, which could underpin the variety of phenotypic differences observed among strains (Bench et al., 2011). Whole genome sequences of six *C. watsonii* strains have shown that *C. watsonii* WH8501 has a larger genome (6.2 Mb vs. 4.5–5.8 Mb), a higher number of transposase genes (1211 genes vs. 165–223 genes), and a higher number of strain-specific transposase genes (71 vs. 4–19) than the five other strains analyzed (Bench et al., 2013). These results suggested that the *C. watsonii* WH8501 strain is an outlier relative to the others examined and may not be the most representative strain of the natural *C. watsonii* population. However, this warrants further study across a broader set of both isolates and field samples.

DISTRIBUTION

Distributions of unicellular diazotrophs, especially in the subtropics, have been conventionally assayed by a combination of phycoerythrin detection from nano-sized cyanobacteria by epifluorescence microscopic observation, flow cytometry, or by fluorometric analysis in the Pacific Ocean (Bonnet et al., 2009; Dore et al., 2002; Dugenne et al., 2020; Falcon et al., 2004; Kitajima et al., 2009; Needoba et al., 2007; Neveux et al., 1999; Stenegren et al., 2018; Wilson et al., 2017), Atlantic Ocean (Foster et al., 2007; Krupke et al., 2013), and Mediterranean Sea (Bonnet et al., 2011). Detection of UCYN-B *nif*H amplicons has been reported from the Pacific Ocean (Berthelot et al., 2017; Bonnet et al., 2008; Church, Jenkins, et al., 2005; Church, Short, et al., 2005; Fong et al., 2008; Grabowski et al., 2008; Gradoville et al., 2020; Halm et al., 2012; Moisander et al., 2010; Needoba et al., 2007; Shiozaki et al., 2015, 2017; Shiozaki, Kondo, et al., 2018; Stenegren et al., 2018; Watkins-Brandt et al., 2011; Zehr et al., 2001), Atlantic Ocean (Detoni et al., 2022; Foster et al., 2007; Foster, Subramaniam, & Zehr, 2009; Goebel et al., 2010; Krupke et al., 2013; Langlois et al., 2008; Martinez-Perez et al., 2016; Mulholland et al., 2012; Turk-Kubo et al., 2011), South China Sea (Kong et al., 2011; Moisander et al., 2008; Shiozaki, Ijichi, et al., 2014; Wen et al., 2017), Indian Ocean (Shiozaki, Ijichi, et al., 2014), Arabian Sea (Mazard et al., 2004), Red Sea (Foster, Paytan, & Zehr, 2009), and Mekong River plume (Bombar et al., 2011). Luo et al. (2012) established the first comprehensive database of diazotrophic *nifH* gene copy number, and these data were used by Tang and Cassar (2019) to compare the distribution of *Trichodesmium*, UCYN-A, UCYN-B, and *Richelia*. Tang and Cassar (2019) reported similar distributions between UCYN-B *nif*H copy number and *Trichodesmium nif*H copy number in the Pacific but lower copy number distribution in the North Atlantic and suggested that this might be the result of a positive relationship of UCYN-B *nif*H copy number and temperature (Moisander et al., 2010) and differences in dissolved inorganic phosphorus (DIP) concentration. Even though no clear separation of diazotrophic groups with depth was observed at the global scale, as all groups decreased with depth, Tang and Cassar (2019) noted that UCYN-B and UCYN-A *nif*H copy number exhibited deeper vertical distribution among these groups of diazotrophs. The validity of *nifH* copy number as a proxy for cell abundance has been recently questioned (Pierella Karlusich et al., 2021); however, the ratio between *nifH* copy number and cell abundance for UCYN-B has been reported as 1.82 with a significant relationship (Gradoville et al., 2022). This showed that while not a direct measure of cells, the *nif*H copy number can be a useful quantitative proxy for *Crocosphaera* abundance (Gradoville et al., 2022).

The comprehensive dataset of UCYN-B *nif*H gene copy number, updated after the study by Tang and Cassar (2019), and by Shao et al. (2023) revealed non-homogeneous distributions (Figure 3a). We note the relative increase in UCYN-B *nif*H gene copy number in the western South Pacific, around Fiji, in the northeastern Pacific, near the Hawaii islands, in the tropical North Atlantic, in the western subtropical South Atlantic, in the Philippine Sea, and in the eastern north Indian Ocean (Figure 3a; Shao et al., 2023 and references therein). The maximum copy number (7.9×10^6 *nifH* gene copies $\cdot$ L^{-1}) was reported from 37 m depth off Fiji (15° S 175° E; Moisander et al., 2010; Figure 3a). Diazotroph distributions can be influenced by many factors including nutrient supply and supply ratios (Shiozaki, Kodama, & Furuya, 2014; Subramaniam et al., 2008), which may be the case for some of the areas of increased UCYN-B *nif*H gene copy number. For example, comparatively low UCYN-B *nif*H gene copy number in the Indian Ocean may be attributed to higher upward nitrate fluxes to the surface because of the shallow nitracline (Sato et al., 2022; Shiozaki, Ijichi, et al., 2014). A negative relationship between a shallow nitracline and UCYN-B *nif*H gene copy number was reported from the northern South China Sea (Shiozaki, Ijichi, et al., 2014). Taken together, identifying the factors that determine the distribution of UCYN-B *nif*H gene copy number is an ongoing area of study.

