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Sediment nitrate manipulation using porewater equilibrators reveals potential for N and S coupling in freshwaters

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ABSTRACT: Anthropogenic nitrogen (N) loading to agricultural and populated landscapes has resulted in elevated nitrate (NO_3^-) concentrations in ground water, streams and rivers, ultimately causing problems in coastal marine environments such as eutrophication, hypoxia and harmful algal blooms. Nitrate removal along hydrologic flow paths through landscapes intercepts much of the N before it reaches coastal zones. We used traditional porewater equilibrators in a novel way to add nitrate to the sediment porewater of 8 wetlands in southwestern Michigan. Nitrate losses and changes in porewater chemistry were examined to elucidate N removal processes, with particular focus on the potential coupling of bacterial sulfur (S) oxidation to (1) dissimilatory nitrate reduction to ammonium (DNRA) and (2) denitrification. We hypothesized that, if S oxidizers utilized the added nitrate, porewater sulfide concentrations should decrease and sulfate concentrations should increase. Additionally, if the nitrate is used in DNRA, ammonium concentrations should increase as well. Nitrate additions caused decreases in dissolved hydrogen sulfide and increases in sulfate relative to controls at all sites. Ammonium also tended to increase, though the response was less consistent due to a high background ammonium pool. These results provide evidence that microbial S transformations may play an important role in nitrate removal in these freshwater wetland sediments.

KEY WORDS: Denitrification · Nitrate removal · Dissimilatory nitrate reduction to ammonium · DNRA · Sulfur oxidation · Porewater equilibrators · Freshwater sediments · Sulfate

INTRODUCTION

Nitrogen (N) loading to aquatic ecosystems has increased dramatically due to anthropogenic activity, particularly in response to the intensification of agricultural practices. This loading has resulted in a considerable increase in nitrate (NO_3^-) transport by many rivers, including the Mississippi River (Turner & Rabalais, 1991), contributing to coastal eutrophication and hypoxia (Rabalais et al. 2002a,b). Increased NO_3^- loading from rivers to marine coastal waters has also been linked to the occurrence of harmful algal blooms (Paerl et al. 2002). However, landscape mass balances for N consistently show that most of the N loaded to landscapes is lost before reaching coastal waters, with both terrestrial soils and freshwaters suspected to be important sites of N removal (Seitzinger et al. 2006).

Once in an aquatic ecosystem, NO_3^- can be assimilated by plants, algae or microbes, or it can undergo dissimilatory transformation to another N form, usually through anaerobic microbial metabolism that is most active at the sediment–water interface. Shallow, productive fresh waters such as wetlands can play a disproportionately important role in NO_3^- removal as

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water moves through landscapes (Zedler 2003). Respiratory denitrification is the most studied anaerobic transformation of NO_3^-, and in sediments this process converts most of the NO_3^- to N_2 under normal circumstances, effectively removing the N from bioavailable pools (Knowles 1982, Seitzinger 1988, Seitzinger et al. 2006).

There are also less well-studied microbial pathways that can remove NO_3^-, such as dissimilatory nitrate reduction to ammonium (DNRA) and denitrification coupled with sulfide oxidation (Burgin & Hamilton 2007). There are at least 2 forms of DNRA, in which NO_3^- is reduced to ammonium (NH_4^+) (Burgin & Hamilton 2007). Fermentative DNRA is thought to be more common in highly reducing environments with high availability of labile C relative to NO_3^-, which favors the reduction of NO_3^- to NH_4^+ as an electron sink for fermentative metabolism (Tiedje 1988, Burgin & Hamilton 2007). In contrast, chemolithoautotrophic DNRA is a redox process wherein NO_3^- is reduced to ammonium (NH_4^+) in conjunction with the oxidation of a reduced inorganic substance to derive energy. In the case of bacterial sulfur oxidation, hydrogen sulfide (H_2S) is converted to either elemental sulfur or sulfate (SO_4^{2-}) (Brunet & Garcia-Gil 1996, Burgin & Hamilton 2007). Brunet & Garcia-Gil (1996) hypothesized that H_2S plays 2 roles in promoting this pathway: (1) as an energy source for S oxidizing bacteria, and (2) as an inhibitor of the enzymes for the terminal steps of denitrification, effectively favoring the DNRA pathway. From the standpoint of excess N loading, DNRA is only a temporary NO_3^- sink because the resultant NH_4^+ is not permanently removed, but remains bioavailable to wetland plants and microbes. Relatively little work on NO_3^- transformations by S oxidizers has been done in freshwater ecosystems, where H_2S concentrations are typically much lower than in marine ecosystems.

The present study investigated the fate of NO_3^- added to the sediment porewaters of 8 wetlands in southwestern Michigan using porewater equilibrators (also known as 'peepers') containing anoxic water with and without added NO_3^-, thereby using porewater equilibrators in a manipulative manner in contrast to their traditional use to describe ambient patterns. NO_3^- removal and changes in porewater chemistry were examined to elucidate NO_3^- removal processes, with particular focus on the coupling of bacterial S oxidation to DNRA and denitrification. We hypothesized that, if dissimilatory NO_3^- transformation by S oxidizers were significant, there should be a decrease in H_2S concentrations concomitant with an increase in SO_4^{2-} concentrations and, if the NO_3^- were used in DNRA, an increase in NH_4^+ concentrations as well.

