Enrichment of nitrate reducing sulfide oxidizers from freshwater sediments

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Abstract:

The removal of nitrogen (N) in aquatic ecosystems is of particular interest because excessive nitrate in ground water and surface water is a growing problem. Enhanced loading of N degrades water quality and is linked to eutrophication and harmful algal blooms, especially in coastal marine waters. Research on nitrate removal processes has emphasized plant or microbial uptake (assimilation) or respiratory denitrification by bacteria. The increasing application of stable isotopes and other tracer techniques to study nitrate removal has yielded a growing body of evidence for alternative microbially mediated processes of nitrate transformation, including chemoautotrophic denitrification via sulfur oxidation. An enrichment technique was developed to screen for the presence of microbes that can couple nitrate reduction to sulfide oxidation in freshwater ecosystems. Clone libraries of enrichments from multiple sites showed that Thiomicrospira denitrificans and Arcobacter may be responsible for this biogeochemical processing. Biogeochemical evidence suggests that the H₂S reduction products can vary between sites for reasons that are not completely known. The presence of microbes that perform this type of metabolism is biogeochemically significant because it provides a direct linkage of the nitrogen and sulfur cycles. Additionally, these findings complicate our understanding what controls NO₃⁻ processing and removal in freshwater ecosystems, where S cycling is largely considered unimportant.
Introduction:
Nitrate (NO$_3^-$) is potentially processed by many different pathways in freshwater ecosystems, including lakes, streams and wetlands. While some of these pathways, such as respiratory denitrification, have been well-studied, others such as dissimilatory nitrate reduction to ammonium (DNRA) and anaerobic ammonium oxidation (anammox) have received relatively little scientific attention in freshwaters. Furthermore, while the linkages between sulfur and nitrogen cycling have been acknowledged and examined in marine-influenced ecosystems, these linkages have not been well studied in freshwaters, in part because of the perception that relatively low sulfate concentrations in freshwaters render S cycling unimportant in overall ecosystem function.

In my dissertation work, I found evidence of linkages between the sulfur and nitrogen cycles in sediments from a diverse set of freshwater streams, lakes and wetlands. I was able to show that the amount of sulfate production relative to nitrate removal varied both within and between different freshwater environments. Based on stoichiometric calculations the sulfate that was produced could explain a substantial fraction of the overall nitrate removal.

The ability of bacteria to couple the reduction of nitrate to the oxidation of sulfur has now been established in a number of taxa with diverse metabolic characteristics (Dannenberg et al. 1992, Bonin et al. 1998, Philippot and Hojberg 1999) including members of the genera *Thiobacillus*, *Thiomicrospora*, and *Thioploca* (Timmernandro 1981, Jorgensen 1982, Kelly 1999, Kelly and Wood 2000). Bacteria with this capability include the “big bacteria” (e.g., *Thioploca*) that are able to store nitrate, sulfur, or calcite in vacuoles (Schulz and Jorgensen 2001). This storage capability, in conjunction with their gliding motility, allows them to take advantage of steep biogeochemical gradients, for example by taking up nitrate from overlying oxic water and utilizing it to oxidize sulfur in sulfide-rich anoxic porewaters (Schulz and Jorgensen 2001).

The biogeochemical importance of nitrate use by sulfur-oxidizing bacteria was first widely recognized in marine sediments, but we are beginning to discover its importance in freshwater ecosystems. For example, much of the nitrate uptake in a groundwater aquifer was ascribed to *Thiobacillus denitrificans* (Bottcher et al. 1990), and *Thioploca* occurs not only in marine sediments, but also in freshwater ecosystems including lakes Erie, Baikal, and Biwa (Megonigal et al. 2004). Furthermore, species of *Beggiatoa*, a genus of sulfur oxidizers common in freshwaters, also appear to be capable of using nitrate to oxidize sulfur (Kamp et al. 2006).

The goal of this project was to develop an enrichment technique to screen for the presence of microbes that can couple nitrate reduction to sulfide oxidation in freshwaters. The development of the populations will be monitored by measuring biogeochemical indicators of microbial activity. The composition of the enrichments was evaluated by creating clone libraries using ARB to estimate the nearest relative.

