REVIEW SUMMARY

NITROGEN FIXATION

Changing perspectives in marine nitrogen fixation

Jonathan P. Zehr* and Douglas G. Capone*

BACKGROUND: Biological dinitrogen (N_2) fixation, the reduction of atmospheric N_2 to ammonia, is important for maintaining the fertility of the oceans by providing biologically useful nitrogen to support primary organic matter production (i.e., carbon dioxide fixation). N_2 fixation offsets the removal of combined nitrogen by microbial denitrification and anaerobic ammonium oxidation (anammox) and export to the deep sea. For several decades, there has been a lack of consensus as to whether losses of N through microbial removal pathways are balanced by biological nitrogen fixation, along with other inputs such as atmospheric nitrogen deposition and terrestrial runoff. N_2 fixation was also generally thought to be largely attributable to only two types of cyanobacteria—the free-living filamentous colony-forming *Trichodesmium* and the symbionts of diatoms, *Richelia*—both of which were found primarily in tropical and subtropical surface waters. Very little was known about when and where N_2 fixation occurred, or whether there were more N_2-fixing species not yet identified. Nor did we know much about the genetic capabilities and adaptations of the marine N_2 fixers and how they acquired other nutrients required for their growth. Hypotheses based on biogeochemical models predicted that regional sites of N losses were linked to N_2 fixation, suggesting a mechanism that could maintain the balance of the global ocean N budget. Understanding the balance of the N cycle in the sea has wide-ranging implications for past, current, and future foodwebs, as well as for the role of marine N_2 fixation in the sequestration of atmospheric CO_2 and the production and consumption of other greenhouse gases such as nitrous oxide. With intensive research over the past several decades, many of these earlier suppositions have been displaced and supplanted by new models as our knowledge of the players and processes has greatly increased. Nonetheless, many new questions have arisen and old ones remain to be fully solved.

ADVANCES: Research on marine N_2 fixation has been aided by the development of new technical and modeling approaches, including genomic and metagenomic analysis of natura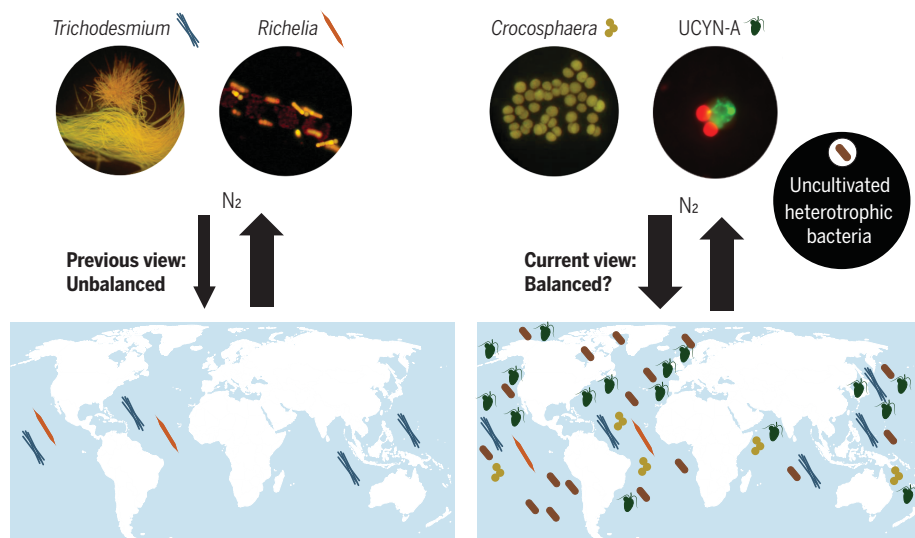l populations, isotope geochemistry, and satellite remote sensing. Global surveys using high-throughput nucleic acid sequencing have uncovered novel organisms and provided new information about the diversity, global distribution, and phylogenetic affiliations of N_2-fixing microorganisms (also referred to as diazotrophs). The known diversity of potential N_2-fixing marine microorganisms has thus greatly expanded and now includes a free-living unicellular cyanobacterium (*Crocosphaera*), a group of novel unicellular cyanobacteria (UCYN-A) symbiotic with a unicellular alga, and many lineages of heterotrophic or photoheterotrophic bacteria. The cyanobacterial symbiosis is important in oceanic N_2 fixation and is characterized by genome reduction and coevolution between cyanobacteria and their eukaryotic hosts. Heterotrophic marine diazotrophs are diverse but have not yet been definitively shown to fix N_2 or to contribute substantially to water column N_2 fixation. Benthic habitats and associations of diazotrophs with macroorganisms have also been identified. Large-scale biogeochemical controls (primarily the inputs and availability of phosphorus and iron) and physical controls on N_2 fixation are beginning to be more fully understood, including how they vary across ocean basins. The local and global contributions of individual species are still not fully understood and remain difficult to assess, which presents important research challenges for the future. Observations and new assessments suggest that N_2 fixation and N removal through denitrification (canonical and anammox) may be in approximate balance, although the stabilizing feedbacks, such as the extent to which input and removal processes are linked in space and time, are still debated.

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OUTLOOK: There are likely to be yet more discoveries of novel organisms and a refined view of where and when N_2 fixation occurs and is controlled, leading to a greatly improved predictive capability for global N_2 fixation. Distributions of N_2-fixing microorganisms and N_2 fixation are expected to respond to chemical and physical alterations resulting from global climate change. Marine N_2 fixers could contribute to the rapidly developing field of marine aquaculture and biotechnology. They have also been suggested as possible targets for upper-ocean fertilization to promote sequestration of atmospheric carbon. ■

Trichodesmium *Richelia* *Crocosphaera* UCYN-A

Uncultivated heterotrophic bacteria

N_2 N_2

Previous view: Unbalanced **Current view: Balanced?**

N_2 fixation in the marine environment and changes in perspectives in recent decades. N_2 fixation was largely attributed to *Trichodesmium* and *Richelia* (upper left). New organisms have been discovered by nucleic acid sequencing, including diverse cyanobacteria (the unicellular symbiotic UCYN-A and free-living *Crocosphaera*) and noncyanobacterial diazotrophs (currently uncultivated and not visualized). N_2 fixation has been found in an increasing array of environments previously not believed to be important regions for N_2 fixation, such as coastal regions and high latitudes. Previously, N losses were believed to exceed N_2 fixation inputs, but whether the N budget of the oceans is balanced remains a matter of controversy.

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Jonathan P. Zehr[1]* and Douglas G. Capone[2]*

Nitrogen fixation, the reduction of atmospheric dinitrogen gas (N_2) to ammonia, is critical for biological productivity but is difficult to study in the vast expanse of the global ocean. Decades of field studies and the infusion of molecular biological, genomic, isotopic, and geochemical modeling approaches have led to new paradigms and questions. The discovery of previously unknown N_2-fixing (diazotrophic) microorganisms and unusual physiological adaptations, combined with diagnostic distributions of nutrients and their isotopes as well as measured and modeled biogeographic patterns, have revolutionized our understanding of marine N_2 fixation and its role in the global nitrogen cycle. Anthropogenic upper-ocean warming, increased dissolved carbon dioxide, and acidification will affect the distribution and relative importance of specific subgroups of N_2 fixers in the sea; these changes have implications for foodwebs and biogeochemical cycles.

