# METHODS FOR MEASURING DENITRIFICATION: DIVERSE APPROACHES TO A DIFFICULT PROBLEM

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*Abstract.* Denitrification, the reduction of the nitrogen (N) oxides, nitrate ($NO_3^-$) and nitrite ($NO_2^-$), to the gases nitric oxide (NO), nitrous oxide ($N_2O$), and dinitrogen ($N_2$), is important to primary production, water quality, and the chemistry and physics of the atmosphere at ecosystem, landscape, regional, and global scales. Unfortunately, this process is very difficult to measure, and existing methods are problematic for different reasons in different places at different times. In this paper, we review the major approaches that have been taken to measure denitrification in terrestrial and aquatic environments and discuss the strengths, weaknesses, and future prospects for the different methods. Methodological approaches covered include (1) acetylene-based methods, (2) $^{15}N$ tracers, (3) direct $N_2$ quantification, (4) $N_2$:Ar ratio quantification, (5) mass balance approaches, (6) stoichiometric approaches, (7) methods based on stable isotopes, (8) in situ gradients with atmospheric environmental tracers, and (9) molecular approaches. Our review makes it clear that the prospects for improved quantification of denitrification vary greatly in different environments and at different scales. While current methodology allows for the production of accurate estimates of denitrification at scales relevant to water and air quality and ecosystem fertility questions in some systems (e.g., aquatic sediments, well-defined aquifers), methodology for other systems, especially upland terrestrial areas, still needs development. Comparison of mass balance and stoichiometric approaches that constrain estimates of denitrification at large scales with point measurements (made using multiple methods), in multiple systems, is likely to propel more improvement in denitrification methods over the next few years.

*Key words: denitrification; greenhouse effect; nitrate; nitric oxide; nitrogen; nitrous oxide; stable isotopes; water quality.*

## Introduction

Denitrification, the reduction of the nitrogen (N) oxides, nitrate ($NO_3^-$) and nitrite ($NO_2^-$), to the gases nitric oxide (NO), nitrous oxide ($N_2O$), and dinitrogen ($N_2$) is important to primary production, water quality, and the chemistry and physics of the atmosphere at ecosystem, landscape, regional, and global scales.

Unfortunately, it is a miserable process to measure. Available methods are problematic for a variety of reasons; they change substrate concentrations, disturb the physical setting of the process, lack sensitivity, or are prohibitively costly in time and expense. Most fundamentally, it is very difficult to quantify the dominant end product ($N_2$) of denitrification given its high background concentration in the environment. Quantification of denitrification is also hindered by high spatial and temporal variation in the process, especially in terrestrial environments.

As the importance of denitrification as a key regulator of water and air quality at regional and global scales has

$NH_4^+$

$NO_2^-$   $C_2H_2$

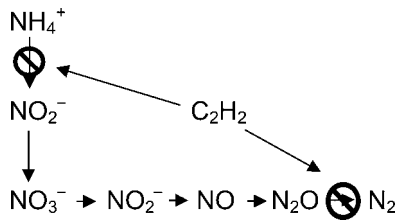$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O$   $N_2$

Fig. 1. Acetylene effects on nitrification and denitrification pathways.

become more obvious (Galloway et al. 2003), concerns about methodological misery have grown. This paper is a result of a U.S. National Science Foundation funded workshop on denitrification methodology that took place in May 2004 that was designed to advance the state of the science so that important questions related to denitrification can be addressed. Here, we review the major approaches that have been taken to measure denitrification in terrestrial and aquatic environments. For each method, we (1) provide a description of the method, (2) review the historical development and use of the method, (3) discuss the strengths and what have we learned using this method, (4) describe the weaknesses of the method and where it has led us astray in our attempts to understand and quantify denitrification, and (5) discuss prospects for future development and use of the method.

An exceptionally wide range of methods and approaches are described in this paper. This range makes it clear that the prospects for improved quantification of denitrification vary greatly in different environments and at different scales. One of our major objectives was to present the diversity of approaches to measuring denitrification so that readers from different disciplines could benefit from, and possibly adapt, methods from different environments, furthering progress on this important topic.

THE ACETYLENE INHIBITION METHOD

The most commonly applied method to measure denitrification is based on the ability of acetylene ($C_2H_2$) to inhibit the reduction of $N_2O$ to $N_2$ (Fig. 1; Balderston et al. 1976, Yoshinari and Knowles 1976). In the presence of $C_2H_2$, $N_2O$ becomes the terminal product of denitrification, which can then be quantified by measuring production of $N_2O$, which is relatively easy due to its low atmospheric concentration and the availability of sensitive detectors for this gas (Fig. 1). Numerous reviews of the $C_2H_2$ method, and its application, have been published (Tiedje et al. 1982, 1989, Keeney 1986, Nieder et al. 1989, Revsbech and Sørensen 1990, von Rheinbaben 1990, Payne 1991, Aulakh et al. 1992, Barton et al. 1999, Groffman et al. 1999).

The development of the $C_2H_2$ method in both terrestrial (Yoshinari and Knowles 1976) and aquatic

(Balderston et al. 1976) contexts stimulated a large body of denitrification research in the late 1970s and early 1980s. Methods involving in situ chambers, recirculation cores, and static cores were developed and applied in both environments. A series of problems with the method were discovered beginning in the late 1970s that have made its use problematic and controversial in several contexts.

In terrestrial environments, an early powerful application of the $C_2H_2$ method was the system for perfusing soil profiles beneath in situ chambers with $C_2H_2$ developed by John Ryden and colleagues (Ryden et al. 1979a, b, Ryden and Dawson 1982). This method was used to produce estimates of field fluxes of denitrification from a series of row crop and grassland ecosystems. Use of the method never became widespread however, because it was laborious and because of the discovery of problems with $C_2H_2$ effects on soil microbial communities (detailed below).

The next major advance in application of the $C_2H_2$ method in terrestrial environments was the development of gas recirculation methods where the atmosphere of a soil core could be amended with $C_2H_2$, oxygen levels could be controlled, and $N_2O$ accumulation rates could be measured with high sensitivity (Parkin et al. 1984, Sexstone et al. 1985, Robertson and Tiedje 1987). Recirculation methods continue to be widely used (with and without $C_2H_2$) to examine relationships between various environmental factors and denitrification (Swerts et al. 1995, Butterbach-Bahl et al. 2002).

Perhaps the most widely applied $C_2H_2$-based approach in both terrestrial and aquatic environments has been "static core" methods where $C_2H_2$ is injected or added to the headspace of a sealed soil/sediment core and $N_2O$ accumulation is measured over incubation times ranging from one to 24 hours (Sørensen 1978, Ryden and Skinner 1987, Tiedje et al. 1989, Groffman et al. 1999). These methods have been applied in a wide variety of ecosystems because they are simple to carry out, allowing large numbers of samples to be run, a major advantage given the high spatial and temporal variability of denitrification rates.

Problems with $C_2H_2$ methods began to emerge in the late 1970s, the most critical of which is that $C_2H_2$ inhibits the production of $NO_3^-$ via nitrification (Fig. 1; Hynes and Knowles 1978, Walter et al. 1979, Mosier 1980). This inhibition can lead to underestimation of denitrification rates in systems with small and/or dynamic $NO_3^-$ pools, especially sediments (Seitzinger et al. 1993). Other concerns that arose in the 1970s and 1980s included (1) slow diffusion of $C_2H_2$ into fine-textured and/or saturated soils and sediments (Jury et al. 1982), (2) enhancement of soil respiration, (3) rapid decomposition of $C_2H_2$ by $C_2H_2$-degrading microbes (Yeomans and Beauchamp 1982, Terry and Duxbury 1985, Topp and Germon 1986, Flather and Beauchamp 1992), and (4) contamination of $C_2H_2$ with other gases that can affect denitrification (Hyman and Arp 1987,

Gross and Bremner 1992). In aquatic systems, there was concern about sulfide interference and low $NO_3^-$ concentrations relieving $C_2H_2$ inhibition of $N_2O$ reductase (Oremland et al. 1984, Slater and Capone 1989, Christensen et al. 1989, Rudolph et al. 1991). More recently, there has been concern about $C_2H_2$ scavenging of NO leading to underestimation of denitrification (Bollmann and Conrad 1997).

The problems with the $C_2H_2$ method caused many investigators to consider abandoning this approach to measuring denitrification, but the lack of a viable alternative method, especially one that allows for large numbers of samples to be run, has fostered its persistence (Groffman et al. 1999). We then need to ask: "Just where is the method valid?", "What have we learned about denitrification from $C_2H_2$ methods?", and "Where have these methods led us 'astray'?"

In a positive sense, $C_2H_2$ methods have given us a large number of denitrification rate estimates. Indeed, the body of denitrification rate estimates, especially for terrestrial systems, is dominated by $C_2H_2$-based methods. And these estimates are reasonably robust, especially for systems with moderate or high $NO_3^-$ levels. We have also used $C_2H_2$-based methods to learn much about environmental regulation of denitrification, e.g., control by oxygen, $NO_3^-$, carbon (C), soil moisture, pH, and other factors. These methods have also produced understanding of the "hotspot" nature of denitrification, where small areas of soil cores, ecosystems and landscapes account for a very high percentage of areal denitrification (Parkin 1987, McClain et al. 2003). A by-product of the hotspot nature of denitrification is high spatial and temporal variability, which has been well studied by taking advantage of the ability to run large numbers of $C_2H_2$-based assays (Foloronuso and Rolston 1984, Burton and Beauchamp 1985, Robertson and Tiedje 1985, Parkin et al. 1987, Robertson et al. 1988).

In a negative sense, $C_2H_2$ methods have caused us to underestimate denitrification in large numbers of primarily native ecosystems, where inhibition of nitrification by $C_2H_2$ creates an entirely inaccurate picture of denitrification. The reliance on extracted cores and disturbed samples (rather than, say, the in situ chamber methods pioneered by Ryden et al.) has led to inaccuracies in cases where the physical structure of the environment strongly regulates the substrates or end-products of denitrification. These physical problems, as well as the general alteration of substrate and product flow by $C_2H_2$, have particularly led us astray in analysis of the relative importance of the denitrification end-products (NO, $N_2O$, $N_2$; Robertson and Tiedje 1987); a topic of increasing interest due to the importance of NO and $N_2O$ to the greenhouse effect and atmospheric ozone levels.

There have been surprisingly few attempts to sort out where and when results of the $C_2H_2$ inhibition method are valid in aquatic systems. The two major potential problems, inhibition of nitrification and incomplete inhibition of $N_2O$ reduction are both very time dependent and therefore likely to become obvious over the time course of an incubation, i.e., the initial $N_2O$ accumulation rate should reflect the true denitrification rate and then gradually decline as $NO_3^-$ levels decline and/or as $N_2O$ diffuses into the $NO_3^-$ free zone and becomes reduced (Christensen et al. 1989). Consistent, linear accumulation of $N_2O$ is therefore strong evidence for the validity of the results. Measurements with $NO_3^-$ microsensors in a freshwater sediment suggested that nitrification was too transient, and $NO_3^-$ pools were too small for the $C_2H_2$ method to be valid (Jensen et al. 1993), while a method comparison study in North Sea sediment indicated that, at least in this case, coupled nitrification–denitrification could be picked up reasonably well by a short term incubation with $C_2H_2$ (Lohse et al. 1996). The maximum possible contribution of coupled nitrification–denitrification to total denitrification in sediments may also be estimated from the availability of ammonium ($NH_4^+$) and $NO_3^-$ in the water and the stoichiometry of $O_2$ consumption and $NH_4^+$ production (Christensen et al. 1990).

Is there still a role for $C_2H_2$-based methods in denitrification studies? In systems with high $NO_3^-$ concentrations, certainly yes. And certainly for measurements of denitrification potential, e.g., the widely used denitrification enzyme activity (DEA) assay (Groffman et al. 1999). This assay is still widely (and validly) used for comparisons of sites and experimental treatments in both terrestrial and aquatic environments, for evaluation of controlling factors, and even as an indirect measure of microbial functional diversity (Cavigelli and Robertson 2000).

On the other hand, would we recommend that a new graduate student, interested in understanding the importance of denitrification to the global "nitrogen cascade" (Galloway et al. 2003) or atmospheric chemistry use $C_2H_2$ methods for their study? Perhaps not, especially if they are working with natural ecosystems. In our view, $C_2H_2$ methods will continue to be used in certain circumstances, and the knowledge that we have gained from these methods will help to guide future studies, but the future is likely to be dominated by the new methods described elsewhere in this paper.

## $^{15}$N TRACER METHODS

Several methods based on $^{15}$N have been applied in studies of denitrification in soil, sediment and water, including isotope fractionation, isotope dilution, $^{15}$N mass balances, and direct measurement of $^{15}$N labeled gases upon addition of $^{15}NO_3^-$ and $^{15}NH_4^+$. The latter method, applied to intact samples of soil and sediment as well as to ground water and surface water, has been the most popular, and will be the focus of this section.

Adding $^{15}$N-labeled $NO_3^-$ to soil and measuring the production of $^{15}$N-labeled $N_2$ was the first method applied for direct quantification of denitrification in
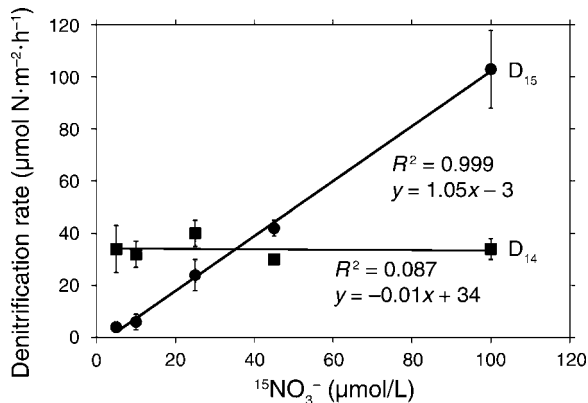
F$_{IG}$. 2. Denitrification of added $^{15}NO_3^-$ ($D_{15}$) and of in situ $NO_3^-$ ($D_{14}$) as a function of water column $^{15}NO_3^-$ concentrations. Error bars indicate standard error. The figure is adapted from Eyre et al. (2002) with permission from the Estuarine Research Federation.

environmental samples (Hauck and Melsted 1956). With some improvements, the $^{15}N$ tracer method has been considered one of the best for soil studies, but its application has been limited by the laborious procedures and expensive instrumentation required for this method compared to approaches based on $C_2H_2$ inhibition or N mass balances. A review and evaluation of the different $^{15}N$ approaches for estimating soil denitrification was given by Myrold (1990). Nishio et al. (1982, 1983) developed a technique for measuring denitrification rates in intact estuarine sediments incubated in a complete flow-through system.

An early problem identified with $^{15}N$ tracer methods was the need to know the ratio of $^{15}N$ to $^{14}N$ in the $NO_3^-$ being denitrified in order to translate tracer data into estimates of total denitrification rates. This problem was rapidly solved for soil studies by Hauck et al. (1958) who demonstrated how the ratio of the two enriched $N_2$ species, $^{14}N^{15}N$ and $^{15}N^{15}N$, reflects the ratio of $^{14}N$ to $^{15}N$ in the source, provided that $N_2$ is formed by random pairing of N atoms. More direct measures of the isotope mixing ratio were later obtained by accurate isotope analysis of $N_2O$, the obligate precursor for $N_2$ (Stevens et al. 1993, Stevens and Laughlin 2001).

In incubations of aquatic sediment, it is straightforward to add $^{15}NO_3^-$ in a controlled mixing ratio with $^{14}NO_3^-$ in the overlying water column. Using that ratio in the calculation, however, will only provide estimates of the denitrification rate of $NO_3^-$ from the water column, while coupled nitrification–denitrification in the sediment may not be detected. The immediate solution to this problem was parallel incubations with $^{15}NH_4^+$ for specific measurement of coupled nitrification–denitrification (Nishio et al. 1983). For that approach it was necessary to assume that nitrification was not $NH_4^+$ limited and that the isotope mixing of $NH_4^+$ in the nitrification zone was homogenous and measurable

(Seitzinger et al. 1993): two assumptions that are difficult to fulfill and test.

Much later, the original solution of Hauck et al. (1958), based on the ratio of $^{14}N^{15}N$ and $^{15}N^{15}N$ production, was implemented in aquatic studies (Nielsen 1992). As the ratio of $^{15}N^{14}N$ and $^{15}N^{15}N$ reflected the isotope ratio of $NO_3^-$ within the denitrification zone and not in the overlying water, the isotope pairing method could measure and differentiate denitrification of $NO_3^-$ from the water column and coupled nitrification–denitrification in one simple incubation (see the review by Steingruber et al. [2001]). Several methods comparison and evaluation studies have been published (vanLuijn et al. 1996, Risgaard-Petersen et al. 1998, Eyre et al. 2002) and a protocol handbook for application in various systems is available (Dalsgaard et al. 2000).

It is interesting that the ideas of Hauck et al. (1958) were applied in aquatic systems only after a 34-year delay, highlighting the limited interaction of terrestrial and aquatic researchers. An additional reason for the delay is probably that the pioneers in aquatic studies by chance only had access to mass spectrometers with dual collectors for $^{14}N^{14}N$ and $^{14}N^{15}N$ (Nishio et al. 1983). Production of $^{15}N^{15}N$ was therefore considered a nuisance that should be minimized by applying $NO_3^-$ with a low $^{15}N\%$. These methods are now much more widely applied in aquatic than terrestrial systems.

Addition of $^{15}NO_3^-$ or $^{15}NH_4^+$ to achieve high levels of $^{15}N$ enrichment increases the availability of N and may result in an overestimation of denitrification in systems with N limitation. These techniques are mostly used in agricultural soils and N rich waters, and here the fertilization problem is less likely to appear. In pristine systems with low in situ concentrations of inorganic N, it is more important to evaluate the levels of $NO_3^-$ present during the experiment.

Myrold (1990) summarized studies comparing $C_2H_2$ inhibition and $^{15}N$ tracer methods in soil, and they deviated by less than 30%. Typically the $C_2H_2$ method estimated about 20% higher rates than the $^{15}N$ tracers which could be explained by the bacteria compensating for the lower number of electrons accepted by $NO_3^-$ reduction to $N_2O$ only. Groffman et al. (1993) found indications of larger discrepancies in a poorly drained soil, but statistically the differences were not significant.

In aquatic applications, parallel incubations with different concentrations of $^{15}NO_3^-$ are generally recommended to check for any problems with addition effects and isotope mixing (Nielsen 1992, Steingruber et al. 2001). Fig. 2 shows a typical result for such a test. Denitrification of $^{15}NO_3^-$ is proportional to the $^{15}NO_3^-$ concentration, thus confirming that addition of extra $NO_3^-$ in the applied range from 2 to 60 μmol/L $NO_3^-$ did not interfere with the inherent denitrification activity. This linearity is typical for aquatic sediments, and reflects that the process is primarily limited by the diffusion of $NO_3^-$ from the water column to the

denitrification zone (Andersen 1977, Christensen et al. 1990). If $NO_3^-$ assimilation in the sediment had been restricting denitrification in situ, the line would intercept the x-axis at a concentration indicating a saturation threshold for assimilation. At the other end, the curve must level off at higher $NO_3^-$ concentrations as diffusion limitation is displaced by limits in process kinetics and extension of the denitrification zone. The uniform estimate of inherent denitrification ($D_{14}$) proves that there was homogenous isotope mixing and confirms the absence of $NO_3^-$ addition effects at all concentrations applied. If there was nonhomogeneous isotope mixing, the $D_{14}$ estimate would have increased with $^{15}NO_3^-$ concentration as more and more of the $^{14}N$ would be captured in the directly measured $^{14}N^{15}N$ fraction and not in the underestimated $^{14}N^{14}N$ fraction.

Theoretically, the ideal instantaneous and homogenous isotope mixing will never be achieved in the application of the $^{15}N$ tracer technique in static cores. Diffusion barriers, structural heterogeneity, bioturbation, isotope fractionation, microoxic zones with overlapping nitrification and denitrification, anammox, temporal variations, and many more phenomena will all generate a more or less nonhomogeneous isotope mixing. Nevertheless, concentration tests have repeatedly offered a comfortable range of $^{15}NO_3^-$ concentrations where the effect of nonhomogeneous isotope mixing is insignificant and low enough to avoid addition effects (see summary by Steingruber et al. [2001]). A simulation model of the method showed that neither isotope fractionation, overlapping nitrification–denitrification, or $NO_3^-$ addition effects should result in errors above 10% (Middelburg et al. 1996a). This model paper also triggered a peculiar dispute on the nature of diffusion and the physical reality of the distinction between denitrification of $NO_3^-$ from the water column and coupled nitrification–denitrification (Nielsen et al. 1996, Middelburg et al. 1996b).

Coupled nitrification–denitrification in the rhizosphere of $O_2$ excreting aquatic plants is not measured by the standard isotope pairing, as added $^{15}NO_3^-$ will not be in contact with the rhizosphere. Therefore, an alternative approach based on injection of $^{15}NH_4^+$ has been developed for the investigation of vegetated sediments (Caffrey and Kemp 1992, Ottosen et al. 1999, Welsh et al. 2000). $^{15}NH_4^+$ has also been used to measure denitrification in exposed intertidal sediments where a water column source of $NO_3^-$ for denitrification is absent (Ottosen et al. 2001).

Recent discoveries of anammox in aquatic systems and the existence of bacterial populations with large and dynamic intracellular pools of $NO_3^-$ deep in the sediment has raised unexpected new questions in our understanding and measures of $N_2$ production (Thamdrup and Dalsgaard 1992, Fossing et al. 1995). A protocol for simultaneous measurement of anammox and denitrification in sediments with $^{15}N$ tracer methods has been presented, but field validation is still lacking

(Risgaard-Petersen et al. 2003). Reduction to $NH_4^+$ seems to be the major pathway of $NO_3^-$ reduction in the $NO_3^-$ accumulating bacteria, but the question is still open whether they may denitrify as well or liberate $NO_3^-$ to denitrifiers in the sediment (see review by Schulz and Jørgensen 2001).

Aquatic systems with a complex matrix of macrophytes, microphytes, and solid substrates might be an overwhelming challenge for $^{15}N$ tracer methods. In addition to the sediment, multiple, more or less ephemeral hotspots of nitrification and denitrification could be present in biofilms, periphyton, and decomposing pieces of leaves and stems in these systems. Here the standard concentration test may well indicate that the isotope pairing method is not applicable, as the assumptions of homogenous isotope mixing and linearity between denitrification rates and bulk water $NO_3^-$ concentration will be far from valid. In these systems typical of many streams, wetlands, and littoral zones, the $C_2H_2$ inhibition technique or other methods may be superior.

