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ENVIRONMENTAL CONTROLS ON DENITRIFYING COMMUNITIES AND DENITRIFICATION RATES: INSIGHTS FROM MOLECULAR METHODS

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Abstract. The advent of molecular techniques has improved our understanding of the microbial communities responsible for denitrification and is beginning to address their role in controlling denitrification processes. There is a large diversity of bacteria, archaea, and fungi capable of denitrification, and their community composition is structured by long-term environmental drivers. The range of temperature and moisture conditions, substrate availability, competition, and disturbances have long-lasting legacies on denitrifier community structure. These communities may differ in physiology, environmental tolerances to pH and O_2, growth rate, and enzyme kinetics. Although factors such as O_2, pH, C availability, and NO_3^- pools affect instantaneous rates, these drivers act through the biotic community. This review summarizes the results of molecular investigations of denitrifier communities in natural environments and provides a framework for developing future research for addressing connections between denitrifier community structure and function.

Key words: denitrification; functional genes; microbial community composition; molecular techniques; N_2O; nar; nirK; nirS; nor; nosZ; proximal and distal controls.

INTRODUCTION

Denitrification is an important component of ecosystem N cycling (Schlesinger 1997) and is likely to be affected by global change (Barnard et al. 2005). It is the respiratory microbial process by which N oxides serve as terminal electron acceptors for respiratory electron transport, resulting in the reduction of NO_3^- to gaseous products, mainly N_2O and N_2. The gaseous products of denitrification are the major biological pathway for N loss from ecosystems, and the gaseous intermediates, NO and N_2O, have implications in atmospheric trace gas chemistry and global warming (Prather et al. 2001). The potential environmental importance of denitrification has led to numerous empirical measurements of the process in a range of habitats (e.g., soils, sediments, marine and freshwater, groundwater, etc.) all around the globe.

Since the pioneering work of Nõmmik (1956), scientists have investigated the factors controlling denitrification in an attempt to better understand the process, interpret the rates they have measured, and extrapolate those rates in space and time. Almost universally, scientists have focused on the roles of NO_3^- availability, O_2, and pH in controlling denitrification rates (Firestone et al. 1979, Davidson and Swank 1986, Weier et al. 1993, Thomas et al. 1994). These are the key regulators of denitrification rates at any particular instant. However, these factors act through a biotic community whose composition reflects and integrates the long-term soil climate, disturbances, and resource availability (Fig. 1). We use the term "distal controls" to refer to these factors that control the composition and diversity of denitrifying communities over the long term. The denitrifying community, in turn, acts as a transducer through which proximal controls on denitrification are realized (Fig. 1). We define proximal controls on denitrification as environmental conditions and resources that affect instantaneous denitrification rates. In this review, we provide evidence that NO_3^- acts primarily as a proximal control on denitrification rates, but has less direct effects on denitrifier communities in the long term.

Investigations of denitrifying communities provide a biological perspective on what is often considered as a biogeochemical process. It has generally been assumed that the diversity and composition of the denitrifying community is not important in determining rates, based on lack of empirical evidence and, to some extent, the fact that denitrification genes are widespread in phylogenetically distant organisms (Linne von Berg and Bothe 1992). However, the possession of denitrifying genes does not necessarily mean that these genes will be expressed in the environment or that the gene products

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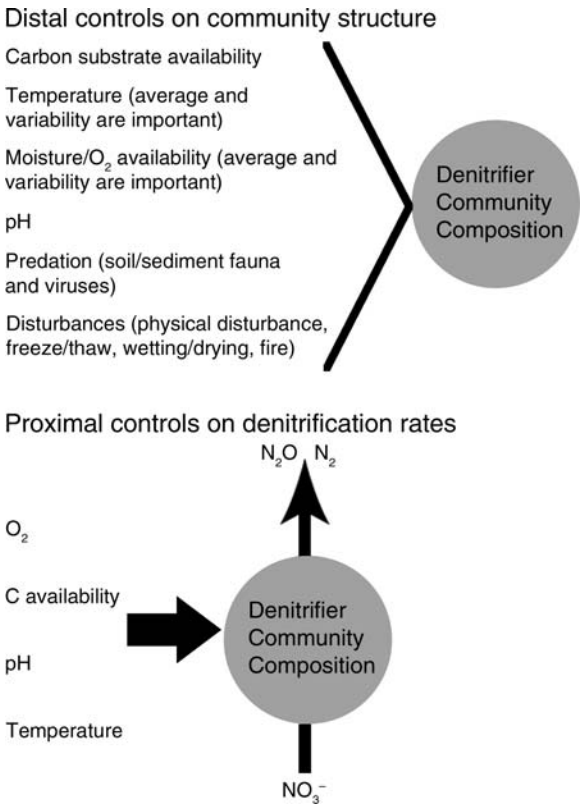

