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Amy J. Burgin
*University of Nebraska-Lincoln & Michigan State University, burginam@ku.edu*

Stephen K. Hamilton
*Michigan State University & University of Nebraska-Lincoln*

Stuart E. Jones
*University of Notre Dame & University of Nebraska-Lincoln*

Jay T. Lennon
*Indiana University & Michigan State University*

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Denitrification by sulfur-oxidizing bacteria in a eutrophic lake

Amy J. Burgin1,2,3,*, Stephen K. Hamilton1,2, Stuart E. Jones1,4, Jay T. Lennon1,5,6

1W. K. Kellogg Biological Station, 2Department of Zoology and 3Department of Microbiology and Molecular Genetics, Michigan State University, 3700 East Gull Lake Dr., Hickory Corners, Michigan 49060, USA

3Present address: School of Natural Resources, University of Nebraska-Lincoln, 3310 Holdredge St., 412 Hardin Hall, Lincoln, Nebraska 68583-0974, USA

4Present address: Department of Biological Sciences, University of Notre Dame, 264 Galvin Hall, Notre Dame, Indiana 46556, USA

6Present address: Department of Biology, Indiana University, 1001 E. 3rd St., Bloomington, Indiana 47405–3700, USA

ABSTRACT: Understanding the mechanistic controls of microbial denitrification is of central importance to both environmental microbiology and ecosystem ecology. Loss of nitrate (NO₃⁻) is often attributed to carbon-driven (heterotrophic) denitrification. However, denitrification can also be coupled to sulfur (S) oxidation by chemolithoautotrophic bacteria. In the present study, we used an in situ stable isotope (¹⁵NO₃⁻) tracer addition in combination with molecular approaches to understand the contribution of sulfur-oxidizing bacteria to the reduction of NO₃⁻ in a eutrophic lake. Samples were incubated across a total dissolved sulfide (H₂S) gradient (2 to 95 µM) between the lower epilimnion and the upper hypolimnion. Denitrification rates were low at the top of the chemocline (4.5 m) but increased in the deeper waters (5.0 and 5.5 m), where H₂S was abundant. Concomitant with increased denitrification at depths with high sulfide was the production of sulfate (SO₄²⁻), suggesting that the added NO₃⁻ was used to oxidize H₂S to SO₄²⁻. Alternative nitrate removal pathways, including dissimilatory nitrate reduction to ammonium (DNRA) and anaerobic ammonium oxidation (anammox), did not systematically change with depth and accounted for 1 to 15% of the overall nitrate loss. Quantitative PCR revealed that bacteria of the Sulfurimonas genus that are known denitrifiers increased in abundance in response to NO₃⁻ addition in the treatments with higher H₂S. Stoichiometric estimates suggest that H₂S oxidation accounted for more than half of the denitrification at the depth with the highest sulfide concentration. The present study provides evidence that microbial coupling of S and nitrogen (N) cycling is likely to be important in eutrophic freshwater ecosystems.

KEY WORDS: Denitrification · Nitrate reduction · Sulfur oxidation · Sulfur-driven denitrification · Sulfurimonas denitrificans · Sulfide · Wintergreen Lake

INTRODUCTION

Denitrification is an important microbial process with beneficial consequences for water quality. More than 75% of the anthropogenic nitrogen (N) entering watersheds is lost along landscape flow paths before reaching the oceans (Alexander et al. 2000). This ‘missing’ N is attributed to heterotrophic denitrifica-
Anaerobic sediments and biofilms of aquatic ecosystems are conducive to NO$_3^-$ reduction; however, $^{15}$N tracer studies often show that less than half of the total NO$_3^-$ disappearance is attributable to direct denitrification (e.g. Mulholland et al. 2008). Such findings suggest that other microbial processes may be important for removing NO$_3^-$ in freshwater ecosystems (Gardner et al. 2006, Burgin & Hamilton 2007, Scott et al. 2008, Gardner & McCarthy 2009). NO$_3^-$ can also be reduced via dissimilatory nitrate reduction (DNRA) to ammonium (NH$_4^+$) by fermentative bacteria as well as via denitrification or DNRA coupled to the chemolithoautotrophic oxidation of either sulfur (Brunet & Garcia-Gil 1996, Otte et al. 1999) or iron (Weber et al. 2006). The relative importance of DNRA and denitrification is germane to understanding the fate of NO$_3^-$ because the NH$_4^+$ produced by DNRA is biologically available, while N$_2$, the predominant end-product of denitrification, is lost from the available N pool.