Earth's carbon and nitrogen cycles are critical for maintaining the fertility and habitability of the planet (1). Nitrogen is the second most abundant element for life on Earth, but availability often limits the growth and productivity of terrestrial and aquatic ecosystems despite the large atmospheric reservoir of N_2 gas. Biological N_2 fixation is an important source of combined N, which ultimately is balanced by losses due to reduction of inorganic forms of N back to N_2 gas by anaerobic microorganisms [canonical denitrification and anaerobic ammonium oxidation (anammox)]. N_2 fixation, atmospheric deposition, terrestrial runoff, and internal redistribution (by mixing or upwelling of nitrate-rich deep water to the surface) are external sources of "new" N to the upper ocean that can support the sinking and loss of N and associated C to the deep ocean. These processes have direct importance for atmospheric CO_2 dynamics and global climate change. Just as human activities have perturbed the carbon cycle, anthropogenic activities have substantially altered the nitrogen cycle by chemically reducing N_2 gas to make N fertilizers in the Haber-Bosch process, with resulting eutrophication of inland waters and coastal seas as well as the increased production of N_2O, a potent greenhouse gas (1, 2). These composite perturbations on both the carbon and nitrogen cycles are directly affecting the overall capacity of the oceans to sequester carbon (1).

Biological N_2 fixation partially constrains the ultimate productivity of the oceans because N is a limiting nutrient throughout large areas of the surface ocean. As recently as a decade ago, there were several widely held hypotheses about where N_2 fixation was localized, which organisms were important, and whether N_2 fixation was balanced by losses at the basin and global scales (3, 4). A wide range of studies, from the level of genes and genomes to that of whole ecosystems, using methods drawn from such fields as molecular biology, nutrient and isotope geochemistry, and satellite remote sensing, have expanded the known habitats and the diversity of microorganisms involved in this process (Fig. 1). N_2 fixation has also been explicitly represented in physiological, ecological, and biogeochemical models that have served to organize and refine our

knowledge of its geographical distribution and quantitative importance in the sea.

These developments have led to major shifts in paradigms and perspectives for our current understanding of the role of N_2 fixation in ocean ecosystems and have provoked new questions (5–7). It will be critical to understand how the effects of human activities, including ocean warming, acidification, and N deposition and runoff, will affect marine biological N_2 fixation and its role in the global nitrogen cycle in the future.

## Diversity of symbiotic and free-living diazotrophic cyanobacteria

Putative N_2-fixing microorganisms have been discovered by the application of molecular biological and genomics approaches. In recent years, nitrogenase genes from N_2-fixing unicellular cyanobacteria and heterotrophic (possibly including photoheterotrophic) bacteria have been amplified and sequenced from open-ocean DNA samples using the polymerase chain reaction (PCR) and quantitative PCR (8). More recently, metagenomics and metatranscriptomics (8, 9) have shown that N_2-fixing microbial assemblages are far more diverse than previously realized (Fig. 2). Prior to these studies, marine N_2 fixation was primarily attributed to the nonheterocyst-forming cyanobacterium *Trichodesmium* (10) and the symbiotic heterocyst-forming cyanobacterium *Richelia* (11, 12). Unlike *Trichodesmium* and diatom symbionts, some of the newly discovered N_2 fixers cannot be visualized macroscopically or even by microscopy, and it has yet to be
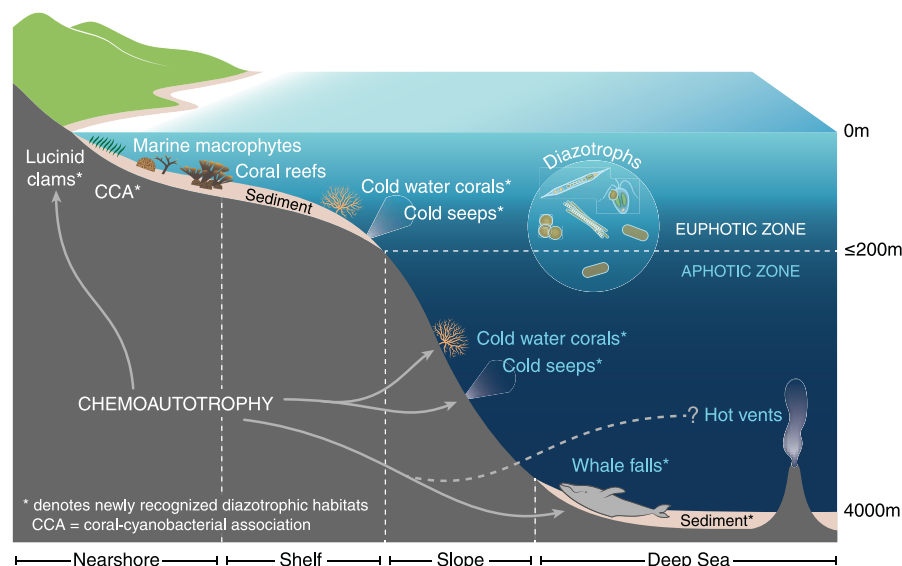
Fig. 1. N_2 fixation in the marine environment. N_2 fixation is distributed throughout marine habitats; recently detected novel metabolic modes supporting N_2 fixation are indicated. N_2 fixers and N_2 fixation have been observed in sediments, hydrothermal vents, corals, and other habitats as well as the surface ocean. Surface-ocean diazotrophs include *Trichodesmium*, *Crocosphaera*, UCYN-A symbionts, diatom symbionts, and noncyanobacterial diazotrophs (NCDs).

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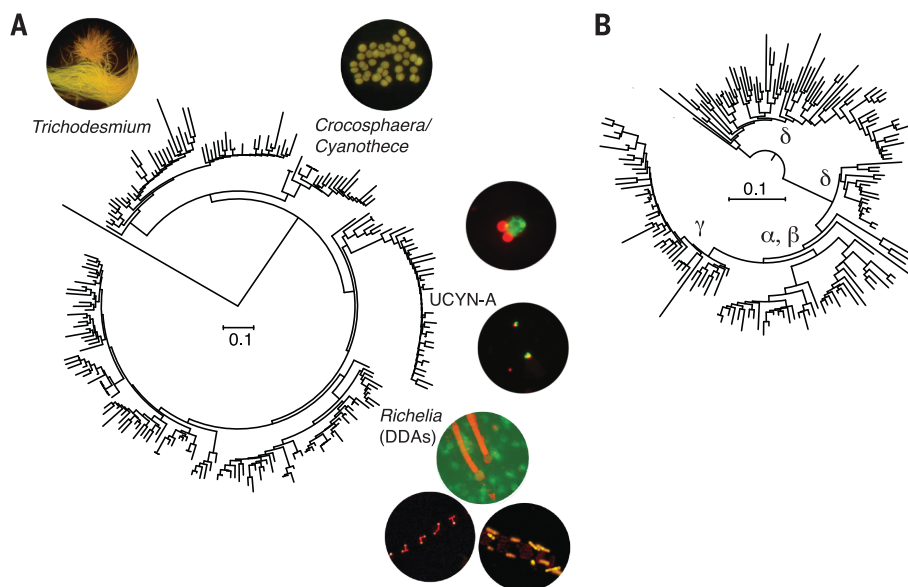
Fig. 2. Phylogeny and microscopy of open-ocean N2-fixing microorganisms. (**A**) Cyanobacteria are now known to include not only *Trichodesmium* and the diatom symbionts *Richelia* and *Calothrix* but also the unicellular symbiont UCYN-A and free-living unicellular *Crocosphaera* (UCYN-B). (**B**) Diverse photoheterotrophic or heterotrophic species have been detected by nucleic acid approaches but are currently being evaluated for their role in N2 fixation. [*Trichodesmium* and UCYN-A micrographs courtesy of R. Foster and A. Cabello Pérez]

