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NO_3^--Driven SO_4^{2-} Production in Freshwater Ecosystems: Implications for N and S Cycling

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Abstract

Massive anthropogenic acceleration of the global nitrogen (N) cycle has stimulated interest in understanding the fate of excess N loading to aquatic ecosystems. Nitrate (NO_3^-) is traditionally thought to be removed mainly by microbial respiratory denitrification coupled to carbon (C) oxidation, or through biomass assimilation. Alternatively, chemolithoautotrophic bacterial metabolism may remove NO_3^- by coupling its reduction with the oxidation of sulfide to sulfate (SO_4^{2-}). The NO_3^- may be reduced to N_2 or to NH_4^+, a form of dissimilatory nitrate reduction to ammonium (DNRA). The objectives of this study were to investigate the importance of S oxidation as a NO_3^- removal process across diverse freshwater streams, lakes, and wetlands in southwestern Michigan (USA). Simultaneous NO_3^- removal and SO_4^{2-} production were observed in situ using modified ''push-pull'' methods in nine streams, nine wetlands, and three lakes. The measured SO_4^{2-} production can account for a significant fraction (25–40%) of the overall NO_3^- removal. Addition of $^{15}NO_3^-$ and measurement of $^{15}NH_4^+$ production using the push–pull method revealed that DNRA was a potentially important process of NO_3^- removal, particularly in wetland sediments. Enrichment cultures suggest that *Thiomicrospira denitrificans* may be one of the organisms responsible for this metabolism. These results indicate that NO_3^--driven SO_4^{2-} production could be widespread and biogeochemically important in freshwater sediments. Removal of NO_3^- by DNRA may not ameliorate problems such as eutrophication because the N remains bio-available. Additionally, if sulfur (S) pollution enhances NO_3^- removal in freshwaters, then controls on N processing in landscapes subject to S and N pollution are more complex than previously appreciated.

Key words: sulfide oxidation; ^{15}N; dissimilatory nitrate reduction to ammonium; denitrification; *Thiomicrospira denitrificans*; sulfate production.

Introduction

Increases in the intensity and extent of agriculture as well as fossil fuel combustion have led to dramatic alterations of the global nitrogen (N) cycle, more than doubling the annual input of bio-available, fixed N to terrestrial ecosystems (Vitousek and others 1997) and dramatically increasing N loading to aquatic ecosystems (Carpenter and others 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 hence most of this N load evidently disappears in transit (Seitzinger and others 2006). These massive changes in N cycling have stimulated interest in

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understanding how the dominant inorganic N form, nitrate (NO_3^-), moves through landscapes, and what controls the fraction that is removed before reaching the coastal marine environment where N causes eutrophication. Most attention has been devoted to the study of NO_3^- removal by respiratory denitrification of organic carbon (C) (Burgin and Hamilton 2007). Some work has also examined dissimilatory nitrate reduction to ammonium (DNRA) conducted by fermentative bacteria (Tiedje 1988).

Recent work in marine and freshwater systems has demonstrated that certain sulfur (S) oxidizing bacteria can use NO_3^- to oxidize sulfide and elemental S to SO_4^{2-} (Megonigal and others 2004; Burgin and Hamilton 2007). The ability of bacteria to mediate these reactions has now been established in a number of taxa with diverse metabolic characteristics (Dannenberg and others 1992; Bonin 1996; Phillipot and Højberg 1999) including members of the genera *Thiobacillus*, *Thiomicrospora*, and *Thioploca* (Timmer-ten Hoor 1981; Jorgensen 1982; Kelly and Wood 2000). The biogeochemical importance of NO_3^- use by S-oxidizing bacteria was first widely recognized in marine sediments, but we are beginning to discover its importance in freshwaters. For example, much of the NO_3^- uptake in a groundwater aquifer has been ascribed to *Thiobacillus denitrificans* (Böttcher and others 1990), and *Thioploca* mats have been discovered not only in marine sediments, but also in lakes Erie, Baikal, and Biwa (Megonigal and others 2004). Studies of groundwater systems have observed NO_3^- reduction coincident with oxidation of sulfide minerals (Korom 1992; Postma and others 1991; Lucassen and others 2002). NO_3^- removal by S-oxidizing bacteria has also been exploited as a water treatment method for removing NO_3^-, for example, in cores packed with elemental S granules (Soares 2002).

Freshwater lakes, streams, and wetlands are often considered to be too low in S for S oxidizers to play a major biogeochemical role. However, in 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. Similarly, NO_3^- additions to pools on the Wisconsin River floodplain also resulted in SO_4^{2-} production (Forshay 2003).

These observations of SO_4^{2-} production coupled to NO_3^- reduction can be explained if S-oxidizing bacteria are utilizing the NO_3^- in the oxidation of reduced S compounds to SO_4^{2-}. The metabolic flexibility of S-oxidizing bacteria has been increasingly appreciated in recent years. *T. denitrificans* couples the oxidation of inorganic S compounds to the reduction of NO_3^- and is common in freshwater and marine sediments (Kelly 1999; Haaijer and others 2006). *Tms. denitrificans* performs a similar metabolic reaction, but as a member of the ϵ-proteobacteria, it is not closely related to *T. denitrificans*, a member of the β-proteobacteria. Both are predominantly anaerobes but are known to tolerate microaerophilic conditions (Timmer-ten Hoor 1981). This N–S coupling capability also extends to the γ-proteobacteria, and a group of *Thioploca* spp., which were first described from Lake Constance in the early 1900s (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 a unique gliding motility that allows them to migrate upward to oxic overlying water of higher NO_3^- concentration, and store NO_3^- intracellularly (Jorgensen and Gallardo 1999).

The microbes responsible for conducting the NO_3^--driven SO_4^{2-} production may be reducing the NO_3^- to either N_2, as a form of denitrification, or to NH_4^+, as a form of DNRA. When the reaction proceeds via denitrification to dinitrogen gas (N_2), the initial oxidation step may have the following stoichiometry (Fossing and others 1995):

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

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 and others 1995):

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

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:

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

However, the NO_3^- may not be completely reduced to N_2, but may be converted to NH_4^+ in a form of DNRA. The conversion of NO_3^- to NH_4^+, assuming direct transformation of sulfide to elemental S [as in equation (1)], has the following stoichiometry (Sayama and others 2005):

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

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

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

Reactions 4 and 5 may occur sequentially producing one mole of SO_4^{2-} for each mole of NO_3^- consumed:

$$4H_2S + 4NO_3^- + 4H_2O \rightarrow 4NH_4^+ + 4SO_4^{2-} \quad (6)$$

The stoichiometry of these two reactions can be used to estimate the magnitude of the SO_4^{2-} production that could be coupled to NO_3^- removal.

The alternative reduction products (N_2 vs. NH_4^+) have different implications for ecosystem N cycling. NO_3^- 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). Furthermore, although freshwater wetlands are low in S compared to marine systems, they often contain enough S to support significant bacterial transformations (Lovley and Klug 1983). The potential for and significance of S-coupled N cycling is all but unknown, particularly for freshwater ecosystems.

In this study, we investigated links between NO_3^- reduction and SO_4^{2-} production in streams, wetlands, and lakes of southwestern Michigan (USA). The objectives of this study were to: (1) confirm that the NO_3^--driven SO_4^{2-} production is a biological phenomenon and not an artifact of experimental methods; (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 when NO_3^--driven SO_4^{2-} production is an important NO_3^- removal process.

