ASSESSING THE ROLE OF ANAMMOX IN A NITROGEN CONTAMINATED AQUIFER

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Chapter 1

The Anammox tale: An in-depth review of the ecology, morphology and ecological impact of anaerobic ammonium oxidation
INTRODUCTION

The Nitrogen Cycle

Nitrogen is an essential element that exists in up to 9 different oxidation states, utilizes several different environmental transport/storage pathways and contains many ways to change species (See review: Jetten 2009). In order to achieve every oxidation state, the nitrogen must pair with an atom of oxygen, hydrogen, or other nitrogen. This flexibility of the nitrogen atom allows for many combinations of unique molecules that represent every oxidation state. Different nitrogen species are used by organisms generating cellular energy by serving as an electron donor or acceptor in metabolic pathways. Nitrogen is then assimilated as a macronutrient, which is evident in the reduced, basic chemical formula of an organism, CH$_2$O$_{0.5}$N$_{0.15}$ (Review: Jetten 2009).

The global nitrogen cycle operates by keeping a smaller, fixed/biologically available pool of nitrogen in constant flux with the monstrous amount of atmospheric nitrogen (in the N$_2$ form). The flux and balance of this system controls the amount of fixed nitrogen in marine and terrestrial ecosystems. This delicate balance is important for many reasons, including the role of nitrogen as a limiting nutrient in primary production (Vitousek and Howarth, 1991).

The nitrogen cycle is mainly composed of five catabolic processes (nitritification, nitrification, denitrification, dissimilatory nitrate reduction to ammonia (DNRA) and anaerobic ammonium oxidation (anammox), three anabolic processes (ammonium uptake, assimilatory nitrate reduction and nitrogen fixation), and ammonification (Figure 1).
Figure 1. Nitrogen cycling in a marine ecosystem. (Francis et al., 2007).
Although nitrogen makes up 78% of our atmosphere, most is unavailable to organisms due to the strong triple bond that holds the two N atoms together. In order to be able to utilize this N, it must be fixed. N fixation can either occur abiotically by lightning or biologically by certain microorganisms such as some bacteria, actinomycetes, cyanobacteria and blue-green algae. One of the biomarkers for this process is the \textit{nifH} gene in microorganisms which codes for the reductase component of nitrogenase, the enzyme necessary to fix N (Zehr et al., 2007). The nitrogenase enzyme complex is inhibited by oxygen, so many N fixing bacteria utilize a symbiotic relationship with plant roots that contain the oxygen-molecule stealing leghaemoglobin to create a low-oxygen environment (Herridge et al., 1995). Once the N is biologically fixed, the new organic N can be mineralized to ammonium by the process of ammonification. The nitrogen is now in an inorganic state. Plants may assimilate the ammonium to turn back into organic nitrogen for cellular processes (Herridge et al., 1995).

Nitrification is an important piece of nitrogen speciation, transport, and removal in soils and watersheds (Hill & Shackleton, 1989; Fisk & Fahey, 1990). During the process of nitrification, ammonium is converted to nitrate. This is a two-step process that involves two different types of bacteria, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). The AOB convert ammonium to nitrite in an aerobic (though not always) environment. The two dominant bacteria genera that carry out the first step in the nitrification process are \textit{Nitrosospira} and \textit{Nitrosomonas}, both betaproteobacteria. Certain Gammaproteobacteria like the \textit{Nitrosococcus} can also oxidize ammonium, but are usually found in marine systems (Teske et al., 1994). Recently it was discovered that Archaea can also oxidize ammonium (AOA). Two of the main enzymes in this process are ammonia monoxygenase (AMO) which catalyzes the
oxidation of ammonium to hydroxylamine, and hydroxylamine oxidoreductase (HAO) which oxidizes hydroxylamine to nitrite.

The second half of the nitrification process is mediated by NOB, which oxidize the intermediate nitrite to nitrate. *Nitrobacter* spp. are the main genus thought to carry out the nitrite oxidation process. Nitrite oxidoreductase (NOR) is the enzyme responsible for the oxidation of nitrite (Meincke et al., 2004).

When oxygen is not present, nitrate becomes a very attractive electron acceptor for nitrate respiring microorganisms. The process of the dissimilatory reduction of nitrate is called denitrification, a very important process in the global geochemical nitrogen cycle. The process is utilized by bacteria, Archaea, and even fungi (Kobayashi et al., 1996). The nitrate respiration begins with the reduction of nitrate to nitrite, using the enzyme nitrate reductase (*nar*). The next stage is the reduction of nitrite to nitric oxide, using the enzyme nitrite reductase (*nor*). The next step is the reduction of nitric oxide to nitrous oxide. This reduction is interesting because the enzyme that catalyzes this reaction, nitric oxide reductase (*nor*), is the enzyme that creates formation of the dinitrogen bond between the two nitrogen atoms of nitrous oxide (Bothe et al., 2000). Nitrous oxide is not always reduced to dinitrogen gas by all denitrifiers because not all denitrifiers possess the ability for that last reduction step (Bothe et al., 2000). However, if the denitrifier does possess the nitrous oxide reductase gene (*nos*), the final step of denitrification is complete and the dinitrogen gas is released to the atmosphere as it is not biologically available. The denitrification process is normally carried out by heterotrophic bacteria such as *Paracoccus denitrificans* and various pseudomonads (Carlson & Ingraham 1983).
Dissimilatory reduction of nitrate to ammonium (DNRA) process occurs in low oxygen settings similar to denitrification, and both processes can even compete for available nitrate. This process is mediated by prokaryotes carrying the \textit{nrfA} gene. (See review: Simon 2002). One of the main concerns about DNRA is that NO$_3^-$ is transformed into another mineral N form which is less mobile and may conserve N in the ecosystem, unlike denitrification (Buresh and Patrick, 1978; Tiedje, 1988). King and Nedell (1985) determined that DNRA may occur in areas with elevated concentrations of organic electron donors and low nitrate. The ratio between the available electron donors and electron acceptors is an important factor to determine if DNRA or denitrification is a dominant (Tiedje et al., 1982; Smith and Zimmerman, 1981) as DNRA, anammox, and denitrification are competing for the same oxidants (Payne 1973; Burgin and Hamilton, 2007). When simply comparing the potential free energy of total denitrification (-2669 kJ mol$^{-1}$ glucose) and DNRA (-1796 kJ mol$^{-1}$ glucose), denitrification should be favored over DNRA (Gottschalk, 1986). However, in certain ecosystems such as anoxic sediments with sulfidic conditions (Christensen et al., 2000), DNRA has the advantage over denitrification since more electrons can be transferred per mole of nitrate (Tiedje et al., 1982). There are several genera of soil bacteria capable of DNRA, which are either obligate anaerobes (\textit{Clostridium}), facultative anaerobes (\textit{Citrobacter, Enterobacter, Erwinia, Escherichia, Klebsiella}) or aerobes (\textit{Bacillus, Pseudomonas}) (Tiedje et al., 1988).

The last process of the N cycle is another form of dinitrogen gas production, where dinitrogen is created through the anaerobic oxidation of ammonium (anammox) (Van de Graaf et al., 1995; Jetten et al., 2001; Dalsgaard et al., 2003; Kuypers et al., 2003). The anammox bacteria are members of \textit{Planctomycetes} phylum in which 5 genera have been described. Anammox bacteria utilize a unique organelle like structure lined with ladderane lipids called the
anammoxosome to carry out the anammox process using the volatile hydrazine as an intermediate. The anammox process is important because it does not create nitrous oxide as a byproduct, unlike denitrification (Jetten 2008).

ANAMMOX UNRAVELED

History

Several decades before the discovery of anammox bacteria, Austrian theoretical chemist Engelbert Broda (1977) put forth the idea that organisms could be capable of oxidizing ammonium by using nitrite or nitrate as an electron acceptor. Richards (1965) even demonstrated the mysterious and unexplainable loss of ammonium in anoxic fjords a decade before Broda’s prediction. In 1995, Mulder et al. discovered the same phenomena in a bioreactor in the Netherlands. It was not until 1999 that Strous et al., (1999) purified anammox cells from an enrichment culture and the anammox bacteria examination could begin in earnest. The first discovered anammox bacterium belongs to the order of Planctomycetales (Strous et al., 1999) and was named Candidatus Brocadia anammoxidans. At the time of this review, five anammox genera have been described, with 16S rRNA gene sequence identities of the species ranging between 87 and 99% (Jetten et al., 2009).

Even with as few as 87% identity to one another in some anammox bacteria, the organisms still exist in the same monophyletic cluster (Brocadiales) and the same order (Planctomycetales). The anammox branch lies inside the phylum Planctomycetes (Strous et al.,
1999; Schmid et al., 2005; 2007) (figure 2). Four “Candidatus” anammox genera have been enriched from bioreactors: “Kuenenia” (Schmid et al., 2000; Strous et al., 2006), “Brocadia” (Strous et al., 1999; Kuenen and Jetten., 2001; Kartal et al., 2008), “Anammoxoglobus” (Kartal et al., 2007b) and “Jettenia” (Quan et al., 2008). The last anammox genus, “Candidatus Scalindua” (Kuypers et al., 2003; Schmid et al., 2003; van de Vossenberg et al., 2008), has often been detected in marine sediments and oxygen minimum zones (Dalsgaard et al., 2005; Schmid et al., 2007). Within the genus “Scalindua”, two marine molecular isolates have been identified.
Figure 2. The 5 genera of anammox bacteria within the planctomycetes phylum (Song, Unpublished)
**Morphology and functionality**

Since their discovery anammox bacteria have been known as slow-growers, with a division time of once every 11-20 days (Strous et al., 1999). *Escherichia coli*, for example, divide once every 20 or so minutes. The anammox bacteria are obligate anaerobes and cannot tolerate oxygen conditions above 2 μM (Strous et al., 1999). With such a slow growth and low oxygen tolerance, it is quite difficult to culture the organisms, but it is possible to grow an enrichment culture of the microbes with the sequencing batch reactor (SBR) method (Strous et al., 1999). This type of reactor can keep a stable batch for more than a year.

