

Temperature Regulates Global Freshwater Diazotrophy

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SUMMARY

Biological dinitrogen (N₂) fixation is increasingly recognized as an important source of nitrogen in freshwater ecosystems; however, the magnitude and drivers of this process are poorly quantified at the global scale. This limited understanding stems from a historical focus on cyanobacterial diazotrophs in a limited number of regions, obscuring the true magnitude, community composition, and environmental drivers of freshwater diazotrophy. This study presents the first standardized global assessment quantifying diazotroph abundance, N₂ fixation rates, and *nifH* gene diversity at 206 freshwater sites across 36 countries on five continents. Diazotrophs were ubiquitous across freshwater ecosystems, comprising up to 16% of the total bacterial abundance, with N₂ fixation rates spanning more than 5 orders of magnitude (0.001–133 nmol N L⁻¹ d⁻¹). Noncyanobacterial diazotrophs (NCDs) dominated these communities at 85% of the sites, challenging the cyanobacteria-centric paradigm. Multilinear regression models revealed that water temperature is a key regulator of diazotrophy, delineating distinct regimes for N₂ fixation rates (21°C) and the relative abundance of NCDs (9°C). These models further indicated that warmer conditions may reduce the relative abundance of NCDs while favoring cyanobacterial diazotrophs. Ultimately, these thermally defined regimes highlight that warming freshwater environments would change the physicochemical and community drivers that currently regulate freshwater N₂ fixation patterns, with major implications for nitrogen cycling models.

INTRODUCTION

Biological nitrogen fixation (BNF) in aquatic systems is the microbial conversion of dissolved dinitrogen (N_2) into reactive N forms. This conversion is catalyzed by the nitrogenase enzyme, which is produced by specific groups of bacterial and archaeal prokaryotes termed diazotrophs¹. BNF is a central biochemical process in marine and freshwater environments, introducing ~170 Tg of fixed nitrogen globally per year and supporting primary producers across the aquatic ecosystem²⁻⁵. Thus, the diversity, metabolic strategies, and environmental distribution of diazotrophs can often affect the magnitude and temporal and spatial influx of available nitrogen for aquatic food webs. Previous studies have reported that aquatic diazotrophs can be classified as cyanobacterial or noncyanobacterial diazotrophs (NCDs) on the basis of the *nifH* gene, which encodes the nitrogenase enzyme^{6,7}. Although ubiquitous, the ecological contribution of diazotrophs is highly heterogeneous across aquatic habitats¹ because N_2 fixation rates vary in response to abiotic conditions, including nutrient stoichiometry⁸, water temperature⁹, light¹⁰, and oxygen saturation¹¹.

Recent work, based on local samples and global metadata analysis, highlights the potential importance of freshwater diazotrophs, with an estimated contribution of ~24 Tg N year⁻¹ to freshwater ecosystems globally⁴. Nonetheless, the extrapolation of diazotrophy-related insights from marine to freshwater ecosystems as well as from local to global scales is not straightforward as these inland waters are extremely dynamic, with complex three-dimensional flow paths¹². Moreover, these freshwater environments can experience abrupt physicochemical shifts due to enhanced anthropogenic inputs and the intensification of climate change¹².

Taken together, N_2 fixation along the freshwater continuum, spanning from rivers and streams (lotic environments) to lakes and reservoirs (lentic environments), has traditionally been attributed to cyanobacterial diazotrophs^{2,13}. However, recent studies have highlighted the potential importance of planktonic NCDs in freshwater systems^{14,15}. Unlike cyanobacterial diazotrophs, NCDs are heterotrophic or chemolithotrophic microorganisms that fix N_2 without direct dependence on light availability, allowing diazotrophic activity to occur across a broad range of aquatic environments and abiotic conditions¹⁶. Although little is known about planktonic freshwater diazotrophs, shifts in dominance between cyanobacteria and NCDs are likely to occur in response to sharp spatiotemporal abiotic changes, given their contrasting traits and environmental tolerances¹⁶. Specifically, cyanobacterial diazotrophs often thrive under warm ($\geq 20^\circ\text{C}$)^{17,18}, stratified, light-replete conditions, whereas NCDs are more metabolically

versatile and remain active across a wider range of temperatures and redox conditions¹⁶. Specifically, NCDs are often associated with organic aggregates such as marine, lake and river snow¹⁹. Regardless of which group is dominant, recent studies have suggested that because freshwater and terrestrial environments are tightly connected, even low inputs of fixed N may alter primary production and thus significantly impact food-web dynamics¹³.

Despite growing evidence of the ecological importance of freshwater N₂ fixation, global trends in diazotroph abundance, activity, and diversity, as well as the environmental drivers controlling freshwater diazotrophy, remain unresolved. Here, we present a global assessment of freshwater diazotrophy based on more than 200 lentic and lotic sites across five continents. We applied a standardized sampling approach to quantify diazotroph abundance, N₂ fixation rates, and *nifH*-based diversity. The results of this comprehensive analysis, in addition to local environmental variables, were used to determine the global drivers of freshwater diazotrophy in lentic and lotic ecosystems through a multiple linear regression approach. Our results provide the first globally standardized framework for understanding freshwater diazotrophy, with implications for nitrogen cycling and the response of freshwater ecosystems to climate change.

RESULTS AND DISCUSSION

Global Patterns of Diazotroph Abundance, N₂ Fixation Rates, and Diversity Across the Freshwater Ecospace

The abundance of diazotrophs across global freshwater ecosystems has been largely unknown. In this research, we determined the average unicellular diazotroph abundance (DA) across the freshwater ecospace to be $4 \pm 4.8 \times 10^7$ cells L⁻¹, whereas the median value was 1.5×10^7 cells L⁻¹ (n=203; Figure 1A). The highest concentrations of diazotrophs were measured in Newmans Lake, Florida, USA and Lake George, Australia ($7.2\text{--}11 \times 10^8$ cells L⁻¹), which exceeded the average by an order of magnitude. Notably, these lentic sites were highly eutrophic (TN: ~ 2.8 mg L⁻¹, exceeding the hypereutrophic threshold of 1.2 mg L⁻¹; ²⁰), suggesting that freshwater diazotrophs could thrive in nutrient-rich ecosystems and not only in oligotrophic environments. The total bacterial and archaeal abundance in these systems (BA) according to flow cytometric analysis ranged from 4.7×10^7 to 1.8×10^{10} cells L⁻¹, with a global average of 1.6×10^9 cells L⁻¹ (Figure S1), yet negligible (<1%) concentrations of microalgae (Figure S10). The proportion of diazotrophs relative to the total bacterial and archaeal abundance (DA:BA) was highly variable, ranging from ~ 0.1 to 16% (Figure 1B). The DA:BA ratio was significantly greater

($p < 0.01$; Wilcoxon rank-sum test) in lotic environments ($n=88$) than in lentic environments ($n=115$), likely reflecting wider physicochemical heterogeneity of flowing systems. These values are consistent with the few local reports of planktonic DA in freshwater environments^{15,21}. Moreover, the diazotroph concentrations measured in this study are similar to those reported in marine environments (ranging from 10^1 to 10^7 cells L^{-1}), which have received considerably more attention^{22,23}. However, abundance alone does not capture the full ecological relevance of diazotrophy, which is also critically related to N_2 fixation rates.

