Nutrient interactions mediate microbial N$_2$O production in salt marsh sediments

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ABSTRACT

Coastal ecosystems such as salt marshes may play a substantial role in the production of N$_2$O, a powerful greenhouse gas, particularly as nutrient loads from anthropogenic sources increase. Controls on N$_2$O production are likely to involve complex interactions between nutrients including nitrate, ammonium, and iron. Slurry experiments were conducted using rhizosphere (root and surrounding sediments) of *Spartina alterniflora* plants from West Falmouth Harbor in Woods Hole. Nitrate (and ferrihydrite) additions significantly stimulated N$_2$O production but levels of enhancement after 4 hours were mediated by ammonium concentrations in the slurries. Ammonium addition alone displayed only a long term (after 5 days) enhancement of N$_2$O production. The relative abundance of ammonia-oxidizing bacteria in 3 slurries and one environmental sample were explored via CARD-FISH and FISH, but no significant populations of known γ-proteobacteria or β–proteobacteria groups were identified. Efforts to isolate ammonia-oxidizing bacteria from the marsh sediments in autotrophic and heterotrophic media were also unsuccessful within 14 days, suggesting that these nitrifying bacteria are rare and slow-growing, and therefore may not be major players in the production of N$_2$O. Future efforts should further characterize the diversity of microbial groups capable of N$_2$O production, including denitrifying bacteria and test the role of ammonium, redox, and other nutrients in mediating their activities in the coastal environment.

INTRODUCTION

Increases in anthropogenic production of fertilizers and industrial combustion processes have led to a cascade of consequences for global biogeochemistry, including enhanced production of nitrous oxide. This gas has increased significantly from pre-industrial (pre 1750) levels of 270 ppb to more than 319 ppb in 2003 (Dow and Downing 2006). As N$_2$O is roughly 300 times more effective than CO$_2$ as a greenhouse gas, as well as being involved in depletion of stratospheric ozone, rising concentrations of this gas may have significant consequences for global climate.

Coastal salt marshes may play substantial roles in global climate via their roles in N$_2$O production. Increased anthropogenic nutrient loading to coastal ecosystems may enhance the activities of nitrifying and denitrifying bacteria that produce N$_2$O in the process of converting NH$_4^+$ to NO$_3^-$, or NO$_3$ to N$_2$, respectively. However, nitrogen inputs to marshes may increase benthic oxygen consumption and ultimately lead to sulfide inhibition of N$_2$O production. In recent slurry experiments with salt marsh sediments, reactive ferric iron was found to protect ammonia mono-oxygenase, the enzyme with which bacteria catalyze nitrification, from sulfide inhibition (Dollhopf et al. 2005). Thus burrowing macrofauna and plants which increase availability of oxidized iron in subsurface marsh sediments may mediate and possibly enhance N$_2$O production.

OBJECTIVES

The purpose of this research was to explore controls on N$_2$O production in intertidal
sediments. The following specific questions were addressed: (A) What are the independent and interactive effects of ferric iron\(^1\) and ammonium on N\(_2\)O production in intertidal sediments? (B) Do changes in N\(_2\)O production occur via shifts in the relative abundance of ammonia-oxidizing bacteria?

A combination of slurry experiments, culturing efforts, and molecular community characterization of slurry contents (and field sample references) from West Falmouth Harbor near Mashapaquit Creek are proposed to test the effects of ferric iron (FeOOH) and NH\(_4\) additions (independently or in that sequence) on: (a) N\(_2\)O production and (b) the relative abundance of nitrifying bacteria. Changes in the relative abundance of nitrifying microbes are hypothesized to underly differences in N\(_2\)O production among slurries with different nutrient treatments. The relative importance and interactive effects of micro- and macro-nutrients in affecting N\(_2\)O production by wetland microbes holds significant consequences for responses of coastal ecosystems to nutrient loading and their roles in global climate change.

**METHODS**

**Field site description and sample collection**

West Falmouth Harbor is a mildly eutrophic bay with significant input of groundwater in intertidal regions (noted during this study with salinity levels of 0.3). Tall *Spartina alterniflora* plants fringe the back portion of the harbor.

Sediment cores approximately 6 cm deep were extracted with a knife from *Spartina alterniflora* beds (between plants but adjacent to stalks) at regular intervals in a square pattern with roughly 6 feet between samples. These were transferred to sterile glass vials, capped with plastic wrap- lined lids, and returned to the lab for further processing within 1 hour of collection.

**Slurry Experiment**

From each of four sediment cores collected from the marsh of West Falmouth Harbor, four subsamples (16 total) of sediment was extracted with a sterile scalpel and compacted to fill a 1.5 ml tube. This sediment was transferred to a 10 ml plastic tube and shaken overnight (approximately 12 hours) in 5 ml of 1X seawater base (3422 mM NaCl, 14.8 mM MgCl\(_2\), 6H\(_2\)O, 1.0 mM CaCl\(_2\), 2H\(_2\)O, 6.71 mM KCl) at room temperature.