Anammox bacteria usually gain their energy from the 1:1 chemolithotrophic conversion of ammonia and nitrite to dinitrogen gas. The overall reaction includes ammonium being oxidized by nitrite to form water and dinitrogen gas (Strous et al., 1999). The free energy of this reaction is -357 kJ/mol (Strous et al., 1999). This is a thermodynamically favorable reaction and is more favorable than the aerobic oxidation of ammonium (Strous et al., 1999). However, the metabolic rate is relatively low, which could account for the slow growth rate (Jetten et al., 2009). Anammox bacteria are known for having a high affinity for their substrates, and can grow at concentrations of less than 5μM (Strous et al., 1999).

Strous et al., (2006) was able to piece together the genome of *Candidatus “Kuenenia stuttgartiensis”* in order to further understand the anammox metabolic processes and genes. These genes include a cd1 type nitric oxide: nitrite oxidoreductase (*nirS*), and nine paralogues of hydroxylamine/hydrazine oxidoreductase (*hao/hzo*) (Hooper et al., 1997). The stepwise combination of ammonium and nitric oxide to form hydrazine gene has yet to be found, but a “hydrazine hydrolase” later to be “hydrazine synthase” enzyme is proposed to combine
ammonium with nitric oxide to form hydrazine (Jetten et al., 2009; Kartal et al., 2011). The current mechanism for the anammox reaction begins with nitrite being reduced to nitric oxide through *nirS*. The second step would be the reduction of NO and its concurrent condensation with ammonium to produce N$_2$H$_4$ (Hydrazine). The next step is the oxidation of hydrazine to dinitrogen gas, which produces four electrons and four protons and thus creates the proton motive force across the anammoxosome membrane (Jetten et al., 2009; Kartal et al., 2011). Recently hydrazine synthase (*hzs*), has been used as a biological marker for anammox discovery and is an integral aspect of the anammox process. *hzs* catalyzes the synthesis of hydrazine from nitric oxide and ammonium.

Anammox bacteria have many extraordinary and unique features that separate this organism from other bacteria, even other *Planctomycetes*. Anammox have a unique cell plan and unique lipids that have been recently studied in the past decade. Anammox bacteria have a unique intracellular structure called the “anammoxosome” (figure 3). This compartment occupies most of the cell by volume. However, this cell plan is not exclusive to anammox bacteria and is similar to other members of the phylum *Planctomycetes* (Kartal et al., 2008). Members of the *Planctomycetes* have intracellular membranes and complex compartmentalization in comparison to a typical bacterium (Fuerst, 2005). The cytoplasm in anammox bacteria is thus divided into three cytoplasmic compartments separated by single bilayer membranes; the outer most is called the “paryphoplasm”, similar to the periplasm in Gram-negative bacteria. They are different in that the periplasm is outside the cytoplasmic membrane and the paryphoplasm is inside the cytoplasmic membrane (Lindsay et al., 2001). In most planctomycetes, the innermost cellular compartment is the “riboplasm.” This compartment contains the nucleoid and the ribosomes and is therefore the center of DNA replication, translation, and transcription (Strous et al., 1999;
Lindsay et al., 2001). When dealing with anammox bacteria however, the third compartment is the anammoxosome, which is the proposed location of the anammox reaction (van Niftrik et al., 2008a; 2008b).

Anammox bacteria contain lipids in the previously aforementioned anammoxosome membrane that are unique to anammox and not found elsewhere in nature. These “ladderane” lipids consist of hydrocarbon chains with linearly concatenated cyclobutane rings with the ladderanes in a cyclohexane ring. The concatenated cyclobutane ring systems are unique in nature. These ladderane lipids have ester-linkages and ether-linkages, which are interesting because ether-linkages are were thought to be exclusive to Archaea while ester-linkages are exclusive to bacteria and eukaryotes (Sinninghe Damsté et al., 2002). Based on molecular modeling, the ladderane lipids were described as tightly-packed in the anammoxosome (Sinninghe Damsté et al., 2002). This unusually high density prevents this membrane from being permeable to apolar compounds. Because the metabolism of anammox bacteria involves gaseous molecules and the toxic intermediate hydrazine, this tightly packed membrane may allow the anammoxosome to minimize the loss of the substrates (Jetten et al., 2009).
Figure 3: Vertical cross-section of the anammox cell and anammoxosome (Kuypers et al., 2003).
**Ecology**

**Molecular detection methods** Many methods are available for the detection of anammox bacteria and their activity in the natural environment and man-made ecosystems (Risgaard-Petersen et al., 2003; Schmid et al., 2005). Anammox have been detected in many environments, including: marine sediments (Dalsgaard and Thamdrup, 2002; Rich et al., 2008), oxygen-minimum zones (Dalsgaard et al., 2003; Kuypers et al., 2003; 2005), freshwater marshes (Penton et al., 2006), permafrost (Penton et al., 2006), rivers (Zhang et al., 2007), river estuaries (Risgaard-Peterson et al., 2004; Dale et al., 2009). PCR amplification with non-specific 16S rRNA gene-targeted primers followed by phylogenetic analysis is a common technique to discover the identity of unknown organisms in environmental samples. However, anammox bacteria may not be fairly represented in general 16S rRNA gene clone libraries since the “universal” primer set for 16S rRNA gene amplification can result in low identification. Using a more specific primer, i.e. amx386F (an anammox specific primer) along with a general eubacterial reverse primer or a specific anammox reverse primer (i.e. amx820R) can increase relative amounts of anammox 16S rRNA gene sequences (Schmid et al., 2000; 2007; Penton et al., 2006).

More recently however, more functional PCR approaches using primers amplifying anammox genes encoding hydroxylamine/hydrazine oxidoreductase (HAO/HZO), hydrazine synthase (HZS), and nitrous oxide reductase (NIR) proteins showed that these genes are suitable targets for molecular ecological studies on both aerobic and anaerobic ammonium-oxidizing bacteria (Haranghi et al., 2012; Li et al., 2010; Quan et al., 2008; Schmid et al., 2008). Li et al., (2010) and Haranghi et al., (2012) found that primers for hzo and hzs genes may provide better coverage of anammox bacteria genera than primers for anammox 16S rRNA genes, respectively.
Another approach to quantifying and qualifying anammox bacteria in the environment is fluorescent in situ hybridization (FISH). FISH is useful for visual identification of anammox cells and has been used successfully in several studies (Helmer-Madhok et al., 2002; Schmid et al., 2000, 2003). Schmid et al., (2003) was able to create a probe that was very effective at hybridizing to 16S rRNA of anammox bacteria. As more valid anammox sequences become available, probe designs will continue to improve (Jetten et al., 2009). Two advanced FISH techniques are; the Raman-FISH, which combines stable-isotope Raman spectroscopy and FISH for the single cell analysis of identity and function (Pätzold et al., 2008), and the CARD-FISH, (Catalyzed reporter deposition) has also been used to detect anammox because it is more sensitive than the traditional FISH technique (Hannig et al., 2007).

$^{15}$N Tracer incubation methods Anammox contribution to the total N$_2$ production (%anammox) can be calculated by measuring the rates of both anammox and denitrification using $^{15}$N tracer incubation techniques (Thamdrup and Dalsgaard, 2002). The $^{15}$N tracer incubation has been the technique of choice for the detection of anammox activity. When environmental samples including soil, sediment and water, are incubated under an anoxic environment with $^{15}$N-substrates, one of several reactions can happen. For example, the addition of $^{15}$NH$_4^+$ paired with $^{14}$NO$_2^-$, or $^{15}$NO$_3^-$/NO$_2^-$ with $^{14}$NH$_4^+$ will generate $^{29}$N$_2$. This specific molecular weight of dinitrogen gas is indicative of the anammox reaction and has been used successfully in several studies since anammox bacteria utilize one molecule each of NH$_4^+$ and NO$_3^-$/NO$_2^-$(Dalsgaard et al., 2003; Kuypers et al., 2003; Thamdrup and Dalsgaard 2002). The addition of $^{15}$NO$_3^-$/NO$_2^-$ with $^{14}$NH$_4^+$ could also generate $^{30}$N$_2$ as an end production of denitrification, which uses two molecules of NO$_3^-$/NO$_2^-$. In this method, rates of N$_2$ production via anammox or denitrification may be measured simultaneously in the same sample via continuous flow isotope ratio mass
spectrometer (Thermo Finnigan Delta V; Thermo Scientific, Waltham, MA) in line with a gas bench interface. The %anammox has been shown to vary across aquatic environments, from being functionally absent to the dominant pathway with up to 79% of the N removed by anammox (Engstrom et al., 2005). The $^{15}$N$_2$ ($^{14}$N$^{15}$N) isotope technique can also be modified to include the addition of inhibitors. Differential effects of acetylene and methanol on anammox and denitrification helped solidify a more solid understanding of the main pathways of N$_2$ production in marine sediments (Jensen et al., 2007).