Anaerobic oxidation of ammonium (anammox) also converts NO$_3^-$ to N$_2$. This chemolithoautotrophic pathway reduces nitrite (NO$_2^-$) (the source of which is presumably incomplete denitrification of NO$_3^-$) with electrons from NH$_4^+$ to produce N$_2$. Anammox can account for high fractions (~50%) of N$_2$ production in marine oxygen-minimum zones (Dalsgaard et al. 2005). Few estimates of anammox exist for lakes, but when measured in Lake Tanganyika, anammox contributed <13% of the overall N$_2$ production in a suboxic layer at 100 to 110 m depth (Schubert et al. 2006).

The various dissimilatory N transformations are subject to different controlling factors (Burgin & Hamilton 2007). Denitrification is known to be influenced by the availability of labile organic carbon (C), NO$_3^-$, and O$_2$ (Cornwell et al. 1999). In addition, H$_2$S may play an important role in regulating N cycling processes. H$_2$S is toxic to sensitive biomolecules, including enzymes (Wang & Chapman 1999), and is known to inhibit N transformations, such as nitrification (Joye & Holibaugh 1995) and heterotrophic denitrification (Senga et al. 2006). However, in habitats where nitrate and reduced sulfur compounds occur concomitantly, the latter can also be used as an energy source by chemolithoautotrophs, with NO$_3^-$ as the electron acceptor, yielding N$_2$ and thus enhancing denitrification rates. Therefore, H$_2$S may have a non-linear effect on rates of nitrogen cycling because it can both enhance and inhibit key N transformation processes.

Denitrification by S-oxidizing bacteria has been documented in marine ecosystems (Brettar & Rheinheimer 1991, Shao et al. 2010) and has been suggested to occur in freshwater wetlands (Burgin & Hamilton 2008, Payne et al. 2009). Our previous work identified *Sulfurimonas denitrificans* as 1 microbe potentially contributing to this process in wetland sediments (Burgin & Hamilton 2008). To our knowledge, that study was the first reported isolation of *S. denitrificans* from freshwater habitats. The *S. denitrificans* genome contains all of the genes necessary for the complete reduction of NO$_3^-$ to N$_2$ and uses the Sox pathway to oxidize reduced sulfur (S) species completely to SO$_4^{2-}$ (Sievert et al. 2008).

Here, we report an experiment that took advantage of a naturally occurring H$_2$S gradient (2 to 95 µM over 1 m of depth) in a thermally stratified, eutrophic lake to examine how H$_2$S concentrations influence N transformations and microbial dynamics. *In situ* incubations of lake water with isotopically enriched $^{15}$NO$_3^-$ allowed us to follow the fate of added NO$_3^-$, simulating natural inputs that could occur via groundwater inflows or surface runoff. We also used quantitative PCR (qPCR) to track the response of populations of a denitrifying *Sulfurimonas* bacterium to the NO$_3^-$ addition at varying H$_2$S concentrations. Our goals were to (1) determine the fate of NO$_3^-$ in a eutrophic lake, including whether the reduction end-products are affected by H$_2$S concentrations, and (2) test if bacteria that actively couple N and S in dissimilatory reactions respond to the H$_2$S gradient in ways that would explain patterns of denitrification.