demonstrated whether or when some of them fix N2.

Perhaps the most striking discovery of the past two decades was the finding of substantial N2 fixation rates in the smaller size fraction of the marine microplankton (*13*, *14*). Unicellular cyanobacteria were not known to be important in diazotrophy in the open ocean until their discovery by nitrogenase gene sequencing in a wide variety of locations (sequence groups UCYN-A, -B, and -C), including the free-living *Crocosphaera* (UCYN-B), (*15*). Even more surprising was an uncultivated species known as UCYN-A (*Candidatus* Atelocyanobacterium thalassa) (Fig. 2). UCYN-A is a very unusual cyanobacterium with a small genome and extreme metabolic streamlining (*16*) that is found in symbiosis with relatives of the unicellular haptophyte alga *Braarudosphaera bigelowii* (*17*, *18*), a eukaryotic photoautotroph. It has diverged and coevolved with its specific host for ~100 million years (*16*, *19*). UCYN-A does not fix CO_2 or perform oxygenic photosynthesis as do typical cyanobacteria, is likely to be an obligate symbiont with the haptophyte (*16*), and has been directly shown to fix N2 [by $^{15}N_2$ isotopic labeling and nanoscale secondary ion mass spectrometry (nanoSIMS)] in divergent geographic locations (*18*, *20*). This discovery has implications for organelle origins and evolution, but it also is an unusual unicellular symbiosis that enables daytime N2 fixation by a unicellular cyanobacterium, which is important in the low-nutrient waters of the oligotrophic ocean.

Another recent surprise is that the major diazotrophic cyanobacterial groups *Trichodesmium* (*21*, *22*), *Richelia/Calothrix* (*12*), and the unicellular cyanobacteria *Crocosphaera* and UCYN-A (*23*) are made up of genetically diverse and morphologically distinct species or sublineages that are likely ecotypes or strains adapted to specific conditions (*24*). All these findings have radically changed our view of how, when, and where N2 fixation occurs and the fate and foodweb pathways that recently fixed N may take. It is thus possible that the magnitude of N2 fixation in the oceans is considerably greater than had previously been estimated from field observations.

Potential role of noncyanobacterial diazotrophs

Along with the unicellular cyanobacterial N2 fixers, noncyanobacterial diazotrophs (NCDs; heterotrophic or photoheterotrophic bacteria) were discovered at Station ALOHA in the North Pacific Ocean in the form of gene sequences and gene transcripts (mRNA) (*25*). More recently, it has become clear that NCDs are distributed widely and are extremely diverse (*25*–*27*), even though their contribution to open-ocean N2 fixation has yet to be demonstrated. These include groups of gammaproteobacteria (*28*, *29*) and multiple genome types found worldwide in the global TARA metagenomic sequence database (*30*).

The discovery of the high diversity and wide distribution of NCDs is intriguing and yet enigmatic (*28*). Although heterotrophic bacteria are common in terrestrial N2 fixation, they inhabit organic-rich soils or live in symbiosis with C-fixing plants. N-limited oligotrophic habitats in the oceans also have very low concen-

trations of dissolved organic matter and are saturated with dissolved O_2, which is inhibitory to nitrogenase. A plausible explanation is that heterotrophic N2-fixing bacteria are largely associated with more organic-rich particles (*31*) where the physical structure of the particle can restrict diffusion, allowing O_2 concentrations to be reduced and N2 fixation to occur. Genes from presumed heterotrophic bacteria have been found associated with detrital aggregates, associated with algal cells (*31*), and in invertebrate plankton (copepod) guts (*32*), some of which are native to the copepod microbiome (*33*). The relative roles and contribution of NCDs to N2 fixation represent a major unknown that needs to be resolved.

Knowledge of the phylogeny and physiology of surface-ocean diazotrophic microorganisms, including free-living, symbiotic cyanobacteria and NCDs, is critical for improving our understanding of marine N2 fixation, yet detailed information on distributions and abundances of diazotrophs, N2 fixation rates, and foodweb fates of N2 fixation remain poorly constrained. We still do not know for certain which microorganism(s) contribute the most to global marine N2 fixation. Rectifying biogeochemical estimates with microbial distributions and activities continues to be a challenge for the future.

New perspectives on physiological strategies and ecological controls

N2 fixation is energetically expensive and very sensitive to inhibition and inactivation by O_2. The roles and quantitative importance of different groups of N2-fixing microorganisms are a function of physiological adaptations and ecological controls on growth and activities, which in turn determine the geographic distribution, ecological competitiveness, and activities of individual species and groups. In recent years, molecular-level understanding of growth and physiological controls coupled with analytical models have shown promise for understanding when and where N2 fixation occurs, but have also raised more questions when model predictions diverge from robust field observations (*6*, *34*).

Critical physiological characteristics for diazotrophs are the mechanisms for protection from O_2 (including the production of O_2 in the case of photoautotrophic cyanobacteria), obtaining energy from light (cyanobacteria) or dissolved organic matter (heterotrophs), non-N nutrient acquisition strategies and relative sensitivity to the presence of fixed inorganic N, and the temperature optima for growth. Although there are now a few cultivated isolates of open-ocean diazotrophs (the unicellular *Crocosphaera*, the heterocyst-forming symbiont *Calothrix* sp. SC01, and the nonheterocyst-forming *Trichodesmium* spp.) whose physiologies can be

studied in the laboratory, a major limitation is that UCYN-A and surface-ocean NCDs have not been obtained in culture. Physiological information for these species depends on novel cultivation-independent approaches with natural populations (20).