The following morning, 1 ml of ferrihydrite solution (300 μM FeOOH) was added into half of the shaken tubes (8 total), while the remainder received only 1 ml of 1X seawater base, and all were shaken for an additional 3 hours. (Ferrihydrite salts were prepared for use in sediment slurry experiments and analyzed following protocol of Kostka and Nealson (1998). However, the starting reagent used for preparation was Fe(NO\(_3\))\(_3\), and perhaps due to incomplete titration or washing, significant levels of nitrate were detected (> 256 μM) after the experiment was already in progress.)

Shaken sediments were subsequently poured into 160 ml glass vials containing an additional 45 ml of 1 X seawater base with HEPES buffer (0.4 g in 100 ml) with or without 300

\(^1\) Ferrihydrite additions included nitrate (> 256 μM) as an unintended byproduct of synthesis from Fe(NO\(_3\))\(_3\) H\(_2\)O, see Methods
μM NH₄⁺ enrichment, to result in a total of 16 bottles with 4 replicates (n=4) of the following treatments:

- **Treatment A**: 300 μM FeOOH, > 256 μM NO₃⁻
- **Treatment B**: 300 μM FeOOH, > 256 μM NO₃⁻, 300 μM NH₄⁺
- **Treatment C**: 300 μM NH₄⁺
- **Control Treatment**: No nutrient additions

In the process of generating slurries, large root material was excluded, so only sediments that washed free into supernatant of shaken tubes was transferred to the bottles. HEPES buffer was added with the intention of maintaining pH fluctuations within a range supportive of ammonia-oxidizing bacteria. Samples were sealed with rubber stoppers and metal crimps. Slurries were incubated in dark conditions and shaken continuously at 30 °C.

**N₂O analyses**

Gas subsamples (5 ml) were taken from only 4 slurry bottles for a “time zero” analysis due to time and transportation limitations within 30 minutes of experiment initiation (sealing of the vials). Additional subsamples of headspace gases were also collected after 4 hours, and 5 days (approx. 120 hours). These were analyzed on a ECD-GC at the USGS Woods Hole laboratory. Standard curves were constructed from mixtures of N₂O (0 ppm, 0.33 ppm, and 5 ppm from commercially available standard gases).

**Porewater analyses**

Porewater subsamples were taken by syringe from each slurry within 24 hours of experiment initiation, passed through 0.45 μm filters, and stored at 4 °C until further analysis (within 48 hours). Ammonium concentrations were determined following procedures described in Paerl (1998). At the end of the slurry experiment, additional porewater was collected (unfiltered) and treated with acid digestion for indirect determination of Fe (III) in ferrozine assays based on procedures of Kostka and Nealson (1998). Also, 6 ml of overlying water were submitted for nutrient analysis at the Woods Hole Oceanographic Institution nutrient analytical laboratory within 24 hours of experiment termination.

**Enrichment methods**

Approximately 1g from one sediment core collected in the field were used to inoculate media designed for isolation of autotrophic and heterotrophic ammonia-oxidizing and nitrite-oxidizing bacteria. Parallel inoculations were conducted that included the media below with either a pH indicator (Phenol Red) or a pH buffer (CaCO₃). Media for enrichment of autotrophic bacteria contained the following ingredients:

- 0.5 ml 100X FW base
- 150 ml 1X SW base
- 5 ml 1 M NH₄Cl or 5ml NaNO₂
- 0.2 ml 1M Na₂SO₄
- 0.2 ml 1M Na₂HPO₄
- 0.2 ml 1M 1000X HCL dissolved trace metals
- 0.75 g CaCO₃ or 15 μL of Phenol Red (0.5%)
Media for heterotrophic bacteria consisted of these same ingredients plus plant root material (less than 0.5 g) that was washed in 1X SW base. Following inoculation, tubes were shaken continuously at 30°C.

Detection of ammonia-oxidizing bacteria

One replicate from nearly all nutrient treatments and one environmental sample were examined for the presence and relative abundance of nitrifying bacteria using 16S DNA probes specific to γ-proteobacteria (NSCOC 128) and β–proteobacteria (NSO 190), respectively (from Mobarry et al. 1996). The latter was employed in CARD-FISH (Catalyzed Reporter Deposition-Fluorescent In Situ Hybridization) while the former was mono-labelled and thus could be employed only in standard FISH procedures.

