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# Nitrification and Denitrification: Probing the Nitrogen Cycle in Aquatic Environments

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**Abstract.** Methods designed to detect microorganisms involved in the biogeochemistry of nitrogen in the marine environment are rapidly being developed and deployed in ecological investigations. Probes based on phylogenetic sequences (usually rRNA) and those based on the sequences of functional genes or proteins have both been demonstrated in the nitrogen cycle. The most progress has been made for ammonia oxidizers; several sets of PCR primers have been described and their specificity may be optimized to allow detection of genetically and ecologically meaningful groups. For denitrifying bacteria, functional probes based on nitrite reductase show most promise. These approaches should complement the more familiar, but no less sophisticated, methods that focus on quantification of in situ transformation rates. Both approaches in combination will be useful in understanding regulation and environmental control of biogeochemical processes.

## Introduction

The nitrogen cycle is of fundamental importance in all ecosystems, but especially so in the marine environment because of the potential for available nitrogen to control the rate or level of primary productivity. Nitrogen fixation (Zehr, this volume) is the most important biological process by which new nitrogen is added to oceanic systems, and many other microbial processes are involved in transformation of nitrogen among several valence states and among a wide variety of organic and inorganic compounds. Research on the biogeochemistry of nitrogen aims to elucidate the distributions of various nitrogen compounds in the environment from the basis of the bacteria that produce and consume them. This requires an understanding of what controls the abundance and activities of the particular kinds of bacteria involved in these processes, in addition to direct measurement of the rates of processes themselves.

Like the sulfur cycle [19], the nitrogen cycle [26] is predominantly controlled

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by bacteria, and their activities determine the distribution of nitrogen compounds. In turn, environmental conditions that regulate the activity of bacteria determine where each process occurs, the degree of exchange among various nitrogen pools, and the physical, chemical, and biological interactions that are possible. Thus, although detailed biochemical information about bacterially mediated reactions is required, such knowledge alone is not sufficient to understand control of or to predict rates of bacterial processes in the environment.

This paper is primarily a review of recent developments in the study of nitrification and denitrification in the marine water column, specifically the use of molecular and immunological methods to study the bacteria involved in these processes. The processes of these two components of the nitrogen cycle are performed by different groups of bacteria: nitrifiers are usually obligate chemoautotrophs and denitrifiers are generally facultatively anaerobic heterotrophs. Nitrification is usually considered to be an obligately aerobic process, although an apparently anaerobic nitrification process has recently been reported [36]. Similarly, denitrification is considered to be an anaerobic process, but the capacity for aerobic denitrification may be more widespread than currently appreciated [44, 45]. Exceptions to these generalizations are discussed near the end of this paper. Despite their considerable physiological differences, nitrifiers and denitrifiers often interact in close proximity in the environment. Interactions between nitrifiers and denitrifiers are often mediated across oxic/anoxic interfaces and involve the diffusion of substrates and products along concentration gradients over scales ranging from microns to meters.

## Ecological Significance of Nitrification and Denitrification in Aquatic Systems

Denitrification is of significance because it is the major biotic loss term for fixed nitrogen in the environment, and it is therefore important in nitrogen budgets on ecosystem to global scales. Denitrification is also a respiratory mode that enables facultatively anaerobic bacteria to continue to mineralize organic carbon in the absence of molecular oxygen. In respiratory denitrification, nitrogen oxides are reduced in stepwise fashion from nitrate through nitrite, nitric oxide, and nitrous oxide to dinitrogen (e.g., [64]). Thus denitrifying bacteria influence both the carbon and nitrogen cycles and their impact varies depending on the local oxygen tension of the environment.

