Heterogeneous nitrogen fixation rates confer energetic advantage and expanded ecological niche of unicellular diazotroph populations

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Nitrogen fixing plankton provide nitrogen to fuel marine ecosystems and biogeochemical cycles but the factors that constrain their growth and habitat remain poorly understood. Here we investigate the importance of metabolic specialization in unicellular diazotroph populations, using laboratory experiments and model simulations. In clonal cultures of Crocosphaera watsonii and Cyanothece sp. spiked with 15N2, cellular 15N enrichment developed a bimodal distribution within colonies, indicating that N2 fixation was confined to a subpopulation. In a model of population metabolism, heterogeneous nitrogen (N2) fixation rates substantially reduce the respiration rate required to protect nitrogenase from O2. The energy savings from metabolic specialization is highest at slow growth rates, allowing populations to survive in deeper waters where light is low but nutrients are high. Our results suggest that heterogeneous N2 fixation in colonies of unicellular diazotrophs confers an energetic advantage that expands the ecological niche and may have facilitated the evolution of multicellular diazotrophs.
Nitrogen (N\textsubscript{2}) fixing microorganisms (diazotrophs) are critical suppliers of bioavailable nitrogen (N) in the world’s oceans. The N\textsubscript{2} fixed by these organisms supports cell growth, but also enters the food web through grazing by zooplankton and excretion of ammonium (NH\textsubscript{3}+) or other dissolved nitrogenous compounds\textsuperscript{1–5}. All diazotrophs have a N\textsubscript{2} fixing enzyme complex, nitrogenase. Since most nitrogenase enzymes are irreversibly damaged by molecular oxygen\textsuperscript{6,7}, diazotrophs are taxonomically diverse and occupy distinct large-scale habitats\textsuperscript{8–12}, suggesting there are multiple strategies for managing the energetic demands of photosynthesis, growth, and N\textsubscript{2} fixation under a wide range of ocean conditions. However, the links between diverse physiological strategies and the resulting ecological niches and spatial distributions remain poorly understood\textsuperscript{10,13–15}.

*Crocosphaera watsonii* (hereafter *Crocosphaera*), a marine unicellular diazotroph, is abundant and widespread in tropical and subtropical oceans\textsuperscript{10,11,16,17}, and its areal N\textsubscript{2} fixation rate is restricted to the dark period. A similar diel cycle is observed in *Trichodesmium*, a filamentous diazotroph abundant in tropical oceans\textsuperscript{16,18,19}. Due to its strong diel alternation between C and N metabolisms, *Crocosphaera* is a promising model for investigating cellular C and N physiology\textsuperscript{17,20–22}. *Cyanothece* is another well studied unicellular diazotroph. It is closely related to the sequence-defined genus, UCYN-C\textsuperscript{24,25}, which has been observed to supply N to other phytoplankton and contribute to vertical POC (particulate organic carbon) transport\textsuperscript{26}. Intensive studies on *Cyanothece* physiology make it a model organism to study the physiology of unicellular diazotrophic cyanobacteria\textsuperscript{27,28}.

When exposed to a light:dark cycle, the peak of N\textsubscript{2} fixation activity of most unicellular photosynthetic diazotrophs is restricted to the dark period. A similar diel cycle is observed in *Crocosphaera*\textsuperscript{20–22,29} and *Cyanothece*\textsuperscript{27,28}, however, both taxa can be forced to fix N\textsubscript{2}, if maintained under constant illumination for an extended period\textsuperscript{30,31,32}. This observation led to speculation that its metabolism is heterogeneously distributed among cells in a population\textsuperscript{14} as observed in *Trichodesmium*\textsuperscript{9}.

Recent technological advances in the visualization of enriched stable isotopes in individual cells using NanoSIMS enable cell level analyses of N\textsubscript{2} fixation activity\textsuperscript{32–38}. With this technology, high variations in C and N\textsubscript{2} fixation activity from in situ *Crocosphaera*-like cell colonies were shown, suggesting heterogeneity of metabolisms\textsuperscript{17}. During the same period of time, similar physiological heterogeneity was observed among the clonal population of *Crocosphaera* (WH8501)\textsuperscript{23}. These observations lead us to question how widely this heterogeneity applies and how it impacts the cellular energetics and resulting ecological niches.

Here, we investigated physiological heterogeneity among clonal populations of multiple genera of cyanobacteria, *Crocosphaera* (PS0609A) and *Cyanothece* (ATCC51142), by quantifying the pattern in N\textsubscript{2} fixation and C uptake at the sub-cellular level. Using clonal populations prevents interference from other N fixing organisms, a potential problem noted in the in situ studies\textsuperscript{17} and using different genera and strains of cyanobacteria allows us to evaluate the generality of the pattern. We have also applied multiple statistical methods to quantify the heterogeneity in C and N\textsubscript{2} fixation. To analyze the energetics and C consumption of the observed cell-level heterogeneity, we present a model of diazotroph population to simulate the advantage of maintaining both nitrogen fixing and non-N\textsubscript{2} fixing cells. We used oceanographic data to predict the implications of metabolically differentiated populations for the ecological niche of unicellular diazotrophs through the photic zone.

