

## Emerging Patterns of Nitrogen Fixation in the Oceans

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The importance of biological nitrogen fixation in ocean biogeochemistry has only recently come to be fully appreciated (Mahaffey et al. 2005, Carpenter & Capone 2008). Results from field studies in the 1980s largely at mid-latitudes and in marginal tropical and subtropical seas, indicated that nitrogen fixation could be of regional significance in some of these areas. However, aggregation of these early studies suggested that there was a relatively limited role for marine nitrogen fixation in the global N cycle and in supporting primary production. However, several lines of geochemical evidence which emerged in the late 1990s suggested otherwise. This has prompted a resurgence in field efforts examining this process which in turn has provided direct evidence to support the biogeochemical significance of nitrogen fixation and its role in supporting net (or new) primary production the oligotrophic ocean. The recognition that oceanic nitrogen fixation may directly promote atmospheric carbon sequestration has fueled further interest in this process (Michaels et al. 2001).

Research on marine nitrogen fixation continues to move rapidly apace. Macro-diazotrophs such as the non-heterocystous cyanobacterium *Trichodesmium* spp. have been recognized to contribute to marine nitrogen fixation since the late 1960's and have been well studied (Capone et al. 1997). Symbiotic associations between oligotrophic diatoms and heterocystous cyanobacteria have also been observed in the past but their quantitative significance in nitrogen fixation has only recently come to be appreciated.

The infusion of molecular biological methods into biological oceanography has allowed scientists to make a more realistic assessment of the diversity of marine diazotrophs and especially those that cannot be seen with the naked eye or using microscopy. Indeed, diazotrophic coccoid cyanobacteria and proteobacteria have been found that can be very abundant in some regions of the ocean (Zehr et al. 2001) and provide substantial inputs (Montoya et al. 2005).

While nitrogen fixation was long ignored in ocean biogeochemical modeling efforts (with a few notable exceptions e.g. Schaffer 1989), many current biogeochemical models are now incorporating nitrogen fixation as an explicit function providing input of new reactive nitrogen into marine ecosystems and evaluating its importance in supporting carbon uptake and sequestration in the sea.

However, there are still major puzzles to be solved. Two current and related conundrums are whether rates of denitrification and nitrogen fixation are near balance in the current ocean, and discerning how closely they are coupled in time and space. Depending on the source, recent scale-ups of oceanic inputs by nitrogen fixation and removal by "denitrifying" processes are either near steady-state, or substantially out of balance (see Carpenter & Capone 2008 and references therein) with removal far exceeding inputs. Open ocean nitrogen fixation is generally

associated with the upper, photic layers of oligotrophic waters which represent about 50-60% of the global ocean. Denitrification (including the anammox reaction) in the oceanic water column is thought to be restricted to the major oxygen minimum zones (OMZ) of the Arabian Sea, eastern tropical north Pacific and eastern tropical South Pacific. The denitrifying processes occurring in OMZs result in deep waters that are enriched in phosphate relative to nitrate. Recent modeling efforts suggest that as these waters reach the surface (e.g. through upwelling) and advect offshore, nitrogen fixers may proliferate by exploiting the excess phosphate thereby providing a potential for close spatial and temporal ( $\sim 10^2$  years) coupling between these opposing processes (Deutsch et al. 2007). In contrast, the excess nitrogen generated in the North Atlantic in the absence of a substantial loss pathway appears to be exported into South Atlantic and beyond to provide an input which offsets deficits over ocean mixing ( $\sim 10^3$  years) time-scales (Moore et al. 2009).

Controls on nitrogen fixation are also being actively explored. Field observations and experimental and modeling results suggest diazotrophs, which are not likely limited by dinitrogen availability, may be limited by other macro and micronutrient factors in different ocean basins. Indeed, a mosaic of factors which may constrain nitrogen fixation *in situ* is emerging. Large dust inputs into the tropical N. Atlantic from the Sahel appear to foster higher availability of iron in the tropical & subtropical N. Atlantic, where phosphate availability may be a more important constraint on nitrogen fixation. In contrast, excess phosphate in surface waters of the eastern tropical and subtropical Pacific (both north & south) coupled with low deposition rates of iron from the atmosphere drives iron limitation with respect to diazotrophic growth and activity. Diazotrophy in the tropical and sub-tropical S. Atlantic & S. Pacific may also be strongly constrained by iron availability although the data density for these areas is substantially lower than from the northern hemisphere. Interestingly, elevated  $\text{CO}_2$  concentrations have been shown to stimulate nitrogen fixation by some cyanobacterial marine diazotrophs.

