Detection of Anammox Bacteria in Ammonium-Contaminated Groundwater

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis, with the exceptions of methods, results, and discussion relating to the ^{15}N-stable isotope labeling experiments. The isotope work and results described in this thesis were performed by Dr. Ian Clark and Dr. Yangping Xing at the University of Ottawa. Assistance with field work and water sampling was provided by B. Lazenby and S. Lanza. Water chemistry analyses were conducted by R. Elgood. M.D.J. Lynch provided assistance with the phylogenetic analysis of anammox-specific clone libraries, and helped generate the associated figure. A manuscript prepared using the data in this thesis has been published in Environmental Science & Technology:

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Abstract

Anaerobic ammonium-oxidizing (anammox) bacteria perform an important step in the global nitrogen cycle: oxidizing ammonium and reducing nitrite to form dinitrogen gas in the absence of oxygen. Anammox bacteria from the Planctomycetes phylum have been identified in a variety of natural environments but their role in groundwater ammonium oxidation has been unclear. Recent isotope studies have suggested that anammox bacteria are likely active in ammonium attenuation at contaminated groundwater sites; however, only limited biomarker-based data confirmed their presence prior to this study. I used complimentary molecular and isotope-based methods to assess the communities of anammox performing organisms at three ammonium contaminated groundwater sites in Canada: quantitative real-time PCR (qPCR), denaturing gradient gel electrophoresis (DGGE), DNA sequencing of 16S rRNA genes (with both Sanger and Illumina technologies), and ^{15}N-tracer incubations. DNA sequencing and qPCR results demonstrated that anammox performing organisms were present at all three contaminated sites, and that they were among the dominant bacterial community members for at least one particular site (Zorra, Ontario). In addition, anammox bacterial diversity was variable. One site possessed four of five known genera of anammox performing organisms although the dominant anammox bacteria at all sites belonged to the *Candidatus* Brocadia genus. Isotope data from two groundwater sites showed that denitrification and anammox occurred jointly and although denitrification was the dominant process, anammox was responsible for maxima of 18 and 36% of N_2 production at these sites. By combining molecular and isotopic results I have demonstrated the diversity, abundance and activity of these anaerobic chemolithoautotrophic bacteria; these results provide strong evidence for their important biogeochemical role in attenuating groundwater ammonium contamination.

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List of Abbreviations & Symbols

%	percent
‰	permil
°C	degrees Celsius
amoA gene	ammonia monooxygenase
Anammox	anaerobic ammonium oxidation
BLAST	Basic Local Alignment Search Tool
C	carbon atom
Ca.	Candidatus
CANON	Completely Autotrophic Nitrogen removal Over Nitrite
CO_2	carbon dioxide
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DNA-SIP	DNA stable-isotope probing
dNTP	deoxynucleoside triphosphate
DO	dissolved oxygen
FISH	Fluorescence *in situ* hybridization
g	gram(s)
g L^{-1}	grams per litre
h	hour
hzo gene	hydrazine oxidoreductase
kb	kilobase
km	kilometer(s)
L	litre
m	metres
mg L^{-1}	milligrams per litre
mg-N L^{-1}	milligrams-nitrogen per litre
min	minute(s)
mL	millilitre
mmol	millimolar
mRNA	messenger ribonucleic acid
N	nitrogen atom
N_2	dinitrogen gas
N_2H_2	hydrazine
NCBI	National Center for Biotechnology Information
ng	nanogram(s)
NH_2OH	hydroxylamine
NH_4^+	ammonium
nirS gene	nitrite reductase
nm	nanometers
nmol L^{-1} h^{-1}	nanomoles per litre per hour
nmol N_2 L^{-1} d^{-1}	nanomoles of dinitrogen gas per litre per day
NO_2^-	nitrite
NO_3^-	nitrate
OLAND	one-stage Oxygen-Limited Autotrophic Nitrification/Denitrification
OMZ	oxygen minimum zone

PCR	polymerase chain reaction	SRA	Short Read Archive
qPCR	quantitative polymerase chain reaction	UPGMA	Unweighted Pair Group Method with Arithmetic Mean
RDP-II	Ribosomal Database Project II	$\mu g\ L^{-1}$	micrograms per litre
rRNA	ribosomal ribonucleic acid	µL	microlitre(s)
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction	µm	micrometer(s)
		µM	micromolar
SHARON	Single reactor system for High Ammonia Removal Over Nitrite	µmol	micromoles

1.0 Introduction and Literature Review

1.1 Initial discovery

Anaerobic ammonium oxidation (anammox) and the bacteria responsible for this reaction were first discovered in 1995, in a reactor designed to favour denitrification (van de Graaf *et al*., 1995; Mulder *et al*., 1995). Until that time, the only known pathway for the autotrophic oxidization of ammonium (NH_4^+) and reduction to dinitrogen (N_2) occurred through sequential aerobic nitrification and anaerobic denitrification (Figure 1). We now know that the anammox reaction is performed by at least five *Candidatus* genera branching deeply within the Planctomycetes division of the Bacteria (Strous *et al*., 1999), all of which oxidize ammonium and reduce nitrite to form N_2 gas in the absence of oxygen ($NH_4^+ + NO_2^- \rightarrow N_2 + H_2O$) This newly discovered nitrogen cycle reaction provided a possible mechanism for previously unexplained N-deficits in anoxic environments (Richards *et al*., 1965); however, anammox bacteria had been detected only in reactor systems. The first two genera of anammox performing organisms (*Candidatus* Brocadia and *Candidatus* Kuenenia) were discovered in reactor systems (Jetten *et al*., 1999; Strous *et al*., 1999) and anammox was identified as having tremendous potential for NH_4^+ removal from N-rich wastewater (Jetten *et al*., 1997; Jetten *et al*., 2001; Schmid *et al*., 2003). Despite the importance of these organisms in engineered reactor systems, the role and importance of anammox in natural environments was not well understood (van Loosdrecht & Jetten, 1998; Zehr & Ward, 2002).

Upon probing the natural environment, researchers discovered anammox-like sequences and N_2 production by a new genus, *Candidatus* Scalindua, in the marine environment (Kuypers *et al*., 2003; Thamdrup & Dalsgaard, 2002). Soon many aquatic

locations were probed for anammox organisms and, in some environments, anammox was considered to be potentially

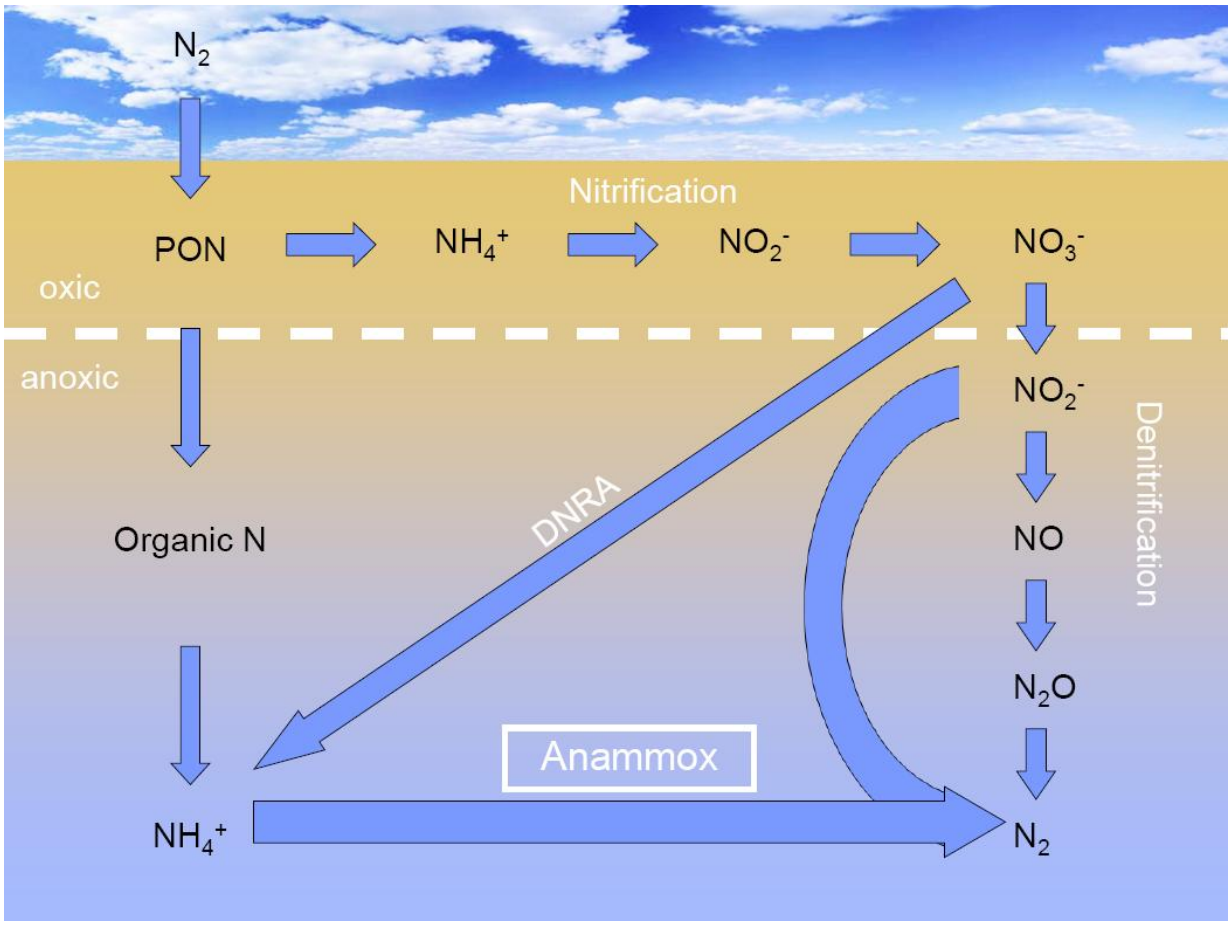

Figure 1: Nitrogen cycling in groundwater; modified from Francis *et al*. (2007); PON: particulate organic nitrogen, DNRA: dissimilatory nitrate reduction to ammonia

more important than denitrification in producing N_2 (Brandes *et al*., 2007; Jetten *et al*., 2009; Schouten *et al*., 2004). Later, two more genera of anammox performing organisms were detected in reactor systems: *Candidatus* Jettenia and *Candidatus* Anammoxoglobus (Kartal *et al*., 2007b; Quan *et al*., 2008). The discovery of these anammox clades provided the knowledge

required to investigate the presence of anammox organisms in natural environments. Research demonstrated that *Ca*. Scalindua is the dominant anammox genus in marine environments across the globe (Woebken *et al*., 2008). Similar to marine environments, freshwater lakes (Schubert *et al*., 2006) and rivers (Zhang *et al*., 2007) also exhibit low diversity of anammox genera, but tend to be associated with *Ca*. Brocadia and *Ca*. Kuenenia populations. Estuaries, however, provide an ideal environment for anammox bacterial niches, exhibiting wider anammox diversity than either marine or freshwater environments (Dale *et al*., 2009; Nicholls & Trimmer, 2009; Trimmer *et al*., 2003). Soil environments possess diverse assemblages of anammox genera, typically belonging to all five *Ca.* genera and are generally more diverse than marine or freshwater environments (Hu *et al*., 2011; Humbert *et al*., 2010; Zhu *et al*., 2011). Recently, anammox bacteria have been discovered in additional habitats including peat soil (Hu *et al*., 2011), fertilized paddy soil (Zhu *et al*., 2011), and deep-sea hydrothermal vents (Byrne *et al*., 2009). Together, these studies have demonstrated widespread distributions of anammox bacteria.