The vertical distribution or UCYN-B *nif*H gene copy number was restricted to above 200 m of depth, with $>10^5$ *nif*H gene copies $\cdot$ L^{-1} observed above 150 m (Figure 4a). Temperature seems to be an important niche trait for diazotrophs, and the UCYN-B *nif*H gene copy number was observed to increase with increasing water temperature in regional and global studies (Moisander et al., 2010; Tang & Cassar, 2019). Although the UCYN-B *nif*H gene copy number was typically highest between 25 and 30°C, the UCYN-B *nif*H gene has been identified in regions as cold as 10.1°C (Goebel et al., 2010; Shao et al., 2023). Further, the UCYN-B *nif*H gene copy number at >25°C was not always high ($>10^5$ *nif*H gene copies $\cdot$ L^{-1}), highlighting that temperature is not the only explanatory factor influencing UCYN-B *nif*H gene copy number (Figure 4b). Laboratory experiments using clonal cultures have reported the optimum temperatures of eight *Crocosphaera watsonii* strains (WH8501, WH8504, WH0002, WH0003, WH0005, WH0006, WH0401, and WH0402) to be between 16 and 36°C (Fu et al., 2014; Qu et al., 2022; Webb et al., 2009); this broad range supports the variation seen in UCYN-B *nif*H gene copies as a function of temperature (Figure 4b). Despite the breadth of the temperature range, UCYN-B *nif*H gene copies were detected at higher temperatures than *Trichodesmium nif*H gene copies by Tang and Cassar (2019), and this is consistent with culture data where growth rates of *C. watsonii* strains WH0005, WH0003, WH0401 and WH0402 with growth optimum temperature at 28–30°C were ~25% less than those of *Trichodesmium* at 24°C and ~50% higher than *Trichodesmium* at 28°C (Fu et al., 2014).

PHYSIOLOGICAL ECOLOGY

The classical view on marine N_2 fixation assumes that the preferred ecological niches of diazotrophs are limited mainly to surface waters of oligotrophic areas, characterized by low DIN, saturated dissolved oxygen, and

