Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways

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The removal of nitrogen (N) in aquatic ecosystems is of great interest because excessive nitrate in groundwater and surface water is a growing problem. High nitrate loading degrades water quality and is linked to eutrophication and harmful algal blooms, especially in coastal marine waters. Past research on nitrate removal processes has emphasized plant or microbial uptake (assimilation) or respiratory denitrification by bacteria. The increasing application of stable isotopes and other tracer techniques to the study of nitrate removal has yielded a growing body of evidence for alternative, microbially mediated processes of nitrate transformation. These include dissimilatory (the reduction of nitrogen into other inorganic compounds, coupled to energy producing processes) reduction of nitrate to ammonium (DNRA), chemoautotrophic denitrification via sulfur or iron oxidation, and anaerobic ammonium oxidation (anammox), as well as abiotic nitrate removal processes. Here, we review evidence for the importance of alternative nitrate removal pathways in aquatic ecosystems and discuss how the possible prevalence of these pathways may alter views of N cycling and its controls. These alternative pathways are of particular importance for the management of excess N in the environment, especially in cases where nitrate is transformed to ammonium, a biologically available and less mobile N form, rather than to dinitrogen gas.

In a nutshell:

- Increased nitrogen loading causes eutrophication of aquatic ecosystems and degrades water quality
- Most of the nitrogen added to landscapes is removed during transit to the ocean, and this removal has been attributed largely to denitrification, with a lesser proportion to assimilation and accumulation in groundwater
- New research has pointed to the importance of alternative microbial pathways for nitrate removal
- The possible prevalence of these pathways has critical implications for managing excess N in aquatic ecosystems

Where does the nitrate go?

Up to 75% of the N added to a landscape may be removed before reaching marine ecosystems (Howarth et al. 1996). The various transformations and eventual fate of this N as it is carried along hydrologic flow paths is a problem that has interested scientific and management communities alike. The current consensus is that the dis-
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Panel 1. An introduction to heterotrophic energy production

Heterotrophic respiration of organic matter can be either aerobic (involving oxygen) or anaerobic (not requiring oxygen). Both forms of respiration are oxidation–reduction reactions, in which simple organic carbon compounds are combined with electron (-) acceptors to yield oxidized carbon (CO₂), reduced products (H₂O in the case of aerobic respiration), and energy. The process of respiratory denitrification we describe in this review is a form of anaerobic respiration in which nitrate serves as the alternate electron acceptor. Various substances can act as electron acceptors in anaerobic respiration, and depending on the electron acceptor and its ultimate product, variable amounts of energy are produced. Some common electron acceptors are listed in order from highest to lowest efficiency of energy yield: those microbes performing the more efficient reactions tend to out-compete others for labile organic matter.

**Aerobic respiration**

**Anaerobic respiration**

In respiratory denitrification, nitrate acts as the terminal electron acceptor for the oxidation of organic matter under anaerobic conditions; in aquatic sediments, most of the nitrate is usually converted to N₂, with a variable but small fraction escaping as nitrous oxide (N₂O; Figure 1). Because N₂ is unavailable for use by most organisms, respiratory denitrification is considered a permanent removal of N from the ecosystem. Denitrification rates have been estimated in soils, wetlands, and surface waters, but estimates vary greatly within and among environments, as well as between different measurement techniques. Nevertheless, denitrification is thought to remove substantial fractions of the total nitrate loads to lakes, rivers, and coastal estuaries (Seitzinger 1988; Cornwell et al. 1999). However, while nitrate disappearance in soils and aquatic sediments is usually assumed to be largely due to denitrification, estimates of denitrification based on direct assays (e.g. acetylene block techniques) often account for less than half of the total nitrate disappearance (e.g. see tables in Seitzinger [1988]).