Genomic data provide a possible explanation for the widespread distribution of anammox bacteria in the environment. The first anammox genome (*Candidatus* Kuenenia stuttgartiensis) was published in 2006, yielding a cultivation-independent view into the metabolic potential of anammox bacteria (Strous *et al*., 2006; Hu *et al*., 2011; Humbert *et al*., 2010; Zhu *et al*., 2011), including approximately 200 genes involved in catabolism and respiration. These results suggested that anammox bacteria may be generalists that are capable of living a versatile lifestyle (Strous *et al*., 2006) and may imply that anammox organisms exist in environments where their presence has not yet been investigated.

1.2 Distinguishing denitrification and anammox

In discussing anammox bacteria in marine and other environments, it is important to note that studies have used the ^{15}N stable-isotope pairing technique (Thamdrup & Dalsgaard, 2002) to assess the relative activities of anammox and denitrification. The bacteria responsible for these two dissimilatory pathways can occupy similar niches, but their activities are controlled by specific environmental parameters. By reviewing the environmental factors known to control anammox bacterial communities, I also highlight factors found to affect denitrifier competition with anammox bacteria.

As in other environments, the distribution of anammox bacteria and denitrifiers in marine sediment and water columns is niche dependent. Anammox activity is typically lower than denitrification activity in marine and estuarine sediments, with a few exceptions (Engström *et al*., 2005; Engström *et al*., 2009; Rich *et al*., 2008; Risgaard-Petersen *et al*., 2004; Trimmer *et al*., 2003), but the water chemistries of oxygen minimum zones (OMZs) favour the dominance of anammox over denitrification, as seen off the coast of Namibia (Kuypers *et al*., 2005), and the OMZs of the Peruvian and Chilean upwellings (Galán *et al*., 2009; Hamersley *et al*., 2007; Lam *et al*., 2009; Thamdrup *et al*., 2006; Ward *et al*., 2009). In these OMZs, anammox activities were highest in zones of NO_3^- depletion and NO_2^- accumulation, despite extremely low or undetectable NH_4^+ concentrations (Hamersley *et al*., 2007; Schmid *et al*., 2005; Thamdrup *et al*., 2006). Anammox activities were highest close to the top of the OMZ (Lam & Kuypers, 2011), and were positively correlated with cell densities (Hamersley *et al*., 2007; Kuypers *et al*., 2005). Although these suboxic water columns harbour potential for heterotrophic denitrification (assessed by the presence of denitrifier *nirS* genes), measured

denitrifier transcription rates were undetectable, indicating that anammox is responsible for the vast majority of N_2 production from marine OMZs (Lam & Kuypers, 2011).

Although one genus of anammox bacteria tends to dominate within a single habitat, natural environments undergo seasonal and even diel shifts in a variety of environmentally relevant parameters. Most evidence indicates that both anammox and denitrifier communities coexist in natural environments for the removal of N. Indeed, these microbial communities undergo regular shifts in their relative abundance and activities at certain times of the year, or in response to environmental changes. Some examples of seasonal variation in the importance of anammox and denitrifier communities are described below. In Lake Rassnitzer, a permanently stratified freshwater lake (Hamersley *et al*., 2009), only anammox was measured in January and October (with no detectable denitrification), and only denitrification activity in May (with no detectable anammox). The controls for the shift between anammox and denitrification were unclear because temperature and NO_3^-/NH_4^+ concentrations remained constant throughout the year. However, their results suggested that anammox activity decreased due to an increase in organic carbon oxidation (and, as such, an increase in competition for NO_2^-) by denitrifiers in the month of May. This is surprising considering that the classic denitrification reaction (involving the step-wise oxidation of NO_3^- to N_2) occurs intracellularly. Later in the year, an increase in activity and growth of the anammox population led to the dominance of anammox during the winter months (Hamersley *et al*., 2009). In comparison to the seasonal shift between anammox and denitrification observed in Lake Rassnitzer, an interannual shift was observed in the central anoxic Baltic Sea, Gotland Basin (Hannig *et al*., 2007). Hannig and colleagues observed a shift from denitrification to anammox between 2002 and 2005; the shift was controlled by the disappearance of the NO_3-H_2S

interface (site of chemoorganoheterotrophic denitrification) due to an influx of oxygenated water in the 2002-2003 years (Hannig *et al*., 2007). Two additional studies examined the seasonal variation between denitrification and anammox in the coastal regions of the Baltic Sea, yielding inconsistent results. Hietanen and Kuparinen (2007) observed a minimal seasonal difference, with anammox contributing 10% and 15% to N_2 production in the spring and autumn, respectively. In a follow-up study, Jäntti and colleagues (2011) concluded that denitrification dominated year-round, and anammox and dissimilatory nitrate reduction to ammonia (DNRA) were not important nitrogen transformation processes in either season.

A controversial paper by Lam and colleagues (2009) documented the nitrogen loss pathways in the Peruvian OMZ, where denitrification was undetectable. They suggested a revision of nitrogen cycle models for OMZs whereby denitrification is unnecessary because DNRA supplies anammox organisms with NH_4^+. This conclusion was treated with skepticism because of studies demonstrating denitrifier activity and gene sequences in OMZs of the Arabian Sea (Bulow *et al*., 2010; Ward *et al*., 2009). Although anammox dominates OMZs, denitrification potential remains high and a natural event could stimulate a major community shift (as seen in the Gotland Basin; Hannig *et al*., 2007), so excluding denitrification involvement with OMZ ecology seems unwise. The relative contributions of anammox and denitrification to N_2 production are clearly variable among anoxic systems and, in the majority of ecosystems, both processes are responsible for N_2 loss.

1.3 Potential niche adaptation by anammox genera

The distribution of particular anammox organisms also appears to depend on environmental conditions, which permit the dominance of one group over another (or growth together). The five known anammox-performing genera (*Candidatus* Brocadia, Kuenenia, Scalindua, Jettenia,

and Anammoxoglobus) each also have their own niche, and one genus tends to dominate in a single environment (Kartal *et al*., 2006; Kartal *et al*., 2007b). Below, I highlight several studies that demonstrate anammox niche development, shifts in anammox-performing populations, or observations about their ecology and response to environmental variables.

The most obvious examples of niche adaptation of anammox bacterial genera within naturally occurring habitats include: *Ca*. Scalindua has frequently been observed as the sole anammox genus detectable in marine environments (Amano *et al*., 2007; Dale *et al*., 2009; Dang *et al*., 2010; Galán *et al*., 2009; Hong *et al*., 2011; Li *et al*., 2011a; Li *et al*., 2010; Rich *et al*., 2008; Schmid *et al*., 2007; Woebken *et al*., 2008), while *Ca*. Brocadia and *Ca*. Kuenenia dominate freshwater environments (Dale *et al*., 2009; Hamersley *et al*., 2009; Hirsch *et al*., 2010; Li *et al*., 2011a; Smits *et al*., 2009; Zhang *et al*., 2007) and artificial reactor systems (Bae *et al*., 2010; van Dongen *et al*., 2001; Innerebner *et al*., 2007; Kartal *et al*., 2006; Kartal *et al*., 2007a; Kartal *et al*., 2010; Kimura *et al*., 2010; Li *et al*., 2009; Park *et al*., 2010; Quan *et al*., 2008; Third *et al*., 2005; Third *et al*., 2001). In estuaries, *Ca*. Brocadia, *Ca*. Kuenenia and *Ca*. Jettenia dominated in the freshwater reaches of the estuary, while *Ca*. Scalindua dominated the saline end of the estuary (Dale *et al*., 2009; Li *et al*., 2011a), although the rate of anammox decreased with increasing salinity in a number of estuary systems (Meyer *et al*., 2005; Rich *et al*., 2008; Trimmer *et al*., 2003). There are, of course, exceptions: *Ca*. Scalindua was the only anammox organism detected in a stratified freshwater lake (Schubert *et al*., 2006), and *Ca*. Brocadia sequences were nearby a deep sea hydrothermal vent (Byrne *et al*., 2009). These observations support the conclusions of Brümmer and colleagues (2004), suggesting that freshwater Planctomycetes species are different than marine species.

Exposure of anammox enrichments to new substrates or environmental conditions reveals additional evidence for niche partitioning. Anammox communities shifted to favour the genera of anammox bacteria better suited for growth under increased salinity (Kartal *et al*., 2006) and changed carbon sources (propionate, Kartal *et al*., 2007b; acetate, Kartal *et al*., 2008). In other cases, a change in environment (e.g. seeding a new reactor with anammox-enrichment biomass) can trigger a shift in the dominant anammox genus (Park *et al*., 2010; van der Star *et al*., 2007), or anthropogenic inputs can drastically alter community structure (Li *et al*., 2011a). Anammox niche development can be influenced by the feed material, affinity of anammox bacteria for nitrite, micronutrient concentrations, or the inclusion of an alternative energy source in either engineered or natural systems (Hu *et al*., 2011; Kartal *et al*., 2007a; Kartal *et al*., 2007b; van de Vossenberg *et al*., 2008). Anammox bacteria possess branched respiratory chains, potentially permitting them to colonize habitats and adapt to new situations using a variety of electron donors and terminal electron acceptors (e.g. manganese and iron oxides, formate; Strous *et al*., 2006). Anammox bacteria have demonstrated the potential for genus-specific niche differentiation in both natural and engineered systems, and dominance in some environments (such as the OMZs discussed above). The activities of other nitrogen-cycling bacteria (aerobic nitrifiers and anaerobic denitrifiers) and archaea (aerobic ammonia oxidizers), however, are also an important consideration because of interactions occurring between these groups.