The occurrence of denitrification can be detected on the basis of distributions of inorganic nitrogen species in the marine water column, typically by anomalous accumulations of nitrite, the first intermediate in the reductive sequence, and by undersaturations in nitrous oxide, the last intermediate before dinitrogen. A nitrite accumulation near the bottom of the euphotic zone (the primary nitrite maximum) is attributable to phytoplankton excretion or nitrification, while a nitrite maximum associated with oxygen-depleted waters below the euphotic zone (the secondary nitrite maximum) is usually attributable to denitrification. Undersaturation of nitrous oxide is interpreted to imply complete denitrification and removal of fixed nitrogen, whereas nitrite accumulation in regions of reduced oxygen concentration implies partial denitrification [5]. In oceanic oxygen minimum zones, the two signals occur in overlapping depth intervals, and imply an imbalance in the reduction

sequence: either the community includes many more bacteria that are capable of the first step, reduction of nitrate to nitrite, than can carry out the entire sequence, or the reactions subsequent to nitrite formation are limiting to the overall rate of dinitrogen production. Both nitrite accumulation and nitrous oxide depletion are accompanied by a loss of nitrate. The severity of the nitrate deficit (up to 5 μM compared to typical deep water concentrations of nitrate of above 30 μM [5]) can indicate the magnitude of the fixed nitrogen loss due to denitrification. These chemical distributions are characteristically found in oxygen minimum zones, such as the eastern tropical North Pacific or coastal Peru, where dissolved oxygen is depleted enough to induce respiratory denitrification. The oxygen concentration necessary to induce denitrification in the environment is difficult to measure directly; culture studies imply that expression of the denitrification enzymes is induced below 5 mg $l^{-1}$ [24].

The significance of nitrification is that the deep ocean is a reservoir of nitrate rather than ammonium, due to the oxidation of ammonium (produced by heterotrophs during mineralization of organic matter) by nitrifiers. Nitrification has no impact on the overall marine nitrogen budget, because it neither adds nor removes nitrogen, but simply changes its oxidation state. However, nitrification links ammonification to denitrification, thus linking organic matter decomposition to loss of fixed nitrogen. Both ammonium and nitrate can be used by phytoplankton for nitrogenous nutrition, although preference for one substrate over the other may exert selection within some communities [11]. Ammonium accumulates only under anoxic conditions in which nitrification does not occur. Nitrification appears to be enhanced by low but nonzero oxygen concentrations and leads to the accumulation of nitrous oxide and nitric oxide in oxygen gradient conditions (see below).

Although the physiology of nitrifiers and denitrifiers suggests that environments that encourage their growth should be mutually exclusive, many environments have oxic/anoxic interfaces across which the two processes can interact. Substrates and products can diffuse across the gradients to support coupled oxidation/reduction of nitrogen. Such interfaces occur in large areas of the open ocean where the oxic/anoxic gradients span several hundred meters, in enclosed basins where the gradients span a few tens of meters, and in microbial mats and sediment/water interfaces where the gradients are microscopic. Generally, the greater the vertical extent of the gradient (i.e., meters vs. microns between oxygen and sulfide), the greater the flux of carbon and nitrogen through denitrification.

## Rates of Nitrification and Denitrification in the Ocean

The depth distribution of nitrification rates in the marine water column is predictable in its general shape from an understanding of the factors that influence the rate (e.g., [53]); the most important of these are light intensity (both ammonia oxidizers and nitrite oxidizers are inhibited by light), substrate flux (ammonium and nitrite availability could limit their oxidation rates), and oxygen concentration (oxygen is required by both kinds of nitrifiers, but low levels are apparently optimal). In a reasonably well-oxygenated water column, the decrease in nitrification rate with depth can be modeled as an exponential or power function [59], similar to that observed for decreasing vertical particle flux as measured by sediment traps [31].

The nitrogen lost between adjacent traps is presumably solubilized by biological/physical processes, decomposed, and then remineralized and nitrified.

Extensive bodies of oxygen deficient water arise in the ocean due to a combination of general circulation patterns and high productivity in surface waters, which leads to excess respiration in the underlying subsurface layers. Regions of oxygen deficient water occupy layers up to several hundred meters thick with horizontal extent of many hundreds of kilometers in the eastern tropical North and South Pacific Oceans, for example. The depth distribution of nitrification is complicated by the occurrence of oxygen minimum zones. The nitrification rate may be enhanced at the boundary of the oxygen minimum zone [30] and is reduced in the most anoxic part of the zone [30, 59]. In low-oxygen conditions, a relatively greater proportion of the products of ammonium oxidation may occur as nitric or nitrous oxide [6, 15, 29, 59]. These gaseous intermediates also contribute to the linkage between nitrification and denitrification, which is suggested to be most intense in the boundary regions of the oxygen minimum zone. Thus, in the oxygen minimum zone, the significance of nitrification in terms of nutrient regeneration is reduced and its significance in contributing to net loss of fixed nitrogen through the denitrification connection is enhanced.

