Evaluation of Denitrification Rates and Mechanisms in Microcosm Experiments with Sediments and Plants

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ABSTRACT

Five sets of microcosm batch experiments were performed to evaluate mechanisms of nitrate removal in constructed wetlands. The study evaluated biological denitrification activity as a function of 1) electron donors carbon and sulfur 2) wetland sediment depth 3) oxygen and abiotic reactions and 4) carbon addition and attached populations from wetland bulrushes. Wetland-water-only controls amended with nitrate showed negligible denitrification rates. Nitrate-only amended wetland sediments demonstrated nitrate removal rates of 17.0-25.8 mg N/L*day (13.6-20.6 kg N/acre*day) for two wetland systems. Autotrophic denitrification occurred simultaneously with heterotrophic nitrate reduction. The addition of carbon via glucose or plants increased rates 24% to 82%; whereas, adding sulfide to batch tests resulted in variable effects dependent on sediment conditions. Sulfide oxidizers appeared to scavenge available oxygen creating reduced conditions when the headspace was purged with air. Bulrushes contained attached populations of denitrifying bacteria and increased nitrate reduction rates by supplying electron equivalents via carbon.

KEYWORDS

Denitrification, Wetlands, Autotrophic Sulfide Oxidation, Acetylene-block

INTRODUCTION

The reuse of nitrate-contaminated water sources is often necessary in arid regions due to the scarcity of economical unpolluted supplies. The United States EPA drinking water maximum contaminant level (MCL) for nitrate is 10 mg NO_3-N/L due to its presumed risk as a cause of methemoglobinemia or blue-baby syndrome (Johnson, 1998; Alexander, 1999) and cancers such as non-Hodgkin's lymphoma (National Cancer Institute, 1996). As an alternative electron acceptor to energetically favorable oxygen, nitrate can be reduced effectively in constructed wetlands to nitrogen gas by microorganisms present in wetland sediments due to low redox conditions and high electron donor quantities such as carbon and reduced inorganic compounds such as

sulfur. Thus, the purpose of this study was to provide insight into relative denitrification rates and the mechanisms of denitrification. Five sets of batch experiments were completed to examine the following:

1. To determine the effect of electron donor (carbon sources and sulfide) on denitrification activity
2. To determine the effect of sediment depth on microbial denitrification activity
3. To determine the influence of oxygen and potential abiotic mechanisms
4. To determine the effects of plants and microbes attached to the plants on denitrification activity.
5. To determine the potential for nitrous oxide production from wetland sediments by adding sulfide and acetylene.

Mechanisms for biological denitrification include heterotrophic activity, with bacteria using carbon as the electron donor and organic carbon as the carbon source, and autotrophic activity using non-organic electron donors. Until the relatively recent acceptance of nitrate removal mechanisms in constructed wetlands without a seasonably sustainable organic carbon supply (Wass, 1999; Nahar, 2000), standard theory for biological denitrification in wetlands was based solely on heterotrophic nitrate reduction. Heterotrophic denitrifying bacteria dominant in soils include *Pseudomonas* and *Archomobacter* (Hammer & Knight, 1994). Equation 1 is a microbial reaction for heterotrophic denitrification using methanol as a carbon source (USEPA, 1993).

(1) $NO_3^- + 0.833\ CH_3OH = 0.5\ N_2 + 0.833\ CO_2 + 1.167\ H_20 + OH^-$

In wetland systems input of particulate and dissolved organic carbon is highest due to macrophyte senescence in the autumn of the year and lowest in the spring due to plant growth (Weisner, et al., 1994). Therefore, organic carbon supplies are highly variable dependent upon the season and can be limiting. End products of particulate carbon degradation pathways include a variety of simple fatty acids, particularly acetic acid, that are ultimately used as a carbon source for nitrate and sulfate reduction (Reddy and Graetz, 1988). Nitrate reduction is favored over sulfate reduction due to increased energy release (difference of approximately 400 mV per mole); however, conditions exist where a carbon source can be used exclusively for sulfate reduction (Burgoon, 1993). Sulfate that is subsequently reduced to sulfide is stored in the anaerobic layers of wetland sediments where it can diffuse into solution (Reddy and Patrick, 1984).

It is of conventional theory that carbon is the limiting factor in the efficacy of biological denitrification in wetland systems based on biomass growth cycles. The addition of sodium acetate to constructed wetlands has been shown to significantly increase denitrification rates (Kozub & Liehr, 1999). Gersberg et al. (1983,1984) supported this theory when they added shredded plant biomass, methanol, or primary effluent to wetland systems and showed some of the highest field nitrate removal rates ever recorded (7.7-12.5 kg N/acre*day). Others have demonstrated this same concept by enhancing denitrification in constructed wetlands by adding glucose or glycerol (Dahab, 1991). Heterotrophic denitrification by an external carbon source is a widely used, proven

biological treatment technology. High cost and safety concerns associated with external carbon addition, coupled with increases in disinfection by-product formation potential, reduce the attractiveness of this treatment option.

Autotrophic denitrification is based on chemolithotrophic respiration of an inorganic carbon source (CO_2). *Thiobacillus denitrificans* and other sulfur-oxidizing species have long been used in industrial biological denitrification processes with a relatively high degree of success (Koenig & Liu, 1996). Bench-scale and laboratory chemostat batch-cultures have also shown adequate nitrate removal rates by autotrophic species (Flere and Zhang, 1998, and Justin and Kelly, 1978a). Aminuddin and Nicholas (1974b) and Flere and Zhang (1998) demonstrated that *T. denitrificans* even reduces nitrate under aerobic conditions due to oxygen uptake inhibition during sulfite oxidation. The denitrification rates of certain *T. denitrificans* strains were found to be the same at 5˚C as 20˚C (Trouve et al., 1998).

Although numerous bench, pilot, demonstration, and full-scale treatment schemes using autotrophic media exist to treat nitrate-contaminated waste streams, only in the past few years has this biological mechanism been established in wetland systems as a method for denitrification during periods of low organic carbon supplies. Equation 2 is an autotrophic denitrification reaction using sulfide as an electron donor and carbon dioxide as a carbon source.

(2) $$NO_3^- + 0.74\ S^{2-} + 0.1886\ CO_2 = 0.74\ SO_4^{2-} + 0.48\ N_2 + 0.037\ C_5H_7O_2N + 0.1H^+ + 0.37H_2O$$

NOTE: Equation 2 calculated thermodynamically assuming:
$R = R - f_e*R_a - f_s*R_c$
where $f_s = 1/(1+A) = 0.18\ e^-$ *and* $f_e = 0.82\ e^-$

As shown in the equation above, the stoichiometric relationship for {(sulfate production)/(nitrate consumption)} is approximately 0.74 for autotrophic denitrification. This ratio was used extensively to quantify the portion of nitrate reduction caused by each type of metabolic pathway (i.e. 100% autotrophic activity corresponds to a $\{-\Delta[NO_3^-] / \Delta[SO_4^{2-}]\} = 0.74$). In addition, headspace carbon dioxide production was linked to heterotrophic denitrification activity, as consumption of carbon dioxide was related to autotrophic activity.

