A Search for Chemolithotrophic Denitrification

A Thesis
Presented to
The Academic Faculty

by

Christopher B. Bartley

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in
The School of Earth and Atmospheric Sciences

Georgia Institute of Technology
February, 2004

A Search for Chemolithotrophic Denitrification

Approved by:

Dr. Ellery Ingall, Advisor

Dr. Judith A. Curry

Dr. Joseph Montoya

Date Approved April 12, 2004

ACKNOWLEDGEMENT

This work reflects the time, input, and effort of many individuals. Without their valuable contributions, this work could not have been completed. Dr. Philip Froelich, my former advisor, now at Florida State University, as well as Dr. Ellery Ingall, my current advisor and my thesis committee have offered invaluable insight and guidance throughout my graduate career. The extraordinary patience and understanding of these individuals have helped me to produce a work of which I am very proud. I would also like to thank my family. They now know more about the nitrogen cycle than they ever thought they would.

Many other individuals contributed to the completion of this project. Patrick Lesley and I spent hours on the river collecting data, and I would like to thank him for his friendship and unique wisdom. Patrick and I had more fun playing in the mud than graduate students should. I owe Judson Partin special thanks. His efforts on my behalf both in the field and in the lab helped me tremendously. Jud helped foster "Chris' Kids" over the months and got to know them almost as well as I did. Also, Dr. Stephany Rubin-Mason was invaluable in the completion of this work due largely to her friendship and her ability to keep me focused. I would also like to acknowledge Meg Grantham and Cynthia Vance-Harris for their time spent helping me in their lab, and Dr. Spyros Pavlostathis for his expertise in the initial design of this experiment.

Lastly, I'd like to thank "Chris' Kids." Without these nine lovable lumps of mud, I would have had nothing to do during my nights and weekends. May they rest in peace.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

DIN	Dissolved Inorganic Nitrogen
ΔG	Gibbs Free Energy
DON	Dissolved Organic Nitrogen
MWCO	Molecular Weight Cut-Off
MW	Molecular Weight
DDW	Doubly Deionized Water
EDTA	Ethylenediaminetetra-acetic acid
GFF	Glass Fiber Filter
LOI	Loss on Ignition
MIMS	Membrane Inlet Mass Spectrometer
EA	Elemental Analyzer
C_{org}	Organic Carbon

SUMMARY

A 165-day batch culture experiment was conducted with anoxic muds collected from the Chattahoochee River, Georgia. The purpose of this experiment was to confirm or deny the presence in the sediments of a process called chemolithotrophic denitrification. Though time series data reflect a simultaneous disappearance of nitrate and ammonium, which might indicate the presence of this process, rate, mass balance and stable isotope tracer experiments failed to completely constrain the fate of ammonium within the system. Despite this failure, this experiment likely rules out chemolithotrophic denitrification is occurring in these sediments.

CHAPTER 1

INTRODUCTION

1.1 Rationale and Objectives

1.1.1 Overview

Dissolved inorganic nitrogen (DIN) is often limiting to primary production in natural waters. When allochthonous processes cause an increase in DIN above natural background levels, eutrophication can result in deleterious environmental effects caused by excessive growth of primary producers. Biological DIN removal is very important in natural systems as a control of primary production, and is also important in engineered systems for effluent DIN removal. Heterotrophic denitrification is the principle pathway by which DIN is biologically removed: the reduction of oxidized DIN species to dinitrogen in anaerobic environments. However, recent research by Mulder et al. (1995), Thamdrup and Dalsgaard (2002), and their colleagues has identified a chemolithotrophic denitrification process that may be important in nature. Chemolithotrophic denitrification is a microbially mediated autotrophic process that couples ammonium and nitrate/nitrite removal and produces dinitrogen. This one-step denitrification pathway could have important implications for wastewater treatment processes, and may, if widespread in nature, be significant in the global nitrogen cycle.

My research attempted to demonstrate that chemolithotrophic denitrification occurs in freshwater lacustrine sediments. I established batch cultures of anoxic riverine muds, deprived them of O_2*, and manipulated their* HCO_3^-*,* NO_3^-*,* NO_2^-*, and* NH_4^+ *sources to favor anaerobic autotrophs at the expense of heterotrophs. My goal was to enrich naturally-occurring microbial chemolithotrophic denitrifiers from lacustrine sediments to* *demonstrate the existence of this pathway in nature. Mass balance investigations of nitrogen partitioning within the cultures were unable to demonstrate the presence of chemolithotrophic denitrifiers within these sediments.*

1.1.2 Background

Denitrification is classically defined as nitrate (or nitrite) reduction by heterotrophic bacteria to N_2 (or N_2O). This pathway (reaction 1) occurs under anoxic conditions, where bacteria oxidize organic matter with nitrate rather than oxygen (Froelich *et al.*, 1979).

$$(CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 94.4HNO_3 \rightarrow 106CO_2 + 55.2N_2 + H_3PO_4 + 177.2H_2O \quad (1)$$

$$\Delta G^{o'}_{(1)} = -567 \text{ kJ (mol N)}^{-1}$$

ΔG is the free energy produced by the reaction and is shown as energy released (kJ) per mole of nitrogen. The more negative ΔG is, the more energy is available for metabolic reactions. Therefore, as ΔG becomes more negative, the more energetically favorable the reaction becomes for microorganisms to utilize as fuel for metabolism.

After oxygen is consumed, nitrate (reaction 1) is one of the next most energetically favorable oxidants available in sediments. Reaction 1 is classically described as being *preceded* by nitrification, the aerobic, microbially-mediated oxidation of ammonium to [ultimately] nitrate (Stumm and Morgan, 1996):

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+ \qquad \Delta G_{(2)} = -290 \text{ kJ (mol } NH_4^+)^{-1} \quad (2)$$

$$2NO_2^- + O_2 \rightarrow 2NO_3^- \qquad \Delta G_{(3)} = -72 \text{ kJ (mol } NO_2^-)^{-1} \quad (3)$$

Until recently, this coupled process (nitrification followed by denitrification) was thought to be the only removal pathway for fixed nitrogen, both in natural settings and in engineered wastewater treatment facilities. However, an autotrophic process has been identified recently that microbially reduces nitrite with ammonium to produce N_2. The anaerobic pathway is thought to proceed thus (Kuenen and Jetten, 2001; Hulth *et al.*, 1999):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad \Delta G_{(4)} = -298 \text{ kJ (mol N)}^{-1} \quad (4)$$

Nitrite is reduced and ammonium is oxidized in a single step directly to N_2 gas. Nitrate may also be reduced by ammonium, as suggested by Thamdrup and Dalsgaard in ^{15}N tracer experiments (Richards, 1965; Bender *et al*, 1977; Hulth *et al*, 1999; Thamdrup *et al.*, 2002):

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+ \qquad \Delta G_{(5)} = -421 \text{ kJ (mol N)}^{-1} \quad (5)$$

Both reactions 4 & 5 denitrify ammonium in a single step, *i.e.* uncoupled from nitrification or carbon oxidation. Ammonium is denitrified *directly* to N_2. Reactions 4 & 5 have been called the "Anammox" process (Mulder *et al.*, 1995).

Additionally, chemolithotrophic denitrification may be catalyzed by Mn-species and/or Fe-species via the following reactions (Luther *et al.*, 1997; Hulth *et al.*, 1999):

$$2NO_3^- + 5Mn^{+2} + 4H_2O \rightarrow N_2 + 5MnO_2 + 8H^+ \quad (6)$$

$\Delta G_{(6)}$= -98 kJ (mol N)$^{-1}$

$$2NH_4^+ + 3MnO_2 + 4H^+ \rightarrow N_2 + 3Mn^{+2} + 6H_2O \quad (7)$$

$\Delta G_{(7)}$= -448 kJ (mol N)$^{-1}$

$$2NO_3^- + 10Fe^{2+} + 24H_2O \rightarrow N_2 + 10Fe(OH)_3 + 18H^+ \quad (8)$$

$\Delta G_{(8)}$= -638 kJ (mol N)$^{-1}$

$$8NO_3^- + 5FeS + 8H^+ \rightarrow 4N_2 + 5SO_4^{2-} + 5Fe^{2+} + 4H_2O \quad (9)$$

$\Delta G_{(9)}$= -3352 kJ (mol N)$^{-1}$

If microbes perform these reactions in nature there could be important implications for the global nitrogen cycle (Bender *et al.*, 1977) and for re-engineering inorganic nitrogen removal from wastewater. Demonstrating that these reactions occur in natural sediments would be very significant because of the potential to remove fixed nitrogen species without the oxidation of labile organic carbon.

Nutrient levels in the Chattahoochee River, downstream of the city of Atlanta and its wastewater treatment plants, are elevated in $\delta^{15}NO_3^-$ and nitrite, nitrate, ammonia, and reactive phosphate concentrations. In fact, nitrate concentrations in the river are always elevated (>100 μM), and at times exceed 200 μM. However, nutrient levels fall, and $\delta^{15}N$ of NO_3^- increases, as the river flows farther south, indicating nutrient loss - almost certainly including benthic denitrification.

The anoxic sediments of the upper riverine portion of West Point Reservoir, an impoundment of the Chattahoochee River, provide the type of environment that would be suitable for organisms performing reactions 1 – 9: anoxic, organic carbon-rich, nutrient-rich muds containing abundant iron and manganese-rich clays. Sediments from this area were incubated in anoxic batch cultures and manipulated for nitrate, nitrite, ammonium, and bicarbonate to search for the activity of these microbially mediated reactions.

1.2 Overview of Results

Batch culture experiments lasting 165 days were designed to test for the presence of chemolithotrophic denitrification to evaluate its potential importance in sediment biogeochemistry. This microbial pathway might be revealed through simultaneous consumption of nitrate or nitrite and ammonium under anoxic conditions.

Denitrification is observed in the anoxic sediments of the Chattahoochee River at West Point Reservoir in the top nine cm of sediment. The average nitrate loss rate in these anoxic muds is –188 μmoles m^{-2} h^{-1}. $^{15}NO_3^-$ tracer amendments were transferred to labeled headspace $^{15}N_2$ and to sediment $^{15}N_{org}$, indicating simultaneous $^{15}NO_3^-$ denitrification and reductive assimilation.

Mass balance indicated ammonium loss. The average ammonium rate in the top six cm of sediment is –67 μmoles m^{-2} h^{-1}. $^{15}NH_4^+$ tracer amendments did not appear in headspace N_2 or sediment-N, ruling out occurrence of Reaction 5 and also probably Reaction 4, as well as ammonium assimilation as N_{org} in the sediment. The only nitrogen pool not measured in these experiments is dissolved organic nitrogen (DON). Inconsistencies in ammonium mass balance and $^{15}NH_4^+$ tracer experiments may be solved through future work including measurement of DON.

These experiments failed to constrain the fate of ammonium loss through either mass balance or isotope tracer experiments. Nitrate and/or nitrite loss observed in these experiments is most likely a heterotrophic process, but loss of ammonia to DON, or another unmeasured nitrogen pool could not be ruled out.

CHAPTER 2

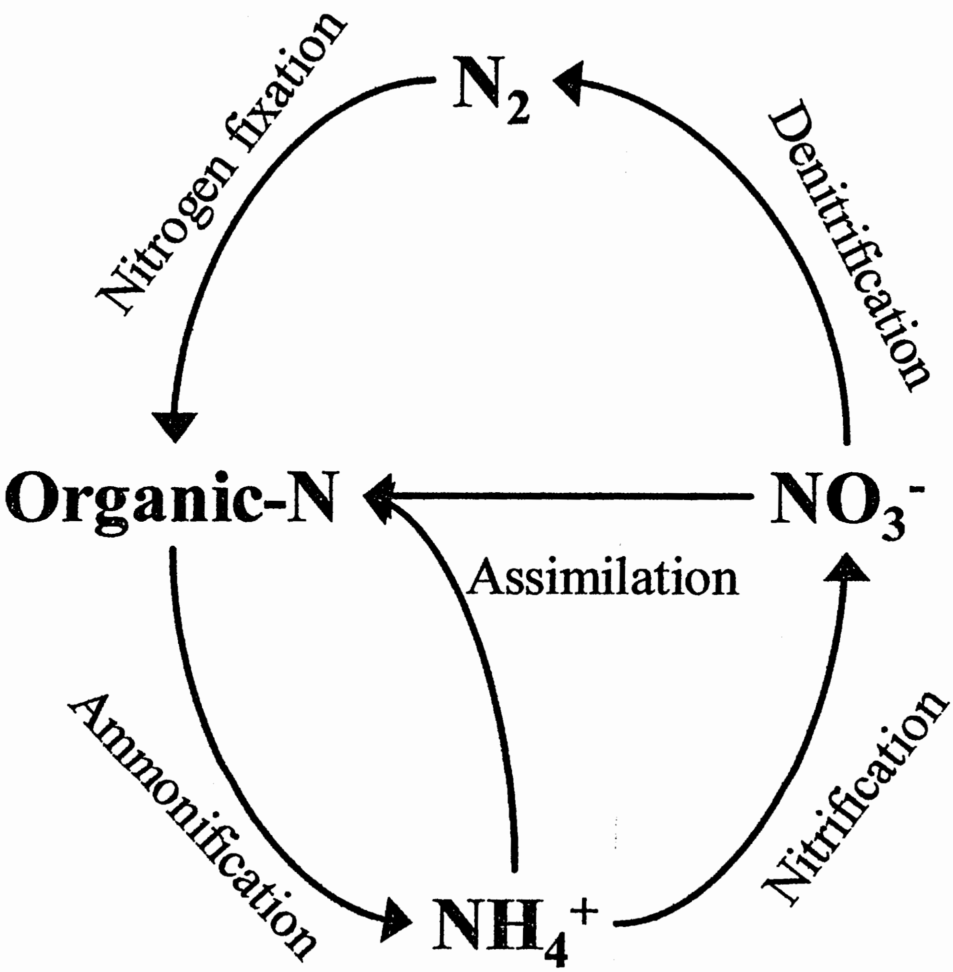
THE NITROGEN CYCLE

2.1 Summary- The Nitrogen Cycle

Nitrogen comprises 78.08 % of Earth's atmosphere. N_2 gas (dinitrogen) in the zero oxidation state is relatively inert. However, nitrogen gas can be "fixed" by microorganisms and made bioavailiable. Nitrogen fixation is the first key step in the nitrogen cycle (see Figure 2-1).

Nitrogen, in its fixed form, is the key component in amino acids, the building blocks of proteins- as well as DNA and RNA. In general, organic fixed nitrogen in solution exists primarily as urea, a waste product. Dissolved inorganic fixed nitrogen can be found in the –III oxidation state in anoxic environments as ammonium (NH_4^+), or in the +III or +V oxidation states as nitrite (NO_2^-) or nitrate (NO_3^-), respectively. Ammonium is generated by a process called ammonification, which occurs when organic matter containing nitrogen is broken down. In the presence of oxygen, ammonium is used as an electron donor by organisms called nitrifiers and oxidized to nitrite and, ultimately, to nitrate. Nitrate is the stable form of oxidized inorganic nitrogen. However, both nitrate and nitrite can be used by some organisms, called denitrifiers, as terminal electron acceptors.

Figure 2-1: The Classic View of the Nitrogen Cycle: Arrows represent the reactions and the transfer of nitrogen from one pool to another. Nitrogen fixation and Nitrification occur in oxic environments. Ammonification and Denitrification are anaerobic processes. Assimilation is the uptake of inorganic fixed nitrogen by biota and incorporation as organic-N.



2.2 Nitrogen Fixation

Nitrogen fixation is the transformation of biologically unavailable N_2 to organic-N. Although nitrogen fixation has an important terrestrial component linked to legume-type plants, here I focus on aquatic and marine nitrogen fixation.

2.2.1 Linking Atmospheric and Organic Nitrogen in Aquatic Environments

Nitrogen fixation is the biologically mediated transformation of N_2 to organic nitrogen by way of the nitrogenase enzyme (Stumm and Morgan, 1996).

$$N_2 + 3H_2(g) + 2H^+ \rightarrow 2NH_4^+ \qquad (10)$$

$\Delta G_{(10)} = -78.8$ kJ (mol N)$^{-1}$

$$3[CH_2O] + 2N_2 + 3H_2O + 4H^+ \rightarrow 3CO_2 + 4NH_4^+ \qquad (11)$$

$\Delta G_{(11)} = -241.2$ kJ (mol N)$^{-1}$

The nitrogenase enzyme is present in a few photosynthetic bacteria such as *Azotobacter* and *Clostridium spp.*, but it is found in aquatic and marine environments primarily in the blue-green algae, also called cyanobacteria. These photosynthetic organisms take up nitrogen gas into specialized cells called heterocysts that exclude oxygen with thick cell walls. Oxygen inhibits the nitrogenase enzyme. Additionally, photosystem II, the photosynthetic step that produces oxygen, is not found in these cells (Horne and Goldman, 1994). As a result, aquatic and marine nitrogen fixation can occur in the upper oxygenated photic zone of the water column. However, nitrogen fixation is not a light limited process, and can proceed without sunlight via organic carbon oxidation as an energy source.

The oceanic cyanobacterium *Trichodesmium* appears to be one important exception to this rule. Oceanic *Trichodesmium* populations can aerobically fix N_2 without heterocysts, and can simultaneously evolve O_2 (Capone *et al.*, 1997). *Trichodesmium,* however, does not fix nitrogen at night, suggesting that this organism does not perform reaction 11. These organisms are probably the most important nitrogen fixers in the ocean, but their global role is debated (Karl *et al,* 1997; Zehr & Ward, 2002).

2.2.2 The Ecological Role and Global Significance of Nitrogen Fixation

The ecological role of nitrogen fixation is very important. In many natural systems, the supply of nitrogen is limited by sources outside of the system. However, in oligotrophic systems, allochthonous nitrogen may be nearly non-existent. Nitrogen fixation provides a source of new, or allochthonous nitrogen to a system. When nitrogen is assimilated as organic-N at the primary production level, new bioavailable nitrogen is supplied to the food web by nitrogen fixation, and nitrogen limitation is avoided.

It is this ecological interaction that makes nitrogen fixation a globally important process. Recent ideas have caused many to believe that marine nitrogen fixation may be much more important on long timescales than once thought (Capone, 2001). Because nitrogen fixation may be limited in part by upper ocean iron supply, changes in iron export to the ocean or changes in phosphorus or silica dynamics can alter the global ocean biological CO_2 pump (Codispoti, 1997; Falkowski, 1997; Falkowski *et al.*, 1998; Boyd *et al.*, 2000; Chisholm, 2002). If the ratio of "the supply side" of the nitrogen cycle (nitrogen fixation) is altered with respect to "the demand side" of the nitrogen cycle (denitrification), then global carbon sequestration and glacial/interglacial climate may be affected (Altabet *et al.*, 2002; Codispoti, 2002; Devol, 2002).

2.3 Nitrification

Nitrification is the microbially mediated oxidation of ammonium to nitrate or nitrite.

2.3.1 Ammonium to Nitrate Conversion

Ammonium (NH_4^+) in aquatic environments arises from the decay of organic matter that contains nitrogen. Often, but not always, this decay and release of ammonium, called ammonification, occurs in anoxic sediments. Chemolithotrophic organisms called nitrifiers oxidize the ammonium that reaches oxic zones. Nitrate (and nitrite) is produced. In fact, two groups of organisms carry out sequential nitrification reactions (collectively reactions 2 & 3).

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+ \qquad (2)$$

$\Delta G_{(2)} = -290.4$ kJ (mol N)$^{-1}$

$$2NO_2^- + O_2 \rightarrow 2NO_3^- \qquad (3)$$

$\Delta G_{(3)} = -72.1$ kJ (mol N)$^{-1}$

Nitrosomonas and Nitrosospira spp. perform reaction 2, the conversion of ammonium to nitrite. *Nitrobacter and Nitrospira spp.* complete the oxidation by converting nitrite into nitrate (reaction 3). Nitrification is inhibited by sulfide (HS^-) generated in anaerobic sediments. This inhibition may be of ecological importance, especially in sulfide-rich coastal marine waters (Joye and Hollibaugh, 1995).

2.3.2 The Ecological role and Global Importance of Nitrification

Nitrification is very important for nutrient availability. Ammonium is not as mobile in the aqueous phase as nitrate, instead tending to adsorb onto organic matter or clay particles, especially at medium and high porosities in sediments of terrigenous origin (Rosenfeld, 1979; Mackin and Aller, 1984). As a result, allochthonous nitrogen supplied to receiving waters may be limited. However, nitrifiers can mobilize nitrogen and make it more available to plants and aquatic ecosystems.

Biological nitrification is also very important in the engineered treatment of wastewater. Aeration of ammonium rich effluents aids in the conversion of ammonium to nitrate by nitrifiers. This nitrate can then be supplied to a denitrifier population for nitrogen removal to N_2.

2.4 Denitrification

Denitrification is the removal of fixed nitrogen from a system as nitrogen gas.

2.4.1 Heterotrophic Denitrification

Heterotrophic denitrification is the microbially mediated conversion of nitrate or nitrite to nitrogen gas using organic carbon as the electron donor.

2.4.1.1 The Classic Paradigm

Denitrification is classically defined as the microbial reduction of nitrate (or nitrite) to N_2 gas, although N_2O may also be produced in small (<10 %) amounts

(Seitzinger *et al*, 1984). A better definition of denitrification is simply the production of N_2 from fixed nitrogen. "Classic" denitrification (NO_3^- or NO_2^- reduction to N_2) is a heterotrophic process that proceeds under anoxic conditions, where organic matter is oxidized using nitrate as the electron acceptor (Reaction 1). Many groups of common bacteria, such as many varieties of *Psuedomonas*, are capable of denitrification via the nitrate reductase enzyme, which may be why many define denitrification as solely nitrate or nitrite reduction to dinitrogen.

2.4.1.2 Ecological and Global Impacts of Denitrification

Denitrification "closes the loop" of the nitrogen cycle. Nitrogen fixation supplies "new" fixed nitrogen, and denitrification removes it. Denitrification is pervasive in environments with high organic matter content. After microbial degradation consumes all oxygen, nitrate is the next most energetically favorable oxidant (Froelich *et al.*, 1979). Much of the denitrification that occurs in aquatic systems occurs in anoxic sediments, especially those with high organic matter content (Saunders & Kalff, 2001).

Coastal margin studies have established that denitrification in estuarine and continental margin environments is responsible for a significant amount of global denitrification (Seitzinger *et al.*, 1984; Devol, 1991; Seitzinger *et al.*, 1993; Ogilvie *et al.*, 1997). As discussed earlier (sec. 2.2.2), it is this "demand side" of the nitrogen cycle, coupled with nitrogen fixation that may in part help drive global climate change on glacial/interglacial timescales.

2.4.2 Coupled Nitrification-Denitrification

Nitrification consumes ammonium released from the microbial degradation of organic matter. The nitrate that is produced can diffuse back into anoxic zones, most commonly into sediments, and be consumed by denitrifiers (Jenkins and Kemp, 1984). This process is globally important because it denitrifies nitrogen produced *within* a system. Over 10% of nitrogen incorporated during primary production over the continental shelf may be denitrified in the sediments through this direct couple (Seitzinger and Giblin, 1996). This process may also explain discrepancies in predicted and observed denitrification rates (Seitzinger *et al.*, 1993). If ammonium produced within sediment is oxidized to nitrate and immediately reduced to dinitrogen gas, then more gas production will be observed than can be accounted for through overlying water nitrate consumption alone (Brandes and Devol, 1997; An and Joye, 2001). There are however, other novel microbial processes that remove fixed nitrogen as N_2 and also may yield more N_2 production than nitrate consumption.

Additionally, anaerobic nitrification may be important in manganese rich environments (Luther *et al.*, 1997; Luther *et al.*, 1998; Hulth *et al.*, 1999). Manganese dioxide can react with ammonium to generate nitrate (anaerobic ammonia oxidation via reaction 12 (Hulth *et al.*, 1999):

$$NH_4^+ + 4MnO_2 + 6H^+ \rightarrow NO_3^- + 4Mn^{+2} + 5H_2O \qquad (12)$$

$\Delta G_{(12)} = -98$ kJ (mol $NO_3^-)^{-1}$

2.4.3 Suboxic Denitrification with Metals

The role of iron and manganese has been shown to be important in early diagenetic processes, especially the oxidation of organic matter by sedimentary bacteria

(Froelich *et al.*, 1979). Recently, iron and manganese have been shown to be important in nitrogen cycling as well (Luther *et al.*, 1997; Luther *et al.*, 1998). There are several reactions in which iron and manganese can oxidize ammonium to nitrate, and reduce nitrate to dinitrogen (Hulth *et al.*, 1999).

$$2NO_3^- + 5Mn^{+2} + 4H_2O \rightarrow 5MnO_2 + N_2 + 8H^+ \qquad (6)$$
$\Delta G_{(6)}$= -98 kJ (mol N)$^{-1}$

$$2NH_4^+ + 3MnO_2 + 4H^+ \rightarrow 3Mn^{+2} + N_2 + 6H_2O \qquad (7)$$
$\Delta G_{(7)}$= -448 kJ (mol N)$^{-1}$

$$2NO_3^- + 10Fe^{2+} + 24H_2O \rightarrow N_2 + 10Fe(OH)_3 + 18H^+ \qquad (8)$$
$\Delta G_{(8)}$= -638 kJ (mol N)$^{-1}$

$$8NO_3^- + 5FeS + 8H^+ \rightarrow 4N_2 + 5SO_4^{2-} + 5Fe^{2+} + 4H_2O \qquad (9)$$
$\Delta G_{(9)}$= -3352 kJ (mol N)$^{-1}$

These manganese and manganese oxide reactions are likely microbially mediated under neutral pH's, but they can proceed abiotically at low pH's (Luther and Popp, 2002). The spatial extent and global importance of these reactions is still under debate (Luther *et al.*, 1998; Dolfing, 1998; Thamdrup and Dalsgaard, 2000). However, manganese-nitrogen interactions seem to be enhanced in bioturbated sediments, where manganese oxides are mixed into deeper redox zones (Aller, 1990; Aller, 1994; Hulth *et al.*, 1999; Anschultz *et al.*, 2000). If these reactions are widespread, then re-evaluation of global balances in the nitrogen cycle may be needed.

2.4.4 Chemolithotrophic Denitrification

There are alternative pathways by which fixed inorganic nitrogen may be converted to dinitrogen without organic carbon oxidation. The oxidation of ammonium by nitrate is thermodynamically feasible (Richards, 1965):

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+ \qquad (5)$$

$\Delta G_{(5)} = -421$ kJ (mol NO_3^-)$^{-1}$

Because this reaction produces $^8/_3$ more N_2-N per mole of nitrate consumed than heterotrophic denitrification, it can explain observed excesses in N_2 production compared to nitrate disappearance in the same system, and has been invoked to explain N imbalances in anoxic sediments (Bender *et al.*, 1977). No direct observations of this reaction occurred until the 1990's. Simultaneous ammonium and nitrate disappearance was observed in a reactor in a European wastewater treatment facility (Mulder *et al.*, 1995). Termed "Anammox" (for ANaerobic AMMonium OXidation), the process was found to consume ammonium and nitrate biologically in the absence of oxygen, which reversibly inhibits the process (van de Graaf *et al.*, 1995; Strous *et al.*, 1997). It was later determined that nitrite, not nitrate, is the ammonium oxidant according to the reaction (Kuenen and Jetten, 2001; Sliekers *et al.*, 2002):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad (4)$$

$\Delta G_{(4)} = -298$ kJ (mol N)$^{-1}$

This reaction yields about 53 % of the free energy per mole of N as reaction 1.

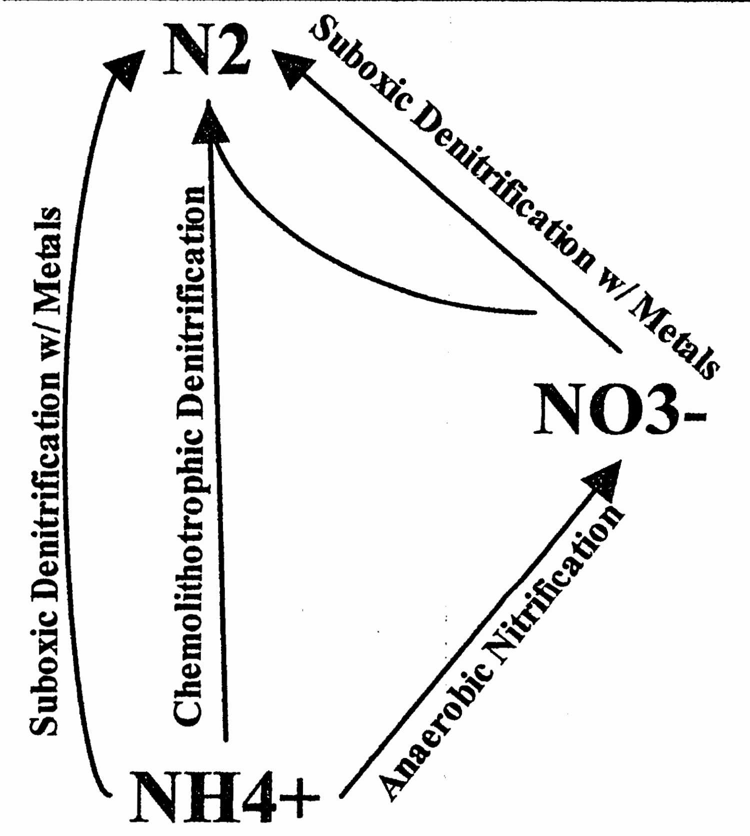
The *Anammox* organism is a member of the order *Planctomycetes.* Identified as the chemolithotroph *Candidatus Brocadia anammoxidans*, this organism shares the 16S rRNA gene with some *Nitrosomonas* species (van de Pas-Schoonen *et al.*, 2000; Casiotti and Ward, 2001; Kuenen and Jetten, 2001). This relationship suggests a common ancestor and the possibility that similar anaerobic ammonium oxidizers might occur in nature (Schmid *et al.*, 2000).

These organisms prefer a pH range of 6.7 – 8.3, and their optimal temperature range is 20 – 40° C (Strous *et al.*, 1999). These unique bacteria grow rather slowly, with a doubling time of 11 days. Hydroxylamine and hydrazine are metabolic intermediates (van de Graaf *et al.*, 1997; Schalk *et al.*, 1998; Jetten *et al*, 1999; Schalk *et al.*, 2000). Specialized organelle-like structures comprised of unusual lipids, dubbed ladderines, seem to allow these organisms to utilize these typically toxic substances in their metabolic pathways (DeLong, 2002).

These organisms often occur in anoxic microzones of biofilms, with autotrophic, aerobic ammonium oxidizers on the outside supplying nitrite (Seigrist *et al.*, 1998; Koch *et al.*, 2000; Egli *et al.*, 2001). Special reactor configurations supporting such biofilms allow for nitrogen removal from wastewater in a single step without oxygen or organic carbon addition (i.e., without coupled nitrification denitrification) (Jetten *et al.*, 1997; Strous *et al.*, 1997; Strous *et al.*, 1998; Verstraete and Philips, 1998).

Recently, targeted gene probes and stable isotope tracer experiments have detected evidence for chemolithotrophic ammonium oxidizing denitrifiers in marine anoxic sediments and water column environments (Thamdrup and Dalsgaard, 2001; Dalsgaard *et al.*, 2003; Devol, 2003; Kuypers *et al.*, 2003). While ammonium oxidation by nitrate is as yet unfound, if similar processes such as *Anammox* are widespread in nature, then previous global nitrogen budgets will need re-evaluation (see Figure 2-2). Where denitrification estimates are based solely on nitrate and nitrite disappearance, up to half of the total fixed nitrogen loss may be unaccounted for if direct ammonium conversion to N_2 gas is not included.

Figure 2-2: Alternative pathways to dinitrogen. All reaction pathways are anaerobic and do not require organic carbon as an electron donor (heterotrophy). Anaerobic nitrification can proceed using manganese oxide as an electron donor. Suboxic Denitrification may use metal cations as electron donors. Chemolithotrophic denitrification is the direct coupling of nitrate (or nitrite) and ammonium to produce nitrogen gas.