Ladderane Lipid analysis The ladderane lipids are so unique and otherwise unknown in nature, they are an ideal biomarker for anammox bacteria (Lanehoff & Karlsson, 2010; Rattray et al., 2008). The intact ladderane glycerolphospholipids are thermally unstable (Li & Gu, 2011; Sinninghe Damsté et al., 2005), however, they degrade into predictable breakdown products (Rush et al., 2011). The $^{13}$C content of anammox lipids is around 45% depleted when compared to their carbon source, while lipids from other autotrophic organisms are around 20 to 30% depleted (Rattray et al., 2010; Rush et al., 2011). Recently, lipid analysis has become a viable method for detecting anammox bacteria in natural environments (Brandsma et al., 2011; Byrne et al., 2009; Hu et al., 2011; Jaeschke et al., 2007; Jaeschke et al., 2009; Lanehoff & Karlsson, 2010).

Importance of anammox in marine ecosystems Nitrogen cycling in marine ecosystems is very important due to N being the limiting agent of primary productivity. The ammonium is converted to nitrate via nitrite through the process of nitrification (as described above), and the nitrite and nitrate are converted to nitrogen gas anaerobically through the process of anammox or denitrification (Kuypers et al., 2003; 2005). The first discovery of anammox in anoxic waters was by Kuypers et al. (2003) in the largest anoxic basin on the planet, the Black Sea. To confirm
the presence of anammox, Kuypers et al (2003) utilized several techniques listed above including nutrient analysis, FISH, $^{15}$N potential rates, and 16S rRNA sequence analysis. After this development, similar ecosystems such as the Peruvian and Benguela upwelling systems also yielded the existence of anammox (Kuypers et al., 2005; Thamdrup et al., 2006). In the Benguela system there was no significant denitrification activity reported, which would leave anammox as the sole sink of fixed nitrogen in the entire system.

The water chemistry of oxygen minimum zones (OMZs) would seem to support anammox over denitrification as seen in the OMZs of the Peruvian and Chilean upwelling (Galán et al., 2009; Hamersley et al., 2007; Lam et al., 2009; Thamdrup et al., 2006; Ward et al., 2009). In these OMZs, anammox rates were highest in zones of depleted nitrate and accumulating nitrite, even with little to no ammonium (Hamersley et al., 2007; Schmid et al., 2005; Thamdrup et al., 2006). Anammox rates were at their highest closer to the top of the OMZ (Lam & Kuypers, 2011), and positively correlated with cell densities (Hamersley et al., 2007; Kuypers et al., 2005).

The discovery of anammox in anoxic waters allowed researchers to explore other anoxic marine ecosystems for anammox presence. Soon after, anammox was found in marine/estuary sediment (Risgaard-Petersen et al., 2004; Risgaard et al., 2004; Meyer et al., 2005; Tal et al., 2005; Amano et al., 2007; Hietanen, 2007; Schmid et al., 2007; Rich et al., 2008). Within these systems the anammox reaction was estimated to contribute more than 50% of all marine nitrogen losses, which would indicate the anammox was far more important to the global nitrogen cycle than previously thought.
**Importance of anammox in freshwater and terrestrial ecosystems** The impact of anammox bacteria have also been explored in freshwater and terrestrial ecosystems such as freshwater marshes (Penton et al., 2006; Humbert et al., 2010), rivers (Zhang et al., 2007), lakes (Schubert et al., 2006), and river estuaries (Risgaard-Peterson, et al., 2004; Trimmer et al. 2003; Dale et al., 2009), various soil types, which include permafrost soils (Penton et al., 2006), reductised, agricultural soils (Humbert et al., 2010), peat soils (Hu et al., 2011) and groundwater (Clark et al., 2008). Humbert et al., (2010) showed that terrestrial anammox diversity is much greater than marine diversity using molecular methods.

The first anammox found in lake ecosystems was in Lake Tanganyika, the second largest lake in the world, by Schubert et al. (2006). Incubations with stable $^{15}$NO$_3^-$ indicated that anammox was discovered in the suboxic water. FISH revealed up to 13,000 anammox bacteria ml$^{-1}$ and the presence of ladderane lipids. Phylogenetic analyses of 16S rRNA genes indicated the presence of sequences most closely related to the known anammox bacterium “Candidatus Scalindua brodae”. Anammox rates were similar to those reported for the marine water column and up to 13% of the produced N$_2$ could be attributed to the anammox bacteria.

Estuarine sediments are an important ecosystem that serves as a “catch” for N runoff from terrestrial ecosystems (Costanza et al., 1998). Up to 26% of N$_2$ production from estuarine sediments is estimated to originate from anammox (Meyer et al., 2005; Risgaard-Petersen et al., 2004; Trimmer et al., 2006). This would make anammox a significant aspect of the estuarine nitrogen cycle.

In regards to agricultural soil, the application of inorganic nitrogen fertilizers has created widespread changes in the global nitrogen cycle. Fertilizer inputs have doubled the rate of
nitrogen uptake in terrestrial environments and increased the activity of denitrifiers, which in turn has increased the production of nitrous oxide (N\textsubscript{2}O), a greenhouse gas. Inorganic nitrogen fertilizers have also contributed to the increased acidification of soils, and increased the rate of nitrogen transfer from streams and rivers to estuaries and oceans (Vitousek et al., 1997).

**Importance of anammox in groundwater** While studies have shown that anammox have been found in ammonium contaminated groundwater (Humbert et al., 2010; Smits et al., 2009), there are very few studies showing the impact of anammox on groundwater, how anammox bacteria interact within an aquifer community, and the amount of N\textsubscript{2} anammox bacteria are responsible for moving. Only one other study, Moore et al. (2011), has addressed the rates, community structure and abundance of anammox bacteria in a contaminated aquifer. Groundwater ecosystems, very much unlike river or marine systems, have very long exposure times to active microbial communities, depending on the aquifer characteristics. Combined with the surplus of ammonium and nitrite/nitrate and low concentrations of organic carbon the slow-growing anammox of this aquifer may be one of the best sites to study anammox bacteria. Groundwater systems are vastly important and the United States uses 79.6 billion gallons per day of fresh groundwater for public supply, private supply, irrigation, livestock, manufacturing, mining, thermoelectric power, and other purposes (USGS, 2009). The removal of nitrogen from contaminated aquifers is understudied, and the possibility of remediation through microbial means must be studied further. This experiment is one of the first studies to couple the rates, abundance, and geochemical factors of anammox in a groundwater ecosystem.

In order to narrow the scope of the experiment, three focused questions were created. Each question was accompanied by a hypothesis based on previously available knowledge and data. To test each hypothesis, three tasks were created. The first research question asked was
which nitrogen removal pathway in a contaminated aquifer, anammox or denitrification, would be the dominant pathway. The question was aimed to explore exactly how important anammox bacteria are with regards to nitrogen removal. Based on preliminary geochemical data we hypothesized that anammox would be the dominant removal process in the aquifer due to the relatively high concentrations of nitrite and ammonium, necessary substrates for the anammox reaction to proceed. In order to test this, the rates of anammox and denitrification will be measured using $^{15}$N isotope pairing techniques. The second research question was to determine which environmental factors would favor anammox in nitrogen removal. We hypothesized that higher concentrations of ammonium and nitrite will enhance anammox contributions to nitrogen removal. This hypothesis is based on the conventional thought that more substrate availability would directly impact the rates of nitrogen removal. To address this, we will compare anammox rates and gene abundance to relevant geochemical features measured by the United States Geological Survey along the same vertical gradient. The final research question is a more ecological focused question, asking which anammox genera are more prevalent within the groundwater ecosystem. Based on research in a similar contaminated aquifer, we hypothesized that bacteria assigned to the genus *Brocadia* would be dominant anammox population in groundwater ecosystem. We tested this by examining the anammox community structures using the 16S rRNA and an anammox specific functional gene as genetic markers. By combining these three results and cross-referencing the data, the mystery of anammox bacterial activity in contaminated aquifers will be revealed. Thus, I have conducted the examination of the importance of anammox in groundwater N cycle for my masters thesis research.
CHAPTER 2

Molecular and stable isotope characterization of anammox and denitrification in a nitrogen-contaminated aquifer, Cape Cod, Massachusetts
ABSTRACT

