

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

E. K. Payne<sup>1,2</sup>, A. J. Burgin<sup>2,3,\*</sup>, S. K. Hamilton<sup>2</sup>

<sup>1</sup>Life Sciences and Society Program, University of Michigan School of Public Health, 109 South Observatory Road, Ann Arbor, Michigan 48105, USA

<sup>2</sup>W.K. Kellogg Biological Station and Department of Zoology, Michigan State University, 3700 Gull Lake Drive, Hickory Corners, Michigan 49060, USA

<sup>3</sup>Cary Institute of Ecosystem Studies, Box AB, Millbrook, New York 12545, USA

**ABSTRACT:** Anthropogenic nitrogen (N) loading to agricultural and populated landscapes has resulted in elevated nitrate ( $\text{NO}_3^-$ ) 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

—Resale or republication not permitted without written consent of the publisher—

## 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 ( $\text{NO}_3^-$ ) transport by many rivers, including the Mississippi River (Turner & Rabalais, 1991), contributing to coastal eutrophication and hypoxia (Rabalais et al. 2002a,b). Increased  $\text{NO}_3^-$  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,  $\text{NO}_3^-$  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  $\text{NO}_3^-$  removal as

\*Corresponding author. Email: burginam@gmail.com

water moves through landscapes (Zedler 2003). Respiratory denitrification is the most studied anaerobic transformation of  $\text{NO}_3^-$ , and in sediments this process converts most of the  $\text{NO}_3^-$  to  $\text{N}_2$  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  $\text{NO}_3^-$ , 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  $\text{NO}_3^-$  is reduced to ammonium ( $\text{NH}_4^+$ ) (Burgin & Hamilton 2007). Fermentative DNRA is thought to be more common in highly reducing environments with high availability of labile C relative to  $\text{NO}_3^-$ , which favors the reduction of  $\text{NO}_3^-$  to  $\text{NH}_4^+$  as an electron sink for fermentative metabolism (Tiedje 1988, Burgin & Hamilton 2007). In contrast, chemolithoautotrophic DNRA is a redox process wherein  $\text{NO}_3^-$  is reduced to ammonium ( $\text{NH}_4^+$ ) in conjunction with the oxidation of a reduced inorganic substance to derive energy. In the case of bacterial sulfur oxidation, hydrogen sulfide ( $\text{H}_2\text{S}$ ) is converted to either elemental sulfur or sulfate ( $\text{SO}_4^{2-}$ ) (Brunet & Garcia-Gil 1996, Burgin & Hamilton 2007). Brunet & Garcia-Gil (1996) hypothesized that  $\text{H}_2\text{S}$  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  $\text{NO}_3^-$  sink because the resultant  $\text{NH}_4^+$  is not permanently removed, but remains bioavailable to wetland plants and microbes. Relatively little work on  $\text{NO}_3^-$  transformations by S oxidizers has been done in freshwater ecosystems, where  $\text{H}_2\text{S}$  concentrations are typically much lower than in marine ecosystems.

The present study investigated the fate of  $\text{NO}_3^-$  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  $\text{NO}_3^-$ , thereby using porewater equilibrators in a manipulative manner in contrast to their traditional use to describe ambient patterns.  $\text{NO}_3^-$  removal and changes in porewater chemistry were examined to elucidate  $\text{NO}_3^-$  removal processes, with particular focus on the coupling of bacterial S oxidation to DNRA and denitrification. We hypothesized that, if dissimilatory  $\text{NO}_3^-$  transformation by S oxidizers were significant, there should be a decrease in  $\text{H}_2\text{S}$  concentrations concomitant with an increase in  $\text{SO}_4^{2-}$  concentrations and, if the  $\text{NO}_3^-$  were used in DNRA, an increase in  $\text{NH}_4^+$  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  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{HCO}_3^-$ . Concentrations of  $\text{NO}_3^-$  and  $\text{SO}_4^{2-}$  in ground waters in the vicinity of KBS average 243  $\mu\text{M}$  (3.4 mg N  $\text{l}^{-1}$ ) and 229  $\mu\text{M}$  (22 mg  $\text{l}^{-1}$ ), 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  $\text{NO}_3^-$  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  $\mu\text{m}$  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  $\pm$  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  $\text{NO}_3^-$  addition, -: concentration decrease in response to the addition, 0: little or no effect of addition. TM: Turkey Marsh; LP: Loosetrife Pond; WGL: Wintergreen Lake; WP: Windmill Pond; PC: Prairieville Creek; LL: Lawrence Lake; 3L: Three Lakes; DLO: Douglas Lake Outflow