Most biological denitrification takes place under reducing (low redox) conditions where oxygen is limited, although some strains of bacteria have been isolated that denitrify in the presence of oxygen; however, the mechanism and physiological advantage gained by this pathway is not completely clear (Carter et al., 1995). Four distinct biological enzymes work in series in the reduction of nitrate. The acetylene-block method is a common technique for quantifying denitrification by inhibiting the biological enzyme

Biological Denitrification Pathway

$NO_{3\,(aq)}$	**->**	**$NO_{2\,(aq)}$**	**->**	**$NO_{(g)}$**	**->**	**$N_2O_{(g)}$**	**->**	**$N_{2\,(g)}$**
(nitrate)		*(nitrite)*		*(nitric oxide)*		*(nitrous oxide)*		*(nitrogen)*

that converts nitrous oxide to nitrogen gas in the final step. Acetylene is generally accepted to block nitrous oxide reduction by all denitrifying organisms, but the enzyme system for autotrophic organisms has not been researched extensively. Therefore, although the acetylene-block method has been proven effective for heterotrophic quantification of nitrate reduction (Kozub and Liehr, 1999), this method has not attempted to distinguish autotrophic from heterotrophic denitrification. Recovery of nitrous oxide in anaerobic serum bottles with 90-120% water-filled pore space when incubated for >1 day is often underachieved due to the delay in diffusion from sediments to the headspace (Aulakh & Doran, 1991). Sulfide has also been linked to the inhibition the nitrous oxide reduction at high concentrations. Nitrous oxide is considered a green-house gas 20 times as detrimental as carbon dioxide.

METHODOLOGY

The Wetlands of Avondale, a 78-acre gravity-flow wetlands system in western Phoenix, AZ, was the primary source of microcosm materials used in this study. Avondale's wetland system, fully operational in August, 1999, receives Salt River Project (SRP) and Central Arizona Project (CAP) surface water supplies contaminated with irrigation runoff from surrounding agricultural land-use. A unique feature of the system is that nitrate is the major form of nitrogen entering the wetlands and is the only contaminant of concern. Inlet concentrations of nitrate varied between 0.5-16 mg NO_3-N/L. Vegetative islands compose approximately 28 acres (two 1-acre vegetative islands/wetland cell) of the 78-acre system. Three species of bulrush macrophytes comprise a majority of the plant biomass on the vegetative islands: *Scirpus perpus* (60%), *Scirpus americanas* (30%), and *Scirpus califonicas* (10%).

The Tres Rios Wetlands, a 14-acre demonstration system in western Phoenix, AZ, was also used in this study to compare the removal efficacy of different types of constructed wetlands systems. The Tres Rios Hayfield Site H1 used in this study received nitrified/denitrified effluent from the 91st Ave. Wastewater Treatment Plant in Tolleson, AZ. The Hayfield Site H1 (3 acres) is located on a former agricultural field consisting of five 9-meter deep zones which occupy approximately 20% of the total wetland area (80% shallow vegetation zones).

Wetland sediment for batch tests was obtained from soil cores using 1-inch diameter hi-density polyethylene tubes. Batch experiments were conducted using 165mL clear glass serum bottles (VWR) filled with wetland soil (20 g wet weight or 5.07 cm^2 equivalent vegetative area), wetland water (80mL from Tres Rios Hayfield Site H1 or Avondale wetland cell 3), and purged with ultrapure nitrogen gas (Praxair). Each bottle was stoppered (blue septum, VWR) and sealed (aluminum caps, Wheaton). Wetland soil was

applied by extruding 1" (2.54 cm) diameter sediment cores obtained from the east vegetative island of Avondale Wetland #3. Each core was extruded and a 2 cm-height core segment (10.1 cm^3 vol., 20 g wet weight) of soil was used for each batch test. Serum bottles were mixed on a table shaker in the dark to simulate field conditions. Periodically, 6 mL liquid aliquots were withdrawn, filtered, and analyzed by ion chromatography (Dionex 120) for chloride, sulfate, nitrite and nitrate. Each volume of liquid sampling volume was replaced with nitrogen gas to equilibrate headspace pressure in the serum bottle. Headspace analysis was performed using a 1000 uL gastight syringe (Hamilton) by withdrawing 700 uL from the headspace, replacing the withdrawn volume with nitrogen gas, and injecting 500 uL into a Gas Chromatograph (Gow Mac 580) for analysis of carbon dioxide, nitrogen, nitrous oxide, and methane.

Nitrate additions were performed to achieve initial aqueous concentrations of 2mM (Avondale batch tests) and 1.5 mM (Tres Rios batch tests). Electron donors added in particular batch experiments consisted of carbon sources glucose (1.6mM) or bulrush (2g chopped, cut, or oven-dried) and sulfur sources in the form of sulfide (1.6 mM). At the Wetlands of Avondale, the top 0-2 cm depth of sediment cores generally consisted of a sandy, sparse plant detritus layer composed of decaying plant stems and roots, the 2-4 cm layer was consistently a black, organic-rich sand, and 4-6 cm layer was a generally a inorganic brown sand. Sediment conditions were consistent for the 6 months of sampling. Plant material for macrophyte experiments was bulrush indigenous to Avondale wetland island 3 (east). Plant material consisted of a 50/50 mixture by mass of dormant (brown) and growing (green) bulrush from between 1'-2' above the stalk base. Plant material was divided into three categories: 1) sterile (oven-dried bulrushes @ 105 C for 24 hours, chopped to <1 cm length brown segments) 2) chopped (fresh bulrushes chopped 1'-2' above the stalk base to <1 cm length white/green segments) 3) cut (fresh bulrushes cut 1'-2' above the stalk base into >3 cm white/green pieces). Enrichment of Avondale sediment in experiments was achieved by adding 1.6 mmol of nitrate every two days for 6 days (three additions) to enhance denitrifying populations present in the sediment and consume existing electron donor supplies. Concentrations of nitrate were monitored by ion chromatograph until stabilized. Acetylene was added to the headspace of specific experiments at a 10% (vol:vol) concentration to block reduction of N_20 to N_2. Sediment from the Tres Rios Wetlands was obtained from 0-5 cm depth of deep zone 5 from the Hayfield Site H1 (Tolleson, AZ). Each serum bottle was filled with 20 g of the black, organic-rich composite soil mixture. Sterilized soil for the abiotic batch experiment was produced via autoclave 3 hours daily for 3 consecutive days. Deionized nanopure water was used for sterile water in the abiotic batch experiment. The headspace was purged with air for the aerobic batch test.

Additional batch experiments were performed using denitrifying sludge from the Globe, AZ wastewater treatment facility to compare growth kinetics and evaluate denitrification mechanisms. The Globe facility is a sequencing batch reactor treating casino waste-flows of 100,000 gallons/day.

RESULTS AND DISCUSSION

Triplicate mean nitrate plus nitrite (NOx) and sulfate concentrations versus time are displayed in Figure 1 for experiments containing wetland water only amended with nitrate. The denitrification rate obtained from the change in NOx concentration versus time was negligible, indicating bacteria populations in the water column were too low to significantly reduce nitrate. Subsequent denitrification rates were related to quantities of sediments and plant material only.