Sediment samples from slurries and environmental samples were initially processed and preserved as described by Ishii et al. (2004). Samples were sonicated 7 times for 30 s in PBS and ethanol (1:1) at low power (0.2 Watts) with a probe sonicator (Microson XL 2007). From the sonicated sample, 200 microL were transferred to 4 ml 1X PBS and filtered onto GTTP filters (Millipore). These filters were subsequently processed following CARD-FISH and FISH procedures in the course lab manual (based on Schonhuber et al. 1997).

RESULTS
N2O production

After 4 hours, N2O production significantly differed among all treatments (F2,8 = 6.31, p=0.02) although only 1 replicate among controls (treatment D) was analyzed (Figure 1). As treatments A and B differed only in terms of ammonium enrichment (none in A, 300 μM in B), their N2O production was examined in further detail. N2O production was higher in slurries of treatment A (ferrihydrite and nitrate enriched) than in treatment B (ferrihydrite and nitrate enriched plus ammonium enrichment) (t3=1.03, one-tailed p=0.04).

Figure 1: Concentration of N2O in headspace of sediment slurries exposed to the following experimental treatments after 4 hours (A) 300 μM FeOOH, >256 μM NO3−; (B) 300 μM FeOOH, >256 μM NO3−, 300 μM NH4+; (C) 300 μM NH4+; Control Treatment: No nutrient additions
Significant differences between slurry treatments also persisted after 5 days (F_{3,11}=4.38, p=0.03, Figure 2) with significantly higher N\textsubscript{2}O production among treatment A (ferrihydrite and nitrate addition) than any other treatments (student’s t=2.20, p=0.05), but no significant differences between treatments A and B after 5 days (t_{3}=1.39, p=0.25).

![Figure 2 (left): Concentration of N\textsubscript{2}O in headspace of sediment slurries exposed to the following experimental treatments after 5 days: (A) 300 μM FeOOH, >256 μM NO\textsubscript{3}⁻; (B) 300 μM FeOOH, >256 μM NO\textsubscript{3}⁻, 300 μM NH\textsubscript{4}⁺; (C) 300 μM NH\textsubscript{4}⁺; Control Treatment: No nutrient additions.](image)

Some variability in N\textsubscript{2}O production was related to ammonium concentrations in slurry waters (Figure 3 A and B). Specifically, the replicate of treatment B (number 4, circled) which demonstrated the highest N\textsubscript{2}O production after 4 hours also had notably less ammonium than other replicates of the same treatment (which all initially contained 300 μM NH\textsubscript{4}⁺) (Figure 3A). In addition, after 5 days, replicate 2 of treatment C showed highest N\textsubscript{2}O production but lowest porewater ammonium of all other treatment Cs (Figure 3B).

![Figure 3: Relationships between N\textsubscript{2}O concentrations in slurry headspaces and ammonium concentrations in water contained within the slurries (A) after 4 hours (top) and (B) 5 days (bottom).](image)
**Enrichment efforts**

Within the time frame of this study (12 days), no significant growth of ammonia- or nitrite-oxidizing bacteria was observed in media for autotrophic bacteria. Among heterotrophic enrichments for ammonia-oxidizers some growth was noted in the primary enrichment and portions were subsequently transferred to a secondary enrichment tube, but the latter did not produce visible growth prior to termination of the experiment. According to pH indication by phenol red, media with NaNO₃ had lower pH than NH₄Cl (Figure 4).

**Figure 4 (left):** Photo of media designed for enrichment of heterotrophic ammonia and nitrite-oxidizing bacteria from salt marsh rhizospheres, with tubes on left containing phenol red (a pH indicator) and tubes on the right containing a pH buffer (see Methods).

**Figure 5:** Images of fixed cells from slurry 1D that were probed via CARD-FISH with EUBI-III, NON 338, and NSO 190. Top images (blue) show all DAPI stained cells, and bottom images show cells with specific labels only.
Relative abundance of Ammonia-oxidizing bacteria

No significant numbers of ammonia-oxidizing bacteria were noted in 3 slurry samples (1A, 1C, 1D) and one environmental sample (replicate 2) to which CARD-FISH and FISH techniques were applied with the NSO 190 and NSCOC 128 probes, respectively.

Images of fixed and filtered cells from a representative slurry 1D that was analyzed via CARD-FISH are illustrated in Figure 5. Cells were targeted with the general EUB I-III probe (for all bacteria) served as positive controls, while the nonspecific probe, NON 338 served as a negative control. The group specific probe, NSO190, for ammonia-oxidizing β−proteobacteria showed similar patterns of non-specific fluorescence as the NON 338 probe. More specifically, none of the fluorescent objects on filters hybridized with the NSO 190 probe appeared to have corresponding fluorescence visible with the DAPI filter, suggesting that these were not real cells, and thus they were not counted.

Results from regular FISH analyses with NSCOC 128 produced similar results and thus are not shown. Patterns observed with the environmental sample were similar to those of slurry samples.