Site	PW $\text{NH}_4^+$ ( $\mu\text{M}$ )	PW $\text{NO}_3^-$ ( $\mu\text{M}$ )	PW $\text{H}_2\text{S}$ ( $\mu\text{M}$ )	PW $\text{SO}_4^{2-}$ ( $\mu\text{M}$ )	SW $\text{NH}_4^+$ ( $\mu\text{M}$ )	SW $\text{NO}_3^-$ ( $\mu\text{M}$ )	SW $\text{SO}_4^{2-}$ ( $\mu\text{M}$ )	Response		
								$\text{SO}_4^{2-}$	$\text{NH}_4^+$	$\text{H}_2\text{S}$
<b>TM</b>										
Shallow	427.5 $\pm$ 54.6	1.2 $\pm$ 0.8	8.3 $\pm$ 1.4	10.4 $\pm$ 0.4	0.8 $\pm$ 0.2	0.2 $\pm$ 0.1	74.2 $\pm$ 18.4	+	+	-
Deep	228.2 $\pm$ 12.7	0.4 $\pm$ 0.1	4.8 $\pm$ 0.6	8.1 $\pm$ 0.7				+	+	-
<b>LP</b>										
Shallow	777.5 $\pm$ 57.3	0.4 $\pm$ 0.1	24.9 $\pm$ 3.2	8.6 $\pm$ 1.2	1.7 $\pm$ 0.4	0.5 $\pm$ 0.4	158.1 $\pm$ 12.5	+	+	-
Deep	1007.2 $\pm$ 22.2	0.6 $\pm$ 0.2	11.4 $\pm$ 0.8	6.1 $\pm$ 0.6				+	0	-
<b>WGL</b>										
Shallow	2240.3 $\pm$ 469.8	0.5 $\pm$ 0.0	172.1 $\pm$ 28.9	28.7 $\pm$ 4.2	16.6 $\pm$ 7.5	2.8 $\pm$ 1.4	95.7 $\pm$ 8.7	+	+	-
Deep	1658.2 $\pm$ 82.9	0.7 $\pm$ 0.2	101.1 $\pm$ 24.9	21.5 $\pm$ 1.2				+/0	+	-
<b>WP</b>										
Shallow	1082.4 $\pm$ 300.0	14.6 $\pm$ 12.5	125.5 $\pm$ 42.4	146.5 $\pm$ 62.1	2.8 $\pm$ 0.3	7.1 $\pm$ 1.3	208.2 $\pm$ 10.6	+/0	+	-
Deep	373.4 $\pm$ 75.3	112.5 $\pm$ 41.5	9.5 $\pm$ 6.0	269.3 $\pm$ 30.1				-/0	+	-
<b>PC</b>										
Shallow	2330.7 $\pm$ 236.5	7.7 $\pm$ 1.9	4.5 $\pm$ 0.3	10.7 $\pm$ 1.9	2.0 $\pm$ 0.5	432.4 $\pm$ 22.0	379.9 $\pm$ 9.7	+	-	-
Deep	523.8 $\pm$ 134.6	8.9 $\pm$ 1.2	9.1 $\pm$ 1.6	18.7 $\pm$ 7.7				+	-	-
<b>LL</b>										
Shallow	4234.7 $\pm$ 210.9	1.3 $\pm$ 0.2	51.3 $\pm$ 1.2	14.3 $\pm$ 1.1	10.5 $\pm$ 3.4	51.0 $\pm$ 7.3	190.3 $\pm$ 5.1	+	+	-
Deep	1939.0 $\pm$ 412.5	0.9 $\pm$ 0.2	72.2 $\pm$ 7.0	25.4 $\pm$ 4.3				+	+	-
<b>3L</b>										
Shallow	3513.6 $\pm$ 331.4	20.9 $\pm$ 1.3	1.2 $\pm$ 0.2	27.0 $\pm$ 1.3	5.3 $\pm$ 2.0	111.3 $\pm$ 8.9	288.1 $\pm$ 8.4	+	-	-/0
Deep	1984.0 $\pm$ 256.3	16.9 $\pm$ 2.7	1.6 $\pm$ 0.2	21.7 $\pm$ 3.1				+	-	-/0
<b>DLO</b>										
Shallow	1508.0 $\pm$ 245.2	0.5 $\pm$ 0.0	22.3 $\pm$ 2.8	9.1 $\pm$ 0.7	0.5 $\pm$ 0.0	0.4 $\pm$ 0.3	93.4 $\pm$ 12.6	+/0	+	-
Deep	586.4 $\pm$ 33.8	1.4 $\pm$ 0.6	24.3 $\pm$ 1.5	10.4 $\pm$ 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  $\text{NO}_3^-$ -N  $\text{l}^{-1}$  (7.14 mM) as  $\text{NaNO}_3$ . After the equilibrators were constructed, they were placed vertically in an acrylic box filled with the same deionized or  $\text{NO}_3^-$  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  $\text{NO}_3^-$ . After a few hours to a full day, the  $\text{NO}_3^-$  concentration in the wells starts to decrease as it diffuses outward and equilibrates (grey arrow in Fig. 1B) with the surrounding porewater (grey semi-