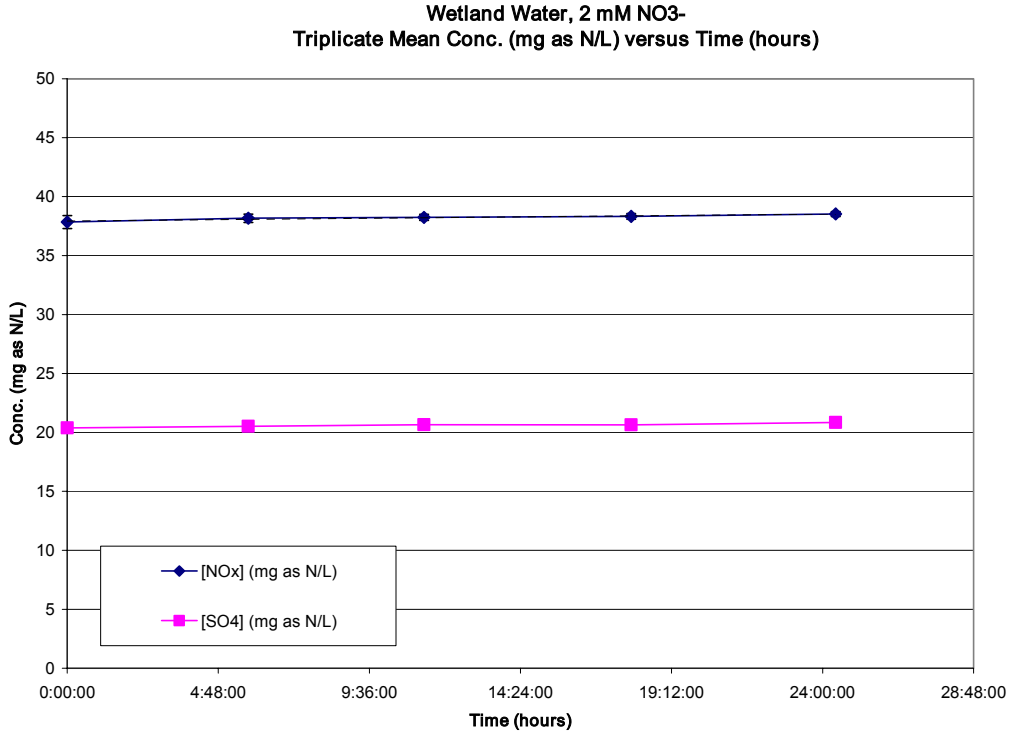

Figure 1. NOx (Nitrate+Nitrite) reduction and sulfate production in a nitrate amended experiment with Avondale wetland water only. Negligible denitrification activity was demonstrated, and rates of nitrate reduction in batch tests were associated with quantity of sediment and plants.

Effects of Electron Donors (Sulfide, Plants, and Soluble Carbon) –
From the Wetlands of Avondale, 2-4 cm depth sediment cores were amended with nitrate only, nitrate plus sulfide, nitrate plus glucose and nitrate plus plant material that was finely chopped (<1 cm length). Figure 2 displays mean aqueous NOx and sulfate concentrations versus time for triplicate nitrate-only amended control experiments. Sulfate is expressed in milligrams as nitrogen per liter to compare stoichiometrically with changes in nitrogen concentrations. The zero-order denitrification rate (13.6 kg N/acre*day) for the nitrate-only amended sediment was similar to other such experiments (15.5-20.6 kg N/acre*day). The {-ΔNOx/ΔSO4} ratio was relatively high (0.79), indicating autotrophic denitrification was the main mechanism for removal. Figure 3 shows the relative mean rates of denitirifcation versus time for the control and three electron donor experiments. Nitrate removal rates appeared to be linear (i.e. zero-order)

with time for nitrate only and nitrate plus sulfide experiments; however, the addition of plants and glucose produced non-linear plots. The addition of sulfide had an inhibitory effect on the total denitrification rate (6.72 kg N/acre*day) comparable to the nitrate only experiment at a depth of 2-4 cm. The addition of carbon, via glucose and chopped plant material, increased denitrification rates significantly (linear trendline equal to 18.7 and 24.8 kg N/acre*day, respectively). The {-ΔNOx/ΔSO4} ratios decreased to 0.41 (glucose) and 0.27 (chopped plants) as autotrophic denitrification became less significant with the addition of carbon substrates.

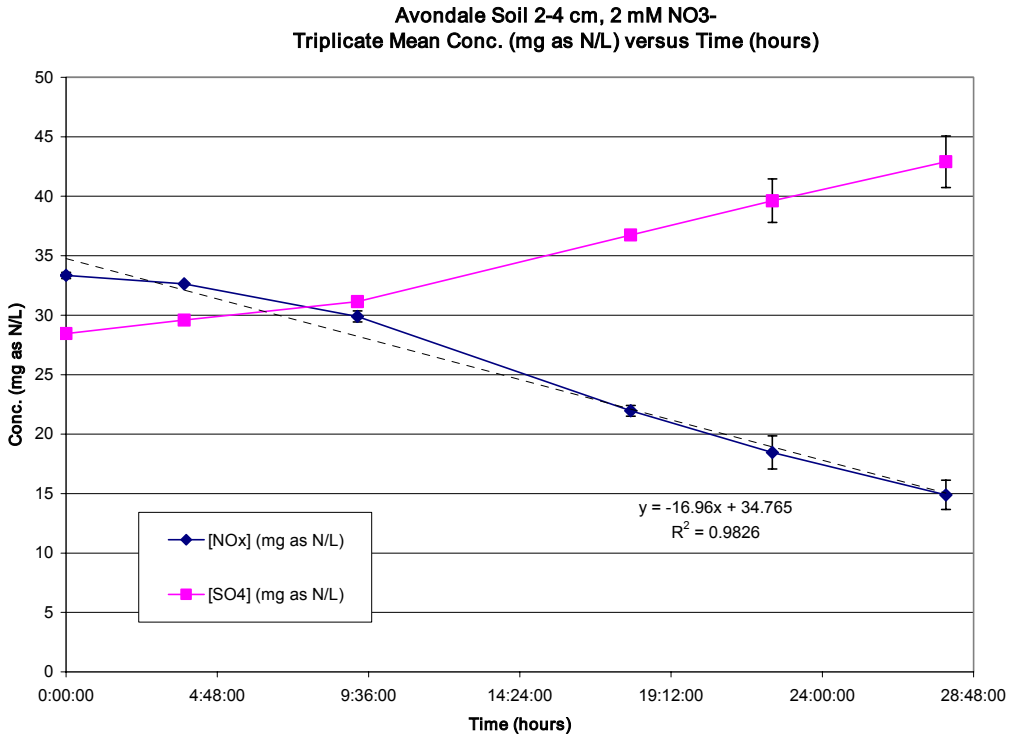

Figure 2. NOx (Nitrate+Nitrite) reduction and sulfate production in a nitrate amended sediment experiment. The sulfate concentrations are expressed as nitrate based on the reducing equivalent of sulfide.

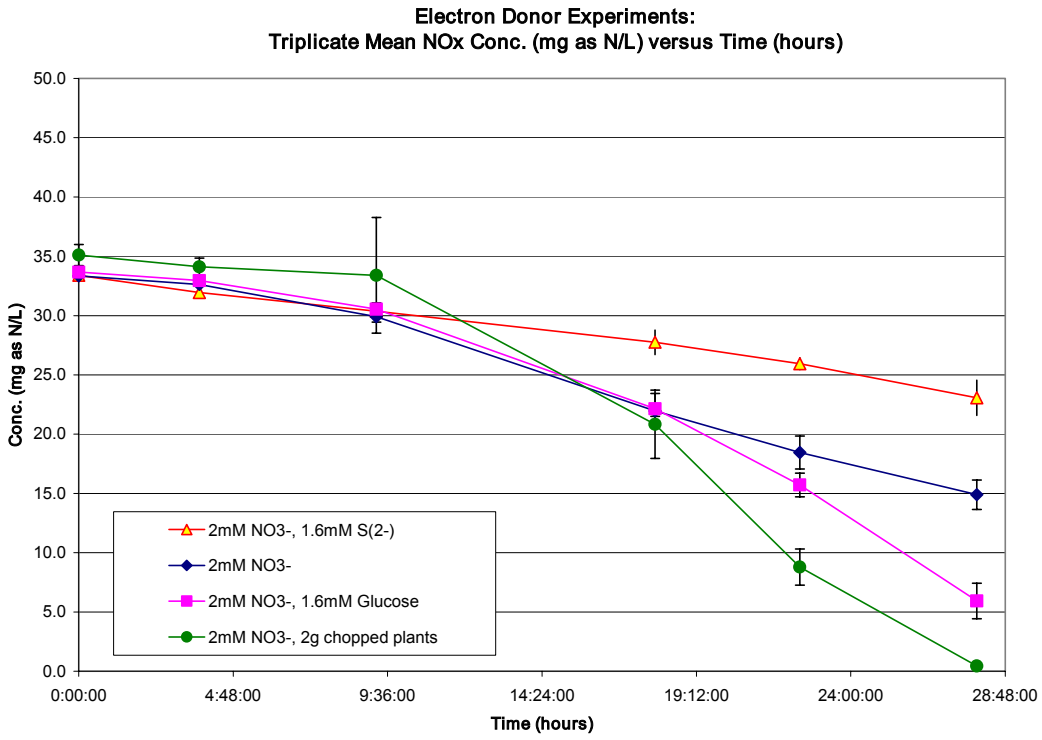

Figure 3. NOx (Nitrate+Nitrite) reduction versus time for sediment experiments amended with nitrate and electron donors sulfide, glucose, and chopped bulrush.