FIG. 1. A conceptual illustration of distal and proximal controls on denitrifiers and denitrification. Denitrifier community composition is structured over the long term by distal controls which include both environmental factors and biotic interactions. The kinetics of denitrification at any particular time are the result of proximal controls that affect the metabolism of the existing microbial community. Denitrifying communities act as a transducer, through which proximal controls act to affect the rate and kinetics of denitrification.

will function equivalently. More recently, the assumption that the composition of the denitrifying community is of minor importance in controlling denitrification has been challenged by findings suggesting that denitrifier communities vary in their tolerances to environmental conditions and stress (Cavigelli and Robertson 2000, Holtan-Hartwig et al. 2000).

The purpose of this review is to briefly summarize key controls on the distribution of denitrifier communities, review what we have learned about the structure of denitrifier communities and how that relates to their function, and to suggest some prospects for future advances in understanding the linkages between denitrifier community structure and function.

Genes of the Denitrification Pathway

Denitrification involves four enzymatically catalyzed reductive steps (Fig. 2): nitrate reduction, nitrite reduction, nitric oxide reduction, and nitrous oxide reduction (Philippot et al. 2002). The genes (indicated by italics) that code for the enzymes (non-italicized) involved in denitrification have each been used as targets for molecular methods to characterize the composition of denitrifier communities. The dissimilatory reduction of nitrate is associated with two homologous enzymes, membrane-bound (Nar) and periplasmic-bound (Nap) nitrate reductases (Fig. 2). Because these nitrate reductases are also present in bacteria that do not denitrify (e.g., nitrate respirers, dissimilatory reducers of nitrate to ammonia), the *narG* and *napA* genes that code for these enzymes have not been widely used to characterize the diversity of denitrifying bacteria (Philippot et al. 2002, Cheneby et al. 2003, 2004). Genes coding for nitrite reductase (Nir) were the first to be used for studies of denitrifier diversity and have subsequently been the most common molecular marker for denitrifier community studies (Braker et al. 1998). These genes have been used because nitrite reduction is the first step in the reaction that results in the production of a gaseous product (Fig. 2). There are two evolutionarily unrelated forms of the nitrite reductase enzyme, a copper and a cytochrome *cd*1-nitrite reductase, coded for by the *nir*K and *nir*S genes, respectively. A few studies have focused on nitric oxide reductase (Nor) using *norB* as a gene target (Braker and Tiedje 2003). Because this enzyme forms the N-N bond, it plays a unique role in the denitrification pathway. Nitrous oxide reductase (*nosZ*)

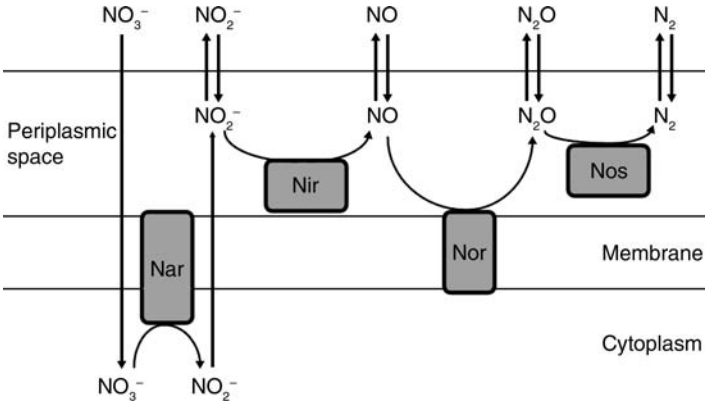

FIG. 2. Sequential reductive pathway of denitrification showing the location of enzymes relative to the cytoplasmic membrane: Nar, nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase (modified from Ye et al. [1994]).

has been quite widely used to describe denitrifier communities because primers were developed by Scala and Kerkhof (1998), in part because this enzyme reduces N_2O, a potent greenhouse gas, to N_2 which is relatively inert in the atmosphere.