While most aquatic N_2 fixation rates have been determined for marine ecosystems¹³, a recent metadata analysis of freshwater measurements highlighted the potential significance of diazotrophs to N budgets across inland waters⁴. In the present study, N_2 fixation rates were directly measured by analyzing the assimilation rates of dissolved $^{15}N_2$ at 139 freshwater sampling locations across the world. Although highly variable, the measured average rates (3.6 ± 14 nmol N $L^{-1} d^{-1}$, median of 0.13 nmol N $L^{-1} d^{-1}$) were within the range of values recently reported for inland waters⁴, but remained one order of magnitude lower than those typical of marine environments²³. Nonetheless, extremely high N_2 fixation rates (70 – 133 nmol N $L^{-1} d^{-1}$), which skewed the average rates, were detected at three eutrophic (TN: 3.1 – 5.5 mg L^{-1}) locations: Newmans Lake, Florida, USA; Lake Mattamuskeet, North Carolina, USA; and Wainscott Pond, New York, USA (Figure 1C). Similarly, a recent study of urban and suburban rivers in China, which are polluted by industrial and anthropogenic activities, reported exceptionally high N_2 fixation rates, ranging from 58 to 189 nmol N $L^{-1} d^{-1}$ ²⁴. These findings suggest that freshwater diazotrophy could be an important source of reactive nitrogen, and more widespread than previously reported, regardless of eutrophic conditions, as has been discussed for other aquatic ecosystems^{15,19,25}. Taken together, the rates measured globally further highlight that freshwater environments can sustain significant N_2 fixation, underscoring the potential importance of diazotrophy as a source of fixed N across freshwater ecosystems. Moreover, the N_2 fixation rates presented in our study were strongly correlated with the immunolabeling-based abundance of active diazotrophs (Pearson correlation coefficient, $R^2=0.7$; $P < 0.01$), indicating a close link between abundance and function.

Normalization of N_2 fixation rates to the number of immunolabeled diazotrophs provided an indirect estimate of cell-specific N_2 fixation (Figure 1D), revealing the isolated effect of cellular activity on bulk rates. The global cell-specific rates were generally consistent across freshwater habitats (0.11 ± 0.41 fmol N cell $^{-1} d^{-1}$). These results indicate that although N_2 fixation rates

have been linked to diazotroph lifestyle (i.e., free-living or aggregate associated) and specific abiotic conditions¹⁵, single diazotroph cells maintain comparable activity across freshwater environments, with no apparent trend. Only a few studies have reported cell-specific N₂ fixation rates ranging from 3×10^{-4} to $0.5 \text{ fmol N cell}^{-1} \text{ d}^{-1}$ in freshwater ecosystems¹⁵ and 0.05 to $9 \text{ fmol N cell}^{-1} \text{ d}^{-1}$ in marine ecosystems²⁶. The cell-specific rates measured herein are within those ranges and represent the first globally distributed freshwater dataset across diverse lentic and lotic ecosystems. These findings suggest that diazotrophs in freshwater and marine environments exhibit comparable levels of activity, although these comparisons should be interpreted cautiously given the differences in environmental conditions and community composition.

Bioinformatics analysis of *nifH* MiSeq revealed that diazotroph richness varied substantially across global freshwater ecosystems, with an average of 175 ± 139 ASVs (Figure 1E) and an average Shannon diversity index of 3.5 ± 1.3 (Figure 1F). Both diversity metrics are within the upper ranges reported for various marine and coastal systems^{27,28}, indicating that freshwater diazotroph assemblages harbor high taxonomic and functional diversity, further highlighting their ecological relevance. While no spatial patterns in diazotroph diversity were detected across the freshwater ecospace, the Shannon index was significantly greater (32%; $p < 0.01$; Wilcoxon rank-sum test) in lotic environments than in lentic environments (Figure 1Fi). Similar patterns have been reported in global bacterial surveys, where the alpha diversity of freshwater bacterioplankton was higher in lotic environments than in lentic environments²⁹. The higher diazotroph diversity observed in lotic systems than in lentic systems is likely due to greater physicochemical heterogeneity, which could be due to flow-driven sediment resuspension as well as the presence of microhabitats that contribute to gradients in oxygen, nutrients, and organic matter, such as different types of free-floating aggregates³⁰.

The diversity of diazotroph communities was further characterized by quantifying the relative abundance of *nifH* phylogenetic clusters (Clusters I–IV), which broadly classify these prokaryotes by evolutionary and functional affiliation³¹. All four *nifH* clusters were detected across the freshwater ecospace, with overall dominance of Clusters I (78%) and III (20%). Moreover, the relative abundance of diazotrophs ascribed to Clusters II and IV was significantly greater ($p < 0.01$; Wilcoxon rank-sum test) in lotic environments than in lentic environments (Figure 1F ii). Notably, Cluster IV may contain *nifH*-like paralogs that do not support N₂ fixation³², potentially introducing bias into estimates of diazotroph community

richness. Further diazotroph classification indicated that NCDs were dominant in all *nifH* clusters (62%–100%), whereas cyanobacterial diazotrophs dominated only Cluster I (34%), despite being present in Clusters I, III, and IV. These results indicate that NCDs play a central role in freshwater N₂ fixation, which is consistent with observations from marine environments³³. Moreover, these findings challenge the traditional view that freshwater diazotrophy is driven by cyanobacteria, instead suggesting a wider range of metabolic strategies to support BNF.