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). In 2005, sites were selected in part based on data from the larger 2004 survey, and in part based on sites from a cross-site study of NO_3^- removal by streams (Mulholland and others 2008). Sites were selected to represent the range of aquatic ecosystems in southwest Michigan, as well as to encompass a gradient of geochemical and hydrologic settings. The number of sites in this study precludes a detailed description of each site, however, the sites are listed and their general chemistry detailed in Table 1. Coordinates for the site locations are listed in Table 1 in Appendix A.

Push–Pull Methodology

Push–pull experiments entail removing a sample of porewater and amending it with one or more reactive solutes (for example, 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 push–pull method has been used to estimate biogeochemical processing rates in aquifers (Istok and others 1997; McGuire and others 2002), lake sediments (Luthy and others 2000), and riparian wetlands (Addy and others 2002).

In this study, push–pull measurements were done using a near-surface push–pull technique similar to the method developed by Whitmire and Hamilton (2005). The experiments were conducted in situ at 5–10-cm depth in the sediments, at three locations per treatment (described below) in each site. The amendment solution was O_2-free and minimal in volume. Generally, 30 ml of porewater was removed from the sediments, and after removing 3 ml of this water for analysis of background concentrations, the remaining 27 ml was amended with 3 ml of anoxic Br^- (control) or NO_3^- + Br^- (treatment) solution (final concentrations were 10 mg l^{-1} [714 μM] NO_3^- –N and 3 mg l^{-1} [37.5 μM] Br^-), and reinjected into the sediments. We added a relatively high NO_3^- concentration to sediment porewaters that are normally much lower in NO_3^- concentration, and therefore these rate constants should be considered potentials; however, the NO_3^- concentration we used is not unrealistic and is similar to groundwater NO_3^- concentrations in the area (Whitmire and Hamilton 2005; see section ''Discussion'' for more detail on the caveats of the push–pull method). The injection line was then flushed with 1 ml of porewater and 1 ml of sample was withdrawn and filtered (0.45 μm, 13 mm Millex syringe filters). In lake and wetland sediments, samples were taken

Table 1. Physico-Chemical Characteristics of the Study Sites

Site	Ecosystem	SW temperature (°C)	SW DO (mg l^{-1})	SW cond. (μS cm^{-1})	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

DO, dissolved oxygen; Cond., conductivity; SW, surface water; PW, porewater; BDL, below detection limits. An asterisk (*) denotes sites that were also studied in 2005 for the $^{15}NO_3^-$ push–pull experiments.

every 10 min for the first hour and then every 30 min for the next 2–3 h. Incubations generally lasted 4 h. Due to the increased hydrologic movement in stream sediments, samples were taken every 5 min for the first 20 min and every 10 min thereafter.

In 2004, we performed push–pull experiments in a diverse set of wetlands (n = 9), streams (n = 9), and lakes (n = 3) (Table 1). In 2005, we 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 N removal 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. Porewater-dissolved N_2 was extracted using static headspace equilibration and analyzed for $\delta^{15}N$ (Hamilton and Ostrom 2007) to quantify denitrification, and the remaining water was filtered for analysis of $\delta^{15}N$ in NH_4^+ to quantify DNRA. The $\delta^{15}N$ of NH_4^+ was measured using the ammonia-diffusion procedure (Holmes and others 1998). These samples 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. The ^{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 background-corrected NO_3^- and Br^- concentrations, and were modeled as first-order reactions using the following exponential function, which relates the concentration (C) of the reactant (NO_3^-) to the tracer (Br^-) at a given point in time (t):

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

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

NO_3^- removal was calculated for each subsample as the difference between the measured concentration and what would be expected based on the concentration of the conservative tracer (Br^-), after

background correction if background concentrations were measurable:

$$[NO_3^-]_{exp'd} = [Br^-]_{obs'd} * ([NO_3^-]/[Br^-])_{injectate} \quad (8)$$

where $[NO_3^-]_{exp'd}$ is the expected concentration if no loss or gain of NO_3^- had taken place, $[Br^-]_{obs'd}$ is the Br^- concentration observed at a given sampling time, and $([NO_3^-]/[Br^-])_{injectate}$ is the ratio of NO_3^- to Br^- in the injectate that was added at the beginning of the experiment. SO_4^{2-} production was calculated similarly. All concentrations are molar.

The stoichiometric model described above [equations (1)–(6)] was applied to compare how NO_3^- removal (yielding either NH_4^+ or N_2) related to the measured SO_4^{2-} production in different aquatic ecosystems under the two alternative reaction stoichiometries (DNRA vs. denitrification). 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^- or elemental S by S oxidizing bacteria.

Testing for Experimental Artifacts

To ensure that the observed SO_4^{2-} production is not an experimental artifact of the push–pull methodology, we performed three 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 direct injection of NO_3^- into anoxic porewaters.

For the temperature test, we collected soil cores from a nearby wetland and placed the cores into 1-quart canning jars along with approximately 100 ml overlying water allowing 1 day in the dark for stabilization. To each jar, we 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, there were 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, we collected sediment from the same site to half-fill a 20-l bucket, leaving approximately 2.5 cm of overlying water to which NO_3^- and Br^- were added. This overlying water was periodically sampled over a longer time period than the push–pull experiments because we 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 colorimetric method (Golterman and Clymo 1969). NH_4^+ was measured colorimetrically using the phenylhypochlorite technique (Aminot and others 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 with a Gelman 0.45 μm Supor membrane filter) for analysis of major solutes and nutrients.

Enrichment and Microbial Characterization

An enrichment culture was created to identify the major organism(s) in these sediments that grow in the presence of NO_3^- and H_2S. Inoculation sources (1–2 g sediment) were taken from three of the sites used in this study. The cultures were grown in 120 ml serum bottles with 50 ml of a complex anaerobic medium without C or N sources (see Appendix A for a detailed description of the medium). These cultures were kept completely anoxic (indicated by resazurin) and all transfers and nutrient additions were conducted in an anaerobic glove bag. Cultures were given H_2S (to a final concentration of 200 μM) and NO_3^- (to a final concentration of 1,000 μM) as needed, typically every other day. The concentrations of H_2S, NO_3^-, and SO_4^{2-} in the enrichment cultures were monitored until the enrichments were harvested for characterization. On the final sampling, water was extracted for analysis of elemental S as in Guerrero and others (1985) and SO_4^{2-} via ion chromatography. DNA from the primary enrichments was extracted using the MoBio Ultraclean Soil DNA isolation kit. The 16S rRNA was PCR amplified using universal bacterial primers (8F and 1498R). The PCR product was cloned into a plasmid vector and transformed into *Escherichia coli* using an Invitrogen TOPO TA Cloning Kit. *E. coli* was plated

onto LB plates. Twenty colonies were picked from each site and a total of 60 colonies were sequenced at the Josephine Bay Paul Center in Woods Hole, Massachusetts. The sequences were manually aligned and analyzed using the ARB package (Ludwig and others 2004) to identify the nearest relative of the isolates. Analyses were compared to the output of BLAST (Basic Local Alignment and Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/) matches.