2.4.5 Mn / MnO_2 Catalysis

Anaerobic nitrification and suboxic denitrification w/ metals can recycle manganese within a closed system. If reactions 6 and 12 are coupled, then the overall net reaction is identical to reaction 5:

$$\textbf{(Reaction 6) x 4} = 20\overset{0}{\cancel{Mn^{+2}}} + \overset{3}{\cancel{8}NO_3^-} + 16\overset{0}{\cancel{H_2O}} \rightarrow 20\overset{0}{\cancel{MnO_2}} + 4N_2 + \overset{2}{\cancel{32}H^+}$$

$$\textbf{(Reaction 12) x 5} = 20\overset{0}{\cancel{MnO_2}} + 5NH_4^+ + \overset{0}{\cancel{30H^+}} \rightarrow 20\overset{0}{\cancel{Mn^{+2}}} + 5\overset{0}{\cancel{NO_3^-}} + \overset{9}{\cancel{25}H_2O}$$

Sum = Reaction 5: $5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 2H^+ + 9H_2O$

Mn^{+2} and MnO_2 are completely recycled. These reactions can mimic anammox-type chemolithotrophic reactions *in situ* with manganese acting as a redox catalyst.

CHAPTER 3

METHODS

3.1 Sampling (Cores)

This section describes the location, materials, and methods used during the collection of sediments from West Point Reservoir (Chattahoochee River, Georgia) and the subsequent sectioning and placement of sediment inocula into culture flasks.

3.1.1 Core Sampling Location

The Chattahoochee River flows from northeast Georgia southwest to the Georgia-Alabama border. The river then bends south and flows into the Apalachicola River in Florida before emptying into the Gulf of Mexico.

The Chattahoochee River basin includes 36 counties in Georgia, and encompasses several large metropolitan areas, including Atlanta, Ga (Cocker, 1998).

Within Georgia, the river flows through two reservoirs. Lanier Reservoir is northeast and upstream of Atlanta. West Point Reservoir is downstream and southwest of Atlanta near the Georgia-Alabama border. West Point Reservoir has a drainage area of 3,440 square miles, and at full stage covers 25,900 acres (Atlantic Mapping, Inc., 2000).

3.1.2.1 Core Collection and Storage

A sediment core, "Core 22", was taken on *August 21, 2002* from the riverine section of north West Point Reservoir south of Franklin, Ga., near channel marker 121 (see Figure 3-1). At the time of sampling, the Southeastern US was near the end of a four-year drought, and the reservoir water level was very low.

The core was taken with a 50 cm long, 9.5 cm diameter Jahnke tube. The Jahnke tube is a clear acrylic tube with a Teflon piston at either end (Jahnke, 1988). These pistons can be slid from either or both ends (to pressurize the core barrel) for core extrusion and/or pore water sampling. Pore water sampling ports are drilled at 1 cm intervals down the side of the tube and sealed with Teflon screws that can be removed for pore water sampling.

To take the core, the pistons were removed from the Jahnke tube and the core tube was pushed into the sediment by hand. The sediment surface was disturbed minimally. Once the core tube was filled with sediment to a level just below the top sampling port, the top piston was inserted and sealed. The core was then slowly removed from the surrounding sediments and the bottom piston was inserted and sealed. The top port was opened when the bottom piston was replaced to allow pressure release. About 5 cm of water remained overlying the sediments in the core tube, and the core had no headspace gas when it was sealed. The outside of the core tube was cleaned and dried, and the plastic end caps were replaced and the core was sealed with tape. The core was placed upright at 4 °C for transport. Gas bubbles, presumably methane, developed within the black-colored sediments and were visible through the clear acrylic Jahnke tube. The core was stored in the sealed tube in the dark at 4°C until sectioning. During storage, the core developed a headspace gas (presumably methane) & overpressure, indicating that the deepest sediments were methanogenic.

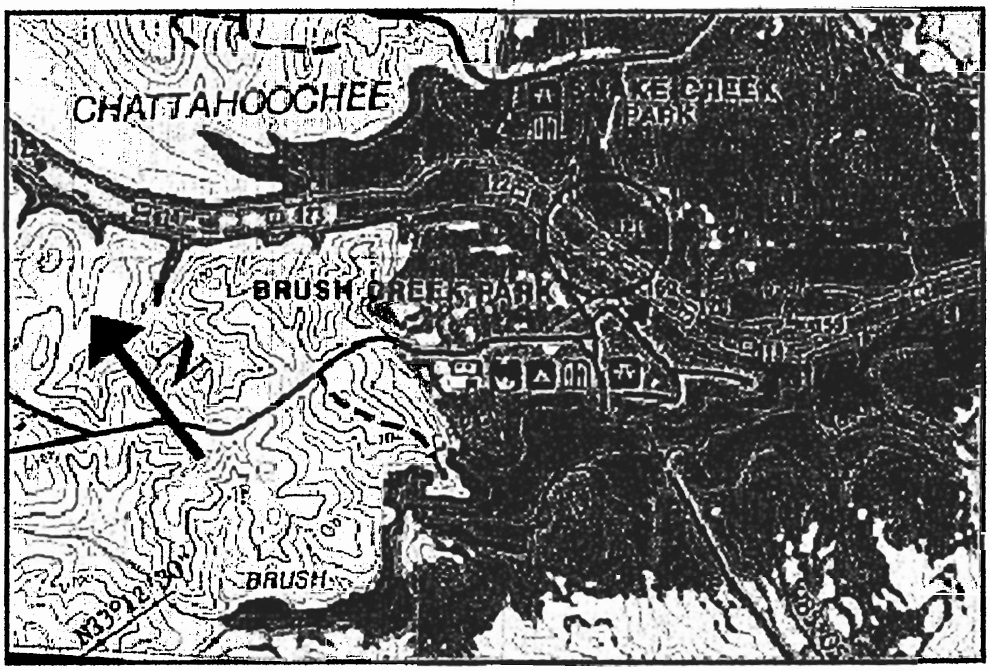


Figure 3-1: Sediment Core Sample Site: The riverine portion of West Point Reservoir shown is approximately 4 km downstream of Franklin, Georgia. The river flows from left to right. The Core 22 sample location is north of Brush Creek Park at channel marker 121 and is circled in red (approximate sample location: N33° 12′ 30″, W85° 05′ 00″). The core was sampled from the south side of the river. The contour interval is 10 feet. NOTE: At this time the reservoir was very low due to drought conditions. Water depth at Core 22 site was approximately 1 m (Atlantic Mapping, Inc., 2000)

3.1.2.2 Core Sectioning

In preparation for sectioning, the core was removed from refrigerated storage on January 17, 2003 and placed in the dark at room temperature for 24 hrs. Small sediment samples were taken downcore at intervals through the pore water sampling ports for carbon content, porosity, and bulk density analysis (see section 3.3.2.7.1)

The top piston of the Jahnke tube was removed, and the overlying water was discarded by sliding the bottom piston up, extruding the sediment in air. Three 3 cm-long sections were sliced from the top of the core by extruding the desired length out of the top of the tube and sectioning with a stainless steel spatula. The top section (A-Section) was taken from 0 – 3 cm downcore. The middle (B-Section) and bottom (C-Section) sections were taken from 3 – 6 cm and 6 – 9 cm downcore, respectively. All these procedures were done in air.

From each 3 cm section, three ~5 g sediment sample aliquots were taken. Wet sample mass was accurately measured, and the sediment was sealed inside a short (approximately 6 – 10 cm) length of 300,000 dalton Molecular Weight Cut-Off (MWCO) regenerated cellulose dialysis tubing (1 dalton = the molecular weight (MW) of hydrogen). Each segment of dialysis tubing had previously been rinsed three times and soaked for 30 minutes in distilled de-ionized water (DDW) to remove traces of the sodium azide storage solution (per the manufacturer's instructions). A single, weighted dialysis tubing closure was placed on the end of the tubing, and the tubing was then placed into a beaker of argon-sparged river water, that had been previously sparged for approximately 1 hr. with high-purity grade argon to minimize oxygen contamination. After the sediment inoculum was placed inside the dialysis tubing and interior gas bubbles were removed, the dialysis tubing was sealed with another weighted closure and placed inside a culture flask filled with argon sparged river water.

3.2 CULTURES

3.2.1 Design

Sealing sediment inocula inside a dialysis membrane serves three purposes. First, since the inoculum is sealed, the sediment is well contained and will not suspend into the culture media and create filtering problems, or be diluted and lost during media replacement. Secondly, the bacterial community is held *within* its natural host sediments, so the target bacterial population is encouraged to grow within the sediment or on the inside of the membrane (at the sediment water interface) and not on the sides of the flask. Third, a MWCO of 3×10^5 daltons allows free diffusion of smaller solutes (products, reactants, cations, anions, gases, dissolved organic matter, etc.) to and from the sediment while retaining clay particles and bacterial cells (see below).

In order to best approximate the largest MWCO that will retain bacterial cells, I reasoned that a 0.3 μm filter will retain clay particles and bacterial cells. If there are approximately 1000 daltons per nm, then a MWCO of 300,000 daltons is approximately 0.3 μm and should retain most clays and bacterial cells.

No attempt was made to sterilize the flask, dialysis tubing, or media. An unspecified microbial community (bacterial and/or fungal) was observed to grow within the flasks on the outside of the sealed inoculum. However, as long as this community did not significantly affect chemical concentrations of observed species within the control inocula, then the presence of these incidental microbes was not a concern. These effects are described later.

The inocula were placed into nine (three replicates from each Section) 125 mL rubber-stoppered Erlenmeyer flasks with ~100 mL unfiltered, de-gassed (discussed later) Chattahoochee river water, sampled on September 2, 2002 from the river upstream of Atlanta as it exits Buford Dam below Lanier Reservoir. This water was stored in the dark at room temperature. Each volume used as culture media was siphoned as needed. Water from the Chattahoochee River below Buford Dam was chosen as stock media for a variety of reasons. A "natural" water, as opposed to de-ionized water manufactured in the lab, contains many of the unspecified minerals, micronutrients, vitamins, and co-factors often required by aqueous microorganisms. Water from below the dam also has low nitrite, ammonia, and phosphate concentrations, and relatively low nitrate concentrations compared to the river below Atlanta (see Table 3-1).

Table 3-1: Stock Chattahoochee River water nutrient concentrations

Species	Concentration (μM)
Nitrate	38
Nitrite	0.01
Ammonium	0.3
Reactive Phosphate	0.1

An additional three flasks were maintained as "active" controls (with dialysis tubing and clamps, but no sediment inoculum). Active controls allow us to distinguish between reactions due to water column and sedimentary organisms. Since the stock river water is not sterile, any microbes present are presumably viable in all three control cultures and nine sediment cultures.

Each set of three replicate sediment cultures and a control are called a *Series* and are given the same label as the section of the core from which they were taken (A, B, or C) as well as a number (1, 2, or 3) to designate each replicate within a series. The control culture within each series is given the same label as that series and a "C" to designate it as a control (e.g., A-C, B-C, C-C).

Argon is slowly bubbled through all twelve flasks to strip atmospheric gases from the aqueous phase and fill the headspace. The outlet from the headspace of each culture is a diffusion loop terminating in an air lock. This loop ensures that the cultures remain anoxic and do not develop a pressure in the headspace different than ambient.

The 12 cultures (9 inocula + 3 controls) were incubated in the dark at room temperature (approximately 20 °C). Nutrient levels in the supernatant were monitored periodically by sampling through a third tube out of the top of the rubber stopper (see Figures 3-2 & 3-3). This sample tube penetrates through a small hole drilled through the stopper to the solution in the flask. In addition to providing a sampling point, this tube also allows fresh enriched river water to be pumped back into the flask to replace nutritionally depleted water and the volumes withdrawn for analyses. All analyses are performed on 10 mL sample volume. During the initial setup of each nutrient enrichment, a large volume of stock media (unfiltered river water spiked with known concentrations of inorganic nutrients) was bubbled with argon to remove oxygen (and N_2) and to replace nutritionally depleted water in the cultures. The composition of this enriched river water was modified to suit the nutritional requirements of the changing bacterial communities in the cultures.

The concentration of each analyte in the sample was multiplied by the volume in the culture flask to estimate a mass balance.

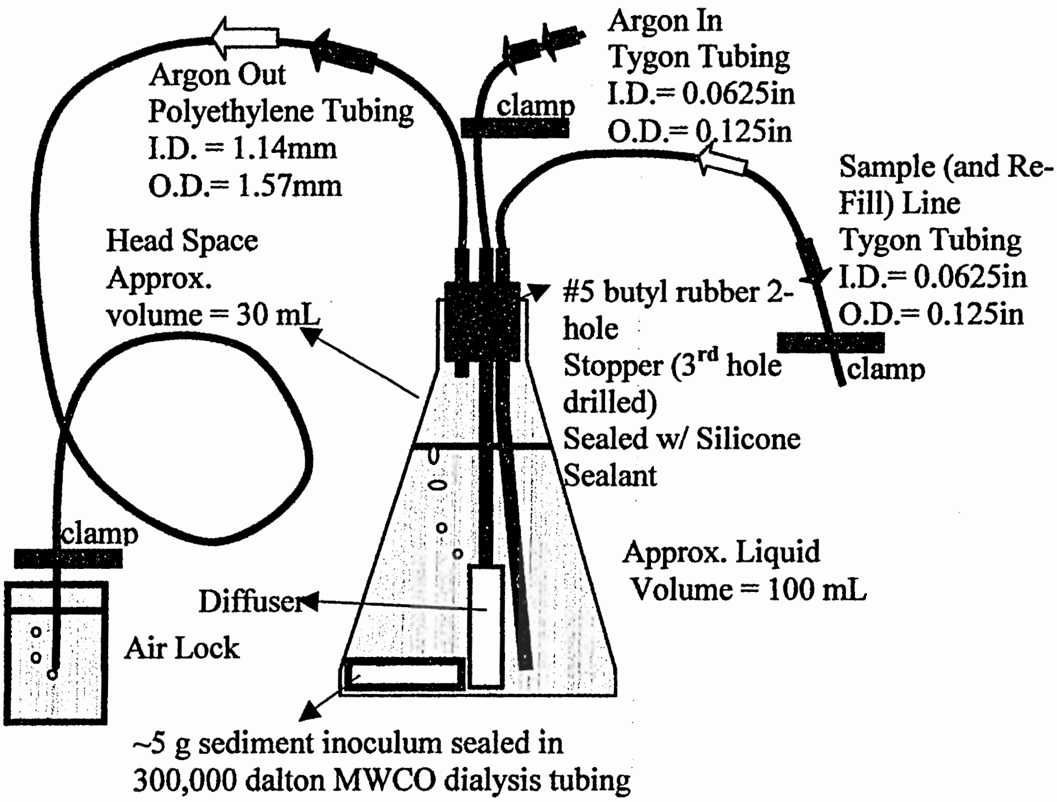


Figure 3-2: 125 mL Erlenmeyer Flasks for Batch Cultures. **(a)** FILL CYCLE: Green Arrows indicate the flow of enriched culture media into the flask through the Sample line and argon out; the clamp on the Argon In line is closed. The stock media flask (under normal [blue route] gas flow) sample line is plumbed to the sample line of each culture for re-filling. Culture media is forced into the flask through the culture flask sample line by argon pressure applied to the stock media flask. The flasks were *not* stirred **(b)** SPARGE CYCLE: Grey Arrows indicate sparge argon gas flow; the clamp on the Sample Line is closed. **(c)** CULTURE CYCLE: all lines are clamped (no argon flow). **(d)** SAMPLE CYCLE: Blue Arrows indicate the flow of gas into the culture flask and the flow of sample out; the clamp on the Argon Out line is closed. Sample volume is replaced by argon.

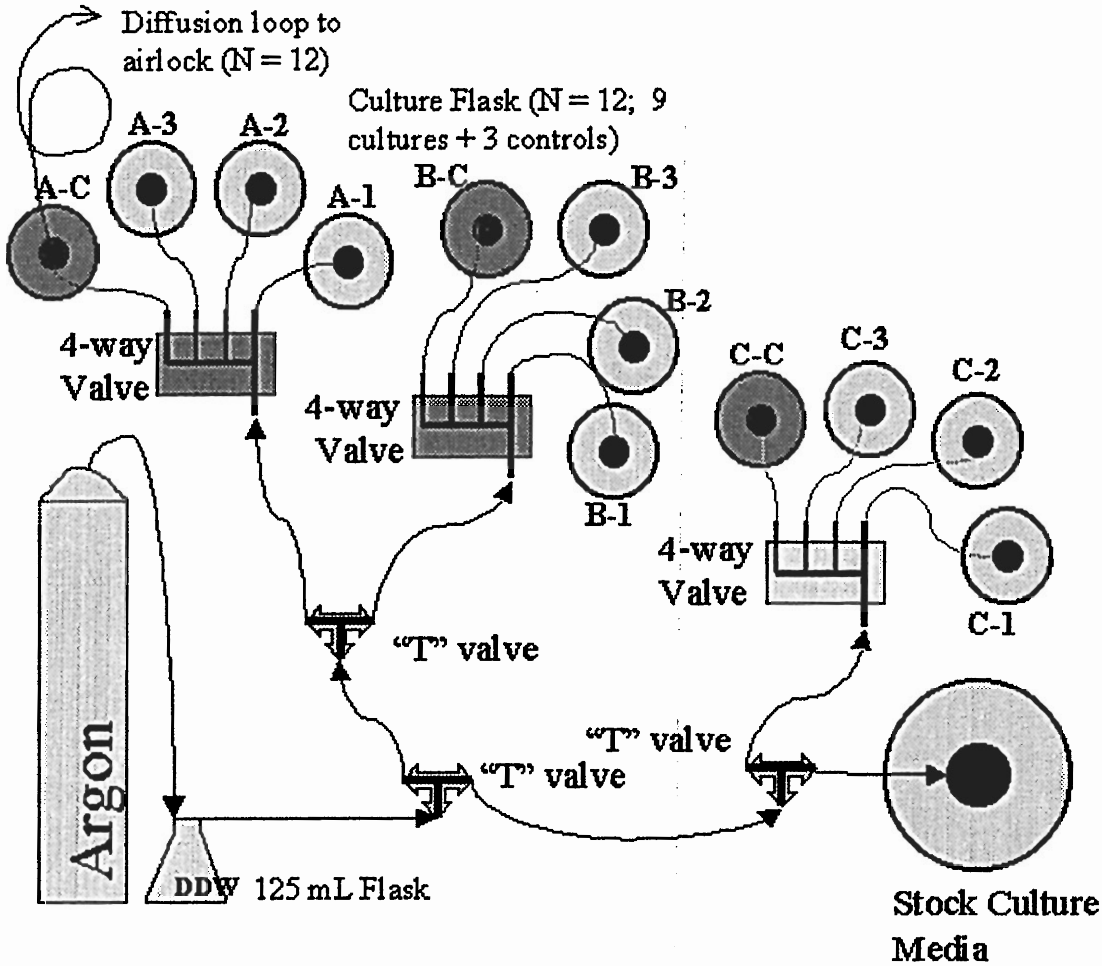


Figure 3-3: *Argon Gas Routing* (Plan view) One 125 mL "saturation" flask is filled with distilled, de-ionized water (DDW). All argon is routed first through this flask to insure that the gas is saturated with water to prevent water loss from the cultures. Sediment cultures are shown in gray; controls, in purple. The A-Series cultures (sediment from 0 – 3 cm downcore) and the A-Control (A-C) are plumbed to the red 4-way valve. The B-Series cultures (sediments from 3 – 6 cm downcore) and the B-Control (B-C) are plumbed to the blue 4-way valve. The C-Series cultures and C-Control (C-C) are plumbed to the green 4-way valve. Each culture has a letter designating the Series to which it belongs and a number to differentiate it from others in that Series. This set-up is incubated in the dark at 20 °C.

3.2.2 Implementation

The following sections describe both the original and modified experimental designs to test for the activity of chemolithotrophic denitrifiers in Chattahoochee River sediments.

3.2.2.1 Sediment Addition

The three replicate cultures containing sediment collected from 0 – 3 cm downcore are termed A-Series cultures. The three replicate cultures containing sediment collected from 3 – 6 cm downcore are termed B-Series cultures. The three replicate cultures containing sediment collected from 6 – 9 cm downcore are termed C-Series cultures. Each series has a fourth flask attached to its argon inlet 4-way valve that is the control for that series. The A-Control is termed A-C; the B-Control is called B-C; and the C-Control, C-C. See Figure 3-3 above for series and control gas routing and set-up.

Approximately 5 g of wet sediment was placed into each segment of dialysis tubing and placed within the appropriate culture flask. Five g of wet sediment allows for a maximal amount of sediment to be added while providing a mass of carbon that could be oxidized within the time scale of this research. Methods of sediment analyses are discussed in section 3.3.2.7.1.

Organic carbon oxidation time is important for the scaling of the experiment. Enough organic carbon must be provided within the sediment inoculum so that trends in denitrification can be observed. However, the addition of too much organic carbon can cause changes in observed chemical species too quickly, as well as prevent heterotrophic limitation (discussed later). In order to estimate organic carbon oxidation times, an average lacustrine denitrification rate, as well as high and low ranges, were tabulated from the literature (see Table 3-2). These rate estimates are used as a range of potential denitrification rates for the cultures. Sample frequency, media exchange, and carbon oxidation times can be estimated from these rates.

Table 3-2: Lacustrine Sedimentary Denitrification Rates estimates. *o = oligotrophic. m = mesotrophic. e = eutrophic. Rates are given in μmol N $m^{-2}h^{-1}$. Average Rate = 105 μmol N $m^{-2}h^{-1}$. Maximum Rate = 480.5 μmol N $m^{-2}h^{-1}$. Minimum Rate = 1 μmol N $m^{-2}h^{-1}$ (after Saunders & Kalff, 2001).

Lake Location	Trophic Status*	Average Denitrification Rate
Denmark	o	37.5
Switzerland	o	7
Switzerland	o	23
Canada	o	7
Canada	o	1
Canada	o	1
Canada	o	9
Canada	o	13
Canada	o	2
Canada	o	5
Canada	o	179
US	o	31.5
Denmark	o-m	27.5
Denmark	o-m	27
US	o-m	50
US	o-m	56
Austria	m	125
US	e	13.5
Netherlands	e	128
Switzerland	e	68
Switzerland	e	237
Switzerland	e	137
Switzerland	e	82
Switzerland	e	37
Denmark	---	267
Denmark	e	480.5
Denmark	e	326
Denmark	e	252
Denmark	e	179
Denmark	e	9
Denmark	e	371
Denmark	e	159.5

A range of possible denitrification rates for the cultures was calculated at the highest (6 cm downcore) and lowest (1 cm downcore) wet bulk density in Core 22 (see Tables 4-9, 4-20, & 4-31 for all values from Core 22) using the high, low, and average rates from Table 3-3. Assuming a 5 g sediment inoculum, a high and low volume was calculated for the sediment inoculum. These volumes are equivalent to the volumes of the dialysis tubing in which the sediments are contained. Taking these volumes as the volumes of cylinders (radii = 0.5 cm), then high and low lengths of dialysis tubing were calculated. From these lengths, high and low surface areas of the dialysis tubing were calculated. These two surface areas, when multiplied by the high, low and average rates from Table 3-2, provide a range of possible denitrification rates for the cultures. If the µmoles of organic carbon in a 5 g sediment inoculum (from 1 cm and 6 cm downcore in Core 22) are equivalent to the µmoles of nitrogen required to fully oxidize it, then the µmoles of nitrogen multiplied by the high, low, and average rates at each surface area yield a range of organic carbon oxidation times.

Based on the range of denitrification rates provided in Table 3-2, it is possible that in some of the warm, artificially eutrophic cultures, all of the labile organic carbon could be oxidized within 20 days if the denitrification rate is very fast, or several years if the rate is relatively slow (see Table 3-3). At the fastest rate, <20 µmoles of nitrate would be consumed in one day. At the slowest rate, it would take several days to detect a change in nitrate concentration. Therefore a sampling frequency (and/or media exchange) within the batch cultures every one to three days was chosen.

Table 3-3: Minimum, Maximum, & Average Nitrate Consumption Rate and Carbon Oxidation Time Estimates. Rate in μmoles $NO_3^- h^{-1}$. Time in days. Wet Bulk Density is 1.05 g cm^{-3} at 1 cm downcore and 1.83 g cm^{-3} at 6 cm downcore.

	1 cm downcore	6 cm downcore
Minimum Rate	0.0019	0.0011
Average Rate	0.115	0.116
Maximum Rate	0.912	0.528
Min. C_{ox} time	9144	3.6×10^5
Avg. C_{ox} time	151	3382
Max. C_{ox} time	19	743

3.2.2.2 Heterotrophic Limitation and Chemolithotrophic Enrichment

The first stage of the experiment, heterotrophic limitation, was designed to enrich chemolithotrophic denitrifiers by satisfying their nutritional requirements and while simultaneously limiting the growth of heterotrophic denitrifiers. Labile organic carbon was oxidized by repeatedly adding 200 μM nitrate to the cultures to encourage heterotrophic denitrifiers to oxidize organic carbon by reducing nitrate. Through this oxidation of labile organic carbon, the system was pushed toward organic carbon limitation and the loss of the ability to use nitrate as an electron acceptor. Any potentially poisonous gaseous waste products, such as H_2S, were removed from the system by argon sparging and media exchange. In addition, any protons produced or consumed (see Reactions 2, 5 - 11), which might cause undesirable changes in pH, were buffered by bicarbonate added during the second stage of the experiment, (chemolithotrophic enrichment: discussed below). Note that not all of the organic carbon needs to be oxidized, only the labile fraction. Regardless of the organic carbon content, as long as heterotrophic denitrifiers are successfully limited, the chemolithotrophic enrichment stage should allow the autotrophs to out-compete the heterotrophs.

Once the cultures reached the stationary phase of growth with respect to nitrate use, the supernatant in the culture was exchanged for the chemolithotrophic enrichment stage stock media containing > 1 mM bicarbonate (to prevent inorganic carbon limitation), 200 μM nitrate (or nitrite), and 100 μM ammonium. once the heterotrophs can no longer use nitrate or nitrite, any subsequent disappearance of nitrate or nitrite and ammonium could be attributed to chemolithotrophic denitrification.

The experiment was conducted as a sequential, stepwise enrichment process. Nutrients were added, and, after a period of time, the cultures were sampled. If analyses revealed that the nutrients within each flask had become depleted, a new sequence was started by adding fresh, nutrient amended water to the flasks. Nutrient levels in the flasks were usually depleted after 7 – 10 days. A total volume change was initiated when nutrient amended stock culture media was added to all cultures at the start of each sequence. Each media replacement step is the start of a new "enrichment sequence." During each enrichment sequence, concentrations were measured every one to three days. During some enrichment sequences, nutrient levels were not monitored. These enrichment sequences are called "stasis sequences." Later enrichment sequences were monitored only for initial and final conditions after sequence dynamics had been well constrained. During each "Culture Cycle" (see Figure 3-2) of both enrichment and stasis sequences, argon flow is off, and the argon inlet and sample lines into the cultures are clamped not only to allow ingrowth of dinitrogen in the headspace, but also to prevent possible atmospheric contamination due to leakage.

Based on measured denitrification rates (nitrate or nitrite loss) during the progress of research, it was determined that denitrification rates were too slow to allow for heterotrophic limitation within the time scale of this work. However, it was observed that once several enrichment sequences of 200 μM nitrate had been added to and used by the cultures, ammonium levels did not increase appreciably during the sequences (see results), as would be expected during anaerobic carbon respiration. This observation prompted a change in experimental design. The second stage of the experiment, chemolithotrophic enrichment, was initiated after day 25. 100 μM ammonium, and later 500 μM bicarbonate, were added to the 200 μM nitrate (or nitrite) media *without* first observing heterotrophic limitation conditions. Subsequent decreases in both nitrate/nitrite and ammonium were taken as evidence for possible activity of chemolithotrophic denitrification.

Over this 165-day incubation, the cultures within the A, B, & C series, as well as the controls, were all treated the same (see Figure 3-4, Table 3-4). In one enrichment sequence, nitrite alone was added to constrain the dynamics of nitrite within the cultures without excess nitrate addition. However, during one enrichment sequence the series were treated differently. The A-Series and control received the "normal" nitrate, ammonium and bicarbonate amendment, but ammonium amendments to the B-Series and control were doubled. The C-Series cultures and control were amended with ammonium and bicarbonate only (no nitrate) in order to further constrain ammonium dynamics. Both series were returned to the "normal" amendments of nutrients during the next enrichment sequence. A complete explanation of each enrichment sequence, including nutrient additions and notable observations of each culture, can be found in *Appendix II*.

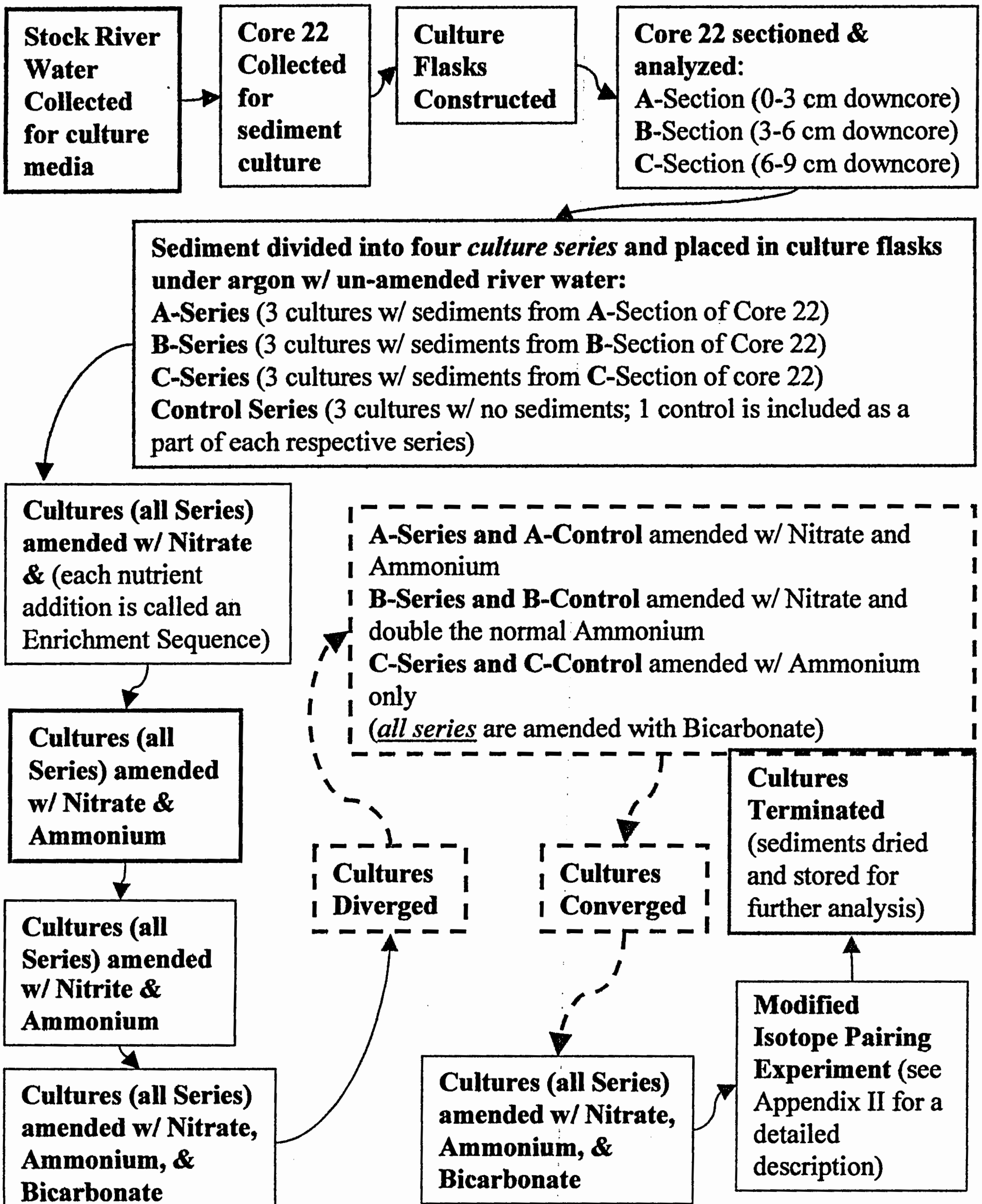


Figure 3-4: Experimental Protocol Flowchart. This chart shows the progression of this experiment. A detailed description of each Enrichment Sequence is available in Appendix II. Black Boxes correspond to all actions up to and including Enrichment Sequence I. Red (Heterotrophic Limitation) corresponds to Enrichment Sequences II – III. Orange (Chemolithotrophic Enrichment begins) corresponds to Enrichment Sequences IV - VI. Blue corresponds to Enrichment Sequence VII. Green corresponds to Enrichment Sequences VIII – XXII. Dashed Green lines = Enrichment Sequence XVII.