**Results**

**Heterogeneity in N enrichment among cells.** 15N enrichment was variable within a single strain of continuous culture grown *Crocosphaera* cells (Figs. 1 and 2) and batch culture grown *Cyanothece* (Fig. 2). Initially, ratios of 13C:13C (≈13C:12C) and 15N:14N (≈15N:14N) were 8.8 ± 0.5‰ and 3.5 ± 0.1‰, respectively in *Crocosphaera* harvested at steady state under continuous culture and were 11.0 ± 0.4‰ and 3.6 ± 0.2‰, respectively in *Cyanothece* harvested at exponential phase under batch culture (Fig. 3 and Supplementary Table 1). After 11 h in the dark, two cells (Fig. 1; white arrows in panel 11D 15N:14N) were more strongly enriched in 15N compared to the other four cells, of which one cell showed the least enrichment (Fig. 1; blue arrow). However, the least 15N enriched cells were actively 13C enriched in the light period (e.g., cells with blue arrow in panel 3L 13C:12C) showing that these cells were alive and metabolically active. The variable 15N enrichment was observed not only under continuous culture but also under exponentially growing batch culture and suggests that heterogeneous 15N\textsubscript{2} fixation happens with or without nutrient stress.

The 15N\textsubscript{2} fixation in the dark was observed based on the temporal changes in cellular 15N:13N ratios in both *Crocosphaera* and *Cyanothece* (Fig. 3a, d and Supplementary Table 1). Calculated per-cell 15N enrichment rates, p, followed similar trends as the 15N:14N ratio, and increased significantly (p = 0.014 by one-way ANOVA\textsuperscript{39}) in *Crocosphaera* from 0 fmol N cell\textsuperscript{−1} h\textsuperscript{−1} at the start of the dark period to a mean of 17.3 fmol N cell\textsuperscript{−1} h\textsuperscript{−1} at 7D, but with a range of 0 fmol N cell\textsuperscript{−1} h\textsuperscript{−1} to 37.7 fmol N cell\textsuperscript{−1} h\textsuperscript{−1} (Supplementary Table 1b). These values are comparable to those reported in earlier studies (Supplementary Table 2). *Cyanothece* also showed a similar trend within population heterogeneity; p varied from 0 fmol N cell\textsuperscript{−1} h\textsuperscript{−1} to 12.7 fmol N cell\textsuperscript{−1} h\textsuperscript{−1} across 84 cells at the time of highest mean 15N enrichment (5.54 fmol N cell\textsuperscript{−1} h\textsuperscript{−1} at 6D) (Supplementary Table 1b). The 15N:14N ratios measured by NanoSIMS were in good agreement with the ratios measured by mass spectrometer (Supplementary Fig. 1).

The proportion of *Crocosphaera* cells that incorporated detectable 15N (i.e., cells with 15N:14N exceeding 2 SD above the mean at time 0: 3.8% for *Crocosphaera*, 4.0% for *Cyanothece*) increased from 40 to 75% in the dark, suggesting that at least ~25% of cells did not detectably fix N\textsubscript{2} (Fig. 3b, e). Higher variability of 15N enriched cells in *Crocosphaera* compared to *Cyanothece* may be the result of low number of observed cells (between 7 to 33 cells at each time point) (Fig. 3b, d). Hotspots of 15N:14N were observed in the dark period. The 15N hotspots started to appear after 5D, and continued to form until the beginning of the light period (2 L), with the peak of 62% at 10D (Fig. 1 and 3c) in *Crocosphaera*. Similar temporal changes were observed for the proportion of cells with 15N hotspot among total cells in *Cyanothece*, from 2D to 6 L, with a peak at 6D. Therefore, the lack of 15N hotspot in at least ~40% of cells again shows that a large fraction of cells did not detectably fix N\textsubscript{2}. N\textsubscript{2} fixation earlier in the diel cycle in *Cyanothece* compared to *Crocosphaera* (Fig. 3) supports previous reports of a peak around 4D in *Cyanothece* and 9D in *Crocosphaera* under 12 L:12D cycle\textsuperscript{21,40}.

