INTRODUCTION

Although nitrogen (N) as dinitrogen gas (N₂) is the most abundant element in the atmosphere, N in this form is unavailable to most organisms. N is a major component of many metabolites and structural molecules (amino acids, proteins, and nucleic acids). N₂ fixation is the conversion of the inert atmospheric gas N₂ into biologically available compounds such as ammonium and nitrogen oxides. Most organisms obtain N from combined forms of N, including nitrate, nitrite, urea, ammonium, or organic N, and these compounds are often in low concentrations relative to other elements required for growth. N₂-fixing microorganisms, also called diazotrophs, are able to draw upon the large reservoir of N₂ in the atmosphere, since it readily dissolves in seawater (about 400–575 μmol/L N₂; Sharp 1983).
In the marine environment, the availability of N is a key factor limiting primary and secondary production (Carpenter and Capone 1983; Dugdale 1967; Ryther and Dunstan 1971). In all marine environments (estuaries, coastal, pelagic), biological demands for N often exceed availability. On ecosystem, regional, and global scales, the availability of N is ultimately controlled by the relative activities of N2 fixation and the processes (e.g., denitrification) that form N2 (Codispoti et al. 2001). Historically, geochemists have argued that N is not the nutrient that ultimately limits production in the oceans, since the presence of the genetic potential for obtaining N from the large atmospheric reservoir should alleviate N deficiencies over long time scales (Redfield 1958; Smith 1984; Tyrrell 1999). However, N2 fixation rates in the ocean are controlled by a myriad of interacting environmental factors, including oxygen tension, turbulence, availability of non-N nutrients and energy needed to sustain this process, and even grazing on short and long time scales (Falkowski 1997; Howarth and Marino 1988; Paerl 1990).

For many years, rates of N2 fixation in the surface waters of the open ocean were assumed to be negligible relative to the other inputs of N, such as upwelling and advection from nitrate-rich deep ocean waters. However, the results of biogeochemical analyses, and the application of molecular biology and high-sensitivity analytical techniques, have led to a new appreciation of the role of N2 fixation in the oceans (Capone 2001; Capone et al. 2005; Codispoti et al. 2001; Karl et al. 2002; Lipschultz and Owens 1996). The goal of this chapter is to review nitrogen fixation from fundamental biochemistry and physiology to the biological and molecular biological aspects, focusing on recent discoveries with respect to the open ocean. Other reviews of N2 fixation, including the biogeochemical perspective, are available (Capone 2000; Herbert 1999; Mahaffey et al. 2005).

CHEMISTRY, BIOCHEMISTRY, AND GENETICS OF N2 FIXATION

N2 fixation requires large amounts of energy, since there is a high activation energy for breaking the triple bond of the N2 (N≡N) molecule (Postgate 1982). Abiological N2 fixation can occur in the upper atmosphere, catalyzed by lightning discharges that result in the production of oxidized forms of N and ammonium (Galloway et al. 2004). Anthropogenic abiological N2 fixation occurs in internal combustion engines (Galloway et al. 2004) and the Haber–Bosch industrial synthesis of nitrogenous fertilizers. Anthropogenic N2 fixation has resulted in global-level perturbations of the N cycle (Galloway et al. 2004; Galloway and Cowling 2002; Howarth et al. 1996; Paerl 1997).

The stoichiometry of biological N2 fixation is

\[ \text{N}_2 + 8\text{H}^+ + 8\text{e}^- + 16\text{ATP} \rightarrow 2\text{NH}_3 + \text{H}_2 + 16\text{ADP} + 16\text{P}_i \]

where ATP is adenosine triphosphate, ADP is adenosine diphosphate, and P_i represents inorganic phosphates. This reaction is catalyzed by the enzyme nitrogenase,
which is composed of two multisubunit metalloproteins. N₂ fixation requires large supplies of ATP and reductant (NADH or NADPH: the reduced forms of nicotinamide adenine dinucleotide and its phosphate, respectively). Hydrogen (H₂) is evolved by nitrogenase, which is recovered by uptake hydrogenases in some microorganisms (Postgate 1998). This reduction of H⁺ to H₂, which consumes ATP, links H₂ production and N₂ fixation, and is not well understood (Rees and Howard 2000). H₂ production has been measured in diazotrophic cyanobacteria (Paerl 1982a), and in the ocean during a *Trichodesmium* bloom (Scranton 1983). H₂ production during N₂ fixation can be an energy loss for the diazotrophs, but may be an energy source for the microbial community (Karl et al. 2002), since many microorganisms can obtain energy through H₂ oxidation. The ATP stoichiometry of the reaction shown above is not necessarily fixed, and ratios of ATP per electron transfer can exceed 2 (Rees and Howard 2000). Differences in nitrogenase efficiencies may affect competition between microorganisms, but the ecological significance of varying efficiencies in the environment is not known.

**Genetics and Enzymology**

The molecular biology and genetics of the N₂ fixation apparatus is beyond the scope of this chapter, but the structural and functional complexity of the apparatus is important for understanding the evolution, adaptation, and ecology of diazotrophic microorganisms. The two nitrogenase proteins are called component I (MoFe (molybdenum–iron) protein, or dinitrogenase) and component II (the Fe protein or dinitrogenase reductase) (Fig. 13.1). The best-described nitrogenase requires Mo in

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**Figure 13.1** Regulation of N₂ fixation at transcriptional, translational and post-translational levels. Different levels of regulation can be assayed using molecular genetic, immunological, and proteomic methods.
a Mo–Fe cofactor (FeMo-co). A discussion of the characteristics of the proteins can be found in Dixon and Kahn (2004) and Rees and Howard (2000).

The **Fe protein** (about 60 kDa) is composed of a pair of identical subunits encoded by the *nifH* gene. The **MoFe protein** (about 200 kDa) is composed of two sets of heterodimers, composed of the α and β subunits encoded by the *nifD* and *nifK* genes. The multisubunit structure coordinates FeS clusters, called the P clusters, and the FeMo cofactor (FeMo-co), which is presumably the site for substrate (N₂) binding (Howard and Rees 1996; Rees and Howard 2000). The primary function of the Fe protein is to reduce the MoFe protein through a series of single electron transfers. Each electron transfer requires docking and undocking of the two proteins and hydrolysis of ATP (Howard and Rees 1996). The Fe protein plays several roles, including maturation and proper insertion of FeMo-co into the MoFe protein (Rubio and Ludden 2005). The substrate-binding site for N₂ reduction is the FeMo-co in the MoFe protein (Howard and Rees 1996; Rees and Howard 2000).

The structural genes *nifHDK* are often present as a single copy in the genome. However, *Clostridium* has multiple copies of *nifH*, as well as having the conventional and alternative nitrogenases. “Alternative” nitrogenases are often present as second and/or third copies of the *nif* operon that encode non-Mo-containing component I proteins (Bishop and Premakumar 1992; Eady 1996). One of these (the “first alternative”) contains Vanadium (V) in place of Mo in the cofactor; the other (the “second alternative”) contains only Fe. The alternative nitrogenases differ from the Mo-containing nitrogenase since they contain a third subunit in component I encoded by *vnfG* or *anfG* (Bishop and Premakumar 1992; Eady 1996). The ecological significance of the alternative nitrogenase genes is not known (Eady 1996). **Gammaproteobacteria** with Mo-independent nitrogenases have been isolated from salt marshes (Loveless et al. 1999).

There is a poorly characterized enzyme isolated from a thermophilic *Streptomyces* that catalyzes the reduction of N₂ via a mechanism that is substantially different from nitrogenases (Ribbe et al. 1997; Rubio and Ludden 2005). Unlike other N₂ fixation reactions, the one catalyzed by the *Streptomyces* nitrogenase involves carbon monoxide (CO). The environmental significance is not yet known, but this *Streptomyces* was isolated from a very unusual environment, and it is unlikely that CO levels in the surface ocean could support activity of this nitrogenase.

The Fe protein of the conventional and alternative nitrogenases is highly conserved among microorganisms (*nifH*, *vnfH*, *anfH*). The structural genes for nitrogenase, *nifHDK*, are often contiguous and expressed as a single operon (Figs. 13.1 and 13.2). The abundance of each of the structural gene transcripts can differ, due to multiple transcript start sites or RNA processing. This has significance for assaying the activities of diazotrophs by *nifH* or *nifD* gene expression in the oceans and other natural environments.
Multiple genes are involved in N$_2$ fixation. Some gene products are involved in cofactor synthesis and insertion, and others in Mo uptake (Dean and Jacobsen 1992). At least 20 nitrogenase (nif) genes have been characterized in *Klebsiella pneumoniae*. The nif genes include the nifJ, nifH, nifD, nifK, nifT, nifY, nifE, nifN, nifX, nifU, nifS, nifV, nifZ, nifM, nifF, nifL, nifA, nifB, and nifQ genes (Postgate 1998), although not all of these are found in all organisms. The structural genes are sometimes on plasmids, for example, the *Desulfovibrio vulgaris* megaplasmid (200 kbp) and the *Sinorhizobium meliloti* plasmid (1.35 Mbp) (http://www.ncbi.nlm.nih.gov/) (Sobecky et al. 1997).