Table 3-4: Culture Nutrient Addition Summary. This table is a summary of the approximate nutrient additions to the cultures during each sequence. A detailed description of each sequence is located in Appendix II. Time series data, including initial masses, are available in Appendix III. Cultures shown in bold are stasis sequences. Initial masses for each stasis sequence are average masses of ~20 μmole nitrate and ~10mmole ammonium amended enrichment sequences. These average initial masses are: 22.87 μmoles NO_3^- & 7.93 μmoles NH_4^+ for the A-Series, 24.18 μmoles NO_3^- & 6.47 μmoles NH_4^+ for the A-Control, 23.87 μmoles NO_3^- & 7.88 μmoles NH_4^+ for the B-Series, 24.76 μmoles NO_3^- & 8.76 μmoles NH_4^+ for the B-Control, 23.67 μmoles NO_3^- & 8.77 μmoles NH_4^+ for the C-Series, 25.47 μmoles NO_3^- & 9.05 μmoles NH_4^+ for the C-Control. Asterisk denotes a single ~20 μmole nitrite addition. During Enrichment Sequence XVIII, the cultures were diverged, and masses added to the A, B, & C-Series (and controls) are shown separately.

Sequence	μmoles NO_3^- added	μmoles NH_4^+ added	μmoles HCO_3^- added
I	0	0	0
II	20	0	0
III	20	0	0
IV	20	10	0
V	20	10	0
VI	20	10	0
VII	0*	10	0
VIII	20	10	0
IX	20	10	0
X	20	10	0
XI	20	10	50
XII	20	10	50
XIII	20	10	50
XIV	20	10	50
XV	20	10	50
XVI	20	10	50
XVII	20	10	50
XVIII (A-Series & Control)	20	10	50
XVIII (B-Series & Control)	20	20	50
XVIII (C-Series & Control)	0	10	50
XIX	20	10	50
XX	20	10	50
XXI	20	10	50

3.3 METHODS

Sampling methods are addressed in section 3.3.1. Section 3.3.2 is devoted to the analytical methods used in this research. Section 3.3.3 covers the isotope pairing method.

3.3.1 Sampling

3.3.1.1 Aqueous Culture Sampling

Aqueous culture samples were taken into polyethylene test tubes via the sample line from each flask (see Figure 3-2). The samples were reacted unfiltered. The unfiltered samples showed no significant turbidity blank. The samples were reacted and analyses completed within 3 hours. Some nitrate samples were refrigerated and analyzed 12 hours later.

3.3.1.2 Culture Headspace Sampling and $\delta^{15}N$ of N_2 Determination

After the final enrichment sequence (XX), culture headspace samples were taken into gas-tight 50 mL glass syringes fitted with a two-way valve and a medium gauge 1.5-inch stainless steel needle. The syringe was first filled with approximately 30 mL of argon-degassed DDW. All lines into and out of the culture flask were double-clamped to prevent atmospheric contamination. The needle was forced through the top of the rubber stopper until it pierced the bottom of the stopper into the headspace of the flask. De-gassed DDW was forced into the headspace, thus supplying an overpressure. When the plunger of the syringe was released the overpressure forced gas headspace back into the syringe. This step was repeated until the entire headspace gas was displaced with water and transferred to the syringe. Most of the DDW remaining in the syringe was wasted, and the valve was closed. It was necessary to leave a small amount of DDW in the syringe to avoid atmospheric contamination. The needle was discarded, and the syringe was plumbed and discharged directly into an elemental analyzer/ mass spectrometer for total N_2 mass and $\delta^{15}N$-N_2 determination.

3.3.2 Analytical Methods

The following section contains the analytical methods used in this research. All culture aqueous samples were reacted unfiltered.

3.3.2.1 Reactive Phosphate

Reactive phosphate was determined by the spectrophotometric phosphomolybdate method (Murphy & Riley, 1962; Parsons *et al.*, 1984). Standards were made such that their concentrations bracket the expected sample concentrations. 4 mL river samples were filtered through 0.45 mm Acrodisk filters and reacted immediately. This method has an error of $\pm$ 0.1 μM.

3.3.2.2 Nitrite and Nitrate Determination

Nitrite was determined colorimetrically on a *Technicon* AutoAnalyzer. This automated system reacted nitrite with sulfanilamide. The resultant complex reacted with an N-1-napthyethylenediamine dihydrochloride solution to form a light purple diazo dye. The absorbance was measured on a colorimeter at 540 nm. Standards were made to bracket expected high and low nitrite and nitrate concentrations. Samples with high nitrite were diluted 10-fold with DDW. This method has an error of ± 0.4 μM.

Nitrate was determined after reduction to nitrite in a *Technicon* AutoAnalyzer. The method for nitrate is the same as nitrite except for the addition of a copper-cadmium reduction column to the AutoAnalyzer, which reduces nitrate to nitrite. Sample nitrate to nitrite conversion was >95%. Sample volumes of < 0.64 mL were required for the sample/rinse ratio of 2:1 that I used. Samples with high nitrite were diluted 10-fold with DDW.

3.3.2.3 Ammonium and Exchangeable Ammonium Determination

Ammonium was determined spectrophotometrically using the indophenol blue method (Parsons *et al.*, 1984). Standards were made to bracket expected high and low concentrations. 3.5 mL samples are reacted with sodium hypochlorite and phenol in a sodium citrate solution. Sodium nitroferricyanide dihydrate acts as a catalyst. River samples are filtered through a 0.45 mm Acrodisk and reacted. Culture samples are reacted unfiltered. Absorbance is measure at 640 nm in a 1 cm cell. This method has an error of ±0.1 μM.

Exchangeable ammonium was determined on wet sediments from the dialysis tubing at the termination of the experiment. The sediment was divided into two fractions. One fraction was sealed and stored at 4 °C for later analysis of Total N (see section 3.3.2.7.2). The other fraction (dried) was washed with 2 M KCl solution (Rosenfeld, 1979; Mackin & Aller, 1984) at a solid to solution ratio of 10:1 (mg: mL). The supernatant 2 M KCl solution was analyzed with the indophenol blue method for ammonium. Exchangeable ammonium is the mass of ammonium found in the supernatant. This ammonium concentration value was multiplied over the entire sediment inoculum on a mass/mass basis to determine the total exchangeable ammonium fraction.

3.3.2.4 Manganese (II) Determination

Mn (II) was determined spectrophotometrically by the formaldoxime reaction, in most cases immediately after sampling (Brewer & Spencer, 1970). In some instances, samples were preserved with 10% hydroxylamine hydrochloride. This method requires a 5% EDTA solution if iron interference is suspected. 0.100 mL of mixed reagent are added to 1 mL of sample and absorbance is read in a 1 cm cell at 459 nm after 30 minutes.

3.3.2.5 pH Determination

pH was determined with a handheld Cole Parmer pH wand calibrated with pH 3, 7, and 10 buffers before and after each sample set.

3.3.2.6 $\delta^{15}N$ of NO_3^- Determination

$\delta^{15}N$ of NO_3^- was measured using the Adapted Ammonia Diffusion Method (Sigman *et al.*, 1997). This method reduced nitrate to ammonia with Devarda's alloy under alkaline conditions. The ammonia diffused out of the solution and was recovered on an acidified glass fiber filter (GFF). This GFF was then combusted for isotopic analyses.

Each GFF, along with NaCl and MgO reagents were combusted at 450 °C for four hours to remove trace organics. Samples must be in 10 % HCl cleaned and DDW rinsed glass bottles with treaded caps. Sample volume is not crucial, but is typically <100 mL, as long as there is sufficient nitrogen mass within the sample for detection. This mass varies between mass spectrometers, but is targeted at 2 μmol N (Sigman *et al.*, 1997). For freshwater samples, 5 g of combusted NaCl must be added to assure osmotic balance between the solution and the filter packet (discussed below). 300 mg of MgO was added to buffer the solution to pH ≈ 9.7. The samples were evaporated to ~100 mL overnight to drive off any ammonia. If volumes were less than 100 mL, samples were diluted with DDW. Filter packets were added along with 0.5 g of Devarda's alloy. Filter packets are constructed of a combusted and acidified (25 μL of 5 N sulfuric acid) GFF sandwiched between two Teflon filter membranes. After packet and Devarda's addition, samples were tightly capped and placed in an oven at 40 °C. After 2 days, samples were shaken at 100 rpm at 40 °C for 5 days. After 5 days filter packets were removed, rinsed of debris, and stored under vacuum in a desiccator with an open vial of concentrated sulfuric acid (to absorb any traces of ammonia) for 3 – 4 days. When dry, the packets were opened under a clean bench, and the GFF was placed inside a compressed tin capsule for combustion and isotopic analysis.

3.3.2.7 Sediment Analyses

The following sections discuss sediment analyses conducted before and after the 165-day incubation.

3.3.2.7.1 Carbon Content, Porosity, & Bulk Density Determination

Carbon content within each sediment inoculum was estimated by simple loss on ignition (LOI). Porosity, as well as wet and dry bulk density, were estimated during the LOI determination. An aliquot of each wet sediment was weighed. The sediments were dried at 130°C for approximately 24 hours. Once cooled, the sediments were weighed again. The weight difference is water content. Porosity was then estimated as water loss per gram of wet sediment. The dried sediments were ground in an agate mortar. A small subset of dry, ground sediment was removed and weighed. This fraction was placed into an oven for 4 hours at 485°C to burn off organic matter, cooled and re-weighed. All pre- and post-combustion masses were recorded.

Equations used to estimate carbon content, porosity, and wet and dry bulk density are:

m_{wet} = wet sediment mass (g)

m_{dry} = dry sediment mass (g) (130 °C dried)

Δ_m = mass of pore water (g) = $m_{wet} - m_{dry}$

W_w = "wet" sediment water content = Δ_m / m_{wet} (g)

ρ_s = grain density (g cm^{-3}) = 2.5 g cm^{-3} (assumed)

ϕ = porosity = $W_w / (W_w + ((1 - W_w) / \rho_s))$ (cm^3/g)

B_w = "wet" bulk density = $\rho_s + \phi - (\phi \times \rho_s)$ (g/cm^3)

m_{pre} = pre-ignition dry sediment mass (g)

m_{post} = post-ignition mass (g) (485 °C)

LOI = mass lost on ignition = $m_{pre} - m_{post}$ (g)

OC = mass of organic carbon in sediment =

$0.40 \times (LOI) + 0.0025 \times (LOI)^2$ (g) (Craft *et al.*, 1991)

$\%OC$ = percent organic carbon= OC / m_{pre} (g/g) x 100

3.3.2.7.2 ΣN and $\delta^{15}N$ of ΣN Determination

The solid fractions of KCl treated and untreated sediments from the exchangeable ammonium determination were placed in an oven, covered and dried at 60 °C. The dry sediments were ground in an agate mortar and pestle and placed in capped 10% HCl cleaned glass test tubes to prevent any contamination with organic matter. A small fraction (~30 mg dry sediment) of each sediment sample is compressed inside a tin capsule and analyzed for nitrogen mass, carbon mass, $\delta^{13}C$, and $\delta^{15}N$.

3.3.2.8 Membrane Inlet Mass Spectrometry

Membrane inlet mass spectrometry (MIMS) is a relatively new technique that allows for quick and precise measurement of dissolved gas (Kotiaho *et al.*, 1991; Kana *et al.*,1994). This technique is a powerful tool for estimating denitrification rates, particularly when coupled with the isotope pairing technique (see section 3.3.3) (Kana *et al.*, 1998; An *et al.*, 2001).

This technique uses a high vacuum to strip dissolved gas from solution through a small membrane. The gas passes through a cryogenic loop submerged in liquid nitrogen that is boiling and open to the atmosphere. The gas is then expanded and accelerated into a quadrupole mass spectrometer. The standard for this technique is N_2 at equilibrium with the atmosphere in two water baths held at two different temperatures. Standards are argon, nitrogen stable isotope, and oxygen stable isotope concentrations in each water bath for the specific temperature, altitude, and atmospheric pressure.

This system seems to yield good results for analyses of nutrient amended natural samples. However, my argon-sparged samples produced an over-response for argon.

Argon concentrations appeared to be up to 4 times over-saturated (400% of the pure Ar-gas solubility). This over-saturation is not possible at the temperature (~20 °C) and pressure (~1 atm) of my cultures, and is probably due to saturation of the detector. Argon in the standard is in relatively small concentrations (atmosphere = ~1 % argon). When relatively large amounts of argon are introduced from my argon-saturated cultures, the effect is noticeable. Because of this unknown "argon effect," the measured masses of all other gas in solution within my cultures are unknown since I have no standard for comparison. As a result, further MIMS determinations on the aqueous phase of my cultures were abandoned.

Additionally, I discovered that a boiling liquid nitrogen or argon cold trap open to the atmosphere in a laboratory may affect gas concentrations and isotopic abundances of air in the lab. There is essentially an artificial source of light isotopes to the air in the room as they escape from the boiling liquid. This boiling liquid (either nitrogen or argon) is located in close proximity to the water baths used for standardization and may have an effect on the isotopic abundances and gas concentrations in the water baths. Typically, the cold trap is liquid nitrogen and the standard used to calibrate the MIMS is argon in equilibrium with the atmosphere. However, when we attempted to use liquid argon in the cold trap, the standard was affected.

3.3.3 The modified isotope pairing technique

The isotope pairing technique is a method with which denitrification rates may be measured using the heavy stable isotope of nitrogen ^{15}N (Neilsen, 1992). When the abundance of $^{15}N\text{-}NO_3^-$ is artificially increased in a system, the labeled nitrate mixes into the ambient nitrogen pool. When denitrifiers utilize the labeled nitrate, labeled N_2 is produced. Denitrification of labeled nitrate results in the isotope pairing of two ^{15}N labeled nitrate molecules resulting in N_2 with mass 30. When a labeled nitrate molecule is paired with an unlabeled nitrate molecule, the result production of N_2 with mass 29. If two unlabeled nitrate molecules are paired during denitrification, then there is production of N_2 of mass 28. By measuring the mass of the N_2 produced, denitrification and coupled nitrification/denitrification rates can be calculated through the application of the following formulae (Neilsen, 1992):

$$D_{15} = (^{14}N^{15}N) + 2(^{15}N^{15}N)$$

$$D_{14} = ((^{14}N^{15}N) / 2(^{15}N15N)) * ((^{14}N^{15}N) + 2(^{15}N^{15}N))$$

where:

D_{15} and D_{14} are the rates of denitrification of $^{15}NO_3^-$ and $^{14}NO_3^-$ respectively.

$^{14}N^{15}N$ is the measured mass of $^{29}N_2$.

$^{15}N^{15}N$ is the measured mass of $^{30}N_2$

This method appears best suited for other ecological settings, such as in coastal and freshwater systems (Risgaard-Petersen *et al.*, 1998; Robinson, 2001). One such limitation seems to occur because of nonuniform mixing of the labeled nitrogen (Neilsen, 1992; Middelburg *et al.*, 1996). In my system, the argon sparging should provide adequate stirring to overcome this concern. One major advantage of the tracer addition is that fractionation can be neglected.

This technique has been used to evaluate marine systems for chemolithotrophic denitrification (Thamdrup and Dalsgaard, 2000; Thamdrup and Dalsgaard, 2002; Dalsgaard *et al.*, 2003; Kuypers *et al.*, 2003).

One problem that I faced is that the pairing of the nitrogen isotopes was lost with the abandonment of the MIMS method for determining $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$. N-isotope determination by coupled elemental analyzer/ MS introduces splitting and re-mixing of the nitrogen isotopes before they reach the mass spectrometer. The $\delta^{15}N$ can be determined, but not the actual fractions of each isotope pair (i.e. % $^{14}N^{14}N$, $^{14}N^{15}N$, and $^{15}N^{15}N$). Another problem I faced was that the nearly 100% argon in my headspace samples caused fractionation in the source inlet that was not accounted for by my isotope standards. This mass bias effect in the mass spectrometer calls into question the quantitative value of the data. Therefore, in my system, I modified the isotope pairing method to provide a qualitative answer for the fates of ammonium and nitrate in my system. By measuring the $\delta^{15}N$ of the dinitrogen produced, I can constrain whether or not ammonium is being denitrified. I added labeled nitrate (240 µM nitrate; $\delta^{15}N \approx 444\ ^{0}/_{00}$) and unlabeled ammonium to some cultures, and labeled ammonium (100 µM ammonium; $\delta^{15}N \approx 469\ ^{0}/_{00}$) and unlabeled nitrate to others (see Appendix II). In cultures that received labeled nitrate and unlabeled ammonium, when nitrate is converted to dinitrogen, the dinitrogen should have enriched ^{15}N and therefore a high $\delta^{15}N$. In cultures that received unlabeled nitrate and labeled ammonium, if the $\delta^{15}N$ of the dinitrogen is high, then ammonium is ending up as dinitrogen. Since the cultures are not oxygenated, then ammonium disappearance should be attributable to chemolithotrophic denitrification. However, if labeled N_2 is not produced, then the tracer should be observed either in the organic fraction of the sediments as a high $\delta^{15}N$ of ΣN (ammonium disappearance can be attributed to biological uptake), in the clay fraction (ammonium disappearance can be attributed to fixation in exchangeable cation sites), or in DON (unmeasured).

CHAPTER 4

RESULTS

This chapter is organized into three major sections. The first section contains the nutrient data collected over a six-month period from the Chattahoochee River. I used these data to choose the location of Core 22. The second section contains time series data from the cultures. These data were used to establish patterns of nutrient utilization within the cultures and as a basis for the third section of this chapter. The third section contains nitrate and ammonium mass and isotopic enrichment data collected from the culture solid, aqueous, and gas phases. This section attempts to constrain the fates of nitrogen through mass balance and stable isotope tracer investigations.

4.1 Chattahoochee River Nutrient Transects

This section is the analytical results of the nutrient transects of the Chattahoochee River collected and averaged over a six-month period. This is the data used to choose the area of the core sample location. *The analytical data presented in this section can be found in Appendix 1.*

The graphs shown in Figure 4-1 are six-month averages of (clockwise from the top left) dissolved reactive phosphate, shown in green with open green circles; nitrate, shown in black with open black circles; nitrite, shown in red with open red circles; and ammonium, shown in blue with open blue circles. The sample dates were December 18, 2001, March 6, 2002, May 8, 2002, and June 5, 2002. The x-axis is km downstream, and the y-axis is the 6 month average concentration (μmol/L). Buford Dam is located at 0 km.

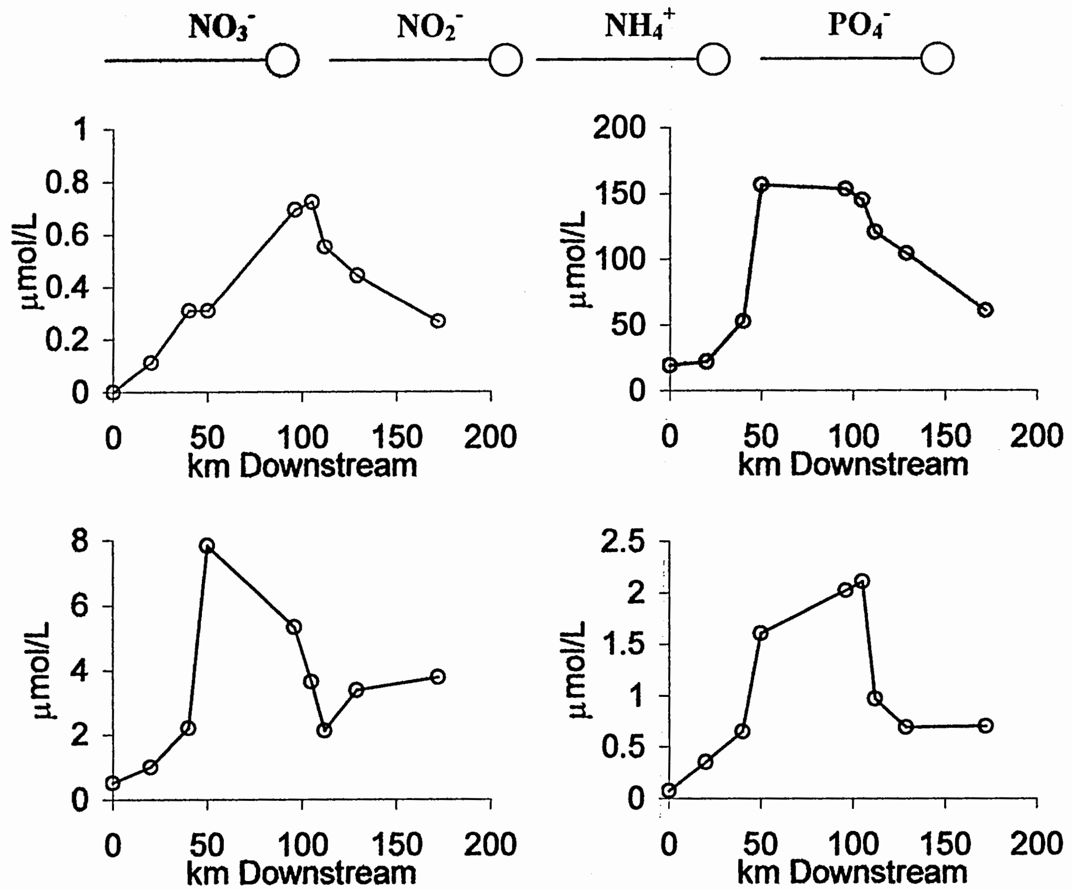


Figure 4-1: Chattahoochee River Nutrient Transects- Six-Month Average Concentration

Water from the river below the dam is the stock river water used in the culture flasks. This site reflects bottom water nutrient concentrations found in Lanier Reservoir. Holcomb Bridge is located 20 km downstream and is near an intake for Atlanta's drinking water treatment facilities. 40 km is upstream of the confluence of Utoy Creek and the Chattahoochee River. Utoy Creek is the receiving water for one of Atlanta's wastewater treatment plants. The 50 km sample site is downstream of the Chattahoochee-Utoy convergence. The 96 km sample site is just upstream of two major coal fired power plants located along the river, Georgia Power's Plants Yates and Wansley. 105 km downstream is between the two power plants, and the 112 km site is downstream of the plants. Franklin, Georgia, is 129 km downstream and is approximately 4 km upstream of the Core 22 sample location. 172 km is downstream of West Point reservoir at the town of West Point, Georgia.

Dissolved reactive phosphate (green) is below detection limits at 0 km downstream. The PO_4^- concentration increases to a maximum of 0.72 μmol/L between two the coal-fired power plants (105 km), and falls to 0.27 μmol/L below the plants and West Point Reservoir at 172 km.

Nitrate (black) is 20 μmol/L at 0 km downstream, and is 22 μmol/L at 20 km downstream. The concentration triples from 53 μmol/L above Utoy Creek (40 km) to a maximum of 157 μmol/L below the Chattahoochee - Utoy confluence (50 km). The concentration falls across the coal-fired power plants to 105 μmol/L at Franklin, Georgia (129 km downstream). Below West Point Reservoir, the NO_3^- concentration is 61 μmol/L, a drop in concentration of roughly 60%.

Nitrite (red) is 0.08 μmol/L at 0 km, and rises to a maximum of 2.11 μmol/L at 105 km downstream between the power plants. The concentration of NO_2^- falls to 0.7 μmol/L as it enters the reservoir at Franklin (129 km) and remains at 0.7 μmol/L below the reservoir.

Ammonium (blue) is 0.53 μmol/L at 0 km. The concentration rises sharply between 40 and 50 km- nearly quadrupling from 2.2 μmol/L to 7.8 μmol/L respectively. The value falls to a local minimum of 2.14 μmol/L below the coal-fired power plants, and rises again across Franklin and the reservoir to 3.8 μmol/L at 172 km. The fall in ammonium concentration from it's maximum coincides with the rise of nitrite to it's maximum, probably reflecting oxidation of ammonium to nitrite- the first step in the nitrification sequence.

These data indicate that riverine portion of West Point reservoir is receiving a high nutrient load. Elevated nitrate, nitrite, and ammonium values, coupled with the anoxic, organic carbon rich muds found here, may provide a suitable environment for chemolithotrophic denitrifiers.

4.2 Time Series Results

The following section contains the analytical results of the A-, B-, & C-Series sediment and control cultures. The A-series sediment cultures contain sediments collected from the top 3 cm of Core 22. The B-Series sediment cultures contain sediments collected from 3 – 6 cm of Core 22. The C-Series sediment cultures contain sediments collected from 6 – 9 cm of Core 22. A description of each enrichment sequence can be found in Appendix II. The time series data for the A, B, C, and Control series can be found in Appendix III.

Each time series is an average of the three replicate sediment cultures within the series (e.g., A1, A2, &A3). Control cultures are shown separately. All "normal" 20 µmole nitrate plus 10 µmole ammonium amended enrichment sequences are plotted together over ten days to establish series behavior during an enrichment sequence. The complete time series plot for each analyte (NO_3^-, NO_2^-, NH_4^+, pH, & Mn^{+2}) during each individual sequence can be found in Appendix IV.

In general, when nitrate is added, it disappears from solution over the course of several days. Over this time, nitrite accumulates to a maximum and then disappears in the sediment cultures- presumably reflecting the reduction of nitrate to nitrite and then subsequent nitrite reduction (nitrite accumulates to a maximum in the control cultures). When ammonium is added, the mass of ammonium in solution decreases with time.

4.2.1 Nitrate Mass vs. Time

Figures 4-2 through 4-7 show average nitrate mass vs. time for the three culture series. The x-axis is time in days. The y-axis is mass in units of µmoles. Nitrate is plotted with open black circles. The first-order rate equation of each trend line is in the form $N=N_0e^{-kt}$, where N is the mass of nitrogen, N_0 is the average initial nitrate addition, t is time in days, and k is the rate constant in day^{-1}. The lines represent the best fit exponential curves to the data.

In the control cultures (Figures 4-2 through 4-4, A-C, B-C, & C-C) nitrate loss was slow and nearly linear with time. While there was considerable scatter ($R^2 \approx 0.5$),

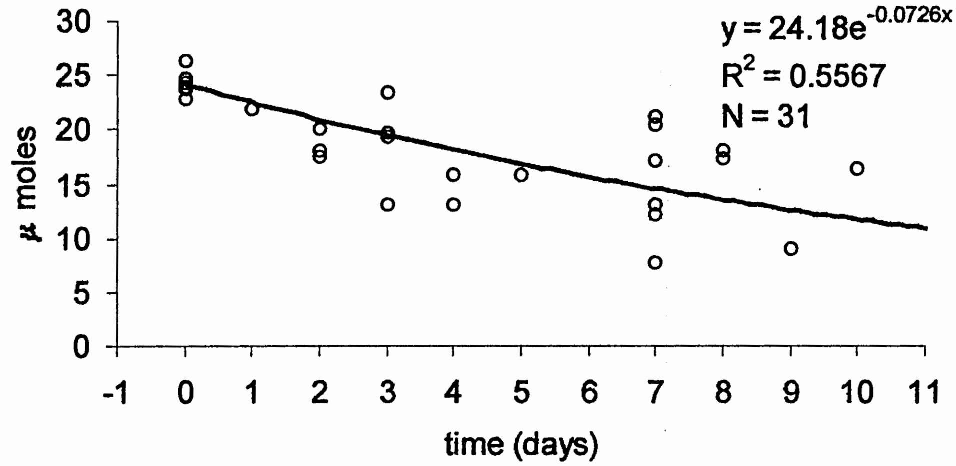


Figure 4-2: A-Control Nitrate Mass versus Time

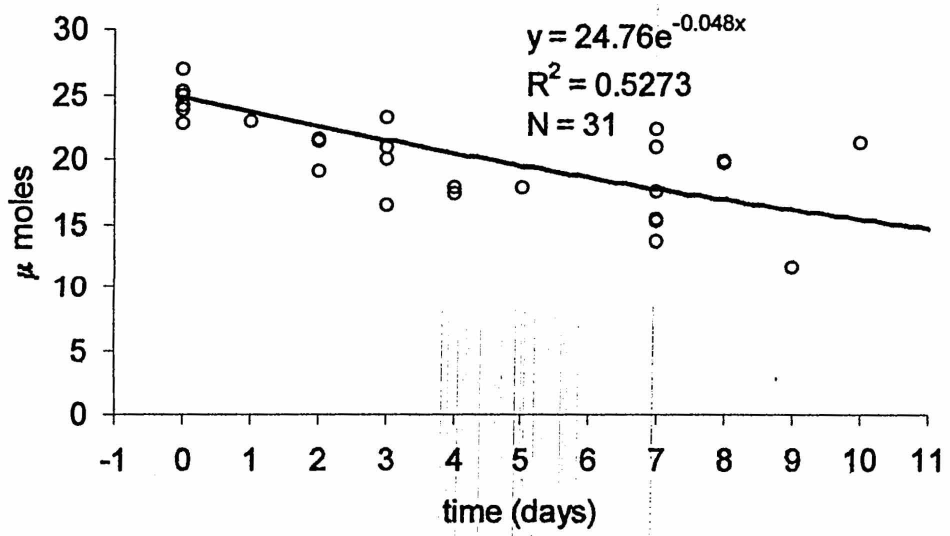


Figure 4-3: B-Control Nitrate Mass versus Time

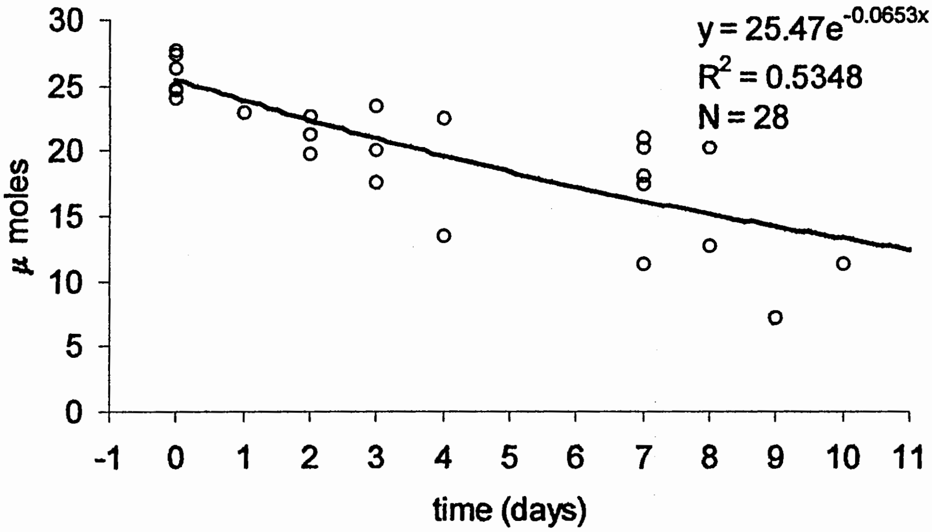


Figure 4-4: C-Control Nitrate Mass versus Time

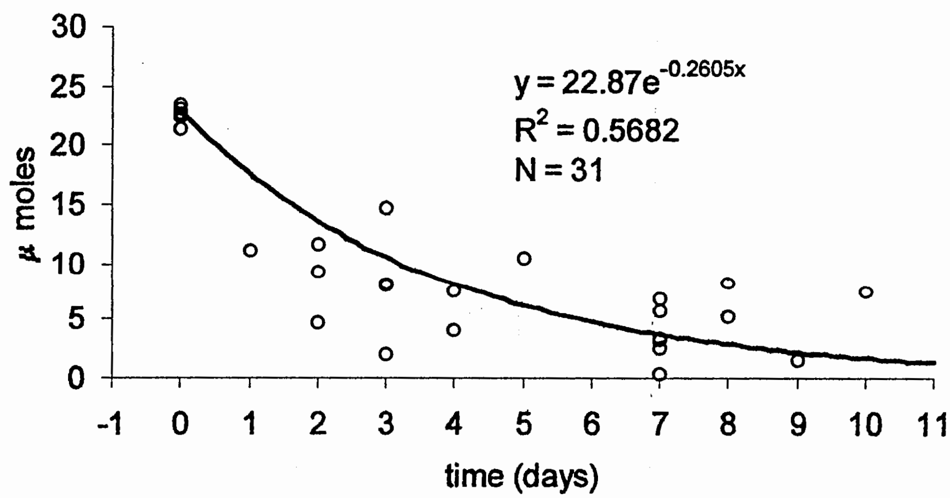


Figure 4-5: A-Series Sediment Culture Nitrate Mass versus Time

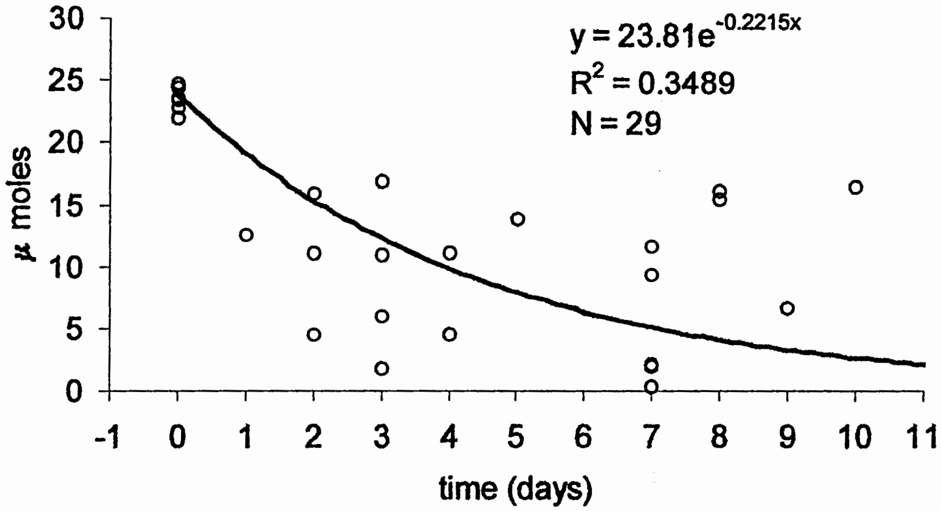


Figure 4-6: B- Series Sediment Culture Nitrate Mass versus Time

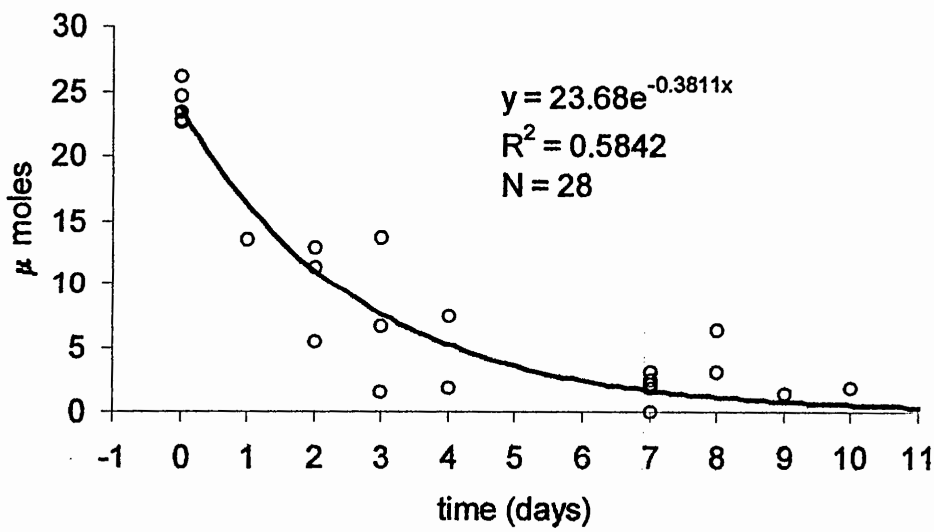


Figure 4-7: C- Series Sediment Culture Nitrate Mass versus Time

nitrate mass in solution always decreased with time. The first order rate constants for A-C, B-C, and C-C were –0.07, -0.05, and –0.07 day^{-1} respectively.