**MATERIALS AND METHODS**

**Study site**

We conducted our study at Wintergreen Lake, Michigan, USA, an 18 ha eutrophic, glacial kettle lake located at the Kellogg Bird Sanctuary (maximum depth = 6.3 m). The lake has been the subject of intensive study for decades (Wetzel 2001), including research on sulfur cycling (King & Klug 1980, 1982) and phototrophic sulfur bacteria (Vila et al. 1998). A vertical thermal profile shortly before our field experiment (July 2006) showed that the lake was stratified with a thermocline at ~5.25 m below the surface (Fig. 1A). The metalimnetic waters (from 4.5 to 5.5 m) contained no detectable NO$_3^-$ (<1 µM), relatively low concentrations of SO$_4^{2-}$ compared to the epilimnetic waters, and high concentrations of NH$_4^+$ (Fig. 1B). We also observed a strong H$_2$S gradient across the 1 m depth range (4.5 m: 1.8 ± 0.9 µM, 5.0 m: 24.5 ± 5.1 µM, 5.5 m: 93.9 ± 6.0 µM). Analytical methods are detailed in the following section.
The lake’s natural chemical gradient allowed us to test the influence of H₂S on N cycling by planktonic bacterial populations under field conditions. In late summer, NO₃⁻ was uniformly low throughout the water column, while NH₄⁺ increased to nearly 400 µM in the bottom waters (Fig. 1B). The pH ranged from 9.05 in surface waters to 6.8 near the bottom, and was 7.2 to 7.9 at the depths under study here (4.5 to 5.5 m). The photosynthetic compensation point (1% of surface irradiance) occurred at 4.2 m. The specific conductance (corrected to 25°C) of the lake water ranged from 304 µS cm⁻¹ at the surface to 683 µS cm⁻¹ near the bottom; precipitation, sedimentation, and redissolution of calcium carbonate over the period of summer stratification produces much of this vertical differentiation in conductance (Hamilton et al. 2009).

Field experiment

We designed a field experiment to examine the responses of the bacterial processes across the metatalimnetic H₂S gradient to added NO₃⁻. The experiment was set up on 21 August 2006, a time when the summer stratification of the water column was close to its maximum vertical differentiation. We used a peristaltic pump (GeoPump) to collect water from depths of 4.5, 5.0, and 5.5 m, transferring the samples to 1 l Nalgene LPE bottles by pumping water into the bottles from the bottom and allowing them to overflow to minimize the entrainment of atmospheric O₂. Measurements of H₂S in both the treatment and control bottles on the following day confirmed that the sample transfer did not alter the H₂S gradient. To test the effects of H₂S concentration and the \textit{in situ} NO₃⁻ reduction processes, bottles were randomly assigned to 3 treatments: (1) live controls (labeled ‘live’) composed of ambient lake water, (2) killed controls (‘killed’) containing added NaCl at a final concentration of 300 g l⁻¹ to arrest biological activity as well as added ¹⁵NO₃⁻ (final concentration of 267 µM), and (3) added ¹⁵NO₃⁻ (¹⁵N; final concentration of 267 µM). We used NaCl for the killed control because H₂S can react with many other poisons (Brock 1978). Three replicate ¹⁵N-treated bottles were positioned at each of the 3 depths; these were grouped with 1 live and 1 killed control per depth for a total of 5 bottles per depth and 15 bottles total per line (a line refers to a string of bottles with an anchor and a float). Four replicate lines were positioned together so that 1 line (15 bottles) could be destructively harvested each day of the experiment (22 to 25 August 2006). The NO₃⁻ addition resulted in higher concentrations than those present in the lake during the study; however, this concentration was well within the range of NO₃⁻ concentrations found in southern Michigan groundwater-fed lakes, such as Wintergreen Lake.

Hydrochemical and isotopic measurements

Upon opening the bottles, water was immediately and carefully removed for analysis of ¹⁵N gases and H₂S, taking care to minimize atmospheric gas exchange. Dissolved gases were extracted using a static headspace equilibrium method (Hamilton & Ostrom 2007), followed by transfer of the headspace gas samples to evacuated Exetainers (Labco) that were sent to the Stable Isotope Facility at the University of California at Davis for analysis of δ¹⁵N in N₂O and N₂ (including ¹⁵N:¹⁴N (²⁹N₂) and ¹⁵N:¹⁵N (³⁰N₂) forms). A subsample of the water was immediately removed and fixed (i.e. colorimetric reagents added) in the field for analysis of dissolved H₂S by the methylene blue spectrophotometric method.