N$_2$ fixation activity is directly regulated by the expression (transcription and translation) of the nifHDK structural genes (Fig. 13.1). Regulation of the N$_2$ fixation apparatus is important, since nitrogenase is rapidly inactivated by oxygen, and the energy expenditure involved in N$_2$ fixation makes it advantageous to use other sources of fixed N when available. In organisms with multiple gene copies, the different nitrogenases can be regulated by Mo and V availability or levels of oxygen (Eady 1996). *Anabaena variabilis* is a heterocyst-forming cyanobacterium containing a nitrogenase that is synthesized in the heterocyst (a specialized cell that fixes N$_2$ and has reduced photosystem II activity) and another that is expressed in vegetative cells under anaerobic or microaerophilic conditions (Thiel et al. 1995). *A. variabilis* also contains a V nitrogenase (Thiel 1993).

**Evolution of N$_2$ Fixation**

The diversity of N$_2$-fixing microorganisms and the distribution in different habitats is partially a legacy of the evolution of life on Earth. The evolution of N$_2$ fixation has been reviewed elsewhere (Berman-Frank et al. 2003; Raven and Yin 1998; Zehr et al. 2006). Although the Earth’s atmosphere has always been dominated by N$_2$ (Galloway 2003), ammonium may have been present in the early Earth’s atmosphere, and this has been argued to preclude the need for N$_2$ fixation (Towe 2002). It is sometimes debated whether nitrogenase evolved early, or was laterally transferred later in evolution (Postgate and Eady 1988; Raymond et al. 2004; Young 1992, 2005). Coincident with changes in oxygen concentrations in the atmosphere and oceans were changes in the chemistry and availability of metals and ions required for N$_2$ fixation. The early oceans are believed to have been anoxic, and hence reduced, with high concentrations of biologically available ferrous ions (Fe$^{2+}$) (Raven and Yin 1998). Thus, the Fe nitrogenase may have been the first nitrogenase, with the Mo and V nitrogenases arising later (Berman-Frank et al. 2003; Raymond et al. 2004). Most phylogenetic analyses conclude that nitrogenase evolved during the early stages of prokaryotic evolution, although there are examples of lateral transfer, and lateral transfer is required to explain the current distribution of Mo and V nitrogenases (Fani et al. 2000; Leigh 2000; Raymond et al. 2004; Young 1992; Zehr et al. 1995).
Figure 13.2  Nitrogenase gene arrangements from genomes of a number of microorganisms. Multiple genes are required for assembly and activity of nitrogenase. There is conservation of gene organization in related organisms, but also differences between major taxonomic groups. Gene arrangements and other \textit{nif} genes could be targets for studying genetic diversity in natural populations.
Nitrogenase, or some form of prenitrogenase genes, could have initially served a function other than for N\textsubscript{2} fixation. It could have played a role in cyanide reduction early in the Earth’s history when cyanide was produced by photooxidation of atmospheric methane (Kasting and Siefert 2001), and then later became functional in N\textsubscript{2} fixation (Fani et al. 2000; Kasting and Siefert 2001; Raymond et al. 2004). It is also conceivable that nitrogenase served to make N biologically available early on, if ammonium in the Archean was depleted by early life, including methanogens, other anaerobic chemoheterotrophs, and autotrophs (Ehrlich 1990; Kasting and Siefert 2002; Nealson and Rye 1994; Raven and Yin 1998; Rosing 1999; Sprent and Raven 1992). These organisms would have been present about 3.8 Ga ago (Nisbet and Fowler 2003). Extant methanogens and sulfate reducers have \textit{nif} genes (Leigh 2000), and were probably present over 3.5 Ga ago (Canfield 1998; Shen et al. 2001).

**Phylogeny of Nitrogenase**

Phylogenetically, the nitrogenase Fe protein (\textit{nifH}) genes form four or five deeply branching clusters (Fig. 13.3) (Zehr et al. 2003). One of these comprises the evolutionarily related protochlorophyllide reductases (see below). Another deep cluster includes \textit{nif}-like genes from \textit{Archaea} that are not involved in N\textsubscript{2} fixation. The three major clusters, Clusters I–III, all contain \textit{nifH} genes that encode active nitrogenases. Cluster II comprises the second alternative \textit{nifH} genes, the Fe-only nitrogenases. The first alternative V-containing nitrogenases do not form a distinct cluster in the \textit{nifH} tree, but can usually be distinguished from their conventional nitrogenase within the same organism (e.g., \textit{Azotobacter vinelandii} or \textit{Anabaena variabilis}).

Within Cluster I, \textit{nifH} and 16S rRNA phylogenies are largely congruent. The phylogeny of \textit{nifH} provides a way to characterize or identify uncultivated N\textsubscript{2}-fixing microorganisms (Zehr et al. 2003). Ultimately, confirming the classification of marine diazotrophs is dependent upon cultivation or by linking \textit{nifH} genes to rRNA genes by genomic approaches, since there are some examples of possible lateral gene transfer of \textit{nifH} (Cantera et al. 2004; Raymond et al. 2004).

**Genomics of N\textsubscript{2} Fixation**

The genomes of relatively few N\textsubscript{2}-fixing microorganisms have been sequenced. Nitrogenase genes have not been found in the genomes of abundant marine organisms, including \textit{Pelagibacter} (Giovannoni et al. 2005), which is a cultured representative of the abundant SAR11 clade, \textit{Silicibacter} (Moran et al. 2004), \textit{Prochlorococcus} (Rocap et al. 2003), and marine \textit{Synechococcus} (Palenik et al. 2003). Chapter 4 discusses genomics in marine microbial ecology.

There are draft genome sequences of two important oceanic diazotrophs: \textit{Trichodesmium} and \textit{Crocosphaera} (http:www.jgi.doe.gov). The nitrogenase gene organizations of these two cyanobacteria are highly conserved along with the genes of other cyanobacteria (Fig. 13.2). The interesting contribution of the
genome sequences is not the nitrogenase genes themselves, but the genomic information that can provide hypotheses as to how these microorganisms compete in the marine environment. For example, genomic analysis has suggested that there may be differences in the phosphorus-utilization pathways of *Trichodesmium* and *Crocosphaera* (Dyhrman et al. 2006; Dyhrman and Haley 2006). The *dps* gene that encodes an Fe-binding protein was found and subsequently expressed in *Escherichia coli* (Castruita et al. 2006). The genomes of several more marine diazotrophic cyanobacteria are currently being sequenced (http://www.moore.org/microgenome, http://www.wust.edu).

There may be differences in regulatory pathways as well. Cells sense and respond to the environment using signal transduction systems composed of sensor and response regulator protein domains. One-component and two-component signal transduction systems have been identified in *Trichodesmium* (Ulrich et al. 2005), but the number of one-component systems is small for its genome size (Ulrich et al. 2005). In the draft sequences, there were many more two component systems in *Crocosphaera* than in *Trichodesmium* (Zehr et al. 2005).
Diversity of N$_2$-Fixing Microorganisms

There are diverse N$_2$-fixing microorganisms in terrestrial and aquatic environments. N$_2$ fixation has not yet been found in Eukarya, but is found in the Bacteria and Archaea domains (Young 1992). N$_2$-fixing Bacteria and Archaea are genetically, physiologically, and ecologically diverse, and N$_2$ fixation appears to be widely scattered throughout prokaryotic lineages (Fig. 13.3). However, the nitrogenase genes are not necessarily conserved within lineages, so that closely related microorganisms may differ in their ability to fix N$_2$. For example, Vibrio diazotrophicus fixes N$_2$, but V. cholerae does not.

N$_2$ fixation and the nitrogenase genes are found in chemoheterotrophs (aerobic, anaerobic, and facultative), chemolithotrophs, photolithotrophs, and photoheterotrophs (see Chapter 1 for definitions of these terms). Diazotrophic representatives include (1) phototrophic bacteria (other than cyanobacteria, such as Chlorobium, Chromatium, and Rhodospirillum), (2) cyanobacteria, including all heterocystous filamentous (Aphanizomenon, Calothrix, and Nodularia), some nonheterocystous filamentous (Oscillatoria, Lyngbya, and Trichodesmium) and unicellular (Gloeothece and Synechococcus) genera, (3) strict anaerobes (e.g., Clostridium and Desulfovibrio), (4) heterotrophs (e.g., Klebsiella, Vibrio, and Azotobacter), (5) Fe oxidizers (e.g., Thiobacillus), and (6) archaeal methanogens.

Examples from all of these groups are found in marine environments, although different groups are most abundant or active only in specific habitats consistent with requirements for oxygen, energy (light and organic matter), and macro- and micronutrients (Capone 1983; Paerl 1990; Potts 1980).