Taxonomic diversity of denitrifiers

Denitrification is a facultative anaerobic microbial process. The ability to denitrify has been identified in a very diverse group of phylogenetically unrelated bacteria, including members of the Aquificae, Deinococcus-Thermus, Firmicutes, Actinobacteria, Bacteroides, and Proteobacteria phyla (Zumft 1997). Some fungi (Shoun et al. 1992, Tanimoto et al. 1992) and Archaea (Philippot 2002) are also capable of denitrification. Furthermore, denitrification can be considered to be a community process, as many denitrifying organisms do not produce the complete suite of enzymes (Fig. 2) to complete the reaction and could work together to complete the process (Zumft 1997).

Most research on the microbiology of denitrifiers has focused on bacteria, which are generally believed to be the dominant denitrifying organisms in most environments. In addition to numerous isolates in pure culture, recent studies have focused on the diversity of noncultured denitrifiers (Scala and Kerkhof 2000, Prieme et al. 2002, Rich et al. 2003, Cheneby et al. 2004, Throback et al. 2004). Because of the wide phylogenetic diversity of denitrifying bacteria, the commonly targeted rRNA genes are impractical for examining denitrifying communities. Therefore, most studies of denitrifiers in natural habitats have targeted the functional genes that code for enzymes involved in denitrification.

The ability of extremophile Archaea to denitrify has been long known, but the extent of this ability in non-extremophile Archaea remains unknown. The *nir*K gene, which codes for the nitrite reductase enzyme, has been identified in several well characterized halophilic Archaea, including *Haloarcula marismortui* and *Halferax denitrificans* (Inatomi and Hochstein 1996, Ichiki et al. 2001), and these sequences differ substantially from those identified in bacteria. More targeted studies are needed to elucidate the importance of Archaeal denitrification.

The contribution of fungi to denitrification has been reconsidered in recent years (Shoun et al. 1992, Laughlin and Stevens 2002). There is some evidence that fungi may be more important than bacteria for denitrification in grassland soils (Laughlin and Stevens 2002). Apparently the functional genes found in fungal genomes differ substantially from bacterial denitrifying functional genes (Zhang et al. 2001), and will also require studies that specifically target these genes.

Molecular tools for studying denitrifier community composition

A variety of molecular methods have been applied to investigations of denitrifying genes in natural environments. In general, these approaches attempt to quantify either the diversity or overall abundance of denitrifiers, and each technique differs in resolution. Most of these approaches begin by selectively amplifying the target functional genes using the polymerase chain reaction (PCR; Fig. 3). In PCR, oligonucleotide primers are designed to target the gene of interest based on sequence alignments found in publicly available databases (e.g., GenBank). For each denitrification gene there are very few full-length sequences, as these are typically generated from genome sequencing of cultured microbes. Therefore, the degree to which these primers target all variants of these genes is difficult to assess, but is improving rapidly as public databases expand and additional genes are sequenced. Any technique that uses PCR is also subject to biases during the amplification process that may affect results, but are difficult to detect and assess (Becker et al. 2000).

