Quantifying Oxygen Management and Temperature and Light Dependencies of Nitrogen Fixation by *Crocosphaera watsonii*

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**ABSTRACT** *Crocosphaera* is a major dinitrogen (N\textsubscript{2})-fixing microorganism, providing bioavailable nitrogen (N) to marine ecosystems. The N\textsubscript{2}-fixing enzyme nitrogenase is deactivated by oxygen (O\textsubscript{2}), which is abundant in marine environments. Using a cellular scale model of *Crocosphaera* sp. and laboratory data, we quantify the role of three O\textsubscript{2} management strategies by *Crocosphaera* sp.: size adjustment, reduced O\textsubscript{2} diffusivity, and respiratory protection. Our model predicts that *Crocosphaera* cells increase their size under high O\textsubscript{2}. Using transmission electron microscopy, we show that starch granules and thylakoid membranes are located near the cytoplasmic membranes, forming a barrier for O\textsubscript{2}. The model indicates a critical role for respiration in protecting the rate of N\textsubscript{2} fixation. Moreover, the rise in respiration rates and the decline in ambient O\textsubscript{2} with temperature strengthen this mechanism in warmer water, providing a physiological rationale for the observed niche of *Crocosphaera* at temperatures exceeding 20°C. Our new measurements of the sensitivity to light intensity show that the rate of N\textsubscript{2} fixation reaches saturation at a lower light intensity (~100 μmol m\textsuperscript{-2} s\textsuperscript{-1}) than photosynthesis and that both are similarly inhibited by light intensities of >500 μmol m\textsuperscript{-2} s\textsuperscript{-1}. This suggests an explanation for the maximum population of *Crocosphaera* occurring slightly below the ocean surface.

**IMPORTANCE** *Crocosphaera* is one of the major N\textsubscript{2}-fixing microorganisms in the open ocean. On a global scale, the process of N\textsubscript{2} fixation is important in balancing the N budget, but the factors governing the rate of N\textsubscript{2} fixation remain poorly resolved. Here, we combine a mechanistic model and both previous and present laboratory studies of *Crocosphaera* to quantify how chemical factors such as C, N, Fe, and O\textsubscript{2} and physical factors such as temperature and light affect N\textsubscript{2} fixation. Our study shows that *Crocosphaera* combines multiple mechanisms to reduce intracellular O\textsubscript{2} to protect the O\textsubscript{2}-sensitive N\textsubscript{2}-fixing enzyme. Our model, however, indicates that these protections are insufficient at low temperature due to reduced respiration and the rate of N\textsubscript{2} fixation becomes severely limited. This provides a physiological explanation for why the geographic distribution of *Crocosphaera* is confined to the warm low-latitude ocean.

**KEYWORDS** *Crocosphaera*, carbon, cell flux model, daily cycle, iron, light, nitrogen, nitrogen fixation, oxygen, photosynthesis, temperature

Nitrogen (N) availability is recognized as a growth-limiting factor for primary producers in the ocean (1–4), controlling the flow of carbon (C) through the ecosystem (5–7). Dinitrogen fixation (N\textsubscript{2} fixation) represents an important source of N that is...
estimated to account for nearly 50% of the fixed N input in the ocean (7). The microorganisms that fix N\textsubscript{2} are termed “diazotrophs” and are physiologically diverse, including unicellular, filamentous, and heterocystous cyanobacteria with life strategies that include symbiotic, free-living, and colonial forms (8). *Crocosphaera watsonii* is a major phototrophic diazotroph and makes a significant contribution to the pool of fixed N in oligotrophic environments of the subtropical and tropical Atlantic and Pacific oceans (9–12). Observations show that the niche of *Crocosphaera* is limited to the region above 20°C (12, 13). Similarly, laboratory culturing experiments show that *Crocosphaera* grow only above 20°C (14, 15).

The enzyme responsible for N\textsubscript{2} fixation, termed “nitrogenase,” is highly sensitive to oxygen (O\textsubscript{2})(16, 17), thus necessitating careful O\textsubscript{2} management by diazotrophs (18). In particular, *Crocosphaera* is a cyanobacterium with oxygen-producing photosynthesis, and fixing both C and N\textsubscript{2} simultaneously would be a challenge. To circumvent the potential problem caused by O\textsubscript{2} production, *Crocosphaera* fix N\textsubscript{2} predominantly during the dark period in both laboratory (19–21) and natural (22) populations, thereby temporally segregating N\textsubscript{2} fixation from O\textsubscript{2}-producing photosynthesis. However, O\textsubscript{2} in the ocean is mostly saturated (~200 μM in low latitudes) (23), and diurnal fluctuation of O\textsubscript{2} is relatively small (~10 μM) (24). Thus, even during the dark period, cells are likely to be exposed to a considerable influx of O\textsubscript{2}. Even at 5% O\textsubscript{2} (~50 μM), nitrogenase activity decreases to less than 30% within 20 min (17). Thus, questions remain about how *Crocosphaera* protects nitrogenase against O\textsubscript{2}.