Regulation in Diazotrophs

N$_2$ fixation provides a means of obtaining N for growth at the expense of ATP and reductant. The nitrogenase enzyme is sensitive to oxygen, yet for some organisms oxygen is required as an electron sink to support the respiration needed to supply energy. The N fixed must balance growth requirements, or excess N will be lost to the environment and to other organisms. The nitrogenase reaction regenerates electron acceptors, and is an electron sink. In general, the major factors that regulate nitrogenase gene expression and activity are energy (e.g., light in the case of some phototrophs and photoheterotrophs), O$_2$ concentrations, and fixed inorganic N availability. Trace-metal availability for the metallocenters (Mo and V) can also control expression of the $nif$ and alternative nitrogenase ($vnf$ and $anf$) genes. Presumably, Fe availability, believed to limit productivity and N$_2$ fixation in some areas of the oceans (Berman-Frank et al. 2001a; Falkowski 1997), may also regulate N$_2$ fixation even at the gene expression level. The specifics of the regulatory networks (e.g., $nif$LA genes) differ among organisms, but the important point is that complex networks have evolved to balance N and carbon metabolism. An in-depth review of the general physiology and regulation of N metabolism and N$_2$ fixation is beyond the scope of this chapter, and is provided elsewhere (Dixon and Kahn 2004; Karl et al. 2002; Martinez-Argudo et al. 2005; Ninfa and Jiang 2005).
Methods for Assessing Diazotroph Diversity, Gene Expression, and N$_2$ Fixation Activity

N$_2$ fixation activity of enzymes or cells can be measured directly by using N$_2$ enriched in the stable isotope $^{15}$N or indirectly using acetylene reduction (Stewart et al. 1967). Rates of N$_2$ fixation are most directly measured using the stable isotope $^{15}$N. Acetylene (CH≡CH) is an analogue of N$_2$ and is reduced by conventional nitrogenases to ethylene, which is easily measured by gas chromatography or, more recently, by photoacoustics (Zuckermann et al. 1997). These methods are reviewed elsewhere (Capone 1983; Capone and Montoya 2001; Paerl 1998; Zehr and Montoya 2007). Recently, stable-isotope techniques have been developed that can assay N$_2$ incorporation at the cell level. Secondary ion mass spectrometry (SIMS) has been used to visualize isotope incorporation of $^{13}$C at cell-level resolution (Orphan et al. 2001), and applications for $^{15}$N are being developed (D. G. Capone, personal communication). The natural abundance of $^{15}$N in particulate material can also be used to estimate the importance of N$_2$ fixation rates in natural samples (Zehr and Montoya 2007). Particulate N that has been fixed from atmospheric N$_2$ has a ratio of $^{15}$N:$^{14}$N slightly lower than atmospheric N$_2$.

Historically, N$_2$ fixers have been identified, enumerated, and characterized by classical microbiological enrichment techniques (Guerinot and Colwell 1985; Wynn-Williams and Rhodes 1974). Cultivation techniques can yield N scavengers (surviving on trace concentrations of fixed N contaminants in media) rather than diazotrophs (Wynn-Williams and Rhodes 1974). For years, the important diazotroph *Trichodesmium* (Fig. 13.4) eluded cultivation, but within the last two decades a few strains have been retained in many laboratories. One of these strains, *Trichodesmium* sp. IMS 101, originally isolated from the Gulf Stream off the North Carolina Atlantic coast (Prufert-Bebout et al. 1993), is now available in culture collections, and a draft genome sequence is also available. *Crocosphaera*, a unicellular cyanobacterium (Fig. 13.4), was cultivated from Atlantic waters in the mid-1980s (Waterbury and Rippka 1989), and related strains have been cultivated from both the Atlantic and Pacific (Falcón et al. 2004b; Zehr et al. 2001a, b). Recently, the symbiont of *Chaetoceros*, *Calothrix* sp. (Fig. 13.4), was isolated and is now maintained asymbiotically in N-free media (Foster et al. 2007).

The high degree of similarity of the *nifH* gene among diverse taxa has made it attractive for use in cultivation-independent molecular studies, since *nifH* can be amplified with “universal” degenerate polymerase chain reaction (PCR) primers (Langlois et al. 2005; Lovell et al. 2000; Mehta et al. 1999; Steppe et al. 1996; Zehr and McReynolds 1989; Zehr et al. 1995, 2003; Zehr and Capone 1996; Zehr and Paerl 1998). Phylogenetic analysis of amplified *nifH* sequences can help to identify diazotrophs (Fig. 13.3), since *nifH* phylogeny largely mirrors rRNA phylogeny (Zehr et al. 2003). Functional gene cultivation-independent approaches are useful, since gene transcription into messenger RNA (mRNA) can be directly assessed (Omoregie et al. 2004b; Zani et al. 2000). Gene transcription does not necessarily lead to N$_2$ fixation, since subsequent protein translation, modification, and transcript degradation can regulate N$_2$ fixation activity.
Figure 13.4 Photomicrographs of cultivated and uncultivated marine cyanobacterial diazotrophs. (a) Uncultivated unicellular cyanobacteria from the open ocean that are likely diazotrophs. (b) Diversity of *Trichodesmium* morphology in natural populations. (c, d) Symbiotic unicellular cyanobacteria. (e) Heterocystous symbiont of diatoms. (f) Heterocystous symbiont of diatoms. (g) Heterocystous symbiont of diatoms. (h, i) Association of bacteria with heterocysts of heterocyst-forming cyanobacteria. (See also in color insert.)
The detection of *nifH* mRNA potentially identifies the organisms likely to be involved in N\(_2\) fixation. The large database of *nifH* gene sequences facilitates the application of other techniques to quantify nitrogenase genes and to assess diversity. Methods such as terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) have been used for *nifH* gene characterization. Quantitative PCR methods have made it possible to target specific groups of microorganisms to determine the distribution and autecology of specific *nifH* phylotypes (Short et al. 2004). This approach can be combined with reverse transcription to identify actively transcribed genes (Church et al. 2005b; Short and Zehr 2007).

DNA macroarrays and microarrays have been used to assay whole-genome expression, or to examine diversity using “phylochips” (Jenkins et al. 2004; Moisander et al. 2006; Steward et al. 2004; Taroncher-Oldenburg et al. 2003; Tiquia et al. 2004). In this approach, different *nifH* gene probes are spotted on the DNA microarray, and the array then hybridized to *nifH* amplification products (Fig. 13.5).

Estimates of N\(_2\) fixation rates in the ocean obtained from biogeochemical analysis have recently been reviewed (Mahaffey et al. 2005). N\(_2\) fixation can be measured by incorporation of N over time during incubation. This method is only applicable when both rates and biomass are high, which occurs in blooms in some estuarine and coastal systems (e.g., the Baltic Sea) (Elmgren and Larsson 2001). Over large spatial scales, the ratios of regeneration of inorganic N and phosphorous (P) have been used to identify regions of N\(_2\) fixation. N\(_2\) fixation rates are estimated by gradients in N/C\(_3\) (see the text box) integrated along isopycnal surfaces over appropriate spatial scales if rates of mixing along the isopycnals are estimated.

![Figure 13.5](image)

**Figure 13.5** (a) A *nifH* microarray hybridization to an oligonucleotide microarray containing 768 *nifH* probes. *nifH* was amplified from a Chesapeake Bay North Bay surface water sample collected in October 2002. (b) Response of individual probe spots in hybridizations to samples from the Chesapeake Bay and Sargasso Sea (Bermuda Hydrostation ‘S’, 5 m depth). The target concentration was the same in each of the hybridizations, and the data were normalized using internal standards.
Elevated dissolved inorganic N : P ratios are produced from regeneration of particulate material with non-Redfield high N : P elemental ratios that result from diazotrophy. $N^*$ is a variable that is a linear function of regenerated N and P (forced to global Redfield ratios of N : P and global $N^* = 0$). Integration of $N^*$ has been used to estimate basin scale $N_2$ fixation and denitrification rates and identify regions of activity (Deutsch et al. 2001; Gruber and Sarmiento 1997). These calculations require multiple assumptions, including the global balance of denitrification and $N_2$ fixation, an estimate of the elemental composition of diazotrophs, and extrapolation along pycnoclines over large spatial scales. Furthermore, $N^*$ does not necessarily reflect the composition of local sinking material due to the time scales of remineralization (Mahaffey et al. 2005). Chapter 14 also discusses $N^*$.

Remote sensing and modeling approaches are needed to scale rate information to regional and ocean basin scales. In the open ocean, blooms of *Trichodesmium* and symbiont-containing diatoms can be detected by remote sensing (Fig. 13.6) (Capone et al. 1998; Carpenter et al. 1999; Dupouy et al. 1998; Hood et al. 2002; Subramaniam et al. 1999, 2002). There are constraints on remote sensing, but blooms of highly reflective microorganisms (such as *Trichodesmium* gas vacuoles) concentrated in the surface waters can be detected by satellite or aircraft sensors. Detection of surface-dwelling blooms of the heterocystous, aggregate-forming cyanobacteria *Nodularia*, *Aphanizomenon*, and *Anabaena*, which are a dominant feature of the surface waters of the Baltic Sea and are a significant source of N (Elmgren and Larsson 2001; Kononen et al. 1996), could also be approached using remote sensing techniques.

![Figure 13.6](image_url) Remote sensing of blooms of *Trichodesmium*. (a) True-color image of surface ocean, indicating position of *Trichodesmium* bloom detected by remote sensing. (b) Remote sensing image of *Trichodesmium* using the algorithm of Subramaniam et al. (2002). Modified from Subramaniam et al. (2002). (See also in color insert.)
Models have focused primarily on *Trichodesmium* (Hood et al. 2001). The model of Hood et al. (2001) assumes that diazotrophic growth is a function of light, and predicts the distribution of *Trichodesmium* in the North Atlantic Ocean. Interestingly, this model predicts high abundances and N₂ fixation rates of *Trichodesmium* as part of successional events from upwelling regions (Hood et al. 2004). Coles et al. (2004) estimated the contribution of N₂ fixation to phytoplankton in the North Atlantic on the basis of relationships between chlorophyll, sea-surface height (SSH), and sea-surface temperature (SST), which were observed by remote sensing. A model for the North Pacific incorporated light, temperature, P, and wind stress, and the N₂ fixation rate was predicted from growth rate and elemental composition (Fennel et al. 2005). N₂ fixation can also be estimated from CO₂ drawdown (Lee et al. 2002).

