Anaerobic ammonium oxidation in groundwater contaminated by fertilizers

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Abstract

Anaerobic ammonium oxidation (anammox) is a pathway that has been known for almost 2 decades, but few studies have investigated its importance in natural groundwaters. This thesis investigated the presence of anammox cells and the groundwater geochemistry of 2 sites (Elmira and Putnam) in southwestern Ontario where groundwaters are contaminated with high levels of nitrate and ammonium. Fluorescence in situ hybridization (FISH) was used to quantify the relative abundance of anammox cells in these waters. Our results showed that anammox cells could be detected in many wells at both sites and that their relative abundance varied between 0.45 and 4.81 % at the Putnam site, whereas it ranged between 0.8 to 8.4 % at the Elmira site. These values are within the same range as those obtained for marine and freshwater environments where anammox cells have been detected. In addition, indirect observations point to the fact that N cycling at the 2 sites might be linked to Fe and Mn reduction, but additional experiments are needed. In summary, our results corroborate the findings of N-labeled microcosm experiments which demonstrated that anammox was an important pathway of N cycling in those groundwaters and molecular analyses that detected important anammox organisms at the same sites.

Résumé

L’oxydation anaérobique de l’ammonium (anaerobic ammonium oxidation (anammox) en anglais) est une réaction qui est connue depuis environ 20 ans, mais peu d’études se sont attardées à son importance dans les eaux souterraines. La présente thèse s’est intéressée à la présence des bactéries anammox et à la géochimie des eaux souterraines de 2 sites (Putnam et Elmira) contaminés au nitrate et à l’ammonium dans le sud-ouest de l’Ontario. La méthode FISH (fluorescence in situ hybridization) a été utilisée pour déterminer l’abondance relative de ces bactéries dans les eaux. Nos résultats indiquent que l’abondance relative des bactéries anammox varie de 0.45 à 4.81% au site Putnam, alors qu’elle varie entre 0.8 et 8.4% au site Elmira. Ces valeurs sont comparables à celles obtenues pour des environnements d’eaux douces et marines où les bactéries anammox ont été détectées. De plus, des observations indirectes indiquent que le cycle de l’azote aux 2 sites étudiés pourrait être lié à la réduction du Fe et du Mn, mais des expériences supplémentaires seront requises. En sommaire, nos résultats corroborent les travaux avec des de microcosmes contenant des composés d’azote isotopiques qui ont démontré que l’anammox est une réaction importante dans ces eaux souterraines, ainsi que des travaux moléculaires qui ont identifié plusieurs organismes capables d’oxyder de façon anaérobique l‘ammonium.

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1. INTRODUCTION

1.1 Nitrogen & Nitrogen reservoirs

Nitrogen is the element that all life forms depend on. In the environment, nitrogen has several oxidation states: –III (NH_3), O (N_2), +I (N_2O), +II (NO), +III (NO_2^-), +IV (NO_2), and +V (NO_3^-). Nitrogen in the form of inert gas, dinitrogen (N_2), has accumulated in the Earth's atmosphere since the planet was formed. Nitrogen gas is continually released into the atmosphere from volcanic and hydrothermal eruptions and the atmosphere represents one of the major global reservoirs of nitrogen. The Earth's crust is another important reservoir where nitrogen is often unavailable to organisms; it must therefore be fixed which is the process called 'nitrogen fixation' whereby atmospheric nitrogen is converted to nitrogen compounds by certain bacteria or by a process called Haber-Borsch, before it can be used by living organisms. Nitrogen fixation is an energy-intensive process and a relatively slow process and it is carried out by a limited number of microorganisms. Smaller reservoirs of nitrogen include the organic nitrogen found in living biomass and in dead organic matter and soluble inorganic nitrogen salts. These small reservoirs tend to be actively cycled, particularly because nitrogen is often a limiting nutrient in the environment. For example, soluble inorganic nitrogen salts in terrestrial environments have turnover rates greater than once per day, while nitrogen in plant biomass turns over approximately once a year and nitrogen in organic matter, turns over once in several decades (Table 1) (Maier et al., 2009).

Table 1: Nitrogen reservoirs in all habitats. (Modified from Maier et al., 2009)

GLOBAL NITROGEN RESERVOIRS		
Nitrogen Reservoir	**Metric tons of N**	**Actively Cycled**
Atmosphere		
N_2	3.9×10^{15}	NO
Oceans		
Biomass	5.2×10^{8}	YES
Dissolved and particulate organics	3.0×10^{11}	YES
Soluble Salts (NO_3^-,NO_2^-,NH_4^+)	6.9×10^{11}	YES
Dissolved N_2	2.0×10^{13}	NO
Land		
Biota	2.5×10^{10}	YES
Organic Matter	1.1×10^{11}	SLOW
Earth's Crust	7.7×10^{14}	NO

1.2 Nitrogen as a contaminant

Approximately 75% of the water used in Canada is taken from surface water sources. In Ontario, groundwater provides about 30% of the water requirements, but the rural population depends almost entirely on the extraction of groundwater by private wells (Goss et al., 1998). Diffuse or nonpoint sources of contamination from intensive agriculture provide a potential threat to groundwater quality in the recharge areas of urban well fields (Table 2). The impact of agricultural land-use practices on regional groundwater quality is however not well understood. Some of the most common concerns for groundwater quality in relation to human health in the

rural environment include contamination with pathogenic microorganisms, nitrate and toxic organic compounds. For instance, introduction of reactive N into the biosphere by humans now exceeds the rate of biological nitrogen fixation in native terrestrial ecosystems (Galloway et al., 2004). Increased inputs of N to aquatic ecosystems from atmospheric deposition, sewage and agricultural runoff can cause eutrophication and a range of associated effects, including damage to fisheries in coastal ecosystems (Rabalais et al., 2002). While human health certainly benefits from the salutary effects of food production made possible by N fertilization, it is also negatively affected by several diseases that have been linked to air-and water- borne N (Townstead et al., 2003). These undesirable "cascading affects" (Galloway et al., 2003) of reactive N moving through aquatic and terrestrial ecosystems and the atmosphere do not stop until the reactive N is eventually converted back to N_2, primarily through the processes of denitrification and anammox. As nitrogen enters soils, rivers, lakes, groundwater, and ultimately, estuaries and coastal areas, denitrification reduces the downstream N loading (Seitzinger et al., 2006). Aquatic ecosystems are particularly vulnerable to N-induced eutrophication, as primary production is frequently limited by N in both marine and freshwaters (Elser et al., 2007).

Table 2: Nitrogen compounds as contaminants and different examples of removal processes. (Modified from Goss et al., 1998)

Contaminant	**Canadian water safety guidelines (mg/L)**	**Treatment methods**
Nitrate (NO_3^-)	10.0 (total nitrate and nitrite measured as N)	Reverse Osmosis with Thin Film Composite Membrane (sensitive pressure) Anion Exchange (Cl^- form, sensitive sulfates) Nitrates "Selective" Anion Exchange Distillation Canon: Completely Autotrophic Nitrogen removal Over Nitrite
Nitrite (NO_2^-)	1.0 (measured as N)	Anammox process (Anaerobic ammonium oxidation)

1.3 Nitrogen Cycling

N is an essential nutrient for all organisms and it is also a critical component for protein synthesis in animals, plants, and bacteria. In the atmosphere, most nitrogen (i.e., ~ 78%) exists as triple-bonded dinitrogen, but the largest reservoir of N is in the hydrosphere and biosphere. For protein synthesis, only a small number of specialized N-fixing microbes or under abiotic high temperature processes (e.g. Haber-Bosch), is it possible to break the triple bond holding the two N atoms together so that N can be used to create biomass (Francis et al., 2007). Nitrogen has several oxidation states but in biological compounds, it is almost exclusively in the ammonium form (Cabello et al., 2004).

In soils, sediments and ocean water, N exists in both inorganic and organic forms. The inorganic forms of N are ammonia, ammonium ion, nitrate and gaseous oxides, whereas important organic forms of N compounds include humic and fulvic acids, proteins, peptides, amino acids, purines, pyrimides, pyridines and other amines and amides. In the environment, inorganic N compounds exist as gases in the atmosphere and as dissolved species in water. Nitrate compounds of sodium containing potassium, calcium, magnesium and ammonium are also found in bird guano, playa or caliche; they are usually formed by N fixing bacteria (e.g., cyanobacteria).

Although N is thought to be the best bio signature for life on other planets (Capone et al., 2006), N cycling on Earth is still being investigated. Most notably, important N cycling pathways have been discovered in the last decades, including anaerobic ammonium oxidation (Kuenen, 2008), which will be called 'Anammox' from now on in this thesis.

The N cycle (Fig. 1) is composed of multiple transformations of nitrogenous compounds that are catalyzed by microbes. Microbes control the availability of nitrogenous nutrients and the main biological productivity in marine systems (Ryther, et al., 1971). They are involved in a complex series of transformations, including: 1) nitrate (NO_3^-) and nitrite (NO_2^-) reduction to nitrous oxide (N_2O); 2) dinitrogen (N_2), ammonium (NH_4^+) and organic-N by one of several assimilatory or dissimilatory processes; 3) production from the decomposition of organic-N (ammonification); 4) NH_4^+ oxidation to NO_2^-, N_2O and NO_3^- (nitrification); and, 5) N_2 reduction

to NH_4^+ and organic-N (N_2 fixation). Most of these processes affect the oxidation state of N, and therefore the free energies of these molecules and compounds. Consequently, most of these biological conversions are either energy-yielding or energy-demanding and are fundamental processes in microbial biosynthesis (e.g. nitrification) and bioenergetics (e.g. N fixation) (Karl et. al, 2002).

The rapid increase of knowledge in molecular biology and genomics has had enormous impact on our understanding of the N cycle by making it possible to study the ecological underpinnings and diversity of microorganisms involved in specific N cycle reactions. Genomic and biochemical investigations have changed our understanding of processes, for instance, denitrification was thought to be restricted to very specific habitats and microbes, however Burgin and Hamilton's work showed that it can exist in various environments in an ecosystem (Burgin et. al, 2007).

The oxidation – reduction reactions that take place in the cycle are used in the energy metabolism of microbes, where prokaryotes play a predominant role. Specific enzymes catalyze many of these reactions. These enzymes and genes provide useful targets for studying microbial processes. Knowledge of the genes encoding enzymes involved in biogeochemical transformations provide useful tools especially, not only for assaying gene expression, but also for determining the diversity of microorganisms involved in specific segments within the N cycle transformations (Fig. 1).

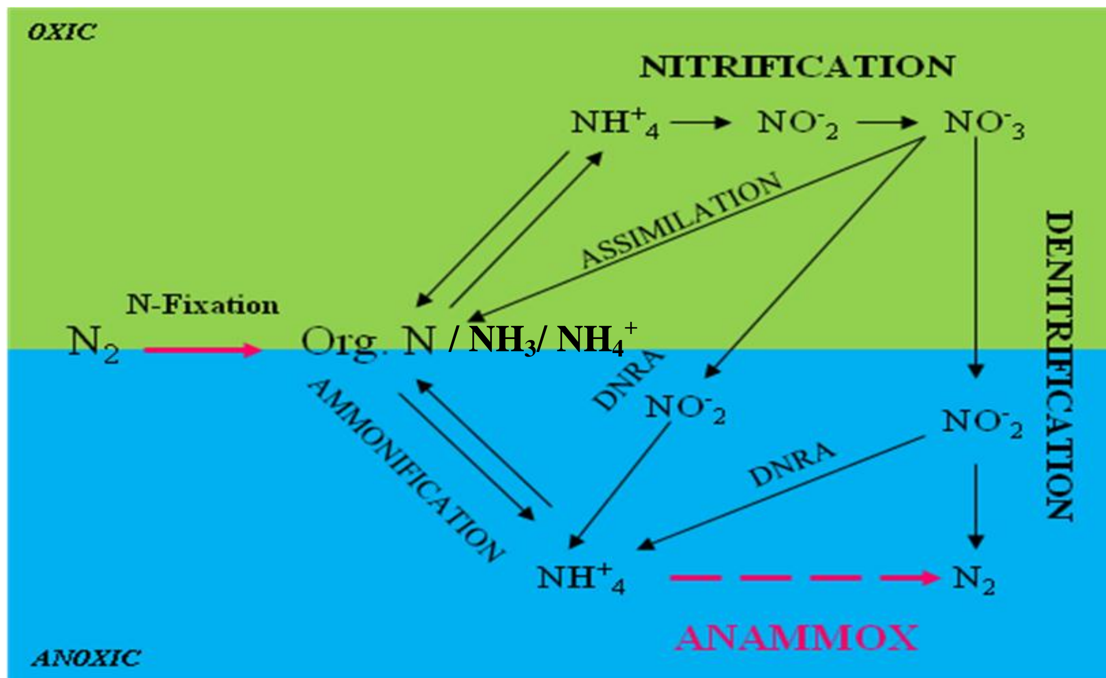


Figure 1: Overall view of the nitrogen cycle (taken from Smith et al., 2008)

1.4 Nitrogen fixation

Nitrogen fixation is a process by which atmospheric N_2 is transformed to N-rich compounds. This process is an enzyme-catalyzing reduction of molecular N to ammonia (NH3), ammonium (NH_4^+), and various organic N forms. This enzyme (nitrogenase) is not specific for dinitrogen; it can also catalyze the reduction of acetylene, hydrogen azide, hydrogen thiocynanate, protons carbonmonoxide (CO) and some other compounds (Smith et. al, 1983). N fixation is a very energy intensive reaction consuming as many as 16 moles of ATP (adenosine-triphosphate) per mole of nitrogen in the reduction of dinitrogen to ammonia (Newton and Burgess, 1983).

In natural processes, biological N fixation is the ultimate source of N for all biota. A number of symbiotic and non-symbiotic microorganisms, both bacteria and algae, have the ability to fix nitrogen. The more quantitatively significant symbiotic N fixative species require

the N-fixing bacteria to be associated with a host plant, or in some rare cases with animals, which utilize the ammonia produced (Ehrlich, 2002).

There are two major limitations to biological N fixation. The first is related to the fact that energy is required to split the triple bond in N_2, as only some organisms with highly developed catalytic systems are capable to. These organisms couple hydrogen oxidation to ATP synthesis, which can be used in CO_2 assimilation via ribulose bisphosphate carboxylase/oxidase system (Alexander, 1977). In N fixation, the ability to couple hydrogen oxidation to ATP synthesis may represent an energy conservation system because the enzyme (nitrogenase) can generate a significant amount of hydrogen during N fixation (Broughton, 1983). The second limitation is connected to obligatory anaerobic conditions for N fixation since it is a reductive process. Eventually, all organisms living in anaerobic environments are capable of creating reducing conditions that can fix nitrogen. Unlike the symbiotic N fixers, non-symbiotic organisms appear to be able to maintain an intracellular environment in which nitrogenase is not activated by oxidizing conditions. The capacity for non-symbiotic N fixation is widespread among prokaryotes, like Azotobacter and Beijerinckia and even amongst some photo and chemolithotrophs (Balows, 1992).

N fixation is very common in the aquatic environment and during the last couple of decades; it has been shown that N fixation by different strains of *Trichodesmioum* (a genus of non-heterocystous filamentous cyanobacteria) balances out N losses by denitrification and anammox in the oceans (Olivia R. Dale, 2007, M.Sc. Thesis). Growth rates of plankton in open ocean surface waters are often limited by the availability of reduced forms of N. New combined N enters surface waters either by advection, diffusion of NO_3^- from deep water, or from biological N_2 fixation. Biological N_2 fixation can be significant in tropical and subtropical seas where large cyanobacteria, Trichodesmioum spp., have been considered the major organisms responsible for it (Capone et al., 1997; Capone et al., 1998; Carpenter et al., 1992). Some estimates have shown that cyanobacteria are responsible for up to 80% of total fixed N in fresh waters (Bashkin 2002).

The special enzyme (nitrogenase) associated with N fixation (Eq. 1) is only found in prokaryotic organisms, including aerobic, anaerobic photosynthetic and non-photosynthetic

bacteria and Archaea. Nitrogenase is an oxygen-sensitive enzyme complex which generates two molecules of NH_3 from the reduction of N_2.It is usually a combination of two iron proteins and molybdenum protein, which receive electrons from an iron protein known as dinitrogenase reductase (Chiswell et al., 1988).

$$N_2 + 6H^+ + 6e^- \rightarrow 2NH_3 \quad \text{(Eq. 1)}$$

Analyses of the genetic diversity in ocean water have shown that organisms other than cyanobacteria can also fix N (Church et al., 2005; Metha et al., 2005). Ongoing research on the identification of other N_2 fixation pathways and alternative pathways in the oceans has pointed out the potential role of certain N_2-fixing unicellular bacterioplankton; this could have a significant impact on the global biogeochemistry of N and C (Dore et al., 2002; Falcón et al., 2004).

In contrast to cyanobacteria, Archaea can thrive in extreme environments because they are able to drive both dissimilatory and assimilatory reactions in the nitrogen cycle. In spite of this, archaeal inorganic nitrogen metabolism is relatively unknown compared to bacterial N metabolism (Cabello et al., 2004). Recent studies have shown that the Earth's earliest organisms may have been similar to the thermophiles (microorganisms that live and grow in extreme environments, such as hydrothermal or geothermal habitats) (Dong et al., 2007). The best known thermophilic microorganism, Methanococcus thermolithotrophicus, is able to fix N_2 at 64°C. Metha et al. (2003) described the first evidence of potential N fixers in diffuse hydrothermal vent fluids and deep-sea water reporting that all of the nitrogenase reductase genes from these samples were closely related to M. thermolithotrophicus.

The importance of N fixation has been underestimated for decades, but the fixation by planktonic organisms appears unimportant as a source of nitrogen to most oligotrophic and mesotrophic lakes (generally <1% of total N inputs), but accounts for 6-82% of the N inputs to eutrophic lakes (Fasham, 2003). Generally, planktonic fixation attains less than 1% of N inputs to the surface waters of the world's oceans but it has been shown to provide more than 20% of N input to the Asko region of the Baltic Sea and 17% of the N input to the Peel- Harvey estuary in Australia (Howarth et al., 1988).

1.5 Ammonia Assimilation (Immobilization) and Ammonification (Mineralization)

The end product of N_2 fixation is ammonia (NH_3). In the environment, there is equilibrium between ammonia and ammonium (NH_4^+), which is driven by pH (Eq. 2) (Worrell et al., 1998). It is the ammonium form that is assimilated by the cells into amino acids to form proteins, cell wall components and purines and pyrimidines to form nucleic acids (Tiedje, 1988). This process is called ammonia assimilation or immobilization. The process that reverses immobilization, the release of ammonia from dead and decaying cells, is called ammonification or ammonium mineralization (Maier et al., 2009). Both immobilization and mineralization of nitrogen occur under aerobic and anaerobic conditions.

$$NH_3 + H^+ \rightarrow NH_4^+ \qquad \text{(Eq. 2)}$$

Ammonification is another major source of reduced nitrogen for living organisms. Ammonification can be defined as the breakdown of organic nitrogen compounds and the release of ammonia (NH_3^+) or ammonium (NH_4^+) (Jaffe et al., 2000). Decomposition of soil or aquatic organic matter is the typical example of this process and heterotrophic bacteria are principally responsible for it (Butcher, 1992). During ammonification, microbes get the carbon source from dead plant or animal biomass and yield the NH_3^+/NH_4^+ system as additional products. Most of this reduced nitrogen is conserved in the biological cycle, but small fractions of it may be volatilized. The more significant source of ammonia is the volatilization during the breakdown of animal excreta and in some regions; these values are comparable to the losses of nitrogen in the denitrification processes (Prosser, 1986). Which of these two processes, immobilization or mineralization, predominates in the environment? It all depends on whether nitrogen is the limiting nutrient. If nitrogen is limiting, then immobilization will become the more important process, on the other hand for environments where nitrogen is not limiting, mineralization will dominate. This limitation of nitrogen is dictated by the C/N ratio. For bacteria, the required C/N ratio is 5 (Zhao, et al., 2006).