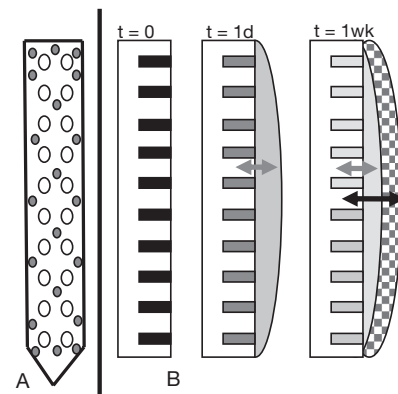


Fig. 1. (A) Schematic of a porewater equilibrator in the vertical deployment position. Grey dots: bolts attaching face plate to acrylic base. Empty circles: well pairs. (B) Conceptual diagram of equilibration dynamics that occur in the equilibrators over 1 d and 1 wk (see 'Materials and methods' for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

circle in Fig. 1B). This equilibration of the added  $\text{NO}_3^-$  continued until we removed the equilibrator 1 wk later. However, at this point,  $\text{NO}_3^-$  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  $\text{O}_2$  diffusion past the membrane. Samples were drawn from each well into a syringe to prevent air contamination. A subsample was taken for dissolved  $\text{H}_2\text{S}$  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  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$  and  $\text{Na}^+$  (AS14A column, Dionex Corporation), and by the indophenol-blue method with long-path-length spectrophotometry for  $\text{NH}_4^+$  (Aminot et al. 1997).

**Statistical analysis.** All statistical analysis was completed using SYSTAT 11. Comparisons of response variables ( $\text{H}_2\text{S}$ ,  $\text{NH}_4^+$  and  $\text{SO}_4^{2-}$  concentrations) between treatment (+ $\text{NO}_3^-$ ) and control (no  $\text{NO}_3^-$ ) 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  $\text{NO}_3^-$  lost from the equilibrator chambers after 7 d of equilibration varied by

depth within a given site and among the different sites (Fig. 2).  $\text{NO}_3^-$  loss would have occurred via diffusion out of the chambers, and microbial removal of  $\text{NO}_3^-$  in the porewater environment would have hastened this diffusive loss. The microbial removal of  $\text{NO}_3^-$  can be estimated by comparing the total  $\text{NO}_3^-$  loss with the loss of a conservative tracer (e.g.  $\text{Na}^+$ ). More than half of the added  $\text{NO}_3^-$  had diffused out of the chambers at all sites. The sites generally fell into 3 groups: (1)  $\text{NO}_3^-$  loss was lowest at TM, LP and WGL, (2) WP, PC, and DLO all had intermediate  $\text{NO}_3^-$  losses (>80 to 98%), and (3) LL and 3L had the most complete  $\text{NO}_3^-$  losses ( $\geq 98\%$ ). Representatives of these 3 groups are shown in Fig. 2, arranged from highest  $\text{NO}_3^-$  loss to lowest  $\text{NO}_3^-$  loss. Also plotted is the % loss of  $\text{Na}^+$ , which was added to the wells in conjunction with  $\text{NO}_3^-$  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),  $\text{Na}^+$  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  $\text{NO}_3^-$  and  $\text{Na}^+$  loss indicates uptake or transformation of  $\text{NO}_3^-$ , and ranged from nearly 0 to 23% of the total loss (Fig. 2). Thus, most of the observed  $\text{NO}_3^-$  loss in the wells was due to diffusion and dispersion, but  $\text{NO}_3^-$  uptake or transformation was also apparent.

At most sites, there was markedly higher %  $\text{NO}_3^-$  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  $\text{NO}_3^-$  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-

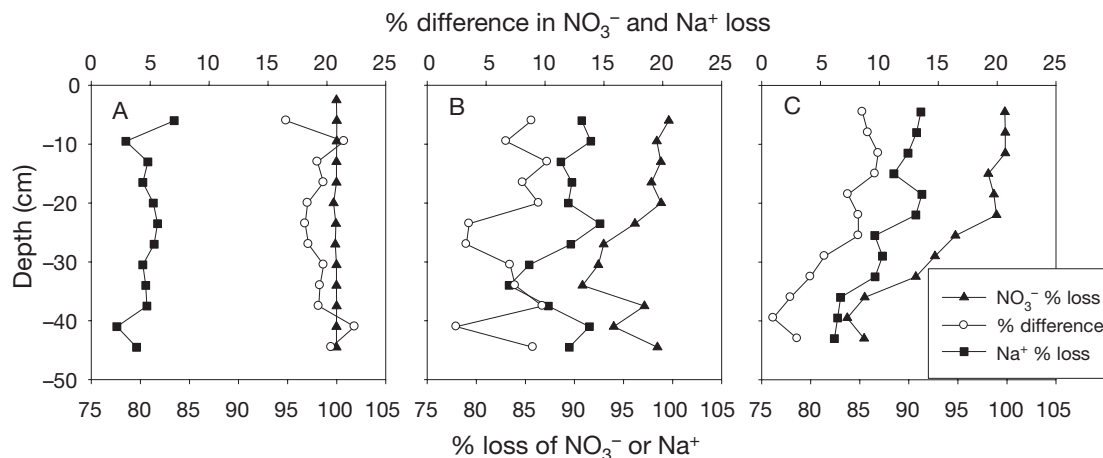


Fig. 2. Relative (%)  $\text{NO}_3^-$  and  $\text{Na}^+$  losses and difference between the two from the equilibrators as a function of sediment depth at 3 representative sites: (A) Lawrence Lake, (B) Windmill Pond and (C) Three Lakes