Materials and Methods:

Site selection

Inoculation sources were taken from four freshwater wetlands and from the thermocline of a lake. 1-2 grams of sediment was used to inoculate from the wetlands. A 50 ml sample of water from the thermocline of the lake was centrifuged down to concentrate the cells and then 2ml of the water was used to inoculate the enrichments. Sider’s Pond in Falmouth and School
Street Marsh in Woods Hole were used as local sites, and a MI wetland, Turkey Marsh, was used for comparison.

**Enrichment media**

I designed an enrichment media that was largely based on the anaerobic media used for the class enrichments, though I omitted the acetate and ammonium sources, and only amended the H$_2$S to 200 µM (rather than 400 µM) for reducing power. NO$_3^-$ was added as an electron donor (either 100 µM or 1 mM final concentration) and thiosulfate (final concentration 1 mM) was added to half of the bottles to act as an alternative electron donor. A more detailed description of the media is in Appendix A.

**Aqueous chemistry**

At each sampling, two ml of culture was removed from the enrichment bottles. The samples were then analyzed for H$_2$S using the microelectrodes. One ml was then filtered for NO$_3^-$/SO$_4^{2-}$ analysis by ion chromatography (Dionex IC; AS14A column) in the Hamilton Lab at KBS/MSU. The other ml was filtered for NH$_4^+$ analysis using a modified indol-blue method adapted from the Hamilton Lab protocol. Colorimetric samples were analyzed on the Verian spectrophotometer at 630 nm. For analysis of elemental S, 2-ml of sample were removed from the enrichments and filtered through a glass fiber filter, disposing of the filtrate. The filters were then deposited in 2 ml of ethanol for extraction of the soluble S. A UV-Vis scan was then done of the spectrophotometer and the absorbance at 260 nm was recorded (citation). More details on the aqueous chemistry methods are in Appendix B.

**Microscopy**

Samples were extracted from all primary enrichments, wet mounted and viewed (generally at 100x) using the Zeiss microscope and photographed with the Zeiss Axiocam.

**Clone library and colony sequencing**

DNA from the primary enrichments (only the high NO$_3^-$ / H$_2$S treatment from all five sites) was extracted using the using the MoBio DNA extraction kit for soil samples. The 16S rDNA was then PCR amplified using universal bacterial primers (8F and 1498R). The PCR product was then cloned into a plasmid vector and transformed into *E. coli* using an Invitrogen TOPO TA Cloning Kit. E. coli were then plated onto LB plates containing 50 µg/ml kanomycin and 40 µg/ml x-Gal. 17-20 colonies were picked from each site and a total of 96 colonies were sequenced. These were aligned in ARB using the xxx PT server.

**Results and Discussion:**

**Sider’s Pond**

The samples from the Sider’s Pond enrichment were taken from approximately ~27 ft near the oxic-anoxic interface (Figure 1). The Sider’s Pond enrichment was supplemented with NO$_3^-$ and H$_2$S on approximately a daily basis. Except for the first few days of enrichment development, the NO$_3^-$ and H$_2$S were removed within 24 hours of the addition. This was also accompanied by an increase in SO$_4^{2-}$ concentrations over time (Figure 2). Microscopic analysis of the samples suggested that the majority of the enrichment was composed of small, highly motile (and fast) rods that produced elemental sulfur globules, but no individual “spindles” (see Turkey Marsh for an explanation) were seen in these samples. Community analysis suggested
that this was a nearly pure culture of *Thiomicrospira denitrificans*, since all 20 clones picked grouped closest to this species (not all clones are shown on the tree in Figure 2).

Stoichiometric data for the oxidation products suggested that most of the added H$_2$S was reduced to SO$_4^{2-}$ rather than S$^0$ (Figure 7). This was true for both the high NO$_3^-$/H$_2$S treatment and the low NO$_3^-$/H$_2$S treatment, although the oxidation to SO$_4^{2-}$ was more complete in the high NO$_3^-$/H$_2$S treatment (that is the ratio of SO$_4^{2-}$:S$^0$ was greater in the low NO$_3^-$ treatment due to more complete oxidation). This is what would be expected based on the relative supply and demand of the electron donors and acceptors in the media. In the low NO$_3^-$/H$_2$S treatment, only half as much electron acceptor was available compared to the donor; therefore to get maximal yield out of the available donor, the microbes would have preferred to completely oxidize the H$_2$S. The high NO$_3^-$/H$_2$S treatment suggests that even when the microbes have 5x more electron acceptor than donor, they still prefer to take the available donor to the most oxidized state.