Rate estimates for nitrification have largely been derived from the use of $^{15}N$ tracer techniques. That approach has so far had very limited application for measurement of denitrification in the water column. Direct measurement of dinitrogen production in the ocean is difficult due to the dilution of signal in dinitrogen-saturated waters. The distribution of denitrification rates in the water column has usually been measured indirectly, using fixed nitrogen distributions to estimate fixed nitrogen loss. The nitrate deficit indicates the cumulative magnitude of denitrification in a water mass. The distribution of nitrous oxide undersaturation and the secondary nitrite maximum are usually correlated with the nitrate deficit, all three being indicators of denitrification [39]. Natural abundance isotope signatures have also been proposed as indicators of denitrification [3]. Electron transport system (ETS) activity has been interpreted as denitrification in suboxic waters [10, 14], on the assumption that the only metabolic activity in the zone was due to denitrifying bacteria. These methods have advanced our understanding of the regional distributions and biogeochemical significance of denitrification, but other approaches are necessary to provide an in situ rate measurement, or to pinpoint the real-time distribution of organisms responsible for the processes.

Incubations at ambient oxygen concentration can be used to measure the first step in denitrification, the reduction of nitrate to nitrite, with $^{15}NO_3$ as a tracer. The distribution of nitrate reduction in the oxygen-deficient water column off Peru was similar to that of nitrate oxidation, suggesting a coupling between the two processes [30]. In the Black Sea, peaks in nitrification and denitrification were interleaved, implying that each was favored under different conditions but still occurring in proximity in the water column [58]. These results were interpreted as consistent with the model of Anderson et al. [1], in which it was hypothesized that nitrite produced in the oxygen minimum zone by partial denitrification could support nitrite oxidation at the edges of the zone, while nitrate produced by nitrification at the edge could support nitrate reduction in adjacent oxygen-depleted waters.

If nitrification and denitrification are closely coupled, the observed nitrate and nitrite concentrations represent net or steady-state concentrations and may not

reflect the dynamic nature of the system. For example, if a significant amount of denitrification is directly coupled to nitrification, then the nitrate deficit underestimates nitrogen turnover because the source of nitrate for denitrification is both the external nitrate pool and in situ nitrate production from nitrification. Greater amounts of fixed nitrogen loss could be occurring accompanied by carbon mineralization than is reflected in the net nitrate deficit. Thus, simple chemical distributions might suggest a different stoichiometry between denitrification and carbon mineralization.

Direct methods to measure in situ rates of nitrification and denitrification with stable isotopes and inhibitor methods continue to be refined and will undoubtedly continue to yield important information [63]. Bottle incubations have shortcomings, however, that include the following: (1) It is necessary to disrupt the natural environment, no matter how carefully the manipulations are done. For example, interface environments, including sediments, where small scale physical structure is important make it impossible to do an incubation that both preserves the structure and accurately traces the process. (2) Such experiments usually involve perturbation of natural substrate levels and supply terms, although the limitations can be minimized with recent improvements in tracer and mass spectrometer technology. (3) The incubation experiments themselves often require hours to perform, and the labor-intensive isotope measurements much longer.

These methods can be supplemented, however, by others that focus on the organisms responsible for the reactions. The "microbiological" approach can address questions that the rate measurements do not: (1) How does the observed rate depend on the species composition and diversity of different denitrifying or nitrifying organisms present in the sample? Diverse bacteria with widely different metabolic pathways for carbon assimilation or tolerance for environmental variables such as oxygen, may all possess the ability to denitrify. The rate of denitrification might therefore depend on variables not directly related to the nitrogen transformation process itself, so the presence of multiple species could have unpredictable effects on the rate of denitrification. (2) Are the biochemical models derived from cultured organisms, which we use to understand rate processes in the environment, accurate? In other words, are cultured organisms representative of the organisms we suspect are present but cannot culture? (3) Does species succession or competition among similar metabolic types influence the observed rates? (4) How is chemical information from the environment reflected in regulation of bacterial processes and therefore in net transformation rates?