Recent molecular evidence suggests that the relative dominance of different diazotrophic groups may also vary among ocean basins. Microdiazotrophs such as *Trichodesmium* spp. appear to play a more predominant role in the N. Atlantic compared to nanodiazotrophs such as *Crocospaera* and other small coccoid cyanobacteria (Langlois et al. 2008, Foster et al. 2007). In contrast, nanodiazotrophs are seemingly more dominant in the central N. Pacific (Church et al. 2008). Diazotrophs may also be constrained by the relative availability of other elemental (e.g. Mo, Co) and organic factors. Some recent evidence suggests that cyanobacterial diazotrophs may specifically contribute to certain trace vitamin (e.g.  $\text{B}_{12}$ ) fluxes in the upper ocean.

With the prospect of upper ocean warming as a result of global climate change, the magnitude, distribution and importance of marine nitrogen fixation is likely to change, although research into how is just beginning. Upper ocean warming, ocean acidification and increases in dissolved inorganic carbon are all likely to affect the extent and distribution of oceanic nitrogen fixation. Finally, atmospheric N deposition to the ocean is rapidly accelerating and will soon exceed current estimates of oceanic nitrogen fixation. With the current state of the nitrogen cycle still poorly constrained, and climatically and human induced changes on the horizon, the continued study of oceanic nitrogen fixation is important to our broader understanding of ocean biogeochemistry now and in the future.

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Free-living and symbiotic marine nitrogen fixing Cyanobacteria

During the last several decades marine cyanobacteria have been shown to play a prominent role in the open ocean. Marine picocyanobacteria belonging to the two closely related genera *Synechococcus* and *Prochlorococcus* are probably the most abundant photosynthetic organisms on earth and are very important at the base of the marine food web. Knowledge of their biology and ecology has been aided by their amenability to culture opening their study to a large array of molecular, genomic and ecological techniques. However, studies have shown that neither of these lineages possesses the ability to fix nitrogen.

In the open ocean nitrogen fixation has been most extensively studied in the nonheterocystous, filamentous genus *Trichodesmium*. This genus has been known for centuries to form extensive surface “blooms” in the tropical oceans and was shown to fix nitrogen by Dugdale in 1961. For more than twenty years studies were limited to field populations because of the difficulty in culturing these extremely sensitive and strict oligotrophs. Within the last dozen years culture techniques have been improved so that representatives of each of the five species can be maintained in culture but they still remain difficult to manipulate experimentally both in the laboratory and in the field.

The introduction of epifluorescence microscopy and the use of nitrogenase genes for phylogenetic analysis of natural populations led to the discovery of *Crocospaera* (Waterbury, 1988) and UCYN-A (Zehr et al., 2001 and 2008) respectively; two groups of nitrogen fixing unicellular cyanobacteria that are present in the tropical Atlantic and Pacific oceans. *Crocospaera* spp. are amenable to culture, have a typical cyanobacterial pigment system and fix nitrogen during the dark. Members of the UCYN-A group are globally distributed and can be periodically abundant but have not yet been cultured. Metagenomic analysis from cells sorted by flowcytometry have shown that they lack: photosystem II, the capacity to fix CO<sub>2</sub>, a complete TCA cycle and the pathways for several amino acids and purines. They do however have a complete nitrogenase system and fix nitrogen during the middle of the day. Their lack of key metabolic pathways suggests that they may live in symbiosis with another organism, however, size fractionation experiments, where they appear in the less than 3µm fraction, suggest they are free living. Further study will be required to determine how this enigmatic organism makes a living.