MATERIALS AND METHODS

Study sites. The experiments were conducted at 8 sites near Michigan State University's W.K. Kellogg Biological Station (KBS) in southwestern Michigan. Groundwater and surface water chemistries at these sites are detailed in Table 1. All are freshwater wetlands or lakes situated on glacial terrain, and groundwater discharge is a predominant influence on their hydrology. Carbonate mineral weathering in the glacial aquifers results in alkaline ground waters that are dominated by Ca^{2+}, Mg^{2+}, and HCO_3^-. Concentrations of NO_3^- and SO_4^{2-} in ground waters in the vicinity of KBS average 243 µM (3.4 mg N l^{-1}) and 229 µM (22 mg l^{-1}), respectively (Rheaume 1990). Loosestrife Pond (LP) 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 to continual groundwater inputs from a spring, which drive surface flow across the wetland. Turkey Marsh (TM) is a 3.1 ha isolated, depressional wetland located at KBS near Gull Lake. The wetland is both precipitation- and groundwater-fed, and its surface water levels fluctuate seasonally. Windmill Pond (WP) is located on KBS grounds next to Gull Lake and receives both ground and lake waters. Prairieville Creek (PC) is a complex of springs and fens that drain into the creek to the north of Gull Lake (Whitmire 2003). Three Lakes (3L) is a series of 3 connected lakes that are largely groundwater fed; our sampling spot was in a marshy area that separates the upper 2 lakes. Douglas Lake outflow (DLO) is a marsh complex that developed at the outflow of a culvert that drains the lake, which is southwest of the Kellogg Forest. Wintergreen Lake (WGL) is a 15 ha hyper-eutrophic lake at the Kellogg Bird Sanctuary with a maximum depth of 6.3 m. Lawrence Lake (LL) is a 5 ha, oligotrophic lake with a maximum depth of 12.6 m. WGL and LL receive substantial groundwater inputs and support outflow streams.

Porewater equilibrators and sample analysis. Porewater equilibrators were used in this experiment to add NO_3^- to anoxic sediment porewaters (Hesslein 1976). The equilibrators were constructed out of acrylic blocks (60 × 6.5 × 3.8 cm) with 14 pairs of wells (2.54 cm diameter × 2.2 cm depth; ~12.5 ml volume per well) extending from the sediment–water interface to approximately 50 cm below the interface (Fig. 1A). The wells were covered with a Biotrans® Nylon membrane (0.20 µm pore size). The membrane was held in place by a thin acrylic faceplate with matching well cut-outs, attached using stainless steel bolts. The equilibrators were assembled in a water bath to minimize the amount of dissolved oxygen present.

Table 1. Porewater and surface-water characteristics of 8 study sites in southwestern Michigan, USA. Shallow: 0 to 20 cm sediment depths, Deep: 21 to 50 cm sediment depths. Porewater values are means ± SE of wells at that depth (n = 3 to 4 in shallow, n = 8 to 9 in deep sediments). SW: surface water, PW: porewater. +: concentration increase in response to NO_3^- addition, –: concentration decrease in response to the addition, 0: little or no effect of addition. TM: Turkey Marsh; LP: Loosestrife Pond; WGL: Wintergreen Lake; WP: Windmill Pond; PC: Prairieville Creek; LL: Lawrence Lake; 3L: Three Lakes; DLO: Douglas Lake Outflow