In the A, B, & C-Series sediment cultures, shown in Figures 4-5 through 4-7, nitrate also always decreased with time. While R^2 showed scatter comparable to the control cultures, the first order rate constants for the A, B, & C-Series sediment cultures were an order of magnitude larger, -0.26, -0.22, and –0.38 respectively. In all three series, the addition of ammonium and bicarbonate appeared to have no observable effect on nitrate disappearance (see Appendix II and Appendix IV).

These data indicate that sediment addition had a clear affect on nitrate dynamics. The control cultures consumed nitrate slowly and are probably zero order with respect to nitrate utilization. Sediment cultures consumed nitrate much faster and nitrate utilization is well represented by a first order loss.

4.2.2 Nitrite Mass vs. Time

Figures 4-8 through 4-13 show the average nitrite mass vs. time for the three culture series. The x-axis is time in days. The y-axis is mass in units of μmoles. Nitrite is plotted with open red circles.

In Figures 4-8 through 4-13, the control culture nitrite accumulated to a maximum at the end of the enrichment sequence. This accumulation probably reflects the reduction of nitrate to nitrite, the first stage of denitrification. However, nitrate loss did not equal nitrite gain. In the sediment cultures, nitrite accumulated to a maximum much earlier in the sequence, usually at one to four days, and then disappeared from solution.

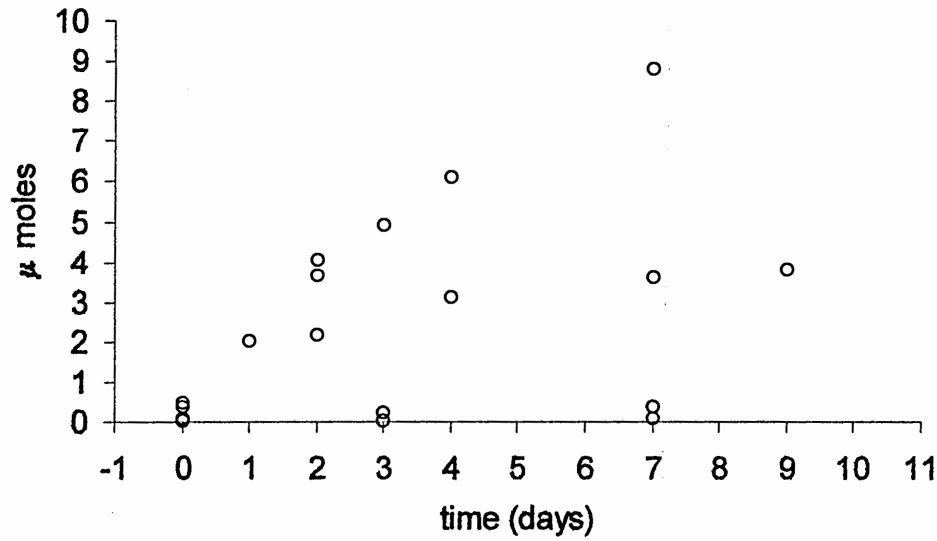


Figure 4-8: A-Control Nitrite Mass versus Time

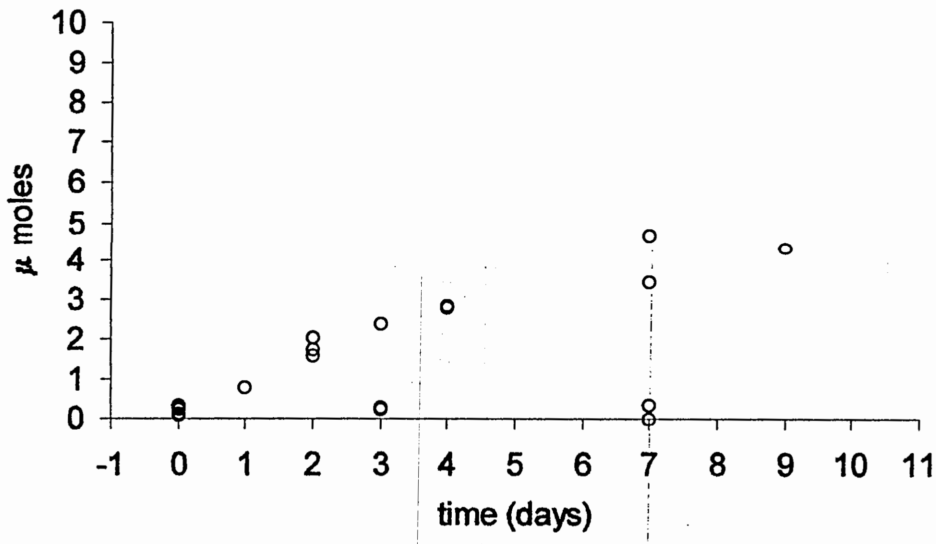


Figure 4-9: B-Control Nitrite Mass versus Time

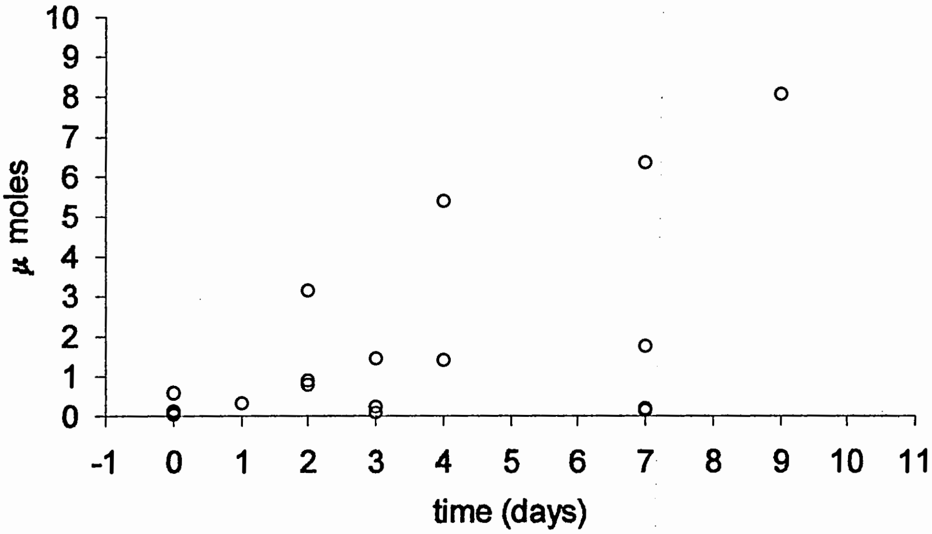


Figure 4-10: C-Control Nitrite Mass versus Time

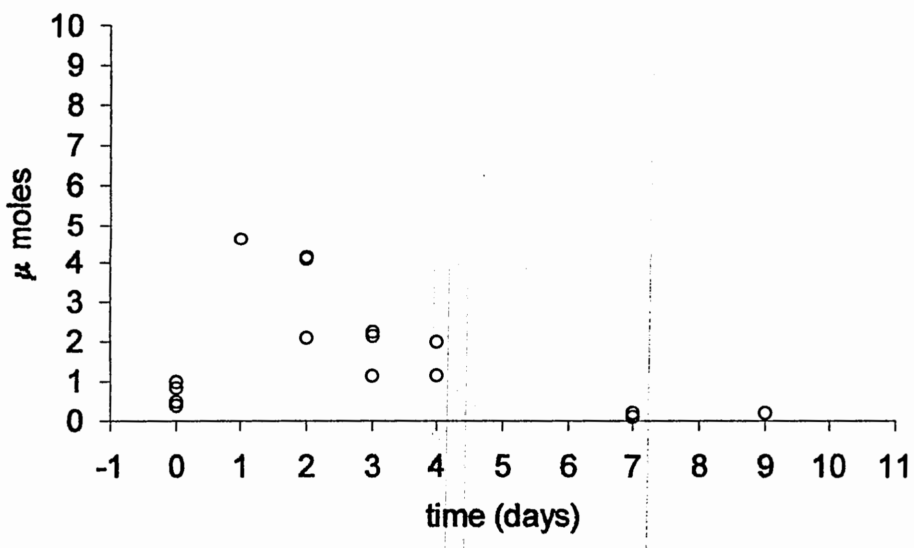


Figure 4-11: A-Series Sediment Culture Nitrite Mass versus Time

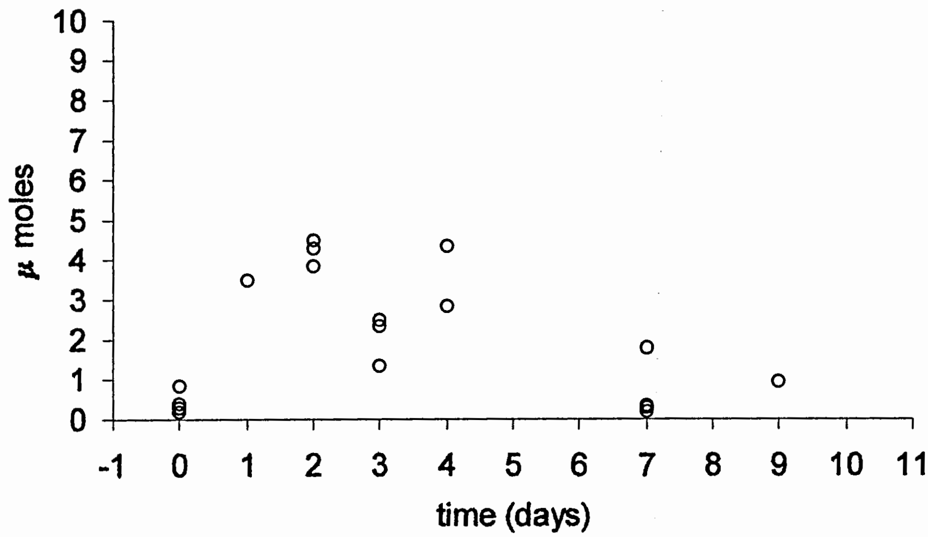


Figure 4-12: B- Series Sediment Culture Nitrite Mass versus Time

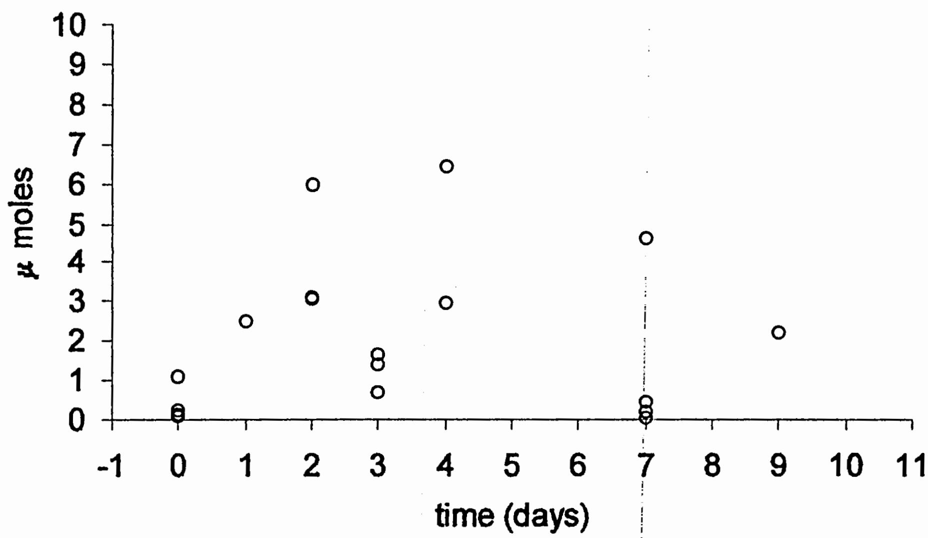


Figure 4-13: C- Series Sediment Culture Nitrite Mass versus Time

This disappearance probably reflects reduction of nitrite to dinitrogen, the second stage of denitrification. Again, nitrate loss did not equal nitrite gain. When ~20 μmoles nitrite were added to the cultures in Enrichment Sequence XVII (see Appendix II and Appendix IV), nitrite disappearance behavior was very similar to nitrate disappearance. The A, B, and C-Series sediment culture first order nitrite rate constants (for nitrite amended Enrichment Sequence XVII only) are -0.64, -0.25, and –0.31 day^{-1} respectively. The A-C, B-C, & C-C first order nitrite rate constants for Enrichment Sequence XVII are –0.06, -0.03, and –0.05 day^{-1} respectively. Unfortunately, the overall observed behavior of the control and sediment cultures was not readily modeled by simple first order rate equations. As a result, nitrite is omitted from mass balance calculations (nitrite-nitrogen addition was <5% of total nitrate-nitrogen addition).

In the control cultures, net nitrite gain was observed. This gain was smaller than nitrate loss, and probably reflects nitrate reduction to nitrite with a small amount of nitrite utilization. This behavior is in stark contrast to the sediment cultures, which showed faster nitrite production and near zero net nitrite gain.

4.2.3 Ammonium Mass vs. Time

Figures 4-14 through 4-19 show the average ammonium mass vs. time for the three culture series. The x-axis is time in days. The y-axis is mass in units of μmoles. Ammonium is plotted with open blue circles. The first-order rate equation of each trend line is in the form $N=N_0e^{-kt}$, where N is the mass of nitrogen, N_0 is the average initial ammonium addition, t is time in days, and k is the rate constant in day^{-1}. All cultures showed ammonium loss with time. A-C & C-C were nearly linear versus time with

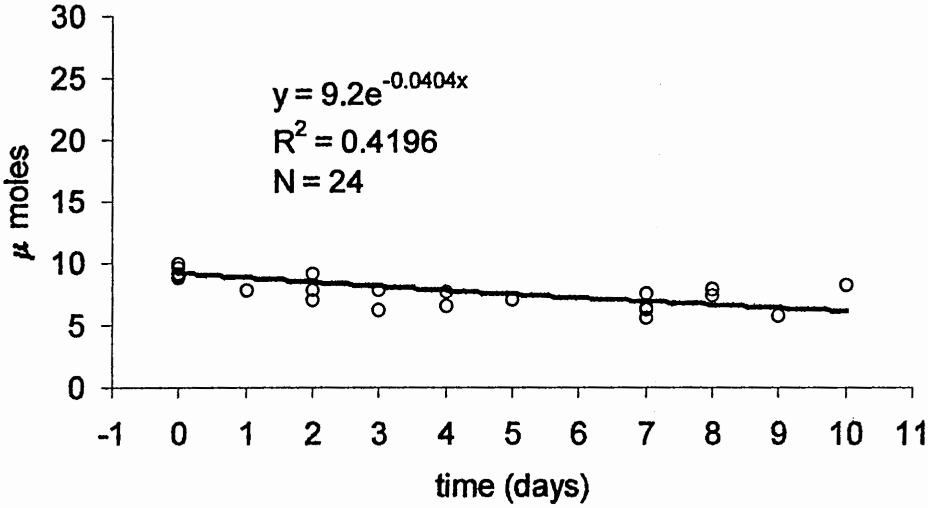


Figure 4-14: A-Control Ammonium Mass versus Time

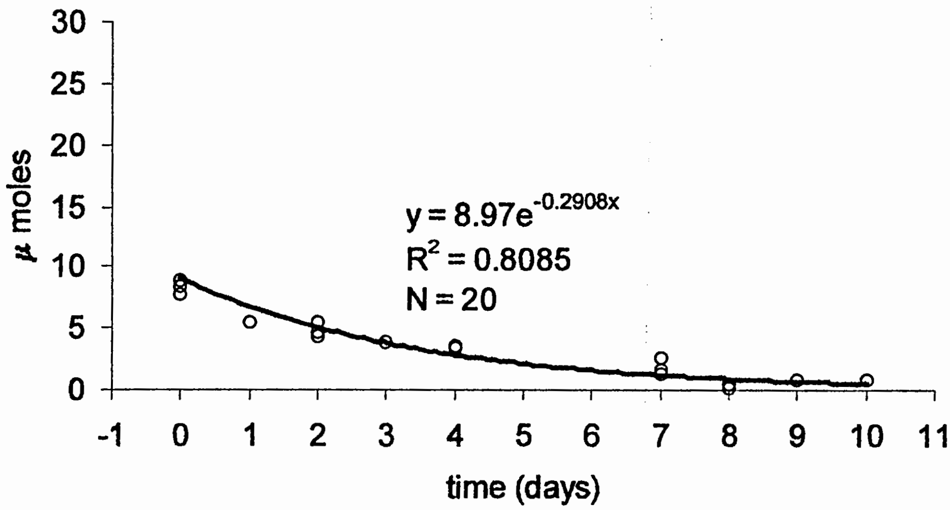


Figure 4-15: B-Control Ammonium Mass versus Time

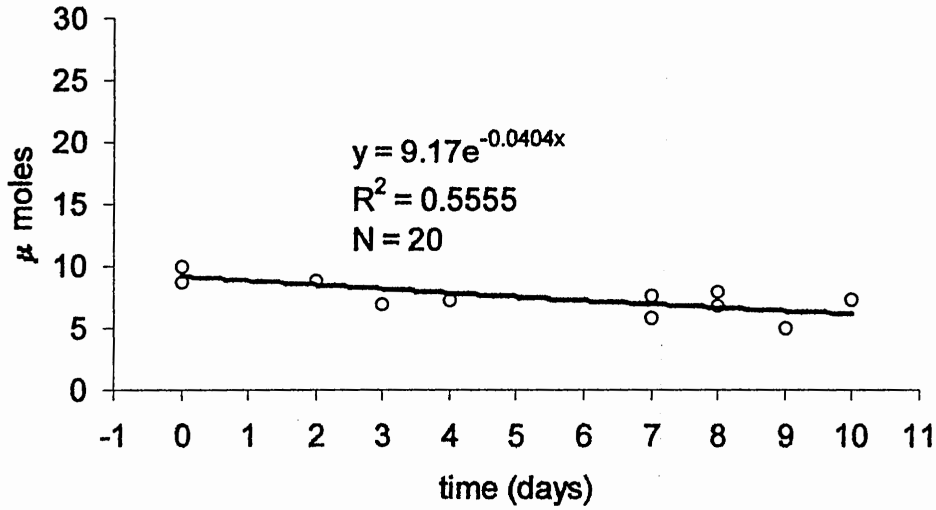


Figure 4-16: C-Control Ammonium Mass versus Time

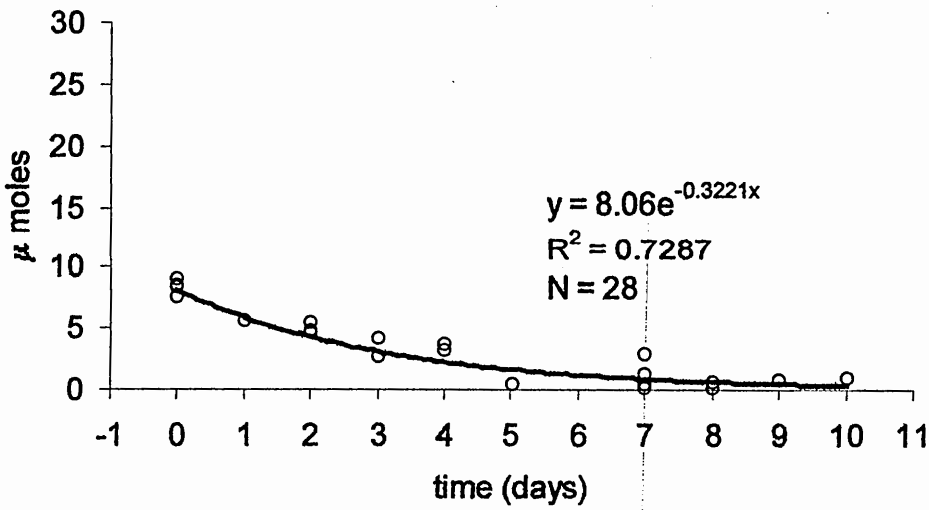


Figure 4-17: A-Series Sediment Culture Ammonium Mass versus Time

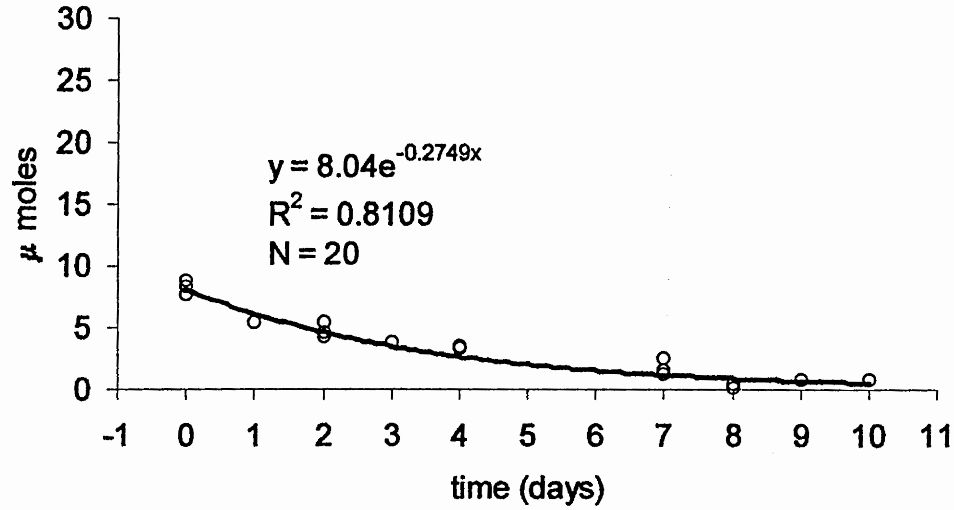


Figure 4-18: B- Series Sediment Culture Ammonium Mass versus Time

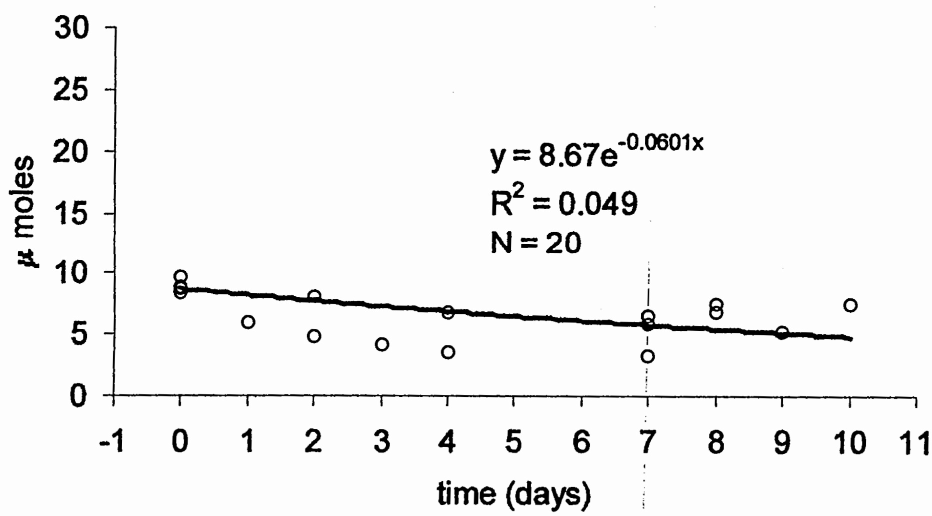


Figure 4-19: C- Series Sediment Culture Ammonium Mass versus Time

respect to nitrate mass. The first order rate constant for A-C and C-C was –0.04 day^{-1}. The first order rate constant for B-C was an order of magnitude larger than the other two controls, -0.29 day^{-1}. This control culture was similar to the A and B-Series sediment cultures with rate constants of –0.32 and -0.27 day^{-1} respectively. The C-Series sediment cultures were very similar to A-C and C-C, showing more linear behavior with respect to ammonium and a smaller rate constant, -0.06 day^{-1}.

4.3 Mass Balance

The following sections attempt to constrain the fates of ammonium and nitrate in the cultures through mass balance and isotope tracer investigations. The first section constrains the masses of nitrate and ammonium that were added to and disappeared from the aqueous phase of the cultures. The second section provides the mass balance of nitrogen in the sediments as exchangeable ammonium in clays and N_{org} in the bulk sediment. The masses of N_{org} and C_{org} and relative isotopic enrichments of sediments labeled with $^{15}NO_3^-$ or $^{15}NH_4^+$ provide clues to the interactions of these species within the solid phase of the cultures (including biological assimilatory activity within the sediment). The third section contains the results of the headspace nitrogen measurements (gas phase) conducted at the end of the isotope tracer experiment. The results of that section, while quantitatively problematic, provide additional qualitative clues to the fates of ammonium and nitrate within the system.

4.3.1 Nutrient Addition and Disappearance

Table 4-1 contains the average mass data of nitrate and ammonium added and lost for the three series. These data reflect the total mass of each nitrogen species added to the system and the total mass of nitrogen that disappeared during the 165-day incubation. The data presented in this section are in units of μmoles.

The A-Series sediment cultures were amended with an average of 441.3 μmoles NO_3^- total. A total of 374.4 μmoles NO_3^- disappeared from the A-Series, a total utilization of 85 %. Similarly, A-C, the A-Series control culture, was amended with 466.4 μmoles NO_3^- total. However, a total loss of only 197.2 μmoles, or 42 %, was observed.

The A-Series showed similar behavior with respect to ammonium. The sediment cultures were amended with 142.9 μmoles NH_4^+ total; while 113.3 μmoles, or 79 %, disappeared. The control culture, A-C, was amended with 138.8 μmoles NH_4^+, and showed a total loss of only 36.2 μmoles NH_4^+, or 26 %.

The B-Series sediment and control cultures were similar in behavior to the A-Series with respect to both nitrate and ammonium. The B-Series sediment cultures were amended with 451.2 μmoles NO_3^-, and 71 %, or 318.6 μmoles NO_3^- disappeared. B-C, the B-Series control, lost 31 %, a total of 149.9 μmoles NO_3^- out of 477.2 μmoles NO_3^- added. Ammonium loss in the B-Series sediment cultures was 112.7 μmoles NH_4^+ out of 150.4 μmoles NH_4^+ added total, a total loss of 75 %. B-C showed a total disappearance of 38.1 μmoles NH_4^+ from 168.5 μmoles NH_4^+ added total, or 23 %.

The C-Series cultures also showed similar behavior with respect to nitrate. A total of 436.4 μmoles NO_3^- were added to the C-Series sediment cultures, and 92 %, or 402.4 μmoles NO_3^-, disappeared. C-C lost 243.6 μmoles NO_3^-, or 52 % of the 469.8 μmoles NO_3^- added total. However, with respect to ammonium, all of the C-Series cultures

Table 4-1: Culture Fixed Nitrogen Addition and Consumption. ΣNO_3^- Added is the total μmoles of nitrate added to the cultures. ΔNO_3^- is the total μmoles of nitrate consumed by the cultures. ΣNH_4^+ Added is the total μmoles of ammonium added to the cultures. ΔNH_4^+ is the total μmoles of ammonium consumed by the cultures. ΔNO_3^- & ΔNH_4^+ for each stasis sequence are calculated using the formula: ($[N]_{init}\,e^{-kt}$), where the average rate constant (k) is used for stasis sequences, $[N]_{init}$ is the mass of nitrogen initially added at the start of the sequence, and t is the length of each stasis sequence. The ΔN for each stasis sequence is added to the total for the series.

Culture	ΣNO_3^- Added (μmoles)	ΔNO_3^- (μmoles)
A-1	Series Avg. =	Series Avg. =
A-2	441.3	-374.4
A-3		
B-1	Series Avg. =	Series Avg. =
B-2	451.2	-318.6
B-3		
C-1	Series Avg. =	Series Avg. =
C-2	436.4	-402.3
C-3		
A-C	466.4	-197.2
B-C	477.2	-149.9
C-C	469.8	-243.6

Culture	ΣNH_4^+ Added (μmoles)	ΔNH_4^+(μmoles)
A-1	Series Avg. =	Series Avg. =
A-2	142.9	-113.3
A-3		
B-1	Series Avg. =	Series Avg. =
B-2	150.4	-112.7
B-3		
C-1	Series Avg. =	Series Avg. =
C-2	157.9	-50.4
C-3		
A-C	138.8	-36.2
B-C	168.5	-38.1
C-C	163.0	-43.8

showed behavior like the A and B-Series control cultures. The C-Series sediment cultures were amended with 157.9 μmoles NH_4^+. Only 50.4 μmoles NH_4^+, a total of 32 %, disappeared. C-C was amended with 163 μmoles NH_4^+, and 43.8 μmoles NH_4^+, or 27 %, disappeared from solution.