Symbiosis is an important feature of N_2 fixation in the oceans as well as in terrestrial environments. *Trichodesmium* has a complex associated microbiome (35). Two groups of open-ocean N_2-fixing cyanobacteria, *Richelia/Calothrix* and UCYN-A, are symbiotic with unicellular algae (diatoms and haptophytes, respectively) (12), which means that the physiological capabilities of the eukaryotic partners (hosts) have critical physiological characteristics defining ecological competitiveness and niches. Although much remains to be known about the unicellular diatom and haptophyte host physiologies, the ability to couple eukaryotic photosynthesis and growth to cyanobacterial N_2 fixation clearly is an advantage in the nutrient-poor, high-light environment of the oligotrophic surface ocean and is an important area of current and future research.

Growth rates of specific diazotrophs are one of the most important characteristics because they define their competitive abilities relative to other N_2-fixing and non–N_2-fixing species. The growth rates of N_2-fixing microorganisms have usually been assumed to be lower than those of non–N_2-fixing microorganisms (36), and this assumption underpins a number of mathematical models (6, 37). However, recent estimates suggest that some diazotrophs, in particular the uncultivated UCYN-A, might have higher growth rates (up to 1.6 day^{-1}) than previously assumed (38, 39). This is of fundamental importance for understanding and predicting N_2 fixation in the oceans, but is very difficult to determine because laboratory experiments with cultures are not necessarily representative of in situ rates. Moreover, many of the newly discovered N_2 fixers remain uncultivated, and growth rates can only be estimated in natural populations by indirect methods. Knowledge of growth rates is a key parameter for models but also is critical for understanding the ecosystem flux and fate of N fixed by N_2-fixing microorganisms. Hence, this is an outstanding and currently important unresolved area of research.

Growth is ultimately balanced by loss processes, which are also poorly known for oceanic N_2 fixers. At least some diazotrophs are grazed, although relatively little is known about diazotroph grazers (33) or their species specificity (40). Possible mortality from viruses has been reported for *Trichodesmium* (41), but viruses of other diazotrophs are not yet known. Some species, such as the symbiotic diatoms (which have silicon tests), are negatively buoyant, can form dense blooms that sink relatively quickly, and can therefore be important vectors in

transporting N (and C) from the surface to the deep ocean (42). A recent mesocosm study in the southwest Pacific showed the direct transfer of N from *Trichodesmium* blooms to various components of the upper foodweb (43). However, the fates of other smaller-sized diazotrophs that are likely to follow distinct pathways in the foodweb are poorly known. Our understanding of the broader ecological influence of N_2 fixation as well as the accuracy of N_2 fixation models will be improved by research to determine the flux of recently fixed N into the foodweb and the loss processes involved in removing these distinct subgroups.

Photosynthesis, oxygen, and marine N_2 fixation

Oxygen is an important factor controlling N_2 fixation in O_2-evolving cyanobacteria. Most cyanobacteria avoid inactivation of nitrogenase by fixing N_2 at night, or by spatially separating photosynthesis in vegetative cells from nitrogenase in specialized heterocyst cells that lack oxygenic photosynthesis (Fig. 3). *Crocosphaera* is a typical unicellular N_2-fixing cyanobacterium that fixes N_2 primarily during the dark period, with nitrogenase gene expression just prior to this period (44) (Fig. 3). The symbionts of diatoms (*Richelia* and *Calothrix*) and free-living *Nodularia* and *Aphanizomenon* fix N_2 in heterocysts, thereby avoiding inhibition by photosynthetically produced O_2. Daily cyanobacterial N_2 fixation is coordinated by a complex daily cycle of gene expression in *Trichodesmium*, *Crocosphaera*, *Richelia*, and UCYN-A (44, 45) (Fig. 3). A model of the cellular costs of N_2 fixation shows that *Crocosphaera* must have an O_2 barrier in addition to using respiration to protect nitrogenase (46). Daily gene expression of the symbiotic cyanobacteria

is coordinated with host gene expression in the diatom-*Richelia* symbiosis (45), thus demonstrating the high level of integration of the association.

Although much has been learned about *Trichodesmium*, it continues to puzzle researchers because it does not have obviously differentiated heterocysts (as does *Richelia*) and yet fixes N_2 primarily in the light (in contrast to *Crocosphaera*). Early studies suggested that colony (aggregate) formation favored the formation of anoxic microzones, but application of new methods shows that this is clearly not the case (47, 48). Some have argued that *Trichodesmium* uses spatial separation of activities along the filament analogous to a heterocyst [reviewed in (49)], as well as fine-scale temporal separation of activities to fix N_2 during the day (49, 50), but there are contradicting results for spatial intercellular distributions of nitrogenase and for the timing and inactivation of photosystem II (PSII)–mediated O_2 evolution (47). Nonetheless, metabolic modeling has been used to evaluate how such spatial separation might work (50, 51). Studies have also shown that although *Trichodesmium* primarily fixes N_2 during the light, it will fix over much of the dark period if provided with nickel (Ni) (52). There is much yet to learn about how marine diazotrophs avoid O_2 inactivation, which is important for understanding diel cycles and the depth distribution of cyanobacterial N_2 fixation. Because marine N_2-fixing symbioses are mostly unicellular, the underlying mechanisms involved may have useful biotechnological implications for developing N_2-fixing plants in agriculture and aquaculture.

The UCYN-A haptophyte symbiosis also fixes N_2 only during the light, but UCYN-A lacks PSII genes for oxygenic photosynthesis
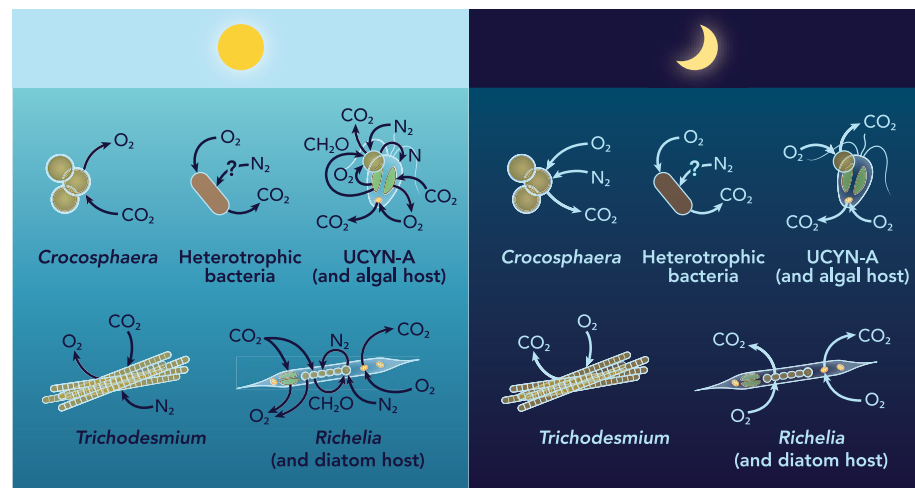
Fig. 3. Physiology of open-ocean N_2-fixing microorganisms showing daily cycles of C and N metabolism, O_2, and nutrients. Cyanobacteria differ in whether they fix N_2 in the light or in the dark and have different adaptations for obtaining non-N nutrients that have implications for ecological distributions and magnitude of N_2 fixation. It is not known whether N_2-fixing bacteria fix during the day or the night.

and does not evolve O_2 itself, although the partner haptophyte alga does. It has not been conclusively demonstrated that the oceanic UCYN-A are truly endosymbiotic (intracellular), but images of the related UCYN-A2 of Japanese coastal waters clearly show that they are (16, 17). UCYN-A lacks two of the three *kai* genes that are important in circadian rhythms in cyanobacteria (53). The cycle of UCYN-A N_2 fixation, although not yet understood, may involve a novel circadian network or coordination with the host cell circadian rhythm, or may simply be dependent on light-driven metabolite production by the host (53). UCYN-A nitrogenase activity is susceptible to inhibition by the O_2 evolved from the haptophyte during the day, and very recent work has shown that hopanoid membrane lipids may be an important barrier for O_2 diffusion in UCYN-A as well as in other N_2 fixers (54). This strategy may also be important in other nonheterocyst-forming cyanobacteria (such as *Trichodesmium* and *Crocosphaera*) and the heterotrophic components of the *Trichodesmium* microbiome and should be a focus of future research efforts.

