ALTERNATIVE MICROBIAL PATHWAYS OF NITROGEN REMOVAL FROM MICHIGAN STREAMS, WETLANDS AND LAKES

By

Amy J. Burgin

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology
and
Program in Ecology, Evolutionary Biology, and Behavior

2007

ABSTRACT

NOVEL MICROBIAL PATHWAYS OF NITRATE REMOVAL FROM FRESHWATER ECOSYSTEMS

By

Amy J. Burgin

The removal of nitrogen (N) in aquatic ecosystems is of particular interest because excessive nitrate in ground and surface waters is a growing problem. Research on nitrate removal processes has emphasized biotic uptake (assimilation) or respiratory denitrification by bacteria. The increasing application of tracer techniques (e.g., stable isotopes) has yielded a growing body of evidence for alternative microbially mediated processes of nitrate transformation, including dissimilatory reduction of nitrate to ammonium (DNRA), chemoautotrophic denitrification via sulfur or iron oxidation, and anaerobic ammonium oxidation (Anammox). In Chapter 1, I 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.

Anaerobic microbial processes are responsible for much of the nutrient cycling in freshwater systems. Nitrate disappearance in sediments is usually assumed to be due to respiratory denitrification. Push-pull tracer experiments entail adding nitrate and a conservative solute to sediment porewater, followed by in-situ incubation with periodic subsampling. While performing

such tracer experiments to quantify rates of nitrate removal in aquatic sediments of Michigan streams and wetlands, I found that nitrate removal coincided with sulfate production. Push-pull experiments in a diverse set of streams, lakes and wetlands revealed a persistent pattern of sulfate production during nitrate removal (Chapter 2). Push-pull experiments done with $^{15}NO_3^-$ also indicate the importance of DNRA to overall nitrate removal in these sediments.

To compare the relative importance of alternative pathways of NO_3^- reduction (e.g., to NH_4^+ or N_2), I again employed the use of stable isotopes in conjunction with a flow-through core technique (Chapter 3). Using a flow-through set up, treatment water ($^{15}NO_3^-$, $^{15}NH_4^+/^{14}NO_3^-$, or control) was pumped over cores from six different sites. Results indicate that conversion to N_2 was the predominant nitrate loss across all six sites. I also found that conversion into the $^{15}NH_4^+$ pool, indicative of DNRA, can account for a variable fraction of the dissimilatory nitrate removal, but that anammox accounted for very little of the overall nitrate removal.

I tested the relative importance of carbon vs. sulfide in regulating DNRA using a laboratory assay (Chapter 4), by adding nitrate along with a gradient of organic carbon (as acetate) and free sulfide to anoxic sediments. I found that both carbon and sulfide were important in controlling nitrate removal rates and end-products in both sites. While denitrification tended to be the more important removal pathway in the low ambient sulfide site, DNRA was of equal importance in the high ambient sulfide site.

DEDICATION

This dissertation is dedicated to the memory of my maternal grandfather,

Virgil Schurman
(1918-2002)

He was a farmer who only finished school through the 8th grade. Shortly before he died, I told him what I was going to study in graduate school, and he replied, "I always knew that nitrogen we added had to go somewhere..."

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Steve Hamilton, for his advice and guidance throughout my dissertation. I would also like to thank my committee members, Dr. Nathaniel Ostrom, Dr. Phil Robertson, Dr. Tom Schmidt, and Dr. Jay Lennon for their feedback and help with many different stages of this process. Even though they are not on my committee, I learned a great deal from interacting with Kay Gross, Gary Mittelbach and Jeff Conner and other KBS and MSU faculty.

This field and lab work would not have been possible without the help of David Weed, Philip Riekenburg, Erin Payne, Melinda Roth, Nicole Reid, Stefanie Whitmire, Eric Thobaben, Amanda Kurzman, Suzanne Sippel, Kathy Crowley. I would also like to thank my newer labmates Jorge Celi, Leila DeSotelle, Lauren Kinsman and Jon O'Brien for the camaraderie and feedback.

KBS is a special place to me, and I made many wonderful life-long friends while I was here. I would especially like to thank Wendy Mahaney and Kurt Smemo for being such great friends and for their help with science and great conversations about how to do science. Thank you to Claire McSwiney, who was also a good friend and role model, and willing to listen when the instruments weren't cooperating. Many other people also made KBS a great place to live and work, including Katie Wharton, Lindsey Walters, Brook and Emily Wilke, Emily Grman, Stuart Grandy, Jay Sobel, Charlotte Reemts, Jarad Mellard, and Todd Robinson.

KBS is such and easy place to live and work because of the help of numerous support staff. I'd like to thank Nina Consollati, Sally Shaw, Alice Gillespie, John Gorentz, and Melissa Yost for their assistance.

I have been fortunate to work with a large number of non-KBS people during my graduate studies as well. I gained a great deal from interacting with our LINX collogues, in particular the Notre Dame students (Jake, Laura, Clay and Denise). Pat Mulholland provided a review of Chapter 1 before it was submitted for publication, and his comments improved the manuscript (and chapter). I owe a great deal to Wayne Gardner and Mark McCarthy at UTMSI for hosting my visit there and training me on the flow-through core system used in chapter 3 (and running all of the gas samples!).

I owe special thanks to my husband and colleague, Terry Loecke, for his encouragement and support (and field assistance, and reading drafts of chapters). My family, Jay and Cindy Burgin and my siblings Jerrod and Sarah, have been wonderfully supportive of my many years of school, and didn't ask *too* often when I was going to finish.

This research was funded by grants from the National Science Foundation (DEB). I obtained additional funds through the LTER network, MSU Zoology Dept, MSU Ecology, Evolutionary Biology and Behavior Program, Environmental Science and Policy Program, and Sigma Xi.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 1

As published in *Frontiers in Ecology and the Environment*:

Burgin, A.J. and S.K. Hamilton. 2007. Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. *Frontiers in Ecology and the Environment* **5** (2): 88-96.

ABSTRACT:

The removal of nitrogen (N) in aquatic ecosystems is of particular interest because excessive nitrate in ground water and surface water is a growing problem. Enhanced loading of nitrate degrades water quality and is linked to eutrophication and harmful algal blooms, especially in coastal marine waters. 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 study nitrate removal has yielded a growing body of evidence for alternative microbially mediated processes of nitrate transformation, including dissimilatory reduction of nitrate to ammonium (DNRA), chemoautotrophic denitrification via sulfur or iron oxidation, and anaerobic ammonium oxidation (Anammox), as well as abiotic N 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 particularly significant for the management of excess N in the environment in cases where they transform nitrate to ammonium, a biologically available and less mobile N form, rather than to dinitrogen gas.

In a nutshell:

- Increasing nitrogen loading causes eutrophication of aquatic ecosystems and degrades water quality for human use.

- 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 ground water.
- New research has pointed to the importance of alternative microbial pathways of nitrate removal.
- The possible *prevalence* of these pathways has critical implications for managing excess N in aquatic ecosystems.

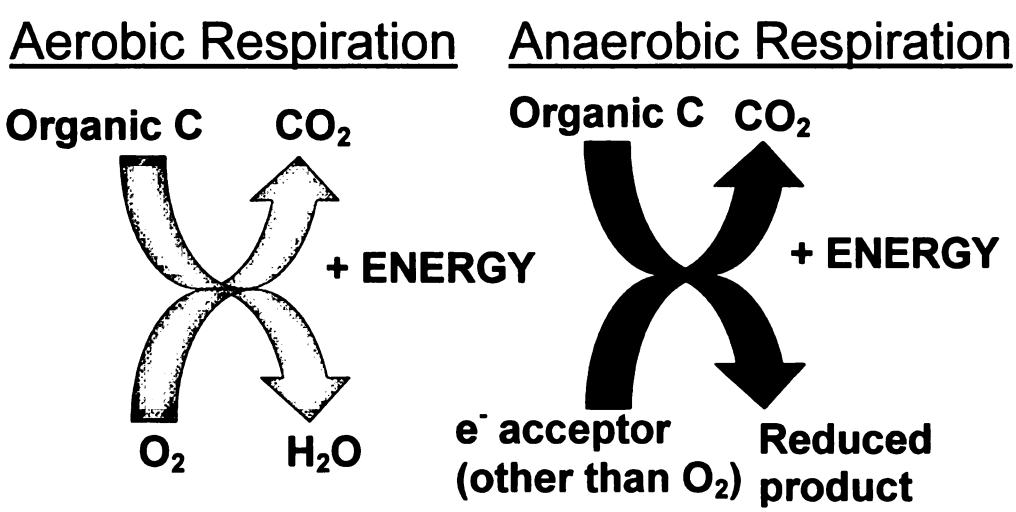
Nitrogen in aquatic ecosystems

Excessive nitrogen (N) concentrations, often largely in the form of nitrate (NO_3^-), present a water-quality problem of growing concern. Nitrate concentrations in ground water and in rivers in developed areas of the world have risen dramatically following the growing use of synthetic N fertilizers and cultivation of N-fixing crops (Turner and Rabalais 2003). Increasing N export from landscapes to coastal waters has been implicated in coastal eutrophication, creating hypoxic zones (such as in the Gulf of Mexico; (Rabalais et al. 2001) and harmful algal blooms (Paerl et al. 2002). There is still some debate over whether or not N alone is always the main driver of these problems (Dodds 2006), but there is no question that the increases in N loading represent a major perturbation of streams, rivers, estuaries and coastal marine waters.

Although N loading to coastal zones has increased, regional watershed mass-balance studies indicate that most of the anthropogenic N that enters watersheds is removed before reaching the oceans (Howarth et al. 1996,

Alexander et al. 2000). Surface and groundwater flow through landscapes often enters riparian wetlands and headwater streams, which can efficiently remove nitrogen (Peterson et al. 2001, Zedler 2003). Thus, key interfaces along landscape flow paths control nitrate export to downstream surface waters, such as large rivers and lakes, and ultimately to estuaries and marine ecosystems. In this article, we discuss the multiple possible fates for this removed nitrate, which include some grossly underestimated and understudied microbial pathways, many of which have recently been gaining attention among the scientific community. The importance and even possible prevalence of these pathways have profound implications for the management of aquatic ecosystems to promote nitrate removal.

Box 1: An introduction to heterotrophic energy production. Heterotrophic respiration of organic matter can be either aerobic (using oxygen) or anaerobic (no oxygen). Both forms of respiration are oxidation-reduction reactions in which simple carbon sources are combined with electron (e^-) acceptors to yield oxidized carbon (CO_2), reduced products (H_2O 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 e^- 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 the order from highest to lowest efficiency of energy yield; those microbes performing the more efficient reactions tend to outcompete others for labile organic matter.



Electron acceptors: $O_2 > NO_3^- > Fe^{3+} > SO_4^{2-}$

Where does the nitrate go?

Up to 75% of the N added to a landscape can 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 disappearance of N is due largely to biological transformations, since increased storage (e.g., in groundwater or biomass accrual) cannot explain most of the "missing N" (Howarth et al. 1996). Biological removal of nitrate from water passing through or over sediments is often assumed to be due to either assimilation into algal or microbial biomass, producing organic N that may be remineralized later, or respiratory denitrification by bacteria, producing gaseous N_2.

In respiratory denitrification, nitrate acts as the terminal electron acceptor for the oxidation of organic matter under anaerobic conditions; in aquatic sediments most the nitrate is usually converted to N_2 with a variable but small fraction escaping as nitrous oxide (N_2O) (Figure 1). Because N_2 is unavailable to 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 we propose that nitrate removal in aquatic ecosystems may entail much more than denitrification and assimilation.

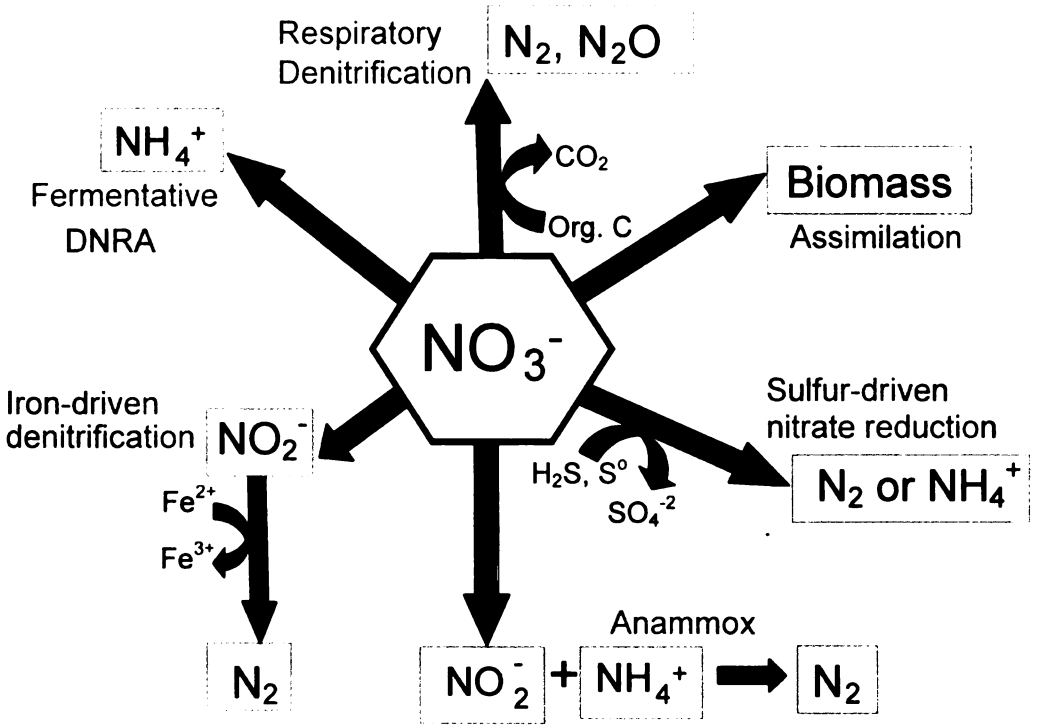


Figure 1: A conceptual diagram of the nitrate removal pathways discussed in this article. This is not meant to represent an exhaustive list of microbial transformations, but rather to illustrate the different possible pathways and fates of nitrate removal. Blue arrows denote autotrophic pathways, while purple arrows denote heterotrophic pathways.

Alternatives to respiratory denitrification

Respiratory denitrification is surely an important nitrate removal pathway, but we will not discuss it in further detail due to the numerous reviews on the process (Knowles 1982, Tiedje et al. 1982, Seitzinger 1988, Cornwell et al. 1999). Our focus is not meant to lead the reader to the conclusion that these alternative pathways are generally more important than denitrification, but to point out that there are several processes that could rival denitrification in significance but have been much less studied until now. While there is some evidence for each of these pathways, much more research is needed, particularly in freshwater ecosystems, to ascertain their importance relative to respiratory denitrification in whole-ecosystem nitrate removal.

Dissimilatory nitrate reduction to ammonium (DNRA)

The existence of DNRA has been widely recognized for at least the past 25 years, though its potential significance 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 (NH_4^+), in contrast to assimilatory processes that incorporate the 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, 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 unknown if the two DNRA pathways are mutually exclusive.

Fermentative DNRA couples electron flow from organic matter via fermentation reactions to the reduction of nitrate (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 (anoxia, available nitrate and organic substrates), fermentative DNRA is thought to be favored in nitrate-limited, labile-carbon rich environments 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 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 (H_2S and S^{2-}) and elemental sulfur (S) (Brunet and Garcia-Gil 1996, Otte et al. 1999). The nitrate may be reduced either to ammonium, making it a form of DNRA, or to N_2 in 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 nitrous oxide and N_2. 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 nitrous oxide, lending support to the idea that the enzymes that support respiratory denitrification may be inhibited by the presence of sulfide. On the other hand, metal-bound sulfides (e.g., FeS), which are often abundant constituents of freshwater sediments (Holmer and Storkholm 2001),

also can be oxidized by these 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 (Dannenberg et al. 1992, Bonin 1996, Philippot and Hojberg 1999) including members of the genera *Thiobacillus*, *Thiomicrospora*, and *Thioploca* (Timmertenhoor 1981, Jorgensen 1982, Kelly and Wood 2000). Bacteria with this capability include the "big bacteria" (e.g., *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 porewaters (Schulz and Jorgensen 2001).

The biogeochemical importance of nitrate use by sulfur-oxidizing bacteria was first widely recognized in marine sediments, but we are beginning to discover its importance in freshwater ecosystems. 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 rapid reaction of the nitrite to N_2. Postma et al. (1991) concluded that this reaction would only remove a significant 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 DON (Davidson et al. 2003); evidence for this reaction was discovered recently in forest soils (Dail et al. 2001), but it is not known 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 (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, though it may be important in areas of high reduced iron and a limited supply of organic C (Weber et al. 2001).

Anaerobic Ammonium Oxidation

*An*aerobic *amm*onium *ox*idation (known as 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 therefore Anammox contributes to permanent nitrate removal. The process was discovered in a wastewater treatment system in the 1990's, and since its discovery, studies have shown it occurs 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), and recently it has 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 Anammox is performed by slow-growing organisms (doubling time is approximately 11 days; Jetten et al. 1999), an idea further upheld 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, a group that has 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 that have an excess of nitrogen relative to carbon inputs. This may include significant parts of the open-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 2/3 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 freshwaters was in Lake Tanganyika where Shubert and others (2006) found that 7-13% of the N_2 production came 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 4 is a flow chart based on what we know about 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.

The relative availability of labile carbon, reduced sulfur, and reduced iron are proposed to be 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, is not known 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 includes 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

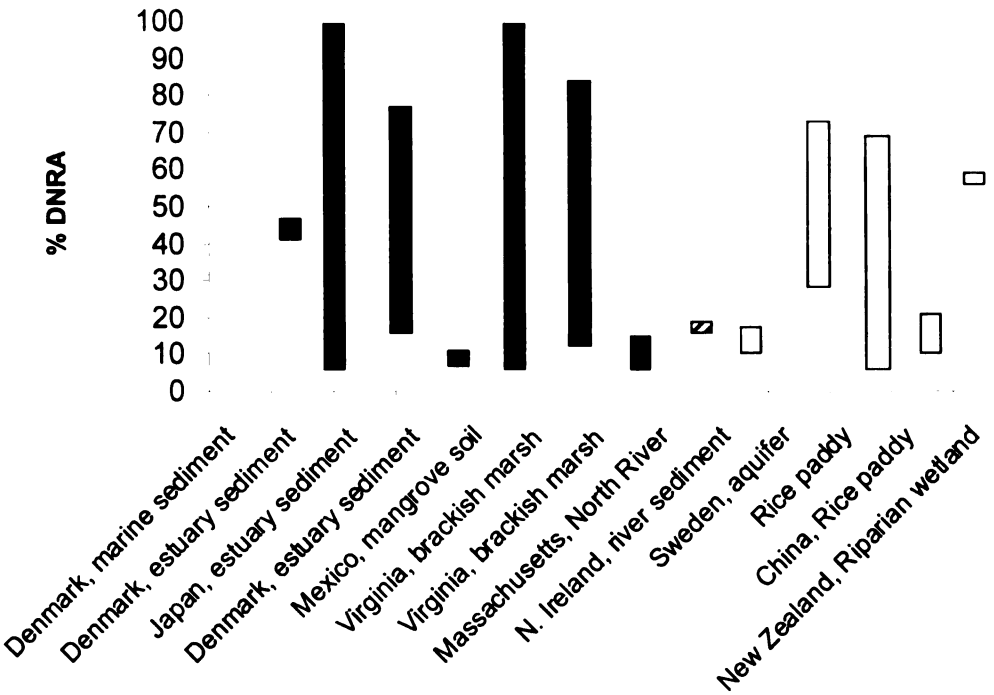


Figure 2: DNRA estimates across a variety of aquatic ecosystems. The bars represent the ranges of DNRA as a percent of the total dissimilatory nitrate removal found in a given study site, with the balance presumably due to denitrification. Closed bars designate marine and brackish ecosystems, open bars 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).

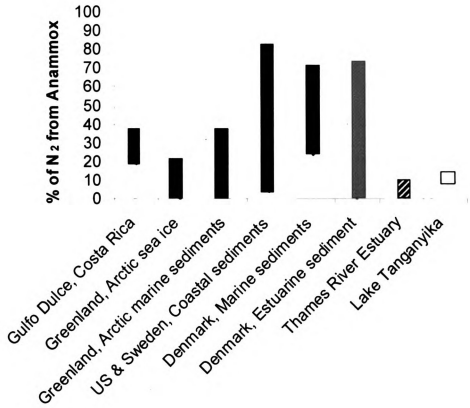


Figure 3: Anammox estimates across a variety of aquatic ecosystems. The bars represent the ranges of total N_2 production that can be attributed to Anammox in a given study site. Closed bars designate marine and brackish ecosystems, open bars designate freshwaters. The Thames River Estuary is hatched because the study spanned a range of freshwater and marine-influenced sites.

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). Evidence for DNRA has been found in freshwaters as well, including river sediments, rice paddies, riparian wetlands and aquifers (Figure 2, blue). 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 we understand 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 Anammox contributes half or more

of the N_2 production in coastal shelves and the deep sea (Figure 3), and possibly is responsible for 1/3 to 2/3 of global oceanic nitrate removal. The role of Anammox in freshwater nitrogen cycling remains speculative since only one study in a natural freshwater ecosystem has been published (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 pore water. 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).

How is it that scientists may 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 through the use of stable isotope and molecular microbial methods. Prior to the widespread use of stable isotopes, the favored method to measure denitrification was the acetylene block technique (ABT) (Tiedje 1988). The ABT typically entails creation of a sediment slurry, de-oxygenation with an inert gas, addition of acetylene to block the transformation of nitrous oxide, N_2O, to N_2, and measurement of the rate of N_2O production over time to indicate the rate of denitrification. This method suffers from a number of problems for trying to detect alternative nitrate removal processes, including the removal of free sulfide by sparging, disruption of the steep sediment redox gradients that may favor certain organisms and reactions, and the incorrect assumption that all of the nitrous oxide produced is from

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

Immense amounts of effort have been expended to study respiratory denitrification and management decisions are being made based on that body of knowledge. The possible importance – or even prevalence – of alternative nitrate removal pathways has profound implications for our management of aquatic ecosystems to reduce nitrate loads. Nitrate is the most mobile N form, so removal of nitrate by any of the processes described above is important to downstream water quality, but permanent removal by denitrification is most desirable.

Removal by other pathways can result in transformation of the nitrate to something other than dinitrogen gas (N_2). Nitrate removal via Anammox still creates dinitrogen gas as an end-product, but removes both a nitrate and an ammonium ion 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. Additionally, if S-oxidizers prove to take up much of the nitrate, then N cycling is closely linked to sulfide availability, which is turn is linked to sulfate reduction. In freshwaters sulfate

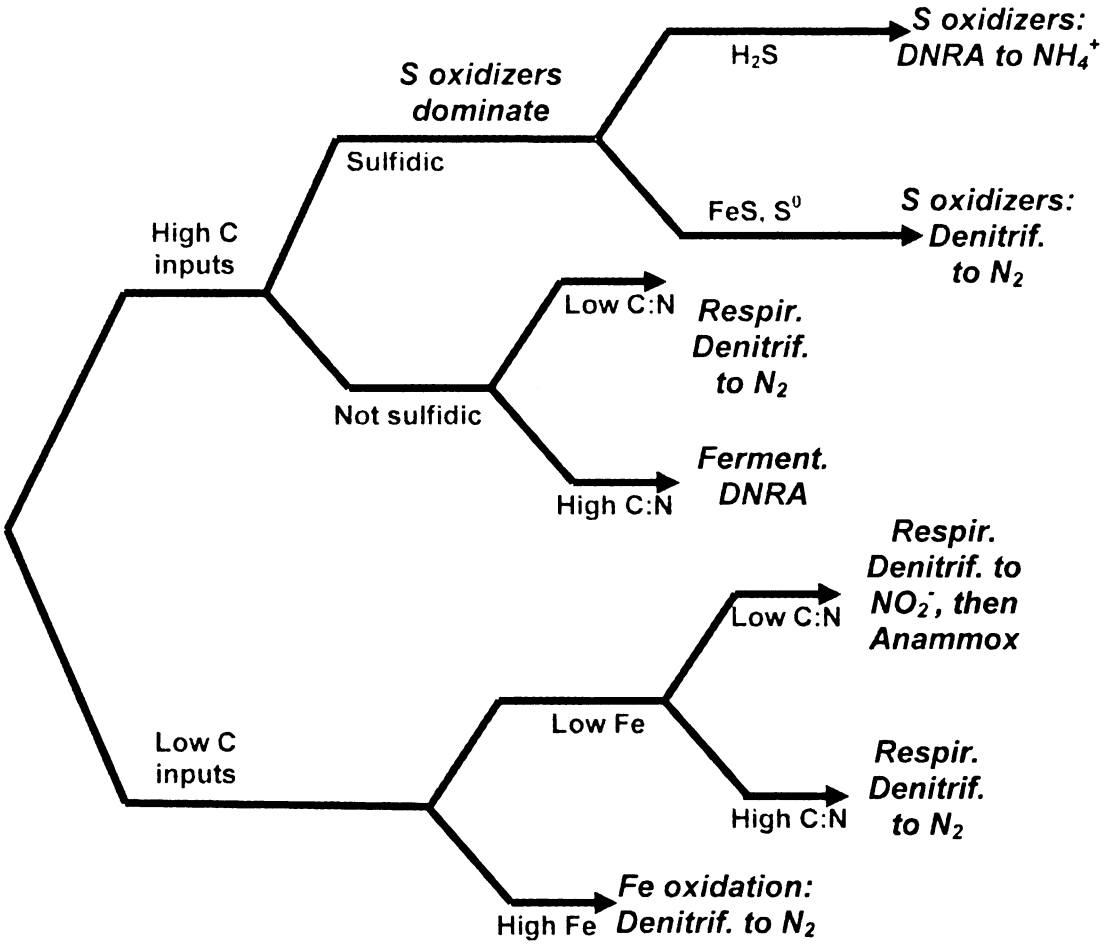


Figure 4: Hypothesized controls on predominant dissimilatory pathways of nitrate removal. This flow chart summarizes the conditions under which we would expect a particular nitrate removal pathway to be important. C inputs refer to labile organic carbon available to microbes. Sulfidic refers to the presence of significant amounts of either free sulfide (H_2S or S^{2-}), elemental S (S^0), 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 inhibit denitrification and thus promotes DNRA. C:N ratios refer to the ratio of labile organic carbon to nitrate. Denitrif. = denitrification; DNRA = Dissimilatory Nitrate Reduction to Ammonium; Anammox = Anaerobic Ammonium Oxidation.

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 freshwaters 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 needs to be done on 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 these processes in freshwater ecosystems subject to elevated nitrate inputs remains incomplete.

Acknowledgements:

We would like to thank the following people for their help and advice: Jay Lennon, Terrance Loecke, Patrick Mulholland, Nathaniel Ostrom, G. Philip Robertson, Thomas Schmidt, Stefanie Whitmire and Nancy Grimm's N reading group at ASU. The comments of two anonymous reviewers also improved this manuscript. This work was supported by U.S. National Science Foundation grants DEB-0111410, 0508704, 0423627, and 0516076. This is contribution #1275 to the W.K. Kellogg Biological Station.

References:

Alexander, R., R. Smith, and G. Schwarz. 2000. Effect of stream channel size on the delivery of nitrogen to the Gulf of Mexico. Nature **403**:758-761.

An, S. M., and W. S. Gardner. 2002. Dissimilatory nitrate reduction to ammonium (DNRA) as a nitrogen link, versus denitrification as a sink in a shallow estuary (Laguna Madre/Baffin Bay, Texas). Marine Ecology-Progress Series **237**:41-50.

Bonin, P. 1996. Anaerobic nitrate reduction to ammonium in two strains isolated from costal marine sediment: A dissimilatory pathway. FEMS Microbiology Ecology **19**:27-38.

Bottcher, 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.

Brettar, I., and G. Rheinheimer. 1991. Denitrification in the Central Baltic - evidence for H_2S-oxidation as motor of denitrification at the oxic-anoxic interface. Marine Ecology-Progress Series **77**:157-169.

Brunet, R. C., and L. J. Garcia-Gil. 1996. Sulfide-induced dissimilatory nitrate reduction to ammonia in anaerobic freshwater sediments. FEMS Microbiology Ecology **21**:131-138.

Cornwell, J. C., W. M. Kemp, and T. M. Kana. 1999. Denitrification in coastal ecosystems: methods, environmental controls, and ecosystem level controls, a review. Aquatic Ecology **33**:41-54.

Dail, D. B., E. A. Davidson, and J. Chorover. 2001. Rapid abiotic transformation of nitrate in an acid forest soil. Biogeochemistry **54**:131-146.

Dalsgaard, T., B. Thamdrup, and D. E. Canfield. 2005. Anaerobic ammonium oxidation (anammox) in the marine environment. Research in Microbiology **156**:457-464.

Dannenberg, S., M. Kroder, W. Dilling, and H. Cypionka. 1992. Oxidation of H_2, organic compounds and inorganic sulfur compounds coupled to reduction of O_2 or nitrate by sulfate-reducing bacteria. Archives of Microbiology **158**:93-99.

Davidson, E. A., J. Chorover, and D. B. Dail. 2003. A mechanism of abiotic immobilization of nitrate in forest ecosystems: the ferrous wheel hypothesis. Global Change Biology **9**:228-236.

Dodds, W. K. 2006. Nutrients and the "dead zone": the link between nutrient ratios and dissolved oxygen in the northern Gulf of Mexico. Frontiers in Ecology and the Environment **4**:211-217.

Fossing, H., V. A. Gallardo, B. B. Jorgensen, M. Huttel, L. P. Nielsen, H. Schulz, D. E. Canfield, S. Forster, R. N. Glud, J. K. Gundersen, J. Kuver, N. B. Ramsing, A. Teske, B. Thamdrup, and O. Ulloa. 1995. Concentration and transport of nitrate by the mat-forming sulfur bacterium *Thioploca*. Nature **374**:713-715.

Holmer, M., and P. Storkholm. 2001. Sulphate reduction and sulphur cycling in lake sediments: a review. Freshwater Biology **46**:431-451.

Howarth, R. W., G. Billen, D. Swaney, A. Townsend, N. Jaworski, K. Lajtha, J. A. Downing, R. Elmgren, N. Caraco, T. Jordan, F. Berendse, J. Freney, V. Kudeyarov, P. Murdoch, and Z. L. Zhu. 1996. Regional nitrogen budgets and riverine N&P fluxes for the drainages to the North Atlantic Ocean: Natural and human influences. Biogeochemistry **35**:75-139.

Jetten, M. S. M. 2001. New pathways for ammonia conversion in soil and aquatic systems. Plant and Soil **230**:9-19.

Jetten, M. S. M., M. Strous, K. T. van de Pas-Schoonen, J. Schalk, U. G. J. M. van Dongen, A. A. van de Graaf, S. Logemann, G. Muyzer, M. C. M. van Loosdrecht, and J. G. Kuenen. 1998. The anaerobic oxidation of ammonium. FEMS Microbiology Reviews **22**:421-437.

Jorgensen, B. 1982. Ecology of the bacteria of the sulphur cycle with special reference to anoxic-oxic interface environments. Philosophical Transitions of the Royal Society of London **298**:543-561.

Kamp, A., P. Stief, and H.N. Schulz-Vogt. 2006. Anaerobic sulfide oxidation with nitrate by a freshwater Beggiatoa enrichment culture. Applied and Environmental Microbiology **72**:4755-4760.

Kelly, D. P., and A. P. Wood. 2000. Confirmation of *Thiobacillus denitrificans* as a species of the genus *Thiobacillus*, in the beta-subclass of the Proteobacteria, with strain NCIMB 9548 as the type strain. International Journal of Systematic and Evolutionary Microbiology **50**:547-550.

Kelso, B. H. L., R. V. Smith, R. J. Laughlin, and S. D. Lennox. 1997. Dissimilatory nitrate reduction in anaerobic sediments leading to river nitrite accumulation. Applied and Environmental Microbiology **63**:4679-4685.

Knowles, R. 1982. Denitrification. Microbiological Reviews **46**:43-70.

Matheson, F. E., M. L. Nguyen, A. B. Cooper, T. P. Burt, and D. C. Bull. 2002. Fate of ^{15}N-nitrate in unplanted, planted and harvested riparian wetland soil microcosms. Ecological Engineering **19**:249-264.

Nijburg, J. W., M. J. L. Coolen, S. Gerards, P. J. A. K. Gunnewiek, and H. J. Laanbroek. 1997. Effects of nitrate availability and the presence of *Glyceria maxima* the composition and activity of the dissimilatory nitrate-reducing bacterial community. Applied and Environmental Microbiology **63**:931-937.

Otte, S., J. G. Kuenen, L. P. Nielsen, H. W. Paerl, J. Zopfi, H. N. Schulz, A. Teske, B. Strotmann, V. A. Gallardo, and B. B. Jorgensen. 1999. Nitrogen, carbon, and sulfur metabolism in natural *Thioploca* samples. Applied and Environmental Microbiology **65**:3148-3157.

Paerl, H. W., R. L. Dennis, and D. R. Whitall. 2002. Atmospheric deposition of nitrogen: Implications for nutrient over-enrichment of coastal waters. Estuaries **25**:677-693.

Peterson, B. J., W. M. Wollheim, P. J. Mulholland, J. R. Webster, J. L. Meyer, J. L. Tank, E. Marti, W. B. Bowden, H. M. Valett, A. E. Hershey, W. H. McDowell, W. K. Dodds, S. K. Hamilton, S. Gregory, and D. D. Morrall. 2001. Control of nitrogen export from watersheds by headwater streams. Science **292**:86-90.

Philippot, L., and O. Hojberg. 1999. Dissimilatory nitrate reductases in bacteria. Biochimica et Biophysica Acta-Gene Structure and Expression **1446**:1-23.

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.

Rabalais, N. N., R. E. Turner, and W. J. Wiseman. 2001. Hypoxia in the Gulf of Mexico. Journal of Environmental Quality **30**:320-329.

Raghoebarsing, A. A., A. Pol, K. T. van de Pas-Schoonen, A. J. P. Smolders, K. F. Ettwig, W. I. C. Rijpstra, S. Schouten, J. S. S. Damste, H. J. M. Op den Camp, M. S. M. Jetten, and M. Strous. 2006. A microbial consortium couples anaerobic methane oxidation to denitrification. Nature **440**:918-921.

Rysgaard, S., and R. N. Glud. 2004. Anaerobic N_2 production in Arctic sea ice. Limnology and Oceanography **49**:86-94.

Rysgaard, S., R. N. Glud, N. Risgaard-Petersen, and T. Dalsgaard. 2004. Denitrification and anammox activity in Arctic marine sediments. Limnology and Oceanography **49**:1493-1502.

Rysgaard, S., N. RisgaardPetersen, and N. P. Sloth. 1996. Nitrification, denitrification, and nitrate ammonification in sediments of two coastal lagoons in Southern France. Hydrobiologia **329**:133-141.

Schlesinger, W. H. 1997. Biogeochemistry: an analysis of global change, 2nd edition. Academic Press, San Diego.

Schubert, C. J., E. Durisch-Kaiser, B. Wehril, B. Thamdrup, P. Lam, M.M.M. Kuypers. 2006. Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika). Environmental Microbiology **8**:1857-1863.

Schulz, H. N., and B. B. Jorgensen. 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.

Senga, Y., K. Mochida, R. Fukumori, N. Okamoto, and Y. Seike. 2006. N_2O accumulation in estuarine and coastal sediments: The influence of H_2S on dissimilatory nitrate reduction. Estuarine Coastal and Shelf Science **67**:231-238.

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

Straub, K. L., and B. E. E. Buchholz-Cleven. 1998. Enumeration and detection of anaerobic ferrous iron-oxidizing, nitrate-reducing bacteria from diverse European sediments. Applied and Environmental Microbiology **64**:4846-4856.

Tiedje, J. M. 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. Pages 179-244 *in* A. J. B. Zehnder, editor. Biology of Anaerobic Microorganisms. Wiley & Sons, New York.

Tiedje, J. M., A. J. Sexstone, D. D. Myrold, and J. A. Robinson. 1982. Denitrification - ecological niches, competition and survival. Antonie Van Leeuwenhoek Journal of Microbiology **48**:569-583.

Timmer-ten-hoor, A. 1981. Cell Yield and Bioenergetics of *Thiomicrospira-Denitrificans* Compared with *Thiobacillus-Denitrificans*. Antonie Van Leeuwenhoek Journal of Microbiology **47**:231-243.

Tobias, C. R., S. A. Macko, I. C. Anderson, E. A. Canuel, and J. W. Harvey. 2001. Tracking the fate of a high concentration groundwater nitrate plume through a fringing marsh: A combined groundwater tracer and in situ isotope enrichment study. Limnology and Oceanography **46**:1977-1989.

Turner, R. E., and N. N. Rabalais. 2003. Linking landscape and water quality in the Mississippi river basin for 200 years. Bioscience **53**:563-572.

Weber, K. A., F. W. Picardal, and E. E. Roden. 2001. Microbially catalyzed nitrate-dependent oxidation of biogenic solid-phase Fe(II) compounds. Environmental Science & Technology **35**:1644-1650.

Weber, K. A., M. M. Urrutia, P. F. Churchill, R. K. Kukkadapu, and E. E. Roden. 2006. Anaerobic redox cycling of iron by freshwater sediment microorganisms. Environmental Microbiology **8**:100-113.

Welsh, D. T., G. Castadelli, M. Bartoli, D. Poli, M. Careri, R. de Wit, and P. Viaroli. 2001. Denitrification in an intertidal seagrass meadow, a comparison of ^{15}N isotope and acetylene-block techniques: dissimilatory nitrate reduction to ammonia as a source of N_2O? Marine Biology **139**:1029-1036.

Zedler, J. B. 2003. Wetlands at your service: reducing impacts of agriculture at the watershed scale. Frontiers in Ecology and the Environment **1**:65-72.

Zopfi, J., T. Kjaer, L. P. Nielsen, and B. B. Jorgensen. 2001. Ecology of *Thioploca* spp.: Nitrate and sulfur storage in relation to chemical microgradients and influence of *Thioploca* spp. on the sedimentary nitrogen cycle. Applied and Environmental Microbiology **67**:5530-5537.

Due to space limitations, we would like to include this supplementary information online:

References from Figure 2 (in order of bars):

Goeyens, L., R. T. P. Devries, J. F. Bakker, and W. Helder. 1987. An experiment on the relative importance of denitrification, nitrate reduction and ammonification in coastal marine sediment. Netherlands Journal of Sea Research **21**:171-175.

Christensen, P. B., S. Rysgaard, N. P. Sloth, T. Dalsgaard, and S. Schwaerter. 2000. Sediment mineralization, nutrient fluxes, denitrification and

dissimilatory nitrate reduction to ammonium in an estuarine fjord with sea cage trout farms. Aquatic Microbial Ecology **21**:73-84.

Koike, I., and A. Hattori. 1978. Denitrification and ammonia formation in anaerobic coastal sediments. Applied and Environmental Microbiology **35**:278-282.

Pelegri, S. P., L. P. Nielsen, and T. H. Blackburn. 1994. Denitrification in estuarine sediment stimulated by the irrigation activity of the amphipod *Corophium volutator*. Marine Ecology-Progress Series **105**:285-290.

Rivera-Monroy, V. H., R. R. Twilley, R. G. Boustany, J. W. Day, F. Veraherrera, and M. D. Ramirez. 1995. Direct denitrification in mangrove sediments in Terminos Lagoon, Mexico. Marine Ecology-Progress Series **126**:97-109.

Tobias, C. R., I. C. Anderson, E. A. Canuel, and S. A. Macko. 2001. Nitrogen cycling through a fringing marsh-aquifer ecotone. Marine Ecology-Progress Series **210**:25-39.

Tobias, C. R., S. A. Macko, I. C. Anderson, E. A. Canuel, and J. W. Harvey. 2001. Tracking the fate of a high concentration groundwater nitrate plume through a fringing marsh: A combined groundwater tracer and in situ isotope enrichment study. Limnology and Oceanography **46**:1977-1989.

Bowden, W.B. 1986. Nitrification, nitrate reduction, and nitrogen immobilization in a tidal freshwater marsh sediment. Ecology **67**: 88-99.

Kelso, B. H. L., R. V. Smith, R. J. Laughlin, and S. D. Lennox. 1997. Dissimilatory nitrate reduction in anaerobic sediments leading to river nitrite accumulation. Applied and Environmental Microbiology **63**:4679-4685.

Bengtsson, G., and H. Annadotter. 1989. Nitrate reduction in a groundwater microcosm determined by ^{15}N gas chromatography-mass spectrometry. Applied and Environmental Microbiology **55**:2861-2870.

Buresh, R. J., and W. H. Patrick. 1978. Nitrate reduction to ammonium in anaerobic soil. Soil Science Society of America Journal **42**:913

Yin, S. X., D. Chen, L. M. Chen, and R. Edis. 2002. Dissimilatory nitrate reduction to ammonium and responsible microorganisms in two Chinese and Australian paddy soils. Soil Biology & Biochemistry **34**:1131-1137.-918.