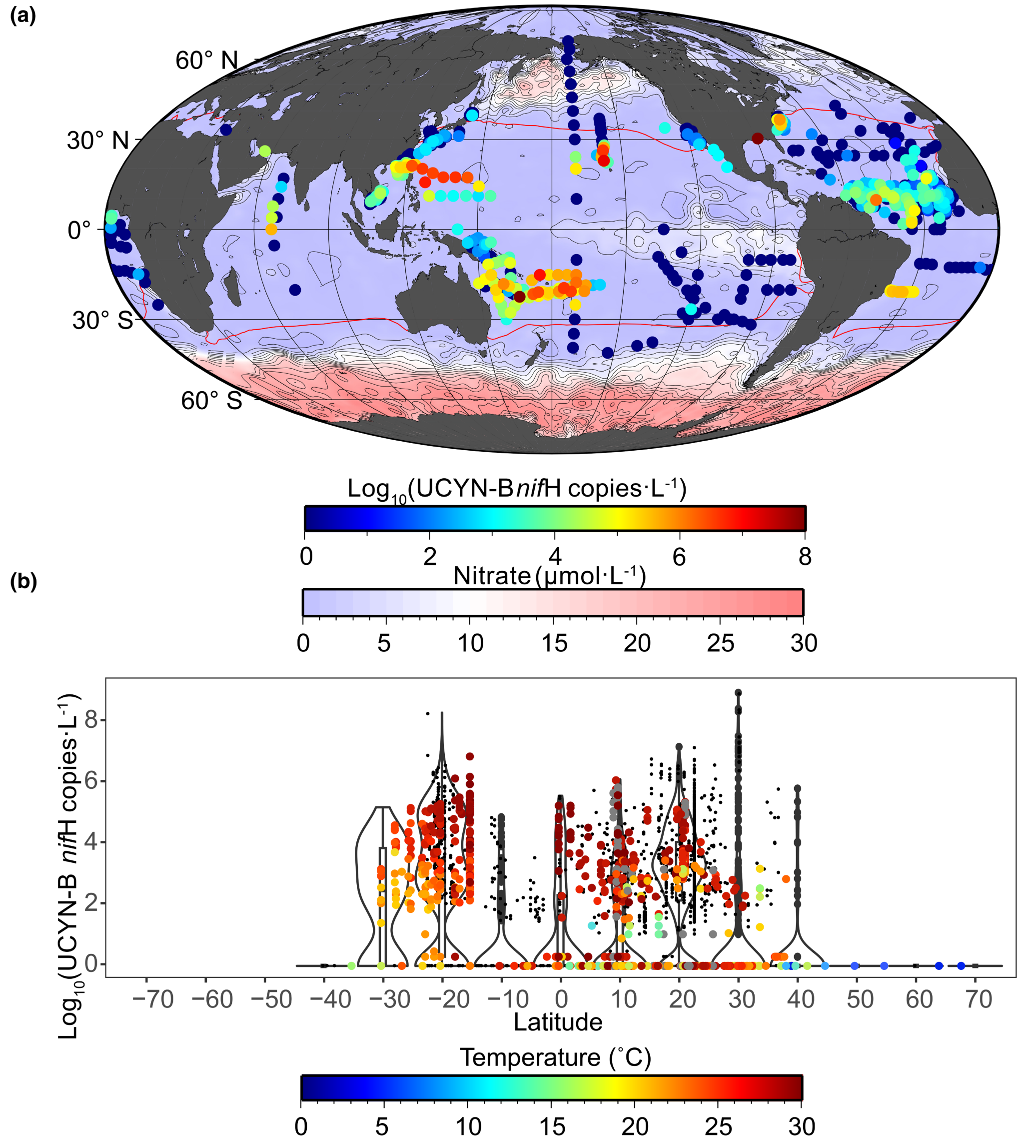

FIGURE 3 A global map of UCYN-B *nif*H copies plotted from Shao et al. (2023). (a) The geographic distribution of the maximum observed UCYN-B *nif*H copies (>10 copies · L^{-1}) at a location. The plots were compiled from 3416 published observations of UCYN-B *nif*H gene copy number from 0 to 200 m depth (Shao et al., 2023 and references therein). The colors in the circles indicate the maximum abundance on a logarithmic scale, the black dots indicate a result reported as below the detection limit by Shao et al. (2023). The background color is the nitrate concentration with contour lines indicating the surface nitrate concentrations obtained from the World Ocean Atlas 2018. Red lines indicate the 20°C isotherm of the sea surface temperature. (b) Latitudinal distribution of the maximum UCYN-B *nif*H copies (>10 copies · L^{-1}), shown with violin plots using data from Shao et al. (2023). The colored dots show the temperature of the seawater at the sampling station. The black dots indicate the absence of temperature data. [Color figure can be viewed at wileyonlinelibrary.com]

high light intensities (Zehr & Capone, 2020). This view is confirmed by patterns in UCYN-B *nif*H gene copy number where the copy number was typically highest (>10^5 *nifH* gene copies · L^{-1}) in the surface under low nitrate and phosphorus concentrations, that is, <0.02 µM of nitrate (*t*-test; $t=-12.115$, $df=405$, $p<0.001$) and <0.14 µM phosphate (*t*-test; $t=-17.306$, $df=253$, $p<0.001$; Figure 4a,c,d). However, copies of UCYN-B *nif*H were detected in the subsurface and at nutrient concentrations above the 0.02 µM nitrate and 0.14 µM phosphate concentrations referenced above (Figure 4a,c,d). Culture studies have shown N_2 fixation is inhibited by increasing the concentration of added NH_4^+ or NO_3^- (Dekaezemacker & Bonnet, 2011; Knapp et al., 2012), and the extent of inhibition of fixed N (both NH_4^+ and NO_3^-) on N_2 fixation is more pronounced under low light intensity (25 µmol photons · m^{-2} · s^{-1}) compared to high light intensity (175 µmol photons · m^{-2} · s^{-1}; Garcia & Hutchins, 2014). These results suggest that *Crocosphaera watsonii* would likely use N_2 in low N surface waters and use combined N in the subsurface, but patterns of N uptake and N_2 fixation are an active area of ongoing study.