These data indicated nitrate dynamics between all control and sediment is very consistent. Observed divergences in ammonium dynamics occurred between the A and B-Series sediment cultures and the C-Series sediment cultures, where the C-Series sediment cultures showed behavior similar to the control cultures.

4.3.2 Sediment Nitrogen Mass Balance

This section constrains nitrogen in the solid phases of the cultures. This section does not include data on the control cultures since they contain no sediment. All data are in μmoles per sediment culture. Exchangeable ammonium is inorganic ammonium released from clays by cation exchange. Sediment total nitrogen is presented as two values, one value before cation exchange and one value (denoted by an asterisk) after cation exchange. Also presented here are the stable isotopic analyses of sediment nitrogen. Cultures in bold were amended with $^{15}NO_3^-$, and all others were amended with $^{15}NH_4^+$. The $\delta^{15}N$ of sediment nitrogen is presented both before and after cation exchange to determine if isotope tracers were present in the organic fraction (sediment $\delta^{15}N^*$) or the inorganic (NH_4^+) fraction. Additionally, sediment analyses of organic carbon are presented both before (as LOI data) and after (as elemental analyzer data) the 165-day incubation. These values provide clues to total organic carbon fixed or remineralized during this experiment.

The data in Table 4-2 indicate that total ammonium in clays is insignificant in the total sediment nitrogen pool. At maximum, ΣNH_4^+ in clays accounted for only 16 % of the total sediment nitrogen. Additionally, the ammonium tracer did not appear in the exchangeable ammonium pool since $\delta^{15}N$ is not significantly different before or after cation exchange.

The data in Table 4-2 indicate that the $^{15}NO_3^-$ was incorporated in the sediment nitrogen pool as shown by elevated $\delta^{15}N$. The elevated $\delta^{15}N$ cannot be an artifact of $^{15}NO_3^-$ that crystallized from pore waters upon drying because the elevated $\delta^{15}N$ of the sediment was retained after the sediment was washed for cation exchange. Therefore, the $^{15}NO_3^-$ was most likely incorporated into the organic fraction, indicating reductive anaerobic nitrate assimilation.

The ammonium isotope tracer did not appear in the sediment fraction in $^{15}NH_4^+$ amended cultures since $\delta^{15}N$ of the $^{15}NH_4^+$ amended cultures is small, and after cation exchange, sediment $\delta^{15}N$ is still relatively low. This low $\delta^{15}N$ indicates that ammonium is not assimilated in the organic fraction of the sediments and is apparently not exchanged into or out of clays.

Table 4-2: Sediment Nitrogen Balance. Mass of nitrogen measured in the culture sediments after 165 days of incubation. Cultures shown in bold italics were amended with ^{15}N enriched nitrate; all others were amended with ^{15}N enriched ammonium. ΣNH_4^+ in clays is total ammonium in clays extracted by cation exchange (exchangeable ammonium determination). Sediment ΣN is total nitrogen in the culture sediments (μmoles) Sediment $\delta^{15}N$ is Delta 15-N of sediment nitrogen. "*" denotes sediment values after cation exchange.

Culture	ΣNH_4^+ in clays (μmoles)	Sediment ΣN (μmoles)	Sediment ΣN* (μmoles)
A-1	***1.38***	***26.60***	***28.64***
A-2	0.97	30.20	25.86
A-3	***1.22***	***29.63***	***29.47***
B-1	***6.07***	***146.97***	***116.16***
B-2	9.95	156.96	139.13
B-3	***32.92***	***205.79***	***188.04***
C-1	24.67	218.82	177.33
C-2	***8.52***	***162.61***	***149.69***
C-3	7.21	164.52	196.08

Culture	Sediment $\delta^{15}N$ ($^0/_{00}$)	Sediment $\delta^{15}N$* ($^0/_{00}$)
A-1	***71.42***	***79.52***
A-2	5.76	6.15
A-3	***47.34***	***40.51***
B-1	***26.80***	***25.01***
B-2	10.75	9.13
B-3	***18.79***	***29.93***
C-1	14.77	15.89
C-2	***146.97***	***165.85***
C-3	15.80	23.96

Since there are relatively large masses of nitrogen in the sediment in Table 4-2, artificial isotopic enrichment of ^{15}N (on a mass basis) is overwhelmed by the natural background. It is necessary to subtract the natural background ^{15}N from the total mass by applying the natural ^{15}N to $^{14}N + ^{15}N$ ratio (3.66 x 10^{-3} atom %) to the sediment total nitrogen mass. This mass is subtracted from the total, and the remainder is that which was artificially introduced (added over the course of this experiment) ^{15}N mass per sediment culture, shown in Table 4-3. ^{15}N mass in $^{15}NO_3^-$ amended cultures is slightly higher than ^{15}N masses observed $^{15}NH_4^+$ amended cultures. Only culture C-2 was different, showing a large ^{15}N sediment component and indicating almost complete assimilation of $^{15}NO_3^-$.

Table 4-3: Sediment ^{15}N- Tracer Component: Mass of ^{15}N in Culture Sediment due to tracer addition. Natural background ^{15}N has been subtracted. "*" denotes sediment values after cation exchange (exchangeable ammonium determination). Cultures shown in bold italic were amended with 128 nmoles of $^{15}NO_3^-$, all others were amended with 54 nmoles of $^{15}NH_4^+$.

Sample	Sediment ^{15}N Tracer Component (nmoles)
A1	***8.80***
A2	2.58
A3	***7.14***
B1	***24.1***
B2	16.3
B3	***27.6***
C1	26.0
C2	***99.8***
C3	20.2
A1*	10.3
A2*	2.24
A3*	6.34
B1*	18.3
B2*	13.6
B3*	33.0
C1*	21.8
C2*	102.4
C3*	30.1

Another crucial component in the constraint of sediment mass balance is the role of organic carbon. Oxidation of organic carbon by heterotrophic processes is reflected by a decrease in organic carbon content from initial LOI values to final elemental analyzer carbon values. Conversely, carbon fixation in the cultures is reflected by a gain of carbon from beginning to end.

In Table 4-4, the A-Series sediment cultures reflect a predominance of carbon fixation shown by an overall increase in carbon content. This indicates that the B and C-Series sediment cultures show an overall decrease from initial to final values, indicative of oxidation of organic carbon and a dominance of heterotrophic processes.

Table 4-4: LOI Carbon Content vs. EA Carbon Content. LOI total carbon content is carbon content per sediment culture in µmoles measured by loss on ignition. These values reflect the initial carbon content of the cultures. EA total carbon content is carbon content per sediment culture in µmoles determined at the end of the experiment. These values reflect the final carbon content in the cultures.

Culture	$\Sigma C_{(LOI)}$ in Sediments (µmoles)	$\Sigma C_{(EA)}$ in Sediments (µmoles)
A1	407	490
A2	429	572
A3	428	543
B1	5732	2604
B2	6690	2727
B3	8403	3646
C1	7606	3606
C2	5845	2760
C3	6019	2892

4.3.3 Headspace Nitrogen Mass Balance

Due to mass bias effects in the mass spectrometer because I used a 100 % argon atmosphere in the cultures that is not accounted for by the isotope standards, the results presented in this section must be evaluated qualitatively. The actual masses and partial pressures presented here are less meaningful than the qualitative relationships that the data imply.

In Table 4-5, total headspace N_2-N mass is presented in μmoles. The masses shown were in excess of the total nitrogen added during the enrichment sequence (~30 μmoles), indicating either an atmospheric leakage into the headspace or an unknown factor introduced by the mass bias effect artificially increasing the mass of nitrogen measured by the mass spectrometer. The most important information shown in Table 4-5 is the isotopic enrichment of the headspace gas. The data show that in $^{15}NO_3^-$ amended sediment cultures (shown in bold), ^{15}N is enriched in the headspace, indicating denitrification of nitrate to dinitrogen. In the $^{15}NO_3^-$ amended control (B-C), the headspace showed little or no enrichment. In the $^{15}NH_4^+$ amended cultures, neither sediment nor control culture headspace gas analyses showed enrichment of ^{15}N, indicating that the headspace was not a sink for ammonium. Therefore, ammonium-denitrification (i.e. chemolithotrophic denitrification) is not occurring. However, mass balance indicated ammonium loss from solution. Since ammonium losses cannot be accounted for in the sediment or gas nitrogen pools, these data offer no definitive answer for the fate of ammonium.

Table 4-5: Culture Headspace Nitrogen. Cultures shown in bold were amended with ^{15}N enriched nitrate; all others were amended with ^{15}N enriched ammonium. Headspace ΣN (µmoles) is total µmoles of N_2 found in the headspace (for enrichment sequence XXI only). $P(N_2)$(atm) is the partial pressure (atm.) of the dinitrogen in the headspace. Headspace $\delta^{15}N$ is isotopic enrichment of ^{15}N in the headspace gas.

Culture	Headspace ΣN (µmoles)	$P(N_2)$(atm)	Headspace $\delta^{15}N$ (‰)
A-1	***72.99***	***0.06***	***401.03***
A-2	66.89	0.06	40.69
A-3	***60.65***	***0.05***	***452.78***
B-1	***67.57***	***0.06***	***774.02***
B-2	192.51	0.17	29.40
B-3	***no data***	***no data***	***no data***
C-1	217.66	0.18	16.73
C-2	***196.32***	***0.17***	***178.41***
C-3	190.56	0.17	19.72
A-C	45.65	0.04	24.15
B-C	***38.72***	***0.03***	***37.57***
C-C	***no data***	***no data***	***no data***

CHAPTER 5

DISCUSSION

5.1 Overview of Rationale and Objectives

The primary focus of this work was to test anoxic freshwater muds for the presence of chemolithotrophic denitrification. A sediment core was collected from the upper, riverine section of West Point Reservoir, an impoundment of the Chattahoochee River. This portion of the reservoir receives a high nutrient load and seemed a likely target to search for chemolithotrophic denitrification. The sediment core was sectioned, and mud inocula from three depths were incubated for 165 days. During this incubation, the concentrations of nitrate, nitrite, ammonium, and bicarbonate were manipulated in an attempt to test for the presence of naturally occurring chemolithotrophic denitrifiers within the sediment inocula. Rate, mass balance, and isotope tracer investigations showing simultaneous denitrification of nitrate and ammonium might indicate the presence of this autotrophic microbial pathway. However, my data indicate that this process in not occurring within these anoxic lacustrine muds.

5.2 Chemolithotrophic Denitrification

Chemolithotrophic denitrification is an alternate pathway to dinitrogen. This microbial process couples nitrate or nitrite reduction and ammonium oxidation to produce dinitrogen without direct oxidation of organic carbon (see Section 2.4). Time series data indicated this that this process was occurring. Simultaneous nitrate (and nitrite) and ammonium disappearance was observed within control and sediment batch cultures (see Section 4.2). These data alone indicate that reactions 4 and 5 are occurring. Nitrate, nitrite, and ammonium rate estimates (discussed below) also indicated that these reactions might be important. However, stable isotope tracer evidence did not support this idea and indicated that another process was responsible for the observed behavior.

5.2.1 Culture Rate

5.2.1.1 Control Culture Nitrate and Ammonium Rates & Rate Constants

Table 5-1 contains nitrate rate constants and maximum rates (rate at t= 0 days) for the cultures. Rate constants were calculated by a best-fit exponential curve through the time series data (see Figures 4-2 – 4-7).

Table 5-1: Sediment and Control Culture Nitrate Maximum Rates and Rate Constants. Rate constants (k), in day^{-1}, are estimated from best-fit curves (see Section 4.2). Maximum Rates (R_0), in μmoles m^{-2} h^{-1}, were calculated from the rate constants: R_0= (k / 24 hours) x (M / 0.0015 m^2) where k is rate constant, M is initial mass (mass at t= 0 days), and 0.0015 m^2 is average surface area of a sediment inoculum.

Culture	k (day^{-1})	R_0 (μmoles m^{-2} h^{-1})
A-Series	-0.26	-165
A-C	-0.07	-49
B-Series	-0.22	-146
B-C	-0.05	-33
C-Series	-0.38	-251
C-C	-0.07	-46

The control cultures represent the contribution of all non-sediment factors to nitrogen dynamics. The control culture rates shown in Table 5-1 are similar to denitrification rates observed in oligotrophic freshwater systems (see Table 3-2). Since heterotrophic denitrification is dependent on organic carbon, and the control cultures have a low organic carbon content, the control culture rates probably reflect organic carbon limited nitrate utilization. Therefore, observed similarities to oligotrophic denitrification rates are fitting.

Table 5-2 contains the ammonium rate constants and maximum rates of the control and sediment cultures. Ammonium rate constants were estimated in the same manner as nitrate rate constants (Figures 4-14 through 4-19).

Table 5-2: Sediment and Control Culture Ammonium Maximum Rates and Rate Constants. Rate constants (k), in day^{-1}, were estimated from best-fit curves (see Section 4.2). Maximum Rates (R_0), in μmoles m^{-2} h^{-1}, are calculated from the rate constants: R_0= (k / 24 hours) x (M / 0.0015 m^2) where k is rate constant, M is initial mass (mass at t= 0 days), and 0.0015 m^2 is average surface area of a sediment inoculum.

Culture	k (day^{-1})	R_0 (μmoles m^{-2} h^{-1})
A-Series	-0.32	-72
A-C	-0.04	-10
B-Series	-0.27	-61
B-C	-0.29	-72
C-Series	-0.06	-14
C-C	-0.04	-10

If the control cultures approximate an oligotrophic system as indicated by nitrate consumption rate, then some ammonium utilization is expected. Indeed, relatively slow ammonium utilization is observed in A-C and C-C. However, the ammonium rate of B-C is seven times faster then the other control cultures. This rate suggests different ammonium dynamics are occurring in the B-Series control culture. The relationship between ammonium and nitrate rates in this control culture suggests that B-C may be supporting a population of microorganisms with different nutritional requirements than those inhabiting the other control cultures. The nitrogen dynamics suggested by B-C's rate relationship appear very similar to reaction 5.

5.2.1.2 Sediment Culture Nitrate and Ammonium Rates & Rate Constants

Nitrate rate constants in the sediment cultures shown in Table 5-1 are an order of magnitude higher than the control cultures. Therefore, maximum nitrate utilization rates are much faster. The sediment culture nitrate utilization rates are in fact similar to denitrification rates observed in eutrophic lakes shown in Table 3-2. The warm, nutrient rich culture media, coupled with sediment inocula rich in organic matter is certainly a suitable environment for heterotrophic denitrifiers, and nitrate rate suggests heterotrophic denitrification is important. However, ammonium utilization rates within the A and B-Series sediment cultures are high as well. This substantial ammonium reaction rate indicates that the nutritional requirements of the organisms in these cultures include ammonium. The relationship between ammonium and nitrate rate in these two sediment cultures suggests that reaction 5, probably in conjunction with reaction 1, may govern nitrogen dynamics.

5.2.1.3 Control Rates vs. Sediment Rates

The following sections compare the rates of nitrate and ammonium reaction in the sediment cultures and the control cultures.

5.2.1.3.1 Nitrate Rates: Sediment Cultures vs. Control Cultures

Nitrate rate constants in the sediment cultures are fully an order of magnitude higher than those of the control cultures. The flat nature of the exponential curves (see Figures 4-2 through 4-7) for nitrate in the controls suggests that nitrate utilization rate in the control cultures is zero-order while nitrate utilization rate in the sediment cultures is first-order.

5.2.1.3.2 Ammonium Rates: Sediment Cultures vs. Control Cultures

Ammonium rate constants in the A and C-Series sediment cultures are an order of magnitude higher than the A and B-Series control cultures. However, the C-Series sediment culture rate constant is very similar to the A and C-Control rate constants. The flat nature of the best-fit exponential curves (see Figures 4-14 through 4-19) indicates that ammonium utilization rates of A-C, C-C, and the C-Series cultures are probably zero-order. The B-Series control culture rate constant is very similar to the A and B-Series sediment culture rate constants. The rates of these cultures are probably first-order.

5.2.2 Rates vs. Depth Downcore

The following sections discuss rates versus depth in the sediment. Since each series contains sediment from a different depth, variations in rates could be attributed to differences in substrate or microbial activities at each depth. The A-Series contains sediment from 0 – 3 cm in Core 22. The B-Series sediment was collected between 3 and 6 cm downcore, and the C-Series sediment was from 6 – 9 cm downcore.

5.2.2.1 Nitrate Rate Constants vs. Depth

Nitrate utilization rate constants vary little in the top six cm (-0.24 ±0.03 day^{-1}). However, the rate constant is 63 % higher in the C-Series, -0.38 day^{-1}. This increase in rate constant reflects an increase in nitrate utilization rate with depth below 6 cm. This increase in rate with depth could be due to a higher organic carbon content in the sediment cultures from this depth. An increased availability of substrate translates into a faster rate.

5.2.2.2 Ammonium Rate vs. Depth

Ammonium rate constants show a trend opposite from nitrate rate constants with depth. In the top 6 cm, ammonium rate constant is –0.30 ±0.03 day^{-1}. Below 6 cm, ammonium rate constant is 80% lower at -0.06 day^{-1}. This trend suggests that the microbial community below 6 cm does not require a reduced nitrogen source and that the utilization rate that we do observe in cultures from this depth downcore is probably due to organisms in the stock culture media.

5.2.3 Rate Constants Reproducibility

5.2.3.1 Control Culture Nitrate & Ammonium Rate Constants Reproducibility

The control culture rate constants show good reproducibility with respect to nitrate. The average control culture nitrate rate constant is –0.06 ± 0.01 day^{-1}. This consistency translates into similar nitrate utilization rates within the control cultures.

Ammonium rate constants show different behavior in all of the control cultures. The A and C-Series controls show good reproducibility; however, the B-Series control culture is markedly different. The A and C-Controls have the same rate constant, -0.04 day^{-1}, but the B-Control has a rate constant of –0.29 day^{-1}, an order of magnitude greater.

5.2.3.2 Sediment Culture Nitrate & Ammonium Rate Constants Reproducibility

The A and B-Series sediment cultures show good agreement with respect to the nitrate rate constants. The average rate constant of these cultures is –0.24 ±0.03 day^{-1}. The C-Series nitrate rate constant (–0.38 day^{-1}) is much greater than the other series.

The A and B-Series sediment cultures also show good reproducibility with respect to ammonium rate constant. The average of the two cultures is -0.30 day^{-1} and $\sigma = 0.03$ day^{-1}. Again, the C-Series is unlike the other series with an ammonium rate constant of –0.06 day^{-1}.

5.3 Mass Balance: Constraining the Fate of Ammonium

The following is a discussion of nitrogen mass balance within the A, B, and C-Series sediment and control cultures. This discussion is an attempt to constrain ammonium loss within the system and thereby confirm or deny the presence of chemolithotrophic denitrification.

Table 4-5 shows that the ammonium and nitrate tracers did not accumulate in the gas phase of the control cultures in any significant amounts (see Table 4-5 and Appendix IV). $\delta^{15}N$ of the headspace gas in both tracer amended control cultures was small. Still, NO_3^- and NH_4^+ disappeared from the control cultures. Neither nitrogen species accumulated in the headspace as labeled N_2, yet they disappeared from solution. This disappearance cannot be attributed to chemolithotrophic denitrification since both reactions 4 and 5 would have produced labeled dinitrogen. Additionally, since labeled N_2 did not appear, mass balance is not satisfied in the control cultures with respect to either species.

Therefore, nitrate nitrogen and ammonium nitrogen must remain in the control cultures in a different, unmeasured nitrogen pool. The near zero-order nitrate utilization rate (and ammonium in A-C and C-C) suggests two possibilities. The first explanation is adsorption to the glass and/or plastics inside of the culture flask. This explanation seems more likely for ammonium than nitrate, given the relatively small mass of ammonium that disappeared over the course of the experiment and the tendency of this cation for adsorption. Another possibility is a small population of heterotrophic nitrate reducers were present in the stock media. When introduced with each enrichment sequence to the anoxic environment of the control culture flasks, these microorganisms were able to carry out assimilatory nitrate reduction using dissolved organic matter in the stock media as a carbon source (and possibly ammonium as an additional reduced nitrogen source). No visible growth was observed in the controls, which suggests suspended growth was occurring. If the population remained suspended, any evidence of this assimilatory activity would have been lost each time the next enrichment sequence was initiated. Without analyses of particulate organic matter in the aqueous phase at the end of the modified isotope tracer experiment, no definitive answer can be given as to the fate of ammonium within the controls.

This theory suggests that the control cultures represent true non-sediment contributions to nitrogen dynamics. If the average nitrate and ammonium masses used by the controls are averaged and subtracted from the sediment cultures, then the remainder can be attributed to activity within the sediment.

The same mechanism operating in the control cultures seems to be occurring with ammonium dynamics in the C-Series sediment cultures. ΔNH_4^+ in the C-Series sediment cultures (Table 4-1) is very similar to the control cultures. In fact, the C-Series sediment cultures only used an average of 50.4 μmoles of ammonium, only 11 μmoles more than average control ammonium uptake. This mass is within error (see Appendix IV). It is likely that ammonium disappearance in the C-Series sediment cultures can be attributed to the stock culture media and not processes in the sediments. Ammonium tracer amended C-Series sediment results seem to support this theory.

Table 4-2 sediment data show little or no ammonium tracer in $^{15}NH_4^+$ amended C-Series sediment; however, ^{15}N does appear in $^{15}NO_3^-$ amended culture C-2 sediment. This ^{15}N enrichment in C-2 suggests assimilatory nitrate reduction within the C-Series sediment with little or no assimilation of ammonium. Additionally, nitrate tracer appears in the gas phase of C-2 while ammonium tracer does not. The absence of labeled headspace N_2 rules out reactions 4 and 5 in the C-Series sediment cultures. The enrichment of ^{15}N in C-2 headspace gas does suggest heterotrophic denitrification (reaction 1) is occurring simultaneously with assimilatory nitrate reduction within the C-Series sediment. Indeed, Table 4-4 suggests a predominance of heterotrophic processes in the C-Series sediment since total carbon mass decreased with time.

Nitrate dynamics in the B-Series sediment cultures closely parallel those of the C-Series sediment cultures. Nitrate tracer appears in the sediment organic matter as well as the headspace gas, suggesting simultaneous assimilatory nitrate reduction and denitrification. However, the B-Series sediment cultures behave differently than the C-Series sediment cultures and the control cultures with respect to ammonium. If the average μmoles of NH_4^+ loss seen in the controls, 39.4 μmoles NH_4^+, is subtracted from the B-Series sediment culture total ammonium loss, 112.7 μmoles NH_4^+, then 73.3 μmoles of NH_4^+ can then be attributed to the activity of the sediments. This value is outside of error. Therefore, ammonium disappears from solution in excess of what can be accounted for by the stock media. However, this mass could certainly be accounted for in the mass of N found in the B-Series sediment in Table 4-2; that is, *if* ammonium assimilation by sediment microorganisms is responsible for the observed ammonium loss. Table 4-2 shows the $\delta^{15}N$ of the sediment N in $^{15}NH_4^+$ amended culture B-2 is only 10.75 $^0/_{00}$. Therefore, little or no ammonium tracer was incorporated into the organic matter during the isotope tracer experiment. Table 4-5 shows that ammonium tracer does not appear in the headspace as labeled N_2. Reactions 4 and 5, as well as assimilation of ammonium by sediment microorganisms can almost completely be ruled out. Ammonium in clays as a potential reservoir for "excess" ammonium disappearance is ruled out because very little mass was observed through cation exchange. Therefore, 70.51 μmoles of ammonium must be attributed to some unknown pool that cannot be determined from this series of experiments. NH_3 is not important at neutral pH. N_2O and NO are also very unlikely products. The only other potentially significant unmeasured nitrogen pool is dissolved organic nitrogen.

The A-Series sediment culture results are similar to the B-Series sediment culture results. Simultaneous assimilatory nitrate reduction and denitrification are both evidenced by the elevated $\delta^{15}N$ values in $^{15}NO_3^-$ amended sediment and headspace results. Again, if the average ammonium loss attributed to the stock culture media, 39.4 μmoles, is subtracted from the total ammonium lost by the A-Series sediment cultures, 113.3 μmoles, then 73.9 μmoles of excess ammonium mass lost from solution must be attributed to activity in the sediments. The A-Series sediments contain an average of 28.8 μmoles of N, so the total ammonium lost from solution, even after the subtraction of the stock culture media ammonium assimilation, can clearly not be accounted for in the sediment organic fraction. Therefore, the activity in the sediments that is consuming the ammonium is clearly not assimilation by microorganisms. Isotope enrichment data support this argument. The $\delta^{15}N$ of the sediment N in the ammonium amended culture A-2 shows very little ^{15}N enrichment at 5.76 $^{0}/_{00}$. Again, the ammonium tracer does not appear in the headspace as labeled gas. These data almost completely rule out reaction 4 and 5, as well as ammonium assimilation by sediment microorganisms. Ammonium in clays cannot account for "excess" ammonium disappearance. Therefore, this 63.66 μmole ammonium loss from the A-Series cultures must also be attributed to the same unknown pool that cannot be determined from this series of experiments.

The A-Series sediments also yield another caveat. In Table 4-4, ~100 μmoles of carbon seem to have been fixed by the A-Series sediment cultures. This carbon fixation could be attributed to any number of autotrophic processes. However, carbon fixation in the "closed" system of my batch cultures should not lower pH, as shown in Appendix IV. I cannot explain this phenomenon.

While all cultures show simultaneous nitrate and ammonium loss from solution, the failure of this series of experiments to fully constrain the fate of ammonium within the system does not allow me to demonstrate the presence of chemolithotrophic denitrification. The data suggest that reactions 4 and 5 are not occurring. However, the A & B-Series both have large masses of "excess" ammonium that remain un-accounted for in the mass balance. Since the ammonium loss in these cultures cannot be explained, no conclusion can be drawn as to the true nature of the nitrate loss that is observed within them. Assimilatory nitrate reduction appears to be important in the nitrogen dynamics of these sediments, as is heterotrophic denitrification (reaction 1). However, ammonium dynamics within both the control and sediment cultures cannot be constrained. As a result, this experiment fails to either confirm or deny the presence of chemolithotrophic denitrification.

CHAPTER 6

CONCLUSIONS

This experiment attempted to constrain the nitrogen dynamics of freshwater sediments collected from the Chattahoochee River in western Georgia. Through a series of nutrient additions to batch cultures, the main goal of this research was to test for the presence of chemolithotrophic denitrification in these sediments. This autotrophic, microbially mediated anaerobic process simultaneously removes oxidized nitrogen as nitrate or nitrite and reduced nitrogen as ammonium through a direct couple to produce N_2 gas.

Time series results were encouraging. In these time series, I observed simultaneous consumption of nitrate and ammonium in anoxic sediment batch cultures. Mass balance and isotope tracer investigations could attribute nitrate loss to reductive assimilation and heterotrophic denitrification. Ammonium loss, though observed, could not be attributed to assimilation or chemolithotrophic denitrification in these sediments. Ammonium loss is attributed to an unmeasured nitrogen pool within the cultures, possibly DON.

One major obstacle in this work was the problem that arose when trying to measure nitrogen through mass spectrometry. The choice of a 100% argon atmosphere seems to have been a mistake. It would seem that a better choice would have been one of the lighter noble gases such as neon or helium. Using one of these gases may have eliminated mass bias effects and help me to better constrain gas phase nitrogen mass balance.

The results of these experiments are promising. That is, while I cannot confirm the presence of chemolithotrophic denitrification, as was my original goal, neither can I completely rule out that this process is occurring in these sediments. The large masses of ammonium allocated by mass balance to an unknown nitrogen pool bear further investigation through continued research.

APPENDIX I

Chattahoochee River Nutrient Data

The following tables contain the nutrient data collected over a six-month period from the Chattahoochee River. Distance downstream is in kilometers below Buford Dam at Lake Lanier reservoir. Each sample date is listed across the top of each table. All data is listed in units of µmoles/L. "nd" indicates that data analysis for this field was *not determined.* "<dl" indicates *below detection limits*. Data not included in *this* appendix are metalloid and silica concentrations, as well as water quality data such as pH, conductivity, and dissolved oxygen. These data can be found included in Lesley (2003).

Table A1-1: Chattahoochee River Dissolved Reactive Phosphate Concentrations

	Sample Date			
Dist. Downstream (km)	12-18-01	3-6-02	5-8-02	6/5/02
0	nd	nd	nd	<dl
20	0.23	0.13	0.09	<dl
40	nd	nd	nd	0.31
50	nd	nd	nd	0.31
96	1.19	0.27	0.57	0.75
105	1.14	0.32	0.53	0.90
112	0.91	0.31	0.53	0.46
129	0.68	0.31	0.57	0.21
172	0.45	0.13	0.38	0.11

Table A1-2: Chattahoochee River Nitrate Concentrations

	Sample Date			
Dist. Downstream (km)	12-18-01	3-6-02	5-8-02	6/5/02
0	nd	nd	nd	19.53
20	21.91	22.17	24.53	21.15
40	nd	nd	nd	52.79
50	nd	nd	nd	156.83
96	204.25	100.68	99.02	210.17
105	179.08	102.42	96.94	201.61
112	159.32	87.44	73.76	163.15
129	133.39	73.33	61.54	150.28
172	61.07	74.78	61.52	47.15

Table A1-3: Chattahoochee River Nitrite Concentrations

	Sample Date			
Dist. Downstream (km)	12-18-01	3-6-02	5-8-02	6/5/02
0	nd	nd	nd	0.08
20	0.72	0.19	0.33	0.18
40	nd	nd	nd	0.65
50	nd	nd	nd	1.61
96	2.39	1.30	1.30	3.10
105	2.43	0.93	1.95	3.14
112	1.55	0.38	0.65	1.31
129	1.15	0.32	0.52	0.78
172	0.80	0.60	0.43	0.99

Table A1-4: Chattahoochee River Ammonium Concentrations

	Sample Date			
Dist. Downstream (km)	12-18-01	3-6-02	5-8-02	6/5/02
0	nd	nd	nd	0.53
20	0.43	nd	2.56	0.06
40	nd	nd	nd	2.22
50	nd	nd	nd	7.86
96	6.01	nd	5.94	4.08
105	3.42	nd	6.12	1.42
112	0.49	nd	4.38	1.56
129	2.75	nd	4.44	2.97
172	0.28	nd	4.77	6.28

APPENDIX II

Enrichment Sequence Labels and Specifics

In the following appendix, each enrichment sequence is given a Roman numeral designation. A short description of that step and any important observations follows. A "stasis" sequence indicates nutrient addition but no nutrient analysis.

I) -6 – 0 days: The initial set-up of culture flasks with un-amended river water and sediment inocula

II) 0 – 9 days: The first enrichment step of the cultures. Approximately 20 μmoles of nitrate added. NOTES: t= 7 & 9 days, water and plastics appears stained. Brown/Black growth appears on the outside of dialysis tubing in B-3, C-2.

III) 9 - 26 days: Approximately 20 μmoles of nitrate added. NOTES: t= 12 days, after sampling, a segment of DDW soaked and rinsed dialysis tubing and two DDW rinsed weighted closures were added to each control. T= 23 & 26 days, more brown staining of the water and plastics within the flasks containing sediments. More brown to red-brown and black growth noticeable on A-2, A-3, B-2, B-3, C-2, C-3.

IV) 26 – 37 days: Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. NOTES: Ammonium was added to increase the ammonium concentration so that we could observe what was happening to ammonium within the cultures, to get the ammonium "baseline concentration" high enough to observe any changes. t= 28 days, dark, red-brown "fuzzy" growth has appeared to increase in some of the cultures. Any growth visible is outside of the dialysis tubing or on the air diffuser where it touches the dialysis tubing. A large gas bubble is visible INSIDE of B-1. t= 30 - 37 days, extensive growth on outside of dialysis tubing in A-3, B-2, & C-3. t= 37 days, very strong H_2S smell from culture A-1.

V) 37 – 40 days: Detailed time series- Analyses every day. Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. NOTES: t= 37 – 40 days Continued visible growth outside of dialysis tubing. Bubble inside dialysis tubing in A-2, B-1.

VI) 40-51 days: STASIS. Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. NOTES: t= 51 No apparent visible changes. H_2S smell from A-1, A-2, and A-3.

VII) 51- 61 days: Approximately 20 μmoles of NITRITE added. Approximately 10 μmoles of ammonium added. NO nitrate added NOTES: t= 54 – 58 days, A-series cultures generating strong H_2S smell.