Once ammonium is released into the environment, it can be taken up by plants, incorporated into living biomass or become bound to nonliving organic matter, such as soil colloids or humus (Nannipieri et al., 2009). Ammonium can also be sequestered inside clay

minerals (Haider, 1992), which essentially traps the molecule and removes the ammonium from active cycling. Finally, because ammonia is volatile, some mineralized ammonium can escape into the atmosphere and it can be utilized by chemoautotrophic microbes in a process known as nitrification (Maier et al., 2009).

1.6 Nitrification

Nitrification is the biological formation of nitrate from the sequential oxidation of ammonium with the intermediate formation of nitrite. These oxidative reactions are catalyzed by two mutually exclusive groups of microorganisms, namely ammonium oxidizers and nitrite oxidizers (Yu et al., 2003). Nitrification is a key process in marine N cycling and is responsible for the formation of the large deep-sea nitrate reservoir. In addition to the above, nitrification connects the recycling of the organic N with the ultimate nitrogen loss from the oceans. The products of this process are substrates for denitrification and anaerobic ammonium oxidation (anammox), the only two presently known N loss processes (Lam et al., 2009).

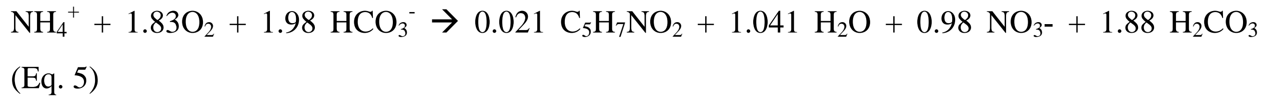
Nitrification consists of two conjugated steps, which start with the oxidation of ammonium to nitrate followed by the oxidation of nitrite to nitrate. The reactions are:

$$NH_4^+ + 3/2\ O_2 \rightarrow NO_2^- + H_2O + 2H^+ \qquad \text{(Eq. 3)}$$

$$NO_2^- + ½\ O_2 \rightarrow NO_3^- \qquad \text{(Eq. 4)}$$

Stoichiometrically, the release of free energy that comes from the oxidation of ammonium to nitrite is around 58-84 kcal per mole of ammonium. The free energy released from the oxidation of nitrite to nitrate is estimated to be between 15.4-20.9 kcal per mole of nitrite (Heidman, 1994). This indicates that ammonium oxidizers under anaerobic conditions obtain more energy than the nitrite oxidizers for the oxidation of one mole of N. The more complete

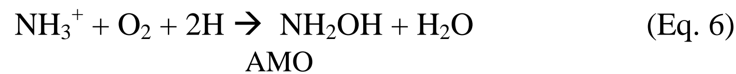
reaction for anammox can be written using the empirical formula $C_5H_7NO_2$ for the formation of biomass as shown below:



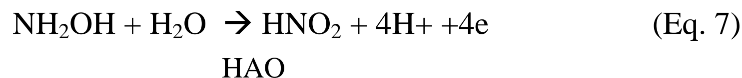
$$NH_4^+ + 1.83O_2 + 1.98\ HCO_3^- \rightarrow 0.021\ C_5H_7NO_2 + 1.041\ H_2O + 0.98\ NO_3- + 1.88\ H_2CO_3$$
(Eq. 5)

This equation shows that the nitrification is an obligatory aerobic process and that 4.18 moles of oxygen is required for the oxidation of one gram ammonium N (Wang et al., 2008).

Several ammonia oxidizing bacteria have been identified in recent years including, Nitrosomonas europaea, Nitrosomonas eutropha, Nitrosococcus oceanus, Nitrosospira briensis, Nitrosovibrio tenuis and Nitrosolobus multiformis and Nitrosolobus europaea (Head et al., 1993). Ammonia oxidation to nitrous acid is carried out by an enzyme called hydroxyl-amine (NH_2OH). Ammonia is first oxidized to hydroxylamine by the catalysis of ammonia monooxygenase (AMO) via molecular oxygen. Hydroxylamine is then oxidized to nitrous acid by hydroxylamine oxidoreductase which is the catalysis of the reaction (Equations 6 and 7). (Bernhard, 2010)



$$NH_3^+ + O_2 + 2H \xrightarrow[\text{AMO}]{} NH_2OH + H_2O \qquad \text{(Eq. 6)}$$



$$NH_2OH + H_2O \xrightarrow[\text{HAO}]{} HNO_2 + 4H+ +4e \qquad \text{(Eq. 7)}$$

A study investigating anammox in the Black Sea showed that maximum nitrate concentrations were only present at the bottom of the oxic zone in the western basin, which was caused by the mineralization of phytoplankton-derived organic N coupled to aerobic nitrification (Kuypers et al., 2003). For decades, only specific groups of β- and γ protobacteria have been found to exhibit this capability (Schleper et al., 2005). More recently, metagenomic studies in the Sargasso Sea, and later in a marine sponge symbiont, have identified that in the marine group

Crenarchaeota (a phylum or a kingdom of the Archaea), there are genes which have the ability to encode proteins resembling ammonia oxidizing bacteria (AOB) (Venter et al., 2004).

In the open ocean, almost 30-50% of all N loss occurs in pelagic oxygen minimum zones (OMZ's), where massive N losses have recently been attributed to anammox (Kuypers et al., 2005). The electron acceptor is NO_2^-, which is either produced by NO_3^- reduction or by aerobic ammonium oxidation which is the first step of nitrification. The first rate limiting step of nitrification is aerobic ammonium oxidation. Therefore it can easily be said that nitrification is particularly very significant process especially in OMZs at anoxic/oxic interfaces in coastal sediments, where the complex interplay between nitrification, denitrification and anammox drives rapid N transformations and large N losses to the atmosphere (Thomson, 2011). Although nitrification is important throughout the ocean, it plays a critical role in the coastal ocean by linking the decomposition of nitrogenous organic matter to N loss via denitrification (Francis et al., 2005). By removing a large percentage of anthropogenic N pollution from estuaries and continental shelf regions before it can reach the open ocean (Seitzinger et al., 1988), coupled nitrification/denitrification effectively isolates the marine N cycle from the heavily altered terrestrial N cycle (Galloway, 2004).

1.7 Denitrification

Denitrification is a reduction process performed by particular groups of heterotrophic bacteria that are ubiquitous in the environment. These heterotrophic microorganisms capable of denitrification are widely distributed in terrestrial and aquatic environments. The recent assumption that the composition of the denitrifying community is of minor importance in controlling denitrification has however been challenged by findings suggesting that denitrifier communities vary in their tolerances to environmental conditions and stress (Cavigelli and Robertson, 2000; Holtan-Hartwig et al., 2000). It is a multi-step process, with N_2 being the end-product. At low oxygen (O_2), denitrifying microbial communities can use nitrate (NO_3^-), nitrite (NO_2^-) or nitrous oxide (N_2O) as alternative electron acceptors to O_2 during anaerobic respiration (Firestone and Davidson, 1989). It is a facultative anaerobic process and it is known that

denitrification occurs in almost all terrestrial, freshwater, coastal and some oceanic ecosystems, as well as human-engineered systems. Denitrification, as opposed to nitrification, occurs under low-oxygen conditions and is widespread among over 50 different genera (Zumft, 1997), including members of the bacteria, Archaea, and even benthic foraminifer (Risgaard-Peterson et al., 2006). Known denitrifying bacteria and Archaea possess several clusters of genes involved in denitrification and most are therefore capable of performing the multi-step process:

$$(NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2).$$

The first denitrifiers were isolated in 1886 (Payne, 1981). Nõmmik's pioneering (1956) work on denitrification opened a new window in nitrogen cycling. Nõmmik and other scientists investigated the factors that might have an effect on the rate of denitrification, such as the role of NO_3^- availability, O_2 and pH (Fig.2) (Firestone et al., 1979). These dominant controlling factors are highly variable over space and time, they give rise to "hot spots" and "hot moments" of denitrification that are difficult to predict (McClain et al., 2003). Understanding these controlling factors is of interest because the intermediate gaseous products are important greenhouse gases, whereas the N_2 final product is highly inert and thus has no adverse environmental consequences. The gaseous products are the major biological pathway for N loss from ecosystems and the intermediates, NO and N_2O, have implications for the atmospheric gas chemistry (Prather et al., 2001).

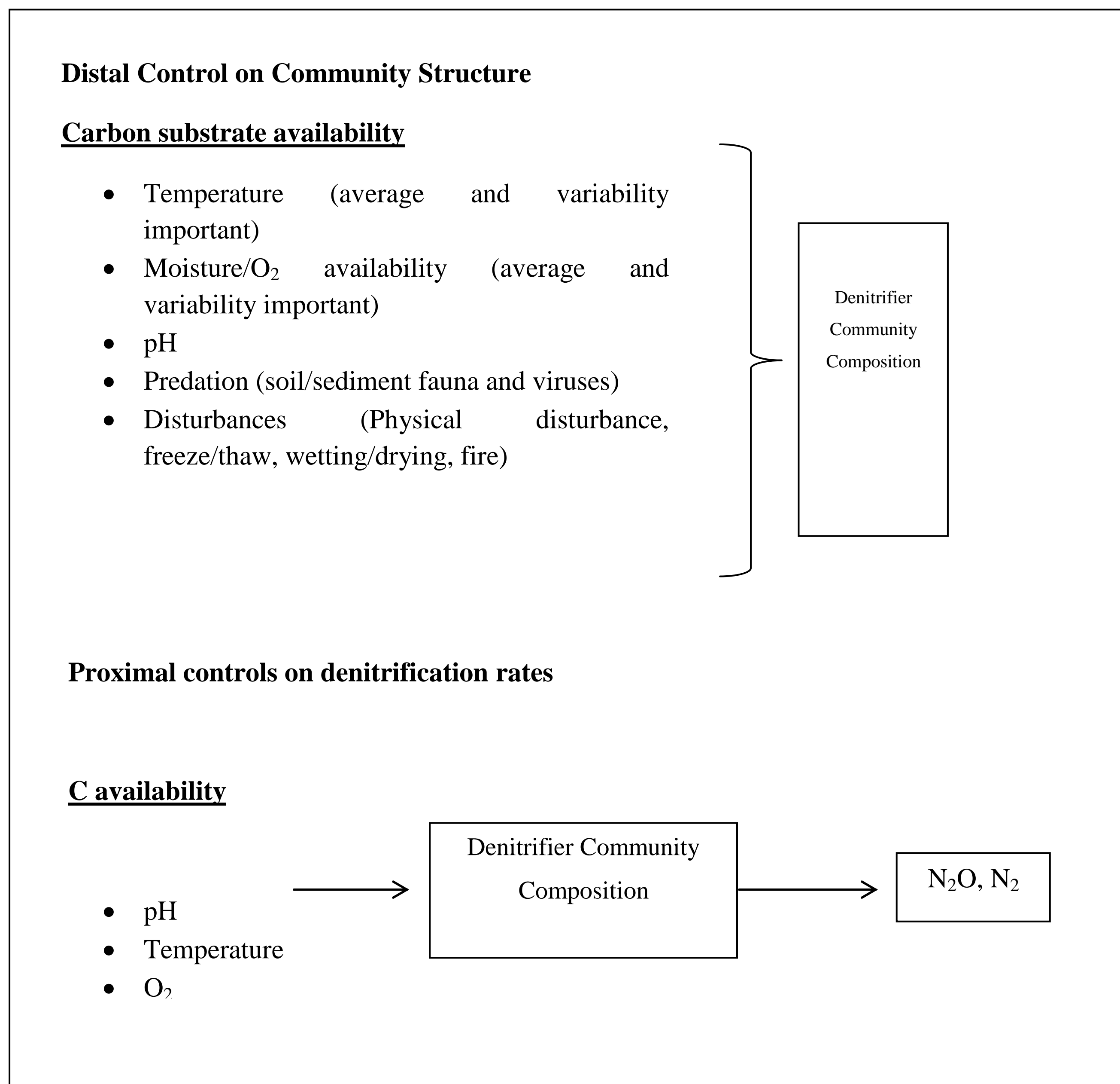


Figure 2.A proposed scheme of distal and proximal controls on denitrification and denitrifers. (Modified from Wallestein D. Matthew et al., 2006)

The proportion of N lost in the form of N_2O during denitrification is small, generally smaller than 1% of total gaseous production (McCracken, 2010, Ph.D. thesis). In terrestrial soils and aquatic sediment ecosystems, relative N_2O production is known to be influenced by the rate of N loading, availability of organic carbon, pH and the concentration of dissolved oxygen

(Firestone et al., 1980; Seitzinger, 1988). McCrackin and Elser (McCracken, 2010, Ph.D. thesis) hypothesized that atmospheric N deposition influences both N_2O fluxes and the relative production of N_2O because of increased denitrification and elevated concentrations of nitrate, respectively. McCrackin and Elser's work added a novel understanding to the large data set of N cycling in streams (Mulholland et al., 2008) in supporting the conclusion that nitrate concentration is "the single" most important predictor of denitrification.

1.7.1 Dissimilatory nitrate reduction to ammonium

Dissimilatory nitrate reduction to ammonium (DNRA) involves the transition of nitrate to ammonium, in contrast to assimilatory processes that incorporate N into cell components. The resultant NH_4+ is biologically more available and less mobile than nitrate. DNRA has 2 known pathways, fermentation and sulfur oxidation, but the link between the two is still unknown. DNRA coupled to sulfur cycling has been documented in marine and freshwater ecosystems. (Brettar et al., 1991). The mechanism in fermentative DNRA is the coupling of electron flow from organic matter to the reduction of nitrate via fermentation reactions (Tiedje, 1988). When conditions promoting fermentative DNRA and respiratory denitrification are similar, e.g., anoxia, nitrate availability and organic substrates, fermentative DNRA is thought to be favored in nitrate-limited environments rich in labile carbon, while respiratory denitrification would be favored under carbon-limited conditions (Kelso et al., 1997). In addition, the redox conditions of the sediments may also be important. It has been suggested that the micro zones of oxygen leakage from roots of emergent plants in wetland sediments may favor aerobic denitrifiers over anaerobic fermentative bacteria (Matheson et al., 2002).

Another DNRA pathway is chemolithoautotrophic; it couples the reduction of nitrate to the oxidation of reduced sulfur forms, including free sulfide and elemental sulfur (H_2S, S_2^-). The most dominant fate of the reduced nitrate can be determined by the ambient concentration of free sulfide, which is known to inhibit the final two reduction steps in the denitrification sequence (Brunet et al., 1996). A similar process that couples the reduction of nitrate to the oxidation of methane was discovered in freshwaters (Raghoebarsing et al., 2006). The discovery of a new

"intra-aerobic" pathway of nitrite reduction has been recently described (Ettwig et al., 2010). This pathway resembles the proposed mechanism of denitrification. The process is often overlooked or lumped together with conventional denitrification (Ettwig et al., 2009).

1.7.2 Nitrate reduction coupled to iron oxidation

The reduction of nitrate coupled to iron (Fe) cycling is thought to take place through both biotic and abiotic pathways (Weber et al., 2006). There are also abiotic pathway whereby nitrate is converted to nitrite (NO_2^-) by ferrous iron (Fe_2^+) and reduced manganese (Mn_2^+), followed by the rapid transformation of NO_2^- to N_2 (Postma et al., 1991). Another abiotic reaction involves the reduction of nitrate to nitrite by Fe or Mn and the nitrogen species are subsequently bound to organic substances to produce dissolved organic nitrogen (DON) (Davidson et al., 2003). Evidence for this reaction was discovered in forest soils (Dail et al., 2001). It is also well known that some microbes can mediate nitrate reduction coupled to iron oxidation in aquatic ecosystems (Weber et al., 2006). This biotic reduction occurs at relatively low temperatures and circumneutral pH (5.5-7.2; Weber et al., 2001) and thus may be more likely to occur in surface waters than the equivalent abiotic reactions.

1.8 Anaerobic ammonium oxidation (Anammox)

The conversion of nitrate (NO_3^-) to gaseous N_2 was believed to be the only important process in oxygen-depleted zones of the open ocean and in anoxic basins and fjords (Dalsgaard et al., 2003), but oceanographers knew that far less ammonium accumulates in anoxic fjords and basins than what is expected from the stoichiometry of heterotrophic denitrification (Richards et al., 1965) (Table 3)

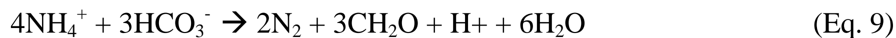
Table 3: Gibbs free energy of several reactions that takes place in autotrophic denitrification and energy yield difference between nitrite and nitrate (Richards et al., 1965).

Reaction Equation	ΔG^0 (kj mol1- NH4+ or NO3-)
$2NO_3^- + 5H_2 + 2H^+ \rightarrow N_2 + 6H_2O$	-560
$8NO_3^- + 5HS^- + 3H^+ \rightarrow 4N_2 + 4H_2O + 5SO_4^{2-}$	-465
$3NO_3^- + 5NH_4^+ \rightarrow 4N_2 + 9H_2O + 2H^+$	-297
$NO_2^- + NH_4^+ \rightarrow N_2 + 2H_2O$	-358
$2O_2 + NH_4^+ \rightarrow NO_3^- + H_2O + 2H^+$	-349
$6O_2 + 8NH_4^+ \rightarrow 4N_2 + 12H_2O + 8H^+$	-315

In recent years, our understanding of the N cycle has been revised with several new pathways, including anaerobic ammonium oxidation (anammox) and anaerobic methane oxidation coupled to denitrification (AMO) (Kuypers et al., 2005). Among these pathways, "anammox" with nitrite is extremely important for N cycling in marine environments.