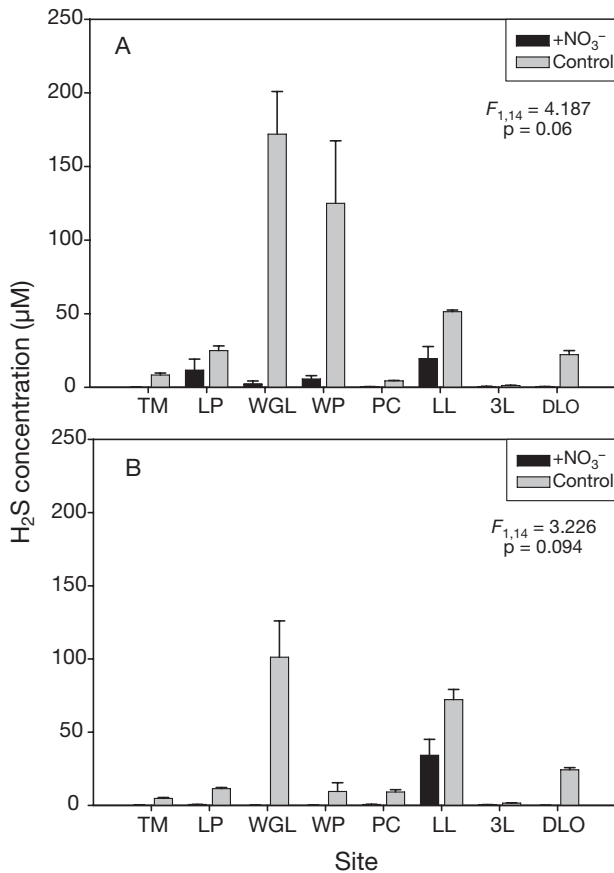


Fig. 3. Treatment and control porewater H<sub>2</sub>S concentrations in (A) the upper 20 cm (shallow sediments) and (B) 20 to 50 cm (deep sediments) at 8 sites. Values are means + SE. See Table 1 for site abbreviations

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

In both the shallow and deep sediments, NO<sub>3</sub><sup>-</sup> addition markedly decreased H<sub>2</sub>S 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<sub>2</sub>S 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<sub>4</sub><sup>+</sup> concentrations were higher in the NO<sub>3</sub><sup>-</sup> 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<sub>4</sub><sup>+</sup> concentrations in the presence of added NO<sub>3</sub><sup>-</sup>, showing increases that represent a substantial fraction of the total observed decrease in NO<sub>3</sub><sup>-</sup> on a molar basis. However, at Sites 3L and PC, NH<sub>4</sub><sup>+</sup> concentrations were

greater in the control equilibrators than in the NO<sub>3</sub><sup>-</sup> amended equilibrators. At Site LP, the treatment and control equilibrators had nearly the same NH<sub>4</sub><sup>+</sup> concentrations, which did not vary with depth.

NO<sub>3</sub><sup>-</sup> amendments significantly increased SO<sub>4</sub><sup>2-</sup> 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<sub>4</sub><sup>2-</sup> 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<sub>4</sub><sup>2-</sup> concentrations, while the other sites did not respond to the NO<sub>3</sub><sup>-</sup> addition as strongly. At Sites TM, LP, and WGL, SO<sub>4</sub><sup>2-</sup> concentrations were greater in the shallow sediments; at Sites WP, LL, and DLO, SO<sub>4</sub><sup>2-</sup> concentrations were at least as great, if not greater, in the deep profile as in the shallow one. At Site PC, the SO<sub>4</sub><sup>2-</sup> concentrations were greater with NO<sub>3</sub><sup>-</sup> addition in the shallow sediments, but at Site 3L, the SO<sub>4</sub><sup>2-</sup> concentrations increased more with NO<sub>3</sub><sup>-</sup> addition in the deeper sediments.