Groundwater nitrogen contamination is a widely known problem and a serious concern for public health and ecosystem management. Denitrification and anaerobic ammonium oxidation (anammox) are microbial pathways capable of removing nitrate and nitrite from groundwater ecosystems. Anammox may have a significant role in removing N since organic carbon in groundwater might be limited to support denitrification. In order to evaluate the importance of anammox in N removal and to identify anammox hot spots in aquifers, groundwater microbial communities were examined at a nitrogen contaminated sand and gravel aquifer on Cape Cod, MA. The U.S. Geol. Survey has constructed multilevel sampling systems and a database of water quality parameters at this aquifer. Groundwater samples were collected from multiple depths of a selected sampling well (F575) spanning from 6.2 m above to 6.7 m below sea level. Molecular methods were used to examine anammox community structures and to quantify the abundance of anammox and denitrifying bacteria along the depth profile. $^{15}$N tracer incubation was conducted to measure the rates of anammox and denitrification. Quantitative PCR (qPCR) assays of hydrazine synthase genes ($hzsA$) estimated that anammox bacterial gene abundance ranged from $2.5 \times 10^3$ to $2.5 \times 10^5$ copies ng$^{-1}$ DNA while abundance of denitrifying bacteria genes were $1.9 \times 10^3$ to $1.1 \times 10^4$ copies ng$^{-1}$ DNA based on qPCR of nitrous oxide reductase genes ($nosZ$). Anammox rates were found to range from negligible to $6.4 \pm 0.048$ nmoles of $N_2$ L$^{-1}$ D$^{-1}$ while denitrification ranged from $0.38 \pm 0.005$ to $34.9 \pm 0.05$ nmoles of $N_2$ L$^{-1}$ D$^{-1}$. Anammox was estimated to contribute up to 64% of the total $N_2$ production in the examined aquifer site. Thus, this study clearly demonstrates that the anammox plays a significant role in microbial nitrogen removal in the aquifer.
INTRODUCTION

Groundwater nitrogen contamination is a widely known problem and a serious concern throughout the United States and other areas of the world. Nitrogen contamination usually occurs in places of heavy agricultural activities through the use of synthetic fertilizers, or of wastewater spills from septic tanks and an old sewer system (Savard et al., 2009). In some parts of the world groundwater is the only source of drinking water, and nitrate contaminated water can cause methemoglobinemia in infants (also known as Blue baby syndrome), due to the presence of methemoglobin in the blood (Umezawa et al., 2008). Nitrates and nitrites in groundwater designated as drinking water have also been linked to several types of cancers in the digestive tract, a result of the carcinogenic nature and formation of nitrosamines (Harte et al., 1991; Nash, 1993; Khaniki et al., 2008). The United States has issued a Federal maximum containment level of 10 mg NO\textsubscript{3}-N L\textsuperscript{-1} (US Environmental Protection Agency, 1986). In lieu of expensive physical removal techniques that could harm the ecosystem, there are only two different microbial pathways that will remove nitrogen from a system biologically via denitrification or anaerobic ammonium oxidation (anammox). It was long thought that denitrification was the main source of removal, but now anammox may be more important significant in nitrogen removal than previously thought (Kuypers et al., 2005).

Denitrification proceeds with nitrate reduction to nitrite, via nitrate reductase. Nitrite reductase will reduce nitrite to nitric oxide and nitric oxide reductase catalyzes the transformation to nitrous oxide (Rinaldo et al., 2007). Denitrification is finally completed when nitrous oxide is reduced to dinitrogen gas via nitrous oxide reductase. A concern with the denitrification is the production of N\textsubscript{2}O (300 times more potent than CO\textsubscript{2} as a greenhouse gas) as a result of incomplete denitrification (IPCC, 2001). N\textsubscript{2}O is a known greenhouse gas, which is
detrimental to the environment. Denitrifying bacteria can be detected by targeting the specific functional genes that facilitate the reduction of nitrate to dinitrogen gas. Because denitrifiers are taxonomically very diverse, 16S rRNA-based phylogenetic analysis is inappropriate to explore their diversity. Functional marker genes such as the nitrite reductase genes nirK and nirS, which code for copper and cytochrome cd1-containing nitrite reductases, respectively, the key enzymes in the denitrification process (Zumft, 1997), had been targeted to study denitrifier communities in several different habitats. These include groundwater (Yan et al., 2003), soils (Priemé et al., 2002; Rösch et al., 2002), river sediments (Taroncher-Oldenburg et al., 2003), marine sediments (Braker et al., 2000; Braker et al., 2001; Liu et al., 2003) and seawater (Jayakumar et al., 2004). The gene encoding nitrous oxide reductase (nosZ) has also been used to examine and quantify N2O respiring denitrifying bacteria in sediments and soils (Henry et al., 2006; Dandie et al., 2008; Chon et al., 2011).

Anammox bacteria were discovered in a waste water reactor in the 1990’s and are mainly recognized for converting ammonium and nitrite into dinitrogen gas (Jetten et al., 1998). Anammox bacteria offer a separate concurrent pathway from denitrifying bacteria and skip the step of producing N2O gas. They produce N2 through anaerobic oxidization of NH4+ by NO2- reduction (Mulder et al., 1995; Van de Graaf et al., 1995). Nitrogen contaminated aquifers are ripe with ammonium and nitrite central to anammox, and can provide ideal microbial habitats for anammox bacteria (Miller et al., 2009). The anammox proceeds in bacteria that are members of the family Brocadiaceae in the phylum Planctomycyes. There are five candidate genera that have been described from enrichment cultures: Candidatus “Brocadia”, Candidatus “Jettenia”, Candidatus “Kuenenia”, Candidatus “Anammoxoglobus”, and Candidatus “Scalindua” (Jetten et al, 2009).
16S rRNA genes have been mainly used for detecting and identifying anammox bacteria in environmental samples (Jetten et al., 2009). However, 16S rRNA is highly conserved, which can limit their use in examining the full range of anammox diversity in an environment (Hirsh et al., 2011). Most of the published PCR methods for anammox detection amplified the 16S rRNA genes of non-anammox bacteria belonging to Planctomycetes as well as anammox bacteria, although some methods of detection work better than others (Dale et al., 2009; Penton et al., 2006). Targeting the functional genes of anammox bacteria provides a more reliable method of detection, especially in environments with low anammox abundance (Kartal et al., 2011). There are three main catalytic proteins that have been explored for biomarkers for anammox functional detection; nitrite reductase (nirS: Strous et al., 2006), hydrazine synthase (hzsA gene: Harhangi et al., 2012) and hydrazine oxidoreductase (hzo gene: Hirsch et al., 2011; Li et al., 2010).

At the MMR study site in Cape Cod, MA a plume of groundwater contamination was created by discharge of treated wastewater from 1936 to 1995. The study aquifer is a shallow sand and gravel aquifer that contains a large contamination plume. This site was chosen out of all the other MMR sites due to its differing biogeochemical nature along the plume gradient. The plume is at least a kilometer wide, 7 kilometers long and up to 25 meters deep (Miller et al., 2009). The USGS began sampling groundwater and recording site data in 1990. The resulting plume is estimated to be around 6 kilometers and 1 kilometer wide. The main sampling site, F575, is located in the ‘Gravel pit’ approximately 100 yards down-gradient from the initial point of contamination site, S469 (figure 4). There are several sampling wells spaced in between the two main sites. This study site has a rich history, with years of geochemical data collection that has been previously collected by the USGS on the site and the unique physiological characteristics that a contaminated aquifer provides.
Figure 4. Aerial depiction of the contaminant plume (Miller et al., 2008).
The first objective of the study was to evaluate the importance of anammox in N removal and identify anammox hotspots in a N contaminated aquifer by quantifying bacterial abundance and N production rates. The hotspots should hypothetically be located in depths where the bacterial abundance and rates are located. The second objective was to determine the geochemical and microbial factors influencing anammox activities in groundwater by cross-referencing the abundance/rates with geochemical data. The anammox hotspots of elevated abundance and rates should be located in the depths where the precursors of the anammox reaction (ammonium and nitrite/nitrate) are located. The third objective was to compare anammox community structures under different geochemical conditions by conducting phylogenetic analyses of 16S rRNA and hzsA genes in different depths. Based on studies from similar contaminated aquifers, the bacteria in the aquifer should be closely related to bacteria from the genus *Brocadia* (Moore et al., 2011).
METHODS AND MATERIALS

Sample Collection in August of 2011

All samples were collected from site F575 located approximately 100 meters down-gradient from the initial treated wastewater seepage infiltration beds. The samples were collected on duplicate 0.20 micron Sterivex cartridge filters (EMD Millipore, Billerica, MA) from two adjacent multi-level samplers located within 2 horizontal meters of one another. The access screens ranged vertically approximately 47ft from an altitude of 24.4 to -21.9ft MSL. The MLS consisted of 14 screened ports evenly spaced inside the anoxic zone of the aquifer. The ports cover a wide range of geochemical features, including a nitrate/nitrite zone, an ammonium zone, and an N-deficient zone with no available inorganic or organic N available (Figure 5). The groundwater is subsequently pumped via peristaltic pump (Geo pump, Geotech Environmental Equipment, Denver, CO). The amount of groundwater pumped through each filter is 4 Liters or until clogged, whichever occurs first. The filters were flushed with an empty syringe to discharge any extra water inside the filter and immediately placed onto dry ice until transferred to a -80ºC freezer for further DNA extraction. Triplicate filter samples were also collected for incubation analyses. Four liters of groundwater were pumped through each filter and submerged inside an airtight glass container filled completely with site water. The containers were kept on ice until stored in a 4ºC cold room. The physical and chemical properties of the water samples were analyzed by Dr. Richard Smith at United States Geological Survey. (USGS, DOI, Reston, VA)

DNA extraction.