The increase in denitrification rates from the addition of carbon is not observed until after 12 hours and the reduction of nitrate as a function of time is non-linear. The non-linear increasing rate of denitrification is consistent with microbial growth. Since heterotrophic denitrifiers can have doubling times of less than 12 hours, heterotrophic microbial population could have more than doubled during the experiment resulting in significant growth. The linear decrease in nitrate concentration observed with nitrate only amended sediments was consistent with zero order kinetics and insignificant growth during the experiment. Since the nitrate concentrations are much higher than the half velocity constant for denitrification, the kinetics of nitrate utilization by the microorganisms should be zero-order throughout the experiment. Autotrophic microorganisms are relatively slow growing as compared to heterotrophs resulting in negligible growth during the experiment when autotrophic denitrification is the major mechanism. Chopped plant material increased denitrification rates more than glucose, indicating microorganism acclimation to plant material degradation products in the field.

Three experiments were performed on “enriched” sediment from the Wetlands of Avondale (nitrate fed to batch experiments containing sediment until denitrification no longer occurred) to isolate populations of 1) heterotrophs (glucose additions) 2) autotrophs (sulfide additions) and 3) combined heterotrophs and autotrophs (glucose and sulfide additions). Triplicate mean denitrification rates were 26 kg N/acre*day for 1.6mM glucose amended experiments and 17.7 kg N/acre*day for 1.6mM sulfide-amended experiments. Thus, the substrate utilization rate of nitrate for autotrophs was

68% of that for heterotrophs. Enriched sediment amended with 1.6 mM glucose and 1.6 mM sulfide resulted in a denitrification rate of 32.0 kg N/acre*day, 73% of the summation of heterotrophic and autotrophic rates.

Effects of Sediment Depth & Electron Donor Concentration–
The effect of sediment depth was evaluated by extruding sediment cores from the Wetlands of Avondale and amending depths of 0-2 cm and 2-4 cm with nitrate and nitrate plus sulfide. Experiments containing sediment from the 4-6 cm depth produced relatively negligible rates of nitrate reduction as compared to the 0-4 cm depth region. Denitrification rates for nitrate-only amended experiments were 11.0 and 15.5 kg N/acre*day for depths of 0-2 cm and 2-4 cm, respectively. Denitrification was mostly autotrophic for these batch tests based on molar ratios of nitrate reduction to sulfate production {-ΔNOx/ΔSO4} ranging between 0.48-0.62, signifying low carbon in the sediment. This experiment was performed September 16, 2000 when dissolved organic carbon in the field was low and field denitrification appeared to also be autotrophic, based on sulfate production. The sandy 0-2 cm depth layer likely displayed lower rates than the 2-4 cm depth due to lower quantities of electron donor, particularly sulfide in the soil matrix. When the 0-2 cm layer was amended with sulfide, however, denitrification rates increased to 17.1 kg N/acre*day, indicating sufficient populations of autotrophs in the 0-2 cm layer were present without adequate electron donor supplies. The 2-4 cm depth layer of sediment amended with sulfide demonstrated decreased rates of denitrification (11.9 kg N/acre*day) and the additional sulfide appeared inhibitory due to existing supplies of sulfide in the 2-4 cm layer. Sulfide, although necessary as an electron donor for sulfur-driven autotrophic microorganisms, has been shown to be inhibitory when present at high concentrations.

Concentrations of sulfide were increased to evaluate the inhibition effect of sulfide for enriched sediment experiments. Increases in sulfide concentrations to 4.8 mM and 8.0 mM decreased rates for autotrophic bacteria by 19% and 37%, respectively. Rates for combined autotrophs and heterotrophs increased by 9% and decreased by 43% when sulfide concentrations were increased to 4.8 mM and 8.0 mM, respectively. The pH in the enriched autotrophic experiments exceeded 10 when sulfide concentrations were increased to 4.8 mM which may have caused decreases in rates. Some carbonate buffering capacity was produced by heterotrophic denitrification which delayed the increase in pH for batch tests containing combined autotrophic and heterotrophic bacteria. Figure 4 displays NOx concentration versus time for wetland sediment amended repeatedly with glucose and sulfide. A lag-phase was apparent for the triplicate enriched sediment containing combined heterotrophic and autotrophic denitrifiers prior to nitrate reduction. Interestingly, unlike the non-linear kinetics demonstrated for heterotrophic experiments, the sulfide plus glucose amended experiment produced zero-order kinetics (see Figure 4). Figure 5 displays triplicate mean NOx concentration versus time for denitrifying wastewater sludge amended with 1) glucose plus nitrate and 2) glucose and sulfide plus nitrate. Despite similar rates of denitrification for both sets of batch tests and zero-order kinetics, a similar lag-phase was evident.

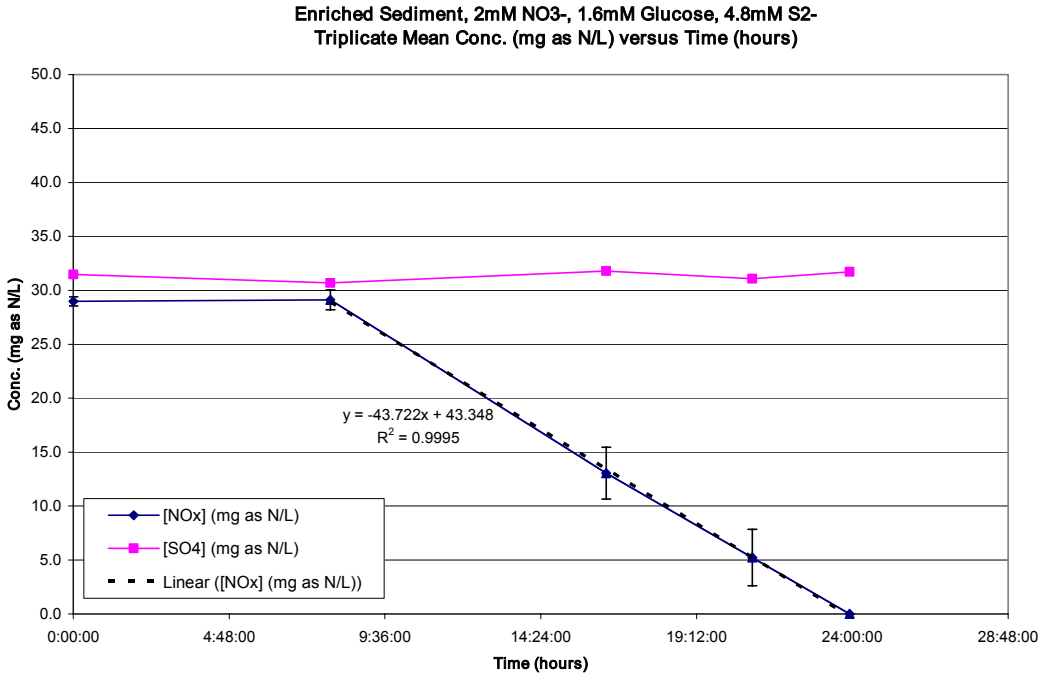

Figure 4. Triplicate mean NOx and sulfate concentration versus time for enriched sediment experiments with repeated additions of nitrate, sulfide, and glucose. A lag-phase prior to nitrate reduction is shown here believed to be caused by sulfide inhibition (initial sulfide concentration at time zero 4.8 mM).