Matheson, F. E., M. L. Nguyen, A. B. Cooper, T. P. Burt, and D. C. Bull. 2002. Fate of ^{15}N-nitrate in unplanted, planted and harvested riparian wetland soil microcosms. Ecological Engineering **19**:249-264.

References from Figure 3 (in order of bars):

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

Rysgaard, S., and R. N. Glud. 2004. Anaerobic N_2 production in Arctic sea ice. Limnology and Oceanography **49**:86-94.

Rysgaard, S., R. N. Glud, N. Risgaard-Petersen, and T. Dalsgaard. 2004. Denitrification and anammox activity in Arctic marine sediments. Limnology and Oceanography **49**:1493-1502.

Engstrom P, Dalsgaard T, Hulth S, and Aller, R.C. 2005. Anaerobic ammonium oxidation by nitrite (anammox): Implications for N_2 production in coastal marine sediments. Geochimica et Cosmochimica Acta **69** (8): 2057-2065.

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**: 1312-18.

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

Trimmer, M., J. C. Nicholls, and B. Deflandre. 2003. Anaerobic ammonium oxidation measured in sediments along the Thames estuary, United Kingdom. Applied and Environmental Microbiology **69**:6447-6454.

Schubert, C. J., E. Durisch-Kaiser, B. Wehril, B. Thamdrup, P. Lam, M.M.M. Kuypers. 2006. Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika). Environmental Microbiology **8**:1857-1863.

CHAPTER 2

THE PREVALENCE OF SIMULTANEOUS NITRATE REMOVAL AND SULFATE PRODUCTION IN STREAM, WETLAND AND LAKE SEDIMENTS

Introduction:

Increases in the intensity and extent of agricultural land use have led to dramatic alterations of the global N cycle, contributing to a doubling of the annual input rate of bio-available, fixed N (Vitousek et al. 1997). N pollution originating from agriculture and other anthropogenic activities has dramatically increased N loading to aquatic ecosystems (Carpenter et al. 1998, Bernot and Dodds 2005). Approximately 75% of the N loading to terrestrial landscapes cannot be accounted for in river exports to the ocean and most of this evidently disappears in transit (Seitzinger et al. 2006). These massive changes in N cycling have stimulated interest in understanding how the dominant inorganic N form, nitrate (NO_3^-), moves through landscapes, and what controls the fraction that is removed in transit through aquatic ecosystems.

Nitrate removal has long been acknowledged to be driven by the heterotrophic microbial metabolism of organic carbon, with NO_3^- serving either as an electron donor for respiratory denitrification, or in a fermentation process wherein nitrate is reduced to ammonium in dissimilatory nitrate reduction to ammonium (DNRA) (Tiedje 1988). More recently, a few studies have reported evidence for apparent linkages between sulfur (S) and N cycling in freshwater ecosystems. With experimental additions of NO_3^- and sulfate (SO_4^{2-}) to wetland sediments in southwest Michigan, Whitmire and Hamilton (2005) found that in

approximately half of their 16 experiments, SO_4^{2-} production occurred during the period of NO_3^- removal. NO_3^- additions on the Wisconsin River floodplain also resulted in an approximately equimolar amount of SO_4^{2-} production (Forshay 2003). Evidence of this phenomenon has also been found in the Netherlands where researchers observed that in an aquifer containing pyrite, SO_4^{2-} concentrations increased while infiltrating NO_3^- was removed (Lucassen et al. 2002). S-driven NO_3^- removal has also been exploited as a water treatment method for removing NO_3^- in cores packed with elemental sulfur granules (Soares 2002).

The metabolic flexibility of S-oxidizing bacteria has been increasingly appreciated in recent years. *Thiobacillus denitrificans* couples the oxidation of inorganic sulfur compounds to the reduction of NO_3^- and is common in freshwater and marine sediments (Kelly 1999, Haaijer et al. 2006). *Thioploca* spp. were first described from Lake Constance in the early 1900s and were later found to occur in several other lakes in northern Germany (Jorgensen and Gallardo 1999). However, their unique influence on biogeochemical cycling was appreciated only after *Thioploca* was found in vast benthic mats off the coast of Chile (Gallardo 1977). Species of *Thioploca* have been intensively studied since the discovery of the marine species, in large part because their distinctive metabolism couples NO_3^- reduction to sulfide (H_2S) oxidation (Jorgensen and Gallardo 1999). Additionally, they possess gliding motility that allows them to migrate upward to oxic overlying water of higher NO_3^- concentration, and store both NO_3^- and elemental S intracellularly (Jorgensen and Gallardo 1999). *Thiomargarita*, known

for its enormous size (750 μm), also utilizes and stores NO_3^- and S^o intracellularly (Schulz et al. 1999, Schulz and Jorgensen 2001). *Beggiatoa* spp., close relatives of *Thioploca* and *Thiomargarita*, and are also gliding, filamentous bacteria that can store S^o and oxidize it with NO_3^- (Kamp et al. 2006). These bacteria have been known to occur in marine ecosystems for some time (Sweerts et al. 1990, McHatton et al. 1996), and have recently been isolated from freshwater ecosystems (Kamp et al. 2006).

This study was inspired by the observations of Whitmire and Hamilton (2005), who examined anaerobic microbial processes including NO_3^- removal in a variety of wetlands. Tracer injections were conducted using a modified "push-pull" method to quantify rates of NO_3^- and SO_4^{2-} uptake (see "Methods"). The injection solution in that work was local groundwater with ambient concentrations of NO_3^- (14 mg N/L) and SO_4^{2-} (53 mg/L) to simulate groundwater inputs to five different wetland sediments. In 9 of her 16 experiments, they observed marked production of SO_4^{2-} relative to the concentration of the conservative tracer (Br^-), but only during the period when NO_3^- was being consumed. Figure 1 shows examples of this phenomenon in a wetland in which the SO_4^{2-} production (triangles) was quite pronounced (top left panel) and a wetland in which the response was less prominent (bottom left panel). In all cases, SO_4^{2-} removal (triangles) commenced soon after the available NO_3^- (circles) was removed, presumably by SO_4^{2-} reduction (Figure 1).

Nitrate removal can be stoichiometrically compared to SO_4^{2-} production by expressing the push-pull experiment data as the difference between observed

and expected concentrations (Figure 1, right panels). Expected concentrations are calculated as the product of the observed Br^- concentration and the ratio of NO_3^- or SO_4^{2-} to Br^- in the injection solution, after background correction (Whitmire 2003, Whitmire and Hamilton 2005). Figure 1 illustrates two examples from the dataset collected for this experiment. The example in the top set of panels is from a site where high rates of sulfate production occurred. The example from the bottom panels is from a site where relatively little SO_4^{2-} production occurred.

The variation observed in Whitmire and Hamilton's work led me to question if this pattern was indeed biological, and if so, how widespread it was across aquatic ecosystems. As part of this study, I performed several tests to show that the occurrence was enzymatically catalyzed (see Results). Once assured that the process is biological, I was faced with discerning what reactions may be driving the SO_4^{2-} production and NO_3^- removal, and in particular, I wanted to know what happens to the nitrate that is reduced? Assuming the reaction proceeds via denitrification to dinitrogen gas (N_2), the initial oxidation step may have the following stoichiometry (Fossing et al. 1995):

$$5\ HS^- + 2\ NO_3^- + 7\ H^+ \rightarrow 5\ S^o + N_2 + 6\ H_2O \qquad (1)$$

The resultant elemental S may be stored in the cells before later being oxidized to SO_4^{2-}. Further oxidation to SO_4^{2-} could occur by the following reaction (Fossing et al. 1995):

$$5\ S^o + 6\ NO_3^- + 2\ H_2O \rightarrow 5\ SO_4^{2-} + 3\ N_2 + 4\ H^+ \qquad (2)$$

If these two reactions occurred sequentially, the molar ratio of NO_3^- consumed to SO_4^{2-} produced would be 8:5 (=1.6) as in this combined reaction:

$$5\ HS^- + 8\ NO_3^- + 3\ H^+ \rightarrow 5\ SO_4^{2-} + 4\ N_2 + 4\ H_2O \qquad (3)$$

However, the nitrate may not be completely reduced to N_2, but may also be converted to NH_4^+ in a form of DNRA. The stoichiometric formula for the conversion of NO_3^- to NH_4^+, assuming direct transformation of sulfide to elemental S (as in equation 1), is (Sayama et al. 2005):

$$4\ H_2S + NO_3^- + 2\ H^+ \rightarrow 4\ S^o + NH_4^+ + 3\ H_2O \qquad (4)$$

The resultant S may be stored, or further oxidized to SO_4^{2-} via:

$$4\ S^o + 3\ NO_3^- + 7\ H_2O \rightarrow 3\ NH_4^+ + 4\ SO_4^{2-} + 2\ H^+ \qquad (5)$$

Reactions 4 and 5 may occur sequentially producing ratio of one mole of nitrate consumed to every one mole of sulfate produced, as in:

$$4\ H_2S + 4\ NO_3^- + 4\ H2O \rightarrow 4\ NH_4^+ + 4\ SO_4^{2-} \qquad (6)$$

The stoichiometry of these two reactions can be used to estimate the magnitude of the sulfate production that could be to nitrate removal.

The alternative reduction products (N_2 vs. NH_4^+) have different implications for ecosystem N cycling. Nitrate reduced to N_2 via denitrification is permanently removed, whereas NO_3^- transformed to NH_4^+ via DNRA is retained within the ecosystem. Few studies have examined the role of DNRA in freshwater systems, and the eventual fate of NH_4^+ produced by DNRA is uncertain (Burgin and Hamilton 2007). Freshwater wetlands are low in S compared to marine systems, but often contain enough to support significant bacterial transformations (Lovley and Klug 1983).

The objectives of this study were to: 1) confirm that the SO_4^{2-} production observed by Whitmire and Hamilton (2005) was a biological phenomenon; 2) ascertain how widespread the phenomenon is across diverse freshwater ecosystems; 3) explore which potential pathways and reactions could be responsible; and, 4) seek a predictive understanding of what types of aquatic ecosystems would exhibit significant coupled NO_3^--S cycling.

Methods:

Site selection

Experiments were conducted in nine streams, nine wetlands and three lakes (Table 1). For the 2004 study, sites were selected to encompass a range of free H_2S concentrations in sediment porewaters (Whitmire 2003; Table 1). In 2005, sites were selected in part based on data from the larger 2004 survey, and in part based on sites from the LINX II experiments (streams only; Mulholland et al., in review).

Push-pull methodology

The push-pull method has been used to estimate biogeochemical processing rates in aquifers (Istok et al. 1997, McGuire et al. 2002), lake sediments (Luthy et al. 2000), and riparian wetlands (Addy et al. 2002). In my work, push-pull experiments were done using a near-surface push-pull technique similar to the method developed by Whitmire and Hamilton (2005), modified slightly to minimize dead volume in the injector system. The experiments were conducted *in situ* at 5-10-cm depth in the sediments, at three points per

treatment (described below) in each site. Sites are described in Whitmire (2003), Whitmire and Hamilton (2005), (Wetzel 2001) and Chapters 3 and 4 of this dissertation.

Push-pull experiments entail removing a sample of porewater and amending it with one or more reactive solutes (e.g., NO_3^-) as well as a conservative solute tracer (Br^-), reinjecting the porewater into the sediments, and withdrawing samples over time to quantify the rates of uptake. The ratio of the reactant to the conservative tracer concentration corrects for dilution and dispersion; use of the conservative tracer together with the biologically active solute allows for calculation of the net production or consumption of the reactant (see Whitmire and Hamilton 2005 for more details on the calculations). The amendment solution is O_2-free and minimal in volume. Generally, 27 mL of porewater were removed from the sediments, amended with 3 mL of anoxic Br^- (control) or NO_3^- + Br^- (treatment) solution (final concentrations were 10 mg L^{-1} NO_3^--N and 3 mg L^{-1} Br^-), and reinjected into the sediments. The injection line was then flushed with 1 mL of porewater and 1 mL of sample was withdrawn and filtered (0.45 µm Millex syringe filters) for analysis by ion chromotrography (NO_3^-, SO_4^{2-}, Br^-). In lake and wetland sediments, samples were taken every 10 minutes for the first hour and then every 30 minutes for the next 2-3 hours. Incubations generally lasted 4 hours. Due to the increased hydrologic movement in streams, samples were taken every five minutes for the first 20 minutes and every 10 minutes thereafter.

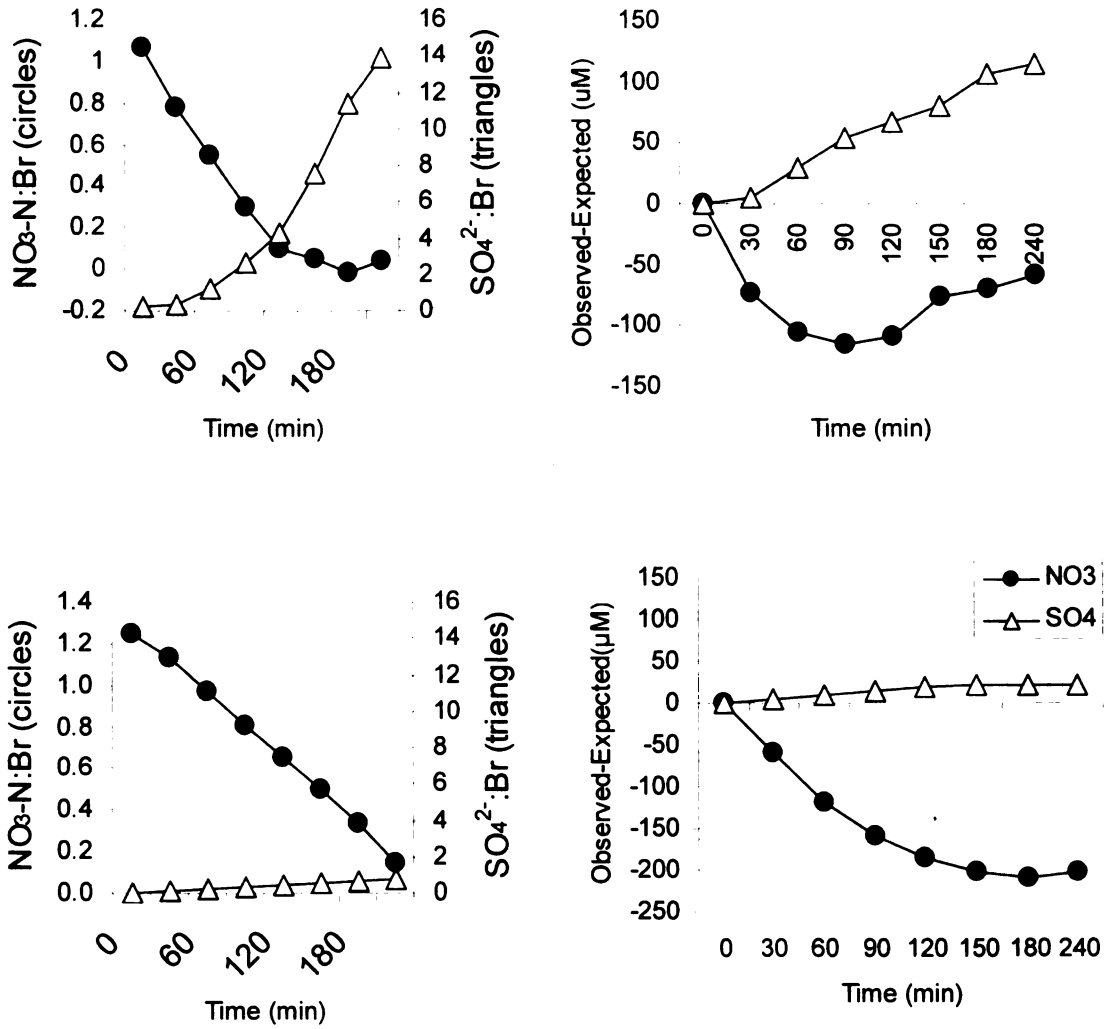


Figure 1: Simultaneous nitrate removal and sulfate production in two different wetlands. The panels on the left are the ratios of the reactants (NO_3^- and SO_4^{2-}) to the conservative tracer (Br^-) in two push-pull experiments. The panels on the right are a comparison of observed and expected concentration (eq. 8) of NO_3^- and SO_4^{2-} in a site with high (top) and low (bottom) sulfate production. The top panels are data from Prairieville Creek Fen and the bottom panels are from Windmill Pond (see Table 1 for more site information). These two examples represent the highest and lowest rates of SO_4^{2-} production relative to NO_3^- removal that I observed.

In 2004, I performed push-pull experiments in a diverse set of wetlands (n=9), streams (n=9) and lakes (n=3), with site details in Table 1. In 2005, I returned to each of three wetlands, lakes and streams and repeated the push-pull experiments in the same locations using both $^{14}NO_3^-$ for the push-pull rate measurements and $^{15}NO_3^-$ to discern the dominant end products (sites marked with asterisks in Table 1). Due to analytical constraints, we could not remove small sub-samples of the $^{15}NO_3^-$ addition; therefore, those injectors were sampled only once at the end of the incubation period. The treatment addition was thus undisturbed for the length of the experiment, and the whole volume (40 mL) was removed at the end of the experiment and the $^{15}N_2$ extracted using the static headspace equilibration (Hamilton and Ostrom 2007) to quantify denitrification and the remaining water was filtered for $^{15}NH_4^+$ analysis to quantify DNRA. The $\delta^{15}N\text{-}NH_4^+$ was measured using the ammonia diffusion procedure (Holmes et al. 1998), in which MgO is added to elevate the pH and the NH_3 diffuses into an acidified glass-fiber filter sealed within a Teflon packet suspended above the surface of the sample. These samples on filters were analyzed for $\delta^{15}N$ either in the Stable Isotope Biogeochemistry Laboratory operated by Nathaniel and Peggy Ostrom at MSU or at the Marine Biological Laboratory's facility in Woods Hole, MA. These ^{15}N tracer data were compared to the NO_3^- removal rates calculated from the $^{14}NO_3^-$ addition at the same site.

NO_3^- removal rate constants were calculated based on NO_3^- and Br^- concentrations, and were modeled as first order rate reactions using the following

exponential function, which relates the concentration of the reactant (NO_3^-) to the tracer (Br^-) at a given point in time:

$$C_{reactant}(t) = C_{tracer}(t) * e^{-kt} \qquad (7)$$

The NO_3^- removal rate constant (k) is the slope of a regression line fit to a plot of $(\ln)(C_{reactant}(t)/C_{tracer}(t))$ versus time (C is concentration). Linear regressions for the calculation of nitrate removal were always significant (i.e., $p < 0.05$).

Stoichiometric calculations

In addition to calculating NO_3^- removal rate constants from the push-pull experiments, I also calculated SO_4^{2-} production. Briefly, SO_4^{2-} production was calculated as the difference between the measured SO_4^{2-} concentration (observed) and what would be expected based on the concentration of the conservative tracer (Br^-):

$$[SO_4^{2-}]_{exp'd} = [Br^-]_{obs'd} * ([SO_4^{2-}]/[Br^-])_{injectate} \qquad (8)$$

where $[SO_4^{2-}]_{exp'd}$ is the expected concentration if no loss or gain of SO_4^{2-} had taken place, $[Br]_{obs'd}$ is the Br concentration observed at a given sampling time, and $([SO_4^{2-}]/[Br^-])_{injectate}$ is the ratio of SO_4^{2-} to Br^- in the injectate that was added at the beginning of the experiment. This expected SO_4^{2-} concentration was subtracted from the measured concentrations, and the difference calculated as SO_4^{2-} production. The same calculations can also be done for NO_3^- removal, substituting NO_3^- for SO_4^{2-} in equation 5. All concentrations are molar.

The SO_4^{2-} production and NO_3^- removal can then be related to the stoichiometry of various possible reactions (equations 1-4) to infer which

processes may be occurring in these experiments. The molar ratio of total SO_4^{2-} production to total NO_3^- removal over the duration of the experiment is hereafter referred to as SP:NR. The "overall ratio" (mean of the ratios taken at each time point) over the duration of the NO_3^- removal period may be a better indicator of reaction stoichiometry because of the potential temporary storage of NO_3^- by S oxidizing bacteria.

Testing for experimental artifacts

In studying this phenomenon, I first thought that perhaps the apparent sulfate production could be an experimental artifact of the push-pull methodology. I therefore performed three experimental artifact tests: 1) a temperature test to verify enzymatic catalysis and thereby rule out the possibility of a strictly abiotic chemical reaction; 2) a test using an alternative injector material to ensure that the phenomenon was not caused by a reaction with the stainless steel injectors; and 3) additions of tracers to water overlying sediments to see if the phenomenon occurred in the absence of the injectors and push-pull experiments.

For the temperature test, I collected soil cores from a nearby wetland (Turkey Marsh, a site that was not used in the in situ push-pull experiments described above) and placed the cores into 1 quart canning jars along with ~100 mL overlying water, allowing one day in the dark for stabilization. To each jar, I added a regular push-pull injector made with a stainless steel mesh tip. Triplicate jars were incubated at 6, 22, or 50°C. For the 22°C treatment, I had three additional jars with injectors made from a Nylon mesh material, to test the

second possible artifact described above. Push-pull experiments were conducted in these jars, using the same methods described for the field experiments. Additionally, for the third experiment, I collected sediment from the same site to half-fill a 20-L bucket, leaving approximately 2.5 cm of overlying water, to which I added NO_3^- and Br^-. This overlying water was periodically sampled over a longer time period than the push-pull experiments because I expected reactions to occur more slowly, limited by sediment-water diffusive exchanges. Water samples for all experiments were analyzed as described below.

Porewater chemistry analysis

In both control (Br) and treatment (NO_3^- + Br) injections, porewaters were sampled for NH_4^+ and H_2S concentrations prior to application of the treatment ("pre") and at the end of the experiment ("post"). H_2S was analyzed by the methylene-blue method (Golterman and Clymo 1969). NH_4^+ was measured colorimetrically using the phenylhypochlorite technique (Aminot et al. 1997). NO_3^-, Br^-, and SO_4^{2-} were measured using membrane-suppression ion chromatography (Dionex 4200 with an AS14A anion column). At each site a sample of surface water was collected (filtered to 0.45 µm with a Gelman membrane filter) for comprehensive hydrochemical analysis (major solutes, nutrients).

Statistical analysis

All statistical analyses were conducted using Systat version 9.0 software. I used a non-parametric Kruskal-Wallis analysis of variance to test the differences in the distributions of NO_3^- removal rate constants and SP:NR ratios across ecosystem types. A parametric analysis of variance (ANOVA) was used to compare differences in porewater chemistry between treatment and control injectors. Stepwise multiple regression (MR; $\alpha = 0.10$) was used to determine which environmental variables (Table 1) best explained the variation in NO_3^- rate constants and SP:NR ratios. Variables were square root transformed when necessary to improve normality. MR was only performed on the 2004 data to avoid including the same sites from two years; MR was not performed on the 2005 sites alone because there were only 9 sites total, which was not enough power for the analysis.

Results:

Experimental Artifact Tests

After observing SO_4^{2-} production in field studies, I attempted to rule out other possible explanations (i.e., to ensure that the production was not an experimental artifact). To rule out a chemical reaction, I performed experiments in microcosms (jars) at various temperatures. Both SO_4^{2-} production and NO_3^- removal revealed an intermediate thermal optimum, indicative of biological mediation via enzymatic activity (below). I found similar results using injectors made of either stainless-

steel screens (closed symbols) or nylon filters (open symbols), indicating that an interaction with the materials was not causing the phenomenon.

To ensure that the tracer ions did not somehow affect sediment ion exchange equilibria (desorbing SO_4^{2-}) I injected only Br^- at various concentrations, and found no SO_4^{2-} production (the NaBr comprised most of the total ions added). My experiments were conducted in sediments free of plant roots, ruling out NO_3^- uptake by vascular plants. Finally, to ensure that the injection was not creating an artificial juxtaposition of reduced and oxidized substances, I added NO_3^- to the water overlying sediments in a wetland field enclosure and in a bucket of organic sediment in the lab. Water-column NO_3^- additions yielded the same results, albeit over a longer time scale (Figure 3).

To summarize the large amount of variation within one ecosystem type, I have placed all of the field sites into an aquatic ecosystem category (stream, S; lake, L; wetland, W) and have created box plots (e.g., Fig. 4) that encompass all of the individual injectors from each site (i.e., the box plots are based on the entire set of experiments, rather than site averages (experiments were conducted at three points within each site). This gives the most complete illustration of the variation within and among ecosystems.

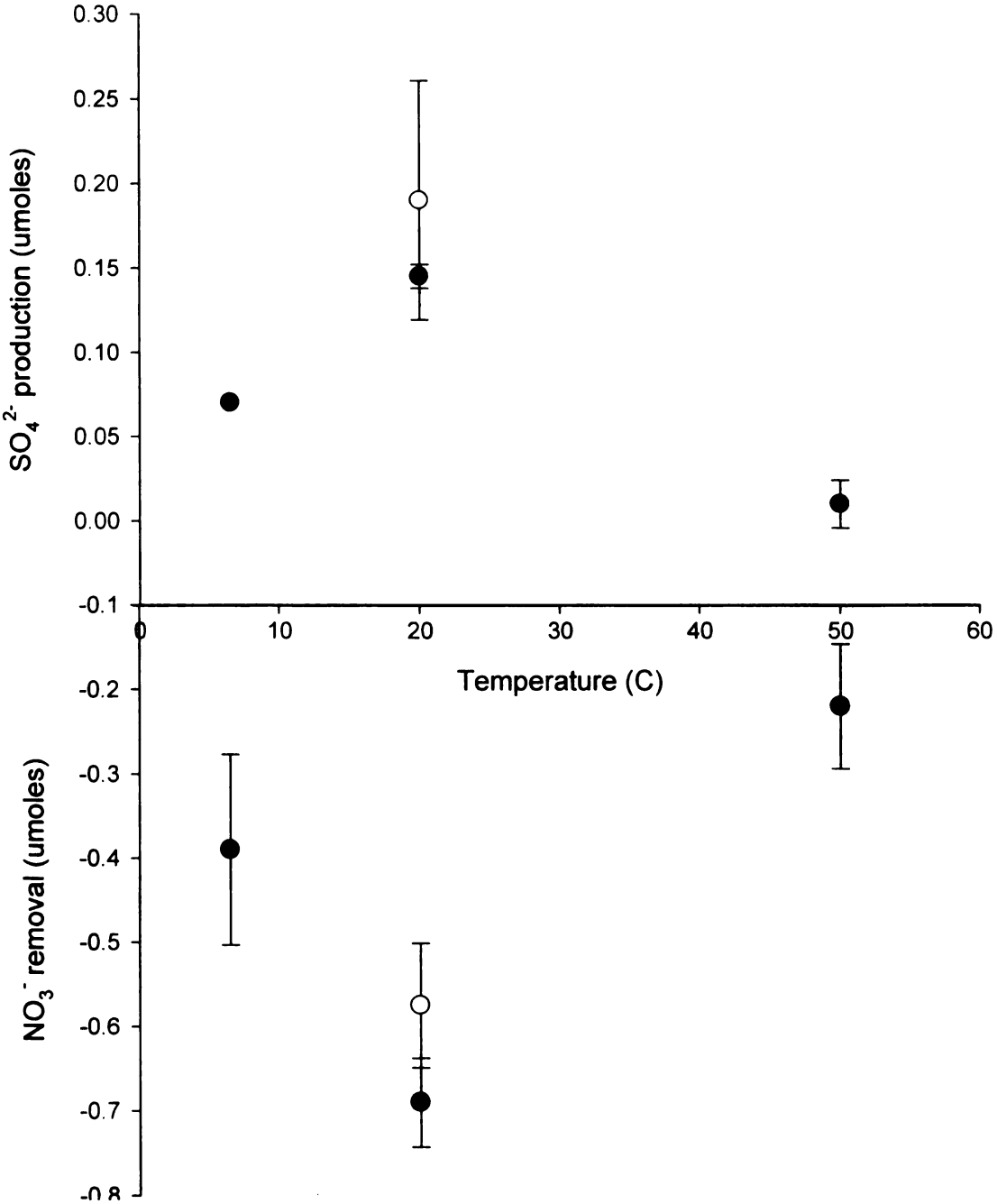


Figure 2: The effect of temperature on nitrate removal and sulfate production (both in µmoles). Open symbols denote the nylon mesh injectors, closed symbols denote the statinless steel injectors that were more commonly used and employed in all of the field experiments.

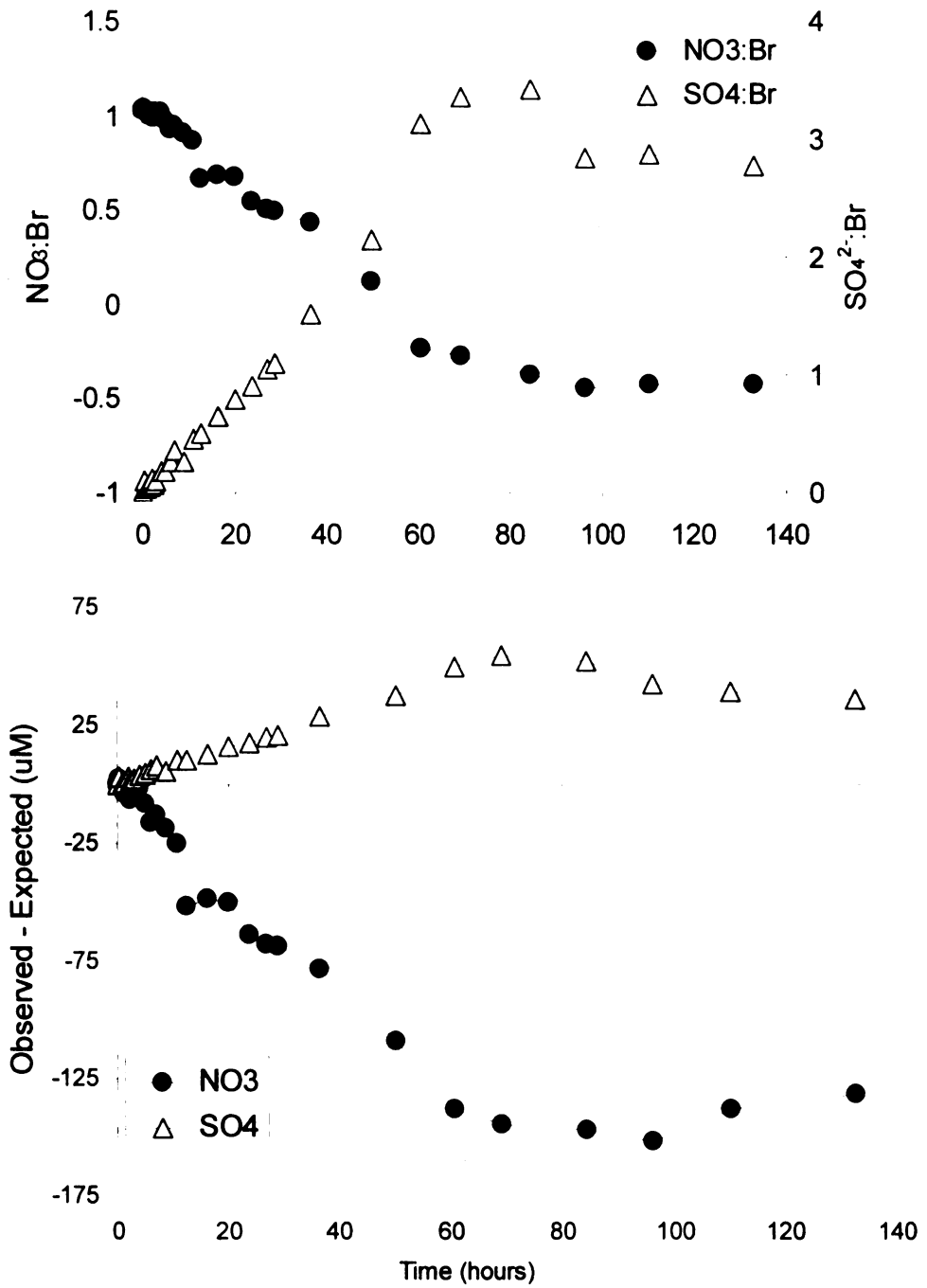


Figure 3: Simultaneous nitrate removal and sulfate production in surface water over sediments after nitrate addition to the surface water (conducted in the lab in a bucket). The top panel shows the ratios of the reactants (NO_3^- and SO_4^{2-}) to the conservative tracer (Br^-). In the top panel NO_3^-:Br^- became negative because the nitrate concentration fell below what was originally present (~0.6 mg/L) before the experimental NO_3^- / Br^- addition. The bottom panel is a comparison of observed and expected concentrations of NO_3^- and SO_4^{2-}.

The NO_3^- removal rate constant is the fraction of the NO_3^- concentration that was removed per unit time (min^{-1}). For example, a rate of -0.01 min^{-1} means that 1% of the nitrate that is present (concentration dependent) is removed every minute. NO_3^- removal rate constants were highly variable both among ecosystem types and within a given ecosystem (Figure 4). Lakes tended to have higher removal rates than did streams and wetlands (Kruskal-Wallis ANOVA 5.639, df = 2; p = 0.06). Stream NO_3^- removal rate constants ranged from -0.006 to -0.0545 min^{-1}, wetlands ranged from -0.0031 to -0.0752 min^{-1}, and lakes ranged from -0.062 to -0.0842 min^{-1}.

To compare the relative amount of SO_4^{2-} production in relation to NO_3^- removal across sites and aquatic ecosystems, I used a ratio of the µmoles of SO_4^{2-} produced (calculated from the observed SO_4^{2-} concentration and the expected concentration based on the Br^- concentration; figures 1 and 3) to the µmoles of NO_3^- removed (also as in Figures 1 and 3). This generates a unitless ratio of sulfate production:nitrate removal (abbreviated "SP:NR"); numbers near 1 reflect a situation wherein nearly all of the NO_3^- removal can be accounted for by the SO_4^{2-} production.

Concurrent SO_4^{2-} production and NO_3^- removal occurred in all freshwater ecosystem types (Figure 5). Streams had the greatest range of ratios of total SO_4^{2-} production to total NO_3^- removal (SP:NR 0.02-2.4). In 6 stream experiments (injectors, in 2 sites), SO_4^{2-} production could account for much more of the NO_3^- removed than actually occurred; this also occurred in 4 wetland injectors seen in wetlands (comprising 2 sites). Wetland SP:NR ranged from

0.005-1.1 and lake SP:NR from 0.03-1.1. Generally, SO_4^{2-} production accounted for 25-50% of NO_3^- removal (estimated from the inter-quartile ranges in Figure 4), and the fraction of removal attributable to SO_4^{2-} production was higher in wetlands and streams than in lakes (KW ANOVA 9.394, df=2; p=0.009).

By applying the stoichiometric model described above (equations 1-4) to the 90 experiments (considering each experiment separately), I compared how NO_3^- removal (yielding either NH_4^+ or N_2) relates to the measured SO_4^{2-} production in different aquatic ecosystems under the two alternative reaction stoichiometries (DNRA vs. denitrification). The amount of SO_4^{2-} produced explained a significant fraction of the NO_3^- removed in all types of aquatic ecosystems (Figure 6). Lakes have the highest S-dependent N removal, followed by streams and wetlands (medians, Figure 6), though there is considerable variation within a given ecosystem type. The 8:5 ratio of NO_3^- consumed to SO_4^{2-} produced (grey boxes, Figure 6) accounts for a greater fraction of the overall NO_3^- removal. When considering the possibility of NH_4^+ as the end product, as would be the case for a 1:1 ratio of NO_3^- consumed to NH_4^+ produced (black boxes, Figure 6), a slightly smaller, but still significant proportion of the NO_3^- removal could be explained by the SO_4^{2-} production.

Table 1: Physico-chemical characteristics of the sites in this study. Abbreviations: DO = dissolved oxygen; Cond. = conductivity; SW = surface water; PW = porewater; BDL = below detection limits. (*) denotes sites that were returned to in 2005 for the $^{15}NO_3^-$ push-pull experiments.

Site	Ecosystem	SW Temp (C)	SW DO mg L^{-1}	SW Cond. µS	SW pH	SW NO_3^- µM	SW NH_4^+ µM	SW SO_4^{2-} µM	PW H_2S µM	PW NH_4^+ µM
Wayland	Stream	24.5	13.2	726	8.23	48.6	0.9	350.2	0.5	131
Sand	Stream	16.4	9.4	488	8.05	23.2	1.7	162.7	0.3	98
Augusta	Stream	23.0	8.6	463	7.81	95.2	1.1	191.9	0.6	55
Buskirk	Stream	19.6	9.3	412	8.23	16.4	1.5	203.4	0.6	44
Ransom	Stream	22.5	8.4	365	8.01	0.5	0.6	85.1	1.6	128
*Arcadia**	Stream	24.1	7.7	600	7.89	21.4	0.7	179.1	0.3	64
*Dorr**	Stream	16.2	8.7	702	8.06	56.8	3.1	881.7	0.4	70
*Bullet**	Stream	14.9	10.6	557	8.04	14.3	1.7	360.1	2.0	67
P'ville Crk.	Stream	16.7	17.1	615	7.78	375.7	2.2	349.6	0.8	198
*Windmill**	Wetland	23.1	9.5	448	7.71	7.1	2.8	208.2	13.8	167
Sanctuary	Wetland	21.4	4.6	59	6.68	9.9	0.4	3.2	0.6	104
*Loosestrife**	Wetland	25.4	12.6	380	8.04	0.7	0.8	204.6	26.7	268
LTER	Wetland	26.5	7.1	23	5.91	0.5	0.7	2.8	4.5	177
KRFP	Wetland	20.0	10.5	651	7.96	58.69	0.4	540.4	0.7	14
LUX 10	Wetland	23.4	9.8	19	7.28	BDL	1.8	11.9	1.5	138
*LUX 23**	Wetland	26.2	2.6	70	7.26	30.3	0.5	44.2	1.2	146
Duck	Wetland	23.2	6.3	142	7.50	BDL	0.3	15.9	4.4	1
P'ville Fen	Wetland	16.6	2.6	681	6.92	366.7	2.3	345.5	2.5	23
*Wintergreen**	Lake	23.2	13.3	377	8.31	BDL	3.6	105.9	42.2	204
*Lawrence**	Lake	22.9	11.5	529	8.53	17.7	1.0	178.2	2.2	94
*Morrow**	Lake	21.9	8.7	403	8.13	0.2	0.2	358.2	1.0	16

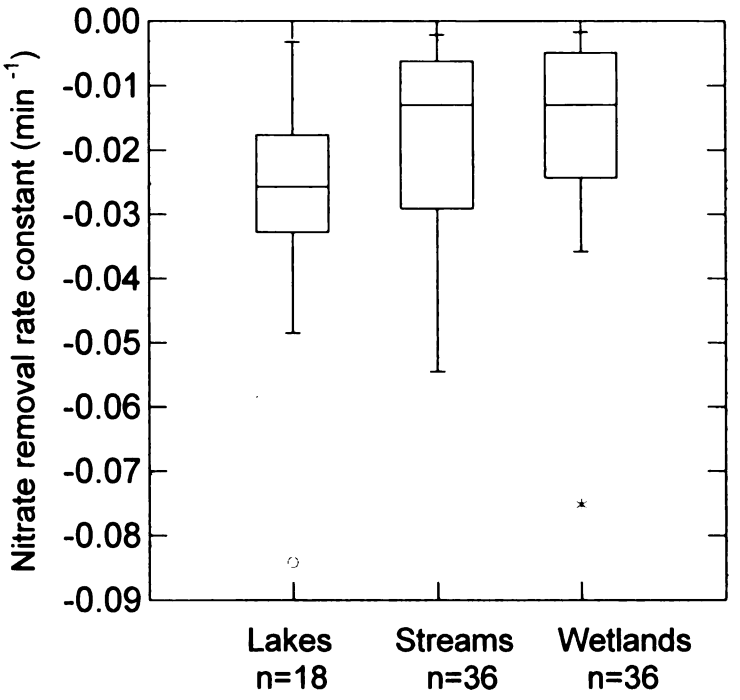


Figure 4: Nitrate removal rate constants by ecosystem. "n" refers to the number of individual experiments (typically 3 per site), not to the number of sites of that particular ecosystem. Boxes encompass the upper and lower quartiles, while the line indicates the median for the dataset. Asterisks are mild outliers and open circles are extreme outliers.