Site	PW NH_4^+ (µM)	PW NO_3^- (µM)	PW H_2S (µM)	PW SO_4^{2-} (µM)	SW NH_4^+ (µM)	SW NO_3^- (µM)	SW SO_4^{2-} (µM)	Response SO_4^{2-}	Response NH_4^+	Response H_2S
TM										
Shallow	427.5 ± 54.6	1.2 ± 0.8	8.3 ± 1.4	10.4 ± 0.4	0.8 ± 0.2	0.2 ± 0.1	74.2 ± 18.4	+	+	–
Deep	228.2 ± 12.7	0.4 ± 0.1	4.8 ± 0.6	8.1 ± 0.7				+	+	–
LP										
Shallow	777.5 ± 57.3	0.4 ± 0.1	24.9 ± 3.2	8.6 ± 1.2	1.7 ± 0.4	0.5 ± 0.4	158.1 ± 12.5	+	+	–
Deep	1007.2 ± 22.2	0.6 ± 0.2	11.4 ± 0.8	6.1 ± 0.6				+	0	–
WGL										
Shallow	2240.3 ± 469.8	0.5 ± 0.0	172.1 ± 28.9	28.7 ± 4.2	16.6 ± 7.5	2.8 ± 1.4	95.7 ± 8.7	+	+	–
Deep	1658.2 ± 82.9	0.7 ± 0.2	101.1 ± 24.9	21.5 ± 1.2				+/0	+	–
WP										
Shallow	1082.4 ± 300.0	14.6 ± 12.5	125.5 ± 42.4	146.5 ± 62.1	2.8 ± 0.3	7.1 ± 1.3	208.2 ± 10.6	+/0	+	–
Deep	373.4 ± 75.3	112.5 ± 41.5	9.5 ± 6.0	269.3 ± 30.1				–/0	+	–
PC										
Shallow	2330.7 ± 236.5	7.7 ± 1.9	4.5 ± 0.3	10.7 ± 1.9	2.0 ± 0.5	432.4 ± 22.0	379.9 ± 9.7	+	-	–
Deep	523.8 ± 134.6	8.9 ± 1.2	9.1 ± 1.6	18.7 ± 7.7				+	-	–
LL										
Shallow	4234.7 ± 210.9	1.3 ± 0.2	51.3 ± 1.2	14.3 ± 1.1	10.5 ± 3.4	51.0 ± 7.3	190.3 ± 5.1	+	+	–
Deep	1939.0 ± 412.5	0.9 ± 0.2	72.2 ± 7.0	25.4 ± 4.3				+	+	–
3L										
Shallow	3513.6 ± 331.4	20.9 ± 1.3	1.2 ± 0.2	27.0 ± 1.3	5.3 ± 2.0	111.3 ± 8.9	288.1 ± 8.4	+	–	–/0
Deep	1984.0 ± 256.3	16.9 ± 2.7	1.6 ± 0.2	21.7 ± 3.1				+	–	–/0
DLO										
Shallow	1508.0 ± 245.2	0.5 ± 0.0	22.3 ± 2.8	9.1 ± 0.7	0.5 ± 0.0	0.4 ± 0.3	93.4 ± 12.6	+/0	+	–
Deep	586.4 ± 33.8	1.4 ± 0.6	24.3 ± 1.5	10.4 ± 1.1				+	+/0	–

For each site, 1 equilibrator was prepared in a bath of deionized water (control equilibrator) and another in deionized water containing 100 mg NO_3^--N l^{-1} (7.14 mM) as $NaNO_3$. After the equilibrators were constructed, they were placed vertically in an acrylic box filled with the same deionized or NO_3^- enriched water and sparged with helium (He) overnight to remove dissolved oxygen. The following day, the equilibrators were transported in their boxes to the wetlands for deployment. The equilibrators were quickly removed from the boxes, keeping a layer of water on top of the equilibrator wells, and placed vertically into the wetland sediment with the uppermost 1 or 2 well pairs remaining above the sediment–water interface. The equilibrators were retrieved 1 wk later.

Porewater equilibrators are typically used in a descriptive fashion to document vertical profiles of solutes in sediments. To our knowledge, this is the first study to use porewater equilibrators in a manipulative fashion to stimulate a microbial process in sediments. To better illustrate what occurs during this manipulation, we have created a conceptual diagram of the changes that occur over time (Fig. 1B). At time zero, the wells are dark because they contain the highest concentration of NO_3^-. After a few hours to a full day, the NO_3^- concentration in the wells starts to decrease as it diffuses outward and equilibrates (grey arrow in Fig. 1B) with the surrounding porewater (grey semi-

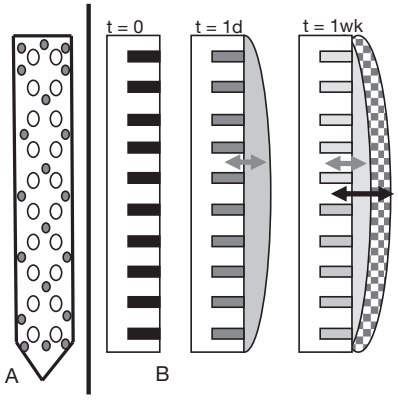

Fig. 1. (A) Schematic of a porewater equilibrator in the vertical deployment position. Grey dots: bolts attaching face plate to acrylic base. Empty circles: well pairs. (B) Conceptual diagram of equilibration dynamics that occur in the equilibrators over 1 d and 1 wk (see 'Materials and methods' for explanation)

circle in Fig. 1B). This equilibration of the added NO_3^- continued until we removed the equilibrator 1 wk later. However, at this point, NO_3^- transformed in the porewater environment had been converted to end-products (checked semi-circle in Fig. 1B), which also exist in equilibrium (black arrow in Fig. 1B) with the surrounding porewater and the water inside the equilibrator wells.

At the time of collection, equilibrators were removed one at a time from the sediment and placed horizontally for collection of water samples from the equilibrator wells. Horizontal placement ensured that a layer of water covered the membrane over the equilibrator wells, restricting the rate of O_2 diffusion past the membrane. Samples were drawn from each well into a syringe to prevent air contamination. A subsample was taken for dissolved H_2S analysis by the methylene blue spectrophotometric method (Golterman & Clymo 1969), adding reagents immediately in the field. Subsamples were analyzed upon return to the laboratory by membrane-suppression ion chromatography for NO_3^-, SO_4^{2-} and Na^+ (AS14A column, Dionex Corporation), and by the indophenol-blue method with long-path-length spectrophotometry for NH_4^+ (Aminot et al. 1997).