Other unicellular nitrogen fixing cyanobacteria are associated with several genera of nonphotosynthetic dinophysoid dinoflagellates (*Dinophysis*, *Histionesis* and *Ornithocercus*) that occur at low cell densities in the tropics. These cyanobacterial epibionts, that occur from 10s to 100s of cells in the girdles of the dinoflagellates, have not been cultured but have been shown to be closely related to *Cyanothece* (Foster and Mullen, 2008).

Free living heterocystous cyanobacteria are not prominent in the open ocean, but two genera *Richelia* and *Calothrix* form associations with diatoms. *Calothrix* spp., growing epiphytically on species of *Chaetoceros*, have been reported from the Indian and western

Pacific oceans and have recently been cultured (Foster and Mullen, 2008). *Richelia intracellularis* occurs widely, often in large blooms, in both the Atlantic and Pacific oceans as an endosymbiont in *Rhizosolenia* spp. and *Hemiaulus* spp. Both of these diatoms with their associated symbionts can be maintained in the laboratory for periods of approximately one year. On one occasion *Richelia* from *Rhizosolenia* sp. was grown briefly independent of its host (T. Villreal) whereas repeated attempts to grow *Richelia* independent of *Hemiaulus* have failed.

Processes controlling the patterns of diazotrophic cyanobacterial distributions, abundances and activities in the oceans remain largely unknown. A recent study at station ALOHA off the coast of O'ahu, Hawaii by Church et al. showed that the nitrogen fixing cyanobacteria varied seasonally with unicellular forms, *Crocospaera* and UCYN-A, dominating in late winter and spring while filamentous forms, *Trichodesmium* and *Richelia* living symbiotically with diatoms appeared episodically during the summer. The results of this study suggested that mesoscale physical forcing was important in controlling diazotroph community structure and the rates of nitrogen fixation in the North Pacific subtropical gyre. Temperature is also a primary determinant of the distribution of both *Trichodesmium* (Breitbarth et al., 2007) and *Crocospaera* (Webb et al., 2009). Both have temperature ranges permitting growth from approximately 20 °C to 35 °C thus limiting their ranges to the tropical and subtropical oceans.

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# One Hundred Years of the Nitrogen Cycle\*

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## Abstract

Studies of the oceanic nitrogen cycle that include isotope ratios, and automated determination of ammonium and nitrate extend backwards < 50 years (e.g. Dugdale and Goering, 1967). Studies of nitrous oxide distributions and cycling in the ocean began in earnest only ~ 35 years ago (Yoshinari, 1976; Cohen and Gordon, 1978). During the next 50 years mankind's impact will continue to perturb the oceanic nitrogen cycle in ways that are unique in the history of our planet. For example, Haber-Bosch production of fixed-nitrogen has ramped up to rates that rival natural fixation. Disruptions of the marine environment by bottom trawling (Watling and Norse, 1998) are now enormous with about 25% of the global continental shelf being swept by bottom trawls every year, etc. Can we employ the knowledge gained during the past 50 years to predict how the oceanic budgets of nitrous oxide and fixed-nitrogen may change and may influence change over the next 50 years? The answer is not with any certainty, but we do know enough to make some suggestions about where to focus more attention. For example, nitrous oxide is a powerful greenhouse gas (Forster et al., 2007) whose production is enhanced in hypoxic waters (dissolved  $O_2 < 2$  ml/l) particularly in the extremely hypoxic waters that occur at the boundaries of suboxic ( $O_2 \sim 0$ ) water masses (Codispoti and Christensen, 1985) and in intermittently suboxic waters (Naqvi et al., 2009). We also know that there are large volumes of water on the verge of hypoxia and suboxia along some oceanic eastern boundaries and in the northern Indian Ocean. Global warming, ocean acidification, dam building and anthropogenic nutrient additions to the ocean all have the potential to increase oceanic nitrous oxide production by causing oceanic hypoxia to intensify and shoal allowing the co-occurrence of conditions that favor high rates of nitrous oxide production (severe hypoxia in high respiration waters and intermittent suboxia in high respiration waters). Warming may cause an intensification of the oxygen minimum zone (Stramma et al., 2008). Acidification which may be occurring at a rate unprecedented in the geologic record (Kump et al., 2009), and dam building may lower the supply of "ballast" and decrease the sinking rate of organic matter. This will, in turn, steepen the exponential increase of respiration rates towards the sea surface and facilitate the occurrence of low oxygen waters at shallow depths where respiration rates are high. Finally, direct nutrient inputs arising from human activities are increasing the incidence of coastal hypoxia (Naqvi et al., 2009), and may be significantly increasing open ocean nitrous oxide production via aerial deposition of nutrients (Duce et al., 2008). An interesting and open question is whether or not warming will intensify upwelling along oceanic eastern boundaries (Vecchi et al., 2008). Some models suggest that it will. Other