The filters were removed from -80ºC storage and the plastic outer cartridge cut open with a commercial glass cutter (Home Depot). The filter paper was removed and divided into halves. Small incisions were made along the paper to facilitate shredding. The filter paper, along with
800μl of lysis solution (5Prime, Gaithersburg, MD) was added to 2mL microcentrifuge tubes and incubated at 70°C for 30 minutes. The contents were decanted into 2mL bead beating tubes (MP Biomedical, Santa Ana, CA) and shaken at max speed (6.5) for 30 seconds in Model FP120 high speed bench top cell disrupter (Thermo Fisher Scientific, Waltham, MA). The tubes were centrifuged at 10,000 x g and 600μl were extracted and placed into new 1.5mL microcentrifuge tubes. RNAse solution (3U: 5Prime, Gaithersburg, MD) was added to each tube and the mixtures were incubated at 37°C for 15 minutes after being inverted several times. Protein precipitation solution (600μl; 5Prime, Gaithersburg, MD) was added and the tubes were spun at 10,000 xg until the protein pellet was compact. The supernatant was poured into new tubes and 600μl of 100% cold isopropanol were added. The tubes were incubated at 4°C overnight. The tubes were centrifuged at 10,000xg for 15 minutes and then aspirated twice. The DNA pellets were hydrated with 50μl of DNA hydration solution (5’). The extracted DNA concentration was measured using a Quant-It™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA).

**PCR amplification, Cloning, and Sanger Sequencing**

PCR was performed to amplify anammox bacterial 16S rRNA genes and hzsA genes in two distinct depths (7.44m MSL and 2.48m MSL). These depths were chosen for the large quantity of NO$_2$/$3(7.44m)$ and NH$_4$ (2.48m), respectively (Table 1). The 16S rRNA genes of anammox bacteria were amplified using the primers amx385F and amx820R (Schmid et al., 2000; 2003). The PCR began with an initial denaturation step of 95°C for 5 min, 30 cycles of denaturation (94°C for 30 sec), primer annealing (45°C for 30 sec), extension (72°C for 1 min) and a final extension step for 5 min at 72°C. Detection of hzsA genes was conducted using the primers hzsA526F and the hzsA1857R (Harhangi et al., 2012). The hzsA PCR mixture was a
25μl reaction containing 12.5μl GoTaq Green Master Mix (Promega, Madison, WI), 1μl of DNA as the template, and 1μl of each primer (10 μM). The thermal profile started with a denaturation step of 5 min at 96°C, followed by 30 cycles of denaturation (1 min at 96°C), primer annealing (1 min), and extension (1.5 min at 72°C), and finally a last extension step of 5 min at 72°C (Harhangi et al., 2012). To differentiate the PCR products, gel electrophoresis on agarose gel (1.0%) was used, which were then purified using the Wizard® SV Gel PCR clean-up System (Promega, Madison, WI) using the manual instructions included. The purified products were cloned using the pGEM®-T Easy Vector Systems PCR Cloning Kit (Promega, Madison, WI). Clone libraries for the M02-10P port (10.5m depth) and M01-01PT port (15.6m depth) were created. The clones were sequenced using BigDye® terminator (Applied Biosystems, Foster City, CA) and an ABI 3130xl automated genetic analyzer (Applied Biosystems, Foster City, CA). NCBI Blast Search (http://www.ncbi.nih.gov) was used to determine which sequences were closely related. The sequences, along with reference sequences that are closely related, were aligned using clustalW (http://www.ebi.ac.uk/clustalw/). MEGA version 5.0 (http://www.megasoftware.net/) was used to create neighbor-joining trees with 1000x bootstrap of anammox 16S rRNA and hzsA gene sequences.

**Quantitative PCR of functional genes in anammox, denitrifying and nitrifying bacteria**

A total of 13 DNA samples extracted from descending sequential depths of groundwater were used to quantify the abundance of anammox, denitrifying and nitrifying bacteria (Table 2). All of the qPCR was conducted with the GoTaq qPCR Master Mix Green (Promega) and a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The anammox bacteria abundance was quantified by the qPCR of the hzsA using the primers hzsA1597F (5’-WTYGGKTATCARTATGTAG-3’) and hzsA1829R (5’-TCATACCACCARTTGTA-3’) as described by Harhangi et al., (2012). The hzsA qPCR started with an initialization step of 3 min
at 96°C, followed by 50 cycles of denaturation (30 sec at 96°C), primer annealing (45 sec at 53°C), and extension (35 sec at 72°C), and finally a signal detection step at 75°C for 35 sec. For the dissociation curve, the temperature ramped from 55°C to 95°C (at the instrument default rate). Denitrifying bacterial abundance was measured by targeting nosZ for N₂O respiring bacteria while nirS and nirK genes were quantified for nitrite respiring denitrifiers. The nosZ qPCR was conducted with the primers nosZ2F (5’- CGCRACGGCAASAGGTSMSGT - 3’) and nosZ2R (5’- CAKRTGCAKSGCRTGCAGAA - 3’) (Henry et al., 2006). The nosZ qPCR began with an initial denaturation step for 10 minutes at 95°C followed by 50 cycles of 95°C for 45 sec, 55°C for 45 sec, 72°C for 35 sec, and a fluorescent signal detection step for 35 sec at 80°C. For the dissociation curve, the temperature ramped from 55°C to 95°C (at the instrument default rate). Q-PCR of nirK gens was performed with the primers nirK-q-F (5’-TCATGGTGCTGCCGCGYGA) (Santoro et al., 2006) and nirK1040 (Henry et al., 2004), while nirS qPCR was conducted with the primers nirS1F (Braker et al., 1998) and nirS-q-R (5’-TCCMAGCCRCRTCRTGCAG) (Mosier et al., 2010). The nirS qPCR began with an initial denaturation step for 15 minutes at 95°C followed by 38 cycles of 95°C for 15 sec, 62.5°C for 30 sec, 72°C for 30 sec, and a fluorescent signal detection step for 35 sec at 84°C. For the dissociation curve, the temperature ramped from °C to 95°C (at the instrument default rate). The nirK qPCR began with an initial denaturation step for 15 minutes at 95°C followed by 9 touchdown cycles of 95°C for 15 sec, 68°C for 30 sec (including an auto-increment of -1.0), 81.5°C for 30 sec, followed by 35 cycles of 95°C for 15 sec, 60°C for 60 sec, 81.5°C for 30 sec, and a fluorescent signal detection step for 10 sec at 86°C. For the dissociation curve, the temperature ramped from 55°C to 95°C (at the instrument default rate). The Eubacterial 16S qPCR for total bacterial abundance used the primers EU 341F and 685R (Muyzer et al., 1993)
The Eubacterial 16S qPCR began with an initial denaturation step for 10 minutes at 95°C followed by 40 cycles of 55°C for 30 sec and a fluorescent signal detection step for 35 sec at 72°C. For the dissociation curve, the temperature ramped from 55°C to 95°C (at the instrument default rate). Abundance of aerobic ammonia oxidizing bacteria was also quantified by targeting ammonium monooxygenase genes (amoA) using the primers AMO1F (5’ - GGGG TTTCTACTGGTGTT - 3’) and AMO2R (5’ - CCCCTCKGSAAGCCCTTCTTC - 3’) (Rotthauwe et al., 1997). The initial denaturation began at 95°C for 10 min followed by 50 cycles at 95°C for 15 sec, primer annealing at 53°C for 45 sec, and extension at 72°C for 30 sec and a fluorescent signal detection step for 35 sec at 86°C. For the dissociation curve, the temperature ramped from 55°C to 95°C (at the instrument default rate). All of the PCR products were run on 1% agarose gels along with a 1kb DNA ladder to confirm the correct size of PCR products. All of the qPCR reactions were completed in triplicates. PCR specificity and primer-dimer formation were monitored with analysis of disassociation curves. QPCR standards of each gene were generated via serial dilution of the PCR product carrying the targeted gene.

\[1^{5}N\text{-Tracer Incubation Experiments}\]

Anammox and denitrification rates in groundwater communities were measured using a modified method of isotope pairing method described by Thamdrup and Dalsgaard (2002). The filter cartridges with concentrated biomass were split open using a commercial glass cutter, and the whole membrane filters were aseptically removed and placed inside 12 mL Exetainer tubes and completely submerged beneath 2mL of the corresponding site water. The Exetainer tubes were capped, sealed air-tight with new rubber septa, and flushed for 10 minutes with Helium gas. The Exetainer tubes were incubated at room temperature overnight to eliminate any residual
NO$_3^-$/NO$_2^-$. After overnight incubation, the presence of residual NO$_3^-$/NO$_2^-$ were examined using Vanadium (III) reduction and chemiluminescent detection developed by Braman and Hendrix (1989) with an Antec 7020 nitric oxide analyzer (Antek Instruments, Houston, TX). After the overnight incubation, the tubes were vacuumed for 5 minutes and flushed completely with Helium gas for 5 minutes. The tubes had Helium-flushed stock solutions of Na$^{15}$NO$_3$ (99.5 atm%; Cambridge Isotope Laboratory, Andover, MA) and $^{14}$NH$_4$SO$_4$ (98 atm%; Sigma-Aldrich, St. Louis, MO) added to give a final concentration of 0.1 mM of $^{15}$NO$_3^- + ^{14}$NH$_4^+$. The incubations proceeded forward in duplicate and were collected in three time points: 0hr, 6hr, and 12hr. At each collection, the incubations were stopped completely with the addition of 0.5 mL of 4M ZnCl$_2$ solution. The samples were stored upside down in water until run simultaneously on a continuous flow isotope ratio mass spectrometer (Thermo Finnigan Delta V; Thermo Scientific, Waltham, MA) connected to a gas bench interface. The production rates of $^{29}$N$_2$ and $^{30}$N$_2$ were measured simultaneously in each sample tube. The $^{29}$N$_2$ production is indicative of anammox activity, while the $^{30}$N$_2$ production shows only denitrification activity based on the gas measurements from $^{15}$NO$_3^-$ and $^{14}$NH$_4^+$ incubation experiments. The rates of $^{29}$N$_2$ and $^{30}$N$_2$ production were calculated using a modified method of Thamdrup and Dalsgaard (2002).