**School Street Marsh**

The samples from the School Street Marsh enrichment were taken from an area of apparent sulfur oxidizer growth near a seep close to the forested area. The School Street Marsh enrichment was supplemented with NO$_3^-$ and H$_2$S on slightly less frequently than the Sider’s Pond enrichment, in part because I mistook residual H$_2$S at 110h as H$_2$S that had not been consumed, when it was really H$_2$S that had been produced by sulfate reducers. This trend of sulfate reduction continued to produce H$_2$S between 110 and 130 hours, with slight leveling in the increase at ~150 h due to the addition of NO$_3^-$. These differences in the biogeochemical signatures probably indicate that a different microbial community has developed than was originally enriched for. Except for the first few days of enrichment development, the NO$_3^-$ and H$_2$S were removed within 24 hours of the addition. This was also accompanied by an increase in SO$_4^{2-}$ concentrations over time (Figure 3), though this increase was not as large as that in Sider’s Pond, probably in part due to some SO$_4^{2-}$ reduction occurring and thus consuming any SO$_4^{2-}$ that would’ve been produced by NO$_3^-$ reducing sulfur oxidizers. Microscopic analysis of the samples suggested that the enrichment contained a much more varied microbial community with multiple morphologies and a noted absence of the small, highly motile (and fast) rods seen in Sider’s Pond and Turkey Marsh (Figure 3).

Community analysis agreed with this visual and biogeochemical assessment (Figure 4). The majority of the clones picked from this enrichment fell closest to Fusibacter paucivorans, a thiosulfate reducer. These are probably responsible for the apparent sulfide production seen in the biogeochemical data. Additionally, one clone grouped closest to *Arcobacter*, a species that is also known for the ability to couple NO$_3^-$ reduction to H$_2$S oxidation to elemental S.

Stoichiometric data for the oxidation products suggested that approximately equivalent amounts of S$^0$ and SO$_4^{2-}$ were produced from H$_2$S oxidation in the high NO$_3^-$/H$_2$S treatment (Figure 7). In the low NO$_3^-$/H$_2$S treatment, only elemental S was produced and no SO$_4^{2-}$ was measured. However, this may be due to the increase in the sulfate reducing community, not because the nitrate dependent sulfide oxidizers weren’t producing SO$_4^{2-}$.

**Turkey Marsh (KBS, MI)**

The samples from the Turkey Marsh enrichment were taken from top 5cm of the wetland sediment. This enrichment was supplemented with NO$_3^-$ and H$_2$S on approximately a daily basis. Except for the first few days of enrichment development, the NO$_3^-$ and H$_2$S were removed within 24 hours of the addition. This was also accompanied by an increase in SO$_4^{2-}$ concentrations over time (Figure 5). Microscopic analysis of the samples suggested that the
majority of the enrichment was composed of small, highly motile (and fast) rods that produced some elemental sulfur globules in addition to individual “spindles” (Figure 5a). Community analysis suggested that this was a nearly pure culture of *Thiomicospira denitrificans*, since 16 of the 20 clones picked grouped closest to this species (not all clones are shown on the tree in Figure 6). The rest of the community was composed of species closely related to *Psuedomonas sp*, *Syntrophus sp*, and *Gemella sp*. Stoichometric data for the oxidation products suggested that the added H$_2$S was reduced to approximately equal amounts of SO$_4^{2-}$ and S$^0$ in both the high NO$_3^-$ and low NO$_3^-$ treatments (Figure 7).

Conclusions

The method employed for this project produced a successful enrichment of sulfide oxidizing denitrifiers in multiple freshwater ecosystems. The majority of the bacteria enriched for were *Thiomicospira denitrificans*, although there were probably some *Arcobacter* at the School Street Marsh site. Most of the sulfide was oxidized to SO$_4^{2-}$, which would explain the apparent sulfate production that has been documented during NO$_3^-$ additions to wetlands in MI. The fate of the reduced NO$_3^-$ was not characterized, though it was probably reduced to N$_2$ because there was no apparent NH$_4^+$ production during the course of the experiments. This study confirms that diverse freshwater sediments contain the biological capacity to couple the nitrogen and sulfur cycles.
Figure 1: Depth profile of Temperature (C), dissolved oxygen (DO; mg/L) and salinity from Sider’s Pond, point C. Collected on 11 July 2007. Samples for enrichments were collected at 27ft.
Figure 2: Clone library tree (a), photograph of cell morphology (1000x; b) and biogeochemical time course data (c) for the high NO₃⁻/H₂S enrichment from Sider’s Pond.
Figure 3: A photograph of cell morphology (1000x; a) and biogeochemical time course data (b) for the high NO₃⁻/H₂S enrichment from School Street Marsh.
Figure 5: A photograph of cell morphology (1000x; a) and biogeochemical time course data (b) for the high NO$_3$/$\text{H}_2\text{S}$ enrichment from Turkey Marsh.
Figure 7: Comparison of the end-products of H$_2$S oxidation across sites. Solid bars are the low NO$_3$/$\text{H}_2\text{S}$ treatments and dotted bars are the high NO$_3$/$\text{H}_2\text{S}$ treatments. Grey bars are elemental sulfur and black bars denote the production of SO$_4^{2-}$ as the end product. SSM = School Street Marsh, SP = Sider’s Pond, TM = Turkey Marsh.
References:
Appendix A. Media details