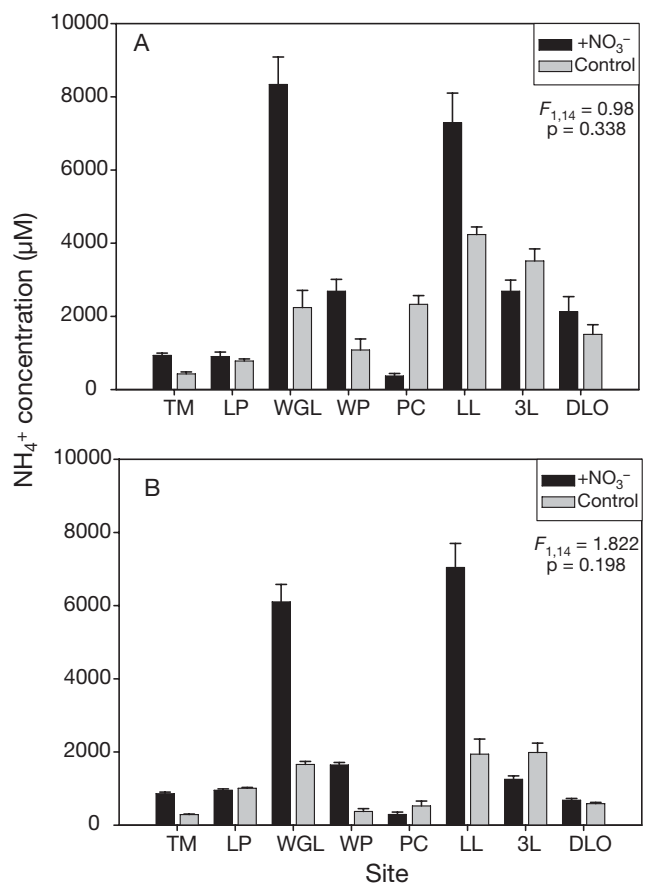


Fig. 4. Treatment and control porewater NH<sub>4</sub><sup>+</sup> concentrations in (A) the upper 20 cm (shallow sediments) and (B) 20 to 50 cm (deeper sediments) at 8 sites. Values are means + SE. See Table 1 for site abbreviations

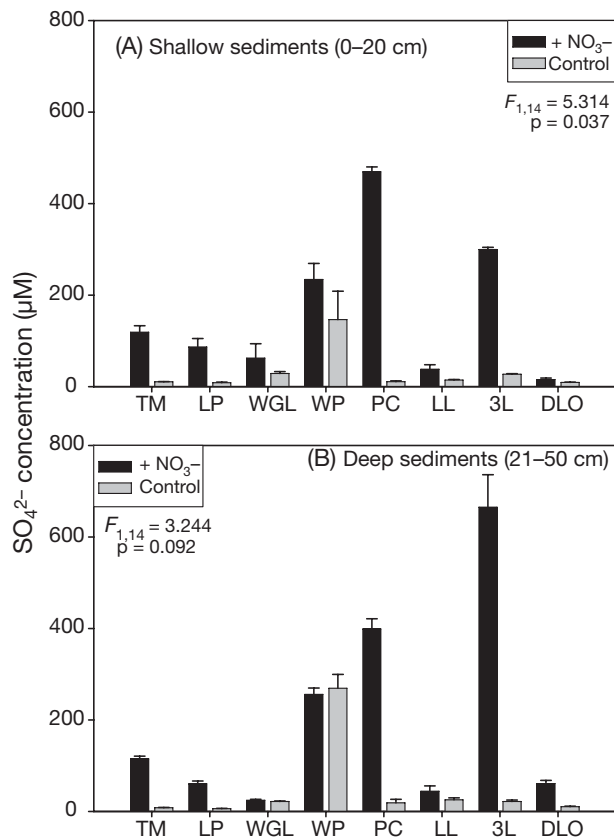


Fig. 5. Treatment and control porewater  $\text{SO}_4^{2-}$  concentrations in (A) the upper 20 cm and (B) 20 to 50 cm depth ranges of sediments at 8 sites. Values are means + SE. See Table 1 for site abbreviations

To better compare the effects of  $\text{NO}_3^-$  addition among sites, the mean concentrations at all depths in the  $\text{NO}_3^-$  amended equilibrator were compared to the mean concentrations at all depths in the control equilibrator for each response variable ( $\text{H}_2\text{S}$ ,  $\text{NH}_4^+$  and  $\text{SO}_4^{2-}$  concentrations), resulting in 1 mean value per site and treatment (Fig. 6).  $\text{H}_2\text{S}$  concentrations were generally lower in the  $\text{NO}_3^-$  amended equilibrator than in the controls at all sites (Fig. 6A), indicating removal of  $\text{H}_2\text{S}$  in the presence of  $\text{NO}_3^-$ . 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 equilibrator.  $\text{SO}_4^{2-}$  concentrations in the  $\text{NO}_3^-$  amended equilibrator were generally greater than in the controls, indicating that  $\text{SO}_4^{2-}$  was produced in the presence  $\text{NO}_3^-$  (Fig. 6B). The response was greatest at Sites 3L and PC, while WP had little to no increase in  $\text{SO}_4^{2-}$ . Sites WGL, LL, and DLO all showed just slight  $\text{SO}_4^{2-}$  production in the treatments.  $\text{NH}_4^+$  concentrations in the  $\text{NO}_3^-$  amended equilibrator tended to increase or remain the same relative to the controls, except for 2 sites where they decreased with  $\text{NO}_3^-$  (Fig. 6C).