**Statistical analyses**

To compare the correlation between rates, abundances, and geochemical parameters, two Canonical Correspondence Analysis (CCA) plots were created to demonstrate possible statistically significantly correlated variables. To further test this, Pearson product-moment correlation coefficient analysis was utilized via Sigma plot 11.0 (Systat Software inc., Germany). The Pearson analysis will measure the strength of the linear dependence between two variables.
RESULTS

*Vertical Geochemical distribution*

The USGS catalogued the geochemistry along the MLS ports sampled for the project. The geochemical data is listed according to decreasing depth and lower altitude MLS ports in Table 1. The dissolved oxygen concentration was below detection throughout the studied MLS ports. The shallowest MLS ports (altitudes 7.40 – 6.20m MSL) contain the largest concentrations of NO$_3^-$ and NO$_2^-$. The highest concentration of NO$_3^-$ is 132 µM at altitude 7.44m MSL and the lowest is negligible. The NO$_3^-$ decreases gradually to a N-deficient zone where very little organic or inorganic N exists. After N-deficient zone, there was an NH$_4^+$ spike (ranging from 7 to 38 µM) from altitudes 0.95 to -3.63m MSL that persisted through four MLS ports. NO$_3^-$ increased steadily in altitudes below -3.63m MSL (30 µM+).
<table>
<thead>
<tr>
<th>Sample Port</th>
<th>Altitude (meters MSL)</th>
<th>Chloride (uM)</th>
<th>Nitrate (uM)</th>
<th>Sulfate (uM)</th>
<th>Ammonium (uM)</th>
<th>Magnesium (uM)</th>
<th>TDN (uM)</th>
<th>Inorganic N (uM)</th>
<th>Organic N (uM)</th>
<th>DOC (uM)</th>
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<tr>
<td>FSW 575-M02-09Y</td>
<td>7.44</td>
<td>187</td>
<td>8</td>
<td>132</td>
<td>120</td>
<td>0</td>
<td>73</td>
<td>158</td>
<td>140</td>
<td>18</td>
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<tr>
<td>FSW 575-M02-10P</td>
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<td>8</td>
<td>91</td>
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<td>0</td>
<td>74</td>
<td>109</td>
<td>99</td>
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<td>246</td>
<td>12</td>
<td>64</td>
<td>135</td>
<td>0</td>
<td>61</td>
<td>85</td>
<td>76</td>
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Table 1. Geochemical vertical distribution of F575 sampling site
Abundance of anammox, denitrifying, and nitrifying bacteria in the aquifer

Abundance of anammox and denitrifying bacteria was measured based on qPCR of functional genes. The qPCR assays of hzsA genes estimated that anammox bacterial gene abundance ranged from $2.5 \times 10^3$ to $2.5 \times 10^5$ copies ng$^{-1}$ DNA (Table 2). The highest copy number of hzs genes was found in the ammonium-rich 2.5 MSL altitude and the lowest in the geochemical N-deficient zone altitude between 6.8 and 4.3 MSL (Figure 5). The abundance of nosZ genes quantified in the aquifer ranged from $1.9 \times 10^3$ to $1.1 \times 10^4$ copies ng$^{-1}$ DNA. The largest quantity of N$_2$O respiring denitrifying bacteria containing the nosZ gene appears at altitude 4.3m MSL, while the lowest abundance appears in the N-deficient zone altitudes (Figure 5). The qPCR results of nirK ($9.23 \times 10^0$ to $2.64 \times 10^3$ copies ng$^{-1}$ DNA) genes and nirS ($3.0 \times 10^2$ to $2.17 \times 10^5$ copies ng$^{-1}$ DNA) genes were combined to estimate the abundance of NO$_2^-$ resiping denitrifiers (Table 2). The highest number of nir genes was found at the 2.5m MSL altitude and the lowest in the N-deficient zone (Table 2). The qPCR of amoA genes quantified AOB abundance ranging from $1.6 \times 10^2$ to $5.4 \times 10^1$ copies ng$^{-1}$ DNA (Table 2). The highest abundance of AOBs were discovered in the ammonium zone (2.5 MSL) and no AOBs were found in several ports, including (7.4 MSL to 6.2 MSL and -2.71 MSL to -3.63 MSL) (Table 2). Eubacterial 16S rRNA gene abundance ranged from $2.6 \times 10^6$ to $1.8 \times 10^7$ copies ng$^{-1}$ DNA. The abundance of anammox bacteria ranged from 1.1 to 40.5% of all bacteria detected by eubacterial 16S rRNA qPCR. Bacteria containing the nirS/nirK genes ranged from 0.25 to 25.6% of all bacteria detected, and bacteria containing nosZ genes only ranged from 0.03 to 1.56% of all bacteria (Table 3).
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<tr>
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<th>eub. 16S</th>
<th>eub. 16S stdev</th>
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<th>nirK stdev</th>
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Table 2. Copy numbers per ng DNA of functional genes and 16S RNA.
Table 3. Percent abundance of bacteria that contain specific functional gene

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<th>Altitude (meters (MSL))</th>
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<th>nirS %</th>
<th>hzsA %</th>
<th>nosZ %</th>
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Potential rates of anammox and denitrification Based on $^{15}N$ Isotope Pairing

In order to determine the effectiveness of microbial activity on N removal in the aquifer, the potential rates of anammox and denitrification were measured using $^{15}$NO$_3^-$ and $^{14}$NH$_4^+$. Any $^{29}$N$_2$ production was considered from anammox activity, while $^{30}$N$_2$ production was considered from denitrification (Figure 5). The $^{29}$N$_2$ production rates varied from as high as 6.39 nmol L$^{-1}$ D$^{-1}$ to as low as below detection. The lowest rate of $^{29}$N$_2$ production occurred at altitude 6.2m MSL, a nitrate rich, zero-ammonium depth. The location of the highest rate of $^{29}$N$_2$ production is altitude -5.2m MSL, where the nitrate plume begins to appear. The second highest rate of $^{29}$N$_2$ production is altitude 2.5m MSL, the transition zone between the N-deficient zone and NH$_4^+$ zone and also the location of the highest abundance of the anammox bacteria via the $hzsA$ gene. The average rate of $^{29}$N$_2$ production inside the ammonium zone is 3.73 ±0.038 nmol L$^{-1}$ D$^{-1}$, while the average rate of production outside of the ammonium zone is 0.40 ±0.007 nmol L$^{-1}$ D$^{-1}$. The $^{30}$N$_2$ production rates varied from as high as 34.9 nmol L$^{-1}$ D$^{-1}$ to as low as 0.38 nmol L$^{-1}$ D$^{-1}$. The lowest rate of $^{30}$N$_2$ production occurred in the N-deficient zone, while the highest $^{30}$N$_2$ production rate appears within the ammonium cloud (Figure 5). By comparing the production of $^{29}$N$_2$ to the total N$_2$ produced, an estimate of % anammox is calculated. The altitude with the largest impact of anammox is in the N-deficient zone at altitude 4.9m MSL, where the anammox constitutes 64% of total N$_2$ production. The altitude with the least impact is in the ammonium zone at altitude 0.95 MSL, where anammox is responsible for only 3% of total N$_2$ production. Overall, the rates of $^{30}$N$_2$ production are much higher in the ammonium and nitrate zones than in the N-deficient zone. Both the rates and gene abundance show decline in the N-deficient zone, and both show marked increases within the zones of either nitrate or ammonium. Based on the gene abundance and rate measurements, the ratios of rates per gene copy can be calculated at
each depth by dividing the rate by the copy number per ng of DNA extracted. Ratio of anammox rate over *hzO* gene abundance ranged from 0 to 289 fmol N₂ per gene copy number. Ratio denitrification rate over *nosZ* gene abundance ranged from $7.6 \times 10^1$ to $1.4 \times 10^4$ fmol N₂ per gene copy number (Figure 6).
Figure 5. Copy number per ng DNA, N₂ production rates and geochemical parameters arranged vertically along the MLS in the aquifer. Bacterial abundance was not available from the -5.2m MSL altitude.
Figure 6. Ratio of rates to gene abundance, N₂ production rates and geochemical parameters arranged vertically along the MLS in the aquifer. Bacterial abundance was not available from the -5.2m MSL altitude.
Table 4. Potential rates compared to percent anammox

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<th>Stdev N₂ production via AMX</th>
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Statistical analysis of Rates, bacterial abundance and geochemical parameters

The statistical analyses using Pearson product-moment correlation coefficients between anammox and denitrification rates against geochemical data and abundance data against geochemical data are tabulated in tables 4 and 5, respectively. In order to determine which variables would be good candidates for statistical inference two Canoco Correspondent Analysis (CCA) plots were created, figures 7 and 8, respectively. Rates and bacterial abundances are tabulated in Table 7.