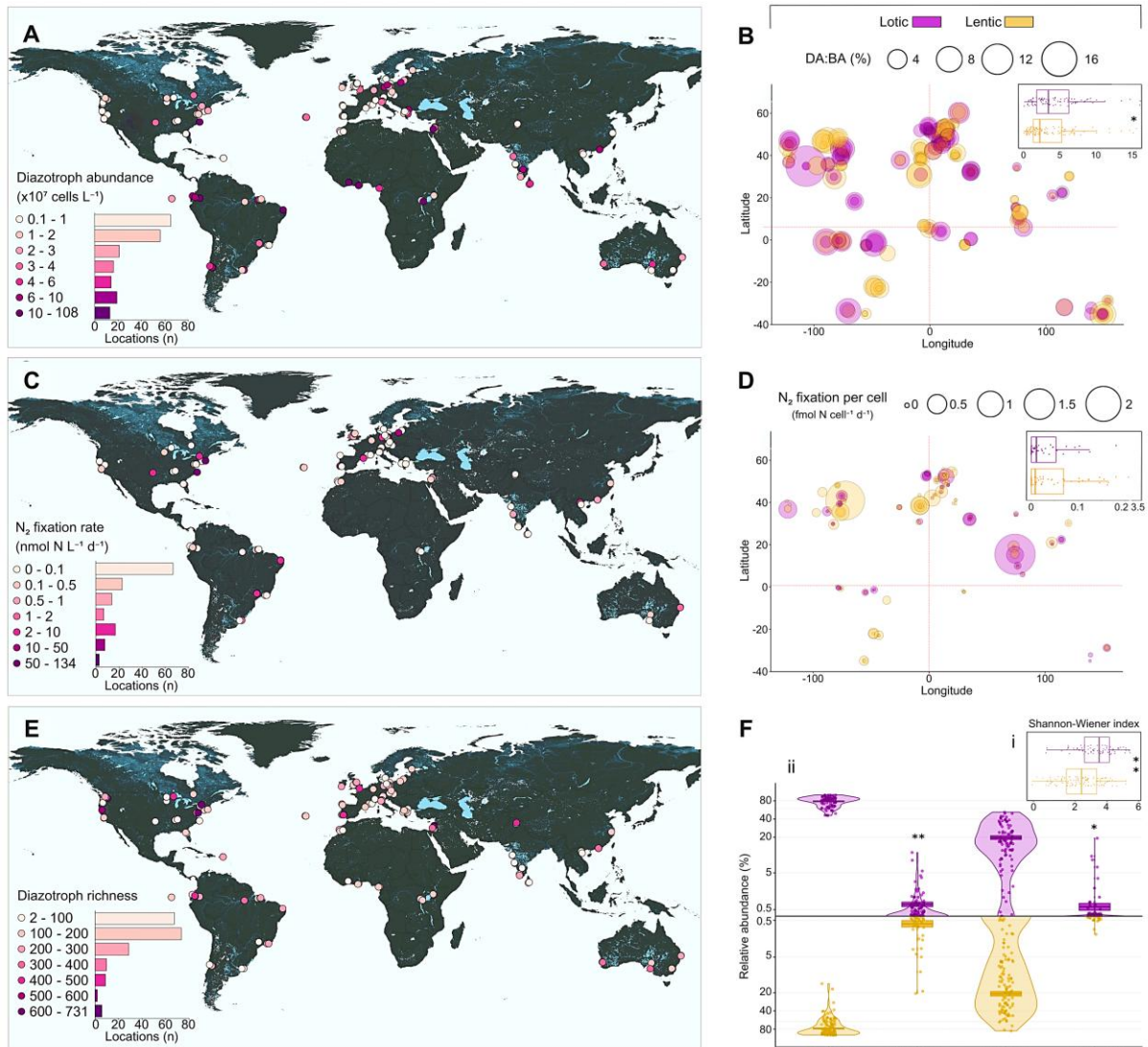


Figure 1. Diazotrophs are widespread across global freshwater ecosystems. (A) Unicellular diazotroph abundance (DA) based on immunolabeled flow cytometry. (B) Ratio of DA to bacterial/archaeal abundance (BA) in lentic and lotic environments in relation to latitude and longitude. (C) N₂ fixation rates based on the ¹⁵N₂ enrichment approach. (D) Cell-specific N₂ fixation rates calculated by dividing the measured rates by the number of immunolabeled diazotroph cells and plotted for lentic and lotic environments. (E) Richness of freshwater diazotroph taxa based on *nifH* sequencing. (F) Shannon–Wiener index calculated for lentic and lotic environments (i) and the relative abundance distribution of each *nifH* gene cluster in lotic (top) and lentic (bottom) environments.

Violin plots show the kernel density distribution of each *nifH* cluster, and the horizontal bars indicate the mean $\pm$ SE. Pseudolog₁₀ scaling was applied to improve the visualization of low-abundance clusters (ii). Inserts within panels (A), (C), and (E) indicate the number of sampling sites (locations) within each category. Asterisks represent significant differences according to Wilcoxon rank-sum tests (*: $p \leq 0.01$, **: $p \leq 0.001$).

NCDs Dominate N₂ Fixation Across the Pelagic Freshwater Ecospace

With respect to the global diversity of freshwater N₂ fixers, specific attention was given to the interplay between NCDs and cyanobacterial diazotrophs, with a focus on their relative abundance, taxonomic composition, and assignment to N₂ fixation categories across freshwater ecosystems. Freshwater diazotroph communities were composed predominantly of NCDs, with relative abundances exceeding 90% at 85% of the sampling locations (Figure 2A), suggesting that heterotrophic and chemolithotrophic diazotrophs, rather than cyanobacteria, are the prime N₂ fixers across these freshwater ecosystems. These findings extend previous local and regional reports of NCD occurrence in lentic¹⁴ and lotic¹⁵ ecosystems to a global scale, as reported across the marine environment³⁴. While no spatial patterns were observed in terms of freshwater NCDs, their relative abundance was 30% greater in lotic ecosystems than in lentic ecosystems (Figure 2B). A similar pattern has been reported for the ratio of planktonic heterotrophic to phototrophic bacteria on the basis of 16S rRNA surveys across various freshwater environments^{35,36}. The greater environmental heterogeneity of lotic than lentic ecosystems observed in our study (PERMANOVA: $F = 5.63$, $p < 0.001$) likely underlies this difference as physicochemical variability of flowing systems may favor NCDs owing to their metabolic and physiological versatility¹⁹.

Despite the environmental heterogeneity between ecosystems, the order-level composition within NCD communities across freshwater environments shared a common compositional backbone, dominated by *Burkholderiales* and *Desulfuromonadales*, whose abundance ranged from 16% to 23% (Figure 2B). These orders are recognized as metabolically versatile, heterotrophic freshwater lineages capable of exploiting diverse substrates, making them plausible core members of NCD assemblages across habitat types^{37,38}. The key order-level compositional change between ecosystem types was the replacement of *Pseudomonadales* (9%) in lentic environments by *Rhodocyclales* (10%) in lotic environments. *Pseudomonadales* are typically associated with organic-rich, relatively stable water columns, whereas *Rhodocyclales* are more frequently linked to dynamic, nutrient-rich settings where denitrification and other nitrogen transformations are active^{37,38}. These community changes, while modest in magnitude, suggest that the distinct environmental setting of lotic compared

with lentic ecosystems selectively favors the NCD specialists superimposed on an otherwise shared backbone of heterotrophic diazotrophs.