During the late 1970's, Broda's description of missing lithotrophs in nature created a discussion about a new microbial reaction carried out by "chemosynthetic bacteria that oxidize ammonia to nitrogen with O_2 or nitrite (NO_2^-) as an oxidant" (Broda. 1977) (Equations 8 and 9).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad \text{(Eq. 8)}$$

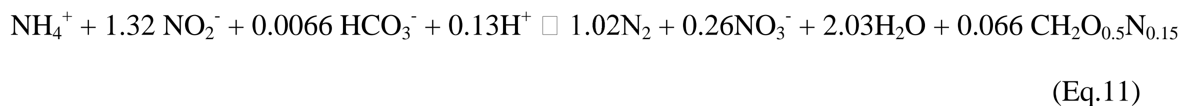


$$4NH_4^+ + 3HCO_3^- \rightarrow 2N_2 + 3CH_2O + H+ + 6H_2O \quad \text{(Eq. 9)}$$

It was long thought that ammonium was chemically inert and that anaerobic oxidation of ammonium would be impossible. The alternative nitrogen removal pathway was first proposed by Richards (1965) who studied ammonium deficits in anoxic marine basins. Early evidence for the presence of anammox was also observed in marine sediment pore water profiles where there was simultaneous disappearance of nitrite and ammonium (Friederich,G. E.,1987; Cline et al., 1972). Later on, the direct evidence for the anaerobic ammonium oxidation was found in an anoxic (denitrifying) plant (Mulder et al., 1995). This proposed reaction was suggested:

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+ \quad (\Delta G° = -1.483,5 \text{ kJ per reaction}) \qquad \text{(Eq. 10)}$$

The Gibbs free energy indicates that the reaction is exergonic "a chemical reaction where the change in the Gibbs free energy is negative, indicating a spontaneous reaction" and thus it is a supply for energy growth (Van de Graff et al., 1995). In addition, mass balance studies showed that anammox organisms use carbon dioxide as a carbon source to produce their biomass and use nitrite not only as an electron acceptor, but also for reduction of carbon dioxide. This is due to their typical chemolithoautotrophic life-style (see Eq. 11).



$$NH_4^+ + 1.32\ NO_2^- + 0.0066\ HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066\ CH_2O_{0.5}N_{0.15} \qquad \text{(Eq.11)}$$

Nitrite is an intermediate in both heterotrophic denitrification and aerobic ammonium oxidation (Third et al., 2001) and the enrichment culture cells indicate that anammox bacteria can also reduce nitrate to nitrite while oxidizing short-chain fatty acids (Guven et al., 2005). However apart from nitrite, it has been shown that anammox bacteria also employ iron (Fe_3^+), manganese oxides (MnOx) and nitrate as electron acceptors for their metabolism (Strous et al., 2006). These characteristics show similarities to denitrification reaction pathways but anammox differs by using nitrite as an electron acceptor, after being oxidized from nitrate in the environment (Kartal et al., 2007).

Classical cultivation techniques have so far failed to enrich and grow anammox bacteria. 15N- labeled compounds are used to identify the intermediates and products of the reaction, and to ascertain whether the reaction is a spontaneous chemical reaction or a biologically mediated reaction (Van de Graaf et al., 1995). After several years of trying to cultivate anammox bacteria, Strous and his colleagues managed to enrich the organisms with a high biomass yield in a sequencing fed-batch reactor (Strous et al., 1998). The exponentially growing culture was up to 70% enriched with anammox bacteria. This enrichment culture was obtained based on a system containing unlimited mineral media containing ammonium and nitrite only (Van de Graaf et al.,

1996). The obtained enriched anammox cells were used for DNA isolation and gene identification, especially for the identification of the 16S rRNA genome sequences. Given the lack of pure culture, DNA analyses remained limited but researchers managed to purify the bacteria responsible for the process by physically purifying from highly enriched cultures with an optimized Percoll density gradient centrifugation (Strous et al., 1999; 2002).

Phylogenetic identification from the purified cells provided the 16S rRNA sequences of the anammox organisms (Kuenen et al., 2001). Results revealed that the anammox bacteria branch very deeply within the order "Planctomycetales" which are a phylum of aquatic bacteria (Strous et al., 1999). The first discovered, planctomycete - like anammox bacterium was named "Candidatus Brocadia anammoxidans" (Kuenen and Jetten, 2003). These novel metabolic organisms have a number of very unique features, including the use of hydrazine (N_2H_4) as a free catabolic intermediate. The other important intermediates include hydroxylamine (NH_2OH), which is a reducing agent (Va der star wouter et al., 2008). In addition, the biosynthesis of ladderane lipids and the presence of a special intracytoplasmic compartment called "anammoxosome" which may protect the cell from toxic intermediates like hydrazine (Jetten et al., 2003) are unique features of anammox bacteria. Anammox bacteria have extremely slow growth rates due to a low substrate conversion rate (doubling time is approximately 13 days; Jetten et al., 1999).

They have received considerable attention in engineered systems, but were assumed to be minor players in the N_2 cycle within all natural ecosystems (Francis et al., 2007). Anammox occurs in anoxic waters where there are suitable concentrations of both nitrate and ammonium and the process is inhibited by many simple organic compounds, including pyruvate ($CH_3COCOOH$- the output of the anaerobic metabolism of glucose known as glycolysis) and ethanol (Jetten et al., 1999). It has been shown that alcohols inhibit anammox bacteria, while short-chain fatty acids were converted by them (Guven et al., 2005). Methanol is the most effective inhibitor to the reaction which leads to a complete and absolutely irreversible loss on the activity if the concentrations are as low as 0.5 mM. Propionate also has negative effects.

1.8.1 Anammox bacteria

As stated earlier, 16S rRNA phylogenetic studies showed that the anammox bacteria branch very deeply within the order "Planctomycetales" which are a phylum of aquatic bacteria (Strous et al., 1999). Anammox bacteria comprise several unique species and cell compartments in its structure. That being said, Anammoxosomes are unique metabolically significant compartment of Planctomycetes performing the anammox process. They comprise Candidatus "a term that is used for taxonomic status for noncultivable organisms" genera: Candidatus Brocadia (Strous et al., 1999; Schmid et al., 2003), Candidatus Kuenenia (Schmid et al., 2000), Candidatus Scalindua (Schmid et al., 2003), Candidatus Anammoxoglobus (Kartal et al., 2007) and Candidatus Jettenia (Quan et al., 2008). "Brocadia", "Kuenenia" and "Scalindua" are mostly from wastewater treatment bioreactors or marine anaerobic habitats and none of which are yet in pure culture. The first anammox planctomycete "Candidatus Brocadia anammoxidans" was discovered to be the dominant member of the microbial community of an anaerobic wastewater treatment bioreactor performing Anammox (Strous et al., 1999). A second genus and species, "Candidatus Kuenenia Stuttgartiensis" (Schmid et al., 2000) has been discovered, and a further probable distinct species of this genus, the Kolliken anammox planctomycete, has been claimed (Schmid et al., 2000; Egli et al., 2001). A third genus, "Candidatus Scalindua" has been described which would appear to have a marine representative "Candidatus Scalindua sorokii" from the anoxic Black sea habitat and two other species, "Candidatus Scalindua brodae" and "Candidatus Scalindua wagneri", from a wastewater plant treating landfill leachate (Jetten et al., 2003; Schmid et al., 2003).

2. Objectives and hypotheses

The main objective of this thesis is to show the existence of anammox microorganisms in ground waters contaminated with fertilizers and nitrogen related compounds, using specific FISH (Fluorescence in situ Hybridization) probes with a unique nucleotide sequence of a known rRNA sequence. Based on the previous nitrogen isotope data (15N) of one of the two sites studied here (Elmira, ON, Clark et al., 2008), it is hypothesized that anammox bacteria are present and active in the contaminated ground waters. The other sampling site, Putnam, ON, also has high concentrations of ammonium and nitrate which originate from a nearby turkey manure site.

The second objective was to characterize the physico-chemical properties of the contaminated groundwaters and to assess the relationship between the water chemistry and the anammox cells' abundance. Evidence of anammox have been linked to the concomitant decline of nitrate, nitrite and ammonium in solution (Kuypers et al., 2006). In addition, the presence of anammox bacteria has also been linked to the geochemistry of Fe and Mn in solution (Strous et al., 2006). We hypothesized that deeper wells showing decreased dissolved oxygen levels and/or high levels of reduced Fe and Mn would correlate with anaerobic conditions and therefore with a higher abundance of anammox bacteria.

3. Methodology

3.1 Study sites

3.1.1 Elmira, ON

The town of Elmira (43^0 35' 38 52'' N, 80^0 33' 06.41'' W) in Ontario, Canada, is the largest community within the Township of Woolwich in the Regional Municipality of Waterloo and it is located 15 km north of the city of Waterloo. The Canagagigue Creek is the major body of water, flowing southeast from the north end of town to the Grand River, some 4 km to the east. Since the Canagagigue creek is connected to the Waterloo city municipal water system, water quality of the creek becomes extremely important. The reason why Elmira is one of our sampling sites is because of high levels of nitrogen species, especially ammonia and nitrate concentrations, in the groundwaters. The chemical company "Chemtura" discharged ammonia to an on-site waste-water pond, which was at the same location as the fertilizer company that also allowed dry spills and discharges to an on-site wastewater pond (Fig. 3). The ammonium from the chemical company was either a chemical manufacturing by-product or a delivered product to the plant for subsequent manufacturing processes. The fertilizer company produced ammonium and nitrate from its products. Both ammonium and nitrate concentrations persist today with ammonium levels approaching 840 ppm of N and nitrate levels around 350 ppm of N in the groundwaters (Clark et al., 2008).

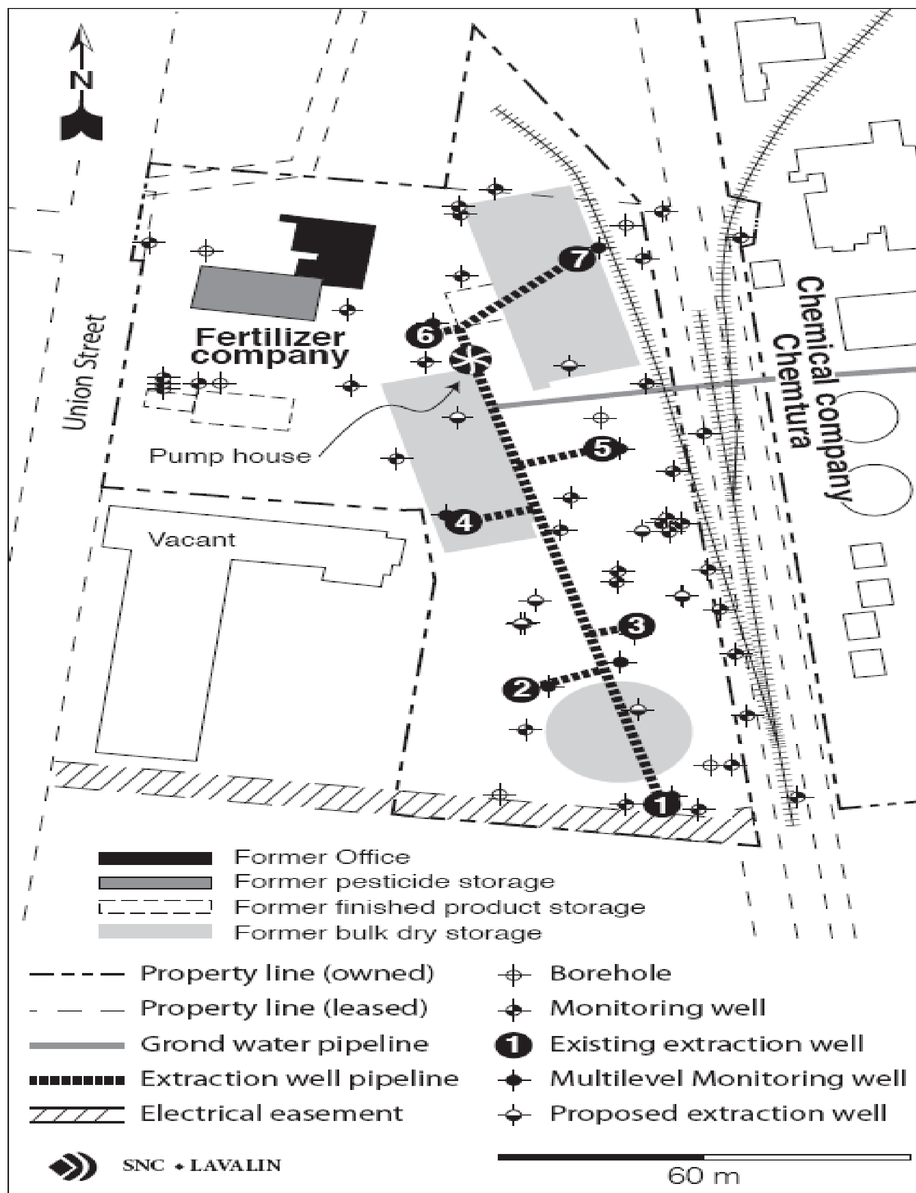


Figure 3: Location of the Elmira site showing the fertilizer company with the existing extraction wells and the chemical company.

3.1.2. Putnam, ON

The second study site is near Putnam, which is located in southern Ontario, west of the city of London (43^{0}00’ 21.31’’ N, 80^{0}57” 21.43’’ W) (Fig. 4).

The Zorra turkey manure factory located in Putnam contains manure and manure/fertilizer piles that drain into man-made lagoons. Due to the elevation of the manure piles, the lagoon and the nearby groundwaters are heavily contaminated with ammonium and nitrate, with concentrations as high as 200 ppm 110 ppm, respectively (Moore et. all, 2011).

Figure 4: The Putnam Turkey manure site seen by satellite imaging. The numbers refer to the well location.

3.2 Field sampling methods

There are several groundwater wells at the Putnam Zorra site. A background water well (PU86) was selected (Figure 4) as a background (control) site as it shows very low levels of ammonium, nitrate and nitrite. PU92 and PU95 were selected due to both their availability during the sampling periods and also to their high concentrations of ammonium and nitrate. There were also other wells that were sampled but during some sampling periods, sampling was unsuccessful due to the low water levels caused by a lack of precipitation. All water wells have different depths (see Table 4). During sampling, water was collected with a simple peristaltic water pump and stored immediately in sterile containers at low temperature storage (-20°C freezer).

At the Elmira site, the fertilizer company's pumping station was used to sample from 7 different separate wells (see Table 4). The depth of the wells was constant, which is 11 meters. The wells were selected based on their concentrations of ammonium and nitrite. However, throughout the sampling periods, some targeted wells had to be replaced with other wells due to low water levels caused seasonal changes in precipitation.

Table 4: Well numbers, depths and sampling times at the Putnam and Elmira sites.

Well ID	Depth (m)	Sampling Times
Putnam-PU86	• 2.3	July,2009 August,2009 October,2009
Putnam-PU92	• 2.2	July,2009 August,2009 October,2009
Putnam-PU92	• 2.6	July,2009 November,2009
Putnam-PU95	• 2.2	July,2009 August,2009
Putnam-PU95	• 2.6	July,2009
Elmira 1	• 11	August,2009 October,2009 November,2009
Elmira 2	• 11	August,2009 November,2009
Elmira 3	• 11	August,2009 October,2009 November,2009
Elmira 4	• 11	July,2009 August,2009 October,2009

		November,2009
Elmira 5	• 11	July,2009 August,2009 October,2009 November,2009
Elmira 6	• 11	August,2009 November,2009
Elmira 7	• 11	July,2009 August,2009 October,2009 November,2009

3.3 Water analysis

3.3.1 On site field measurements

For a better understanding of the groundwater geochemistry during sampling, on site measurements of pH, dissolved oxygen (DO) and temperature were performed. The temperature and pH measurements were done with a pH meter (VMR) and a pH probe (Fisher Scientific) calibrated with pH 4 and 7 standards. Calibration was done at the same temperature as the groundwater samples.

3.3.2 Groundwater geochemistry

For both total concentrations and dissolved concentrations, groundwater samples (50 mL each) were collected from each well and field-filtered (Whatman syringe filter, 0.45µm) prior to their analysis. Filtered samples were prepared for ICP-MS and ion chromatography (DX100 IC,

Dionex) with a prior acidification in the laboratory for the analysis of major cations (with the exception of NH_4^+ and NO_2^-). For the concentration of NH_4^+, the salicylate colorimetric method (Crooke and Simpson, 1971) was used, where NO2- concentrations were determined with the sulfanilamide colorimetric method (Egli et al., 2001). The intermediate products of anammox reaction (hydroxylamine (NH_2OH) and hydrazine (N_2H_4) were analyzed according to the methods of Frear and Burrel (1955) and Watt and Chrisp (1952), respectively. The detection limits for NH_2OH and N_2H_4 were 7.5 and 1.5 µmol L.

3.4 Fixation of cells and storage

100 mL of groundwater was sampled from each sampling station and cell fixation was performed on site using 4% (w/v) paraformaldehyde. Filtration of the groundwater samples was performed within 12 hours after sampling. For better imaging, G&E Water & process Technologies Polycarbonate black background filter papers (pore size of 0.22µm) were used. Millipore filter devices were used for the filtration step. After the filtration, the filters were washed with different concentrations of ethanol (50%, 80%, and 95%) and stored at -20°C.

3.5 Fluorescent in situ hybridization (FISH)

Standard identification of anammox bacteria with 16S rRNA-targeted fluorochromes-labeled oligonucleotide probes was carried out according to a protocol that was modified from Amann et al. (1990) , Glockner et al. (1996) and Manz et al. (1992). A group of species specific oligonucleotide probes were used according to their abundance and environmental existence (Table 5). All probes were purchased from IDT technologies. S-P-Planc-0046-a-A-18 and S-Amx-0368-a-A-18 were tested. The hybridization temperature from the Amann et al. protocol was slightly modified, 48°C was judged more suitable for the denaturation of the gene sequences

than the proposed 46°C. In addition, the temperature of the washing step was changed from 48°C to 50°C. The protocol that we have used can be found in the appendix. (APPENDIX II)

Table 5: Oligonucleotide probes used for FISH analyses.

Fluorochrome	Excitation (nm)	Emission (nm)	Fluorescence color
CY3	~550	~570	Orange-Red
Alexa Fluor 532	532	554	Yellow
Alexa Fluor 488	495	519	Green
DAPI	358	460	Blue

For the microscopic observations, the oligonucleotide probe gene sequences were labeled with different fluorochromes (Table 6). Planc-0046-a-A-18 labeled with Alexa Fluor 532 yellow and Amx-0368-a-A-18 labeled with Alexa fluor 488 green to their 5' to 3' ends were used. EUBI +EUBII "all bacteria oligonucleotide" was labeled with Cy3 red fluorochrome. After the hybridization, the filters were counter stained with 4, 6-diamidino-2-phenylindole (DAPI), which targets all DNA (for total cell counts). For imaging, a conventional epifluorescent microscope (Zeiss Axiophot) was used with proper filter sets matching the fluorochromes. Images were taken with a camera that is attached to the microscope.

Table 6: Fluorescence characteristics of fluorochromes under an epifluorescence microscope.