Statistical Analysis

To summarize the large amount of variation in NO_3^- removal and NO_3^--driven SO_4^{2-} production within each ecosystem type, we created box plots that encompass all of the individual injectors from each site (that is, the box plots are based on the entire set of experiments, rather than site averages, and experiments were conducted at three points within each site). This gives the most complete illustration of the variation within and among ecosystems. All statistical analyses were conducted using Systat version 11.0 software. One-way analysis of variance (ANOVA) was used to test the differences in NO_3^- removal rate constants (k), % of NO_3^- removal by DNRA, % overall NO_3^- removal, and SP:NR ratios across ecosystem types. One-way ANOVA was also used to compare pre- and post-manipulation 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^- removal 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 2 years; MR was not performed on the 2005 data alone because there were only nine sites total, which was not enough power for the analysis.

Results

Figure 1 shows examples (from this study) of this phenomenon in a wetland in which the SO_4^{2-} production (triangles) was quite pronounced (Figure 1A) and a wetland in which the response was less prominent (Figure 1C). NO_3^- removal can be stoichiometrically compared to SO_4^{2-} production by expressing the data as the difference between observed and expected concentrations (Figure 1B, D). The example in panels A and B is from a site where high rates of SO_4^{2-} production occurred. The example in panels C and D is from a site where relatively little SO_4^{2-} production occurred.

Field Push–Pull Experiments

The NO_3^- removal rate constant is the fraction of the NO_3^- concentration that was removed per unit time (min^{-1}), with removal indicated by a negative constant. NO_3^- removal rate constants were highly variable both among ecosystem types and within a given ecosystem (Figure 2A). Lakes had higher removal rates than did streams and wetlands (ANOVA, $F_{2,97} = 3.09$; $P = 0.05$). 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, we used a ratio of the micromoles of SO_4^{2-} produced (calculated from the observed SO_4^{2-} concentration and the expected concentration based on the Br^- concentration; Figure 1) to the micromoles of NO_3^- removed (also as in Figure 1). This generated a unitless ratio of SP:NR.

Concurrent SO_4^{2-} production and NO_3^- removal occurred in all freshwater ecosystem types (Figure 2B). Streams had the greatest range of ratios of total SO_4^{2-} production to total NO_3^- removal (SP:NR 0.02–2.4). Wetland SP:NR ranged from 0.005 to 1.1 and lake SP:NR from 0.03 to 1.1. Generally, SO_4^{2-} production accounted for 25–50% of NO_3^- removal (estimated from the inter-quartile ranges in Figure 2B), and the fraction of removal attributable to SO_4^{2-} production did not differ among ecosystem types (ANOVA, $F_{2,84} = 1.45$; $P = 0.24$). In six injectors from two stream sites, SO_4^{2-} production greatly exceeded the stoichiometric equivalent of NO_3^- removed under the model assumptions; this also occurred in four wetland injectors from two sites (<10% of the experiments we conducted).

The amount of SO_4^{2-} produced accounted for a substantial fraction of the NO_3^- removed in all types of aquatic ecosystems (Figure 2C). Lakes had the highest potential NO_3^--driven SO_4^{2-} production, followed by streams and wetlands (Figure 2C), though there was considerable variation within a given ecosystem type. The reaction in which NO_3^- is denitrified (to N_2) to oxidize SO_4^{2-} (gray boxes, Figure 2C) accounts for a greater fraction of the overall NO_3^- removal because of the 8:5 stoichiometry. The DNRA reaction with 1 mole of NH_4^+ as the end product for every 1 mole of NO_3^- removed (black boxes, Figure 2C) can explain a smaller, but still significant proportion of the NO_3^- removal.

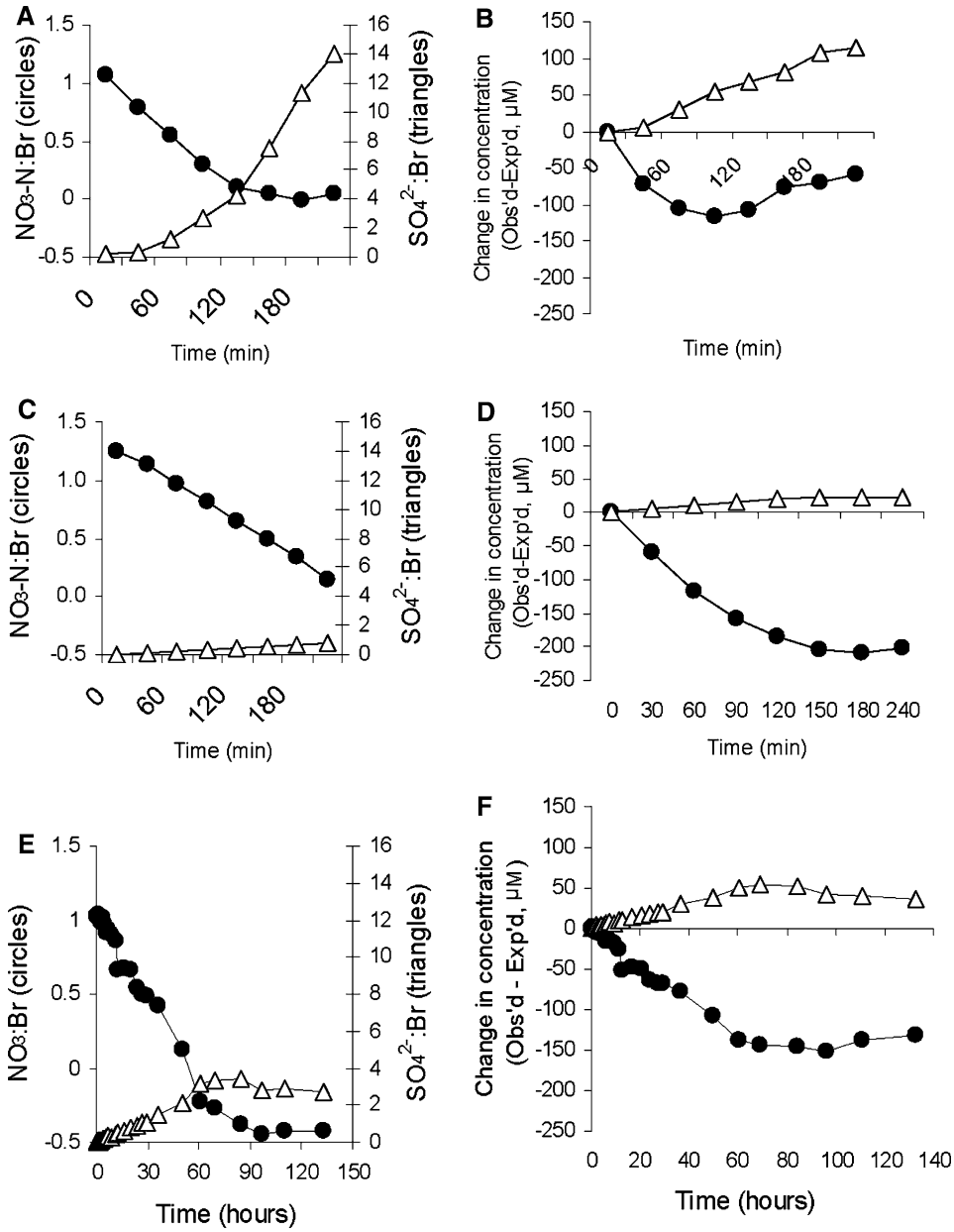

Figure 1. Nitrate removal and sulfate production in two different wetlands (**A** and **B**; **C** and **D**). The panels on the left are the ratios of the reactants (NO_3^- and SO_4^{2-}) to the conservative tracer (Br^-) in two push–pull experiments. The panels on the right are a comparison of observed and expected concentrations of NO_3^- [equation (8)] and SO_4^{2-} in a site with high (top) and low (bottom) relative 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 were observed. Panels **E** and **F** are NO_3^- removal and SO_4^{2-} production in surface water over Turkey Marsh sediments after NO_3^- addition to the surface water (conducted in the lab in a bucket). NO_3^-:Br^- became negative because the NO_3^- concentration fell below what was originally present (~0.6 mg N/L) just prior to the experimental NO_3^-/Br^- addition. Note the difference in time scales between panels **A–D** and panels **E**, **F.**