1.4 Relationship between anammox and other N-cycling organisms

Aerobic ammonia oxidation is performed by members in the ammonia-oxidizing bacteria (AOB) and archaea (AOA); these autotrophic microorganisms live together with anammox

bacteria in environments experiencing nitrogen input, and can act together to cycle N through the system (Vlaeminck *et al*., 2010).

Sediments of freshwater or marine systems provide an optimal environment for anammox bacteria (or denitrifiers) to live in close association with AOB and AOA. These groups can be affected by a variety of environmental parameters (Trimmer *et al*., 2005; Dalsgaard *et al*., 2002), but here I discuss oxygen and nitrite/nitrate. Oxygen (O_2) is the terminal electron acceptor for nitrifier metabolism but it inhibits anammox activity. In sediments, activity in the aerobic layers depletes available O_2 and causes a sharp gradient of O_2 to develop, permitting close proximity between aerobic AOA/AOB and anaerobic anammox/denitrifiers (Jenkins & Kemp, 1984; Jensen *et al*., 1993). Anammox bacteria in sediments exist in close association with AOA and AOB under microaerobic conditions (Dalsgaard *et al*., 2005), and these associations can persist despite specifically enriching for anammox organisms under anaerobic conditions. Yan *et al*. (2010) exposed an anammox enrichment to very low oxygen levels, and developed a co-enrichment of a marine nitrifier and anammox bacteria, suggesting that during the process of enriching marine anammox bacteria, a small proportion of nitrifying bacteria remained inactive until oxygen levels were restored. In the marine water column, a similar O_2 gradient can develop in marine snow (Shanks & Reeder, 1993), perhaps providing viable niches for anammox bacteria in the water column of OMZs. Nitrite and nitrate are the products of partial and complete nitrification, respectively, and are substrates for a variety of N-cycling processes including anammox, denitrification, and DNRA. Nitrite is often found at much lower concentrations than nitrate and is quickly consumed or oxidized by microbial (or algal) activity (Grant & Turner 1969). The anammox reaction uses nitrite, and it is often supplied by the activity of other organisms. In paddy soils where

nitrification activity is greater than denitrification, partial nitrification is the likely supply of nitrite for anammox (Zhu *et al*., 2011). Partial nitrification has also been suggested as the source of nitrite for anammox in the Black Sea (Lam *et al*., 2007) and the Benguella upwelling in the Namibian OMZ (Kuypers *et al*., 2005). Anammox has also been linked to archaeal nitrification and partial bacterial nitrification in the Black Sea and the Peruvian OMZ (Lam *et al*., 2007, 2009). The distribution of anammox in marine waters and sediments is often positively correlated with the concentration of NO_2^- (Dang *et al*., 2010; Jaeschke *et al*., 2007) and NO_3^- (Nicholls & Trimmer, 2009; Rich *et al*., 2008), which are likely affected by the metabolism of other organisms. To date, the coexistence of AOA, AOB, and anammox remains untested in terrestrial systems.

The potential for interaction between anammox and AOA exists; however, their distributions are rarely determined in tandem. Archaeal *amoA* genes are pervasive in a number of environments important for the global nitrogen cycle including suboxic water columns, and coastal and estuarine sediments (Francis *et al*., 2005), and anammox organisms coexist with AOA in reactors (Bae *et al*., 2010), so it seems likely that anammox and archaeal nitrification processes are linked in more than one natural environment. However, one particular study of Arabian Sea samples revealed that an aerobic archaeal nitrifying layer was more than 400 meters separated from a layer performing anammox (Pitcher *et al*., 2011); it is unlikely that anammox and archaeal nitrification were linked in this habitat. Anammox bacteria commonly live in close association with other (bacterial and archaeal) aerobic organisms in aerobic reactors. However, the anammox process only operates under anoxic or microaerobic conditions (Jensen *et al*., 2008; Strous *et al*., 1997). Under aerobic conditions, anammox bacteria benefit from close associations with AOB in flocculant material (Third *et al*., 2001),

granules (Vlaeminck *et al*., 2009), or biofilms (Vlaeminck *et al*., 2009) because aerobic activity by AOB develops an anoxic zone where anammox can grow. The outermost layers of the floc are composed of aerobic AOA and AOB, and anammox organisms inhabit the anoxic center (Vlaeminck *et al*., 2010), or AOA/AOB and anammox live together in suspended solids and sludge (Bae *et al*., 2010). Full-scale wastewater treatment plants treat wastewater in a one-stage process via partial nitrification and anammox involving cooperation between AOB and anammox organisms (Third *et al*., 2001; Vlaeminck *et al*., 2009). On a smaller scale, preparing and maintaining enrichment cultures in reactor environments facilitates the study of cooperation between anammox and other bacteria. In attempting to enrich a cyclohexane degrader (~75% of cells), Musat *et al*. (2010) also succeeded in enriching anammox cells to ~18% of the reactor biomass. In this situation, anammox organisms used NO_2^- and NH_4^+ generated by cyclohexane-dependent nitrate reduction. Further study is required to determine the parameters which regulate the distribution of these bacterial and archaeal groups.

1.5 Anammox in freshwater and terrestrial environments

Studies of marine anammox dominate the literature and relatively few studies have examined the role of anammox in natural freshwater systems, with a few exceptions (Hamersley *et al*., 2009; Humbert *et al*., 2010; Schubert *et al*., 2006). Studies of freshwater anammox bacteria (mainly *Ca*. Brocadia and Kuenenia) have involved reactor environments (Kartal *et al*., 2007a; Kartal *et al*., 2008; Kartal *et al*., 2007b; Kartal *et al*., 2010; Kimura *et al*., 2010; Park *et al*., 2010; Quan *et al*., 2008; van der Star *et al*., 2008). Nitrogen cycling is important in terrestrial and freshwater systems, and recent discoveries of anammox in new and unexpected environments (e.g. fertilized paddy soil, Zhu *et al*., 2011; peat soil, Hu *et al*., 2011) suggest that anammox bacteria are widely distributed beyond marine systems. A molecular survey of

anammox bacteria revealed greater diversity in terrestrial systems compared to marine systems (Humbert *et al*., 2010). This study also sampled other environments for anammox-like sequences, including those environments associated with water or nitrogen input (Humbert *et al*., 2010). Anammox organisms are not ubiquitously distributed in terrestrial environments—for example, anammox sequences were detected only at particular depths in a soil profile (Humbert *et al*., 2010), similar to the narrow range of anammox activity observed in layers of marine sediments (Dalsgaard *et al*., 2003, Dalsgaard *et al*., 2005).

1.5.1 Anammox bacteria and the groundwater environment

Groundwater is an essential resource for 8.9 million people in Canada, and makes up more than 30% of the world's freshwater supply (Environment Canada, 2011). Groundwater is also important for its contribution to the flow of most streams and rivers, and for its influence on the quality of lake water. Contamination of groundwater by anthropogenic activities (such as landfilling, Erksine 2000; manure stockpiling and fertilizer storage, Barcelona & Naymik 1984; Böhlke *et al*., 2006; and septic system effluent, Aravena & Robertson, 1998) can lead to contamination of surface waters and receiving water bodies, or impact groundwater serving as a source of drinking water. Groundwater has a long residence time, meaning that it can take many weeks to thousands of years for water to be completely refreshed, and for contamination to be removed from the groundwater system; this stable environment might be habitable by anammox bacteria.

The presence of anammox in NH_4^+-contaminated groundwater has been documented previously (Humbert *et al*., 2010; Smits *et al*., 2009), and groundwater provides a likely site for anammox activity. Nitrifying organisms in the unsaturated oxic zones oxidize NH_4^+ to NO_2^- and then to NO_3^-. Movement of the groundwater through the sediment matrix then carries the

products of partial nitrification (NH_4^+, N_2O and NO_2^-/NO_3^-) into the groundwater flow system. Given the relatively long exposure time of groundwater to metabolically active microbial communities, contaminated groundwater environments will likely favour the anammox reaction when both NO_2^- and NH_4^+ are present in areas of low oxygen. Although the presence of anammox-like sequences in groundwater has been confirmed (Ca. Brocadia, Humbert *et al*. 2010; *Ca*. Brocadia and Kuenenia, Smits *et al*., 2009), more work is required to explore anammox activity and distributions in contaminated groundwater environments, and to determine how these communities differ from other freshwater anammox populations. A recent discovery of anammox in peat soils by Hu and colleagues (2011) demonstrated the potential importance of *Ca*. Brocadia and *Ca*. Jettenia bacteria within groundwater environments. In this study, a peat bog fed by nitrate-rich groundwater encountered ammonium-rich peat soil. Other organisms rapidly consumed available O_2, providing anammox organisms with an ideal environment for growth (Hu *et al*., 2011).

1.6 Methods for detecting anammox abundance and activity

Anammox organisms are difficult to enrich and no pure cultures exist. As a result, most anammox community characterization relies on culture-independent methods. The majority of characterization of anammox organisms is done via three main techniques: molecular amplification and detection of target DNA and RNA sequences, isotope analysis, and ladderane lipid analysis.

Detection and identification of anammox bacteria is commonly performed using two molecular techniques, PCR amplification of the 16S rRNA gene, and fluorescence *in situ* hybridization (Song & Tobias 2011). Primers and probes with different specificities (genus and species) are used to detect and quantify anammox bacteria in environmental samples, but

provide no indication of activity. Stable isotope studies permit measurement of the relative activities of anammox and coupled nitrification-denitrification, which are otherwise poorly distinguishable from one another based on substrates and final products. Ladderane lipids are unique to anammox bacteria, so the presence of these lipids is considered to be diagnostic of the presence of anammox cells. Certain phospholipids can be used as biomarkers to detect live anammox bacteria, and distinguish between live and dead anammox cells based on measurements of predictable lipid breakdown products (Rush *et al*., 2011).

Ideal approaches for studying anammox bacteria include both molecular and isotopic components. Alone, molecular techniques can only verify the presence of anammox organisms (or their nucleic acids) and ^{15}N-stable isotope labeling studies cannot definitively state whether anammox bacteria are involved, or identify those metabolically active organisms. Recently, lipid analyses have been used for detecting anammox in naturally occurring environments and to distinguish between the different anammox genera by the proportions of core lipid fractions (Rattray *et al*., 2008). When used together these molecular and isotopic methods can provide strong evidence that anammox organisms are present, viable, and metabolically active. Subsequent sections will discuss each approach in further detail.