The CCA plot showing possible correlations between bacterial abundance and geochemical variables displays a positive correlation between abundance of nirS and hzsA genes, and inorganic N compounds such as ammonium and nitrite (Figure 7). However, Pearson correlation analysis shows no significance between the gene abundances and ammonium and nitrite. Alternatively, there are significant negative relationships between nirK and nosZ gene abundances to TDN and inorganic N, which maintaining a positive correlation with organic carbon.

CCA also demonstrates possible correlations among anammox and denitrification rates, and geochemical variables (Figure 8). Anammox rates are significantly negatively correlated with organic carbon. Percent anammox showed is significant negative correlation with TDN and inorganic N. Denitrification rates are significantly correlated with the concentration of ammonium (Table 6).

The correlation between potential rates of anammox and denitrification the respective genes abundances responsible for the N₂ production (ie; hzs vs anammox rate, and denitrification rate vs nosZ, nirK, and nirS) were tested using Pearson's product-moment to determine whether any significant trends occurred (Table 7).
Figure 7. CCA plot showing possible correlations between bacterial abundance and geochemical variables.
Table 5. Pearson product-moment correlation coefficient analysis between the log of AMX/DNF bacterial abundance and geochemical variables.

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Figure 8. CCA plot showing possible correlations between bacterial abundance and geochemical variables.
Table 6. Pearson product-moment correlation coefficient analysis between anammox and denitrification rates, % anammox and geochemical variables.

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Table 7. Pearson product-moment correlation coefficient analysis between anammox and denitrification rates and relevant bacterial abundances.

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Phylogeny of Anammox Bacterial communities

In order to elucidate anammox community structure for the aquifer, two depths were selected for anammox community analysis based on 16S rRNA and hzsA genes. Each depth was selected specifically for their geochemical properties and the effect on the community. The first altitude, 7.44 MSL, contains a surplus of TDN including quantities of NO$_3^-$ and NO$_2^-$, while the second altitude, 2.48 MSL, has very low TDN and is in the transition zone between the N-deficient zone and ammonium-rich plume. Primarily, anammox bacterial 16S rRNA genes were targeted to determine which genera were dominant. Based on the anammox 16S rRNA detection, the sequences obtained are closely related to the terrestrial Candidatus Kuenenia spp., and Candidatus Brocadia spp. and clustered further away from Candidatus Anammoxoglobus and Candidatus Jettenia spp.. The “Deep” (2.48m MSL) community and “Shallow” (7.44m MSL) community are 5 meters apart, yet contain 2 slightly distinct bacterial communities (96.0-99.8% similarity). The Deep anammox 16S rRNA community and Shallow community clustered away from established anammox species. The communities are most similar to the Candidatus Kuenenia spp. (Shallow: 94.5-95.0%; Deep: 94.4-94.7%) and Candidatus Brocadia spp. (Shallow: 95.2-95.8%; Deep: 95.0-95.6%) while less similar from Candidatus Jettenia spp. (Shallow: 93.3-93.7%; Deep: 93.1-93.5%) and other anammox species (Figure 10). The hzsA genes were independently used to compare the anammox community structures in both MLS’s. Anammox bacteria were similar to Candidatus Brocadia spp., while maintaining a large distance from the Candidatus Kuenenia spp.. The “Deep” (2.48m MSL) hzsA community and “Shallow” (7.44m MSL) hzsA community are 5 meters apart, yet contain 2 distinct bacterial communities (87.6-98.1% similarity). The Deep hzsA community and the Shallow hzsA community both cluster away from other known anammox genera. The Cape Cod sequences related relatively
closest to *Candidatus* Brocadia spp. (Shallow: 83.4-83.6%; Deep: 82%) and *Candidatus* Jettenia spp. (82.0-82.4%) than the *Candidatus* Kuenenia spp. (Shallow: 79.2-79.3%; Deep: 79.3-79.8%) (Figure 9). Both phylogenies clustered away from the out group *Candidatus* Scalindua spp., the marine sediment genera, which was not expected to be in a freshwater aquifer. The phylogenetic tree of *hzsA* genes used 15 sequences from the 7.44m depth and 16 sequences from the 2.48m depth. The 16S rRNA tree used 11 sequences from the 7.44m depth and 5 sequences from the 2.48m depth. The discrepancy between the numbers of sequences between the two trees lays in the failure of the anammox specific 16S rRNA gene primers to only create true anammox sequences. In the anammox 16S rRNA tree, both depths align close to the groundwater communities in the UK (Smits et al., 2009), and are similar, albeit much less so, to sediment samples taken from Cape Fear River estuary sediment (Dale et al., 2009). Comparative analysis of anammox communities using the *hzsA* gene is difficult due to lack of *hzsA* sequence databases because the primers have not been available for long.
Figure 9. Phylogenetic tree of hzsA gene sequences detected from two different depths.
Figure 10. Phylogenetic tree of anammox bacterial 16S rRNA sequences detected from two different depths.
DISCUSSION

Anammox and denitrifier Quantification

Anammox bacterial quantification was mainly conducted with fluorescent in situ hybridization of 16S rRNA (FISH) and qPCR of 16S rRNA genes (Schmid et al., 2005). However, non-specific detection of 16S rRNA genes may lead to overestimation of anammox bacterial abundance in various environmental samples and similar flaws (Amano et al., 2007; Dale et al., 2009; Li et al., 2010). The functional genes including *nirS*, *hzo* and *hzs* have been recently used as alternative genetic markers to detection and quantify anammox bacteria (Strous et al., 2006; Harhangi et al., 2012; Hirsch et al., 2011; Li et al., 2010). The use of the *hzsA* gene as a functional marker for anammox bacterial detection is more advantageous than using hydrazine oxidase (*hzo*), as a single anammox bacterial cell carries a single copy of *hzsA* genes unlike *hzo* (Harhangi et al., 2012). The *hzsA* genes are unique for anammox bacteria while homologues genes of the *hzo* or *nirS* genes can be found in non-anammox bacteria (Li et al., 2010; Schmid et al., 2008). Because the abundance of the *hzsA* gene maintains a 1:1 ratio with the number of anammox cells, it is possible to compare the abundances of *hzsA* genes alongside the total cell counts provided from the FISH enumerations or *hzsB* quantification (Table 8).
Table 8. Estimated gene abundance in 1L of groundwater samples.

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Overall, the abundance of anammox bacteria calculated from hzsA quantification (6.5x10^5 to 7.6x10^7 copies L^{-1}) proved to be lower than in the freshwater Pearl river (via hzsB quantification:1.3 x10^8 to 2 x10^9 copies g^{-1})(Wang et al., 2012), the Golfo Dulce (via FISH:1.4 x10^{10} - 8.4 x10^{10} anammox cells L^{-1}) (Schmid et al., 2007), but an order of magnitude less than the numbers found via FISH in the Black Sea (Kuypers et al., 2003) and the Benguela OMZ (Kuypers et al., 2005).

Abundance of denitrifiers in the aquifer was measured using qPCR of nirS, nirK and nosZ genes. The nirS gene copies were one to two-fold lower than those measured in the Arabian Sea Oxygen Minimum zone (1.9 to 5.6 x 10^8 L^{-1} seawater) (Jayakumar et al., 2009). However, nirK abundance was comparable to that of the Arabian Sea OMZ (1 x 10^4 to 4 x 10^5 L^{-1} seawater) (Jayakumar et al., Unpublished). The nosZ quantification was surprisingly low, given the relatively (relative to anammox) high denitrification rates in the aquifer. The nosZ relative abundance compared to total 16S rRNA copy numbers was around 0.5%, similar to denitrifier abundance described in several different environments (Kandeler et al., 2006; Henderson et al., 2010; Henry et al., 2006). Eubacterial 16S rRNA genes ranged from 2.44 x10^7 to 3.11 x10^8, an order of magnitude higher than the abundance found in a similar aquifer (Moore et al., 2011). AmoA copy numbers were much lower when compared to Eastern Tropical South Pacific (ETSP) and the Arabian Sea OMZs (Bouskill et al., 2012). This could be due to the very low concentration of dissolved oxygen in this segment of the aquifer, which would inhibit ammonium oxidation. When correlation analysis was conducted, several obvious patterns were sought after, though for little gain. Dissolved organic carbon, important for the denitrification showed no clear trend when correlated to the nosZ abundance, but was significantly correlated with nirK abundance. Oddly enough, both nirK and nosZ abundance were negatively correlated
with DIN and TDN, which is a surprising finding. Nitrate is a necessary component of the denitrification process and it is counterintuitive that the correlations would be negative.

Anammox gene abundance (hzs) showed no clear trend when compared to dissolved organic nitrogen and other environmental parameters.

When functional gene abundances are compared to total eubacterial 16S rRNA gene abundances, the hzsA gene was found in up to 40% of all bacterial cells detected. However, in the depths where the hzsA gene relative abundance was highest, the ²⁰N₂ production rates were not always elevated as a result. This would further support that idea that potential rates do not increase with abundance, and possibly that the hzsA qPCR could detect anammox bacteria that are simply inactive, or less efficient in N₂ production.