This discrepancy between local denitrification estimates and the large losses of nitrate at the landscape scale remains difficult to reconcile. One possible explanation is that we have not yet designed adequate methods to extrapolate from site-specific rates to entire ecosystems (Cornwell et al. 1999). An alternative explanation is that much of the nitrate removal can be attributed to processes other than respiratory denitrification or assimilation. New research has pointed to the importance of processes that remove nitrate in freshwater ecosystems, including dissimilatory nitrate reduction to ammonium (Tiedje 1988), anaerobic ammonium oxidation (Jetten et al. 1998; Jetten 2001), denitrification coupled to sulfide oxidation (Dannenberg et al. 1992; Fossing et al. 1995; Brunet and Garcia-Gil 1996; Otte et al. 1999), and reduction of nitrate coupled to abiotic or biotically mediated oxidation of iron (Davidson et al. 2003; Weber et al. 2006). Here, we review mounting scientific evidence for the importance of these alternative nitrate removal pathways and propose that nitrate removal in aquatic ecosystems may entail much more than denitrification and assimilation.

### Alternatives to respiratory denitrification

Respiratory denitrification is surely an important nitrate removal pathway, but we will not discuss it in further detail as it is thoroughly examined elsewhere (e.g. Knowles 1982; Tiedje et al. 1982; Seitzinger 1988; Cornwell et al. 1999). This paper is not meant to lead the reader to the conclusion that these alternative pathways are generally more important than denitrification, but to show that there are several processes that could rival denitrification in importance, which have been much less studied until now. While there is some evidence for the importance of each of these pathways, much more research is needed, particularly in freshwater ecosystems, to ascertain their value relative to respiratory denitrification in whole-ecosystem nitrate removal.

### Dissimilatory nitrate reduction to ammonium (DNRA)

The existence of dissimilatory nitrate reduction to ammonium (DNRA) has been widely recognized for at least the past 25 years, although its potential importance as a nitrate removal pathway on an ecosystem scale has generated increased interest within the past decade. This microbially mediated pathway involves the dissimilatory transformation of nitrate to ammonium (NH₄⁺), in contrast to assimilatory processes that incorporate N into cellular constituents. Compared to nitrate, the resultant ammonium is a more biologically available and less mobile form of inorganic N (Figure 1). Little is known about the eventual fate of the nitrate that is converted to ammonium via DNRA pathways, but it is possible that, under appropriate conditions, the ammonium is converted back.
to nitrate via nitrification. The resultant ammonium may also be assimilated into plant or microbial biomass.

There are two recognized DNRA pathways: one involving fermentation and the other linked to sulfur oxidation. Early work on DNRA suggested that it was mainly carried out by fermentative bacteria (Tiedje 1988), though in recent years the existence of DNRA coupled to sulfur cycling has been documented in marine and freshwater ecosystems (Brettar and Rheinheimer 1991; Brunet and Garcia-Gil 1996). It is not known whether the two DNRA pathways are mutually exclusive.

Fermentative DNRA couples electron flow from organic matter to the reduction of nitrate via fermentation reactions (Tiedje 1988; Megonigal et al. 2004). Many microbes perform fermentative DNRA, including species of Clostridia, Desulfovibrio, Vibrio, and Pseudomonas; these organisms can also carry out fermentation without using nitrate (Tiedje 1988). Although the conditions promoting fermentative DNRA and respiratory denitrification are similar (ie anoxia, available nitrate, and organic substrates), fermentative DNRA is thought to be favored in nitrate-limited environments rich in labile carbon (carbon forms that are more easily broken down), while respiratory denitrification would be favored under carbon-limited conditions (Kelso et al. 1997; Silver et al. 2001). Tiedje (1988) argued that high labile carbon availability would favor organisms that used electron acceptors most efficiently; DNRA transfers eight electrons per mole of nitrate reduced, whereas denitrification only transfers five. Some studies have supported Tiedje’s (1988) hypothesis that DNRA is more important in high-carbon, low-nitrate systems, including Bonin (1996) and Nijburg et al. (1997). The oxidation state of the sediments may also be important. For example, Matheson et al. (2002) hypothesized that microzones of oxygen leakage from roots of emergent plants in wetland sediments may favor the facultatively aerobic denitrifiers over the obligately anaerobic fermentative bacteria. Much more work is needed to understand where and when DNRA is prevalent in ecosystems before we can fully understand what factors govern its importance relative to other nitrate removal processes.