Probe	Target	Sequence (5'-3')	Reference
EUB I+II	All bacteria	GCA GCC ACC CGT AGG TGT	(Amann et al., 1990)
Pla46	Planctomycetales	GAC TTG CAT GCC TAA TCC	(Neef et al., 1998)
Amx368	All anammox organisms	CCT TTC GGG CAT TGC GAA	(Schmid et al., 1999)

4. Results

4.1 Physico-chemical characteristics of the groundwater samples

4.1.1. Putnam – Elmira Sampling Sites

Throughout the seasonal sampling, groundwater samples from Putnam were taken from several wells. The wells were chosen according to their nitrate, nitrite and ammonium concentrations and organic matter content. A background well was chosen, i.e., well number PU86, for comparison. Due to the various sampling times throughout the year and variable precipitation levels prior to sampling, problems were encountered while pumping waters from some wells. We replaced them with other wells while taking into consideration their similar geochemical characteristics and positions relative to the man-made lagoon and groundwater flow direction.

The Elmira samples were taken from a pump station located in the fertilizer company area. There were 7 different wells, i.e., Elmira 1 to 7. Groundwater pumped into the wells in the pump station (Elmira 1-7) is from the Canagagigue Creek. Elmira 6 is connected to a water tank where water is pumped into from the other 6 groundwater wells. At this sampling site, there were no depth variations. All wells are approximately 11 meter deep. Variable precipitation levels during the different sampling seasons were also a problem, but we did not have other well options to replace the ones lacking water. Missing well numbers represent wells those where we were unable to pump water from.

4.1.1.1. Dissolved Oxygen (D.O.)

At the Putnam site, the lowest dissolved oxygen value was 0.16 mg/L whereas the highest was 5.18 mg/L (Fig. 5). From the results, we can see that the background well PU86 showed the highest dissolved oxygen concentration in July and August, but not in October and November (Fig. 5). The concentration of dissolved oxygen in the other wells was below 2 mg/L throughout the sampling months, with the exception of PU122 in October, which was around 3 mg/L.

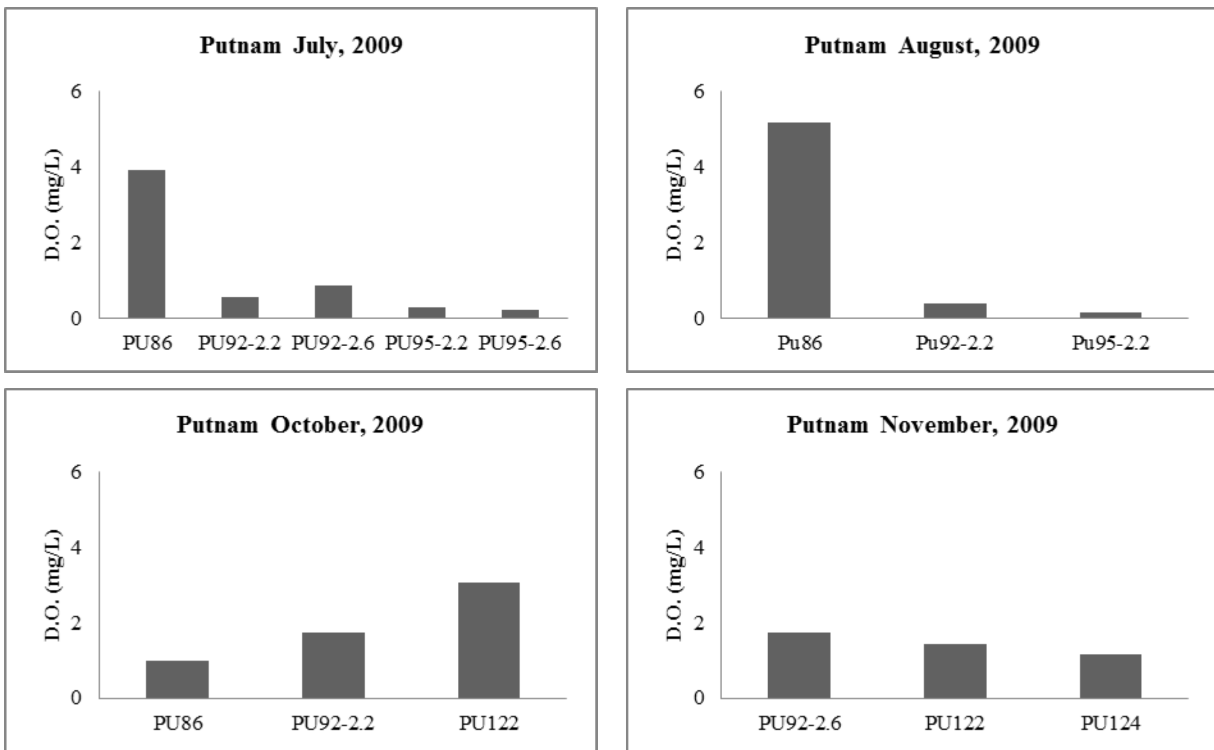


Figure 5: Dissolved oxygen in the various wells at the Putnam site over the course of 4 months

At Elmira, the lowest average dissolved oxygen concentration for all wells was 1.02 mg/L in the month of October, 2009. For the other sampling dates, average dissolved oxygen concentrations varied between 3.01 mg/L , 1.53 mg/L and 1.90 mg/L in July, August and November, respectively.

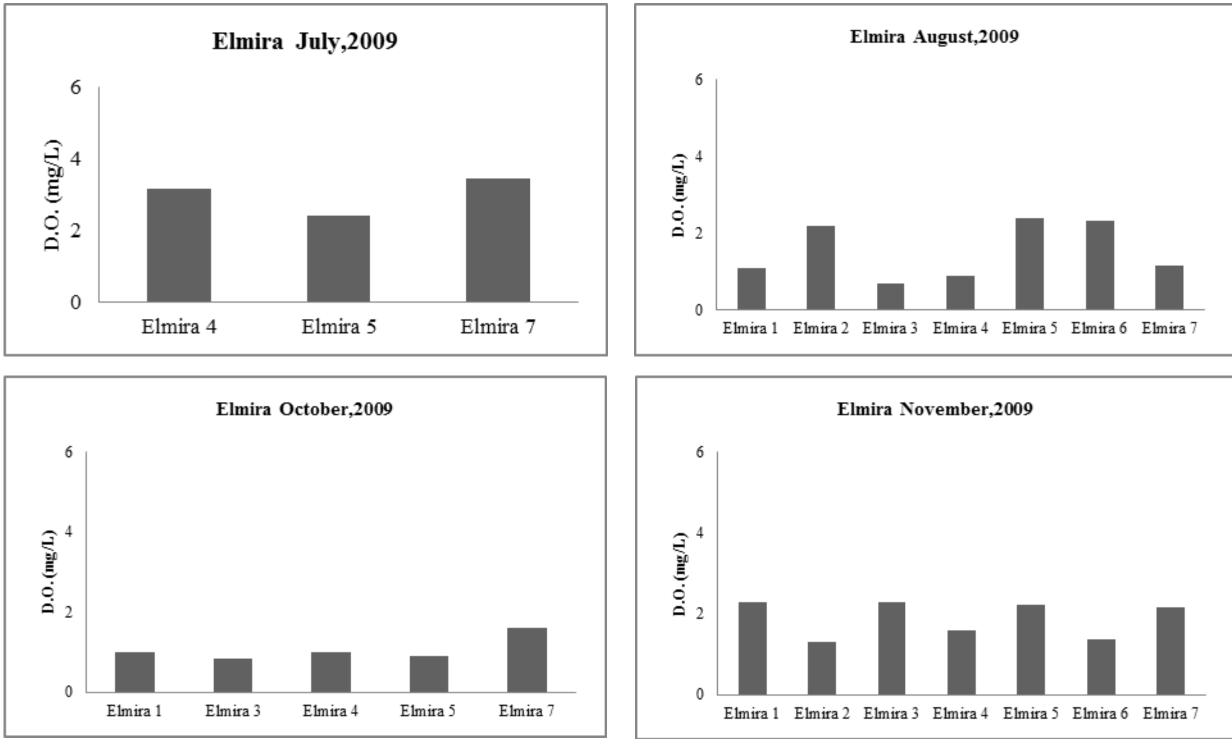


Figure 6: Dissolved oxygen values at the various wells of the Elmira site over 4 months of sampling.

4.1.1.2 pH

At the Putnam site, pH measurements did not vary much between wells, sampling periods and depth variations (Fig. 7). pH values stayed very close to neutral values, varying between 6.75 and 8.10.

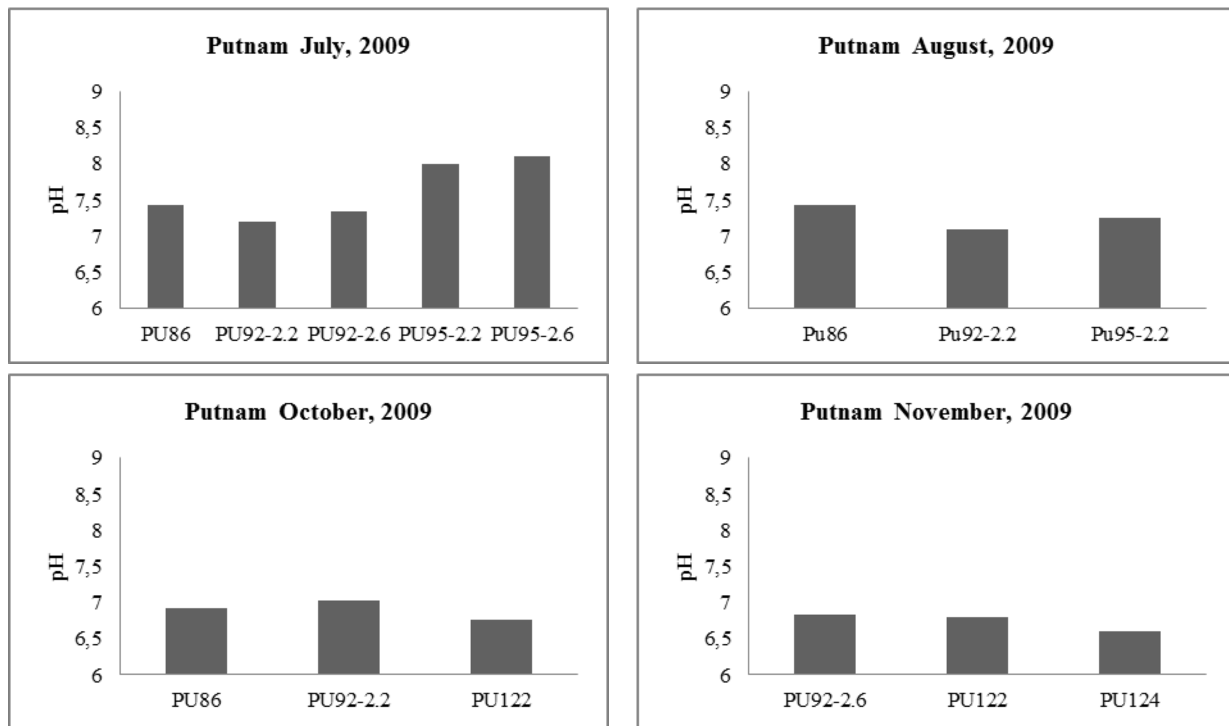


Figure 7: pH variations as a function of the sampling times and wells at Putnam.

At the Elmira site, the groundwater samples showed near neutral pH values. pH values ranged from 6.84 as the lowest value in the month of November to 7.01 as the highest value in the month of October (Fig. 8).

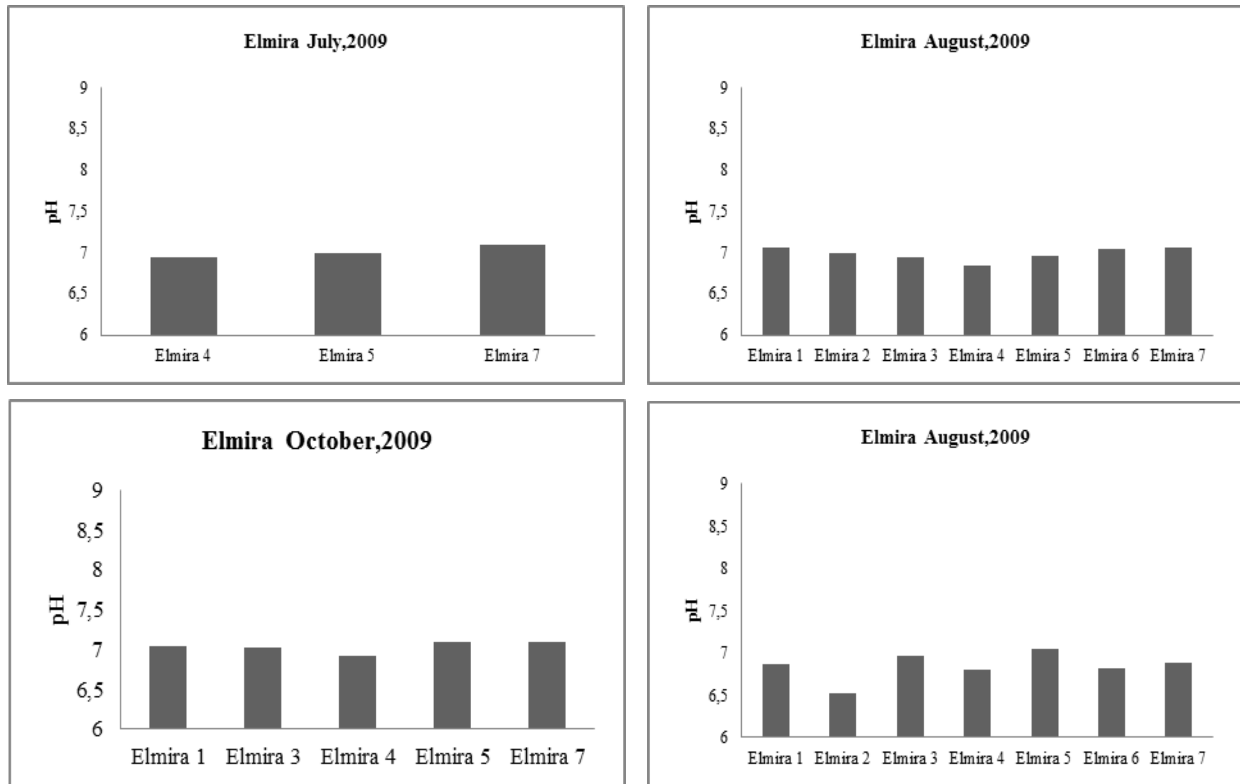


Figure 8: pH variation during the different months and in different wells at the Elmira site.

4.1.1.3 Nitrate (NO_3^-)

In the Putnam PU86 background well, nitrate concentrations were higher than in the other wells in July and October 2009, averaging 49,18 mg/L and 58,13 mg/L (Fig. 9). Between different wells closer to the lagoon, concentrations decreased and became in some cases, below the detection limit. NO_3^- concentrations generally decreased in the wells having a lower depth (PU92 showed a nitrate concentration below detection limit at 2.2 m whereas at the 2.6 m, PU92 had 1.3 mg/L in the month of July, 2009). In deeper wells (PU122 and PU124), nitrate levels varied between 38.8 mg/L and 4.4 mg/L.

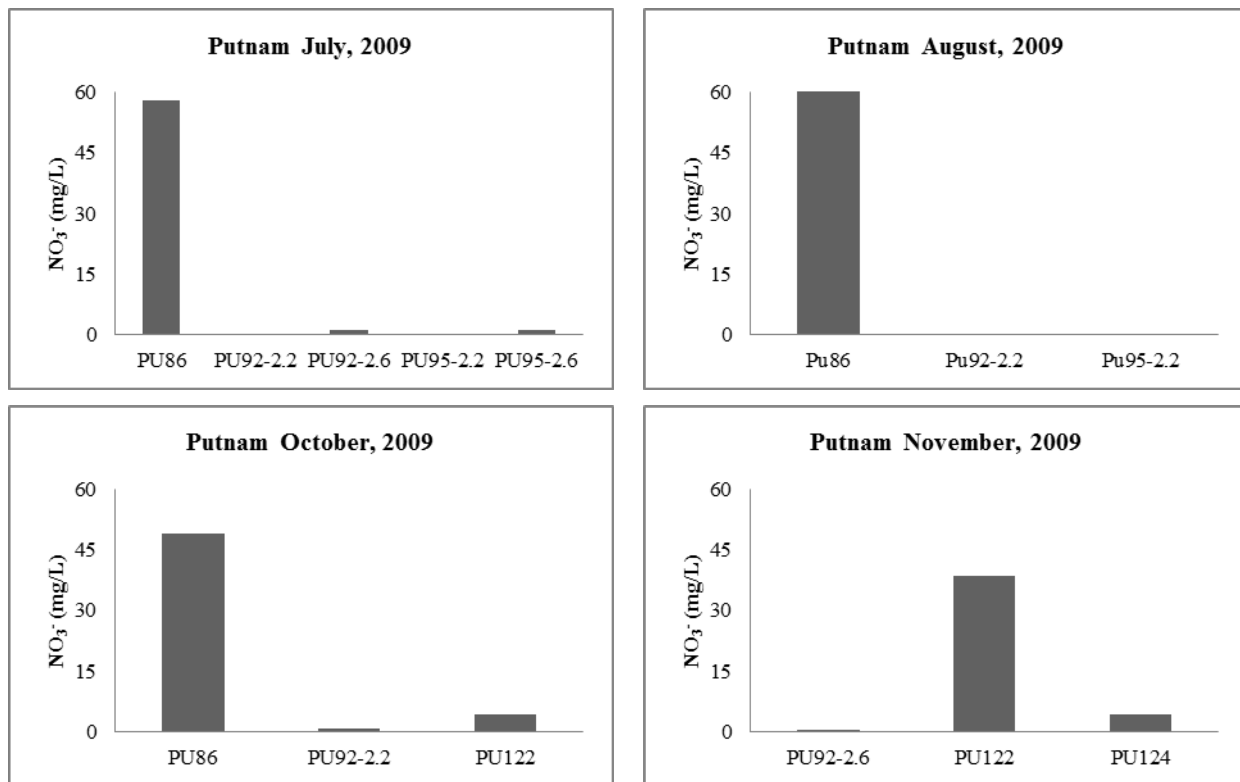


Figure 9: NO_3^- concentrations (mg/L) at the Putnam site as a function of wells and sampling times.

Nitrate concentrations were extremely high in all Elmira groundwater wells (Fig. 10). During the different sampling seasons, the lowest nitrate concentration was 109.5 mg/L at Elmira 7 well in November, 2009. On the other hand, the highest concentration was 933.7 mg/L at the Elmira 1 well in November 2009. The Elmira 2, 6 and 7 wells were at the low end for nitrate concentrations (average values for these well numbers are 170.6, 308.5 and 192.5 mg/L), while Elmira wells 1, 3, 4 and 5 were on the high end (average values for these well numbers are 914.1, 764.0, 808.0 and 792.6 mg/L).