Following amplification of the gene, there are a number of strategies for investigating the diversity and community composition of that gene in a mixed community (Fig. 3). In general, there is a tradeoff between efficiency and detail. For example, one relatively efficient technique is terminal restriction fragment length polymorphism (T-RFLP), where the PCR-amplified gene fragments are cut with restriction enzymes, separated by gel electrophoresis, and the length of the cut fragments measured quantitatively using a DNA sequencer (Liu et al. 1997, Blackwood et al. 2003). This technique quantifies the relative abundance of the dominant gene variants, presumably representing the dominant denitrifiers in the community. T-RFLP is a powerful technique for comparing communities, though it provides very little information about the identity of specific members of the community. Another widely used fingerprinting technique is denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993, Throback et al. 2004). This technique separates the amplified DNA based on its length and GC (guanine and cytosine) content, resulting in a series of bands on a gel, each band representing a unique variant of the gene, and therefore a unique member of the denitrifying community. DGGE is less quantitative than T-RFLP, but has the advantage that intact gene fragments can be extracted and sequenced to provide insight into the phylogenetic identity of specific denitrifiers. Therefore, DGGE has the potential to provide a more detailed phylogenetic assessment of denitrifier community structure. Finally, the overall relative abundance of any particular gene fragment can be measured by quantitative PCR (qPCR) (Gruntzig et al. 2001, Henry et al. 2004, Wallenstein and Vilgalys 2005). This technique provides precise quantification of the gene of interest, but no information on community composition.

In contrast to DNA fingerprinting techniques, other techniques, which involve gene sequencing, provide much more specific information about variations of the gene between organisms and within mixed commun-

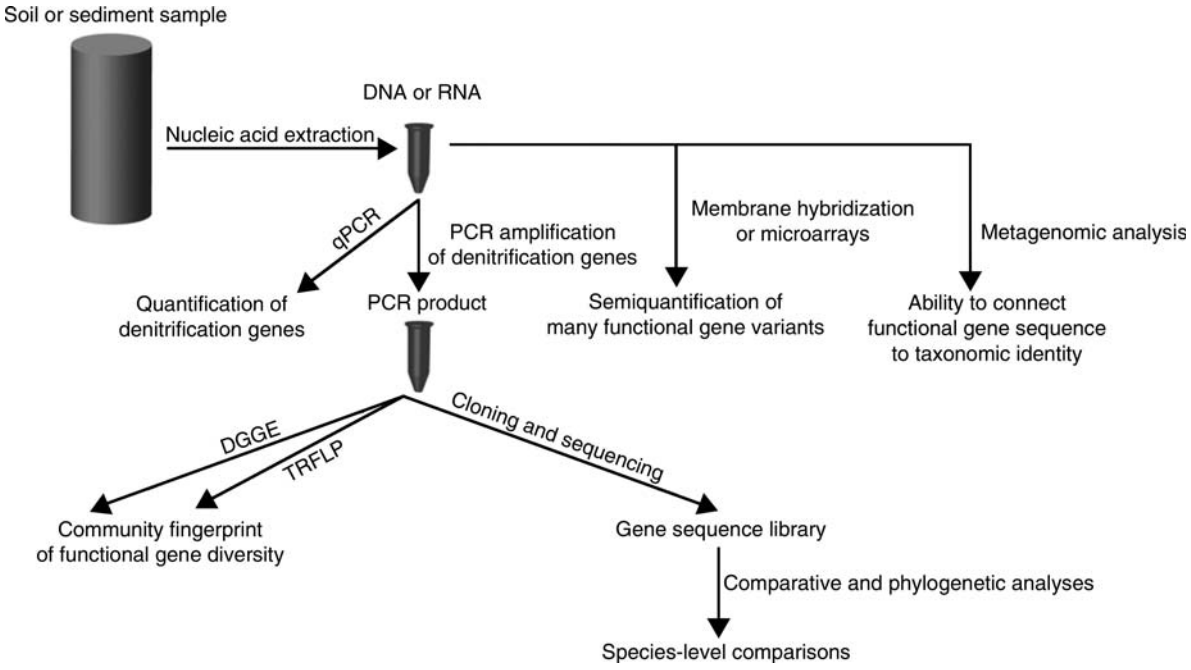

FIG. 3. An illustration of alternative molecular techniques for examining denitrifier communities. After nucleic acids are extracted from environmental samples, they can be assayed directly by hybridization techniques, or the denitrification genes can be amplified by the polymerase chain reaction (PCR). A modified PCR technique (qPCR) can be used to quantify the relative abundance of denitrifying genes. PCR products can also be used to assess the overall community structure or diversity by denaturing gel gradient electrophoresis (DGGE) or terminal-restriction fragment length polymorphism (TRFLP). Sequences of uncultured organisms can be obtained by cloning and sequencing techniques.