Models of N₂ fixation cellular processes have begun to be devised to predict daily N₂ fixation patterns in individual populations or species (Rabouille et al. 2006; Stephens et al. 2003). These models are useful for generating hypotheses for how and why diazotrophs fix N₂ during certain periods and to understand the energetics of N₂ fixation and photosynthesis. Ultimately, models at this cellular scale will provide a necessary link between ecosystem models and organismal biology.

**ECOPHYSIOLOGICAL ASPECTS OF N₂ FIXATION**

N₂ fixation rates in the environment are controlled by microbial community structure, grazing, and environmental factors that regulate gene expression and activity of populations. Environmental factors that have been hypothesized or shown to regulate and control the activity, abundance and distribution of marine diazotrophs include (1) energy in the form of light or organic matter; (2) oxygen inhibition; (3) nutrient availability, including silica, N and P, N : P ratios, chemical forms and sources; (3) Fe and other trace elements (e.g., Mo); (4) organic matter composition and concentration; (5) salinity; (6) fixed N availability; (7) temperature; (8) water column turbulence, stratification and stability; and (9) biotic interactions (symbiosis, synergism, and grazing) (cf. Paerl 1990).

Cyanobacterial N₂ fixation is supported directly, in the light, by photosynthesis, or indirectly through the metabolism of fixed carbon compounds. Cyanobacteria that fix N₂ in the light are primarily filamentous cyanobacteria that form heterocysts—specialized cells for N₂ fixation that lack photosynthetic O₂ evolution. *Trichodesmium* also fixes N₂ primarily during the day. Most unicellular or filamentous non-heterocyst-forming cyanobacteria fix N₂ during the night, using respiration of carbon storage compounds for energy production. A recent study in hot spring mats indicated that fermentation may support dark N₂ fixation in a unicellular cyanobacterium (Steunou et al. 2006). Since increasing light intensity increases photosynthetic rates up to a point, increasing light intensity increases N₂ fixation rates in cyanobacteria that fix N₂ during the light. Both photosynthesis-versus-irradiance curves (P vs. I) and photosynthesis versus N₂ fixation curves exhibit saturating behavior, with inhibition at higher light intensities. Presumably, energy does not limit
phototrophic diazotrophs in the upper euphotic zone or the surfaces of microbial mats. In the case of N₂ fixation, inhibition at higher light intensities is partially due to the inhibition of nitrogenase activity by oxygen evolved in photosynthesis.

Heterotrophic N₂ fixation can be supported by a wide variety of organic substrates, metabolized aerobically (e.g., *Azotobacter*) or anaerobically (e.g., clostridia, methanogens, and sulfate reducers). Energy is probably an important factor regulating heterotrophic diazotrophs in the oligotrophic ocean, but perhaps is not as important in microbial mats. Interestingly, the expression of heterotrophic *nifH* genes exhibited a slight diel cycle (Church et al. 2005b), which may reflect the link between heterotrophic metabolism and organic matter substrates provided by photoautotrophs.

Several different metabolic strategies are used by diazotrophic microorganisms to obtain energy while avoiding inactivation by oxygen. Aerobic heterotrophs (*Azotobacter*) use polysaccharide capsules to restrict influx of O₂ from the environment, while maintaining high respiratory rates to maintain low intracellular O₂ concentrations and generate ATP. Strict anaerobes, such as *Clostridium*, support N₂ fixation with fermentative metabolism in anoxic habitats. Purple bacteria, including *Rhodospirillum* and *Rhodobacter*, can support N₂ fixation by photoautotrophic (anaerobic in the light) or photoheterotrophic (microaerophilic) metabolism (Postgate 1998). Under some circumstances, nitrogenase can act as an electron sink rather than a N source, such as in *Rhodobacter*, when other electron acceptors are unavailable (Tichi and Tabita 2001).

The cyanobacteria have a number of strategies for fixing N₂ while depending upon a phototrophic metabolism that generates oxygen (Bergman et al. 1997; Berman-Frank et al. 2003; Gallon 1992). Some cyanobacteria spatially separate N₂ fixation from photosynthesis by forming heterocysts. Many cyanobacteria separate the two processes in time, and they fix N₂ primarily in the dark. Organisms such as *Trichodesmium* fix N₂ during the day, yet do not have heterocysts. This paradox has been the subject of many studies.

Patterns of localization of the nitrogenase protein in different cells along filaments (termed diazocytes: El-Shehawy et al. 2003; Fredriksson and Bergman 1995) indicate that there may be some differences in distribution of nitrogenase in cells along the filaments, but the expected inverse relationship between nitrogenase activity and photosystem II oxygen evolution in individual cells has not yet been demonstrated. Paerl et al. (1989) used immunolocalization to demonstrate that nitrogenase was widely distributed along and among individual trichomes of *Trichodesmium*. Microelectrode and cellular C14CO₂ microautoradiographic studies of photosynthetic activity indicated that localized differences in oxygen tension and reductant supplies, may be regulating enzyme activity in aggregates (Paerl and Bebout 1988b). Berman-Frank et al. (2001b) suggested that the Mehler reaction is elevated during the middle part of the light period to alleviate O₂ inactivation of nitrogenase during the day. This mechanism was hypothesized to work in concert with a complex mechanism involving temporal (short time interval) and spatial (different cells) separation of photosynthesis and N₂ fixation (Berman-Frank et al. 2001b; Küpper et al. 2004). Clearly, the mechanisms involved are still only partially understood, and, as a result, remain unclear.
Since N\textsubscript{2} fixation is generally more energetically expensive than the metabolism of fixed dissolved inorganic N, the presence of dissolved inorganic N should affect the selection for diazotrophs and the expression of nitrogenase activity. In general, \textit{nif} gene transcription is repressed in the presence of ammonium, although there are exceptions. Nitrate and ammonium inhibit nitrogenase activity and gene expression in a number of cyanobacteria, including heterocystous and unicellular strains (Flores and Herrero 1994). Interestingly, the reduction of nitrate appears to be thermodynamically less favorable than N\textsubscript{2} fixation (Karl et al. 2002): it is the high activation energy required to break the triple bond of N\textsubscript{2} that makes N\textsubscript{2} fixation energetically expensive. Interestingly, Holl and Montoya (2005) showed that \textit{Trichodesmium} rapidly shifts to use of nitrate even at relatively low concentrations (micromolar), and shifts back to diazotrophy when nitrate concentrations fall below micromolar levels. N\textsubscript{2} fixation by \textit{Nodularia} in the presence of low concentrations of nitrate has been observed in culture (Sanz-Alférez and Campo 1994). Since some microorganisms continue to fix N\textsubscript{2} in the presence of nitrate, it is perhaps not surprising that cyanobacterial \textit{nifH} expression was detected in the Chesapeake Bay in the freshwater reaches where nitrate concentrations were micromolar (Short and Zehr 2007). Intriguingly, the diversity of the genes and organisms is not necessarily correlated with the degree of N limitation (Fig. 13.7) (Zehr et al. 2003). Environments that are not extremely N-limited, such as sediments with high concentrations and gradients of ammonium (see Chapter 14), can have a high diversity of \textit{nifH} genes (Burns et al. 2002). Mats have diverse \textit{nifH} genes. Here, N\textsubscript{2} fixation is largely carried out by phototrophs, which provide the bulk of the biomass (Bebout et al. 1993).

![Figure 13.7](image)

**Figure 13.7** Diversity of \textit{nifH} genes and expression in open-ocean (a), Chesapeake Bay Estuary (b), and Guerrero Negro cyanobacterial mat (c) habitats. Circles indicate genes that are expressed, highlighting differences in genetic potential and the number of genes that are expressed in different habitats.
The Chesapeake Bay (Maryland and Virginia) and Neuse River (North Carolina) Estuaries have a very high diversity of \textit{nifH} genes (Affourtit et al. 2001; Jenkins et al. 2004). Manipulations of salt marsh sediments with additions of fixed N resulted in little perturbation of the diazotroph community (Bagwell and Lovell 2000), which suggests that the diazotrophs are not selected or retained because of the function of their \textit{nif} genes. \textit{nifH} phylotypes have their own spatial and temporal distributions (Short et al. 2004), which would suggest that microorganisms containing the different \textit{nifH} gene types (\textit{nifH} phylotypes) are selected by the environmental factors. However, since most of the genes are not expressed (Fig. 13.7), the distribution is not due to the selective advantage of the \textit{nif} genes themselves.

Specific nutrient deficiencies may restrict the activities and geographic extent of N$_2$ fixation. The trace metals Mo and Fe (and potentially V) are important components of nitrogenase. Mo has been investigated as a nutrient potentially limiting N$_2$ fixation (Cole et al. 1993; Howarth and Cole 1985). Howarth and Cole (1985) proposed that the relatively high (>20 mmol/L) concentrations of sulfate (SO$_4^{2-}$), which is a structural analogue of the most common form of molybdenum found in seawater, namely, molybdate (MoO$_4^{2-}$), could competitively (via the uptake process) inhibit N$_2$ fixation. Competitive inhibition of MoO$_4^{2-}$ uptake by high SO$_4^{2-}$ concentrations was shown by Cole et al. (1993). However, MoO$_4^{2-}$ is highly soluble in seawater, with concentrations on the order of 100 m$mol/L$ (Collier 1985). Ter Steeg et al. (1986) and Paulsen et al. (1991) showed that, despite the potential for SO$_4^{2-}$ competition, Mo availability was ensured at concentrations much lower than 100 m$mol/L$. The presence of “alternative” non-Mo-requiring nitrogenases in bacterial and cyanobacterial diazotrophs (Bishop and Premakumar 1992) could provide a mechanism by which Mo limitation can be circumvented.