O\textsubscript{2} management by *Crocosphaera* must be considered within the context of their distinct daily physiological cycle. One metabolic strategy to constrain O\textsubscript{2} is to sustain high rates of respiration in excess of the energetic demand during the night by using C stored from photosynthesis during the day (25). This distinct physiological cycle is paralleled by management of intracellular iron (Fe). *Crocosphaera* shuttles intracellular Fe between photosystems and nitrogenase during the daytime and the dark period to support photosynthesis and N\textsubscript{2} fixation, respectively. The nitrogenase complex in particular is completely degraded and resynthesized each day (21). Despite the significance of the intracellular Fe cycling, the quantitative models have not mechanistically included the cycling or have they linked it with other metabolisms, i.e., those affecting C, N, and O\textsubscript{2}. Developing a model which explicitly links Fe cycling to cellular metabolisms allows us to test how O\textsubscript{2} can be managed within the context of the distinct daily cycle of Fe, C, N, and O\textsubscript{2}.

**Physiological model of *Crocosphaera***.

First, we describe a new model of the C, N, Fe, and O\textsubscript{2} budgets of *Crocosphaera*, and we then use it as a tool to explore the role of different O\textsubscript{2} protection mechanisms. A more detailed description of the model is found in Materials and Methods and in Text S1 in the supplemental material.

**Simulating the daily cycle of metabolism based on Fe translocation.** We quantify the daily metabolic cycle for *Crocosphaera* by developing a coarse-grained model of *Crocosphaera* (cell flux model of *Crocosphaera* [CFM-Croco]), as depicted in Fig. 1 (see Materials and Methods and Text S1 for details). A previous dynamic model of *Crocosphaera* was developed to examine the daily cycle of C and N in *Crocosphaera* driven by a cellular clock and time-dependent functions (26). In our model, we explicitly link Fe cycles to C, N, and O\textsubscript{2} metabolisms. As a starting point, we assume the total Fe constant within the cell and simulate the temporal variation of the fraction of Fe in different pools. During the light period, a predominant amount of Fe exists in photosystems contributing to photosynthesis. After sunset, Fe moves to nitrogenase, increasing the rate of N\textsubscript{2} fixation. Before sunrise, Fe moves back to photosystems, preparing for daytime photosynthesis. We linearly link the amount of Fe in photosystems with the rate of photosynthesis and that in nitrogenase with the rate of N\textsubscript{2} fixation leading to diurnal fluctuation of these metabolic rates. During the daytime, with photosynthesis, C storage (starch) is accumulated and fuels nitrogen fixation during the night. Night-time metabolism also includes respiratory depletion of intracellular O\textsubscript{2}, which depends on the amount of C storage and the temperature-dependent metabolic capacity.
Quantifying the rate of $N_2$ fixation. $N_2$ fixation is modeled as explicitly dependent on the intracellular concentration of nitrogenase, the size of the intracellular carbohydrate and fixed-N stores, the intracellular $O_2$ concentration, and the temperature. During the dark period at a fixed temperature, the rate of $N_2$ fixation ($N_{2fix}$, mol N cell$^{-1}$ h$^{-1}$) is assumed to be affected by the fluctuation of Fe, the respiratory depletion of intracellular $O_2$, and the storage of N and C as depicted in Fig. 2:

$$N_{2fix} = A_{N2fix}FeNf_N(C_S, N_S, O_2^{cell})$$ (1)

where $A_{N2fix}$ is a rate constant (mol N mol Fe$^{-1}$ h$^{-1}$), $FeN$ is the mass of Fe in nitrogenase (mol Fe cell$^{-1}$), and $f_N(C_S, N_S, O_2^{cell})$ scales the rate of N fixation between zero and its maximum value per nitrogenase ($A_{N2fix}$), based on the available C storage ($C_S$; mol C cell$^{-1}$) and N storage ($N_S$; mol N cell$^{-1}$) and the presence of intracellular $O_2$. 

![Diagram of modeled C, N, O$_2$, and Fe fluxes.](image1)

**FIG 1** Schematics of modeled C, N, O$_2$, and Fe fluxes. (A and B) C, N, and O$_2$ fluxes for light and dark periods, respectively. Black solid arrows represent material fluxes, red dashed arrows represent energy fluxes, and a black dotted arrow represents photon flux. C-based molecules are in yellow, N-based molecules are in pink, $O_2$ is in red, and $H_2O$ is in blue. $C_S$ and $N_S$ C and N storage, respectively; Chl, chlorophyll. (C and D) Fe fluxes (black solid arrows) for light and dark periods, respectively. The large circular frame indicates the cellular boundary.

![Graphs showing how each factor influences $N_{2fix}$ in the model.](image2)

**FIG 2** Schematics of how each factor influences $N_{2fix}$ in the model. (A) $FeN$; (B) $C_S$; (C) $N_S$; (D) $O_2^{cell}$. $N_S^{max}$ is the maximum N storage capacity, and $O_2^{cell}_{cri}$ is the critical $O_2$ concentration.
(O$_2^{\text{cell}}$; mol O$_2$ cell$^{-1}$). Increasing nitrogenase concentrations (as proxied by Fe$_{\text{N}}$) increase the encounter rate of N$_2$ gas, with nitrogenase proportionally/linearly increasing N$_2$fix (Fig. 2A). C$_5$ positively influences N$_2$fix, since it provides energy for N$_2$ fixation. We have assigned a saturating dependence (Michaelis-Menten type curve) to C$_5$ (Fig. 2B), the C substrate. On the other hand, we assume that N$_8$ negatively influences N$_2$ fixation (Fig. 2C), as reactive N is often observed to inhibit N$_2$ fixation (27–29). Intracellular O$_2$ (O$_2^{\text{cell}}$) also negatively influences N$_2$ fixation, since the proteins in the nitrogenase complex are sensitive to O$_2$ (16, 17) (Fig. 2D). We assign a critical O$_2$ concentration (O$_2^{\text{crit}}$) above which N$_2$ fixation does not occur, below which we assumed that N$_2$fix increases linearly with decreasing O$_2^{\text{cell}}$. This assumption is to represent an in vitro experiment of nitrogenase activities, where there are rather gradual negative correlations between O$_2$ and activities of nitrogenase subunits (17).