ities. A common strategy is to develop a library of gene sequences from an environmental sample by cloning gene fragments that have been PCR amplified from mixed community nucleic acids. Randomly selected clones are sequenced and aligned, giving a census of the amplified gene fragments within a given sample. By collecting detailed sequence information for a large number of clones, the relative proportion of each gene variant can be assessed, providing a measure of overall gene diversity and specific community composition. This technique provides a very specific description of community structure, but is more expensive than the two fingerprinting approaches described above.

There are also approaches that avoid the biases associated with PCR amplification of functional genes. For example, denitrification genes can also be measured directly by hybridization of nucleic acids to gene probes (complementary single-stranded DNA fragments of the target gene), either on nylon membranes or using microarrays (Mergel et al. 2001*b*, Wu et al. 2001, Taroncher-Oldenburg et al. 2003). Following hybridization, quantitative detection of a fluorescent or radiometric signal indicates the presence of genes complementary to the probes. Molecular biology continues to advance at a rapid pace, and new techniques will continue to arise that improve our ability to assess microbial community structure.

Although the presence of denitrifying genes in DNA suggests only the capability of an organism to denitrify, their detection in mRNA suggests that the organism was actively expressing the gene and producing the associated enzyme. Because mRNA precedes protein production, its detection is a direct indicator of gene expression. There are many more difficulties associated with extracting and stabilizing RNA from environmental samples, though these have been overcome by several labs (Borneman and Triplett 1997, Hurt et al. 2001, Sessitsch et al. 2002, Burgmann et al. 2003) and offer exciting possibilities for future research. For example, Nogales et al. (2002) examined the expression of five denitrifying genes by analyzing mRNA from estuarine sediments. In this study, all five genes were detectable in DNA; however, they were only able to detect *nirS* and *nosZ* genes in mRNA, suggesting that many genes that were present in the microbes at this site were not being expressed. This study indicates that, although the ability to denitrify is widespread, it is facultative and only induced under particular conditions.

Distal controls on denitrifier community structure: case studies

Molecular approaches provide the tools necessary to evaluate the role of distal controls (Fig. 1) in structuring denitrifier communities. To date, most studies in natural environments have focused either on soils, estuarine

sediments, or marine sediments, with little attention given to stream sediments.

Soils

The community composition of denitrifiers is very diverse and appears to vary in different soil environments. In soils, denitrifier communities appear to be structured in the long-term by C availability, pH, and the range of moisture and temperature they experience (Fig. 1). Although it is difficult to compare among studies due to differences in primer selection, and the limitations of fingerprinting techniques such as DGGE and TRFLP, comparative studies of denitrifier communities from soils that differ in one or more of these factors generally find large differences in both their composition and diversity, supporting the concept of distal environmental controls on community structure. For example, Prieme et al. (2002) compared the diversity of *nirK* and *nirS* gene fragments in forested upland and wetland soils using a variation of the T-RFLP technique. One of their most striking findings was that there was very little overlap in *nirK* gene sequences between the two soils, although the gene sequences were closely related. They also found that the *nirK* gene pool in marsh soils was more diverse than in upland soils. Similarly, Chenby et al. (1998), using T-RFLP to characterize cultured isolates, observed very little overlap in *nosZ* sequences in two soils with contrasting N_2O:N_2 ratios. These studies suggest a strong role for distal controls on denitrifier community structure, but do not provide enough data to parse the effects of individual environmental controls on community structure.

In contrast to the results from studies targeting the *nirK* and *nirS* genes, studies investigating *nosZ* gene distributions have found fewer differences between contrasting environments. Rich et al. (2003) found that soils of adjacent forests and meadows shared most of the *nosZ* gene variants in common using T-RFLP, although differences in relative abundance of *nosZ* gene types, as well as a few habitat-specific variants, were sufficient to differentiate between the two vegetation types. In a study of adjacent agricultural and riparian soils, and creek sediment, Rich and Myrold (2004) also found most *nosZ* gene variants were in common, with a few rare gene types associated with a specific habitat. These studies may indicate that the distribution of *nosZ* genes is more cosmopolitan than other genes involved in denitrification, and less affected by distal controls.