Fig. 1. Depth profiles of (A) dissolved O₂ (DO) and temperature and (B) water chemistry (NO₃⁻, NH₄⁺, SO₄²⁻, and H₂S) from Wintergreen Lake, Michigan, USA, at the initiation of the experiment in August 2006.
(Golterman & Clymo 1969). The gas-extracted water was then filtered through 0.45 µm polyethersulfone membrane filters. Samples for $^{15}$NH$_4^+$ were collected by a modified diffusion method (Holmes et al. 1998), trapping the NH$_4^+$ on filters, which were analyzed on a stable isotope ratio mass spectrometer at Michigan State University’s Isotope Biogeochemistry Laboratory. Sub-samples were also taken to determine the NH$_4^+$ concentration using the phenyl-hypochlorite method (Aminot et al. 1997) and for NO$_3^-$, N$_2$, and SO$_4^{2-}$ on a Dionex membrane-suppression ion chromatograph.

Stable N isotope ratios were converted to mole fractions and multiplied by N pool sizes to yield masses of $^{15}$N tracer in each pool (N$_2$, N$_2$O, and NH$_4^+$), thereby facilitating comparison of the flux rates. In the case of dissolved N$_2$, we used the atmospheric equilibrium concentration at the temperature of the sampling depth.

**Molecular methods**

From previous work, we determined that a close relative of *Sulfurimonas denitrificans* was possibly responsible for coupled N–S cycling documented in a freshwater ecosystem near Wintergreen Lake (Burgin & Hamilton 2008). Therefore, we used primers to target bacteria related to *S. denitrificans* (Labrenz et al. 2004, Höfte et al. 2005, Brettar et al. 2006). Our goal in employing these primers was to link the measured biogeochemical processes (e.g. denitrification) with the population dynamics of bacteria known to couple sulfur oxidation with NO$_3^-$ reduction to N$_2$. Specifically, we used OST 1F (5′-TCA GAT GTG AAA TCC AAT GGC TCA-3′) and OST 1R (5′-CTT AGC GTC AGT TAT GTT CCA GG-3′). These primers were designed to target the genus *Sulfurimonas* in the Baltic Sea; analysis of the PCR products showed that the amplified partial 16S rRNA gene sequences from Wintergreen Lake were closely related to *S. autotrophica* (A. Burgin unpubl. data). Of our 8 sequences, 7 were identical to each other, and the eighth had 96% similarity to the other 7 sequences. Because organisms sharing >95% 16S rRNA gene sequence identity are commonly considered to be of the same genus, throughout the remainder of the paper we refer to the amplified organisms as denitrifying *Sulfurimonas*.

The aforementioned primers were used to amplify DNA that had been extracted from filters using a MoBio UltraClean Water DNA isolation kit following the manufacturer's instructions. For PCR amplification, 50 µl reactions were carried out with HotStart Buffer and Taq polymerase at the recommended final concentrations (Promega). A gradient PCR was used to optimize PCR thermal conditions, and an optimal annealing temperature was found to be between 57 and 59°C. The reaction ran an initial denaturing step at 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, and the optimized annealing temperatures of 58°C for 40 s and 72°C for 2 min. The OST-amplified DNA was gel-purified using a Novagen SpinPrep Gel DNA kit following the manufacturer's instructions. The target DNA was then cloned into *Escherichia coli* using an Invitrogen TOPO-TA kit per the manufacturer's instructions. Eight colonies were selected and PCR-amplified using M13 primers and manufacturer-suggested PCR conditions. M13 PCR product was purified with a QIAquick PCR purification kit (Qiagen). Sequencing confirmed the taxonomic identity of our PCR amplicons as members of the genus *Sulfurimonas*. Sequencing was conducted at the Research Technology Support Facility at Michigan State University. All sequences obtained in the present study were deposited in GenBank (accession numbers GU937440 to GU937447). The sequences, along with reference sequences obtained from GenBank, were aligned and trimmed using ClustalX. The 150 bp alignment was then used as the basis for a phylogenetic tree constructed using MrBayes, run on the CIPRES Web Portal (www.phylo.org).