If the species composition of natural assemblages is accurately reflected in our culture collections, then extrapolation from laboratory studies of physiological and environmental regulation of reaction rates is a powerful way to study the environment. If not, it is necessary to investigate the diversity of organisms capable of a particular biogeochemical reaction, and this may not be possible using conventional culture-based approaches. It is even difficult to assess how representative cultured organisms are when using this approach.

Most attempts to describe microbial species diversity rely on enrichment and cultivation to identify individual species present in the environment. Smorczewski and Schmidt [48] used a sophisticated version of this approach to investigate the diversity of nitrifying bacterial populations in a temperate lake. Organisms that grew in MPN tubes were screened with polyclonal species-specific antisera, and

the diversity of the cultures evaluated in terms of serological reactivity. This study revealed the presence of previously unknown strains of nitrifiers and also documented the persistence of nitrifiers in anoxic sediments, an environment thought to be incompatible with their metabolism. With the advent of epifluorescence microscopic enumeration methods in the mid 1970s, it was recognized that most of the cells present in aquatic and soil systems cannot be cultivated, although it is not known if this applies equally well to all bacterial types [46]. Thus, culture methods attest to the ability of bacteria to perform almost any imaginable metabolic process but do not reliably indicate the importance or prevalence of those processes or strains in the environment.

Molecular biological techniques make it possible to investigate the diversity of bacterial communities without culturing the organisms, and even without actually identifying the species present. Lee and Fuhrman [27, 28] used percent hybridization of total DNA extracted from natural assemblages to estimate the similarity of microbial communities in different parts of the marine water column. This method is difficult to normalize and thus difficult to quantify. It did, however, indicate that community composition probably did differ depending on depth and location. Moyer et al. [34, 35] investigated the diversity of microbial assemblages in a hydrothermal vent system using sequence and RFLP analysis of a clone library of ribosomal RNA genes. They were able to evaluate the relatedness of the different clones and to estimate their ancestry within the ribosomal phylogeny generated from rRNA sequences of known bacteria. Delong et al. [9] used PCR amplification and sequencing of rRNA genes from DNA extracted from seawater and marine snow to show that the two habitats contained different species groups. Fuhrman et al. [13] compared the phylogenetic diversity of 16S rRNA partial sequences obtained from cloned PCR fragment and found evidence both of considerable diversity and broad spatial distribution of some types. The power of PCR and sequence analysis is that organisms can be compared at a genetic level without culturing them, so the genetic diversity of unculturable populations can be investigated.

## Probes for Bacteria in the Nitrogen Cycle

Probes based in ribosomal RNA sequences are the most commonly applied methods for detection and characterization of both isolates and natural communities. These sequences lend themselves to development of both specific detection techniques and investigation of evolutionary relationships. A focus on the biogeochemical processes themselves, however, encourages the use of probes based on enzymes or genes directly involved in the transformation processes. Functional probes can yield information on the distribution and expression of genes involved in a certain biogeochemical pathway without regard to the taxonomic identity of the organisms that might possess those genes in nature. Both kinds of approaches to studying bacteria involved in the nitrogen cycle are being developed and applied in aquatic environments.