Statistical analysis. All statistical analysis was completed using SYSTAT 11. Comparisons of response variables (H_2S, NH_4^+ and SO_4^{2-} concentrations) between treatment (+NO_3^-) and control (no NO_3^-) porewater equilibrators were made by 1-way ANOVA for both shallow and deep wells using each site as a statistical replicate (n = 8).

RESULTS

The percentage of added NO_3^- lost from the equilibrator chambers after 7 d of equilibration varied by depth within a given site and among the different sites (Fig. 2). NO_3^- loss would have occurred via diffusion out of the chambers, and microbial removal of NO_3^- in the porewater environment would have hastened this diffusive loss. The microbial removal of NO_3^- can be estimated by comparing the total NO_3^- loss with the loss of a conservative tracer (e.g. Na^+). More than half of the added NO_3^- had diffused out of the chambers at all sites. The sites generally fell into 3 groups: (1) NO_3^- loss was lowest at TM, LP and WGL, (2) WP, PC, and DLO all had intermediate NO_3^- losses (>80 to 98%), and (3) LL and 3L had the most complete NO_3^- losses (≥98%). Representatives of these 3 groups are shown in Fig. 2, arranged from highest NO_3^- loss to lowest NO_3^- loss. Also plotted is the % loss of Na^+, which was added to the wells in conjunction with NO_3^- and served as a conservative tracer to indicate solute loss by dilution and dispersion in the absence of microbial transformations. At some sites (e.g. LL), Na^+ loss was similar over the entire depth of the equilibrator (Fig. 2A); however, other sites had less diffusive loss in deeper sediments (e.g. 3L, Fig. 2C). The percent difference in NO_3^- and Na^+ loss indicates uptake or transformation of NO_3^-, and ranged from nearly 0 to 23% of the total loss (Fig. 2). Thus, most of the observed NO_3^- loss in the wells was due to diffusion and dispersion, but NO_3^- uptake or transformation was also apparent.

At most sites, there was markedly higher % NO_3^- removal in wells <20 cm from the sediment–water interface (wells that were above the interface are not included in Fig. 2). Only LL departed from this pattern. For further analysis, given the differences in NO_3^- removal between the deeper and near-surface sediments, we split the sediment profiles into shallow (0 to 20 cm) and deep (20 to 50 cm) depth ranges, represented by 3 to 4 and 9 well pairs, respectively. This difference in activity between shallow and deep sedi-

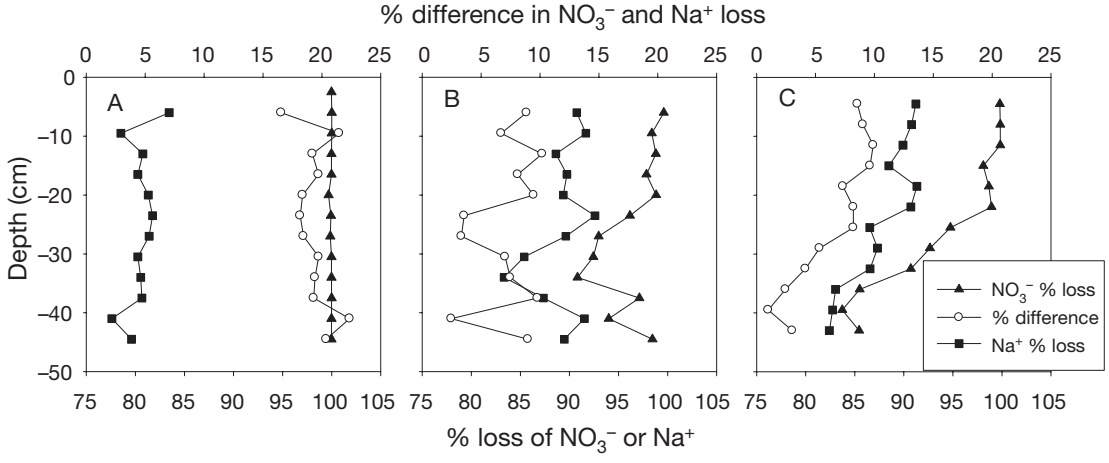

Fig. 2. Relative (%) NO_3^- and Na^+ losses and difference between the two from the equilibrators as a function of sediment depth at 3 representative sites: (A) Lawrence Lake, (B) Windmill Pond and (C) Three Lakes