Quantification of denitrifying *Sulfurimonas* populations was performed using BioRad iQ SYBR Green Mastermix and an Eppendorf Mastercycler ep realplex $^2$ qPCR thermocycler. The qPCR mixtures (15 µl) contained master mix, 900 nM OST 1F, and 300 nM OST 1R. The assay included an initial denaturing step at 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 58°C for 40 s, and 72°C for 50 s. Our cloned and purified PCR product was also used as a standard for our qPCR assay after being quantified with a Nanodrop spectrophotometer. We also used qPCR primers that targeted the bacterial 16S rRNA gene as a way to estimate the relative contribution of denitrifying *Sulfurimonas* populations to the total bacterial community in the bottle incubations. The bacterial 16S rRNA qPCR reactions (15 µl) contained master mix and 667 nM each of the 340f/533r primers. The total bacterial reaction included an initial denaturing step at 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 68°C for 40 s, 72°C for 50 s, and data collection at 83.5°C (Jones & Lennon 2009). The qPCR amplification efficiencies were always between 0.9 and 1.1, and there was no evidence for primer dimers based on the melting curves.
Statistical analysis

We used 1-way analysis of variance (ANOVA) to compare rates of denitrification and DNRA across depths (SYSTAT 11 software). To compare changes in the abundance of denitrifying *Sulfurimonas* populations over time and depth, we conducted repeated measures (RM) ANOVA (SAS PROC MIXED) with covariance structure selected using the Bayesian Information Criterion (Wolfinger & Chang 1999).

RESULTS

Microorganisms processed the added NO$_3^-$ differently at the 3 depths in Wintergreen Lake. At the most oxic and shallow depth (4.5 m), nearly all of the added NO$_3^-$ (~267 µM) was removed by Day 3 (Fig. 2). During this time, we observed a large increase in NH$_4^+$, but isotopic evidence indicates that this was not a product of DNRA. The water at 4.5 m depth had very low concentrations of H$_2$S (2.2 ± 0.2 µM), which changed little over the course of the experiment. The SO$_4^{2-}$ concentrations decreased slightly from 120 to 108 µM. At the intermediate depth (5.0 m), more than half (185 µM) of the added NO$_3^-$ remained on the last day (Fig. 2). The 25 µM H$_2$S present in the beginning was quickly removed (to below our detection limit of 1.0 µM) and remained low. This drop in H$_2$S was accompanied by an increase of ~10 µM in SO$_4^{2-}$. At the most H$_2$S-rich (95 µM) and deepest depth (5.5 m), the NO$_3^-$ was completely removed, with a concomitant increase of 85 µM in SO$_4^{2-}$ (Fig. 2). At 5.5 m, the microbial populations converted H$_2$S to SO$_4^{2-}$ in a nearly 1:1 stoichiometric ratio; discrepancy from this expected 1:1 ratio at 5.0 m could be accounted for via intracellular storage (as elemental S) or incomplete conversion via unmeasured intermediates (elemental S or thiosulfate).

The NO$_3^-$ added to the bottles was reduced mainly to N$_2$ via denitrification (Fig. 3A) and to a lesser extent to NH$_4^+$ via DNRA (Fig. 3B). Rates of NO$_3^-$ reduction, denitrification, and DNRA were calculated over Days 0 to 3 of the experiment because all of the reactants and products of the focal processes were measurable during this time period, and changes in concentrations, where they occurred, were approximately linear. Denitrification rates increased significantly with depth (and with increasing H$_2$S concentrations), ranging from 1 µM ¹⁵N d$^{-1}$ at 4.5 m to 17 µM ¹⁵N d$^{-1}$ at 5.5 m (Fig. 3A; $F_{2,6} = 252.5$, $p < 0.001$). A larger fraction of overall NO$_3^-$ reduction (Fig. 3C) could be attributed to denitrification at the 5.0 and 5.5 m depths, whereas a much smaller fraction (~1%) was denitrified at 4.5 m. DNRA accounted for 1 to 15% of the NO$_3^-$ reduction (Fig. 3B,C). DNRA rates were not affected by increasing depth or H$_2$S concentration, though there were significant differences in rates among depths (Fig. 3B) ($F_{2,6} = 10.4$, $p = 0.01$). Anammox was not a significant source of the tracer ¹⁵N in N$_2$ because 96 to 100% of the tracer ¹⁵N appeared in the ¹⁵N:¹⁴N form, whereas anammox would have produced ¹⁵N:¹⁴N through the partial denitrification of the added ¹⁵NO$_3^-$ to ¹⁵NO$_2^-$ followed by its reaction with ¹⁴NH$_4^+$ (data not shown). Dissolved N$_2$O accounted for a very minor proportion of the overall NO$_3^-$ reduction (<0.1%) and did not
show any clear patterns over time or among depths (data not shown).