**Simulating O$_2$ management.** To simulate the intracellular O$_2$ concentration, we assume a balance between diffusive flux into the cell and the respiratory O$_2$ consumption within it, which can be expressed as follows (18):

\[
[O_2^{\text{cell}}] = [O_2] - \frac{r^2 \gamma_{\text{net}}}{3 \kappa_{O_2}}
\]

where [O$_2^{\text{cell}}$] (mol O$_2$ m$^{-3}$) represents the intracellular concentration of O$_2$, [O$_2$] (mol O$_2$ m$^{-3}$) is the environmental concentration of O$_2$, $r$ is the cell radius (m), $\gamma_{\text{net}}$ is the net respiration rate (respiration rate – photosynthesis) per cell volume (mol O$_2$ m$^{-3}$ s$^{-1}$), and $\kappa_{O_2}$ is the effective O$_2$ diffusion coefficient (m$^2$ s$^{-1}$) which accounts for the diffusivity in both the molecular boundary layer surrounding the cell and a semipermeable cell membrane layers. In order to minimize [O$_2^{\text{cell}}$], cells may live in low [O$_2$] environments, increase cell size (increasing $r$), increase respiration (increasing $\gamma_{\text{net}}$), or decrease O$_2$ diffusivity through the cell membrane layers (decreasing $\kappa_{O_2}$).

**Temperature dependence of metabolic processes.** To study why *Crocosphaera*'s niche exists mostly above 20°C, we use a commonly used temperature factor [$f_T(T)$] based on the Arrhenius equation (30, 31):

\[
f_T(T) = \exp \left( A_1 \left( \frac{1}{T_{\text{ref}}} - \frac{1}{T} \right) \right)
\]

where $T$ is temperature (K), $T_{\text{ref}}$ is a reference temperature (K), and $A_1$ is a constant factor (K$^{-1}$). This factor independently modulates three key metabolic functions, namely, N$_2$ fixation, photosynthesis, and respiration, and simulates the daily integrated rates of metabolisms. We explore the significance of the temperature dependence of each metabolic component.

**Quantifying light dependence of metabolisms with laboratory measurements.**

We model the dependence of photosynthesis on light using the commonly employed saturating functional form (32, 33) with photoinhibition:

\[
f_l(I) = 1 - e^{\lambda I} - \Omega(I)
\]

where $I$ is light intensity (μmol m$^{-2}$ s$^{-1}$), $A_1$ is a light absorption/processing factor (μmol$^{-1}$ m$^2$ s), and $\Omega(I)$ is a photoinhibition term (dimensionless). We have also conducted laboratory measurements of N$_2$ fixation rates and photosynthetic electron transfer rates by *Crocosphaera watsonii* WH8501 for various light intensities (see Materials and Methods) and compared them with the model.

**RESULTS AND DISCUSSION**

**Analysis of daily metabolic cycles.** We have simulated time-dependent laboratory cultures of *Crocosphaera* (25) and the linked Fe allocation within the cell (21). The model accurately predicted rates of photosynthesis and respiration both qualitatively and quantitatively for different O$_2$ concentrations in the culture (Fig. 3A): 20% (186 μM, the normal atmospheric composition and surface ocean concentration in tropics) and 5% (46 μM, one-quarter of the normal composition), as used in the previous laboratory experiment (25). The rate of photosynthesis was maximal during the middle of the day for both 20% and 5% O$_2$ values.
The rate of photosynthesis was correlated with Fe cycles, since the rate is proportional to Fe in photosystems (FeP) (Fig. 3F); as the sun rises, Fe moves from the buffer (an intracellular Fe storage, e.g., ferritin and bacterioferritin [21]) to the photosystems, but in the afternoon, it starts moving back to the buffer, which was predicted by relating Fe to CS. As CS increases and approaches maximum storage levels, there is no benefit to further photosynthesis. Therefore, the amount of photosystems was downgraded, and Fe was moved back to the buffer. We predicted a limited difference in photosynthesis between 5% and 20% O2, consistent with laboratory data.

We also reproduced the observed daily cycle of respiration and showed that higher respiration rates occurred at 20% O2 during the dark period due to respiratory protection (25). Because of this respiratory protection, intracellular O2 concentrations decreased to almost zero during the middle of the night (Fig. 3B), leading to peak N2 fixation during this period (Fig. 3C). The data showed that N2 fixation increased more quickly at 5% O2 than at 20% O2 during the early dark period and can be explained by the concentration of intracellular O2. Based on the model, cellular O2 was eliminated more quickly at 5% (Fig. 3B), reaching zero before 14 h, while such elimination occurs after 15 h for 20% O2, thereby delaying N2 fixation. This delay in N2 fixation under normal O2 conditions is widely observed both in the laboratory (19, 34) and in natural populations (22). This delay may reflect the extra time required for O2 elimination, given that Crocosphaera’s nifH gene is transcribed following the initiation of the dark period (19, 21, 22), and both the model and laboratory data showed a much smaller delay in N2 fixation in a low-O2 environment. We capture this trend with the O2 dependence of N2 fixation (equation 1) (Fig. 2D); at 5% O2, the intracellular O2 is depleted quickly (Fig. 3B) and thus the rate of N2 fixation increases earlier (Fig. 3C).