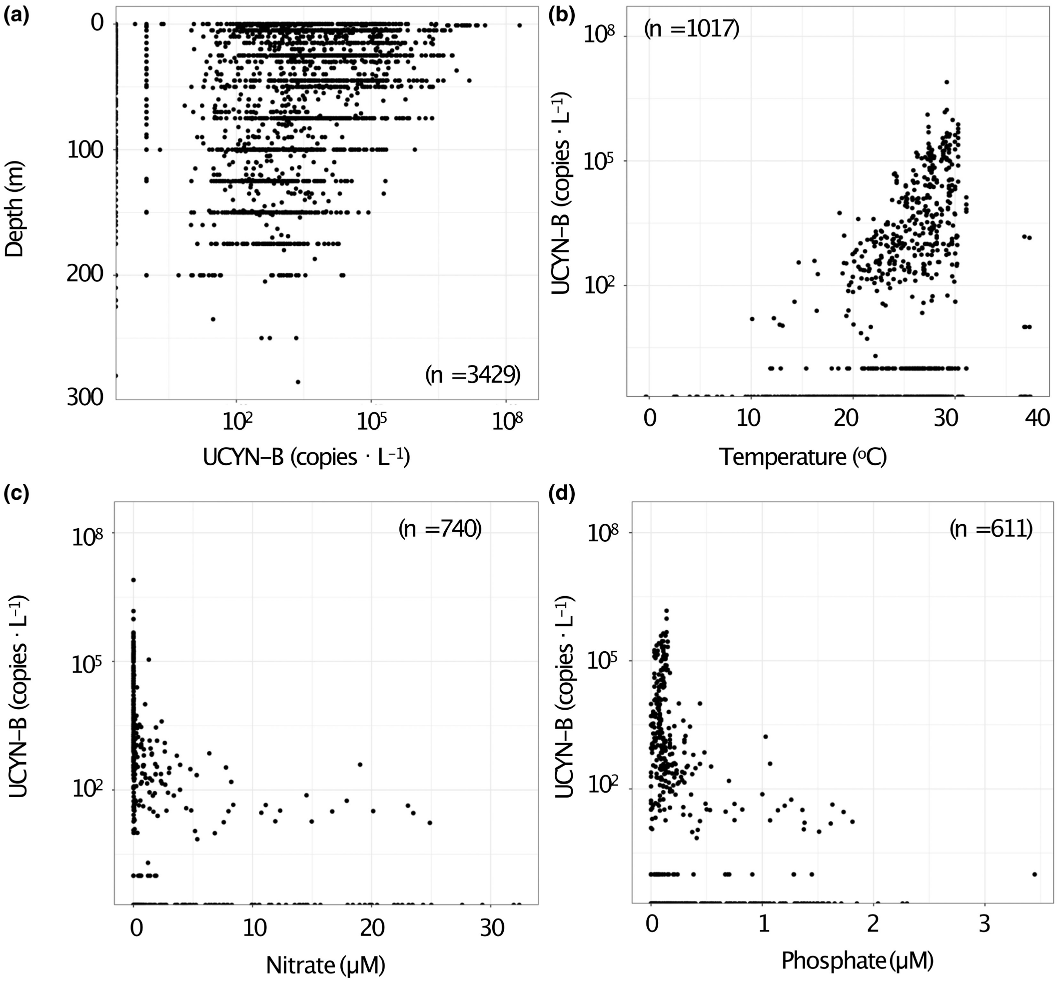

FIGURE 4 Relationship of UCYN-B *nif*H gene copies · L^{-1} and physical and chemical parameters based on the data set used for Figure 3 (Shao et al., 2023 and references therein). UCYN-B *nif*H gene copies · L^{-1} against (a) depth, (b) temperature, (c) nitrate concentrations, and (d) phosphorus concentrations.

Both NH_4^+ and NO_3^- assimilation can save energy (ATP) relative to N_2 fixation, but *Crocosphaera watsonii* keeps fixing N_2 in the presence of NH_4^+ and NO_3^- (Dekaezemacker & Bonnet, 2011; Großkopf & Laroche, 2012; Masuda et al., 2013; Rabouille et al., 2021). Although *C. watsonii* is known as free-living, self-aggregates of multiple cells have been reported both from field observations (Foster et al., 2013) and clonal cultures (Mohr et al., 2013). Aggregation of cells may be related to extracellular polysaccharide (EPS) production (Foster et al., 2013). Observations of concurrent combined N assimilation and N_2 fixation may be related to a division of labor observed among *C. watsonii* sub-populations in self-aggregates in which some cells in the population fix N_2 and release NH_4^+ while other cells take up NH_4^+ (Masuda et al., 2020). Cellular heterogeneity in N_2 fixation activity was observed from in situ *Crocosphaera*-like cells (Foster et al., 2013) and *C. watsonii* WH8501 and PS0609 isolates (Masuda et al., 2020; Mohr et al., 2013) using a combination of stable isotope labeling and NanoSIMS analysis. Interestingly about 30% of cells in the culture study of Masuda et al. (2020) did not perform N_2 fixation, but these non-N_2-fixing cells did perform photosynthesis (Masuda et al., 2020). Genomic information supports both NH_4^+ and NO_3^- assimilation (Shi et al., 2010). In sum, these observations suggest *C. watsonii* can utilize both N_2 and combined N as nitrogen sources.

The excretion of NH_4^+ is estimated at a range of up to 26%–66% of fixed N_2 (Dron, Rabouille, Claquin, Le Roy, et al., 2012; Masuda et al., 2013). This is a potentially substantial source of so-called new N into low N systems. It had previously been shown that NH_4^+ release was particularly active for an isolate with larger cell size (*Crocosphaera watsonii* WH0003) compared to a small-celled isolate (*C. watsonii* WH8501; Dekaezemacker & Bonnet, 2011), and elevated amounts of EPS have been reported from larger cells (>4 μm; Sohm et al., 2011; Webb et al., 2009), which could influence patterns in self-aggregation. Such self-aggregation may influence broader aspects of N or C cycling by sinking fixed C and N. For example, *C. watsonii* was observed to be one of the sinking particles during an elevated carbon flux event (Poff et al., 2021), and self-aggregation may play a role in this carbon flux. Multiple mechanisms for acclimation to low dissolved inorganic phosphate (DIP) environments have been identified in *C. watsonii*. For example, *C. watsonii* can grow on dissolved organic phosphorus (DOP) instead of DIP as a sole phosphorus source including both phosphomonoesters and phosphodiesters (Yamaguchi et al., 2020), and its genome (e.g. strain WH8501) contains genes that encode for the alkaline phosphatase enzymes needed to hydrolyze dissolved organic phosphoesters (Bench et al., 2013; Dyhrman & Haley, 2006). When extracellular P is in excess, *C. watsonii* uses the constitutively expressed phosphate inorganic transport (PIT) system to transport P into the cell. Under P-limiting conditions, a high-affinity phosphate-specific transport (PST) system uses ATP-mediated transport, and the high-affinity phosphate-binding protein (PstS) is upregulated to maximize phosphate uptake to meet cellular P demand (Dyhrman & Haley, 2006; Pereira et al., 2016). Given its regulation pattern, expression of the *pst*S gene may be a good molecular marker of *C. watsonii* P stress (Pereira et al., 2016, 2019). Notably, some *C. watsonii* strains have up to seven copies of the *pst*S gene (Bench et al., 2013). In low DIP environments, toxic arsenate could be transported into cells through the PST system, but *C. watsonii* has an arsenate detoxification pathway in its genome (Dyhrman & Haley, 2011). Unlike other cyanobacteria (i.e., *Prochlorococcus*, *Synechococcus*, and *Trichodesmium*), *C. watsonii* does not substitute sulpholipids for phospholipids, nor utilize phosphonates in low P environments (Dyhrman & Haley, 2006; Pereira et al., 2016; Van Mooy et al., 2009). Recently, a study of the interaction between temperature and P limitation showed that at elevated temperatures, genes for sulfolipid biosynthesis (*sgd*B) were upregulated, suggesting that *C. watsonii* may substitute sulpholipids for phospholipids under certain conditions (Deng et al., 2021). This potential P sparing mechanism may, in part, underpin the similar low-P and P-replete growth rates observed at high temperatures by Deng et al. (2021) and warrants further study for how this might relate to *C. watsonii* fitness with future global warming.