A very different form of DNRA is chemolithoautotrophic and couples the reduction of nitrate to the oxidation of reduced sulfur forms, including free sulfide (H₂S and S²⁻) and elemental sulfur (S; Brunet and Garcia-Gil 1996; Otte et al. 1999). The nitrate may be reduced either to ammonium, as a form of DNRA, or to N₂, as a form of denitrification, although not all species can do both (Zopfi et al. 2001). In this pathway, the predominant fate of the reduced nitrate may be determined by the ambient concentration of free sulfide, which is known to inhibit the final two reduction steps in the denitrification sequence. Sulfide inhibition of these terminal steps may drive the reduction to ammonium rather than to N₂O and N₂. Brunet and Garcia-Gil (1996) studied the effects of various sulfur forms as potential electron donors, and found that only free sulfide yielded ammonium and N₂O, lending support to the idea that the enzymes that sustain respiratory denitrification may be inhibited by the presence of sulfide. On the other hand, metal-bound sulfides (eg iron sulfide, [FeS]), which are often abundant constituents of freshwater sediments (Holmer and Storkholm 2001), can also be oxidized by such bacteria, but these compounds may not inhibit denitrification (Brunet and Garcia-Gil 1996). A similar process that couples the reduction of nitrate to the oxidation of methane was recently discovered in freshwaters (Raghoebarsing et al. 2006), though it is not yet clear if this process is important to whole-ecosystem nitrate removal.

The ability of bacteria to couple the reduction of nitrate to the oxidation of sulfur has now been established in a number of taxa with diverse metabolic characteristics (eg Dannenberg et al. 1992; Bonin 1996; Philippot and Hojberg 1999), including members of the genera Thiothrix, Thiobacillus, and Thioploca (Timmer-ten-hoor 1981; Jorgensen 1982; Kelly and Wood 2000). Bacteria with this capability include the “big bacteria” (eg Thioploca) that are able to store nitrate, sulfur, or calcite in vacuoles (Schulz and Jorgensen 2001). This storage capability, in conjunction with their gliding motility, allows them to take advantage of steep biogeochemical gradients, for example by taking up nitrate from overlying oxic water and utilizing it to oxidize sulfur in sulfide-rich anoxic porewater (interstitial sediment water; Schulz and Jorgensen 2001).

Figure 1. A conceptual diagram of the nitrate removal pathways discussed in this article. This is not meant to represent an exhaustive list of microbial transformations, but rather to illustrate the different possible pathways and fates of nitrate removal. Blue arrows denote autotrophic pathways, while purple arrows denote heterotrophic pathways.
The biogeochemical importance of nitrate use by sulfur-oxidizing bacteria was first widely recognized in marine sediments, but its importance in freshwater ecosystems is beginning to be revealed. For example, much of the nitrate uptake in a groundwater aquifer was ascribed to *Thiobacillus denitrificans* (Bottcher et al. 1990), and *Thioploca* occurs not only in marine sediments but also in freshwater ecosystems, including Lakes Erie, Baikal, and Biwa (Megonigal et al. 2004). Furthermore, species of *Beggiatoa*, a genus of sulfur oxidizers common in freshwaters, also appear to be capable of using nitrate to oxidize sulfur (Kamp et al. 2006).

**Nitrate reduction coupled to iron oxidation**

The reduction of nitrate coupled to iron (Fe) cycling is thought to take place through both biotic and abiotic pathways (Weber et al. 2006; Davidson et al. 2003). In Figure 1, we depict one example of an abiotic pathway in which nitrate is converted to nitrite (NO$_2^-$) by ferrous iron (Fe$^{2+}$; this could also be done by reduced manganese, Mn$^{2+}$), followed by the rapid reaction of the NO$_2^-$ to N$_2$. Postma et al. (1991) concluded that this reaction would only remove a major proportion of nitrate from groundwater in areas with low nitrate inputs. Another abiotic reaction has been proposed, in which nitrate is reduced to nitrite by reaction with Fe or Mn and the nitrite binds with organic substances to produce dissolved organic nitrogen (DON; Davidson et al. 2003). Evidence for this reaction was discovered recently in forest soils (Dail et al. 2001), but it has not, so far, been shown to occur in aquatic ecosystems.