VIII) 61- 65 days: STASIS; Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. NOTES: t= 65 days large volume removed from A-1, B-

2, C-3 and sealed in glass ampoule for carbon analysis in M. Purdue lab. Several connections re-glued where glue was peeling from wear.

IX) 65 – 72 days: STASIS; Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. NOTES: lab temp variations from 19 – 24 °C

X) 72 – 79 days: STASIS; Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. NOTES: none

XI) 79 – 89 days: STASIS; Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. Approximately 50 μmoles sodium bicarbonate added NOTES: none

XII) 89 – 96 days: STASIS; Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. Approximately 50 μmoles sodium bicarbonate added NOTES: none

XIII) 96 – 101 days: STASIS; Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. Approximately 50 μmoles sodium bicarbonate added NOTES: 101 days, A-1 generating H_2S smell. Noticeable growth on outside of dialysis tubing in B-3

XIV) 101 – 110 days: Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. Approximately 50 μmoles sodium bicarbonate added NOTES: 110 days: A-1, A-2, A-3 strong H_2S smell

XV) 110 – 118 days: STASIS; Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. Approximately 50 μmoles sodium bicarbonate added NOTES: none

XVI) 118 – 126 days: Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. Approximately 50 μmoles sodium bicarbonate added NOTES: none

XVII) 126 – 133 days: Diverge Cultures; A-Series: Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. Approximately 50 μmoles sodium bicarbonate added. B-Series: Approximately 20 μmoles of nitrate added. Approximately 20 μmoles of ammonium added. Approximately 50 μmoles sodium bicarbonate added. C-Series: No nitrate added. Approximately 10 μmoles of ammonium added. Approximately 50 μmoles sodium bicarbonate added NOTES: 133 days, visible outside growth in B-3 has become rusty, brown/black.

XVIII) 133 – 140 days: Approximately 20 μmoles of nitrate added. Approximately 10 μmoles of ammonium added. Approximately 50 μmoles sodium bicarbonate added NOTES: 140 days, MIMS analysis on cultures: analysis FAILED

XIX) 140 – 150 days: Approximately 20 µmoles of nitrate added. Approximately 10 µmoles of ammonium added. Approximately 50 µmoles sodium bicarbonate added NOTES: none

XX) 150 – 158 days: Approximately 20 µmoles of nitrate added. Approximately 10 µmoles of ammonium added. Approximately 50 µmoles sodium bicarbonate added NOTES: none

XXI) 158 – 165 days: *Modified Isotope Pairing Experiment*: Approximately 20 µmoles of nitrate added. Approximately 10 µmoles of ammonium added. Approximately 50 µmoles sodium bicarbonate added; A-1, labeled nitrate & unlabeled ammonium; A-2, labeled ammonium & unlabeled nitrate; A-3, labeled nitrate & unlabeled ammonium; B-1, labeled nitrate & unlabeled ammonium; B-2, labeled ammonium & unlabeled nitrate; B-3, labeled nitrate & unlabeled ammonium; C-1, labeled ammonium & unlabeled nitrate; C-2, labeled nitrate & unlabeled ammonium; C-3, labeled ammonium & unlabeled nitrate; A-C, labeled ammonium & unlabeled nitrate; B-C, labeled nitrate & unlabeled ammonium; C-C, labeled nitrate & unlabeled ammonium.

XXII) 166 days: cultures terminated, dried and prepared for exchangeable ammonium analysis.

APPENDIX III

Culture Time Series Data

The following tables contain the time series aqueous chemistry data for each culture in the A, B, C, and Control series. The tables are listed by day for each enrichment sequence. All nitrate, nitrite, ammonium, and manganese data is in units of μmoles. pH is in units of $-\log[H^+]$. "*ND*" indicated "not determined." t is time in days. Stasis sequences were not monitored and are not included here.

Enrichment Sequence I

t= -6

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	3.80	0.00	0.03	*ND*	*ND*
A-2	3.80	0.00	0.03	*ND*	*ND*
A-3	3.80	0.00	0.03	*ND*	*ND*
A-C	3.80	0.00	0.03	*ND*	*ND*
B-1	3.80	0.00	0.03	*ND*	*ND*
B-2	3.80	0.00	0.03	*ND*	*ND*
B-3	3.80	0.00	0.03	*ND*	*ND*
B-C	3.80	0.00	0.03	*ND*	*ND*
C-1	3.80	0.00	0.03	*ND*	*ND*
C-2	3.80	0.00	0.03	*ND*	*ND*
C-3	3.80	0.00	0.03	*ND*	*ND*
C-C	3.80	0.00	0.03	*ND*	*ND*

t= -3.42

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.07	0.32	*ND*	*ND*	*ND*
A-2	0.47	0.39	*ND*	*ND*	*ND*
A-3	0.67	0.32	*ND*	*ND*	*ND*
A-C	3.19	0.02	*ND*	*ND*	*ND*
B-1	1.28	0.46	*ND*	*ND*	*ND*
B-2	0.00	0.59	*ND*	*ND*	*ND*
B-3	0.00	0.46	*ND*	*ND*	*ND*
B-C	2.14	0.87	*ND*	*ND*	*ND*
C-1	1.00	0.87	*ND*	*ND*	*ND*
C-2	0.00	0.87	*ND*	*ND*	*ND*
C-3	1.54	0.80	*ND*	*ND*	*ND*
C-C	3.02	0.19	*ND*	*ND*	*ND*

Enrichment Sequence I (continued)

t= -1.68

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.18	0.07	3.27	*ND*	*ND*
A-2	0.00	0.07	6.13	*ND*	*ND*
A-3	0.06	0.13	5.60	*ND*	*ND*
A-C	2.70	0.07	0.20	*ND*	*ND*
B-1	0.36	0.19	8.77	*ND*	*ND*
B-2	0.09	0.05	5.13	*ND*	*ND*
B-3	0.03	0.05	9.99	*ND*	*ND*
B-C	1.55	1.10	0.70	*ND*	*ND*
C-1	0.07	0.24	7.39	*ND*	*ND*
C-2	0.12	0.07	6.38	*ND*	*ND*
C-3	0.19	0.47	6.58	*ND*	*ND*
C-C	2.53	0.30	0.22	*ND*	*ND*

Enrichment Sequence II

t= 0.51

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	23.8	0.43	0.03	*ND*	*ND*
A-2	24.9	0.70	0.03	*ND*	*ND*
A-3	19.7	0.36	0.03	*ND*	*ND*
A-C	26.2	0.03	0.03	*ND*	*ND*
B-1	25.4	0.17	0.03	*ND*	*ND*
B-2	24.7	0.23	0.03	*ND*	*ND*
B-3	24.0	0.23	0.03	*ND*	*ND*
B-C	26.9	0.10	0.03	*ND*	*ND*
C-1	26.2	0.10	0.03	*ND*	*ND*
C-2	26.2	0.10	0.03	*ND*	*ND*
C-3	26.1	0.17	0.03	*ND*	*ND*
C-C	27.6	0.03	0.03	*ND*	*ND*

Enrichment Sequence II (continued)

t= 3.71

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	4.45	5.15	0.68	*ND*	*ND*
A-2	10.3	1.10	0.60	*ND*	*ND*
A-3	9.66	0.55	1.47	*ND*	*ND*
A-C	23.4	0.06	0.14	*ND*	*ND*
B-1	12.2	0.43	1.26	*ND*	*ND*
B-2	9.37	3.86	1.04	*ND*	*ND*
B-3	11.2	3.25	1.40	*ND*	*ND*
B-C	23.2	0.31	0.17	*ND*	*ND*
C-1	12.9	0.37	2.49	*ND*	*ND*
C-2	13.9	3.56	1.43	*ND*	*ND*
C-3	14.1	0.31	1.74	*ND*	*ND*
C-C	23.4	0.12	0.14	*ND*	*ND*

t= 7.54

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	1.40	0.27	0.81	*ND*	*ND*
A-2	2.49	0.33	0.21	*ND*	*ND*
A-3	3.93	0.04	0.88	*ND*	*ND*
A-C	21.2	0.10	0.04	*ND*	*ND*
B-1	3.30	0.10	1.23	*ND*	*ND*
B-2	1.28	0.39	1.36	*ND*	*ND*
B-3	1.28	0.39	1.55	*ND*	*ND*
B-C	20.9	0.33	0.05	*ND*	*ND*
C-1	2.61	0.21	2.44	*ND*	*ND*
C-2	4.05	1.07	1.79	*ND*	*ND*
C-3	2.72	0.10	2.08	*ND*	*ND*
C-C	21.0	0.21	0.12	*ND*	*ND*

Enrichment Sequence III

t= 9.63

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	21.0	0.59	0.03	*ND*	*ND*
A-2	21.8	0.39	0.03	*ND*	*ND*
A-3	21.4	0.19	0.03	*ND*	*ND*
A-C	22.8	0.09	0.03	*ND*	*ND*
B-1	22.0	0.19	0.03	*ND*	*ND*
B-2	22.0	0.26	0.03	*ND*	*ND*
B-3	22.1	0.13	0.03	*ND*	*ND*
B-C	22.8	0.13	0.03	*ND*	*ND*
C-1	22.8	0.13	0.03	*ND*	*ND*
C-2	22.1	0.13	0.03	*ND*	*ND*
C-3	22.8	0.13	0.03	*ND*	*ND*
C-C	24.1	0.13	0.03	*ND*	*ND*

t= 12.54

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	3.21	1.71	0.17	*ND*	*ND*
A-2	8.35	1.48	0.11	*ND*	*ND*
A-3	12.0	0.26	0.10	*ND*	*ND*
A-C	19.4	0.26	0.09	*ND*	*ND*
B-1	9.78	0.66	0.38	*ND*	*ND*
B-2	6.48	0.90	0.80	*ND*	*ND*
B-3	1.78	2.53	0.80	*ND*	*ND*
B-C	20.0	0.26	0.10	*ND*	*ND*
C-1	8.29	0.31	1.22	*ND*	*ND*
C-2	3.55	1.36	0.96	*ND*	*ND*
C-3	8.23	0.37	1.14	*ND*	*ND*
C-C	20.0	0.26	0.09	*ND*	*ND*

Enrichment Sequence III (continued)

t= 16.50

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	1.06	0.04	0.21	*ND*	*ND*
A-2	3.36	0.19	0.11	*ND*	*ND*
A-3	5.31	0.07	0.09	*ND*	*ND*
A-C	17.2	0.42	0.12	*ND*	*ND*
B-1	3.74	0.42	0.19	*ND*	*ND*
B-2	2.14	0.19	0.74	*ND*	*ND*
B-3	0.17	0.01	0.53	*ND*	*ND*
B-C	17.6	0.01	0.09	*ND*	*ND*
C-1	2.69	0.25	0.52	*ND*	*ND*
C-2	0.30	0.19	0.85	*ND*	*ND*
C-3	2.75	0.19	0.84	*ND*	*ND*
C-C	18.1	0.13	0.09	*ND*	*ND*

t= 23.58

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.21	0.01	0.07	*ND*	*ND*
A-2	0.76	0.01	0.09	*ND*	*ND*
A-3	0.76	0.01	0.10	*ND*	*ND*
A-C	8.36	5.08	0.08	*ND*	*ND*
B-1	0.21	0.01	0.11	*ND*	*ND*
B-2	0.76	0.01	0.10	*ND*	*ND*
B-3	0.21	0.01	0.43	*ND*	*ND*
B-C	13.8	1.31	0.11	*ND*	*ND*
C-1	0.21	0.01	0.49	*ND*	*ND*
C-2	0.00	0.01	0.76	*ND*	*ND*
C-3	0.00	0.01	0.81	*ND*	*ND*
C-C	15.6	0.61	0.09	*ND*	*ND*

Enrichment Sequence IV

t= 26.47

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	21.2	1.62	8.20	*ND*	*ND*
A-2	23.1	1.02	8.38	*ND*	*ND*
A-3	23.1	0.35	8.51	*ND*	*ND*
A-C	24.3	0.48	9.60	*ND*	*ND*
B-1	23.1	0.41	8.33	*ND*	*ND*
B-2	24.6	0.21	8.45	*ND*	*ND*
B-3	22.9	0.61	8.52	*ND*	*ND*
B-C	25.2	0.25	9.53	*ND*	*ND*
C-1	25.2	0.28	8.63	*ND*	*ND*
C-2	23.9	0.21	8.81	*ND*	*ND*
C-3	24.5	0.28	8.66	*ND*	*ND*
C-C	27.4	0.15	10.07	*ND*	*ND*

t= 28.69

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	6.49	3.23	4.61	*ND*	*ND*
A-2	6.69	6.76	4.89	*ND*	*ND*
A-3	14.7	2.46	5.10	*ND*	*ND*
A-C	18.12	4.06	7.96	*ND*	*ND*
B-1	12.21	3.11	5.00	*ND*	*ND*
B-2	15.4	1.74	4.71	*ND*	*ND*
B-3	5.45	6.76	4.20	*ND*	*ND*
B-C	21.4	1.74	8.31	*ND*	*ND*
C-1	9.52	3.94	4.52	*ND*	*ND*
C-2	9.44	3.70	4.92	*ND*	*ND*
C-3	15.3	1.62	5.05	*ND*	*ND*
C-C	22.6	0.79	8.51	*ND*	*ND*

Enrichment Sequence IV (continued)

t= 30.50

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.47	0.04	3.63	*ND*	*ND*
A-2	0.80	3.67	4.05	*ND*	*ND*
A-3	10.63	2.30	3.62	*ND*	*ND*
A-C	13.0	6.11	6.58	*ND*	*ND*
B-1	4.19	3.67	3.81	*ND*	*ND*
B-2	9.14	1.54	3.14	*ND*	*ND*
B-3	0.05	3.28	3.22	*ND*	*ND*
B-C	17.4	2.85	7.05	*ND*	*ND*
C-1	1.00	2.90	3.40	*ND*	*ND*
C-2	0.69	2.36	3.84	*ND*	*ND*
C-3	3.73	3.56	3.60	*ND*	*ND*
C-C	22.5	1.40	7.72	*ND*	*ND*

t= 33.79

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.00	0.01	2.73	*ND*	*ND*
A-2	0.00	0.01	3.80	*ND*	*ND*
A-3	0.81	0.51	2.35	*ND*	*ND*
A-C	7.65	8.82	5.63	*ND*	*ND*
B-1	0.24	0.53	3.49	*ND*	*ND*
B-2	0.81	0.51	1.87	*ND*	*ND*
B-3	0.00	0.03	2.62	*ND*	*ND*
B-C	13.7	4.69	6.14	*ND*	*ND*
C-1	0.00	0.01	3.18	*ND*	*ND*
C-2	0.00	0.01	3.33	*ND*	*ND*
C-3	0.00	0.08	3.02	*ND*	*ND*
C-C	17.5	1.75	6.93	*ND*	*ND*

Enrichment Sequence V

t= 37.58

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	22.3	0.74	8.30	1.23	*ND*
A-2	22.6	0.48	8.53	0.78	*ND*
A-3	22.8	0.28	8.55	0.36	*ND*
A-C	23.7	0.41	9.02	0.02	*ND*
B-1	22.8	0.25	8.55	0.35	*ND*
B-2	22.8	0.28	8.13	0.34	*ND*
B-3	22.7	0.38	8.36	0.63	*ND*
B-C	23.8	0.28	8.98	0.02	*ND*
C-1	22.8	0.22	8.51	0.56	*ND*
C-2	22.8	0.22	8.43	0.74	*ND*
C-3	22.8	0.28	8.33	0.33	*ND*
C-C	26.3	0.12	9.01	0.02	*ND*

t= 38.63

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	8.11	6.20	5.34	0.31	*ND*
A-2	13.1	5.21	6.14	0.38	*ND*
A-3	12.0	2.58	5.55	0.24	*ND*
A-C	21.9	2.06	7.96	0.03	*ND*
B-1	15.7	2.70	6.40	0.24	*ND*
B-2	10.5	3.49	4.77	0.38	*ND*
B-3	11.2	4.33	5.39	0.41	*ND*
B-C	22.9	0.78	8.08	0.04	*ND*
C-1	14.2	2.64	6.09	0.39	*ND*
C-2	12.0	2.61	6.03	0.43	*ND*
C-3	14.5	2.32	5.84	0.18	*ND*
C-C	23.0	0.37	8.42	0.03	*ND*

Enrichment Sequence V (continued)

t= 39.56

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	3.14	4.04	4.56	0.30	*ND*
A-2	6.51	6.26	5.23	0.42	*ND*
A-3	4.68	1.94	4.39	0.25	*ND*
A-C	17.5	3.66	7.16	0.03	*ND*
B-1	9.20	4.42	5.42	0.30	*ND*
B-2	2.83	3.23	3.63	0.41	*ND*
B-3	1.44	5.19	4.19	0.44	*ND*
B-C	19.0	1.59	7.27	0.03	*ND*
C-1	6.42	3.56	4.96	0.40	*ND*
C-2	3.84	2.51	4.99	0.47	*ND*
C-3	6.10	3.05	4.67	0.14	*ND*
C-C	19.7	0.90	7.67	0.03	*ND*

t= 40.52

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	1.49	0.08	4.12	0.30	*ND*
A-2	3.10	5.67	4.63	0.42	*ND*
A-3	1.37	0.71	3.73	0.23	*ND*
A-C	13.1	4.93	6.23	0.04	*ND*
B-1	4.08	4.69	4.58	0.29	*ND*
B-2	0.42	1.15	3.11	0.39	*ND*
B-3	0.57	1.25	3.71	0.40	*ND*
B-C	16.4	2.37	6.38	0.05	*ND*
C-1	2.02	2.37	4.27	0.35	*ND*
C-2	0.88	0.69	4.37	0.44	*ND*
C-3	1.79	1.84	4.01	0.14	*ND*
C-C	17.6	1.45	6.87	0.04	*ND*

Enrichment Sequence VII

t= 51.54

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	2.85	20.8	6.96	*ND*	*ND*
A-2	3.19	20.8	6.97	*ND*	*ND*
A-3	2.85	20.8	6.81	*ND*	*ND*
A-C	3.22	21.5	6.89	*ND*	*ND*
B-1	3.19	20.8	6.79	*ND*	*ND*
B-2	2.53	21.2	6.70	*ND*	*ND*
B-3	2.85	20.8	6.86	*ND*	*ND*
B-C	2.88	21.5	7.27	*ND*	*ND*
C-1	2.85	20.8	7.18	*ND*	*ND*
C-2	3.17	20.5	6.96	*ND*	*ND*
C-3	3.19	20.8	6.75	*ND*	*ND*
C-C	3.55	21.5	6.94	*ND*	*ND*

t= 54.50

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.00	0.32	6.80	0.47	*ND*
A-2	0.07	10.3	6.15	0.23	*ND*
A-3	0.98	10.6	5.00	0.06	*ND*
A-C	0.33	20.0	6.48	0.03	*ND*
B-1	4.42	15.0	4.04	0.07	*ND*
B-2	0.38	10.6	6.14	0.14	*ND*
B-3	0.89	7.37	6.12	0.08	*ND*
B-C	1.25	20.6	6.35	0.04	*ND*
C-1	0.93	8.54	6.83	0.38	*ND*
C-2	0.00	4.43	6.89	0.55	*ND*
C-3	2.32	15.6	6.06	0.08	*ND*
C-C	3.00	18.2	6.37	0.05	*ND*

Enrichment Sequence VII (continued)

t= 56.54

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.00	0.22	5.54	*ND*	*ND*
A-2	0.49	2.38	5.59	*ND*	*ND*
A-3	0.48	2.11	4.79	*ND*	*ND*
A-C	0.00	17.0	5.90	*ND*	*ND*
B-1	5.42	12.4	2.10	*ND*	*ND*
B-2	0.26	3.74	5.64	*ND*	*ND*
B-3	0.42	0.76	5.63	*ND*	*ND*
B-C	0.90	18.9	5.58	*ND*	*ND*
C-1	0.46	1.84	6.37	*ND*	*ND*
C-2	0.13	0.49	6.35	*ND*	*ND*
C-3	0.89	12.13	5.25	*ND*	*ND*
C-C	3.33	16.5	5.58	*ND*	*ND*

t= 58.54

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.00	0.22	4.54	*ND*	*ND*
A-2	0.00	0.22	4.68	*ND*	*ND*
A-3	0.00	0.22	4.07	*ND*	*ND*
A-C	0.00	13.9	5.44	*ND*	*ND*
B-1	1.77	11.0	1.57	*ND*	*ND*
B-2	0.00	0.22	5.24	*ND*	*ND*
B-3	0.00	0.22	4.92	*ND*	*ND*
B-C	0.00	17.3	5.13	*ND*	*ND*
C-1	0.00	0.22	5.67	*ND*	*ND*
C-2	0.00	0.22	5.60	*ND*	*ND*
C-3	0.04	6.81	4.96	*ND*	*ND*
C-C	1.98	15.3	5.02	*ND*	*ND*

Enrichment Sequence XIV

t= 101.45

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	21.7	1.15	8.93	0.05	*ND*
A-2	23.2	0.71	8.86	0.04	*ND*
A-3	23.1	0.74	9.27	0.07	*ND*
A-C	24.7	0.51	10.0	0.02	*ND*
B-1	23.1	1.11	9.17	0.06	*ND*
B-2	23.6	0.61	9.05	0.05	*ND*
B-3	24.0	0.88	8.30	0.07	*ND*
B-C	24.2	0.34	9.85	0.01	*ND*
C-1	23.8	0.78	9.79	0.07	*ND*
C-2	22.9	0.98	9.82	0.10	*ND*
C-3	23.3	1.55	9.50	0.06	*ND*
C-C	24.6	0.61	10.0	0.02	*ND*

t= 103.45

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	5.33	2.31	5.95	0.06	*ND*
A-2	14.8	0.74	4.51	0.16	*ND*
A-3	14.5	3.26	6.17	0.04	*ND*
A-C	20.1	2.18	9.12	0.03	*ND*
B-1	11.7	7.41	6.08	0.16	*ND*
B-2	17.4	2.60	5.67	0.07	*ND*
B-3	18.7	3.55	4.68	0.06	*ND*
B-C	21.5	2.05	8.95	0.02	*ND*
C-1	14.4	4.40	8.24	0.09	*ND*
C-2	11.6	6.23	8.38	0.26	*ND*
C-3	12.6	7.41	7.80	0.06	*ND*
C-C	21.3	3.16	8.93	0.03	*ND*

Enrichment Sequence XIV (continued)

t= 105.54

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	1.94	0.81	4.05	0.04	*ND*
A-2	10.9	0.37	1.93	0.14	*ND*
A-3	9.56	2.26	3.72	0.03	*ND*
A-C	15.8	3.16	7.80	0.03	*ND*
B-1	6.78	7.03	4.09	0.09	*ND*
B-2	12.5	1.87	3.31	0.05	*ND*
B-3	13.9	4.19	3.27	0.05	*ND*
B-C	17.9	2.77	7.64	0.02	*ND*
C-1	8.48	5.05	6.86	0.05	*ND*
C-2	4.80	7.31	6.95	0.22	*ND*
C-3	8.77	7.03	6.40	0.04	*ND*
C-C	13.5	5.42	7.33	0.03	*ND*

t= 108.50

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.76	0.13	2.19	0.08	*ND*
A-2	4.20	0.08	0.07	0.06	*ND*
A-3	5.26	0.33	1.43	0.05	*ND*
A-C	12.2	3.61	6.50	0.02	*ND*
B-1	3.95	3.99	2.19	0.05	*ND*
B-2	9.60	0.43	1.04	0.04	*ND*
B-3	14.5	1.04	1.41	0.05	*ND*
B-C	15.2	3.41	6.32	0.02	*ND*
C-1	3.84	3.06	5.88	0.04	*ND*
C-2	0.34	4.47	6.20	0.23	*ND*
C-3	2.11	6.36	5.31	0.04	*ND*
C-C	11.3	6.36	5.84	0.03	*ND*

Enrichment Sequence XIV (continued)

t= 110.54

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.76	0.17	1.25	0.09	*ND*
A-2	1.75	0.11	0.12	0.06	*ND*
A-3	2.06	0.26	0.90	0.04	*ND*
A-C	8.94	3.83	5.80	0.03	*ND*
B-1	2.32	1.40	1.27	0.04	*ND*
B-2	6.18	0.32	0.43	0.03	*ND*
B-3	11.3	1.20	0.90	0.04	*ND*
B-C	11.7	4.34	5.57	0.01	*ND*
C-1	1.86	1.16	5.16	0.04	*ND*
C-2	0.21	0.72	5.74	0.15	*ND*
C-3	1.96	4.78	4.76	0.03	*ND*
C-C	7.26	8.06	5.07	0.02	*ND*

Enrichment Sequence XVI

t= 118.67

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	23.8	0.76	7.57	0.01	*ND*
A-2	23.2	0.70	7.36	0.06	*ND*
A-3	23.3	0.64	7.60	0.05	*ND*
A-C	23.9	0.33	8.93	0.01	*ND*
B-1	24.2	0.70	7.39	0.03	*ND*
B-2	24.1	0.45	8.01	0.11	*ND*
B-3	24.9	0.30	7.60	0.12	*ND*
B-C	25.0	0.27	8.61	0.01	*ND*
C-1	23.7	0.58	8.84	0.09	*ND*
C-2	23.7	0.58	8.92	0.10	*ND*
C-3	22.9	1.01	8.66	0.10	*ND*
C-C	24.8	0.76	8.77	0.01	*ND*

Enrichment Sequence XVI (continued)

		t= 126.45			
Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.25	0.18	0.66	0.21	7.16
A-2	3.03	0.15	0.00	0.10	7.30
A-3	12.6	0.06	0.00	0.04	7.23
A-C	18.0	2.02	7.45	0.02	7.36
B-1	15.9	0.09	0.00	0.05	7.12
B-2	9.36	0.55	0.54	0.07	7.38
B-3	22.7	0.35	0.00	0.07	7.72
B-C	19.6	2.19	7.37	0.02	8.32
C-1	5.43	0.81	7.49	0.11	7.98
C-2	2.30	6.39	8.11	0.15	7.86
C-3	1.66	6.72	6.79	0.07	8.33
C-C	12.8	7.54	6.83	0.02	8.34

Enrichment Sequence XVII

		t= 126.45			
Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	22.7	1.04	8.73	0.08	8.35
A-2	23.2	0.82	8.75	0.04	8.45
A-3	23.3	0.76	8.37	0.03	8.40
A-C	24.3	0.38	9.27	0.02	8.49
B-1	24.2	0.54	18.0	0.04	8.29
B-2	24.4	0.32	16.8	0.11	8.06
B-3	24.7	0.69	14.2	0.07	8.61
B-C	24.9	0.19	19.5	0.02	8.34
C-1	3.61	0.51	9.99	0.09	8.11
C-2	3.30	0.82	10.2	0.07	8.14
C-3	3.18	0.94	9.40	0.05	8.42
C-C	3.98	0.48	10.3	0.03	8.50

Enrichment Sequence XVII (continued)

t= 129.67

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	12.1	0.88	2.70	0.07	*ND*
A-2	13.9	1.12	2.70	0.11	*ND*
A-3	17.8	0.56	2.70	0.02	*ND*
A-C	19.6	2.63	7.97	0.01	*ND*
B-1	13.8	3.00	7.16	0.05	*ND*
B-2	18.4	0.53	7.16	0.06	*ND*
B-3	18.2	2.57	4.47	0.05	*ND*
B-C	20.9	1.93	16.7	0.01	*ND*
C-1	0.00	0.07	6.83	0.75	*ND*
C-2	0.00	0.07	7.45	0.20	*ND*
C-3	0.00	0.07	6.39	0.34	*ND*
C-C	0.55	2.51	7.80	0.02	*ND*

t= 131.42

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	7.07	0.39	1.49	0.05	*ND*
A-2	10.0	0.13	0.00	0.05	*ND*
A-3	14.5	0.21	0.20	0.03	*ND*
A-C	15.9	3.85	7.13	0.02	*ND*
B-1	9.53	1.14	4.74	0.04	*ND*
B-2	14.8	0.16	5.39	0.03	*ND*
B-3	17.0	1.17	2.57	0.05	*ND*
B-C	17.9	2.92	15.6	0.01	*ND*
C-1	0.45	0.08	6.41	0.64	*ND*
C-2	0.00	0.08	7.20	0.29	*ND*
C-3	1.25	0.08	5.97	0.58	*ND*
C-C	0.00	2.53	7.12	0.03	*ND*

Enrichment Sequence XVII (continued)

t= 133.54

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	3.46	0.46	0.33	0.05	6.79
A-2	5.73	0.15	0.00	0.05	6.89
A-3	11.1	0.13	0.00	0.04	7.07
A-C	13.1	4.53	6.33	0.03	7.42
B-1	7.11	0.49	2.63	0.07	7.55
B-2	12.1	0.18	3.30	0.05	7.43
B-3	15.4	0.75	0.91	0.08	7.29
B-C	15.3	3.81	13.7	0.02	8.04
C-1	0.16	0.08	5.88	0.46	7.75
C-2	0.41	0.08	6.42	0.22	7.48
C-3	0.65	0.08	5.08	0.32	8.06
C-C	0.64	1.56	6.44	0.04	8.16

Enrichment Sequence XVIII

t= 133.54

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	23.8	0.76	7.57	0.06	8.35
A-2	23.2	0.70	7.36	0.06	8.45
A-3	23.3	0.64	7.60	0.03	8.40
A-C	23.9	0.33	8.93	0.02	8.49
B-1	24.2	0.70	7.39	0.05	8.29
B-2	24.1	0.45	8.01	0.04	8.06
B-3	24.9	0.30	7.60	0.06	8.61
B-C	25.0	0.27	8.61	0.01	8.34
C-1	23.7	0.58	8.84	0.76	8.11
C-2	23.7	0.58	8.92	0.34	8.14
C-3	22.9	1.01	8.66	0.69	8.42
C-C	24.8	0.76	8.77	0.03	8.50

Enrichment Sequence XVIII (continued)

t= 140.69

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.76	0.15	1.57	0.28	7.75
A-2	1.71	0.18	0.00	0.11	7.70
A-3	15.0	0.12	0.00	0.03	7.65
A-C	20.4	1.93	7.54	0.02	7.44
B-1	28.7	0.21	0.05	0.03	7.80
B-2	14.8	0.59	3.89	0.12	7.52
B-3	26.2	0.24	0.00	0.05	7.78
B-C	22.3	1.22	8.10	0.02	8.04
C-1	5.42	0.86	6.91	0.29	7.84
C-2	1.45	8.61	6.80	0.07	7.55
C-3	0.73	7.12	5.83	0.22	8.06
C-C	20.1	3.41	7.53	0.03	8.13

Enrichment Sequence XIX

t= 140.69

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	23.8	0.76	7.57	0.06	8.35
A-2	23.2	0.70	7.36	0.06	8.45
A-3	23.3	0.64	7.60	0.03	8.40
A-C	23.9	0.33	8.93	0.02	8.49
B-1	24.2	0.70	7.39	0.05	8.29
B-2	24.1	0.45	8.01	0.04	8.06
B-3	24.9	0.30	7.60	0.06	8.61
B-C	25.0	0.27	8.61	0.01	8.34
C-1	23.7	0.58	8.84	0.76	8.11
C-2	23.7	0.58	8.92	0.34	8.14
C-3	22.9	1.01	8.66	0.69	8.42
C-C	24.8	0.76	8.77	0.03	8.50

Enrichment Sequence XIX (continued)

t= 150.45

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	0.49	0.19	2.66	0.59	7.96
A-2	0.49	0.19	0.07	0.51	7.95
A-3	21.3	0.22	0.00	0.03	7.78
A-C	16.3	3.96	8.23	0.02	8.09
B-1	15.2	0.92	0.00	0.03	7.76
B-2	6.84	1.27	2.49	0.23	7.71
B-3	27.1	0.28	0.00	0.02	8.00
B-C	21.2	2.03	8.61	0.02	8.10
C-1	4.07	0.78	7.65	0.26	7.91
C-2	0.28	3.38	7.82	0.36	7.73
C-3	1.12	0.75	6.92	0.20	8.24
C-C	11.4	9.53	7.27	0.03	8.09

Enrichment Sequence XX

t= 150.45

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	23.8	0.76	7.57	0.06	8.35
A-2	23.2	0.70	7.36	0.06	8.45
A-3	23.3	0.64	7.60	0.03	8.40
A-C	23.9	0.33	8.93	0.02	8.49
B-1	24.2	0.70	7.39	0.05	8.29
B-2	24.1	0.45	8.01	0.04	8.06
B-3	24.9	0.30	7.60	0.06	8.61
B-C	25.0	0.27	8.61	0.01	8.34
C-1	23.7	0.58	8.84	0.76	8.11
C-2	23.7	0.58	8.92	0.34	8.14
C-3	22.9	1.01	8.66	0.69	8.42
C-C	24.8	0.76	8.77	0.03	8.50

Enrichment Sequence XX (continued)

t= 158.54

Culture	NO_3^-	NO_2^-	NH_4^+	Mn	pH
A-1	1.13	0.20	2.04	0.08	7.91
A-2	5.36	0.20	0.00	0.05	7.93
A-3	18.1	0.20	0.00	0.04	7.83
A-C	17.2	0.92	7.83	0.03	8.06
B-1	5.98	0.65	0.02	0.04	7.60
B-2	16.3	0.24	1.60	0.05	7.84
B-3	23.8	0.20	0.00	0.02	7.90
B-C	19.9	0.42	8.55	0.02	8.07
C-1	7.29	0.27	7.10	0.22	7.99
C-2	5.88	2.88	6.70	0.12	7.68
C-3	5.78	0.90	6.34	0.08	8.34
C-C	20.3	0.24	7.83	0.03	8.13

APPENDIX IV

Time Series Plots

The following graphs are the time series plots for each series and control culture. These data are presented as mass in µmoles versus time in days of sequential enrichment sequences.