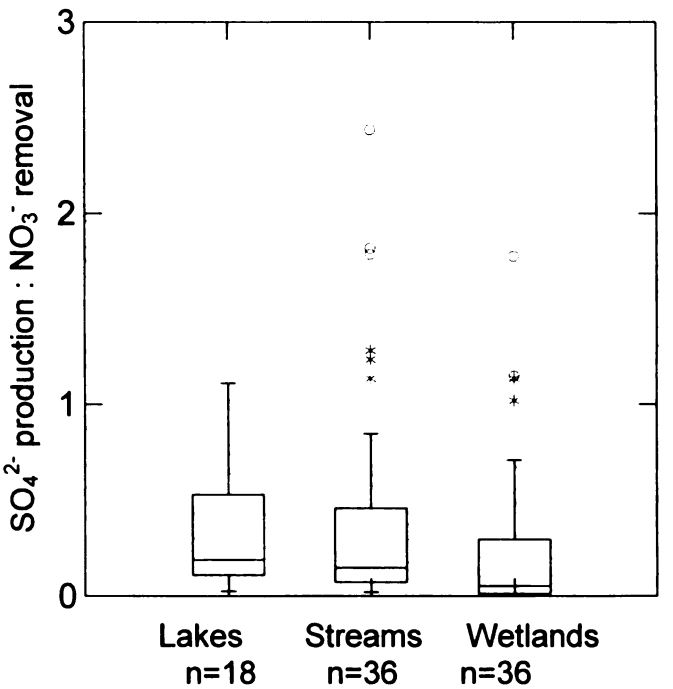


Figure 5: Ratios of total SO_4^{2-} production : total NO_3^- removal (SP:NR) by ecosystem. "n" refers to the number of individual injectors (typically 3 per site). A value of 1 indicates that all of the nitrate removal can be explained by the sulfate production. Values greater than one indicate that sulfate production can account for more nitrate removal than was measured.

The pore waters of each injector were sampled for NH_4^+ and H_2S prior to application of the treatment (NO_3^- + Br^- or the Br^--only "control") and at the end of the experiment. Figure 7 illustrates the pre and post treatment NH_4^+ (top) and H_2S (bottom) concentrations in all experiments. Generally there was more NH_4^+ production and H_2S depletion in NO_3^--amended sediments (solid) than in the control injections (open). This pattern reflects what would be expected if DNRA was coupled with S oxidation, there is no significant difference between the "post" control and treatment injectors for either the NH_4^+ concentrations (ANOVA $F_{1,77}$=1.8; p =0.19) or H_2S concentrations (ANOVA $F_{1,67}$=0.44; p=0.51).

Additional evidence for the importance of DNRA as a NO_3^- removal pathway in aquatic ecosystems comes from the 2005 experiments in which I added $^{15}NO_3^-$ to the sediments. Wetlands had the greatest variation in the percent of NO_3^- removal that could be attributed to DNRA, ranging from 5-110%. Streams and lakes had comparable amounts of NO_3^- removal attributable to DNRA, ranging from 3.5-37% and 0.5-42%, respectively (Figure 8).

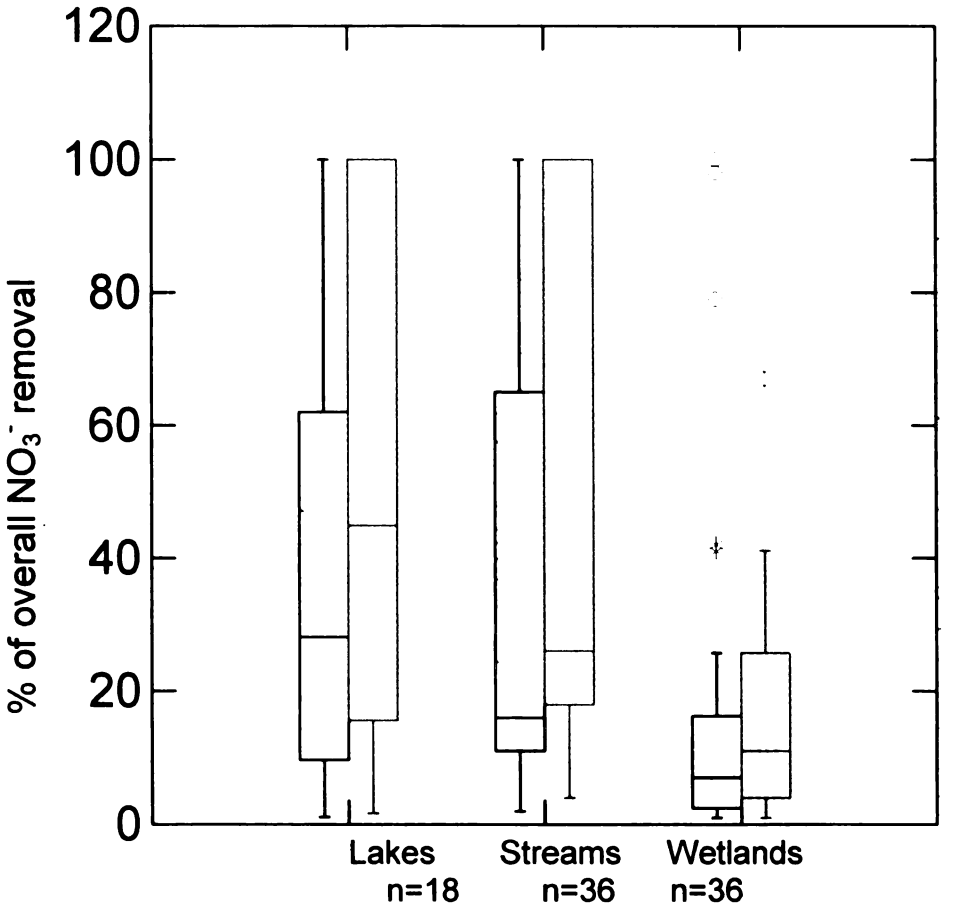


Figure 6: The fraction of measured nitrate removal that can be explained by sulfate production using equations 1-3 (grey boxes) and equation 4-6 (black boxes). "n" refers to the number of individual injectors (typically 3 per site), not to the number of sites of that particular ecosystem.

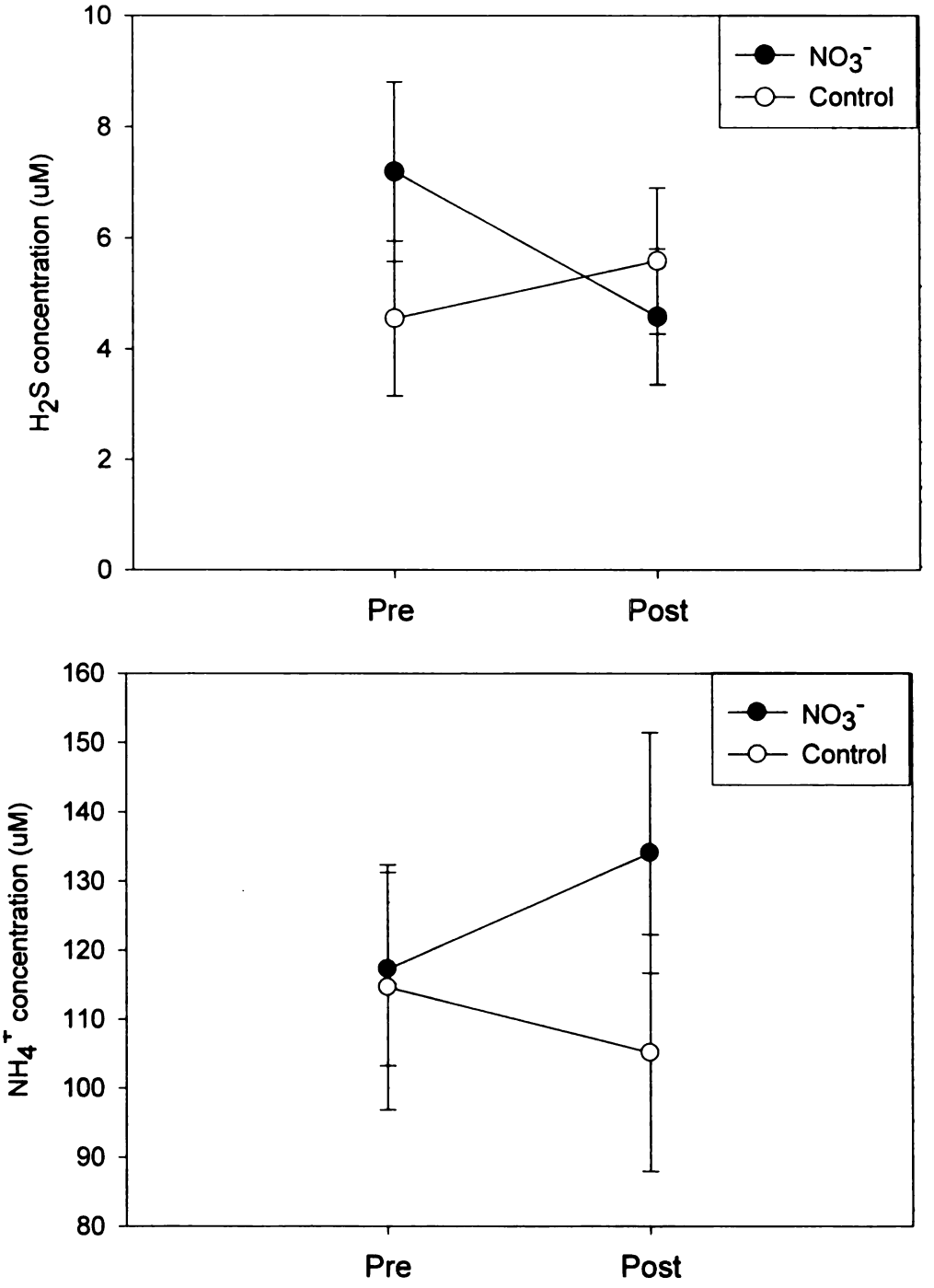


Figure 7: Pre and post treatment NH_4^+ concentrations (bottom) and H_2S concentrations (top) for all injectors at all sites in both 2004 and 2005. Closed symbols denote injectors that received NO_3^- additions and open symbols denote control injectors that only received a Br^- addition. Error bars are standard errors of the mean.

As part of the 2005 experiments, I added both $^{14}NO_3^-$ and $^{15}NO_3^-$ in separate injectors within the same site. In both treatment types I measured the pre and post treatment NH_4^+ concentration. The top panel of Figure 9 shows a positive albeit weak relationship between the μmoles of $^{15}NH_4^+$ produced (measured from the $^{15}NO_3^-$ treatment) and the change in NH_4^+ concentration (post—pre NH_4^+) in the same injectors ($F_{1,16} = 4.2$; $p=0.056$). The bottom panel compares the $^{15}NH_4^+$ produced to the change in NH_4^+ concentration in the $^{14}NO_3^-$ injectors within the same site (i.e., same site, but different injector). There is no relationship between these two variables ($F_{1,16}=0.76$; $p=0.39$).

Surface and porewater chemical characteristics were measured at each site where a push-pull experiment was conducted (Table 1). These data were used in a stepwise multiple regression model to examine what site characteristics best predicted both NO_3^- rate constants and SP:NR across sites. NO_3^- rate constants and SP:NR ratios were square-root transformed to meet the requirement for a normal distribution.

NO_3^- rate constants were best predicted by surface water (SW) NO_3^- and NH_4^+ concentrations and porewater (PW) H_2S concentrations (Table 2). Sites with higher surface water NO_3^- had higher NO_3^- removal rate constants. Additionally, sites with lower surface water NH_4^+ and lower H_2S had higher NO_3^- removal rate constants. These three variables explained 58% of the variation in NO_3^- removal rate constants across sites.

SP:NR ratios, indicative of the relative importance of SO_4^{2-} production in NO_3^- removal, were best predicted by a combination of surface water SO_4^{2-} and

porewater NH_4^+ concentrations (Table 2). Sites with higher SP:NR ratios had higher surface water SO_4^{2-} and porewater NH_4^+ concentrations, whereas sites with greater SP:NR ratios had lower porewater NH_4^+ concentrations. The combination of these two variables explained 44% of the variation in SP:NR ratios across sites.

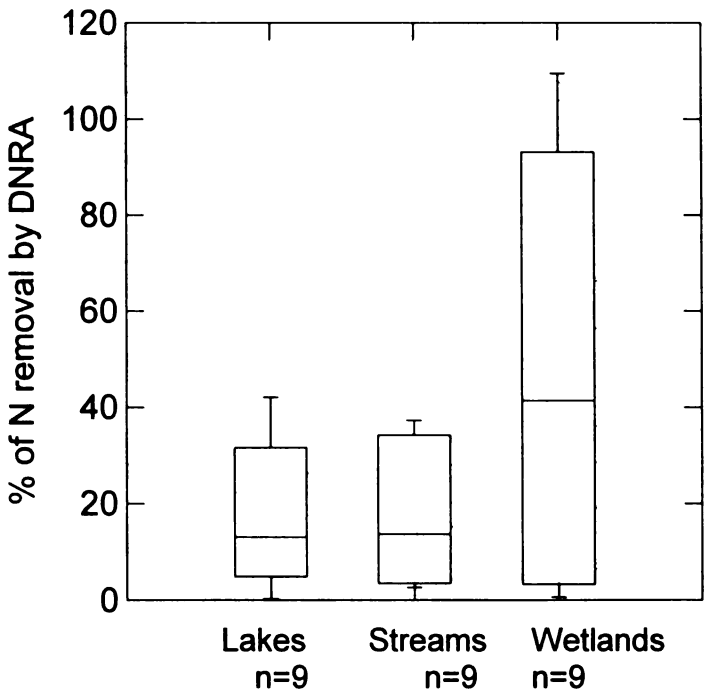


Figure 8: Percentages of NO_3^- removal attributed to DNRA as measured using $^{15}NO_3^-$additions in combination with push-pull techniques. "n" refers to the number of individual injectors (typically 3 per site).

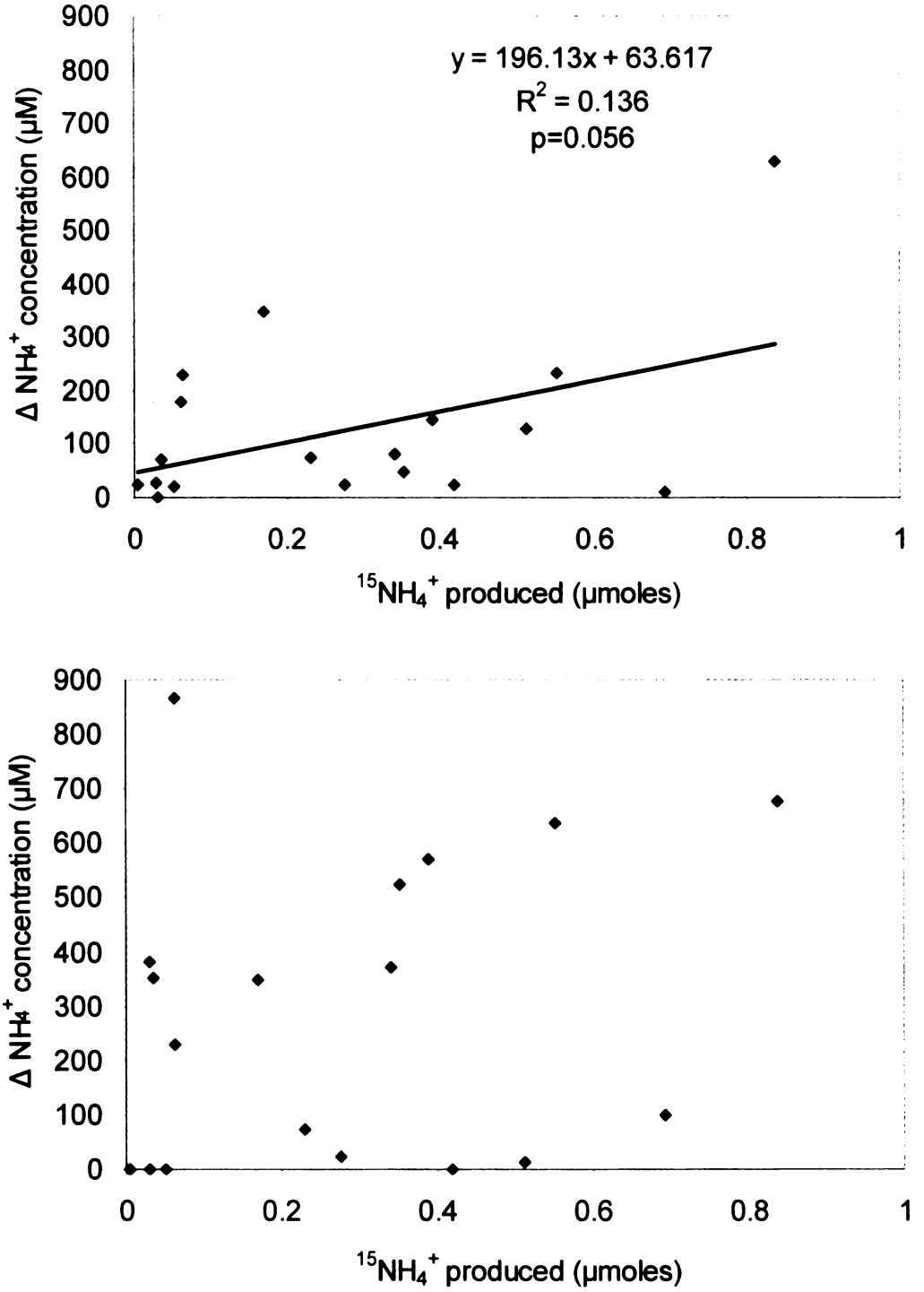


Figure 9: The relationship between measured $^{15}NH_4^+$ production (x-axis) and the change in NH_4^+ concentration in the $^{15}NO_3^-$ injectors (same injectors, top panel) and the $^{14}NO_3^-$ injectors (different injectors within the same site, bottom panel).

Table 2: Stepwise multiple regression model ($\alpha = 0.01$) results to predict the best indicators of NO_3^- removal rate constants and SO_4^{2-} production : NO_3^- removal ratios (SP:NR).

Effect	*Coefficient*	*Std Error*	*t statistic*	*p*
NO_3^- rate constants				**model R^2 = 0.582**
Model constant	0.199	0.018	11.11	0.000
SW NO_3^-	0.013	0.006	2.27	0.039
SW NH_4^+	-0.003	0.001	-3.26	0.006
PW H_2S	-0.021	0.006	-3.44	0.004
SO_4^{2-} production : NO_3^- removal (SP:NR)				**model R^2 = 0.440**
Model constant	0.309	0.119	2.59	0.018
SW SO_4^{2-}	0.006	0.003	2.43	0.025
PW NH_4^+	-0.001	0.000	-2.06	0.055

Discussion:

Nitrate removal across aquatic ecosystems

Nitrate removal is often considered to be a beneficial service provided by aquatic ecosystems (Zedler 2003), particularly in agricultural landscapes such as those in SW Michigan. This study is consistent with that generalization, quantifying how quickly NO_3^- can be removed in sediments of many different types of aquatic ecosystems (Figure 4). Additionally, all ecosystem types exhibited a similar amount of variation in NO_3^- removal rates, indicating that perhaps there is nothing distinctive about NO_3^- removal in sediments of streams, lakes, or wetlands. Nitrate removal may be driven by the transport of NO_3^- (or lack thereof) into the sediment pore waters. This premise, however, would predict that there should be higher rates of NO_3^- removal in streams, due to their greater turbulence and hydrologic connectivity to pore waters. I did not find evidence for higher NO_3^- removal rates in streams as compared to wetland or lake sediments.

Evidence of linkage between NO_3^- removal and SO_4^{2-} production

This study revealed that NO_3^- removal and concurrent SO_4^{2-} production is a biologically mediated process that is found across diverse freshwater ecosystems in southwest Michigan. Evidence to support the assertion that the process is biological can be found in the intermediate temperature optimum seen for both NO_3^- removal and SO_4^{2-} production (Figure 2). If the reaction was not enzymatically mediated, one would expect increasing rates of SO_4^{2-} production with increasing temperatures. Evidence that this is not merely an experimental

artifact of the push-pull experimental method can be found from the NO_3^- additions to a sediment mesocosm (Figure 3). In this experiment, I found that the same NO_3^--induced SO_4^{2-} production occurred, albeit over a longer time-scale.

Further evidence of the common occurrence of nitrate-driven sulfate production can be found both in the large number of sites in which it was observed and in the substantial fraction of NO_3^- removal that could be attributed to SO_4^{2-} production (Figure 5). Sulfate production commonly explained 25-50% of the NO_3^- removal across aquatic ecosystems (based on the interquartile range of SP:NR ratios). Lakes and streams generally had higher amounts of SO_4^{2-} production relative to NO_3^- removal than did wetlands (Figure 5), though there was a great deal of variation within each ecosystem type. Wetland porewaters tended to have higher H_2S concentrations (the hypothesized electron donor for NO_3^- reduction). This relative abundance of electron donors may have caused H_2S to be oxidized only to elemental sulfur (as in equations 1 and 4) rather than all the way to SO_4^{2-} (as in equations 3 and 6), which may have led to the overall decrease in the importance of SO_4^{2-} production relative to NO_3^- removal (SP:NR).

A final line of evidence for the importance of NO_3^- reduction coupled to H_2S oxidation comes from examining the concentrations of NH_4^+ (a potential product) and H_2S (the hypothesized reactant) before and after the NO_3^- additions (Figure 7). Across all of the individual experiments from both years, NH_4^+ concentration increased after adding NO_3^- compared to the control, while the H_2S concentrations simultaneously decreased. While the differences illustrated in Figure 7 are not statistically significant, they are consistent with the general

pattern I would expect to see if N-S coupling was present. This is a further line of evidence that simultaneous NO_3^- and H_2S removal coincide with SO_4^{2-} and NH_4^+ production, all of which indicate the presence and importance of a pathway linking S and N cycling in these freshwater sediments. This study adds to the observations of others (Lucassen et al. 2002, Soares 2002, Forshay 2003, Whitmire 2003, Whitmire and Hamilton 2005) that there may be important but relatively unexplored linkages between the N and S cycles.

What kinds of sites have NO_3^- linked SO_4^{2-} production?

Multiple linear regression models suggested that of the surface and porewater chemistry variables measured, surface water SO_4^{2-} and porewater NH_4^+ were the best predictors of a given site's SP:NR, which in turn indicates the amount of SO_4^{2-} production relative to NO_3^- removal. Specifically, sites with higher SP:NR ratios had a higher surface water SO_4^{2-} concentrations and lower porewater NH_4^+ concentrations (Table 2). The positive relationship between SP:NR and surface water SO_4^{2-} makes intuitive sense since sites with high surface water SO_4^{2-} may be sites with higher rates of S cycling, and in particular higher SO_4^{2-} reduction. H_2S is the product of SO_4^{2-} reduction, so both would be necessary to support populations of S oxidizers. However, the negative relationship between SP:NR and porewater NH_4^+ may be because sites with higher SO_4^{2-} reduction potential and more porewater NH_4^+ are more thoroughly and consistently anoxic, and thus may have less S oxidation potential.

The same approach was used to predict which variables would be good indicators of a site's NO_3^- removal rate. The model showed a positive

relationship between NO_3^- removal rates and surface water NO_3^- concentrations (Table 2), suggesting that sites with higher NO_3^- also have higher removal rates. It also showed a negative relationship between NO_3^- removal rates and surface water NH_4^+ and porewater H_2S concentrations. Higher NO_3^- removal rates at lower porewater H_2S concentrations would make sense if indeed H_2S was the reactant to drive N-S coupled cycling. On the other hand, it may reflect the site's antecedent conditions of low redox state, corresponding with low NO_3^- availability. The relationship between higher NO_3^- removal rates and lower surface water NH_4^+ is less obvious, but may be linked through high rates of nitrification, which would decrease the NH_4^+ pool, particularly in the more oxygenated surface water, while simultaneously producing NO_3^-. The higher NO_3^- production (via nitrification) could effectively prime the NO_3^- reducing communities and be reflected in the higher reduction rates.

NO_3^- removal end-products

In this study, I use two methods to estimate nitrate removal and its end-products: a stoichiometric approach (eq. 1-6) and ^{15}N tracer methods. Stoichiometric methods inherently rely on a mass balance approach, but relate the fluxes of N to another element (Groffman et al. 2006), sulfur in this case. Rates of DNRA can be directly measured using stable isotopes to track the flow of ^{15}N from NO_3^- to NH_4^+.

Sulfate production can account for a variable but significant fraction of overall NO_3^- removal when I apply the stoichiometric model outlined in equations 1-3 (for S coupled denitrification; Figure 6 grey boxes) and 4-6 (for S-coupled

DNRA; Figure 6 black boxes). In general, the sulfate production explained between 25-40% of nitrate removal in lakes, 15-25% of removal in streams, and 10-15% of removal in wetlands (medians, Figure 6). There was, however, a great deal of variation in both streams and lakes, and a smaller degree of variation in the amount of sulfate production that could account for nitrate removal in wetlands.

To elucidate these pathways with greater clarity than our stoichiometric model can provide, I performed push-pull experiments with $^{15}NO_3^-$ in the same stream, lake and wetland sites that were sampled in 2004 (Figure 8). Wetlands had the greatest range of nitrate removal attributable to DNRA, whereas streams and lakes had comparable ranges of DNRA. Tiedje (1988) hypothesized that fermentative DNRA should occur in the most biologically productive sites where sediments were most highly reducing, which could explain why wetlands had higher DNRA than either lakes or streams (though the pattern was not statistically significant), which generally don't have as reduced conditions as wetlands.

The median amount of NO_3^- removal predicted for the reduction to NH_4^+ (equations 4-6; Figure 6 black boxes) agrees reasonably well with the DNRA measured via ^{15}N methods for lakes and streams (about 20-30% in both cases). However, there is a large discrepancy between the amount of nitrate removal to NH_4^+ as predicted by the SO_4^{2-} production (Figure 6, black boxes) and the DNRA measured via ^{15}N methods in wetlands. The ^{15}N approach estimated that roughly half of the NO_3^- was converted to NH_4^+ in wetlands (Figure 8) whereas

the sulfate production only estimated 10% of the overall nitrate removal was to NH_4^+. This suggests that the stoichiometric model explained above (Figures 4-6) and represented in Figure 6 may drastically underestimate the amount of NO_3^- being lost to DNRA, particularly in wetlands. On the other hand, the stoichiometric model may overestimate the amount of NO_3^- being lost to DNRA in some streams and wetlands (see outliers in Figure 6). However, while there is a great deal of variation, it is important to note that the median values of measured DNRA (Figure 8) and the median values of the stoichiometric model (Figure 6, black bars) are very similar, explaining ~20% of the nitrate removal. In this regard the two methods for estimating the importance of N-S coupled cycling (via either measured sulfate production or measured DNRA) agree well.

Measuring DNRA using $^{14}NO_3^-$ vs. $^{15}NO_3^-$ additions

In this study I attempted to measure DNRA by quantifying the increase in porewater NH_4^+ concentrations after NO_3^- injection (Figure 7), and by using stable isotope enrichment experiments (Figure 8). I can then compare the DNRA measured via the stable isotope enrichments to the NH_4^+ increase in the same injector that the $^{15}NO_3^-$ was added to (top panel, Figure 9), and also to the increase of NH_4^+ in injectors that were in the same site, but at a slightly different location (Figure 9, bottom panel). There is a significant positive relationship in the first comparison, suggesting that within the same site, injectors with high DNRA also displayed increases in NH_4^+ concentration. However, injectors that were also placed in a given site, but received $^{14}NO_3^-$, did not have a significant positive relationship with the measured DNRA in that site. The results of both

comparisons (Figures 7 and 9) emphasize how difficult it is to measure DNRA by quantifying changes in NH_4^+ concentration alone. This is in large part due to the high degree of variation in NH_4^+ production both within a given site and between sites of a specific ecosystem.

I also measured $^{15}N_2$ production (indicative of denitrification) as part of the $^{15}NO_3^-$ experiments, which would have allowed me to directly compare it to DNRA. However, we concluded that since I was adding 99% $^{15}NO_3^-$ in the experiments, in many cases large amounts of $^{30}N_2$ may have been produced. The $^{30}N_2$ was produced over $^{29}N_2$ because there is little ambient nitrate ($^{14}NO_3^-$) in the pore water for the $^{15}NO_3^-$ that was added to be mixed. Isotope ratio mass spectrometers (IRMS) are often only tuned to measure 28 and 29 N_2 because the atmospheric amounts of $^{30}N_2$ are very low, and that was the case when samples from these experiments were analyzed. Thus, I was not able to get accurate estimates of denitrification from the $^{15}N_2$ measurements.

Implications for aquatic ecosystem N cycling

The existence and relative importance of denitrification versus DNRA has profound implications for N cycling in aquatic ecosystems (Burgin and Hamilton 2007). Whereas nitrate that is converted to N_2 is permanently removed from biological availability, nitrate that is converted to NH_4^+ becomes more biologically available to many plants and bacteria, and is more likely to be retained within the ecosystem. DNRA is a relatively understudied pathway compared to denitrification, and it is not clear what happens to the resultant NH_4^+. In well-oxygenated ecosystems, such as streams, the NH_4^+ may be re-nitrified (via

nitrification), or it could be stored temporarily as sorbed ions in the sediments or as organic N assimilated into biomass.

Nitrate is the most soluble N form, so removal of NO_3^- by either of these processes is important to water quality; however, permanent removal (to N_2 gas) by denitrification is most desirable. If S-oxidizers are taking up and transforming much of the NO_3^- in surface or ground waters, then NO_3^- removal is closely linked to S cycling, and specifically to SO_4^{2-} inputs. Sulfate is a ubiquitous pollutant in industrialized regions and atmospheric deposition of SO_4^{2-} is greatly enhanced over pre-industrial times (Schlesinger 1997). If excess SO_4^{2-} in freshwaters actually enhances NO_3^- removal, by stimulating H_2S formation through SO_4^{2-} reduction, then the controls on N processing in landscapes subject to S and N pollution become more complex than previously thought.

References:

Addy, K., D. Q. Kellogg, A. J. Gold, P. M. Groffman, G. Ferendo, and C. Sawyer. 2002. In situ push-pull method to determine ground water denitrification in riparian zones. Journal of Environmental Quality **31**:1017-1024.

Aminot, A., D. S. Kirkwood, and R. Kerouel. 1997. Determination of ammonia in seawater by the indophenol-blue method: Evaluation of the ICES NUTS I/C 5 questionnaire. Marine Chemistry **56**:59-75.

Bernot, M. J., and W. K. Dodds. 2005. Nitrogen retention, removal, and saturation in lotic ecosystems. Ecosystems **8**:442-453.

Burgin, A. J., and S. K. Hamilton. 2007. Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Frontiers in Ecology and the Environment **5**:89-96.

Carpenter, S. R., N. F. Caraco, D. L. Correll, R. W. Howarth, A. N. Sharpley, and V. H. Smith. 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecological Applications **8**:559-568.

Forshay, K. J. 2003. Nitrogen dynamics in floodplain water bodies following inundation on the Wisconsin River floodplain. M.S. Thesis. University of Wisconsin, Madison, WI.

Fossing, H., V. A. Gallardo, B. B. Jorgensen, M. Huttel, L. P. Nielsen, H. Schulz, D. E. Canfield, S. Forster, R. N. Glud, J. K. Gundersen, J. Kuver, N. B. Ramsing, A. Teske, B. Thamdrup, and O. Ulloa. 1995. Concentration and transport of nitrate by the mat-forming sulfur bacterium *Thioploca*. Nature **374**:713-715.

Gallardo, V. A. 1977. Large benthic microbial communities in sulfide biota under Peru Chile subsurface countercurrent. Nature **268**:331-332.

Golterman, H.L. and R.S. Clymo. 1969. Methods for chemical analysis of freshwaters. Blackwell, Oxford.

Groffman, P. M., M. A. Altabet, J. K. Bohlke, K. Butterbach-Bahl, M. B. David, M. K. Firestone, A. E. Giblin, T. M. Kana, L. P. Nielsen, and M. A. Voytek. 2006. Methods for measuring denitrification: Diverse approaches to a difficult problem. Ecological Applications **16**:2091-2122.

Haaijer, S. C. M., M. E. W. Van der Welle, M. C. Schmid, L. P. M. Lamers, M. S. M. Jetten, and H. J. M. Op den Camp. 2006. Evidence for the involvement

of betaproteobacterial *Thiobacilli* in the nitrate-dependent oxidation of iron sulfide minerals. FEMS Microbiology Ecology **58**:439-448.

Holmes, R. M., J. W. McClelland, D. M. Sigman, B. Fry, and B. J. Peterson. 1998. Measuring N-$^{15}NH_4^+$ in marine, estuarine and fresh waters: An adaptation of the ammonia diffusion method for samples with low ammonium concentrations. Marine Chemistry **60**:235-243.

Istok, J. D., M. D. Humphrey, M. H. Schroth, M. R. Hyman, and K. T. OReilly. 1997. Single-well, "push-pull" test for in situ determination of microbial activities. Ground Water **35**:619-631.

Jorgensen, B. B., and V. A. Gallardo. 1999. Thioploca spp: filamentous sulfur bacteria with nitrate vacuoles. FEMS Microbiology Ecology **28**:301-313.

Kamp, A., P. Stief, and H. N. Schulz-Vogt. 2006. Anaerobic sulfide oxidation with nitrate by a freshwater *Beggiatoa* enrichment culture. Applied and Environmental Microbiology **72**:4755-4760.

Kelly, D. P. 1999. Thermodynamic aspects of energy conservation by chemolithotrophic sulfur bacteria in relation to the sulfur oxidation pathways. Archives of Microbiology **171**:219-229.

Lovley, D. R., and M. J. Klug. 1983. Sulfate reducers can out-compete methanogens at freshwater sulfate concentrations. Applied and Environmental Microbiology **45**:187-192.

Lucassen, E. C. H. E. T., A. J. P. Smolders, and J. G. M. Roelofs. 2002. Potential sensitivity of mires to drought, acidification and mobilisation of heavy metals: the sediment S/(Ca+Mg) ratio as diagnostic tool. Environmental Pollution **120**:635-646.

Luthy, L., M. Fritz, and R. Bachofen. 2000. In situ determination of sulfide turnover rates in a meromictic alpine lake. Applied and Environmental Microbiology **66**:712-717.

McGuire, J. T., D. T. Long, M. J. Klug, S. K. Haack, and D. W. Hyndman. 2002. Evaluating behavior of oxygen, nitrate, and sulfate during recharge and quantifying reduction rates in a contaminated aquifer. Environmental Science & Technology **36**:2693-2700.

McHatton, S. C., J. P. Barry, H. W. Jannasch, and D. C. Nelson. 1996. High nitrate concentrations in vacuolate, autotrophic marine *Beggiatoa spp.* Applied and Environmental Microbiology **62**:954-958.

Sayama, M., N. Risgaard-Petersen, L. P. Nielsen, H. Fossing, and P. B. Christensen. 2005. Impact of bacterial NO_3^- transport on sediment biogeochemistry. Applied and Environmental Microbiology **71**:7575-7577.

Schlesinger, W. H. 1997. Biogeochemistry: an analysis of global change, 2nd edition. Academic Press, San Diego.

Schulz, H. N., T. Brinkhoff, T. G. Ferdelman, M. H. Marine, A. Teske, and B. B. Jorgensen. 1999. Dense populations of a giant sulfur bacterium in Namibian shelf sediments. Science **284**:493-495.

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

Soares, M. I. M. 2002. Denitrification of groundwater with elemental sulfur. Water Research **36**:1392-1395.

Sweerts, J. P. R. A., D. Debeer, L. P. Nielsen, H. Verdouw, J. C. Vandenheuvel, Y. Cohen, and T. E. Cappenberg. 1990. Denitrification by sulfur oxidizing *Beggiatoa Spp* mats on freshwater sediments. Nature **344**:762-763.

Tiedje, J. M. 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. Pages 179-244 *in* A. J. B. Zehnder, editor. Biology of Anaerobic Microorganisms. Wiley & Sons, New York.

Vitousek, P. M., J. D. Aber, R. W. Howarth, G. E. Likens, P. A. Matson, D. W. Schindler, W. H. Schlesinger, and D. G. Tilman. 1997. Human alteration of the global nitrogen cycle: Sources and consequences. Ecological Applications **7**:737-750.

Wetzel, R. G. 2001. Limnology: Lake and River Ecosystems, 3 edition. Academic Press, San Diego.

Whitmire, S. L. 2003. Anaerobic biogeochemical functions of Michigan wetlands and the influence of water source. Ph.D. Dissertation. Michigan State University, East Lansing, MI.

Whitmire, S. L., and S. K. Hamilton. 2005. Rapid removal of nitrate and sulfate in freshwater wetland sediments. Journal of Environmental Quality **34**:2062-2071.

Zedler, J. B. 2003. Wetlands at your service: reducing impacts of agriculture at the watershed scale. Frontiers in Ecology and the Environment **1**:65-72.

CHAPTER 3

THE RELATIVE IMPORTANCE OF DENITRIFICATION, DISSIMILATORY NITRATE REDUCTION TO AMMONIUM (DNRA), AND ANAEROBIC AMMONIUM OXIDATION (ANAMMOX) TO NITRATE REMOVAL IN FRESHWATER SEDIMENTS

Introduction:

Excessive nitrogen (N) concentrations, often in the form of nitrate (NO_3^-), present a water-quality problem of growing concern. Increasing problems with eutrophication of coastal marine waters are linked to the export of N from terrestrial landscapes. Surface and groundwater flow within the landscape often passes through wetlands and headwater streams, where much of the NO_3^- present in the water can be removed (Peterson et al. 2001, Zedler 2003). Thus, key interfaces along landscape flow paths control N export to downstream surface waters, such as large rivers and lakes, and ultimately to estuaries. The removal of NO_3^- by wetlands and streams is of particular interest in agricultural landscapes where N export by rivers has been implicated in creating hypoxic zones (such as in the Gulf of Mexico) and harmful algal blooms (Rabalais et al. 2001, Paerl et al. 2002).

As NO_3^- rich water moves through a landscape, many different processes can remove the nitrate, reducing the amount of loading to downstream ecosystems. These pathways include respiratory denitrification, dissimilatory nitrate reduction to ammonium (DNRA), and anaerobic ammonium oxidation (anammox; indirectly through the reduction of NO_3^- to NO_2^-). Respiratory

denitrification has long been thought to be the predominant microbial pathway for nitrate removal in freshwater sediments. Respiratory denitrification is a microbially mediated transformation that reduces nitrate coupled to the oxidation of organic carbon. The NO_3^- is sequentially reduced to nitrite (NO_2^-), nitrous oxide (N_2O) and di-nitrogen gas (N_2). Many factors influence rates of respiratory denitrification in surface waters, including oxygen, NO_3^- and carbon availability, light, and the presence of plants (Knowles 1982, Golterman 2004).

There have been many studies of denitrification in diverse ecosystems, and attempts have been made to uncover broad patterns across ecosystem types through meta-analyses (Pina-Ochoa and Alvarez-Cobelas 2006) and compilations and analyses of published rate measurements (Seitzinger 1988, Cornwell et al. 1999). Denitrification rates have been assessed in soils, wetlands, and surface waters, but estimates vary greatly within and among environments, as well as between different measurement techniques. Wetlands can be particularly efficient NO_3^- sinks and support high rates of denitrification (Peterjohn and Correll 1984, Hedin et al. 1998, Tobias et al. 2001, Whitmire and Hamilton 2005). Headwater streams are also efficient N processing sites (Peterson et al. 2001, Mulholland et al. *in prep*). However, in a large study of 72 streams across the US, denitrification accounted for a relatively small proportion (median of 16%) of the overall nitrate removal (Mulholland et al. *in prep*). Seitzinger (1988) and Cornwell et al. (1999) concluded that denitrification removes highly variable but significant fractions of the total N loading to lakes, rivers, and coastal estuaries. While denitrification can often account for a

significant fraction of the overall nitrate removal, it rarely accounts for the entire amount. The balance of this removal is often ascribed to biological assimilation (Burgin and Hamilton 2007).

An alternative pathway of nitrate removal that has received relatively little study is dissimilatory nitrate reduction to ammonium (DNRA). This pathway involves the transformation of NO_3^- to ammonium (NH_4^+) either by fermentative bacteria utilizing carbon substrates (Tiedje 1988) or by chemolithoautotrophic bacteria that can utilize NO_3^- to oxidize reduced sulfur compounds, such as sulfide (H_2S), producing sulfate (Fossing et al. 1995, Brunet and Garcia-gil 1996). Tiedje (1988) suggested that fermentative DNRA would be most important in highly reducing environments that maintain anoxic conditions for long time periods. Additionally, DNRA is thought to be favored in NO_3^--limited, labile-carbon rich environments while respiratory denitrification would be favored under carbon-limited conditions (Tiedje, 1988). Sulfur-driven DNRA, on the other hand, may be controlled by the availability of reduced sulfur compounds, including H_2S and S^0, to use as electron donors. Furthermore, H_2S may be a key driver in determining the relative importance of denitrification and DNRA by denaturing the final denitrification reductases, thus shunting the pathway over to DNRA (Brunet and Garcia-gil 1996).

DNRA has mostly been studied in wetlands and marine-influenced ecosystems (Bowden 1986, Tobias et al. 2001, Gardner et al. 2006), and in freshwater Lake Vilar (Brunet and Garcia-gil 1996). In Ringfield Marsh, a brackish coastal marsh, DNRA accounted for 7-70% of the overall NO_3^-

reduction; the relative importance of DNRA and denitrification varied between seasons depending on groundwater inputs (Tobias et al. 2001). Bonin (1996) found that DNRA accounted for 80% of the total NO_3^- consumption in sediments of the Mediterranean coast of France, even though bacterial biomass estimates suggested that ammonium-producers were 100-fold less abundant than respiratory denitrifiers. An and Gardner (2002) demonstrated the relative importance of DNRA in many Texas estuaries; in a few of the systems, DNRA was more important than denitrification to overall NO_3^- removal. In Lake Vilar (Spain), Brunet and Garcia-Gil (1996) found that adding NO_3^- to the H_2S-rich, anoxic hypolimnion resulted in H_2S removal and NH_4^+ production. In a study of a freshwater tidal marsh in MA, Bowden (1986) found that DNRA accounted for less than 10% of the overall NO_3^- reduction. DNRA has apparently not yet been investigated in freshwater streams.