To quantify the differentiation of rates within each population, we examined the statistical distribution of C and N isotope enrichments among all cells. Intercellular metabolic heterogeneity was defined as the coefficient of variation (CV; ref. \textsuperscript{41}) in each isotope ratio. The variations in 15N enrichment are observable in cell level 15N:14N ratios, which varied from 3.2% to 6.2% (4.4 ± 1.0‰, CV = 23.6%) at 6D in *Crocosphaera*, 3.5% to 18.9% (8.3% ± 4.4‰, CV = 53.8%) at 6D in *Cyanothece* (Figs. 2 and 3 and Supplementary Table 1). In contrast, 13C uptake (13C:12C
ratio) was generally similar across cells, in both $^{15}$N-enriched cells and non-enriched cells (Figs. 1 and 2 and Supplementary Table 1). For example, in the $^{13}$C:$^{12}$C ratio of 3 L in Fig. 1, all 14 cells are enriched similarly, with $^{13}$C:$^{12}$C ratios between 7.9‰ and 9.4‰ (8.7‰ ± 0.5‰, CV = 6.2%) in Crocosphaera (Fig. 2). The CV for $^{15}$N:$^{14}$N (23.6 to 31.4% during 6D to 12D, 25.4 to 48.0% during 6 L to 12 L) were greater than those estimated for $^{13}$C:$^{12}$C during 6 L to 12 L (4.8 to 10.6%), suggesting higher heterogeneity in $^{15}$N$_2$ fixation compared to $^{13}$C fixation. The same trend was observed in Cyanothece (46.4 to 56.2% during 6D to 12D, 45.5 to 48.9% during 6 L to 12 L in $^{15}$N:$^{14}$N, 24.0 to 40.1% in $^{13}$C:$^{12}$C during 6 L to 12 L) (Supplementary Table 1a).
Fig. 3 Observed heterogeneity in $^{15}\text{N}$ uptake by two different unicellular diazotrophs. a, d Diel change in $\text{N}_2$ fixation ($^{15}\text{N} : ^{14}\text{N}$), b, e diel change in percentage of $^{15}\text{N}$-enriched cells, c, f diel change in percentage of cells with $^{15}\text{N}$ hotspot in a to c Crocosphaera and d to f Cyanothece. Black and white bars at the bottom of each graph indicate dark and light period, respectively. NO DATA at 4 L in Crocosphaera shows no data was collected.

Fig. 4 Statistical analysis of heterogeneous uptake of $\text{N}$ and $\text{C}$. Frequency distribution of a, b $^{15}\text{N} : ^{14}\text{N}$ for Crocosphaera and Cyanothece (6 L to 12 L) and c, d $^{13}\text{C} : ^{12}\text{C}$ for Crocosphaera and Cyanothece (6 L to 12 L), respectively. Red dashed curve is the normal distribution for the initial condition ($t = t_0$) with the special $y$-axis on the right (that on the left is for other plotted values). Green dashed curve is the normal distribution based on the mean value and standard deviation of the probability density. $S$, bimodal separation; Dev, deviation from the normal distribution; $n$, number of samples. Both for Crocosphaera and Cyanothece, $S$ and Dev are larger for $^{15}\text{N} : ^{14}\text{N}$, suggesting stronger heterogeneity for $\text{N}$ uptake.
The distribution of isotope ratios among cells reveals qualitatively different enrichment trends for C compared to N, for both Crocosphaera and Cyanothece (Fig. 4). The distribution of 15N:14N reveals two distinct peaks after 12 h, one that remains near the initial ratio and a second that develops at enriched levels of 15N, for both Crocosphaera and Cyanothece (Fig. 4a, b). In contrast, few cells remain at the initial ratio 13C:12C (Fig. 4c, d), and only a single broad peak is evident. To evaluate this bimodality, we calculated the “bimodal separation” (S; ref. 45), a distance between the means of two Gaussian distributions fit to the data (see Methods). The separations of peaks in 15N:14N was consistent with that for 13C:12C, both for Crocosphaera (S = 1.45 for N, vs. S = 0.42 for C) and for Cyanothece (S = 0.79 for N, vs. S = 0.004 for C). We have also applied the bimodal curve fit to 15N:14N to 13C:12C with the curve shape maintained (relative relation between two normal distributions and S are maintained); even after the curve is fitted to 13C:12C, the difference between the data and the curve is statistically significant for both diazotrophs (p < 0.001), indicating a significant difference between 15N:14N and 13C:12C.

To confirm that the distribution of N isotopes develops a bimodal structure indicative of distinct rates among sub-populations, we compared the observed frequency distributions to a single Gaussian distribution with the same mean value and standard deviation (Fig. 4). For 15N:14N, the peak of the normal distribution appears near the local minima between the two peaks of the data (Fig. 4a, b), again indicating strong bimodal separation. In contrast, the normal distribution largely overlaps with the data for 13C:12C (Fig. 4c, d). We computed the deviation (Dev) from the normal distribution by adapting a commonly used form of Chi square (χ²) normalized by the sample number (n) (see Methods). The deviations from a single Gaussian distribution are stronger for 15N:14N than for 13C:12C for both diazotrophs (Dev = 1.97 vs. 0.42 for Crocosphaera and 0.81 vs. 0.33 for Cyanothece). These results qualitatively and quantitatively support stronger heterogeneity in N uptake than for C uptake, indicating a clear separation between N2 fixing cells and non-N2 fixing cells, without a comparable separation of C fixation.