There are no NCD isolates representative of the types found in the oligotrophic surface ocean. As a result, it remains unknown whether they are active and how they may be adapted to fixing N_2 under aerobic conditions. N_2-fixing bacteria have been shown to be active in particles enriched in organic matter and perhaps in microzones of reduced O_2 (55), but this has not yet been shown to be a general phenomenon. A physiological model of N_2 fixation in the bacterium *Azotobacter* applied to hypothetical marine heterotrophs found that bacteria need several mechanisms to avoid O_2 inhibition, or must exist in relatively low O_2 habitats (46). Some of the diverse heterotrophic diazotrophs might contain proteorhodopsins and are functionally photoheterotrophic rather than chemoheterotrophic, which may be of relevance particularly given the recent establishment of the ubiquity of this type of metabolism among marine heterotrophic bacteria in general. Understanding whether heterotrophic or photoheterotrophic NCD bacteria are active in the surface ocean, where they get their energy, and how they avoid O_2 inhibition are important remaining questions in understanding oceanic N_2 fixation.

Nutrient controls on the distribution of N_2 fixation and diazotrophs

The ability of N_2 fixers to compete for non-N nutrients determines the ecological success of N_2 fixers and the biogeography of N_2 fixation. Different species have different responses and adaptations to low nutrient availability, as shown in culture (56), and nutrients limiting diazotroph growth vary with space and time. Physiological traits include basic cell size and growth rate relative to nutrient resources; the cell-size trait may explain the seasonal bloom, sinking of diazotrophic diatoms, and transitions to smaller, unicellular species (11). Assays for expression of high-affinity transporters (*pstS*) (57) in *Crocosphaera* indicate possible stress and growth limitation caused by low P availability. Organic P compounds comprise multiple classes and are assumed to be a labile source for microorganisms (58). One class of organic P compounds are phosphonates, which have a C-P bond and are abundant in *Trichodesmium* (59). Thus, the use of organic forms of P as a nutrient source provides an ecological advantage to some species. Less is known about how P limits growth in the uncultivated diazotrophs, but the addition of inorganic P has been shown to stimulate UCYN-A growth (38).

Fe availability is particularly important if cell quotas for Fe of N_2 fixers are greater as a result of the FeS-rich centers in nitrogenase. Fe occurs largely in the oxidized (ferric) forms in oxic seawater and can also be complexed with organic ligands (60, 61), and most focus has been on dissolved Fe. However, other sources that have been overlooked, such as fluxes from sediments, may also be important (62). Key genes involved in Fe metabolism are involved in transport, such as *FutA/idiA*, which are more highly expressed under Fe limitation (63). An efficient adaptation for Fe use has been demonstrated in *Crocosphaera*, where switching Fe from one protein to another may help to decrease Fe requirements overall by providing Fe to photosynthetic reaction (PS1) centers during the day and reusing the Fe in nitrogenase at night (64).

The relative availabilities of P and Fe are important in determining the geographic distributions, activities, and species composition of N_2-fixing assemblages. Fe appears to be a controlling factor for diazotrophic growth in some regions (e.g., South Atlantic and South Pacific), whereas P may be the more critical element in others (e.g., North Atlantic) (65) (Fig. 4). Intriguingly, there is an interaction between P and Fe availability and species responses, where low Fe can increase the relative growth rates of some cyanobacteria under P stress (56).

It was long assumed that N_2 fixation was important where fixed N concentrations were low, and that N_2-fixing microorganisms are not ecologically competitive where combined N resources were available. Energetically, fixing N_2 is costly, although it is only marginally (~25%) more costly than using NO_3^- (66), and evidence has been accumulating from culture work and the environment that N_2-fixing microorganisms can be present and active when modest concentrations of fixed N in the form of NO_3^- are present (66). The UCYN-A symbiosis
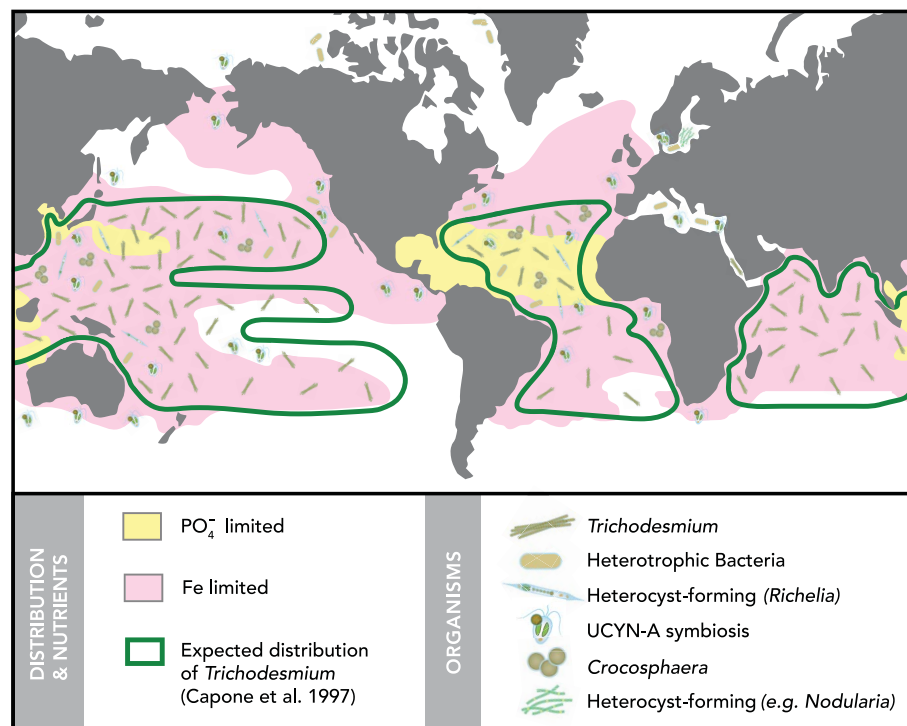
Fig. 4. Map of known N_2 fixation. Map is based on previously known *Trichodesmium* distributions from Capone et al. (10) (green borders) and now-recognized general distributions of diverse diazotrophs including coastal regions and the Arctic Ocean. Rough areas of P and Fe limitation are indicated, based on (37, 97). For specific sampling stations and detailed data, see Luo et al. (132).

has been found in many environments with elevated NO_3^- (67). Recent studies have also shown that it is beneficial for heterotrophs to use both fixed inorganic N and N_2 fixation under some conditions (68) and that it can be energetically favorable for *Trichodesmium* to use fixed N (69). This change in the N_2 fixation paradigm has expanded the environments where N_2 fixation may be expected, as well as the global biogeography of N_2 fixation more generally.