Preparation:
1. I will use FW base already made for lab. To it I will add:
   a. 1 ml of 0.1% rezasurin
   b. 70 ml of 1M NaHCO$_3$ (buffer) = 5.98 g
   c. 20 ml of 1M MOPS (buffer)
   d. 10 ml of trace element and vitamin mix
2. Boil for 10 mins and cool under N$_2$/CO$_2$ flow.
3. Transfer solution to chamber.
4. Transfer 50 ml of the final solution into the ~120ml bottles (already degassing in the bag). Cap with septa and crimp seal. Autoclave for 40 mins on liquid cycle.
5. H$_2$S solution: 2.02 g crystal into 100 ml anaerobic water.
Thiosulfate solution: 50 mM (0.78 g NaS$_2$O$_3$ into 100 ml). Filter sterilized.

Appendix B: Protocol for analysis of H$_2$S, NO$_3^-$, and NH$_4^+$ in enrichment cultures:

1. Set up H$_2$S microelectrodes. Use 1mM H$_2$S solution and FW base to calibrate the electrodes.
   a. Remove 2ml of solution from each vial. Measure H$_2$S immediately afterwards to preserve H$_2$S concentration in solution. Use a fresh needle to extract to prevent cross-contamination of enrichments.
   b. Filter 1 ml into a NO$_3^-$ “A” vial and 0.5 ml into an eppendorf tube and an IC vial. Cover the IC vial with parafilm to prevent evaporation.
2. NH$_4^+$ run
   a. Set up std curve using stock stds and using sterile media as the blank (to correct for any absorbance due to reagents or rezasurin. Blank A will be for media and blank B will be for DI.
   b. Dilute the 0.5 ml of sample with 0.5 ml of DI for a total sample volume of 1 ml.
   c. Add 33 uL of reagent A. Shake.
   d. Add 33 uL of reagent B. Shake.
   e. Store for 3-6 hours to allow color to develop.
   f. Measure at 630nm on a spec, recording absorbance. Calculate concentration based on the std. curve, subtracting for the reagent and DI blank.

Reagents for ammonium analysis:
- **Reagent A.** Dissolve 3.5 g of Phenol and 0.040 g of sodium nitroferricyanide dihydrate (sodium nitroprusside) in 100 mL of DI water. Store in a clean HDPE bottle in the refrigerator. **Do not weigh the phenol in a plastic dish; use a glass beaker, or aluminum pan.** Avoid inhalation of phenol. Solution is stable for one month and should be discarded as waste when the solution turns dark brown or greenish. For best results, use freshly made Reagent A.
• **Reagent B** (these are mixed together just before use; see “Procedure” above).

Alkaline reagent: dissolve 20 g of sodium citrate and 2 g of sodium hydroxide (analytical reagent grade) in 200mL of DI water. This solution is stable indefinitely.

Sodium hypochlorite solution (ordinary Clorox bleach (~6%NaOCl) works well)

<table>
<thead>
<tr>
<th>Rgt volume needed:</th>
<th>10 mL</th>
<th>20 mL</th>
<th>25 mL</th>
<th>30 mL</th>
<th>50 mL</th>
<th>100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Alkaline Reagent</td>
<td>8</td>
<td>16</td>
<td>20</td>
<td>24</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>(2) Clorox</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>10</td>
<td>20</td>
</tr>
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3. NO$_3^-$ run: these were run by ion chromatography because the red color produced by the colorimetric method overlapped with the red color produced by oxidized rezasurin.