The distribution of diazotrophs is often constrained by the availability of iron (Mills et al., 2004; Monteiro et al., 2010; Moore et al., 2009). In the case of *Crocosphaera watsonii* WH8501, Fe limitation led to a significant decrease in growth rate; C, N, and Chl *a* contents per cell; and N_2- and CO_2-fixation rates (Jacq et al., 2014). Under Fe stress, *C. watsonii* induces the IdiA protein as a cellular Fe scavenging mechanism (Webb et al., 2001). In addition, Fe limitation is known to reduce cell size, and this effect has been documented for *C. watsonii* strains WH8501 and WH0003 (Garcia et al., 2015; Jacq et al., 2014). Two phases of response have been observed depending on the degree of Fe limitation: (i) Under a moderate Fe limitation, the biovolume of *C. watsonii* was strongly reduced, but metabolic activity remained (Jacq et al., 2014), and (ii) with increasing Fe deprivation, biovolume remained unchanged but the growth and N_2- and CO_2-fixation rates decreased (Jacq et al., 2014). Advantages of decreased cell size in a low nutrient environment have been proposed, such as a high cell surface area-to-volume ratio and thin diffusion boundary layer, as well as reduced material and energetic investment for cell division (Garcia et al., 2015). Interestingly, under P-deficient conditions, *C. watsonii* WH0003 grew and fixed N_2 faster when Fe was deficient than when Fe was replete, where decreases in cell size were only achieved when Fe and P were co-limiting (Garcia et al., 2015). In addition, Fe and P colimitation appears to support higher growth when compared to Fe or P limitation alone (Yang et al., 2022). This finding suggests the complex influence both Fe and P availability may have on *C. watsonii* growth and N_2 fixation.

The Fe half-saturation constant for growth of *Crocosphaera watsonii* was twice as low as that of *Trichodesmium* (Bucciarelli et al., 2013; Jacq et al., 2014), indicating *C. watsonii* may have a competitive advantage over *Trichodesmium* in low Fe environments. One study on the interactive effect of Fe and temperature showed that at optimum temperature, nitrogen-specific iron-use efficiency was higher under Fedepleted conditions than Fe replete conditions (Yang et al., 2021). Results of proteomic analysis have suggested that internal Fe recycling between photosynthesis and N_2 fixation occurs in *C. watsonii* (Saito et al., 2011). The Fe availability is also important in the response of *C. watsonii* to future increased CO_2 levels: Growth and N_2 fixation are- predicted to increase with future increasing CO_2 concentration (Gradoville et al., 2014) but only if Fe is not limiting (Fu et al., 2008). Much remains unknown regarding the physiological mechanisms that help *C. watsonii* cope with Fe and P limitation.

TEMPORAL SEPARATION OF N_2 FIXATION AND PHOTOSYNTHESIS

A crucial aspect of *Crocosphaera watsonii* biology is the temporal separation of N_2 fixation and photosynthesis (Mohr et al., 2010; Shi et al., 2010; Tuit et al., 2004; Waterbury & Rippka, 1989; Wilson et al., 2017). Oxygenic photosynthesis during the day generates cellular energy and reductant power, and *C. watsonii* restrains the peak of N_2 fixation to the night period when there is no light-dependent photosynthesis (Dron, Rabouille, Claquin, Le Roy, et al., 2012; Masuda et al., 2018; Shi et al., 2010). The temporal segregation helps to solve the problem of nitrogenase being inactivated by oxygen, a byproduct of oxygenic photosynthesis (Fay, 1992;