## DISCUSSION

The experimental addition of  $\text{NO}_3^-$  to the sediment porewaters of these freshwater wetlands using porewater equilibrator showed variable rates of  $\text{NO}_3^-$  disappearance (Fig. 2), and this corresponded with

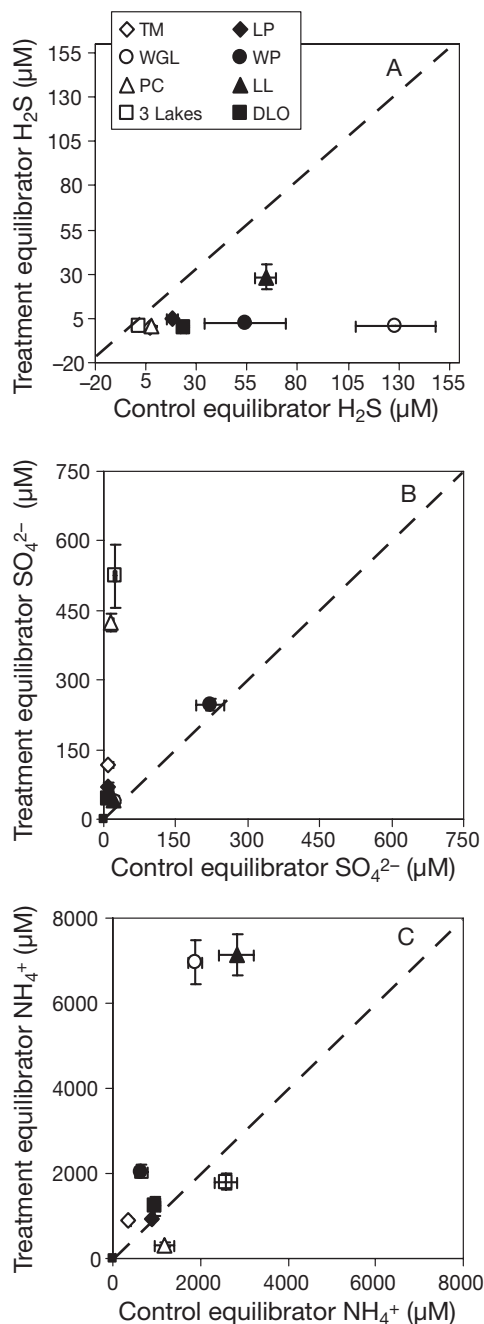


Fig. 6. Comparison of (A)  $\text{H}_2\text{S}$ , (B)  $\text{SO}_4^{2-}$ , and (C)  $\text{NH}_4^+$  concentrations in paired control (no  $\text{NO}_3^-$ ) and treatment ( $\text{NO}_3^-$  added) equilibrator at each site. Data points above the 1:1 line (dashed) indicate an increase in treatment concentrations compared to the control. Values are mean  $\pm$  SE of the 14 wells in each equilibrator. See Table 1 for site abbreviations



decreases in  $\text{H}_2\text{S}$  (Fig. 3), increases in  $\text{SO}_4^{2-}$  (Fig. 4) and, in many cases, increases in  $\text{NH}_4^+$  as well (Fig. 5). One explanation for these observations is that the increased  $\text{NO}_3^-$  availability stimulated bacterial S oxidation, presumably by serving as an alternate oxidant in the absence of  $\text{O}_2$ . 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  $\text{NO}_3^-$  as an electron acceptor (Burgin & Hamilton 2008).

The S concentrations we report here are not exceptionally high for freshwaters, and the  $\text{SO}_4^{2-}$  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  $\text{NO}_3^-$  stimulated respiratory denitrification, which effectively outcompeted the  $\text{SO}_4^{2-}$  reducers for labile products of organic matter decomposition and generated more  $\text{NH}_4^+$  through more efficient heterotrophic activity. That alone could halt  $\text{SO}_4^{2-}$  consumption, but would not explain the observed increase in  $\text{SO}_4^{2-}$  and decrease in  $\text{H}_2\text{S}$  upon  $\text{NO}_3^-$  additions, which may be explained by  $\text{O}_2$ -driven S oxidation that occurred simultaneously with  $\text{SO}_4^{2-}$  reduction under ambient conditions. The source of  $\text{O}_2$  at these sediment depths is difficult to identify.

High initial concentrations of  $\text{NO}_3^-$  in the wells were necessary to enable measurement of the direct products of  $\text{NO}_3^-$  transformations prior to the complete depletion of the added  $\text{NO}_3^-$ . Once  $\text{NO}_3^-$  is depleted,  $\text{SO}_4^{2-}$  reduction commences, consuming any  $\text{SO}_4^{2-}$  that was produced by  $\text{NO}_3^-$  driven S oxidation (Whitmire & Hamilton 2005). We recognize that our experiments began with an unnaturally high concentration of  $\text{NO}_3^-$  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 treat-

ment (+  $\text{NO}_3^-$ ) to a control (no  $\text{NO}_3^-$ ), but cannot be used to infer rates of  $\text{NO}_3^-$  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  $\text{NO}_3^-$  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.  $\text{NO}_3^-$  reduction coupled to  $\text{SO}_4^{2-}$  production), we recognize that other processes simultaneously occurred since these are *in situ* experiments with diverse microbial communities. In addition to the hypothesized chemolithoautotrophic  $\text{NO}_3^-$  removal coupled to S oxidation by microbes such as *Thiomicrospira denitrificans*,  $\text{NO}_3^-$  removal likely occurs via heterotrophic (respiratory) denitrification. Additional  $\text{NO}_3^-$  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.  $\text{NO}_3^-$  removal may also be coupled to chemolithoautotrophic oxidation of manganese (Mn) or iron (Fe) (Weber et al. 2006). Furthermore, it is possible that some of the  $\text{SO}_4^{2-}$  production occurred near the surface (i.e. in the uppermost of the 14 well sets) where  $\text{O}_2$  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  $\text{SO}_4^{2-}$  production measured throughout the 50 cm depth of sediment, and thus  $\text{SO}_4^{2-}$  production was more likely directly coupled to the dissimilatory reduction of  $\text{NO}_3^-$  added to the porewaters.