models suggest the opposite. Some recent observations suggest that upwelling is intensifying (Chan et al., 2008; McGregor et al., 2007) along eastern boundaries. Intensified upwelling in the Eastern Tropical Pacific will have the effect of making the system more el Nina like with an elevated thermocline that brings low oxygen waters high onto the respiration rate curve. In 1985, extremely high nitrous oxide concentrations were observed off Peru during a la Nina (Codispoti et al., 1986; Codispoti et al., 1988; station 68 in Codispoti et al., 1992).

Climate change is occurring most rapidly in the Arctic (Serreze et al., 2009). We know that denitrification and anammox remove globally significant quantities of fixed-N in extensive Arctic shelf sediments (e.g. Devol et al., 1997; Chan and Devol, 2009). We also know that these processes occur in brine pockets in Arctic ice (Rysgaard and Glud, 2004). The data, however, are sparse and little to no attention has been paid to zones of high methane release. Studies of nitrous oxide cycling in the Arctic are rarer still. In addition to looking for nitrous oxide releases from Arctic sediments, it would be interesting to examine nitrogen cycling during winter in Arctic estuaries since oxygen concentrations in Arctic rivers can be low when the rivers are ice-covered (e.g. Chambers et al., 1997).

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\*With apologies to G. G. Márquez

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### “Ecological stoichiometry of nutrient limitation in lakes and beyond”

Ecological stoichiometry is the study of the balance of energy and multiple chemical elements in ecological interactions (Sterner and Elser 2002). This work places special emphasis on the elements nitrogen (N) and phosphorus (P) in ecosystems and in living things because N and P are frequently limiting to growth of microbes, plants, and animals and because significant amounts of N and P must be allocated to critical molecules, such as proteins and nucleic acids, in all living things. Thus, nutrient limitation is a delicate dance between environmental supply and biological demand in space and time.

Various internal and external factors affect N/P limitation in ecosystems, including food web structure (Figure 1; via differential nutrient recycling by consumer species that differ in body N:P ratio; Elser et al. 1988) and atmospheric N deposition. In the latter case (Figure 2), new data show that the stoichiometry of overall N and P supplies is severely skewed in lakes in Colorado and in Scandinavia that receive atmospheric N inputs from urbanization and agriculture (Elser et al. 2009). Furthermore, these elevated N inputs appear to have shifted phytoplankton nutrient limitation from predominantly N-limitation in unaffected lakes to predominantly P-limitation in high deposition lakes. Finally, internal feedback processes, such as the balance of N-fixation and denitrification (Figure 3), can produce and maintain nutrient limitation of autotrophs in an ecosystem in N- or P-limited state depending on a variety of complex physical, hydrological, and biogeochemical factors that are yet poorly understood.

N and P limitation may have a more direct role to play in the future of humanity. Recent population expansion of *Homo sapiens* has been made possible by the massive increase in agricultural production known as the “Green Revolution.” While the Green Revolution encompassed a variety of agronomic innovations, ultimately its success was made possible by large increases in fertilizer use. For N, this has been met by industrial N-fixation via the Haber-Bosch reaction. However these large increases in crop production could not have been achieved without simultaneous application of P; however, P cannot be pulled out of thin air. Instead, the P supply has been amplified by a massive expansion of mining of high-grade fossil P deposits located in just a few countries (USA, China, Morocco). The economic longevity of these P deposits, especially in light of expanding human population size, increasing affluence (and thus a P-rich diet) in the developing world, and a burgeoning biofuel industry, has recently become a cause for concern (Cordell et al. 2009) and indeed raises the possibility of a “phosphorus famine” in coming decades. Thus, the long-term sustainability of the human enterprise itself may come down to a question of phosphorus limitation and will challenge human ingenuity in ways not yet contemplated by many.