Novel habitats for N_2 fixation

Within the past two decades, N_2 fixers have been reported to be present and active in regions not previously thought to be important in N_2 fixation, including nutrient-enriched coastal (5, 70) and low-temperature higher-latitude waters (67, 71, 72). Both have recently been shown to have substantial and active populations of UCYN-A (Figs. 1 and 2). N_2 fixers and low but measurable N_2 fixation rates have also been detected in the oxygenated sub–euphotic zone (7) as well as in hypoxic and anoxic oxygen-deficient zones (ODZs) along with NCD *nifH* genes (73–75). ODZs, with their reduced O_2 and combined N levels, are potentially ideal habitats for certain NCD diazotrophs. However, other recent studies have not found active N_2 fixation in sub–euphotic zone stations across the North Pacific (8) or in the eastern tropical South Pacific ODZ (76), despite the presence of *nifH* sequences of putative NCDs (77).

Rates of N_2 fixation, when reported, in sub–euphotic zone systems are often close to the limit of detection for the tracer $^{15}N_2$ isotope uptake method (8). Nonetheless, if the reported directly measured rates in the sub–euphotic zone are real, as argued by Benavides *et al.* (7) and others, the scaled-up integrated rates of N_2 fixation from these direct measurements would be quantitatively important on a global scale, given the large volumes of these habitats. However, these inputs should be included in basin-scale analyses using nutrients and isotopes, and so should not change the estimated budgets derived by those methods. Although the presence of NCD N_2 fixers in the sub-euphotic zone and low O_2 waters extends the spectrum of marine habitats that host diazotrophs, the spatial and temporal variability of this input and the agents involved remain largely unknown, as does their quantitative importance to global marine N_2 fixation.

N_2 fixation has been extensively examined in a range of coastal habitats, including coral reefs (78), seagrasses, and sediments (79) (Fig. 1). Over the past decade, deeper benthic sites have also been found to host active and previously undescribed diazotrophic consortia. Diazotrophy in deep hydrocarbon seeps was demonstrated with the enriched stable isotope uptake method coupled with nanoSIMS.

"Whale falls" (microbial "oases" defined by the decaying carcass of a whale) have also been shown to have active populations of diazotrophs (80). Diazotrophy in the hydrocarbon seeps is mainly associated with the methanogen partner of the anaerobic methane-oxidizing (ANME) consortia of a methanogen and sulfate-respiring bacteria (in which the oxidation of methane occurs by reverse methanogenesis by the methanogen partner). In the whale-fall environments as well as several other deep sediment environments, a more diverse diazotrophic flora was evident, suggesting other metabolic pathways that support observed diazotrophic activity (81). N_2 fixation has also been documented in the sulfur-oxidizing diazotrophic partners of deep-water corals (82). Curiously, sulfur-oxidizing bacteria symbiotic with deep hot vent animals have also been implicated in diazotrophy, but with only indirect evidence to date (83). Although the range of benthic habitats with diazotrophic inhabitants has expanded substantially, the deep-sea benthos is the most understudied of all marine habitats, and there are likely novel sites and microorganisms yet to be discovered.

Global distribution of N_2 fixation and balance with N losses

Maintaining the balance of global ocean inputs of N, including N_2 fixation, with losses is critical for maintaining the fertility of the seas over longer time scales (Fig. 5). However, oceanographic research paid little attention to N_2 fixation for a considerable time until convenient means of determining N_2 fixation rates were available (see Box 1) (84). In contrast, reactive N losses back to N_2 (now known to be through both anammox and canonical denitrification) had been broadly studied and

appeared to be predominant in shelf sediments and in the major ODZs (3). By the 1990s, extrapolation of available field measurements of N_2 fixation to the basin scale indicated that N_2 fixation was much less than losses, suggesting that there were as yet unidentified sources and organisms, that there were errors in the estimates (e.g., large overestimates of denitrification), or that the budget was indeed unbalanced (3). An imbalanced budget would have important implications for contemporary marine productivity and greenhouse gas production (such as nitrous oxide) and consumption (2) and cannot be sustained over long time scales without major effects on ocean productivity. However, obtaining accurate estimates of basin-scale rates by extrapolating from measurements remains an ongoing challenge given the relatively low density and limited geographical range of such observations.

A correspondence between the elemental composition of plankton of the surface ocean with the ratios of key nutrients, and in particular nitrate and phosphate (N:P), in the deep ocean resulting from the remineralization of surface-derived organic matter was first noted by Redfield (85) and is referred to as the Redfield ratio (C:N:P = 106:16:1). It was long assumed that these ratios were relatively invariant in the oceans. However, extensive global surveys of ocean nutrients over the past several decades showed that in fact these ratios ranged substantially in value (4) (Fig. 5). One consequence of N_2 fixation is an enrichment of N relative to P in organic matter, and high subsurface N:P ratios are associated with areas of substantial N_2 fixation. Similarly, denitrification and anammox can decrease the ratio of N to P in major nutrient
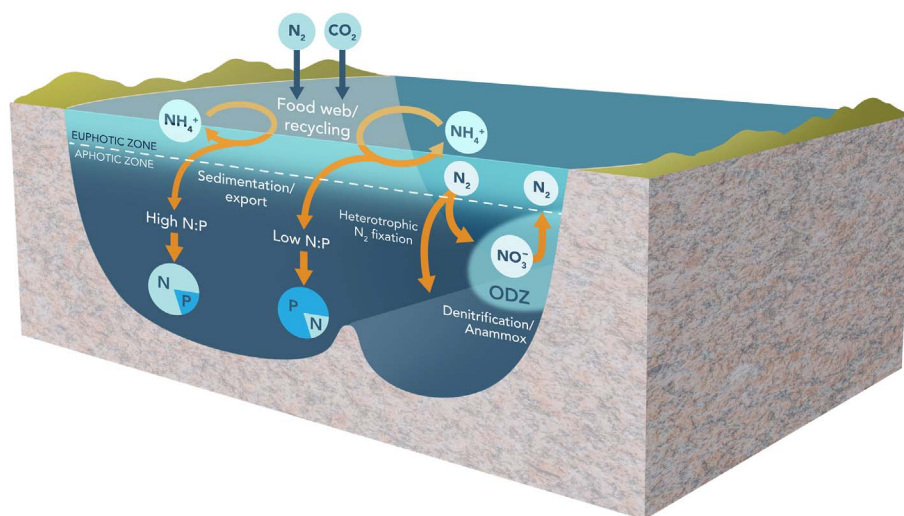
Fig. 5. Conceptual model of N flows in the surface ocean. The model links N_2 fixation, regeneration in deep water, and effect on deep-water N:P ratios that are used to estimate N_2 fixation rates, taking into account known global circulation and integrating over large-scale ocean sections.