*Probes for Nitrifiers*

For groups that are monophyletic, rRNA sequences can be used to infer physiological attributes, and thus to identify unculturable organisms at a functional level. While the nitrifiers are not monophyletic, the ammonia oxidizers cluster into closely interrelated groups in which the ribosomal RNA phylogenies correlate extremely well with the functional grouping. Ribosomal RNA sequence data [16, 61, 62] discerned two main lines of descent within the ammonia-oxidizing nitrifiers. One line is in the beta subclass of the Proteobacteria and includes two clusters: *Nitrosomonas* (including the previously identified groups *Nitrosomonas* and *Nitrosococcus mobilis*) and *Nitrosospira* (including *Nitrosospira, Nitrosovibrio,* and *Nitrosolobus*). The other line of descent is in the gamma subclass (*Nitrosococcus oceanus* and *Nitrosococcus halophilus*). Teske et al. [49] recently described the nitrifiers, both ammonia and nitrite oxidizers, as decendents of several branches of the purple photosynthetic phenotype. The result is that the nitrifiers cluster as several small, closely related groups that are not closely related to each other. Most work so far has emphasized the ammonia oxidizers, but phylogenetic studies on nitrite oxidizers have also been done [40] and sequence availability makes probe development imminent [49].

The phylogeny of nitrifiers makes them ammenable to probe development based on their ribosomal RNA sequences. Voytek and Ward [51] reported a set of PCR primers that specifically amplify all of the nine known species of ammonia oxidizers in the beta subclass of the Proteobacteria. Another set of primers has been designed to amplify selectively the known members of the gamma subclass of ammonia oxidizers (Voytek and Ward, submitted).

Although these primer sequences were developed on the basis of sequences obtained from cultured organisms, they can now be used to discover and characterize uncultured nitrifiers that are phylogenetically related to the ones in culture. The power of this approach was demonstrated in a permanently ice-covered lake in Antarctica, where for four years a variety of enrichment and isolation techniques failed to yeild nitrifying bacterial isolates. The chemical distributions in the lake strongly imply that nitrification occurs and has a dominant effect on the distribution of nitrogen in the lake [42]. Nitrifier sequences were amplified from DNA extracted from lake samples, thus verifying the presence of unculturable nitrifiers [51].

Direct amplification with these primers has detected nitrifier DNA in several lakes in northern Germany, the Baltic sea, sewage treatment plant samples, seawater from coastal and oceanic sites, and enrichment cultures from various environments including microbial mats and marine sediments ([59], Ward et al., in press). The example shown in Fig. 1 illustrates the detection of the beta-subclass ammonia oxidizers as a function of depth in a temperate lake. Samples were collected prior to fall overturn, while the water column was stratified into an oxygenated epilimnion and an anoxic hypolimnion. Primer set NitAB amplifies all members of the beta-subclass ammonia oxidizers [51], and primer set NitDB is specific for *Nitrosomonas europaea* (Ward et al., in press). Although quantification of the relative abundance of different targets has not been attempted, amplification results suggest that the species composition of the nitrifier assemblage changes with depth, with *N. europaea* undetectable everywhere except near the oxic/anoxic interface. Ammonia

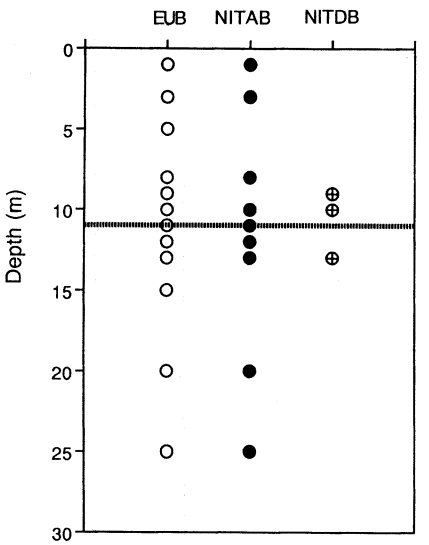


**Fig. 1.** Symbols denote successful amplification of total DNA extract using EUB (eubacterial universal, *open circles*), NITAB (beta-subclass ammonia oxidizers, *filled circles*), and NITDB (*Nitrosomonas europaea*–specific) primers in a depth profile of the Plußee, a lake in northern Germany. Amplification with the EUB primers was used as a postive control, to verify that amplifiable DNA was obtained from the extraction at every depth. The *bar* at 11 m denotes the depth of the oxic/anoxic interface at the time of sampling.

oxidizer targets were detected below the interface, under conditions incompatible with the growth of the obligately aerobic nitrifiers. Nitrifiers are capable of prolonged inactive but viable existance, which may under these conditions enable them to persist over annual cycles.