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Figure 1.

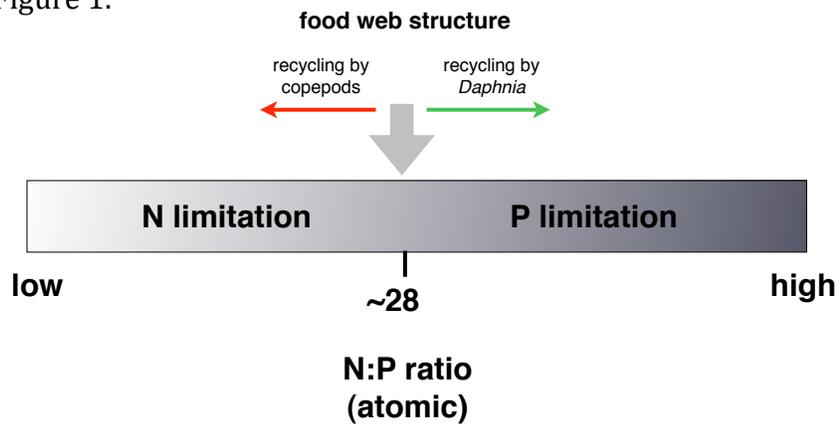


Figure 2.

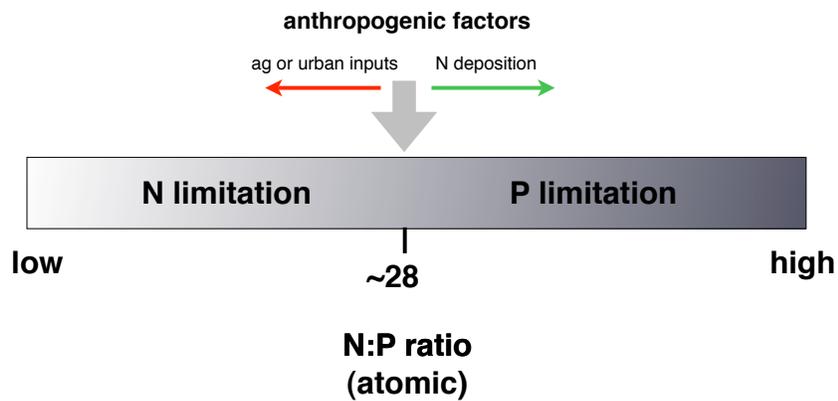
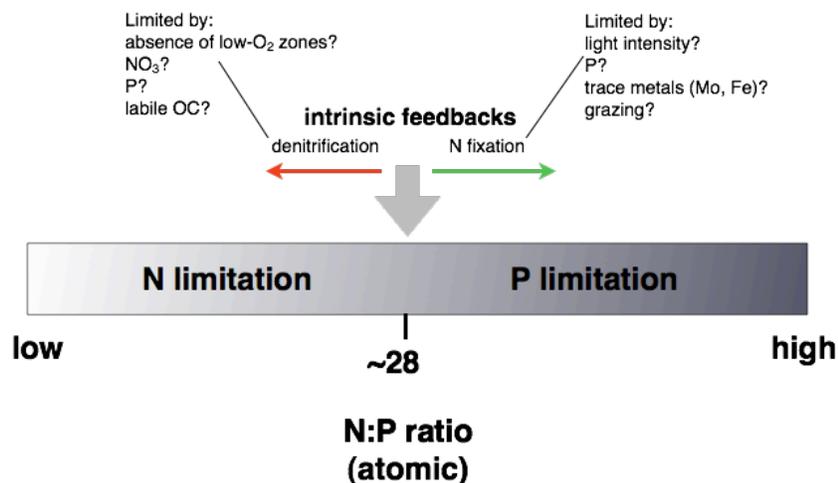


Figure 3.