For several reasons, our methods do not permit estimation of the relative importance of  $\text{NO}_3^-$  use by S oxidizers to the overall  $\text{NO}_3^-$  transformation in the sediments, although rough comparisons of the decrease in  $\text{NO}_3^-$  concentrations (always >4000  $\mu\text{M}$ ) to the changes in concentrations of the other potential reactants suggest that S oxidation may be a significant contributor to  $\text{NO}_3^-$  transformations. The observed decrease in  $\text{NO}_3^-$  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  $\text{H}_2\text{S}$  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  $\text{SO}_4^{2-}$  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  $\text{NH}_4^+$  concentration reflects not only DNRA, but also potentially increased N reminer-

alization activity by stimulation of respiratory denitrification; a potentially large  $\text{NH}_4^+$  pool sorbed on the sediment ion exchange complex could have buffered changes in porewater dissolved  $\text{NH}_4^+$ . Finally, we were not able to estimate the production of  $\text{N}_2$  from the added  $\text{NO}_3^-$ , and S oxidizers are known to denitrify  $\text{NO}_3^-$  to  $\text{N}_2$  as well as conduct DNRA. In spite of these caveats, the changes we observed provide evidence for  $\text{NO}_3^-$  driven S oxidation as a potential  $\text{NO}_3^-$  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  $^{15}\text{N}$ -labelled  $\text{NO}_3^-$  in water flowing over sediment cores (A. Burgin & S. Hamilton, unpubl. data).

Nearly all sites had more  $\text{NO}_3^-$  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  $\text{NO}_3^-$ . This pattern of greater N cycling in shallower sediments has also been observed in streams with high  $\text{NO}_3^-$  availability (Inwood et al. 2007), where greater denitrification was correlated with shallower depth, more organic matter and higher  $\text{NO}_3^-$  concentrations.

The relative importance of denitrification and DNRA as  $\text{NO}_3^-$  sinks cannot be estimated from the present study, but the addition of  $\text{NO}_3^-$  to freshwater sediments generally resulted in an increase in  $\text{NH}_4^+$  and  $\text{SO}_4^{2-}$ , with a concomitant decrease in  $\text{H}_2\text{S}$ , demonstrating the potential contribution of DNRA by S oxidizers (Fig. 6).  $\text{NO}_3^-$  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  $\text{NO}_3^-$  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  $\text{H}_2\text{S}$  coupled with the reduction of  $\text{NO}_3^-$  to  $\text{NH}_4^+$ . *D. desulfuricans* consumed one mole of  $\text{H}_2\text{S}$  per mole of  $\text{NO}_3^-$  and produced equimolar amounts of  $\text{SO}_4^{2-}$  and  $\text{NH}_4^+$ . Additionally, Burgin & Hamilton (2008) found evidence for  $\text{NO}_3^-$  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  $\text{SO}_4^{2-}$  for every 8 moles of  $\text{NO}_3^-$  converted to  $\text{N}_2$  in a form of chemolithoautotrophic denitrification (Burgin & Hamilton 2008). These investigations have further emphasized the potential importance of sulfur cycling in  $\text{NO}_3^-$  removal from aquatic ecosystems.

If S-oxidizers are responsible for a significant portion of the  $\text{NO}_3^-$  removal from surface or ground waters, then  $\text{NO}_3^-$  removal should be linked to S cycling and, specifically, to  $\text{SO}_4^{2-}$  availability.  $\text{SO}_4^{2-}$  is a ubiquitous pollutant in industrialized regions, and atmospheric deposition of  $\text{SO}_4^{2-}$  as well as concentrations in ground waters and rivers are greatly elevated over pre-industrial times (Schlesinger 1997). Excess  $\text{SO}_4^{2-}$  in freshwaters may indirectly enhance  $\text{NO}_3^-$  removal by stimulating  $\text{H}_2\text{S}$  formation through  $\text{SO}_4^{2-}$  reduction. The increased abundance of  $\text{H}_2\text{S}$  then fosters the development of populations of S oxidizing bacteria at redox gradients, and these bacteria are able to use  $\text{NO}_3^-$  as an alternate oxidant when  $\text{O}_2$  is not available. Yet the ultimate fate of  $\text{NO}_3^-$  used by S oxidizers remains unclear; whether it becomes  $\text{NH}_4^+$  that remains in bioavailable form or is denitrified to  $\text{N}_2$  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.