Although Crocosphaera and Cyanothece are generally referred to as free-living unicellular cyanobacteria, they have been reported in colonies of more than two cells17,23 (Fig. 1). In the Crocosphaera culture, 55% of total cells were observed as colonies of 3 to 5 cells in this measurement (Supplementary Fig. 2). Colonial Crocosphaera cells were shown in earlier culture studies23, and an in situ study found that 45 to 85% of Crocosphaera-like cells were observed as colonies of 3 to 242 cells17. Colonial formation of cells might increase the efficiency of excreted NH₄⁺ transfer among cells.

Simulating population heterogeneity of N₂ fixation. The strong concentration of newly fixed N in a sub-population of colonial unicellular diazotrophs suggests that localizing the costly process of N₂ fixation may confer an advantage to the population as a whole. A large part of the energetic cost of N₂ fixation is incurred in the protection of nitrogenase from O₂, which is achieved through excess respiration of C (refs. 33,44). We therefore hypothesize that having only a limited proportion of cells to pay the oxygen management cost could reduce community C requirements, potentially leading to overall higher growth. To evaluate the potential benefits of confining N₂ fixation to a sub-population, we used a Cell Flux Model of a N₂ fixer44. The model uses a coarse-grained metabolic flux network including core metabolisms of respiration, biosynthesis and N₂ fixation, which are constrained by mass, electron and energy balance (Fig. 5) (see Methods for details). We simulate a steady state environment where cells grow at a rate of μ (d⁻¹). To maintain the prescribed rate of growth, energy must be provided by respiration, with distinct rates allocated to N₂ fixation and biomass production44,45. In turn, the total respiration rate predicts the intracellular O₂ concentrations, for a given diffusivity of O₂ across the cell membrane. Additional respiration is added as needed to maintain axonix inside the cell, thus protecting the nitrogenase enzyme and enabling N₂ fixation44. The total carbon consumption rate per cell is computed to satisfy the sum of all 3 demands: biomass growth, N₂ fixation, and respiratory protection against O₂.

Here we adapted this cellular model44 to represent a heterogeneous colony of cells (the model version named CFM-Colony, with a fraction fN that fix N₂, and a remaining fraction 1 − fN that do not. The two sub-populations share a common medium, allowing N₂-fixing cells to transfer fixed nitrogen (NH₄⁺) to non-N₂-fixing cells. The transfer of newly fixed N is prescribed by an efficiency parameter, E_N, with the remaining fraction (1 − E_N) of excreted NH₄⁺ being lost from the entire colony.

To quantify the impact of heterogeneous rates of N₂ fixation, we compare its population-scale rate of C consumption (denoted Cₛ), to the rate that would apply to a homogeneous population of the same size (denoted C_H). When Cₛ/C_H < 1, the colony has lower C consumption with heterogeneous N₂ fixation than homogeneous N₂ fixation. The rate of N₂ fixation by a heterogeneous community, Nₛ, relative to a population with uniform rates, N_H (when fN = 1) can be expressed as follows:

\[
Nₛ/N_H = f_N \left( 1 + \frac{1 - f_N}{f_N E_N} \right)
\]

The ratio of C consumption associated with N₂ fixation and respiratory protection follows the ratio of N fixation rates by heterogeneous versus homogeneous populations (Eq. 1).

Modeled colonies with N₂ fixation confined to a sub-population benefit from a substantial drop in overall C consumption, due to lower community level requirements for respiratory protection of nitrogenase (Fig. 6). For typical Crocosphaera growth rates (μ = 0.2) and a low efficiency of NH₄⁺ transfer (E_N = 0.2) C savings amount to ~8 fmol C cell⁻¹ h⁻¹, which is >30% of the C budget of a population with homogeneous rates (Fig. 6a, b). Total C consumption reaches a minimum value at an intermediate value of fN, due to two opposing factors; as fN initially decreases below 1, respiratory protection is reduced. However, as fN decreases, a larger portion of cells must also rely on transferred NH₄⁺, which allows more NH₄⁺ to be dissipated into the environment, requiring higher C consumption to replace it. This effect is represented by (Eq. 1) where increasing fN leads to increasing Nₛ. At an intermediate value of fN, these two factors minimize Cₛ, and respiratory protection is covered by energetically balanced productive flows of respiration.