Enclosure effects are a major problem shared by all methods relying on incubations. It is essential to keep hydrodynamics and concentrations of $O_2$, $NH_4^+$, and $NO_3^-$ near in situ conditions to produce accurate estimates of in situ denitrification rates. An extreme demonstration of this was seen when significant discrepancies between lake N mass balances and denitrification measured in static cores lead to the set up of large, flexible in situ enclosures for $^{15}N$ tracer experiments (Risgaard-Petersen et al. 1999). Denitrification rates in the enclosures were an order of magnitude higher than rates measured in cores because wave forces in situ forced the sediment into a semifluid state with greatly enhanced solute transport. These conditions could not be reproduced in the cores, even with vigorous stirring. In estuaries with more consolidated sediment, comparisons of denitrification rates obtained from system mass balance and static core incubations have been much more satisfactory (Nielsen et al. 1995, 2001).

Enclosure effects are not a concern in aquifer and small stream studies, and $^{15}N$ tracer methods have been applied in these systems with relatively little disturbance of in situ conditions. Smith et al. (2004) describe a method that includes measurement and modeling of the isotopic evolution of nitrate, nitrite, nitrous oxide, and nitrogen gas along natural flow paths between injection and recovery sites. Böhlke et al. (2004) and Mulholland et al. (2004) describe similar experiments at the reach scale in small streams, which are complicated by nitrogen gas inputs from air and discharging ground water. Istok et al. (1997) developed a "push–pull" method to determine in situ groundwater denitrification where a single mini-piezometer is used for both dosing and sampling.

Provided that standard tests and measures of isotope mixing ratios are integrated in the application of the $^{15}N$ tracer method in soil and sediment, there is little doubt
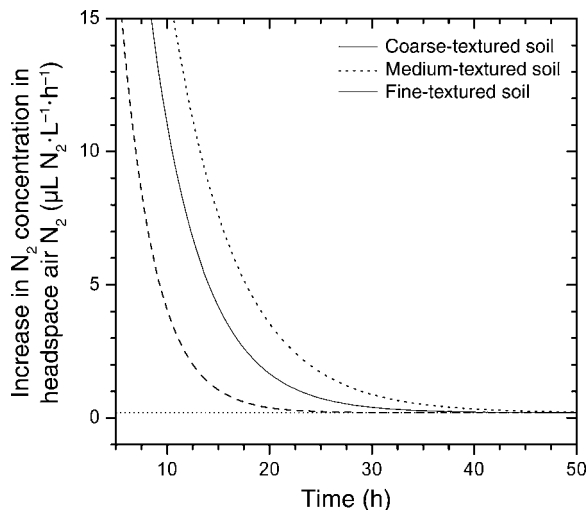
FIG. 3. Time needed to reduce the background emission of $N_2$ from a sterilized soil column to the system inherent leakage rate (0.2 $\mu$L $N_2 \cdot L^{-1} \cdot h^{-1}$) in soils of different texture. Purging rate with an $N_2$-free He–$O_2$ gas mixture (80% to 20%) is 200 mL/min; soil volume is approximately 3 L; and soil moisture is high.

that it can be used to generate reliable estimates of denitrification rates within the sampled unit. The results obtained so far have also provided a much better understanding of the regulation of denitrification and interactions with other processes and environmental conditions. The major question left for the method is how to do representative sampling in space and time and how to successfully simulate in situ conditions in experimental enclosures. Integration with landscape scale measures and models may eventually provide an answer to these challenges.

## DIRECT $N_2$ QUANTIFICATION IN TERRESTRIAL SYSTEMS

Direct measurements of $N_2$ emissions from soils are a major challenge. It is not currently possible to quantify small changes in $N_2$ concentrations due to denitrification against the atmospheric background of 79% $N_2$. Because of these analytical difficulties (which have been noted for five decades [Nõmmik 1956]), one needs to apply enclosure techniques in which either mixed or intact core samples are incubated under conditions of reduced atmospheric $N_2$ to allow for quantification of $N_2$ (and $N_2O$) production by denitrification (Scholefield et al. 1997a, Butterbach-Bahl et al. 2002) without addition of labeled substrates (e.g., $^{15}N$ compounds) or inhibitors such as $C_2H_2$. Maintaining a partial pressure of $N_2$ of a few microliters per liter requires a very gas-tight system, built from material which does not outgas $N_2$, as well as complete removal of $N_2$ stored in aggregates and pores of the soil or sediment sample before measurements begin. Such incubation systems were first developed in the early 1970s and have recently been improved (greater sensitivity; Scholefield et al. 1997a, Butterbach-Bahl et

al. 2002, Cárdenas et al. 2003). In these methods, intact or mixed samples are enclosed in a gas tight vessel which is then flushed with an artificial gas mixture (He or Ar based) to remove $N_2$. The purging time needed to ensure that all $N_2$ is removed depends on the soil/sediment texture, moisture content and the volume of the sample (Fig. 3). The entire process can be accelerated substantially by varying pressure.

In most cases, the incubation vessels are constructed so that temperature can be controlled and so that substrate or moisture additions (e.g., to imitate rainfall events) can be made. Because of the need to maintain a gas-tight system, design, construction and operation of these gas flow incubation systems can be complex and difficult. However, gas flow core methods are well suited for parameterization experiments where the effects of temperature or moisture on $N_2O$ and $N_2$ emission from soils are quantified and then used as a basis for estimating $N_2$ losses due to denitrification (Scholefield et al. 1997b, Butterbach-Bahl et al. 2002).

The need to develop "undisturbed" soil, sediment, or soil–plant incubation systems for direct measurement of N gas production was first noted by Nõmmik (1956). Since then, several attempts have been made to develop gas-tight systems with reduced partial pressure of $N_2$ for both terrestrial (e.g., Ross et al. 1964, Stefanson 1970, 1972, Van Cleemput 1971, Scholefield et al. 1997a, Butterbach-Bahl et al. 2002) and aquatic (Barbaree and Payne 1967, Seitzinger 1988) systems. In most of these studies, $N_2$ was replaced with inert gases such as He or Ar (e.g., Stefanson and Greenland 1970, Stefanson 1972, Galsworthy and Burford 1978, Wickramasinghe et al. 1978) or the $N_2$ partial pressure was reduced to <30 000 kPa and an $^{15}N$ tracer was used (e.g., Ross et al. 1964, Kralova et al. 1992). Until recent years, the major shortcoming of these methods was a rather low sensitivity, since mostly robust katharometers or thermal conductivity detectors were used for $N_2$ analysis, yielding detection limits for denitrification of at best 50 g N·ha$^{-1}$·d$^{-1}$ (e.g., Galsworthy and Burford 1978, Scholefield et al. 1997a). For this reason, the methods were mainly used in studies with highly fertilized agricultural or grassland soils (Stefanson and Greenland 1970, Wickramasinghe et al. 1978, Scholefield et al. 1997b) or in sediments with high denitrification rates (e.g., Seitzinger 1988). Recently, development of the nonradioactive pulsed-discharged helium ionization detector has significantly improved sensitivity for $N_2$, allowing for measurement of $N_2$ fluxes from soils due to denitrification as low as 2.4 g N·ha$^{-1}$·d$^{-1}$ (Butterbach-Bahl et al. 2002, Cárdenas et al. 2003).

Gas flow core techniques that allow for direct estimation of $N_2$ emission from soil due to denitrification have several advantages: (1) they are nondestructive; (2) no label (e.g., $^{15}N$) needs to be introduced to the soil or sediment system, which is of significant importance for studies of semi-natural and natural environments; (3) no inhibitor needs to be used; and (4) it can be

a very sensitive method for measurements of $N_2$ emissions, with a detection limit <1 kg $N_2$-N·ha$^{-1}$·yr$^{-1}$. Furthermore, provided that an adequate gas-tight incubation system is available, it is a rather simple method, which can easily be used for quantification and parameterization experiments of denitrification in soils, sediments and open water. Based on a series of experiments and a comparison of $N_2O$ fluxes measured under ambient and $N_2$-free atmospheres, Scholefield et al. (1997$a$) and Butterbach-Bahl et al. (2002) concluded that alteration of the soil atmosphere to remove $N_2$ does not affect the magnitude of microbial processes involved in N trace gas production. Shortcomings of gas flow core techniques are mainly associated with the time needed to establish an $N_2$ free atmosphere and with the complex technology needed to ensure the gas-tightness of the incubation vessel and the precise and uncontaminated sampling of gas samples from the headspace of the vessel.

The gas flow core technique is increasingly being used for the quantification of $N_2$ (and $N_2O$) production by denitrification in a wide range of terrestrial and marine ecosystems. There are also strong prospects for combining gas flow core methods with inhibitors and $^{15}N$ tracers to address specific research questions related to environmental regulation of denitrification or in validation studies of other methods. A major advance will be the development of methods to measure NO flux in gas flow core techniques so that all three gaseous denitrification products can be measured simultaneously.

## Direct $N_2$ and $N_2$:Ar Measurements in Aquatic and Marine Systems

It is generally easier to measure the end-product of denitrification in aquatic and marine systems than it is in terrestrial systems. This is partly a result of the fact that soils exchange gas freely with the atmosphere, whereas deep saturated systems tend to be more closed to gas exchange. Also, because of lower background concentrations in water, denitrification typically produces a $N_2$ concentration gradient on the order of 1000 times greater than that produced in air, making it possible to measure realistic denitrification rates with an analytical precision in the range of 0.03–0.3%. This is attainable using mass spectrometry (Kana et al. 1994) or, at the lower precisions, gas chromatography (An and Joye 1997). Such measurements were made in the early 1960s, but subsequent development and application of direct $N_2$ methods was slowed by the limited availability of instrumentation with the requisite precision.

Seitzinger et al. (1980) made the first extensive direct $N_2$ flux measurements using enclosed core incubations with a pre-incubation period to remove $N_2$ from the core by flushing with $N_2$-free gases. These methods were instrumental in defining the problems with acetylene inhibition of coupled nitrification-denitrification, but there were concerns about the long pre-incubation

period required to remove $N_2$ creating "bottle effects" and decoupling of the sediment sample from water column $NO_3^-$ and C flow (Seitzinger et al. 1993, Nowicki 1994).

In recent years, low-cost mass spectrometers have become available and this has spurred interest among limnologists and oceanographers in measuring denitrification by $N_2$ production without altering background $N_2$ concentrations. Because mass spectrometric measurements are more precise for gas ratio data, most direct $N_2$ studies are actually based on $N_2$:Ar ratio measurements, and the technique is also referred to as the $N_2$:Ar method.

A number of approaches have been taken to detect $N_2$ production from denitrification in aquatic and marine systems. These approaches fall into two general categories: enclosure experiments designed to measure $N_2$ fluxes and open system measurements of ambient $N_2$:Ar. It is worth noting that both approaches measure *net* denitrification which is the difference between gross denitrification and gross N fixation. Methods have been described that separate these fluxes by the use of isotopes (An et al. 2001) or inhibitors (Joye and Paerl 1993). A brief description of each direct $N_2$ approach as used in contemporary studies follows.

Enclosure experiments are typically used for studies involving sediment, which is the site of most denitrification activity. The method usually involves either a benthic chamber (Devol 1991) or closed-chamber sediment core sample (Cornwell et al. 1999) that is subsampled over time for dissolved gases in the water above the sediment. The $N_2$ flux is determined from the rate of change in $N_2$ concentration. If $N_2$:Ar data are used, the change in $N_2$ ($\Delta N_2$) is determined from $\Delta N_2$:Ar) $\times$ [Ar], where [Ar] is the Ar concentration at air saturation determined from solubility equations (Weiss 1970, Colt 1984). In practice, changes in [$N_2$] can usually be resolved in incubations lasting <12 h. For oxic water, it is desirable to measure changes in oxygen concentration within the chambers and confine the analysis of $\Delta[N_2]$ to the period when [$O_2$] is greater than ~50% of saturation. This minimizes the effect of reduced oxygen availability on coupled nitrification denitrification activity (Jenkins and Kemp 1985). This approach is readily scaled to multiple cores for site replication and/or for sampling multiple environments.

A second type of enclosure experiment involves sediment cores with flow-through headwater (Kana et al. 1998). Denitrification rates are determined from measurements of the difference in $N_2$:Ar between inlet and outlet water, flow rate, and core surface area. This type of setup allows the experiment to be run under steady-state conditions and allows perturbation experiments that are not possible with batch experiments. For routine determinations of denitrification rates there are disadvantages of flow-through experiments including a more elaborate setup and greater incubation time (minimum of several days).

With proper care, enclosure experiments can provide accurate rates of denitrification in environmental samples. For studies of ecosystem denitrification, the rates determined in centimeter- and hour-scale experiments must be extrapolated to kilometer- and months-scale estimates. There is inherent uncertainty in this process, because it is usually not possible to accurately assess the environmental heterogeneity of the system. An alternative approach, one that integrates large scale systems, is through the measurement of $N_2$ in water samples taken from the environment in question. We refer to this approach as in situ or environmental measurement studies.

In situ measurement studies depend on the ability to detect, in the ambient water, $N_2$ that is contributed by the denitrification process. This is made difficult by the high concentration of $N_2$ in the aqueous environment (400–800 μmol/L) and physical processes related to mixing, air exchange, and water volume in contact with the area of denitrification. Nevertheless, some environments have proved amenable to this approach and there is active interest in exploiting this inherently large-scale integrated measurement approach. Several different systems have been studied using in situ measurements. Within the oceans, suboxic zones exist within the water column in specific regions and $N_2$:Ar ratios within those zones have been found to be up to 3% in excess of that expected from physical processes owing to in situ water column denitrification (Codispoti et al. 2001). In river reaches, two related approaches have been taken to study denitrification. One study measured the change in $N_2$:Ar with distance (and related time) and corrected for air exchange measured using a volatile tracer (Laursen and Seitzinger 2002). Another study used time-series measurements of diel $N_2$:Ar and corrected for air exchange and groundwater discharge (McCutchan et al. 2003).

In contrast to surface waters that may be turbulently mixed and in exchange with air, water trapped in sediments and aquifers may be somewhat more closed with respect to external fluxes. Some of the earliest studies of excess $N_2$ production in sediments were done by analyzing dissolved gases in pore water (Reeburgh 1969, Barnes et al., 1975, Nishio et al. 1981). Denitrification in aquifers has been studied from dissolved gas analyses of groundwater samples from a variety of hydrogeologic environments in which the gases were under sufficient hydrostatic pressure to prevent major loss or exchange with air (Vogel et al. 1981, Wilson et al. 1990, Böhlke and Denver 1995, Böhlke et al. 2002, McMahon et al. 2004*a, b*). Blicher-Mathiesen et al. (1998) describe a study of shallow groundwater in a riparian-wetland system in which denitrification was accompanied by substantial degassing. In this case, changes in groundwater $N_2$ and Ar along a flow path were used to adjust the apparent $N_2$ losses for ebullition (determined from Ar losses) and determine the amount of $N_2$ excess from denitrification.

Measurement technologies play an important role in these studies. Dissolved $N_2$ has been measured using gas chromatography (GC) and mass spectrometry (MS). The GC technique has precision on the order of 0.1–0.3% (An and Joye 1997), and has been used to measure $N_2$ flux in sediment core experiments kept at ambient $[N_2]$ (Devol 1991) and reduced $[N_2]$ (Seitzinger et al.1980, Nowicki 1994), as well as in groundwater, where denitrification contributions to $N_2$ commonly accumulate in a closed system. GC is used less frequently now for surface-water studies because of the availability of comparably priced mass spectrometers that offer better precision and water handling. There are two approaches to measuring dissolved gases by mass spectrometry. One method involves an off-line degassing step and subsequent analysis of a head-space gas sample (Emerson et al. 1991, 1999). A second method involves an inline degassing process using a membrane separator between a flowing sample stream and the MS vacuum inlet (Kana et al. 1994). The later membrane inlet (or introduction) mass spectrometry (MIMS) technique has become popular for denitrification measurements because it provides rapid (<2 min) measurements on small (<7 mL) samples with no preprocessing and provides high precision (~0.03% for $N_2$:Ar ratios) (Kana et al. 1994).

Although determinations of denitrification using $N_2$:Ar data go back to the 1950s (Richards and Benson 1961), the mass spectrometry technique was not available to most laboratories. GC measurements were applied in the 1960s and 1970s on sediment pore water samples, which exhibited large gradients with depth (Reeburgh 1969, Barnes et al. 1975). The first direct flux measurements on sediment cores were done by Seitzinger et al. (1980) using a technique involving the reduction in $N_2$ in the chamber (cf. part 4). This allowed detection of $N_2$ fluxes by GC. The introduction of the relatively simple MIMS technique (Kana et al. 1994) coupled to low-cost MS detectors has led to growing interest in the development of both core incubation techniques (e.g., Kana et al. 1998, Cornwell et al. 1999) and open system measurements (Laursen and Seitzinger 2002, McCutchan et al. 2003). A MIMS "probe" was also developed to measure depth profiles of sediment gases (Thomas and Lloyd 1995).

Detection of the end product ($N_2$) is a direct measure of net denitrification. Interferences are limited in closed systems to biological N fixation when experiments are properly controlled for solubility-driven fluxes. Current incubation methods allow replication and relatively efficient use of resources and time. The method is also amenable to large-scale integrated measurements of aquatic and marine systems.

Eyre et al. (2002, but see also Kana and Weiss 2004) pointed out a number of concerns with the core incubation technique where care is needed to minimize errors due to uncontrolled physical fluxes. For core incubations, there is no completely adequate control

that can account for all solubility flux errors and this generally sets the lower limit of detection. For some environments (e.g., oceanic oxygen minimum zones), open system measurements of $N_2$ excess can only be converted to rates when water mass age tracers are also measured to estimate the time variable associated with the accumulated $N_2$. In some cases (e.g., river systems), the time variable is built into the method, but the environmental signal may be dominated by physical transport.

The prospects for methods based on $N_2$ detection of denitrification in aquatic and marine systems are excellent. The combination of direct $N_2$ measurements with isotope ($^{15}N_2$) methods is just now being explored for separating denitrification from N fixation in systems where they coexist (An et al. 2001, Eyre et al. 2002). Currently, there are only a handful of in situ measurements in open systems (streams and rivers), but the prospect for expanding these measurements, and improving techniques to account for physical transport and time units, are good. The great challenge remains to apply these methods to unsaturated terrestrial systems (e.g., soils), where the $N_2$ background is high and exchanges rapidly with air.

### Mass Balance Approaches

Mass balance has long been used to estimate various terrestrial N fluxes at the field scale (Allison 1955), and more recently at stream reach, watershed, or regional scales (e.g., Goolsby et al. 1999, David and Gentry 2000, Pribyl et al. 2005). In some studies, denitrification is estimated using literature values and is one component of the mass balance (Goolsby et al. 1999). Others estimate denitrification by difference in the mass balance (e.g., David and Gentry 2000, Pribyl et al. 2005).

In the marine environment, Johnstone (1908) first speculated that the imbalance between the amount of N entering and leaving the North Sea could be due at least in part to denitrification but did not calculate a rate. Nearly 50 years later, Emery et al. (1955) used the difference in a mass balance of the world's oceans to estimate that nearly $70 \times 10^{12}$ g of N per year were lost by denitrification. More recently, estimates of denitrification by the difference in a mass balance have been carried out in semi-enclosed seas and bays (Billen et al. 1985, Wulff et al. 1990, Kamp Neilsen 1992), however, due to the difficulty of accurately measuring water fluxes it is more common to compare laboratory measurements of denitrification to mass balance calculations of major fluxes to assess the magnitude of denitrification (e.g., reviewed for estuarine systems by Nixon et al. 1996). In rare cases, both laboratory measurements and mass balance calculations have been compared in the same system (Nielsen et al. 1995, Kelly 1997).

To correctly estimate denitrification by mass balance, direct measurements or estimates of all other N fluxes and changes in storage for the system are needed. Frequently, it is assumed that the system is at steady state with regard to large internal pools and only inputs and outputs are quantified. Major inputs to terrestrial systems typically include fertilizer, biological $N_2$ fixation, and atmospheric deposition, with outputs including crop or biomass harvest and export from the area and leaching/runoff/riverine export (Fig. 4). Typically measured inputs to marine systems include: atmospheric deposition, inputs from "nonpoint" sources such as road runoff, and direct sewage discharge; with outputs assumed to be due to fisheries harvest, oceanic flushing, and sediment burial. More recently the potential importance of biological $N_2$ fixation and N inputs from the oceanic endmember are being recognized and included in budgets.

For most terrestrial fertilized systems considered using mass balance, inputs are typically much greater than outputs, and the difference can be attributed to denitrification (often both terrestrial and in-stream or reservoir/lake, depending on the scale). Generally the change in storage is assumed to be very small and is ignored, because it is difficult to accurately estimate across the short time scales of most mass balance studies. However, storage pools of N in many ecosystems, particularly midwestern agricultural watersheds, are often extremely large (e.g., 10 000 to 20 000 kg N/ha in the upper 1 m of soil [David et al. 2001]), so that even relatively minor changes in storage could affect flux estimates of denitrification. In addition, groundwater residence times may be long enough (decades or more) that recharge and discharge fluxes of N may be imbalanced simply because of changes in land use and agricultural practices on decadal time scales (Böhlke 2002, Lindsey et al. 2003). For pristine terrestrial systems, where external N inputs and outputs are small, mass balance approaches lack the sensitivity to pick up small, but important denitrification fluxes.