1.6.1 Molecular techniques

The 16S rRNA gene is a common target for assessing community composition in natural environments because this gene is critical for cellular function in all life, and the conserved DNA sequence makes most organisms detectable with universal primers. Indeed, the 16S rRNA gene has been the main target for detection of anammox bacteria by PCR (Jetten *et al*., 2003); however, there is a high level of sequence dissimilarity between 16S rRNA genes of anammox performing genera (e.g. ~85% similarity between "*Candidatus* S. sorokinii" and

either "*Candidatus* B. anammoxidans" or "*Candidatus* K. stuttgartiensis"; Jetten *et al*., 2003); therefore it has been difficult to develop primers targeting all known genera of anammox bacteria, and importantly, these may not target as-yet unknown anammox genera. The 16S rRNA gene is a common gene target for quantitative studies of anammox and other groups (Schmid *et al*., 2001) for assessing anammox cell numbers relative to the bacterial community because there is only one copy per genome in known anammox bacteria (Strous *et al*., 2006). Nonetheless, extracellular DNA (present without live cells) is amplifiable using DNA detection methods, meaning that detection of 16S rRNA genes does not necessarily predict the presence of only live cells (Schmid *et al*., 2005). It is possible to detect live anammox cells when a functional gene transcript (i.e. RNA) is targeted in (quantitative) PCR based assays, as described below.

In theory, actively transcribing anammox bacteria can be targeted via the internal transcribed spacer (ITS) region between the 16S and 23S subunits of bacterial ribosomal RNA. This technique would permit detection of actively transcribing cells because the ITS region is only detectable in the short interval between transcription and gene processing to create the mature transcript. When combined with FISH, the fluorescent signal is only present when the organisms are active, but high transcription rates are necessary for successful detection of anammox cells (Schmid *et al*., 2001). The spacer region is not under selective pressure so nonspecific probe binding is likely (Schmid *et al*. 2005), but higher-resolution diversity can be revealed by sequencing through this region, potentially down to the subspecies level (Woebken *et al*. 2008).

The first candidate functional biomarker gene for anammox abundance was the hydrazine oxidoreductase (*hzo*) gene, encoded by all known anammox genera and used to

oxidize hydrazine to N_2. HZO enzymes have conserved functions and can be targeted for detection of anammox organisms in mixed communities. This gene has been used as an alternative phylogenetic marker for anammox bacteria—in a study performed by Dang and colleagues, phylogenetic analysis of *hzo* genes revealed a more diverse community of anammox organisms than that observed by analyzing 16S rRNA genes (Dang *et al*. 2010). Some genera of anammox bacteria possess more than one copy of the *hzo* gene, so this gene is less useful for quantification studies (Dong *et al*., 2009; Lam *et al*., 2009; Li *et al*., 2011b). Still, *hzo* gene sequences can be used to create reliable phylogenetic trees (Li *et al*. 2009).

Another anammox specific functional gene has been identified recently as a reliable target for quantification studies: the *nirS* gene. The *nirS* gene codes for the cytochrome cd_1 containing nitrite reductase in anammox bacteria; this enzyme mediates the reduction of nitrite to nitric oxide, which then reacts with ammonium to generate hydrazine (Li *et al*., 2011b). Recently the *nirS* gene was suggested as a new biomarker for detection of anammox in natural environments, and was confirmed as a viable tool for quantitatively assessing active anammox via reverse transcriptase PCR (Li *et al*., 2011b; Lam *et al*. 2009; Dong *et al*. 2009). The anammox *nirS* sequence is distinct from denitrifier *nirS* genes ($\leq$ 63% sequence identity; Lam *et al*. 2009), and the gene provides consistent phylogenies as revealed by *hzo* and 16S rRNA genes (Li *et al*., 2011b). There is only one copy of the *nirS* gene per cell, making this gene more useful for quantification studies than the *hzo* gene (Strous *et al*. 2006).

Fluorescence *in situ* hybridization (FISH) has been referred to as the "gold standard" method for anammox detection (Schmid *et al*. 2005) because it can be used to collect quantitative and qualitative information on anammox bacteria in environmental samples (Schmid *et al*. 2000; Schmid *et al*. 2003). Anammox-FISH has drawbacks including, i) probes

target the 16S rRNA gene (limitations mentioned above) ii) high detection threshold, and iii) low signal-to-noise ratio. Nonetheless, this technique is the only widely used non nucleic-acid-based method for the detection of anammox bacteria (Schmid *et al*. 2005), and has been used in many studies of anammox in both natural environments and engineered reactors (Schmid *et al*., 2005).

Catalyzed reporter deposition (CARD)-FISH has also been used to detect anammox because it is more sensitive than the traditional FISH technique (Hannig *et al*. 2007). The anammox probe (targeting the 16S rRNA gene) is linked to a reporter enzyme—peroxidase. When peroxidase is provided with fluorescently labeled tyramide, covalent bonds form between tyramide and tyrosine amino acid residues within the cell. This signal amplification provides CARD-FISH with greatly enhanced sensitivity, and has been used to detect anammox cells in environmental samples where they are typically present in low relative abundances (Hannig *et al*. 2007; Lam *et al*. 2007; Woebken, *et al*., 2007; Hamersley *et al*. 2009).

1.6.2 Stable isotope incubations

Stable N-isotopes are frequently used when assessing the activities of anammox and denitrification in natural environments. The paired isotope technique was developed to permit measurement of anammox- and denitrification-derived N_2 (Thamdrup & Dalsgaard, 2002). Without paired isotope studies, anammox and nitrification/denitrification are indistinguishable by their substrates and end products. The end product of both anammox and denitrification is dinitrogen gas, and the paired isotope experiment relies on the fact that anammox requires both NH_4^+ and NO_3^- to produce N_2, while denitrifiers require only N-oxides (e.g. NO_2^-/NO_3^-). When an airtight incubation vial is supplied with $^{15}NO_3^-$ and $^{14}NH_4^+$, the anammox reaction generates $^{15,14}N_2$ while the denitrification reaction generates $^{15,15}N_2$. When supplied with

$^{14}NO_3^-$ and $^{15}NH_4^+$, anammox generates $^{15,14}N_2$ while denitrification generates $^{14,14}N_2$. Stable isotope labeling was first used to determine anammox bacterial activity (van de Graaf *et al*., 1995; Thamdrup & Dalsgaard, 2002), and is very commonly used to study anammox in reactor and natural environments, often in combination with the above techniques.

Using isotope-based techniques, Clark and colleagues (2008) were able to determine that anammox activity was the likely process responsible for N-loss at a contaminated groundwater site in Elmira, Ontario, Canada even before biomarker-based characterization was performed by the research in this thesis. The native $\delta^{15}N$- and $\delta^{18}O$ compositions of NH_4^+ and NO_2^-/NO_3^- were measured at the site, and evidence for anammox activity was supported by the following observations: (i) loss of NH_4^+ and NO_3^- (respective to K^+ and Cl^-) along the groundwater flowpath, indicating metabolic activity by anammox bacteria; (ii) a progressive enrichment in $\delta^{15}N_{NH4}$ and $\delta^{15}N_{NO3}$, indicating enzymatic preference for the lighter isotope; (iii) $\delta^{15}N_{NH4}$ values were less than $\delta^{15}N_{NO3}$, indicating that nitrification was not responsible for NH_4^+ loss; and (iv) increasing N_2 overpressuring above atmospheric equilibrium with increasing $\delta^{15}N\text{-}NH_4^+$.

Like the work of Clark and colleagues, early studies of anammox in a variety of environments involved only isotope work (Thamdrup & Dalsgaard 2002, Dalsgaard *et al*., 2003, Trimmer *et al*., 2003, Rysgaard *et al*., 2004). Now that more anammox sequences have been identified and are submitted to publically available databases, isotope studies can be readily adapted to include a molecular component to determine the genera of anammox bacteria present, and to measure their abundance in the bacterial community.

1.6.3 Ladderane lipid analysis

Anammox bacteria possess an internal compartment called an anammoxosome. The anammoxosome membrane is highly enriched in ladderane lipids—lipids possessing linearly concatenated butane rings, which stack to form a dense membrane (Sinninghe Damsté *et al*., 2002). Hydrazine, a reactive metabolic intermediate, is maintained within the anammoxosome to prevent damage to cellular machinery (Sinninghe Damsté *et al*., 2002). Although other (non-anammox performing) planctomycetes exhibit internal compartmentalization, they lack ladderane lipids (Jetten *et al*., 2009). These lipids are otherwise unknown in nature, making them an ideal biomarker for anammox bacteria (Lanekoff & Karlsson, 2010; Rattray *et al*., 2008). Although the intact ladderane glycerolphospholipids are thermally unstable (Li & Gu, 2011; Sinninghe Damsté *et al*., 2005), they degrade into predictable breakdown products (Rush *et al*., 2011), making it possible to discriminate between intact lipids from live organisms and breakdown products (e.g. ladderane core lipids), which may have been carried from elsewhere (Rattray *et al*., 2010; Rush *et al*., 2011). Over the course of the last five years, lipid analysis has emerged as 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; Lanekoff & Karlsson, 2010), but is commonly used in conjunction with isotope and nucleic-acid based techniques.

1.7 Research Hypothesis and Objectives

I hypothesize that the anammox reaction occurs in ammonium contaminated groundwater when both NO_2^- and NH_4^+ are present in anoxic regions, and that contaminated groundwater sites harbour specific anammox performing communities that affiliate more closely with freshwater anammox genera than saltwater genera. I also hypothesize that anammox organisms

will comprise a substantial proportion of the total bacterial community in these sites given the existence of conditions that are expected to favour the growth of anammox bacteria.

This thesis will present data on the distribution and activity of anammox organisms in contaminated groundwater environments, with a focus on site-to-site variability. Although anammox organisms have been identified in groundwater environments (Smits *et al*., 2009, Humbert *et al*., 2010) and isotope evidence for the anammox reaction in NH_4^+-impacted groundwater was shown at the site of a former fertilizer operation in Elmira, Ontario, Canada, (Clark *et al*., 2008), the presence and activity of anammox organisms has yet to be confirmed with coupled ^{15}N labeling incubations and biomarker-based evidence. This study is among the first investigations of anammox presence and activity in contaminated groundwater.

2.0 Methods and Materials

Samples from the three ammonium-impacted groundwater sites (Zorra, Elmira, and Southland Dog Park, SDP; Table 1) were collected and analyzed for total bacterial composition and for anammox-specific molecular and isotopic signatures. For consistency, representative samples from each site were subjected to the same analyses (e.g. qPCR, DGGE, clone libraries), except for Illumina sequencing (Zorra site only), and isotope incubations (Zorra and Elmira sites only).