During the dark period, as the nitrogenase enzyme is synthesized, Fe moves from the buffer to nitrogenase, initiating N2 fixation. As the dark period approaches dawn, Fe
begins moving back to the buffer, preparing for daytime photosynthesis through
degradation of the nitrogenase protein complex (19, 21), accompanied by decreased
respiration (Fig. 3A), leading to the drop in the rate of N₂ fixation (Fig. 3C) and increased
intracellular O₂ (Fig. 3B). We can use the model to consider how Crocosphaera’s diel
cycle might be regulated. If the C storage reservoir were to be the trigger for Fe transfer,
we would expect C storage to be significantly reduced during the dark period.
However, even at the end of the dark period, we predict a significant amount of C
storage remaining in the cell. If N storage is the trigger, Fe should start moving earlier
in the case of 5% O₂ than in the case of 20% O₂, as the cell fixes N₂ faster under low
O₂. The data show, however, that the rate of N₂ fixation drops at almost the same time
for the two O₂ cases. Also, the peak of N₂ fixation appears during similar time ranges
among different studies (19, 21, 34). Finally, since Crocosphaera maintains the daily
cycle even under continuous light (35, 36), it seems that the Fe transfer within Crocosphaera
is largely controlled by a circadian clock that regulates key cellular functions. To further
examine what controls Fe transfer, higher-resolution measurement of N₂ fixation (34)
under various O₂ concentrations would also be useful.

Based on the metabolic rates (respiration, photosynthesis, cellular growth, and N₂
fixation), we computed the cellular C and N quotas (Fig. 3D and E). During the light
period, the cells accumulate C, while during the dark period, C storage decreases due
to respiration and N₂ fixation. During the light period, the data show that C accumulation is slightly greater for 20% O₂ than for 5% O₂; the model predicts this trend, with
increased cell size for higher O₂ (thus, larger cell size for 20% O₂). During the dark
period, however, it gets lower for 20% O₂ than for 5% O₂ due to higher respiration for
O₂ management. Due to the larger cell size (discussed in “Sensitivity studies”), cells
under 20% O₂ have higher N. However, at the end of the dark period, cellular N levels
under these different O₂ concentrations get closer since the rate of N₂ fixation is higher
for 5% O₂. The slight decrease in N during the early light period is due to cell division.

Sensitivity studies. In the following three sections, we describe sensitivity studies
performed with the model to probe the significance of different O₂ protection strate-
gies. To examine the effect of each strategy, we specifically turned off each O₂ manage-
ment mechanism (size change, respiratory protection, and diffusion management) and the
model results (cellular C, N, O₂ fluxes, and N₂ fixation rates) were compared with the default
run with all the mechanisms present.

Relationship between cell size and O₂. An increase in cell size is a potential
physiological strategy for Crocosphaera, since it decreases the surface-to-volume ratio,
thereby decreasing passive O₂ uptake per volume (equation 2) (18). Adjusting the cell size
based on O₂ concentrations facilitates replication of the laboratory data (Fig. 4).
During the light period, if the cell size is independent from O₂ concentration, the model shows identical values for C and N per cell for different O₂ concentrations, while the data show generally higher values for 20% O₂; as a result, more predictions are outside the error bars of the data, especially for C per cell (Fig. 4A). During the dark period, the data show a reverse effect in relation to C: the C per cell in 20% O₂ starts at a higher value but ends up at a lower value. This trend was reproduced only by including the size variation (Fig. 4A). N per cell is almost the same value at the end of the dark period, but the model with a fixed cell size shows much lower values in 20% O₂ outside the error bar (Fig. 4B). These results indicate that the cells acclimate to higher O₂ environments by adjusting their size. Recent studies have shown that there are two size classes of Crocosphaera (14, 22, 37). The model indicates that the larger cells have a significant advantage in O₂ management and might be a result of adapting to the high O₂ environments widespread in oceanic surface waters.

Respiratory protection against O₂. In order to examine whether respiratory protection is essential, we ran the model without respiratory protection (Fig. 5; see Fig. S1 in the supplemental material). In this case, respiration serves only to provide energy for N₂ fixation. At 5% O₂, the N₂ fixation rate is almost the same as that for the simulation with respiratory protection (Fig. 5B). Once the dark period initiates and photosynthesis stops, the cellular O₂ concentration drops low enough for N₂ fixation. As N₂ fixation initiates, respiration increases to provide energy, further decreasing intracellular O₂ to zero, until N₂ fixation peaks. On the other hand, at 20% O₂, even after the initiation of the dark period, the cellular O₂ concentration was still high (Fig. S1A), preventing N₂ fixation (Fig. 5B). The respiration rate was much lower than the laboratory values (Fig. 5A), especially at 20% O₂. Since there is no flux that consumes C at 20%, the model overestimated intracellular C (Fig. S1B). Together these results imply that respiratory protection is occurring in Crocosphaera and is essential for N₂ fixation at normal O₂ concentrations in the environment.