The value of fN that maximizes C savings tends to increase with decreasing E_N due to increased costs for N₂ fixation [Eq. 1] (Fig. 7b). When E_N = 0.1, Cₛ (thus Cₛ/C₇) reaches a minimum at fN ~ 0.56 (Supplementary Fig. 3), a level of heterogeneity similar to that seen in the culture experiments, in which about a half of cells fix N₂. This optimum fN also increases with the growth rate μ due to increased energy costs for biomass production and N₂ fixation (Fig. 7d). The 2D plot of Cₛ and Cₛ/C₇ for various fN and E_N shows that up to 55% of C can be saved at high E_N and low fN (Fig. 7a, b). On the other hand, even at E_N = 0.1, heterogeneity can still save carbon (Fig. 7b), due to the small cost of N₂ fixation relative to respiratory protection44. Considering the fact that C is one of the limiting factors for the growth for diazotrophs28,46,47,
Fig. 5 Schematic of cell flux model simulating heterogeneity of *Crocosphaera* during the dark period. Green space, cytoplasmic space; peach frames, cell membrane layers; circular blobs, chemical compounds; solid arrows, material fluxes; dashed arrows, energy fluxes. C store represents C storage accumulated during the preceding light period, which is used for multiple purposes. The use of C store is represented by solid arrows of different colors by C fluxes and the different energy fluxes from respiration are colored differently; see the list at the bottom. \( f_N \) represents the fraction of \( N_2 \) fixing cells; thus that of non-\( N_2 \) fixing cells becomes \( 1 - f_N \). The \( O_2 \) concentration of \( N_2 \) fixing cell \( \frac{1}{2} O_2 \) is kept small through respiratory protection at the expense of C store. Contrarily, only biosynthetic respiration occurs in non- \( N_2 \) fixing cells. Excreted, fixed \( N \text{NH}_4^+ \) is transferred to non- \( N_2 \)-fixing cells with efficiency of \( E_N \). \( 1 - E_N \) is the fraction of excreted \( \text{NH}_4^+ \) lost to the environment. Cells grow at the rate of \( \mu (d^{-1}) \).

**Fig. 6** Carbon use of heterogeneous population, \( C_S \) and \( C_S \) relative to non-heterogeneous population \( C_0 \). a, \( C_S \) for various \( f_N \). b, \( C_S / C_0 \) for various \( f_N \). c, \( C_S \) for various \( \mu \). d, \( C_S / C_0 \) for various \( \mu \). For a and c the legend in c shows the colors used for each fluxes; Dark green, biosynthesis; Bright green, respiratory energy production for biosynthesis; Orange, electron donation for \( N_2 \) fixation; Yellow, respiratory energy production for \( N_2 \) fixation; Cyan, respiratory protection. See Fig. 5 for more detail where similar colors are used for each C flux. Black solid lines at the top of a and c represent the total C fluxes. Black dotted lines in b and d are for \( C_S / C_0 \) = 1, \( f_N = 0.5 \), \( E_N = 0.2 \), and \( \mu = 0.2 (d^{-1}) \) unless they are variable on the x-axes. Temperature \( T = 26 ^\circ C \) and \( O_2 \) concentration in the environment \( [O_2] = 208 \mu M \), representing saturated concentration at this temperature and salinity of 35ppt.

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heterogeneity of N₂ fixation might be an important strategy to increase their growth rates. Because unicellular diazotrophs can use NHi, growth efficiency should be maximized when cells can meet their N demand from NHᵢ in the environment, thus saving the considerable cost of N₂ fixation (Fig. 6a, c). If cells rely solely on the N₂ fixation for their N source, higher growth rate would render respiratory protection negligible, yielding higher growth efficiency. For example, the cell flux model (Fig. 6) predicts that as growth rate increases beyond ~0.28 (d⁻¹), respiratory protection is no longer needed and the growth efficiency reaches its highest level. This occurs at a specific fN where respiratory protection is minimized with minimum loss of N to the environment (Cₛ and Cₛ/Cₛ° at fN ~ 0.38 in Fig. 6a, b respectively and cyan dashed curve in Fig. 7b).

The amount of C saved by heterogeneous N₂ fixation depends only slightly on the poorly known value of EN. This insensitivity is based on the relatively small cost for N₂ fixation⁴⁴. While N₂ fixation requires 16 ATP per N₂, when EN = 1, the cost is predicted to be low relative to the whole cell energy requirement for biosynthesis since N₂ fixation is just one reaction and there are many other pathways where ATP is consumed in the process of biosynthesis. In addition, cost for O₂ management is overwhelming. As EN decreases, the cost for N₂ fixation increases inversely proportional to EN, but due to the relatively low costs of N₂ fixation, the whole cell C costs (thus Cₛ and Cₛ/Cₛ°) are relatively insensitive to EN.

The energetic advantage of heterogeneous N₂ fixation rates increases as growth rates decline (Figs. 6c, d and 7c, d). Slower growth rates reduce the costs of biomass synthesis and N₂ fixation, thus making respiratory protection a dominant energetic and C cost (Fig. 6c). Since heterogeneous populations can lower this cost by focusing N₂ fixation in a fraction of cells, more C can be saved at lower μ. Over 90% of C can be saved at low μ and low fN (Fig. 7d). On the other hand, when μ > 0.35 (d⁻¹), Cₛ/Cₛ° can go above 1 (Figs. 6d and 7d) due to high costs for growth and N₂ fixation, and N loss to the environment. The growth rates of Crocosphaera compiled from laboratory studies have a mean value of μ < 0.3 (d⁻¹) (ref. ⁴⁸). In the ocean, nutrients such as iron and phosphorus are generally more limited compared to culture conditions leading to even lower μ. Thus, with a typical growth rate in the ocean, it is likely that population heterogeneity in N₂ fixation can save a considerable fraction of population C costs.