Regional denitrification has recently been estimated by mass balance in the midwestern United States, where large fluxes of N are present, and can be used to illustrate the method. In this example, the net anthropogenic N input (NNI) approach used by Howarth et al. (1996) was extended further by using estimates of riverine export and obtaining denitrification by difference as the only remaining, important, N flux unaccounted for. The NNI approach estimates residual N that could be lost from an ecosystem by riverine export or other fates, and focuses on relating inputs to riverine losses. In this midwestern example, David and Gentry (2000) and David et al. (2001) developed recent and historical N mass balances treating the state of Illinois as a large watershed. In their approach, major inputs and outputs were estimated, including denitrification (Fig. 4). Inputs included fertilizer N, soybean $N_2$ fixation, other $N_2$ fixation, and atmospheric deposition, whereas outputs included net grain export, animal product export, riverine export, and field + in-stream denitrification. Denitrification was assumed to be equal to inputs not lost to grain/animal exports + riverine losses,
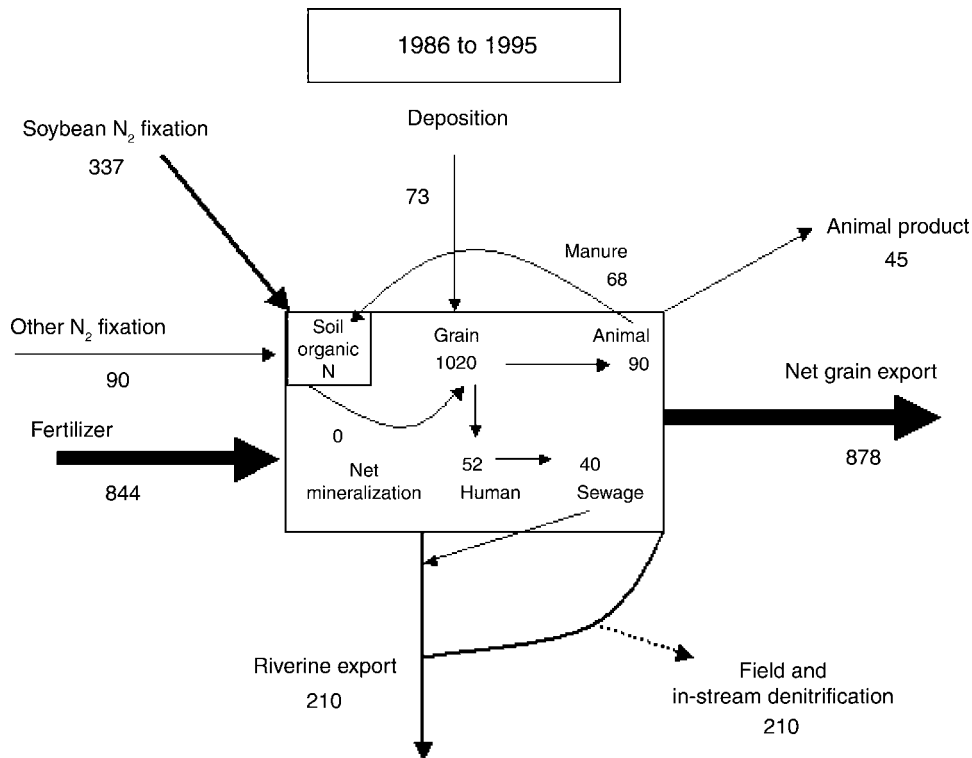
Fig. 4. Nitrogen mass balance for treating the state of Illinois, USA, as a watershed, showing average fluxes for a ten-year period. Line thickness is proportional to flux rate. Units are Gg N/yr (1 Gg = $10^9$ g). The figure is adapted from David et al. (2001) with permission from the *The Scientific World*.

balancing the inputs and estimated outputs in the mass balance. In this approach, net soil mineralization for the ten-year period was assumed to be zero, indicating that soils were not gaining nor losing N. An earlier version of this mass balance (David and Gentry 2000) estimated denitrification using literature values, and developed values of 87 000 and 132 000 Mg N/yr for field and in-stream denitrification, respectively. However, recent measurements of denitrification in Illinois streams reported by Royer et al. (2004) suggested that in-stream denitrification may have been overestimated by the mass balance approach. Therefore, the lumped value of David et al. (2001) is probably a better indication of our current knowledge of the magnitude of denitrification in agricultural landscapes, and our uncertainty of whether it is occurring primarily in fields or in streams/reservoirs. Mass balance at this scale doesn't tell us much about where denitrification is occurring or about the temporal dynamics of denitrification (McIsaac and Hu 2004).

All of the issues of using mass balance to measure denitrification in terrestrial systems apply to aquatic systems and can be illustrated with the estimates of the global oceanic N budget (Table 1). Since Emery et al.'s (1955) initial budget we have not only discovered the importance of N fixation but also revised our estimates of all of the original sources and sinks by at least a factor of 2. Estimates of N fixation have varied by more than a

factor of 10 over the last 15 years (see review by Karl et al. 2002) so measuring denitrification using mass balance becomes problematic. However, perhaps a more serious obstacle is that it isn't known whether or not the oceans are at steady state. McElroy (1983) has proposed that the oceans oscillate between storing N during glacial periods and losing N during interglacial periods. Gruber and Sarmiento's budget (1997; Table 1), suggests that the ocean is more or less in balance while Codispoti et al. (2001), (Table 1), argue that denitrification greatly exceeds N inputs due to human disturbances of the global N cycle. Ocean pools are so large there is little chance that changes in oceanic N stores due to human activities over the last 100 years could be measured (Galloway et al. 1995). Hence, estimates of denitrification by difference in mass balances are not likely to resolve the critical question of whether or not the oceans are at steady state.

In conclusion, the mass balance approach is quite limited in providing quantitative rate measurements of denitrification. We have chosen examples that may represent "worst cases." In systems where inputs, outputs, and storages can be very well constrained, such as reservoirs, there have been successes in measuring denitrification by mass balance (e.g., Jossette et al. 1999). For all systems, mass balance can provide some insight into the potential importance of denitrification.

TABLE 1. A comparison of global nitrogen budgets since 1955 showing how the calculated magnitude of denitrification would have changed over time if estimated by mass balance.

| | Global N (Tg), by study | | | |
| --- | --- | --- | --- | --- |
| | | Gruber and Sarmiento (1997) | | |
| Source | Emery et al. (1955) | Preindustrial | Modern | Codispoti et al. (2001) |
| Inputs | | | | |
| Land drainage | 19 | 41 | 76 | 76 |
| Precipitation | 59 | 15 | 30 | 86 |
| N fixation | | 125 | 125 | 125 |
| Total inputs | 78 | 181 | 231 | 287 |
| Sinks (excluding denitrification) | | | | |
| Sediment burial | 8.6 | 15 | 25 | 25 |
| Fisheries, guano | | | | 1 |
| N₂O loss | | | 4 | 46 |
| Total non-denitrification sinks | 8.6 | 19 | 29 | 32 |
| Difference | 69.4 | 162 | 202 | 255 |
| Denitrification estimate | | 165 | 175 | 450 |

*Notes:* Emery et al. (1955) estimated denitrification by difference; the other authors used independent estimates of denitrification. All values are in Tg ($=1 \times 10^{12}$ g). Empty cells indicate variables that were not measured in the analysis in that column.

These approximations can be improved if multiple years of data are used and averaged, rather than trying to estimate balances for each year or on even shorter time scales. The method is also attractive in that it is one of the few that can be applied at a range of scales, from small plots to large watersheds, lakes, estuaries, and open marine systems. Indeed, it is the large amounts of "missing N" in regional-scale mass balances (Howarth et al. 1996) that has motivated much of the increased recent interest in denitrification. However, until we are able to improve upon our ability to quantify all fluxes accurately, and to accurately determine changes in N storage pools, the estimates by mass balance will have to be verified by direct measurements. Therefore, the future application of mass balance for providing a benchmark measurement of denitrification rates in terrestrial and aquatic ecosystems is still quite poor. Likely they are most useful for constraining estimates produced by other methods, e.g., if a new laboratory method suggests that denitrification is much larger than estimates of system inputs or changes in storage determined from mass balance, this would be a sure sign that there is a problem with the new method.

### Stoichiometric Approaches

Stoichiometric methods have been extensively used to estimate denitrification in aquatic environments. The basis of the stoichiometric method arose from the observations of Redfield (1934) that marine phytoplankton had the same elemental ratio as that found dissolved in deep ocean seawater. This ratio (106 C:16 N:1 P, in molar units), is now referred to as the Redfield ratio. Estimates of denitrification using stoichiometery still rely on a mass balance approach but because of this consistency in elemental composition, the behavior of N is referenced to another element whose fluxes and/or storages can be more accurately determined. The method has been largely restricted to use in aquatic systems where the elemental concentration of aquatic primary producers is believed to be far more constrained than in the terrestrial environment. In the terrestrial environment, the presence of woody plants leads to wide variations in C:N:P ratios, and even non-woody species show considerable plasticity in elemental ratios (Sterner and Elser 2002). Recent analyses have confirmed that the elemental ratio of marine phytoplankton varies within relatively narrow ranges, however, freshwater phytoplankton are far less constrained in their elemental ratios than previously assumed (Sterner and Elser 2002, Hecky et al. 1993), hence there is more potential for error when applying stoichiometric techniques in freshwaters.

In practice, the stoichiometric method assumes that when organic matter with a known elemental ratio is decomposed using oxygen, or some other electron acceptor, there will be a set of products with a defined elemental composition (Table 2). Based upon the calculated consumption of electron acceptors, or the appearance of phosphate or $CO_2$, the expected dissolved inorganic N can be calculated. The difference between the expected and the observed DIN is then used to calculate denitrification. When applied to large oceanic water masses it is normally assumed that the water was originally in equilibrium with the atmosphere at the temperature and salinity observed in situ and the difference between the observed and measured oxygen concentration is the apparent oxygen utilization (AOU) for decomposition. A correction must also be made for the quantities of nutrients present in the water mass (preformed nutrients) at the time it sank from the

TABLE 2. Stoichiometric reactions used for the calculation of denitrification rates by various authors.

| Eq. no. | Equation |
| --- | --- |
| 1 | $(CH_2O)_{106}(NH_3)_{16}(H_3PO_4)_1 + 138O_2 \rightarrow 106CO_2 + 16NO_3^- + H_2PO_4^- + 122H_2O + 17H^+$ |
| 2 | $(CH_2O)_{106}(NH_3)_{16}(H_3PO_4)_1 + 84.8NO_3^- + 99.8H^+ \rightarrow 106CO_2 + 42.2N_2 + 16NH_4^+ + H_2PO_4^- + 148.4H_2O$ |
| 3 | $(CH_2O)_{106}(NH_3)_{16}(H_3PO_4)_1 + 94.4NO_3^- + 93.4H^+ \rightarrow 106CO_2 + 55.2N_2 + H_2PO_4^- + 177.2H_2O$ |
| 4 | $(CH_2O)_{106}(NH_3)_{16}(H_3PO_4)_1 + 53SO_4^{-2} + 121H^+ \rightarrow 106CO_2 + 53H_2S + 16NH_4^+ + H_2PO_4^- + 106 H_2O$ |
| 5 | $C_{61}H_{97}O_{42}N_{16} + 60.2NO_3^- + 60.2H^+ \rightarrow 61CO_2 + 38.1N_2 + 78.6H_2O$ |
| 6 | $(CH_2O)_{106}(NH_3)_{16}(H_3PO_4)_1 + 106O_2 + 15H^+ \rightarrow 106CO_2 + 16NH_4^+ + H_2PO_4^- + 106H_2O$ |

*Notes:* Eqs. 1–4 are essentially those of Richards (1965), and Eq. 5 is presented in Codispoti et al. (2001). Eq. 6 is used by Nixon et al. (1976). All equations except Eq. 5 assume organic matter of a Redfield stoichiometry. The equations have been slightly rewritten to more clearly represent the changes in alkalinity and the form of the products at seawater pH. The ratios of some of the products and reactants are reported below. DIC represents all forms of inorganic carbon ($CO_2$, $H_2CO_3$, $CO_3$) and is represented by the product $CO_2$ in the equations. Following convention, the ratio of DIC:$O_2$ (often referred to as the RQ) is calculated on a mole : mole basis, while the ratio of oxygen consumed to DIN or P produced is calculated on an atom : atom basis. DIN refers to either $NH_4$ or $NO_3$ released by the reaction. The notations ($NO_3$) and ($SO_4$) are used to denote nitrate and sulfate when they are consumed as electron acceptors. Ratios involving $N_2$ produced by the reaction are also expressed on an atom : atom basis. NC indicates that the ratio cannot be calculated because the denominator is 0. Notes on the individual equations are as follows:

Eq. 1.—The reaction for aerobic respiration where the mineralized N is nitrified. Note that it is assumed in this reaction that there is no $CO_2$ uptake by nitrifying bacteria. Fenchel and Blackburn (1979) suggest that ($CO_2$ fixed)/($NH_4$ oxidized) = 0.2. Ratios: DIC:$O_2$ = 0.768, DIC:DIN = 6.625, O:DIN = 17.25, DIC:P = 106, O:P = 276, DIN:P = 16.

Eq. 2.—The reaction for decomposition using nitrate reduction where the N in the organic matter is mineralized to $NH_4$. Ratios: DIC:($NO_3$) = 1.25, DIC:DIN = 6.625, DIC:($N_2$-N) = 1.25, DIC:P = 106, ($N_2$-N):P = 84.8, DIN:P = 16.

Eq. 3.—The reaction for decomposition using nitrate reduction where the N mineralized from the organic matter is converted to $N_2$ though the reaction $5NH_3 + 3HNO_3 \rightarrow 4N_2 + 9H_2O$. Ratios: DIC:($NO_3$) = 1.12, DIC:DIN = NC, DIC:($N_2$-N) = 1.12, DIC:P = 106, ($N_2$-N):P = 110.4, DIN:P = 0.

Eq. 4.—The reaction for decomposition using sulfate reduction. Ratios: DIC:($SO_4$) = 2, DIC:DIN = 6.625, ($SO_4$):DIN = 3.31, DIC:P = 106, ($SO_4$):P = 53, DIN:P = 16.

Eq. 5.—The reaction for the decomposition of ideal proteins using nitrate reduction and assuming all of the mineralized N is converted to $N_2$ though the reaction shown in the footnote for Eq. 3. Ratios: DIC:($NO_3$) = 1.01, DIC:DIN = NC , DIC:($N_2$-N) = 1.01, DIC:P = NC, ($N_2$-N):P = NC, DIN:P = NC.

Eq. 6.—The reaction for decomposition using oxygen where the mineralized DIN is not nitrified. Ratios: DIC:$O_2$ = 1, DIC:DIN = 6.625, O:DIN = 13.25, DIC:P = 106, O:P = 212, DIN:P = 16.

surface. The preformed nutrient content is usually estimated from hydrographic data, and phosphate : AOU relationships.

The stoichiometric approach to calculating denitrification was first illustrated using previously published data from the Black Sea and the Cariaco Trench by Redfield et al. (1963) and expanded upon by Richards (1965). Samples were taken from the anoxic bottom water in these relatively stagnant basins, where relatively high concentrations of metabolites build up facilitating the measurements. It is impressive to see that the authors were able to compare these stoichiometric estimates to direct $N_2$ measurements with reasonable success. However, because water turnover times were not well known they did not calculate an areal rate. Later, Cline and Richards (1972) calculated a denitrification rate in an oxygen minimum zone of the Eastern Tropical North Pacific Ocean from apparent oxygen utilization (AOU), $NO_x$, and phosphate relationships and demonstrated that denitrification in the oxygen deficient regions of the world was likely of sufficient magnitude to close the oceanic N budget of Emery et al. (1955). For these calculations atmospheric exchange and interactions with the sediments are assumed to be unimportant, and denitrification and fixation are assumed to take place in different water masses. Since then, stoichiometric relationships have been widely applied to calculate denitrification in stratified water

bodies and in oceanic oxygen minimum zones. Modern estimates have been improved by better analytical methods and by the development of tracers such as $^3H{:}^3He$ to determine the turnover time of the water masses. However, in spite of these improvements there is still considerable uncertainty in the estimates of global oceanic water column denitrification due to uncertainties in the stoichiometry of the reactions, the stoichiometry of the organic matter being decomposed, and possible interactions with sediments and the atmosphere (e.g., see Table 2; Codispoti et al. 2001).

Stoichiometric relationships have also been used to calculate denitrification occurring within sediments. Typically cores or benthic chambers are used to enclose a small quantity of sediment and overlying water and the exchange of gases and nutrients across the sediment–water interface is measured. Nixon et al. (1976) was among the first to note that in these incubations the ratio of oxygen consumption to N release, was often much greater than expected based upon the Redfield ratio and suggested that the missing N may have been denitrified. Many studies have now confirmed that benthic fluxes are usually deficient in N and that the amount of missing N can be considerable. Independent methods of measuring denitrification have shown that coupled nitrification/denitrification is a major process in many sediments (Seitzinger 1988, Devol 1991).

When used to measure denitrification in sediment cores or chambers, rather than open water columns, the stoichiometric method has the advantage that atmospheric exchange is eliminated and water turn over is either zero or easily measured. However, the technique still relies on the same assumptions as those described for oceanic water masses, which may be less likely to be met in sediment systems. First, seasonal or permanent storage of some compounds is more likely to occur in sediments than in the open water, altering the expected ratios. Because of difficulties measuring small changes in DIC, oxygen historically was used as a measure of denitrification. Oxygen uptake includes anaerobic metabolism if the reduced end products are reoxidized. If reduced species from anaerobic respiration are permanently stored or lost in a reduced form, oxygen uptake will underestimate the decomposition rate. In marine systems dominated by sulfate reduction, the permanent storage of reduced sulfur is usually rather small, less than 10% (Jorgensen 1982), but there can be substantial discrepancies in sediments with high sedimentation rates (Chanton et al. 1987, Zimmerman and Benner 1994). Seasonal storage and reoxidation can be extremely important even in sediments where there is little permanent burial (Cornwell and Sampou 1995, Giblin et al. 1997), so calculated denitrification rates will be in error unless integrated over an annual time frame. Difficulties with using oxygen as a measure of decomposition have lead investigators to rely on DIC fluxes as techniques have improved but DIC fluxes are subject to potential artifacts from carbonate precipitation or dissolution. In some cases, this can be checked by using high precision measurements of sediment calcium fluxes.

Nixon et al. (1976) also noted aberrant N:P flux ratios from sediments and this has been widely confirmed. However, phosphorus fluxes are rarely used to calculate denitrification in core incubations. Although P fluxes do appear to follow Redfield stoichiometery in some systems (Nixon et al. 1976, S. V. Smith et al. 1991); in other systems DIC:P and O:P ratios indicate permanent P burial (Hopkinson et al. 1999). Large seasonal changes in P storage and release due to redox processes have been well documented in lakes (Mortimer 1941) and can also occur in fully marine systems that do not experience water column anoxia (Giblin et al. 1997, Hopkinson et al. 2001). Finally, while DIN usually does not exhibit much change in storage over the season in marine or freshwater systems, in sediments undergoing seasonal changes in salinity there may be a fairly large temporary storage and release of adsorbed $NH_4^+$ from particles leading to aberrant O:N or DIC:N ratios (Hopkinson et al. 1999).

A second problem in using stoichiometric methods in sediments is uncertainty about the C:N ratio of the substrate being metabolized. In lakes and estuaries, there may be substantial inputs of allochthonous terrestrial material of a high C:N ratio and, in deep systems, the sinking particles may have undergone substantial decomposition in the water column. In the deep ocean, sinking particles have a higher C:N ratio than do live phytoplankton (Martin et al. 1987). If the material being decomposed has a C:N ratio substantially above the Redfield ratio, stoichiometric techniques will overestimate the denitrification rate. One approach to this problem is to use the C:N value found in the sediments themselves to determine the ratio of the decomposing organic matter (Hopkinson et al. 1999). Using the C:N ratio of the sediment may yield a minimum denitrification rate as the few studies which have measured the mineralization rate directly have usually found that the C:N ratio of material mineralized from surface sediments is substantially lower than that from bulk sediment (Burdige 1991, Zimmerman and Benner 1994).

Both water column suboxic zones and sediments are somewhat physically constrained systems. There has also been an effort to use a combination of mass balance and stoichiometric calculations to assess denitrification in open bays and estuaries. S. V. Smith et al. (1991) used water and salt budgets to estimate water exchange and mixing between Tomales Bay, California, and the open ocean and then calculated the net exchange of C, N, and P. High atmospheric exchange precluded direct measurement of water fluxes of $O_2$ and $CO_2$ and therefore total net metabolism could not be measured directly. Instead, they used the net P export as a measure of net ecosystem production (primary production minus respiration) and concluded that the bay was heterotrophic. The mismatch from Redfield stoichiometry in N:P fluxes was then used to calculate net denitrification (N fixation minus denitrification). This approach has been expanded to form the Land Ocean Interactions in the Coastal Zone (LOICZ) guidelines for constructing such budgets (Gordon et al. 1996) and more than 100 budgets have been published (LOICZ budgets; *available online*).[11] The simple box modeling approach used in this project has potentially large errors associated with it (Webster et al. 2000). But in systems where the hydrodynamics are well constrained and there is good spatial and temporal coverage of nutrient data, whole-system stoichiometry may be a useful way to constrain estimates of the importance of the net difference between denitrification and N fixation over large areas.