Although NO_3^- supply is known to be a strong control on denitrification rates, Mergel et al (2001*b*) found little correlation between NO_3^- concentrations and denitrifier abundance, based on DNA hybridization with denitrification gene probes, in an acidic forest soil depth profile. This is consistent with the concept that denitrifier community structure and abundance is primarily controlled by factors other than NO_3^- supply (Fig. 1).

Marine Ecosystems

It is likely that the relative importance of various distal regulators on denitrifier communities differs between soils and sediments. Whereas soil microbes are generally considered C limited, N may more often be a limiting resource in marine sediments. In sediments within an oxygen-deficient zone off the Pacific coast of Mexico, Liu et al. (2003) used a PCR-cloning approach to evaluate the diversity of *nirS* and *nirK* genes, and detected distinct communities along the environmental gradient. A principal components analysis (PCA) of biogeochemical controls on the relative abundance of distinct gene variants indicated that NO_3^- and oxygen levels were the strongest controls on the distribution of denitrifiers.

Other studies of marine sediment denitrifiers have not focused on the relative importance of specific factors in controlling their distribution, but generally suggest that community composition is regulated by a broad set of environmental controls. For example, the input of organic matter appears to drive community differences between Puget Sound and Washington coast sediments (Braker et al. 2000, 2001). In these same sediments, there was no indication that redox gradients occurring with sediment depth structure denitrifier communities (based on T-RFLP), however these sediments may be frequently mixed by marine invertebrates (Braker et al. 2001). The sequences of *nosZ* genes from Atlantic shelf sediments were distinguishable using phylogenetic analyses from those collected from Pacific shelf sediments, but it is unclear whether this is due to differences in environmental controls, or long-term evolution of distinct communities (Scala and Kerkhof 1999).

Denitrification has also been observed in water columns located in upwelling zones, which are likely to differ from soils and sediments in the factors that regulate denitrifier communities. The diversity of *nirS* genes was investigated by sequencing and RFLP analyses in a region of high denitrification rates in the Arabian Sea in relation to NO_2^- and NO_3^- distribution (Jayakumar et al. 2004). Most of the *nirS* sequences from these waters were not closely related to previously sequenced *nirS* genes from other habitats. A potential relationship between *nirS* diversity and denitrification rates was also observed, although the rates were inferred from NO_2^- concentrations. Diversity, based on rarefaction curves, was greater in a sample with high NO_2^- concentration than in one with undetectable levels of NO_2^-. The significance of these findings is unclear, but may indicate that in these waters, denitrifier communities are regulated by the supply of N, in contrast to soils and sediments.

Are denitrifying communities stable?

It is well known that denitrification rates vary over a variety of temporal scales (Groffman and Tiedje 1989, Weier et al. 1991, Van Kessel et al. 1993, Wolf and

Brumme 2002), however, the population dynamics of denitrifier communities are not well understood. A few studies have evaluated temporal dynamics of denitrifier community structure. In arable soils, Wolsing and Prieme (2004) observed strong seasonal shifts in community composition, based on T-RFLP of *nirK* and *nirS* genes, suggesting that different denitrifiers are active under different conditions. Mergel et al. (2001*a*) also found that denitrifiers were less abundant in the summer compared to other seasons. Interannual variations in *nosZ* T-RFLP were observed by Boyle et al. (2006) for forest and meadow soils sampled each fall during a five-year period. Whether these communities function differently remains to be tested.

How do communities respond to environmental change?