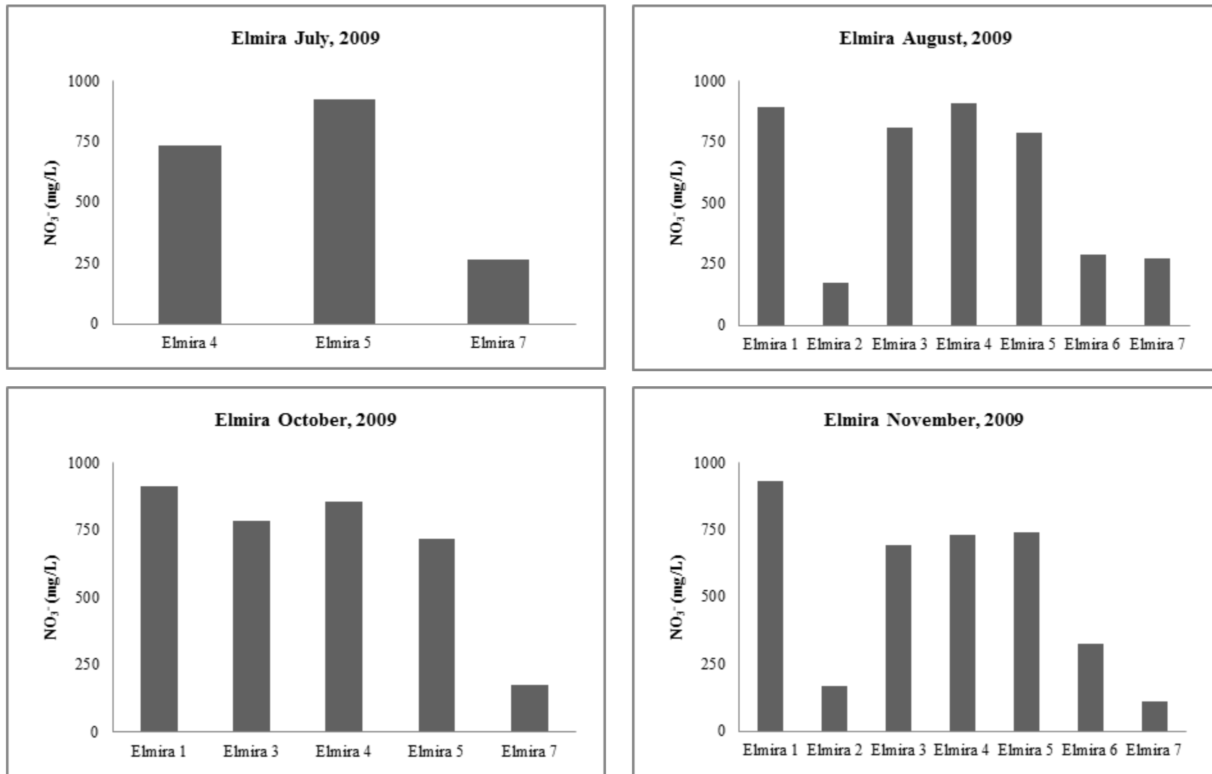


Figure 10: NO_3^- concentrations (mg/L) at the Elmira site as a function of the wells and sampling dates. Note that the concentrations are much higher at this site than at the Putnam site (Fig. 9).

4.1.1.4 Nitrite (NO_2^-)

At the Putnam site, NO_2^- concentrations were generally very low or below the detection limit, with the exception of October 2009 (Fig. 11). In July and November 2009, very low concentrations were measured, ranging from below the detection limit to 0.05 mg/L on average. The highest nitrite concentrations were observed in October 2009 at most sites. Concentrations varied between 0.65 and 3.53 mg/L.

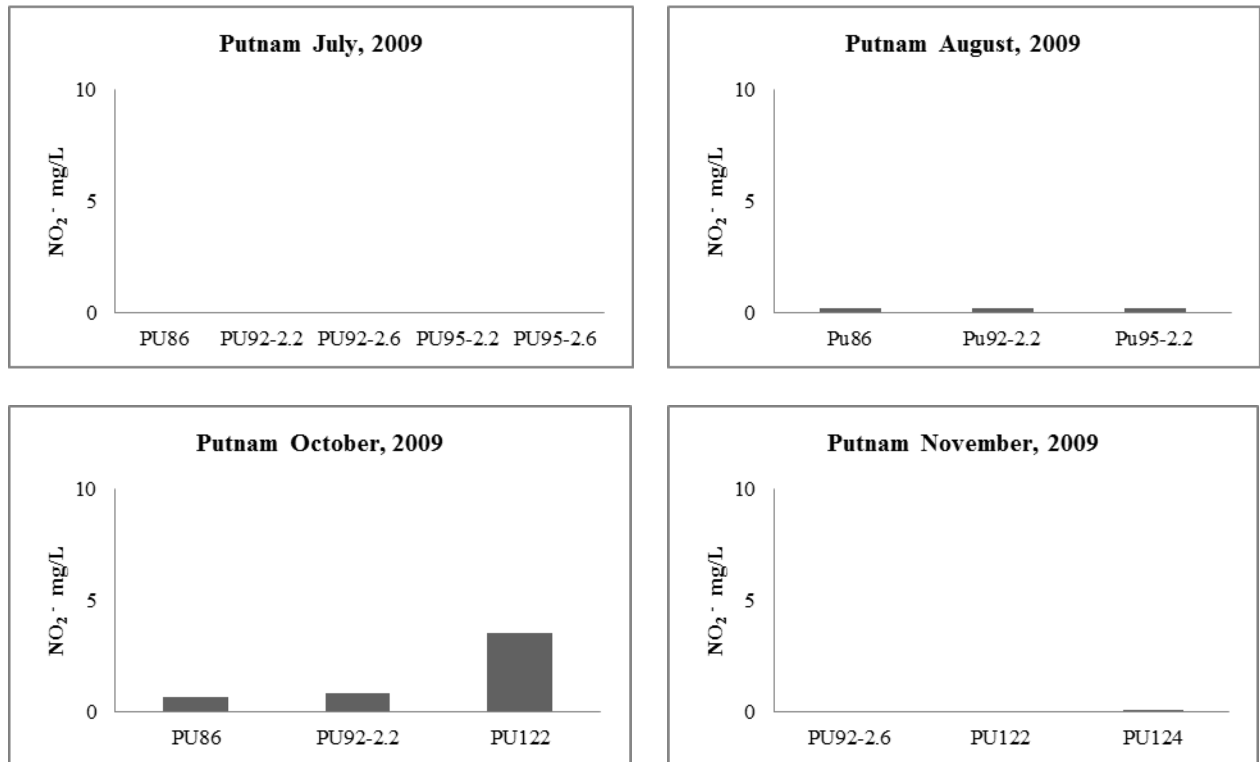


Figure 11: Nitrite concentrations at the Putnam site as a function of the wells and sampling dates.

At the Elmira site, nitrite concentrations were also very low for most sampling dates, with the exception of October 2009 (Fig. 12). Concentrations for July and August 2009 were below the detection limit, whereas the lowest nitrite concentration was 0,01 mg/L in Elmira 5 in November 2009 and the highest was measured in Elmira 1 on October 2009, i.e., 18.30 mg/L.

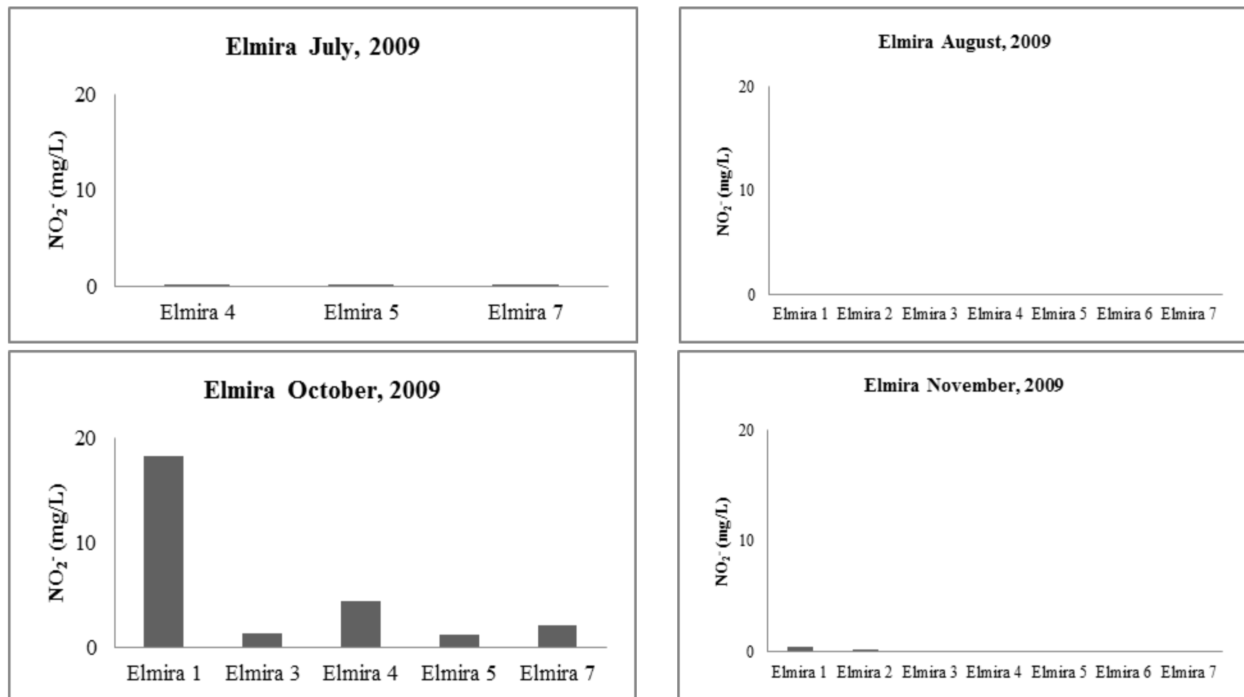


Figure 12: Nitrite concentrations at the various Elmira groundwater wells and sampling seasons.

4.1.1.5 Ammonium (NH_4^+)

Water samples from PU122 (October 2009) and PU124 (November 2009) (both chosen to replace dry wells closer to the lagoon) from deeper depths showed the highest ammonium concentrations (Fig. 13). PU92 and PU95 wells, which were away from the lagoon showed lower concentrations, i.e., ammonium concentrations from PU92 at a depth of 2.2 m was 27.7 mg/L in July 2009, but at a depth of 2.6 m, it was 16.6 mg/L in July, 2009. The ammonium concentration for PU95 at a depth of 2.2 m was 49.6 mg/L in July, 2009, whereas it was 45.5 mg/L at a depth of 2.6 m again in July, 2009. The ammonium concentrations in the background well PU86 always showed low concentrations, close to 0.02 mg/L in July 2009, 0.09 mg/L in August 2009 and 0.21 mg/L in October 2009.

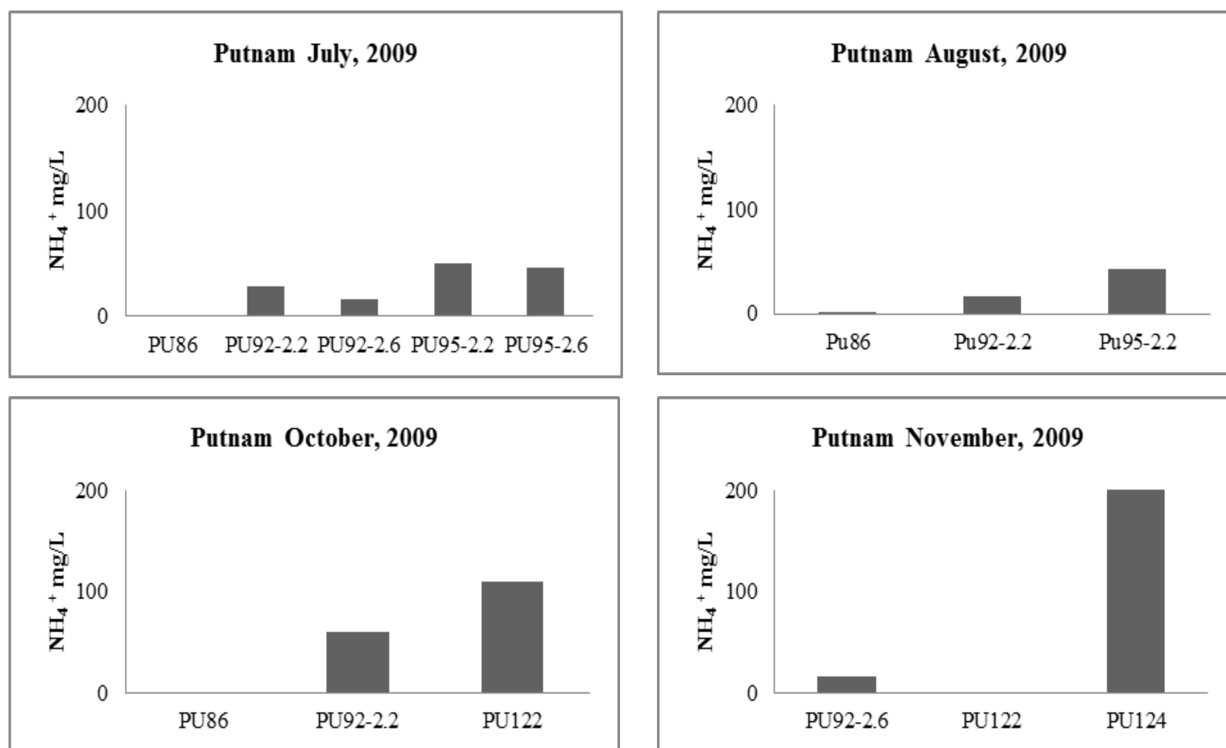


Figure 13: Ammonium concentrations in the various wells at Putnam during the various samplings times.

Very high ammonium concentrations were measured in several wells throughout the sampling season at Elmira. For instance, the highest concentrations were measured at Elmira 3 in August, October and November 2009 (conc. > 450 mg/L; Fig. 14). Very low concentrations were also present, including well 7 in October and November 2009. Ammonium concentrations also varied greatly between wells for each sampling time (Fig. 14).

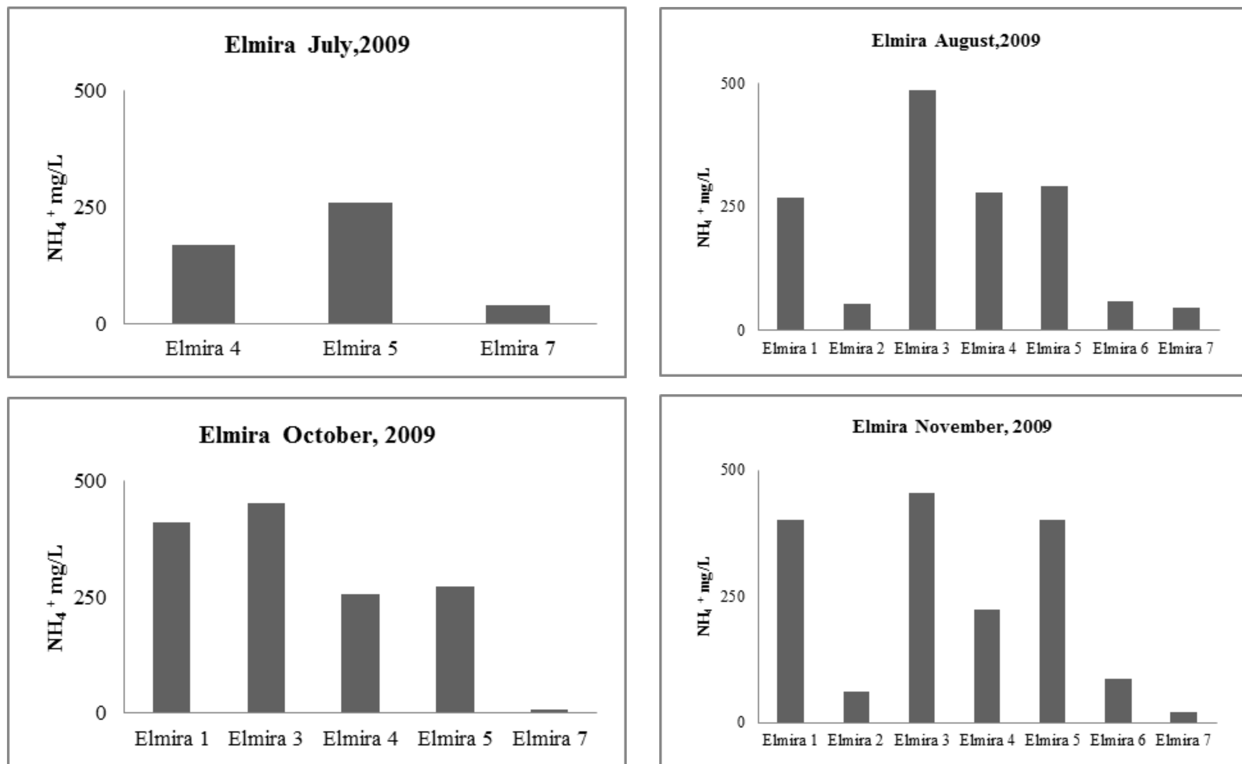


Figure 14: Ammonium concentrations in the various wells of the Elmira site during the various samplings times. Note that the ammonium concentrations at this site are much higher than those at the Putnam site.

4.1.1.6 Iron and Manganese (Fe – Mn)

In Putnam, the background groundwater well PU86 showed very low concentrations of Fe and Mn at all sampling times (Fig. 15). Fe and Mn concentrations from other wells which interacted with the permeated turkey manure piles showed slightly higher concentrations. For instance, PU92 in October 2009 at a depth of 2.2 m showed Mn and Fe concentrations of 8.50 and 10.58 mg/l, respectively. PU95 (at all sampling times and for both depths) generally showed higher Fe-Mn concentrations than the other wells (Fig. 15). PU124 well also displayed higher Fe concentrations than the other wells, with the exception of PU95.