2.1 Sampling sites

Groundwater sites within Canada were chosen with the occurrence of both NH_4^+ and NO_3^- /NO_2^- contamination as a first criterion (Table 1). Samples were collected from Zorra township, Ontario (Zorra site, Z), which is a manure composting facility where runoff from compost piles collects in a lagoon. Lagoon NH_4^+-N was 150 mg L^{-1} and NO_3^--N was 3.4 mg L^{-1}; groundwater NH_4^+-N was 2.5 mg L^{-1} and NO_3^--N was 3.2 mg L^{-1} at the Z106 piezometer nest; N concentrations were uncharacteristically low for this site in the 2008 season, compared to previous years (data not shown). The second site was in Elmira, Ontario (Elmira site, E), which is adjacent to a decommissioned fertilizer company, with groundwater NH_4^+-N concentrations of >350 mg-N L^{-1} and NO_3^- -N of >200 mg L^{-1} (Table 1). The third site was in Calgary, Alberta (Southland Dog Park, SDP), and is 2.5 km downgradient from a decommissioned plant involved in munitions production and fertilizer mixing from 1942 to 1992, with groundwater NH_4^+-N concentrations of >55 mg L^{-1} and NO_3^- -N concentrations of >30 mg L^{-1} (Lanza, 2009). For comparison to NH_4^+-impacted groundwater sites, one "background" piezometer nest was sampled at three depths from the Zorra site (Z86).

Table 1. Field metadata for groundwater samples included in this study.

Sample	$[NH_4^+]$ mg-N/L	$[NO_3^-]$ mg-N/L	EC (μS/cm)	pH	[DO] mg/L
Z103 (3.1 m)	2.5	3.2	1675	7.22	<1
Z103 (5.1 m)	1.5	4.0	1503	7.13	<1
Z103 (7.5 m)	8.3	0.2	1675	7.13	<1
Z106 (3.1 m)	1.6	4.6	2450	6.86	<1
Z106 (5.1 m)	1.5	2.6	1445	7.08	<1
Z106 (7.5 m)	1.3	2.3	1440	7.13	<1
Z86 (1.9 m)	ND	ND	ND	ND	ND
Z86 (2.3 m)	0.01	7.4	541	6.59	4.47
Z86 (3.1 m)	0.03	5.0	877	6.73	7.73
E1	16.1	28.0	ND	6.88	2.17
E2	67.1	83.3	ND	6.81	1.38
E3	313.5	189.7	ND	7.05	2.23
E4	175.3	186.7	ND	6.80	1.60
E5	354.4	177.5	ND	6.97	2.29
E6	47.0	42.7	ND	6.54	1.32
E7	313.9	238.8	ND	6.87	ND
E (composite)	ND	ND	ND	ND	ND
SDP (4.0 m)	31.4	86.2	2220	7.00	0.35
SDP (5.0 m)	34.9	32.1	2140	7.03	0.50
SDP (6.0 m)	69.9	29.0	2140	7.07	0.42

Z = Zorra, ON; E = Elmira, ON; SDP = Southland Dog Park, Calgary AB; ND = not determined

The Zorra site overlies a shallow aquifer system comprised of heterogeneous glaciofluvial outwash including everything from fine silt and clay to very coarse sand and gravel (Robertson & Schiff, 2008). Continuous core sampling at Z103 and Z106 revealed a sequence of heterogeneous and increasingly coarse-grained sediments with depth (unpublished data). The Elmira site is situated in gently sloping glacial outwash sediments, with surficial drainage toward a perennial creek. The regional carbonate bedrock in the region is overlain by approximately 30 m of unconsolidated glaciofluvial deposits. The SDP site is a river-connected alluvial aquifer primarily consisting of sands, gravels, and cobbles. The alluvial aquifer is underlain by bedrock (at 4 to 9 m below ground surface), and overlain by 1.4 m of silty sand, with a water table depth ranging from 2.5 to 4 m below ground surface (Lanza, 2009). Sonic drill cores in the immediate vicinity of the multi-level well showed three distinct units: sand (1.0 to 2.5 m below ground surface), gravelly sand (2.5 to 3.2 m below ground surface), and gravel (3.2 to 4.2 m below ground surface).

Samples from the Zorra site (Z) were taken from multi-level piezometers. Piezometer nest Z106 is closest to the manure lagoon source (10 m), and piezometer nest Z103 is 50 m downgradient. Both Z103 and Z106 were sampled at 3.1 m, 5.1 m, and 7.5 m below ground surface in October 2008; these piezometers screen groundwater over approximately 5 cm at intervals of 1 m. Piezometer Z92 is 30 m away from Z103, but remains the same distance from the manure lagoon; similarly Z122 is 30 m away from Z106. Z95 is 30 m downgradient from Z122, and Z124 is 35 m downgradient from Z122. Z86 is 90 m away from Z95, outside of the contaminant plume. These piezometers were sampled between 2.6 m and 7.5 m below ground surface in November 2009. Z86, Z92, Z95, Z122, Z103, and Z124 piezometer nests screen groundwater over approximately 2 cm at intervals of 0.4 m. At the Elmira site (E), a seven-well

composite sample was taken in April 2009 (coverage ~5100 m^2), in addition to the individual wells that were represented within the composite (wells 1 through 7); wells were screened over several meters. At the Southland Dog Park site (SDP), a single multi-level piezometer was sampled at 4 m, 5 m, and 6 m below ground surface in May 2009.

2.2 Molecular techniques

Between 240 mL and 1 L samples of groundwater were collected and filtered onto 0.22-µm Sterivex filters (Millipore). Filters were stored at –70°C until DNA extraction. Nucleic acids were extracted from the filter surface using a lysis and purification technique described previously (Neufeld *et al*., 2007).

Quantitative real-time PCR (qPCR) was performed using a C1000 thermal cycler with a CFX96 real-time system (Bio-Rad). PCR was performed using the following reaction components in 12.6-µl volumes: 6 µl of SYBR-green Supermix (Bio-Rad), 0.05 µl equivalent of each forward and reverse primer (100 mM), 0.5 µl of bovine serum albumin (10 mg mL^{-1}; Kreader *et al*., 1996), and 0.5 µl of extracted and quantified environmental nucleic acids (1 to 10 ng μl^{-1}) or DNA standards. Anammox-specific qPCR was conducted similarly to a previously published protocol (Ward *et al*., 2009) using primers Amx368f (Schmid *et al*., 2003) and Amx820r (Schmid *et al*., 2000), listed in Table 2. General bacterial qPCR used primers 341f and 518r (Table 2; Muyzer *et al*., 1993). The qPCR thermal program for Amx368f and Amx820r involved an initial denaturation of 3 minutes at 95°C followed by 40 cycles of 45 seconds at 95°C, 1 min at 62°C and 1 min at 72°C. Melt curve analysis used a gradient of 62°C to 95°C with 0.5°C temperature increments. For qPCR with 341f and 518r, the reaction involved an initial denaturation step of 3 min at 95°C, followed by 40 cycles of 45

Table 2. List of PCR primers used in this study

Primers	Target gene	Reference
An7f, An1388r	Anammox 16S rRNA gene	Penton *et al*., 2006
Amx368f, Amx820r	Anammox 16S rRNA gene	Schmid *et al.*, 2000, Schmid *et al*., 2003
GC-341f, 341f, 518r	Bacterial 16S rRNA gene	Muyzer *et al*., 1993
27f, 1492r	Bacterial 16S rRNA gene	Lane *et al*., 1991

seconds at 95°C, 1 min at 55°C, and 1 min at 72°C. The melt curve analysis involved an increase of temperature from 55°C to 95°C in 0.5°C increments. The efficiency of general bacterial and anammox qPCRs were 88.9% and 84.6%, with R^2 values of 1.00 and 0.998, respectively. All qPCR products were run on a 1% agarose gel along with a 1 kb Plus DNA ladder (Invitrogen) to confirm the size and quality of PCR products.

PCR products from groundwater samples were used to generate standard curves for qPCR. PCR products were purified using a MinElute kit (Qiagen) and quantified with a spectrophotometer (Nanodrop ND-1000). Products were diluted to 10 ng μl^{-1} and eight serial 10-fold dilutions were performed using sterile distilled and deionized water. All qPCR amplifications were conducted in duplicate.

In order to compare community composition for all samples, DGGE was conducted at the level of all bacteria and also adapted to target anammox bacteria. General bacterial 16S rRNA gene profiles were generated using DGGE primers (GC-341f and 518r; Muyzer *et al*., 1993). Anammox-specific 16S rRNA gene profiles were generated using a "nested" PCR technique: PCR products from amplification with An7f and An1388r (Penton *et al*., 2006) were diluted (10^{-2}) in water, and products were used as template for PCR with bacterial DGGE

primers, using only 20 cycles for the nested amplification. PCR for DGGE and cloning was performed using the following reaction components in 25-µl volumes: 2.5 µl of Thermal Polymerase Buffer (10X; NEB), 0.05 µl dNTPs (100 mM; NEB), 0.05 µl equivalent of each forward and reverse primer (100 µM), 1.5 µl of 10 mg ml^{-1} bovine serum albumin, 0.1 µl of *Taq* DNA polymerase (5 U μl^{-1}; NEB), and 1 µl extracted environmental nucleic acids (1 to 10 ng μl^{-1}).

PCR with general bacterial 16S rRNA gene primers GC-341f and 518r used the following thermal profile: initial denaturation of 5 minutes at 95°C, then 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C, with a final extension step of 7 min at 72°C. PCR with anammox specific primers (An7f, An1388r; Table 2) used the following thermal profile: initial denaturation of 5 min at 95°C, then 30 cycles of 1 min at 95°C, 1 min at 63°C, and 1 min at 72°C, with a final extension step of 7 min at 72°C. All PCR products were run on 1% agarose gels along with a 1 kb Plus DNA ladder (Invitrogen) to confirm the size and quantity of PCR products.

DGGE used a 30% to 70% denaturing gradient in 10% acrylamide gels (C.B.S. Scientific Company). Gels were run for 14 hours at 85V according to a previously published protocol (Green *et al*., 2010). Bands were cut from the gel and sequenced at The Center for Applied Genomics (TCAG; ABI 3730XL sequencer). DGGE band sequences were manually edited to correct base miscalls, and primer sequences were removed prior to analysis.

Anammox DGGE community profiles were subjected to UPGMA (Unweighted Pair Group Method with Arithmetic mean) cluster analysis using Pearson correlations of fingerprint densitometric curves with Gelcompar II (Applied Maths).