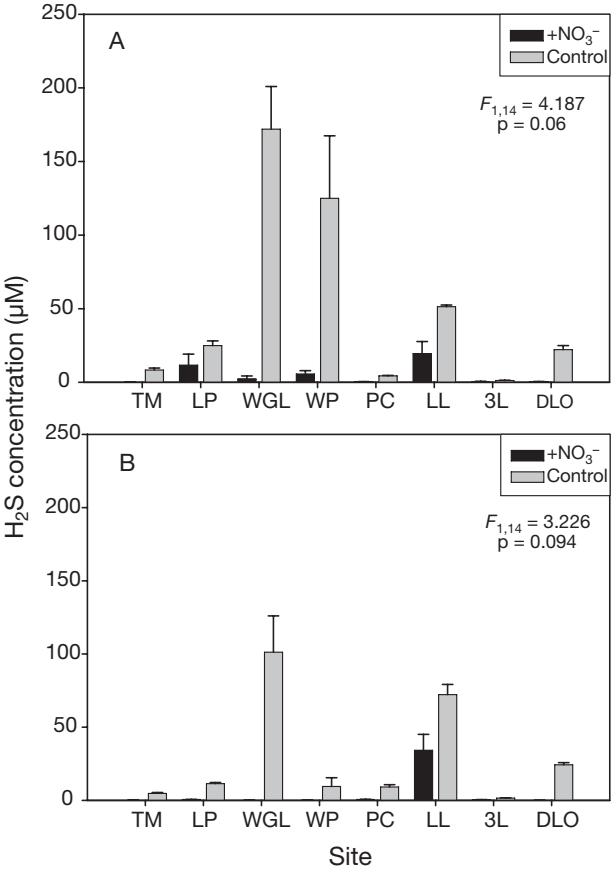

Fig. 3. Treatment and control porewater H_2S concentrations in (A) the upper 20 cm (shallow sediments) and (B) 20 to 50 cm (deep sediments) at 8 sites. Values are means + SE. See Table 1 for site abbreviations

ments was also observed by Whitmire (2003) for many similar local wetlands.

In both the shallow and deep sediments, NO_3^- addition markedly decreased H_2S concentration compared to the controls (Table 1, Fig. 3; $F_{1,14} = 4.19$, p = 0.06, and $F_{1,14} = 3.23$, p = 0.09, respectively). In the controls, H_2S was detectable but variable among sites, and typically at higher concentration in the shallow part of the profile (e.g. sites WGL and LP), though Sites LL and PC were exceptions to this.

At most of the sites, NH_4^+ concentrations were higher in the NO_3^- amended equilibrators compared to the controls (Table 1, Fig. 4: Sites TM, WGL, WP, LL and DLO) in both the shallow and deep sediments, though this trend was not significant overall ($F_{1,14}$ = 0.98, p = 0.33, and $F_{1,14} = 1.82$, p = 0.19, respectively). Sites LL and WGL had the greatest increase in NH_4^+ concentrations in the presence of added NO_3^-, showing increases that represent a substantial fraction of the total observed decrease in NO_3^- on a molar basis. However, at Sites 3L and PC, NH_4^+ concentrations were greater in the control equilibrators than in the NO_3^- amended equilibrators. At Site LP, the treatment and control equilibrators had nearly the same NH_4^+ concentrations, which did not vary with depth.

NO_3^- amendments significantly increased SO_4^{2-} concentrations compared to the controls in shallow sediments (Fig. 5A and Table 1; $F_{1,14} = 5.31$, p = 0.037). This same effect also occurred in the deep sediments and, although the increase in SO_4^{2-} agreed with our prediction, it was not statistically significant ($F_{1,14} = 3.24$, p = 0.09). Sites PC, 3L, LP and TM had the greatest increases in SO_4^{2-} concentrations, while the other sites did not respond to the NO_3^- addition as strongly. At Sites TM, LP, and WGL, SO_4^{2-} concentrations were greater in the shallow sediments; at Sites WP, LL, and DLO, SO_4^{2-} concentrations were at least as great, if not greater, in the deep profile as in the shallow one. At Site PC, the SO_4^{2-} concentrations were greater with NO_3^- addition in the shallow sediments, but at Site 3L, the SO_4^{2-} concentrations increased more with NO_3^- addition in the deeper sediments.

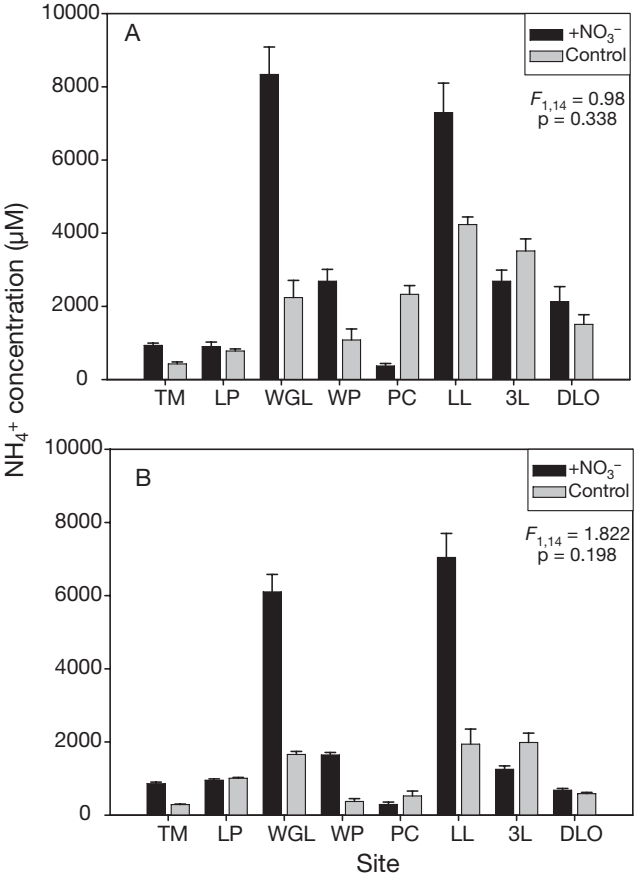

Fig. 4. Treatment and control porewater NH_4^+ concentrations in (A) the upper 20 cm (shallow sediments) and (B) 20 to 50 cm (deeper sediments) at 8 sites. Values are means + SE. See Table 1 for site abbreviations