Methane Fueled Syntrophs Fix Nitrogen in the Deep Sea  
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Nitrogen fixing microorganisms play an instrumental role in the global nitrogen cycle and can regulate overall productivity in diverse marine and terrestrial ecosystems. Mediating the energetically demanding conversion of  $N_2$  to  $NH_4$ , they provide bioavailable nitrogen for the rest of the biosphere unable to directly assimilate  $N_2$ , and close the nitrogen cycle by counteracting  $N_2$ -generating catabolic microbial processes (i.e. denitrification and anammox). The recent discovery of new phylogenetic and physiological diversity of environmental microbes capable of nitrogen fixation, including hyperthermophilic methanogens from hydrothermal vents (8), has expanded our understanding of the range of habitats where new nitrogen production occurs (3, 5). These extreme environments and other localized habitats of enhanced productivity in the deep-sea could represent underappreciated sources of fixed nitrogen which may aid in offsetting the predicted imbalance in the oceanic N cycle (1, 7).

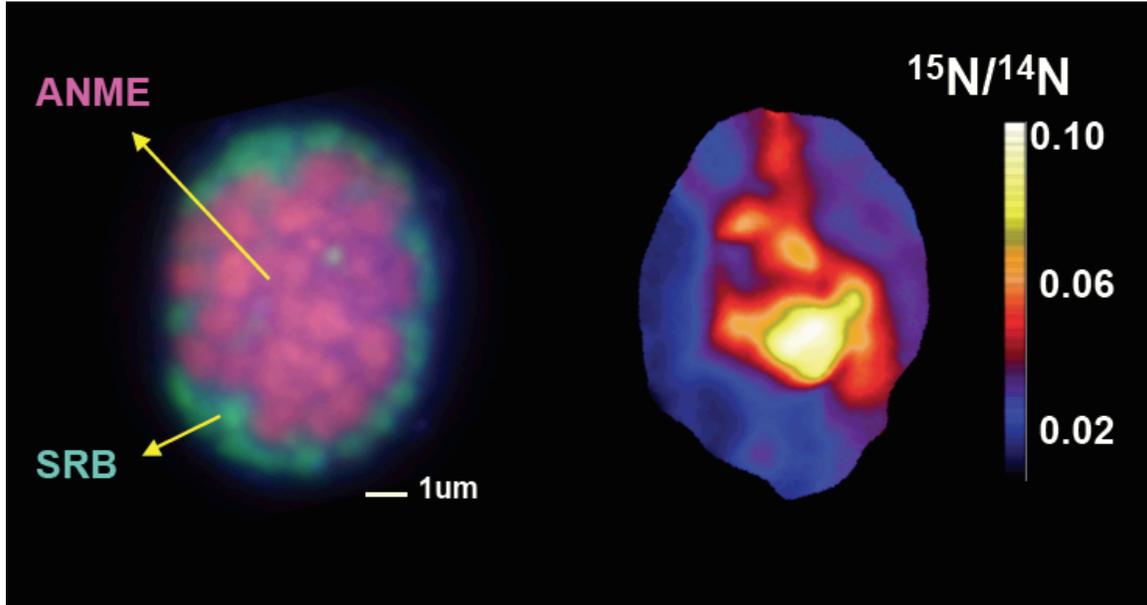
In a previous metagenomic study, we detected the genes required for nitrogen fixation, the *nif* genes, in a concentrated sample of the symbiotic microorganisms mediating the anaerobic oxidation of methane (AOM) in marine sediment (10). This was an intriguing result, considering the high energy requirements of nitrogen fixation (~800 kJ/mol  $N_2$ ) in contrast to the extremely low energy yield of AOM (~40 kJ/ mol  $CH_4$  at methane seeps), in addition to the assumed rarity of benthic nitrogen fixation due to the presence of other bioavailable nitrogen sources. We tested the hypothesis that these uncultured microbial consortia were able to fix nitrogen using  $^{15}N$ - stable isotope tracers and high-resolution ion imaging (2). Indeed, the single-cell resolution afforded by nano-