Alternatively, microbes can mediate nitrate reduction coupled to iron oxidation in aquatic ecosystems (Weber et al. 2006). This biotic reduction occurs at relatively low temperatures and circumneutral pH (between 5.5–7.2; Weber et al. 2001), and thus it may be more likely to occur in surface waters than the equivalent abiotic reaction. Microbes that can perform this process have been isolated from a diverse array of aquatic sediments (Straub and Buchholz-Cleven 1998). The majority of the work in this area has focused on describing the microbes capable of the reaction, and we could not find an estimate of the potential importance of the reaction as an ecosystem-level process compared to other N removal processes. The controls on the process remain poorly understood, although it may be important in areas of high reduced iron and a limited supply of organic carbon (Weber et al. 2001).

**Anaerobic ammonium oxidation**

Anaerobic ammonium oxidation (the acronym for which is anammox) is a chemolithoautotrophic process by which ammonium is combined with nitrite under anaerobic conditions, producing N$_2$. The nitrite is derived from the reduction of nitrate, possibly by denitrifying bacteria, and anammox therefore contributes to permanent nitrate removal. The process was discovered in a wastewater treatment system in the 1990s, and since then, studies have shown it to occur in anoxic wastewater, oxygen-depleted zones of the ocean, temperate shelf sediments, sea ice, and cold Arctic shelf sediments (Jetten et al. 1998; Rysgaard and Glud 2004; Rysgaard et al. 2004). It has also recently been reported in one freshwater ecosystem (Lake Tanganyika; Schubert et al. 2006).

Scientists still know relatively little about the bacteria that carry out anammox, and no pure cultures exist (Strous et al. 2006). This may be because the process is carried out by slow-growing organisms (doubling time is approximately 11 days; Jetten et al. 1999), an idea further supported by evidence that the process has a low thermal optimum (12°C compared to 24°C for denitrification; Jetten 2001). Those anammox bacteria that have been identified belong to the Planctomycetes, an order of aquatic bacteria that have evolved internal compartmentalization (similar to eukaryotes) and a specialized structure called an anammoxosome, which may protect the cell from toxic anammox intermediates such as hydrazine (Jetten et al. 2003; Strous et al. 2006).

Anammox occurs in anoxic waters where there are suitable concentrations of both nitrate and ammonium, and the process is inhibited by many simple organic compounds, including pyruvate, ethanol, and glucose (Jetten et al. 1999). Thus, anammox may be most important in ecosystems with limited labile carbon or an excess of nitrogen relative to carbon inputs. This may include substantial parts of the pelagic ocean and continental shelves (Dalsgaard et al. 2005). A recent synthesis of anammox studies suggests that in marine ecosystems, water depth is important in regulating the relative importance of anammox to total nitrate removal, with anammox producing up to two-thirds of the N$_2$ in areas over 20 m deep. Although anammox seems to be less important to overall nitrate removal in shallower marine and estuarine waters (< 1 m), many of these areas have higher absolute rates of anammox (Dalsgaard et al. 2005). While little is known about anammox in freshwaters, based on what is known about the process in marine ecosystems, one might expect that it would be more important in very deep, large, oligotrophic lakes. The only study to date on anammox in freshwaters was conducted in Lake Tanganyika, where Schubert and others (2006) found that 7–13% of the N$_2$ production was derived from anammox.

**How important are these pathways in aquatic ecosystem N cycling?**

This is a particularly difficult question to answer at the present time, because many of the pathways we described are just beginning to be studied in detail. In this section, we provide evidence for the importance of alternative pathways in marine and freshwater ecosystems (Figures 2 and 3). We also describe the conditions under which we might expect a particular pathway to be important. Figure
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Figure 2. DNRA estimates across a variety of aquatic ecosystems. The bars represent the ranges of DNRA as a percent of the total dissimilatory nitrate removal found in a given study site, with the balance presumably due to denitrification. Purple bars designate marine and brackish ecosystems; blue bars designate freshwaters. The North River site is hatched because it was alternatively freshwater-dominated and oligohaline. Many of these studies were originally compiled by Megonigal et al. (2004). (References corresponding to these studies can be found in the Web-only material.)