Box 1. Overcoming challenges for N_2 fixation research.

Measurement of N_2 fixation rates and quantification of abundances of diazotrophs, particularly if they are uncultivated, can be challenging. Quantitative polymerase chain reaction (qPCR) (117) is commonly used to quantify even uncultivated microorganisms by the presence of the nitrogenase gene (specifically *nifH*). Rates are usually measured in bulk water by acetylene reduction (technically simple but less sensitive and indirect) or the more sensitive but analytically more tedious $^{15}N_2$ technique. Recently, difficulties have been identified in the $^{15}N_2$ technique [(118, 119), but see also (120)] and the analysis of the abundances of genes (121). Other approaches for measuring activity have been proposed, such as using highly purified acetylene to improve sensitivity (122), measuring H_2 evolution (113, 123), or using isotopically labeled acetylene in an acetylene reduction method (124).

Advanced methods make it possible to measure taxon- and cell-specific N_2 fixation. As diazotroph 16S rRNA gene sequences become available, individual diazotroph cells such as UCYN-A can be visualized and enumerated by catalyzed reporter deposition fluorescence in situ hybridization [CARD-FISH (125)]. Nanoscale secondary ion mass spectrometry (nanoSIMS) has been used to directly identify active diazotroph cells (126). Stable isotope probing (SIP) (127) directly identifies active diazotrophs and has been used widely in soils and recently in a marine setting (81).

The inability to sample the vast oceans with sufficient spatial and temporal resolution remains a primary challenge for determining how factors such as critical nutrients control N_2-fixer distributions and activities. Advanced remote instrumentation promises to provide high-resolution sampling over large spatial scales (42) and short time intervals that approximate the Lagrangian behavior of water parcels. New technologies have been adapted to directly determine in situ patterns of nitrogenase activity (5, 128). Satellite color remote sensing has contributed greatly to our knowledge of the basin-scale distributions of certain diazotrophs, such as *Trichodesmium* and diatom-diazotroph associations (DDAs) (129, 130). The use of color sensors on unmanned aerial vehicles (UAVs; drones) from land and ships will provide a new level of spatial resolution (131).

Innovative mathematical models are critical for evaluating and predicting N_2 fixation over basin scales by using diazotroph size, growth, and nutrient relationships (97) (Movie 1). These models provide important approximations of regional N_2 fixation by different size classes of N_2 fixers, although they are based on assumptions of lower maximum growth rate of N_2 fixers and elevated non-N nutrient requirements (36).

pools. Thus, regional imbalances in N_2 fixation and denitrification drive these deviations from the Redfield ratio. Robust basin- and global-scale estimates of N_2 fixation and denitrification have been derived from geochemical gradients of these ratios (4, 86). Similarly, isotopic signatures of N in nitrate integrated over time and space (87) have been used to infer integrated rates of N_2 fixation and denitrification.

On the basis of these basin-scale analyses, some have concluded that the nitrogen cycle has strong homeostatic controls and feedbacks and is close to balanced (4). Deutsch *et al.* (86) noted that the excesses in phosphate relative to nitrate in waters upwelled through the ODZ zones of denitrification or anammox in the eastern tropical Pacific showed progressive decreases in the large P excess as those waters were transported westward in surface waters. They ventured that these regions downstream of the ODZs should coincide with high N_2 fixation rates, thereby providing a mechanism for coupling inputs and losses over relatively short space and time scales (86). However, this model does not agree with the spatial distribution of directly measured N_2 fixation in field studies in the region (88). Furthermore, zones of high N_2 fixation have been observed far from these areas in the western Pacific (89). Others

still assert that an imbalance exists (90) or that plasticity in nutrient ratios in organisms can make these feedbacks weaker than predicted from assuming constant elemental ratios in organisms (6, 91, 92), as many earlier models have done.

A very recent study using two independent modeling approaches predicted N_2 fixation regions more consistent with field observations (6) but still yielded relatively high rates in the South Pacific and South Atlantic gyres inconsistent with several earlier field studies. This study also predicted low rates in the North Atlantic equatorial upwelling region, which contrasts with recent observations (93) and geochemical inferences of relatively high rates of N_2 fixation based on basin-scale trends in the isotopic signature of nitrate (34).

All methods for estimating basin- and global-scale N_2 fixation rates have substantial biases and assumptions (94). Nonetheless, modeling approaches based on the distributions of nutrients and their isotopes continue to be the best current way to make estimates at these scales. Thus, the state of the relative balance of inputs and removal in the oceanic nitrogen cycle, and the strength and time scale of coupling of N_2 fixation and denitrification, are still widely debated (6, 34, 86, 91, 95) and have yet to be conclusively resolved.

The distribution of N_2-fixing microorganisms, direct measurements of N_2 fixation rates, and determination of which nutrients constrain N_2 fixation provide complementary perspectives on when and where N_2 fixation occurs (Fig. 4). Knowing the identities of the N_2-fixing microorganisms matters because the physiological subgroups vary greatly in size and will therefore have different ecological consequences and fates. New models based on size classes of N_2-fixing microorganisms (37) provide the ability to predict how different groups respond to nutrients and ocean circulation, as well as the ability to visualize where different species grow (Movie 1). These models can be used to predict distributions and dynamics even over long time intervals and are useful in forecasting the possible effects of global climate change (Movie 1).

Fe and P availability (the inputs, concentrations, and chemical forms) are believed to be two major factors controlling the distribution of specific diazotrophs and N_2 fixation rates (65, 91). The global patterns of atmospheric deposition of Fe in aerosol dust may be a key determinant of the structure of diazotrophic communities and the magnitude of surface-ocean N_2 fixation (65). Physical processes can be important in providing nutrients by advection, such as in the North Atlantic, where the nature of nutrient limitation drives species composition of microbial communities including diazotrophs (96). New approaches to measure and model N, P, and Fe limitation have begun to allow us to predict broad geographic patterns (97) (Fig. 4 and Movie 1). Comprehensive sampling of ocean metal distributions is currently under way through the international GEOTRACES Program (98) and will provide a quantum jump in our understanding of how metal dynamics affect plankton populations in the sea in general and diazotrophy in particular.

Marine N_2 fixation, global change, and the future

Diazotroph communities are now recognized to be much more diverse than previously appreciated, including uncultivated symbiotic cyanobacteria and heterotrophs. Although these discoveries have resulted in major changes in perspectives, many questions remain to be resolved before we can understand and predict N_2 fixation in the oceans, including information on the organisms, interactions, and factors controlling N_2 fixation (99). The next decade promises to yield exciting new insights and yet more shifts in paradigms of marine N_2 fixation. Understanding N_2 fixation and its role in the nitrogen cycle of the oceans is critical for understanding and predicting the effects of global environmental changes on the biology and chemistry of the seas.