**Implications for vertical habitat range.** Fixed C is required for N₂ fixation, respiration and cellular growth, providing energy, electrons and reduced C. In the open subtropical ocean, chlorophyll concentrations typically reach a maximum at the bottom of the photic zone, and the top of the nutricline, ~100 m depth, where both light and nutrients are adequate for growth, albeit at low rates. Below these depths, available light becomes so low that it prevents cells from fixing enough C to be viable (here we define maximum viable depth, MVD). Since heterogeneous N₂ fixation reduces the overall C requirement of such populations, it could act to extend their MVD deeper into the nutricline.

We simulated the depth variation of the growth rate for Crocosphaera populations with homogeneous versus heterogeneous N₂ fixation rates (see Methods). The model result shows that MVD of the heterogeneous population is ~ 25 m deeper than that of the homogeneous population (Fig. 8a). This expanded MVD may be important because the available nutrient typically increases with depth and expanding MVD allows Crocosphaera to utilize the higher concentration of the growth-essential nutrient. For example, at the Hawaii Ocean Time-series (HOT) site at 22° South Pacific Gyre at 25°S, 170°W, where the highest nitrate concentration of Crocosphaera have also been observed⁵⁰. Under those conditions, the model predicts heterogeneous population...
would utilize up to ~90% higher concentration of PO$_4^{3-}$ (Supplementary Fig. 4a).

In these observations, NO$_3^-$ concentrations also increase with depth, which may lead to a partial suppression of N$_2$ fixation (Fig. 8c and Supplementary Fig. 4b). However, the NO$_3^-$ concentrations in the expanded vertical niche remain well below what would cause full suppression$^{51-53}$. For example, the concentration of NO$_3^-$ at MVD is below 2 µM whereas even 5 µM does not fully suppress N$_2$ fixation of Crocosphaera$^{51-53}$.

Since respiratory protection is required regardless of the level of N$_2$ fixation (thus required even when N$_2$ fixation is partially suppressed)$^{54}$, heterogeneous population would still save C even at depths near the MVD.

**Discussion**

The results of our laboratory observations demonstrate that unicellular diazotrophic cyanobacteria form colonies in which the key metabolic function of N$_2$ fixation is confined to a distinct subpopulation. Guided by these observations, metabolic modeling shows that this functional specialization may provide an energetic advantage, especially in oligotrophic regions where nutrient availability increases as light diminishes. These findings have important implications for role of metabolic specialization in the evolution of multi-cellularity, and the biogeography of unicellular diazotrophs and their role in biogeochemical cycles. Evaluating these broader implications will require a more complete understanding of the mechanisms and economics of material transfers within colonies, and the environmental factors that influence and sustain them.

Our results suggest that the exchange of newly fixed N within colonies is key to reducing population carbon costs, potentially explaining why the cells are often observed in aggregations (Fig. 1). However, the mechanisms of NH$_4^+$ transfer between cells and its overall efficiency ($E_N$) within each colony remain poorly constrained. It is likely that the surface-volume ratio of the cell and the size of aggregated colonies can both influence $E_N$. The diffusivity between the cellular spaces, might be affected by the production of extracellular polymeric substances. Also, the uptake properties of the cells (i.e. the maximum uptake rate and the half saturation constants of NH$_4^+$) influence how effectively they obtain NH$_4^+$. For example, if the N is transported with intercellular transporters, $E_N$ would decrease considerably. To understand what regulates population heterogeneity of N$_2$ fixation, it may be useful to examine the heterogeneity of N$_2$ fixation under varying growth conditions, including different ambient NH$_4^+$ and O$_2$ concentrations. Also, it is possible that N$_2$ fixation is tied to specific phase of the cell cycle, which requires further experiments.

Recent ocean ecological and biogeochemical models simulate various functional groups of diazotrophs including unicellular types$^{55,56}$, but diazotrophs within the same functional groups are generally represented as a uniform metabolic population. Given the observed bimodality of N$_2$ fixation and its impact on C cost, our study suggests that resolving such heterogeneity and its underlying causes may be essential to simulating the ocean ecosystems and predicting the niche of unicellular diazotrophs. In particular, the dependence of C savings on cellular growth rate would help to test the model predictions for expanded vertical habitat. On the other hand, our model shows that population C savings are relatively insensitive to uncertainties in $E_N$, especially at low growth rate, where the rate of N assimilation becomes small and costs of respiratory protection dominate (Fig. 7c and Supplementary Fig. 5). Thus, while the expansion of vertical niche depends on the growth rate dependence of C savings, it appears robust to uncertainty in N transfer efficiency, $E_N$.