Fe is required for the metallocenters of nitrogenase, and diazotrophic microorganisms exhibit a relatively (compared with Mo) high demand for this metal. Fe was initially suggested to be important in controlling N$_2$ fixation in \textit{Trichodesmium} (Rueter 1988). It appears to be generally assumed that Fe controls N$_2$ fixation in the oceans (Berman-Frank et al. 2001a). The chemistry of Fe in seawater is complex. Interestingly, Fe limitation appears to be far less commonplace in the benthos than in the planktonic environment (Paerl and Prufert 1987; Paerl et al. 1994; Paerl 2000). This is likely because Fe is sequestered from the water column as either precipitated or particle-associated Fe, which is then solubilized in periodically reduced sediments.

Dissolved organic matter (DOM) content has been suggested to be a possible modulator of N$_2$-fixing cyanobacterial growth and bloom potential. The hypothesized mechanism for DOM-stimulated cyanobacterial growth is that DOM induces nutrient assimilatory enzymes and heterotrophy (Antia et al. 1991) and/or provides energy and nutrition for associated heterotrophic bacteria, which are known to optimize the growth of “host” cyanobacteria (Paerl and Pinckney 1996a, b). Heterotrophic N$_2$-fixing microorganisms have been discovered in the open ocean, based on the
presence and expression of \textit{nifH} genes (Bird and Karl 1991; Church et al. 2005a; Falcón et al. 2004a; Langlois et al. 2005; Zehr et al. 1998). Although these organisms have not been cultivated, these bacteria are likely to be heterotrophic \textit{Gamma-} or \textit{Alphaproteobacteria} that rely at least partially on DOM for energy. DOM availability in the surface ocean may limit heterotrophic N\textsubscript{2} fixation.

Salinity, per se, is not a strong modulator of either the establishment or activities of diazotrophs. A wide variety of active N\textsubscript{2}-fixers has been observed in the plankton and benthos of estuarine, coastal, and open-ocean environments, and even hypersaline lakes and lagoons (Paerl et al. 2000, 2003; Potts 1980). However, hypersaline conditions, where salinity greatly exceeds (by more than fourfold) seawater levels (35 psu), have been shown to inhibit N\textsubscript{2} fixation (Paerl et al. 2003; Pinckney and Paerl 1997). Factors other than salinity, including nutrient limitation, turbulence, organic matter supply, and grazing (Marino et al. 2006), appear to control N\textsubscript{2} fixation activity along the freshwater to open ocean salinity continuum.

Temperature is an important factor regulating enzymes and the distribution of organisms. N\textsubscript{2} fixation is found in a wide spectrum of temperatures, from Antarctic lakes to hot springs (Paerl and Priscu 1998; Postgate 1998; Steunou et al. 2006). It is interesting that the most abundant cyanobacterial diazotrophs in the oceans are either filamentous nonheterocystous cyanobacteria or unicellular cyanobacteria. It is not clear why free-living heterocystous cyanobacteria are not more abundant in the tropical open ocean, although heterocystous strains are often found in symbiotic relationships in the plankton (Villareal 1992). An interesting hypothesis implicates a possible role of temperature in selecting for filamentous nonheterocystous cyanobacteria. Staal et al. (2003) showed that the diffusion of oxygen into N\textsubscript{2}-fixing cells is constrained by the solubility of oxygen (which is a function of temperature and salinity) and that there was no advantage for a heterocyst cell wall in the tropical open ocean, since oxygen diffusion would limit the respiration required to support N\textsubscript{2} fixation.

Turbulence exerts a strong impact on phytoplankton growth and structural integrity (Fogg et al. 1973; Thomas and Gibson 1990). Increased levels of turbulence may inhibit growth of diazotrophs (Fogg et al. 1973; Fogg 1982). Aquatic environments with persistent elevated turbulence may have a lower abundance of active N\textsubscript{2}-fixing heterocystous cyanobacteria. In laboratory experiments where shear rates representative of surface wind-mixed conditions were applied to bloom-forming cyanobacteria (\textit{Anabaena} and \textit{Nodularia}), Kucera (1996) and Moisander (2002a) showed that rates of N\textsubscript{2} fixation and photosynthesis can be suppressed by strong turbulence. However, other studies did not find a negative relationship between turbulence and N\textsubscript{2} fixation (Burford 2006; Gervais et al. 1997; Howarth et al. 1993; Keto et al. 1992; Nakano et al. 2001). The negative impacts of elevated shear could be due to (1) breakage or weakening of cyanobacterial filaments, specifically at the delicate heterocyst–vegetative cell junction, causing O\textsubscript{2} inactivation of nitrogenase in heterocysts (Fogg 1969); (2) disruption of bacterial–cyanobacterial associations (Paerl 1990).

Diazotrophs form many symbiotic relationships with organisms in terrestrial and aquatic environments. These can be mutualistic interactions or loose associations. There are a number of symbiotic relationships between heterocystous cyanobacteria
and the diatom genera *Rhizosolenia*, *Hemiaulus*, *Bacteriastrum*, and *Chaetoceros* in the marine environment (Villareal 1992) (Fig. 13.4). Numerous other symbioses have been reported (Carpenter and Foster 2002; Foster et al. 2006a, b), including unicellular cyanobacterial associations with tintinnids, dinoflagellates, and radiolarians (Foster et al. 2006a, b). Heterotrophic bacterial symbionts in the diatoms *Rhizosolenia* have also been reported (Martinez et al. 1983). Evidence of N\textsubscript{2} fixation was found in symbionts of a dinoflagellate (*Histioneis* sp.) host using immunolabeling with nitrogenase coupled to transmission electron microscopy (TEM) (Foster et al. 2006a). In addition, 16S rRNA sequences obtained from another *Histioneis* sp. host were similar to the diazotroph *Cyanothece* sp. ATCC, and provided evidence for N\textsubscript{2}-fixing potential in the cyanobiont (Foster et al. 2006b).

Cyanobacterial–microbial associations are commonly observed among naturally occurring and cultured bloom forming genera, including *Anabaena*, *Aphanizomenon*, *Nodularia*, and *Trichodesmium* (Paerl and Kellar 1978; Paerl 1982b, 2000; Paerl and Millie 1996). Examples of consortial N\textsubscript{2} fixation are the aggregates of the common heterocystous cyanobacterium *Nodularia* in the Baltic Sea, and species of the nonheterocystous aggregate-forming *Lyngbya* as planktonic and benthic aggregates (Paerl and Kuparinen 2002). Associated microorganisms include eubacteria, fungi, protozoans, and eukaryotic algae. Many associations appear to be intimate, occurring within and around colonies and aggregates of filaments, and within fibrillar–mucilaginous sheaths, capsules, and exuded slimes (Paerl 1982b) (Fig. 13.4).

Specific associations, where certain microbial populations exclusively attach to specific types of cyanobacterial host cells (i.e., akinetes and heterocysts), have also been observed (Fig. 13.4). The mechanistic basis for cyanobacterial–bacterial synergism is poorly understood, and remains a subject of intense scrutiny. Proposed mutually beneficial mechanisms include exchange of metabolites and growth factors, as well as detoxifying roles of associated bacteria (Paerl 1982b; Paerl et al. 2000).

**Ecology of Diazotrophs in the Open Ocean**

Biological evidence on the rates and distribution of diazotrophs points to higher diversity, and wider geographical distributions and activity, than previously appreciated (Capone et al. 2005; Church et al. 2005a; Langlois et al. 2005; Montoya et al. 2004; Zehr et al. 2001b). Several diazotrophic cyanobacterial genera have long been recognized even without cultivation-independent approaches. These organisms include *Trichodesmium*, which has been mentioned several times already. Long before molecular studies and its cultivation in the laboratory, this microbe was well recognized to be a filamentous, non-heterocyst-forming cyanobacterium that forms aggregates or colonies visible to the unaided eye (Capone et al. 1997; LaRoche and Breitbarth 2005) (Fig. 13.4). Key characteristics of *Trichodesmium* are its colonial growth (although it also occurs as free filaments), the ability to fix N\textsubscript{2} simultaneously with O\textsubscript{2} evolution from oxygenic photosynthesis, the formation of dense surface blooms, and buoyancy regulation by gas vesicles (LaRoche and Breitbarth 2005). There appear to be several species of *Trichodesmium*, based on
Bloom of *Trichodesmium* have been observed frequently in calm conditions, suggesting that water column stability and stratification selects for *Trichodesmium* (Carpenter and Capone 1992; Karl et al. 1995, 1999). *Trichodesmium* is most abundant during summer stratified conditions in the North Pacific Gyre at Station ALOHA (Karl et al. 1995). The reason for a correlation between N2 fixation and *Trichodesmium* and lack of turbulence or water column stability was assumed to be due to the effect of turbulence in mixing O2 within the colony (Paerl and Bebout 1988a), but it is now known that *Trichodesmium* colony formation is not absolutely necessary for N2 fixation, although it may affect the efficacy of this process.