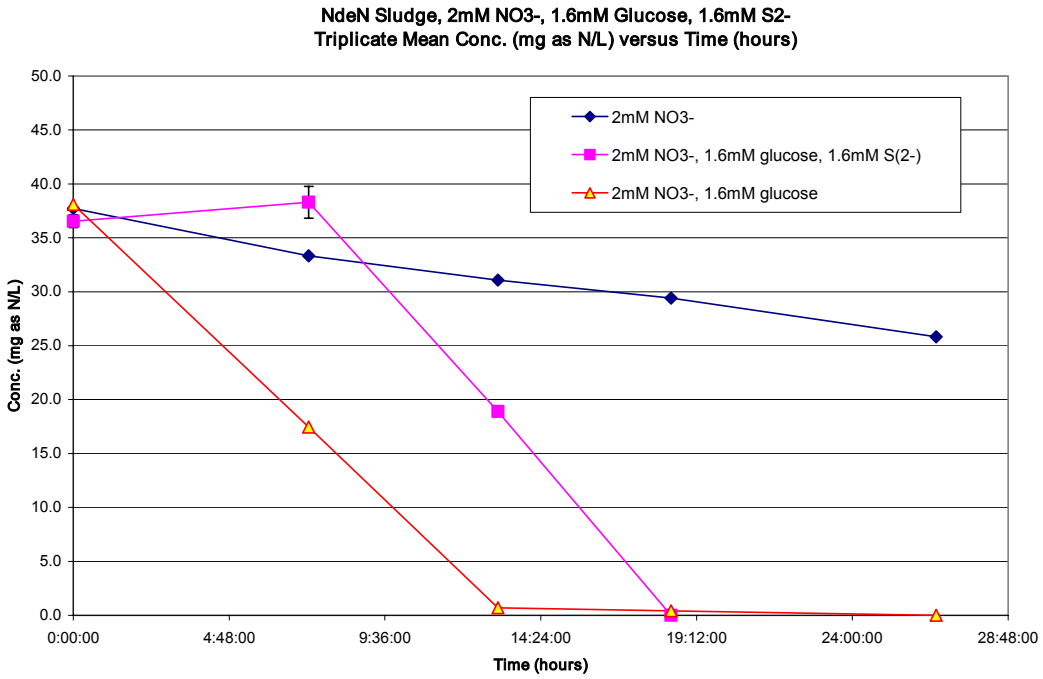

Figure 5. Triplicate mean NOx concentration versus time for denitrifying wastewater sludge. A lag-phase appears with the addition of 1.6 mM sulfide although zero-order denitrification rates are similar.

Effect of Oxygen and Abiotic Conditions –
Batch tests were done by amending sediments from the Tres Rios Wetlands with nitrate only and nitrate plus sulfide. Additional experiments were done using air instead of nitrogen gas for headspace and sediments sterilized by autoclaving. No abiotic reactions were observed with sterilized sediments as both nitrogen and sulfate concentrations did not change. When no nitrate was used to amend sediments, significant sulfate reduction (> 100 mg/L decrease) occurred indicating that there was ample organic carbon in the sediments (Figure 6).

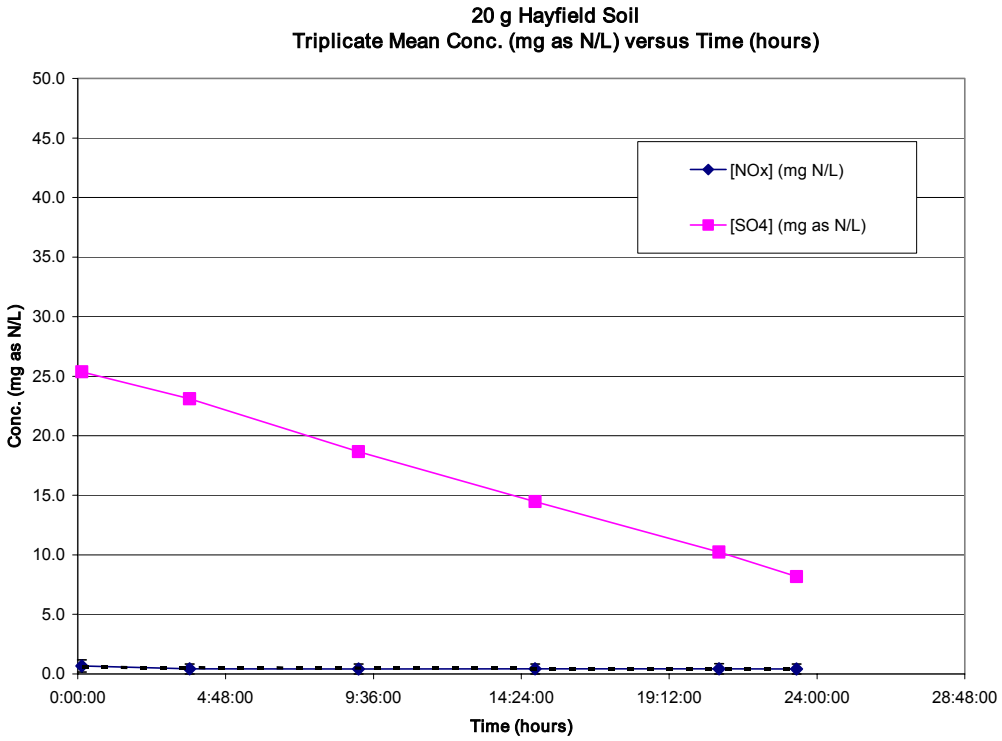

Figure 6. Triplicate mean NOx and sulfate concentrations versus time for Tres Rios wetland sediment with no amendments. Significant sulfate reduction was demonstrated when nitrate concentrations were low, indicating electron equivalents being transferred from carbon to reduced sulfur.

The addition of nitrate-only resulted in a linear decrease in nitrate concentration, and sulfate production was observed as sulfides in the sediment were used for denitrification along with organic carbon. The addition of sulfide increased rates of denitrification by approximately 60%, verifying the presence of autotrophic denitrifiers. The use of air in the headspace resulted in denitrification rates almost identical as compared to the experiments using nitrogen gas with headspace. A relatively large increase in sulfate (> 150 mg/L) was observed and most likely aerobic sulfide oxidizing bacteria scavenged the available oxygen and maintained low redox conditions for denitrifying bacteria. In practice, aerobic sulfide oxidizers could provide a mechanism for maintaining low oxygen supplies in wetland sediments conducive to denitrification by either autotrophic or heterotrophic organisms even when bulk solution oxygen concentrations are high.