The porewaters of each injector were sampled for NH_4^+ and H_2S prior to the application of the treatment (NO_3^- + Br^- or the Br^- only "control") and at the end of the experiment. Generally there was more NH_4^+ production (Figure 3A) and H_2S depletion (Figure 3B) in NO_3^- amended sediments than in the control injections. This pattern reflects what would be expected if DNRA was coupled with S oxidation, although there is no significant difference between the "post" control and treatment

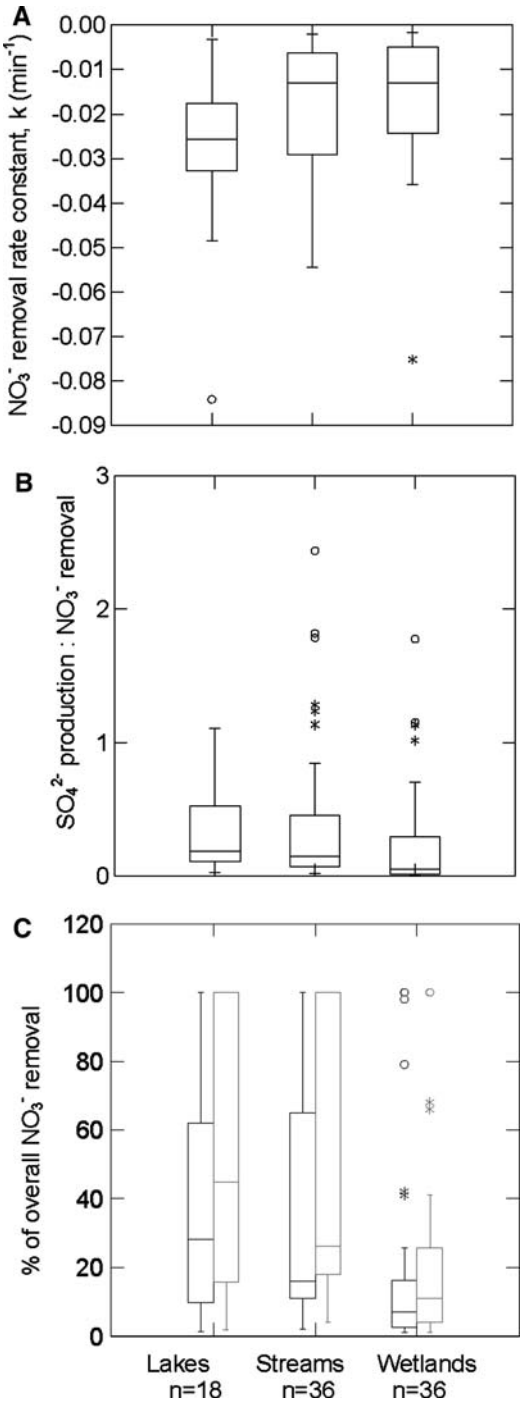

Figure 2. Nitrate removal by lakes, streams, and wetlands. ''*n*'' refers to the number of individual experiments (typically three per site), not to the number of sites of that particular ecosystem. *Boxes* encompass the upper and lower quartiles, while the *line* indicates the median. *Asterisks* are mild outliers and *open circles* are extreme outliers. Shown here are: (**A**) NO_3^- removal rate constants (min^{-1}); (**B**) ratios of total SO_4^{2-} production to NO_3^- removal (SP:NR); and (**C**) the fraction of measured NO_3^- removal that can be explained by SO_4^{2-} production using equations (1)–(3) (*gray boxes*) or equations (4)–(6) (*black boxes*).

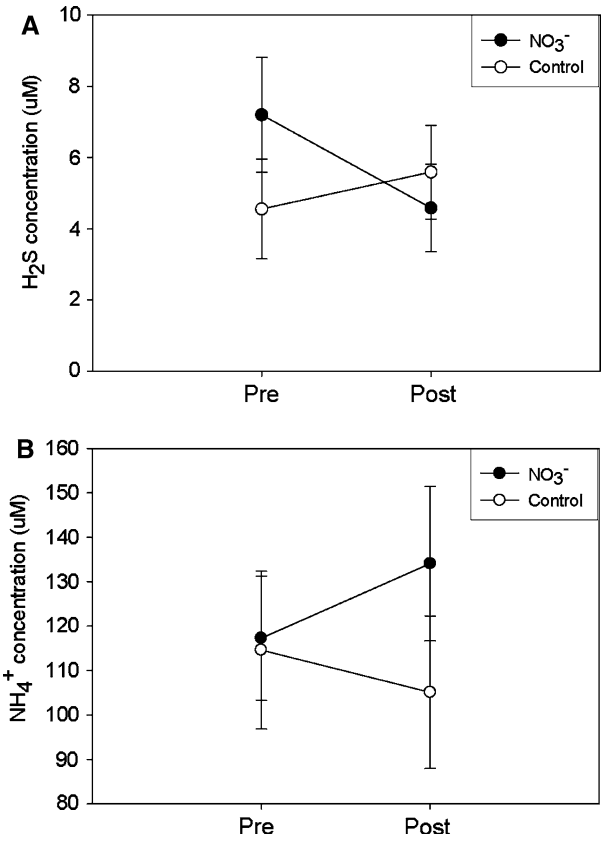

Figure 3. Pre- and post-treatment NH_4^+ concentrations (*bottom*) and H_2S concentrations (*top*) for all injectors at all sites in both 2004 and 2005. *Closed symbols* denote injectors that received NO_3^- additions and *open symbols* denote control injectors that only received a Br^- addition. Error bars are ±1 standard error of the mean.

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 we added $^{15}NO_3^-$ to the sediments during push–pull experiments (Figure 4). Although there were no significant differences in the amount of DNRA among ecosystem types (ANOVA, $F_{2,15} = 2.46$; $P = 0.119$), wetlands tended to have a higher fraction of NO_3^- removal attributable to DNRA. Additionally, wetlands had the greatest variation in the percent of NO_3^- removal that could be attributed to DNRA. Streams and lakes had comparable amounts of NO_3^- removal attributable to DNRA, ranging from 3.5 to 37% and 0.5 to 42%, respectively (Figure 4). This DNRA assay would reflect the sum of both fermentative and chemolithoautotrophic metabolisms.