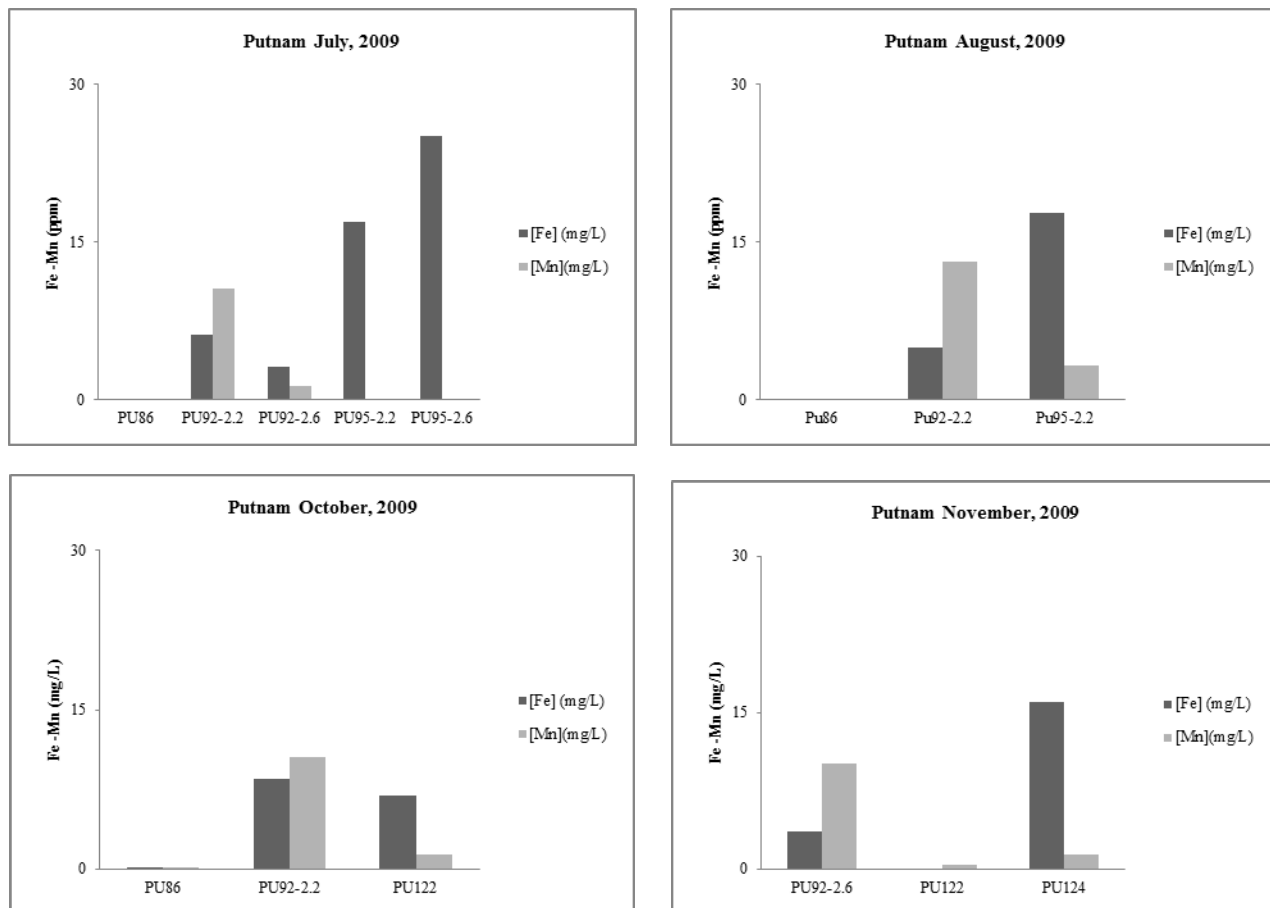


Figure 15: Fe and Mn concentrations as a function of the sampling time and wells at the Putnam site.

At Elmira, Fe and Mn concentrations were very low when compared to those observed at Putnam (Fig. 16). The highest Fe concentration was measured at Elmira 3 in October 2009 (i.e., 0.90 mg/L), whereas the highest Mn concentration was found at Elmira 5 in July 2009 (i.e., 0.52 mg/L).

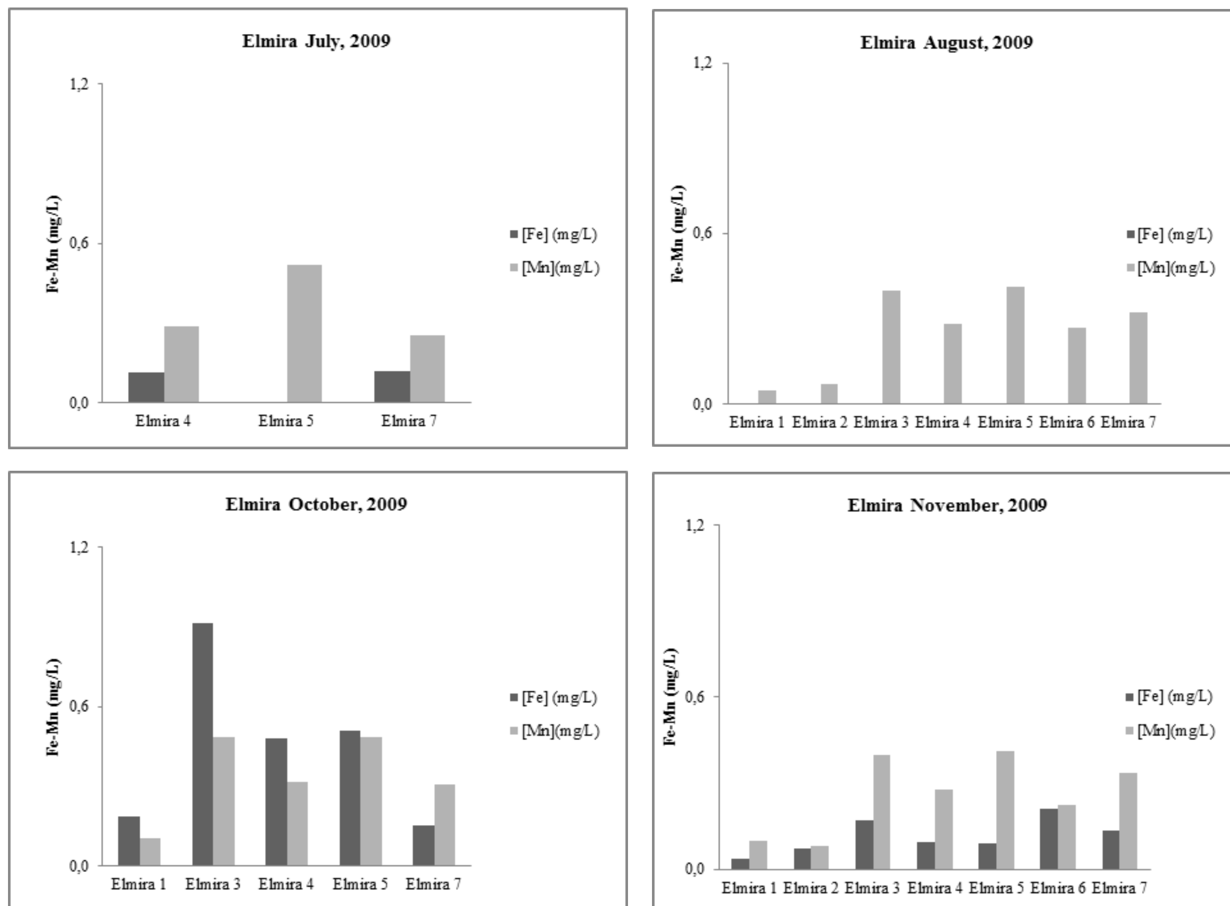


Figure 16: Fe and Mn concentrations as a function of the sampling time and wells at the Elmira site. Note that the concentrations at the Elmira site are much lower than those at the Putnam site (Fig. 15).

4.2 Bacterial abundance in ground waters using FISH

4.2.1. Putnam

FISH analyses were carried out to assess the presence of anammox organisms. Bacteria were detected in all Putnam samples for all sampling times. Cell counts indicated that the number of anammox cells was very low (generally below 200 cells/mL) (Fig. 17). The highest anammox cells counts were observed in July, 2009 at the PU95 well at the depth of 2.6m. In addition, cell count numbers from deeper wells (PU922.6 and PU95-2.6) and from those closer to the manmade lagoon showed the highest numbers (Fig. 17). Despite the low cell counts, the relative abundance of anammox bacteria (i.e., Amx/EUB ratio) varied between 0.45 and 4.81 % (Fig. 17) in the various sampling wells over the course of the sampling period.

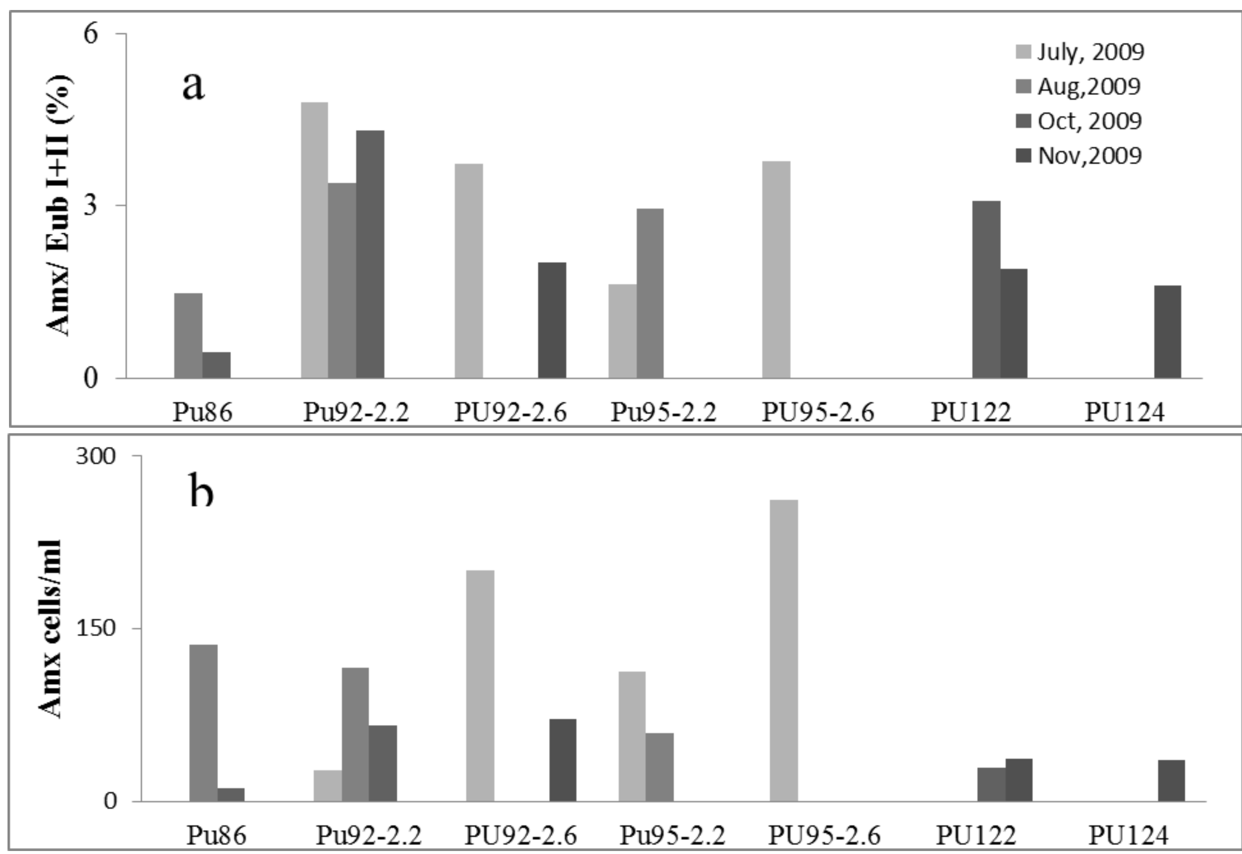


Figure 17: Relative abundance of anammox cells (with respect to total bacterial counts) for all sampling sites and dates (a).Total abundance of anammox cells in the various Putnam wells for all sampling times (b).

4.2.2 Elmira

FISH analysis showed the presence of anammox cells at all sampling wells and sampling dates at Elmira (Fig. 18). Direct cell counts indicated that the number of anammox cells varied between sites and sampling times (Fig. 17). The highest number of anammox cells was observed in November 2009 at Elmira 3, where it reached 1199 cells/ml. Cell counts were generally higher in that same month for the other sites, with the exception of Elmira 1. The relative abundance of anammox bacteria with respect to the total cell counts varied between 0.8 to 8.4 % (Fig. 18). The highest relative abundance was observed at Elmira 7 in August 2009.

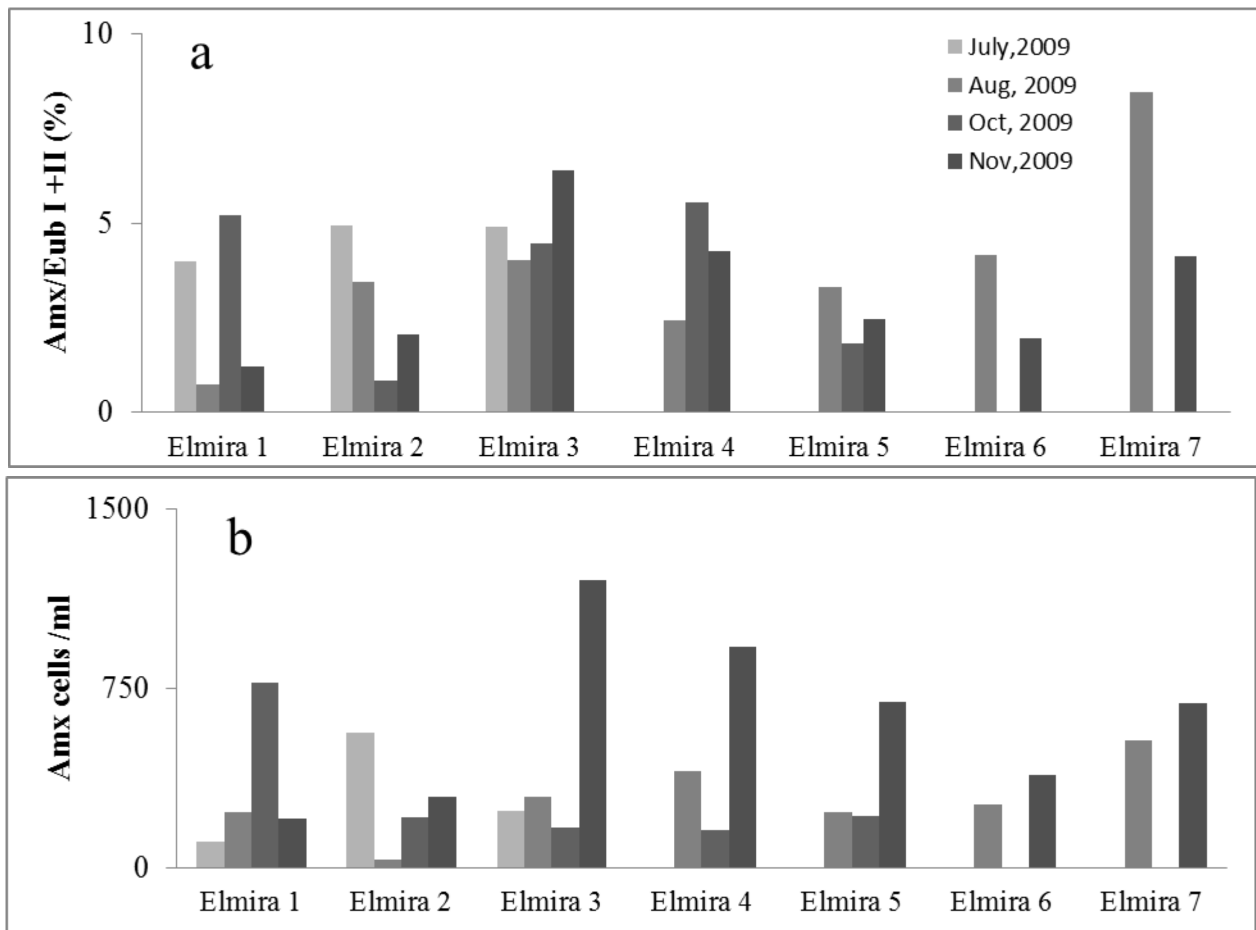


Figure 18: Relative abundance of anammox cells (with respect to total bacterial counts) for all sampling sites and dates (a). Total abundance of anammox cells in the various Elmira wells for all sampling times (b).

4.3 Relationship between the water chemistry and the relative abundance of anammox cells

4.3.1 Putnam

Basic linear regressions between the relative abundance of anammox cells (% of Amx/EUB) and the chemistry of the Putnam well waters from all sites and sampling times showed that there was a weak correlation ($R^2 = 0.351$) between the percentage of Amx/EUB and nitrate (NO_3^-) concentrations indicating that when the relative abundance of the cells increases, the nitrate concentrations decrease (Fig. 19). On the other hand, there was no correlation between Amx/EUB and the concentration of ammonium in the well waters (Fig. 20). Another weak relationship ($R^2 = 0.4709$) was observed between the relative abundance of the anammox cells and the concentration of dissolved Mn (Fig. 21), whereas no relationship was present with dissolved Fe (Fig. 22). No attempts were made to find correlations between the relative abundance of anammox cells and nitrite (NO_2^-) because most nitrite measurements were below the detection limit (Fig. 12).

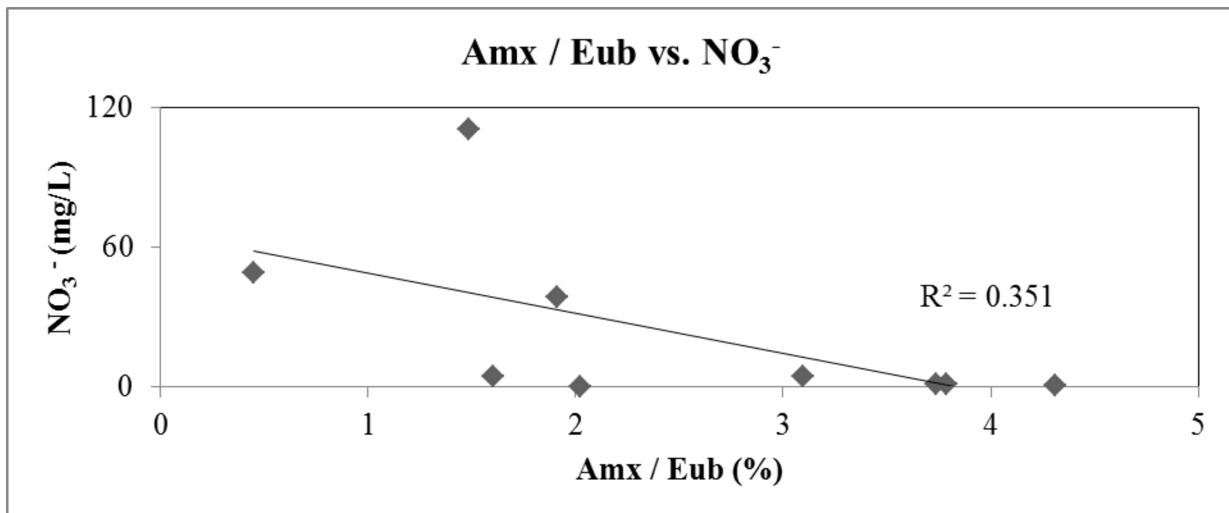


Figure 19: Weak relationship between the concentrations of soluble nitrate in the various wells of Putnam and the relative abundance of anammox cells. (All wells and sampling dates are included).