Anaerobic oxidation of ammonium (anammox) is the process by which NH_4^+ (electron donor) is oxidized by NO_2^- (electron acceptor) under anaerobic conditions, producing N_2. Anammox was discovered in sludge reactors, and has since been shown to occur in anoxic wastewater, temperate shelf sediments, sea ice, and more recently in cold Arctic shelf sediments (Jetten et al. 1998, Megonigal et al. 2004, Rysgaard and Glud 2004, Rysgaard et al. 2004), where it has been estimated to account for a wide range (1-65%) of total N_2 production (Dalsgaard et al. 2005). In marine sediments the source of the NO_2^- for anammox is hypothesized to be incomplete denitrification of NO_3^-. It is apparent that anammox occurs in areas where there are suitable concentrations of both

NO_3^- and NH_4^+, but no O_2, and is inhibited by many simple organic compounds including pyruvate, ethanol, and glucose (Jetten et al. 1998).

There are very few published studies of anammox in freshwater ecosystems. The exception to this is Schubert et al.'s (2006) study of anammox activity in Lake Tanganyika, the second largest freshwater lake in the world. Schubert's study found that up to 13% of the N_2 produced could be attributed to anammox activity. Because Lake Tanganyika is a unique freshwater system, generalizations about the role of anammox in freshwater N cycling remains speculative (Megonigal et al. 2004). However, anammox would be expected to occur where sufficient NO_3^- and NH_4^+ co-occur, which could include certain interfaces between surface and ground waters.

In this study, I examined the relative importance of denitrification, DNRA and anammox to overall nitrate removal rates in sediments taken from two streams, lakes and wetlands in southwestern Michigan. While denitrification has been extensively studied in these types of systems, very few studies have addressed whether DNRA and anammox are important to overall nitrate removal in freshwater ecosystems. Additionally, no published studies quantify the relative contribution of all three processes simultaneously. I also examined whether the relative contribution of these processes to overall nitrate removal can be predicted by ecosystem properties (e.g., ecosystem types).

Sites:

Two wetlands, lakes and streams were chosen as sites for this experiment. Sites were selected to represent the biogeochemical diversity of the local landscape, based largely on previous work in the Hamilton Lab. The two wetland sites were Loosestrife Fen (also called Loosestrife Pond, LP) and Turkey Marsh (TM). Loosestrife Fen is a small (0.4 ha) fen created from sediment infilling behind a small earthen dam located in the W.K. Kellogg Experimental Forest. It is dominated by *Chara* sp. and has a few centimeters of surface water year-round due in part to the continuous groundwater inputs from a spring that drives surface flow across the wetland. Turkey Marsh is a 3.1-ha isolated, depressional wetland located near the Kellogg Biological Station. The wetland is both precipitation- and groundwater-fed, and its surface water levels fluctuate more than those in Loosestrife Fen.

The two lake sites were Wintergreen Lake (WGL) and Lawrence Lake (LAW). Wintergreen Lake is approximately 15 ha in area with a maximum depth of 6.3 m and a mean depth of 3.5 m. The lake is hyper-eutrophic due in part to its location within the Kellogg Bird Sanctuary. Lawrence Lake is an oligotrophic, hard-water lake 5 ha in area with a maximum depth of 12.6 m. For further background see (Wetzel 2001).

The two stream sites were Arcadia Creek (ARC) and Bellingham Drain (BEL); both streams were also part of the Michigan Lotic Intersite Nitrogen eXperiment (LINX2) whole-stream $^{15}NO_3^-$ additions in 2005. Arcadia Creek is a small stream with a largely urban catchment located in the city of Kalamazoo, MI.

Groundwater maintains its baseflow, but many urban drains also empty into it making it a typically "flashy" (e.g., highly variable discharge) urban stream. In contrast, Bellingham Drain was excavated for agricultural land drainage and its catchment is largely covered by row-crop production (corn and soy). It is a tributary of the Gun River, which in turn empties into the Kalamazoo River.

Methods:

A flow-through core incubation method was used to determine the relative importance of DNRA, denitrification and Anammox to total nitrate removal in sediments (An and Gardner 2002). Eight 7.6-cm I.D. by 30-cm long undisturbed sediment cores were collected from each of the six locations at each site, along with approximately 100-L of surface water. Cores were collected by hand where the water was shallow, or in the lakes from a boat using a coring device fitted with a one-way rubber valve. The wetland cores were collected on 16 July and the experiments were run 17-19 July 2006. The lake cores were collected on 13 August and the experiments were run from 14-16 August 2006. The stream cores were collected on 29 Sept and the experiments run from 30 Sept -2 Oct 2006.

Once the cores were collected and returned to the lab some of the overlying water was drained off, leaving approximately 2.5-3 cm of the overlying water on the core. The core was fitted with a tight-fitting, O-ring plug that contained Teflon inflow and outflow lines embedded into the plug. The inflow lines were connected to a peristaltic pump that transferred the treatment water from 20-L reservoirs to the cores at a rate of 1.2 ml min^{-1}. The treatments were

site surface water 1) without any ^{15}N (control – 2 cores per site); 2) with added $^{15}NO_3^-$ to examine the relative importance of DNRA and denitrification (increased concentration by ~0.66 mgL^{-1}; 3 cores per site); and 3) with added $^{15}NH_4^+$ to test for the presence of anammox (increased the concentration by ~0.33 mg L^{-1}; 3 cores per site). If sites had little ambient NO_3^- in the surface water (TM, LP, WGL; Table 1), enough NO_3^- was added to the NH_4^+ treatment to increase the NO_3^- concentration to 0.2 mg N L^{-1} to provide adequate concentrations for anammox to occur. The treatment reservoirs (inputs) were aerated to maintain oxic conditions. The cores were kept in the dark at ambient (lab) temperature. The water from the outflow lines was collected in 1-L bottles that were repeatedly filled and emptied throughout the experiment.

The treatments were begun on the same day as the cores were collected. After one day of incubation to allow the cores to equilibrate, duplicate gas and water samples were collected for three successive days. Gas samples were collected directly from the outflow lines into long, narrow tubes fitted with glass stoppers. These samples were then shipped overnight to the Gardner lab (University of Texas Marine Science Institute, Port Aransas, TX) to be analyzed by membrane inlet mass spectrometry (MIMS) for dissolved Ar, O_2, $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$. Samples for nutrients were taken at approximately the same time as the gas samples, filtered with a syringe filter (0.45 µm), and analyzed for NH_4^+ concentration using the phenylhypochlorite method (Aminot et al. 1997) and for anions and cations (including NO_3^- and SO_4^{2-}) on a Dionex membrane-suppression Ion Chromatograph. Samples were collected for $^{15}NH_4^+$ and were

analyzed by a modified diffusion method (Holmes et al. 1998). In addition to the later method, water samples were collected and filtered (0.22 μm) for $^{15}NH_4^+$ analysis by an HPLC technique (Gardner et al. 1991, Gardner et al. 1996).

All flux measurements were corrected for any background fluxes (e.g., $^{14}NO_3^-$ removal in sites with high ambient NO_3^- concentrations) and converted to tracer ^{15}N based on the ^{15}N atom ratio [AR; $^{15}N/(^{15}N+^{14}N)$]. Fluxes are generally reported as activity of the ^{15}N component of the NO_3^- pool, with the exception being the overall NH_4^+ fluxes. Nitrate removal rates were calculated as the difference between $^{15}NO_3^-$ concentrations in the outflow and the inflow on a surface area basis. I assumed that the ^{15}N AR in the outflow was the same as the AR in the inflow water, and calculated the $^{15}NO_3^-$ removal rate as the difference in the ^{15}N component of the NO_3^- pool between the inflow and outflow. Denitrification rates were calculated as the sum of ^{15}N in the forms of $^{29}N_2$ and $^{30}N_2$ that were produced in the presence of $^{15}NO_3^-$. DNRA rates were calculated as the $^{15}NH_4^+$ produced in the presence of $^{15}NO_3^-$. Anammox rates were calculated as the ^{15}N in $^{29}N_2$ produced in the presence of $^{15}NH_4^+$ and $^{14}NO_3^-$.

Statistical Analysis

Samples were analyzed for changes over time (3 day incubation time) using a standard analysis of variance (ANOVA) with time as a covariate. For most response variables there was no significant effect of time, so the measurements from all three days were pooled into a site average. Further comparisons were made with ANOVAs (SAS PROC GLM) using Tukey's

comparisons to perform *a posteriori* comparisons of individual sites or ecosystem types (e.g., streams, wetlands, lakes).

Results:

Physical and chemical characteristics

Experiments were conducted in the mid to late summer months, and thus surface water temperatures were generally high, ranging from 18.6-25.4 °C (Table 1). All sites also had high dissolved oxygen (DO) in overlying water at the time of core collection. Nitrate concentrations were highest in the two stream sites. Nitrate was measurable in both of the lakes, but was below detection limits (~0.01 mg N L^{-1}) in the two wetland sites. Ammonium concentrations were generally ≤ 50 µg N L^{-1} with the exception of Loosestrife Fen (wetland), where the NH_4^+ concentration was more than triple of that at most sites (Table 1). Surface water sulfate concentrations were lowest in Turkey Marsh (wetland) at 1.9 mg L^{-1} and highest in Bellingham Drain (stream) at 123.9 mg L^{-1}; most however were between 10-20 mg L^{-1}.

Sediment Oxygen Demand

The sediment oxygen demand (SOD) indicates the rate at which the sediments are removing O_2 from the water flowing over the sediment cores. It reflects all of the O_2 consuming processes that occur in the sediment-surface water interface, including decomposition, chemical oxidation (e.g. Fe or H_2S),

and microbial processes such as nitrification. All sites consumed O_2 (Figure 1). There were no statistically significant changes in SOD rates over time, so all measurements from the three days were combined for statistical analysis (n=6 per site). Arcadia Creek and Loosestrife Fen were two sites with especially high SOD (Figure 1). Although there were significant differences in SOD between particular sites, there were no differences in SOD between ecosystem types, i.e., streams vs. lakes vs. wetlands ($df=2, 51$; $F = 0.15$; $p=0.86$). Adding NO_3^- to the overlying water did not significantly change the SOD relative to controls ($df=1, 88$; $F = 1.37$; $p=0.2446$).

Nitrogen fluxes

Measured nitrogen flux and transformation rates are illustrated in Figures 2-6 and summarized in Figure 7 and Table 2. Fluxes are in μmoles m^{-2} h^{-1} from 2-3 replicate cores per treatment. Positive values indicate net increase, or production, whereas negative values indicate net removal or loss. All fluxes are background corrected to account for any activity in the control cores, as well as corrected for the ^{15}N atom ratio (AR) so that only the flux of the added ^{15}N was measured and compared to other fluxes.

Site	Temp. °C	SW DO ($mg\ L^{-1}$)	SW NH_4^+ ($\mu g\ N\ L^{-1}$)	SW NO_3^- ($mg\ N\ L^{-1}$)	SW SO_4^{2-} ($mg\ L^{-1}$)
Arcadia Creek (S)	20.3	6.6	12.9	0.29	18.7
Bellingham Drain (S)	18.6	9.2	19.5	1.65	123.6
Loosestrife Fen (W)	25.4	12.5	165.8	BDL	10.5
Turkey Marsh (W)	23.0	7.6	50.1	BDL	1.9
Lawrence Lake (L)	22.9	11.5	26.1	0.20	18.1
Wintergreen Lake (L)	23.2	13.3	49.2	0.01	10.4

Table 1: Physical and chemical characteristics of the sites at the time the cores were collected. Abbreviations: BDL = below detection limits, SW = surface water.

Nitrate removal rates varied considerably among sites, ranging from 114-961 μmoles NO_3^- m^{-2} hr^{-1} (Figure 2). There were no significant changes in rates over time, so all measurements were combined for analysis (n=9 per site). Streams had higher rates of NO_3^- removal than either wetlands (Tukey's p=0.023) or lakes (Tukey's p=0.010), though this pattern is driven in large part by the high NO_3^- removal rates in Arcadia Creek, which were greater than in any other site (Figure 2). One wetland (Loosestrife Fen) also had relatively high removal rates. Generally sites with high SOD (Figure 1) also had high NO_3^- removal rates.

Denitrification rates were also variable among sites ranging from 49-361 μmoles $^{15}N_2$ generated m^{-2} hr^{-1} (Figure 3). There were no statistically significant changes in denitrification rates over time, so all measurements from the three days were combined for statistical analysis (n=9 per site). Streams had much higher rates of denitrification compared to wetland (Tukey's p<0.0001) or lake sites (Tukey's p<0.0001). Wetlands and lakes had relatively similar rates of denitrification ranging from ~50-100 μmoles ^{15}N-N_2 m^{-2} hr^{-1} and were not statistically different from each other (Tukey's p=0.6763). These general patterns were the same when individual sites were compared (Figure 3).

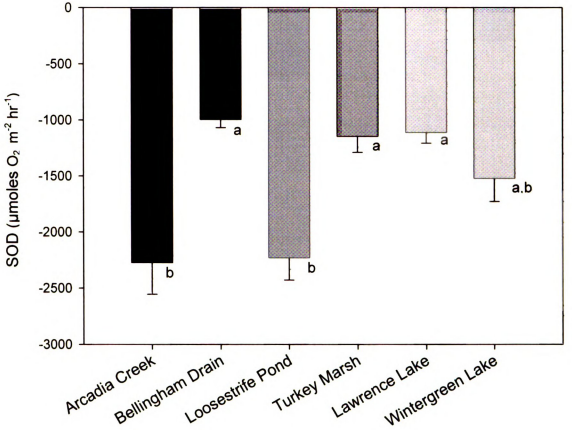


Figure 1: Average sediment oxygen demand (SOD) in the control cores from each site over the three day incubation time (n=6). Error bars represent 1 S.E. of the mean. The ecosystem types are colored as: streams (black), wetlands (dark grey), and lakes (light grey). Sites with the same letter are not statistically different from each other (p=0.05).

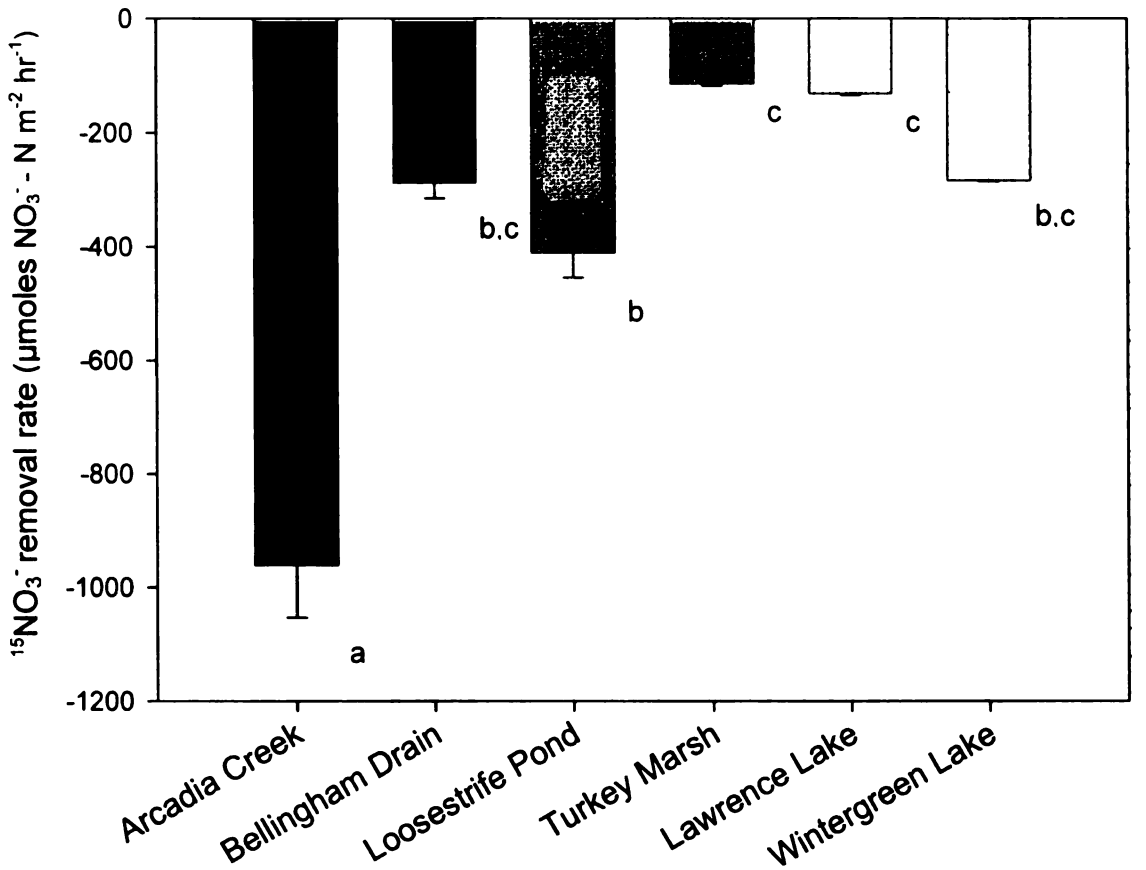


Figure 2: $^{15}NO_3^-$ flux rates from each site over the three day incubation time (n=9). Error bars represent 1 S.E. of the mean. The ecosystem types are colored as: streams (black), wetlands (dark grey), and lakes (light grey). Sites with the same letter are not statistically different from each other (p=0.05).

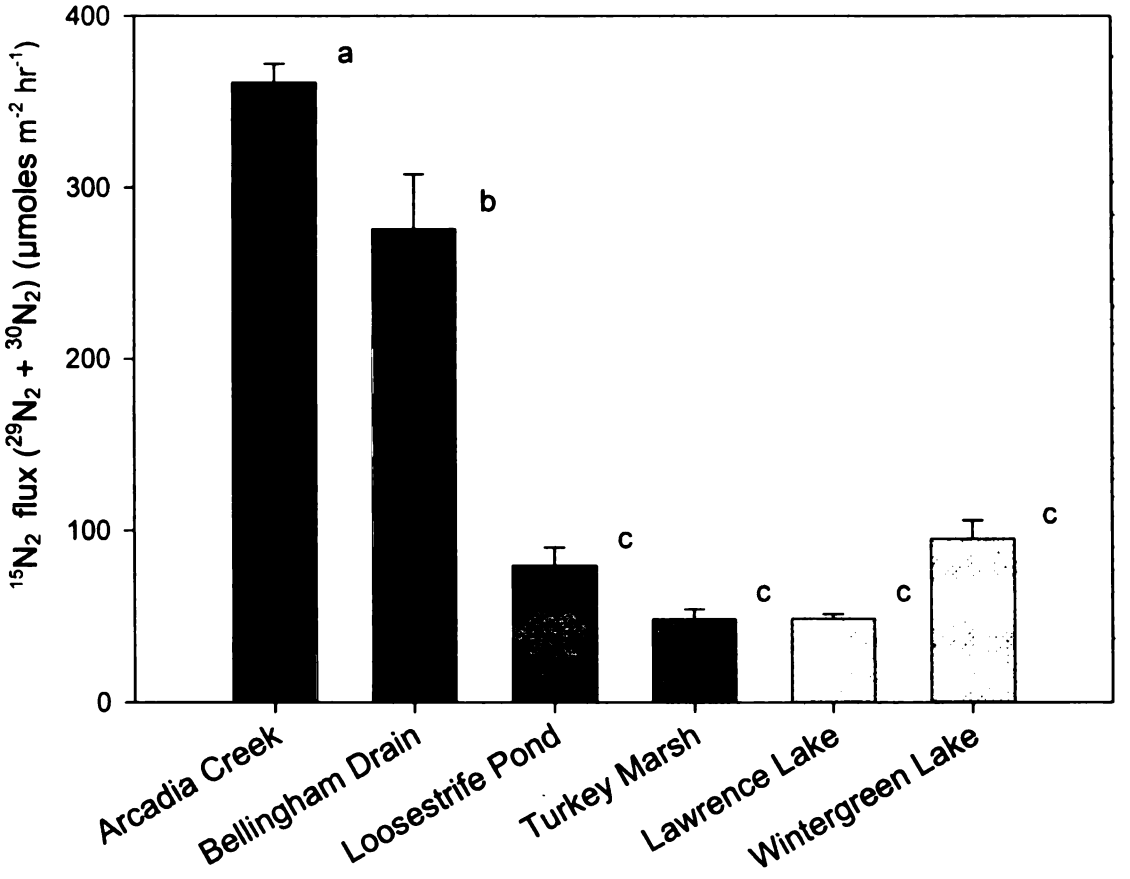


Figure 3: Denitrification rates measured as average ^{15}N-N_2 flux ($^{29}N_2$+ $^{30}N_2$) from the $^{15}NO_3^-$ amended cores from each site over the three day incubation time (n=9). Error bars represent 1 S.E. of the mean. The ecosystem types are colored as: streams (black), wetlands (dark grey), and lakes (light grey). Sites with the same letter are not statistically different from each other (p=0.05).

Anammox rates were calculated as the amount of $^{29}N_2$ produced in the treatment containing $^{15}NH_4^+$ and $^{14}NO_3^-$. Rates were typically low, ranging from 1-27 μmoles $^{29}N_2$ m^{-2} hr^{-1} (Figure 4). There were no statistically significant changes in anammox rates over time, so all measurements from the three days were combined for statistical analysis (n=9 per site). Streams had higher anammox rates than either wetlands (Tukey's p=0.0032) or lakes (Tukey's p=0.0011), though this is in part driven by the very large anammox rates at Bellingham Drain (Figure 4). Lake sediments had small amounts of anammox (~2.5 μmoles $^{29}N_2$ m^{-2} hr^{-1}), and wetlands had the lowest rates of anammox activity though the two groups were not statistically different (Tukey's p=0.75).

Ammonium production was measurable in all six sites in both the control (solid bars, Figure 5) and $^{15}NO_3^-$ treatment cores (hatched bars, Figure 5). However, the flux from the $^{15}NO_3^-$-amended cores was sometimes not different than the flux occurring in the same site's control cores (e.g., Bellingham Drain, Turkey Marsh and Lawrence Lake; p>0.05). Ammonium production was stimulated by adding NO_3^- to the overlying water compared to the control in Arcadia Creek (df = 1, 13; F = 8.52; p=0.012), Loosestrife Fen (df=1,13; F=8.35; p=0.013), and Wintergreen Lake (df=1,13; F=7.22; p=0.019) (Figure 5).

Generally, there were no statistically significant changes in NH_4^+ flux rates over time, so all measurements from the three days were combined for statistical analysis (n=9 per site for treatment, and n=6 for controls). The exception to this is Arcadia Creek, which produced a pulse of high (mostly ^{14}N) NH_4^+ in all three $^{15}NO_3^-$ treatment cores on the first day, but then the NH_4^+ decreased considerably

on days 2 and 3. Though there was a significant effect of time at this site, the data from all three days were combined for graphical and statistical analysis to maintain consistency between sites. This NH_4^+ pulse is the driver of the high variation in NH_4^+ flux at Arcadia Creek (Figure 5).

Rates of DNRA, measured as the $^{15}NH_4^+$ produced in the presence of $^{15}NO_3^-$ in the overlying water, was measurable at all six sites, ranging from 7-72 μmoles $^{15}NH_4^+$ produced m^{-2} hr^{-1} (Figure 6). Though there were no differences in DNRA rates among ecosystem types, there was considerable variation among different sites (Figure 6). The sites with high DNRA (Arcadia, Loosestrife and Wintergreen) were also sites that had a measurable increase in NH_4^+ production (Figure 5) and the sites with the highest SOD (Figure 1).

The relative importance of these three nitrate removal pathways to overall $^{15}NO_3^-$ removal is illustrated by site in Figure 7 and broken down as percentages of the overall $^{15}NO_3^-$ removal in Figure 8 and Table 2. Denitrification is the dominant removal process in most sites; however, with the exception of Bellingham Drain, denitrification only accounted for 20-40% of overall NO_3^- removal (Table 2). The amount of removal that DNRA could account for was of a slightly smaller range at 2-18% of overall NO_3^- removal. Anammox accounted for the smallest fraction of NO_3^- removal (0-10%). In 5 of the 6 sites, a considerable proportion (~50%) of the NO_3^- flux that could be explained by the ^{15}N budgets and rates I measured (Figure 8). In Bellingham Drain, I could account for slightly more (~7%) of the overall nitrate removal than was measured by individual process rates.

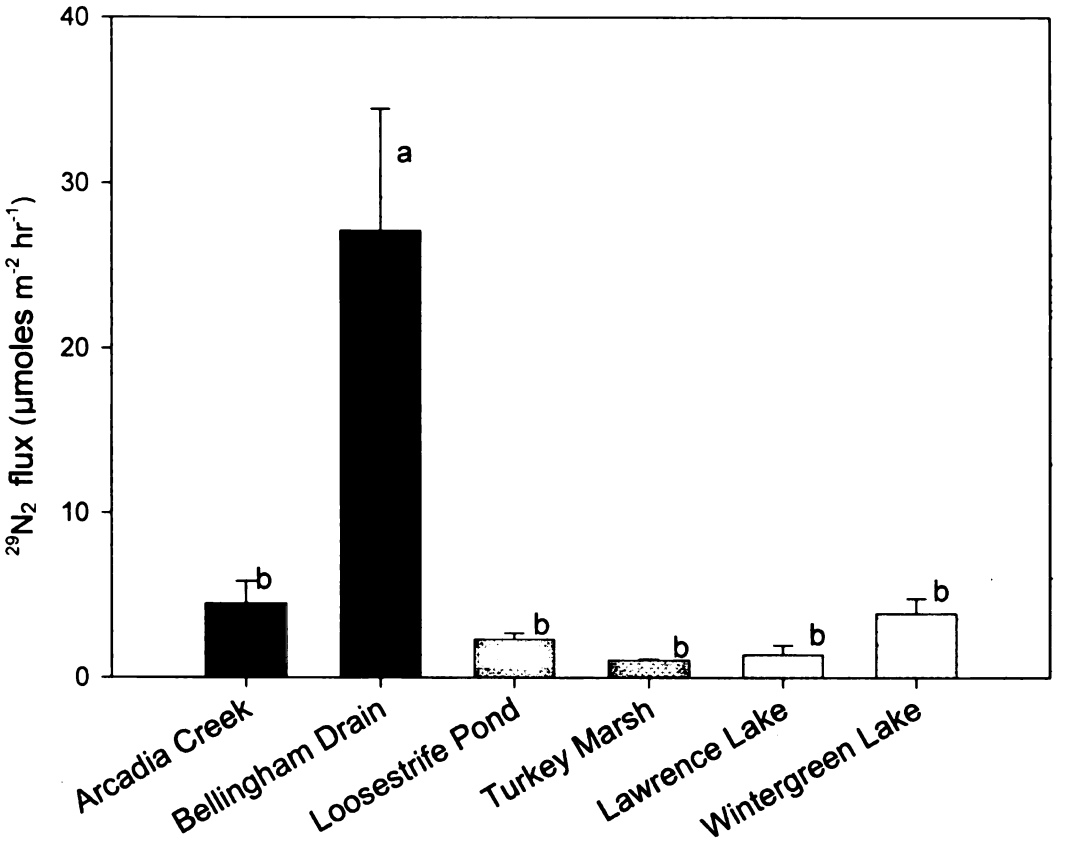


Figure 4: Anammox as control corrected average $^{29}N_2$ flux from the $^{15}NH_4^+$ amended cores from each site over the three day incubation time (n=9). Error bars represent 1 S.E. of the mean. The ecosystem types are colored as: streams (black), wetlands (dark grey), and lakes (light grey). Sites with the same letter are not statistically different from each other (p=0.05).

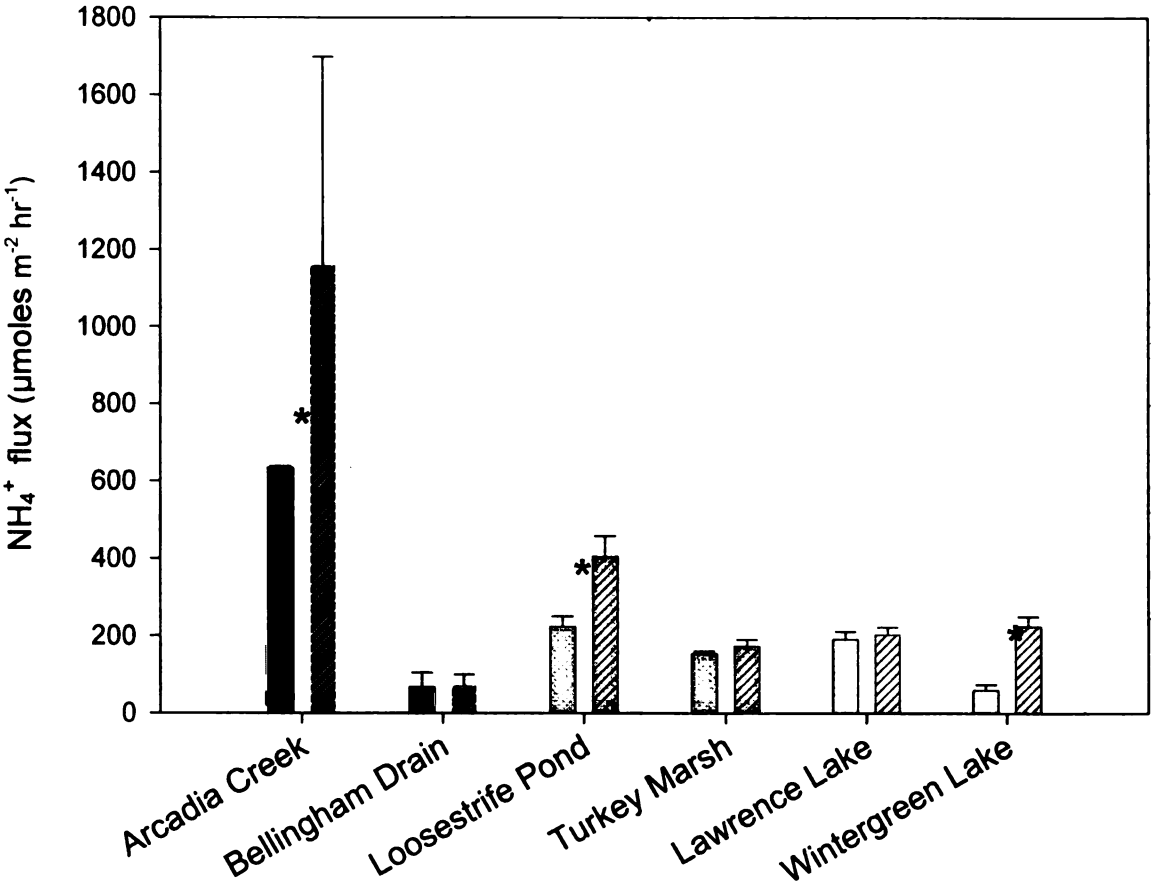


Figure 5: Average NH_4^+ flux from the control cores (n=6; solid) and $^{15}NO_3^-$ amended cores (n=9; hatched) from each site over the three day incubation time. Error bars represent 1 S.E. of the mean. The ecosystem types are colored as: streams (black), wetlands (dark grey), and lakes (light grey). (*) indicates that the control and treatment cores had statistically different ($p<0.05$) NH_4^+ fluxes.

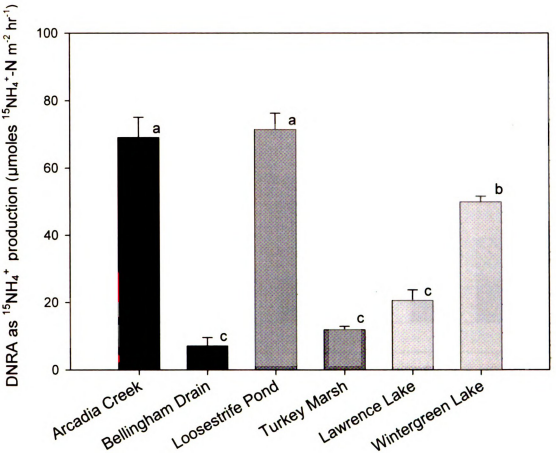


Figure 6: DNRA rates measured as $^{15}NH_4^+$ production in the $^{15}NO_3^-$ amended cores from each site over the three day incubation time (n=9). Error bars represent 1 S.E. of the mean. The ecosystem types are colored as: streams (black), wetlands (dark grey), and lakes (light grey).

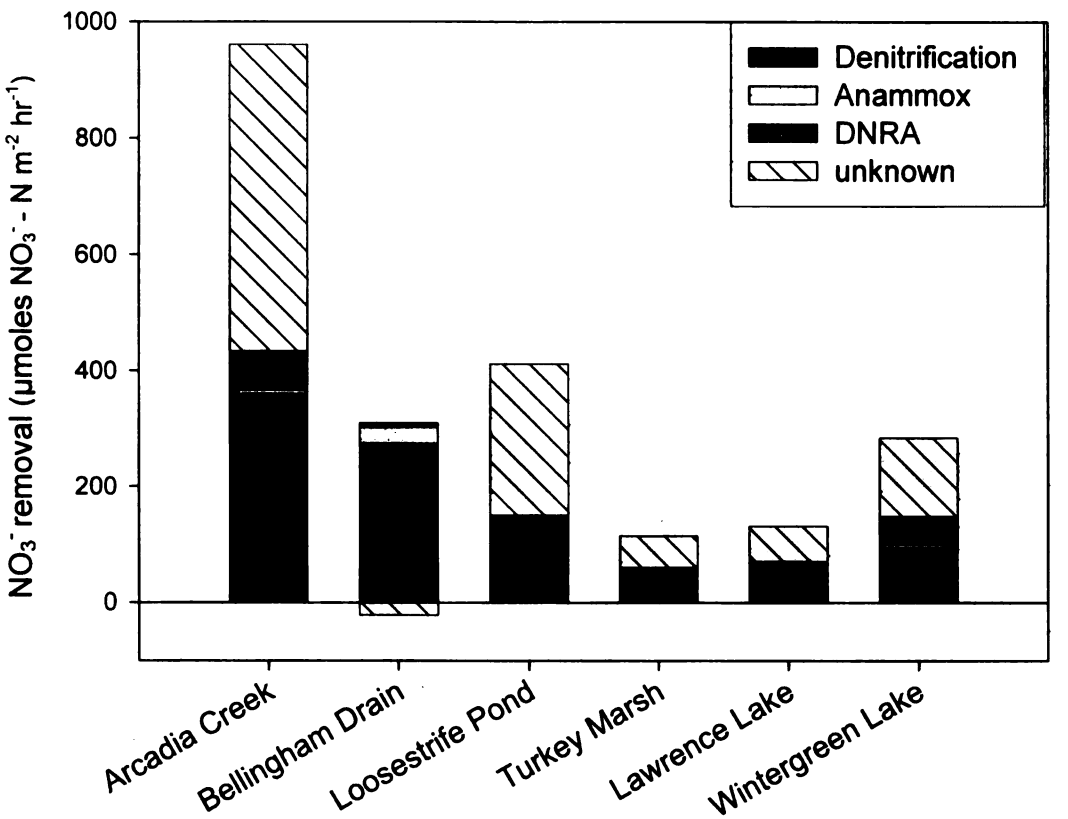


Figure 7: The relative importance of dissimilatory nitrate removal pathways to overall nitrate removal in sediments from streams (black), wetlands (dark grey) and lakes (light grey).

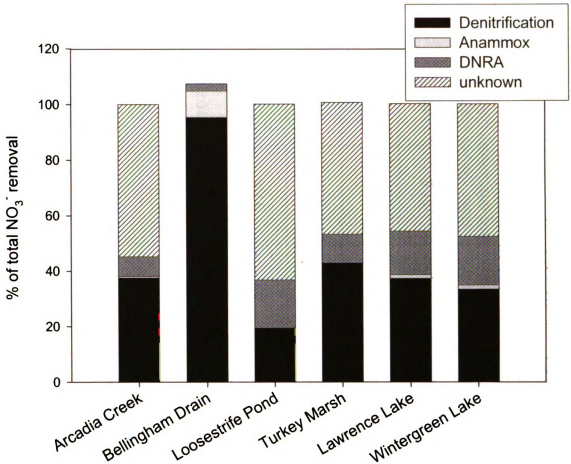


Figure 8: The relative importance of dissimilatory nitrate removal pathways as a percentage of the overall nitrate removal rate from each of the six sites. The hatched area represented the amount of "unknown" NO_3^- removal, i.e., the NO_3^- was removed but cannot be accounted for by the N end-products measured.

Site	$^{15}NO_3^-$ flux µmoles m^{-2} hr^{-1}	Den. µmoles m^{-2} hr^{-1}	Anam-mox µmoles m^{-2} hr^{-1}	DNRA µmoles m^{-2} hr^{-1}	Den. % of NO_3^- removal	Anammox % of NO_3^- removal	DNRA % of NO_3^- removal	Total NO_3^- removal accounted
Arcadia Creek (S)	-961 ± 92	361 ± 11	4.5 ± 1.3	69 ± 6.0	37.6	0.5	7.2	45.3
Bellingham Drain (S)	-288 ± 27	275 ± 32	27.1 ± 7.4	7.1 ± 2.5	95.4	9.5	2.5	107.4
Loosestrife Fen (W)	-411 ± 43	80 ± 11	2.3 ± 0.4	72 ± 4.8	19.5	0.0	17.4	36.7
Turkey Marsh (W)	-114 ± 3.2	49 ± 5.8	1.1 ± 0.1	12 ± 1.0	42.6	0.0	10.4	53.0
Lawrence Lake (L)	-131 ± 3.2	49 ± 2.8	1.4 ± 0.6	21 ± 3.1	37.4	1.3	15.7	54.8
Wintergreen Lake (L)	-284 ± 1.5	95 ± 11	3.9 ± 0.9	50 ± 1.7	33.5	1.4	17.6	52.5

Table 2: NO_3^- processing rates and percent of overall removal of each process by sites. Values are means of all measurements (n=9) with ±1 S.E. DEN = denitrification, Anammox = anaerobic ammonium oxidation, DNRA = dissimilatory nitrate removal to ammonium.

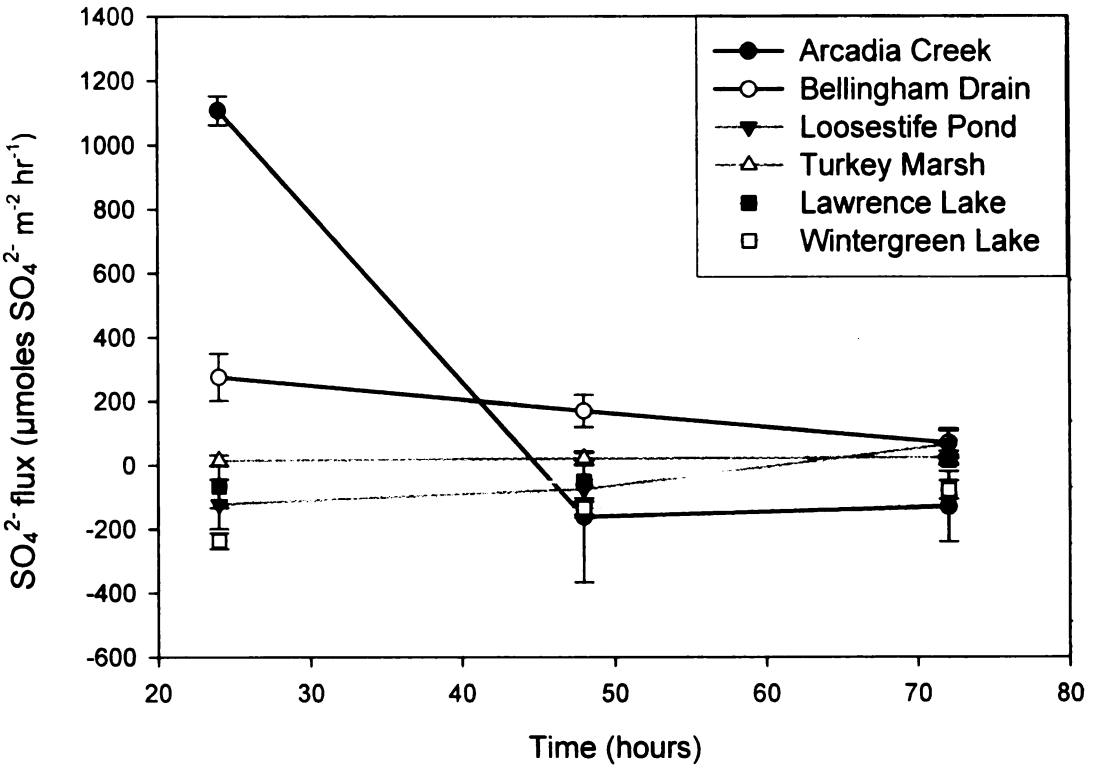


Figure 9: Sulfate flux in the $^{15}NO_3^-$ amended cores from each site and sampling time over the three day incubation time (n=3). Error bars represent 1 S.E. of the mean. The ecosystem types are colored as: streams (black), wetlands (dark grey), and lakes (light grey).