Figures A4-1 through A4-6. Control and Sediment Series Nitrate and Nitrite Mass vs. Time.

Mass in µmoles.

Time in days

Nitrate is in the upper graph and is represented by open black circles. Nitrite is in the lower graph and is represented by open red circles. The Roman numerals in green represent the enrichment sequence (see Appendix II). 0 days is the date of the first nutrient addition (Enrichment Sequence II). Negative values of time reflect a single addition of stock river water with no nutrients added (Enrichment Sequence I). Error bars are in black and represent one standard deviation of the mean of the three replicates within the sediment series.

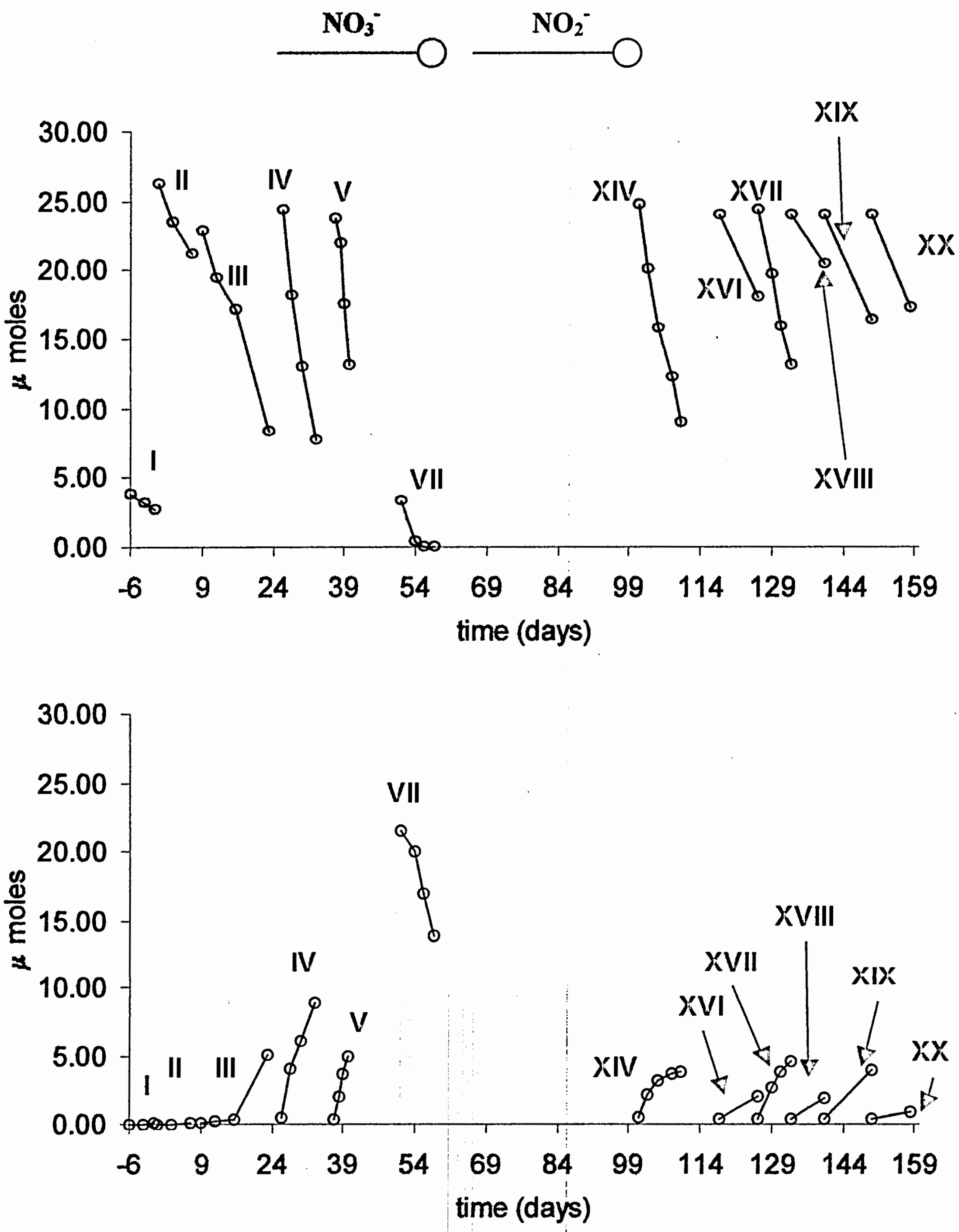


Figure A4-1: A-Control Nitrate (upper plot) and Nitrite (lower plot) Mass vs. Time

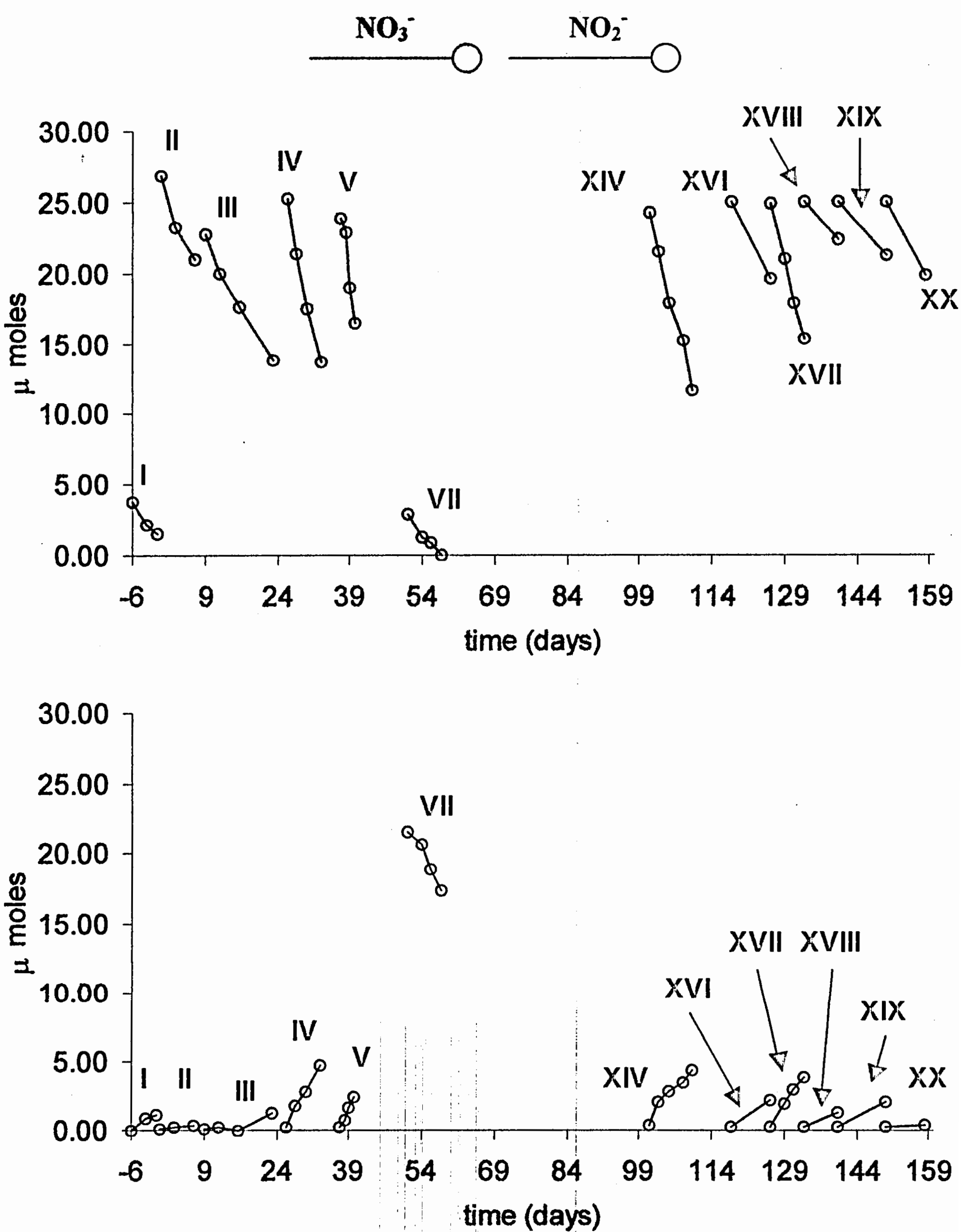


Figure A4-2: B-Control Nitrate (upper plot) and Nitrite (lower plot) Mass vs. Time

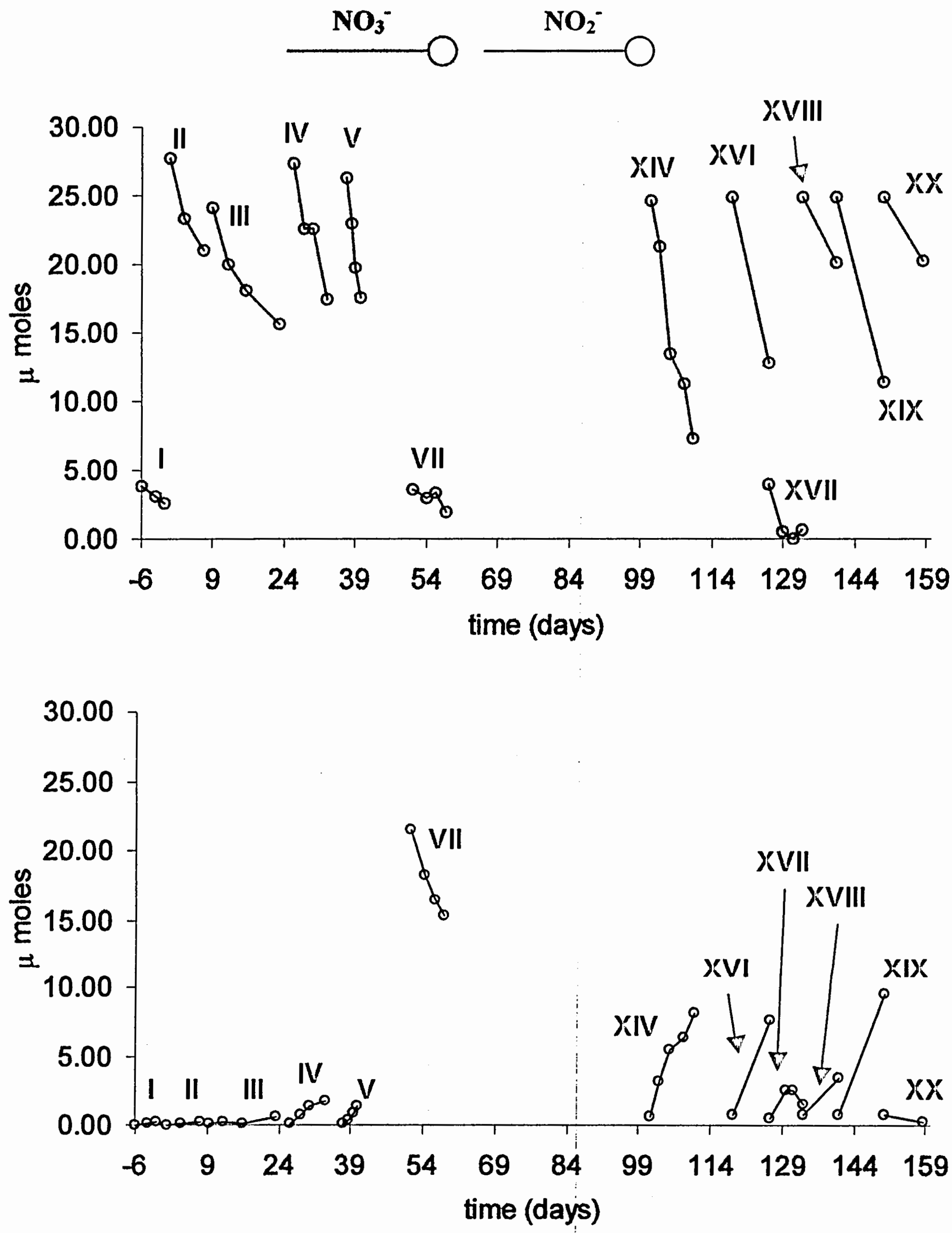


Figure A4-3: C-Control Nitrate (upper plot) and Nitrite (lower plot) Mass vs. Time

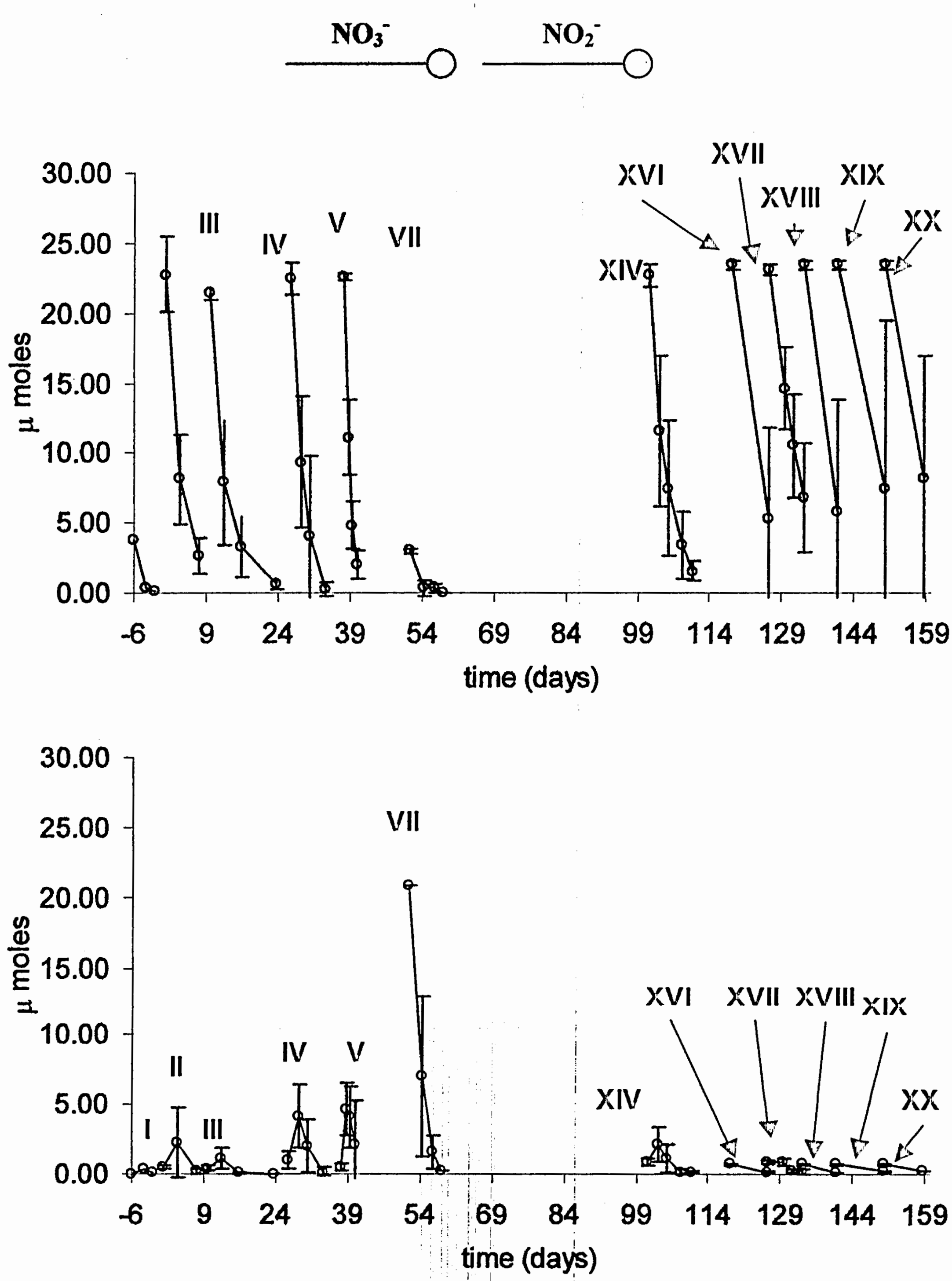


Figure A4-4: A-Series Average Nitrate (upper plot) and Nitrite (lower plot) Mass vs. Time

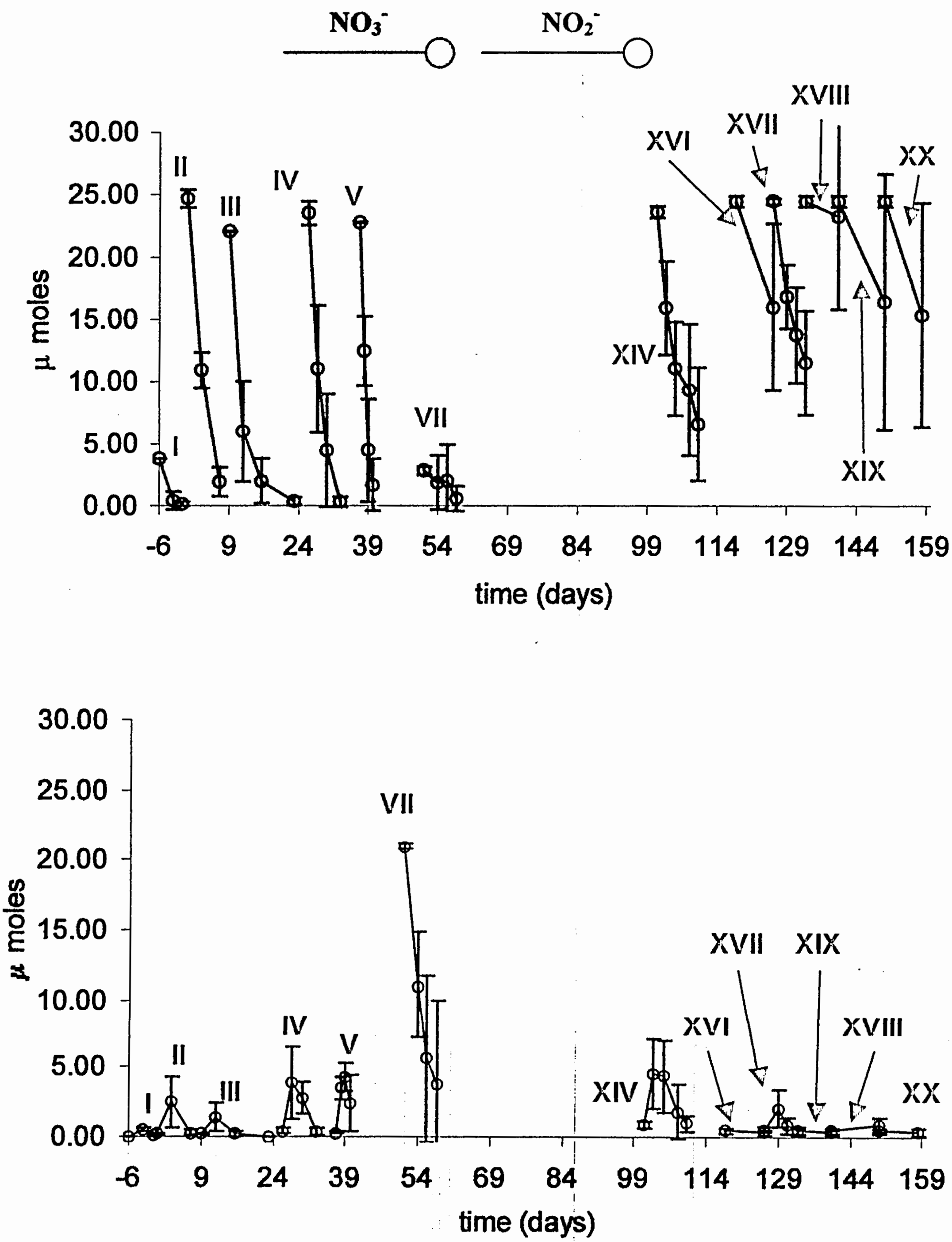


Figure A4-5: B-Series Average Nitrate (upper plot) and Nitrite (lower plot) Mass vs. Time

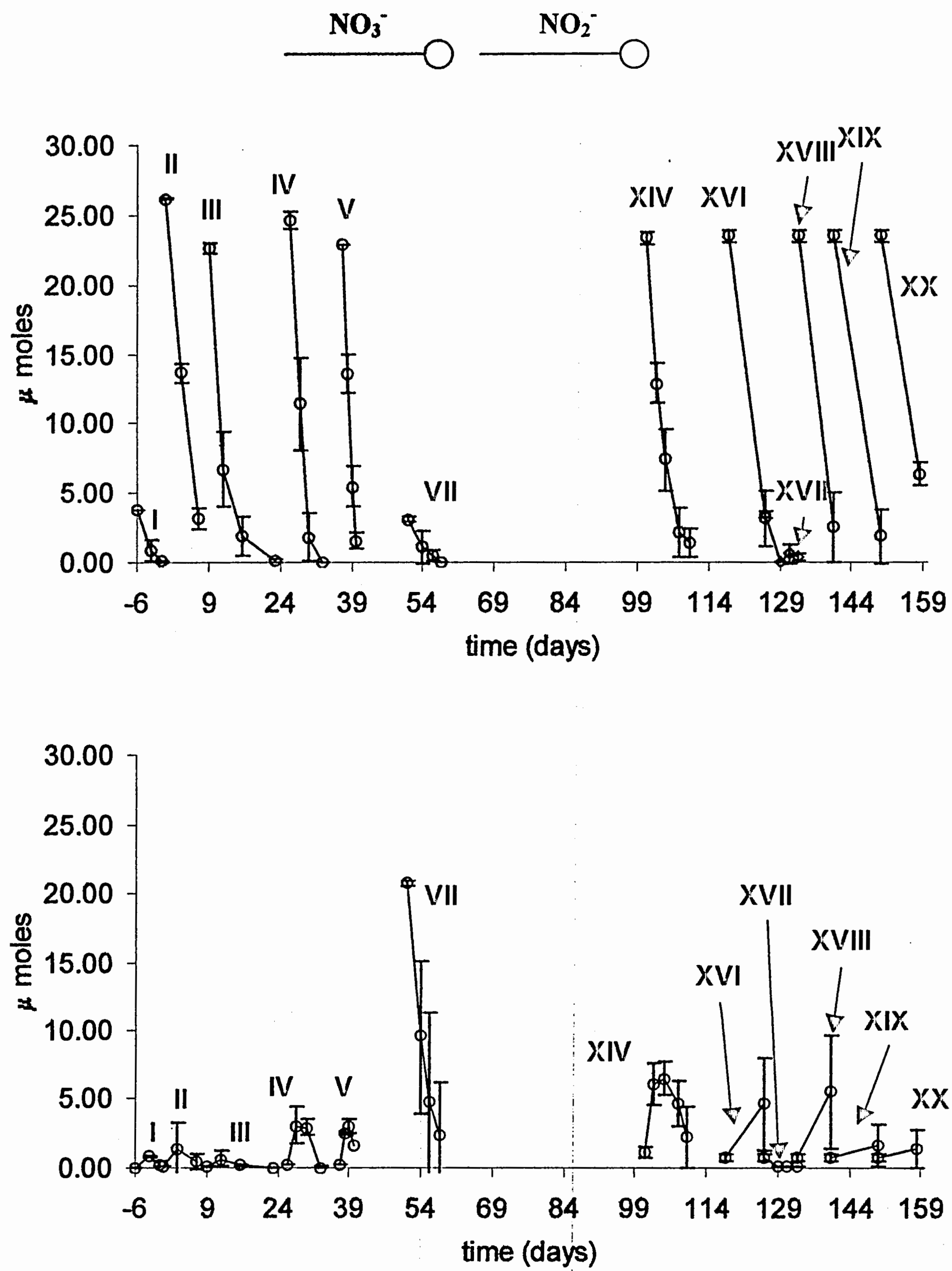


Figure A4-6: C-Series Average Nitrate (upper plot) and Nitrite (lower plot) Mass vs. Time

Figures A4-7 through A4-12. Control and Sediment Series Average Ammonium Mass vs. Time.

Mass in μmoles.

Time in days

Ammonium is represented by open blue circles. The Roman numerals in green represent the enrichment sequence (see Appendix II). 0 days is the date of the first nutrient addition (Enrichment Sequence II). Negative values of time reflect a single addition of stock river water with no nutrients added (Enrichment Sequence I). Error bars are in black and represent one standard deviation of the mean of the three replicates within each sediment series.

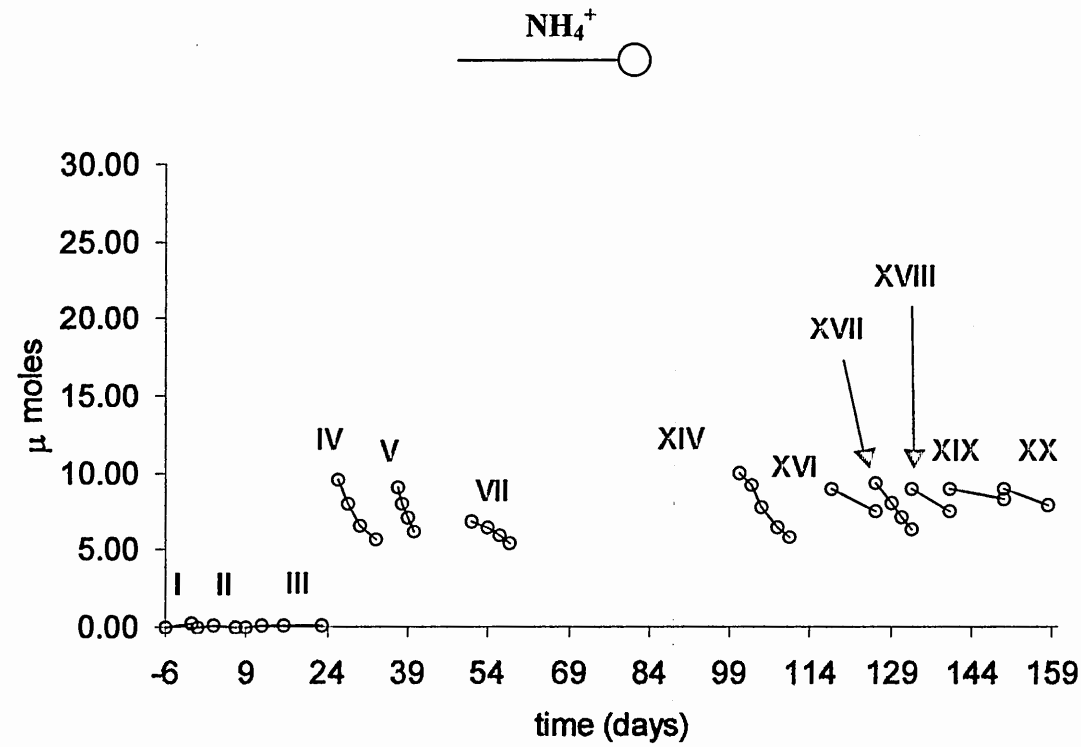


Figure A4-7: A-Control Ammonium Mass vs. Time

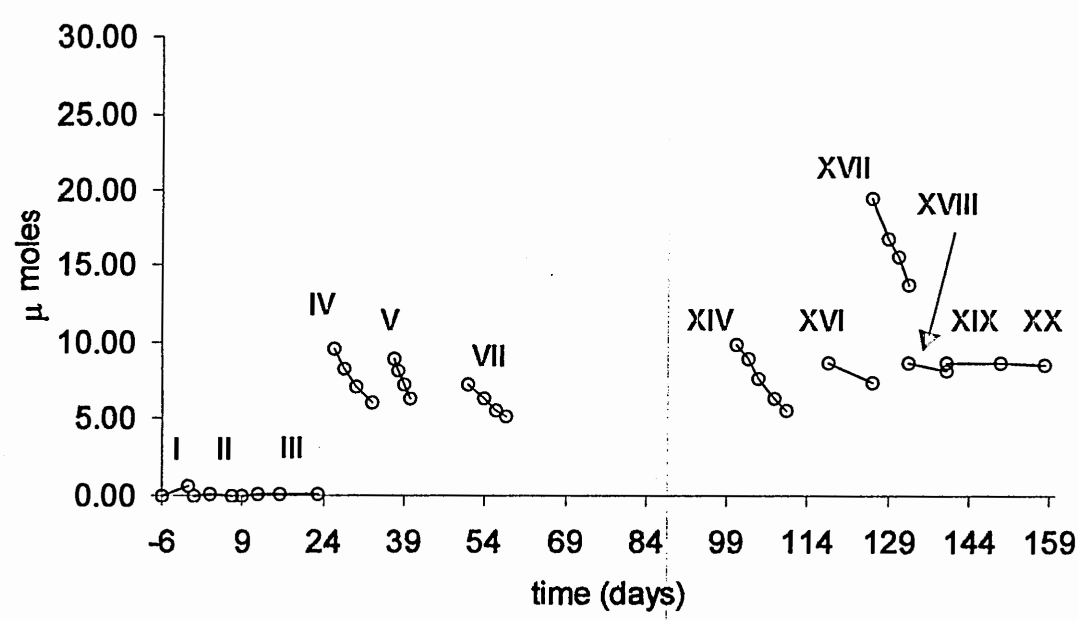


Figure A4-8: B-Control Ammonium Mass vs. Time

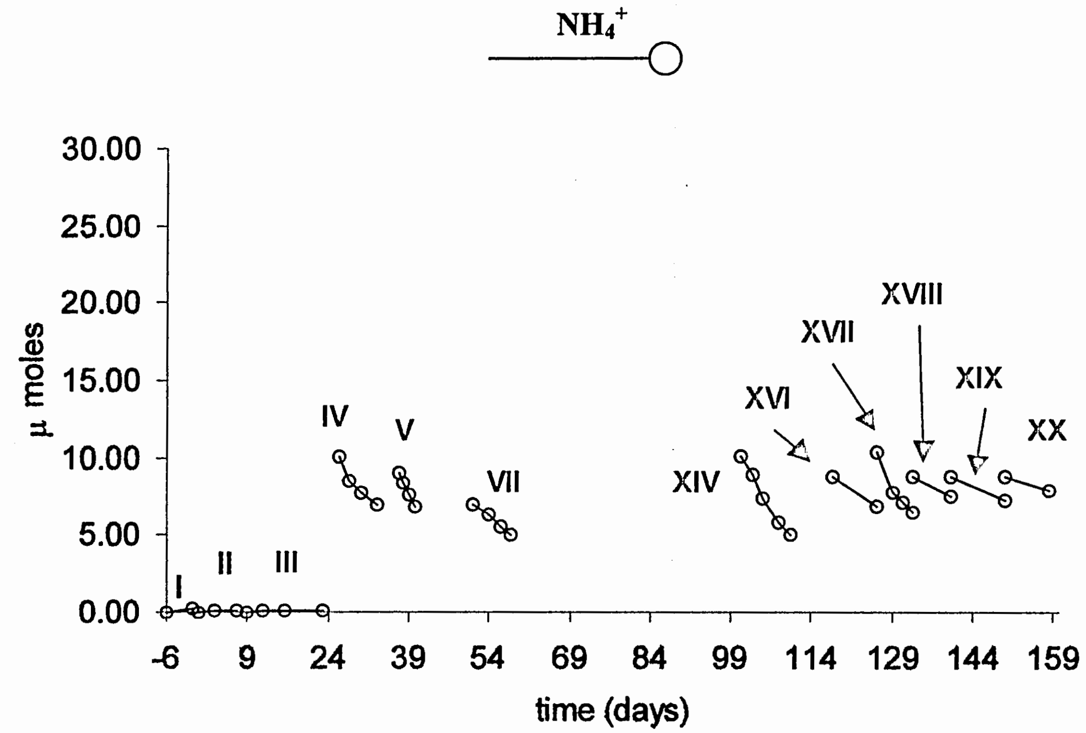


Figure A4-9: C-Control Ammonium Mass vs. Time

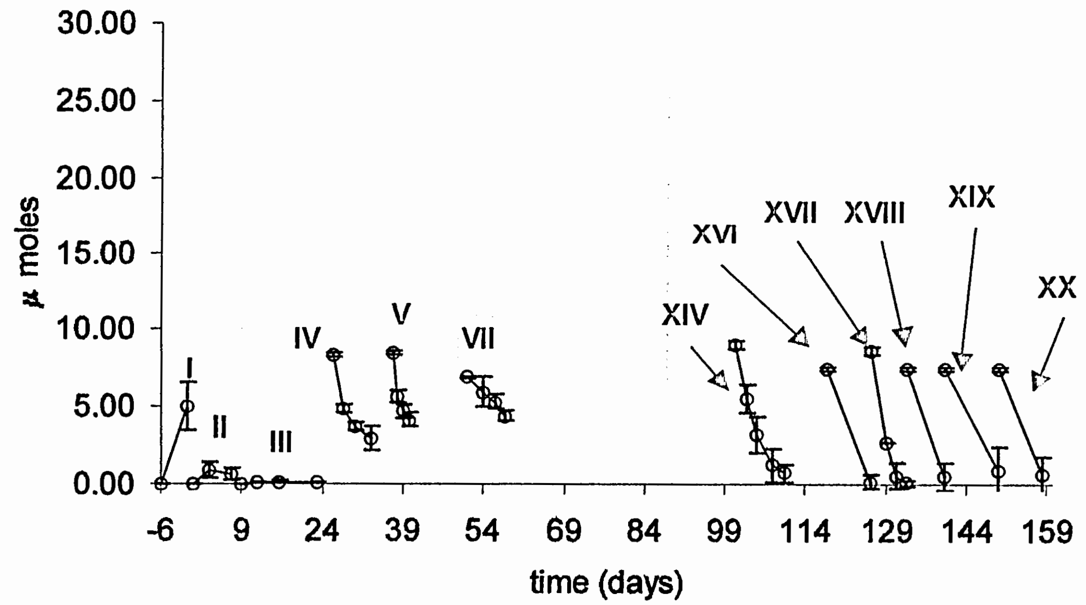
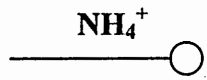