Gallon, 1992). Whole-genome expression analysis on *C. watsonii* grown under a 12:12 h light:dark cycle using a microarray identified that 47.4% of genes had a diel expression pattern (Shi et al., 2010). This diel expression pattern was also observed in *Crocosphaera* field populations, underpinning diel changes in N_2 fixation (Wilson et al., 2017). During periods of light, *C. watsonii* fixes inorganic carbon (Masuda et al., 2018; Mohr et al., 2010) and accumulates storage carbohydrates (Dron, Rabouille, Claquin, Chang, et al., 2012). A part of photosynthetically fixed carbon is exuded outside of cells as EPS and transparent exopolymeric particles (TEP; Rabouille et al., 2017; Sohm et al., 2011). The internally stored carbohydrates are then degraded and used for respiration at the end of the light phase or at the beginning of the dark phase (Dron, Rabouille, Claquin, Chang, et al., 2012) to decrease cellular O_2 concentrations, supply ATP, and reduce the power required to fuel N_2 fixation (Großkopf & Laroche, 2012). Without the presence of additional stressors, N_2 fixation starts at the end of the day, peaking in the middle of the night (Dron, Rabouille, Claquin, Le Roy, et al., 2012; Mohr et al., 2010). This is also the time of DNA synthesis (Dron, Rabouille, Claquin, Le Roy, et al., 2012). Cell division in *C. watsonii* occurs in the middle of the light period (Dron et al., 2013; Wilson et al., 2017) in parallel to peaks of carbon fixation and growth. The timing of these metabolic processes in cyanobacteria is often assumed to be regulated by the circadian clock based on the circadian-related *kai* genes (Shi et al., 2010), which are proposed to vary with light-driven changes in energy metabolism (Rust et al., 2011). However, in the case of *C. watsonii* WH8501, cyclic patterns of gene expression related to N_2 fixation (*nif*H), photosynthesis (*psa*C, *psb*A, and *psb*O), and circadian clock dynamics (*kai*A and *kai*C) continues in constant light (Mohr et al., 2013; Pennebaker et al., 2010).

PHOTOSYNTHESIS

The photosynthetic apparatus of *Crocosphaera watsonii* is comparable to other unicellular cyanobacteria (Zehr et al., 2007), such as the model *Synechocystis* sp. PCC 6803. There is strong diel periodicity both in *C. watsonii*'s structure and function. Like in other cyanobacteria, light is absorbed by pigments of photosynthetic antennae that involve membrane proteins containing chlorophyll *a* and the extramembrane phycobilisomes (Ting et al., 2002). The dominant phycobilins of *C. watsonii* are the short-wavelength-absorbing phycoerythrin and phycourobilin (Webb et al., 2009). This is in contrast to UCYN-C (*C. subtropica*; Mareš et al., 2019) which predominantly contains the longer-wavelength-absorbing phycocyanin. According to genome analysis, *C. watsonii* strains WH8501 and WH0401 lack the Orange Carotenoid Protein (OCP) that is required for the proper function of the energy dissipative photoprotection mechanism modulating the flow of energy from phycobilisomes to reaction centers in most of the sequenced cyanobacteria (Bao et al., 2017; Kerfeld et al., 2017). The OCP photoprotective mechanism is also absent in other ecologically important marine cyanobacteria from oligotrophic regions (*Prochlorococcus*, *Synechococcus*, *Trichodesmium*; Bao et al., 2017; Kelman et al., 2009), suggesting that the homeostasis of energy flow under conditions of severe nutrient limitations is achieved by regulation at other levels, e.g., by investing the excess energy into exporting carbon as EPS or TEP. We note that *C. watsonii* WH0003 does possess OCP (Kerfeld et al., 2017), so there is variability between strains in terms of photoprotection mechanisms.

Another mechanism known for optimizing the energy flow in cyanobacteria under changing light conditions is the so-called state transition (Kirilovsky et al., 2014), a process that modulates the coupling and distribution of absorbed energy flow from phycobilisomes to photosystems I and II (PSI and PSII). The presence and diel rhythms of state transitions have been reported for UCYN-C (*Crocosphaera subtropica*, formerly *Cyanothece*; Meunier et al., 1997). We have observed a similar diel rhythm for state transitions in *C. watsonii* (S. Rana et al., unpublished data). Recently, another photoprotective, energy-quenching mechanism mediated by the IsiA protein has been proposed to operate in cyanobacteria, not only under limited Fe concentrations but also under high light (Chen et al., 2017, 2021). *Crocosphaera watsonii* contains the *isi*A gene. Whether this photoprotective mechanism operates in *C. watsonii* under the low Fe and high light conditions that exist in oligotrophic areas remains to be elucidated.

The direct effect of illumination on decomposition and lack of recovery of PSII in *Crocosphaera watsonii* has been confirmed by biochemical protein analysis together with variable chlorophyll fluorescence measurements (Masuda et al., 2018; Rabouille & Claquin, 2016). The decline of PSII activity and decreased PSII abundance in the dark phase has been explained by a combination of monomerization of PSII and gradual disassembly of a large portion of PSII core complexes (Masuda et al., 2018). Specifically, PSII reaction center D1 protein (PsbA) abundance decreased in the dark, and the non-functional version of D1 (due to the absence of key amino acid residues essential for binding the oxygen-evolving $CaMn_4O_5$ clusters; the so-called "rogue" D1 or rD1; Murray, 2012; Sheridan et al., 2020; Wegener et al., 2015) was detected only in a small percentage of PSII during the dark period (Masuda et al., 2018). The role of rD1 remains unknown. Suppressed N_2 fixation activity under a subjective dark period (i.e., a time when cells were kept in a continous light condition and not returned to the expected dark phase; Mohr et al., 2013) together with a decreasing trend of N_2 fixation activity at the beginning of the light period (Compaoré &

Stal, 2010; Shi et al., 2010) suggests that nitrogenase synthesis is regulated by the circadian clock and is directly entrained by the energy metabolisms, as Rust et al. (2011) posited in relation to *Synechococcus elongatus*, as well as by inactivation of the nitrogenase complex by O_2 generated during photosynthesis.