Sulfate fluxes in two of the six sites changed significantly over the time course of the experiments (Arcadia Creek and Wintergreen Lake). Thus, rather than representing sulfate fluxes as a grand mean of all measurements over the experimental time course, they are displayed as daily means by site (Figure 9). These are not compared to the controls because the lack of NO_3^- in the control cores would have favored sulfate reduction, whereas the NO_3^- added to all of the treatments would have suppressed sulfate reduction. Sulfate fluxes did not consistently change in the same direction over time; for example, in Arcadia Creek SO_4^{2-} flux decreased over time, whereas in Wintergreen Lake the SO_4^{2-} flux increased over time.

Discussion:

Are there differences in NO_3^- processing among aquatic ecosystem types?

The two stream sites had higher rates of NO_3^- removal, denitrification and anammox than did the wetland or lake sites (figures 2, 3, 4). This could be in part due to their higher ambient NO_3^- load compared to the wetlands and lakes and thus their higher background NO_3^- removal rates, as illustrated by the relatively high rates in the control cores (data not shown). Loosestrife Fen (W), however, had no background NO_3^-, but had a NO_3^- removal rate nearly as high as the stream sites (Figure 2). The denitrification and anammox rates at that site, however, were relatively low compared to the other lake and wetland sediments (Figures 3 and 4). Therefore, high ambient NO_3^- is not necessarily a

predictor of higher NO_3^- removal rates, but may be related to higher rates of denitrification and anammox.

The rates of NO_3^- removal are similar to those measured in other aquatic ecosystems, both freshwater and marine. These NO_3^- removal rates fall into the same range as reported by (Kellman 2004) for streams in a similarly human-dominated landscape (273-1041 μmoles m^{-2} hr^{-1}). Furthermore, Arcadia Creek and Bellingham Drain were part of the Lotic Intersite Nitrogen eXperiment 2 (LINX2), which conducted whole stream $^{15}NO_3^-$ enrichments. The results of that study showed that NO_3^- removal rates were 120 μmoles m^{-2} hr^{-1} for Arcadia Creek and 43 μmoles m^{-2} hr^{-1} for Bellingham Drain. The values I report for these same streams are much higher. Due to logistical constraints, I could only sample one area of a given ecosystem, and I tended to sample areas that were not sandy. Thus, the discrepancy in rates may be due to differences of scales or increased spatial heterogeneity. Sediments collected for my experiments may represent a relatively "hot" patch within the larger stream matrix, which would have been more equally represented with the whole-stream $^{15}NO_3^-$ experiments.

Denitrification rates in lakes vary considerably, with reported values ranging from 0.4 - 383 μmoles N m^{-2} hr^{-1} (Seitzinger 1988). Some of this variation is driven by differences in nitrogen loads, oxygen levels, and primary productivity in the waters (Seitzinger 1988). Denitrification rates in the two lakes studied here ranged from 50-100 μmoles m^{-2} hr^{-1}, with approximately twice as much denitrification in the highly eutrophic Wintergreen Lake than in oligotrophic Lawrence Lake. These denitrification rates are similar to those found in the

sediments from a reservoir draining an agriculturally dominated landscape, which ranged from 62-225 μmoles m^{-2} hr^{-1} (David et al. 2006).

Wetlands have been particularly well-studied with regards to denitrification because of their oft cited ability to provide the valuable ecosystem service of nutrient processing and retention, including NO_3^- removal (Zedler 2003). Wetlands have exceptional variation in denitrification rates both within a given system (Poe et al. 2003) and across time within the same site (Smith et al. 2000). Studies of wetlands receiving agricultural drainage have found denitrification rates ranging from 50 – 650 μmoles m^{-2} hr^{-1} in a North Carolina constructed wetland (Poe et al. 2003), 21 - 1414 μmoles m^{-2} hr^{-1} in a California constructed wetland (Smith et al. 2000), and 15 – 25 μmoles m^{-2} hr^{-1} in a minerotrophic fen in Denmark (Hoffmann et al. 2000). The two sites used in this study are neither constructed nor restored ecosystems, which may in part explain why the denitrification rates were relatively low (50-80 μmoles m^{-2} hr^{-1}) and relatively similar between the two sites, as was found by Hoffmann et al. (2000).

The rates of denitrification measured in this study also generally agree with those found in meta-analyses across aquatic ecosystems. Piña-Ochoa and Álvarez-Cobelas (2006) reported a mean denitrification rate of ~350 μmoles m^{-2} hr^{-1} in lakes and ~170 μmoles m^{-2} hr^{-1} in rivers, though overall, rivers had the highest rates of denitrification accompanied by the most variation. While these rates were of the same order of magnitude, I found that streams had much higher rates of denitrification than did lakes (Figure 3). These findings agree better with those reported in Seitzinger (1988), who also found that streams generally had

higher denitrification rates (0-345 μmoles m^{-2} hr^{-1}) than lakes (10-171 μmoles m^{-2} hr^{-1}). The LINX2 studies reported a denitrification rate of 2.38 μmoles m^{-2} hr^{-1} for Bellingham Drain and 7.8 μmoles m^{-2} hr^{-1} for Arcadia Creek. The wetland denitrification rates reported here also agree approximately with rates reported for wetlands of intermediate and high disturbance (5-135 μmoles N m^{-2} hr^{-1}) (Seitzinger 1994).

It is more difficult to compare my estimated DNRA rates to other studies in similar ecosystems because DNRA is not measured as frequently as denitrification. An additional complication is that DNRA is reported as both rates and as percentages of nitrate removal. The rates of DNRA measured here are in the same range and slightly higher (13 – 99 μmoles m^{-2} hr^{-1}; Table 3) compared to those measured in the estuaries studied by Gardner et al. (2006) (3 – 50 μmoles m^{-2} hr^{-1}; Table 3). However, the rates I measured were much lower than those measured in the marine-influenced Ringfield Marsh (1370 – 4230 μmol m^{-2} hr^{-1}) (Tobias et al. 2001) and the rates measured in estuarine sediments (800 – 50,000 μmol m^{-2} hr^{-1}) (Koike and Sorensen 1988).

Most studies of anammox have been done in marine ecosystems. The only study performed in freshwaters was by Schubert et al. (2006) in Lake Tanganyika. Schubert et al. measured rates of anammox in the anoxic water column (>100 m depth) of up to 10 nM N_2 hr^{-1}, which are much lower than the absolute rates of anammox measured in this study. Dalsgaard et al. (2005) also found higher anammox rates but lower relative importance of anammox in overall NO_3^- removal when comparing near-surface anammox measurements to deep

ocean measurements. Although the Dalsgaard et al. (2005) synthesis was reported results in a per volume unit (nmol cm^{-3} hr^{-1}) and my rates were measured on an areal basis (μmoles m^{-2} hr^{-1}), if we assume that the active depth of sediment in the cores is 2 cm, then the rates of annamox in these sediments translate to 10-50 nmoles cm^{-3} hr^{-1} (300 nmoles cm^{-3} hr^{-1} in Bellingham), which are considerably higher than those reported from various studies in the review by Dalsgaard et al. (2005), which tended to range from 1-10 nmoles cm^{-3} hr^{-1}. Dalsgaard et al. (2005) suggested that water depth was a key driver in the relative importance of anammox compared to denitrification, wherein at deeper depths with lower mineralization (and C) anammox bacteria can compete effectively with denitrifers. My sites seem to also fit this pattern for near-surface environments, with relatively little NO_3^- consumed by Anammox..

These rates may be artificially high due to an enrichment or fertilization effect created by increasing the concentration of NO_3^- in the overlying water. Adding NO_3^- to the cores, even when some background NO_3^- was present, increased NO_3^- removal rates in some sites (e.g., in Arcadia Creek; data not shown) but not all. This could explain some of the disparity in the rates of NO_3^- removal and denitrification found in the LINX2 experiments compared to my results. The increase in NO_3^- availability in these experiments, however, was well within the range of what these ecosystems might normally encounter from either agricultural run-off or high nitrate groundwater. Thus, the rates measured here should be well within realistic rates for these ecosystems.

Hypotheses to explain the missing tracer ^{15}N

These experiments did not fully quantify all of the N end products, as indicated by the substantial fraction of NO_3^- removal that could not be accounted for by measuring the end-products accounted for in this study. I was able to account for roughly half in most cases, which is comparable to other studies where one or more nitrate removal processes have been measured (Seitzinger 1988). The unaccountable ^{15}N from NO_3^- could be explained by a number of possibilities: 1) underestimated DNRA rates (discussed below) due to NH_4^+ sorption or exchange with bound NH_4^+ pools; 2) microbial storage of NO_3^-, as has been found in some strains of nitrate-reducing bacteria (Kamp et al. 2006); and 3) other processes not measured, including assimilative uptake for biomass incorporation, although microbial use of NO_3^- seems unlikely given available NH_4^+ and for the same reason N fixation would seem unlikely in the sediment environment.

Denitrification and anammox result in dissolved gaseous nitrogen (N_2), which can readily be quantified with careful sample collection and handling because it behaves conservatively in pore waters. On the other hand, NH_4^+ in sediment pore waters is known to be in equilibrium with the ion exchange complex. Exchange of dissolved NH_4^+ with a large sorbed reservoir would result in an underestimation of the tracer ^{15}N in NH_4^+ and thus an underestimate of DNRA rates because I sampled water flowing over the sediments. The importance of this sorption remains to be investigated.

What is the relative importance of various N removal processes?

Differences in measurement methods and rate units make it difficult to compare processes across different ecosystems and studies. To examine the relative importance of the various processes in this study compared to a number of other published reports, I have taken a ratio of the rates of denitrification to either DNRA (Table 3) or anammox (Table 4). The actual rates reported in each study are reported in the tables, along with their respective units (as footnotes), and the ratio is a unitless number wherein a value less than one indicates that the alternative process is more important than denitrification, and greater than one indicates that denitrification is more important.

DNRA accounted for a significant proportion of nitrate removal in the six sites used in this study, although denitrification was the dominant nitrate removal process (Table 3). The exception to this was in streams where denitrification was much more important than DNRA. The denitrification:DNRA ratios found in these sites are similar to other sites, both marine and freshwater, where both processes have been measured. This is particularly interesting because DNRA has been shown to be an important component of the nitrogen cycle in marine systems, but has received less study in freshwater systems. However, data in Table 3 indicate that DNRA can be quite important relative to denitrification in many freshwater sites (Bowden 1986, Storey et al. 2004, McCarthy et al. 2007a, McCarthy et al. 2007b). This comparison demonstrates that the relative importance of the two pathways is similar in both freshwater and marine ecosystems. Also, in many cases these DNRA estimates are based on isotope

tracer studies that potentially suffer from the same problem of sorption in the sediments that was discussed above, and thus may underestimate true rates.

Anammox tended to be much less important to NO_3^- processing than denitrification in the six sites used in this study. The relative importance of the two processes has received a great deal of study in marine and oceanic ecosystems, but little is known about anammox in freshwaters. Thus, there are fewer available estimates of denitrification:anammox ratios (Table 4), and only one freshwater study for comparison (Schubert 2006). Generally, the relative importance of anammox to denitrification is much lower in these sites than has been measured in other sites (Trimmer et al. 2003, Engstrom et al. 2005), but is similar in range to ratios from other freshwater sites (Trimmer et al. 2003, site 6; McCarthy and Gardner unpublished data). The exception to this is Bellingham Drain, where anammox accounted for approximately 10% as much NO_3^- removal as did denitrification, making the denitrification:anammox ratio more similar to that of the other studies.

Conclusions

In this study, I measured rates of nitrate removal from six freshwater ecosystems and partitioned the nitrate removal end-products to N_2, indicating denitrification activity or NH_4^+ indicating DNRA. Additionally, by using the isotope pairing method, I estimated rates of anammox, which could also be indirectly reliant on nitrate reduction. Denitrification was an important pathway of nitrate removal in all six ecosystems. However, in certain sites, DNRA could be as

important as denitrification to overall nitrate removal. Anammox was not an important pathway of nitrate removal in any of the six sites. The rates of denitrification and DNRA were comparable to those estimated from other freshwater and marine sites where both processes have been measured. Anammox rates are more difficult to compare to other studies due to differing methodologies, but the anammox:denitrification ratio is much higher in these sites than as been measured from other (mostly marine) ecosystems, indicating that anammox is relatively unimportant to nitrate removal in near-surface freshwater sediments.

Site	Reference	Method	Ecosystem type	Season	Den. rate	DNRA rate	Den: DNRA
Arcadia Creek	this study	^{15}N cores	Stream	Summer	361†	69†	**5.2**
Bellingham Drain	this study	^{15}N cores	Stream	Summer	275†	7†	**39.3**
Loosestrife Fen	this study	^{15}N cores	FW Fen	Summer	80†	72†	**1.1**
Turkey Marsh	this study	^{15}N cores	FW Marsh	Summer	49†	12†	**4.1**
Lawrence Lake	this study	^{15}N cores	Lake	Summer	49†	21†	**2.3**
Wintergreen Lake	this study	^{15}N cores	Eutrophic Lake	Summer	95†	50†	**1.9**
Ringfield Marsh	Tobias et al. 2001 MEPS	ABT & ^{15}N	Brackish Marsh	Spring	1.8$^{\#}$	2.4$^{\#}$	**0.8**
Ringfield Marsh	Tobias et al. 2001 MEPS	ABT & ^{15}N	Brackish Marsh	Fall	17.6$^{\#}$	1.6$^{\#}$	**11.0**
Nueces River Mouth	Gardner et al. 2006 L&O	^{15}N cores	Estuary	Summer	21.2†	3.2†	**6.6**
Corpus Christi Bay	Gardner et al. 2006 L&O	^{15}N cores	Estuary	Summer	69.8†	31.3†	**2.2**
East Matagorda Bay	Gardner et al. 2006 L&O	^{15}N cores	Estuary	Winter	12.1†	2.3†	**5.3**
Sabine Lake	Gardner et al. 2006 L&O	^{15}N cores	Estuary	Summer	31.4†	1.1†	**28.5**
Laguna Madre	Gardner et al. 2006 L&O	^{15}N cores	Estuary	Winter	47†	51†	**0.9**

Table 3: A comparison of the relative importance of denitrification to DNRA in studies where both have been simultaneously measured. Abbreviations: (FW) freshwater, (ABT) acetylene block technique, (Den) denitrification, (DNRA) dissimilatory nitrate reduction to ammonium. Units from the different studies are as follows: (†) μmoles m^{-2} hr^{-1}; (‡) umol L^{-1} hr^{-1}; (*) nmol cm^{-3} hr^{-1}, (#) ng N g $sediment^{-1}$ hr^{-1}, ($^{\wedge}$) ng N g^{-1} dry wt hr^{-1}. Journal abbreviations: (MEPS) Marine Ecology Progress Series, (L&O) Limnology and Oceanography, and (FEMS-ME) FEMS Microbiology Ecology.

Site	Reference	Method	Ecosystem type	Season	Den. rate	DNRA rate	Den: DNRA
North River Marsh	Bowden 1986 Ecology	ABT & ^{15}N	FW Tidal Marsh	N/A	0.1*	0.3*	**0.3**
Old Woman Creek Lake Erie	McCarthy et al. 2007a J. Great Lakes Research	^{15}N cores	FW Estuary Mouth – "closed"	Summer	250†	120†	**2.1**
Old Woman Creek Lake Erie	McCarthy et al. 2007a J. Great Lakes Research	^{15}N cores	FW Estuary Wetland – "closed"	Summer	200†	20†	**10**
Lake Taihu, China	McCarthy et al. 2007b Hydrobiologia	^{15}N cores	Eutrophic Lake Main Lake site	Fall	110†	3†	**36.7**
Lake Taihu, China	McCarthy et al. 2007b Hydrobiologia	^{15}N cores	Eutrophic Lake River site	Fall	400†	250†	**1.6**
Speed River, Ontario	Storey et al. 2004 Biogeochemistry	^{15}N cores	Riparian downwelling	N/A	1.9#	1.9#	**1.0**
Speed River, Ontario	Storey et al. 2004 Biogeochemistry	^{15}N cores	Riparian Upwelling	N/A	0.5#	2.6#	**0.2**

Table 3: continued

Site	Reference	Method	Ecosystem type	Season	Den. rate	Anammox rate	Den: Anam -mox
Arcadia Creek	this study	^{15}N cores	Stream	Summer	361†	4.5†	**80.2**
Bellingham Drain	this study	^{15}N cores	Stream	Summer	275†	27.1†	**10.1**
Loosestrife Fen	this study	^{15}N cores	FW Fen	Summer	80†	2.3†	**34.8**
Turkey Marsh	this study	^{15}N cores	FW Marsh	Summer	49†	1.1†	**44.5**
Lawrence Lake	this study	^{15}N cores	Lake	Summer	49†	1.4†	**35.0**
Wintergreen Lake	this study	^{15}N cores	Lake	Summer	95†	3.9†	**24.4**
Skagerrak, Sweden, 700m (S4)	Engstrom et al. 2005 GCA	^{15}N slurry IPT	Ocean	May	14‡	0.6‡	**0.3**
Skagerrak, Sweden (S1)	Engstrom et al. 2005 GCA	^{15}N slurry IPT	Ocean	May	4.1‡	1.4‡	**2.9**

Table 4: A comparison of the relative importance of denitrification to anammox in studies where both have been simultaneously measured. Abbreviations: (FW) freshwater, (IPT) isotope pairing technique, (Den) denitrification. Units from the different studies are as follows: (†) μmoles m^{-2} hr^{-1}; (‡) umol L^{-1} hr^{-1}; (*) nmol N_2 ml wet sed^{-1} hr^{-1}; ($^{\#}$) nM hr^{-1}. Journal abbreviations: (AEM) Applied and Environmental Microbiology, (EM) Environmental Microbiology, and Geochimica et Cosmochimica (GCA).

Site	Reference	Method	Eco-system type	Season	Den. rate	Anam-mox rate	Den: Anam-mox
Long Island Sound, 34m (SD)	Engstrom et al. 2005 GCA	^{15}N slurry IPT	Ocean	August	13‡	1.2‡	**10.8**
Long Island Sound, 16m (SP)	Engstrom et al. 2005 GCA	^{15}N slurry IPT	Ocean	August	16‡	0.9‡	**17.6**
Thames River FW (site 2)	Trimmer et al. 2003 AEM	^{15}N slurry IPT	Estuary	Nov.	120*	10*	**12.0**
Thames River Estuary (site 6)	Trimmer et al. 2003 AEM	^{15}N slurry IPT	Estuary	Nov.	35*	0.5*	**70.0**
Lake Tanganyika (110 m)	Schubert et al. 2006 EM	^{15}N IPT	Lake	July	65#	10#	**6.5**
Lake Erie Central Basin (AS)	McCarthy and Gardner unpublished data	^{15}N cores	Lake	Sept.	241†	12.6†	**19.1**
Lake Erie Central Basin (BW)	McCarthy and Gardner unpublished data	^{15}N cores	Lake	Sept.	513†	15.6†	**32.8**

Table 4 continued

References:

Aminot, A., D. S. Kirkwood, and R. Kerouel. 1997. Determination of ammonia in seawater by the indophenol-blue method: Evaluation of the ICES NUTS I/C 5 questionnaire. Marine Chemistry **56**:59-75.

An, S. M., and W. S. Gardner. 2002. Dissimilatory nitrate reduction to ammonium (DNRA) as a nitrogen link, versus denitrification as a sink in a shallow estuary (Laguna Madre/Baffin Bay, Texas). Marine Ecology-Progress Series **237**:41-50.

Bonin, P. 1996. Anaerobic nitrate reduction to ammonium in two strains isolated from costal marine sediment: A dissimilatory pathway. FEMS Microbiology Ecology **19**:27-38.

Bowden, W. B. 1986. Nitrification, nitrate reduction, and nitrogen immobilization in a tidal freshwater marsh sediment. Ecology **67**:88-99.

Brunet, R. C., and L. J. Garcia-gil. 1996. Sulfide-induced dissimilatory nitrate reduction to ammonia in anaerobic freshwater sediments. FEMS Microbiology Ecology **21**:131-138.

Burgin, A. J., and S. K. Hamilton. 2007. Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Frontiers in Ecology and the Environment **5**:89-96.

Cornwell, J. C., W. M. Kemp, and T. M. Kana. 1999. Denitrification in coastal ecosystems: methods, environmental controls, and ecosystem level controls, a review. Aquatic Ecology **33**:41-54.

Dalsgaard, T., B. Thamdrup, and D. E. Canfield. 2005. Anaerobic ammonium oxidation (anammox) in the marine environment. Research in Microbiology **156**:457-464.

David, M. B., L. G. Wall, T. V. Royer, and J. L. Tank. 2006. Denitrification and the nitrogen budget of a reservoir in an agricultural landscape. Ecological Applications **16**:2177-2190.

Engstrom, P., T. Dalsgaard, S. Hulth, and R. C. Aller. 2005. Anaerobic ammonium oxidation by nitrite (anammox): Implications for N_2 production in coastal marine sediments. Geochimica et Cosmochimica Acta **69**:2057-2065.

Fossing, H., V. A. Gallardo, B. B. Jorgensen, M. Huttel, L. P. Nielsen, H. Schulz, D. E. Canfield, S. Forster, R. N. Glud, J. K. Gundersen, J. Kuver, N. B. Ramsing, A. Teske, B. Thamdrup, and O. Ulloa. 1995. Concentration and transport of nitrate by the mat-forming sulfur bacterium *Thioploca*. Nature **374**:713-715.

Gardner, W. S., L. R. Herche, P. A. St. John, and S. P. Seitzinger. 1991. High-Performance Liquid Chromatographic determination of $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] ion ratios in seawater for isotope dilution experiments. Analytical Chemistry **63**:1838-1843.

Gardner, W. S., M. J. McCarthy, S. M. An, D. Sobolev, K. S. Sell, and D. Brock. 2006. Nitrogen fixation and dissimilatory nitrate reduction to ammonium (DNRA) support nitrogen dynamics in Texas estuaries. Limnology and Oceanography **51**:558-568.

Gardner, W. S., P. A. StJohn, C. Evans, and J. F. Cavaletto. 1996. HPLC retention-time-shift determination of nitrogen isotope ratios in enriched water. American Laboratory **28**:C17.

Golterman, H. L. 2004. The chemistry of phosphate and nitrogen compounds in sediments. Kluwer Academic Publishers, London.

Hedin, L. O., J. C. von Fischer, N. E. Ostrom, B. P. Kennedy, M. G. Brown, and G. P. Robertson. 1998. Thermodynamic constraints on nitrogen transformations and other biogeochemical processes at soil-stream interfaces. Ecology **79**:684-703.

Hoffmann, C. C., S. Rysgaard, and P. Berg. 2000. Denitrification rates predicted by nitrogen-15 labeled nitrate microcosm studies, in situ measurements, and modeling. Journal of Environmental Quality **29**:2020-2028.

Holmes, R. M., J. W. McClelland, D. M. Sigman, B. Fry, and B. J. Peterson. 1998. Measuring N-$^{15}NH_4^+$ in marine, estuarine and fresh waters: An adaptation of the ammonia diffusion method for samples with low ammonium concentrations. Marine Chemistry **60**:235-243.

Jetten, M. S. M., M. Strous, K. T. van de Pas-Schoonen, J. Schalk, U. G. J. M. van Dongen, A. A. van de Graaf, S. Logemann, G. Muyzer, M. C. M. van Loosdrecht, and J. G. Kuenen. 1998. The anaerobic oxidation of ammonium. Fems Microbiology Reviews **22**:421-437.

Kamp, A., P. Stief, and H. N. Schulz-Vogt. 2006. Anaerobic sulfide oxidation with nitrate by a freshwater *Beggiatoa* enrichment culture. Applied and Environmental Microbiology **72**:4755-4760.

Kellman, L. 2004. Nitrate removal in a first-order stream: reconciling laboratory and field measurements. Biogeochemistry **71**:89-105.

Knowles, R. 1982. Denitrification. Microbiological Reviews **46**:43-70.

Koike, I., and J. Sorensen. 1988. Nitrate reduction and denitrification in marine sediments. Pages 251-270 *in* T. H. Blackburn and J. Sorensen, editors. Nitrogen cycling in the coastal marine environments. John Wiley & Sons, London.

McCarthy, M. J., W. S. Gardner, P. J. Lavrentyev, K. M. Moats, F. J. Jochem, and D. M. Klarer. 2007a. Effects of hydrological flow regime on sediment-water interface and water column nitrogen dynamics in a Great Lakes coastal wetland (Old Woman Creek, Lake Erie). Journal of Great Lakes Research **33**:219-231.

McCarthy, M. J., P. J. Lavrentyev, L. Yang, L. Zhang, Y. Chen, B. Qin, and W. S. Gardner. 2007b. Nitrogen dynamics and microbial food web structure during a summer cyanobacterial bloom in a subtropical, shallow, well-mixed, eutrophic lake (Lake Taihu, China). Hydrobiologia **581**:195-207.

Megonigal, J., M. Hines, and P. Visscher. 2004. Anaerobic Metabolism: Linkages to Trace Gases and Aerobic Processes. Pages 317-424 *in* W. H. Schlesinger, editor. Biogeochemistry. Elsevier-Pergamon, Oxford, UK.

Paerl, H. W., R. L. Dennis, and D. R. Whitall. 2002. Atmospheric deposition of nitrogen: Implications for nutrient over-enrichment of coastal waters. Estuaries **25**:677-693.

Peterjohn, W. T., and D. L. Correll. 1984. Nutrient dynamics in an agricultural watershed - observations on the role of a riparian forest. Ecology **65**:1466-1475.

Peterson, B. J., W. M. Wollheim, P. J. Mulholland, J. R. Webster, J. L. Meyer, J. L. Tank, E. Marti, W. B. Bowden, H. M. Valett, A. E. Hershey, W. H. McDowell, W. K. Dodds, S. K. Hamilton, S. Gregory, and D. D. Morrall. 2001. Control of nitrogen export from watersheds by headwater streams. Science **292**:86-90.

Pina-Ochoa, E., and M. Alvarez-Cobelas. 2006. Denitrification in aquatic environments: A cross-system analysis. Biogeochemistry **81**:111-130.

Poe, A. C., M. F. Pichler, S. P. Thompson, and H. W. Paerl. 2003. Denitrification in a constructed wetland receiving agricultural runoff. Wetlands **23**:817-826.

Rabalais, N. N., R. E. Turner, and W. J. Wiseman. 2001. Hypoxia in the Gulf of Mexico. Journal of Environmental Quality **30**:320-329.

Rysgaard, S., and R. N. Glud. 2004. Anaerobic N_2 production in Arctic sea ice. Limnology and Oceanography **49**:86-94.

Rysgaard, S., R. N. Glud, N. Risgaard-Petersen, and T. Dalsgaard. 2004. Denitrification and anammox activity in Arctic marine sediments. Limnology and Oceanography **49**:1493-1502.

Schubert, C. J., E. Durisch-Kaiser, B. Wehrli, B. Thamdrup, P. Lam, and M. M. M. Kuypers. 2006. Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika). Environmental Microbiology **8**:1857-1863.

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

Seitzinger, S. P. 1994. Linkages between organic matter mineralization and denitrification in 8 riparian wetlands. Biogeochemistry **25**:19-39.

Smith, L. K., J. J. Sartoris, J. S. Thullen, and D. C. Andersen. 2000. Investigation of denitrification rates in an ammonia-dominated constructed wastewater-treatment wetland. Wetlands **20**:684-696.

Storey, R. G., D. D. Williams, and R. R. Fulthorpe. 2004. Nitrogen processing in the hyporheic zone of a pastoral stream. Biogeochemistry **69**:285-313.

Tiedje, J. M. 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. Pages 179-244 *in* A. J. B. Zehnder, editor. Biology of Anaerobic Microorganisms. Wiley & Sons, New York.

Tobias, C. R., I. C. Anderson, E. A. Canuel, and S. A. Macko. 2001. Nitrogen cycling through a fringing marsh-aquifer ecotone. Marine Ecology-Progress Series **210**:25-39.

Trimmer, M., J. C. Nicholls, and B. Deflandre. 2003. Anaerobic ammonium oxidation measured in sediments along the Thames estuary, United Kingdom. Applied and Environmental Microbiology **69**:6447-6454.

Wetzel, R. G. 2001. Limnology: Lake and River Ecosystems, 3 edition. Academic Press, San Diego.

Whitmire, S. L., and S. K. Hamilton. 2005. Rapid removal of nitrate and sulfate in freshwater wetland sediments. Journal of Environmental Quality **34**:2062-2071.

Zedler, J. B. 2003. Wetlands at your service: reducing impacts of agriculture at the watershed scale. Frontiers in Ecology and the Environment **1**:65-72.

CHAPTER 4

ORGANIC CARBON AND SULFIDE AS CONTROLS ON NITRATE REMOVAL AND NITRATE REDUCTION END-PRODUCTS IN FRESHWATER SEDIMENTS

Introduction:

Nitrogen (N), as a fundamental constituent of biomass, is critically important to ecosystem productivity because it is often a growth-limiting nutrient. Human activities have approximately doubled the amount of reactive nitrogen globally (Vitousek et al. 1997). This anthropogenic perturbation has led to increased loading of nitrate (NO_3^-) to many surface- and ground-water ecosystems. As nitrate-rich water passes through or over freshwater sediments, the concentration of nitrate typically decreases. The prevailing scientific belief is that this removal is largely due to either assimilation into microbial, algal or plant biomass, or to di-nitrogen (N_2) via bacterial respiratory denitrification. While denitrification has been intensively examined, few studies have investigated other known processes that could directly compete with denitrification and may well be as important for overall nitrate removal (Megonigal et al. 2004, Burgin and Hamilton 2007).

We are only starting to understand the complexity of microbial N cycling, especially with regard to factors that control the relative importance of multiple potential pathways. With regard to NO_3^- utilization by bacteria, multiple pathways are known to simultaneously occur, including respiratory denitrification and dissimilatory nitrate reduction to ammonium (Burgin and Hamilton 2007). Availability of free sulfide (hereafter referred to has H_2S), NO_3^-, and organic

carbon (OC) have all been put forth as potential controls on one or both of these processes (Tiedje et al. 1982, Tiedje 1988, Brunet and Garcia-gil 1996).

In respiratory denitrification, nitrate is used in the terminal oxidation of organic matter under anaerobic conditions; most of the nitrate is transformed to N_2, a biologically unavailable form of N. The enzymatic sequence in nitrate reduction is well studied (Paul and Clark 1996); these enzymes can be inhibited by O_2 and low pH. Incomplete reduction can result in the accumulation of intermediates including nitrite (NO_2^-) and nitrous oxide (N_2O). Therefore, favorable conditions for respiratory denitrification include anoxia, NO_3^-, and labile OC.

In contrast to denitrification, DNRA is a relatively understudied NO_3^- removal pathway. Two forms of DNRA are known to occur: fermentative DNRA, thought to occur under conditions of high labile carbon availability (Tiedje et al. 1988), and sulfur-driven DNRA (Brunet and Garcia-gil 1996, Otte et al. 1999), thought to occur wherever nitrate and reduced sulfur compounds coincide. Fermentative DNRA couples electron flow from organic matter via fermentation reactions to the reduction of NO_3^- (Tiedje 1988, Megonigal et al. 2004). Tiedje (1988) suggested that heterotrophic DNRA would be most important in highly reducing environments that maintain anoxic conditions for long time periods. Although the conditions promoting fermentative DNRA and respiratory denitrification are similar (anoxia, available NO_3^- and labile organic matter), fermentative DNRA is thought to be favored in NO_3^--limited, labile-carbon rich environments while respiratory denitrification would be favored under relatively

carbon-limited conditions (Kelso et al. 1997, Silver et al. 2001). A few studies have supported this hypothesis (Nijburg et al. 1997, Bonin et al. 1998, Christensen et al. 2000), but none to our knowledge has directly tested it by manipulating C:N ratios.

Recent work in marine and freshwater systems has demonstrated that certain S-oxidizing bacteria can use NO_3^- to oxidize H_2S and elemental S to sulfate (SO_4^{2-}) (Fossing et al. 1995, Brunet and Garcia-gil 1996). These S-oxidizing bacteria can reduce nitrate to either N_2 or ammonium (NH_4^+) (Dannenberg et al. 1992, Brunet and Garcia-gil 1996, Otte et al. 1999). The predominant fate of the reduced N (NH_4^+ vs. N_2) may be determined by the ambient concentration of H_2S, which is known to inhibit denitrification (Brunet and Garcia-Gil 1996). High ambient H_2S can inhibit the final two reduction steps of the denitrification sequence, in which case the sulfide can be oxidized to elemental S or SO_4^{2-} with a simultaneous reduction of NO_3^- to NH_4^+. On the other hand, metal-bound sulfides such as FeS also can be oxidized by these bacteria, but do not show the enzymatic inhibition of denitrification (Brunet and Garcia-Gil 1996), and these often are abundant constituents of freshwater sediments (Holmer and Storkholm 2001). Therefore, the importance of sulfur-driven nitrate reduction in a given site may be regulated by the availability of electron donors (H_2S, Thiosulfate, elemental S) as well as the ambient concentration of H_2S, which may inhibit key denitrification enzymes.

In this study, I investigated the relative importance of labile OC and H_2S as controls on NO_3^- removal and its end-products (indicative of dominant

pathways) in a laboratory setting using anoxic wetland sediments. I selected sediments from a high ambient H_2S site and a low ambient H_2S site, assuming that they would have different microbial communities which might differentially utilize the NO_3^-. My goals were: 1) to examine the relative importance of OC and H_2S in regulating N_2 and NH_4^+ production from NO_3^-, and 2) to investigate the time course of N transformations after additions of OC and H_2S. I have no *a priori* reason to believe either OC or H_2S will be a more important regulator of nitrate removal and the end-products; the two are not necessarily mutually exclusive controls on nitrate processing. If H_2S controls DNRA, I would expect to see increasing NH_4^+ production with increasing added H_2S. Additionally, if H_2S inhibits denitrification enzymes, I would expect to see a decrease in N_2 production with increasing H_2S. If OC is an important regulator of DNRA, I would expect to see increasing NH_4^+ production with increasing OC.

Methods:

Site selection:

To compare NO_3^- transformations by bacterial communities charactersitic of different levels of ambient H_2S and organic carbon availability, sediment samples were collected from a high H_2S site (Loosestrife Fen, also called Loosestrife Pond; LP) and a low H_2S site (Windmill Pond; WP). Loosestrife Fen is a small groundwater-fed fen at the Experimental Forest of the Kellogg Biological Station (KBS). Groundwater from a spring enters the site and drives flow across the wetland, which has a residence time of ~24-48 hours. This site tends to have high sediment porewater H_2S concentrations; near-surface

porewater often contains >150 μM H_2S (Whitmire 2003). Windmill Pond (WP) is a shallow pond along and connected to Gull Lake at KBS, which in combination with groundwater inputs provides a source of water for the site. This site has an organic layer of sediment overlying a sandy bottom layer. Windmill Pond sediments have sand colored black by iron sulfides, but concentrations of free sulfide tend to be low in the near surface sediment porewater (<10 μM H_2S: Burgin, unpublished data). Neither site had detectable reduced iron (Fe^{2+}) in the porewaters.

Assay procedure:

Two types of assays were conducted using sediments from these sites. In the first set, which will be referred to as the "gradient" assays, I subjected the sediments to levels of labile organic carbon (OC) and free sulfide (H_2S) in a full factorial experimental design, which also included controls wherein NO_3^- but not OC nor H_2S were added (NO_3^--only treatment). On day 1 and day 3 of the experiment, sediments were collected from each site, brought back to the lab and gently mixed, then allowed to sit for at least 24 hours to return to anaerobic conditions. To conduct the assays, 10 mL of sediments and 20 mL of site surface water were placed into a 40 mL vial and capped with a silicon septum. Five replicate vials of each treatment were sparged with He for 20 min. After sparging, the given treatment assignment of labile OC [as sodium acetate, NaAc; high = 10 mg C/L (46.7 μmoles), med = 5 mg C/L (23.3 μmoles), low = 1 mg C/L (2.3 μmoles)] and sulfide [as Na_2S; high = ~200 μM (7-15 μmoles), med = ~100

μM (2.5-5 μmoles), low = ~20 μM (0.5-1 μmoles)] or a combination of the two were added to the anoxic sediments. The sulfide solution was made and added to vials in a glove bag purged with high-purity He immediately prior to the experiment. All OC and NO_3^- solutions were also prepared fresh for each experiment. All sediments except for controls received a NO_3^- amendment of 14.3 μmoles to yield a final concentration of 10 mg N/L or 0.7 mM. Controls without any added C, H_2S or NO_3^- were also prepared to ensure that air leakage was not a significant source of O_2 and N_2. Sediment vials were then incubated for approximately 24 hours with the caps and septa underwater to minimize air contamination.

After 24 hr of incubation, vials were destructively analyzed as follows: 3 mL of He-sparged water were injected into the bottom of each vial to displace 3 mL of vial headspace into a Shimadzu gas chromatograph containing a Porapak-Q® packed column and a thermal conductivity detector for O_2+Ar and N_2 quantification. Tests showed that N_2 and O_2 contamination via air entry from this procedure was insignificant. Another 4 mL of water was used to displace more headspace into a He-filled exetainer (Labco®) for N_2O, CH_4 and CO_2 quantification via gas chromatography (electron capture, flame ionization, and infrared gas detectors). The vial cap was then removed and a 10-mL water sample was immediately filtered (0.45 μm membrane) for H_2S analysis by the colorimetric method of Golterman and Clymo (1969). Another 5 mL was filtered (0.45 μm) for analysis of NH_4^+, NO_3^-, SO_4^{2-}, and NO_2^- via membrane-suppression ion chromatography (Dionex). A final 3 mL subsample was filtered (Millex sterile

0.22 μm) and frozen for future acetate analysis. All other samples (e.g., gas and water chemistry) were analyzed as soon as they were generated.

The same technique was used to examine the time course of NO_3^- transformations in sediment samples from the same two sites; these will be called "time-course assays". Due to logistical constraints for these experiments, only one level of OC (as acetate; 23.3 μmoles for a final concentration of 415 μM) and of H_2S (~1.5 μmoles for a final concentration of 55 μM) were added to the sediments in a full-factorial design. Fifteen replicates of each of the five treatments (control; NO_3^- only; H_2S + NO_3^-; OC + NO_3^-; OC + H_2S + NO_3^-) were started at approximately the same time, and were destructively harvested at time points chosen based on previous experiments. LP was sampled 12, 24 and 48 hrs after the start of the experiment; for WP sampling took place at 6, 12 and 24 hrs because its sediments took up NO_3^- more rapidly.

All data were corrected for dilution and background concentrations; graphs indicate net fluxes wherein a positive value denotes production and a negative value is removal. The gradient assays were analyzed by two-way analysis of variance (ANOVA) with levels of carbon and sulfide as fixed factors. Because factors and interactions were often significant, the magnitude of the effect (ω^2; effect size) of each factor (treatment) was also calculated. The effect size is based on estimating the variance in a response variable that can be explained by the factor, and then relates that fraction of the variance to the total variance in a response variable (Graham and Edwards 2001). The effect size is not directly dependent on the sample size, and it does not necessarily covary with statistical

significance (p values) (Graham and Edwards 2001). Effect sizes are calculated as described by Graham and Edwards (2001) for a two-way ANOVA of fixed factors. The variance of each factor is related to the overall variance, and the resulting figure is termed the "variance component" (Tables 1 and 2). The variance component for a factor is then related to the overall variance (factors + interactions + error), and multiplied by 100 to reflect the percent of variance attributable to a factor, or the magnitude of that factor's effect (ω^2) (Tables 1 and 2).

The time-course assays were analyzed by a two-way ANOVA at each individual time point (3 time points per experiment). Contrast statements were used to compare the effects of various treatments of interest. Statistical analysis was performed in SAS using PROC MIXED. When appropriate, I used the LSMEANS/PDIFF procedure to make a priori pairwise comparisons. In both experiments, five replicates of each treatment were run.

Results:

OC and H_2S Gradient Assays

Sediments from both sites removed a large proportion of the added 14.3 µmoles of nitrate within 24 hrs (Figure 1). LP sediments removed ~66% of the added nitrate during the 24 hr incubation, whereas the WP sediments removed nearly all added NO_3^-. In the LP sediments, stimulation of NO_3^- removal by added H_2S was dependent on OC availability, as indicated by the significant interaction between the treatments (Table 1). At low to intermediate levels of

H_2S, NO_3^- removal was stimulated by the presence of OC, but at high H_2S levels, OC inhibited NO_3^- removal (Figure 1). The H_2S treatment and the interaction of H_2S and OC had a larger magnitude of effect on NO_3^- removal than did OC alone (Table 1). In the WP gradient experiments, I could not test the effects of OC nor H_2S on overall NO_3^- removal because nearly all of the added nitrate was gone by the end of the 24-hour incubation period, precluding estimation of the removal rate.