Illumina libraries were constructed according to a previously published protocol (Bartram *et al*., 2010) for both 7.5-m samples from the Zorra site (Z103 and Z106) in order to obtain in-depth coverage of the bacterial communities at this site. In brief, the hypervariable region (V3) was amplified with modified 341f and 518r primers (Bartram *et al*., 2010; Muyzer *et al*., 1993) in PCR amplifications using 20 cycles. For each library, triplicate PCR products with unique indexes were mixed in equal ng quantities, quantified and sent to Illumina (Hayward, CA) for 125-nucleotide paired-end multiplex sequencing. After sequencing, image analysis, base calling and error estimation were performed using Illumina Analysis Pipeline (version 2.6). Paired-end reads were assembled to form consensus ~200 base sequences (Bartram *et al*., 2010). All Illumina sequence data were submitted to the Short Reads Archive (SRA; NCBI) under accession number SRA030448.

PCR for cloning was performed using the same reaction components as were used for DGGE (listed above). PCR with 27f and 1492r (Table 2; Lane 1991) used the following thermal profile: initial denaturation of 5 minutes at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C, with a final extension step of 15 minutes at 72°C. All PCR products were run on 1% agarose gels along with a 1 kb Plus DNA ladder (Invitrogen) to confirm the size and quantity of PCR products. PCR products were cloned using a TOPO-TA cloning kit (Invitrogen) according to the manufacturer's instructions. DNA sequencing of plasmid inserts was performed at the University of Washington (ABI 3700 sequencer) and the University of Waterloo sequencing facility (Applied Biosystems 3130xl Genetic Analyzer). The following number of clone sequences were obtained from cloning with An7f and An1388r: Zorra (Z103 and Z106 at 7.5 m depths): 171 sequences; Elmira composite: 146 sequences; SDP (all depths): 27 sequences. All DNA chromatograms from clone libraries and DGGE

bands were manually edited to verify base accuracy and trimmed to ensure only high-quality reads were included in subsequent analyses. Chimeric sequences were identified and removed using Bellerophon (Huber *et al*., 2004). To provide potential taxonomy for experimental sequences in phylogenetic analysis the top 100 *blastn* results (Altschul *et al*., 1997) for each of 25 divergent experimental sequences were filtered to remove redundancy and added to the sequence data set. A Planctomycete outgroup sequence (EU703486) was also added for phylogenetic analysis. Sequences were aligned to a model of the bacterial 16S rRNA secondary structure using the program ssu-align v.0.1 (Nawrocki, 2009). The resulting alignment was trimmed to the majority consensus length of the experimental sequences and spanned 619 characters. The nucleotide model of sequence evolution used in phylogenetic analysis was determined by the AIC test implemented in jModelTest v.0.1.1 (Posada, 2008). A Maximum Likelihood phylogeny was derived using the PhyML v.3.0.1 (Galtier *et al*., 1996; Guindon & Gascuel, 2003), with the GTR model of sequence evolution (Tavaré, 1986) and estimated optimized parameters for the gamma distribution estimating rate variation (GTR+G). The approximate likelihood ratio test (aLRT) statistic in PhyML and 1000 Neighbour Joining bootstrap iterations using PAUP v.4b10 (Swofford, 2003) were used to provide support for the Maximum Likelihood topology. All DNA sequences were deposited in Genbank with the following accession numbers: clone libraries (Z, HQ595389-HQ595557; SDP, HQ595362-HQ595388; E, HQ595558-HQ595705) and DGGE bands (Z, HQ595721-HQ595722; SDP, HQ595706-HQ595708, HQ595718-HQ595720; E, HQ595709-HQ595717).

2.3 N-isotope incubations

For N-isotope incubations, groundwater was collected from the Zorra and Elmira sites. Anammox rates were calculated using incubations of Elmira composite water and groundwater

from piezometer Z92 at Zorra. Estimates of N_2 production represent an average among Elmira groundwater, wells 1, 3, 4, 5, 6, and 7, and an average among Zorra groundwater, wells Z92, Z95, Z122, Z124.

Water was collected for measurement of N-species concentrations and for ^{15}N-tracer incubation experiments. Groundwater samples for concentration measurements were filtered through a 0.45 µm syringe filter (Whatman) into polypropylene vials, and were stored on ice for transport to the laboratory, then were frozen (-20°C) until analysis. For ^{15}N-labelling experiments, the protocol was modified from a previous publication (Dalsgaard *et al*., 2003). Briefly, groundwater was collected directly to 12-mL "Exetainers" (Labco). In order to minimize oxygenation, the Exetainer was submerged into a container completely filled with ground water and neither headspace nor bubbles were observed in the Exetainer. Triplicate samples were collected for ^{15}N-labeling experiments. Water temperature, dissolved oxygen (DO) and pH were measured *in situ* with portable multi-parameter field meters (VWR).

The ^{15}N-labeling experiments were begun immediately after return to the laboratory (less than 2 hours). For these, 3 mL of water was withdrawn by syringe to generate a headspace. Each Exetainer was flushed with high purity helium for at least 15 min to remove background N_2 and dissolved O_2 and N_2. ^{15}N-enriched compounds were added by syringe to a final concentration of 100 µmol in 10 mL as $^{15}NH_4Cl$ and $Na^{15}NO_3$ (all >99% ^{15}N; Sigma-Aldrich). Although the final concentration of enriched ^{15}N varied in previous studies (ranging from 40 µmol L^{-1} to 10 mmol L^{-1}; Dalsgaard *et al*., 2003; Galán *et al*., 2009; Thamdrup *et al*., 2006), the concentration in the current study was higher because background ^{14}N mixing ratios in the collected samples can reach up to 20 mmol L^{-1}. An additional incubation was carried out without any tracer addition as a control to confirm that the incubation system functioned as

expected. A killed (autoclaved) control was included and was supplied with $^{15}NH_4^+$ and monitored for three days to confirm an absence of gas production. ^{15}N-labelling experiments were performed in November 2009 and were incubated in the dark at 15°C, which was an approximation of the *in situ* groundwater temperature (data not shown). $^{14}N^{15}N$:$^{14}N^{14}N$ and $^{15}N^{15}N$:$^{14}N^{14}N$ ratios were determined by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) and calculated as excess above their natural abundances (Thamdrup & Dalsgaard, 2000) (GG Hatch isotope laboratory, University of Ottawa). Samples were incubated with labeled isotopes for three months, during which time the N_2 headspace gas in the Exetainer was measured approximately every two weeks.

The concentration of NH_4^+ was measured before and after incubation by a salicylate colorimetric method on a spectrophotometer at 690 nm (Crooke & Simpson, 1971). The concentrations of NO_3^- and NO_2^- were determined by ion chromatography (DX100 IC, Dionex). The intermediate products of anammox reaction, hydroxylamine and hydrazine, were determined according to the methods of Frear and Burrel (1955) and Watt and Chrisp (1952), respectively. The detection limits for hydroxylamine and hydrazine are 0.25 and 0.05 mg L^{-1}, respectively. The detection limits for NO_3^-, NO_2^-, and NH_4^+ are 0.025 mg L^{-1}, 10 μmol L^{-1}, and 5 μmol L^{-1}, respectively. The molar fractions of ^{15}N label in NH_4^+ and NO_3^- (Fx) were calculated by comparing the initial concentrations of each nitrogen species and the increase in concentration subsequent to the addition of labeled ^{15}N.

The rates of anammox were extrapolated from linear regression of $^{14}N^{15}N$ accumulation as a function of time in the incubation with $^{15}NH_4^+$ and the rates of denitrification were determined from the slope of linear regression of $^{15}N^{15}N$ accumulation over time in the incubation with $^{15}NO_3^-$. The anammox contribution to total N_2 production was calculated by

assuming that the $^{15}NH_4^+$ pool turns over at the same rate as the ambient $^{14}NH_4^+$ pool. In addition to the above, a mathematical methodology for calculating the contribution of anammox, denitrification and atmosphere to an N_2 mixture was applied to $^{15}NH_4^+$ and $^{15}NO_3^-$ incubation experiments (Spott & Stange, 2007).

3.0 Results

3.1 Quantitative PCR

Quantitative PCR using anammox-specific primers (Amx368f and Amx820r) was used to provide an estimate of abundance of anammox bacteria relative to the total bacterial community (Figure 2). The qPCR demonstrated that anammox 16S rRNA gene copies represented 5.2 – 20.8% of the bacterial 16S rRNA gene copy numbers at the Zorra site, 5.1 – 10.1% of the 16S rRNA gene copies at the SDP site, and 4.6% of the 16S rRNA gene copies in the Elmira composite sample (with values ranging between <0.00% and 15% among the samples derived from individual wells at Elmira). Subsequent sampling of wells Z103 and Z106 in 2009 showed similar abundances of anammox targets (data not shown). The relative abundance of anammox 16S rRNA gene copies at Elmira was lower on average than that of Zorra, but was approximately equal to that of the SDP site. A background piezometer nest (Z86) at the Zorra site, which was influenced by agricultural activity but not the manure lagoon, had between one and five orders of magnitude fewer anammox gene copies than for the ammonium-impacted wells. PCR products from Z86 were not cloned and sequenced, so it is possible that amplification in the background well is not specific for anammox sequences.

The efficiency of general bacterial and anammox qPCRs were 88.9% and 84.6%, with R^2 values of 1.00 and 0.998, respectively. Clone library analysis of qPCR products demonstrated that the assay was highly specific: more than 97% of cloned amplicons were identified as anammox 16S rRNA sequences (data not shown).