Many studies have focused on the ability of *Trichodesmium* to fix N2 in the light without heterocysts. One hypothesis is that there is combined temporal and spatial separation of activities (Berman-Frank et al. 2001b), with reduced photosynthetic activities from uncoupling of photosystem II (PSII) (which produces O2) from photosystem I (PSI) on short time scales that would allow N2 fixation to occur (Küpper et al. 2004). Direct evidence that cells are active in N2 fixation but not in photosynthesis is lacking, as there is not yet a way to measure N2 fixation at the cellular level. This may become possible in the near future using SIMS technology (D. Capone, personal communication). Other mechanisms that have been proposed to be involved in facilitating N2 fixation are the Mehler reaction and the high respiration rate in *Trichodesmium* (Berman-Frank et al. 2001b; Kana 1993), reviewed by LaRoche and Breitbarth (2005). Intriguingly, it has been ignored that unicellular cyanobacterial cultures will fix N2 under constant, albeit low levels of illumination, even though it is not possible to differentiate cells even in a unicellular culture. This has been observed in a *Synechococcus* sp. (really a *Cyanothece* sp.) (Mitsui et al. 1987), a *Cyanothece* sp. (Schneegurt et al. 1994), and a *Gloeothece* sp. (Ortega-Calvo and Stal 1991).

Filamentous heterocyst-forming cyanobacteria are symbiotic with some genera of diatoms, but are not well characterized, since they have not been brought into culture (Gómez et al. 2005; Janson et al. 1999b). By comparing microscopic observations with PCR results, Foster and Zehr (2006) were able to show that the different symbiotic associations harbor phylogenetically distinct populations. Quantitative PCR data demonstrate that they are widely distributed (Church et al. 2005a; Foster et al. 2007). The diatom symbionts are important diazotrophs, since diatoms can rapidly sink out of the water column and export C and N to deep water.

A number of other genera of diazotrophic cyanobacteria have been reported, including the heterocystous cyanobacterium *Anabaena gerdii* (Carpenter and Janson 2001). These cyanobacteria are not reported frequently, although a heterocystous cyanobacterial *nif*H was previously amplified from Station BATS in the North Atlantic (Zehr et al. 1998). Undoubtedly, more diazotrophs will be discovered to be widely distributed as quantitative methods are used for mapping nitrogenase genes in the sea.
PCR amplification of nifH genes from ocean samples has yielded a number of non-cyanobacterial phylotypes (Bird et al. 2005; Langlois et al. 2005; Zehr et al. 1998). Recent work has shown that some of these phylotypes are present and expressing the nifH gene in the mesopelagic (Hewson et al. 2007). One group of nifH sequences has repeatedly been found in ocean surveys—the group called UMB by Bird et al. (2005). Phylogenetically, these bacteria appear to be Gammaproteobacteria, and the nifH gene is expressed (Bird et al. 2005; Church et al. 2005a). However, the modes of metabolism of these bacteria, and how they can support N2 fixation are unknown.

In the early 1990s, there was considerable debate as to whether members of the common cyanobacterial genus *Synechococcus* could fix N2 in the plankton. This was partially due to the reports of a marine cyanobacterium, called *Synechococcus* (now believed to be a *Cyanothecaceae* sp.), that fixed N2 (Mitsui et al. 1987). A marine unicellular cyanobacterium, then called *Erythrosphaera* (also called marine *Synechocystis*, or *Crocosphaera watsonii*), had been isolated from the Atlantic Ocean (Waterbury and Rippka 1989). This unicellular cyanobacterium, however, was not a *Synechococcus*, but rather a larger cell, 2–8 μm in diameter, that contained phycoerythrin. A similarly sized unicellular cyanobacterium, *Cyanothece* sp. ATCC 51142, was isolated from the Gulf of Mexico (http://www.atcc.org) (Reddy et al. 1993). It was not generally believed at that time that the unicellular cyanobacteria of this morphology were abundant in the open ocean plankton. However, reports of large (larger than marine *Synechococcus* spp.) phycoerythrin-containing cells were made by Neveux et al. (1999) in the South Pacific and by Campbell et al. (1997) at Station ALOHA in the North Pacific.

When molecular approaches targeting the nifH gene were used to investigate planktonic N2 fixation, nanoplanktonic cyanobacterial and picoplanktonic noncyanobacterial nifH genes were found in the Atlantic and Pacific Ocean (Zehr et al. 1998). Cyanobacterial nifH sequences were repeatedly detected in the Pacific Ocean from Station ALOHA (Zehr et al. 1998). These gene sequences were of two types, called Groups A and B, and were shown to be expressed using reverse-transcriptase (RT)-PCR (Zehr et al. 2001b). Some of the cyanobacterial nifH sequences were closely related to sequences from *Crocosphaera*.

Quantitative PCR assays show that unicellular cyanobacteria and diatom-associated symbionts can be at least as abundant as *Trichodesmium* in the North Pacific Ocean (Church et al. 2005b). Similarly, analysis of PCR amplification products in the North Atlantic Ocean indicate that unicellular cyanobacterial diazotrophs are present, and may have a greater temperature range than *Trichodesmium* (Langlois et al. 2005). The diazotrophs have different daily patterns of nifH gene expression and activity (Church et al. 2005b). Intriguingly, the Group A unicellular cyanobacterial phylotype has maximum nifH gene expression during the day. Although nifH gene expression can be temporally offset from N2 fixation, the pattern suggests N2 fixation during the day. This would be the first unicellular cyanobacterium to fix N2 during the day of a light–dark cycle. It could be that different daily patterns of N2 fixation have
implications for competition, since the turnover of nitrogenase involves cycling of Mo and Fe on a daily basis (Tuit et al. 2004). The fact that Trichodesmium fixes N\textsubscript{2} during the day, along with the proposed Group A unicellular cyanobacteria, may suggest that there is an ecological advantage to daytime N\textsubscript{2} fixation in warm oligotrophic oceans.

**N\textsubscript{2} Fixation Rates: From Cells to Basins**  Perhaps one of the most important and challenging aspects of marine N\textsubscript{2} fixation is to integrate N\textsubscript{2} fixation inputs from scales of microorganisms to ocean basins with coincident temporal variability in biology and physical processes. Rates of N\textsubscript{2} fixation by Trichodesmium and the diatom symbionts have been estimated for cultured and natural populations (Capone et al. 2005; Carpenter et al. 1999; Letelier and Karl 1998). Estimates of N\textsubscript{2} fixation in Crocosphaera have been obtained in culture (Tuit et al. 2004), but N\textsubscript{2} fixation rates of natural populations of smaller unicellular cyanobacterial cells (Crocosphaera and the uncultivated Group A presumed unicellular cyanobacteria) can only be obtained indirectly by size fractionation (Montoya et al. 2004). Rates of N\textsubscript{2} fixation in Trichodesmium can be on the order of 10–30 fmol N cell\textsuperscript{-1} h\textsuperscript{-1}, which may be higher than cellular rates of unicellular cyanobacteria (Mahaffey et al. 2005). In experiments with Trichodesmium and Crocosphaera, daily maximum per cell rates in Trichodesmium ranged from approximately 4- to 15-fold higher in Trichodesmium (Tuit et al. 2004). The variability in rates highlights the difficulty in extrapolating rates obtained in culture to the environment, where gradients in light and nutrients, as well as hydrographic factors, affect the spatial and temporal distribution of N\textsubscript{2} fixation rates.

Areal and basin scale N\textsubscript{2} fixation rates have been estimated by a variety of means, including tracer methods. These estimates have been comprehensively summarized by Mahaffey et al. (2005). N\textsubscript{2} fixation by Trichodesmium ranges from 1.4 to 898 \mu mol N m\textsuperscript{-2} d\textsuperscript{-1}, with the highest rates in the tropics (Mahaffey et al. 2005). Rates up to 4000 \mu mol N m\textsuperscript{-2} d\textsuperscript{-1} for the small-size fraction were reported by Montoya et al. (2004) near the Australian coast. Diatom symbionts can also be responsible for high rates, up to 3500 \mu mol N m\textsuperscript{-2} d\textsuperscript{-1} (Mahaffey et al. 2005). Biogeochemical estimates, based for example on N\textsuperscript{15}/C\textsuperscript{3} (Gruber and Sarmiento 1997; Michaels et al. 1996), can exceed these rates (Mahaffey et al. 2005).

In the past decade, the conceptual framework of N\textsubscript{2} fixation in the open ocean has changed, and it is now generally believed that N\textsubscript{2} fixation is an important process in the N cycle that is involved in supporting carbon export (Gruber and Sarmiento 1997; Karl et al. 2001b; Michaels et al. 1996). Several independent lines of evidence have been interpreted to be indications of N\textsubscript{2} fixation, including low \textsuperscript{15}N: \textsuperscript{14}N ratios in surface waters, increasing concentrations of dissolved organic nitrogen at Station ALOHA, attempts to balance carbon budgets (Lee et al. 2002), and high N\textsuperscript{\textsuperscript{15}}/ N\textsuperscript{\textsuperscript{14}} ratios in some regions (Capone 2001; Mahaffey et al. 2005). Modeling studies indicate that N\textsubscript{2} fixation and surface ocean nitrification are adequate to explain observed productivity at the BATS site in the North Atlantic (Bissett et al. 1999).