Hiorns et al. [17] used a different set of PCR primers to estimate the relative abundance of ammonia oxidizers in DNA extracted from a temperate lake in England and from nitrifier isolates obtained from the lake. They found that the sequence most commonly detected by PCR was not from the species that was most easily cultured, verifying the inherently selective bias of cultivation as a basis for understanding natural populations.

McCaig et al. [32], using yet another permutation of PCR primers to amplify ammonia-oxidizer rRNA sequences, were able to perform a phylogenetic analysis of nitrifiers from enrichment cultures, demonstrating the use of PCR to identify organisms of interest that can be grown in the lab but are difficult to obtain and maintain in pure culture.

It is evident that several different sets of primer sequences can be designed to possess appropriate specificity for this group of ammonia oxidizers. Detection of the beta-subclass nitrifiers in such different environments means that sequences amplified from the environment were probably in the same rRNA group as those culturable ammonia oxidizing bacteria from which the primers were developed, and that ammonia oxidizers have a global distribution, at least at the genus level as determined by rRNA sequences. PCR primers for use as probes for nitrite oxidizers have not yet been reported; with the publication of more sequences [49] their development is only a matter of time.

Prior to the advent of molecular methods for the detection of individual types or groups of bacteria, immunological methods offered the greatest sensitivity and specificity. In particular, immunofluorescence has a long history of application for the study of nitrifying bacteria, both in soils (e.g., [2, 20]) and aquatic systems (e.g., [52, 56]). The main disadvantage of immunofluorescence is that the target

organism must be culturable in order for an antibody to be produced. Even with culturable organisms, the epitopes that induce antibody production may be peripheral phenotypic traits that are not fundamentally related to the functional phenotype of interest, and may not reflect the phylogeny of the organisms. Whole cell immunofluorescence relies on the specificity of antigens on the outside of the cell envelope and these may be subject to selection independent of selection for other aspects of the genotype.

Nevertheless, for culturable organisms, immunofluorescence is a very specific and sensitive method for quantification of particular strains in complex environmental samples, and has been particularly widely applied in clinical situations. The specificity of the antiserum can be optimized and characterized by testing other culturable strains. Because individual cells are enumerated microscopically, interference from noncellular fluorescence is minimized. Some workers have found the benefits of specificity to outweigh the tedium of microscopic enumeration (e.g., [4, 47]). Others reported that the inability to discern live cells from dead was a drawback in predicting activity of the target strain [41]. In practice, nonspecific cell staining is usually not a problem. In fact, extremely narrow specificity is often obtained even with polyclonal antibodies. While this lends credence to specific abundance estimates, the antisera are sometimes so strain specific that they don't react even with closely related strains. Thus, abundance estimates are specific for the target strain, not all members of a species or genus, much less all members of a functional group (e.g., [38]). Excess specificity has been less of a problem for nitrifiers [56] than for other physiologically defined groups, perhaps because of the physiological constraints of nitrifier metabolism, or due to their interrelatedness. Voytek and Ward [51] showed that the *Nitrosomonas* serogroup defined by Ward and Carlucci [56] on the basis of immunoreactivity of polyclonal antisera was a subset of the rRNA group detected by the beta-subclass ammonia-oxidizer PCR primers.

The same or similar primers as those used for PCR detection of nitrifiers have also been used to enumerate individual cells (Voytek, in preparation) in natural environments with fluorescence in situ hybridization. This method combines the specificity characteristics of PCR, which can be optimized along meaningful phylogenetic lines, with the single cell information (and tedium) of immunofluorescence. The development of quantitative PCR for complex environmental samples and its interpretation in terms of cell numbers (from which population distributions can be deduced) would be a major advance.