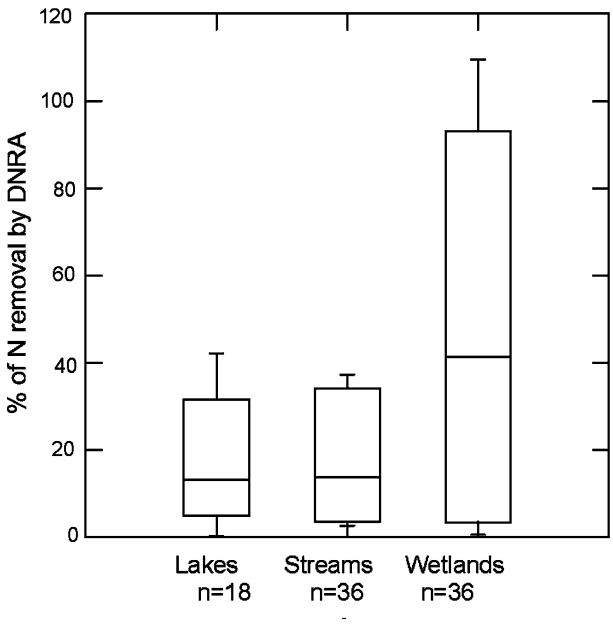

Figure 4. Percent of nitrate removal attributable to DNRA by lakes, streams, and wetlands. ''*n*'' refers to the number of individual experiments (typically three per site), not to the number of sites of that particular ecosystem. *Boxes* encompass the upper and lower quartiles, whereas the *line* indicates the median for the dataset.

We also measured $^{15}N_2$ production (indicative of denitrification) as part of the $^{15}NO_3^-$ injections, which would have allowed for a direct comparison of denitrification to DNRA. However, we concluded that the addition of 99% $^{15}NO_3^-$ in the experiments produced mostly $^{30}N_2$ (as opposed to $^{29}N_2$) because there was little ambient $^{14}NO_3^-$ in the porewater. Isotope ratio mass spectrometers 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, we were not able to get accurate estimates of denitrification from the $^{15}N_2$ measurements.

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 MR model to examine which site characteristics best predicted both NO_3^- removal rate constants and SP:NR across sites. NO_3^- removal rate constants were best predicted by surface water NO_3^- and NH_4^+ concentrations and porewater H_2S concentrations (Table 2). Sites with higher surface water NO_3^- had higher NO_3^- removal rate constants, as did sites with lower surface water NH_4^+ and lower H_2S concentrations. 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 lower porewater NH_4^+ concentrations. The combination of these two variables explained 44% of the variation in SP:NR ratios across sites.

Experimental Artifact Tests

In the tests of SO_4^{2-} production and NO_3^- removal across a temperature gradient, both processes had an intermediate thermal optimum, which is indicative of a biologically mediated (enzymatic) reaction (Figure 5). Results were similar for injectors made of either stainless-steel screens (closed symbols) or nylon filters (open symbols), indicating that an interaction with the steel was not causing the phenomenon.

To ensure that the tracer ions did not somehow affect sediment ion exchange equilibria (desorbing SO_4^{2-}), we injected only Br^- at various concentra-

Table 2. Stepwise MR Model ($\alpha = 0.1$) to Predict NO_3^- Removal Rate Constants and Ratios of SO_4^{2-} Production to NO_3^- Removal (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

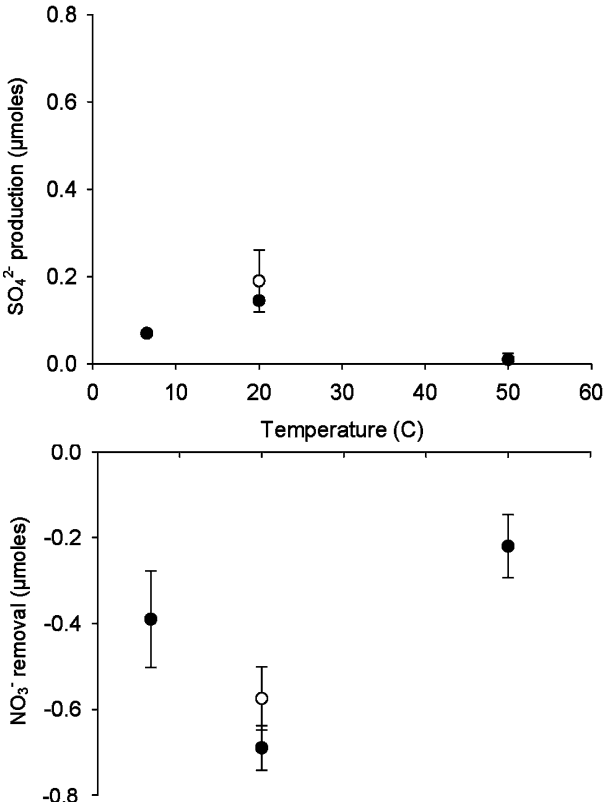

Figure 5. The effect of temperature on nitrate removal and sulfate production (both in micromoles). *Open symbols* denote the nylon mesh injectors, and *closed symbols* denote the stainless-steel injectors that were employed in all of the field experiments. These incubations lasted 4 h.

tions, and found no SO_4^{2-} production (the NaBr comprised most of the total ions added; Figure 1 in Appendix A). All experiments were conducted in sediments free of plant roots, ruling out NO_3^- uptake by vascular plants. Finally, to ensure that the injection of NO_3^- directly into anoxic porewaters was not creating an artificial juxtaposition of reduced and oxidized substances, we added NO_3^- to the water overlying sediments in a bucket of organic sediment in the lab. Water-column NO_3^- additions yielded the same results as the push–pull experiments from the lab and field, albeit over a longer time scale (Figure 1E, F).

Enrichment and Microbial Characterization

To confirm that freshwater wetlands had the biological capacity to conduct NO_3^--driven SO_4^{2-} production, we enriched for and identified microbes responsible for the process. In the cultures from three wetland sites, the added NO_3^- and H_2S were generally removed within 24 h of the addition (Figure 2 in Appendix A). This was also accompanied by an increase in SO_4^{2-} concentration, as was also seen in the push–pull experiments. The majority of the added H_2S was oxidized to SO_4^{2-}; on average, at the end of the experiment more than 90% of the added H_2S was found as SO_4^{2-} with the remainder as elemental S (Figure 3 in Appendix A). Community analysis from the three different enrichments suggested that the cultures contained predominantly *Tms. denitrificans*, because 17, 17, and 14 of the 20 clones picked per site grouped closest to this species for the respective enrichment cultures. BLAST analysis confirmed that these enrichment sequences were largely *Tms. denitrificans*.

Discussion

NO_3^- Removal Across Aquatic Ecosystems

NO_3^- removal is often considered to be a beneficial service provided by aquatic ecosystems (Zedler 2003), particularly in agricultural landscapes such as those in southwestern Michigan. This study, like an earlier one by Whitmire and Hamilton (2005), demonstrates how quickly NO_3^- can be removed in sediments of many different types of aquatic ecosystems (Figures 1 and 2A). 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, and that all aquatic ecosystems have some capacity to remove NO_3^-. NO_3^- removal may be driven by the transport of NO_3^- (or lack thereof) into the sediment porewaters. This hypothesis, however, would predict that there should be higher rates of NO_3^- removal in streams, due to their greater turbulence and hydrologic connectivity to porewaters. We did not find evidence for higher NO_3^- removal rates in streams as compared to wetland or lake sediments.

Our push–pull methodology reveals potential rates of microbial transformations because the background NO_3^- concentration in the porewater was increased to ensure that NO_3^- removal was readily measurable over the time course of the experiment. However they are not maximum potential rates because other potential limiting factors (for example, sulfide, labile organic matter) were not altered. We added 10 mg N/L (714 µM) of NO_3^- to porewaters with relatively low ambient NO_3^- concentrations (generally 1–35 µM, though as high as 550 µM). Although this represents a

large increase over the background concentration, it is not out of the realm of possibility for these sediments to receive water inputs with such high NO_3^- concentrations. Domestic water supply wells in this agricultural region commonly contain ground water with NO_3^- concentrations as high as in our injectate, and most of our study sites were largely groundwater-fed aquatic ecosystems (Whitmire 2003). An additional limitation to the push–pull method is that the rates of the reaction cannot readily be reported on an areal or volumetric basis because the sphere of influence of the tracer injection is unknown and dependent on factors that vary across sites, including the porosity and bulk density of the sediments.