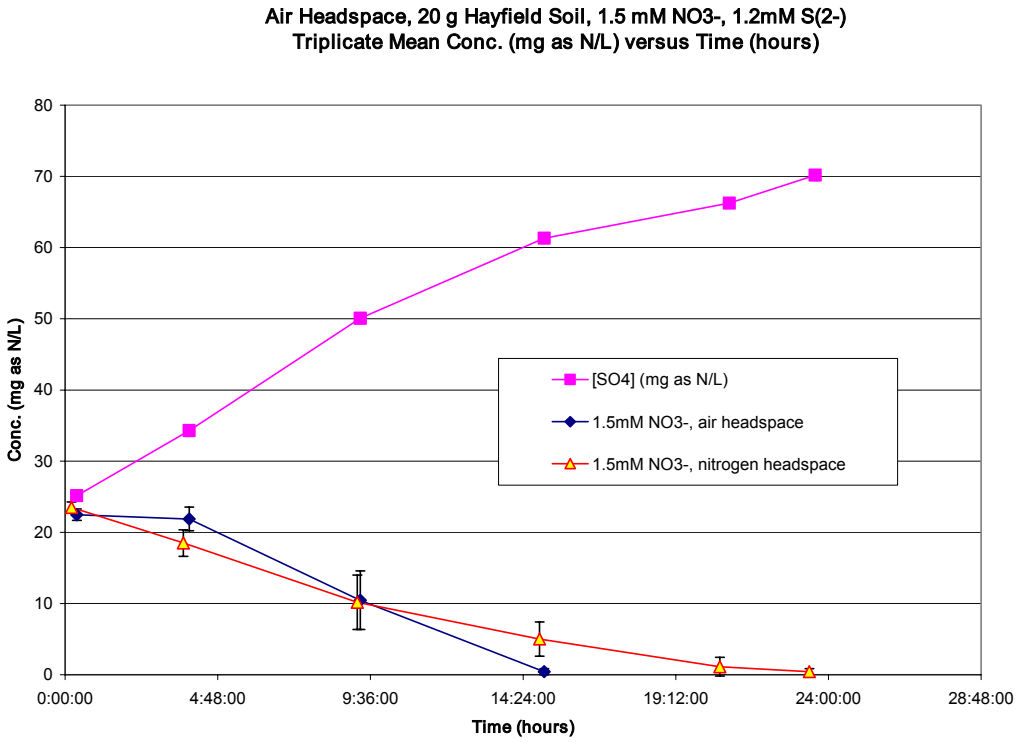

Figure 7. Triplicate mean NOx and sulfate concentrations versus time for Tres Rios wetland sediment amended with nitrate and headspace purged with air. Denitrification rates were comparable with nitrogen purged headspace. High increases in sulfate thought to be caused by aerobic sulfide oxidizing bacteria appeared to scavenge available oxygen in air headspace experiments.

Effects of Plants: Microbial Attachment and Carbon Addition

Batch experiments were done with Wetland of Avondale sediments at depths of 2-4 cm. Plant material was divided into three categories: 1) sterile (oven-dried bulrushes @ 105°C for 24 hours, chopped to <1 cm length brown segments) 2) chopped (fresh bulrushes chopped 1'-2' above the stalk base to <1 cm length white/green segments) 3) cut (fresh bulrushes cut 1'-2' above the stalk base into >3 cm white/green pieces). An additional variable was inadvertently added as 2 of the 3 cut plant material triplicates experiments were greenish leafy bulrush segments taken approximately 2' above the bulrush stalk base; conversely, the remaining serum bottle consisted of a white/green bulrush segment from approximately 1' above the stalk base.

The plant material additions enhanced denitrification kinetics by increasing dissolved organic carbon supplies and by augmenting populations of microorganisms attached to wetland macrophytes. Table 1 displays dissolved organic carbon concentrations in batch experiments amended with the different variations of bulrush. Microbial populations in the bulk wetland water had essentially no effect on denitrification when wetland water was used as a control (see Figure 1). Thus, the denitrification activity by microorganisms attached to plant surfaces could be isolated when plants were added. The oven-dried plants were assumed to be microbially sterile in order to test the effect of organic carbon addition from plants solely.

Table 1. Dissolved Organic Carbon Concentrations for Batch Tests with Bulrush Amendments

Batch Test Components	**[DOC]** *(mg/L)*
80mL wetland water	2 (+/- 1)
80mL wetland water, 20g wetland sediment	4 (+/- 1)
80mL wetland water, 2g chopped fresh plants	55 (+/- 20)
80mL wetland water, 2g cut fresh plants	30 (+/- 5)
80mL wetland water, 2g oven-dried plants (sterile)	325 (+/- 75)

Denitrification rates of wetland water with 2g chopped fresh plants yielded the highest rates of denitrification (linear trendline 25.0 kg N/acre*day) due to relatively high carbon additions and attached microorganisms. The chopped plants released more aqueous carbon than the coarsely cut plants which yielded denitrification rates of 14.9 kg N/acre*day. The location of the coarsely cut plant stalk used was varied inadvertently and affected denitrification rates significantly, as the stalk closest to the sediment yielded rates similar to finely chopped plants. The chopped sterile (or oven-dried) plants, yielded the lowest denitrification rate (12.8 kg N/acre*day) likely due to low populations of living microorganisms even though the organic carbon concentration was extremely high.

Experiments were done consisting of 80mL wetland water, 20g wetland sediment, and plant material similar the experiments with no sediment. The addition of cut and chopped fresh bulrushes and sediment increased denitrification rates (linear trendline 31.9 - 34.2 kg N/acre*day). The increase was not additive since the sediment alone had a rate of 20.6 kgN/acre-day. Figure 8 displays NOx concentration versus time for batch experiments with combinations of wetland water, sediment, bulrush, and sediment plus bulrush.

When plants were added alone, the reduction in nitrate concentration was highly non-linear consistent with the growth of heterotrophs (Figure 8). Since the growth of heterotrophs will be limited by the concentration of organic carbon, increasing organic carbon concentrations increased the rate of growth and consequently, the apparent denitrification rate. The observed non-linear reduction in nitrate concentration was not as significant when sediments and plants were mixed together indicating a less heterotrophic growth.

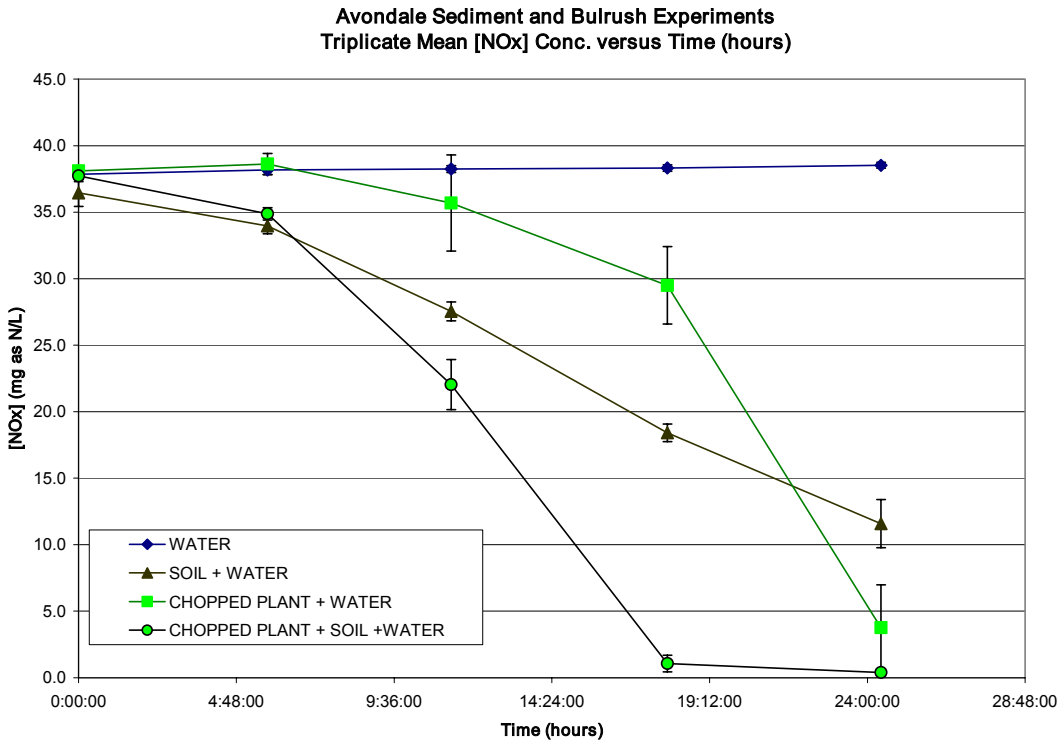

Figure 8. Triplicate mean NOx concentration versus time for batch tests with sediment and chopped bulrush. Carbon addition via bulrush resulted in nitrate reduction consistent with microbial growth (i.e. non-linear). Denitrification rates were not increased substantially in the first 10 hours by bulrush-attached microbial populations.