Diffusion management. A model-data comparison indicates that the diffusivity of the cell membrane layers must be extremely low relative to both the diffusivity in the molecular boundary layer and to the diffusivity of the cell membrane layers inferred from other N₂-fixing organisms. To achieve the results illustrated in Fig. 3, the effective diffusivity of O₂ across the cell membrane layers must be set to 1/(6.45 × 10⁴) of the diffusivity of O₂ in water. Previous studies of the heterotrophic and photoautotrophic diazotrophs Azotobacter vinelandii and Trichodesmium also inferred low cell wall permeability [1/(1.27 × 10⁴)] (18, 38) and [1/(1.60 × 10⁴)] (39) of the diffusivity of O₂ in water, respectively, but the above predicted value for Crocosphaera is even lower. In contrast, the permeability of non-N₂-fixing bacterial cells is much higher [1/(5.30 × 10²)] (40).
Applying the value inferred for *Azotobacter* results in poor simulations of the laboratory data (Fig. S2), significantly overestimating the respiration at 5% O2 (Fig. S2A) and suppressing N2 fixation (Fig. S2B) because the O2 influx could not be matched. We conclude that *Crocosphaera* and other N2-fixing microbes necessarily control the cell wall permeability for O2; otherwise, N2 fixation would be impossible. However, the required protection varies between species, and the details of this adjustment need to be further investigated.

One possibility is that carbohydrate storage may act as an O2 barrier. As shown previously (41), during cell division, starch granules are accumulated near the cell membranes rather than spread evenly in the cytoplasm. Our original electron microscopy images of *Crocosphaera* ultrathin sections revealed that the location of the granules close to the membranes is well preserved in both the light and dark periods (Fig. 6). Since the starch granules are relatively rigid and have dense hydrophilic structures, it is likely that they act as a barrier against O2 during the night. The images also show thylakoid membranes surrounding the granules (Fig. 6) (see also reference 41). Since respiration occurs on the thylakoid membranes as well as cellular membranes (42), such localization may make it possible for the cells to consume O2 before it reaches the inner cytoplasm, as well as further physically decreasing the diffusivity of O2.

Another possible barrier against O2 diffusion is the production of extracellular polymeric substances (EPS), which may create a thick hydrophilic layer, where the diffusion of O2 molecules is reduced. It has been hypothesized that *Azotobacter vinelandii* excrete alginate (one kind of EPS) to decrease the passive O2 uptake and thus protect nitrogenase (43). Its effect on N2 fixation has been further studied using quantitative modeling (18) and laboratory studies (44), supporting the hypothesis. In a batch culture, *Crocosphaera* produce EPS roughly proportional to their growth (37) and the production of EPS increases during the dark period (41). Given these findings, we hypothesize that the EPS produced by *Crocosphaera* plays an important role in reducing O2 diffusion. Recent observations suggest that hopanoid lipids may also play a role in diffusion management, as they have biochemical properties that potentially decrease...

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**FIG 6** Transmission electron micrographs of *Crocosphaera* cells harvested at the 6-h time point during the light period (A to C) and at the 6-h time point during the dark (D to F). Starch granules (SG) and thylakoid membranes (THY) are observed mostly on the edge of the cytosol. More-detailed images (C and F) show that SG are observed mostly between THY.
the permeability to extracellular O\textsubscript{2} and hopanoid synthesis genes are specifically observed in non-heterocyst-forming cyanobacterial diazotrophs (45).

Temperature dependence of N\textsubscript{2} fixation. As we illustrate below, the model suggests that the temperature dependence of N\textsubscript{2} fixation, and thus the fitness of Crocosphaera, is largely explained by the temperature dependence of respiration. N\textsubscript{2} fixation by Crocosphaera is observed to have a strong dependence on temperature. Cell-specific rates of N\textsubscript{2} fixation are maximal at approximately 30°C and decrease to almost zero at 22°C (15). These laboratory-derived physiological observations are supported by field observations of Crocosphaera being most prevalent in warm oceanic regions above 20°C (12, 13).

In the model, N\textsubscript{2} fixation, photosynthesis, and respiration are each modeled with independent, Arrhenius equation-like temperature dependence (equation 3). By employing these in combination, the model replicates the observed temperature dependence of N\textsubscript{2} fixation (Fig. 7A). We tested which temperature dependence has the strongest effect by applying equation 3 to only one of the metabolisms (Fig. 7B). The results show that the temperature dependence on respiration has the strongest effect, closely representing the predicted results with all the temperature dependences. This indicates that the negative effect of temperature on N\textsubscript{2} fixation is largely due to decreased rates of respiration being insufficient to draw down intracellular O\textsubscript{2}, and therefore nitrogenase is unable to fix N\textsubscript{2}. A decrease in the rate of respiration will also decrease the supply of energy for nitrogenase. Decreased temperature will also have effects on other related metabolic processes, such as a direct effect on the rate of enzymatic activity of nitrogenase and a decrease in photosynthesis and therefore less C storage for respiration (Fig. 7B). All of these impacts, however, turned out to be smaller than the decrease in respiratory protection against O\textsubscript{2}.

The rate of N\textsubscript{2} fixation maximized at moderate light intensity. To examine light dependence on N\textsubscript{2} fixation for Crocosphaera, we have simulated the rate of N\textsubscript{2} fixation at various light intensities. In the model, the light intensity influences the rate of photosynthesis based on the equation of light saturation and photoinhibition (equation 4). The model shows that the rate of N\textsubscript{2} fixation increases at low light intensity due to increased photosynthesis and, thus, increased C storage (Fig. 8A). However, despite photosynthesis rates increasing with light intensities up to \textasciitilde 700 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1} (Fig. 8B), N\textsubscript{2} fixation saturates at a relatively low light intensity (\textasciitilde 100 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1}) (Fig. 8A), since it becomes limited by the availability of nitrogenase (here proxied by Fe\textsubscript{N}). This prediction is confirmed by our original measurements of the rates of
Acetylene reduction (proxy for N₂ fixation) and ETR (photosynthetic electron transfer rate) for various light intensities from this study. Above 140 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), the rate of N₂ fixation becomes stable despite the significant increase in ETR.