Evidence for NO_3^--Driven SO_4^{2-} Production

SO_4^{2-} production coincident with NO_3^- removal was observed in most of the freshwater sediments we examined and appears to be a biologically mediated process, as indicated by the intermediate temperature optimum seen for both NO_3^- removal and SO_4^{2-} production (Figure 5). If the reaction was abiotic (that is, 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 water overlying a sediment mesocosm (Figure 1E, F), wherein we saw the same NO_3^--induced SO_4^{2-} production, albeit over a longer time-scale. Further evidence for the biological nature of this reaction is the molecular characterization of *Tms. denitrificans* in enrichment cultures from three of the sites used in this study. The cultures largely produced SO_4^{2-} rather than elemental S. We were not able to ascertain the ultimate fate of the added NO_3^- in the enrichment cultures (that is, denitrification or DNRA).

SO_4^{2-} production often accounted for 25–50% of the NO_3^- removal across aquatic ecosystems (based on the interquartile range of SP:NR ratios; Figure 2B, C). Lakes and streams generally had higher amounts of SO_4^{2-} production relative to NO_3^- removal than did wetlands, although 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; Table 1). This relative abundance of electron donors may have caused H_2S to be oxidized only to elemental S [as in equations (1) and (4)] rather than all the way to SO_4^{2-} [as in equations (3) and (6)], which would have led us to underestimate the importance of SO_4^{2-} production relative to NO_3^- removal (SP:NR). The occasional cases where SO_4^{2-} production exceeded NO_3^- removal might be explained by bacterial use of NO_3^- that had been previously taken up and stored; we are confident that these sediments did not contain enough O_2 to drive the SO_4^{2-} production.

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 3). Across all of the individual experiments from both years, NH_4^+ concentrations increased after adding NO_3^- compared to the control, whereas the H_2S concentrations simultaneously decreased. Although the differences illustrated in Figure 3 are not statistically significant, they are consistent with the expected pattern if NO_3^--driven SO_4^{2-} production was occurring. This study adds to the observations of others (Lucassen and others 2002; Soares 2002; Forshay 2003; Whitmire and Hamilton 2005) that there may be important but relatively unexplored linkages between N and S cycles in freshwaters.

An alternative explanation of our observed SO_4^{2-} production is that the addition of NO_3^- promotes denitrification, and denitrifiers out-compete SO_4^{2-} reducers for labile substrates. Meanwhile, SO_4^{2-} continues to accumulate via another form of sulfide oxidation (for example, O_2-driven SO_4^{2-} production). To get the increases in SO_4^{2-} that we commonly measured would require large amounts of O_2. An increase of just 10 μM SO_4^{2-} (on the low end of what we measured in this study), would require a concentration of 235 μM O_2 in the water, which we believe is impossibly high given our methods. Additionally, we have measured SO_4^{2-} production of similar magnitude when we add NO_3^- to completely anoxic slurries in lab incubations and in the enrichment cultures (Figure 2 in Appendix A), as opposed to the in situ field methods described here. By simultaneously adding NO_3^- and $^{35}SO_4^{2-}$ in a radioisotope pool dilution experiment, we have also demonstrated that this is new SO_4^{2-} produced (Figure 4 in Appendix A). Thus we conclude that this alternative explanation of the SO_4^{2-} production could not account for a significant proportion of the phenomenon we report on herein.

Chemolithoautotrophic oxidation of iron (Fe) and possibly manganese (Mn) could potentially utilize NO_3^- as an oxidant in a manner analogous to NO_3^--driven S oxidation, representing additional potential NO_3^- removal processes that have

been little explored in fresh waters (Hauck and others 2001; Weber and others 2006; Haaijer and others 2006, 2007). Although we did not measure Fe or Mn in our study sites, data from anoxic peats of nearby wetland sites in southwest Michigan indicate that reduced Fe^{2+} and total dissolved Mn are present at low concentrations (5–50 and 10–30 μM, respectively; Koretsky and others 2007). In sediment pore waters free Fe^{2+} and H_2S are somewhat mutually exclusive due to the insolubility of iron sulfide minerals, and thus a given sediment may be more influenced by either Fe or S chemolithoautotrophy. S oxidation (for example, SO_4^{2-} production) can also be coupled to the reduction of oxidized forms of these metals (for example, Fe^{3+} or Mn^{4+}: Lovley 1991), but this reaction is not nearly as energetically favorable as NO_3^--driven S oxidation, and microbes are not able to harvest enough energy from the reaction to support growth (Lovley 1991). We cannot rule out chemolithoautotrophic Fe or Mn oxidation as a contributing sink for NO_3^- in our experiments, and this is an area that warrants further research. Nonetheless, the production of SO_4^{2-} that we observed appears most likely to result from NO_3^--driven S oxidation.

What Predicts NO_3^--Driven SO_4^{2-} Production?

Multiple linear regression models suggested that surface water SO_4^{2-} and porewater NH_4^+ were the best predictors of the amount of SO_4^{2-} production relative to NO_3^- removal (SP:NR). Sites with higher SP:NR had 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-} is understandable because 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, thus providing the sulfide that supports S oxidizing bacterial populations. The negative relationship between SP:NR and porewater NH_4^+ may be because sites with more porewater NH_4^+ are more thoroughly and consistently anoxic, and thus may have lower populations of S oxidizers.

Multiple linear regression showed a positive relationship between NO_3^- removal rates and surface water NO_3^- concentrations (Table 2), as might be expected. The model also revealed negative relationships between NO_3^- removal rates and porewater H_2S concentrations and surface water NH_4^+ concentrations. The negative relationship with H_2S could reflect the negative feedback of high H_2S concentrations, which tend to inhibit microbial S transformations (Jorgensen 1982). Alternatively, particularly high H_2S in porewaters could correspond with a lack of S oxidation potential due to an antecedent absence of oxidants. The inverse relationship between NO_3^- removal rates and surface water NH_4^+ is harder to explain; it could reflect a tendency for surface waters high in NO_3^- to be more oxic and less apt to contain high NH_4^+ concentrations, but such a relationship is not apparent from the measurements in Table 2.

NO_3^- Removal End-Products

We used two methods to estimate NO_3^- removal end-products: a stoichiometric approach [equations (1)–(6)] and ^{15}N tracer methods. Stoichiometric methods inherently rely on a mass balance approach (Groffman and others 2006), but allow comparison of the fluxes of N and S. Rates of DNRA can be directly measured using stable isotopes to track the flow of ^{15}N from NO_3^- to NH_4^+.

Using the stoichiometric approach, SO_4^{2-} production can account for a variable but significant fraction of overall NO_3^- removal [equations (1)–(3) for denitrification or equations (4)–(6) for DNRA; Figure 2C]. In general, SO_4^{2-} production explained between 25 and 40% of NO_3^- removal in lakes, 15–25% of removal in streams, and 10–15% of removal in wetlands (medians, Figure 2C). There was, however, a great deal of variation in the degree to which SO_4^{2-} production could account for NO_3^- removal in streams and lakes, with less variation in wetlands.