The molecular data corroborate the isotope data and suggest that denitrification was coupled to the activity of S-oxidizing bacteria. The abundance of denitrifying Sulfurimonas bacteria changed through time as a function of depth (RM-ANOVA, Time × Depth, $F_{6,10} = 10.1, p = 0.001$; Fig. 4). The denitrifying Sulfurimonas was present at low levels in the live controls, ranging from 8 to 22 cells l$^{-1}$. In contrast, the highest abundance measured in the 5.5 m depth $^{15}$N-treated bottles was nearly 60,000 cells l$^{-1}$, indicating a rapid increase of the population. When expressed as a fraction of the 16S rRNA gene copy number (Fig. 4), the relative abundance of denitrifying Sulfurimonas increased from 3 to 65% in the 5.5 m treatment, whereas the relative abundance of denitrifying Sulfurimonas in the 4.5 and 5.0 m treatments was generally much lower (3 to 5%), particularly after the first day.

**DISCUSSION**

The addition of nitrate to anoxic, sulfidic lake water stimulated rapid, microbially mediated biogeochemical reactions in which sulfur oxidation was linked to nitrate reduction to N$_2$. This conclusion is supported by stoichiometric comparisons of NO$_3^-$ and H$_2$S concentration changes, stable isotope tracing, and molecular evidence for an increase in populations of sulfur-oxidizing bacteria capable of denitrification. We elaborate on these lines of evidence below.
Evidence for sulfur-driven denitrification in freshwater ecosystems

Stoichiometric calculations provide an estimate of the fraction of NO$_3^-$ removal due to S oxidation as indicated by SO$_4^{2-}$ production. In the S oxidation reaction, wherein NO$_3^-$ is reduced to N$_2$, 8 moles of NO$_3^-$ are removed for every 5 moles of SO$_4^{2-}$ produced (Fossing et al. 1995, Burgin & Hamilton 2008):

$$5 \text{HS}^- + 8 \text{NO}_3^- + 3 \text{H}^+ \rightarrow 5 \text{SO}_4^{2-} + 4 \text{N}_2 + 4 \text{H}_2\text{O} \quad (1)$$

Given this stoichiometry and the amount of SO$_4^{2-}$ produced, we estimated that SO$_4^{2-}$ production did not account for NO$_3^-$ removal at 4.5 m but accounted for 6% of the total NO$_3^-$ removal at 5.0 m and 51% of the total NO$_3^-$ removal at 5.5 m (Fig. 2). We combined the fraction of NO$_3^-$ removal to denitrification (Fig. 3A) with the fraction of NO$_3^-$ removal attributable to SO$_4^{2-}$ production to estimate the fraction of denitrification linked to SO$_4^{2-}$ production. Therefore, at 5.5 m, the majority of the denitrification could be coupled to chemolithoautotrophic S oxidation rather than anaerobic respiration of organic matter. These contributions were much lower at the 5 and 4.5 m depths because rates of denitrification (Fig. 3A) and SO$_4^{2-}$ production (Fig. 2) were substantially lower or undetectable. However, estimates based on SO$_4^{2-}$ production alone are conservative because partial oxidation of H$_2$S to elemental S or significant intracellular accumulation of either NO$_3^-$ or elemental S could account for additional coupling not measured by our methods (e.g. Kamp et al. 2006).

Coupled N–S cycling has been known for more than a decade to occur in certain marine ecosystems (Brettar & Rheinheimer 1991, Fossing et al. 1995) and has been incorporated into bioreactor engineering (Cardoso et al. 2006), and our study suggests that reduced S can be a major driver of denitrification in natural freshwater ecosystems. Evidence for sulfur-driven denitrification has been reported in marine ecosystems, including oceanic redoxclines (Brettar et al. 2006, Grote et al. 2008), near-coastal upwelling zones (Fossing et al. 1995, Schulz et al. 1999), and engineered mariculture systems (Cytryn et al. 2005a, Sher et al. 2008). The patterns of biogeochemical activity that we observed over depth are very similar to those documented in the Baltic Sea (Brettar & Rheinheimer 1991, Brettar et al. 2006), albeit over a much smaller spatial scale (1 m vs. 10s of meters). Concentrations of H$_2$S in Wintergreen Lake were comparable to those measured in the Baltic Sea, ranging from 0 to 100 µM in the water column, whereas the highest reported H$_2$S concentration in the Gotland Deep area of the Baltic was ~150 µM (Brettar & Rheinheimer 1991). In laboratory experiments, H$_2$S additions increased denitrification (Brettar & Rheinheimer 1991) by as much as we saw across the ambient H$_2$S gradient in Wintergreen Lake (Fig. 3A). The occurrence, importance, and distribution of these N–S coupling processes in freshwater ecosystems, however, remain almost completely unexplored.