Estimates of N\textsubscript{2} fixation rates from biogeochemical calculations initially appeared to be much larger than could be explained by N\textsubscript{2} fixation by Trichodesmium alone.
Analysis of extensive datasets of *Trichodesmium* indicates that the two estimates are closer than originally thought, and that N$_2$ fixation by *Trichodesmium* can be as great or greater than nitrate advection from deep water (Capone et al. 2005). *Trichodesmium* rates may still be underestimated, since rates are usually measured in *Trichodesmium* aggregates, and, in some cases, free trichomes can be a significant fraction of the biomass (Chang 2000; Letelier and Karl 1998; Orcutt et al. 2001). Including N$_2$ fixation by free trichomes tripled the estimates of the annual input in the Sargasso Sea (Orcutt et al. 2001). There has also been recognition of the presence (Church et al. 2005b; Zehr et al. 2001b), gene expression (Church et al. 2005b), and activity (Montoya et al. 2004) of unicellular cyanobacteria. Although there are several problems in obtaining geographical information on N$_2$ fixation by *Trichodesmium* (LaRoche and Breitbarth 2005), the contribution of the other diazotrophs is even harder to study, because of their small size. The geochemically derived rates of N$_2$ fixation based on N/C$_3$ have been questioned, as there are many assumptions including the region over which to integrate N/C$_3$ values (Hansell et al. 2004). If the estimates of N$_2$ fixation by *Trichodesmium* and from biogeochemical budgets converge (Mahaffey et al. 2005), then a new enigma will emerge, since the diatom symbionts (Carpenter et al. 1999) and unicellular diazotrophic cyanobacteria (Montoya et al. 2004) have been shown to contribute substantially to N$_2$ fixation rates.

It is now clear that N$_2$ fixation plays an important role in “new” production in the oligotrophic gyres. Studies of N$_2$ fixation at the two oceanic long-term monitoring sites, using different approaches, reached the conclusion that N$_2$ fixation is important in the North Atlantic and North Pacific gyres (Karl et al. 1997; Michaels et al. 2000; Orcutt et al. 2001).

Dore et al. (2002) used natural abundance of $^{15}$N and enriched $^{15}$N$_2$ tracer experiments to assess the significance of N$_2$ fixation at Station ALOHA. N$_2$ fixation was strongly seasonal, but there was a large discrepancy between the rates determined from sinking particles and from tracer rate measurements. However, the rates determined from sinking particles agreed well with biogeochemically derived rates (Deutsch et al. 2001) and accounted for a mean of 48 percent of the annual N export from the surface layer. Sources of P and Fe (P from remineralization in advected deep water, and Fe from terrestrially derived aeolian dust) have implications for the ultimate limiting factor and how N$_2$ fixation has responded, and will respond, to climate changes (Karl 2002). This may partially explain why N$_2$ fixation can be affected by El Niño events, for example (Karl 1999, 2002).

**Controls on N$_2$ Fixation** Most likely, oceanic N$_2$ fixation is controlled by a complex interplay of physical, chemical, and biological factors. Correlation studies indicate that N$_2$ fixation in the open ocean appears to be in roughly 20 or 25 °C waters (Capone et al. 1997; LaRoche and Breitbarth 2005). The unicellular diazotrophic cyanobacterium *Crocosphaera* appears also to have a high temperature optimum (J. Waterbury, personal communication). However, diazotroph genes and transcripts have been detected in waters at least as low as 20 °C, and perhaps occur at even lower temperatures. Temperature was not correlated with *Trichodesmium*
abundance in low latitudes north and south of the equator in the eastern tropical North Atlantic (Tyrrell et al. 2003).

Inorganic N and P concentrations are low in the North Atlantic and Pacific gyres (Wu et al. 2000), and soluble reactive P concentrations appear to be decreasing in the North Pacific (Karl et al. 2001b). There is evidence for P limitation in the North Pacific (Björkman et al. 2000; Karl et al. 2001b), and diazotrophy may cause the decreasing P (Karl et al. 2001a). P stress was shown in natural populations of Trichodesmium using a cell-level assay (Dyhrman et al. 2002). There was a correlation between P and N$_2$ fixation, indicating that P, and light, may be major factors controlling N$_2$ fixation in the central North Atlantic (Sañudo-Wilhelmy et al. 2001). This may be due to the fact that P is in much lower concentrations in the North Atlantic than in the Pacific (Wu et al. 2000). Although P additions stimulated N$_2$ fixation by Trichodesmium in the Great Barrier Reef lagoon (Fu and Bell 2003), additions of inorganic P to North Pacific Subtropical Gyre water did not consistently stimulate unicellular cyanobacterial gene expression, abundance, or activity (Zehr et al. 2007).

Different diazotrophs may have different strategies for obtaining P. Genomics studies indicate that Trichodesmium may be able to utilize a previously little studied group of P compounds, the phosphonates (Dyhrman et al. 2006). It has also been proposed that Trichodesmium could migrate to the phosphocline (Karl et al. 1992; Villareal and Carpenter 1990). Experimental and modeling evidence indicates that this is feasible (White et al. 2006), but direct evidence is lacking. Crocosphaera has high- and low-affinity P transporters (Dyhrman and Haley 2006). The relative abilities to compete for sources, in concert with the forms and availability of P, may determine the spatial distribution and succession of diazotroph species.

Fe limits productivity of nondiazotrophs in HNLC regions, and should be important in controlling diazotrophs, since the nitrogenase proteins contain Fe (Kustka et al. 2002, 2003). On a theoretical basis, it has been predicted that Trichodesmium Fe requirements are roughly 10-fold greater than non-N$_2$-fixing phytoplankton (Kustka et al. 2003). The effect of Fe on Trichodesmium was tested experimentally (Paerl et al. 1994; Rueter et al. 1992). Rueter et al. (1992) suggested that Trichodesmium colonies were able to intercept Fe derived from aeolian sources (volcanic emissions, dust from desertification, and air pollution from industrial and automotive emissions) by trapping particulate Fe in the weblike matrix of trichomes. Although the estimates of Fe needs for N$_2$ fixation in Trichodesmium have been reduced from those originally reported (see Kustka et al. 2003; Sañudo-Wilhelmy et al. 2001), there is still a high Fe requirement, presumably to support the Fe metallocenters in nitrogenase. Highest rates in the eastern tropical Atlantic Ocean and in the Amazon River plume correlated with Fe concentrations (Galloway et al. 2004; Mahaffey et al. 2005; Voss et al. 2004). Analysis of the distribution of atmospheric Fe deposition suggested that Fe could limit N$_2$ fixation in 75 percent of the world’s oceans (Berman-Frank et al. 2001a). However, in the North Atlantic Ocean, there was no correlation between Fe and N$_2$ fixation activity (Sañudo-Wilhelmy et al. 2001).
The requirements and quotas of Mo in *Trichodesmium* and *Crocosphaera* have been evaluated in cultures and natural populations (Tuit et al. 2004). Field populations had Mo:C ratios far in excess of that needed for N$_2$ fixation as determined in culture experiments, indicating that Mo does not limit N$_2$ fixation in the open ocean (Tuit et al. 2004).

**Role of N$_2$ Fixation in Food Webs**

N$_2$ fixation is important ecologically in several respects. The fixed N can enter the food chain, is exported to deep waters, or is released into dissolved pools. We probably know the most about the fate of N in *Trichodesmium* biomass, but this is still unclear. *Trichodesmium* has been implicated in the release of ammonium (Mulholland and Capone 2000) and organic N (Bronk et al. 1994). Release of N (and other nutrients) from *Trichodesmium* (and diatom) blooms has been suggested to support secondary blooms of eukaryotic algae (Lenes et al. 2001; Wilson 2003). *Trichodesmium* is rarely found in sediment traps, but symbiotic diatoms are. Thus, there may be large differences in the contribution of individual diazotrophs to carbon and N export. Similarly, there are likely to be substantial differences in transmission through the food chain. Unicellular cyanobacteria could conceivably be very actively grazed by a number of organisms, whereas the large, sometimes toxic (Hawser et al. 1992; Preston et al. 1998), aggregates of *Trichodesmium* appear to be principally grazed by planktonic harpacticoid copepods such as *Macrosetella*.

There are still major gaps in our understanding of the role of N$_2$ fixation in the open ocean. Estimates of abundances and rates of N$_2$ fixation are severely limited by the number of samples. Research cruises to study diazotrophy usually sample regions of known diazotroph occurrences, rather than employing random sampling to map distributions. Another complication is that the highly seasonal and bloom-forming nature of some diazotrophs means that they are often missed even in the monthly sampling programs at Stations ALOHA and the Bermuda Atlantic Time Series (BATS). The factors affecting N$_2$ fixation, even Fe, are still poorly understood. Finally, the pathways of fixed N$_2$ are not well known—for example, whether the different diazotrophs contribute unequally to export or the food chain.