For groups that are not monophyletic or closely related by ribosomal RNA sequences, a more powerful approach for probe development would be the use of a unique functional gene that is specifically associated with the transformation of interest, and this is one approach that has been taken with the denitrifiers (see below). Recent developments have made it possible to focus on the functional gene in ammonia oxidation, ammonia monooxygenase (AMO). A partial sequence of the AMO gene was published [33] and PCR primers and probes for the AMO gene are under investigation in several laboratories. Identifying ammonia oxidizers by means of the functional gene would address directly the presence or absence in the environment of the genetic capability for the process, regardless of the phylogenetic identity of the organism. It might lead to the discovery of currently unknown organisms that possess this capability but are not related to the organisms we have in culture.

The main obstacle to the AMO probes at present is the high level of homology between AMO and the analogous enzyme in methanotrophic bacteria, the particulate methane monooxygenase (MMO) [18]. While these similarities pose problems at present for ecological applications of molecular probes for these functional genes, they may shed light on the evolutionary development of the processes and allow comparison between RNA phylogenies and functional relationships.

### *Probes for Denitrifiers*

The key step in the denitrification process is the reduction of nitrite to nitric oxide, which results in the release of gaseous products. Therefore, the enzyme that mediates this reaction, nitrite reductase, has been the focus of physiological and biochemical studies on denitrification. Dissimilatory nitrite reductase (NiR) occurs very widely in the bacterial kingdom [64] and apparently is absent only in the obligate anaerobes, Gram-positive organisms other than *Bacillus,* and in most of the *Enterobacteriacea.* For denitrification, then, the much greater phylogenetic diversity of bacteria capable of denitrification makes the rDNA approach unrealistic. rDNA sequences might form the basis of species-specific probes for individual denitrifiers [12, 22], but they are not the most efficient approach to the study of denitrifiers as a functional group. A functional probe for denitrification based on NiR offers a more general approach for identification and detection of denitrifying bacteria in the environment.

There are two forms of the enzyme, a copper nonheme time and a cd heme type, which are both common among isolates. The Cu-NiR appears to be present in a wider range of physiological types, but the cd-NiR appears to be numerically dominant overall [7]. These conclusions were based on surveying isolates and soil enrichments and thus may reflect the biases of culture methods. Even so, this is more information than has been available concerning the occurrence and diversity of marine denitrifying bacteria.

Even within the two main types of NiR enzymes, immunological studies indicate important divergences among the proteins. The cd-heme, dNiR, of *Pseudomonas stutzeri* is the most thoroughly studied and it has repeatedly been shown to posses highly antigenic epitopes that are apparently not present in closely related organisms [8, 25, 60]. Even among strains whose NiRs have the same number and size of subunits, considerable differences in the characteristics of purified native protein were detected, both biochemically and immunologically [60]. Similar kinds of variability were reported by Coyne and Tiedje [7] for the Cu-dNiR.

Nevertheless, the diversity of nitrite reductase enzymes must be less than the phylogenetic diversity of the organisms that express this enzyme, and it remains a promising approach for production of group-specific probes. Still, few data have been collected on the expression or distribution of the enzyme in the environment. This may be due to its inducible nature. Although NiR can comprise a very significant portion of the total cell protein under certain laboratory conditions, field studies may not have been performed in the marine water column under conditions that favor its expression.

DNA probes for fragments of the *nir* gene, based on portions of the structural gene itself, have also been described [21, 47, 60]. The gene probes have been used

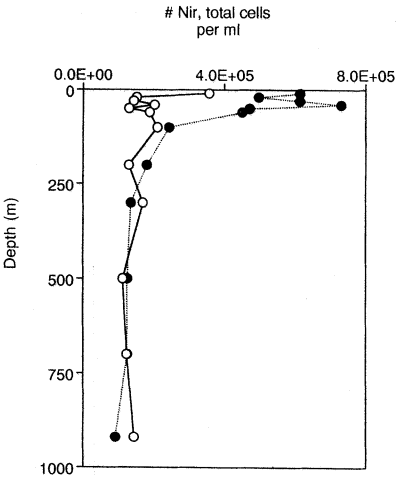


**Fig. 2.** Abundance of NiR fragments (*open circles*) estimated by quantitative hybridization, and total cell abundance (*closed circles*) at a station in Monterey Bay, California.