Effect of Acetylene and Sulfide on Nitrous Oxide Production -
Acetylene was added to block the transformation of nitrous oxide to nitrogen gas to determine if autotrophic denitrification would be impacted by acetylene. In addition, mixtures of glucose, nitrate, and sulfide with and without acetylene were tested. Acetylene had no impact on heterotrophic denitrification rates and linear increases in headspace CO_2 were observed as glucose was used as a carbon source for denitrification. The acetylene blocked nitrous oxide transformation as nitrate decreased linearly and nitrous oxide increased linearly.

Heterotrophic bacteria were first tested by using 1) denitrifying sludge and 2) wetland sediments where existing donors were exhausted prior to feeding bacteria with carbon. As stated previously, the effect of sulfide amendments with glucose resulted in a similar denitrification rate (65.8 kg N/acre*day) for heterotrophic bacteria in sludge experiments to those with glucose only. However, sulfide blocked nitrous oxide reduction when no acetylene was used for both types of experiments (see Figures 9 and 10). The possibility for nitrous oxide production in wetland sediments where heterotrophic denitrification may take place in the presence of sulfide exists. Nitrous oxide production is of concern due to its potential as a greenhouse gas.

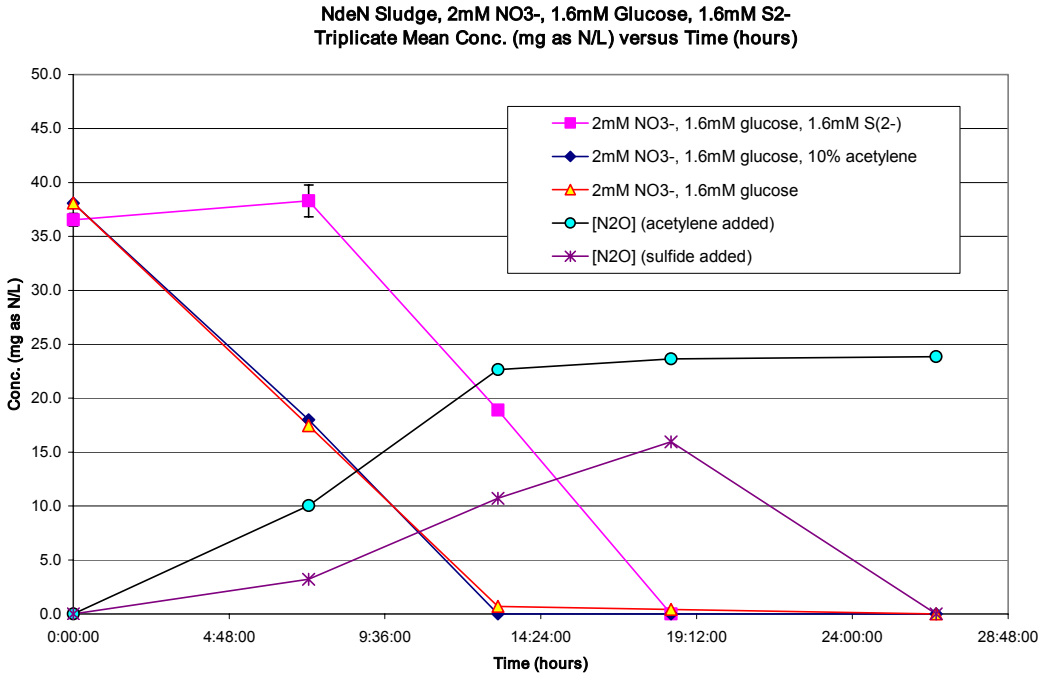

Figure 9. Triplicate mean liquid NOx and gaseous N2O concentration versus time for denitrifying sludge amended with combinations of glucose, sulfide, and acetylene. Acetylene did not effect rates of denitrification and produced nitrous oxide. The addition of sulfide also blocked nitrous oxide temporarily.

Autotrophic inhibition by acetylene was examined by amending sediments with nitrate in the presence of 10% acetylene gas. Figure 10 displays aqueous NOx concentration and gaseous nitrous oxide concentration for the acetylene-amended autotrophic experiment. Acetylene had no effect on autotrophic NOx reduction rates similar to heterotrophic denitrification and the acetylene "blocked" the transformation of nitrous oxide similar to heterotrophs. The enzyme system for autotrophic denitrifiers appeared to be similar to heterotrophs in that acetylene blocked the reductase enzyme, which converts nitrous oxide to nitrogen.

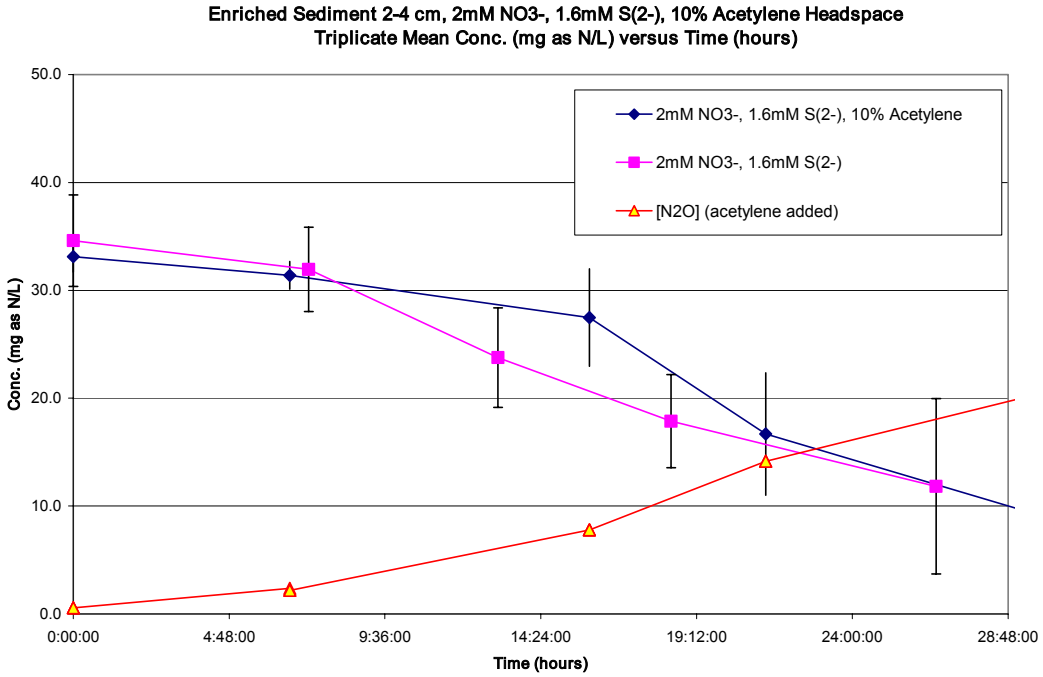

Figure 10. Triplicate mean liquid NOx and gaseous N2O concentration versus time for enriched autotrophic wetland sediment amended with combinations of sulfide and acetylene. Acetylene blocked nitrous oxide for autotrophs similar to heterotrophic experiments signifying a similar enzyme system. Rates of denitrification for the acetylene-amended autotrophic experiment was comparable to the non-acetylene-amended batch test.