The response of microbial communities to climate change, pollution, and other environmental change is an important component of ecosystem response, and recently, several studies have investigated the response of denitrifiers to environmental change. One subject of interest is the response of denitrifiers to anthropogenic N deposition, which has increased in many regions and can impact the health of both terrestrial and aquatic ecosystems (Fenn et al. 1998, Erisman and Vries 2000, Barnard et al. 2005). It is well recognized that N deposition is likely to increase denitrification rates (Gundersen 1991, Aerts 1997, Mohn et al. 2000) and possibly increase the N_2O:N_2 ratio (Firestone et al. 1979, Monaghan and Barraclough 1993), but the long-term direct and indirect effects of N deposition on denitrifying communities have only recently been studied.

Inorganic N supplied through deposition acts as a proximal control to increase denitrification rates, however it appears that the indirect effects of N deposition on decomposition and plant processes are likely to be more important in structuring denitrifier communities over the long term. Wallenstein (2004) measured the abundance of *nirS*, *nirK*, and *nosZ* gene fragments using quantitative PCR in two long-term forest N fertilization experiments. They found that the abundance of denitrifiers in these soils was apparently altered by chronic fertilization. The abundance of *nirK* was lower in fertilized plots compared to control plots at Mt. Ascutney, Vermont, USA. At Harvard Forest, Massachusetts, USA, the *nirS* gene was less abundant in fertilized plots compared to control plots. Despite the greater availability of NO_3^- at these sites, the abundance of denitrifiers appears to have decreased, possibly in response to changes in C availability, moisture, or pH. Another recent study suggested that N fertilization in an agriculture soil caused a shift in the community structure of denitrifiers (Wolsing and Prieme 2004). They analyzed the community composition of *nirK* fragments using T-RFLP analyses, and found that fertilized plots differed in composition from control plots, but they could not attribute these differences to any single abiotic factor. It appears that the response of denitrifier communities to increased N is likely to be affected by both the direct effects of increased N, and indirect effects on other distal controls.

Denitrifiers as transducers: effects of community composition on denitrification rates and kinetics

One of the most outstanding questions facing microbial ecologists is whether microbial communities that differ in composition also differ in function. There is some limited evidence that different microbial communities denitrify at different rates and with different ratios of products, and have varying responses to other factors that indirectly control denitrification rates. For example, Cheneby et al. (1998) found significant differences in denitrifier community composition for two soils with contrasting molar ratios of denitrification products. Similarly, Holtan-Hartwig (2000) found intrinsic differences in enzyme kinetics of NO_3^- and N_2O reduction at three different sites. Cavigelli and Robertson (2000, 2001) found significant differences in enzyme activity, microbial species composition, and enzyme sensitivity to pH and oxygen concentration for two soils differing in plant community composition and disturbance regime. Rich et al. (2003) also found a strong relationship between denitrifier community composition using *nosZ* T-RFLP and denitrifying enzyme potential when comparing soils from forests and meadows. But linkages between denitrifier community structure and function have not always been observed, even when using the same techniques. For example, Rich and Myrold (2004) found that although *nosZ* profiles and N_2O reductase activity varied among soils from an agricultural field, riparian area, and creek bed, gene profiles and activity did not vary in the same fashion, suggesting that denitrifier activity and community composition were uncoupled across this agroecosystem. In their reciprocal transfer study, Boyle et al. (2006) also failed to find a relationship between denitrifier community composition and denitrifier activity. Although denitrification enzyme activity was affected by the experimental manipulations, *nosZ* profiles did not change. This suggests that the relative activity of the enzymes involved in denitrification may sometimes be affected by denitrifier composition but in other cases environmental factors may be the dominant determinants of activity.

Future Prospects

As in the recent past, investigations of denitrifier communities will expand both in scope and sophistication with the advent of new technologies. As our knowledge of denitrifier diversity, physiology, and community ecology improves, the role of biology in controlling denitrification rates should become integrated into both conceptual and quantitative models. Here, we describe several specific aspects of molecular technology that are likely to drive further research into denitrifier communities.

Perhaps the most fundamental need for molecular studies of denitrifier communities is an improved database of functional genes. It is common practice to construct phylogenetic trees of functional gene sequences obtained from an environmental sample in an attempt to identify their species origin. However, very few of the functional gene sequences in publicly available databases belong to known (i.e., cultured) organisms. Because primers and probes can only be designed based on known sequences, we need to employ techniques to recover unknown denitrification gene sequences that do not rely on these primer sets. There are two technologies that promise to increase the number and diversity of denitrification gene sequences, genome sequencing of cultured microorganisms, and metagenomics sequencing of whole communities.