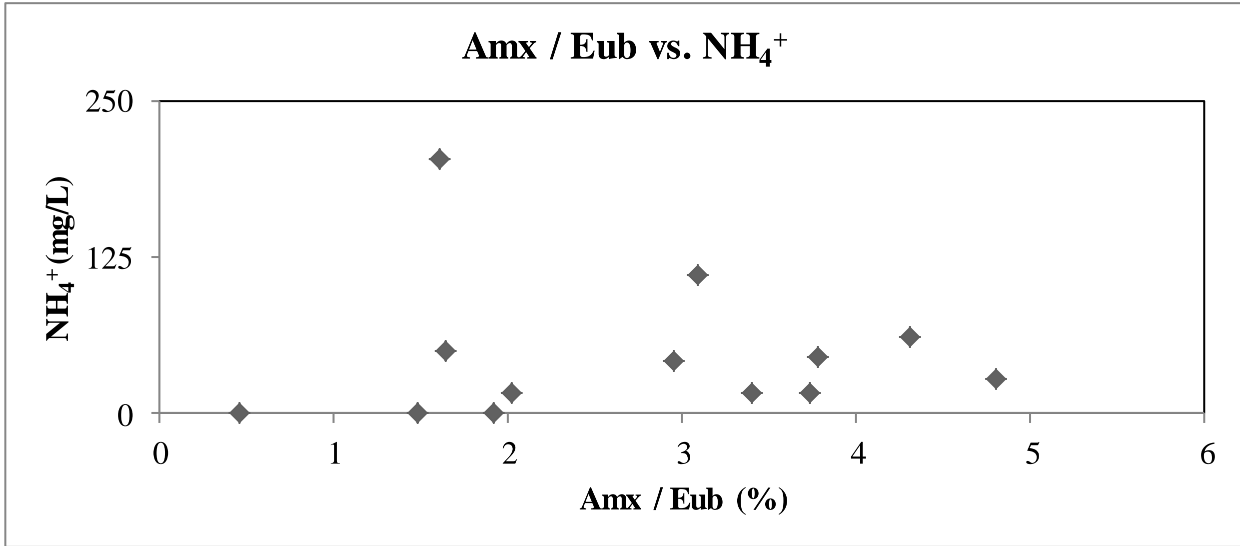


Figure 20: Absence of relationship between the ammonium concentrations in the various wells of Putnam and the relative abundance of anammox cells. (All wells and sampling dates are included).

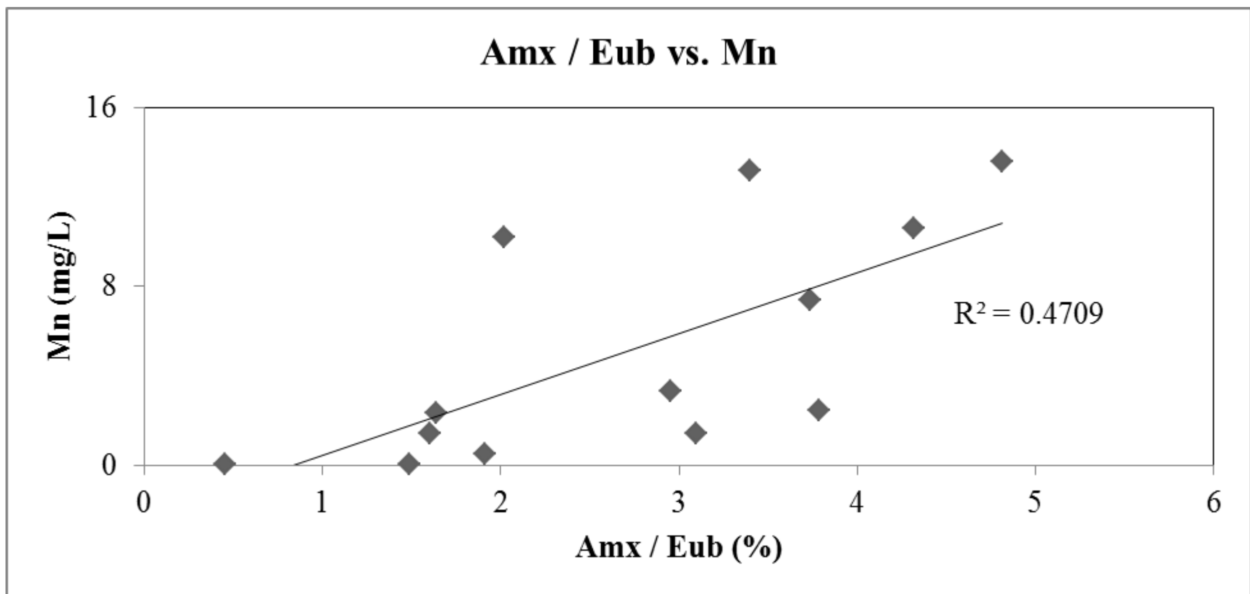


Figure 21: Weak relationship between the concentrations of dissolved Mn in the various wells of Putnam and the relative abundance of anammox cells. (All wells and sampling dates are included).

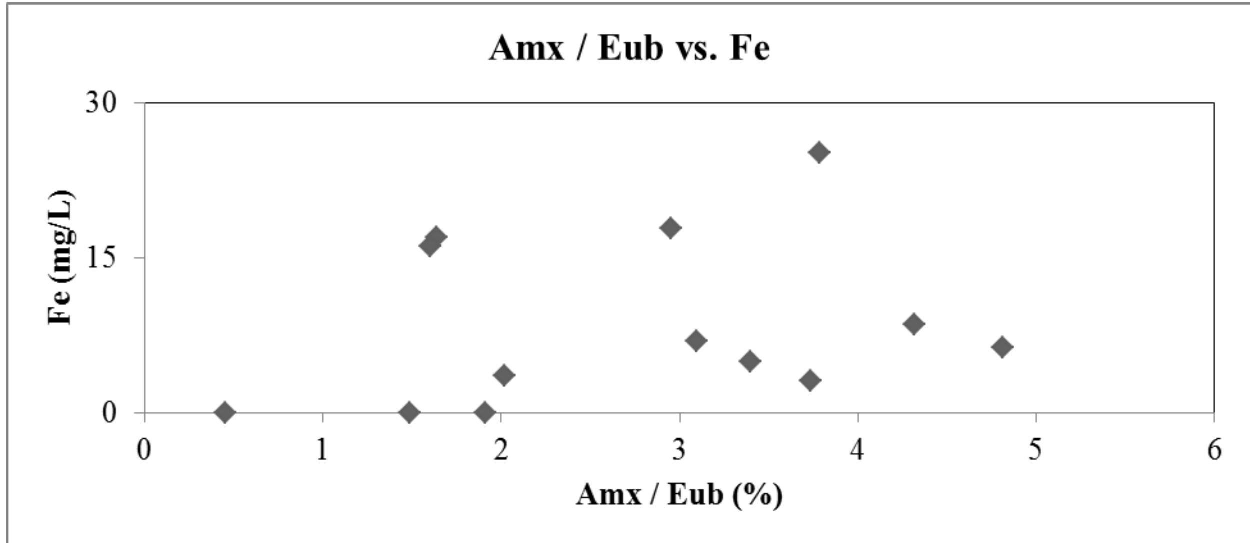


Figure 22: Absence of relationship between the concentrations of dissolved Fe in the various wells of Putnam and the relative abundance of anammox cells. (All wells and sampling dates are included).

4.3.2 Elmira

Basic linear regressions were also performed to find relationships between the relative abundance of anammox cells and the various geochemical properties of the well waters for the Elmira site. Results (Figures 23 to 26) indicate that no linear relations exist between the various parameters, with the exception of a very weak relationship ($R^2 = 0.2158$) between Fe and the Amx/EUB ratio (Fig. 26).

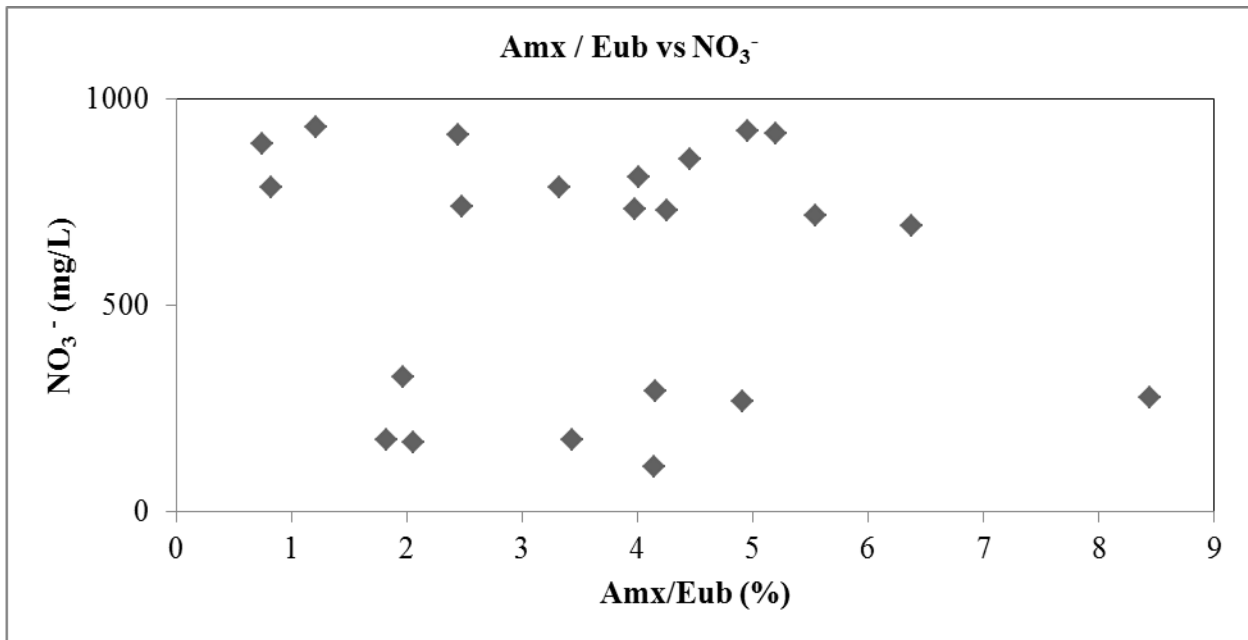


Figure 23: Absence of relationship between the concentration of soluble nitrate in the various wells of Elmira and the relative abundance of anammox cells. (All wells and sampling dates are included)

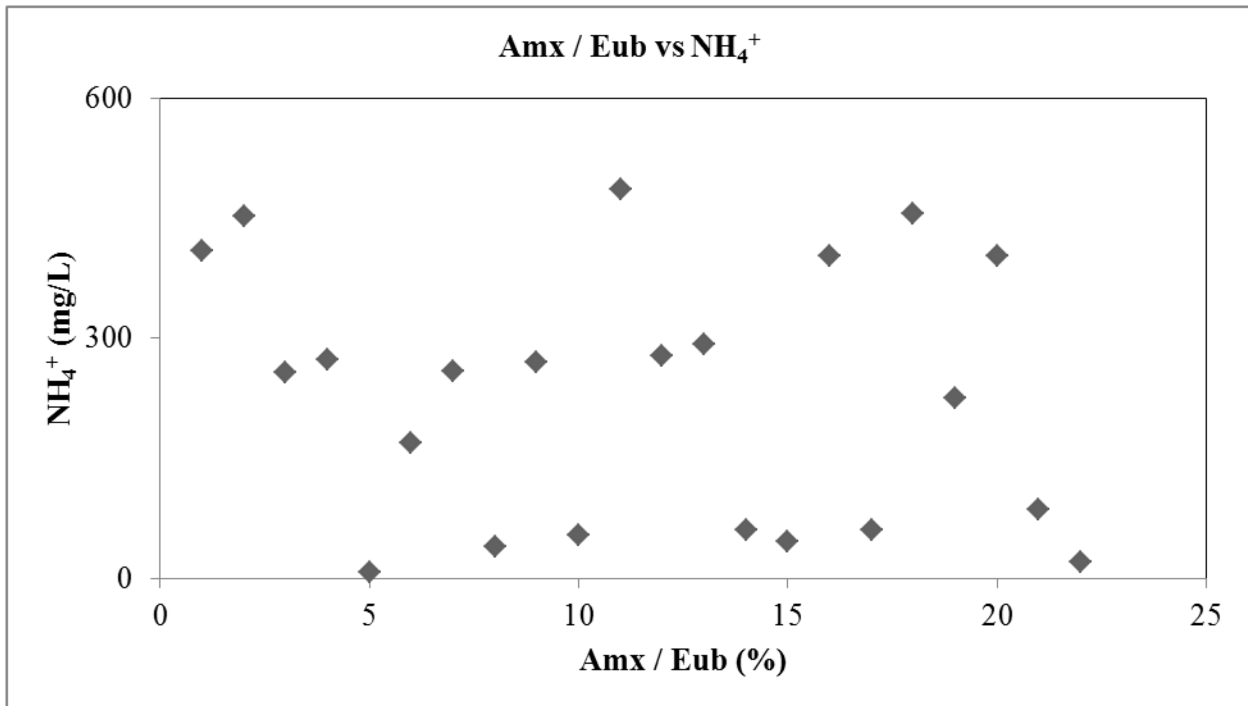


Figure 24: Absence of relationship between the ammonium concentration in the various wells of Elmira and the relative abundance of anammox cells. (All wells and sampling dates are included)

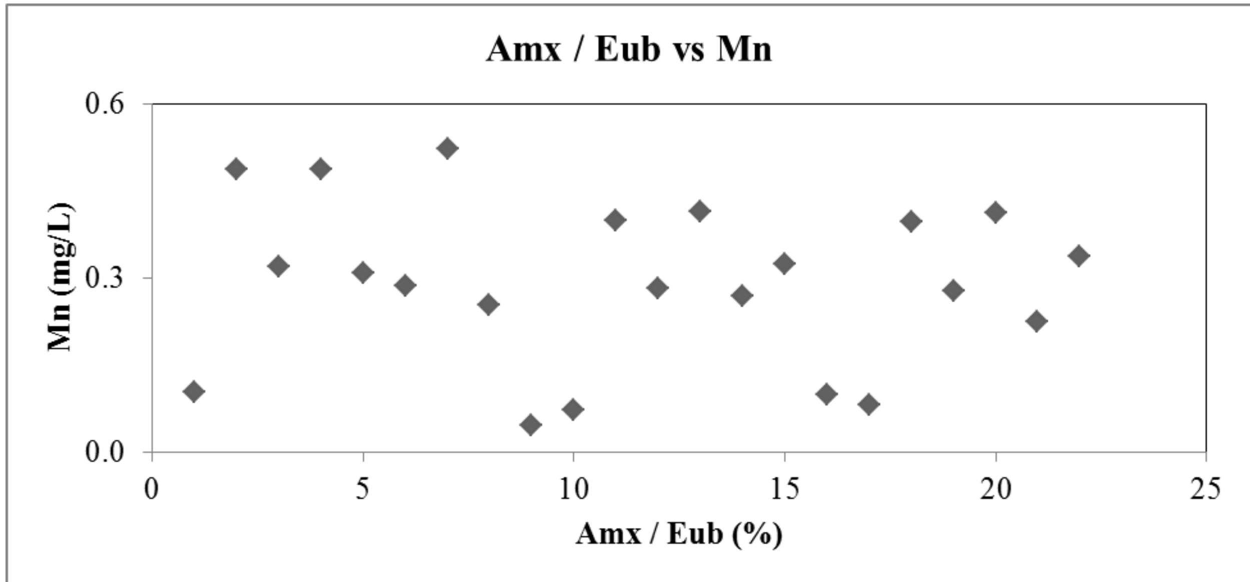


Figure 25: Absence of relationship between the concentrations of dissolved Mn in the various wells of Elmira and the relative abundance of anammox cells. (All wells and sampling dates are included)

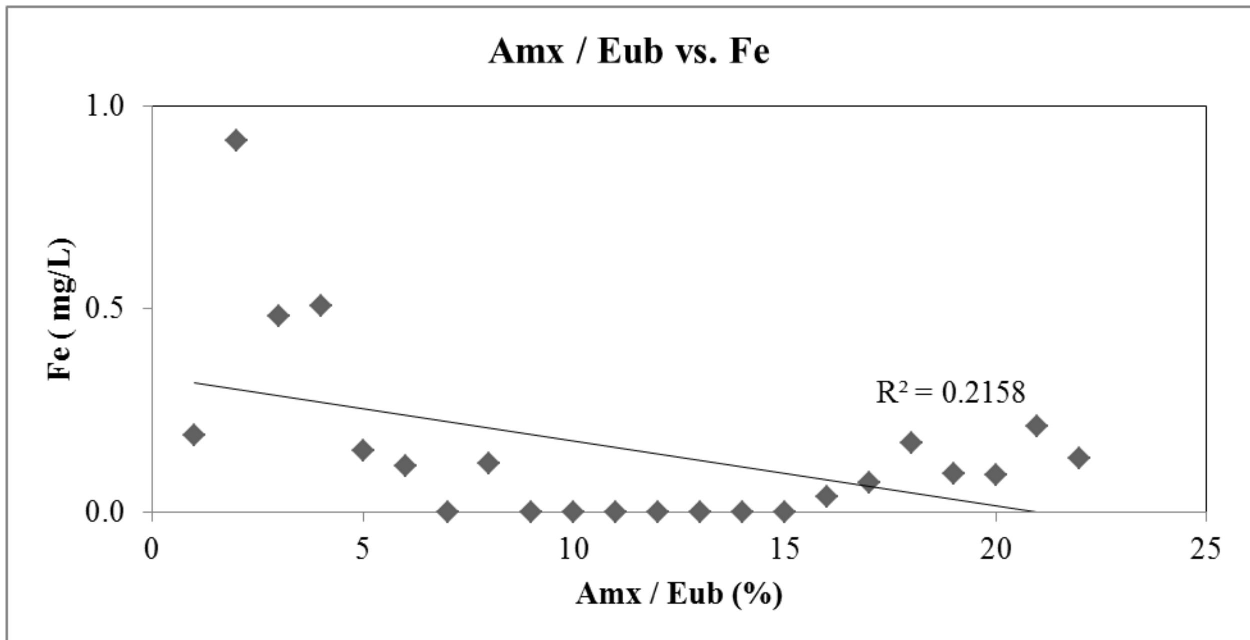


Figure 26: Weak relationship between the concentrations of dissolved Fe in the various wells of Elmira and the relative abundance of anammox cells. (all wells and sampling dates are included)

5. Discussion

5.1 Anammox presence in contaminated groundwaters

The advance of sophisticated molecular techniques in the field of microbiology has enabled the scientific community to better assess the presence and abundance of various bacteria, including those capable of performing anaerobic ammonium oxidation (anammox) (Jetten et al., 2009). In the last decade or so, several studies have shown that anammox bacteria are becoming ubiquitous in the environment. Penton et al. (2006) reported that anammox cells were present in a wide range of environments, ranging from soils, wetlands and lakes (pristine and eutrophic). More recently, Humbert et al. (2009) used a two-step molecular screening approach to determine their distribution and diversity in shallow aquifers, permafrost and agricultural soils. The authors reported that the diversity of anammox bacteria in terrestrial environments exceeded that of marine ecosystems, likely as a result of the presence of more suitable niches in soils. Very recently, Robertson et al. (2012) used N isotope analysis and molecular techniques to show that ammonium was being naturally attenuated in a septic system in southern Ontario as a result of anaerobic ammonium oxidation and, that two *Candidatus* genera of the Planctomycetales (Brocadia and Jettenia) were present in the sediments. The use of molecular techniques also revealed the presence of anammox bacteria in the two contaminated groundwater sites studied here (Moore et al., 2011). Using quantitative PCR technique, Moore's work showed that anammox 16S rRNA gene copies of *Candidatus* genera Brocadia and Kuenenia represented between 5.2 and 20.8% of the bacterial 16S rRNA gene copy numbers at the Putnam site and between 0 and 15% at the Elmira site.