Few studies have examined the effects of H$_2$S concentrations on the multiple processes that contribute to NO$_3^-$ removal, including denitrification, DNRA, anammox. Our results suggest that in the hypolimnetic lake water where H$_2$S was available, denitrification was the most important measured removal process; however, despite the use of $^{15}$N tracers, we were not able to account for the full mass of added nitrate. This is likely because we were not able to measure assimilation, a potentially important process. There was, however, substantial variation in the fate of NO$_3^-$ over the 1 m depth range we investigated. Furthermore, NO$_3^-$ removal rates varied over the depth range, with the majority of NO$_3^-$ removed quickly at 4.5 and 5.5 m, but much less NO$_3^-$ removal at 5.0 m (Figs. 2 & 3C). These differences in biogeochemical processes (Figs. 2 & 3) and microbial population dynamics (Fig. 4) highlight the high degree of spatial variation that can develop over relatively small distances (1 m) in a seasonally stratified water column.

Bacterial population response to NO$_3^-$ addition

The bacteria implicated in linking the N and S cycles in Wintergreen Lake appear to be closely related to bacteria that have been shown to perform similar functions in the Baltic Sea (Brettar et al. 2006). All 8 of our sequences shared >95% sequence identity with a sequence obtained from similar incubation experiments conducted in the Baltic Sea (AJ810529) (Brettar et al. 2006, A. Burgin unpubl. data) and were identical to other environmental sequences recovered from freshwater, anoxic, and sulfidic environments (Briée et al. 2007, Amaral-Zettler et al. 2008, Porter et al. 2009) (Fig. 5). Our results are consistent with the interpretation that the primers we used targeted populations of denitrifying *Sulfurimonas* bacteria that couple the N and S cycles by reducing NO$_3^-$ to oxidize H$_2$S, creating N$_2$ and SO$_4^{2-}$ (Figs. 2 & 3). The ambient populations of the bacteria, as inferred from the gene copies on Day 1 of the experiment (Fig. 4), showed higher abundance with increased H$_2$S. The
populations targeted by the Brettar et al. (2006) primers clearly responded to the NO$_3^-$ addition, with the most growth in the presence of the highest H$_2$S and NO$_3^-$, as in the bottles incubated at 5.5 m depth (Fig. 4).

Based on whole-genome sequencing of *Sulfuriomonas denitrificans*, we know that this bacterium has the genetic capacity to link the N and S cycles through chemolithoautotrophic denitrification (Sievert et al. 2008). We do not know, however, if other microorganisms in the Wintergreen Lake samples also carried out this metabolism. For example, the coupling of N–S cycling is performed by other bacteria besides *S. denitrificans*, including *Thiothrix* (Nielsen et al. 2000, Cytryn et al. 2005b), *Thioploca* (Fossing et al. 1995, Jorgensen & Gallardo 1999), *Beggiatoa* (Kamp et al. 2006), and *Thiomargarita* (Schulz et al. 1999). Many of these are either Gammaproteobacteria or Epsilonproteobacteria, and most have been isolated from marine ecosystems. However, one study suggested that *Beggiatoa* species (also Gammaproteobacteria) from freshwater ecosystems can perform a similar reaction (Kamp et al. 2006). While we found evidence for the importance of denitrifying *Sulfuriomonas* in Wintergreen Lake, the diversity of taxa with the potential for this metabolism suggests there may be other microorganisms that can couple the N and S cycles in other freshwaters. Given that the denitrifying *Sulfuriomonas* was the numerically dominant member of the bacterial community (Fig. 4) at depths where a high degree of coupled N–S cycling occurred (Fig. 2), our study has identified one important group of organisms responsible for the coupling of N–S in eutrophic freshwaters. Future research, however, should focus on identifying other freshwater microorganisms that may further contribute to coupled N–S cycling.