In conclusion, although stoichiometry is essentially a mass balance technique, referencing N to other elements provides additional constraints for estimating denitrification that make it useful in some aquatic situations. It is important to recognize that in many cases, however, the measurement may be yielding the net difference between fixation and denitrification, not simply denitrification. Large errors can be introduced if the stoichiometry of the decomposing organic matter differs from what is assumed or if the sediment is acting as an unmeasured sink or source of key elements. Whenever possible these should be measured. For small scales,
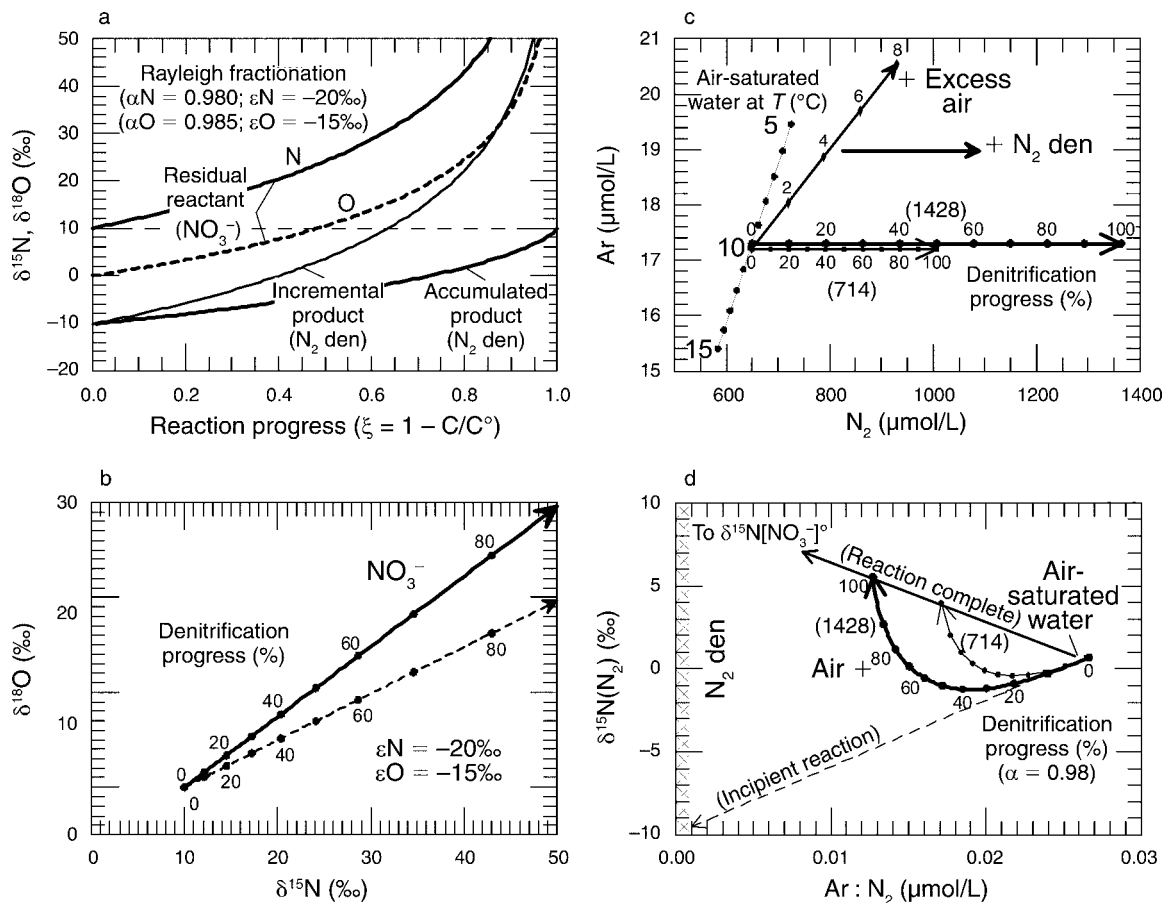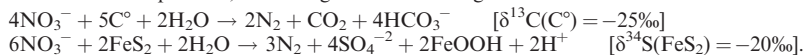
[11] ⟨http://data.ecology.su.se/MNODE/⟩

FIG. 5. Hypothetical stable isotope effects of denitrification in closed aquatic systems (modified from Böhlke 2002). Isotopic compositions and fractionation factors in these diagrams are realistic but not necessarily representative. (a, b) Isotope fractionation of N and O in nitrate and $N_{2den}$ with reaction progress (see Eqs. 3–6). Qualitatively similar effects could be caused by assimilation of nitrate during primary production. (c, d, e, f) Changes in the concentration and isotopic composition of nitrate and $N_2$, where the $N_2$ is a mixture of the denitrification component and typical atmospheric component (10°C, no excess air, at sea level). (g, h) Changes in the concentration and isotopic composition of DIC and sulfate in mixtures of pre-denitrification components and denitrification components, according to the following stoichiometric mass transfers:

$$4NO_3^- + 5C° + 2H_2O \rightarrow 2N_2 + CO_2 + 4HCO_3^-  \quad [\delta^{13}C(C°) = -25‰]$$
$$6NO_3^- + 2FeS_2 + 2H_2O \rightarrow 3N_2 + 4SO_4^{-2} + 2FeOOH + 2H^+ \quad [\delta^{34}S(FeS_2) = -20‰].$$

Panels (c)–(h) include hypothetical reaction paths for two waters, one with initial nitrate concentration equal to the maximum contaminant level of 10 mg/L as N (714 μmol/L) and one with twice that amount (1428 μmol/L). $\delta^{15}N[NO_3^-]°$ and $\epsilon[N]_{den}$ are assumed to be +10‰ and −20‰, respectively.

such as sediment cores, some of the newer isotopic tracer and direct gas techniques should provide superior results to stoichiometry. For larger scale oceanic observations, stoichiometry may provide the best way to scale up estimates of denitrification, especially if the assumptions can be locally verified with direct measurements of dissolved gases, or by using stable isotopic measurements to further constrain the systems.

METHODS BASED ON STABLE ISOTOPE ABUNDANCES

Stable isotopes that are used in various ways in denitrification studies include those of H and O in water; N and O in ammonium, nitrate, nitrite, nitrous oxide, and dissolved nitrogen gas; S and O in sulfate and sulfide; C in oxidized and reduced dissolved carbon; O in dissolved

oxygen gas; and C, N, and S in organic and inorganic solids. These data provide evidence for the occurrence of denitrification along with information about sources of water and the various reactants (e.g., electron donors) and products of denitrification. The same isotopes also can be used as tracers of movement and reaction of nitrate when injected deliberately into laboratory or field systems, but this section is focused on collection and analysis of environmental samples to assess in situ conditions.

The stable isotope ratios typically are expressed in "delta" (δ) notation, for example,

$$\delta^{15}N = \left[ \left( {}^{15}N/{}^{14}N \right)_{sam} \Big/ \left( {}^{15}N/{}^{14}N \right)_{air} - 1 \right] \times 1000‰$$

$$(1)$$
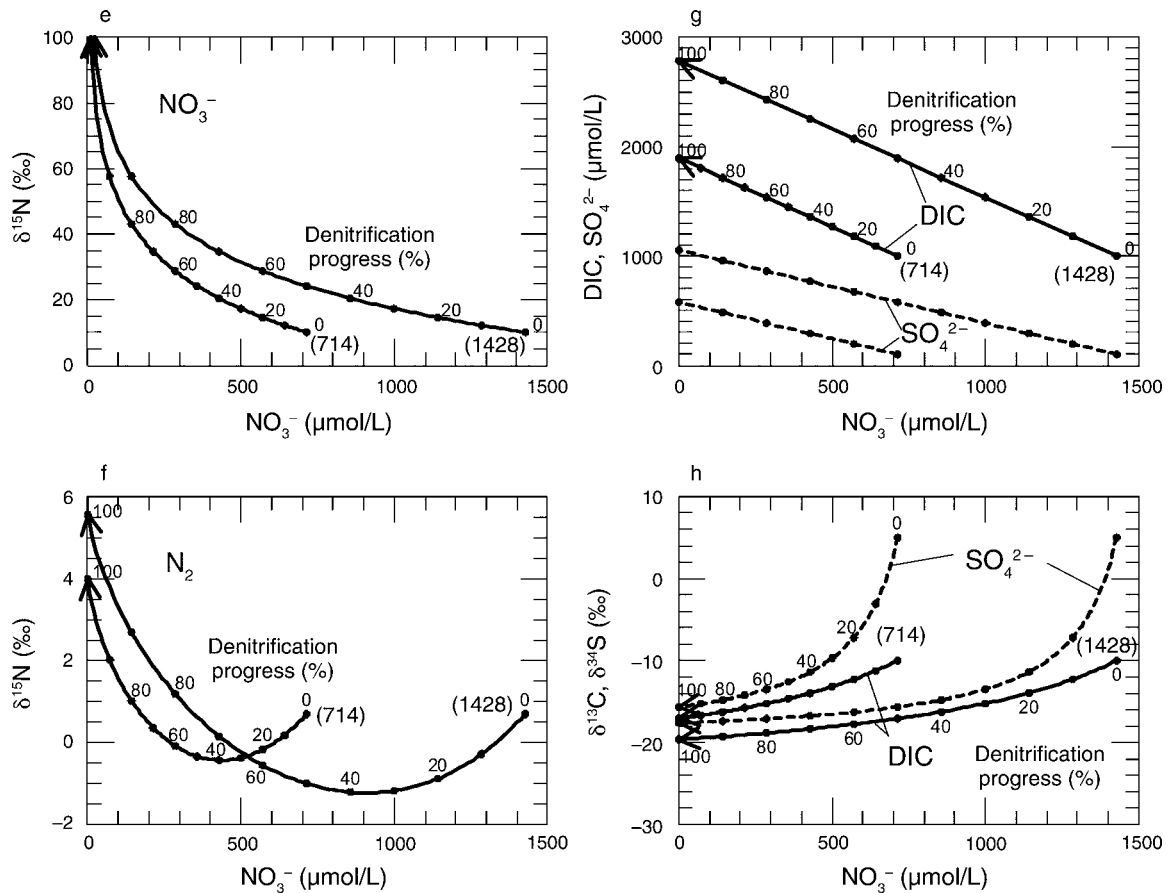
FIG. 5.   Continued.

$$\delta^{18}O = \left[\left(^{18}O/^{16}O\right)_{sam} \Big/ \left(^{18}O/^{16}O\right)_{VSMOW} - 1\right] \times 1000\%$$

(2)

where the isotope ratios reflect molar abundances and "air" (atmospheric $N_2$) and "VSMOW" (Vienna standard mean ocean water) are reference materials for which δ equals 0‰ by consensus (the subscript "sam" stands for sample). The effects of isotopic fractionation on reactants (r) and products (p) during processes such as denitrification commonly are described by a version of the Rayleigh distillation equation (Rayleigh 1896, Clark and Fritz 1997):

$$\left(^{15}N/^{14}N\right)_r = \left(^{15}N/^{14}N\right)_{r,ini} \times \left(^{14}N_r/^{14}N_{r,ini}\right)^{(\alpha-1)} \quad (3)$$

(the subscript "ini" stands for initial). In Eq. 3, the fractionation factor (α) is given by the instantaneous relation between reactant and product isotope ratios,

$$\alpha = \left(^{15}N/^{14}N\right)_p \Big/ \left(^{15}N/^{14}N\right)_r \quad (4)$$

which can also be used to describe simple kinetic fractionation effects:

$$\alpha = k_r\left[^{15}N\right] \Big/ k_r\left[^{14}N\right] \quad (5)$$

and

$$\varepsilon = (\alpha - 1) \times 1000\% \quad (6)$$

where $k_r$ refers to the relative first-order reaction rate constant for reactant molecules containing either $^{15}N$ or $^{14}N$. In some publications, a negative sign has been added to the right side of Eq. 6 to make ε values positive.

Denitrification causes changes in the concentrations and isotopic compositions of many different aqueous species and solids in aquatic systems, for example (Fig. 5): (1) the $\delta^{15}N$ and $\delta^{18}O$ values of $NO_3^-$ typically increase together because of kinetic isotope fractionations during $NO_3^-$ reduction (Amberger and Schmidt 1987, Böttcher et al. 1990), (2) the $\delta^{15}N$ value of $N_2$ may decrease or increase as a result of adding denitrification product $N_2$ ($N_{2den}$) to other components of $N_2$ in the system (Vogel et al. 1981), (3) the $\delta^{34}S$ value of sulfate or the $\delta^{13}C$ value of DIC may change as oxidation products of reduced S or C compounds are added to the existing reservoirs during denitrification (Strebel et al. 1990, R. L. Smith et al. 1991), and (4) the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$

values of organisms (and derivative sediments and soils) may change as a result of changes in the isotopic compositions of their C, N, and S sources during denitrification (e.g., Altabet et al. [1999] for N isotope changes).

Kinetic isotopic fractionation of $NO_3^-$ during bacterial denitrification has been documented in laboratory cultures and field settings since the 1960s (Richards and Benson 1961, Wellman et al. 1968, Delwiche and Steyn 1970, Miyake and Wada 1971, Cline and Kaplan 1975, Mariotti et al. 1981, 1988, Brandes et al. 1998, Altabet et al. 1999, Barford et al. 1999, Voss et al. 2001). Apparent N isotope fractionation factors (ε) ranging from 0‰ to about −30‰ (possibly −40‰) have been reported, indicating that $k_r[^{15}N] \leq k_r[^{14}N]$ and that the $\delta^{15}N$ value of the residual unreacted $NO_3^-$ increases by varying amounts as denitrification products with low $\delta^{15}N$ values are formed. The relatively large negative values of ε have been obtained from water-column studies and from suspended cultures in the laboratory, whereas the smaller values commonly are derived from studies of ground waters and of surface waters that interact with ground waters as well as in marine sediments.

One explanation for the variability of apparent isotope effects is that the maximum kinetic fractionation caused by enzyme-catalyzed reaction at the microscale is expressed fully when the reaction itself limits the rate of $NO_3^-$ reduction in the measurable reservoir, whereas the effect is suppressed when the rate of $NO_3^-$ reduction is limited by transport to the reaction site and when the reaction goes largely to completion locally without exchanging freely with the external reservoir. For example, Mariotti et al. (1988) concluded that relatively small apparent ε values of around −5‰ in partially denitrified ground water could be a result of relatively complete reaction in dead-end pores that had limited diffusional exchange with more mobile $NO_3^-$ bearing water. Similarly, apparent ε values as small as −3‰ to 0‰ have been reported in marine surface water overlying the sediment–water interface where denitrification was limited by diffusion into the sediment (Brandes and Devol 2002, Sigman et al. 2003). Therefore, although isotopic fractionation can be a useful indicator of denitrification, the magnitude of the isotope effect may not reflect the progress of the reaction or the amount of $NO_3^-$ reduced in physically heterogeneous systems.

Regardless of the magnitude of the apparent fractionation factors, the effects of denitrification also can be deduced from correlations between $\delta^{15}N$ and $\delta^{18}O$ and the relative ε values for N and O in $NO_3^-$. Several of the early studies indicated that the ratio εN:εO during denitrification in ground water is around 2.1 (Böttcher et al. 1990, Aravena and Robertson 1998). Subsequent studies in a variety of aquatic settings with a wide range of reaction rates have indicated a range of εN:εO ratios during denitrification that could extend from about 1.3 to 2.1 (Cey et al. 1999, Mengis et al. 1999, Fukada et al.

2003, Lehman et al. 2003; McMahon et al. 2004a), and it has been suggested that values close to 1.0 may be realized during $NO_3^-$ reduction by phytoplankton in some marine systems (Granger et al. 2004). It is not clear at present how much of the reported variability in this ratio is due to analytical errors, misinterpretation of field data, or real variations in the fractionation effects. It is important to recognize that some variation in the isotopic composition of $NO_3^-$ in a given field situation may be related to variations in the $NO_3^-$ source, rather than to subsequent fractionation effects (Böhlke et al. 2002). Nevertheless, positively correlated variations in N and O isotopes of $NO_3^-$ can provide valuable evidence for denitrification in many situations.

Quantitative field studies of denitrification in ground water, and to a lesser extent in surface water, include analyses of the $N_2$ product. If the concentration and $\delta^{15}N$ value of the reaction product $N_2$ can be resolved from the atmospheric components of $N_2$, then the reaction progress and initial $NO_3^-$ concentration and isotopic composition can be reconstructed from partially or completely reacted samples (Vogel et al. 1981, Wilson et al. 1990, Böhlke and Denver 1995, Böhlke et al. 2002, Griggs et al. 2003, McMahon et al. 2004a; Fig. 5c, d, f). This information is especially important where the $NO_3^-$ inputs have changed over time, in which case both the concentration and isotopic composition of $NO_3^-$ could change along a flow path independently of denitrification (Böhlke et al. 2002). A major limitation of this approach in ground water is that the initial concentrations of atmospheric gases including $N_2$ may vary by more than the analytical uncertainties as a result of varying equilibration temperatures and incorporation of excess air during recharge (Sugisaki 1959, Heaton and Vogel 1981, Aeschbach-Hertig et al. 2000, Stute and Schlosser 2000). This means that improvements in analytical precision will not necessarily yield improvements in the estimation of $N_{2den}$. Complete resolution of the atmospheric $N_2$ component generally will require analyses of one or more nonreactive atmospheric gases like Ar, Ne, Kr, and Xe. Argon data have been used extensively for this purpose in denitrification studies, but uncertainties of the order of 20–40 μmol/L or more for $N_{2den}$ commonly result from necessary assumptions about recharge conditions. Addition of Ne data can reduce uncertainties substantially (e.g., 10–20 μmol/L $N_{2den}$; Verstraeten et al. 2001a, McMahon et al. 2004a), and it is likely that further work with other noble gases will be helpful.

Isotopic analyses of S and C can provide indirect evidence for denitrification as well as information about the major electron donors for the coupled redox reaction (Strebel et al. 1990, R. L. Smith et al. 1991, Böhlke and Denver 1995, Aravena and Robertson 1998, Böhlke et al. 2002; Fig. 5g, h). Indirect evidence for variation in the importance of denitrification in terrestrial and freshwater systems also can be derived from variations in the $\delta^{15}N$ values of plants, peat, soils, and aquatic

sediments. For example, if denitrification causes an increase in the $\delta^{15}N$ value of $NO_3^-$ that is subsequently assimilated by organisms, then the organic matter produced with that N also may exhibit an increase in $\delta^{15}N$. Thus, variations in the intensity of denitrification may result in N isotopic variations in organic matter preserved in aquatic sediments.

In the open ocean, the isotopic discrimination associated with denitrification produces large isotopic signals. Here, the $^{15}N$ enrichment in residual $NO_3^-$ clearly propagates throughout the marine N cycle via $NO_3^-$ uptake by phytoplankton. In particular, high $\delta^{15}N$ values are transferred to sinking organic matter derived from phytoplankton production that is ultimately incorporated in the underlying sediments. As shown below, the sedimentary record thus provides a history of past variation in overlying water column denitrification. In addition, because denitrification is the primary sink for combined N from the oceans, another important consequence is that denitrification raises oceanic average $\delta^{15}N$ above the average of the combined N sources.

The clearest examples of denitrification-produced isotope enrichment in the deep, open ocean are found in the main regions of water column denitrification; the Arabian Sea and the Eastern Tropical Pacific (ETP) to the north off Mexico (ETNP) and to the south off Peru (ETSP), respectively. Each of these regions is characterized as having a suboxic oxygen minimum zone (OMZ) between 100 m and almost to 800 m depth, a vertical region also known as the $O_2$ minimum zone (OMZ). While the oceanic average for $\delta^{15}NO_3^-$ is near 5‰ relative to atmospheric $N_2$, depths with greatest intensity of denitrification reach >20‰. In each of the water column denitrification regions, $^{15}N$ enrichment in $NO_3^-$ is clearly associated with the core of the OMZ, and the greatest removal of $NO_3^-$. It is only with the recent advent of methodology suitable for seawater samples (Casciotti et al. 2002) that the coupled enrichment in $^{18}O$ and $^{15}N$ in the residual $NO_3^-$ can be studied and such data are appearing rapidly in the literature.

To study the quantitative relationship between denitrification and isotopic enrichment, the removal of $NO_3^-$ may be estimated by examining the anomaly in observed $NO_3^-$ (and $NO_2^-$) concentration relative to the value expected from Redfield stoichiometry with $PO_4^{-3}$:

$$N' = NO_3^- + NO_2^- - 16 \times PO_4^{-3}. \qquad (7)$$

$N'$ can be used to estimate the residual fraction $NO_3^-$ remaining after denitrification:

$$f = (NO_3^- + NO_2^-)/(NO_3^- + NO_2^- - N') \qquad (8)$$

permitting fits to an approximation of the Rayleigh equation (Eq. 3),

$$\delta^{15}NO_3^- \approx \delta^{15}NO_3^-{}_{ini} + \varepsilon \times \ln(f) \qquad (9)$$

in a closed system, or

$$\delta^{15}NO_3^- \approx \delta^{15}NO_3^-{}_{ini} - \varepsilon \times (1 - f) \qquad (10)$$

in a steady-state open system. Note the above equations adopt the convention of negative $\varepsilon$ values for kinetic effects in which products are enriched in the light isotope (Eq. 6).

Using the Arabian Sea as an example, $\delta^{15}NO_3^-$ maxima within the OMZ vary between stations suggesting variable isotopic fractionation (Fig. 6). Nevertheless, when all station data are plotted together, strong linear correlations are found between $\delta^{15}NO_3^-$ and its fractional removal as estimated by Eq. 8 (Fig. 6). Neither the goodness of fit nor the value for $\varepsilon_{den}$ estimated from the slope appears sensitive to whether the system is assumed to be open or closed. $\varepsilon_{den}$ is between −30 and −20‰, consistent with both laboratory and field observations. Similar results have been reported for the ETNP off Mexico (Voss et al. 2001).

The most successful marine applications to date for natural N isotope abundance in denitrification studies have been in historical reconstruction, particularly in relation to climate change. Sediment core bulk $\delta^{15}N$ has been used to both identify and understand past changes in water-column denitrification on time scales from hundreds to millions of years. Water-column denitrification is regionally isolatable, produces large isotopic signals, and is strongly coupled to past climate change. Suboxic intermediate waters overlying continental margin sediments with high organic matter content input ensure both fidelity of the preserved $\delta^{15}N$ signal and the availability of sediment cores with high accumulation rates for good to excellent temporal resolution. In these sediments, N content is high enough that only tens of milligrams of material are necessary for isotopic analysis.

Detailed denitrification records based on sediment $\delta^{15}N$ have been published for the Arabian Sea (Altabet et al. 1995, 1999, 2002) and the ETNP (Ganeshram et al. 1995, 2000, Kienast et al. 2002). In the latter case, sites along the western American margin are included since upwelled waters are derived from the California Undercurrent, which in turn carries denitrification-influenced water northward from the ETNP. Current studies now also include the ETSP off Peru (Higginson and Altabet 2004), the third of the ocean's major water column denitrification zones.