N₂ Potential Rates via Anammox and Denitrification

The potential rates of N₂ production varied greatly among the ports, based on the geochemical availability of DON and DIN. Ports located in the geochemical N-deficient zone clearly demonstrated much lower rates of N₂ production than ports located in either the nitrate/nitrite cloud or the ammonium plume (Figure 5). The anammox rates in the N-deficient zone ranged from 0 to 0.67 ±0.01 nmol L⁻¹ D⁻¹. Outside of the N-deficient zone, the rates ranged from 1.04 ±0.01 nmol L⁻¹ D⁻¹ to 5.58 ±0.05 nmol L⁻¹ D⁻¹. Anammox potential rates in similar environments are much higher, including a study with 751 nmol L⁻¹ d⁻¹ from a similar contaminated aquifer (Moore et al., 2011), 72 - 438 nmol L⁻¹ d⁻¹ from anoxic bottom water (Dalsgaard et al., 2003), and other anoxic marine systems such as the Benguela upwelling system (96 – 192 nmol L⁻¹ d⁻¹)(Kuypers et al., 2005). The most similar rate findings are in the anoxic waters off northern Chile where anammox rates ranged from 4 nmol L⁻¹ d⁻¹ up to 16.8 nmol L⁻¹
Potential denitrification rates in the aquifer are much higher compared to anammox potential rates. The highest rates are found in the ammonium zone, with the lowest rates found in the N-deficient zone, a common theme found throughout the experiment. Denitrification is by far the greater producer of total N\textsubscript{2} in the aquifer. At the nitrate-rich depth, denitrification actually contributes 100\% of all N\textsubscript{2}. In the same aquifer, Smith et al. (2004) used a one dimensional transport model to elucidate a denitrification rate and determined that in a provided nitrate-rich depth, the rate of N\textsubscript{2} production was 6.5-10 nmol N\textsubscript{2} L\textsuperscript{-1} d\textsuperscript{-1}, which is comparable, albeit lower than the measured potential rates in this experiment. DeSimone and Howes (1996) used several different methods for determining \textit{in situ} denitrification rates in the same aquifer (though from a different area) and determined rates to be from 100-350 nmol N\textsubscript{2} L\textsuperscript{-1} d\textsuperscript{-1}, an order of magnitude higher than rates established in this study. In a wastewater-contaminated karst aquifer in Florida, Griggs et al. (2003) found an average rate of 2 x10\textsuperscript{3} nmol N\textsubscript{2} L\textsuperscript{-1} d\textsuperscript{-1}, several orders of magnitude greater than rates in the Cape Cod aquifer. A groundwater\textsuperscript{15}N addition study by Tobias et al. (2001) found rates ranging from 180 – 235 nmol N\textsubscript{2} L\textsuperscript{-1} d\textsuperscript{-1}.

Anammox does control the majority of N\textsubscript{2} in one site, with 64\% of all N\textsubscript{2} being created by anammox. At 8 of the 11 ports, anammox contributes between 10 – 30\% of all N\textsubscript{2} production. This contribution is within the low range of N\textsubscript{2} production by anammox in anoxic bottom water (19-35\%) (Dalsgaard et al., 2003) and comparable to the two contaminated aquifers sampled by Moore et al. (2011) ranging between 18.0 ±6.5\% and 35.7 ±13.6\%. The data shows that depths lacking DIN have the lowest rates of denitrification and highest percentages of anammox, which could indicate that anammox has a better tolerance for substrate loss than denitrifying bacteria.

When Pearsons product-moment correlation coefficient was calculated, neither the abundance of the genes encoding for denitrification or anammox correlated to their respective
rate counterparts. It should seem intuitive that larger abundances of bacteria would yield higher rates of $\text{N}_2$ production, but it appears that is not the case. This finding might indicate that individual bacteria may not be consistent in single cell $\text{N}_2$ production, or that individual bacteria are not as efficient as others. When geochemical parameters such as total dissolved (TDN) and inorganic dissolved (DIN) nitrogen concentrations were correlated with the rates, per cell rates, and % anammox the conventional thinking would be that the rates would all increase as the TDN/DIN increased. No significant correlations were found within the rates, however percent anammox shows a negative correlation with both TDN and DIN. This may be indicative of strong competition with denitrifying bacteria over the available nutrients. The denitrification rate does not correlate with DIN or TDN, but with the ammonium concentration. Denitrifiers to not require ammonium, but if ammonium oxidation is present perhaps the denitrifiers will quickly consume any available nitrate created by the ammonium oxidizers. This would leave behind the believable scenario of large amounts of inactive denitrifying organisms waiting in depths of low nitrate and high ammonium. Anammox overall rates and anammox per cell rates significantly decreased as DOC increased, which aligns with the idea that when DOC is present in the aquifer denitrifying bacteria are dominant. These results suggest that merely looking at geochemical data may provide a starting point for analysis but is not a direct indication of rates/gene abundance.

*Diversity and phylogeny of Anammox bacteria*

Two depths were sampled for phylogenetic analysis based upon their geochemical properties, one depth containing a large amount of nitrate/nitrite whereas the other contained small amounts of ammonium, yet no nitrate/nitrite. Cloning and sequencing revealed an interesting split in the diversity of the anammox community. Harhangi et al. (2012) reported that
when using the hzsA_526F/hzsA_1857R primers for the hzsA gene the sequences reportedly yielded very similar findings to 16S rRNA sequences, both agreeing on a “Candidatus Brocadia spp.”. However, upon phylogenies of two different sites using anammox 16S rRNA and hzsA sequences it is clear that the results are not congruent. The hzsA phylogenetic tree (Figure 9) shows a clear separation between the Cape Cod sequences and known anammox genera, with the deeper, ammonium-rich community showing similarity to the Candidatus Brocadia spp. clade, while clustering farthest from the Candidatus Kuenenia spp. clade (Figure 9). The 16S rRNA phylogeny shows the both the shallow and deep communities clustering away from previously established genera, but relatively similar to the Candidatus Kuenenia spp. and Candidatus Brocadia spp. (Figure 9). The Phylogenies are inconsistent with regards to the Candidatus Kuenenia spp. placement, differing from the results reported by Harhangi et al. (2012). It was first hypothesized that the anammox of the Cape Cod aquifer would be mostly dominated by Candidatus Brocadia spp., due to Brocadia being the dominant genera in similar sites (Moore et al., 2011), however there is a discrepancy between the phylogenies of 16S rRNA and hzsA genes. Schmid et al. (2007) illustrated that when it comes to displaying diversity, the 16S rRNA gene has the distinct disadvantage of lacking any functional analysis. However, due to detection of the hzsA gene being relatively new and 16S rRNA having been universally used to classify anammox bacteria we can confirm that the dominant anammox genera in the aquifer are similar related to Candidatus Brocadia spp. based on 16S rRNA genes.

However while that supports the third hypothesis, it does not tell the entire story. The <95% similarities between the Cape Cod communities and previously established genera and the placement of the separate phylotypes on the phylogenetic tree would indicate the possibility of a new genera or species of anammox bacteria in the aquifer.
CONCLUSION

The specific MLS ports used were chosen for several reasons; limited availability of dissolved oxygen which would inhibit microbial nitrate reduction and a robust range of organic and inorganic N concentrations that would provide substrate for anammox or denitrification hotspots. The vertical geochemistry was cross-referenced with potential rate data, functional gene quantification distribution, and anammox population analyses to elucidate a complete survey of the sample aquifer. Contrary to conventional thinking, rates and bacterial abundance show no correlation. It has been shown before that rates and abundance do not necessarily match the in situ activity (Penton et al., 2006). It is clear that potential rates or bacterial abundance do not tell the entire story of the aquifer by themselves, but combining rates with abundance and geochemical features show a more complete picture. The anammox potential rates, while low, do not complement the high bacterial abundance yet in low DIN altitudes contribute up to 64% of all N\textsubscript{2} production which makes anammox a very important feature in the Cape Cod aquifer.

Future considerations

Another way to compare the rates and abundances would be as a ratio. The ratio of denitrification rates and anammox rates is a normalization process that better tells the story of the individual anammox or denitrification cell. While merely glancing at the rates alone, it would appear that several depths in the ammonium available zones show decreased rates, where rates should be higher. When normalized to the copy numbers available and compared the rates per copy numbers in the previously low rates depths are higher, illustrating that the rates are lowered from a lack of gene abundance. The flaw in DNA extraction is the efficiency of the process, where not all DNA may be recovered which may repress the abundance numbers. By
normalizing the process to rates per copy numbers, the ‘true’ rates can be analyzed and the depths show a much more cohesive and likely trend when compared to the geochemical vertical nitrogen distribution. It is possible that each anammox cell or denitrification cell possibly utilize available nitrate/nitrite/ammonium more or less efficiently, depending on the geochemical concentration (Figure 6).
LITERATURE CITED


Meybeck, M., (1979). Pathways of major elements from land to ocean through rivers. River Inputs to Ocean Systems United nations environment programme, Intergovernmental oceanographic commission, Scientific committee on oceanic research, Rome Italy.