Genome sequencing of cultured organisms is an endeavor that provides data useful to the entire scientific community, from biomedical researchers to ecologists. As more microbial genomes are sequenced, we will learn more about the physiology and regulation of denitrification genes. Complete genomes of denitrifiers will result in full-length sequences of functional genes, which will aid primer and probe design, as well as in assigning phylogenetic affiliations to functional genes.

Most denitrifiers are not easily culturable. Consequently, genome sequencing alone will not be adequate to survey the diversity of denitrification genes. However, it is possible to reconstruct the genome of uncultured organisms through metagenomics (Rondon et al. 2000, Handelsman et al. 2002, Rodriguez-Valera 2004). The general approach of metagenomics is to sequence randomly selected gene fragments from nucleic acids extracted from a mixed community, and assemble them into genomes using sophisticated bioinformatic tools. Metagenomic approaches could be developed to specifically target denitrifying genes. For example, long fragments of community DNA could be inserted into BAC or fosmid libraries, and the transformed clones could be screened for expression of these genes (Rondon et al. 2000). Clones that show activity could then be targeted for shotgun sequencing. This would enable sequencing of full-length genes, and possibly entire operons. In some cases, we may get lucky and find rRNA genes in the same fragment as the functional gene, enabling species identification.

With current technology, a large amount of resources are needed to adequately survey the diversity of denitrifiers in a single sample. The only high-throughput community analyses available are fingerprinting techniques, such as DGGE (Throback et al. 2004) and T-RFLP analyses, which are limited in their ability to provide specific information about the identification of denitrifying species and gene sequences. One promising technology for high throughput analysis of functional genes is microarrays, which are slides or chips printed with gene probes that target specific genes (Zhou 2003). Microarrays allow for the simultaneous detection and quantification of thousands of genes or gene variants. Though this technology holds great promise, its application has been limited because of a number of technical challenges including sensitivity, consistency, and quantification (Zhou and Thompson 2002). Nonetheless, microarrays have been developed to analyze denitrifier community structure in marine sediments (Taroncher-Oldenburg et al. 2003, Tiquia et al. 2004). In the future, microarrays may provide researchers with a relatively inexpensive (excluding development and instrumentation costs) and efficient technique for assessing gene diversity, abundance, and expression. Like other primer or probe based techniques, microarray design will also benefit from an improved database of functional genes.

Technological developments and improved databases will continue to drive research on denitrifier communities; however, the technology now exists to evaluate the role of microbial community composition in controlling denitrification rates and processes. We see several categories of specific questions that need to be addressed to elucidate the links between denitrifier community composition and function:

1) What subset of the total pool of denitrifying genes in microbial communities is able to be expressed? What factors control their expression?

2) How do other environmental factors (C availability, micronutrients, pH) control community structure and influence gene expression? How does environmental stress (e.g., wetting/drying, freeze–thaw) affect denitrifier community structure? How do soil food web dynamics affect denitrifiers?

3) How does denitrifier community structure respond to environmental change?

These questions should be addressed with a combination of field surveys, controlled lab experiments, and process-based modeling. It is very difficult to assess the role of environmental factors in controlling community composition by looking for correlations in field-based surveys where many factors co-vary. Controlled lab studies may offer more promise for elucidating these controls at a mechanistic level. The role of microbial diversity or community composition in controlling rates is very difficult to address, as it is difficult to manipulate microbial communities.

The advent of molecular approaches to investigate microbial community structure has opened a new frontier in denitrification research. Though the role of denitrifier community structure has largely been ignored in the past, it is now clear that it is one of many factors that affect denitrification rates and the ratio of denitrification products. Future research will determine the conditions under which microbial community structure is important in controlling denitrification, and further elucidate the factors that affect denitrifier communities. These goals can best be achieved through simultaneous studies of denitrifier communities, deni-

trification rates, and environmental conditions in a variety of ecosystems.

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