Our measurements also show that above certain light intensities, both of the rates (N₂ fixation and photosynthesis) start dropping (Fig. 8), likely due to photoinhibition. These results may explain why the maximum population of *Crocosphaera* is often observed below the surface but above a depth of 50 m (12, 13), where they can receive moderate light intensities (100 to 400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)), enough for N₂ fixation without photoinhibition. The model resolves photoinhibition, capturing the trend in ETR (Fig. 8B). However, it does not predict the observed decrease in N₂ fixation under strong light, despite the drop in photosynthesis above 700 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), meaning that the rate of photosynthesis or the level of C storage cannot explain the decreasing trend in N₂ fixation (Fig. 8A). This indicates that high light intensity rather directly inhibits nitrogen fixation, potentially by damaging machinery for nitrogenase synthesis or subunits of nitrogenase before its assembly.

**Conclusions.** We have developed a coarse-grained model of *Crocosphaera* (CFM-Croco) and simulated a daily cycle of *Crocosphaera* metabolism with distinct C, N, O₂, and Fe fluxes. The model mechanistically links the diurnal cycle of Fe with that of C, N, and O₂, reproducing published observations (21, 25). This indicates that including the Fe cycle is essential for simulating the diurnal cycle of *Crocosphaera* metabolism and nutrient fluxes. The model results suggest that *Crocosphaera* employs multiple mechanisms to manage intracellular O₂: size change, respiratory protection, and decreased diffusivity. Since respiratory O₂ management is crucial for N₂ fixation, the temperature dependence of respiration has a significant impact on N₂ fixation, which provides a hypothesis for the strong temperature constraint on their growth and niche in the environment. The light dependence of photosynthesis does not give an advantage to N₂ fixation under extremely high light due to photoinhibition and limitations on nitrogenase concentration, the latter of which may be constrained by the intracellular space. This indicates that the optimum depth is likely not at the very surface, despite the potentially highest availability of Fe dust.

**MATERIALS AND METHODS**

**Cell flux model of *Crocosphaera***. Here, we describe the algorithms employed to implement the model. The model schematic is depicted in Fig. 1. Time-dependent equations are given to describe the rates of change in each of the macromolecular pools (Table 1). The fluxes between macromolecular pools are quantified at each time step. The time-dependent equations (Tables 1 and 2) are advanced in finite time steps, updating the status of the cells. Parameter values and nomenclature with units are available in Tables S1 and S2 in the supplemental material, respectively.

**C, N, and O₂ metabolism.** In order to compute the time variation of intracellular C, N, and O₂ pools and the cell density, we consider the chemical fluxes that impact them. Specifically, we include time variation of C<sub>c</sub>, cell population density (X) based on biomass production, N<sub>p</sub>, and O₂ (equations given in...
TABLE 1 Fundamental relations of C, N, and O₂-based molecules

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<th>Equation no.</th>
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</thead>
<tbody>
<tr>
<td>( \frac{dC}{dt} = P_{\text{Chl}} - \lambda(1 + E) - \frac{P_{\text{CO}<em>2}^{\text{N2fix}}}{P</em>{\text{CO}_2}} - \text{Exc} )</td>
<td>5</td>
</tr>
<tr>
<td>( \frac{dX}{dt} = \frac{\lambda X}{Q_c} )</td>
<td>6</td>
</tr>
<tr>
<td>( \frac{dN}{dt} = N_{\text{bio-all}} - \lambda Y_{\text{bio-all}} )</td>
<td>7</td>
</tr>
<tr>
<td>( \frac{dO_2}{dt} = P_{O_2} - R_{O_2} + V_{O_2} )</td>
<td>8</td>
</tr>
</tbody>
</table>

\( C_\text{st}, \text{C storage}; t, \text{time}; P_{\text{Chl}}, \text{photosynthesis rate per chlorophyll}; \text{Chl}, \text{chlorophyll}; \lambda, \text{biomass production rate}; E, \text{conversion factor of biomass production to biosynthetic CO}_2 \text{ production}; N_{\text{bio}}, \text{N2 fixation rate}; P_{\text{CO}_2}^{\text{N2fix}}, \text{CO}_2 \text{ production due to N2 fixation}; P_{\text{CO}_2}^{\text{R}} \text{, CO}_2 \text{ production due to respiratory protection}; \text{Exc}, \text{C excretion rate}; X, \text{population density of cells}; Q_c, \text{cellular C quota}; N_p, \text{N storage per cell}; Y_{\text{bio-all}} \text{, NC of biomass including storage}; O_2, \text{O}_2 \text{ per cell}; P_{\text{O}_2}, \text{O}_2 \text{ production rate}; R_{\text{O}_2}, \text{respiration rate}; V_{\text{O}_2}, \text{O}_2 \text{ exchange by diffusion.} 

Table 1 and schematics given in Fig. 1A and B. The balance in C storage pool \( C_\text{st} \) (equation 5) is based on the balance among photosynthesis \( (P_{\text{Chl}}, \text{biomass production (\( \lambda \)), biosynthetic CO}_2 \text{ production (\( E \))}, \text{CO}_2 \text{ production for N2 fixation (\( P_{\text{CO}_2}^{\text{N2fix}} \))}, \text{CO}_2 \text{ production due to respiratory protection (\( P_{\text{CO}_2}^{\text{R}} \))}, \text{and C excretion (\( \text{Exc} \))}. \) Cellular C and N are the sum of the baseline biomass and the C and N storages, respectively.