QUANTITATIVE MODELING

There are a growing number of studies that use quantitative models to evaluate *Crocosphaera watsonii* biogeography and physiology. This research had its start with the numerical modeling of diazotroph biogeography as a function of light and resource limitation (Monteiro et al., 2010, 2011; Saito et al., 2011). For example, Saito et al. (2011) used a numerical model and proteomics experimental data from *C. watsonii*, to argue that modulation of iron-metalloenzyme inventory allowed *Crocosphaera* to inhabit a broader range of regions low in Fe, predicting a wide distribution in the subtropical gyres (Saito et al., 2011). Further use of numerical models in subsequent studies have been expanded to look at broader patterns of diversity and biogeography in diazotrophs in general and with *Crocosphaera*-like analogs (Dutkiewicz et al., 2015, 2020).

Separately, more detailed physiological models have been developed (Grimaud et al., 2014; Inomura, Deutsch, et al., 2019; Inomura, Masuda, & Gauglitz, 2019; Nicholson et al., 2018), which Inomura et al. (2020) have termed “coarse-grained models” (Inomura et al., 2020). These types of models started with the representation of C and N fluxes (Grimaud et al., 2014), and recently, O_2 and iron fluxes were included (Inomura, Deutsch, et al., 2019; Nicholson et al., 2018). Models focused on day-night cycles have shown that the many metabolic pathways in *Crocosphaera watsonii* can be explained by a cellular clock (Grimaud et al., 2014). In particular, the metabolisms of N_2 fixation, respiration, and photosynthesis are strongly coupled with the time-dependent allocation of intracellular Fe (Inomura, Deutsch, et al., 2019; Figure 5). Also, the temperature dependencies of respiration may explain why *C. watsonii* is constrained to warm regions (Church et al., 2009; Fu et al., 2014; Moisander et al., 2010): Lower temperature may increase the intracellular O_2 (Inomura, Deutsch, et al., 2019), which may prevent N_2 fixation. This trait differs from what is observed in UCYN-A: UCYN-A may tolerate low temperatures (Harding et al., 2018; Shiozaki et al., 2020; Shiozaki, Fujiwara, et al., 2018). Another coarse-grained model resolved broad-brush macromolecular allocation (Inomura, Masuda, & Gauglitz, 2019) based on the chemostat culture study of *C. watsonii* by Masuda et al. (2013). Inomura, Masuda, & Gauglitz (2019) showed the that by fixing nitrogen, *C. watsonii* can increase their population sizes and expand their niche despite the presence of ammonium. A recent model of O_2 diffusion coupled with simple metabolic fluxes suggested that a strong barrier against oxygen exists in *C. watsonii* (Inomura et al., 2017; Inomura, Deutsch, et al., 2019). This barrier is consistent with the idea that hopanoid-enriched membranes, which are a found in non-heterocyst-forming cyanobacterial diazotrophs like *C. watsonii*, might limit O_2 permeability and thus help to maintain N_2 fixation (Cornejo-Castillo & Zehr, 2019; Sáenz, 2010). Last, a recent model resolves a