DISCUSSION

The relative importance of nitrification and denitrification in the production of N₂O remains to be determined in many environments, particularly in salt marsh sediments, but is of central importance to elucidating mechanisms controlling emissions of this greenhouse gas. In this study, stimulation of N₂O production in the presence of nitrate additions is consistent with N₂O production from denitrifying nitrate-reducing bacteria. These bacteria might be expected to be abundant in salt marsh sediments that contain organic rich sediments with complex fluctuations in oxygen availability that would favor organisms capable of at least facultative nitrate reduction as a means of energy production. Nitrate has been previously reported to stimulate N₂O production in marshes and a suite of other environments.

Impacts of metals on nitrogen cycling have recently been explored and may be relevant to N₂O production. Manganese, for example has been suggested to be coupled to anaerobic nitrification and denitrification (Luther et al. 1997, Newton 2006). In addition, the availability of oxidized iron was also recently suggested to alleviate sulfide reduction of nitrifying bacteria in coastal salt marshes, with undetermined consequences for N₂O production (Dollhopf et al. 2005). Metals may also play key roles in abiotic production of N₂O, as ferrous iron reacts with nitrite under anaerobic conditions to form N₂O (Moraghan and Buresh 1977). Unfortunately, effects of oxidized iron (ferrihydrite) could not be distinguished from those of nitrate due to errors in synthesis of the ferrihydrite reagent, but future work may readily address this question by using commercially available ferric chloride in similar enrichment experiments, and by observing or manipulating patterns of iron availability in marsh environments.
Nutrient interactions, particularly between oxidized and reduced forms of nitrogen, may have significant impacts on N$_2$O production by microbial communities. Most studies have focused independently on NO$_3^-$ (i.e. Lee et al. 1997) or NH$_4^+$ (i.e. Avrahami et al. 2002) as stimuli of denitrification or nitrification respectively. However, significantly less N$_2$O production observed in nitrate-enriched slurries when in the presence of ammonium suggests that the latter may have a key role as a mediator of microbial metabolisms.

Mechanisms for significantly lower N$_2$O production in the presence of ammonium (Figure 1) may involve repression of nitrite reductase. Ammonia and other reduced forms of nitrogen (casamino acids) have been known to repress the nitrite reductase in the fungus *Neurospora crassa* (Yordy and Ruoff 1985). Nitrite reductase is the enzyme which some bacteria use to catalyze reduction of nitrite to nitric oxide, an intermediate preceding N$_2$O and N$_2$ in denitrification. Notably, nitrifiers such as *Nitrosomonas europaea*, are known which can possess the nitrite reductase enzyme (Poth and Focht 1985). Nitrite reductase is used not only in denitrification (or nitrifier denitrification) but also in assimilatory processes from which ammonia is generated. Ammonia repression of nitrite reductase has been reported in this latter context (Yordy and Ruoff 1985). If the same nitrite reductase enzymes can be employed in reduction of nitrite to either nitric oxide or ammonia, then addition of ammonium may repress both reduction processes and consequently block N$_2$O production (Figure 6).

![Diagram](image)

**Figure 6**: Diagram of the role of nitrite reductase in catalyzing part of the denitrification pathway (black) and in catalyzing reduction of nitrite to ammonium (orange); modified from Ingraham 1983 and Yordy and Ruoff 1983).

The reduction of nitrite to ammonium is not thought to be a source of ATP-yielding energy but rather a detoxification mechanism, and a means of dissipating excess electrons.
The presence of excessive nitrate ions (in treatments A and B) may have provided excessive electrons for dissipation by nitrite reduction to ammonium.

Future work is required to address the extent to which ammonium, nitrate, and other nutrients interact in coastal environments to affect microbial metabolism, community structures, and N2O production. Redox and pH have been implicated as key controlling factors affecting the magnitude of N2O production in flooded soils (Yu and Patrick 2003), and these properties might reflect physiological responses of microbes to the relative availability of ammonium and nitrate. In particular, further efforts to characterize microbial communities involved in production of N2O may expand focus beyond ammonia oxidizers which did not seem to be key constituents of slurry sediments or of environmental samples (although further replication is warranted). The low relative abundance (absence) of ammonia oxidizing bacteria in slurry and environmental samples, combined with the failed ability to isolate them from the environment, nonetheless suggests that they are minor and slow-growing members of the community in the West Falmouth Spartina alterniflora marsh. As such, nitrifying bacteria may not be likely to produce high levels of N2O, though they may play significant, indirect roles on the production of this gas via influences on ammonium availability and coupling with denitrifying bacteria. The relative absence of nitrifying bacteria in West Falmouth Harbor may be a consequence of long term nitrate additions via groundwater to the region, and this possibility warrants further exploration.

References