Cyanobacterial diazotrophs showed a consistent order-level composition across both lentic and lotic environments, including *Nostocales* (~57%), *Oscillatoriales* (~38%), and *Chroococcales* (~5.5%) (Figure 2B). Despite representing a small fraction of the diazotroph community, these cyanobacterial orders include filamentous, physiologically plastic taxa that are well adapted to persist in freshwater environments^{39,40}. *Nostocales* in particular include heterocytous diazotrophs and buoyant species that can form dense blooms, often outcompeting other phototrophs⁴¹. The members of this order have high tolerance to variable light conditions, as many of these species can grow under low light and possess UV-absorbing compounds that make them resistant to extreme irradiance⁴¹. These traits may explain why *Nostocales* and *Oscillatoriales* consistently constitute the cyanobacterial diazotroph fraction across both ecosystem types, even in environments where NCDs dominate. Importantly, both cyanobacterial orders include taxa known to produce toxins and form harmful blooms under environmental conditions that spur extensive cyanobacterial growth⁴⁰. While our dataset does not directly assess bloom dynamics, the consistent presence of these bloom-forming orders across the freshwater ecospace suggests that environmental shifts favoring cyanobacterial diazotrophs over NCDs (such as warming, increased stratification, or nutrient loading) could have implications for freshwater quality on the basis of their known ecological roles.

The distribution patterns described above were further examined across four categories of N₂ fixation rates (nmol N L⁻¹ d⁻¹): Zero, Low (0.001–0.2), Medium (0.21–1.4), and High (1.5–133) to estimate whether community composition shifted alongside diazotrophic activity. NCDs were significantly more prevalent than cyanobacterial diazotrophs ($p < 0.01$) regardless of the measured N₂ fixation rates, with relative abundances of 79%, 86%, 82%, and 67% in the Zero, Low, Medium, and High categories, respectively (Figure 2Ci). Notably, the dominance of NCDs was most pronounced at low N₂ fixation rates and progressively decreased as the rates increased, suggesting that at higher N₂ fixation rates (>1.5 nmol N L⁻¹ d⁻¹), the role of cyanobacterial diazotrophs in driving bulk fixation activity increased. We further deduce that this trend reflects environmental conditions (such as warm temperatures, stratification, and eutrophication) that favor cyanobacterial diazotrophs over NCDs, leading to higher N₂ fixation rates.

Across all N₂-fixation categories, *Nostocales* remained the dominant cyanobacterial order (Figure 2Cii), whereas *Desulfuromonadales* and *Burkholderiales* dominated the NCD fraction (Figure 2Ciii), confirming that the core community structure identified across ecosystem types is independent of fixation activity. The persistence of *Desulfuromonadales* and *Burkholderiales* even at low N₂ fixation rates is consistent with the broad metabolic versatility of these NCDs^{42,43}, allowing them to prevail regardless of whether N₂ fixation provides an additional fitness advantage. The dominance of *Desulfuromonadales* and *Burkholderiales* within NCDs suggests that their diazotrophic potential, combined with broad ecophysiological versatility, underpins their abundance across diverse environmental regimes. Together, these results emphasize that freshwater N₂ fixation is primarily supported by metabolically versatile NCDs, whereas cyanobacterial diazotrophs play a more limited role, yet become increasingly important under high-fixation conditions dominated by bloom-forming orders.

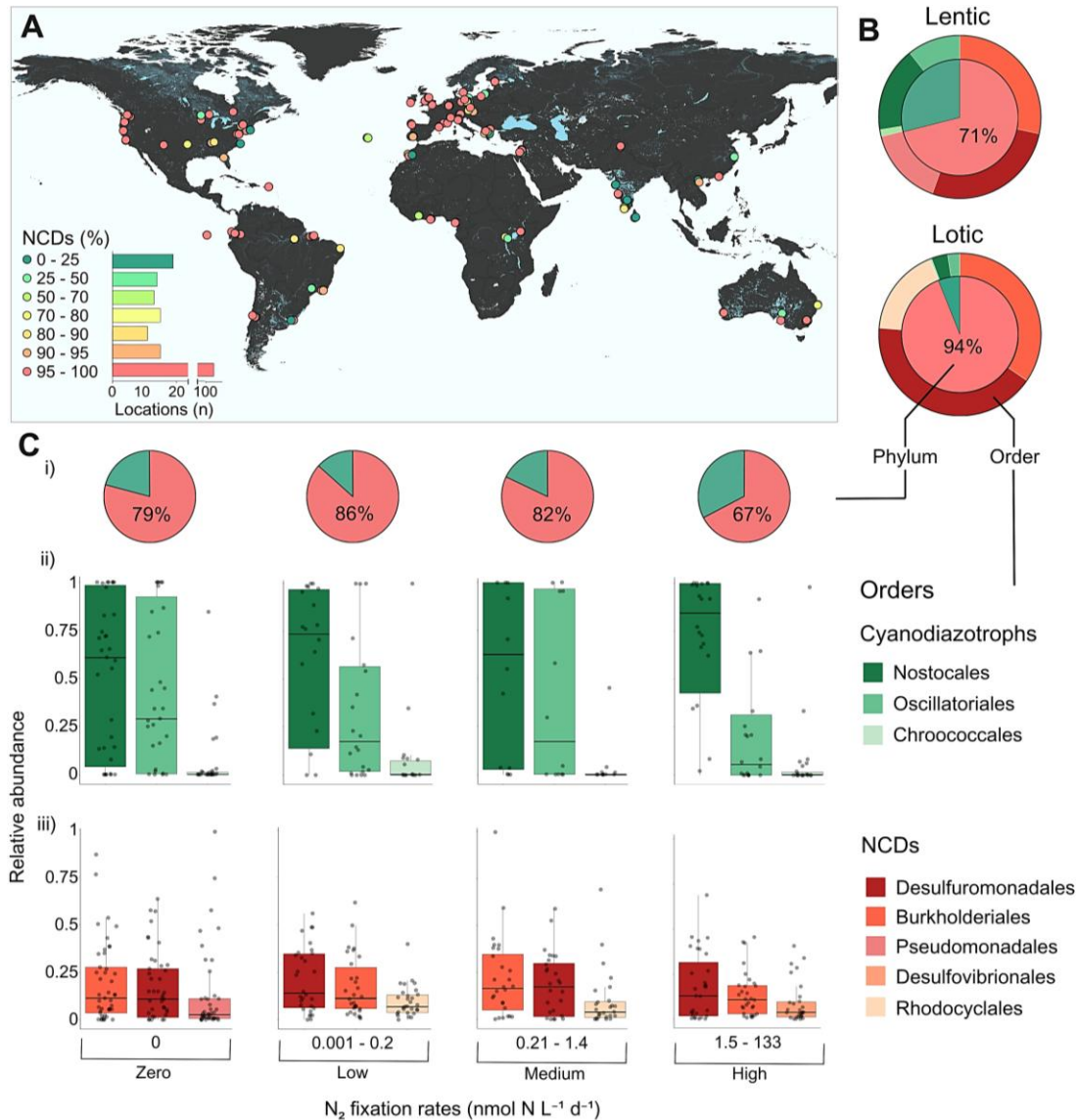


Figure 2. Noncyanobacterial diazotrophs (NCDs) dominate the global distribution of nitrogen fixers over cyanobacterial diazotrophs. (A) Proportion of NCDs. Sampling locations with a clear majority of NCDs are highlighted by red tones, while those with a majority of cyanobacterial diazotrophs are denoted by green tones. The inset indicates the number of sampling sites (locations) within each category. (B) Composition (top 3 orders) of cyanobacterial diazotrophs and NCDs at lentic and lotic sampling locations. (C) Proportion of cyanobacterial diazotrophs and NCDs (i) and the relative abundance of their dominant (top 3) orders (ii, iii) in the four categories of N₂ fixation rates.