Figure A4-10: A-Series Ammonium Mass vs. Time



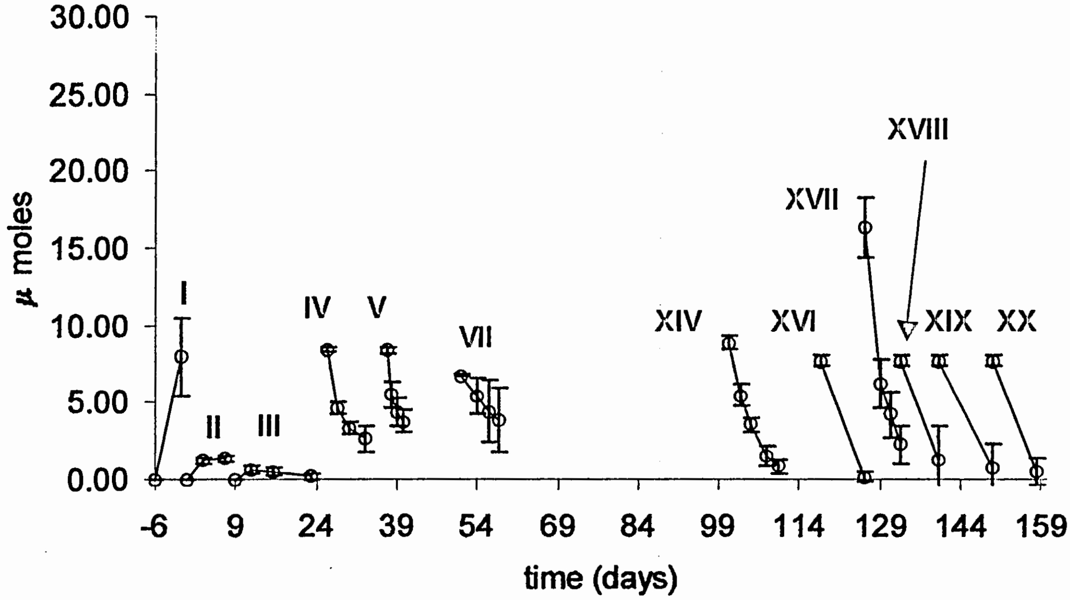


Figure A4-11: B-Series Ammonium Mass vs. Time

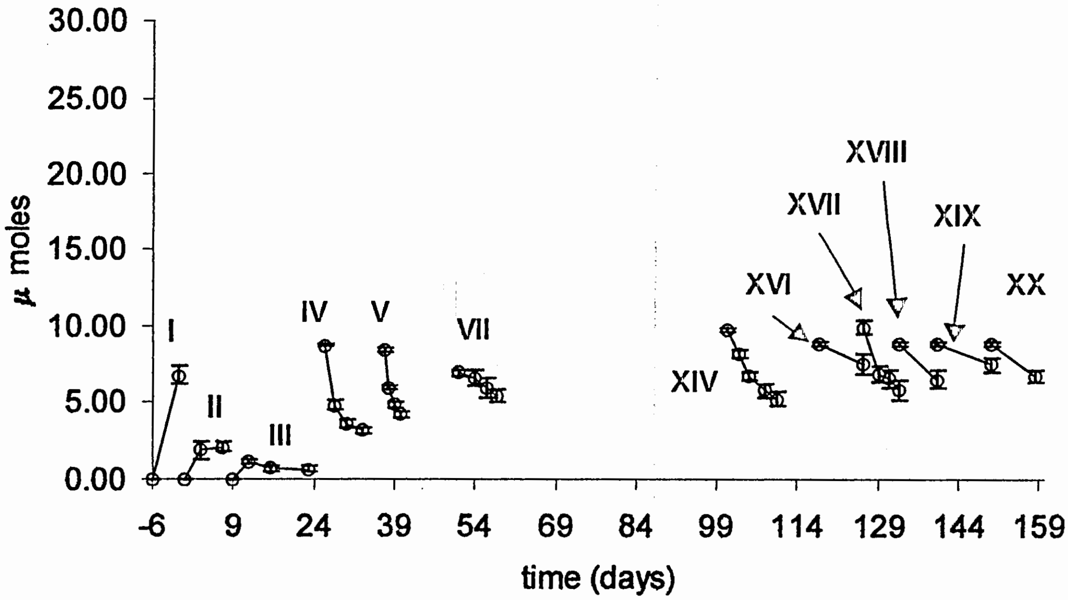


Figure A4-12: C-Series Ammonium Mass vs. Time

APPENDIX V

Sediment Data

Table A5-1 contains the data collected from Core 22 by the Loss on Ignition (LOI) method. Table A5-2 is sediment mass added to each sediment culture (g). Table A5-3 contains data collected by elemental analysis on culture sediment after the isotope tracer experiment.

Table A5-1: Core 22 Values. The values shown in this table were measured and/or calculated by the LOI method. A description of each measurement or calculation is found in Chapter 3, section 3.3.2.7.1.

cm Downcore	Wet Sediment Weight (g)	Mass of Water in sediment (g)
0.00	11.36	11.17
3.00	9.82	8.12
4.00	0.91	0.95
5.00	1.01	0.95
6.00	0.69	0.81
7.00	0.69	0.83
8.00	1.14	1.01
9.00	1.13	1.03
10.00	1.40	1.11

cm Downcore	Pre-Ignition Mass (mg)	Post-Ignition Mass (mg)
0.00	109.50	83.10
3.00	180.80	155.50
4.00	160.50	132.10
5.00	203.90	173.20
6.00	297.20	259.90
7.00	240.80	207.30
8.00	261.40	226.60
9.00	233.90	201.90
10.00	222.60	194.00

Table A5-1 continued

cm Downcore	Mass "Lost" (mg)	% Organic Matter
0.00	26.40	24.11
3.00	25.30	13.99
4.00	28.40	17.69
5.00	30.70	15.06
6.00	37.30	12.55
7.00	33.50	13.91
8.00	34.80	13.31
9.00	32.00	13.68
10.00	28.60	12.85

cm Downcore	% C_{dry}	% C_{wet}
0.00	10.13	0.10
3.00	5.88	0.11
4.00	7.43	1.31
5.00	6.32	1.28
6.00	5.27	2.26
7.00	5.84	2.03
8.00	5.59	1.28
9.00	5.75	1.19
10.00	5.40	0.86

cm Downcore	% Porosity (mL/cm^3)	Wet Bulk Density (g/cm^3)
0.00	96.89	1.05
3.00	88.79	1.17
4.00	54.11	1.69
5.00	49.88	1.75
6.00	44.78	1.83
7.00	44.88	1.83
8.00	51.79	1.72
9.00	54.16	1.69
10.00	53.68	1.69

Table A5-1 continued

cm Downcore	Dry Bulk Density (g/cm3)
0.00	2.65
3.00	2.65
4.00	2.65
5.00	2.65
6.00	2.65
7.00	2.65
8.00	2.65
9.00	2.65
10.00	2.65

Table A5-2: Wet and Dry Sediment Mass, Wet Sediment Volume added per culture

culture	$mass_{wet}$ (g)	$mass_{dry}$ (g)	Volume (cm^3)
A1	4.65	0.80	4.19
A2	4.91	0.84	4.42
A3	4.90	0.84	4.41
B1	4.25	3.24	2.41
B2	4.96	3.78	2.82
B3	6.23	4.74	3.54
C1	6.09	4.59	3.48
C2	4.68	3.53	2.67
C3	4.82	3.64	2.75

Table A5-3: Elemental Analyzer Nitrogen and Carbon Data. This data was collected from the sediment inocula after the 165-day incubation. "*" denotes data collected from sediments after cation exchange.

Sample	$Mass_{dry}$ Sediment Added (mg)	$\delta^{15}N$	µmoles N
A1	36.5	71.42	1.22
A2	29.5	5.76	1.06
A3	31.7	47.34	1.12
B1	27.3	26.8	1.24
B2	24.3	10.75	1.01
B3	32.5	18.79	1.41
C1	27.5	14.77	1.31
C2	34.3	146.97	1.58
C3	31.6	15.8	1.43
A1*	33.9	79.52	1.22
A2*	37.7	6.15	1.16
A3*	33.3	40.51	1.17
B1*	35.1	25.01	1.26
B2*	36.1	9.13	1.33
B3*	28	29.93	1.11
C1*	27.2	15.89	1.05
C2*	31.6	165.85	1.34
C3*	26.7	23.96	1.44

Sample	$\delta^{13}C$	µmoles C	C:N ratio
A1	-25.21	22.45	18.40
A2	-24.51	20.08	18.94
A3	-23.69	20.54	18.34
B1	-23.21	21.97	17.72
B2	-24.36	17.55	17.38
B3	-23.36	24.98	17.72
C1	-22.93	21.59	16.48
C2	-23.2	26.82	16.97
C3	-23.29	25.14	17.58
A1*	-25.2	24.99	20.48
A2*	-24.43	21.3	18.36
A3*	-24.11	22.14	18.92
B1*	-24.2	22.81	18.10
B2*	-25.04	25.04	18.83
B3*	-24.29	20.19	18.19
C1*	-24.14	18.32	17.45
C2*	-23.82	23.3	17.39
C3*	-24.13	22.6	15.69

APPENDIX VI

Headspace Data

Table A6-1 contains the data collected from the headspace of the cultures at the end of the isotope label experiment. Table A6-2 contains headspace volumes of each culture and the mass of ^{15}N found in each sediment culture. The quantitative nature of these data is uncertain because of the argon mass bias effect.

Table A6-1: Headspace Gas Analysis Data. Culture data shown in bold italics was collected from $^{15}NO_3^-$ amended cultures. All other cultures were amended with $^{15}NH_4^+$.

Sample	$\delta^{15}N$	Beam Area	μmoles N
C-C	-	-	-
B-C	***37.57***	***1.05E-07***	***13.57***
A-C	24.15	1.22E-07	16.00
A-3	***452.78***	***1.65E-07***	***22.14***
A-2	40.69	1.75E-07	23.57
A-1	***401.03***	***1.89E-07***	***25.57***
B-1	***774.02***	***1.74E-07***	***23.43***
B-2	29.4	5.02E-07	70.29
B-3	-	-	-
C-1	16.73	5.45E-07	76.43
C-2	***178.41***	***4.92E-07***	***68.86***
C-3	19.72	4.97E-07	69.57

Table A6-2: Culture Headspace Tracer Component. The mass ^{15}N found in the $^{15}NO_3^-$ and $^{15}NH_4^+$ amended control cultures are considered background values and have been subtracted from the values shown. Background concentrations subtracted from sediment cultures amended with $^{15}NO_3^-$ are 0.005 µM ^{15}N. Background concentrations subtracted from sediment cultures amended with $^{15}NH_4^+$ are 0.006 µM ^{15}N. Cultures shown in bold italics were amended with $^{15}NO_3^-$, all others were amended with $^{15}NH_4^+$.

Culture	$V_{headspace}$ (mL)	µmoles $^{15}N_2$ Tracer
C-C	***28.47***	ND
B-C	***28.53***	-
A-C	28.53	-
A-3	***27.39***	***0.185016958***
A-2	28.38	0.086089511
A-1	***28.54***	***0.23168371***
B-1	***28.84***	***0.296402793***
B-2	27.39	0.570889765
B-3	***28.81***	-
C-1	28.48	0.650347143
C-2	***28.51***	***0.712547931***
C-3	27.39	0.556493647

REFERENCES

Aller, R.C. (1990). Bioturbation and manganese cycling in hemipelagic sediments. *Phil. Trans. R. Soc. Lond.* **331**, 51-68.

Aller, R.C. (1994). The sedimentary Mn cycle in Long Island Sound: Its role as intermediate oxidant and the influence of bioturbation, O_2, and C_{org} flux on diagenetic reaction balances. *Journal of Marine Research.* **52**, 259-295.

Altabet, M.A., Higginson, M.J., Murray, D.W. (2002). The effect of millennial-scale changes in Arabian Sea denitrification on atmospheric CO_2. *Nature*. **415**, 159-162.

An, S., Gardner, W.S., Kana, T. (2001). Simultaneous Measurement of Denitrification and Nitrogen Fixation Using Isotope Pairing with Membrane Inlet Mass Spectrometry Analysis. *Applied and Environmental Microbiology*. **67**, 1171-1178.

An, S., Joye, S.B. (2001). Enhancement of coupled nitrification-denitrification by benthic photosynthesis in shallow estuarine sediments. *Limnology and Oceanography*. **46(1)**, 62-74.

Anschultz, P., Sundby, B., LeFrancois, L., Luther, G.W., Mucci, A . (2000). Interactions between metal oxides and species of nitrogen and iodine in bioturbated marine sediments. *Geochimica et Cosmochimica Acta*. **64**, 2751-2762.

Atlantic Mapping, Inc. (2000).Lake West Point. Marietta, Ga, Atlantic Mapping Inc.

Bender, M.L., Fanning, K.A., Froelich, P.N., Heath, G.R., Maynard, V. (1977). Interstitial Nitrate profiles and oxidation of sedimentary organic matter in the Eastern Equatorial Atlantic. *Science*. **198**, 605-609

Black, J. (1999). Microbiology. Upper Saddle River, New Jersey, Prentice Hall.

Boyd, P.W., Watson, A., Law, C., Abraham, E., Trull, T., Murdoch, R., Bakker, D., Bowie, A., Buesseler, K., Chang, H., Charette, M., Croot, P., Downing, K., Frew, R., Gall, M., Hadfield, M., Hall, J., Harvey, M., Jameson, G., LaRoche, J., Liddicoat, M., Ling, R., Maldonado, M., McKay, R., Nodder, S., Pickmere, S., Pridmore, R., Rintoul, S., Safi, K., Sutton, P., Strzepek, R., Tanneberger, K., Turner, S., Waite, A., Zeldis, J. (2000). A mesoscale phytoplankton bloom in the polar Southern Ocean stimulated by iron fertilization. *Nature*. **407**, 695-733.

Brandes, J.A., Devol, A.H. (1997). Isotopic fractionation of oxygen and nitrogen in coastal marine sediments. *Geochimica et Cosmochimica Acta.* **61,** 1793-1801.

Brewer & Spencer. (1970). Colorimetric determination of manganese in anoxic waters. *Limnology and Oceanography.* **16**, 107-110.

Capone, D.G., Zehr, J., Paerl, H., Bergman, B., Carpenter, E. (1997). *Trichodesmium*, a Globally Significant Marine Cyanobacterium. *Science.* **276,** 1221-1229.

Capone, D.G. (2001). Marine Nitrogen Fixation: what's the fuss?. *Current Opinion in Microbiology.* **4,** 341-348.

Casciotti, K.L., Ward, B.B. (2001). Dissimilatory Nitrite Reductase Genes from Autotrophic Ammonia-Oxidizing Bacteria. *Applied and Environmental Microbiology.* **67,** 2213-2221.

Chisholm, S.W. (2000). Stirring times in the Southern Ocean. *Nature.* **407,** 685-687.

Codispoti, L.A. (1995). Is the ocean losing nitrate? *Nature.* **376,** 724.

Codispoti, L.A. (1997). The limits to growth. *Nature.* **387,** 237-238.

Cocker, M.D. (1998). Distribution of Selected Elements in Stream Sediments, Stream Hydrogeochemistry, Lithogeochemistry, and Geology of the Chattahoochee River Basin, Georgia and Alabama. *Bulletin 128.* Atlanta, Georgia. Georgia Department of Natural Resources, Environmental Protection Division, Georgia Geologic Survey.

Craft, C.B., Seneca, E.D., Broome, S.W. (1991). Loss on ignition and Kjeldal digestion for estimating organic-Carbon and total Nitrogen in estuarine marsh soils – Calibration with dry combustion. *Estuaries.* **14** (2), 175-179.

Dalsgaard, T., Canfield, D., Petersen, J., Thamdrup, B., Acuna-Gonzalez, A. (2003). N_2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. *Nature.* **422,** 606-608.

DeLong, E.F. (2002). All in the packaging. *Nature.* **419,** 676-677.

Devol, A.H. (1991). Direct measurement of nitrogen gas fluxes from continental shelf sediments. *Nature.* **349,** 319-321.

Devol, A.H. (2002). Getting cool with nitrogen. *Nature.* **415,** 131-132.

Devol, A.H. (2003). Solution to a marine mystery. *Nature.* **422,** 575-576.

Dolfing, J. (1998). Comment on "Interactions of manganese with the nitrogen cycle: Atlternative pathways to dinitrogen". *Geochimica et Cosmochimica Acta.* **62**, 2217.

Egli, K., Fanger, U., Alvarez, P., Siegrist, H., van de Meer, J., Zehnder, A. (2001). Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. *Arch. Microbiology.* **175**, 198-207.

Falkowski, P.G. (1997). Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean. *Nature.* **387**, 272-275.

Falkowski, P.G., Barber, R.T., Smetacek, V. (1998). Biogeochemical Controls and Feedbacks on Ocean Primary Production. *Science.* **281**, 200-206.

Froelich, P.N., Klinkhammer, G., Bender, M., Luedtke, N., Heath, G., Cullen, D., Dauphin, P., Hammond, D., Hartman, B., Maynard, V. (1979). Early oxidation of organic matter in pelagic sediments of the eastern equatorial Atlantic: suboxic diagenesis. *Geochimica et Cosmochimica Acta.* **43**, 1075-1090.

Horne, A.J, Goldman, C.R. (1994). Limnology 2nd ed. New York, NY, McGraw Hill, Inc.

Hulth, S., Aller, R.C., Gilbert, F. (1999). Coupled anoxic nitrification/manganese reduction in marine sediments. *Geochimica et Cosmochimica Acta.* **63**, 49-66.

Jahnke, R.A. (1988). A Simple, Reliable, and Inexpensive Pore-Water Sampler. *Limnology and Oceanography.* **33**, 483 – 487.

Jenkins, M.C., Kemp, W.M. (1984). The coupling of nitrification and denitrification in two estuarine sediments. *Limnology and Oceanography.* **29(3)**, 609-619.

Jetten, M.S.M., Strous, M., van de Pas-Schoonen, K., Shalk, J., Dongen, U., van de Graaf, A., Logemann, S., Muyzer, G., van Loosdrect, M., Kuenen, J. (1999). The anaerobic oxidation of ammonium. *FEMS Microbiology Reviews.* **22**, 421-437.

Jetten, M.S.M., Horn, S.J., van Loosdrect, M.C.M. (1997). Towards a more sustainable municipal wastewater treatment system. *Water Science and Technology.* **35**, 171-180.

Joye, S.B., Hollibaugh, J.T. (1995). Influence of Sulfide Inhibition of Nitrification on Nitrogen Regeneration in Sediments. *Science.* **270**, 623-625.

Kana, T.M. Darkangelo, C., Hunt, M., Oldham, J., Bennett, G., Cornwell, J. (1994). Membrane Inlet Mass Spectrometer for Rapid High-Precision Determination of

N_2, O_2, and Ar in Environmental Water Samples. *Analytical Chemistry.* **66**, 4166-4170.

Kana, T.M., Sullivan, M., Cornwell, J., Groszkowski, K. (1998). Denitrification in estuarine sediments determined by membrane inlet mass spectrometry. *Limnol. Oceanogr.* **43**, 334-339.

Karl, D., Letelier, R., Tupas, L., Dore, J., Christian, J., Hebel, D. (1997). The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean. *Nature.* **388**, 533-538.

Koch, G., Egli, K., van de Meer, J., Siegrist, H. (2000). Mathematical Modeling of autotrophic denitrification in a nitrifying biofilm of a rotating biological contactor. *Water Science and Technology.* **41**, 191-198.

Kotiaho, T., Lauritsen, F., Choudhury, T., Cooks, G., Tsao, G. (1991). Membrane Introduction Mass Spectrometry. *Analytical Chemistry.* **63**, 875-883.

Kuypers, M.M.M., Sliekers, O., Lavik, G., Schmid, M., Jorgensen, B., Kuenen, J., Damste, J., Strous, M., Jetten, M. (2003). Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature.* **422**, 608-611.

Kuenen, J.G., Jetten, M.S.M. (2001). Extraordinary Anaerobic Ammonium-Oxidizing Bacteria. *ASM News.* **67**, 456-463.

Lesley, M.P. (2003). The Fluxes and Fates of Arsenic, Selenium, and Antimony from Coal Fired Power Plants to Rivers. Master's Thesis presented to the Academic Faculty, Georgia Institute of Technology.

Luther III, G.W., Sundby, B., Lewis, L., Brendel, P., Silverberg, N. (1997). Interactions of manganese with the nitrogen cycle: Alternative pathways to dinitrogen. *Geochimica et Cosmochimica Acta.* **61**, 4043-4052.

Luther III, G.W., Brendel, P., Lewis, B., Sundby, B., Lefrancois, L., Silverberg, N., Nuzzio, D. (1998). Simultaneous measurement of O_2, Mn, Fe, I^-, and S(-II) in marine pore waters with a solid-state voltammetric microelectrode. *Limnology and Oceanography.* **43**, 325-333.

Luther III, G.W., Popp, J.I. (2002). Kinetics of the Abiotic Reduction of Polymeric Manganese Dioxide by Nitrite: An Anaerobic Nitrification Reaction. *Aquatic Geochemistry.* **8**, 15-36.

Mackin, J.E., Aller, R.C. (1984). Ammonium adsorption in marine sediments. *Limnology and Oceanography.* **29**, 250-257.

Middelburg, J.J., Soetaert, K., Herman, P.M.J. (1996). Evaluation of the nitrogen isotope pairing method for measuring benthic denitrification. *Limnology and Oceanography*. **41**, 1839 – 1844.

Mulder, A., van de Graaf, A., Robertson, L., Kuenen, J. (1995). Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbial Ecology*. **16**, 177-184.

Murphy, J., Riley, J.P. (1962). A modified single solution method for the determination of phosphates in natural waters. *Anal. Chim. Acta*. **27**, 31-36.

Nielsen, L.P. (1992). Denitrification in sediment determined from nitrogen isotope pairing. *FEMS Microbiology Ecology*. **86**, 357 – 362.

Ogilvie, B., Nedwell, D., Harrison, R., Robinson, A., Sage, A. (1997). High nitrate, muddy estuaries as nitrogen sinks: the nitrogen budget of the River Colne estuary (United Kingdom). *Marine Ecology Progress Series*. **150**, 217-228.

Parsons, T.R., Maita, Y., Lalli, C.M. (1984). A Manual Method of Chemical and Biological Methods for Seawater Analysis. New York, NY, Pergamon Press.

Pilson, M.E.Q. (1998). An Introduction to the Chemistry of the Sea. Upper Saddle River, New Jersey, Prentice Hall.

Richards, F. A. (1965). In Chemical Oceanography. Riley, J.P., Skirrow, G. Eds. London, Academic Press. **1**, 611-645.

Risgaard-Petersen, N., Meyer, R., Enrich-Prasy, A., Revsbech, N. (1998). Simultaneous Measurement of benthic denitrification, with the isotope pairing technique and the N_2 flux method in a continuous flow-through system. *Water Research*. **32**, 3371 – 3377.

Robinson, D. (2001). $\delta^{15}N$ as an integrator of the nitrogen cycle. *TRENDS in Ecology & Evolution*. **16**, 153 – 162.

Rochelle-Newall, E.J., Fisher, T., Fan, C., Gilbert, P. (1999). Dynamics of chromophoric dissolved organic matter and dissolved organic carbon in experimental mesocosms. *Int. J. Remote Sensing*. **20**, 627-641.

Rochelle-Newall, E.J., Fisher, T.R. (2002). Production of chromophoric dissolved organic matter fluorescence in marine and estuarine environments: an investigation into the role of phytoplankton. *Marine Chemistry*. **77**, 7-21.

Rosenfeld, J.K. (1979). Ammonium adsorption in nearshore anoxic environments. *Limnology and Oceanography*. **24**, 356-364.

Sauders, D.L., Kalff, J. (2001). Denitrification rates in the sediments of Lake Memphremagog, Canada-USA. *Wat. Res.* **35**, 1897-1904.

Schalk, J., Oustad, H., Kuenen, J.G., Jetten, M.S.M. (1998). The anaerobic oxidation of hydrazine: a novel reaction in microbial nitrogen metabolism. *FEMS Microbiology Letters*. **158**, 61-67.

Schalk, J., de Vries, S., Kuenen, J.G., Jetten, M.S.M. (2000). Involvement of a Novel Hydroxlamine Oxidoreductase in Anaerobic Ammonium Oxidation. *Biochemistry*. **39**, 5405-5412.

Schmid, M., Twachtmann, U., Klien, M., Strous, M., Juretschko, S., Jetten, M., Metzger, J., Schleifer, K., Wagner, M. (2000). Molecular Evidence for Genus Level Diversity of Bacteria Capable of Catalyzing Anaerobic Ammonium Oxidation. *Systematic and Applied Microbiology*. **23**, 93-106.

Siegrist, H., Reithaar, S., Koch, G., Lais, P. (1998). Nitrogen loss in a nitrifying rotating contactor treating ammonium-rich wastewater without organic carbon. *Water Science and Technology*. **38**, 241-248.

Seitzinger, S.P., Nixon, S., Pilson, M. (1984). Denitrification and nitrous oxide production in a coastal marine ecosystem. *Limnology and Oceanography*. **29** (1), 73-83.

Seitzinger, S.P., Neilsen, L., Caffrey, J., Christensen, P. (1993). Denitrification measurements in aquatic sediments: A comparison of three methods. *Biogeochemistry*. **23**, 147-167.

Seitzinger, S.P., Giblin, A.E. (1996). Estimating denitrification in North Atlantic continental shelf sediments. *Biogeochemistry*. **35**, 235-260.

Sigman, D.M., Altabet, M.A., Michener, R., McCorkle, D.C., Fry, B., Holmes, R.M. (1997). Natural abundance-level measurement of the nitrogen isotopic composition of oceanic nitrate: an adaptation of the ammonia diffusion method. *Marine Chemistry*. **57**, 227-242.

Sliekers, A.O., Derwort, N., Campos Gomez, J., Strous, M., Kuenen, J., Jetten, M. (2002). Completely autotrophic nitrogen removal over nitrite in one single reactor. *Water Research*. **36**, 2475-2482.

Strous, M., van Gerven, E., Kuenen, J.G., Jetten, M. (1997). Effects of Aerobic and Microaerobic Conditions on Anaerobic Ammonium-Oxidizing (Anammox) Sludge. *Applied and Environmental Microbiology*. **63**, 2446-2448.

Strous, M., van Gerven, E., Zheng, P., Kuenen, J., Jetten, M. (1997). Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation (anammox) process in different reactor configurations. *Water Research*. **31**, 1955-1962.

Strous, M., Heijnen, J., Kuenen, J., Jetten, M. (1998). The sequencing batch reactor as a powerful tool for the study of slow growing anaerobic ammonium-oxidizing microorganisms. *Applied Microbiol. Biotechnology*. **50**, 589-596.

Strous, M., Kuenen, J.G., Jetten, M.S.M. (1999). Key Physiology of Anaerobic Ammonium Oxidation. *Applied and Environmental Microbiology*. **65**, 3248-3250.

Strous, M., Fuerst, J., Kramer, E., Logemann, S., Muyzer, G., van de Pas-Schoonen, K., Webb, R., Kuenen, J., Jetten, M. (1999). Missing lithotroph identified as new planctomycete. *Nature*. **400**, 446-449.

Stumm, W., Morgan, J.J. (1996). Aquatic Chemistry. New York, NY, John Wiley & Sons, Inc.

Technicon Instrument Corp. (1972). Method #158/71W. Tarrytown, NY.

Thamdrup, B., Dalsgaard, T. (2000). The fate of ammonium in anoxic manganese oxide-rich marine sediment. *Geochimica et Cosmochimica Acta*. **64**, 4157-4164.

Thamdrup, B., Dalsgaard, T. (2002). Production of N_2 through Anaerobic Ammonium Oxidation Coupled to Nitrate Reduction in Marine Sediments. *Applied and Environmental Microbiology*. **68**, 1312-1318.

van de Graaf, A.A., Mulder, A., de Bruijn, P., Jetten, M., Robertson, L., Kuenen, J. (1995). Anaerobic Oxidation of Ammonium is a Biologically Mediated Process. *Applied and Environmental Microbiology*. **61 (4)**, 1246-1251.

van de Graaf, A.A., de Bruijn, P., Robertson, L., Jetten, M., Kuenen, J. (1997). Metabolic pathway of anaerobic ammonium oxidation on the basis of ^{15}N studies in a fluidized bed reactor. *Microbiology*. **143**, 2415-2421.

van de Pas-Schoonen, K., van Dongen, U., Yildirim, E., Overeijnder, M., Schmid, M., Wagner, M., Strous, M., Kuenen, J., Jetten, M. (2000). Molecular identity and survey of anaerobic ammonium-oxidizing bacteria. Proceedings of the 4th International Symposium on Biotechnology. European Federation of Biotechnology.

Verstraete, W., Philips, S. (1998). Nitrification-denitrification processes and technologies in new contexts. *Environmental Pollution*. **102**, 717-726.

Zehr, J.P., Ward, B.B. (2002). Nitrogen Cycling in the Ocean: New Perspectives on Processes and Paradigms. *Applied and Environmental Microbiology.* **68**, 1015-1024.