*Acknowledgements.* We thank C. Arango, T. Loecke, P. Riekenberg, M. Roth, D. Weed and S. L. Whitmire for help and advice. This work was supported by US National Science Foundation grants DEB-0508704, 0423627, and 0516076 (including an REU supplement to E.K.P.). This is contribution #1468 of the W.K. Kellogg Biological Station.

#### LITERATURE CITED

- 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
- Brettar I, Rheinheimer G (1991) Denitrification in the central Baltic: evidence for  $\text{H}_2\text{S}$  oxidation as motor of denitrification at the oxic-anoxic interface. *Mar Ecol Prog Ser* 77: 157–169
- Brettar I, Labrenz M, Flavier S, Botel J, Kuosa H, Christen R, Hofle MG (2006) Identification of a *Thiomicrospira denitrificans*-like epsilonproteobacterium as a catalyst for autotrophic denitrification in the central Baltic Sea. *Appl Environ Microbiol* 72:1364–1372
- Brunet RC, Garcia-Gil LJ (1996) Sulfide-induced dissimilatory nitrate reduction to ammonia in anaerobic freshwater sediments. *FEMS Microbiol Ecol* 21:131–138
- 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
- Burgin AJ, Hamilton SK (2008)  $\text{NO}_3^-$  driven  $\text{SO}_4^{2-}$  production in freshwater ecosystems: implications for N and S cycling. *Ecosystems* 11:908–922
- Dannenberg S, Kroder M, Dilling W, Cypionka H (1992) Oxidation of  $\text{H}_2$ , organic-compounds and inorganic sulfur-compounds coupled to reduction of  $\text{O}_2$  or nitrate by sulfate-reducing bacteria. *Arch Microbiol* 158:93–99
- Garcia-Gil LJ, Golterman HL (1993) Kinetics of FeS-mediated denitrification in sediments from the Camargue (Rhône Delta, southern France). *FEMS Microbiol Ecol* 13:85–91



- Golterman HL, Clymo RS 1969. Methods for chemical analysis of fresh waters. Blackwell, Oxford
- Hesslein RH (1976) *In situ* sampler for close interval pore water studies. *Limnol Oceanogr* 21:912–914
- Inwood SE, Tank JL, Bernot MJ (2007) Factors controlling denitrification in Midwestern streams of varying land use. *Microb Ecol* 53:247–258
- Kelly DP (1999) Thermodynamic aspects of energy conservation by chemolithotrophic sulfur bacteria in relation to the sulfur oxidation pathways. *Arch Microbiol* 171:219–229
- Knowles R (1982) Denitrification. *Microbiol Rev* 46:43–70
- Paerl HW, Dennis RL, Whitall DR (2002) Atmospheric deposition of nitrogen: implications for nutrient over-enrichment of coastal waters. *Estuaries* 25:677–693
- Rabalais NN, Turner RE, Wiseman WJ (2002a) Gulf of Mexico hypoxia, a.k.a. 'The dead zone'. *Annu Rev Ecol Syst* 33: 235–263
- Rabalais NN, Turner RE, Dortch Q, Justic D, Bierman VJ, Wiseman WJ (2002b) Nutrient-enhanced productivity in the northern Gulf of Mexico: past, present, and future. *Hydrobiologia* 475-476:39–63
- Rheume SJ 1990. Geohydrology and water quality of Kalamazoo County, Michigan, 1986-88. US Geological Survey Water-Resources Investigations Report 90-4028. US Department of the Interior, Washington, DC
- Schlesinger WH 1997. Biogeochemistry: an analysis of global change, 2nd edn. Academic Press, San Diego, CA
- Seitzinger S (1988) Denitrification in freshwater and coastal marine ecosystems: ecological and geochemical significance. *Limnol Oceanogr* 33:702–724
- Seitzinger S, Harrison JA, Bohlke JK, Bouwman AF and others (2006) Denitrification across landscapes and waterscapes: a synthesis. *Ecol Appl* 16:2064–2090
- Smith MS (1982) Dissimilatory reduction of  $\text{NO}_2^-$  to  $\text{NH}_4^+$  and  $\text{N}_2\text{O}$  by a soil *Citrobacter* sp. *Appl Environ Microbiol* 43: 854–860
- Tiedje JM 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In: Zehnder AJB (ed) *Biology of anaerobic microorganisms*. Wiley & Sons, New York, p 179–244
- Turner RE, Rabalais NN (1991) Changes in Mississippi River water-quality this century. *Bioscience* 41:140–147
- Weber KA, Urrutia MM, Churchill PF, Kukkadapu RK, Roden EE (2006) Anaerobic redox cycling of iron by freshwater sediment microorganisms. *Environ Microbiol* 8:100–113
- Whitmire SL 2003. Anaerobic biogeochemical functions of Michigan wetlands and the influence of water source. PhD 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* 34:2062–2071
- Zedler JB (2003) Wetlands at your service: reducing impacts of agriculture at the watershed scale. *Front Ecol Environ* 1: 65–72

*Editorial responsibility: Rutger de Wit, Montpellier, France*

*Submitted: April 17, 2008; Accepted: November 27, 2008  
Proofs received from author(s): February 17, 2009*