OC and H_2S additions caused NH_4^+ production in the presence of added NO_3^- in sediments from the two sites (Figure 2). In LP, while there was an increase in NH_4^+ production with addition of either H_2S or OC (~0.5-1 µmole), the greatest NH_4^+ accumulation occurred when the two were added together (up to 3.5 µmoles). H_2S and the interaction of OC and H_2S had much larger effect on NH_4^+ flux than did OC alone (Table 1). WP sediments showed net increases of 0-6 µmoles of NH_4^+; the largest change occurred by adding the smallest amount of H_2S in combination with OC, with the most NH_4^+ production occurring at the lowest levels of OC and H_2S combined (Figure 2). In contrast to LP, no change in NH_4^+ flux was seen across the H_2S- or OC-only gradients. Again, in WP as in LP, H_2S had a larger magnitude of effect than did OC, though the difference between the two effect sizes was much smaller than was the case in LP.

N_2 was produced in the sediments from both sites, but at rates up to 3 times greater in WP than in LP (Figure 3). In LP, both OC and H_2S significantly increased N_2 production with no interaction between the treatments,and H_2S had a greater magnitude of effect on N_2 production than did OC (Table 1). Adding

H_2S to 0.5 and 2.5 µmoles increased N_2 production across all OC additions, but the increase did not extend to the 5.5 µmoles H_2S addition. In the WP sediments, N_2 production was dependent on the interaction of H_2S and OC (Table 2). However, both OC and H_2S had similar magnitudes of effect on N_2. Maximal N_2 production in the WP sediments occurred in the low OC (zero ambient sulfide) and 0 µmoles OC and 0 µmoles H_2S (NO_3^- only) treatments; adding OC and H_2S had an inhibitory affect on N_2 production (Figure 3).

N_2O production accounted for a small faction of the nitrate removal in the LP sediments (Figure 4). In LP, the addition of both OC and H_2S significantly decreased N_2O production, though there was also a significant interaction between these main effects (SAS). Even smaller amounts of N_2O were produced in the WP sediments. Adding the highest amount of H_2S increased N_2O production, whereas smaller amounts of H_2S did not affect N_2O production.

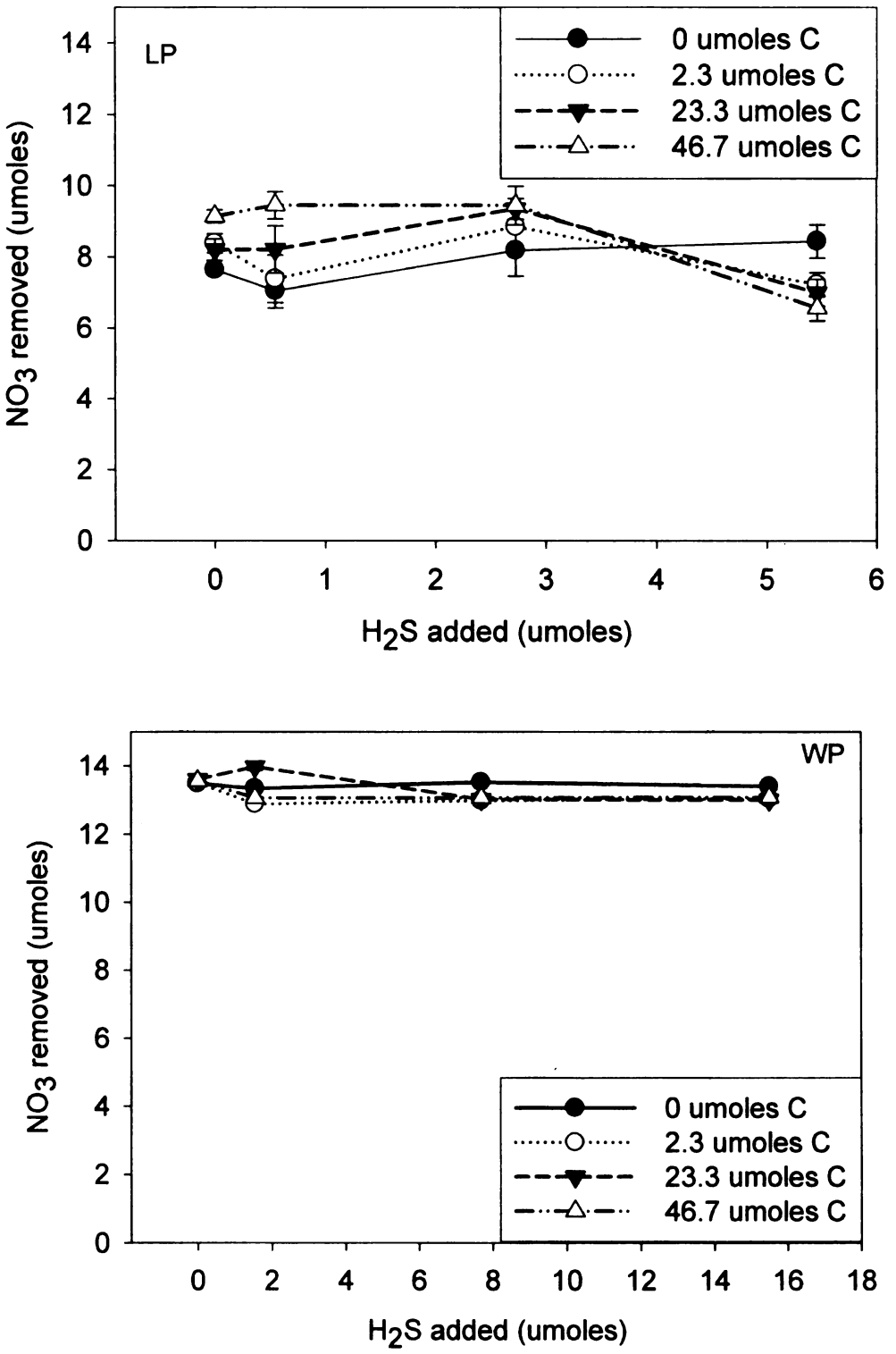


Figure 1: Nitrate removal across a gradient of added sulfide for Loosestrife Fen (top; high ambient H_2S) and Windmill Pond (bottom; low H_2S). All treatments began with 14.3 µmoles of added nitrate and were sampled after 24 hours.

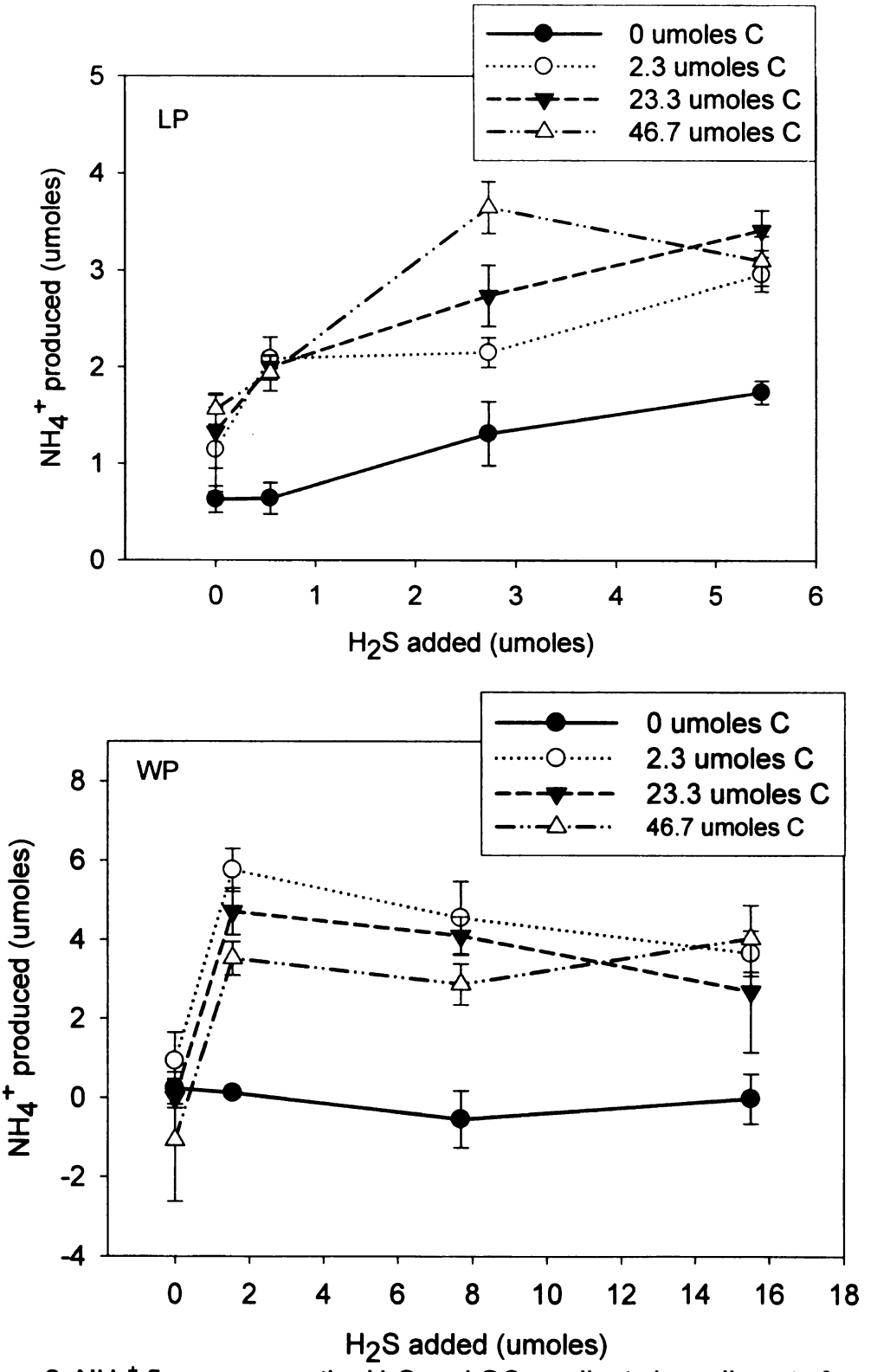


Figure 2: NH_4^+ fluxes across the H_2S and OC gradients in sediments from Loosestrife Fen and Windmill Pond. Positive fluxes indicate net production. Note the differences in the y-axis ranges.

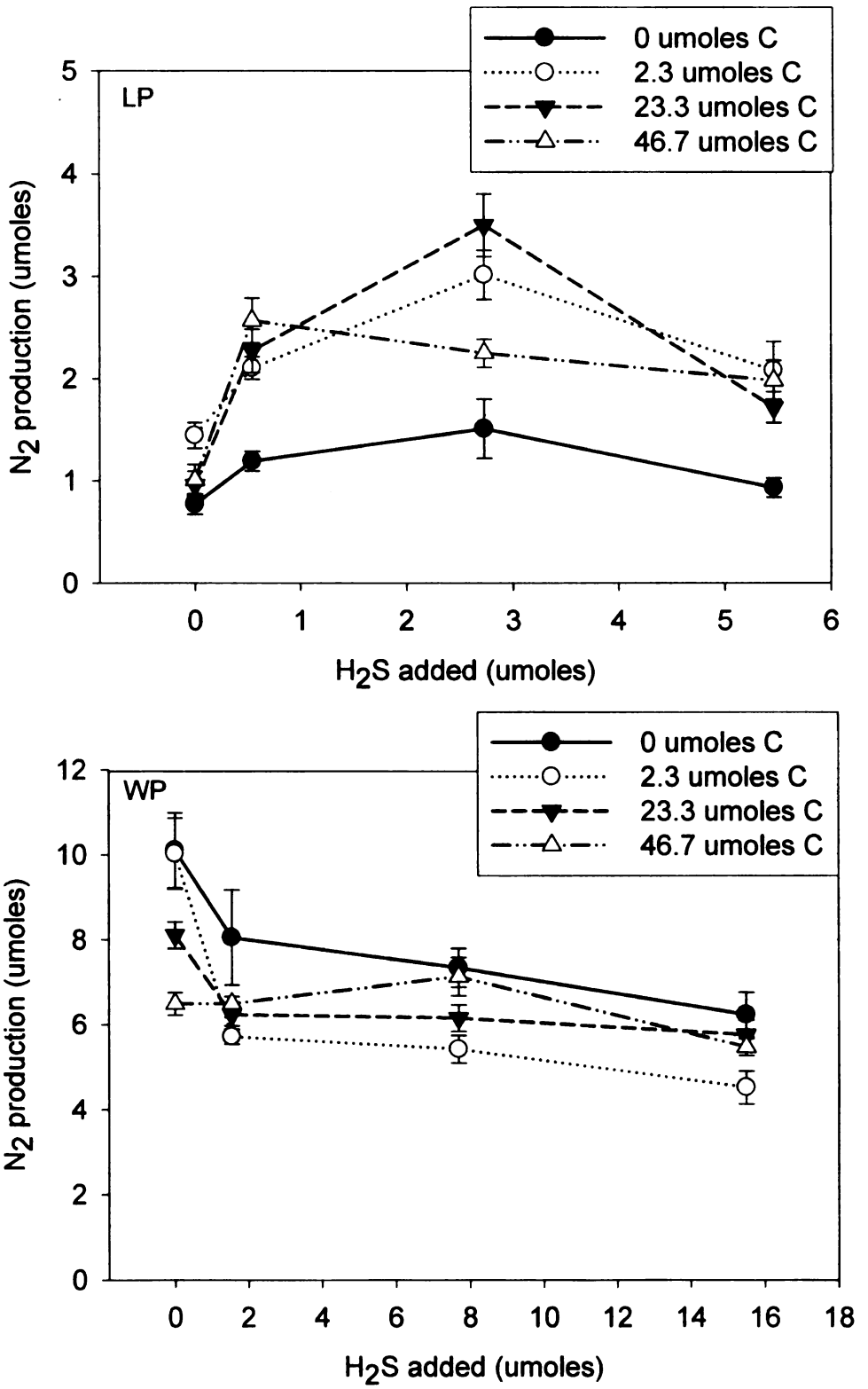


Figure 3: N_2 production for Loosestrife Fen (top) and Windmill Pond (bottom) across a gradient of OC and H_2S. Note the differences in the y-axis ranges.

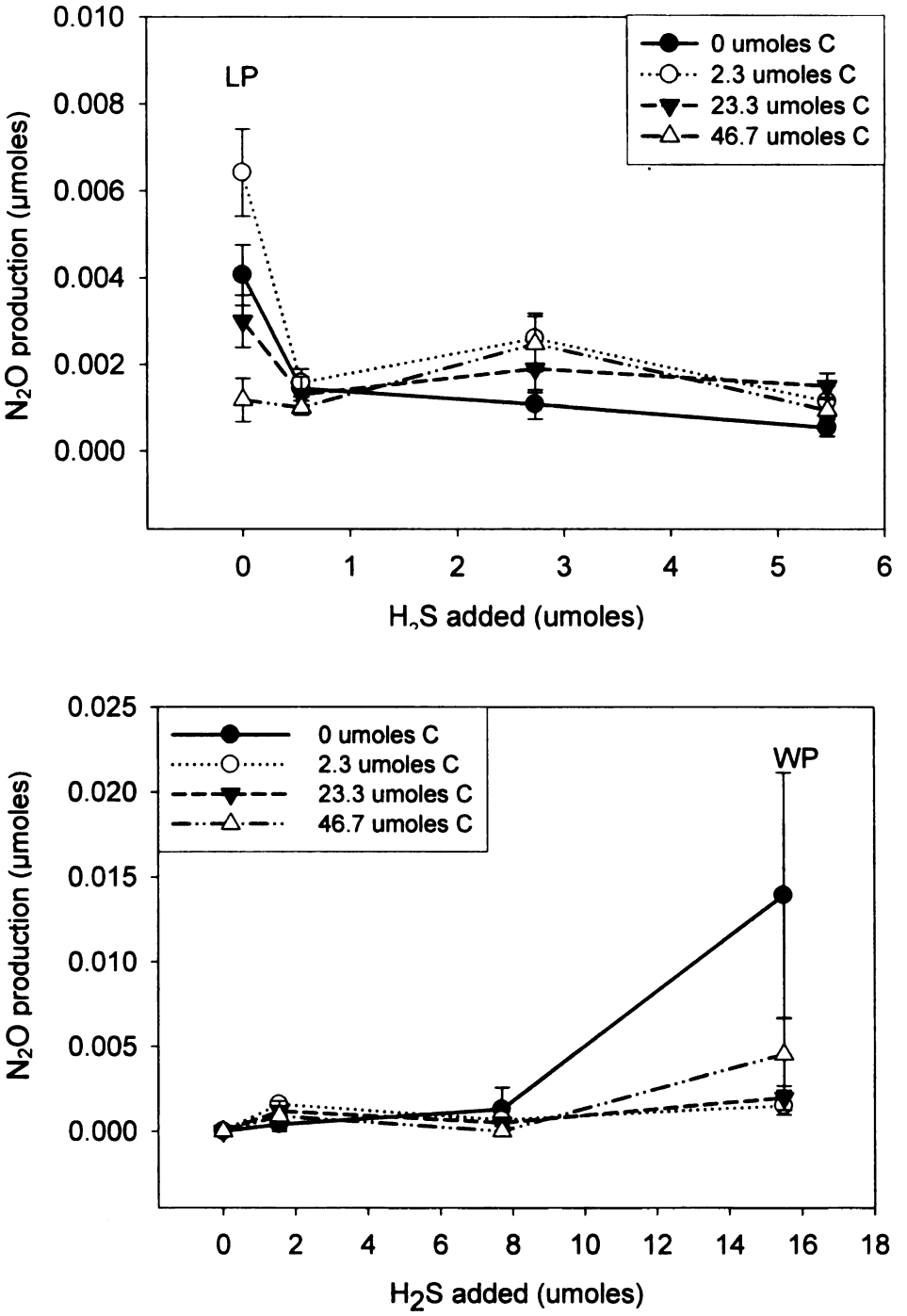


Figure 4: N_2O production (µmoles) for Loosestrife Fen (top) and Windmill Pond (bottom) across a gradient of OC and H_2S.

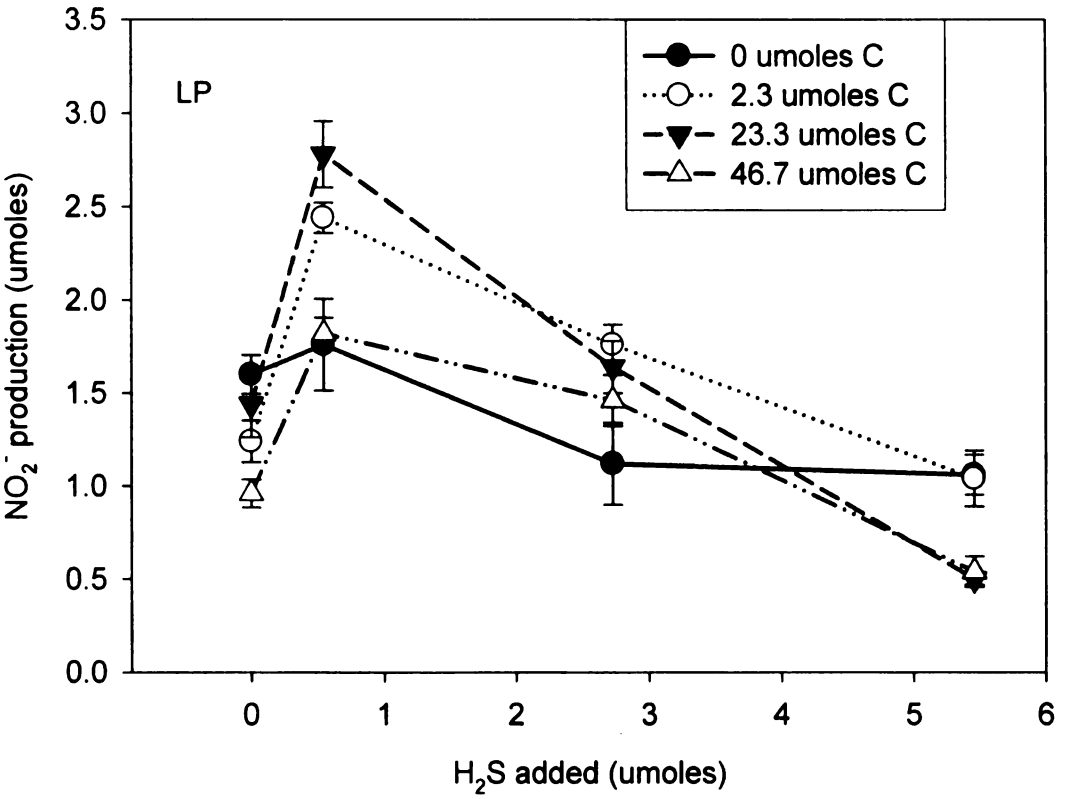


Figure 5: NO_2^- flux for Loosestrife Fen across a gradient of OC and H_2S.

Conversion to NO_2^- accounted for approximately 25% of the overall NO_3^- removal in the LP sediments (Figure 5). The response of NO_2^- to the OC gradient was dependent on the level of the H_2S treatment, as indicated by the interaction between the two factors (Table 1). The effect size of H_2S on NO_2^- production was 10 times greater than the effect size of OC, and 6 times greater than the effect size for the interaction factor (Table 1). Adding small amounts of H_2S increased NO_2^- production (compare 0 and 0.5 µmoles H_2S on Figure 5); however, adding larger amounts of H_2S decreased NO_2^- production. There was no net NO_2^- production in the WP sediments, possibly because of the difference in processing times between the two sediments; transient production of NO_2^- may not have been detected with the more rapid NO_3^- disappearance in WP sediments.

In sediments from both sites, H_2S showed net increases in the controls (i.e., zero added H_2S and OC in Figure 6), whereas in treatments where H_2S was added most of it disappeared by the end of the incubation (Figure 6). In LP, all of the added H_2S was removed in the 0.5 and 2.5 µmole H_2S treatments; however, in the 5.5 µmole H_2S treatment, the addition of OC inhibited further H_2S removal. In WP, a different trend is seen wherein adding OC did not stimulate H_2S removal, but may have inhibited it. In the medium (7.5 µmoles H_2S) and high (15.5 µmoles H_2S) treatments, the zero added OC treatment removed nearly all of the H_2S that was added. In the OC treatments, however, H_2S was still removed, but not to the degree that occurred in the zero OC treatments. In both

LP and WP, H_2S had a much larger magnitude of effect on H_2S flux than did OC (Tables 1 and 2).

SO_4^{2-} concentrations in the assays reflect the balance between production by oxidation of reduced S, potentially coupled to NO_3^- reduction, and consumption by SO_4^{2-} reduction. Both OC and H_2S additions significantly affected SO_4^{2-} concentrations in LP sediments; however, there was a significant interaction between treatments (Table 1). In LP, increased additions of OC progressively inhibited SO_4^{2-} production. When OC was not present (Figure 7, solid line, top panel), SO_4^{2-} concentrations significantly increased with increasing H_2S. Additionally, in the absence of added H_2S, adding additional OC progressively decreased SO_4^{2-} concentrations. In WP sediments, a similar general pattern is seen, wherein the addition of H_2S led to an increase in SO_4^{2-} when OC was not added; when OC was added, SO_4^{2-} was consumed rather than produced. Both OC and H_2S significantly affected SO_4^{2-} concentrations, though there is a significant interaction between the treatments (Table 2). This interaction may also be caused by a similar effect of OC inhibition of SO_4^{2-} production as seen in the LP sediments. When OC was not present (Figure 7, solid line, bottom panel), SO_4^{2-} concentration significantly increased in the 0 µmoles added H_2S treatment as compared to the 0.5, 2.75 and 5.5 µmoles H_2S treatments (pairwise comparisons df = 3, 58; t = -2.51, -2.08, -3.41; p=0.01, 0.04, 0.00 respectively). In both LP and WP, OC had a much larger magnitude of effect than H_2S on SO_4^{2-} production (Tables 1 and 2).

I can account for a majority of the nitrate that was added in most treatments in the LP and WP gradient assays (Figures 8, 9, 10), and the recovery varies by treatment type. Figures 8-10 represent different ways to account for the N removal. Figure 8 illustrates the various fluxes across the low and high H_2S + NO_3^- treatments in both sites, which omits the possibility of the H_2S interacting with OC to mask patterns. Figure 9 is the same as 8, but is averaged across the OC treatments, with no H_2S in the averages. Figure 10 averages all OC treatments (low, medium, high) at a given H_2S level, and therefore incorporates any interactions that may occur between H_2S and OC. Figure 10 generally has less N in the "unknown" category, which means that by adding in factors I can account for more of the added NO_3^-. Conversely, in the H_2S or OC only treatments (Figures 8 and 9) I did not recover the same amount of the added N as in the treatments where both H_2S and OC were added.

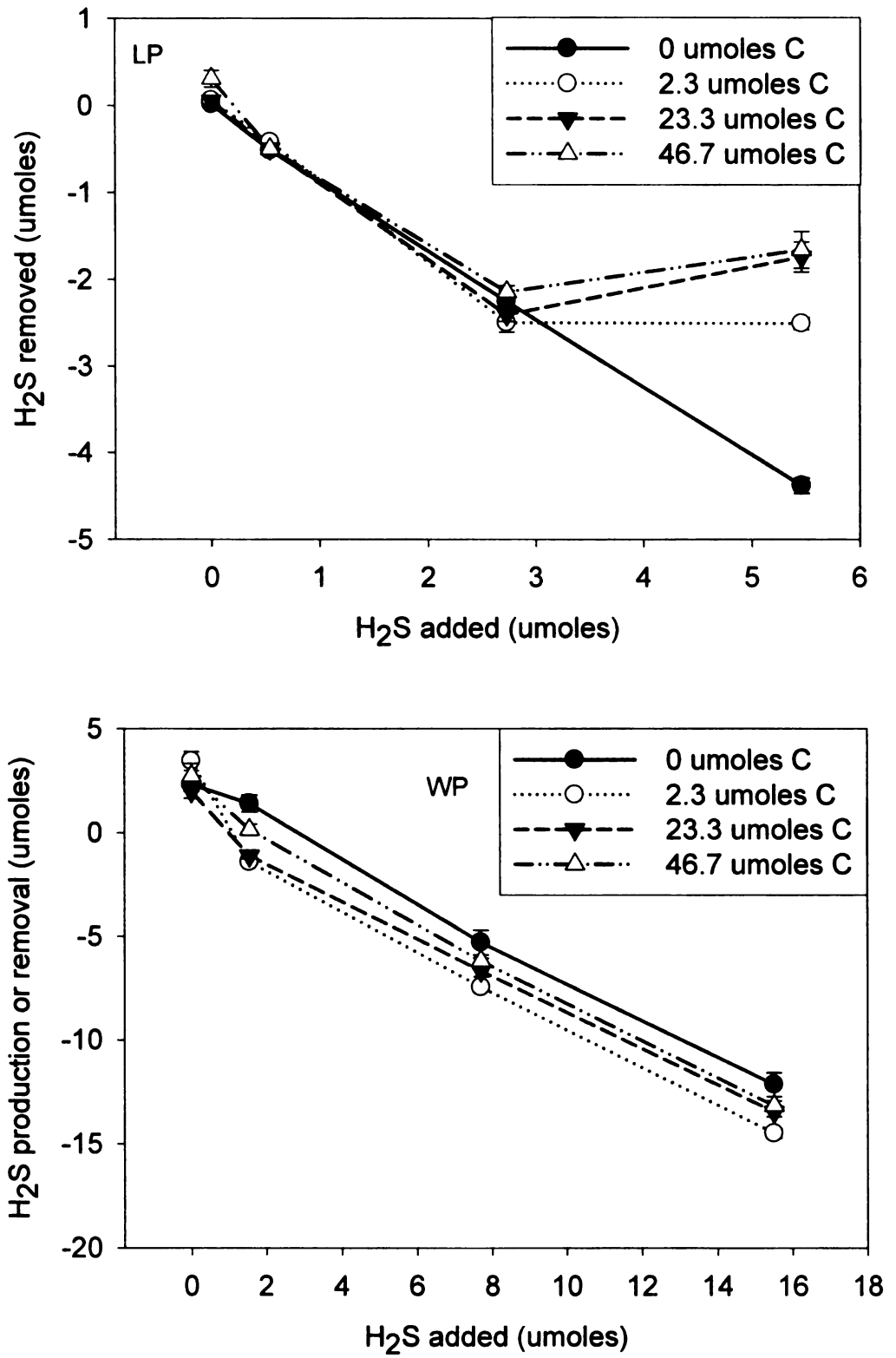


Figure 6: H_2S flux for Loosestrife Fen (top) and Windmill Pond (bottom) across a gradient of OC and H_2S. Negative fluxes indicate net removal.

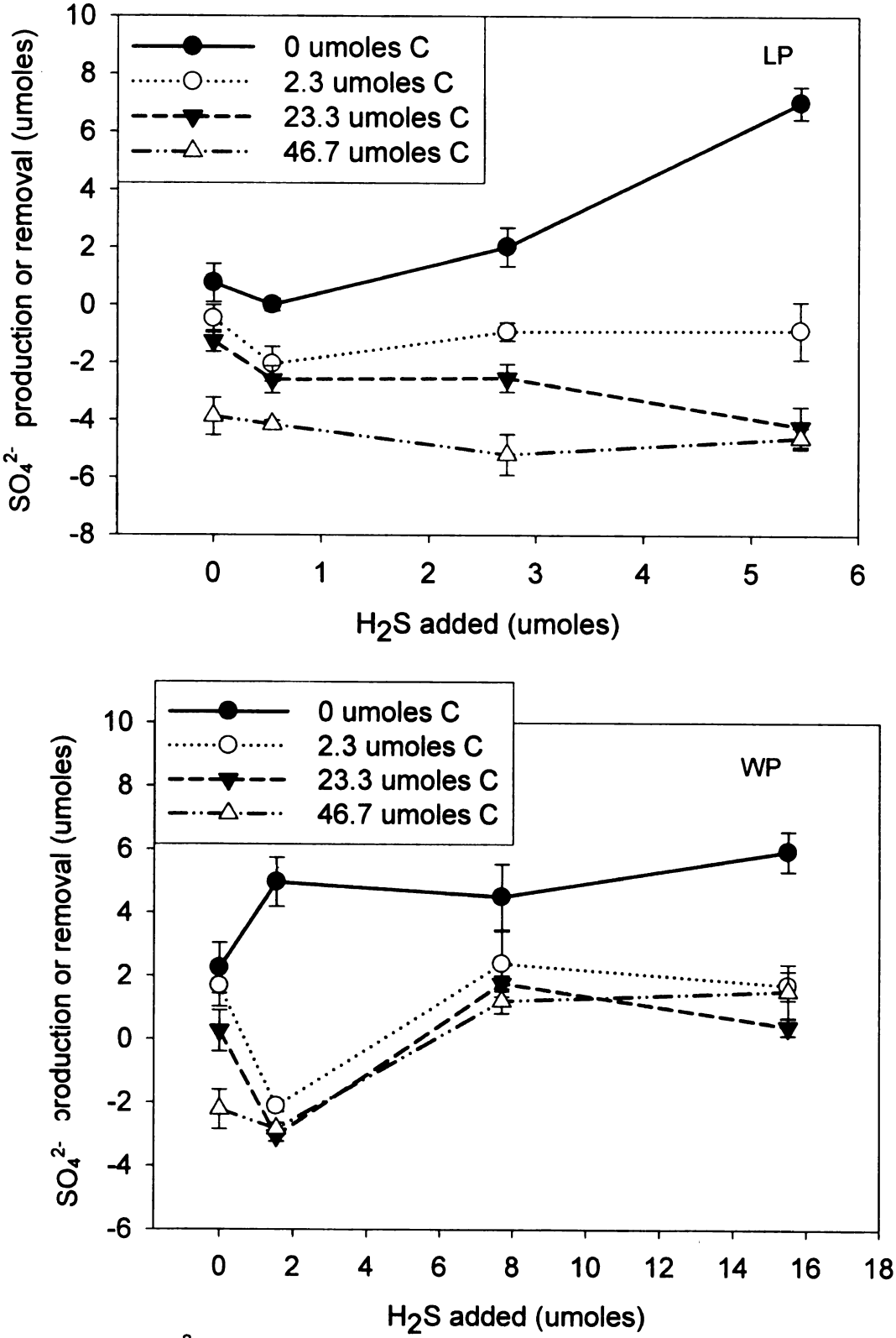


Figure 7: SO_4^{2-} flux for Loosestrife Fen (top) and Windmill Pond (bottom) across a gradient of OC and H_2S.

Table 1. Analysis of variance for individual response variables (NO_3^- and H_2S removal; NH_4^+, N_2, N_2O, NO_2^-, SO_4^{2-} flux) by factor (H_2S and OC) from LP (high ambient H_2S). ω^2 is the fit of a factor to the ANOVA model, also called magnitude of effect or effect size.

Response	Factor	Sum of Squares	Degrees of freedom	Mean square error	F ratio	p value	variance component	ω^2
NO_3^- removal	C	7.875	3	2.625	2.6	0.064	0.059	3.6
	S	27.687	3	9.229	8.9	0.000	0.307	18.5
	C*S	30.005	9	3.334	3.2	0.003	0.259	15.6
	Error	64.867	63	1.030			1.030	62.2
NH_4^+ flux	C	5.2	3	1.720	4.2	0.009	0.006	1.0
	S	8.5	3	2.837	6.9	0.000	0.104	17.0
	C*S	7.7	9	0.860	2.1	0.041	0.097	15.8
	Error	25.9	64	0.406			0.406	66.2
N_2 flux	C	2.708	3	0.903	6.7	0.001	0.029	13.3
	S	4.484	3	1.495	11.1	0.000	0.051	23.6
	C*S	1.332	9	0.148	1.1	0.379	0.001	0.5
	Error	8.649	64	0.135			0.135	62.6

Response	Factor	Sum of Squares	Degrees of freedom	Mean square error	F ratio	p value	variance component	ω^2
N_2O flux	C	0.000024	3	0.0000081	6.5	0.0007	0.0000026	2.9
	S	0.000082	3	0.0000274	21.9	<0.0001	0.0000018	2.1
	C*S	0.000054	9	0.0000061	4.9	<0.0001	0.0000063	7.3
	Error	0.000241	61	0.0000761			0.0000868	87.7
NO_2^- flux	C	1.381	3	0.460	8.1	0.000	0.015	5.9
	S	12.308	3	4.103	72.4	0.000	0.152	59.8
	C*S	2.935	9	0.326	5.8	0.000	0.030	11.8
	Error	3.630	64	0.057			0.057	22.4
H_2S removal	C	7.673	3	2.558	99.7	0.000	0.095	3.2
	S	222.441	3	74.147	2890.4	0.000	2.779	93.1
	C*S	7.028	9	0.781	30.4	0.000	0.085	2.8
	Error	1.642	64	0.026			0.026	0.9
SO_4^{2-} flux	C	308.195	3	102.732	106.6	0.000	3.816	63.6
	S	14.835	3	4.945	5.1	0.003	0.149	2.5
	C*S	94.560	9	10.507	10.9	0.000	1.074	17.9
	Error	60.702	63	0.964			0.964	16.1

Table 1: cont'd.

Table 2. Analysis of variance for individual response variables (H_2S removal; NH_4^+, N_2, N_2O, SO_4^{2-} flux) by factor (H_2S and OC) from WP (low ambient H_2S). ω^2 is the fit of a factor to the ANOVA model, also called the magnitude of effect. NO_2^- was not produced during WP experiments, so it is not a response variable in these analyses. Also, because NO_3^- was gone in all treatments at the end of the experiment, NO_3^- removal is not considered as a response variable. Abbreviations: SS = sum of squares, DF = degrees of freedom, MSE = mean square error, VC = variance component.

Response	Factor	SS	DF	MSE	F ratio	p value	VC	ω^2
NH_4^+ flux	C	19.2	3	6.4	4.9	0.004	0.19	9.7
	S	27.5	3	9.2	6.9	0.000	0.30	15.0
	C*S	25.0	9	2.8	2.1	0.041	0.17	8.4
	Error	81.4	64	1.3			1.31	66.9
N_2 flux	C	14.9	3	5.0	13.3	0.000	0.18	21.4
	S	26.0	3	8.7	33.5	0.000	0.32	28.2
	C*S	8.3	9	0.9	3.6	0.001	0.09	9.1
	Error	15.5	64	0.2			0.25	31.3
N_2O flux	C	1.2×10^{-4}	3	4×10^{-5}	3.4	0.024	2×10^{-5}	3.1
	S	2.9×10^{-4}	3	9.9×10^{-4}	8.3	0.001	1×10^{-5}	1.6
	C*S	3.3×10^{-4}	9	4×10^{-5}	3.1	0.004	6×10^{-5}	7.4
	Error	6.9×10^{-4}	59	6.7×10^{-4}			6.7×10^{-4}	87.8
H_2S removal	C	107.8	3	35.9	56.4	0.000	1.3	4.2
	S	2168.1	3	722.7	1133.1	0.000	27.0	86.2
	C*S	195.0	9	21.6	33.9	0.000	2.3	7.5
	Error	40.8	64	0.6			0.6	2.0
SO_4^{2-} flux	C	182.7	3	60.9	51.7	0.000	2.2	44.7
	S	87.1	3	29.1	24.7	0.000	1.0	20.9
	C*S	54.7	9	6.1	5.2	0.000	0.5	11.0
	Error	75.3	64	1.2			1.1	23.5

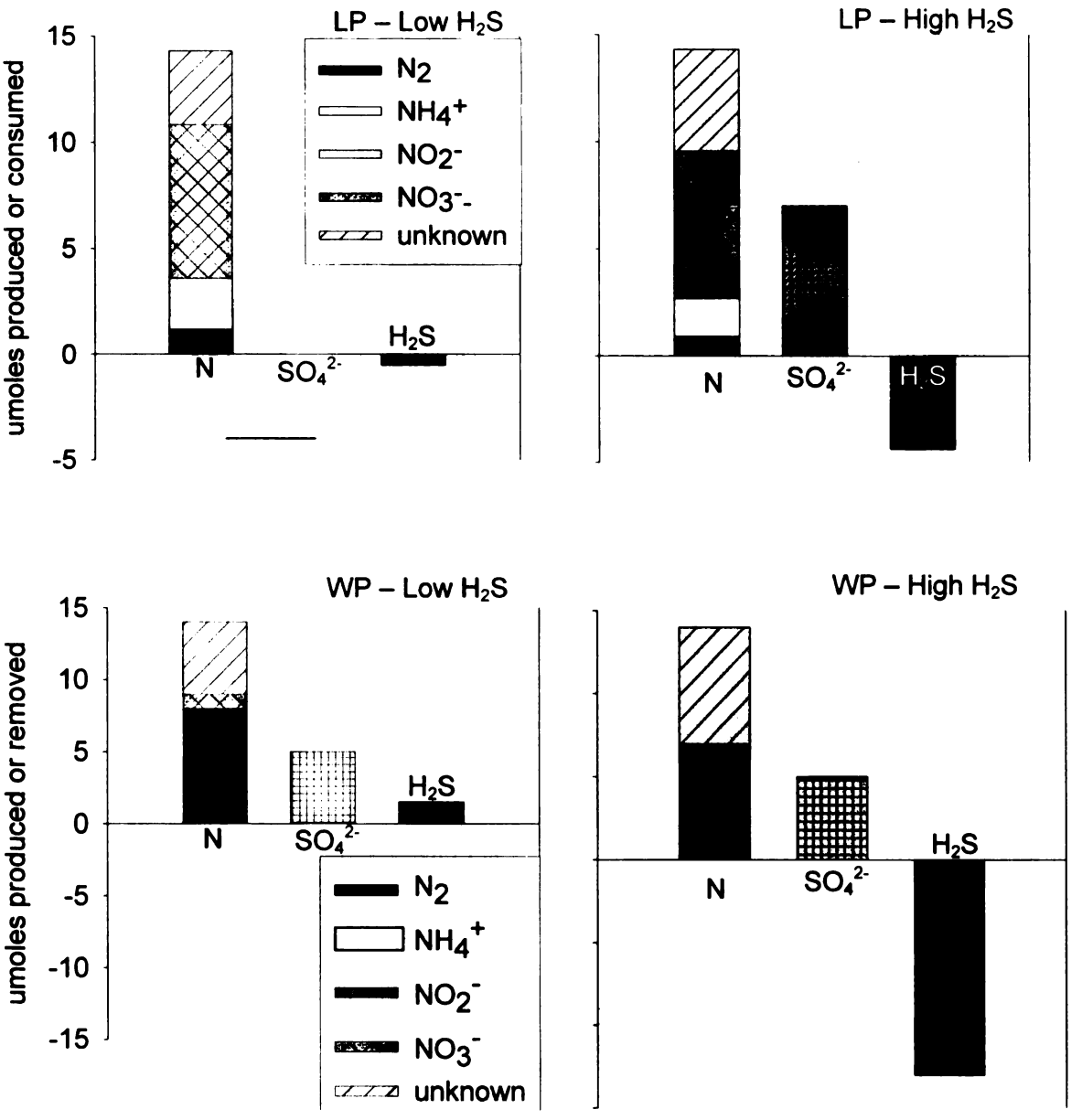


Figure 8: Fractions of each N end-product from the gradient assays. These are means across the H_2S only gradient (i.e., they do not include treatments with added OC). Total N flux is based on the NO_3^- removal that was observed. Also shown for comparison are the SO_4^{2-} and H_2S fluxes. Quantities added: LP low H_2S (0.5 µmoles) , LP high H_2S (5.5 µmoles), WP low H_2S (1.5 µmoles), and WP high H_2S (15.5 µmoles).