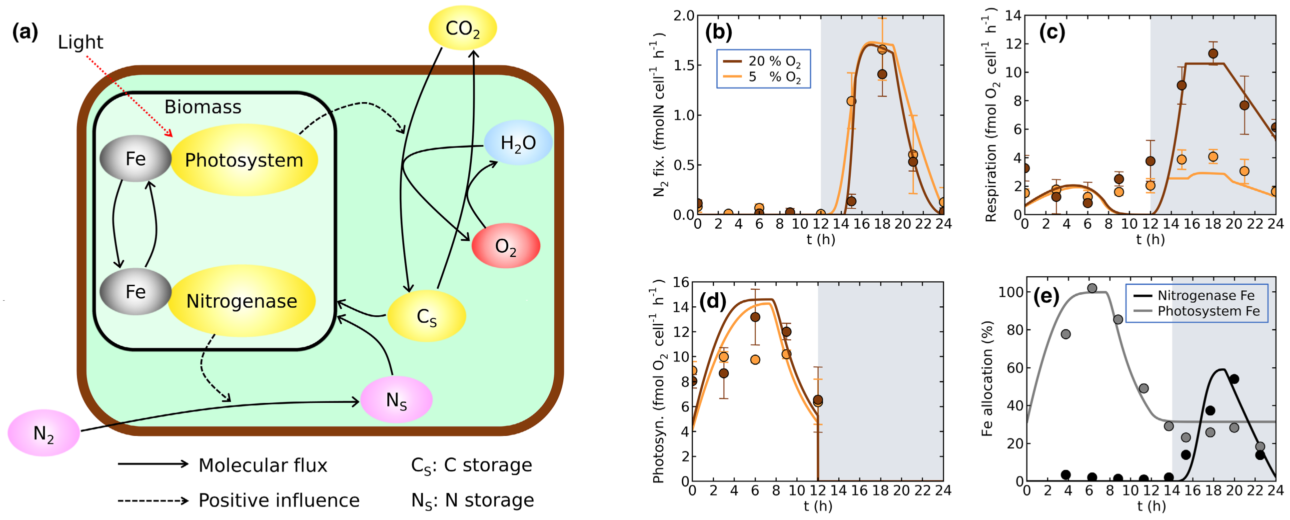

FIGURE 5 An example of a recent quantitative model of *Crocosphaera watsonii* (Cell Flux Model of *Crocosphaera*: Inomura, Deutsch, et al., 2019). (a) A schematic of the model. (b–e) Model results of diurnal patterns are compared with data. Model results and data are represented by curves and circles, respectively. (b–d) N_2 fixation, respiration, and photosynthesis under two O_2 conditions. Data are from Großkopf and LaRoche (2012). (e) Fe allocation within the cell. Gray shading indicates the dark period. Fe allocation data are from Saito et al. (2011). [Color figure can be viewed at wileyonlinelibrary.com]

heterogeneous metabolic population of *C. watsonii*, suggesting that heterogeneous N_2 fixation may reduce the C requirement of the population, extending its viable depth range (Masuda et al., 2020).

CONCLUSIONS

The availability of the environmental *nif*H sequences shed light on the previously unappreciated small unicellular cyanobacterial nitrogen fixer *Crocosphaera watsonii* (Zehr et al., 2001). Extensive field observations, recently compiled by Shao et al. (2023), have corroborated the wide distribution of UCYN-B *nif*H genes. In combination with chemical and physical parameters, we now know that UCYN-B *nif*H gene copies are highest in warm (greater than ~25°C), low nutrient surface waters; however, its distribution is not limited to these regions, and the regulation mechanisms of its distribution are not yet well known. Measuring the physiological responses of *C. watsonii* to chemical and/or physical changes in laboratory experiments, combined with theoretical modeling, is a promising new area of study. Thus, with the increasing amount of both field observations and controlling parameters, knowledge of the global distribution pattern of *C. watsonii* and its drivers is expected to increase rapidly in the near future.

Over the past decade, multiple approaches have been applied and extensive effort has been put toward estimating the contribution of *Crocosphaera watsonii* to global N_2 fixation, which has led to remarkable discoveries. In the future, it will be important to combine in situ observations, culture experiments, and modeling to better estimate the contribution of *C. watsonii* to global C and N cycling. For example, current UCYN-B *nif*H gene copy abundances do not directly represent cell abundances (Gradoville et al., 2022; Sargent et al., 2016; White et al., 2018), the ratios of *nif*H copies per cell abundances are likely different among different diazotroph groups, and not all the cells fix N_2, even though all cells fix C (Foster et al., 2013; Masuda et al., 2020; Mohr et al., 2013). Even though UCYN-B *nif*H gene copy measurements via qPCR are based on the detection of genomic DNA, the significant diel changes in DNA topology (Pennebaker et al., 2010), in gene expression, and in metabolic activities (Dugenne et al., 2020; Shi et al., 2010) indicate that the time of the day should be taken into account in future analyses of *C. watsonii* distribution. Furthermore, intriguing but not yet fully understood phenomena have been discovered, such as (1) intracellular recycling of Fe (Saito et al., 2011), photosystem II inactivation in the dark (Masuda et al., 2018; Rabouille & Claquin, 2016), (2) the division of metabolic activities in the cells of self-aggregates (Masuda et al., 2020), and (3) increased growth under co-limitation compared to single nutrient limitation (Yang et al., 2022). Although most of the studies have focused on the response of *C. watsonii* to physical and chemical conditions, we do not fully understand the effects of co-limitation (e.g., temperature, light, and nutrient source or multiple nutrient sources) nor know the full extent of the organisms that are grazing *C. watsonii* (Dugenne et al., 2020; Wilson et al., 2017).

Even though most of the culture studies have been based on the *Crocosphaera watsonii* WH8501 strain, there is intra-specific *C. watsonii* diversity (Bench et al., 2013). Together with continuous efforts to isolate new strains, the comparison of physiological studies based on multiple clonal cultures may be important. Furthermore, future models of oceanic nutrient cycling should consider the phylogenetic and physiological diversity of unicellular marine diazotrophs within the *Crocosphaera* genus as well as other related free-living and symbiotic lineages. In sum, the drastic diurnal variation in metabolisms, including the circadian clock, the relationship between photosynthesis and N_2 fixation, and intercellular heterogeneity in metabolic activity, make *C. watsonii* not only an important model for learning about diazotrophs but also a model organism for studying cyanobacteria in general.

AUTHOR CONTRIBUTIONS

Takako Masuda: Conceptualization (equal); data curation (equal); funding acquisition (equal); writing – original draft (equal); writing – review and editing (equal). **Jan Mareš:** Data curation (equal); resources (equal); writing – original draft (equal); writing – review and editing (equal). **Takuhei Shiozaki:** Data curation (equal); resources (equal); writing – review and editing (equal). **Keisuke Inomura:** Conceptualization (equal); visualization (equal); writing – original draft (equal). **Amane Fujiwara:** Data curation (equal); visualization (equal). **Ondřej Prášil:** Conceptualization (equal); funding acquisition (equal); writing – original draft (equal); writing – review and editing (equal).

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DATA AVAILABILITY STATEMENT

The data supporting this study are available on request from the corresponding author (TM).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1.

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