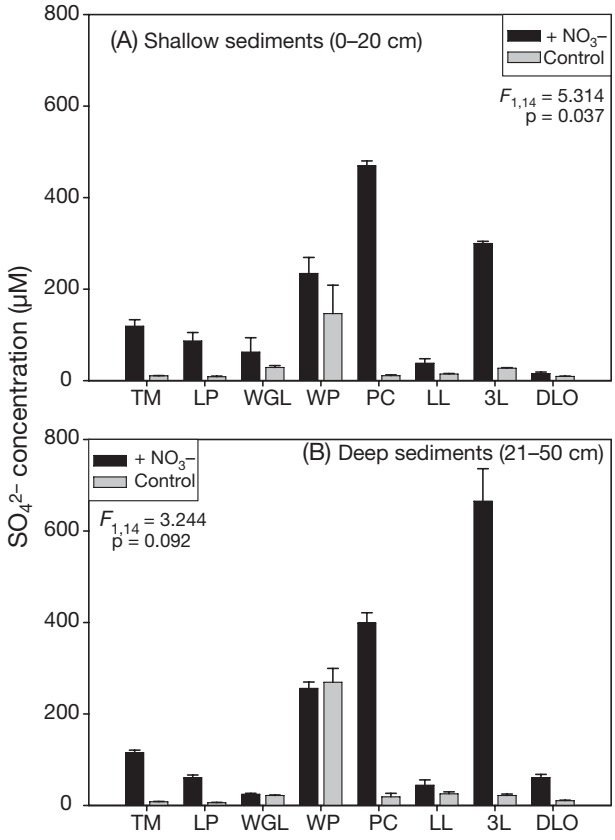

Fig. 5. Treatment and control porewater SO_4^{2-} concentrations in (A) the upper 20 cm and (B) 20 to 50 cm depth ranges of sediments at 8 sites. Values are means + SE. See Table 1 for site abbreviations

To better compare the effects of NO_3^- addition among sites, the mean concentrations at all depths in the NO_3^- amended equilibrators were compared to the mean concentrations at all depths in the control equilibrators for each response variable (H_2S, NH_4^+ and SO_4^{2-} concentrations), resulting in 1 mean value per site and treatment (Fig. 6). H_2S concentrations were generally lower in the NO_3^- amended equilibrators than in the controls at all sites (Fig. 6A), indicating removal of H_2S in the presence of NO_3^-. The response was greatest at Site WGL, followed by WP and LL. Site 3L was the closest to the 1:1 line, indicating the smallest difference between the treatment and control equilibrators. SO_4^{2-} concentrations in the NO_3^- amended equilibrators were generally greater than in the controls, indicating that SO_4^{2-} was produced in the presence NO_3^- (Fig. 6B). The response was greatest at Sites 3L and PC, while WP had little to no increase in SO_4^{2-}. Sites WGL, LL, and DLO all showed just slight SO_4^{2-} production in the treatments. NH_4^+ concentrations in the NO_3^- amended equilibrators tended to increase or remain the same relative to the controls, except for 2 sites where they decreased with NO_3^- (Fig. 6C).

DISCUSSION

The experimental addition of NO_3^- to the sediment porewaters of these freshwater wetlands using porewater equilibrators showed variable rates of NO_3^- disappearance (Fig. 2), and this corresponded with