Conclusions

- Abiotic reactions were negligible and sulfide appeared to be an important sink for molecular oxygen helping to maintain anoxic conditions in sediments.
- The majority of denitrification occurs in the top 4 cm of sediment and autotrophic denitrification plays an important role in wetland sediment denitrification.
- The addition of soluble organic carbon sources will result in the growth of heterophic denitrifiers resulting in apparent increases in denitrification rates.
- Plants have attached microorganisms; however, the addition of plants to sediments did not significantly increased denitrification rates.
- Sulfate reduction was demonstrated when no nitrate was present, showing evidence of sulfide production during periods of high organic carbon in sediments.
- Sulfide, a necessary electron donor for autotrophic denitrification, was shown to produce a lag-phase for heterotrophs and temporarily blocked nitrous oxide reduction.
- Acetylene did not influence denitrification rates and blocked the transformation of nitrous oxide for both autotrophic and heterotrophic denitrifiers.
- Denitrification rates and mechanisms were similar in a constructed wetlands receiving reclaimed water as compared to a constructed wetland receiving surface water contaminated with agricultural return flow.

ACKNOWLEDGEMENTS

Credits. Support for this research was provided by the Arizona Department of Water Resources and the City of Avondale, Arizona. The authors would like to thank Roland Wass for research analysis and his wetlands knowledge base.

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REFERENCES

Aminuddin, M., Nicholas, D.J. (1973) Sulphide oxidation linked to the reduction of nitrate and nitrite in Thiobacillus denitrificans. *Biochemica et Biophysica Acta.* **325**:81-93.

Aminuddin, M., Nicholas, D.J. (1974a) An AMP-independent sulphite oxidase from Thiobacillus denitrificans: purification and properties. *Journal of General Microbiology.* **82**:103-113.

Aminuddin, M., Nicholas, D.J. (1974b) Electron transfer during sulphide and sulphite oxidation in Thiobacillus denitrificans. *Journal of General Microbiology.* **82**:115-123.

Aulakh, M.S., Doran, J.W. (1991) Effectiveness of acetylene inhibition of nitrous oxide reduction for measuring denitrification in soils of varying wetness. *Communication in Soil Science and Plant Analysis.* **22** (1&2):11-21.

Burgoon, P.S. (1993) Oxidation of carbon and nitrogen in the root zone of emergent macrophytes grown in wetland microcosms. Ph.D. Dissertation, University of Florida, Gainesville.

Carter, P et al. (1995) Soil and sediment bacteria capable of aerobic nitrate respiration. *Applied and Environmental Microbiology.* **61** (8): 2852-2858.

Dahab, M.D. (1991) Nitrate treatment methods: an overview. NATO ASI Series, G30, Nitrate Contamination. Springer-Verlag, Berlin Heidelberg. 349-368.

Desimone, L.A., Howes, B.L. (1996) Denitrification and nitrogen transport in a coastal aquifer receiving wastewater discharge. *Environmental Science and Technology.* **30**:1152-1162.

Dorge, J. (1994) Modelling nitrogen transformations in freshwater wetlands. Estimating nitrogen retention and removal in natural wetlands in relation to their hydrology and nutrient loadings. *Ecological Modelling.* **75/76**: 409-420.

Flere, J.M., Zhang, T.C. (1998) Sulfur-based autotrophic denitrification pond systems for in-situ remediation of nitrate-contaminated surface water. *Water Science Technology.* **38** (1): 15-22.

Frederickson, J.K, Balkwill, D.L., Zachara, J.M., Li, S.-M. W. Brockman, F. J., Simmons, M. A., (1991). Physiological diversity and distributions of heterotrophic bacteria in deep Cretaceous sediments of the Atlantic coastal plain. *Applied and Environmental Microbiology* **57**:402-411.

Fox, P. (2000) Evaluation of flow fields in wetlands using physical models. Agreement # 98 C810051 Desalination Research and Development Program, U.S. Department of the Interior Bureau of Reclamation Final Report.

Gale, P.M., Reddy, K.R., Graetz, D.A. (1993a) Nitrogen removal from reclaimed water applied to constructed and natural wetland microcosms. *Water Environment.* **65**: 162-168.

Gersberg, R.M., Elkins, B.V., Goldman, C.R. (1983) Nitrogen removal in artificial wetlands. *Water Research.* **17** (9): 1009-1014.

Gersberg, R.M., Elkins, B.V., Goldman, C.R. (1984) Use of artificial wetlands to remove nitrogen from wastewater. *Journal of the Water Pollution Control Federation.* **56**: 152-156.

Grady, C.P.L, Daigger, G., Lim, H.C. (1999) *Biological Wastewater Treatment*, 2nd Edition, Marcel Dekker, New York, New York.

Justin, P., Kelly, D.P. (1978a) Growth kinetics of Thiobacillus denitrificans in anaerobic and aerobic chemostat culture. *Journal of General Microbiology.* **107**: 123-130.

Justin, P., Kelly, D.P. (1978b) Metabolic changes in Thiobacillus denitrificans accompanying the transition from aerobic to anaerobic growth in continuous chemostat culture. *Journal of General Microbiology.* **107**: 131-137.

Kadlec, R.H., Knight, R.L. (1996) *Treatment Wetlands*. Boca Raton, FL: Lewis-CRC Press.

Koenig, A., Liu, L.H. (1996) Autotrophic denitrification of landfill leachate using elemental sulfur. *Water Science Technology*. **34** (5-6): 469-476.

Kozub, D.D., Liehr, S.K. (1999) Assessing denitrification rate limiting factors in a constructed wetland receiving landfill leachate. *Water Science Technology.* **40** (3): 75-82.

Martin, J., Reddy, K (1997) Interaction and spatial distribution of wetland nitrogen processes. *Ecological Modelling.* **105**: 1-21.

Nahar, S., Fox, P. (2000) Sulfur driven autotrophic denitrification in constructed wetlands. Water Environment Federation Technical Conference 2000, Anahiem, CA.

Reddy, K.R., Graetz, D.A. (1988) Carbon and nitrogen dynamics in wetland soils. The Ecology and Management of Wetlands. Croom Helm, London: D.D. Hook. 307-318.

Reddy, K.R., Patrick, W.H. (1984) Nitrogen transformations and loss in flooded soils and sediments. *CRC Critical Reviews in Environmental Control.* **13**: 273-309.

Trouve, C. et al. (1998) Denitrification by new strains of Thiobacillus denitrificans under non-standard physiochemical conditions. Effect of temperature, pH, and sulphur source. *Environmental Technology.* **19**: 601-610.

U.S. Environmental Protection Agency (1993) Nitrogen Control Manual. Office of Research and Development. EPA/625/R-93/010.

Wass, R. (1999) The occurrence of sulfur-driven autotrophic denitrification in demonstration scale treatment wetlands. Ph.D Dissertation. Arizona State University, Tempe, AZ.

Willems, H. et al. (1997) Nitrate removal in riparian wetland soils: effects of flow rate, temperature, nitrate concentration, and soil depth. *Water Resources.* **31** (4): 841-849.

Wooley, D., Jones, G.L., Happold, F.C. (1962) Some metabolic differences between Thiobacillus thioparus, T. denitrificans, and T. thiocyanoxidans. *Journal of General Microbiology*. **29**: 311-316.

Zhang, T.C., Lampe, D.G. (1999) Sulfur:limestone autotrophic denitrification processes for treatment of nitrate-contaminated water: batch experiments. *Water Resources*. **33** (3): 59