Given the ubiquity of phenotypic heterogeneity$^{57}$ and intercellular cooperation$^{58}$, metabolic heterogeneity may be a general strategy for maximizing fitness among diazotrophic cyanobacteria. It remains an open question whether filamentous diazotroph Trichodesmium separates N$_2$ fixing cells (diazocytes) and cells responsible for photosynthesis$^{59-61}$ or not$^{55,62}$. If Trichodesmium separates N$_2$ fixation and photosynthesis on cellular level, the observation of heterogeneity of N$_2$ fixation in both Crocosphaera and Cylanothece together with the heterogeneity in N$_2$ fixation in Trichodesmium$^{35}$ suggest an evolutionary relationship between unicellular and filamentous diazotrophs. However, it remains an open question whether there is connection between heterogeneity in N$_2$ fixation in unicellular diazotrophs and multicellular diazotrophs, as well as whether unicellular or multicellular diazotrophs evolved first in the cyanobacterial lineage$^{63-65}$. The finding that heterogeneity in N$_2$ fixation occurs in both unicellular and multicellular diazotrophs may support the hypothesis that the division of labor is a key factor driving multi cellular cooperation in evolutionary transitions$^{56,67}$.

**Methods**

**Phytoplankton cultures.** A Crocosphaera strain isolated from the surface of the western subtropical Pacific$^{68}$ was grown in a continuous 1.2 L culture in N-free medium. To closely represent their habitat (the euphotic zones of subtropical gyres), the culture was maintained in a chemostat with a dilution rate 0.20 d$^{-1}$ (40% of the maximum growth rate), at a temperature of 26°C, an irradiance of 200 µmol photons m$^{-2}$ s$^{-1}$, and a dark/light cycle of 12:12 h (1D to 12D, 1L to 12L). The beginning of the dark period was considered time 0 (0D). The N-free medium was prepared from seawater collected from the surface of the western North Pacific...
**Ocean** (34°20'N, 138°40'W), enriched with 20 µM of NaH2PO4, 0.2 vitamins, and 0.2 trace metals93,94. *Cyanothece* sp. ATCC51142 was grown in a 1.0 L culture in N2-free ASP2 medium17 which contains 28.7 µM of K2HPO4, a temperature 26 °C, an irradiance of 400 µmol photons m−2 s−1, and a darklight cycle of 12:12 h (1D to 12D, 1 L to 12 L) at growth rate (μ) of 0.30 d−1.

**15N and 13C uptake.** N2 fixation was measured following the method described by Mohr et al.16. Briefly, N-free medium was degassed and rapidly transferred to 125 mL glass bottles with minimal agitation until the maximum volume of the bottles was reached. These were septum-capped and enriched by injecting 1 ml of N2 gas (99.8 atom% 15N; lot #11059; SI Science Co., Ltd., Tokyo, Japan) into the 24 vials. Previous study confirmed no contamination of 15NO and 13NH4 in the 15N2 gas. The vials were incubated under the same conditions as previously described and harvested one vial every hour beginning at the start of the dark period (6 PM), and split into three aliquots for NanoSIMS, PON and mass spectrometry, and flow cytometry. Samples prior to isotope injection were also collected and analyzed as time 0. Samples at 4 L were lost. Cells observed under NanoSIMS analysis were from 7 to 37 cells at each time point.

In *Cyanothoece*, 15N and 13C uptake were analyzed as described for *Crocosphaera*, except for small differences in the source of 15N2 gas (98 atom% 15N; lot MBBB0968V; Sigma-Aldrich, St. Louis, Missouri, USA), culture volume (4.0 mL of 1.7 × 106 cells mL−1 in 5 mL serum vials), final enrichment (13.6 atom% 15N and 1.7 atom% for 13C, respectively), sampling frequency (2 h), and the 87 to 220 cells were analyzed per each time point. Contamination of dissolved inorganic nitrogen in N2 was not analyzed in the 15N gas.

**NanoSIMS imaging.** Cells (1 mL) were fixed in 2% w/v glutaraldehyde, and collected using 0.2-µm Isopore™ GTTP Millipore Membrane filters (Merck Millipore, Billerica, Massachusetts, USA), which were then washed with Milli-Q ultrapure water and stored at −20 °C until further processing. For analysis, samples were sputtered with gold and secondary ions were imaged in 5 or 10 serial images (layers) on a NanoSIMS 50 (Cameca, Gennevilliers, France) to quantify 12C, 13C, 12C14N, and 12C15N in 7 to 220 cells per time point, following earlier studies34,73. To observe 15N and 13C uptake, 0.5 mL of the 15N2-enriched medium gas (99.8 atom%15N, lot #11059; SI Science Co., Ltd., Tokyo, Japan) into the 24 vials. Previous study confirmed no contamination of 15NO and 13NH4 in the 15N2 gas.

**Calculation of carbon and nitrogen uptake rates.** Images obtained by NanoSIMS were processed in ImageJ17 following methods described by Popa et al.30. Briefly, the metric properties in each cell delineated by the 12C14N images, were integrated over 5 or 10 serial images, corrected against reference standards, and converted to percentage uptake with a measurement precision of 0.8–1.5%. Cells with a 12C14N/12C15N (14N/15N) ratio exceeding 2 standard deviations above the average at time 0 (at which 15N2; 1.7 atom% for Crocosphaera) or 4.0 atom% for *Cyanothece*) were considered 15N-enriched. Similarly, cells with a 12C14N/12C13C (13C/12C) ratio exceeding 2 standard deviations above the average at time 0 (at which 12C13C was 9.8 atom% for *Crocosphaera*, 11.8 atom% for *Cyanothece*) were considered 13C-enriched.