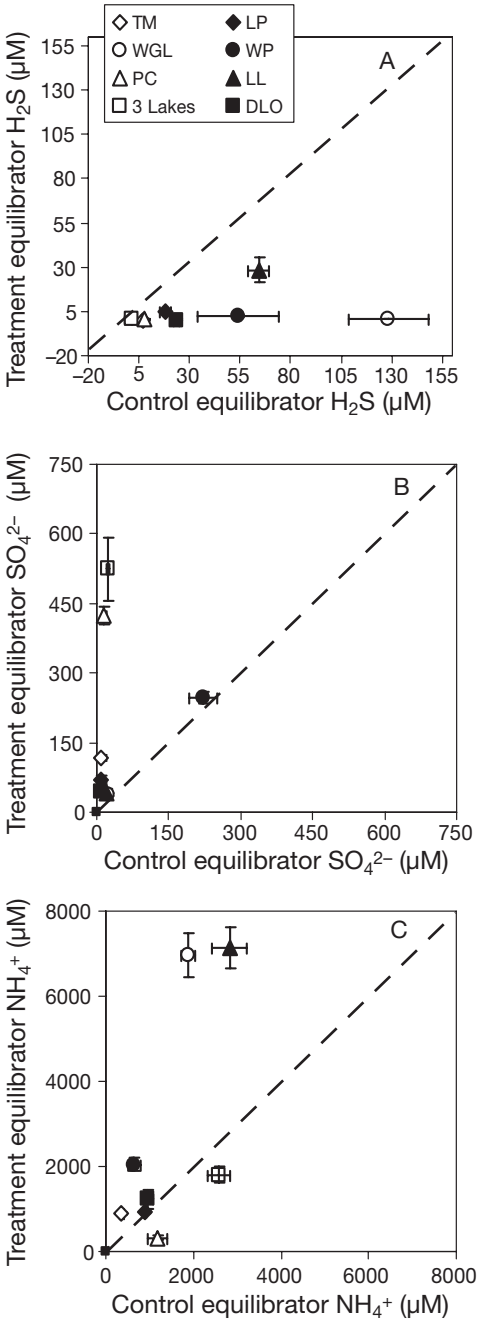

Fig. 6. Comparison of (A) H_2S, (B) SO_4^{2-}, and (C) NH_4^+ concentrations in paired control (no NO_3^-) and treatment (NO_3^- added) equilibrators at each site. Data points above the 1:1 line (dashed) indicate an increase in treatment concentrations compared to the control. Values are mean ± SE of the 14 wells in each equilibrator. See Table 1 for site abbreviations

decreases in H_2S (Fig. 3), increases in SO_4^{2-} (Fig. 4) and, in many cases, increases in NH_4^+ as well (Fig. 5). One explanation for these observations is that the increased NO_3^- availability stimulated bacterial S oxidation, presumably by serving as an alternate oxidant in the absence of O_2. From previous work at 2 of these sites, we know that S oxidizing bacteria (*Thiomicrospira denitrificans*) are active in these freshwater environments and are capable of using NO_3^- as an electron acceptor (Burgin & Hamilton 2008).

The S concentrations we report here are not exceptionally high for freshwaters, and the SO_4^{2-} concentrations in the surface waters of our sites were 2 orders of magnitude below those of seawater (Table 1). Despite the relatively low S availability in these freshwaters compared to marine environments, there is a small but growing body of literature showing that S oxidizing bacteria can be important in freshwater N cycling (Brunet & Garcia-Gil 1996, Burgin & Hamilton 2007, 2008) via processes that are analogous to those often studied in marine ecosystems (e.g. Brettar & Rheinheimer 1991, Brettar et al. 2006).

An alternative explanation for our experimental results is that the addition of NO_3^- stimulated respiratory denitrification, which effectively outcompeted the SO_4^{2-} reducers for labile products of organic matter decomposition and generated more NH_4^+ through more efficient heterotrophic activity. That alone could halt SO_4^{2-} consumption, but would not explain the observed increase in SO_4^{2-} and decrease in H_2S upon NO_3^- additions, which may be explained by O_2-driven S oxidation that occurred simultaneously with SO_4^{2-} reduction under ambient conditions. The source of O_2 at these sediment depths is difficult to identify.

High initial concentrations of NO_3^- in the wells were necessary to enable measurement of the direct products of NO_3^- transformations prior to the complete depletion of the added NO_3^-. Once NO_3^- is depleted, SO_4^{2-} reduction commences, consuming any SO_4^{2-} that was produced by NO_3^- driven S oxidation (Whitmire & Hamilton 2005). We recognize that our experiments began with an unnaturally high concentration of NO_3^- although, upon diffusion into the porewater environment, the concentrations would decrease greatly, approaching levels common in groundwater inputs to these water bodies.

We used equilibrators with rather deep wells, which had a high well volume-to-diffusion area ratio. For other applications of equilibrators, such as describing porewater chemistry, the profiles would come to equilibrium faster if the volume-to-diffusion area ratio were smaller. Thus, researchers should consider the diffusion geometry of their porewater equilibrators in deciding the application of the method. This method is best in a comparative sense, i.e. comparing the effect of a treatment (+ NO_3^-) to a control (no NO_3^-), but cannot be used to infer rates of NO_3^- removal or end-product formation. Furthermore, the potential differences in diffusion between the reactant and the product make it difficult to stoichiometrically compare products and reactants to theoretical values. We therefore restrict our analysis to comparing the effect of adding NO_3^- in the treatments to the controls, and inferring which processes may be occurring based on those differences.

While we were interested in gathering evidence for the existence and potential importance of one particular form of microbial metabolism (i.e. NO_3^- reduction coupled to SO_4^{2-} production), we recognize that other processes simultaneously occurred since these are *in situ* experiments with diverse microbial communities. In addition to the hypothesized chemolithoautotrophic NO_3^- removal coupled to S oxidation by microbes such as *Thiomicrospira denitrificans*, NO_3^- removal likely occurrs via heterotrophic (respiratory) denitrification. Additional NO_3^- removal may be undertaken by DNRA-performing bacteria utilizing a fermentative metabolism (e.g. *Citrobacter* sp., Smith 1982), rather than the S-driven chemolithotrophic DNRA. NO_3^- removal may also be coupled to chemolithoautrophic oxidation of manganese (Mn) or iron (Fe) (Weber et al. 2006). Furthermore, it is possible that some of the SO_4^{2-} production occurred near the surface (i.e. in the uppermost of the 14 well sets) where O_2 may penetrate into the first 1 to 2 cm of sediment. However, we think it is unlikely that another oxidant could be responsible for all of the SO_4^{2-} production measured throughout the 50 cm depth of sediment, and thus SO_4^{2-} production was more likely directly coupled to the dissimilatory reduction of NO_3^- added to the porewaters.