We assume that biomass production is used for the production of new cells, which drives the time change in population density (equation 6). Biomass production is supported not only by C but also by N. Thus, we consider the effect of biomass production on \( N_\text{st} \) (\( \lambda Y_{\text{bio-all}} \)) (equation 7), which is balanced by \( N_\text{fix} \) (\( N_{\text{bio}} \)). Finally, the \( O_2 \) budget is based on photosynthesis \( (O_2 \text{ production rate (\( P_{\text{O}_2} \))}) \), the respiration rate \( (R_{\text{O}_2}) \), and the diffusive exchange of \( O_2 \) \( (V_{\text{O}_2}) \) (equation 8).

Since the metabolism differs between the light and dark periods, we employed different parameterizations of the fluxes in Table 1 at different times of day. Specifically, \( P_{\text{Chl}} \), \( \lambda \), \( \text{Exc} \), and \( P_{\text{O}_2} \) are unique to the light period, and \( N_{\text{bio}}, P_{\text{CO}_2}^{\text{R}} \), and \( P_{\text{CO}_2}^{\text{N2fix}} \) are specific to the dark period.

To solve the model equations, we have applied a finite-difference method to equations 5 to 7. Since the time scale of \( O_2 \) concentration is small relative to that of other metabolites \( (C, N, Fe) \), we have assumed a pseudo-steady state; thus, \( O_2 \) uptake and \( O_2 \) production are always balanced by respiration. For the calculation of fluxes that influence the time variation of each pool, we consider the size of the elemental pools and \( O_2 \) concentrations (the details of the flux calculations are described in Text S1 in the supplemental material).

**Fe metabolism.** The rates of C and N fixation both depend on the Fe allocation to the enzymes which mediate those processes. Here, we model the time-dependent allocation to those Fe pools. The time-dependent equations for the Fe system are given in Table 2. We assume that the exchange of Fe
between photosystems and nitrogenase is mediated by an Fe buffer, such as bacterioferritin protein (Fig. 1C and D) (21). Thus, the time variation of Fe in the photosystems is based on its exchange with the buffer Fe pool (equation 9). The buffer Fe pool is influenced not only by the Fe from the photosystems but also by the exchange of Fe with nitrogenase (equation 10). The Fe allocation to nitrogenase results from the balance between the loss to and gain from the buffer (equation 11).

**Connecting Fe fluxes to C, N, and O₂ fluxes.** The amount of Fe in photosystems (Feₚ) proportionally influences photosynthesis, thus impacting C and O₂ fluxes. We have assumed that the ratio of chlorophyll to Fe in a photosynthetic apparatus (mol C mol Fe⁻¹) is constant (Y₈₅/₈₅Fe). Thus, the balance in chlorophyll is proportional to the balance in photosystem Fe (equation 12). The amount of chlorophyll in turn influences the rate of photosynthesis (equation 5). The rate of N₂ fixation (N₂fix) is assumed to be proportional to the amount of nitrogenase proxied by Fe in nitrogenase (Feₙ). Thus, through the rate of N₂ fixation, Feₚ influences C and N fluxes, and through the associated respiration providing energy for N₂ fixation, it can influence O₂ fluxes.

In order to calculate the amount of Fe pools and chlorophyll, we applied a finite-difference method to equations 9 to 12. For the computation of Fe fluxes, we considered various factors, such as the size of the Fe pool of the origin and the destination, time, O₂ concentration, and carbohydrate storage. Fe fluxes are parameterized based on these factors to reproduce the laboratory observations (21) (Fig. 3F) (see Text S1 in the supplemental material).

**Differentiating light and dark periods.** To reflect a distinct diurnal cycle of *Crocosphaera*, we resolve differences in metabolic configuration during the day and night; some fluxes in Tables 1 and 2 are specific to a certain time of day. The schematics of which flux applies to each time period are illustrated in Fig. 1. The following section broadly describes the day-night differentiation. The detailed fluxes are described in Text S1.

**Light period.** During the light period, cells can harvest light and fix C, accumulating C storage and producing biomass (Fig. 1A). However, N₂ fixation is small and accordingly respiratory protection is also small. For the N source for biomass production, the cell relies on N storage. To reflect this, all the terms in equations 5 to 8 (Table 1) are used except Nₘₚ, N², and NCO₂ (–0). Also, we assume that the translocation of Fe from the buffer to nitrogenase (F₈₅) is –0, since no Feₚ was observed during the light period (21); this assumption depletes Feₚ (Fig. 1C).