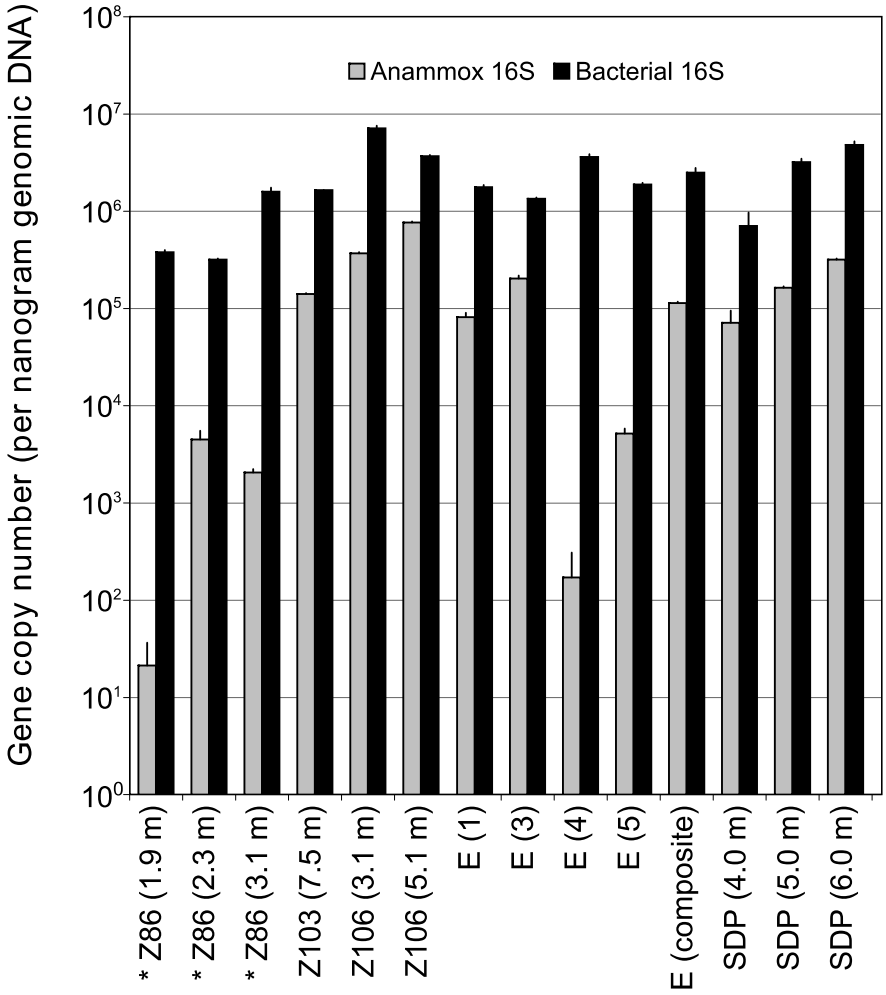

Figure 2. Comparison of anammox or bacterial 16S rRNA gene copies per nanogram genomic DNA at all three sites. Z = Zorra; E = Elmira; SDP = Calgary; * = "background" well located outside of ammonia plume. Error bars represent standard deviation for samples run in duplicate.

3.2 DGGE

The prevalence of anammox bacteria was confirmed with the DGGE data generated in this study (Figure 3). Dominant bands in the anammox-specific and general bacterial 16S rRNA gene profiles were chosen for sequencing. Of 25 sequenced DGGE bands, 18 sequences grouped closely with putative anammox sequences, or with known anammox reference sequences, and 5 of 6 non-anammox bands were chosen from general bacterial 16S rRNA gene DGGE profiles. Anammox bands were detected by sequencing bands from general bacterial DGGE for samples SDP (4.0 m), Z103 (5.1 m), and Elmira well 3. DGGE data suggest that anammox organisms were prevalent members of the microbial community at all three sites because bands with anammox identity (Table 3) were visible in general bacterial 16S rRNA gene profiles (Figure 3). Community profiles from piezometers Z103 and Z106 were highly similar and agreed with results from Illumina libraries (see below); there was little apparent difference between these two piezometers. DGGE patterns from three depths at the SDP site were nearly identical, and showed very little variation in community composition with depth (Figure 3). For the Elmira site, DGGE profiles indicate that the communities of bacteria at each well were distinct and that these communities within the plume were more heterogeneous than at the Zorra or SDP sites. Most of the predominant bands in the individual Elmira wells were also present in the composite sample.

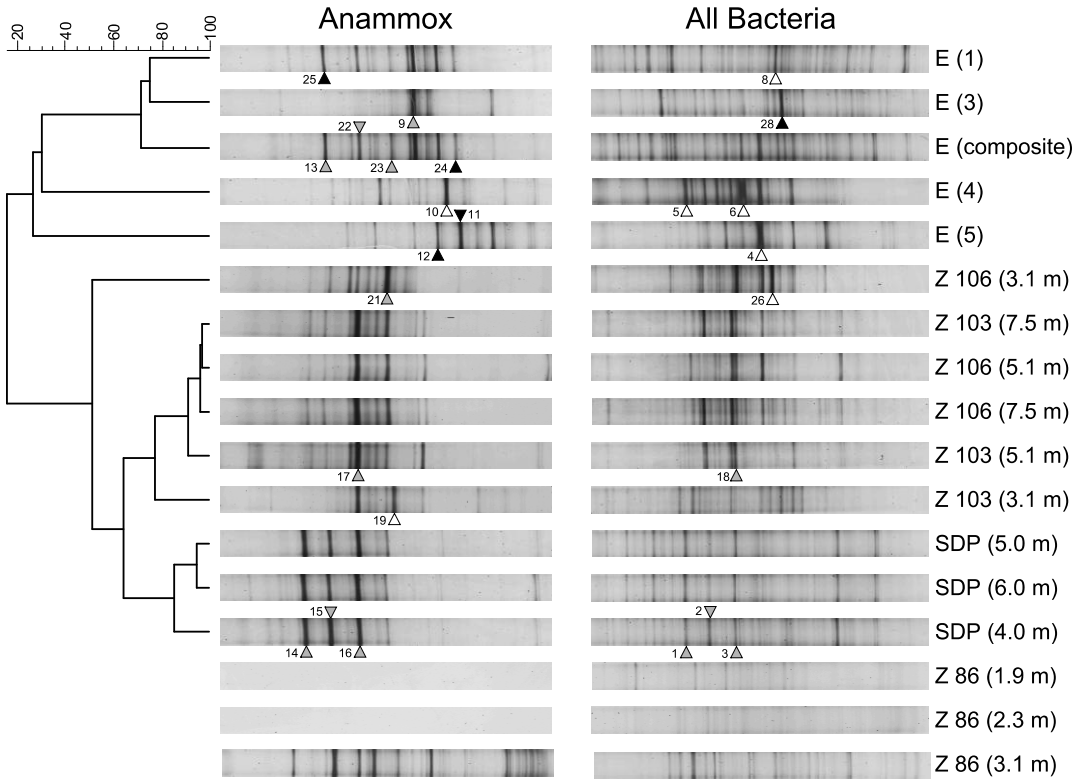

Figure 3. Anammox and general bacterial 16S rRNA gene fingerprints from all three field sites, obtained by denaturing gradient gel electrophoresis (DGGE). Black triangles indicate sequenced bands clustering within 95% similarity to known anammox reference sequences. Gray triangles indicate bands with putative anammox identity (i.e. closest BLAST search result of "uncultured anaerobic ammonium oxidizing clone" or similar). White triangles indicate bands that yielded sequences that did not affiliate with anammox bacteria. See Table 3 for band identities. Z = Zorra; SDP = Calgary; E = Elmira

Table 3. Closest anammox relatives to sequenced DGGE bands according to Genbank search results.

DGGE band	Similarity (%)	Genbank reference sequence (accession number)*
1	97%	Uncultured anaerobic ammonium oxidizing bacterium (FJ213995)
2	100%	Uncultured anaerobic ammonium oxidizing bacterium (FJ213995)
3	97%	Uncultured anaerobic ammonium oxidizing bacterium (FJ213995)
4	100%	*Sphingomonas* (HM346205)
5	98%	Uncultured bacterium clone (EU746741)
6	97%	Uncultured bacterium clone (HM593811)
8	100%	Uncultured bacterium clone (HM146607)
9	100%	Uncultured anaerobic ammonium oxidizing bacterium (FJ213992)
10	98%	Uncultured bacterium clone (GQ495463)
11	98%	*Candidatus* Kuenenia stuttgartiensis (AF375995)
12	96%	*Candidatus* Kuenenia stuttgartiensis (AF375995)
13	97%	*Candidatus* Scalindua sp. enrichment culture clone (EU142947)
14	97%	Uncultured anaerobic ammonium oxidizing bacterium (FJ213995)
15	98%	Uncultured anaerobic ammonium oxidizing bacterium (FJ213995)
16	99%	Uncultured anaerobic ammonium oxidizing bacterium (FJ213995)
17	99%	Uncultured anaerobic ammonium oxidizing bacterium (FJ213995)
18	99%	Uncultured anaerobic ammonium oxidizing bacterium (FJ213995)
19	100%	Uncultured bacterium clone (EU431780)
20		Failed sequencing reaction
21	100%	Uncultured anaerobic ammonium oxidizing bacterium (FJ213995)
22	100%	Uncultured anaerobic ammonium oxidizing bacterium (FJ213995)
23	100%	Uncultured anaerobic ammonium oxidizing bacterium (FJ213995)
24	97%	*Candidatus* Brocadia fulgida (DQ459989)
25	98%	*Candidatus* Scalindua marina clone (EF602039)
26	100%	Uncultured bacterium clone (HQ271309)
28	97%	*Candidatus* Brocadia fulgida (DQ459989)

* Closest "*Candidatus*" known anammox sequence present in BLAST search results having highest match (i.e. 100%) to full length DGGE band sequences. Refer to Fig. 3 for DGGE band numbers.

3.3 Clone libraries

In order to investigate the "background" bacterial communities associated with DNA extracts from each of the samples included in this study, small clone libraries were created with general bacterial 16S rRNA gene primers (27f and 1492r; Figure 4). The samples from each of the sites generated distinct clone libraries. Although Betaproteobacteria represented the dominant phylum in most of the libraries, other phyla varied widely in their relative abundances (e.g. Acidobacteria, Firmicutes, Deltaproteobacteria and Gammaproteobacteria). Notably, Planctomycete sequences were predominant in seven of ten sample libraries, possibly increasing with depth at the Zorra site. Although Planctomycete sequences were detected in most libraries, only one putative anammox sequence was identified, affiliated with one of the sample libraries (Z103, 7.5 m; 92% similarity to *Ca.* Brocadia fulgida). Subsequent bioinformatic analysis revealed several mismatches of the general bacterial primers (27f, 1492r) to anammox type strains (data not shown), which would result in anammox being underestimated in these libraries.

In order to better characterize the diversity of anammox bacterial communities, clone libraries were constructed with primers An7f and An1388r, which target the 16S rRNA gene of the anammox organisms *Ca.* Scalindua, *Ca.* Brocadia and *Ca.* Kuenenia (Penton *et al*., 2006). The following number of clone sequences were obtained from cloning with An7f and An1388r: Zorra (Z103 and Z106 at 7.5 m depths): 171 sequences; Elmira composite: 146 sequences; SDP (all depths): 27 sequences. The retrieved 16S rRNA gene sequences were aligned to known anammox sequences to construct a phylogenetic tree (Figure 5). For the samples included in this study, this primer set was specific to anammox bacteria (Table 4). Anammox sequences from all three contaminated groundwater sites demonstrated that the community

compositions differed between sites. At the Southland Dog Park field site, one phylotype of anammox bacteria (belonging to the *Candidatus* Brocadia genus) dominated the clone library. The dominance of *Ca.* Brocadia was also evident at the Zorra site, where the vast majority (164 of 171) of anammox sequences also grouped with known *Ca.* Brocadia reference sequences, and five of the remaining clones grouped with known *Ca.* Scalindua anammox sequences. The diversity of anammox sequences was highest in the Elmira composite library, with four of the five known genera of anammox sequences represented. Nonetheless, *Ca.* Brocadia clones outnumbered all other anammox genera sequences combined in the Elmira library.