4 is a flow chart based on what we know about the controls of each pathway; its purpose is to synthesize the work we have summarized to this point, and to generate testable hypotheses about when and where certain nitrate removal processes are likely to be important.

We suggest that the relative availability of labile carbon, reduced sulfur, and reduced iron are the key determinants of nitrate removal pathways. Anammox and respiratory denitrification have been shown to be important nitrate removal pathways in areas of relatively low labile carbon; at this time, sulfur, and particularly free sulfide, has not been shown to affect anammox. However, because of its effect on key enzymes in the denitrification sequence, we believe that free sulfide may be a key variable in determining nitrate removal processes in relatively high-carbon environments, which include many freshwater and near-coastal ecosystems. When there is sulfide in close proximity to oxic waters, as, for example, in surficial sediments of many shallow waters, we hypothesize that nitrate removal coupled to microbially mediated sulfur oxidation may be important; in anoxic settings with relatively low sulfide, we expect that respiratory denitrification and perhaps fermentative DNRA could be more important (Figure 4).

DNRA has been measured in a few studies of whole-system nitrate removal (Bonin 1996; Rysgaard et al. 1996; Silver et al. 2001; Tobias et al. 2001; Welsh et al. 2001; An and Gardner 2002), although none of these studies determined if the apparent DNRA was chemolithoautotrophic or fermentative. Figure 2 summarizes data from the literature to show that DNRA is potentially as important as respiratory denitrification in diverse environments. Most work on this pathway has been done in marine ecosystems, including marine and estuarine sediments, brackish marsh sediments, and mangroves, where DNRA can account for a very wide range (0–100%) of the total nitrate removal (Figure 2, purple bars). Evidence for DNRA has been found in freshwater ecosystems as well, including river sediments, rice paddies, riparian wetlands, and aquifers (Figure 2, blue bars). DNRA may be relatively more important in marine than freshwater ecosystems, but this is a tenuous conclusion because of the small number of studies of DNRA in freshwaters (Figure 2). Evidence for DNRA has also been found in certain soils, where it can account for a large fraction (up to 75%) of total nitrate removal (Silver et al. 2001). The observation that DNRA can be important in soils, which are not thoroughly anoxic like aquatic sediments, highlights how little is understood about the process and suggests that DNRA may occur in many other environments that have yet to be investigated.

Research on anammox in marine ecosystems was synthesized by Dalsgaard et al. (2005), who concluded that this process contributes half or more of the N₂ production in coastal shelves and the deep sea (Figure 3), and possibly is responsible for one- to two-thirds of global oceanic nitrate removal. The role of anammox in freshwater

Figure 3: Anammox estimates across a variety of aquatic ecosystems. The bars represent the ranges of total N₂ production that can be attributed to anammox in a given study site. Purple bars designate marine and brackish ecosystems; blue bars designate freshwaters. The Thames River estuary is hatched because the study spanned a range of freshwater and marine-influenced sites. (References corresponding to these studies can be found in the Web-only material.)
nitrogen cycling remains speculative, since only one study in a natural freshwater ecosystem has been published to date (Schubert et al. 2006). Anammox would be expected to occur where nitrate and ammonium coexist, which could perhaps include interfaces between surface water and sediment porewater. However, due to inhibition by simple organic carbon compounds, anammox may be limited to areas that are relatively low in labile carbon, which may not often be the case for near-surface freshwater sediments that support high biological productivity (Figure 4).

Figure 4: Hypothesized controls on predominant dissimilatory pathways of nitrate removal. This flow chart summarizes the conditions under which we would expect a particular nitrate removal pathway to be important. C inputs refer to labile organic carbon available to microbes. Sulfidic refers to the presence of significant amounts of either free sulfide (H₂S or S⁻), elemental S (S⁰), or metal-bound sulfides such as FeS, all of which tend to be abundant in sediment environments with moderate to high sulfate in overlying water and high labile C inputs to support microbial sulfate reduction. Of these S forms, only free sulfide inhibits denitrification and thus promotes DNRA. C:N ratios refer to the ratio of labile organic carbon to nitrate. Respir = respiratory; denitrif = denitrification; DNRA = dissimilatory nitrate reduction to ammonium; anammox = anaerobic ammonium oxidation; ferment = fermentative.