Variation in Arabian Sea denitrification occurs on a variety of climatologically relevant time scales. The longest record from the Owen Ridge off Oman reaches back nearly 1 Myr from the present (Altabet et al. 1999). Variation is primarily at the Milankovitch periods of Earth's orbital variation (23, 41, and 100 kyr), which are also found in the glacial cycles of Pleistocene climate. In general, lower $\delta^{15}N$ and denitrification is found during cold periods of glacial maxima. A detailed study of the last 60 kyr at centennial resolution (Fig. 7) reveals strong millennial variability during MIS 3 (30 to 60 kyr before present, BP), with transitions from low $\delta^{15}N$ to $\delta^{15}N$
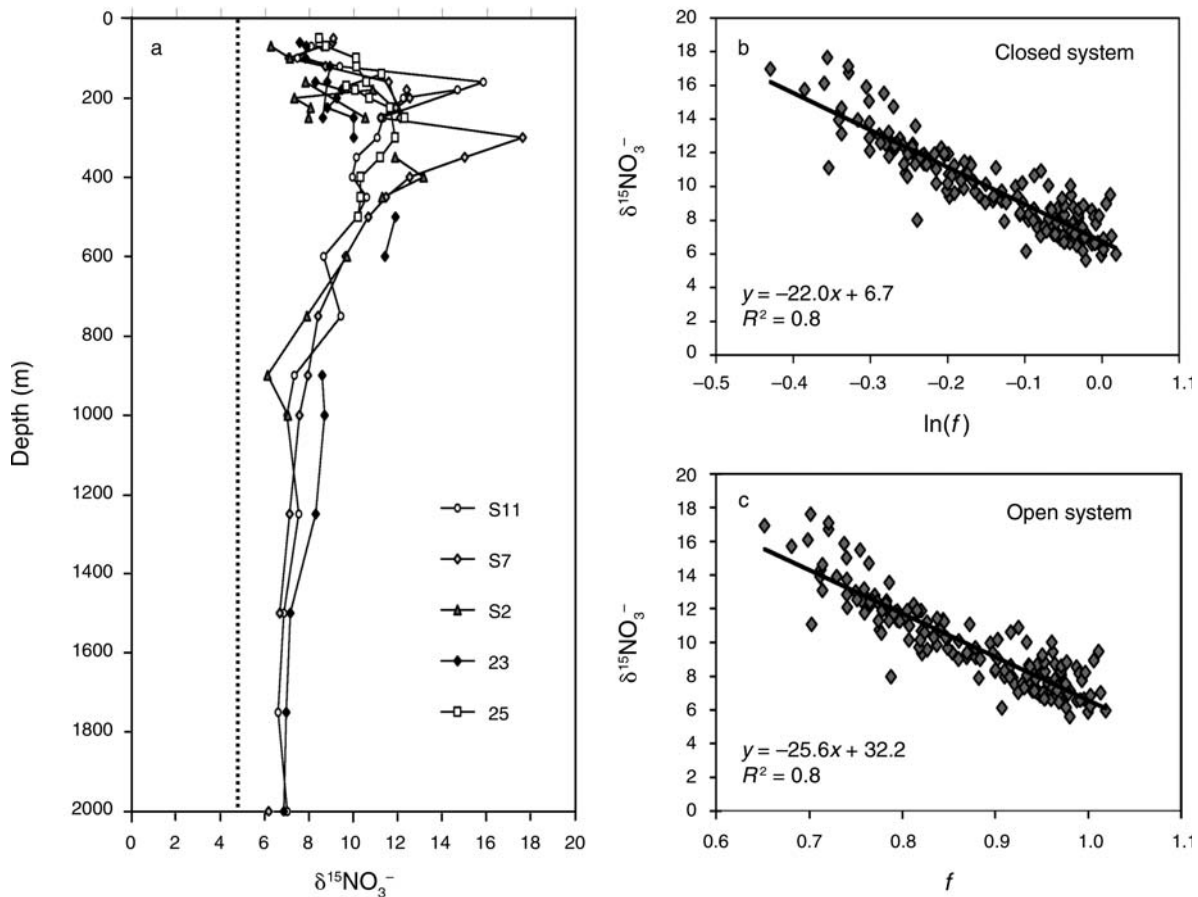
FIG. 6. (a) Depth profiles for $\delta^{15}NO_3^-$ from several stations in the Arabian Sea denitrification zone. The maxima between 200 and 400 m are in the core of this OMZ and co-occur with the most negative N′ values. The vertical dashed line represents average oceanic $\delta^{15}NO_3$ for reference. (b, c) Cross plots of the $\delta^{15}NO_3^-$ data with either the estimated fraction of $NO_3^-$ remaining after denitrification ($f$) or ln($f$) as prescribed by the open- and closed-system Rayleigh equations (Eqs. 9 and 10), respectively. The regression slopes estimate the fractionation factor ($\varepsilon$).

maximum occurring relatively abruptly, on the order of a few hundred years or less (Altabet et al. 2002).

We can make use of nature's "past experiments" to probe the causes of changing denitrification intensity by analysis of synoptic proxies of relevant processes. A leading hypothesis linking N. Hemisphere climate to Arabian Sea denitrification is that climate change modulates the strength of the summer South Asian monsoon and upwelling favorable winds off Oman. Corresponding oscillation in productivity influences subsurface suboxia and thus denitrification intensity in the Arabian Sea. Studies in the ETNP off Mexico have shown similar linkages between past-climate change and denitrification with glacial periods marked by lowered levels (Fig. 7). Recent work, though, indicates significant phase lags between the productivity and denitrification (Kienast et al. 2002). It has been argued that changes in the proportion and ventilation of intermediate waters in the ETNP exert the major proximal control on ETNP OMZ oxygenation (Hendy and Kennett 2003). The ETSP off Peru is the third and last of the major water

column denitrification regions to be subjected to paleoceanographic reconstruction. A priori, we may expect differences in climate response as compared to the Arabian Sea and ETNP because this region is only one of the three located in the Southern Hemisphere. As elsewhere, margin sediments overlaid by low $O_2$ present the best targets due to excellent OM preservation and high accumulation. Not surprisingly, large variations in $\delta^{15}N$, and hence denitrification, are observed over the last 50 kyr. As in the Arabian Sea and ETNP, upon deglaciation there is a dramatic ramp up in denitrification. However, the history of Peru denitrification departs from its northern hemisphere counterparts in several striking respects. First, the deglacial peak in denitrification occurs very early at between 15 and 16 ka, when most of the planet is still very much experiencing glacial conditions. We suspect this is the result of S. Hemisphere forcing of intermediate water ventilation. Second, MIS 3 (30–60 ka) shows overall low denitrification similar to last glacial maximum (LGM) levels except for modest maxima that may be related to
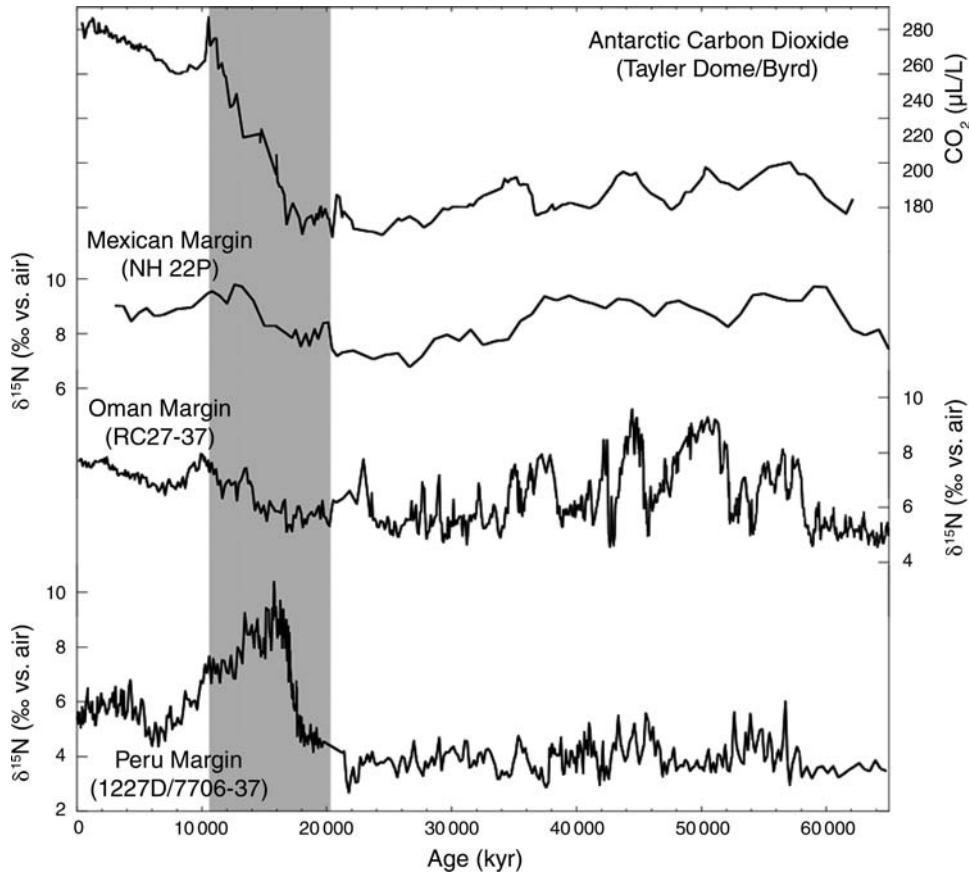
Fig. 7. Comparison of $\delta^{15}N$ records from the three major water column denitrification zones covering the last 60 kyr. Note the relatively low $\delta^{15}N$ values for the ETSP (Eastern Tropical South Pacific off Peru; Peru Margin) record during MIS 3 (Marine Isotope Stage; 30–60 kyr BP) and the early and sharp rise during the early part of the last deglacial period (14—18 ka BP). The ice core record for atmospheric $CO_2$ is shown for reference (Indermuhle et al. 2000). The ETP (Eastern Tropical Pacific off Mexico; Mexican Margin) data are from Ganeshram et al. (2000).

Antarctic Climate Events (ACEs) as observed in Antarctic ice cores. Third, there is substantial late Holocene (0–5 ka) variability in denitrification with excursions in $\delta^{15}N$ almost as large as upon deglaciation occurring over centuries or less. Of the three oceanic denitrification regions, Peru is most impacted by ENSO (El Niño/La Niña southern oscillation) variability, and while individual ENSO events cannot be observed at our temporal resolution, changes in the overall frequency and amplitude would be.

In sum, $\delta^{15}N$ variations in the open ocean are very sensitive to water column denitrification intensity. These $\delta^{15}N$ variations are preserved in underlying sediments, permitting past reconstruction at timescales limited only by the fidelity and resolution of sedimentary records. Results to date show large variations in each of the major oceanic denitrification regions that appear sensitive to regional and global climate change. Though there are significant differences in denitrification history between the ETP off Mexico and Peru, and the Arabian Sea (Oman Margin; Fig. 7), they all show deglacial increases in $\delta^{15}N$ and denitrification between 20 and 10

kyr before present. The combined global increase in denitrification would have resulted in a significant decrease in combined N inventory for the ocean that in turn may have contributed to the increase in atmospheric $CO_2$ through reduction in marine productivity.

## DENITRIFICATION RATES FROM IN SITU GRADIENTS USING ENVIRONMENTAL TRACERS

Although in situ chemical and isotopic data commonly indicate that denitrification has occurred, and in some cases they may indicate how much denitrification has occurred, they do not indicate directly the rate of denitrification, which requires additional information about time. Because apparent denitrification rates range over at least eight to 10 orders of magnitude, approaches for measuring them require some flexibility. This section summarizes some of the approaches for determining denitrification rates using analyses of atmospheric environmental tracers as indicators of elapsed time. Environmental tracers used for dating purposes in denitrification studies include anthropogenic atmospher-

ic constituents such as [3]H (and tritiogenic [3]He), chlorofluorocarbons (CFCs), sulfur hexafluoride ($SF_6$), and other species that may be present in varying concentrations in waters of different ages (e.g., ^{14}C). These data provide information about water transport paths and velocities that are needed to determine denitrification rates from reaction progress data. They also provide historical context for $NO_3^-$ mass gradients that may be independent of denitrification. All of these chemicals and isotopes can be used in the form of deliberate injected tracers, but the current discussion is mainly about the ambient ("in situ") concentrations, which are not necessarily natural in origin but are naturally distributed (hence, the term "environmental tracer"). The guiding principle of this approach is that measurements are done without any deliberate attempt to introduce tracers or to alter the system being studied in any way. The following discussion refers mainly to ground water, but many of the statements also are applicable to water-column studies, for example in lakes and oceans.

Relatively low rates of denitrification in aquifers can be estimated by comparing measured gradients of groundwater age and reaction progress along transport pathways. For example, depending on the situation, the progress of the reaction might be described by

$$(C_{t2} - C_{t1}) = -k^0 \times (t2 - t1) \qquad (11)$$

or

$$(C_{t2}/C_{t1}) = \exp\left[-k^1 \times (t2 - t1)\right] \qquad (12)$$

where $C_t$ is the concentration of $NO_3^-$ in water with age $t$ ($t2 > t1$), $k^0$ refers to a zero-order reaction rate, and $k^1$ refers to a first-order rate constant. This approach has some important advantages over experimental determinations and deliberate tracer applications, for example: (1) by using environmental tracers as age indicators, one can retrieve temporal information about processes that last far longer than the time scales that normally are accessible to experimentation and monitoring, and (2) the spatial scale of observation may be much larger and more integrative with respect to local heterogeneity. The environmental tracer approach also has some important disadvantages, for example: (1) manipulations are not possible, (2) local controls may not be resolvable, (3) systems may be too complex to interpret, and (4) environmental tracer data may be ambiguous.

Numerous reviews have been published recently describing principles and applications of atmospheric environmental tracers for groundwater dating (e.g., Cook and Herczeg 2000). Several different dating methods have been applied to denitrification studies over a range of time scales from years to millennia. Major limitations of these dating methods include degassing, uncertainties about recharge conditions (e.g., temperature, elevation, excess air, initial [14]C:C), incomplete confinement of tritiogenic [3]He, degradation

of compounds like CFCs, excess terrigenic (non-tritiogenic) [3]He, and water–rock reactions affecting DIC in the saturated zone. In addition, mixing of waters of varying ages in aquifers or wells, and atmospheric tracer gradients in thick unsaturated zones, require careful interpretation with respect to solute travel times or mean residence times.

The age gradients used to evaluate denitrification rates must be considered in the broader context of flowpaths and reaction zonation. Because redox reactions in aquifers commonly are ordered according to thermodynamic constraints, reaction zones for individual aqueous species commonly occupy distinctive regions within a flow system. For example, in the saturated zone beneath an oxic recharge area, electron acceptors typically are reduced in a sequence such as $O_2$, $NO_3^-$, $Fe^{3+}$, $SO_4^{2-}$ (Appelo and Postma 1996, Stumm and Morgan 1996). Thus, the zone of active denitrification will largely be downgradient from the $O_2$ reduction zone and upgradient from the $SO_4^{2-}$ reduction zone. In this case, the value of $t$ (Eqs. 11 and 12) for a given sample will not be the time since recharge but rather the time since entering the denitrification zone, which will be less than the groundwater age.

Another important consideration is that the initial concentration of $NO_3^-$ in a series of denitrified samples may not have been the same. In many areas of the world, the recharge fluxes and concentrations of $NO_3^-$ have changed dramatically over decadal time scales as a result of changing agricultural practices (Hallberg and Keeney 1993, Böhlke 2002). Variations can occur over a range of time scales because of land use or climate changes. Some aquifers have preserved records of those changes, in which case the ground water may be considered as a transient reservoir in which gradients occur, and inputs are not balanced by outputs, even if there are no reactions producing or consuming $NO_3^-$. Therefore, the reaction progress parameter is best derived from measurements of reactant ($NO_3^-$) and product ($N_{2den}$) in individual samples (see other sections of this report), or in combination with other information about changes in the initial conditions (Postma et al. 1991).

Some examples of denitrification rates derived from in situ gradients are listed in Table 3. These examples are summarized for comparison as zero-order reaction rates, although there is evidence that first-order rate constants may represent field data more reliably in some cases (Böttcher et al. 1989, Frind et al. 1990). Because the measurable time scales largely are limited to those of the common environmental dating tracers, it is possible that reaction rates derived by this approach will not be evenly distributed over the range. For example, denitrification rates of the order of $1 \times 10^{-5}$ to $1 \times 10^{-4}$ $\mu mol \cdot L^{-1} \cdot d^{-1}$ have been derived from transport paths that are in the range of 1000–20 000 yr long; that is, where natural ^{14}C abundances can be used for dating. In contrast, denitrification rates of the order of $1 \times 10^{-3}$ to

$1 \times 10^{-1}$ $\mu mol \cdot L^{-1} \cdot d^{-1}$ have been derived from transport paths that are in the range of 0–60 yr long; that is, where the modern anthropogenic atmospheric environmental tracers can be applied. Given typical uncertainties in the concentration of $N_{2den}$, clustering of rates and scarcity of some ranges of values are likely to result from a lack of dating methods for certain time scales (e.g., 60–1000 yr) and not to any real feature of natural systems. Similarly, the distribution of in situ environmental denitrification rates at the high end may not merge smoothly with the distribution of rates derived from laboratory experiments and deliberate tracers. Entries in Table 3 include some groundwater studies that fill these gaps by combining numerical simulations with hydraulic or environmental tracer data. Cline and Kaplan (1975) report denitrification rates in ocean water that were estimated by combining reaction progress data with transport velocities based on advection–dispersion modeling with $^{226}Ra$ ($t_{1/2} = 1600$ yr) as the internal chronometer. Expansion of efforts to analyze a wide variety of environmental tracers and to calibrate numerical models with environmental tracer data should increase the range of applicability of the in situ gradient approach for estimating low reaction rates.

In aquatic systems with low rates of reaction, $NO_3^-$ can be transported for years to millennia or longer before denitrification becomes a substantial $NO_3^-$ sink, and then lose $NO_3^-$ gradually over comparable time scales. In contrast, there are many situations in which water and solutes move from an oxidized environment to a reduced environment abruptly, and where $O_2$ reduction, denitrification, and perhaps other redox reactions occur over short travel times and may not be amenable to the approaches described above (Postma et al. 1991, Robertson et al. 1996). In these situations, it is likely that the rate of denitrification is largely controlled by the rate of transport of $NO_3^-$ to the reactive sites at the redox boundary. In aquifers, concentrations of solid electron donor phases may be large compared to the concentrations of electron acceptors in the water, in which case the rate of migration of the denitrification boundary itself may be several orders of magnitude slower than the rate of movement of water delivering $NO_3^-$ to the boundary. Where a sharp boundary is present, the rate of denitrification may be expressed as the flux of $NO_3^-$ in ground water crossing the boundary ($F_{den}$), in the same way that the recharge rate of $NO_3^-$ is given by the flux across the water table:

$$F_{den} = C_{in} \times V_{in} \times n \qquad (13)$$

where $F_{den}$ is measured in $\mu mol \cdot m^{-2} \cdot d^{-1}$, $C_{in}$ is the $NO_3^-$ concentration at the upgradient edge of the redox boundary, $V$ is the component of groundwater velocity normal to the redox boundary, and $n$ is aquifer porosity. It is important to recognize that the velocity of ground water across a redox boundary may be substantially different from the recharge velocity at the water table, even in simple aquifers with horizontal redox boundaries (Cook and Böhlke 2000). Furthermore, because of changes in the recharge rate of $NO_3^-$ over time, the concentration of $NO_3^-$ entering the redox boundary commonly is not the same as the concentration at the water table.

The distinction between gradual and abrupt redox boundaries and reaction rates is related both to the scale of accessible observation (Table 3) and to the scale of the information needed. For example, the reaction boundary approach (Eq. 13) may be more widely applicable for assessing the importance of groundwater denitrification at the watershed scale, whereas the reaction rate approach (Eq. 11 or 12) may be relatively important for predicting contaminant trends at specific locations within the system (Frind et al. 1990). In either case, the empirical information derived from the environmental tracer data can be used to guide various types of model simulations, which may be largely unconstrained otherwise.

## Molecular Approaches

Denitrification rates are a result of complex interactions between physical, hydrological, and chemical parameters in the environment. Many studies have tried to identify the important factors in various systems and model these controls based on what we know from laboratory and field studies. However, abiotic factors alone often do not provide sufficient information for accurate assessment and prediction of rates of N transformations in natural environments. The metabolic response in a given environment to these controllers is largely dependent on characteristics of the microbial community. The extant microbial community reflects the recent history of environmental conditions which has in part framed the community composition. The primary goal of current application of molecular methods to denitrification is to understand how the composition and physiology of the microbial community affects N transformations in the environment and vice versa.

Because denitrification is catalyzed by a diverse group of bacteria, archaea, and fungi (Zumft 1997), research on the abundance and diversity of denitrifiers in the environment has focused on the functional genes involved in the denitrification pathway. Although some studies have utilized the periplasmic and membrane-bound $NO_3^-$ reductase genes (napA and narG) (Flanagan et al. 1999, Gregory et al. 2000, Mergel et al. 2001) and a gene encoding nitrous oxide reductase (nosZ; Michotey et al. 2000, Scala and Kerkhof 2000, Mergel et al. 2001, Rich et al. 2003), most molecular investigations of bacterial denitrification in natural environments (see review by Bothe et al. 2000) have utilized nitrite reductase (nirS and nirK; Linne von Berg and Bothe 1992, Hallin and Lindgren 1999, Braker et al. 2000, 2001, Michotey et al. 2000, Mergel et al. 2001), because it catalyses the first committed step that leads to a

TABLE 3. Examples of denitrification rates derived from in situ gradients of reaction progress and measurements or estimates of groundwater or surface-water age.

| Denitrification ($\mu$mol N·L$^{-1}$·d$^{-1}$) | Evidence used | Scale of gradient |
| --- | --- | --- |
| Groundwater; $^{14}$C time scale | | |
| $6 \times 10^{-5}$ | NO$_3^-$, N$_2$ gradient + $^{14}$C ages | 80 km horizontal, 27 kyr |
| $1.4 \times 10^{-5}$ | NO$_3^-$, N$_2$ gradient + $^{14}$C ages | 100 m vertical, 12 kyr |
| $7 \times 10^{-5}$ | NO$_3^-$, N$_2$ gradient + $^{14}$C ages | 60 m vertical, 7 kyr |
| Groundwater; $^{3}$H, $^{3}$H/$^{3}$He, CFC, and SF$_6$ time scales | | |
| $1–4 \times 10^{0}$ | NO$_3^-$ gradient + hydraulic data | 10 km horizontal, 1–3 yr |
| $2 \times 10^{0}$ | NO$_3^-$ gradient + hydraulic data | 1–2 m vertical, 10 yr |
| $\leq 3 \times 10^{-2}$ | NO$_3^-$, N$_2$ gradient + CFC ages | 10 m vertical, 10 yr |
| $1 \times 10^{-2}$ to $1 \times 10^{0}$ | NO$_3^-$, Cl, $^{3}$H gradient + dispersion model | 2 m vertical [years–decades] |
| $\geq 1 \times 10^{-1}$ to $1 \times 10^{1}$ | NO$_3^-$, N$_2$ gradient + flow model (+CFC) | 2 km horizontal, 10 yr |
| $4 \times 10^{-2}$ | NO$_3^-$, N$_2$ gradient + $^{3}$H, CFC ages | 2 m vertical, 15 yr |
| $\leq 3 \times 10^{-3}$ | NO$_3^-$, N$_2$ gradient + $^{3}$H–$^{3}$He ages | 10–30 m vertical, 30 yr |
| Ocean; $^{226}$Ra time scale | | |
| $2–22 \times 10^{-3}$ | NO$_3^-$ deficit + $^{226}$Ra + advection-dispersion model | 500 m vertical, [decades–centuries] |

*Note:* Measurements are made on water samples; thus, rates are given per liter of water.

gaseous intermediate. The reduction of nitrite is catalyzed by either one of two entirely different enzymes in terms of structure and the prosthetic metal, the cytochrome *cd*1 (encoded by the gene *nirS*) and the copper-containing nitrite reductases (encoded by the gene *nirK*). About three-quarters of cultured strains collected worldwide have the tetraheme protein cytochrome *cd*1 as the respiratory nitrite reductase (Zumft 1997). The copper containing reductase catalyzes the reaction in a greater variety of physiological groups. Studies so far indicate that both reductases are found in all environments but that *nirS* appears to be numerically dominant in most environments (Bothe et al. 2000). The genes encoding nitrite reductase (*nirK*) and nitrous oxide reductase (*norB*) have been found in some nitrifiers (Casciotti and Ward 2001, Casciotti et al. 2003), and the little work that has been done on the genes involved in eukaryotic denitrification have shown the presence of reductases for NO$_x$, nitrite, and NO in a few species (Zumft 1997).