Temperature-Dependent Factors control Freshwater N₂ Fixation Rates

The environmental factors that drive freshwater N₂ fixation were identified by applying a threshold-based multilinear regression (MLR) framework using a prescreened set of variables (detailed in the Supplementary Information). The importance of each variable was quantified using the Lindeman–Merenda–Gold (LMG) method, which involves partitioning the modeled R² among predictors ⁴⁴. Testing temperature thresholds from 15 to 25°C after excluding MLRs that were overfitted or had unrepresentative sample sizes indicated that 21°C generated the strongest models (above 21°C: Adj. R² = 0.92, AIC = -85.3; below 21°C, Adj. R² = 0.34, AIC = -42) (Figure 3A). The MLR results indicated that different environmental variables were associated with freshwater N₂ fixation rates above and below a temperature threshold of 21°C (Figure 3A). Across both temperature regimes, diazotroph abundance (DA) was the primary predictor and positively covaried with global freshwater N₂ fixation rates (Figure S2 and S3), although its relative importance differed across this threshold (59% above and 27% below 21°C, Figure 3B). Regardless of the predictors, a greater proportion of N₂ fixation rates were explained above 21°C, suggesting that the environmental controls of such rates are better captured at warmer sites.

Below 21°C, the correlation between DA and N₂ fixation rates was weaker ($r=0.41$, $p<0.001$) than above the threshold ($r=0.93$, $p<0.001$), indicating that although DA remains the strongest global predictor, it alone explains less of the variation in N₂ fixation rates at lower temperatures. These results suggest that in addition to DA, N₂ fixation rates are also affected by the rate at which the nitrogenase enzyme catalyzes N₂ fixation, as evidenced in previous research ¹⁸. The substrate concentration required for half-maximal activity of the nitrogenase increases with temperature, plateauing at 23–25°C ⁴⁵; below this range, nitrogenase efficiency depends on temperature. Therefore, below 21°C, N₂ fixation rates likely depend not only on community size but also on enzymatic efficiency.

Both electrical conductivity (EC) and the relative abundance of NCDs were also identified as important predictors of N₂ fixation rates (explaining 5% and 6%, respectively), both of which negatively covary with N₂ fixation rates below 21°C, which is consistent with NCDs being proportionally more abundant at sites with lower total fixation (Figure 2C). Given the weak association between N₂ fixation rates and the relative abundance of NCDs while dominating across all these fixation categories, the observed relationship likely reflects a shift in the diazotrophic community composition rather than reduction in their contribution to fixed nitrogen. Indeed, marine N₂ fixation rates in low-temperature environments (< 21°C), which span high latitudes (> 60° N) yet thermally stratified systems, has been recently attributed primarily to planktonic or particle-associated NCDs rather than cyanobacterial diazotrophs⁴⁶, given their ability to tolerate a broad range of temperature, light, and redox conditions¹⁶.

Above the 21°C threshold, the number of phototrophs (<10 µm) and the fraction of total bacteria composed of diazotrophs (DA:BA) were identified as secondary predictors (explaining 16% and 8% of the variation in N₂ fixation, respectively), both of which negatively covaried with N₂ fixation rates (Figures 3B and S3). The negative link between DA:BA and N₂ fixation rates under warm temperatures (>21°C) contrasts with the positive relation between these rates and DA only. This contrasting pattern suggests that high fixation rates were driven by a small subset of highly active diazotrophic taxa rather than by a proportionally large diazotrophic community. This conceptual premise is consistent with the dominance of metabolically versatile NCDs, such as *Desulfuromonadales*, as the core freshwater diazotrophs. Concomitantly, the abundance of phototrophs in warmer environments can indirectly constrain N₂ fixation via intensified competition for dissolved inorganic nutrients. These types of microbial dynamics are well documented in marine ecosystems, where micro-phytoplankton and diazotrophs compete for dissolved inorganic nutrients, such as phosphorus⁴⁷, which may similarly occur in freshwater environments.

MLR analysis was complemented by PERMANOVA and PCA to assess whether N₂ fixation rates clustered in the multivariate space according to the 21°C threshold, providing general patterns of site similarity rather than predicting fixation rates directly. N₂ fixation rates and the associated conditions clustered significantly above and below 21°C (PERMANOVA, $p < 0.01$; Figure 3C), confirming that the two temperature regimes are characterized by distinct multivariate profiles. The environmental vectors in the ordination, displayed as arrows indicating the direction and magnitude of association between variables and site distributions,

largely aligned with the predictors identified in the respective MLRs. The convergence between the multivariate ordination and MLR approaches supports the interpretation of 21°C as an ecological threshold and indicates that both analytical frameworks independently converge on the same temperature-dependent environmental associations. These findings are consistent with localized studies reporting that water temperature is a primary driver of aquatic diazotrophy, affecting the abundance, N₂ fixation rates, and diversity of these microbes⁹, while at the same time extend these insights to the global scale. We hypothesize that under climate change, as more freshwater sites exceed the 21°C threshold, the environmental control of N₂ fixation rates will shift from abiotically dominated to biologically driven dynamics, potentially reshaping the diazotrophic community and its balance with NCDs.