**Freshwater sulfide as a control of denitrification**

We argue that the role of NO$_3^-$ reduction coupled to S oxidation should be considered in building a mechanistic understanding of how S cycling affects N availability in freshwater aquatic ecosystems. Our finding of S-driven denitrification in a freshwater lake represents a departure from our current understanding of freshwater biogeochemistry. The general belief, particularly from an ecosystem perspective, is that all freshwater denitrification is organic carbon-driven (Burgin & Hamilton 2007). Our study provides strong evidence that H$_2$S controls denitrification in a type of ecosystem where it has heretofore not been regarded as important to the overall nitrogen cycle.

Sulfate concentrations in lakes can range from <10 µM to >800 µM in the region where our study was conducted (S. Hamilton unpubl. data), while H$_2$S concentrations in near-surface sediment porewaters can range from 0 to >200 µM (Whitmire 2003). A number of studies in the 1970s and 1980s elucidated the mechanisms and controls of SO$_4^{2-}$ reduction; however, we know relatively little about the oxidative side of S cycling and its role in freshwater ecosystems (Holmer & Storkholm 2001). Tightly coupled N–S cycling may occur in many hypolimnetic zones of lakes or otherwise seasonally or ephemeral anoxic waters, which often originate as groundwater, precipitation, or runoff that is relatively high in both NO$_3^-$ and SO$_4^{2-}$. As illustrated in Fig. 6, potential zones of coupled N–S cycling (denoted by the white-dashed boxes) may first develop at the sediment-water interface of a lake during spring overturn but eventually shift up into the water column as stratification sets in and an anoxic hypolimnion develops. Furthermore, NO$_3^-$ reduction coupled to S oxidation may be particularly important in areas with high groundwater NO$_3^-$ and SO$_4^{2-}$ concentrations and abundant groundwater-fed ecosystems (e.g. the glacial terrain in which Wintergreen Lake is situated). The potential importance of coupled N–S cycling in freshwaters may not have been recognized in many studies of denitrification because assays are typically
performed in lab slurries that alter conditions, including sparging, which greatly reduces the availability of H$_2$S.

Our results suggest that the controls of NO$_3^\text{−}$ removal are spatially variable. H$_2$S becomes more important as an electron donor with increasing concentration (i.e., with depth in the present study), whereas labile organic C is presumably a more important control at shallower depths (Fig. 3). The corollary to this result is that there may also be temporal variability in how labile C and reduced S competitively control NO$_3^\text{−}$ removal and denitrification. We hypothesize that labile C availability may control denitrification more during spring and fall when the lake is completely mixed, whereas S may become an increasingly important control through the development of summer stratification (Fig. 6). Both labile C and H$_2$S are important electron donors for denitrification in the Baltic Sea (Brettar & Rheinheimer 1991). However, in freshwaters, C and available NO$_3^\text{−}$ are considered to be proximal controls of denitrification in groundwaters (Rivett et al. 2008), streams (Arango et al. 2007), and wetlands (Hill & Cardaci 2004). Paradoxically, some studies have also demonstrated a counterintuitive lack of stimulation of denitrification with the addition of labile C (Merrill & Zak 1992, Davidsson & Stahl 2000). Therefore, it seems plausible that some of these conflicting results could be explained by variable and interacting influences of C and S on denitrification.

Results from our in situ experiment in eutrophic Wintergreen Lake demonstrate how distinct microbial populations (Fig. 4) are coupled to biogeochemical functioning across a naturally existing H$_2$S gradient (Figs. 2 & 3). We confirmed that S oxidation can be an important driver of denitrification, contributing over half of the NO$_3^\text{−}$ removal at the higher H$_2$S concentrations. Denitrification rates increased with increasing H$_2$S concentrations (Fig. 3), and this was accompanied by increased abundance of denitrifying Sulfurimonas (Fig. 4). Therefore, the role of NO$_3^\text{−}$ reduction coupled to S oxidation should be considered in building a mechanistic understanding of how S cycling affects N availability in freshwater aquatic ecosystems. Future work should focus on the spatial and temporal variation in organic carbon vs. sulfide as controls of denitrification in freshwater aquatic ecosystems.

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