In the present study, FISH analysis revealed that anammox organisms represented 0.8 to 8.4 % of the total number of cells at the Elmira site (Fig. 18), whereas at the Putnam site, they represented between 0.45 and 4.81 % of the total number of cells (Fig. 17). Their detection by FISH is in agreement with the findings of Moore et al. (2011) and the isotopic study of the Elmira site by Clark et al. (2008). The detection of anammox cells at Elmira is also in line with the results of Xing et al. (2012) who used microcosm tracer incubation experiments with ^{15}N-NH_4^+,^{15}N-NO_3^- and ^{15}N-NO_2^- to assess anammox reaction rates in groundwater and sediments

from Elmira. These authors showed that the highest rates were observed with the samples from Elmira 3 and 7, where we observed the highest relative proportion of anammox cells (Fig. 18).

The relative abundance of anammox cells at both sites is also comparable, but slightly higher, than the values of 0.75 and 1% reported from the Black Sea and the oxygen minimum zone of the Benguela upwelling system, respectively (Kuypers et al., 2003; Kuypers et al., 2005), and 0.98- 1.4% from freshwater systems (Schubert et al., 2006; Hamersley et al., 2009). It is unclear as why our relative abundance is higher than what is being reported in the literature, but we suspect that it might be related to the chemical characteristics of the contaminated groundwaters at both of our sites. Concentrations of nitrate and ammonium at both Elmira and Putnam are one to two orders of magnitude greater than the values reported for marine and freshwater environments in the studies by Kuypers et al. (2003; 2005) and Shubert et al. (2006). We believe that such elevated levels of nitrate and ammonium likely lowered the overall abundance and diversity of several bacteria generally found in groundwaters. We also observed that the overall number of anammox cells in our water samples was generally lower than the values reported in the literature. Hamersley et al. (2009) found 2.7-5.2 $X10^4$ anammox cells/mL in the N_2-production zone of the water column of a stratified lake in Germany, whereas as Kuypers et al. (2005) reported around 8000 cells/mL in the oxygen minimum zone of the Benguela upwelling system and roughly 1900 cells/mL in the Black Sea (Kuypers et al., 2003). In our study, the maximum number of anammox cells counts was around 1200 cells/mL (Fig. 18). Several problems were encountered with the FISH method, which might have lowered the overall number of anammox cells in the samples from Elmira and Putnam, as explained in section 5.3. Finally, FISH results do not necessary indicate that the cells are active but their constant presence during the several months of sampling suggests that might be.

5.2 Relationship between the presence of anammox cells and in situ aqueous geochemistry

Several physico-chemical factors are known to inhibit or slow down anammox reaction rates in natural environments and bioreactors (Dalsgaard et al., 2005; Meyer et al., 2005; Jung et al., 2007; Liao et al., 2008; Kalvelage et al, 2011; Kawagoshi et al., 2012). Such factors include dissolved oxygen (DO), free ammonia (NH_3), nitrite, nitrate, DOC (dissolved organic carbon) and DIC (dissolved inorganic carbon) concentrations and temperature. Given the fact that anammox is a process that occurs under anaerobic conditions, dissolved oxygen concentrations play a key role in the activity of anammox microorganisms. A recent study by Kalvelage et al. (2011) indicated that the anammox reaction is inhibited by levels of oxygen greater than 0.32 mg/L in oxygen minimum zones in the ocean, whereas Jung et al. (2007) found that concentrations below 0.2 mg/L were detrimental in continuous bioreactors. Such concentrations are much smaller than many of the concentrations measured for both the Putnam and Elmira groundwaters. Dissolved oxygen values for Putnam, excluding the background well (PU86), varied between 0.16 mg/L and 1.74 mg/L and between 0.68 mg/L and 3.41 mg/L in the Elmira wells (Figures 5 and 6). One explanation for these high DO levels is that at Elmira, it is very likely that the water samples became oxygenated while being pumped for sampling. For the Putnam site, it is also likely that oxygenation of the well waters occurred after the waters were sampled and as a result, the values do not represent “in situ” conditions. Overall, the bulk dissolved oxygen concentrations reported for both of our sites are clearly in disagreement with what is being reported in the literature, but in the end, the cell numbers and relative abundance of anammox bacteria remain comparable to what has been observed in natural environments where anammox takes place (see section 5.1). In addition, our results, i.e., the presence and activity of anammox bacteria in the contaminated groundwaters of Elmira and Putnam also corroborate the molecular work and isotopic studies done on the same sites (Moore et al., 2011; Clark et al., 2008; Xing et al. 2012).

The abundance of various nitrogen-rich compounds (i.e., free ammonia, nitrite and nitrate) has also been shown to have inhibitory effects on anammox bacteria, but the actual threshold concentrations reported in the literature are highly variable. For instance, ammonia concentrations greater than 2 mg of N/L can inhibit anammox cells in a bioreactor (Jung et al.,

2007), but a more recent study by Aktan et al. (2012) showed that anammox cells in a biofilm within a bioreactor could tolerate NH_3 levels up to 150 mg/L. The actual levels of NH_3 in the Elmira and Putnam waters were not measured (only NH_4^+ was), so it remains difficult to ascertain the effect of ammonia on anammox activity. It is known that the abundance of NH_3 is pH dependant and that concentration increases with higher pH (8 and up) (Jaroszynski et al., 2012). Given the fact that the average pH values of the Elmira and Putnam waters were around 6.84 to 7.01(Fig. 8) and 6.75 to 8.1 (Fig. 7), respectively, it is unlikely that the presence of ammonia affected the anammox cells at both sites. Nitrite is also known to inhibit anammox cells at levels greater than 35 mg/L, as reported by Strous et al. (1999) and Jung et al. (2005). However, Jaroszynski et al. (2012) observed a much higher tolerance to nitrite in their anammox bioreactor, i.e., 120 mg of N/L. These concentrations far exceed the levels measured in the Elmira and Putnam waters. The highest concentration of nitrite at Elmira was 18.3 mg/L (Fig.12), whereas it was much lower at Putnam, i.e., 3.5 mg/L (Fig. 11). Based on these observations, it is clear that nitrite had little or no inhibitory effect on the anammox cells at both sites. High levels of nitrate (> 4340 mg/L) can also hamper anammox rates in bioreactors (Strous et al., 1999), but these concentrations remain far greater than those measured at the Elmira (110-933 mg/L (Fig. 10)) and Putnam (0-60 mg/L (Fig. 9)) sites. We noted a weak relationship between the relative abundance of anammox cells and the concentrations of nitrate at Putnam, which showed that nitrate levels declined as the number of cells increased, suggesting that nitrate was possibly used in the anammox reaction (Fig. 19). However, Xing et al. (2012) showed that denitrification also removed nitrate in microcosms containing water and sediments from the same sites, indicating that both processes are concurrent. No relationship was however observed for Elmira (Fig. 23), possibly because the nitrate levels were greater than those of Putnam. Finally, it has been reported that large concentrations of DIC can lower anammox rates in bioreactors (Liao, et al., 2008), whereas higher levels of DOC favor denitrification over anammox in the deeper parts of the oceans (Dalsgaard et al., 2005). DIC levels in excess of 2g/L can be detrimental to anammox activity in bioreactors (Liao et al., 2008), but these concentrations are superior to what was measured at the Elmira wells, i.e., around 0.48 g/L (Xing et al., 2012). DIC concentrations for the Putnam sites are also lower, in the range of 0.02-0.1 g/L (Y. Xing, pers. comm.). With respect to DOC, recent work suggests that the type of organic

carbon, more than the concentrations themselves, plays a role in anammox activity. Güven et al. (2005) investigated the effects of various organic compounds on the anammox cells in a bioreactor. Their results indicated that methanol was a potent inhibitor of anammox activity, whereas propionate was consumed at a faster rate than acetate. With respect to both of our sites, the concentration of specific organic compounds is not known, only bulk DOC analyses were performed. DOC concentrations in the Elmira waters never exceeded 24 mg/L (Xing et al., 2012), which is one order of magnitude lower than the concentrations used in the Güven et al. study (~210 mg/L). Similar concentrations were also obtained for the Putnam waters (Y. Xing, pers. comm.). Overall, weak correlations can be a sign that water fluxes might be important.

Kawagoshi et al. (2012) investigated the effect of temperature on nitrogen removal performance and bacterial community in culture of marine anammox bacteria and found that the maximum nitrogen removal was achieved at 25^0 C, but no changes in bacterial community was noticed when the anammox culture was grown at temperatures between 5 and 37 ^{0}C. Groundwaters are typically cold, with temperatures around 8-10 ^{0}C (the temperature in the Elmira wells typically varied between 10 and 17 ^{0}C, depending on the sampling season; data not shown). It is then likely that colder temperature lowered anammox rates at the Elmira site. No temperature values are available for the Putnam site.

Finally, there have been some recent studies investigating the relationship between Fe and Mn reduction and nitrate, nitrite and N_2 production as a result of anaerobic ammonium oxidation (Clément et al., 2005; Shrestha et al., 2009; Yang et al., 2012; Javanaud et al., 2011). This is not surprising since both Fe and Mn are redox sensitive. The recent work of Yang et al. (2012) on tropical upland soils refers to the process of iron reduction coupled to anaerobic ammonium oxidation as "Feammox". The authors used labeled iron and ammonium to assess the presence of Feammox in their experiments. In the case of Elmira and Putnam, it remains difficult to ascertain the importance of iron Feammox because microcosm experiments have not been performed. The only indirect observation that we have is that there is weak relationship between the production of soluble Fe and the increasing relative abundance of anammox cells in the Elmira sites (Fig. 26). Given the complexity of the nitrogen cycle, Feammox remains a potential reaction that will need to be investigated in the future. The same can be said for the

role of Mn reduction in N cycling. Interestingly, we observed at the Putnam site that as the relative abundance of anammox cells increased, a larger concentration of soluble Mn was present in the groundwaters (Fig. 21). Such relationship was however not observed for the Elmira site because the soluble Mn concentrations were almost 2 orders of magnitude lower than those at Putnam (Fig. 25). Javanaud et al. (2011) showed anaerobic Mn-oxide-mediated nitrification of ammonium coupled with denitrification in muddy intertidal sediments. Their study indicates that a complex series of nitrogen transformations occur simultaneously in natural sediments. To our knowledge, this new pathway has not been observed in groundwaters yet, which warrants more research given the relationship observed at the Putnam site.

5.3 Improvements and future work

Fluorescence *in situ* hybridization (FISH) is an excellent tool to collect both qualitative and quantitative data of bacteria in environmental samples. It can also be used to validate the findings of clone libraries (Jetten et al., 2009), but it remains very tedious, time consuming and susceptible to several biases. Quantitative FISH has provided novel insights into the structure and dynamics of several microbial communities but it suffers from limited accuracy when the samples contain low numbers of cells (Daims et al., 2001). This was by far the biggest hurdle we faced in this study because groundwater from both sites had extremely low numbers of total and anammox cells (Figures 17 and 18). Organic detritus, such as flocs or biofilm can also cause fluorescence and bias the cell count. This was particularly true for the Putnam site because the groundwater located near the lagoon was dark and contained organic material. As a result, a lot fluorescent material was visible which masked the actual cells in the water samples. Another problem that we encountered is linked to the fixation step. We performed the fixation in the field with paraformaldehyde (PFA), however for most environmental samples, it is recommended to fix with PFA as well as with ethanol (Meier et al, 1999). We did not performed a pre-filtration step prior to the fixation step, which would have concentrated our samples and increased the likelihood of finding more cells with FISH. It is also known that many gram positive bacteria fail to show hybridization if the standard formaldehyde fixation is applied (De Los Reyes et al., 1997). We suggest that other fixation procedures and in some cases, enzymatic pre-treatments, should be performed.

6. Conclusions

The present thesis was part of a much larger project investigating the importance of the anammox process as a natural attenuation strategy to remove ammonium and nitrate from contaminated groundwaters. Previous work by Clark et al. (2008) had indicated that nitrogen isotopic analyses pointed to the fact that anammox was occurring at the Elmira site. Wells were sampled over the course of several months at 2 contaminated sites (Putnam and Elmira) in southwestern Ontario in order to determine the presence and abundance of anammox cells and the chemistry of the waters.

Chemical analyses revealed that both sites contained elevated levels of ammonium and nitrate, and to a lesser extent, some nitrite. FISH analyses did confirm the presence of anammox cells in the groundwaters of both sites but the overall number of cells remained relatively low when compared to that of other environments where anammox occurs. This was likely caused in part by the fixation protocol that we used, which was not optimal. Their detection by FISH however complements the work of Moore et al. (2011) who used molecular techniques to track their presence, and the recent study of Xing et al. (2012) who used N isotope tracers to quantify anammox rates of reaction in microcosm experiments. The relative abundance of anammox cells was however comparable to values reported in the literature for freshwater and marine environments. Given the complexity of nitrogen cycling at both sites, no direct relationship could be clearly established between the total number of anammox cells or their relative abundance and the concentrations of ammonium and nitrate in the waters. Finally, our results indicate that there might be a link between anammox and Fe and Mn cycling, but additional work is required to fully ascertain the relationship.

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APPENDIX I

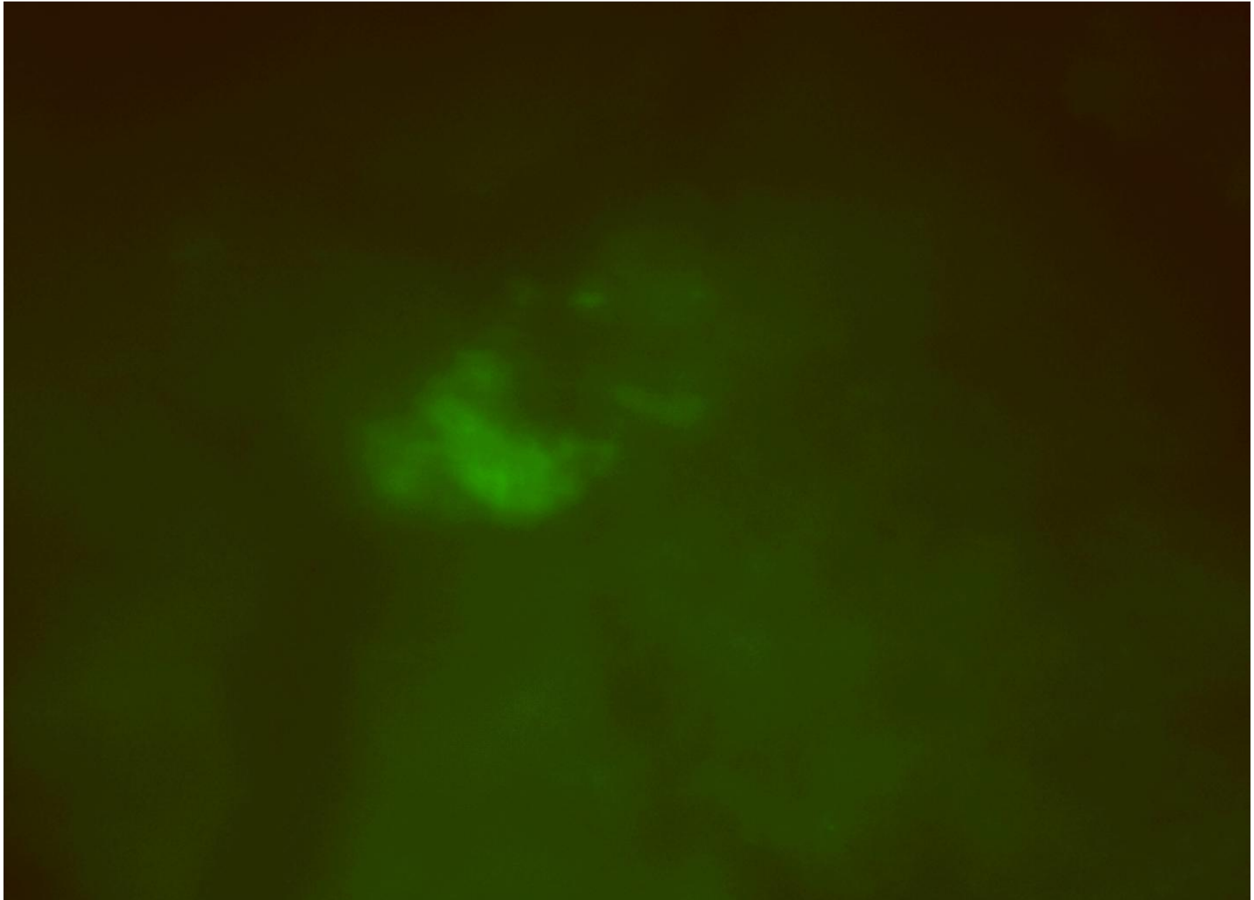

Figure A: FISH image obtained for the Putnam waters showing aggregates of cells and possible exopolysaccharides (EPS)

APPENDIX II

FLUORESCENCE IN SITU HIBRIDIZATION OF WATER SAMPLES by USING POLICARBONATE FILTERS

This protocol is based mainly on the protocol of Amann (Amann, 1995).

Protocol

1) Fix the sample with cold 4% paraformaldehyde solution in phosphate buffered saline solution (PBS). (This solution must always be fresh) Add 3 volumes of paraformaldehyde fixative to one volume of sample and FIX for a minimum of 1 hour and up to 24 hours at 4°C.

2) Filter a known volume of fixed sample through a 0.22 – 0.45 um polycarbonate filter (25 mm) (placed on top a GF/C under filter). Rinse the sample 3 times (applying gentle vacuum at each step) with filtered >1mL of 1x PBS and then dehydrate the cells for 3 minutes each time with >1mL of 50% ethanol, then 80% ethanol and finally 99.5 % ethanol. After the last dehydration step, air dry the filter and store at -20 C in a sealed container. The samples can be kept in the freezer for several months. However, avoid repetitive thawing and freezing cycles.

3) Take the filter out of the freezer (it can be cut into smaller sections (four or eight) and place them on glass slides coated with gelatin. Cover each piece of filter with 16 uL of hybridization buffer and add 2 uL (50ng/uL) of the respective fluorescent probe (the probes should be stored in the freezer and thawed just prior to use).

4) Put the slides quickly into the pre-warmed hybridization (set at 45°C) chamber with a peace of blotting paper soaked in 2 mL of hybridization buffer for 90 min.

5) After 90 minutes, remove the filter from the glass slide with tweezers and quickly rinse with ~ 5 ml of pre-warmed (at 46°C) washing buffer specific to each probe then put the filter pieces in a scintillation vial containing about 15 ml of the same warm washing buffer. Place the vials in a water bath set a 48 C for 15 min. Rinse the filters briefly with distilled water, remove quickly the excess of water and air dry and place on a glass slide for optical microscopy.

6) Each filter is counterstained with 3uL of DAPI (0.1 ug/mL) on glass slides for 5 min and air-dried. One drop of Anti-fading solution is then put on the filter sample and covered with a cover slip.

7) The glass slides should be covered with glycerol medium (Citifluor, Canterbury, England) or glycerol + PBS (pH > 8.5) (4:1) prior to being observed by epifluorescence microscopy.