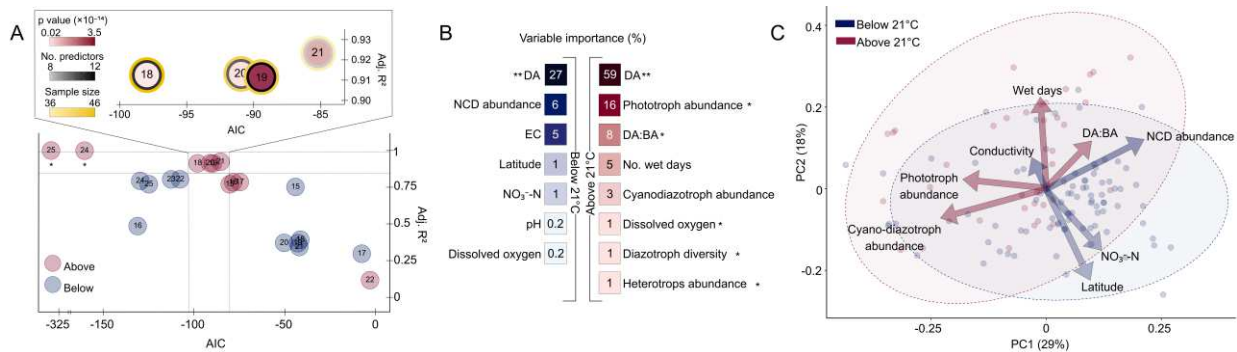


Figure 3. Global drivers of N₂ fixation. (A) Adjusted R² and AIC values of the best-performing MLR models for predicting N₂ fixation across potential temperature thresholds ranging from 15 to 25°C at 1°C intervals. The inset highlights the strongest-performing models at thresholds between 18 and 21°C (above-threshold group). The selected threshold was based on the explanatory power and parsimony of the model. (B) Relative importance of predictors in the MLR models below and above 21°C, with shade intensity indicating the level of importance and the exact importance values shown inside the boxes. (C) Principal coordinate analysis (PCA) of N₂ fixation rates showing sample distributions below and above 21°C, with environmental vectors representing the MLR predictors of importance $\geq 2\%$.

Temperature Structures the Drivers of Freshwater Diazotroph Diversity

Determining the environmental factors that control the relative abundance of NCDs is important because of their global dominance. A threshold-based MLR approach, matching the methodology described in the preceding section, revealed that a water temperature of 9°C affects the environmental factors that control the relative abundance of NCDs (Figure 4A; Supporting Information). This temperature threshold provided the best explanatory power for both temperature regimes (above 9°C: Adj. R² = 0.52, AIC = 217.6; below 9°C: Adj. R² = 0.98; AIC = -31.8). The stronger fit below 9°C indicates that the relative abundance of NCDs in cold

freshwater systems is characterized by a specific set of environmental conditions, whereas at warmer sites ($>9^{\circ}\text{C}$), the drivers remain partially unexplained.

At cold sites ($< 9^{\circ}\text{C}$), water temperature was the strongest predictor of the relative abundance of NCDs (explaining 18%), with the two covariates positively correlated (Figures 4B and S4). At cold sites, even small increases in water temperature provide metabolic advantages to NCDs over cyanobacterial diazotrophs, likely through enhanced ATP production and enzyme efficiency^{16,25}. Diazotroph diversity and phototrophic abundance were also identified as important drivers of the relative abundance of NCDs below 9°C (explaining 17% and 13%, respectively), both of which were negatively covaried with it (Figures 4B and S4). These statistical associations suggest that in cold ecosystems, increases in diazotroph diversity and phototrophic biomass reduce the proportional dominance of NCDs. The dissolved oxygen concentration, N:P ratio, bacterial abundance, and ammonium concentration were also recognized as the drivers for the relative abundance of NCDs below 9°C (explaining between 5% and 11%), all of which positively covaried with it (Figure S4). Traditionally, dissolved oxygen and high ammonium concentrations impaired N_2 fixation⁵; however, the positive relationship observed in this study coincides with research reporting high fixation rates by NCDs under such adverse conditions^{25,48}. Under such environmental constraints, NCD communities are governed by the availability of organic matter⁴⁹, which is often associated with marine, lake, or river snow due to high C:N ratio comprising these particles and the microaerophilic zones within them¹⁹.

Above the 9°C threshold, diazotroph diversity emerged as the strongest predictor (14%), positively covarying with the relative abundance of NCDs (Figures 4B and S5). This positive link indicates that once temperature is no longer a constraint, NCDs encompass greater taxonomic and niche diversity. This assumption is supported by prior studies attributing greater diazotroph diversity to NCD niche partitioning¹⁶. Concurrently, phototroph abundance negatively covaried with the relative abundance of NCDs (11%), whereas the DA:BA ratio and orthophosphate concentration were positively covaried with it (each explaining 5% and 4%, respectively) (Figures 4B and S5). These relationships indicate that at temperatures $> 9^{\circ}\text{C}$, nutrient competition likely becomes a significant structuring force, with NCDs limiting the abundance of cyanobacterial diazotrophs as competition for available phosphorus intensifies. Similar patterns have been reported in marine environments, where increasing temperatures intensify competition for dissolved inorganic nutrients (e.g., orthophosphate) between

phototrophs and NCDs, reducing their relative abundance⁵⁰. Together, these results suggest that above 9°C, the relative abundance of NCDs is determined by community-level diversity and nutrient competition rather than direct thermal constraints.

PERMANOVA and PCA further revealed that different sampling sites significantly clustered (PERMANOVA: $R^2=0.049$, $F=5.55$, $p=0.001$) above and below a water temperature of 9°C (Figure 4C). Colder sites (< 9°C) were oriented in the direction of the temperature and latitude vectors, whereas warmer sites (> 9°C) were strongly aligned with diazotroph diversity and DA:BA vectors (Figure 4C), which are consistent with the predictors identified in the respective MLRs. Variables appearing as gray arrows (Figure 4C) were identified as predictors in both MLRs, indicating an association with the relative abundance of NCDs regardless of the temperature threshold. Although the array of predictors controlling the relative abundance of NCDs differs above and below 9°C, few variables, such as diazotroph diversity and phototroph abundance, are relevant across the full temperature range. Notably, samples below 9°C formed a cluster embedded within the broader distribution of samples above 9°C, suggesting greater similarity between NCD communities across colder ecosystems than among sites with warmer water temperatures.