DNA probes and PCR primers have been developed for these genes and have been used to study the distribution, abundance, diversity, and, to a lesser extent, activity of denitrifying bacteria. Various techniques are used to assess abundance such as direct DNA probing (Linne von Berg and Bothe 1992), quantitative PCR or real-time PCR (Gruntzig et al. 2001, Henry et al. 2004), which measure gene copies and relates that back to cell numbers, or T-RFLP (terminally labeled restriction-length fragment polymorphism, commonly used for estimates of diversity), which has been shown to be semiquantitative (Liu et al. 1997). Community-level information can be obtained by analysis of PCR products by T-RFLP (Liu et al. 1997) or denaturing gradient gel electrophoresis; DGGE (Moeseneder et al. 1999). These techniques provide a profile of the gene population that reflects the diversity and relative abundance of each of the target sequences and can be

used to detect changes in community dynamics related to geographic distance and biogeochemical properties. For denitrifiers, analysis on a functional level with PCR-amplified functional genes such as *nirS* and *nosZ* have been used with subsequent T-RFLP analysis (Scala and Kerkhof 2000, Braker et al. 2001, Rich et al. 2003, Wolsing and Prieme 2004) or by DGGE (Throback et al. 2004) to monitor community differences. DNA microarray technology holds great promise for microbial ecology and the evaluation of bacterial functional guilds (Guschin et al. 1997). The technology is based on the DNA–DNA hybridization principle and probes can be designed for a variety of targets, including variants of the same gene, that allow for the determination of the presence and abundance of specific genes. The quantitative detection of denitrification genes by microarray using current technology poses several problems. The technique has a relatively high detection limit and requires sample amplification. Both cross hybridization and target interference can be a problem since environmental DNA contains DNA originating from a huge diversity of bacteria, archaea, protozoa, etc. Despite these technical issues, the development and evaluation of functional gene arrays for the detection of denitrification genes in the environment is well underway (Cho and Tiedje 2002, Taroncher-Oldenburg et al. 2003).

In general, molecular studies have shown that the number of denitrifiers in the environment is high and the composition of denitrifying communities is complex and apparently subject to large fluctuations, both in time and in space. Studies have revealed a much higher diversity of denitrification genes in the environment than expected which are often divergent from the genes of cultured denitrifiers (Braker et al. 2000, Flanagan et al. 1999, Scala and Kerkhof 1999, Gregory et al. 2000). Clone libraries from a variety of different terrestrial and aquatic environments demonstrate that most species present are not found in our culture collections. In other

TABLE 3. Extended.

| Flow-path characteristics and location | Reference |
| --- | --- |
| confined sandstone aquifer, Kalahari Desert, southwest Africa | Vogel et al. (1981) |
| unconfined fluvial sand aquifer, High Plains, Kansas, USA | McMahon et al. (2004a) |
| unconfined fluvial sand aquifer, High Plains, Texas, USA | McMahon et al. (2004b) |
| confined limestone aquifer, Lincoln, UK | Lawrence and Foster (1986) in Hiscock et al. (1991) |
| unconfined sand aquifer, Fuhrberger Feld, Germany | Böttcher et al. (1989), Frind et al. (1990) |
| unconfined South Platte alluvial aquifer, Colorado, USA | McMahon and Böhlke (1996) |
| shale aquitard beneath South Platte alluvial aquifer, Colorado, USA | McMahon et al. (1999) |
| unconfined glacial outwash sand aquifer, southern British Columbia, Canada | Tesoriero et al. (2000) |
| unconfined glacial outwash sand aquifer, Princeton, Minnesota, USA | Böhlke et al. (2002) |
| unconfined North Platte alluvial aquifer, Nebraska, USA | Verstraeten et al. (2001a, b) |
| upwelling, oxygen minimum zone, Eastern Tropical North Pacific Ocean | Cline and Kaplan (1975) |

words, most organisms in the environment are not similar to the organisms we have used in laboratory experiments designed to test our ideas of how denitrifying communities respond to environmental factors. In addition, the abundance of denitrifiers in the environment has been determined to be higher than that detected by culture techniques alone (Michotey et al. 2000).

Studies have also shown that different populations of denitrifying organisms develop under the selection of differing environmental conditions such as the oxygen level at the sediment surface, the water depth, and the $NO_3^-$ concentration (Braker et al. 2000, Scala and Kerkhof 2000, Liu et al. 2003). Greater differences are observed between communities from distant geographic locations rather than those present at discrete depths along steep vertical redox gradients. There are also reports of denitrifier communities containing nirK responding differently to environmental factors than those containing nirS (i.e., $NO_3^-$ concentration; Liu et al. 2003). Although community abundance and diversity respond to environmental controls this has not necessarily translated into a change in denitrification rates. No correlation between denitrification rates and measurable features of the denitrifying community has been documented. In some environments, genes for denitrification are often detected where there is no measurable denitrification activity (e.g., Tuomainen et al. 2003). This may not be a surprising result. Denitrification is part of the bioenergetic apparatus of the bacterial cell, where N oxides serve as alternative terminal acceptors. The bacterial process is nearly exclusively a facultative trait and a facultative respiratory pathway is not necessarily critical to the survival of a species (Tiedje 1988).

Most of the studies to date have relied on gene detection. A few have attempted to extract mRNA from environmental samples and use reverse transcriptase-PCR (RT-PCR) to measure the active denitrifying community. Detection of mRNAs, which typically have a short half-life, provides a strong indication of specific gene expression at the time of sampling that can be correlated with the physicochemical conditions and may provide a better link to denitrification activity. A few studies have demonstrated that both the presence and the expression of denitrification genes can be explored in complex environmental samples, but results have been somewhat unsatisfactory. For example, Nogales et al. (2002) looked at the presence and diversity of mRNA for the five key genes, narG, napA, nirS, nirK, and nosZ. Although samples from sites exhibiting high denitrification rates were analyzed in this study, they were unable to detect transcripts for three of the five denitrification genes detected in the sediments (narG, napA, and nirK) and the levels of nirS and nosZ were relatively low. Although detection of mRNAs in environmental samples by RT-PCR is potentially a powerful approach for analyzing gene expression in the environment, the technique suffers from inherent biases and limitations, primarily a function of template quantity, nontarget competition, stability of transcript, and enzyme (RT) inhibition.

In addition to the technical difficulties mentioned here, there are limits imposed by the unknown behavior and contribution of nonclassical denitrifiers. In addition to denitrification, alternative mechanisms for $NO_3^-$ removal and $N_2O$ and $N_2$ production have been reported. These reactions are carried out by organisms that often fill a different niche and that respond differently to environmental conditions than classical denitrifiers. The importance of fungal denitrification has not been ascertained but it is probably more of a consideration in terrestrial systems (Laughlin and Stevens 2002). Few studies have been done and far less is known about the enzymes responsible and the environmental factors controlling fungal denitrification. There is some indication that the genes involved and the

response to environmental conditions in fungi is different than for prokaryotic cells (Zumft 1997, Zhang et al. 2001), and will hence require new primers and probes to target them. For example, early reports were equivocal with respect to an inhibitory or stimulatory effect of oxygen, association of the reaction with $NO_3^-$ assimilation, and the use of $NO_3^-$ or nitrite as the active substrate. The importance of denitrifying metabolism by nitrifying bacteria in the environment and its significance to the physiological ecology of these organisms is unknown. In nitrifiers, and even in some methylotrophs, the enzymes responsible for catalyzing the reactions are encoded by nitrite reductase and NO reductase genes that are homologous to the *nirK* and *norB* genes of conventional denitrifying bacteria (Casciotti and Ward 2001, Ye and Thomas 2001). This complicates the interpretation of molecular data but may also provide an opportunity to refine or modify existing molecular techniques in order to evaluate the contribution of these other reactions. Further work is needed to determine if molecular tools can be used to apportion the relative contributions of these nonconventional denitrifiers.

Last, the roles of anammox and dissimilatory $NO_3^-$ reduction are unclear in most environments. Dissimilatory reduction of $NO_3^-$ to $NH_4^+$ (DNRA) is often ignored in aquatic and upland soil systems but has been shown to be important in sediments and wet tropical soils in which fermentative bacteria (with whose metabolism it is often associated) are likely to be found (Tiedje 1988, Silver et al. 2001). In low-redox soils, DNRA competes with denitrification as a fate for $NO_3^-$ resulting in N conservation not gaseous N loss. At this time, there are no specific primers for bacteria carrying out DNRA and hence the relationships between denitrifying and DNRA communities has yet to be examined. Anammox is a newly discovered process, in which ammonia and nitrite are converted anaerobically to dinitrogen gas, and has recently been reported from anaerobic wastewater systems and oxygen minimum zones (Strous et al. 1999, Dalsgaard et al. 2003). An organism responsible for this novel metabolism has been identified; it is a relative of *Planctomyces* and molecular probes have been developed (Kuypers et al. 2003).

In general, there are few differences in the application of molecular techniques to aquatic or terrestrial environments. Molecular analysis is limited by the quantity and quality of nucleic acids recovered from an environmental sample. One difference between these systems is due to the scales of the process and impacts the sampling strategies. In systems where denitrification is occurring in the surface water column or groundwater, the dimensions of denitrification activity is on the order of meters to kilometers. In aquatic sediments or terrestrial soils, localized zones of denitrification occur on the 100s of microns to cm scale. Sediment and soil samples tend to have a higher density of organisms and the typical sample size is a few grams so care must be taken to sample the appropriate sediment layers. Whereas, water

samples are commonly in liters collected onto a filter. DNA extractions of filtered samples are straightforward, and yield high quality DNA ready for further analyses. The extraction of nucleic acids, particularly RNA, from soils and sediments can be difficult in part because the soil/sediment matrix can interfere with efficient extraction and often substances inhibitory to PCR are co-extracted. Subsequent cleanup, if successful, can result in significant loss of total nucleic acids or preferential loss from certain organisms.

In summary, molecular characterization combined with quantification of environmental controllers and denitrification rates will allow us to evaluate abiotic and biotic controls responsible for the N transformations that we observe. Understanding the diversity of denitrifying bacterial populations in aquatic and terrestrial environments, the responses of microbial communities to environmental factors and the impact of changes in microbial community structure and composition on the rate of denitrification may be critical to understanding how environmental change will impact rates of the process. The development of molecular techniques has contributed enormously to the rapid recent progress in the field. While PCR and probing with nucleotides have been used to study the structure of denitrifying communities, studies of their function in natural habitats, which require quantification at the transcriptional level, are currently not routine. Future improvements in molecular techniques are needed to provide meaningful data for natural, noncultured assemblages. These data should eventually provide a complete picture of the potential for denitrification.

## CONCLUSIONS

Scientists have struggled with denitrification methodology for many decades, and it appears that their efforts have produced mixed results. Our review indicates that in some systems, (e.g., aquatic sediments, unconsolidated aquifers), current methodology allows for the production of useful estimates of denitrification at scales relevant to water quality and ecosystem fertility questions. Advances in denitrification measurement methodology have been driven partly by improvements in instrumentation, e.g., membrane inlet and other types of mass spectrometry, but also by clever reasoning about isotopic and hydrologic dynamics. Unsaturated terrestrial systems (soils) remain the greatest challenge, largely because of the high atmospheric background levels of $N_2$ that exchange rapidly with air. But even there, progress is being made, and new gas flow soil core and tracer level $^{15}N$ methods are producing estimates of denitrification relevant to large scale questions about atmospheric deposition and coastal eutrophication. Given that the ultimate test of methods is their ability to provide data useful to answering such questions, mass balance and stoichiometric approaches that constrain estimates of denitrification at large scales, will continue to be

important components of denitrification methodology. Continued comparison of mass balances with point measurements (made using multiple methods), in multiple systems, is likely to propel more improvement in denitrification methods over the next few years.

### Literature Cited

Aeschbach-Hertig, W., F. Peeters, U. Beyerle, and R. Kipfer. 2000. Paleotemperature reconstruction from noble gases in ground water taking into account equilibration with trapped air. Nature 405:1040–1044.

Allison, F. E. 1955. The enigma of soil nitrogen balance sheets. Advances in Agronomy 7:213–250.

Altabet, M. A., R. Francois, D. W. Murray, and W. L. Prell. 1995. Climate-related variations in denitrification in the Arabian Sea from sediment 15N/14N ratios. Nature 373:506–509.

Altabet, M., M. Higginson, and D. W. Murray. 2002. The effect of millennial-scale changes in Arabian Sea denitrification on atmospheric $CO_2$. Nature 415:159–162.

Altabet, M. A., C. Pilskaln, R. Thunell, C. Pride, D. Sigman, F. Chavez, and R. Francois. 1999. The nitrogen isotope biogeochemistry of sinking particles from the margin of the Eastern North Pacific. Deep-Sea Research 46:655–679.

Amberger, A., and H. L. Schmidt. 1987. Natürliche isotopengenhalte von nitrat als indikatoren für dessen herkunft. Geochimica et Cosmochimica Acta 51:2699–2705.

An, S., W. S. Gardner, and T. M. Kana. 2001. Simultaneous measurement of denitrification and nitrogen fixation using isotope pairing with membrane inlet mass spectrometer (MIMS) analysis. Applied and Environmental Microbiology 67:1171–1178.

An, S. M., and S. B. Joye. 1997. An improved chromatographic method to measure nitrogen, oxygen, argon and methane in gas or liquid samples. Marine Chemistry 59:63–70.

Andersen, J. M. 1977. Rates of denitrification of undisturbed sediment from six lakes as a function of nitrate concentration, oxygen, and temperature. Archives of Hydrobiology 80:147–159.

Appelo, C. A. J., and D. Postma. 1996. Geochemistry, groundwater and pollution. A. A. Balkema, Rotterdam, The Netherlands.

Aravena, R., and W. D. Robertson. 1998. Use of multiple isotope tracers to evaluate denitrification in ground water: study of nitrate from a large-flux septic system plume. Ground Water 36:975–982.

Aulakh, M. S., J. W. Doran, and A. R. Mosier. 1992. Soil denitrification: significance, measurement, and effects of management. Advances in Soil Science 18:1–52.

Balderston, W. L., B. Sherr, and W. J. Payne. 1976. Blockage by acetylene of nitrous oxide reduction in Pseudomonas perfectomarinus. Applied and Environmental Microbiology 31:504–508.

Barbaree, J. M., and W. J. Payne. 1967. Products of a marine bacterium as revealed by gas chromatography. Marine Biology 1:136–139.

Barford, C. C., J. P. Montoya, M. A. Altabet, and R. Mitchell. 1999. Steady-state nitrogen isotope effects of $N_2$ and $N_2O$ production in Paracoccus denitrificans. Applied and Environmental Microbiology 65:989–994.

Barnes, R. O., K. K. Bertine, and E. D. Goldberg. 1975. $N_2$:Ar, nitrification and denitrification in southern California borderland basin sediments. Limnology and Oceanography 20:962–970.

Barton, L., C. D. A. McLay, and L. A. Schipper. 1999. Annual denitrification rates in agricultural and forest soils: a review. Australian Journal of Soil Research 37:1073–1093.

Billen, G., M. Somville, E. deBecker, and P. Servais. 1985. A nitrogen budget of the Scheldt hydrographical basin. Pages 191–285 in F. Colijn and W. Helder, editors. Nutrients in estuaries: eutrophication and other effects. EBSA/Hydrobiological Society Symposium: Nederlands Instituut voor Onderzoek, Texel, The Netherlands.

Blicher-Mathiesen, G., G. W. McCarty, and L. P. Neilsen. 1998. Denitrification and degassing in groundwater estimated from dissolved dinitrogen and argon. Journal of Hydrology 208:16–24.

Böhlke, J. K. 2002. Groundwater recharge and agricultural contamination. Hydrogeology Journal 10:153–179 [Erratum, 2002, Hydrogeology Journal 2010:438–439].

Böhlke, J. K., and J. M. Denver. 1995. Combined use of groundwater dating, chemical, and isotopic analyses to resolve the history and fate of nitrate contamination in two agricultural watersheds, Atlantic coastal plain, Maryland. Water Resources Research 31:2319–2339.

Böhlke, J. K., J. W. Harvey, and M. A. Voytek. 2004, Reach-scale isotope tracer experiment to quantify denitrification and related processes in a nitrate-rich stream, midcontinent United States. Limnology and Oceanography 49:821–838.

Böhlke, J. K., R. Wanty, M. Tuttle, G. Delin, and M. Landon. 2002. Denitrification in the recharge area and discharge area of a transient agricultural nitrate plume in a glacial outwash sand aquifer. Minnesota. Water Resources Research 38:1105. [doi: 10.1029/2001WR000663]

Bollmann, A., and R. Conrad. 1997. Acetylene blockage technique leads to underestimation of denitrification rates in oxic soils due to scavenging of intermediate nitric oxide. Soil Biology and Biochemistry 29:1067–1077.

Bothe, H., G. Jost, M. Schloter, B. B. Ward, and K. P. Witzel. 2000. Molecular analysis of ammonia oxidation and denitrification in natural environments. FEMS Microbiology Review 24:673–690.

Böttcher, J., O. Strebel, and W. H. M. Duynisveld. 1989. Kinetik und modellierung gekoppelter stoffumsetzungen im grundwasser eines lockergesteins-aquifers. Geologishes Jahrbuch 51:3–40.

Böttcher, J., O. Strebel, S. Voerkelius, and H.-L. Schmidt. 1990. Using isotope fractionation of nitrate-nitrogen and nitrate-oxygen for evaluation of microbial denitrification in a sandy aquifer. Journal of Hydrology 114:413–424.

Braker, G., H. L. Ayala-del-Rio, A. H. Devol, A. Fesefeldt, and J. M. Tiedje. 2001. Community structure of denitrifiers, bacteria, and Archaea along redox gradients in Pacific northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase

(*nir*S) and 16S rRNA genes. Applied and Environmental Microbiology **67**:1893–1901.

Braker, G., J. Z. Zhou, L. Y. Wu, A. H. Devol, and J. M. Tiedje. 2000. Nitrite reductase genes (*nir*K and *nir*S) as functional markers to investigate diversity of denitrifying bacteria in Pacific northwest marine sediment communities. Applied and Environmental Microbiology **66**:2096–2104.

Brandes, J. A., and A. H. Devol. 2002. A global marine-fixed nitrogen isotopic budget: implications for Holocene nitrogen cycling. Global Biogeochemical Cycles **16**:1120. [doi: 10. 1029/2001GB001856]

Brandes, J. A., A. H. Devol, T. Yoshinari, D. A. Jayakumar, and S. W. A. Naqvi. 1998. Isotopic composition of nitrate in the central Arabian Sea and eastern tropical North Pacific: a tracer for mixing and nitrogen cycles. Limnology and Oceanography **43**:1680–1689.

Burdige, D. 1991. The kinetics of organic matter mineralization in anoxic marine sediments. Journal of Marine Research **49**: 727–761.

Burton, D. L., and E. G. Beauchamp. 1985. Denitrification rate relationships with soil parameters in the field. Communications in Soil Science and Plant Analysis **16**:539–549.

Butterbach-Bahl, K., G. Willibald, and H. Papen. 2002. Soil core method for direct simultaneous determination of $N_2$ and $N_2O$ emissions from forest soils. Plant and Soil **240**:105–116.

Caffrey, J. M., and W. M. Kemp. 1992. Influence of the submersed plant, *Potamogeton perfoliatus*, on nitrogen cycling in estuarine sediments. Limnology and Oceanography **37**:1483–1495.

Cárdenas, L. M., J. M. B. Hawkins, D. Chadwick, and D. Scholefield. 2003. Biogenic gas emissions from soils measured using an automated incubation system. Soil Biology and Biochemistry **35**:867–870.

Casciotti, K. L., D. M. Sigman, M. Galanter Hastings, J. K. Böhlke, and A. Hilkert. 2002. Measurement of the oxygen isotopic composition of nitrate in marine and fresh waters using the denitrifier method. Analytical Chemistry **74**:4905–4912.

Casciotti, K. L., D. M. Sigman, and B. B. Ward. 2003. Linking diversity and stable isotope fractionation in ammonia-oxidizing bacteria. Geomicrobiology Journal **20**:335–353.

Casciotti, K. L., and B. B. Ward. 2001. Nitrite reductase genes in ammonia oxidizing bacteria. Applied and Environmental Microbiology **67**:2213–2221.

Cavigelli, M. A., and G. P. Robertson. 2000. The functional significance of denitrifier community composition in a terrestrial ecosystem. Ecology **81**:1402–1414.

Cey, E. E., D. L. Rudolph, R. Aravena, and G. Parkin. 1999. Role of the riparian zone in controlling the distribution and fate of agricultural nitrogen near a small stream in southern Ontario. Journal of Hydrology. **37**:45–67.

Chanton, J. P., C. S. Martens, and M. B. Goldhaber. 1987. Biogeochemical cycling in an organic-rich coastal marine basin. 7. Sulfur mass balance, oxygen uptake and sulfide retention. Geochimica et Cosmochima Acta **51**:1187–1199.

Cho, J.-C., and J. M. Tiedje. 2002. Quantitative detection of microbial genes by using DNA microarrays. Applied and Environmental Microbiology. **68**:1425–1430.

Christensen, P. B., L. P. Nielsen, N. P. Revsbech, and J. Sørensen. 1989. Microzonation of denitrification activity in stream sediments as studied with a combined oxygen and nitrous oxide microsensor. Applied and Environmental Microbiology **55**:1234–1241.

Christensen, P. B., L. P. Nielsen, J. Sorensen, and N. P. Revsbech. 1990. Denitrification in nitrate-rich streams: diurnal and seasonal-variation related to benthic oxygen metabolism. Limnology and Oceanography **35**:640–651.

Clark, I. D., and P. Fritz. 1997. Environmental isotopes in hydrogeology. Lewis Publishers, New York, New York, USA.

Cline, J. D., and I. R. Kaplan. 1975. Isotopic fractionation of dissolved nitrate during denitrification in the eastern tropical North Pacific Ocean. Marine Chemistry **3**:271–299.

Cline, J. D., and F. A. Richards. 1972. Oxygen deficient conditions and nitrate reduction in the Eastern Tropical North Pacific Ocean. Limnology and Oceanography **17**:885–900.