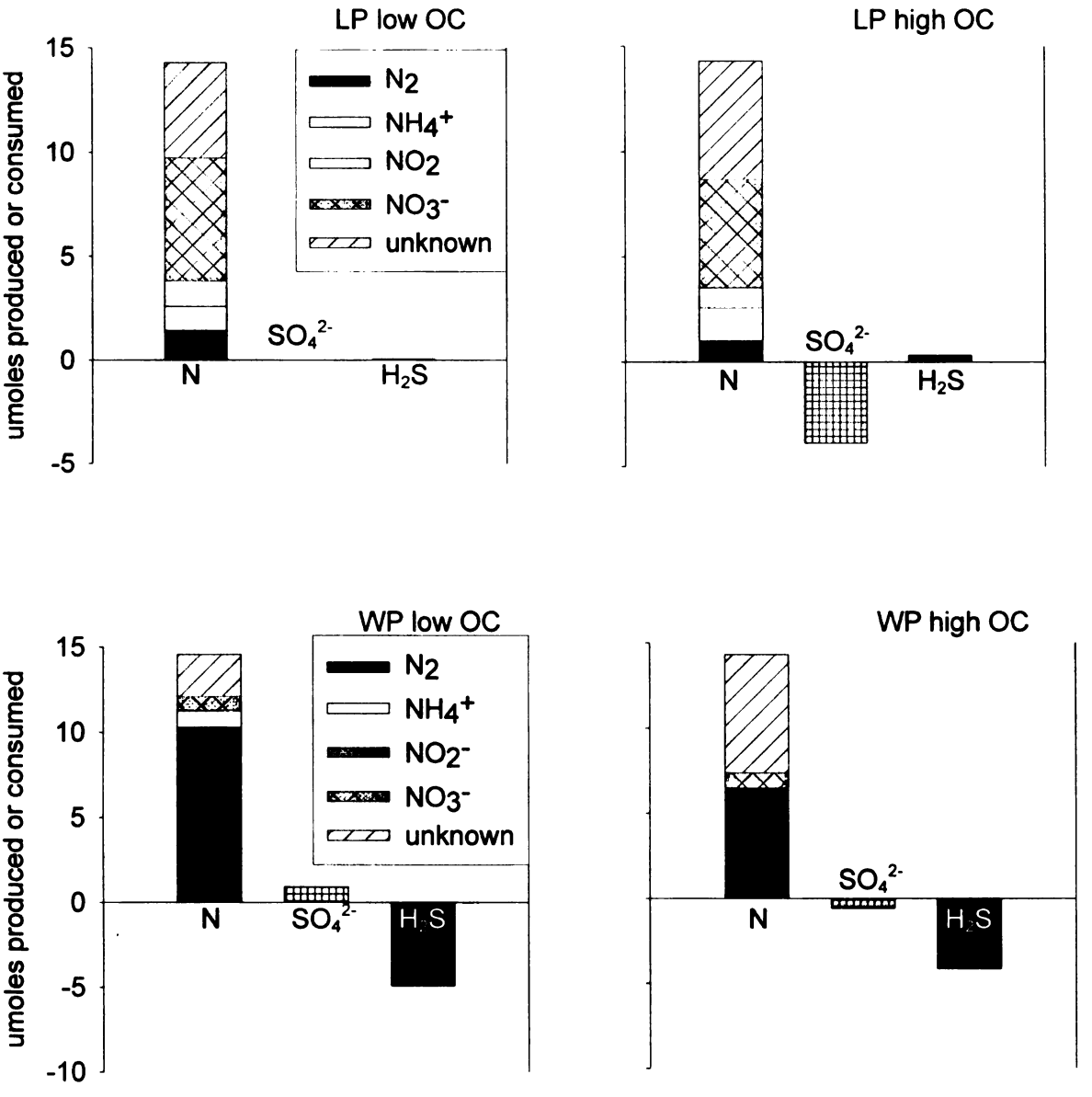


Figure 9: Fractions of each N end-product from the gradient assays. These are means across OC treatments (i.e., they do not include H_2S in the averages). Total N flux is based on the NO_3^- removal that was observed. Also shown for comparison are the SO_4^{2-} and H_2S fluxes. Quantities added: LP low OC (2.3 µmoles), LP high OC (46.7 µmoles), WP low OC (2.3 µmoles), and WP high OC (46.7 µmoles). Positive fluxes indicate net production.

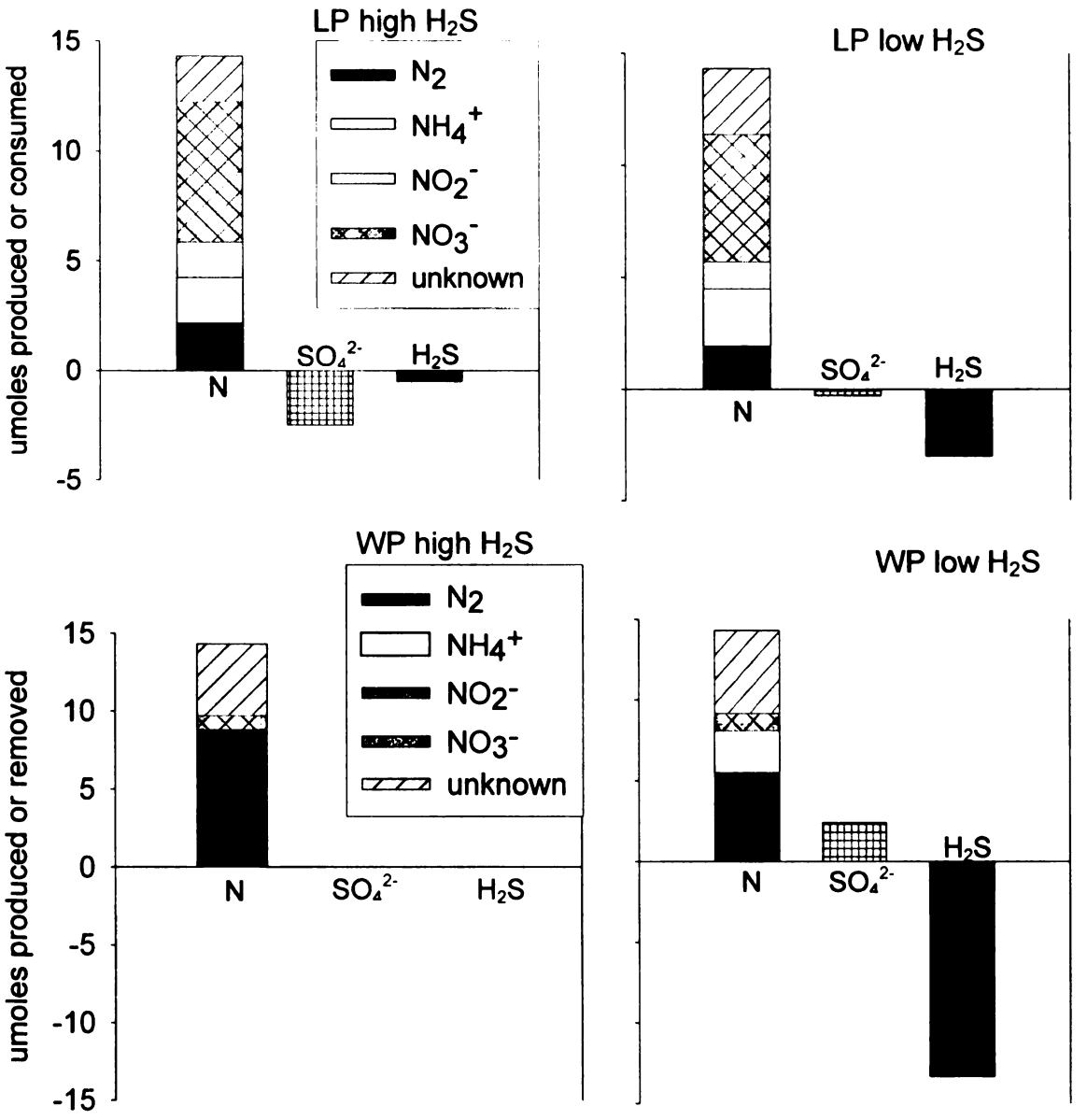


Figure 10: Fractions of each N end-product from the gradient assays. These are means across all treatments (i.e., all levels of OC in a given H_2S treatment were included). Total N flux is based on the NO_3^- removal that was observed. Also shown for comparison are the SO_4^{2-} and H_2S fluxes. Quantities added: LP low H_2S (0.5 µmoles), LP high H_2S (5.5 µmoles), WP low H_2S (1.5 µmoles), and WP high H_2S (15.5 µmoles).

In sediments from both sites, NO_3^- was converted to both N_2 and NH_4^+, though under different circumstances. For example, increasing amounts of NH_4^+ production occurred in LP with increasing levels of H_2S only (no OC interaction; Figure 8, top two panels). However, in WP, NH_4^+ production only occurred when H_2S was added together with OC. For an illustration of this, compare the bottom two panels of Figures 8 and 10. In Figure 8, across the H_2S-only gradient (no OC in averages), no NH_4^+ production occurred. However, Figure 10 shows that adding high levels of H_2S together with OC produced NH_4^+. Adding more OC resulted in an increase in NH_4^+ production in LP (Figure 9, top panels), though the increase was not as great as the increase in NH_4^+ between the low and high H_2S treatments. Adding OC in WP, however, resulted in a decrease in NH_4^+ production (Figure 9, bottom panels). Therefore, conversion to NH_4^+ was a significant NO_3^- sink in both sites but was evidently subject to different controls.

WP consistently had more of the added NO_3^- converted to N_2 compared to LP (Figures 8-10). Adding more H_2S decreased the N_2 produced in both sites (Figures 8 and 10). Adding more H_2S also decreased NO_2^- production, which was only generated in significant amounts in the LP sediments (Figure 8). Adding more OC increased NO_2^- production in LP (Figure 9).

Both LP and WP also had evidence of H_2S consumption and SO_4^{2-} production, but again, the expression of these responses was dependent on H_2S and OC interactions. In LP H_2S + NO_3^- treatments, all of the added H_2S was consumed. However, adding OC tended to decrease H_2S removal (compare top right panels of Figures 8 and 10). The interactions between OC and H_2S are

also apparent in the SO_4^{2-} flux patterns. In LP, adding more H_2S resulted in an increase in SO_4^{2-} (Figure 8, top two panels). However, this response disappeared when H_2S was added together with OC (Figure 10, top two panels) or in the OC only gradient (Figure 9). This pattern of OC inhibiting SO_4^{2-} production was also seen in WP. WP sediments produced SO_4^{2-} in both the low and high H_2S + NO_3^- treatments (Figure 8, bottom panels), but this production was reduced when OC was added to the treatments (Figure 10, bottom panels).

Time-course assays

The time-course assays were designed to examine the dynamics and transient changes in NO_3^- transformations as added NO_3^- is removed. As in the gradient assays, NO_3^- was removed much faster from WP than from LP (Figure 11; note difference in x-axis time scales). In WP, ~75% of the added NO_3^- was gone after 12 h; in LP, ~75% was gone in 24 h. In LP, the H_2S + NO_3^- treatment lagged behind the other treatments in NO_3^- removal early in the experiment, but nearly all of the added NO_3^- was gone by the last sampling time. The treatments wherein either OC or H_2S were added had more NO_3^- removed by the final sampling time than did the NO_3^- only treatment. In LP, NO_3^- removal in the OC treatment was greater than in the NO_3^- only treatment only at the final time point, and LP sediments tended to have higher amounts of NO_3^- removed than when NO_3^- was added alone (Table 3). H_2S significantly decreased NO_3^- removal early in the experiment, but by the last sampling point, the H_2S additions significantly

increased NO_3^- removal (Table 3). The OC treatment consistently produced more NO_3^- removal in LP compared to the H_2S treatment.

Adding OC or H_2S, together or separately, stimulated NO_3^- removal in the WP sediments as well; these treatments consistently removed nitrate faster than did the NO_3^- only treatment (Figure 11). Both the OC and H_2S treatments increased NO_3^- removal compared to the NO_3^- only treatments at most time points, and were never significantly different from each other in their effects on NO_3^- removal (Table 4).

The OC and H_2S treatments had different effects on NH_4^+ fluxes in the two sediments (Figure 12). In LP, neither the H_2S nor OC treatments differed significantly from the NO_3^- treatment in their NH_4^+ production (Table 3). They did, however, generally differ significantly from each other because the H_2S treatment stimulated NH_4^+ production, whereas the OC treatment stimulated NH_4^+ removal. Additionally, the H_2S treatment steadily increased in NH_4^+ production over time, whereas the OC treatment did not show any production of NH_4^+ until the 48-hr sampling time. The results from LP in these assays are similar in the magnitude of NH_4^+ production compared to the LP gradient experiments (Figure 2). In the WP timed assays, NH_4^+ was not produced, as was seen in the WP gradient assays (Figure 2), but was removed or did not change. Addition of H_2S generally produced more NH_4^+ than did NO_3^- alone, whereas the addition of OC stimulated NH_4^+ removal compared to NO_3^- only (Table 4).

N_2 was produced in nearly all treatments in both LP and WP (Figure 13). In the LP sediments, addition of H_2S significantly stimulated N_2 production

compared to the NO_3^- only treatment (Table 3). The OC treatment stimulated N_2 production, which was significantly greater than that in the NO_3^- only treatment only at the 2nd sampling point. Generally, the H_2S treatment produced more N_2 than did the OC treatment across sampling times (Table 3). In WP, the H_2S treatment initially had greater N_2 production, but had the lowest N_2 production by the last sampling time (Table 4). In WP the addition of OC and H_2S significantly stimulated N_2 at the second sampling time, but then significantly decreased it compared to NO_3^- at the final sampling point, making it difficult to discern which had the greater effect. However, the OC treatment generally stimulated N_2 production to a greater degree than the H_2S treatment (Table 4).

N_2O production was low in both LP and WP (Figure 14), and as in the gradient assays, accounted for a very small fraction of the overall NO_3^- removal. LP produced more N_2O than WP (as was also seen in the gradient assays; Figure 4). Neither H_2S nor OC significantly affected N_2O production compared to the NO_3^- only treatment (Table 3). Treatments with added H_2S typically had greater N_2O production in LP sediments (Table 3). WP had the opposite trend, wherein N_2O was produced early on in the experiment, and more production occurred when treatments included H_2S. OC never had a significant affect on N_2O production in WP (Table 4).

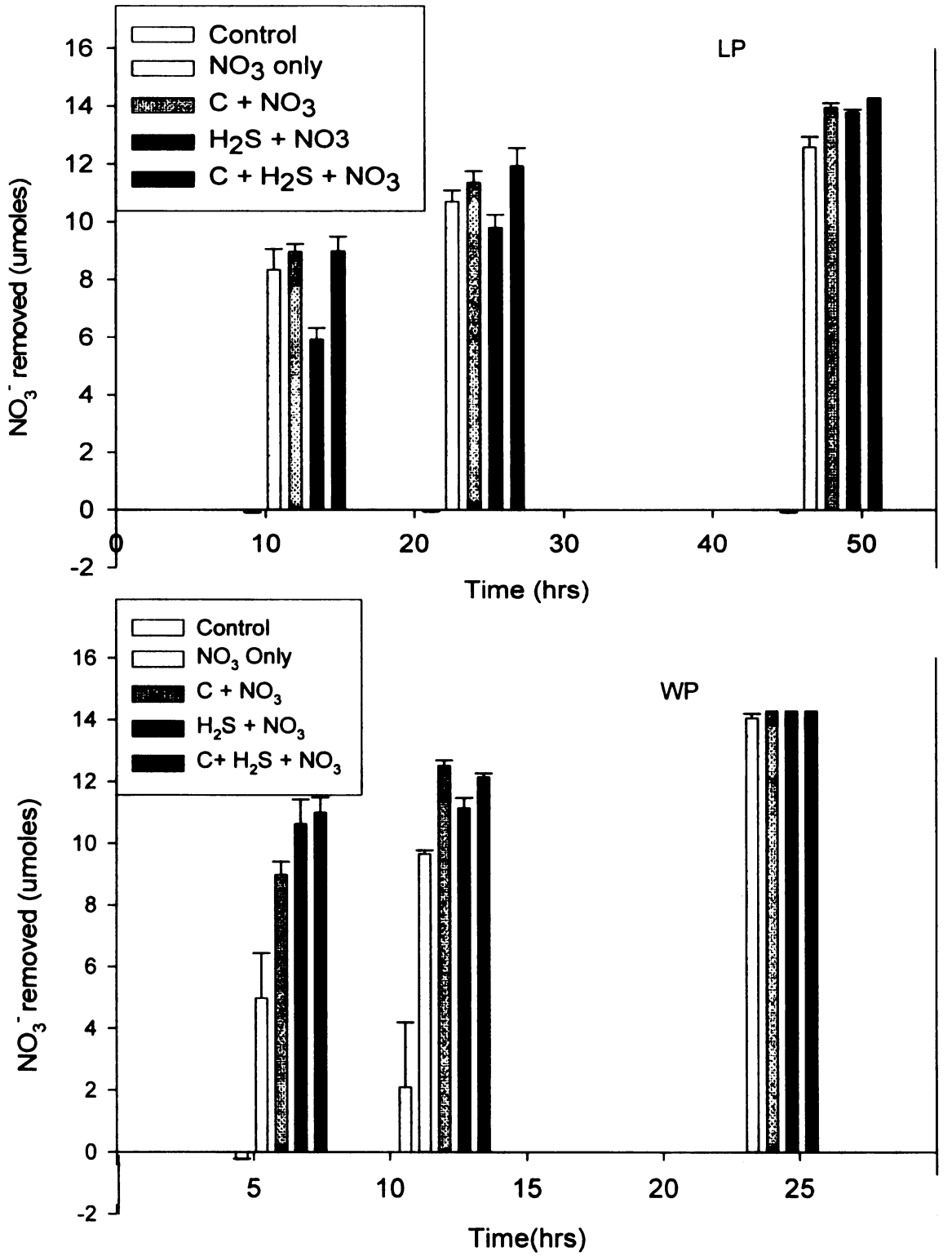


Figure 11: Nitrate removal over time in both Loosestrife Fen (LP) and Windmill Pond (WP). 14.3 µmoles of NO_3^- were added to each treatment at the start of the experiment.

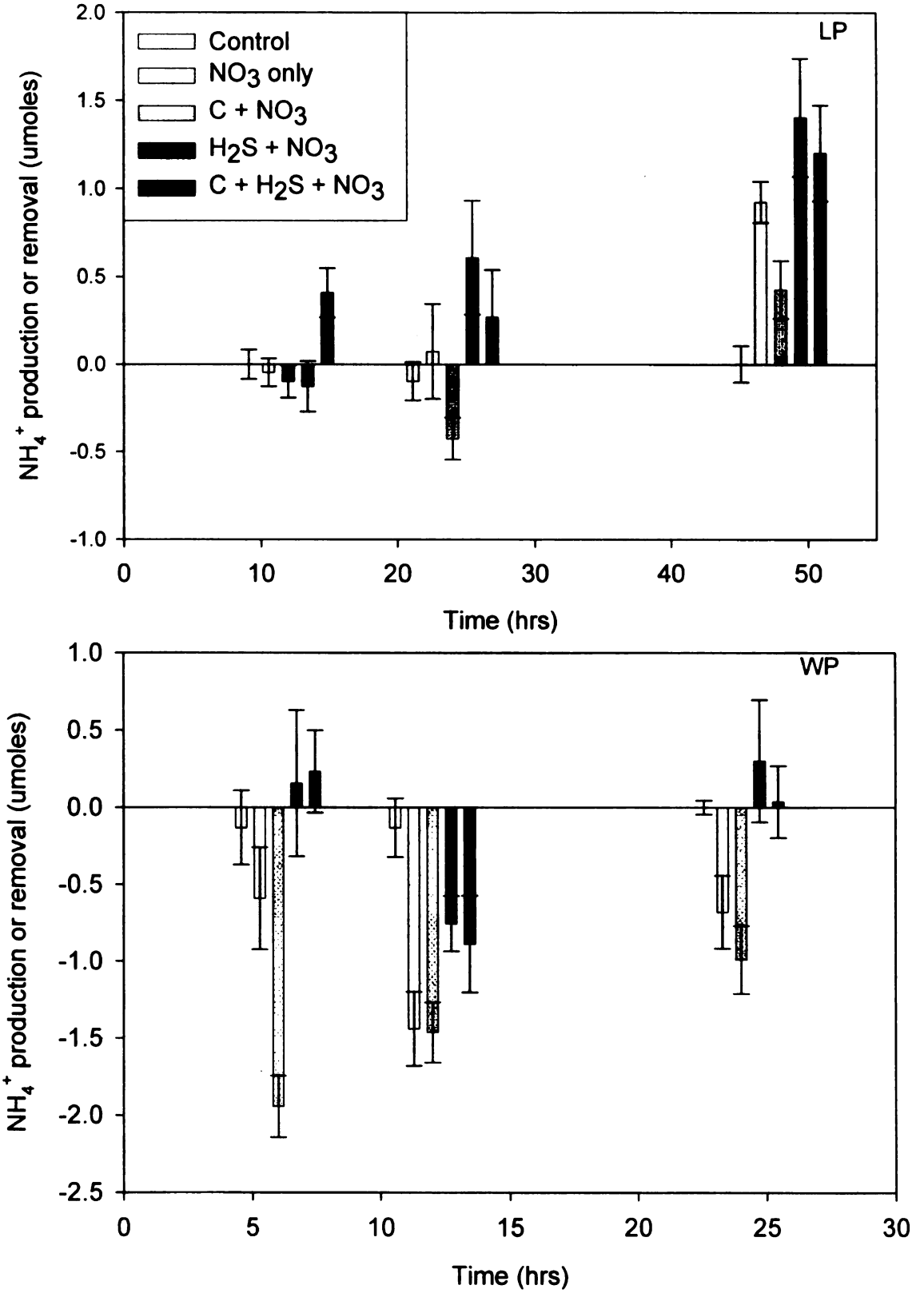


Figure 12: Ammonium flux over time in both Loosestrife Fen (LP) and Windmill Pond (WP). 14.3 µmoles of NO_3^- was added to each treatment at the start of the experiment.

NO_2^- was produced in both LP and WP during the time series assays (Figure 15), in contrast to the gradient assays where only LP showed production (Figure 5). LP produced a much greater amount of NO_2^- throughout the experiment, whereas NO_2^- production in WP was two orders of magnitude lower. In the first sampling at LP, the NO_3^--only treatment produced more NO_2^- than the OC or H_2S treatments, though the effect was not statistically significant. In the final two time points at LP, the H_2S treatment significantly stimulated NO_2^- production compared to NO_3^- only and compared to the OC treatment (Table 3). In WP, both H_2S and OC stimulated NO_2^- production, particularly early in the experiment where both treatments had greater NO_2^- production than NO_3^- alone. NO_2^- production declined over time. At the final sampling point, neither treatment was statistically different from the NO_3^- treatment, though the H_2S treatment had significantly higher NO_2^- than the OC treatment (Table 4).

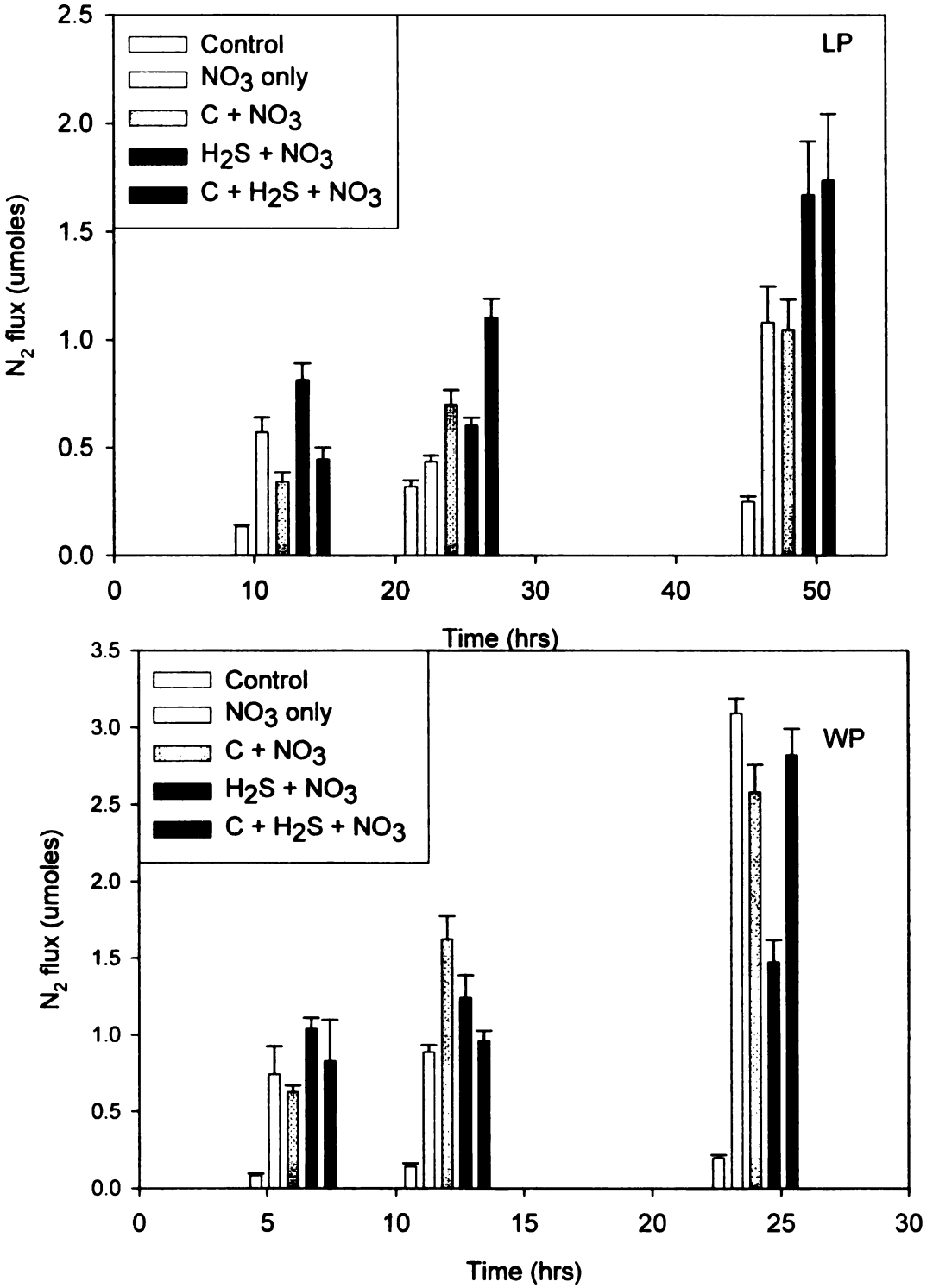


Figure 13: N_2 production over time in both Loosestrife Fen (LP) and Windmill Pond (WP). 14.3 µmoles of NO_3^- was added to each treatment at the start of the experiment. Controls received no NO_3^-, and all vials were maintained underwater to ensure that atmospheric N_2 and O_2 did not leak into the vials.

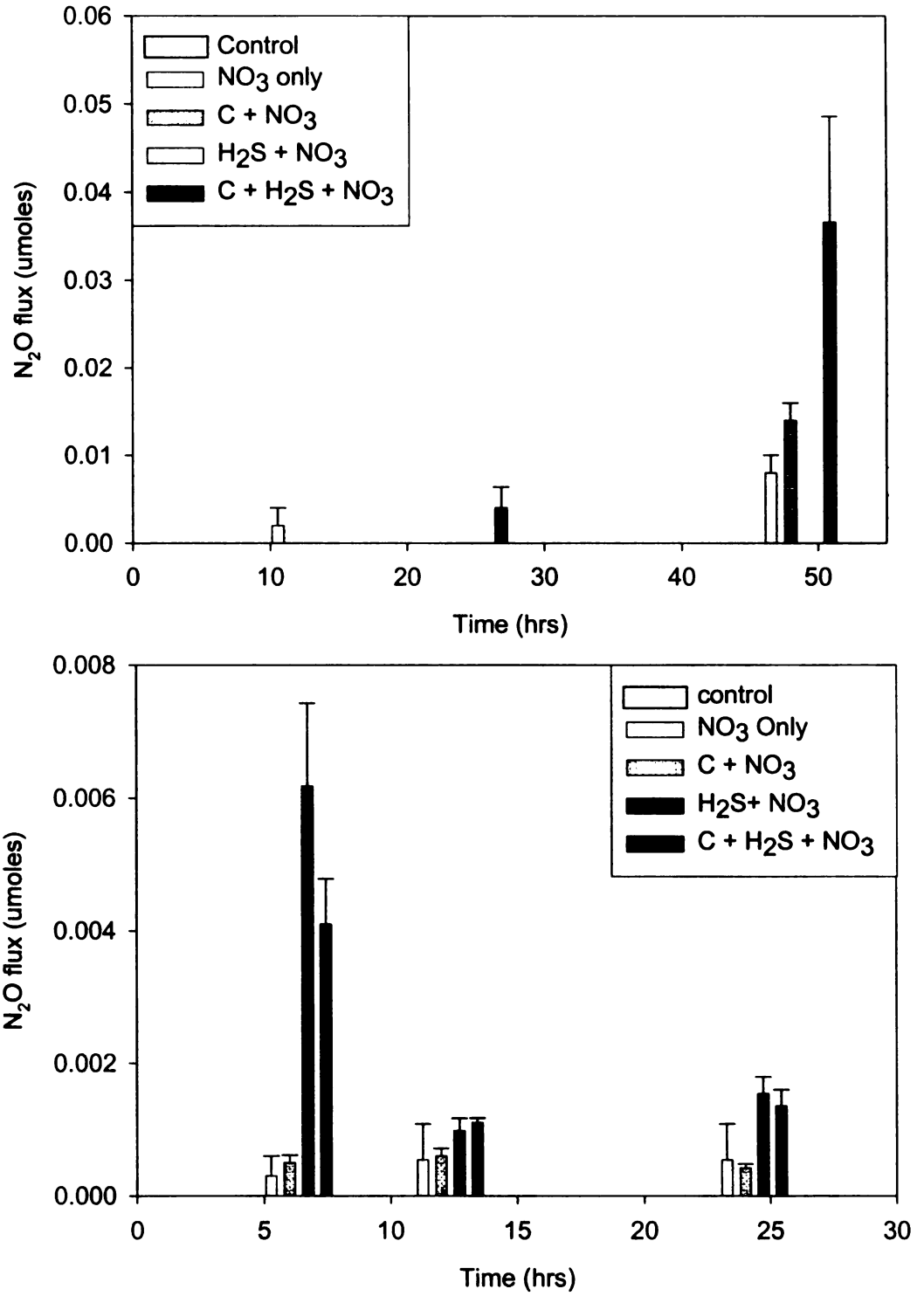


Figure 14: N_2O production over time in both Loosestrife Fen (LP) and Windmill Pond (WP). 14.3 µmoles of NO_3^- was added to each treatment at the start of the experiment. Controls received no NO_3^-, and all vials were maintained underwater to ensure atmospheric N_2 and O_2 did not leak into the vials.

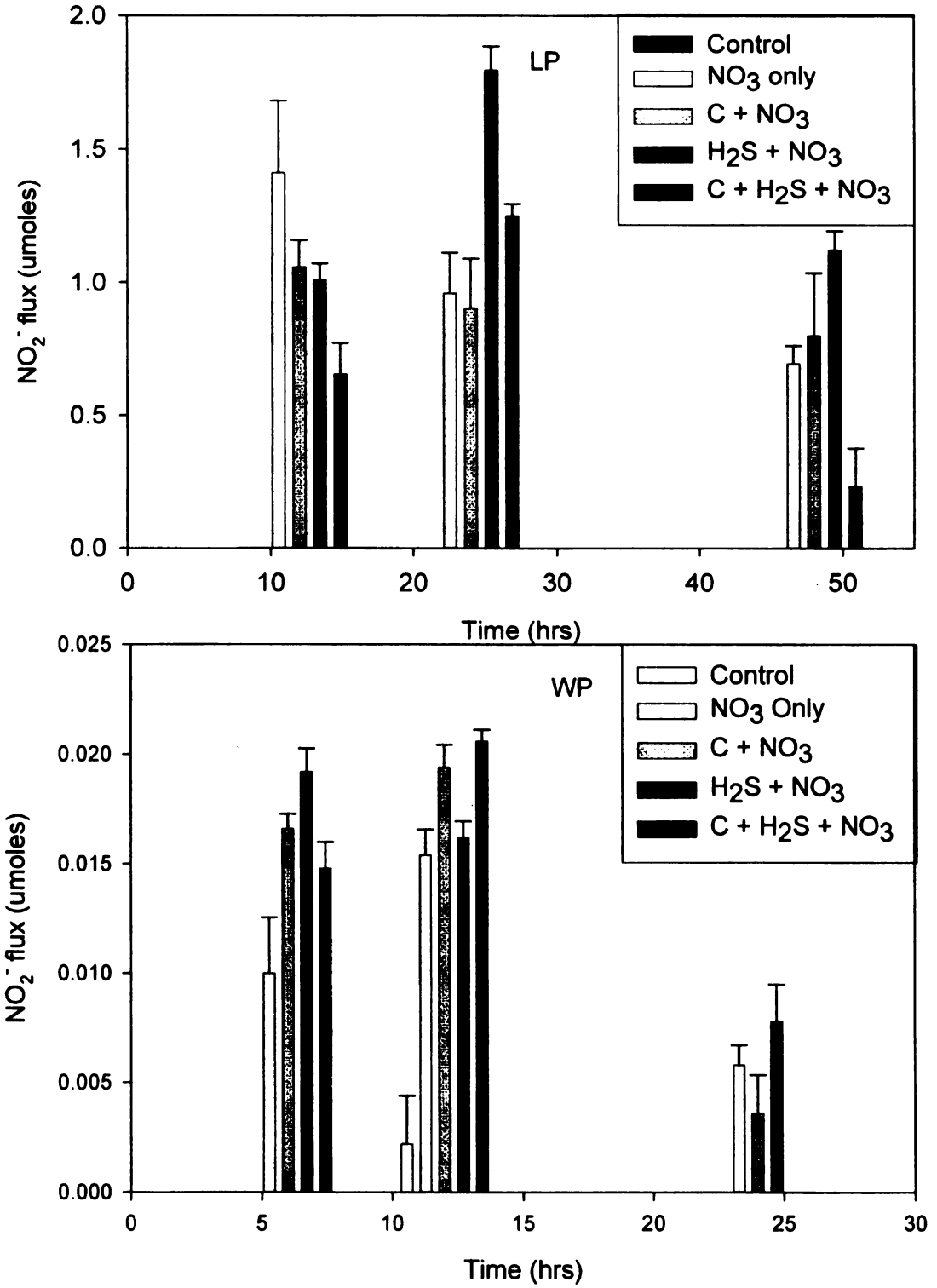


Figure 15: NO_2^- production over time in both Loosestrife Fen (LP) and Windmill Pond (WP). 14.3 µmoles of NO_3^- was added to each treatment at the start of the experiment.

SO_4^{2-} flux was also affected differently by the various treatments in sediments from both sites (Figure 16). Both OC and H_2S significantly affected SO_4^{2-} flux at the second two sampling points, though H_2S increased SO_4^{2-} and OC decreased SO_4^{2-}, as was also seen in the gradient experiments (Table 3). In WP, neither H_2S nor OC had a consistent effect on SO_4^{2-} flux (Table 4). At 24 hrs, however, it is clear that treatments with H_2S added had slightly higher final SO_4^{2-} concentrations than the NO_3^- only or OC treatments (Table 4). However, I do not see the clear increase in SO_4^{2-} that was seen in LP. These trends are consistent with the results from the gradient assays (Figure 7). In both LP and WP, the controls (no OC, NO_3^- or H_2S) removed SO_4^{2-} at the first sampling point because all of the other treatments had NO_3^- added, which would have inhibited SO_4^{2-} reduction (Figure 16). The decreased SO_4^{2-} concentration at the first sampling point is due to SO_4^{2-} reduction that has already occurred by that time.

This sulfate reduction is also evident in the H_2S production in the same controls (Figure 17). The NO_3^- added to the other treatments suppressed this reaction until the added NO_3^- was largely removed. In LP, nearly all of the added H_2S was gone by the first sampling point. By the 2nd sampling point some SO_4^{2-} reduction may have been occurring in some of the treatments. It is clear that across all treatments in both sites, SO_4^{2-} reduction had commenced by the last sampling point leading to an increase in H_2S by the end of the experiment, particularly in the OC and NO_3^- only treatments. This pattern of a rapid decrease in the added H_2S, followed by an increase after NO_3^- is depleted is different than the steady increase in H_2S concentration seen in the controls.

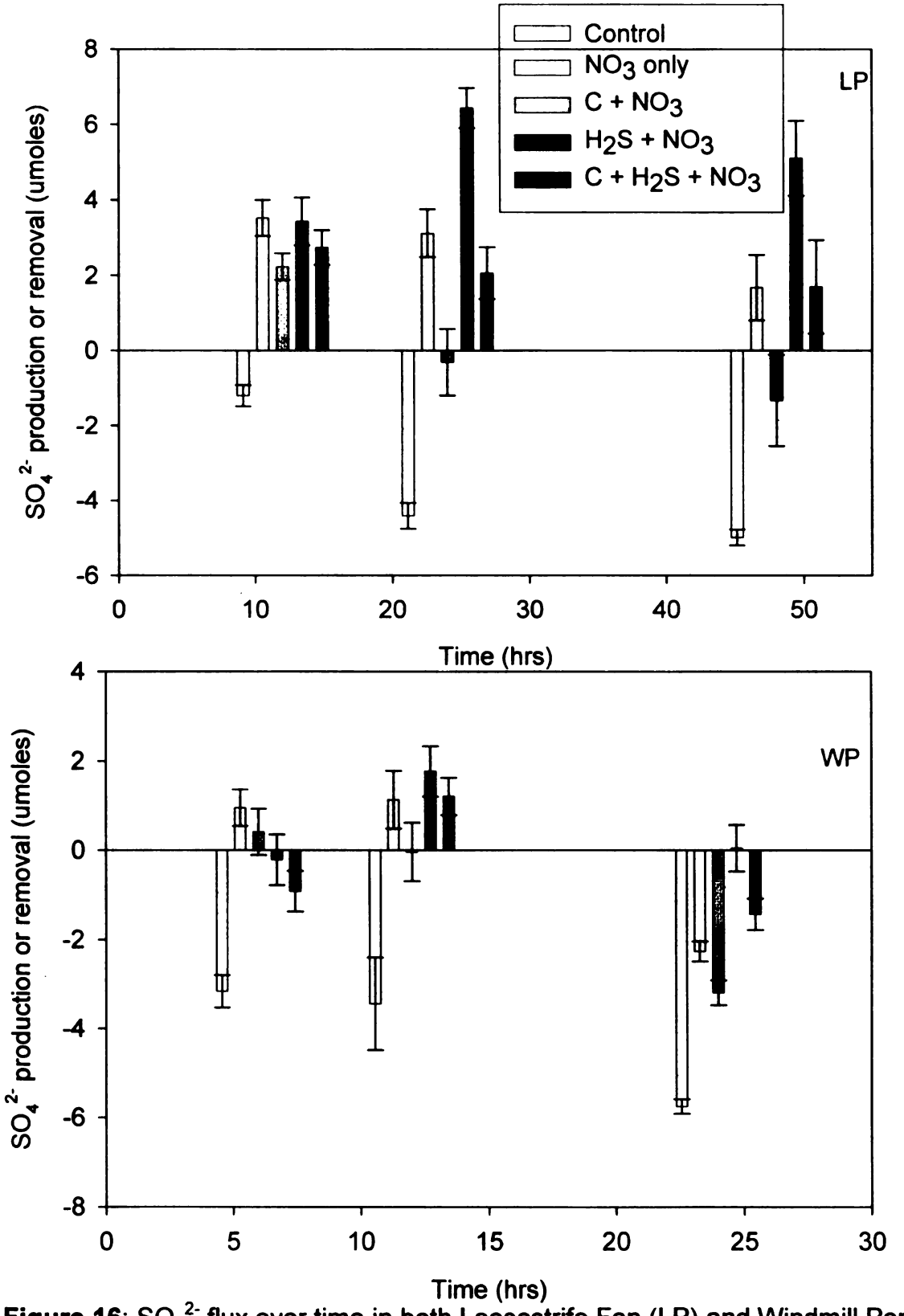


Figure 16: SO_4^{2-} flux over time in both Loosestrife Fen (LP) and Windmill Pond (WP). Controls received no NO_3^-, H_2S or OC, but were maintained to evaluate how SO_4^{2-} changed in the absence of these factors over time.