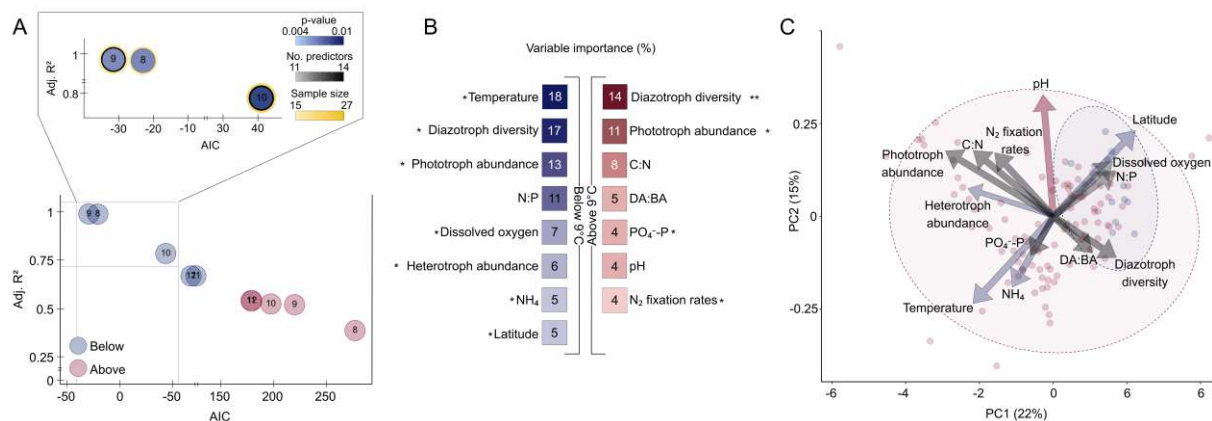


Figure 4. Temperature-dependent environmental drivers of NCD abundance. (A) Adjusted R^2 and AIC values of the best-performing multiple linear regression (MLR) models for predicting the relative abundance of NCDs (% of the total diazotrophic community) across temperature thresholds ranging from 8 to 12°C. The inset highlights the strongest-performing models at thresholds of 8 and 9°C (above-threshold group). The 9°C threshold was selected because it provides the optimal balance of explanatory power and model parsimony. (B) Relative importance of predictors in the MLR models below and above 9°C. Shading intensity indicates predictor importance, with the exact importance values shown inside the boxes. (C) PCA of the relative abundance of NCDs showing the sample distribution below and above 9°C, with environmental vectors representing the MLR predictors of importance $\geq 5\%$. The gray arrows indicate variables that were identified as predictors in both MLR

models. PERMANOVA was used to test whether the relative abundance of NCDs at each site significantly clustered in the PCA ordination according to the 9°C threshold ($R^2=0.049$, $F=5.55$, and $p=0.001$).

Freshwater Diazotrophy During an Era of Climate Change

The measurements reported here indicate that freshwater N_2 fixation is globally ubiquitous, with rates reaching as high as $133 \text{ nmol N L}^{-1} \text{ d}^{-1}$. Together with recently published global estimates of up to $32.3 \text{ Tg of fixed N per year}^4$, our results highlights that freshwater N_2 is a significant source of N, far exceeding the findings of previous paradigms. Notably, the global importance of freshwater diazotrophy may even extend beyond what is captured in this study, as recent work has reported high N_2 fixation rates in previously overlooked environments such as the freshwater subsurface (up to $585 \text{ nmol N L}^{-1} \text{ h}^{-1}$)⁵¹ and streambeds (up to $4 \times 10^7 \text{ nmol N m}^{-3} \text{ d}^{-1}$)²¹, which are not included here or in any ^{15}N assimilation-based global estimates.

Moreover, our results indicate that NCDs dominate global freshwater diazotrophs (>90% relative abundance at 85% of sites), revising the traditional cyanobacteria-centric paradigm⁵². Yet, these NCDs, are not only heterotrophs but could also be found as chemolithotrophs or mixotrophs, capable of coupling N_2 fixation to inorganic energy sources. Such metabolic plasticity, especially during an era of changes in water temperature, may increase the ability of diazotrophs to fix N_2 and dominate environments that were previously ignored. Indeed, water temperature emerged as the key factor structuring the drivers of freshwater diazotrophy, with thresholds at 21°C and 9°C delineating distinct regimes for total N_2 fixation rates and the relative abundance of NCDs. These thresholds are directly relevant under projected climate warming, as freshwater temperatures have risen substantially across both lentic and lotic systems in recent decades^{53–55}. Lakes have warmed at a global mean rate of 0.34°C per decade, with high-latitude lakes reaching rates of up to 0.72°C per decade⁵³. Rivers have warmed at 0.16°C per decade on average, with some regions exceeding 1°C per decade⁵⁴. These trends are projected to accelerate, with 27% of rivers and 66% of lakes expected to shift to warmer thermal regimes by the end of the century⁵⁵ under intermediate and high greenhouse gas concentration trajectories (RCPs 6.0 and 8.5, respectively). As cold sites transition above the 9°C threshold, NCD communities currently structured by thermal limitation may shift toward diversity- and nutrient-driven dynamics. Concurrently, warming favors cyanobacterial diazotrophs through higher thermal optima and competitive advantages for phosphorus acquisition⁵⁶, potentially increasing the occurrence of harmful diazotrophic blooms associated with toxin release⁵⁷, thus jeopardizing freshwater quality. Finally, it is expected that thermal

shifts at the global scale would affect N₂ fixation patterns, with marked implications for nitrogen cycling models.

MATERIALS AND METHODS

Sampling Strategy

Surface freshwater samples (depths of 0.2–1 m) were collected from 206 individual sites across 36 countries globally (Figure S6; specific locations are provided in the Supporting Information). Surface water was selected and defined to standardize sample collection across diverse global hydrologic systems, allowing a uniform sampling strategy. The environments were selected through an international collaborative network, with each group contributing samples from one to three sites of ecological relevance within their region, collectively spanning diverse climatic zones, land uses, and geographic gradients (detailed in the Supporting Information). Freshwater samples were collected following standardized procedures provided to participants in a video manual (Figure S7, Supplementary video), and using a custom-made kit (Table S1). All the reported analyses included independent quality controls to ensure the validity of the results (Figures S9–S11, Table S2). All the sites were sampled between April and May 2023, covering spring in the Northern Hemisphere and autumn in the Southern Hemisphere, providing a broad global coverage. The samples were shipped in disposable coolers preloaded with ice packs to maintain low temperature during transit. The bacterial and diazotrophic abundance as well as diversity were measured in all the samples, while the N₂ fixation rates were determined in dedicated microcosms. Additional freshwater samples were collected for all sites to determine the total organic carbon, total nitrogen, dissolved nitrite, nitrate, ammonium and orthophosphate concentrations. Samples that required immediate preservation were fixed after collection (detailed in the Supporting Information). Auxiliary metadata included the sampling time, solar irradiance, water temperature, salinity, dissolved oxygen concentration, and land use (details are provided in the Supporting Information). These variables were used to determine the physicochemical trends and correlations with global freshwater diazotrophy to define potential drivers.

Freshwater Analyses

Quantifying diazotroph and bacterial abundances using flow cytometry. Briefly, surface water (12 mL) was fixed with glutaraldehyde for 10 min at room temperature (final concentration of

0.02%). Total bacterial abundance was quantified from 300 μ L subsamples by staining cells with LightCycler 480 SYBR Green I Master (excitation wavelength: 480 nm; emission wavelength: 530 nm; catalog no. 04707516001; Roche Diagnostics, Mannheim, Germany). Diazotrophs were identified and quantified by nitrogenase immunolabeling using fluorescent antibodies targeting the nitrogenase enzyme to selectively label and distinguish active diazotrophic cells from total bacteria⁵⁸. Stained and immunolabeled samples were identified and quantified using a flow cytometer (Attune NxT, Thermo Fisher Scientific) at a flow rate of 100 μ L min⁻¹. Unstained samples were also analyzed to quantify the abundance of cyanobacteria and pico- to micro-sized algae, which were identified on the basis of their characteristic autofluorescence signals detected by flow cytometry (chlorophyll a red fluorescence and phycoerythrin orange fluorescence). Notably, despite being present, the average percentage of micro-pico algae was less than 1% from the total BA (Figure S10). Additional details related to the specific staining procedures used during immunolabeling, corresponding controls and flow cytometry protocols are provided in the Supporting Information. Cell-specific N₂ fixation rates were calculated by dividing the N₂ fixation rate to the number of diazotrophic cells.