**Estuarine and Coastal Waters**

Estuaries are characterized by hydrological, salinity, and biogeochemical gradients. Typically, phosphorus availability tends to limit primary production in the upstream, freshwater segments of estuaries, while N assumes a more dominant role as a limiting nutrient in the more saline, downstream marine waters (cf. Fisher et al. 1988). Some estuaries also have brackish regions where N and P co-limitation occur, and there can be seasonal shifts between N and P limitation (Elmgren and Larsson 2001; Fisher et al. 1988; Paerl et al. 1995a). Coastal waters draining estuaries or isolated from estuaries tend to be strongly N-limited (Nixon 1995; Paerl 1997). One would therefore expect these more meso- to euhaline lower estuarine and coastal waters to be potential habitats for N$_2$-fixers. Indeed, in some systems, including coastal and open water regions of the brackish Baltic Sea, as well as lagoonal estuarine and coastal...
ecosystems, N₂-fixers can account for a significant and at times dominant fraction of phytoplankton and benthic microalgal biomass. In addition, bacterial N₂-fixers may be found in these systems, most often in the benthos (Paerl and Zehr 2000). Molecular studies, based on nifH analyses, indicate that a diverse taxonomic potential exists for N₂ fixation in these waters (Affourtit et al. 2001; Burns et al. 2002; Jenkins et al. 2004). Despite the genetic potential, N₂ fixation activity is frequently absent or present at ecologically insignificant rates, and, if it is present, it is confined to sedimentary or biofilm habitats. Recent studies indicate that there may be physical barriers to the establishment and dominance of N₂-fixers in N-limited estuaries, especially in the water column. The relatively turbulent properties of estuarine waters, which include strong wind and tidal mixing, and high rates of small-scale shear, may restrict the establishment and proliferation of diazotrophic cyanobacterial and bacterial communities (Moisander et al. 2002b; Paerl et al. 1995b, 1996). In particular, strong and persistent vertical mixing of near-surface waters prevents dominance by bouyant filamentous diazotrophic cyanobacterial bloom genera (e.g., Anabaena, Aphanizomenon, Nodularia, and Trichodesmium). Lastly, periodic hypoxia in stratified estuaries can result in large fluxes of ammonium from the sediments into the water column. These ammonium pulses may act to repress nitrogenase activity and thus negate potential competitive advantages of N₂-fixing cyanobacteria and bacteria.

In summary, estuarine and coastal waters have a diverse genetic potential for N₂ fixation, which, under favorable conditions (e.g., midsummer stratified conditions in the Baltic Sea), can be readily expressed. However, more often, persistently wind-mixed surface waters and periodically flushed and nutrient-pulsed conditions in these environments represent physical and chemical barriers to N₂-fixers, thus restricting their dominance and bloom potentials. This, combined with the fact that estuarine and coastal systems are frequent sites of active denitrification and P sufficiency, helps explain why these systems exhibit chronic N deficiency.

**Benthic Habitats, Including Microbial Mats and Reefs**

Because of their strong and extensive biogeochemical zonation, microbial mats support metabolically and taxonomically diverse diazotrophic communities (Paerl et al. 2000). These include cyanobacteria (nonheterocystous filamentous, heterocystous, and coccoid), anaerobic photosynthetic bacteria, microheterotrophs, and a range of chemolithotrophs (Omorogbie et al. 2004a; Steppe et al. 1996; Steppe and Paerl 2002; Zehr et al. 1995). Microbial mats are widespread in shallow marine environments, including estuaries, coastal lagoons, salt marshes, mudflats, and reefs. In deeper waters, mats inhabit specialized environments where organic matter and energy sources for sustaining N₂ fixation may abound. These include hydrothermal vents and seeps (see below). Chapter 14 also discusses N₂ fixation in the benthos.

In benthic sediment and mat environments, N₂ fixation can account for significant and at times dominant sources of "new" N supporting primary and secondary production (Paerl et al. 2000). Very high rates in *Spartina* salt marshes can be due to
cyanobacterial mats or epiphytes (Carpenter et al. 1978; Currrin and Paerl 1998; Moisander et al. 2005). Diazotroph diversity is high in the rhizosphere (Lovell et al. 2000). Sulfate-reducing bacteria have been implicated in N₂ fixation of salt marshes (Nielsen et al. 2001). Interestingly, diazotroph diversity remains high even when inorganic N is added, indicating that the diazotrophs are competitive with non-diazotrophs (Piceno and Lovell 2000a), and reverse sample genome probing showed that individual populations were not displaced by fertilization (Bagwell and Lovell 2000). Acetylene reduction activity was detectable in control treatments where interstitial ammonium concentrations are approximately 5 μmol/L even in the control plots (Bagwell et al. 1998). Diazotroph populations appear to be different between different grasses (Lovell et al. 2000). The results of a variety of perturbations indicate that the diazotroph assemblages are stable (Bagwell and Lovell 2000; Lovell et al. 2001; Piceno and Lovell 2000a, b).

Coral reefs are relatively productive ecosystems that are commonly found in N-depleted, oligotrophic tropical and subtropical marine waters. It has been suggested that the relatively productive nature of these ecosystems is likely due to N₂ fixation (D’Elia and Wiebe 1990). Coral reefs are sites of both cyanobacterial and heterotrophic bacterial N₂ fixation (Capone 1983), although measured rates are few and environmental regulation of this process is not well understood (Charpy-Roubaud et al. 2001). Nitrogenase activity has been measured on surfaces of benthic organisms (Charpy-Rouband and Larkum 2005), in sediments (Capone et al. 1992; Charpy-Roubaud et al. 2001; Koop et al. 2001; Mayajima et al. 2001), and in filamentous cyanobacteria endemic to coral reefs (Kayanne et al. 2005). Rohwer et al (2002) observed several cyanobacterial ribotypes associated with coral surfaces, of which at least three are known to fix N₂. Phycoerythin was found in a scleractinian coral that was due to a symbiotic unicellular cyanobacterium (Lesser et al. 2004). Cyanobacteria of the genus Oscillatoria have been observed in benthic microalgal assemblages of coral reefs (Heil et al. 2004). Unpublished data suggest that water column N₂ fixation in coral reef lagoons is mostly attributable to the size fraction smaller than 5 μm (see Charpy 2005), indicating that unicellular cyanobacteria may be an ecologically important component of the coral reef N cycle. While the phylogeny of N₂-fixing microorganisms on coral reefs has not previously been studied at the nifH level, a recent fingerprinting study of sediment diazotrophic assemblages, utilizing terminal restriction fragment length polymorphism (TRFLP) of the nifH gene, indicated that diverse and spatially variable assemblages persist within reef sediments (Hewson and Fuhrman 2006). Thus, diazotrophic prokaryotes may be an important source of new N in nutrient-impoverished coral reef ecosystems.

Deep Water and Hydrothermal Vents

Deep-water environments typically have high concentrations of inorganic and organic N, and thus traditionally N₂ fixation was not believed to occur in these environments. However, recent studies have found N₂-fixing microorganisms in deep-water environments. Some hydrothermal vent fluids contain relatively low concentrations of
combined N (Karl et al. 1988; Lilley et al. 1993), and have been the target for studies of \textit{nifH}. Mehta et al. (2003) reported a wide suite of diazotrophic phylotypes within hydrothermal vent fluids, where the majority of phylotypes were in Clusters II and III, which contain sequences from alternative \textit{nifH}, anaerobic bacteria, and Archaea. Mehta and Baross (2006) recently described a new thermophilic N$_2$-fixing isolate. Additional studies of deep-sea bacteria in seafloor rift valleys have found the presence of more Cluster I, II, and IV sequences, and another group of divergent, coldwater (nonthermophilic) archaean-like genes (Mehta et al. 2005). A recent study of meso- to abyssopelagic diazotroph assemblages found that endemic, low-abundance, and actively transcribed phylotypes were present in waters of the Sargasso Sea, beneath the Costa Rica Upwelling dome, near O’ahu, and in eastern North Pacific waters (Hewson et al. 2007). Anomalously light $\delta^{15}N$ values suggested that N$_2$ fixation may occur in sediments and biomass near cold methane seeps in the Gulf of Mexico (Brooks et al. 1987; Joye et al. 2004). This is consistent with the observation of type II methanotrophs, which can contain nitrogenase genes (Auman et al. 2001). Thus, N$_2$ fixation may be common in deep-water environments, despite the presence of high concentrations of combined N.

**SUMMARY**

1. Biological N$_2$ fixation is an energetically expensive process (requiring ATP and reductant), and is sensitive to oxygen.
2. Nitrogenase is an ancient enzyme, and the evolution of the enzyme and the organisms that contain it reflect the anoxic–oxic transitions in the ocean in Earth’s history.
3. N$_2$-fixing microorganisms are diverse, represented by taxa throughout the Archaea and Bacteria, and by many physiological groups. Most cultivated marine N$_2$-fixing Bacteria have come from sediments or mats. Three open-ocean cyanobacteria have been cultivated: unicellular (\textit{Crocosphaera}), filamentous nonheterocystous (\textit{Trichodesmium}), and a heterocystous symbiont of \textit{Chaetoceros}.
4. N$_2$ fixation and the nitrogenase genes are highly regulated in response to fixed N. Regulation, which involves many genes and gene products, can include a circadian rhythm in cyanobacteria such as \textit{Trichodesmium}.
5. Methods for studying N$_2$ fixation include tracer and natural-abundance approaches, but now include a wide variety of genetic and genomic techniques, such as DNA microarrays and gene expression assays, and remote sensing technology.
6. Recent N$_2$ fixation estimates for the open ocean are higher than previously believed. Revised estimates for \textit{Trichodesmium} approach these higher estimates, and unicellular cyanobacteria and diatom symbionts may contribute substantial to global N$_2$ fixation. New organisms continue to be discovered (Zehr et al. 2001b).
7. A variety of factors control N₂ fixation, ranging from turbulence and grazing to nutrient and trace element availability. Major knowledge gaps exist on the spatial and temporal distributions of N₂-fixing microorganisms in the sea, and the factors that control their activity. The roles of P and Fe in constraining N₂ fixation are still unclear, as are the conditions that select for different diazotroph groups.

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