Environmental changes are challenging the oceans in multiple ways, including upper-ocean
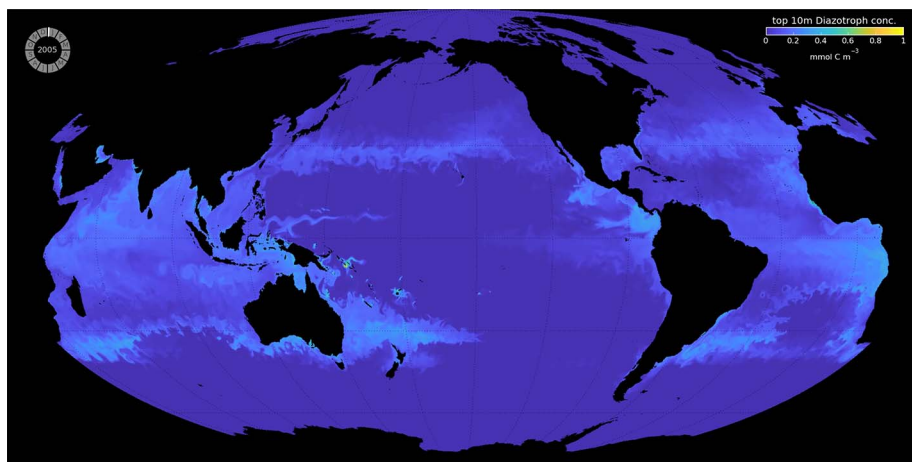
Movie 1. Predicted global distribution of biomass of different size classes of diazotrophic cyanobacteria. Distribution (several size classes) is estimated by the DARWIN biogeochemical model (https://dataverse.harvard.edu/dataverse/GUD_CS510), based on global ocean circulation, estimated growth characteristics, grazing, and nutrient distributions. [Credit: O. Jahn, C. Hill, S. Dutkiewicz, M. Follows (MIT)]

warming from increasing atmospheric CO_2 concentrations, increases in dissolved CO_2 with resulting acidification (100), and atmospheric deposition of inorganic nutrients (2). ODZs, major sites of N losses, are expanding in extent (101). Marine N_2 fixation, along with other components of the marine nitrogen cycle, will be affected by these stressors.

Upper-ocean warming may benefit the growth of some marine diazotrophs such as *Trichodesmium* (102). Increased CO_2 concentrations have also been shown to stimulate N_2 fixation and CO_2 fixation in *Trichodesmium* and some unicellular cyanobacteria [(22) and references therein], although other reports did not observe such stimulation for UCYN-A–dominated assemblages (103, 104). New studies have focused on elucidating the underlying mechanisms of these responses through transcriptomics and modeling (105, 106). Long-term exposure of *Trichodesmium* to elevated CO_2 results in adaptive responses encoded in their underlying genome (107), which may help N_2-fixing organisms adapt to long-term environmental change.

The flux of fixed N to the ocean from the atmosphere and terrestrial runoff is also rapidly accelerating as a result of expanding nitrogen fertilizer synthesis for agriculture (1), and increases in surface combined N related to atmospheric deposition and runoff have already been detected in coastal waters (108). Although N_2 fixation has been noted in the presence of low levels of combined N (109), sufficient deposition of combined N could ultimately suppress N_2 fixation in the upper ocean and serve as a negative, stabilizing feedback (2). Determining the overall effects of this accelerating N flux on the oceanic N balance (including pathways of N removal) and on marine productivity should be a research priority in the upcoming decade.

Looking forward, marine N_2 fixation could play a role in three critical applied areas for humankind: marine aquaculture, biotechnology, and geoengineering. Aquaculture, both of fish and seaweeds, is an emergent enterprise globally. As wild fisheries decrease precipitously, aquaculture will increasingly meet the need for nutritional protein of the swelling human population as well as contributing to the "blue economy" of the sea in terms of carbon sequestration (110). Aquaculture has been well established and is surging in many countries and rapidly expanding in others. Coastal aquaculture systems often require the addition of exogenous nutrients to succeed, and the development of N_2-fixing partners to subsidize nitrogenous nutrient needs (111), paralleling their importance in agricultural systems, seems an obvious future direction. Isolates of hyperthermophilic diazotrophic archaea have been obtained (112) and could be models for an ammonium biosynthesis approach less costly than the Haber-Bosch process (1), perhaps coupled to H_2 production by nitrogenase (113).

Ocean fertilization with Fe has previously been proposed as a potential mechanism to mitigate atmospheric increases in CO_2 through stimulation of surface phytoplankton populations leading to carbon sequestration (114). The primary areas considered for ocean fertilization have been the high-nutrient (or nitrate), low-chlorophyll (HNLC) regions of the oceans, such as areas of the equatorial Pacific and the Southern Ocean where iron is in short supply relative to macronutrients. Low-nutrient, low-chlorophyll (LNLC) regions may also be susceptible to Fe fertilization through the stimulation of cyanobacterial diazotrophs and have also been considered in the context of carbon sequestration (60). However, the area of purposeful ocean

fertilization has a long history of debate concerning the ecological and ethical implications of such an approach. Numerous science-driven open-ocean experiments have shown the stimulatory effect of Fe additions on phytoplankton in some of these regions, although the aggregate results with regard to net carbon sequestration remain ambiguous (115). Furthermore, analogous direct stimulation of N_2 fixers by Fe additions in situ has not yet been observed (116).

The past decade has brought new knowledge and a new understanding of marine N_2 fixation. We now know more about the composition of marine N_2-fixing species and their roles in the nitrogen cycle. We also have better estimates and mathematical models for estimating N_2 fixation as well as the growth and distribution of N_2-fixing microorganisms. Yet questions remain as to whether the N budget is balanced and which species or group is the major contributor to fixed N in the oceans, and how these will change under future global environmental change scenarios. With new microbiological, genomic, and isotopic methods in hand, the ensuing decade is likely to bring new discoveries and further changes to our perspectives and paradigms of marine N_2 fixation.

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Science

Changing perspectives in marine nitrogen fixation

Jonathan P. Zehr and Douglas G. Capone

Changing views and a changing ocean

As a component of many biomolecules, nitrogen is a crucial element for life, especially in nutrient-poor environs such as the open ocean. Atmospheric dinitrogen gas (N_2) is abundant but must be fixed by reduction to ammonia, a process limited to certain organisms and environments. Zehr and Capone review changes in our understanding of what marine microorganisms are fixing N_2, where they live, and what environmental features influence their activity. N_2 fixation is more widely distributed than previously thought, and we still have much to learn about the physiology and regulation involved. We now have better estimates of global- and basin-scale inputs and outputs, but questions remain as to whether the oceanic N cycle is balanced. New tools are enabling better understanding of ocean N_2 fixation despite disruptive consequences from human activities, including ocean acidification and warming.
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