Quantification of N₂ fixation rates. On-site, the samples were transferred into three gas-tight clear bottles and spiked with ¹⁵N₂-enriched freshwater (7.5% v:v; preparation detailed in the Supporting information and Figure S8). Two additional bottles, without enrichment, were kept as controls. All bottles were incubated for 24 h under field conditions (temperature and light exposure), either at the sampling site or reproduced in the laboratory by the partners. At the end of the incubation, the samples were filtered onto precombusted GF/F filters (15–1500 mL). The filtration volumes were highly variable, ranging from 15 mL to 1500 mL, reflecting variability in particulate matter concentration across sites. These volumes were recorded and used to normalize the fixation rates. Dried filters were analyzed using a mass spectrometer (Flash 2000 HT, Thermo Scientific, Waltham, MA, USA) coupled to an isotope ratio mass spectrophotometer (Delta V Plus, Thermo Scientific) according to the method described by Geisler *et al.* 2023⁵⁸. N₂ fixation was calculated on the basis of the difference in ¹⁵N abundance between the enriched samples and those without enrichment (¹⁵N natural abundance) and normalized by incubation time and filtration volume. Additional details are provided in the Supporting Information.

DNA extraction, nifH gene sequencing, and bioinformatics. Water samples (up to 1 L) were filtered on-site using 0.2- μ m Sterivex™ filter units (Millipore®, Cat. #SVGPL10RC) and immediately preserved with 3 mL of RNAlater™ (Invitrogen™, Cat. #AM7021). Upon arrival at the laboratory, the filters were stored at -80°C until further processing. DNA was extracted using a DNeasy PowerWater Sterivex Kit (QIAGEN, Cat. #14600-50-NF) according to the manufacturer's protocol. DNA sequencing was performed at the RUSH Genomics and Microbiome Core Facility. Downstream bioinformatics, including quality filtering, ASV inference, and taxonomic annotation, was performed locally following the procedure of Morando *et al.* (2025), with a few modifications (detailed in the Supporting Information). The main pipeline outputs were used to calculate alpha diversity indices (Chao1 richness and Shannon–Wiener indices) and the taxonomic composition, which revealed the main contributors to global freshwater N_2 fixation, and were incorporated into the regression analyses described below. The comprehensive methodological details and parameters used are detailed in the Supporting Information.

Nutrient analyses: Organic carbon and nitrogen. Water samples were collected on-site using acid-washed glass tubes and immediately acidified (1 M hydrochloric acid; final concentration: 0.1%) to halt biological activity. Upon arrival at the laboratory, the samples were stored at -20°C until further analysis. Each sample was analyzed for total and dissolved organic carbon and nitrogen following filtration through 0.45- μ m PVDF filters (Millex® -GV) using a Multi N/C analyzer (Analytik Jena, Jena, Germany; detection limit = 0.3 mg L^{-1}). Additional details are provided in the Supporting Information.

Nutrient analyses: Inorganic nutrients. Water samples were prefiltered on-site through 0.2 μ m filters (Millipore® Sterivex™, Cat # SVGPL10RC) into 50 mL Falcon tubes. Upon arrival at the laboratory, the samples were stored at -20°C until further analysis. Orthophosphate (PO_4^{3-}), $\text{NO}_3^- + \text{NO}_2^-$, and ammonium (NH_4^+) concentrations were measured using a segmented flow autoanalyzer system (AA-3; Seal Analytical, Wrexham, UK) equipped with a colorimetric detector. Additional details are provided in the Supporting Information.

Multiple linear regression (MLR) modeling to identify diazotrophy drivers. All available in situ physicochemical measurements, laboratory-derived variables, and additional globally available gridded geophysical and climatic data (detailed in the Supporting Information) were used as potential predictors of N_2 fixation rates and of the relative abundance of NCDs, maximizing the environmental information available to the MLRs. A two-step screening

procedure was applied to reduce multicollinearity, retaining variables with pairwise correlations $|r| \leq 0.8$ and variance inflation factors $\leq 10^{59}$. Temperature-dependent patterns motivated a stratified modeling framework in which datasets were partitioned across a range of temperature thresholds and all possible predictor combinations were evaluated. Model selection was based on Akaike's information criterion (AIC), with the optimal threshold chosen according to model fit, parsimony, and sample size criteria. The relative contribution of each driver to freshwater diazotrophy was ranked on the basis of its importance as a predictor within the MLR. Predictor importance was quantified using the Lindeman–Merenda–Gold (LMG) method, which partitions total model R^2 among predictors by averaging their marginal contributions across all possible predictor orderings⁴⁴, providing a robust importance metric in the presence of correlated predictors. A principal component analysis (PCA) biplot was generated using the environmental and biological variables with LMG importance $>2\%$ in either the above- or below-threshold MLR for N_2 fixation, or $>5\%$ in either MLR for NCD relative abundance. Permutational multivariate analysis of variance (PERMANOVA) was applied to assess significant differences in multivariate composition between temperature range groups. Comprehensive methodological details for each MLR are detailed in the Supporting Information.

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AUTHOR CONTRIBUTIONS

EB-Z, SA and ER conceived this study. EB-Z and AG designed, organized and launched the sampling campaign. AG, EG and CV analyzed all the biological data. HS and EG were responsible for all the IRMS analysis including quality controls and standards. GSV analyzed the samples and provided nutrient concentrations for all the sampling sites. CV and TT developed the MLR and further analyzed the data. The manuscript was written mainly by EB-Z, CV and TT. However, SA, AG, HPG, MB, LB and ER critically revised early versions.

Nonetheless, all authors commented and contributed to previous versions of the manuscript.
All authors read and approved of the final manuscript.

COMPETING INTERESTS

There are no competing interests. Attached is Nature's formal form.

ETHICS STATEMENT

This study involved the collection and analysis of environmental freshwater samples and did not involve human participants, human-derived materials, or experiments on animals. All sampling was conducted in accordance with applicable local and national regulations and with the relevant permissions and permits required at each sampling location. Local researchers and collaborators were involved in the design, implementation, and interpretation of the research, where applicable.

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