The $^{15}NO_3^-$ tracer push–pull experiments allow estimation of the fraction of NO_3^- converted to NH_4^+ (Figure 4). Wetlands had the greatest range of NO_3^- removal attributable to DNRA, whereas streams and lakes had smaller ranges. Tiedje (1988) hypothesized that fermentative DNRA should occur in the most biologically productive sites where sediments were most highly reducing. This could explain why wetlands had higher DNRA than either lakes or streams (although the pattern was not statistically significant). However, the most productive sites are also likely to have high organic loading to the sediments and high SO_4^{2-} reduction rates, creating the source of sulfide to support S oxidizers.

The median amount of NO_3^- removal that can be accounted for by the stoichiometry of reduction to NH_4^+ [equations (4)–(6); Figure 2C black boxes] agrees reasonably well with the total 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 NO_3^- removal to NH_4^+ as predicted by the SO_4^{2-} production (Figure 2C, 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 4), whereas the SO_4^{2-} production only estimated 10% of the overall NO_3^- removal was to NH_4^+. This suggests that the stoichiometric model for NO_3^--driven SO_4^{2-} production via DNRA may underestimate the total 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 2C). However, although there is a great deal of variation, the median values of measured DNRA (Figure 4) and the median values of the stoichiometric model (Figure 2C, black bars) are very similar, explaining approximately 20% of the NO_3^- removal. In this regard the two methods for estimating the importance of N–S coupled cycling (via either measured sulfate production or measured DNRA) are in approximate agreement.

We attempted to measure DNRA by quantifying the increase in porewater NH_4^+ concentrations after NO_3^- injection (Figure 3), and by using stable isotope enrichment experiments (Figure 4). Figure 3 demonstrates 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 within a particular ecosystem. However, by using stable isotope techniques (Figure 4), we found that DNRA can be a significant pathway of NO_3^- removal. The isotope tracer experiments provide a conservative estimate of DNRA because our measurements of $^{15}NH_4^+$ production did not include any tracer ^{15}N that equilibrated with the sorbed NH_4^+ pool, which can be significant in many sediments.

The temporary increase in porewater NO_3^- concentrations produced by our experimental methods may have stimulated microbial N transformation rates, and this raises the question of whether a particular NO_3^- removal pathway was favored over others. Several studies have suggested that increasing NO_3^- availability should stimulate denitrification at the expense of DNRA (King and Nedwell 1985; Smith 1982; Fazzolari and others 1990; Laverman and others 2006; Matheson and others 2003; Morkved and others 2005). Therefore, although the absolute rates of DNRA may be stimulated upon NO_3^- addition, these should be conservative estimates of the importance of DNRA relative to overall NO_3^- removal.

Implications for Aquatic Ecosystem N Cycling

The existence and relative importance of alternative N removal processes besides respiratory denitrification have profound implications for N cycling in aquatic ecosystems (Burgin and Hamilton 2007). Whereas NO_3^- that is denitrified to N_2 is permanently removed from biological availability, NO_3^- 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 of microbial metabolism, especially compared to denitrification. The ultimate fate of the resultant NH_4^+ from DNRA is unclear; the NH_4^+ may be nitrified if it reaches oxic environments, or it could be stored temporarily as sorbed ions in the sediments or as organic N assimilated into biomass.

NO_3^- is the most mobile N form in hydrologic systems, and removal of NO_3^- by any of these microbial processes can improve water quality, but permanent removal (to N_2 gas) by denitrification is most desirable. Nitrous oxide (N_2O) is a byproduct of denitrification that contributes to climate change, but the other N transformations including DNRA and NO_3^--driven S oxidation are also potential sources of N_2O, with unknown importance for N_2O emissions to the atmosphere. Our hypothesis that S-oxidizing bacteria are taking up and transforming a significant fraction of the NO_3^- in surface or ground waters implies that NO_3^- removal is closely linked to S cycling, and specifically to SO_4^{2-} inputs. SO_4^{2-} is a ubiquitous pollutant in industrialized regions and freshwater SO_4^{2-} concentrations are greatly enhanced over pre-industrial times (Schlesinger 1997). If anthropogenic SO_4^{2-} loading to freshwaters enhances sulfide availability through SO_4^{2-} reduction, thereby indirectly increasing the importance of NO_3^--driven S oxidation, then the controls on N processing in landscapes subject to S and N pollution become more complex than previously thought.

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REFERENCES

Addy K, Kellogg DQ, Gold AJ, Groffman PM, Ferendo G, Sawyer C. 2002. In situ push-pull method to determine ground water denitrification in riparian zones. J Environ Qual 31:1017–24.

Aminot A, Kirkwood DS, Kerouel R. 1997. Determination of ammonia in seawater by the indophenol-blue method: evaluation of the ICES NUTS I/C 5 questionnaire. Mar Chem 56:59–75.

Bernot MJ, Dodds WK. 2005. Nitrogen retention, removal, and saturation in lotic ecosystems. Ecosystems 8:442–53.

Bonin P. 1996. Anaerobic nitrate reduction to ammonium in two strains isolated from costal marine sediment: a dissimilatory pathway. FEMS Microbiol Ecol 19:27–38.

Bottcher J, Strebel O, Voerkelius S, Schmidt HL. 1990. Using isotope fractionation of nitrate nitrogen and nitrate oxygen for evaluation of microbial denitrification in a sandy aquifer. J Hydrol 114:413–24.

Burgin AJ, Hamilton SK. 2007. Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Front Ecol Environ 5:89–96.

Carpenter SR, Caraco NF, Correll DL, Howarth RW, Sharpley AN, Smith VH. 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecol Appl 8:559–68.

Dannenberg S, Kroder M, Dilling W, Cypionka H. 1992. Oxidation of H_2, organic compounds and inorganic sulfur compounds coupled to reduction of O_2 or nitrate by sulfate-reducing bacteria. Arch Microbiol 158:93–9.

Fazzolari E, Mariotti A, Germon JC. 1990. Dissimilatory ammonia production vs. denitrification in vitro and in inoculated soil samples. Can J Microbiol 36:786–93.

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

Fossing H, Gallardo VA, Jorgensen BB, Huttel M, Nielsen LP, Schulz H, Canfield DE, Forster S, Glud RN, Gundersen JK, Kuver J, Ramsing NB, Teske A, Thamdrup B, Ulloa O. 1995. Concentration and transport of nitrate by the mat-forming sulfur bacterium *Thioploca*. Nature 374:713–5.

Gallardo VA. 1977. Large benthic microbial communities in sulfide biota under Peru Chile subsurface countercurrent. Nature 268:331–2.

Golterman HL, Clymo RS. 1969. Methods for chemical analysis of freshwaters. Oxford: Blackwell.

Groffman PM, Altabet MA, Bohlke JK, Butterbach-Bahl K, David MB, Firestone MK, Giblin AE, Kana TM, Nielsen LP, Voytek MA. 2006. Methods for measuring denitrification: diverse approaches to a difficult problem. Ecol Appl 16:2091–122.

Guerrero RE, Montesinos E, Pedrós-Alió C, Esteve I, Mas J, vanGemerden H, Hofman PAG, Bakker JF. 1985. Phototrophic sulfur bacteria in two Spanish lakes: vertical distribution and limiting factors. Limnol Oceanogr 30:919–31.