The rate of N2 fixation was defined as the change in % 15N h−1 relative to the initial measurement. Per-cell net N uptake rates (µ mol N cell−1 h−1) were calculated using a method adapted from Popa et al.30, described in [Eq. 2].

\[
p = \frac{F_{\text{net}} \times \text{Cell/o}}{\Delta t}
\]

where \(F_{\text{net}}\) is the ratio between 15N in a cell after and the initial 15N content, and Cell/o is the cellular N quota calculated as the sum of particulate organic 15N and 15N normalized to the cell mass. As N2 fixation in *Crocosphaera* occurs only at night10,19, 15N enrichment in the dark (0–12 h) and during light (13–24 h) was treated as N2 fixation and re-uptake of excreted dissolved 15N, respectively.

**Statistics and reproducibility.** 15N13N ratios were compared by one-way ANOVA with 25 time points as factor levels, and individual cells in a sample as independent replicates. Differences were considered significant if \(p < 0.05\). Heterogeneity was defined by the coefficient of variation (CV; ref. 65):

\[
CV = 100 \times \frac{\sigma}{\bar{x}}
\]

where \(\bar{x}\) is the mean and \(\sigma\) is the standard deviation among the cells. Normality assumptions were confirmed after logarithmic transformation (\(p > 0.05\) by K-S test, \(n = 7–37\) for *Crocosphaera*, \(n = 87–220\) for *Cyanothoece*) and residuals had a mean of zero. Dunnett’s T-3 multiple comparisons59 were used to compare background ratios.

To compute the bimodal separation, we first fit the sum of two Gaussian distributions to the histogram42:

\[
F(x) = A_1 \exp\left(\frac{-(x - \bar{x}_1)^2}{2\sigma_1^2}\right) + A_2 \exp\left(\frac{-(x - \bar{x}_2)^2}{2\sigma_2^2}\right)
\]

(4) where \(F(x)\) is the probability density function, \(A_1\) and \(A_2\) are the areas under the two Gaussian curves, \(\bar{x}_1\) and \(\bar{x}_2\) are the means, and \(\sigma_1\) and \(\sigma_2\) are the standard deviations.

To calculate the deviation from the normal distribution, we applied the Kolmogorov-Smirnov test (K-S test).

\[
E(x) = \frac{A}{\sqrt{16\pi^3}} \exp\left(\frac{-(x - \bar{x})^2}{2\sigma^2}\right)
\]

(6) where \(E(x)\) is the expected probability density for value \(x\) based on the normal distribution, \(A\) is the total area of the histogram, \(\sigma\) is the observed standard deviation, and \(\bar{x}\) is the observed mean value. If the CO or N uptake of the population is heterogeneous, we expect stronger deviation from Eq. [6]; we calculate the deviation from the Chi squared (χ2) statistic79, normalized by the sample number:

\[
Dev = \frac{\sum_{n=1}^{n} (O(x) - E(x))^2}{E(x)}
\]

(7) where \(O(x)\) is observed probability density for the value \(x\). The normalization by \(n\) makes results with different sample numbers comparable (here *Crocosphaera* and *Cyanothoece*).

Reproducibility was confirmed by analyzing 7 to 37 independent *Crocosphaera* cells, and 87 to 220 independent *Cyanothoece* cells (Supplementary Table 1).

**Numerical model of heterogenous metabolisms.** To represent heterogenous metabolisms within a single clonal population of unicellular diazotrophs, we have modified the Cell Flux Model of diazotrophs44 by simulating two types of cell N2-fixing and non-N2 fixing (Fig. 5). The model resolves a coarse-grained metabolic network based on mass, electron, and energy (ATP) balance. These balances quantify stored C use for 3 cellular functions: biosynthesis, electron donation for N2 fixation, and respiration. Respiration can be further classified into three uses: respiration for biosynthesis, for N2 fixation and for respiratory protection (Fig. 5). The model was parameterized for *Crocosphaera* based on a respiration budget44 by reducing the diffusivity of the cell membrane44. We use cellular N of 30 fmol N cell−1 and a diameter of 3μm and temperature of 28 °C to better represent *Crocosphaera* per-cell net N uptake rates (µ mol N cell−1 h−1) were calculated using a method adapted from Popa et al.30, described in [Eq. 2].
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Author contributions

T.M. and K.F. designed the experiments and T.M. performed them with help from T.S. T.M. and K.F. performed the computation. T.M. and K.I. statistically analyzed the data with the help of T.S. T.M. prepared the original draft, which is revised by all the co-authors.
Competing interests
The authors declare no competing interests.

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