Codispoti, L. A., J. A. Brandes, J. P. Christensen, A. H. Devol, S. W. A. Naqvi, H. W. Pearl, and T. Yoshinari. 2001. The oceanic fixed nitrogen and nitrous oxide budgets: moving targets as we enter the anthropocene. Scientia Marina **65**:85–105.

Colt, J. 1984. Computation of dissolved gas concentrations in water as functions of temperature, salinity, and pressure. American Fisheries Society Symposium Special Publication 14.

Cook, P. G., and J. K. Böhlke. 2000. Determining timescales for groundwater flow and solute transport. Pages 1–30 *in* P. G. Cook and A. Herczeg, editors. Environmental tracers in subsurface hydrology. Kluwer Academic Publishers, Boston, Massachusetts, USA.

Cook, P. G., and A. L. Herczeg, editors. 2000. Environmental tracers in subsurface hydrology. Kluwer Academic Publishers, Boston, Massachusetts, USA.

Cornwell, J. C., W. M. Kemp, and T. M. Kana. 1999. Denitrification in coastal ecosystems: environmental controls and aspects of spatial and temporal scale. Aquatic Ecology **33**:41–54.

Cornwell, J. C., and P. A. Sampou. 1995. Environmental controls on iron sulfide mineral formation in a coastal plain estuary. Pages 2224–2242 *in* M. A. Vairavamuthy and M. A. Schoonen, editors. Geochemical transformations of sedimentary sulfur. American Chemical Society, Washington, D.C., USA.

Dalsgaard, T., D. E. Canfield, J. Pederesen, B. Thamdrup, and J. Acuna-Gonzalez. 2003. $N_2$ production by the anammox reaction in the anoxic water column of the Golfo Dulce, Costa Rica. Nature **422**:606–608.

Dalsgaard, T., et al., 2000. Protocol handbook for NICE—nitrogen cycling in estuaries: a project under the EU research programme: Marine Science and Technology (MAST III). National Environmental Research Institute, Silkeborg, Denmark.

David, M. B., and L. E. Gentry. 2000. Anthropogenic inputs of nitrogen and phosphorus and riverine export for Illinois, USA. Journal of Environmental Quality **29**:494–508.

David, M. B., G. F. McIsaac, T. V. Royer, R. G. Darmody, and L. E. Gentry. 2001. Estimated historical and current nitrogen balances for Illinois. Scientific World **1**:597–604.

Delwiche, C. C., and P. L. Steyn. 1970. Nitrogen isotope fractionation in soils and microbial reactions. Environmental Science and Technology **4**:929–935.

Devol, A. H. 1991. Direct measurements of nitrogen gas fluxes from continental shelf sediments. Nature **349**:319–321.

Emerson, S., P. Quay, C. Stump, D. Wilbur, and M. Knox. 1991. $O_2$, Ar, $N_2$, and $^{222}Rn$ in surface waters of the subarctic ocean: net biological $O_2$ production. Global Biogeochemical Cycles **5**:49–69.

Emerson, S., C. Stump, D. Wilbur, and P. Quay. 1999. Accurate measurement of $O_2$, $N_2$, and Ar gases in water and the solubility of $N_2$. Marine Chemistry **64**:337–347.

Emery, K. O., W. L. Orr, and S. C. Rittenberg. 1955. Nutrient budgets in the ocean. Pages 299–310 *in* Essays in the natural sciences in honor of Captain Alan Hancock. University of Southern California Press, Los Angeles, California, USA.

Eyre, B. D., S. Rysgaard, T. Dalsgaard, and P. B. Christensen. 2002. Comparison of isotope pairing and $N_2$/Ar methods for measuring sediment denitrification: assumptions, modifications, and implications. Estuaries **25**:1077–1087.

Fenchel, T., and T. H. Blackburn. 1979. Bacteria and mineral cycling. Academic Press, New York, New York, USA.

Flanagan, D. A., L. G. Gregory, J. P. Carter, A. Karakas-Sen, D. J. Richardson, and S. Spiro. 1999. Detection of genes for periplasmic nitrate reductase in nitrate respiring bacteria and in community DNA. FEMS Microbiology Letters **177**:263–270.

Flather, D. H., and E. G. Beauchamp. 1992. Inhibition of the fermentation process in soil by acetylene. Soil Biology and Biochemistry **24**:905–911.

Foloronuso, O. A., and D. E. Rolston. 1984. Spatial variability of field-measured denitrification gas fluxes and soil properties. Soil Science Society of America Journal **49**:1087–1093.

Fossing, H., et al. 1995. Concentration and transport of nitrate by the mat-forming sulfur bacterium *Thioploca*. Nature **374**:713–715.

Frind, E. O., W. H. M. Duynisveld, O. Strebel, and J. Boettcher. 1990. Modeling of multicomponent transport with microbial transformation in groundwater: the Fuhrberg case. Water Resources Research **26**:1707–1719.

Fukada, T., K. M. Hiscock, P. F. Dennis, and T. Grischek. 2003. A dual isotope approach to identify denitrification in groundwater at a river-bank infiltration site. Water Research **37**:3070–3078.

Galloway, J. N., J. D. Aber, J. W. Erisman, S. P. Seitzinger, R. W. Howarth, E. B. Cowling, and B. J. Cosby. 2003. The nitrogen cascade. BioScience **53**:341–356.

Galloway, J. N., W. H. Schlesinger, H. Levy, A. Michaels, and J. L. Schnoor. 1995. Nitrogen fixation: anthropogenic enhancement-environmental response. Global Biogeochemical Cycles **9**:235–252.

Galsworthy, A. M., and J. R. Burford. 1978. A system for measuring the rates of evolution of nitrous oxide and nitrogen from incubated soils during denitrification. Journal of Soil Science **29**:537–550.

Ganeshram, R. S., T. F. Pedersen, S. E. Calvert, G. W. McNeill, and M. R. Fontugne. 2000. Glacial-interglacial variability in denitrification in the world's oceans: causes and consequences. Paleoceanography **15**:361–376.

Ganeshram, R. S., T. F. Pedersen, S. E. Calvert, and J. W. Murray. 1995. Large changes in oceanic nutrient inventories from glacial to interglacial periods. Nature **376**:755–757.

Giblin, A. E., C. S. Hopkinson, and J. Tucker. 1997. Benthic metabolism and nutrient cycling in Boston Harbor, Massachusetts, U.S.A. Estuaries **20**:346–364.

Goolsby, D. A., W. A. Battaglin, G. B. Lawrence, R. S. Artz, B. T. Aulenbach, R. P. Hooper, D. R. Keeney, and G. J. Stensland. 1999. Flux and sources of nutrients in the Mississippi–Atchafalaya River basin. Topic 3. Report of the integrated assessment on hypoxia in the Gulf of Mexico. NOAA Coastal Ocean Program Decision Analysis Series No. 17. NOAA Coastal Ocean Program, Silver Spring, Maryland, USA.

Gordon, D. C., Jr., P. R. Boudreau, K. H. Mann, J.-E. Ong, W. L. Silvert, S. V. Smith, G. Wattayakorn, F. Wulff, and T. Yanagi. 1996. LOICZ biogeochemical modelling guidelines. LOICZ Reports and Studies **5**:1–96.

Granger, J., D. M. Sigman, J. A. Needoba, and P. J. Harrison. 2004. Coupled nitrogen and oxygen isotope fractionation of nitrate during assimilation by cultures of marine phytoplankton. Limnology and Oceanography **49**:1763–1773.

Gregory, L. G., A. Karakas-Sen, D. J. Richardson, and S. Spiro. 2000. Detection of genes for membrane-bound nitrate reductase in nitrate-respiring bacteria and in community DNA. FEMS Microbiology Letters **183**:275–279.

Griggs, E. M., L. R. Kump, and J. K. Böhlke. 2003. The fate of wastewater-derived nitrate in the subsurface of the Florida Keys: Key Colony Beach, Florida. Estuarine, Coastal and Shelf Science **58**:517–539.

Groffman, P. M., E. Holland, D. D. Myrold, G. P. Robertson, and X. Zou. 1999. Denitrification. Pages 272–288 *in* G. P. Robertson, C. S. Bledsoe, D. C. Coleman, and P. Sollins, editors. Standard soil methods for long term ecological research. Oxford University Press, New York, New York, USA.

Groffman, P. M., D. R. Zak, S. Christensen, A. R. Mosier, and J. M. Tiedje. 1993. Early spring nitrogen dynamics in a temperate forest landscape. Ecology **74**:1579–1585.

Gross, P. J., and J. M. Bremner. 1992. Acetone problem in use of the acetylene blockage method for assessment of denitrifying activity in soil. Communications in Soil Science and Plant Analysis **23**:1345–1358.

Gruber, N., and J. L. Sarmiento. 1997. Global patterns of marine nitrogen fixation and denitrification. Global Biogeochemical Cycles **11**:235–266.

Gruntzig, V., S. C. Nold, J. Z. Zhou, and J. M. Tiedje. 2001. *Pseudomonas stutzeri* nitrite reductase gene abundance in environmental samples measured by real-time PCR. Applied and Environmental Microbiology. **67**:760–768.

Guschin, D. Y., B. K. Mobarry, D. Proudnikov, D. A. Stahl, B. E. Rittmann, and A. D. Mirzabekov. 1997. Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. Applied and Environmental Microbiology **63**:2397–2402.

Hallberg, G. R., and D. R. Keeney. 1993. Nitrate. Pages 297–322 *in* W. M. Alley, editor. Regional ground-water quality. Van Nostrand Reinhold, New York, New York, USA.

Hallin, S., and P. E. Lindgren. 1999. PCR detection of genes encoding nitrite reductase in denitrifying bacteria. Applied and Environmental Microbiology. **65**:1652–1657.

Hauck, R. D., and S. W. Melsted. 1956. Some aspects of the problem of evaluating denitrification in soils. Soil Science Society of America Proceedings **20**:361–364.

Hauck, R. D., S. W. Melsted, and P. E. Yankwich. 1958. Use of N-isotope distribution in nitrogen gas in the study of denitrification. Soil Science **86**:287–291.

Heaton, T. H. E., and J. C. Vogel. 1981. "Excess air" in ground water. Journal of Hydrology **50**:201–216.

Hecky, R. E., P. Campbell, and L. L. Hendzel. 1993. The stoichiometry of carbon, nitrogen, and phosphorus in particulate matter of lakes and oceans. Limnology and Oceanography **38**:709–742.

Hendy, I. L., and J. P. Kennett. 2003. Tropical forcing of North Pacific Intermediate water distribution during late Quaternary rapid climate change. Quaternary Science Review **22**:673–689.

Henry, S., E. Baudoin, J. C. Lopez-Gutierrez, F. Martin-Laurent, A. Brauman, and L. Philippot. 2004. Quantification of denitrifying bacteria in soils by *nir*K gene targeted real-time PCR. Journal of Microbiological Methods **59**:327–335.

Higginson, M. J., and M. A. Altabet. 2004. Initial test of the silicic acid leakage hypothesis using sedimentary biomarkers. Geophysical Research Letters **31**:L18303. [doi: 10.1029/2004GL020511]

Hiscock, K. M., J. W. Lloyd, and D. N. Lerner. 1991. Review of natural and artificial denitrification of groundwater. Water Resources **25**:1099–1111.

Hopkinson, C. S., A. E. Giblin, and J. Tucker. 2001. Benthic metabolism and nutrient regeneration on the continental shelf of Eastern Massachusetts, USA. Marine Ecology Progress Series **224**:1–19.

Hopkinson, C. S., A. E. Giblin, J. Tucker, and R. H. Garritt. 1999. Benthic metabolism and nutrient cycling along an estuarine salinity gradient. Estuaries **22**:863–881.

Howarth, R. W., et al. 1996. Regional nitrogen budgets and riverine N and P fluxes for the drainages to the North Atlantic Ocean: natural and human influences. Biogeochemistry **35**:75–139.

Hyman, M. R., and D. J. Arp. 1987. Quantification and removal of some contaminating gases from acetylene used to study gas-utilizing enzymes and microorganisms. Applied and Environmental Microbiology 53:298–303.

Hynes, R. K., and R. Knowles. 1978. Inhibition by acetylene of ammonia oxidation in Nitrosomonas europaea. FEMS Microbiology Letters 4:319–321.

Indermuhle, A., E. Monnin, B. Stauffer, T. F. Stocker, and M. Wahlen. 2000. Atmospheric CO$_2$ concentration from 60 to 20 Kyr BP from the Taylor Dome ice core, Antarctica. Geophysical Research Letters 27:735–738.

Istok, J. D., M. D. Humphrey, M. H. Schroth, M. R. Hyman, and K. T. O'Reilly. 1997. Single-well, "push-pull" test for in situ determination of microbial activities. Ground Water 35: 619–631.

Jenkins, M. C., and W. M. Kemp. 1985. The coupling of nitrification and denitrification in two estuarine sediments. Limnology and Oceanography 29:609–619.

Jensen, K., N. P. Revsbech, and L. P. Nielsen. 1993. Microscale distribution of nitrification activity in sediment determined with a shielded microsensor for nitrate. Applied and Environmental Microbiology 59:3287–3296.

Johnstone, J. 1908. Conditions of life in the sea. Cambridge University Press, Cambridge, UK.

Jorgensen, B. B. 1982. Mineralization of organic matter in the sea bed: the role of sulfate reduction. Nature 296:643–645.

Jossette, G., B. Leporcq, N. Sanchez, and X. Philippon. 1999. Biogeochemical mass-balances (C, N, P, Si) in three large reservoirs of the Seine Basin (France). Biogeochemistry 47: 119–146.

Joye, S. B., and H. W. Paerl. 1993. Contemporaneous nitrogen fixation and denitrification in intertidal microbial mats: rapid response to runoff events. Marine Ecology Progress Series 94: 267–274.

Jury, W. A., J. Letey, and T. Collins. 1982. Analysis of chamber methods used for measuring nitrous oxide production in the field. Soil Science Society of America Journal 46:250–255.

Kamp-Nielsen, L. 1992. Benthic-pelagic coupling of nutrient metabolism along an estuarine eutrophication gradient. Hydrobiologia 235/236:457–470.

Kana, T. M., C. Darkangelo, M. D. Hunt, J. B. Oldham, G. E. Bennett, and J. C. Cornwell. 1994. A membrane inlet mass spectrometer for rapid high precision determination of N$_2$, O$_2$, and Ar in environmental water samples. Analytical Chemistry 66:4166–4170.

Kana, T. M., M. B. Sullivan, J. C. Cornwell, and K. Groszkowski. 1998. Denitrification in estuarine sediments determined by membrane inlet mass spectrometry. Limnology and Oceanography 43:334–339.

Kana, T. M., and D. L. Weiss. 2004. Comment on "comparison of isotope pairing and N$_2$/Ar methods for measured sediments" by B. D. Eyre, S. Rysaard, T. Dalsgaard, and P. Bondo Christensen. (2002. Estuaries 25:1077–1087.) Estuaries 27:157–160.

Karl, D., A. Michaels, B. Bergman, D. Capone, E. Carpenter, R. Letelier, F. Lipshultz, H. Pearl, D. Sigman, and L. Stal. 2002. Dinitrogen fixation in the world's oceans. Biogeochemistry 57/58:47–98.

Keeney, D. R. 1986. Critique of the acetylene blockage technique for field measurement of denitrification. Pages 103–115 in R. D. Hauck and R. W. Weaver, editors. Field measurement of dinitrogen fixation and denitrification. Soil Science Society of America, Madison, Wisconsin.

Kelly, J. R. 1997. Nitrogen flow and the interaction of Boston Harbor with Massachusetts Bay. Estuaries 20:365–380.

Kienast, S. S., S. E. Calvert, R. W. Collier, and T. F. Pedersen. 2002. Nitrogen isotope and productivity variations along the northeast Pacific margin over the last 120 kyr: surface and subsurface paleoceanography. Paleoceanography 17:1055. [doi: 10.1029/2001PA000650]

Kralova, M., P. H. Masscheleyn, C. W. Lindau, and W. H. Patrick. 1992. Production of dinitrogen and nitrous oxide in soil suspensions as affected by redox potential. Water, Air and Soil Pollution 61:37–45.

Kuypers, M. M. M., A. O. Sliekers, G. Lavik, M. Schmid, B. B. Jorgensen, J. G. Kuenen, J. S. Sinninghe Damsté, M. Strous, and M. S. M. Jetten. 2003. Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. Nature 422:608–611.

Laughlin, R. J., and R. J. Stevens. 2002. Evidence for fungal dominance of denitrification and codenitrification in a grassland soil. Soil Science Society America Journal 66: 1540–1548.

Laursen, A. E., and S. P. Seitzinger. 2002. Measurement of denitrification in rivers: an integrated, whole reach approach. Hydrobiology 485:67–81.

Lehman, M. F., P. Reichert, S. M. Bernasconi, A. Barbieri, and J. A. McKenzie. 2003. Modelling nitrogen and oxygen isotope fractionation during denitrification in a lacustrine redox-transition zone. Geochimica et Cosmochimica Acta 67: 2529–2542.

Lindsey, B. D., S. W. Phillips, C. A. Donnelly, G. K. Speiran, L. N. Plummer, J. K. Böhlke, M. J. Focazio, W. C. Burton, and E. Busenberg. 2003. Residence times and nitrate transport in ground water discharging to streams in the Chesapeake Bay Watershed. U.S. Geological Survey Water Resources Investigations Report 03-4035. U.S. Geological Survey, New Cumberland, Pennsylvania, USA.

Linne von Berg, K.-H., and H. Bothe. 1992. The distribution of denitrifying bacteria in soils monitored by DNA-probing. FEMS Microbiology Ecology 86:331–340.

Liu, W.-T., T. L. Marsh, H. Cheng, and L. J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Applied and Environmental Microbiology 63:4516–4522.

Liu, X., S. M. Tiquia, G. Holguin, L. Wu, S. C. Nold, A. H. Devol, K. Luo, A. V. Palumbo, J. M. Tiedje, and J. Zhou. 2003. Molecular diversity of denitrifying genes in continental margin sediments within the oxygen-deficient zone off the Pacific coast of Mexico. Applied and Environmental Microbiology 69:3549–3560.

Lohse, L., H. T. Kloosterhuis, W. van Raaphorst, and W. Heldere. 1996. Denitrification rates as measured by the isotope pairing method and by the acetylene inhibition technique in continental shelf sediments of the North Sea. Marine Ecology Progress Series 29:169–179.

Mariotti, A., J. C. Germon, P. Hubert, P. Kaiser, R. Letolle, A. Tardieux, and P. Tardieux. 1981. Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. Plant and Soil 62:413–430.

Mariotti, A., A. Landreau, and B. Simon. 1988. N isotope biogeochemistry and natural denitrification process in groundwater: application to the chalk aquifer of northern France. Geochimica et Cosmochimica Acta 52:1869–1878.

Martin, J. H., G. A. Knauer, D. M. Karl, and W. W. Broenkow. 1987. VERTEX: carbon cycling in the northeast Pacific. Deep Sea Research Part A 34:267–285.

McClain, M. E., E. W. Boyer, C. L. Dent, S. E. Gergel, N. B. Grimm, P. M. Groffman, S. C. Hart, J. W. Harvey, C. A. Johnston, E. Mayorga, W. H. McDowell, and G. Pinay. 2003. Biogeochemical hot spots and hot moments at the interface of terrestrial and aquatic ecosystems. Ecosystems 6: 301–312.

McCutchan, J. H., J. F. Saunders, A. L. Pribyl, and W. M. Lewis. 2003. Open-channel estimation of denitrification. Limnology and Oceanography Methods 1:74–81.

McElroy, M. B. 1983. Marine biological controls on atmospheric CO$_2$ and climate. Nature 302:328–329.

McIsaac, G. F., and X. Hu. 2004. Net N input and riverine N export from Illinois agricultural watersheds with and without extensive tile drainage. Biogeochemistry **70**:251–271.

McMahon, P. B., and J. K. Böhlke. 1996. Denitrification and mixing in a stream-aquifer system: effects on nitrate loading to surface water. Journal of Hydrology **186**:105–128.

McMahon, P. B., J. K. Böhlke, and B. W. Bruce. 1999. Denitrification in marine shales in northeastern Colorado, USA. Water Resources Research **35**:1629–1642.

McMahon, P. B., J. K. Böhlke, and S. C. Christenson. 2004a. Geochemistry, radiocarbon ages, and paleorecharge conditions along a transect in the central High Plains aquifer, southwestern Kansas, USA. Applied Geochemistry **19**:1655–1686.

McMahon, P. B., J. K. Böhlke, and T. M. Lehman. 2004b. Vertical gradients in water chemistry and age in the southern High Plains Aquifer, Texas, 2002. U.S. Geological Survey Scientific Investigations Report 2004-5053. U.S. Geological Survey, Reston, Virginia, USA.

Mengis, M., S. L. Schiff, M. Harris, M. C. English, R. Aravena, R. J. Elgood, and A. MacLean. 1999. Multiple geochemical and isotopic approaches for assessing ground water $NO_3^-$ elimination in a riparian zone. Ground Water **37**:448–457.

Mergel, A., O. Schmitz, T. Mallmann, and H. Bothe. 2001. Relative abundance of denitrifying and dinitrogen-fixing bacteria in layers of a forest soil. FEMS Microbiology Ecology **36**:33–42.

Michotey, V., V. Mejean, and P. Bonin. 2000. Comparison of methods for quantification of cytochrome cd1-denitrifying bacteria in environmental marine samples. Applied and Environmental Microbiology **66**:1564–1571.

Middelburg, J. J., K. Soetaert, and P. M. J. Herman. 1996a. Evaluation of the nitrogen isotope-pairing method for measuring benthic denitrification: a simulation analysis. Limnology and Oceanography **41**:1839–1844.

Middelburg, J. J., K. Soetaert, and P. M. J. Herman. 1996b. Reply to the comment by Nielsen et al. Limnology and Oceanography **41**:1846–1847.

Miyake, Y., and E. Wada. 1971. The isotope effect on the nitrogen in biochemical, oxidation-reduction reactions. Records of Oceanographic Works in Japan **11**:1–6.

Moeseneder, M., J. M. Arrieta, G. Muyzer, C. Winter, and G. Herndl. 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Applied and Environmental Microbiology **65**:3518–3525.

Mortimer, C. H. 1941. The exchange of dissolved substances between mud and water. I and II. Journal of Ecology **29**:280–329.

Mosier, A. R. 1980. Acetylene inhibition of ammonium oxidation in soil. Soil Biology and Biochemistry **12**:443–444.