Haaijer SCM, Van der Welle MEW, Schmid MC, Lamers LPM, Jetten MSM, den Op Camp HJM. 2006. Evidence for the involvement of betaproteobacterial *Thiobacilli* in the nitrate-dependent oxidation of iron sulfide minerals. FEMS Microbiol Ecol 58:439–48.

Haaijer SCM, Lamers LPM, Smolders AJP, Jetten MSM, den Camp HJMO. 2007. Iron sulfide and pyrite as potential electron donors for microbial nitrate reduction in freshwater wetlands. Geomicrobiol J 24:391–401.

Hamilton SK, Ostrom NE. 2007. Measurement of the stable isotope ratio of dissolved N_2 in ^{15}N tracer experiments. Limnol Oceanogr Methods 5:233–40.

Hauck S, Benz M, Brune A, Schink B. 2001. Ferrous iron oxidation by denitrifying bacteria in profundal sediments of a deep lake (Lake Constance). FEMS Microbiol Ecol 37:127–34.

Holmes RM, McClelland JW, Sigman DM, Fry B, Peterson BJ. 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. Mar Chem 60:235–43.

Istok JD, Humphrey MD, Schroth MH, Hyman MR, OReilly KT. 1997. Single-well, ''push-pull'' test for in situ determination of microbial activities. Ground Water 35:619–31.

Jorgensen B. 1982. Ecology of the bacteria of the sulphur cycle with special reference to anoxic-oxic interface environments. Philos Trans R Soc Lond 298:543–61.

Jorgensen BB, Gallardo VA. 1999. Thioploca spp: filamentous sulfur bacteria with nitrate vacuoles. FEMS Microbiol Ecol 28:301–13.

Kelly DP. 1999. Thermodynamic aspects of energy conservation by chemolithotrophic sulfur bacteria in relation to the sulfur oxidation pathways. Arch Microbiol 171:219–29.

Kelly DP, Wood AP. 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. Int J Syst Evol Microbiol 50:547–50.

King D, Nedwell DB. 1985. The influence of nitrate concentration upon the end-products of nitrate dissimilation by bacteria in anaerobic salt-marsh sediment. FEMS Microbiol Ecol 31:23–8.

Koretsky CM, Haveman M, Beuving L, Cuellar A, Shattuck T, Wagner M. 2007. Spatial variation of redox and trace metal geochemistry in a minerotrophic fen. Biogeochemistry 86:33–62.

Korom SF. 1992. Natural denitrification in the saturated zone—a review. Water Resour Res 28:1657–68.

Laverman AM, Van Cappellen P, van Rotterdam-Los D, Pallud C, Abell J. 2006. Potential rates and pathways of microbial nitrate reduction in coastal sediments. FEMS Microbiol Ecol 58:179–92.

Lovley DR. 1991. Dissimilatory Fe(III) and Mn(IV) reduction. Microbiol Rev 55:259–87.

Lovley DR, Klug MJ. 1983. Sulfate reducers can out-compete methanogens at freshwater sulfate concentrations. Appl Environ Microbiol 45:187–92.

Lucassen ECHET, Smolders AJP, Roelofs JGM. 2002. Potential sensitivity of mires to drought, acidification and mobilisation of heavy metals: the sediment S/(Ca + Mg) ratio as diagnostic tool. Environ Pollut 120:635–46.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH. 2004. ARB: a software environment for sequence data. Nucleic Acids Res 32:1363–71.

Luthy L, Fritz M, Bachofen R. 2000. In situ determination of sulfide turnover rates in a meromictic alpine lake. Appl Environ Microbiol 66:712–7.

Matheson FE, Nguyen ML, Cooper AB, Burt TP. 2003. Short-term nitrogen transformation rates in riparian wetland soil determined with nitrogen-15. Biol Fertil Soils 38:129–36.

McGuire JT, Long DT, Klug MJ, Haack SK, Hyndman DW. 2002. Evaluating behavior of oxygen, nitrate, and sulfate during recharge and quantifying reduction rates in a contaminated aquifer. Environ Sci Technol 36:2693–700.

Megonigal J, Hines M, Visscher P. 2004. Anaerobic metabolism: linkages to trace gases and aerobic processes. In: Schlesinger WH, Ed. Biogeochemistry. Oxford, UK: Elsevier-Pergamon. pp 317–424.

Morkved PT, Sovik AK, Klove B, Bakken LR. 2005. Removal of nitrogen in different wetland filter materials: use of stable nitrogen isotopes to determine factors controlling denitrification and DNRA. Water Sci Technol 51:63–71.

Mulholland PJ, Helton AM, Poole GC, Hall RO, Hamilton SK, Peterson BJ, Tank JL, Ashkenas LR, Cooper LW, Dahm CN, Dodds WK, Findlay S, Gregory SV, Grimm NB, Johnson SL, McDowell WH, Meyer JL, Valett HM, Webster JR, Arango C, Beaulieu JJ, Bernot MJ, Burgin AJ, Crenshaw C, Johnson L, Merrian J, Niederlehmer BR, O'Brien JM, Potter J, Sheibley RW, Sobota DJ, Thomas SM. 2008. Excess nitrate from agricultural and urban areas reduces denitrification efficiency in streams. Nature 452:202–6.

Philippot L, Hojberg O. 1999. Dissimilatory nitrate reductases in bacteria. Biochimica Et Biophysica Acta Gene Struct Expression 1446:1–23.

Postma D, Boesen C, Kristiansen H, Larsen F. 1991. Nitrate reduction in an unconfined sandy aquifer—water chemistry, reduction processes, and geochemical modeling. Water Resour Res 27:2027–45.

Sayama M, Risgaard-Petersen N, Nielsen LP, Fossing H, Christensen PB. 2005. Impact of bacterial NO_3^- transport on sediment biogeochemistry. Appl Environ Microbiol 71:7575–7.

Schlesinger WH. 1997. Biogeochemistry: an analysis of global change. 2nd edn. San Diego: Academic Press.

Seitzinger S, Harrison JA, Bohlke JK, Bouwman AF, Lowrance R, Peterson B, Tobias C, Van Drecht G. 2006. Denitrification across landscapes and waterscapes: a synthesis. Ecol Appl 16:2064–90.

Smith MS. 1982. Dissimilatory reduction of NO_2^- to NH_4^+ and N_2O by a Soil *Citrobacter Sp*. Appl Environ Microbiol 43:854–60.

Soares MIM. 2002. Denitrification of groundwater with elemental sulfur. Water Res 36:1392–5.

Tiedje JM. 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In: Zehnder AJB, Ed. Biology of anaerobic microorganisms. New York: Wiley. pp 179–244.

Timmer-ten-Hoor A. 1981. Cell yield and bioenergetics of *Thiomicrospira denitrificans* compared with *Thiobacillus denitrificans*. Antonie Van Leeuwenhoek J Microbiol 47:231–43.

Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman DG. 1997. Human alteration of the global nitrogen cycle: sources and consequences. Ecol Appl 7:737–50.

Weber KA, Achenbach LA, Coates JD. 2006. Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction. Nat Rev Microbiol 4:752–64.

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

Whitmire SL, Hamilton SK. 2005. Rapid removal of nitrate and sulfate in freshwater wetland sediments. J Environ Qual 34:2062–71.

Zedler JB. 2003. Wetlands at your service: reducing impacts of agriculture at the watershed scale. Front Ecol Environ 1:65–72.