SIMS revealed localized N<sub>2</sub> fixation within anaerobic methane-oxidizing archaea (ANME-2) and apparent sharing of fixed nitrogen with their closely-associated sulfate-reducing bacterial symbionts (*Desulfosarcina/Desulfococcus*) (Figure 1). Inhibition experiments indicate that diazotrophic growth requires the active metabolism of both partners, methane-oxidation and sulfate-reduction, effectively linking the carbon, nitrogen, and sulfur cycles within these globally important microbial consortia. Cyanide, a well-known *in vitro* substrate for nitrogenase, also served as nitrogen source for these organisms, suggesting the enzyme mediating dinitrogen reduction may confer additional functions. We found that although growth was significantly slowed when growing on N<sub>2</sub>, the rate of sulfate reduction, and by proxy the rate of methane oxidation, remained nearly constant. This suggests that the organisms compensate for the energetic burden of diazotrophy by maintaining respiration rates despite the decreased energy requirement for growth. It also implies that rate of methane oxidation *in situ* may be constant despite fluctuations in nitrogen source.

Molecular data supported an archaeal diazotroph in these sediments. Near complete *nif* operons were retrieved from the same sediment incubations probed with <sup>15</sup>N tracers and nano-SIMS. The operon contained the structure typical of that in methanogenic archaeal diazotrophs (“C-type”: *nifH*, *nifI1*, *nifI2*, *nifD*, and *nifK*) and the *nifD* gene clustered with those from archaea. Additionally, the *nifH* genes from this operon were found within a clade of the *nifH* phylogeny that appears to be specific to methane seeps, including samples collected worldwide (2). This is consistent with the global presence of the ANME-2 species at methane seeps, and suggests that ANME-2 diazotrophy may be widespread.

Whether or not these organisms fix nitrogen *in situ* is yet to be demonstrated, however we contend that it is unlikely that these energy-limited organisms would maintain functional genetic machinery if it was not at least periodically employed. Although marine sediment is generally thought to be replete with bioavailable nitrogen, and therefore inhibitory of nitrogen fixation, it is possible that at methane seeps, where there is an abundant carbon source unaccompanied by nitrogen, nitrogen limitation does occur. This is supported by the recovery of abundant *nifH* transcripts in methane rich deep sea sediment (9), as well as  $\delta^{15}\text{N}$  measurements near 0‰ at methane seeps (4), both of which are suggestive of nitrogen fixation. Preliminary studies of the concentration of ammonia at methane seeps shows high spatial variability, including regions where the concentration is well below that known to inhibit nitrogen fixation in pure cultures of diazotrophic methanogens (6). Our preliminary studies indicate that the ANME-2 nitrogen fixation machinery is inhibited in the presence of ammonia, leading us to suggest that ANME-2 nitrogen fixation may vary temporally and spatially as the concentration of ammonia varies.

More studies must be performed to determine the global significance benthic diazotrophy, but this study bolsters the growing dataset implying that unexpected sources of fixed nitrogen are present in the ocean. Our understanding of the marine nitrogen cycle may therefore be improved if we reconsider some of our assumptions about where and when nitrogen fixation can occur. This result is the first demonstration of  $\text{N}_2$  fixation and resource sharing within an archaeal-bacterial syntrophic association and extends the known lower limits of respiratory energy capable of fueling this energetically intensive anabolic process.



**Figure 1.** *Left:* Microbial consortium of archaeal (ANME, interior) and bacterial (SRB, exterior) cells mediating the anaerobic oxidation of methane. Cells have been hybridized with species-specific probes such that the ANME cells appear pink and the SRB cells appear green. The consortium imaged was incubated in bulk sediment for 6 months under a headspace of methane and  $^{15}\text{N}_2$ . *Right:*  $^{15}\text{N}/^{14}\text{N}$  map generated with a Cameca nanoSIMS 50L of the same consortia pictured at left. Hot colors indicate increasing  $^{15}\text{N}$  incorporation, and therefore the location of nitrogen fixation. Natural abundance  $^{15}\text{N}/^{14}\text{N}$  is approximately 0.0036, indicating that the entire consortium is enriched, and sharing of the reduced  $^{15}\text{N}$ -labelled nitrogen occurs.

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