**Dark period.** During the dark period, photosynthesis does not occur, but the cell uses stored C for respiration and N₂ fixation (Fig. 1B). Also, we assume that biomass production and excretion do not occur. Thus, in equation 5, P = λ = Exc = 0. This assumption allows accumulating N storage with N₂ fixation, as observed previously (25). According to the observed targeted proteomics (21), there is limited Fe flux to the photosystems, and we assume that the translocation of Fe from the buffer to the photosystem (F₈₅) is zero. This assumption creates the flow of Fe from photosystems to nitrogenase (Fig. 1D), increasing the rate of N₂ fixation during the early dark period. During the later dark period, we assume that F₈₅ is –0, and the model forces the movement of Fe from nitrogenase to buffer as predicted (21).

**Simulating temperature and light dependences on metabolisms.** The temperature dependence is simulated based on applying a temperature factor fₚ(T) (equation 3) to N₂ fixation, photosynthesis, and respiration. To test the effect of temperature dependence on each metabolism, we applied fₚ(T) to only one of these metabolisms and plotted the rate of N₂ fixation (Fig. 7B). To represent the light dependence of photosynthesis, we have applied a light factor, f_I(0), (equation 4) to the maximum rate of photosynthesis.

**Preparing Crocosphaera watsonii WH8501.** Stock cultures of *Crocosphaera watsonii* WH8501 were obtained from the Culture Collection Yerseke (The Royal Netherlands Institute for Sea Research, Yerseke, The Netherlands; strain number CCY 0601). The cells were maintained in N-free YBC-II medium (46) at 28°C in glass flasks under constant white light of 150 μmol µm⁻² s⁻¹ using a 12-h:12-h light-dark (12L:12D) cycle. At the beginning of each experiment, the cultures were transferred into flat-panel photobioreactors (FMT150; Photon System Instruments, Brno, Czech Republic) (47) with a sinusoidal 12L:12D growth irradiance peaking at 400 μmol m⁻² s⁻¹ with aeration. Cultures were acclimated to these conditions and maintained in exponential growth for at least 5 generations (about 15 days).

**Transmission electron microscopy.** *Crocosphaera* cells (~10⁶ cells ml⁻¹) were harvested by centrifugation (5 min at 5,000 × g). The cells were resuspended in 1 volume of the growth medium mixed 1:1 with 5% (vol/vol) glutaraldehyde fixative in 0.2 M cacodylate buffer, pH 7.2. After 15 min of rotary shaking at room temperature, cells were transferred to 0.1 M cacodylate buffer containing 2.5% (vol/vol) glutaraldehyde and fixed overnight at 4°C. Pelleted cells were washed with cacodylate buffer and postfixed with 1% (wt/vol) osmium tetroxide for 2 h. After washing steps with the same buffer, cells were dehydrated through a graded series of acetone, embedded in low-viscosity Spurr resin (EMS), and polymerized at 60°C for 48 h. Ultrathin sections of 60 nm were cut using an ultramicrotome (UCT, Leica). Sections were collected on Formvar-coated copper grids and stained with 1% (wt/vol) aqueous uranyl acetate for 10 min and with Sato’s lead citrate for 3 min (48). Prepared sections were examined in a JEOL 1010 transmission electron microscope (JEOL) equipped with a Mega View III camera (SIS). Acquired pictures were analyzed by ImageJ software (49).

**N₂ fixation measurements.** To determine the rates of N₂ fixation by acetylene reduction assays (50), 5 ml of cell suspensions grown under different light intensities were dispensed into HCl-rinsed glass vials. After each vial was sealed with a septum, 10 ml of acetylene gas (99.7% [vol/vol]; Linde Gas) was injected by replacing the same volume of headspace. The samples were incubated at 28°C in the dark for 12 h. Subsamples of the headspace were taken immediately after acetylene addition and then at the end of the incubation to measure their ethylene content with a flame ionization gas chromatograph (HRGC...
Parameters were then used to calculate the ETR (54): where $E$ is the light intensity, $F_{v}/F_{m}$ versus $E$ curve, and the light saturation point of the ETR ($E_{\text{TR}1}$). These parameters were then used to calculate the ETR (54):

$$\text{ETR} = \alpha_{\text{PSII}} \times n_{\text{PSII}} \times F_{v}/F_{m} \times \Phi_{\text{RCII}} \times E$$

where $E$ is the intensity of the actinic light, $\Phi_{\text{RCII}}$ is the quantum yield of photochemistry within RCII (taking constant values of 1 mol e$^{-}$ (mol photons)$^{-1}$), $F_{v}$ is variable fluorescence in the light ($F_{v} - F_{o}$), $F_{m}$ is the maximal variable fluorescence in the light ($F_{m} - F_{o}$), and $n_{\text{PSII}}$ is the ratio of functional reaction centers of PSII to total chlorophyll $a$ (55, 56).

The ETR values were then plotted versus irradiance and modeled after the reports of Eilers and Peeters (57) and Silsbe and Kromkamp (58) to derive the maximum ETR ($\text{ETR}_{\text{max}}$), the initial slope of the $P$ versus $E$ curve, and the light saturation point of the ETR ($E_{L}$).

**Data availability.** The model developed in this paper as well as the plotted data have been uploaded in Zenodo/GitHub and is freely available from https://zenodo.org/record/3265448.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/msphere.00531-19.

**TEXT S1**, PDF file, 0.3 MB.

**FIG S1**, TIF file, 0.4 MB.

**FIG S2**, TIF file, 0.7 MB.

**TABLE S1**, PDF file, 0.2 MB.

**TABLE S2**, PDF file, 0.2 MB.

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Erratum for Inomura et al., “Quantifying Oxygen Management and Temperature and Light Dependencies of Nitrogen Fixation by Crocosphaera watsonii”

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