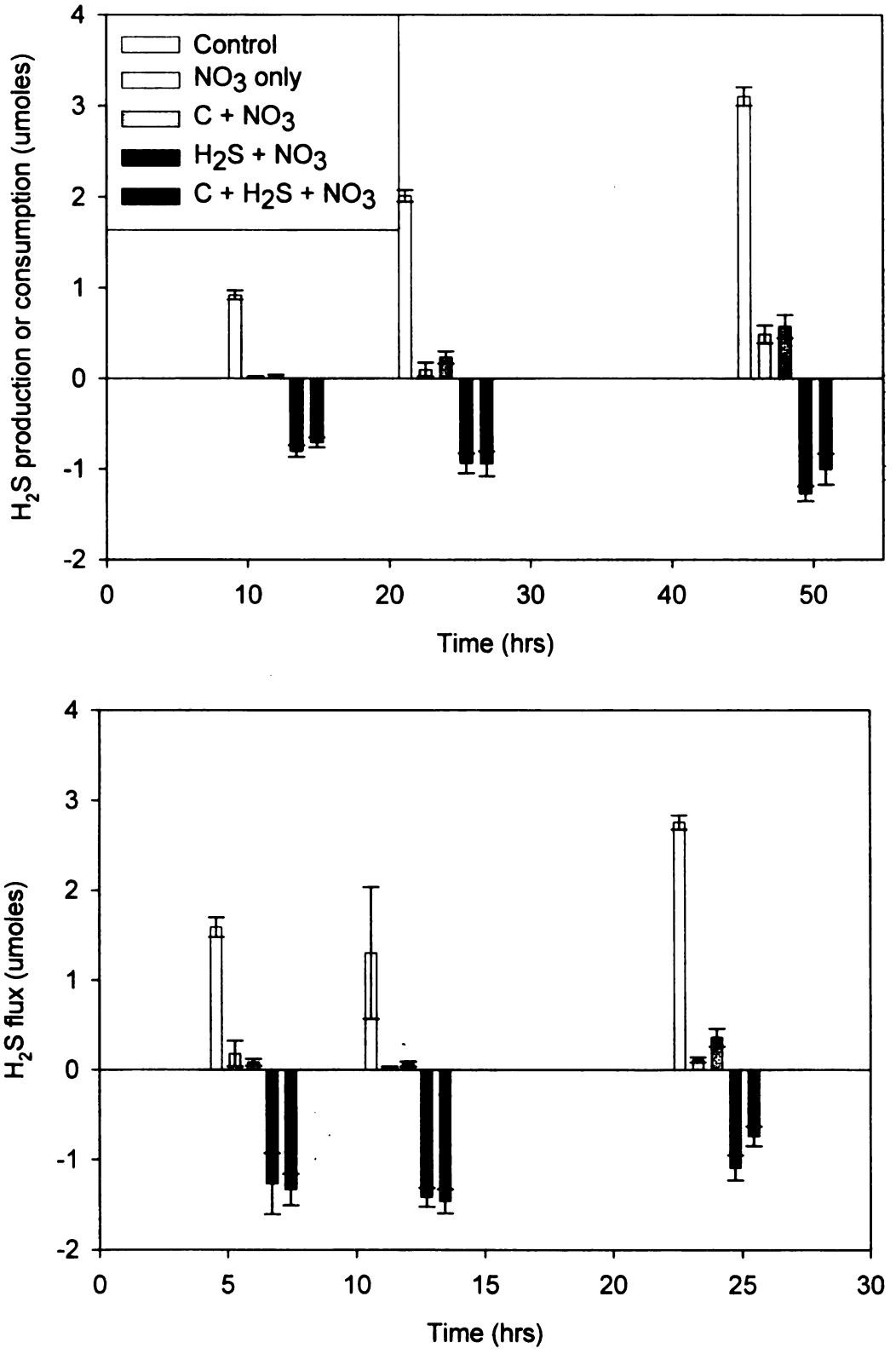


Figure 17: H_2S flux over time in both Loosestrife Fen (LP) and Windmill Pond (WP). Controls received no NO_3^-, H_2S or OC, but were maintained to evaluate how H_2S changed in the absence of these factors over time.

Table 3. Independent contrasts within the analysis of variance (ANOVA) to compare treatments across time in LP. The estimated flux (Estimate) is in µmoles, and indicates the difference between the two treatments. A positive flux denotes that the effect of the first factor listed (e.g, H_2S in H_2S vs. NO_3^- only) on that flux was greater than the second factor. NS = not significant.

Response	Factor	Hour	$F_{1,20}$	P	Estimate (µmoles)
NO_3^- removal	H_2S vs. NO_3^- only	12	14.9	0.0010	-2.43
	H_2S vs. NO_3^- only	24	3.51	NS	-0.89
	H_2S vs. NO_3^- only	48	21.5	0.0020	1.20
	OC vs. NO_3^- only	12	0.98	NS	0.62
	OC vs. NO_3^- only	24	1.85	NS	0.65
	OC vs. NO_3^- only	48	28.2	<0.0001	1.38
	H_2S vs. OC	12	23.49	<0.0001	-3.05
	H_2S vs. OC	24	10.44	0.0042	-1.55
	H_2S vs. OC	48	0.45	NS	-0.17
NH_4^+ flux	H_2S vs. NO_3^- only	12	0.26	NS	-0.08
	H_2S vs. NO_3^- only	24	2.57	NS	0.53
	H_2S vs. NO_3^- only	48	2.46	NS	0.48
	OC vs. NO_3^- only	12	0.11	NS	-0.05
	OC vs. NO_3^- only	24	2.24	NS	-0.49
	OC vs. NO_3^- only	48	2.65	NS	-0.49
	H_2S vs. OC	12	0.03	NS	-0.03
	H_2S vs. OC	24	9.60	0.0057	1.03
	H_2S vs. OC	48	10.23	0.0047	0.98
N_2 flux	H_2S vs. NO_3^- only	12	9.14	0.0073	0.24
	H_2S vs. NO_3^- only	24	6.50	0.0202	0.17
	H_2S vs. NO_3^- only	48	5.69	0.0282	0.59
	OC vs. NO_3^- only	12	8.26	0.0101	-0.23
	OC vs. NO_3^- only	24	16.04	0.0008	0.26
	OC vs. NO_3^- only	48	0.02	NS	-0.03
	H_2S vs. OC	12	34.78	<0.0001	0.47
	H_2S vs. OC	24	2.12	NS	-0.10
	H_2S vs. OC	48	6.37	0.0212	0.62

Table 3: cont'd.

Response	Factor	Hour	$F_{1,20}$	P	Estimate (µmoles)
NO_2^- flux	H_2S vs. NO_3^- only	12	4.04	NS	-0.40
	H_2S vs. NO_3^- only	24	25.76	<0.0001	0.84
	H_2S vs. NO_3^- only	48	7.37	0.0142	0.43
	OC vs. NO_3^- only	12	3.13	NS	-0.35
	OC vs. NO_3^- only	24	0.12	NS	-0.06
	OC vs. NO_3^- only	48	0.40	NS	0.11
	H_2S vs. OC	12	0.06	NS	-0.05
	H_2S vs. OC	24	29.32	<0.0001	0.89
	H_2S vs. OC	48	3.71	NS	0.32
SO_4^{2-} flux	H_2S vs. NO_3^- only	12	0.02	NS	-0.09
	H_2S vs. NO_3^- only	24	13.38	0.0016	3.32
	H_2S vs. NO_3^- only	48	7.34	0.0144	3.43
	OC vs. NO_3^- only	12	3.97	NS	-1.29
	OC vs. NO_3^- only	24	14.30	0.0012	-3.43
	OC vs. NO_3^- only	48	5.00	0.0382	-3.00
	H_2S vs. OC	12	3.42	NS	1.19
	H_2S vs. OC	24	55.33	<0.0001	6.74
	H_2S vs. OC	48	22.95	0.0001	6.43

Table 4. Independent contrasts within the analysis of variance (ANOVA) to compare treatments across time in LP. The estimated flux (Estimate) is in µmoles, and indicates the difference between the two treatments. A positive flux denotes that the effect of the first factor listed (e.g, H_2S in H_2S vs. NO_3^- only) on that flux was greater than the second factor. NS = not significant.

Response	Factor	Hour	$F_{1,20}$	P	Estimate (µmoles)
NO_3^- removal	H_2S vs. NO_3^- only	6	25.45	<0.0001	5.66
	H_2S vs. NO_3^- only	12	1.21	NS	1.48
	H_2S vs. NO_3^- only	24	6.45	0.0195	0.22
	OC vs. NO_3^- only	6	12.79	0.0019	4.01
	OC vs. NO_3^- only	12	4.46	0.0474	2.85
	OC vs. NO_3^- only	24	6.45	0.0195	0.22
	H_2S vs. OC	6	2.16	NS	1.65
	H_2S vs. OC	12	1.03	NS	-1.37
	H_2S vs. OC	24	0.00	NS	0.00
NH_4^+ flux	H_2S vs. NO_3^- only	6	2.78	NS	0.75
	H_2S vs. NO_3^- only	12	4.44	0.0480	0.68
	H_2S vs. NO_3^- only	24	7.54	0.0125	0.98
	OC vs. NO_3^- only	6	9.09	0.0068	-1.35
	OC vs. NO_3^- only	12	0.01	NS	-0.02
	OC vs. NO_3^- only	24	0.75	NS	-0.31
	H_2S vs. OC	6	21.93	0.0001	2.10
	H_2S vs. OC	12	4.76	0.0413	0.71
	H_2S vs. OC	24	13.07	0.0017	1.29
N_2 flux	H_2S vs. NO_3^- only	6	1.94	NS	0.29
	H_2S vs. NO_3^- only	12	5.57	0.0305	0.35
	H_2S vs. NO_3^- only	24	59.1	<0.0001	-1.62
	OC vs. NO_3^- only	6	0.29	NS	-0.11
	OC vs. NO_3^- only	12	24.27	0.0001	0.73
	OC vs. NO_3^- only	24	7.84	0.0118	-0.51
	H_2S vs. OC	6	3.72	NS	0.41
	H_2S vs. OC	12	6.59	0.0200	-0.38
	H_2S vs. OC	24	27.69	<0.0001	-1.11
N_2O flux	H_2S vs. NO_3^- only	6	37.49	<0.0001	0.0059
	H_2S vs. NO_3^- only	12	8.64	0.0081	0.0004
	H_2S vs. NO_3^- only	24	22.18	0.0002	0.0010
	OC vs. NO_3^- only	6	0.04	NS	0.0002
	OC vs. NO_3^- only	12	0.16	NS	0.0001
	OC vs. NO_3^- only	24	0.32	NS	-0.0001
	H_2S vs. OC	6	39.36	<0.0001	0.0057
	H_2S vs. OC	12	6.45	0.0195	0.0004
	H_2S vs. OC	24	27.82	<0.0001	0.0011

Table 4 cont'd.

Response	Factor	Hour	$F_{1,20}$	P	estimate (µmoles)
NO_2^- flux	H_2S vs. NO_3^- only	6	22.18	0.0001	0.009
	H_2S vs. NO_3^- only	12	0.20	NS	0.001
	H_2S vs. NO_3^- only	24	1.48	NS	0.002
	OC vs. NO_3^- only	6	11.42	0.0030	0.007
	OC vs. NO_3^- only	12	4.96	0.0375	0.004
	OC vs. NO_3^- only	24	1.80	NS	-0.002
	H_2S vs. OC	6	1.77	NS	0.003
	H_2S vs. OC	12	3.18	NS	-0.003
	H_2S vs. OC	24	6.54	0.0188	0.004
SO_4^{2-} flux	H_2S vs. NO_3^- only	6	3.09	NS	-1.17
	H_2S vs. NO_3^- only	12	0.41	NS	0.63
	H_2S vs. NO_3^- only	24	24.30	<0.0001	2.31
	OC vs. NO_3^- only	6	0.67	NS	-0.54
	OC vs. NO_3^- only	12	1.42	NS	-1.17
	OC vs. NO_3^- only	24	3.95	NS	-0.93
	H_2S vs. OC	6	0.89	NS	-0.62
	H_2S vs. OC	12	3.36	NS	1.80
	H_2S vs. OC	24	47.85	<0.0001	3.24

Discussion:

NO_3^- processing in high and low H_2S sites

The different NO_3^- removal rates and end-products imply that different microbially mediated reactions are responsible for the NO_3^- processing in sediments from these two sites. This may be due to the biogeochemistry of the sediments and particularly to differences in ambient H_2S concentration, or it may be due to other environmental factors. While H_2S can be an electron donor via chemolithotrophic oxidation, it is also acutely toxic to microbes (Wang and Chapman 1999, Senga et al. 2006). The high H_2S site (LP) may show evidence of this toxic effect in its slower NO_3^- removal rates compared to the low H_2S site (WP; Figure 1). However, the time-course experiments showed that all of the added NO_3^- will be removed given sufficient time (Figure 11).

The NO_3^- removal at both sites resulted in different N end-products, presumably due to different reduction pathways, possibly including both DNRA and denitrification. I hypothesize that DNRA explains the NH_4^+ production in both sites, despite the fact that the responses to the OC and H_2S treatments were different between sites. In LP NH_4^+ production increased across the H_2S gradient when OC was absent (black line, Figure 2); adding OC stimulated more NH_4^+ production, though not as consistently as in the H_2S gradient. There is further support for the idea that H_2S is directly influencing NH_4^+ production from the timed assays (Figure 12). In this case, treatments with added H_2S (as well as the NO_3^- only treatments) produced more NH_4^+ production than in the OC + NO_3^- treatment. In WP, there was no apparent increase in NH_4^+ across gradients of

either H_2S or OC alone; instead, I found substantial NH_4^+ production only when OC and H_2S were added together (Figure 2). This perhaps argues that the NH_4^+ is being generated from two different processes, and is therefore influenced in a different manner by both H_2S and OC. The timed assays give a different view of the NH_4^+ flux in WP. Here, we do not see any apparent net NH_4^+ production, but rather see NH_4^+ uptake, even in the OC + H_2S + NO_3^- interaction treatment. In the gradient assays from both sites, the magnitude of the H_2S effect was larger than the OC effect (Tables 1 and 2). Similarly, in the timed experiments, H_2S either caused more NH_4^+ production than OC, as in LP (Table 3), or caused less NH_4^+ removal over time compared to either NO_3^- only or OC treatments, as in WP (Table 4). These findings argue that NH_4^+ flux in both of these sites is directly linked to H_2S oxidation, possibly through DNRA.

An alternative explanation for the increased NH_4^+ fluxes is that increasing OC and NO_3^- availability increased denitrification, generating more OM breakdown and leading to greater NH_4^+ remineralization and increased NH_4^+ fluxes. I cannot exclude the possibility that the NH_4^+ produced is from DNRA or increased OM remineralization, though earlier (Chapter 3) I measured substantial DNRA, including in the sites studied herein, when measured using ^{15}N methods. Follow-up work using ^{15}N tracer methods in combination with the assay technique would help elucidate the mechanisms.

N_2 production in the WP gradient experiments was generally 3 times greater in LP, potentially indicating that denitrification was a more important NO_3^- removal process at WP (Figure 3). In the gradient experiments from both sites,

H_2S and OC had similar magnitudes of effect on N_2 production, indicating that both are of similar importance to N_2 generation (Tables 1 and 2). OC, however, affected the two sites differently. In LP, adding OC increased N_2 production over the H_2S treatments, whereas OC additions in WP decreased N_2 production compared to the H_2S only gradient (black line, Figure 3). And in WP the addition of H_2S further influenced N_2 flux by decreasing N_2 production over the H_2S gradient (Figure 3); the same inhibition of OC and H_2S on N_2 production was also seen at the final sampling point (24 hr) on the WP timed experiment, wherein the NO_3^- only treatment produced the highest N_2, followed by the two treatments with OC added, and finally by the H_2S + NO_3^- treatment. This OC/H_2S inhibition contrasts with LP, wherein adding intermediate amounts of H_2S, particularly in combination with OC, stimulated N_2 production (Figure 13). This same effect is seen in the LP timed experiment (Figure 13), where the H_2S treatments consistently produced significantly more N_2 than the NO_3^- only treatments, and generally produced more N_2 than the OC treatment (Table 3). For unknown reasons, the N_2 production rate in the WP timed assays was about half of what it was in the gradient assays.

N_2O consistently accounted for only a very minor fraction of the overall nitrate removal and transformation (<1%). Its production, however, was influenced by both time and the gradients of OC and H_2S. In LP, adding any H_2S decreased the amount of N_2O produced (Figure 4). This inhibition was partially relieved by adding OC in combination with H_2S, but the N_2O levels did not get as high as when OC was added alone. In the WP gradient experiments, N_2O

production is only stimulated at the highest levels of H_2S and OC, although there is a high degree of variability (Figure 4). The same is true for the WP timed assay N_2O flux, wherein the highest N_2O flux was observed in treatments with H_2S together with OC (Figure 14).

Both sites had large differences in NO_2^- production. LP consistently had high amounts of NO_2^- produced (>1 µmole), whereas NO_2^- production in WP was far lower (~ 0.01 µmoles), and not detectable at the end of the 24 hr incubation of the gradient experiment. In LP, small amounts of H_2S stimulated NO_2^- production, and adding OC increased the production as well (Figure 5), though H_2S clearly had a larger magnitude of the effect compared to OC (Table 1). A similar pattern was seen in the LP timed assays, wherein the H_2S only treatment had generally had the highest NO_2^- production compared to the NO_3^- only treatments (Figure 15, Table 3). Though both OC and H_2S affected NO_2^- production in WP, there were no discernible patterns to the effects (Table 4).

Thus, it is clear that both DNRA and denitrification were important NO_3^- removal processes in both sites, but under different conditions. Denitrification was consistently the more important NO_3^- removal pathway in WP, whereas in LP both denitrification and DNRA were equally important. Indeed, at high H_2S levels, DNRA (conversion to NH_4^+) accounted for more NO_3^- removal than denitrification (conversion to N_2). LP consistently showed measurable DNRA across the H_2S and OC gradients as well as when both H_2S and OC were added. DNRA in WP, on the other hand, only appeared to be important when H_2S and OC were added together.

Relative effects of OC and H_2S on NO_3^- reduction end-products

The complex interactions between OC and H_2S make it difficult to discern their relative importance as controls on NO_3^- removal end-products based on significance of the main effects alone. However, by partitioning the variation that can be attributed to a given factor and calculating the magnitude of a factor's effect I can gain more insight into the relative importance of OC and H_2S in N cycling. While it is clear that both OC and H_2S are influencing NH_4^+ production (Figures 2 and 12), H_2S consistently had a larger magnitude of effect in both sites (Tables 1 and 2). Additionally, the H_2S treatment consistently produced significantly more NH_4^+ than did the OC treatment in both sites (Tables 3 and 4). Both OC and H_2S affected N_2 production equally, based both on their magnitudes of effects in the gradient assays (Tables 1 and 2); however, in the LP timed assays, the H_2S treatment produced more N_2 than the OC treatment, but in WP, the H_2S treatment produced less N_2 than the OC treatment (Tables 3 and 4). While OC has long been known to affect N_2 production by stimulating denitrification, the influence of H_2S on N_2 production has not been previously noted, particularly in freshwater ecosystems. From these experiments, I can infer that both H_2S and OC contribute to NO_3^- removal and to the ultimate end-product of the NO_3^- reduction.

Few other studies have simultaneously examined the effects of both OC and H_2S on N cycling. Published studies to date have been largely carried out in batch reactors that are very different from natural ecosystems. In one of these examples, Reyes-Avila et al. (2004) examined the effects of acetate and H_2S on

denitrification. They found that denitrification rates were highest with acetate as the sole electron donor. H_2S as a sole electron donor resulted in lower denitrification rates by an order of magnitude, but the combination of the two resulted in intermediate denitrification rates (Reyes-Avila et al. 2004). Perhaps one of the greatest questions from my study arises from noting that upon adding a large amount of labile OC, we did not see more of an effect, as was the case in Reyes-Avila et al. (2004). While I added very large amounts of OC by ecological standards (1.5 mM or 10 mg/L), Reyes-Avila added 12,400 mg/L acetate, over 100-fold more than this study, which may in part explain why he saw a more dramatic difference between the acetate additions. However, Cardoso et al. (2006) added 500 µM acetate to slurries, and that only supported small amounts of denitrification in sediments isolated from reactors that had been conditioned with S compounds. Kelso et al. (1999) compared the effect of different OC sources (including acetate and glucose) on NO_3^- reduction and the relative importance of DNRA and denitrification. She found that the form of C matters to the relative importance of the two pathways, and to overall NO_3^- removal (Kelso et al. 1999). In that study, acetate inhibited NO_3^- removal relative to the NO_3^--only control treatments, and glucose favored NH_4^+ as an end-product more so than the other C sources (Kelso et al. 1999). Thus, the reaction of a particular sediment to acetate may in large part depend on the microbial community that is adapted to those conditions.

A few more studies have examined the effects of H_2S concentrations on NO_3^- reduction without also accounting for the effects of a carbon source. Senga

et al. 2006 isolated two strains of N_2O producers from brackish sediments and subjected them to a range of sulfide concentrations (0->1500 μM). They found that the amount of NH_4^+ produced increased over the H_2S gradient, and the greatest NH_4^+ production occurred at 313 μM H_2S, after which increasing H_2S concentrations led to little or no NO_3^- reduction (Senga et al. 2006). This is similar to what was found in the H_2S only gradient in LP, wherein adding increasing amounts of H_2S resulted in increasing NH_4^+ production. However, I cannot compare the effects of H_2S on N_2 production because Senga et al. 2006 did not directly measure N_2 (it was estimated as the difference between the N added and N accounted for) to make any inferences about the effect of H_2S on N_2 flux. Cardoso et al. 2006 did, however, examine the effects of H_2S on N_2 production. They found that increasing H_2S from 250 to 1000 μM decreased denitrification by 21-fold (Cardoso et al. 2006). The low H_2S concentration in this study, however, was higher than the highest H_2S concentration in my gradient assays, which makes it difficult to compare the two studies.

Carbon and the Tiedje DNRA Hypothesis

It has been hypothesized that DNRA, which at one time was thought to be predominantly fermentative, would be most important in highly reducing environments that maintain anoxic conditions for long time periods (Tiedje et al. 1982, Tiedje 1988). Tiedje et al. (1982) also hypothesized that DNRA would be favored in NO_3^--limited, labile-carbon rich environments while respiratory denitrification would be favored under carbon-limited conditions. This hypothesis

has been supported by some studies examining DNRA across sites of differing organic matter availability (Bonin 1996, Christensen et al. 2000), but has not been tested in a manipulative fashion as in this study. If this hypothesis were true, then we would expect that increasing levels of OC would lead to increasing amounts of DNRA. There is some evidence for this in the LP sediments, but none from the WP sediments (Figure 2). While adding OC in the WP sediments stimulated DNRA, it only occurred when the OC was added together with H_2S. Across the OC gradient there was no increase in NH_4^+ production (all symbols at zero H_2S added in bottom panel of Figure 2). Also, adding increasing amounts of OC with H_2S in WP, generally decreased the amount of NH_4^+ produced. OC clearly does influence DNRA in WP, but DNRA in this site is driven by the interaction between OC and H_2S, not by either one alone. Additional support for the assertion that DNRA is influenced by both OC and H_2S comes from examining the magnitude of the effects of both factors in both sites. H_2S is clearly as important (in the case of WP, Table 2) if not more important (as in LP, Table 1) than OC in determining NH_4^+ production (DNRA).

Evidence of S-linked NO_3^- removal

Evidence for linkages between sulfur and nitrogen cycling has been found in marine ecosystems (Fossing et al. 1995) where sulfate exists in much higher concentrations. Work by Fossing et al. (1995) showed that large mats of *Thioploca* off the coast of Chile were able to take up nitrate and store it in vacuoles, and use it to oxidize sulfide in a chemolithoautotrophic reaction.

Brunet and Garcia-Gil (1996) invoked a similar explanation for patterns they saw in a freshwater lake in Spain, wherein NH_4^+ production coincided with NO_3^- and H_2S depletion. Upon additions of the various S species as potential electron donors in the presence of NO_3^-, they found that H_2S produced only NH_4^+ and N_2O, lending support to the idea that denitrification is inhibited by sulfide. Brunet and Garcia-Gil (1996) further hypothesized that the presence of sulfide was the main factor determining whether NO_3^- was reduced to N_2 or NH_4^+. The experiments I report here provide additional evidence that the presence of H_2S influences NO_3^- reduction, particularly in freshwater ecosystems.

In sediments from both wetlands, adding H_2S resulted in an increase in SO_4^{2-} in both the gradient and timed experiments (Figures 7 and 16). This production was much more pronounced in LP, where adding increasing amounts of H_2S led to increasing amounts of SO_4^{2-} in nearly a 1:1 molar ratio. WP, in contrast, had SO_4^{2-} production stimulated by slight amounts of H_2S, but the trend did not continue to increase over the H_2S gradient. In both sites and both experiments, adding OC inhibited SO_4^{2-} production (Figures 7 and 15), and had a larger magnitude of effect on SO_4^{2-} flux than H_2S in part due to this inhibitive effect (Tables 1 and 2). This is especially clear in the LP gradient experiment wherein adding increasing amounts of OC increasingly inhibited SO_4^{2-} production, and actually stimulated SO_4^{2-} reduction (removal). A similar effect was also noted by Reyes-Avila et al. (2004), who also found that adding acetate in combination with H_2S forced the incomplete oxidation of H_2S to S^o rather than to SO_4^{2-}.

Adding NO_3^- in combination with H_2S stimulated H_2S removal, and this H_2S removal often occurred by the first sampling time in the timed assays (Figure 17). In LP, over half of the added H_2S was gone within 12 hours, and in WP, the added H_2S disappeared by 12 hours. Between 12 and 24 hours, NO_3^- was exhausted and SO_4^{2-} reduction began, which increased the H_2S in the vial slightly by the end of the experiment (Figure 17). There was no change in the H_2S flux in either the NO_3^- only or OC treatments. In the controls (no added OC, NO_3^-, or H_2S), reduction of SO_4^{2-} from the added surface water caused an increase in H_2S concentration over the time course. Adding increasing amounts of H_2S in the gradient assays also led to increasing removal of H_2S in both LP and WP. In LP, nearly all of the added H_2S was removed along the H_2S gradient (Figure 6). However, at the highest level of H_2S addition, adding OC increasingly inhibited H_2S removal. This effect was not seen in WP, where OC stimulated more H_2S removal compared to the H_2S gradient (black line, Figure 6). In both sites, treatments along the OC gradient (no H_2S added) resulted in an increase in H_2S, presumably from SO_4^{2-} reduction.

The simultaneous SO_4^{2-} production and H_2S removal in the presence of NO_3^- strongly suggests a link between the two cycles, but what happens to the NO_3^-? Some have hypothesized that the NO_3^- is reduced to NH_4^+ (Brunet and Garcia-gil 1996, Otte et al. 1999, Sayama 2001), while others have determined that the conversion can be to N_2 (Sweerts et al. 1990). The stoichiometry of these two reactions is different and could provide insight into which is occurring in these experiments. If the NO_3^- is converted to NH_4^+, there should be 1 mole of

NO_3^- and H_2S consumed per mole of SO_4^{2-} and NH_4^+ produced (Sayama et al. 2005). However, if N_2 is produced, 8 moles of NO_3^- consumption results in 5 moles of SO_4^{2-} production (Fossing et al. 1995). These stoichiometric equations are described in more detail in Chapter 2.

If we examine, for example, the high-H_2S-only treatment from LP, we see that 4 µmoles of NO_3^- and 5 µmoles of H_2S were consumed and approximately 7 µmoles of SO_4^{2-} were produced (Figure 8, top right). While this doesn't match perfectly with either of the two reactions discussed above, it more closely reflects what we would expect if the reduction to NH_4^+ was occurring. LP had further evidence of this reaction in the increasing NH_4^+ production along the H_2S gradient (Figure 2 and Figure 8). In this case, adding ~5 µmoles H_2S resulted in 2-3 umole increase in NH_4^+ produced. While one reaction may dominate, it is also likely that the two are not mutually exclusive and both are occurring to some degree, perhaps shifting in response to changing H_2S concentrations over the course of the experiment. This lack of exclusivity between the two pathways is even more likely in natural near-surface sediment environments which vary greatly in H_2S concentrations over both space and time. We have evidence for both pathways (H_2S coupled to both NH_4^+ and N_2 production) occurring because both end-products are affected by H_2S in LP. In the LP gradient experiment, addition of medium amounts of H_2S resulted in increased N_2 production (Figure 3); in the timed experiment, addition of medium amounts of H_2S resulted in the most N_2 production at the end of the experiment (Figure 13).

Conclusions

Denitrification was the more important NO_3^- reduction pathway in the low ambient H_2S site (WP), whereas DNRA and denitrification were of equal importance in the high ambient H_2S site (LP). DNRA, however, occurred in both sites, but under different conditions and was particularly stimulated by adding either OC or H_2S. This study also provides evidence for the influence of H_2S on N_2 production, presumably from S-linked denitrification, which is something that has not been experimentally demonstrated in freshwaters to my knowledge. This work highlights that there are other pathways of NO_3^- removal besides carbon-driven denitrification in freshwater ecosystems. Thus the S and N cycles may be linked in ways previously not appreciated. Since both N and S are heterogeneous components of our landscape, it is safe to assume that not all wetlands process NO_3^- the same way and different pathways may be more or less important under differing conditions. Greater efforts need to be made to understand what controls N processing in addition to OC and O_2, which have mainly been studied in the context of denitrification.

References:

Bonin, P. 1996. Anaerobic nitrate reduction to ammonium in two strains isolated from costal marine sediment: A dissimilatory pathway. FEMS Microbiology Ecology **19**:27-38.

Bonin, P., P. Omnes, and A. Chalamet. 1998. Simultaneous occurrence of denitrification and nitrate ammonification in sediments of the French Mediterranean Coast. Hydrobiologia **389**:169-182.

Brunet, R. C., and L. J. Garcia-gil. 1996. Sulfide-induced dissimilatory nitrate reduction to ammonia in anaerobic freshwater sediments. FEMS Microbiology Ecology **21**:131-138.

Burgin, A. J., and S. K. Hamilton. 2007. Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Frontiers in Ecology and the Environment **5**:89-96.

Cardoso, R. B., R. Sierra-Alvarez, P. Rowlette, E. R. Flores, J. Gomez, and J. A. Field. 2006. Sulfide oxidation under chemolithoautotrophic denitrifying conditions. Biotechnology and Bioengineering **95**:1148-1157.

Christensen, P. B., S. Rysgaard, N. P. Sloth, T. Dalsgaard, and S. Schwaerter. 2000. Sediment mineralization, nutrient fluxes, denitrification and dissimilatory nitrate reduction to ammonium in an estuarine fjord with sea cage trout farms. Aquatic Microbial Ecology **21**:73-84.

Dannenberg, S., M. Kroder, W. Dilling, and H. Cypionka. 1992. Oxidation of H_2, organic compounds and inorganic sulfur compounds coupled to reduction of O_2 or nitrate by sulfate-reducing bacteria. Archives of Microbiology **158**:93-99.

Fossing, H., V. A. Gallardo, B. B. Jorgensen, M. Huttel, L. P. Nielsen, H. Schulz, D. E. Canfield, S. Forster, R. N. Glud, J. K. Gundersen, J. Kuver, N. B. Ramsing, A. Teske, B. Thamdrup, and O. Ulloa. 1995. Concentration and transport of nitrate by the mat-forming sulfur bacterium *Thioploca*. Nature **374**:713-715.

Golterman, H.L. and R.S. Clymo. 1969. Methods for chemical analysis of freshwaters. Blackwell, Oxford.

Graham, M. H., and M. S. Edwards. 2001. Statistical significance versus fit: estimating the importance of individual factors in ecological analysis of variance. Oikos **93**:505-513.

Holmer, M., and P. Storkholm. 2001. Sulphate reduction and sulphur cycling in lake sediments: a review. Freshwater Biology **46**:431-451.

Kelso, B. H. L., R. V. Smith, and R. J. Laughlin. 1999. Effects of carbon substrates on nitrite accumulation in freshwater sediments. Applied and Environmental Microbiology **65**:61-66.

Kelso, B. H. L., R. V. Smith, R. J. Laughlin, and S. D. Lennox. 1997. Dissimilatory nitrate reduction in anaerobic sediments leading to river nitrite accumulation. Applied and Environmental Microbiology **63**:4679-4685.

Megonigal, J., M. Hines, and P. Visscher. 2004. Anaerobic Metabolism: Linkages to Trace Gases and Aerobic Processes. Pages 317-424 *in* W. H. Schlesinger, editor. Biogeochemistry. Elsevier-Pergamon, Oxford, UK.

Nijburg, J. W., M. J. L. Coolen, S. Gerards, P. J. A. K. Gunnewiek, and H. J. Laanbroek. 1997. Effects of nitrate availability and the presence of Glyceria maxima the composition and activity of the dissimilatory nitrate-reducing bacterial community. Applied and Environmental Microbiology **63**:931-937.

Otte, S., J. G. Kuenen, L. P. Nielsen, H. W. Paerl, J. Zopfi, H. N. Schulz, A. Teske, B. Strotmann, V. A. Gallardo, and B. B. Jorgensen. 1999. Nitrogen, carbon, and sulfur metabolism in natural *Thioploca* samples. Applied and Environmental Microbiology **65**:3148-3157.

Paul, E. A., and F. E. Clark. 1996. Soil Microbiology and Biochemistry, 2nd edition. Academic Press, San Diego.

Reyes-Avila, J. S., E. Razo-Flores, and J. Gomez. 2004. Simultaneous biological removal of nitrogen, carbon and sulfur by denitrification. Water Research **38**:3313-3321.

Sayama, M. 2001. Presence of nitrate-accumulating sulfur bacteria and their influence on nitrogen cycling in a shallow coastal marine sediment. Applied and Environmental Microbiology **67**:3481-3487.

Sayama, M., N. Risgaard-Petersen, L. P. Nielsen, H. Fossing, and P. B. Christensen. 2005. Impact of bacterial NO3- transport on sediment biogeochemistry. Applied and Environmental Microbiology **71**:7575-7577.

Senga, Y., K. Mochida, R. Fukumori, N. Okamoto, and Y. Seike. 2006. N2O accumulation in estuarine and coastal sediments: The influence of H2S on dissimilatory nitrate reduction. Estuarine Coastal and Shelf Science **67**:231-238.

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

Sweerts, J. P. R. A., D. Debeer, L. P. Nielsen, H. Verdouw, J. C. Vandenheuvel, Y. Cohen, and T. E. Cappenberg. 1990. Denitrification by sulfur oxidizing *Beggiatoa spp* mats on freshwater sediments. Nature **344**:762-763.

Tiedje, J. M. 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. Pages 179-244 *in* A. J. B. Zehnder, editor. Biology of Anaerobic Microorganisms. Wiley & Sons, New York.

Tiedje, J. M., A. J. Sexstone, D. D. Myrold, and J. A. Robinson. 1982. Denitrification - Ecological Niches, Competition and Survival. Antonie Van Leeuwenhoek Journal of Microbiology **48**:569-583.

Vitousek, P. M., J. D. Aber, R. W. Howarth, G. E. Likens, P. A. Matson, D. W. Schindler, W. H. Schlesinger, and D. G. Tilman. 1997. Human alteration of the global nitrogen cycle: Sources and consequences. Ecological Applications **7**:737-750.

Wang, F. Y., and P. M. Chapman. 1999. Biological implications of sulfide in sediment - A review focusing on sediment toxicity. Environmental Toxicology and Chemistry **18**:2526-2532.

Whitmire, S. L. 2003. Anaerobic biogeochemical functions of Michigan wetlands and the influence of water source. Ph.D. Dissertation. Michigan State University, East Lansing, MI.

CHAPTER 5

CONCLUSIONS

Nitrate is potentially processed by many different pathways in freshwater ecosystems, including lakes, streams and wetlands. While some of these pathways, such as respiratory denitrification, have been well-studied, others such as dissimilatory nitrate reduction to ammonium (DNRA) and anaerobic ammonium oxidation (anammox) have received relatively little scientific attention in freshwaters. Furthermore, while the linkages between sulfur and nitrogen cycling have been acknowledged and examined in marine-influenced ecosystems, these linkages have not been well studied in freshwaters, in part because of the perception that relatively low sulfate concentrations in freshwaters render S cycling unimportant in overall ecosystem function. The dissimilatory transformation of nitrate by chemolithoautotrophs, including anammox as well as use of nitrate as an oxidizing agent by sulfur- and iron-oxidizing bacteria, are so little known in freshwaters that thay can be considered novel pathways of nitrate removal.

I found evidence of linkages between the sulfur and nitrogen cycles in sediments from a diverse set of freshwater streams, lakes and wetlands based on the push-pull tracer experiments (Chapter 2). I was able to show that the amount of sulfate production relative to nitrate removal varied both within and between different freshwater environments. Based on stoichiometric calculations the sulfate that was produced could explain a substantial fraction of the overall

nitrate removal. Furthermore, by using stable isotopes (^{15}N-labeled NO_3^-), I found that dissimilatory nitrate reduction to ammonium (DNRA) could account for a large fraction of the overall nitrate removal, particularly in wetlands.

The push-pull experiments in Chapter 2 helped to convince me that the phenomenon of sulfate production was indeed biologically driven and potentially linked to N cycling, but I needed to conduct further experiments because I could not directly compare multiple nitrate removal pathways, including denitrification and DNRA. To discern the end-products of NO_3^- reduction (e.g., NH_4^+ or N_2), I again employed the use of stable isotopes, particularly of $^{15}NO_3^-$ and $^{15}NH_4^+$, to elucidate the relative importance of multiple pathways operating simultaneously (Chapter 3). This is the first study, to my knowledge, that simultaneously estimates three pathways of nitrate removal. Additionally, it is one of a small handful of studies that have examined the importance of anammox in freshwater ecosystems. While I did not find evidence of substantial rates of anammox in any of my sites, I did discover that DNRA can be an important nitrate removal pathway, and in some cases, could rival denitrification as a nitrate sink.

Once I understood that DNRA in particular was potentially important in many of these sites, I sought to understand what factors control the relative importance of denitrification vs. DNRA in freshwater sediments (Chapter 4). Two forms of DNRA are known to occur: fermentative DNRA, thought to occur under conditions of high labile carbon availability, and sulfur-driven DNRA, controlled by H_2S and other reduced sulfur compounds. Therefore, I wanted to test the effects of carbon vs. sulfide in controlling both denitrification and DNRA, along the lines

of the hypotheses outlined in Chapter 1. I did this using an assay technique I developed that could simultaneously manipulate carbon and sulfide gradients, as well as estimate different end-products of nitrate reduction, and I conducted these assays in both a high and low ambient sulfide site. I found that both carbon and sulfide were important in controlling nitrate removal rates and end-products in both sites. While denitrification tended to be the more important removal pathway in the low ambient sulfide site, DNRA was of equal importance in the high ambient sulfide site. DNRA occurred in both sites, but under different conditions and was particularly stimulated by adding both OC and H_2S. This work also provided evidence for the influence of H_2S on N_2 production, presumably from S-linked denitrification, which is something that has not been experimentally demonstrated in freshwaters to my knowledge. I also saw sulfate production increase over an increasing sulfide addition gradient, which further suggests that the nitrogen and sulfur cycles are intricately linked, as I found in Chapter 2.

While this study (Chapter 4) has given new insight to nitrate reduction and processing, it also raises new questions regarding our understanding of N cycling in freshwater ecosystems. In particular, I have become interested in investigating whether the ambient sulfide concentration of a site changes how nitrogen is cycled in that ecosystem. Stream sediments, for example, tend to be lower in sulfide compared to wetland or lake sediments. Do high sulfide ecosystems inherently cycle nitrogen, particularly nitrate, differently than low sulfide ecosystems? We are currently performing the same assay used in

Chapter 4 on more sites, aiming for a total of 4-5 low and high ambient sulfide sites, which will help me answer the previous question. Additionally, because we know that sulfide and nitrate inputs are variable over time, there may be differences in the relative importance of various nitrate removal pathways over seasonal scales. This would be an interesting question to address using either an assay approach, or ^{15}N methods, or a combination of both.

These findings have significant implications for our current understanding of N cycling in freshwater ecosystems. The possible importance – or even prevalence – of alternative nitrate removal pathways (DNRA, anammox) has profound implications for our management of aquatic ecosystems to reduce nitrate loads. Nitrate is the most mobile N form, so removal of nitrate by any of the processes is important to downstream water quality, but permanent removal by denitrification is most desirable.

Removal by other pathways can yield N_2 in an alternative form of denitrification, or they may result in transformation of the nitrate to something other than dinitrogen gas (N_2). Nitrate removal via Anammox still creates dinitrogen gas as an end-product, but removes both a nitrate and an ammonium ion 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. Additionally, if S-oxidizers prove to take up much of the nitrate, then N cycling is closely linked to sulfide availability, which is 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. If excess sulfate loading to freshwaters actually enhances nitrate removal, then the controls on nitrate removal in landscapes subject to S and N pollution become more complex than previously thought. Much more research needs to be done on 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 these processes in freshwater ecosystems subject to elevated nitrate inputs remains incomplete.