For several reasons, our methods do not permit estimation of the relative importance of NO_3^- use by S oxidizers to the overall NO_3^- transformation in the sediments, although rough comparisons of the decrease in NO_3^- concentrations (always >4000 µM) to the changes in concentrations of the other potential reactants suggest that S oxidation may be a significant contributor to NO_3^- transformations. The observed decrease in NO_3^- concentration reflects not only transformations but also dispersion and dilution of solutes in the pore waters outside the wells as equilibration took place. The measured decrease in H_2S concentration is a minimal indicator of the total pool of reduced sulfide because metal sulfides were likely to be important, and these are potentially oxidizable by S oxidizers (Garcia-Gil & Golterman 1993). The measured increase in SO_4^{2-} concentration is a minimal indicator of total S oxidation since, with an abundance of sulfide, the S oxidizers may produce elemental S (Kelly 1999). The measured increase in NH_4^+ concentration reflects not only DNRA, but also potentially increased N reminer-

alization activity by stimulation of respiratory denitrification; a potentially large NH_4^+ pool sorbed on the sediment ion exchange complex could have buffered changes in porewater dissolved NH_4^+. Finally, we were not able to estimate the production of N_2 from the added NO_3^-, and S oxidizers are known to denitrify NO_3^- to N_2 as well as conduct DNRA. In spite of these caveats, the changes we observed provide evidence for NO_3^- driven S oxidation as a potential NO_3^- removal process in these sediments, and they are consistent with other methods we have employed to examine this process, such as push-pull tracer additions (Whitmire & Hamilton 2005, Burgin & Hamilton 2008) and experiments with ^{15}N-labelled NO_3^- in water flowing over sediment cores (A. Burgin & S. Hamilton, unpubl. data).

Nearly all sites had more NO_3^- removal in shallow sediments than in deeper sediments (Fig. 2), perhaps indicating that the microbial community in the shallow sediments is better poised to remove NO_3^-. This pattern of greater N cycling in shallower sediments has also been observed in streams with high NO_3^- availability (Inwood et al. 2007), where greater denitrification was correlated with shallower depth, more organic matter and higher NO_3^- concentrations.

The relative importance of denitrification and DNRA as NO_3^- sinks cannot be estimated from the present study, but the addition of NO_3^- to freshwater sediments generally resulted in an increase in NH_4^+ and SO_4^{2-}, with a concomitant decrease in H_2S, demonstrating the potential contribution of DNRA by S oxidizers (Fig. 6). NO_3^- driven DNRA by S oxidizing bacteria has been observed in the epilimnion of a freshwater lake in Spain (Brunet & Garcia-Gil 1996), as well as in various freshwater ecosystems in Michigan that our laboratory has investigated using push-pull NO_3^- tracer additions (Whitmire & Hamilton 2005, Burgin & Hamilton 2008). Dannenberg et al. (1992), and Brunet & Garcia-Gil (1996) found that S reducing bacteria, such as *Desulfovibrio desulfuricans* and *D. propionieus*, are able to fully oxidize H_2S coupled with the reduction of NO_3^- to NH_4^+. *D. desulfuricans* consumed one mole of H_2S per mole of NO_3^- and produced equimolar amounts of SO_4^{2-} and NH_4^+. Additionally, Burgin & Hamilton (2008) found evidence for NO_3^- removal coupled to S oxidation at many sites in the general area where the present study was conducted. They attributed this, at least in part, to *Thiomicrospira denitrificans*, which was isolated from sediments using enrichment cultures. *T. denitrificans* is thought to produce 5 moles of SO_4^{2-} for every 8 moles of NO_3^- converted to N_2 in a form of chemolithoautotrophic denitrification (Burgin & Hamilton 2008). These investigations have further emphasized the potential importance of sulfur cycling in NO_3^- removal from aquatic ecosystems.

If S-oxidizers are responsible for a significant portion of the NO_3^- removal from surface or ground waters, then NO_3^- removal should be linked to S cycling and, specifically, to SO_4^{2-} availability. SO_4^{2-} is a ubiquitous pollutant in industrialized regions, and atmospheric deposition of SO_4^{2-} as well as concentrations in ground waters and rivers are greatly elevated over pre-industrial times (Schlesinger 1997). Excess SO_4^{2-} in freshwaters may indirectly enhance NO_3^- removal by stimulating H_2S formation through SO_4^{2-} reduction. The increased abundance of H_2S then fosters the development of populations of S oxidizing bacteria at redox gradients, and these bacteria are able to use NO_3^- as an alternate oxidant when O_2 is not available. Yet the ultimate fate of NO_3^- used by S oxidizers remains unclear; whether it becomes NH_4^+ that remains in bioavailable form or is denitrified to N_2 has critically different ecological implications. Hence, the controls on N processing in freshwaters subject to S and N pollution may be more complex than previously appreciated.

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