How is it that scientists have overlooked these pathways for so many years? We believe this is due in large part to methodological limitations. The importance of these pathways has recently been appreciated, thanks to the use of stable isotope and molecular microbial methods. Prior to the widespread use of stable isotopes, the favored method for measuring denitrification was the acetylene block technique (ABT; Tiedje 1988). The ABT typically entails the creation of a sediment slurry, de-oxygenation with an inert gas, addition of acetylene to block the transformation of N₂O to N₂, and measurement of the rate of N₂O production over time, indicating the rate of denitrification. There are a number of problems when this method is used to detect alternative nitrate removal processes, including the removal of free sulfide by sparging (bubbling with an inert gas to create anoxia), disruption of the steep sediment redox gradients that may favor certain organisms and reactions, and the incorrect assumption that all N₂O produced is the result of denitrification (Welsh et al. 2001, Senga et al. 2006). The widespread use of the ABT, as well as other, less sensitive techniques may have led to an overestimation of the importance of denitrification and an underestimation of other nitrate removal pathways.

Conclusions and implications for management

A great deal of effort has been expended in studying respiratory denitrification, and management decisions are being made based on that body of knowledge. The possible importance – or even prevalence – of alternative

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**Glossary**

See Panel 1 for an introduction to heterotrophic energy production and further explanation of these terms

- **Assimilatory**: a process that leads to incorporation of a nutrient into organic (cellular) compounds
- **Dissimilatory**: a process coupled to energy production that transforms inorganic compounds but does not lead to their assimilation
- **Chemolithoautotrophic**: organisms that obtain energy by the dissimilatory oxidation of inorganic compounds and use it for the fixation of carbon dioxide as the carbon source
- **Oxidation**: the process wherein a substance gives up electrons, thereby becoming oxidized
- **Reduction**: the process wherein a substance receives electrons, thereby becoming reduced
- **Electron acceptor**: a substance that can accept electrons via transfer from another substance (electron donor), thereby becoming reduced in the process
- **Fermentation**: an anaerobic microbial pathway that derives energy from the degradation of organic matter, using organic compounds as both the primary electron donor and the ultimate electron acceptor
Nitrate removal pathways has profound implications for our management of aquatic ecosystems when attempting to reduce nitrate loads. Nitrate is the most mobile form of N, so removal of nitrate by any of the processes described above is important to downstream water quality, though permanent removal by denitrification is the most desirable method.

Removal by other pathways can result in transformation of the nitrate to something other than dinitrogen gas. Nitrate removal via anammox still creates dinitrogen gas as an end-product, although both a nitrate and an ammonium ion are removed in the process. In contrast, the conversion of nitrate to ammonium, as in DNRA, creates an even more bioavailable N form, and one that tends to be less mobile in soils and sediments. This converted ammonium can also be transformed back to nitrate via nitrification. Furthermore, if S-oxidizers are shown to take up much of the nitrate, then N cycling is closely linked to sulfide availability, which in turn is linked to sulfate reduction. In freshwaters, sulfate reduction may be controlled by sulfate inputs, and sulfate is a ubiquitous pollutant in industrialized and agricultural regions (Schlesinger 1997). If excess sulfate loading to freshwater systems actually enhances nitrate removal, then the controls on nitrate removal in landscapes subject to S and N pollution become more complex than previously thought.

Ecologists and managers should accept that nitrate disappearance is no longer synonymous with denitrification and that there are many other pathways that potentially remove nitrate. Much more research is required with regard to these alternative nitrate removal pathways, across a diversity of aquatic ecosystems. Most of what we know about them is based on research done in marine ecosystems, and thus our understanding of what controls such processes in freshwater ecosystems subject to elevated nitrate inputs remains incomplete.

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