Mulholland, P. J., H. M. Vallett, J. R. Webster, S. A. Thomas, L. N. Cooper, S. K. Hamilton, and B. J. Peterson. 2004. Stream denitrification and total nitrate uptake rates measured using a field 15N tracer addition approach. Limnology and Oceanography **49**:809–820.

Myrold, D. D. 1990. Measuring denitrification in soils using $^{15}N$ techniques. Pages 181–198 in N. P. Revsbech and J. Sørensen, editors. Denitrification in soil and sediment. Plenum Press, New York, New York, USA.

Nieder, R., G. Schollmayer, and J. Richter. 1989. Denitrification in the rooting zone of cropped soils with regard to methodology and climate: a review. Biology and Fertility of Soils **8**:219–226.

Nielsen, K., L. P. Nielsen, and P. Rasmussen. 1995. Estuarine nitrogen retention independently estimated by the denitrification rate and mass balance methods: a study of Norsminde Fjord, Denmark. Marine Ecology Progress Series **119**:275–283.

Nielsen, K., N. Risgaard-Petersen, B. Sømod, S. Rysgaard, and T. Bergo. 2001. Nitrogen and phosphorus retention estimated independently by flux measurements and dynamic modelling in the estuary, Randers Fjord, Denmark. Marine Ecology Progress Series **219**:25–40.

Nielsen, L. P. 1992. Denitrification in sediment determined from nitrogen isotope pairing. FEMS Microbiology Ecology **86**:357–362.

Nielsen, L. P., N. Risgaard-Petersen, S. Rysgaard, and T. H. Blackburn. 1996. Reply to the note by Middelburg et al. Limnology and Oceanography **41**:1845–1846.

Nishio, T., I. Koike, and A. Hattori. 1981. $N_2$/Ar and denitrification in Tama Estuary sediments. Geomicrobiology Journal **2**:193–209.

Nishio, T., I. Koike, and A. Hattori. 1982. Denitrification, nitrate reduction and oxygen-consumption in coastal and estuarine sediments. Applied and Environmental Microbiology **43**:648–653.

Nishio, T., I. Koike, and A. Hattori. 1983. Estimates of denitrification and nitrification in coastal and estuarine sediments. Applied and Environmental Microbiology **45**:444–450.

Nixon, S. W., et al. 1996. The fate of nitrogen and phosphorus at the land-sea margin of the North Atlantic Ocean. Biogeochemistry **35**:141–180.

Nixon, S. W., C. A. Oviatt, and S. S. Hale. 1976. Nitrogen regeneration and the metabolism of coastal marine bottom communities. Pages 269–283 in J. M. Anderson and A. MacFadyen, editors. The role of terrestrial and aquatic organisms in decomposition processes. Blackwell, Oxford, UK.

Nogales, B., K. N. Timmis, D. B. Nedwell, and A. M. Osborn. 2002. Detection and diversity of expressed denitrification genes in estuarine sediments after reverse transcription-PCR amplification from mRNA. Applied and Environmental Microbiology **68**:5017–5025.

Nõmmik, H. 1956. Investigations on denitrification in soils. Acta Agriculturae Scandinavica **6**:195–228.

Nowicki, B. L. 1994. The effect of temperature, oxygen, salinity, and nutrient enrichment on estuarine denitrification rates measured with a modified nitrogen gas flux technique. Estuarine and Coastal Shelf Science **38**:137–156.

Oremland, R. S., C. Umberger, C. W. Culbertson, and R. L. Smith. 1984. Denitrification in San Francisco Bay intertidal sediments. Applied and Environmental Microbiology **47**:1106–1112.

Ottosen, L. D. M., N. Risgaard-Petersen, and L. P. Nielsen. 1999. Direct and indirect measurements of nitrification and denitrification in the rhizosphere of aquatic macrophytes. Aquatic Microbial Ecology **19**:81–91.

Ottosen, L. D. M., N. Risgaard-Petersen, L. P. Nielsen, and T. Dalsgaard. 2001. Denitrification in exposed intertidal mud-flats, measured with a new N-15-ammonium spray technique. Marine Ecology Progress Series **209**:35–42.

Parkin, T. B. 1987. Soil microsites as a source of denitrification variability. Soil Science Society of America Journal **51**:1194–1199.

Parkin, T. B., H. F. Kaspar, A. J. Sexstone, and J. M. Tiedje. 1984. A gas-flow soil core method to measure field denitrification rates. Soil Biology and Biochemistry **16**:323–330.

Parkin, T. B., J. L. Starr, and J. J. Meisinger. 1987. Influence of sample size on measurements of soil denitrification rates. Soil Science Society of America Journal **51**:1492–1501.

Payne, W. J. 1991. A review of methods for field measurements of denitrification. Forest Ecology and Management **44**:5–14.

Postma, D., C. Boesen, H. Kristiansen, and F. Larsen. 1991. Nitrate reduction in an unconfined sandy aquifer: water chemistry, reduction processes, and geochemical modeling. Water Resources Research **27**:2027–2045.

Pribyl, A. L., J. H. McCutchan, Jr., W. M. Lewis, Jr., and J. F. Saunders. 2005. Whole-system estimation of denitrification in a plains river: a comparison of two methods. Biogeochemistry **73**:439–455.

Rayleigh, L. 1896. Theoretical considerations respecting the separation of gases by diffusion and similar processes. Philosophical Magazine **42**:493–498.

Redfield, A. C. 1934. On the proportions of organic derivatives in sea water and their relation to the composition of plankton. Pages 177–192 in R. J. Daniel, editor. James Johnstone Memorial Volume. Liverpool University Press, Liverpool, UK.

Redfield, A. C., B. H. Ketchum, and F. A. Richards. 1963. The influence of organisms on the composition of sea-water. Pages 26–77 in M. N. Hill, editor. The sea. Volume 2. John Wiley and Sons, New York, New York, USA.

Reeburgh, W. S. 1969. Observation of gases in Chesapeake Bay sediments. Limnology and Oceanography **14**:368–375.

Revsbech, N. P., and J. Sørensen. 1990. Measurement of sediment denitrification using 15-N tracer method. Pages 259–275 in N. P. Revsbech and J. Sørensen, editors. Denitrification in soil and sediment. Plenum Press, New York, New York, USA.

Rich, J. J., R. S. Heichen, P. J. Bottomley, K. Cromack, Jr., and D. D. Myrold. 2003. Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. Applied and Environmental Microbiology **69**:5974–5982.

Richards, F. A. 1965. Anoxic basins and fjords. Pages 611–645 in J. P. Riley and G. Skirrow, editors. Chemical oceanography. Volume 1. Academic Press, New York, New York, USA.

Richards, F. A., and B. B. Benson. 1961. Nitrogen/argon and nitrogen isotope ratios in two anaerobic environments, the Cariaco Trench in the Caribbean Sea and Dramsfjord, Norway. Deep-Sea Research **7**:254–264.

Risgaard-Petersen, N., L. P. Nielsen, and T. H. Blackburn. 1998. Simultaneous measurement of benthic denitrification, with the isotope pairing technique and the $N_2$ flux method in a continuous flow-through system. Water Research **32**:3371–3377.

Risgaard-Petersen, N., L. P. Nielsen, S. Rysgaard, T. Dalsgaard, and R. L. Meyer. 2003. Application of the isotope pairing technique in sediments where anammox and denitrification coexist. Limnology and Oceanography Methods **1**:63–73.

Risgaard-Petersen, N., S. Skårup, and L. P. Nielsen. 1999. Denitrification in a soft bottom lake: evaluation of laboratory incubation. Aquatic Microbial Ecology **17**:279–287.

Robertson, G. P., M. A. Huston, F. C. Evans, and J. M. Tiedje. 1988. Spatial variability in a successional plant community: patterns of nitrogen availability. Ecology **69**:1517–1524.

Robertson, G. P., and J. M. Tiedje. 1984. Denitrification and nitrous oxide production in old growth and successional Michigan forests. Soil Science Society of America Journal **48**:383–389.

Robertson, G. P., and J. M. Tiedje. 1987. Nitrous oxide sources in aerobic soils: nitrification, denitrification and other biological processes. Soil Biology and Biochemistry **19**:187–193.

Robertson, W. D., B. M. Russell, and J. A. Cherry. 1996. Attenuation of nitrate in aquitard sediments of southern Ontario. Journal of Hydrology **180**:267–281.

Ross, P. J., A. E. Martin, and E. F. Henzell. 1964. A gas-tight growth chamber for investigating gaseous nitrogen changes in the soil : plant : atmosphere system. Nature **204**:444–447.

Royer, T. V., J. L. Tank, and M. B. David. 2004. The transport and fate of nitrate in headwater, agricultural streams in Illinois. Journal of Environmental Quality **33**:1296–1304.

Rudolph, J., P. Frenzel, and N. Pfennig. 1991. Acetylene inhibition technique underestimates in situ denitrification rates in intact cores of freshwater sediment. FEMS Microbiology Ecology **85**:101–106.

Ryden, J. C., and K. P. Dawson. 1982. Evaluation of the acetylene-inhibition technique for the measurement of denitrification in grassland soils. Journal of the Science of Food and Agriculture **33**:1197–1206.

Ryden, J. C., L. J. Lund, and D. D. Focht. 1979a. Direct measurement of denitrification loss from soils: I. Laboratory evaluation of acetylene inhibition of nitrous oxide reduction. Soil Science Society of America Journal **43**:104–109.

Ryden, J. C., L. J. Lund, J. Letey, and D. D. Focht. 1979b. Direct measurement of denitrification loss from soils: II. Development and application of field methods. Soil Science Society of America Journal **43**:110–118.

Ryden, J. C., and J. H. Skinner. 1987. Soil core incubation system for the field measurement of denitrification using acetylene inhibition. Soil Biology and Biochemistry **19**:753–757.

Scala, D. J., and L. J. Kerkhof. 1999. Diversity of nitrous oxide reductase (nosZ) genes in continental shelf sediments. Applied and Environmental Microbiology. **65**:1681–1687.

Scala, D. J., and L. J. Kerkhof. 2000. Horizontal heterogeneity of denitrifying bacterial communities in marine sediments by terminal restriction fragment length polymorphism analysis. Applied and Environmental Microbiology. **66**:1980–1986.

Scholefield, D., J. M. B. Hawkins, and S. M. Jackson. 1997a. Development of a helium atmosphere soil incubation technique for direct measurement of nitrous oxide and dinitrogen fluxes during denitrification. Soil Biology and Biochemistry **29**:1345–1352.

Scholefield, D., J. M. B. Hawkins, and S. M. Jackson. 1997b. Use of a flowing helium atmosphere incubation technique to measure the effects of denitrification controls applied to intact cores of a clay soil. Soil Biology and Biochemistry **29**:1337–1344.

Schulz, H. N., and B. B. Jørgensen. 2001. Big bacteria. Annual Review of Microbiology **55**:105–137.

Seitzinger, S. 1988. Denitrification in freshwater and coastal marine ecosystems: ecological and geochemical significance. Limnology and Oceanography **33**:702–724.

Seitzinger, S. P., L. P. Nielsen, J. Caffrey, and P. B. Christensen. 1993. Denitrification measurements in aquatic sediments: a comparison of three methods. Biogeochemistry **23**:147–167.

Seitzinger, S. P., S. W. Nixon, M. E. Q. Pilson, and S. Burke. 1980. Denitrification and $N_2O$ production in nearshore marine sediments. Geochimica Cosmochima Acta **44**:1853–1860.

Sexstone, A. J., T. B. Parkin, and J. M. Tiedje. 1985. Temporal response of soil denitrification rates to rainfall and irrigation. Soil Science Society of America Journal **49**:99–103.

Sigman, D. M., R. Robinson, A. N. Knapp, A. van Geen, D. C. McCorkle, J. A. Brandes, and R. C. Thunell. 2003. Distinguishing between water column and sedimentary denitrification in the Santa Barbara Basin using the stable isotopes of nitrate. Geochemistry Geophysics Geosystems **4**:1–20.

Silver, W. L., D. L. Herman, and M. K. Firestone. 2001. Dissimilatory nitrate reduction to ammonium in upland tropical forest soils. Ecology **82**:2410–2416.

Slater, J. M., and D. G. Capone. 1989. Nitrate requirement for acetylene inhibition of nitrous oxide reduction in marine sediments. Microbial Ecology **17**:143–157.

Smith, R. L., J. K. Böhlke, S. P. Garabedian, K. M. Revesz, and T. Yoshinari. 2004. Assessing denitrification in groundwater using natural gradient tracer tests with $^{15}N$: in situ measurement of a sequential multistep reaction. Water

Resources Research **40**:W07101. [doi: 10.1029/2003WR002919]

Smith, R. L., B. L. Howes, and J. H. Duff. 1991. Denitrification in nitrate-contaminated ground water: occurrence in steep vertical geochemical gradients. Geochimica et Cosmochimica Acta **55**:1815–1825.

Smith, S. V., J. T. Hollibaugh, S. J. Dollar, and S. Vink. 1991. Tomales Bay metabolism: C–N–P stoichiometry and ecosystem heterotrophy at the land-sea interface. Estuarine Coastal and Shelf Science **33**:223–257.

Sørenson, J. 1978. Denitrification rates in a marine sediment as measured by acetylene inhibition technique. Applied and Environmental Microbiology **36**:143–189.

Stefanson, R. C. 1970. Sealed growth chambers for studies of the effect of plants on the soil atmosphere. Journal of Agriculture and Engineering Research **15**:295–301.

Stefanson, R. C. 1972. Soil denitrification in sealed soil–plant systems. I. Effect of plants, soil water content and soil organic matter content. Plant and Soil **33**:113–127.

Stefanson, R. C., and D. J. Greenland. 1970. Measurement of nitrogen and nitrous oxide evolution from soil–plant systems using sealed growth chambers. Soil Science **109**:203–206.

Steingruber, S. M., J. Friedrich, R. Gachter, and B. Wehrli. 2001. Measurement of denitrification in sediments with the N-15 isoptope pairing technique. Applied and Environmental Microbiology **67**:3771–3778.

Sterner, R. W., and J. J. Elser. 2002. Ecological stoichiometry: the biology of elements from molecules to the biosphere. Princeton University Press, Princeton, New Jersey, USA.

Stevens, R. J., and R. J. Laughlin. 2001. Lowering the detection limit for dinitrogen using the enrichment of nitrous oxide. Soil Biology and Biochemistry **33**:1287–1289.

Stevens, R. J., R. J. Laughlin, G. J. Atkins, and S. J. Prosser. 1993. Automated determination of nitrogen-15-labeled dinitrogen and nitrous oxide by mass spectrometry. Soil Science Society of America Journal **57**:981–988.

Strebel, O., J. Boettcher, and P. Fritz. 1990. Use of isotope fractionation of sulfate-sulfur and sulfate-oxygen to assess bacterial desulfurication in a sandy aquifer. Journal of Hydrology **121**:155–172.

Strous, M., J. A. Fuerst, E. H. M. Kramer, S. Logemann, G. Muyzer, K. T. van de Pas-Schoonen, R. Webb, J. G. Kuenen, and M. S. M. Jetten. 1999. Missing lithotroph identified as new planctomycete. Nature **400**:446–449.

Stumm, W., and J. J. Morgan. 1996. Aquatic chemistry. Third edition. Wiley, New York, New York, USA.

Stute, M., and P. Schlosser. 2000. Atmospheric noble gases. Pages 349–377 *in* P. G. Cook and A. L. Herczeg, editors. Environmental tracers in subsurface hydrology. Kluwer Academic Publishers, Boston, Massachusetts, USA.

Sugisaki, R. 1959. Measurement of effective flow velocity of ground water by means of dissolved gases. American Journal of Science **259**:144–153.

Swerts, M., G. Uytterhoeven, R. Merckx, and K. Vlassak. 1995. Semicontinuous measurement of soil atmosphere gases with gas-flow soil core method. Soil Science Society of America Journal **59**:1336–1342.

Taroncher-Oldenburg, G., E. M. Griner, C. A. Francis, and B. B. Ward. 2003. Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. Applied and Environmental Microbiology **69**:1159–1171.

Terry, R. E., and J. M. Duxbury. 1985. Acetylene decomposition in soils. Soil Science Society of America Journal **49**:90–94.

Tesoriero, A. J., H. Liebscher, and S. E. Cox. 2000. Mechanism and rate of denitrification in an agricultural watershed: Electron and mass balance along groundwater flow paths. Water Resources Research **36**:1545–1559.

Thamdrup, B., and T. Dalsgaard. 2002. Production of N$_2$ through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Applied and Environmental Microbiology **68**:1312–1318.

Thomas, K. L., and D. Lloyd. 1995. Measurement of denitrification in estuarine sediment using membrane inlet mass spectrometry. FEMS Microbiology Ecology **16**:103–113.

Throback, I. N., K. Enwall, A. Jarvis, and S. Hallin. 2004. Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiology Ecology **49**:401–417.

Tiedje, J. M. 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. Pages 170–244 *in* A. J. B. Zehnder, editor. Biology of anaerobic microorganisms. John Wiley and Sons, New York, New York, USA.

Tiedje, J. M., A. J. Sexstone, D. D. Myrold, and J. A. Robinson. 1982. Denitrification: ecological niches, competition and survival. Antonie van Leeuwenhoek **48**:569–583.

Tiedje, J. M., S. Simkins, and P. M. Groffman. 1989. Perspectives on measurement of denitrification in the field including recommended protocols for acetylene based methods. Plant and Soil **115**:261–284.

Topp, E., and J. C. Germon. 1986. Acetylene metabolism and stimulation of denitrification in an agricultural soil. Applied and Environmental Microbiology **52**:802–806.

Tuomainen, J. M., S. Hietanen, J. Kuparinen, P. J. Martikainen, and K. Servomaa. 2003. Baltic Sea cyanobacterial bloom contains denitrification and nitrification genes, but has negligible denitrification activity. FEMS Microbiology Ecology **45**:83–96.

Van Cleemput, O. 1971. Etude de la dénitrification dans le sol. Pedologie **21**:367–376.

Van Luijn, F., P. C. M. Boers, and L. Lijklema. 1996. Comparison of denitrification rates in lake sediments obtained by the N$_2$ flux method, the $^{15}$N isotope pairing technique and the mass-balance approach. Water Research **30**:893–900.

Verstraeten, I. M., G. V. Steele, J. K. Böhlke, T. F. Kraemer, K. E. Wilson, and A. E. Carnes. 2001*a*. Selected field and analytical methods and analytical results in the Dutch Flats area, western Nebraska, 1995–99. U.S. Geological Survey Open-File Report 00-413. U.S. Geological Survey Information Services, Denver, Colorado, USA.

Verstraeten, I. M., G. V. Steele, J. C. Cannia, D. E. Hitch, K. G. Scripter, J. K. Böhlke, T. F. Kraemer, and J. S. Stanton. 2001*b*. Interaction of surface water and ground water in the Dutch Flats area, western Nebraska, 1995–99. U.S. Geological Survey Water-Resources Investigations Report 01-4070. U.S. Geological Survey Information Services, Denver, Colorado, USA.

Vogel, J. C., A. S. Talma, and T. H. E. Heaton. 1981. Gaseous nitrogen as evidence for denitrification in groundwater. Journal of Hydrology **50**:191–200.

von Rheinbaben, W. 1990. Nitrogen losses from agricultural soils through denitrification: a critical evaluation. Zeitschrift fur Pflanzenernahrung und Bodenkunde **153**:157–166.

Voss, M., J. W. Dippner, and J. P. Montoya. 2001. Nitrogen isotope patterns in the oxygen-deficient waters of the Eastern Tropical North Pacific Ocean. Deep Sea Research II **48**:1905–1921.

Walter, H. M., D. R. Keeney, and I. R. Fillery. 1979. Inhibition of nitrification by acetylene. Soil Science Society of America Journal **43**:195–196.

Webster, I. T., J. S. Parslow, and S. V. Smith. 2000. Implications of spatial and temporal variation for biogeochemical budgets of estuaries. Estuaries **23**:341–350.

Weiss, R. F. 1970. The solubility of nitrogen, oxygen and argon in water and seawater. Deep-Sea Research **17**:721–735.

Wellman, R. P., F. D. Cook, and H. R. Krouse. 1968. Nitrogen-15: microbiological alteration of abundance. Science **161**:269–270.

Welsh, D. T., M. Bartoli, D. Nizzoli, G. Castaldelli, S. A. Riou, and P. Viaroli. 2000. Denitrification, nitrogen fixation, community primary productivity and inorganic-N and oxygen fluxes in an intertidal *Zostera noltii* meadow. Marine Ecology Progress Series **208**:65–77.

Wickramasinghe, K. N., O. Talibudeen, and J. F. Witty. 1978. A gas flow-through system for studying denitrification in soils. Journal of Soil Science **29**:527–536.

Wilson, G. B., J. N. Andrews, and A. H. Bath. 1990. Dissolved gas evidence for denitrification in the Lincolnshire limestone groundwaters, eastern England. Journal of Hydrology **113**:51–60.

Wolsing, M., and A. Prieme. 2004. Observation of high seasonal variation in community structure of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of *nir* gene fragments. FEMS Microbiology Ecology **48**:261–271.

Wulff, F., A. Stigebrandt, and L. Rahm. 1990. Nutrient dynamics of the Baltic Sea. Ambio **19**:126–133.

Ye, R. W., and S. M. Thomas. 2001. Microbial nitrogen cycles: physiology, genomics and applications. Current Opinion in Microbiology **4**:307–312.

Yeomans, J. C., and E. G. Beauchamp. 1982. Acetylene as a possible substrate in the denitrification process. Canadian Journal of Soil Science **62**:137–144.

Yoshinari, T., and R. Knowles. 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochemical and Biophysical Research Communications **69**:705–710.

Zhang, L., N. Takaya, T. Kitazume, T. Kondo, and H. Shoun. 2001. Purification and cDNA cloning of nitric oxide reductase cytochrome P450nor (CYP55A4) from *Trichosporon cutaneum*. European Journal Biochemistry **268**:3198–3204.

Zimmerman, A. R., and R. Benner. 1994. Denitrification, nutrient regeneration and carbon mineralization in sediments of Galveston Bay, Texas, USA. Marine Ecology Progress Series **114**:275–288.

Zumft, W. G. 1997. Cell biology and molecular basis of denitrification. Microbiology and Molecular Biology Review **61**:533–616.

Zumft, W. G. 1999. The denitrifying prokaryotes. *In* M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt, editors. The prokaryotes: an evolving electronic resource for the microbiological community. Third edition, release 3.0. Springer-Verlag, New York, New York, USA. ⟨http://141.150.157.117:8080/prokPUB/index.htm⟩