to detect the genetic potential for denitrification in known denitrifying strains from soil [47] and marine environments [60] and in scores of isolates from marine systems ([52]; Ward, unpublished). Hybridization with DNA extracted from natural assemblages in Monterey Bay, the Southern California Bight, and hypersaline Antarctic lakes was also detected (Ward, unpublished; [54]). The distribution of the gene in seawater from a nondenitrifying environment is shown in Fig. 2. The number of copies of the gene was estimated by comparison of unknown samples to a standard curve generated by nonradioactive probing of DNA extracts. (Reproducibility of replicate hybridization samples is excellent (C.V. $< 5\%$), but total error associated with extraction efficiency and complete sample is more difficult to assess). In this profile, the abundance of *nir* fragments did not vary over the depth profile nearly as much as did the total bacterial population (estimated by DAPI direct counts). It appeared that the abundance of the *nir* gene approximately equaled the abundance of total cells below the surface layer where the ratio was closer to 20–30%. These data imply that a surprisingly high fraction of the total cells possess the genetic capability for denitrification. While the estimates may be refined upon further research, they nevertheless suggest that a significant portion of the population may possess this inducible characteristic. The possibility that NiR is used in some other reaction not related to denitrification, which might explain its prevalence, cannot be ruled out completely. The proportion of the total cells that can be identified by immunofluorescence as belonging to a single strain of denitrifiers is much less than 1% [57], suggesting that the natural assemblage of denitrifiers is very diverse.

The presence of the *nir* gene, as determined by DNA probing, indicates the distribution of the genetic potential for denitrification, regardless of whether environmental conditions have induced enzyme expression. The abundance of the *nir* gene is equivalent to the number of cells that possess the genetic capacity for denitrification, assuming there is only one copy of the gene per cell. No correction was made for the observation that the degree of homology between the probe fragment and target gene sequences in diverse denitrifiers varies. Less than 100%

homology would weaken the hybridization signal and, in turn, cause an underestimate in the number of fragments present. It is difficult to estimate how much partial homology would affect the abundance estimates. Hybridization at relatively high stringency (62°C) probably minimizes this problem. The DNA probe for the *nir* fragment was reported to be the least specific of three kinds of probes described for the *nir* gene/enzyme and tested against a suite of proven denitrifiers [55]. Polyclonal antisera for the NiR protein were almost species specific; detection of the gene fragment by PCR amplification recognized more strains as possessing *nir* than did the antisera; and the hybridization probe detected homology in the most strains. Using RFLP analysis of ribosomal genes, it was shown that relationships derived from ribosomal similarities among denitrifying isolates were not correlated with homology with the hybridization probe for nitrite reductase [55]. This finding directly addresses the relative usefulness of functional vs. phylogenetic probes for denitrifiers.

## Conclusions and Future Directions

As more and more sequences for ribosomal and functional genes are obtained, the development of probes for phylogenetically and functionally defined groups will become easier and more accessible. Although the field is developing rapidly, the appropriate specificity and sensitivity have already been demonstrated both for probes based on ribosomal RNA sequences and on sequences encoding functional genes of biogeochemical significance. Quantification of both kinds of probes is also well under way and represents another avenue of rapid advancement in the near future.

These approaches have allowed major new progress in the study of bacteria involved in the nitrogen cycle in terms of their phylogenetic relationships, detecting them and identifying them in the environment, and deducing evolutionary relationships among organisms and pathways. In the future, these tools, may also lead to the discovery of new pathways and new organisms. *Thiosphaera pantotropha,* the organism characterized as being capable of simultaneous nitrification and denitrification [43], may be a very powerful system in which to explore the genetics, evolution, and regulation of both processes. Our vision of denitrification has been expanded to include denitrification under fully aerobic conditions [44, 45]. Likewise, the conventional identification of nitrifiers as obligately aerobic obligate autotrophs will probably be modified. Other physiologies currently under study in ammonia oxidizers include anaerobic ammonium oxidation [50] and dinitrogen production [37]. Heterotrophic nitrification [23] has not been considered in this review and its importance in aquatic systems remains to be determined. The application of both immunological and nucleic acid technologies, in combination with biochemical and physiological studies, promises an exciting future for this field of research.

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