Table 4. Percentage of anammox-specific (An7f, An1388r) clone library sequences with anammox identity.

Sample	Proportion of anammox clones (%)
Z103 (7.5 m)	98
Z106 (7.5 m)	100
E (composite)	86
SDP (4.0 m)	100
SDP (5.0 m)	100
SDP (6.0 m)	100

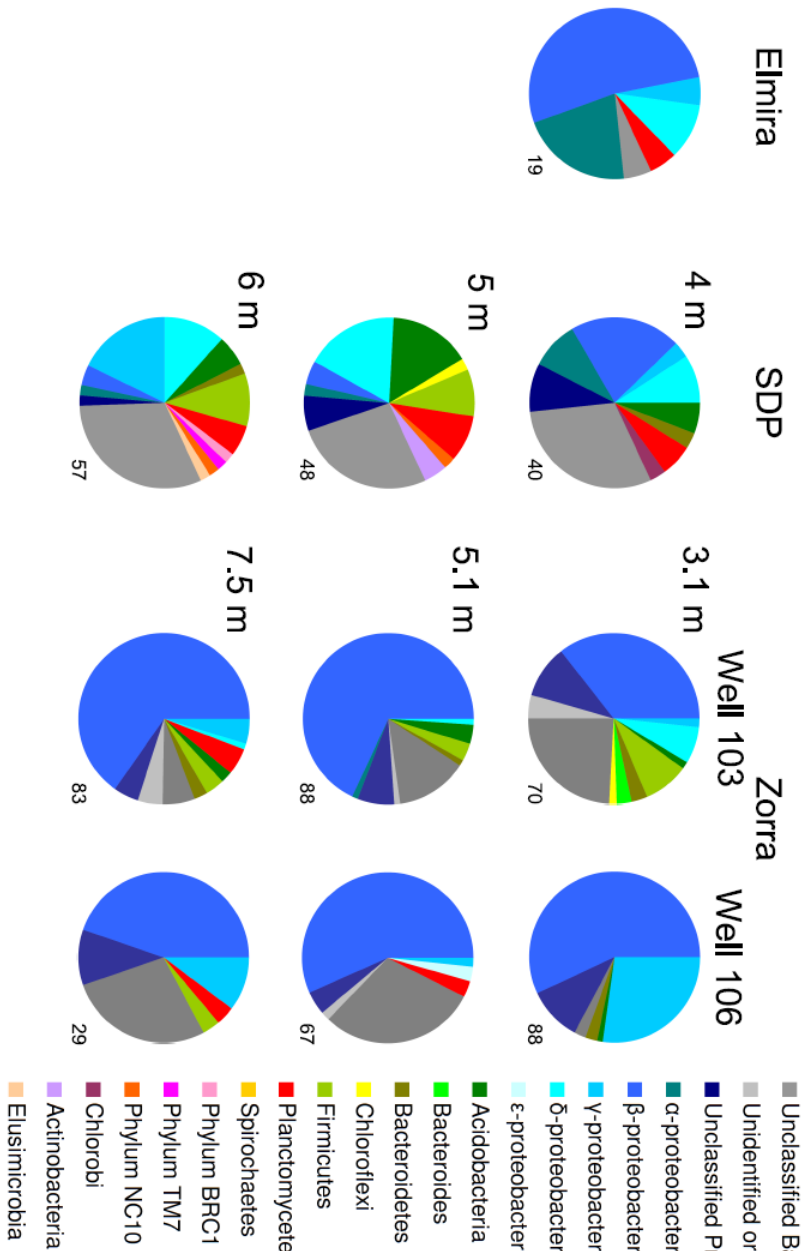

Figure 4. Distribution of phyla in general bacterial 16S rRNA gene clone libraries from Elmira, SDP, and Zorra sites. Small numbers beside pie charts represent the number of clones in each library.

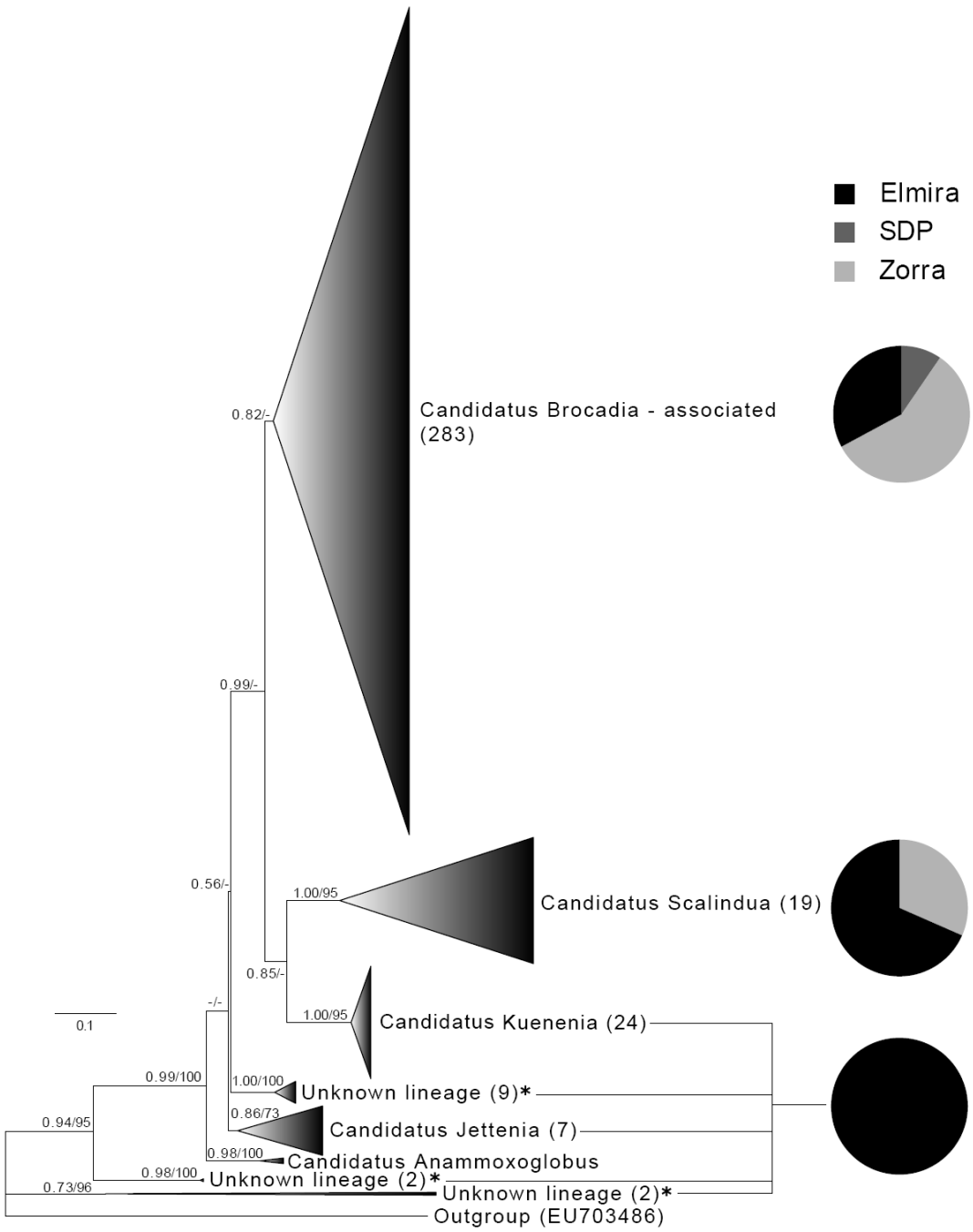

Figure 5. Maximum Likelihood phylogeny of groundwater anammox sequences from all three sites aligned with known anammox reference sequences acquired from GenBank. Numbers in brackets represent the number of clones identifying with each cluster. Pie chart insets represent the proportion of sequences within the corresponding clade that were obtained from each field site. Node support values correspond to aLRT and neighbour joining bootstrap values respectively, with dashes representing values < 0.5 (aLRT) or 50 (bootstrap). Scale bar represents the number of nucleotide substitutions per site. Asterisks represent unknown lineages of exclusively experimental sequences.

3.4 Illumina libraries

Circumventing the limitations of using 27f and 1492r, in addition to qPCR of all samples (see above), I used primers with near perfect matches to most anammox sequences (341f and 518r) to amplify 16S rRNA genes from all bacteria. This approach generated approximately 100,000 ~200-base sequences from each of the two 7.5-m depths in the Zorra site (see Appendix A). Both Illumina libraries were set up as local BLAST databases. These BLAST databases were queried with anammox sequences (retrieved from these samples using anammox specific primers An7f and An1388r; see below). Zorra anammox sequences represented 6.8% (7342 of 107 777) and 6.7% (6427 of 95 873) of the total number of sequences in Illumina libraries from Z103 (7.5 m) and Z106 (7.5 m), respectively. Anammox bacterial identity was associated with the fourth most abundant bacterial sequence in both groundwater Illumina libraries. Still, these proportions may be underestimates of anammox bacterial relative abundance if the 16S rRNA copy numbers in anammox bacteria (e.g. one copy in *Ca*. Kuenenia stuttgartiensis genome; Strous *et al*., 2006) are compared to the average copy number of other bacterial community members (4.02 copies per cell; Klappenbach *et al*., 2001).

The Illumina libraries also suggested the presence of denitrifying bacteria at the Zorra site (Appendix A). Sequences were sorted according to their frequency in Illumina libraries; for both libraries the top 10 most abundant sequences included sequences belonging to orders with cultured denitrifying representatives. In the Z103 and Z106 Illumina libraries, the most abundant ten sequences in both libraries included members of the genera *Herbaspirillum*, *Pseudomonas* and *Methylophilus*. Sequences from these genera were previously associated with denitrifying reactors, or have known denitrifying activity (Ginige *et al*., 2004; Ishii *et al*., 2009; Körner & Zumft, 1989). The groundwater chemistry provides additional support for the

possibility of denitrification. Apart from the anammox sequences that were not correctly assigned within the RDP-II (i.e. anammox sequences classified as "Chlamydiae"), the other sequences in the top 10 had genus-level confidence scores (S_{AB}) below 0.5, so their identities remain unknown.

3.5 Isotope incubations.

N-isotope incubation experiments were performed for Zorra and Elmira sites. Incubation of soil and groundwater samples from both sites with $^{15}NH_4^+$ yielded $^{14}N^{15}N$ (Figure 6a,c), which

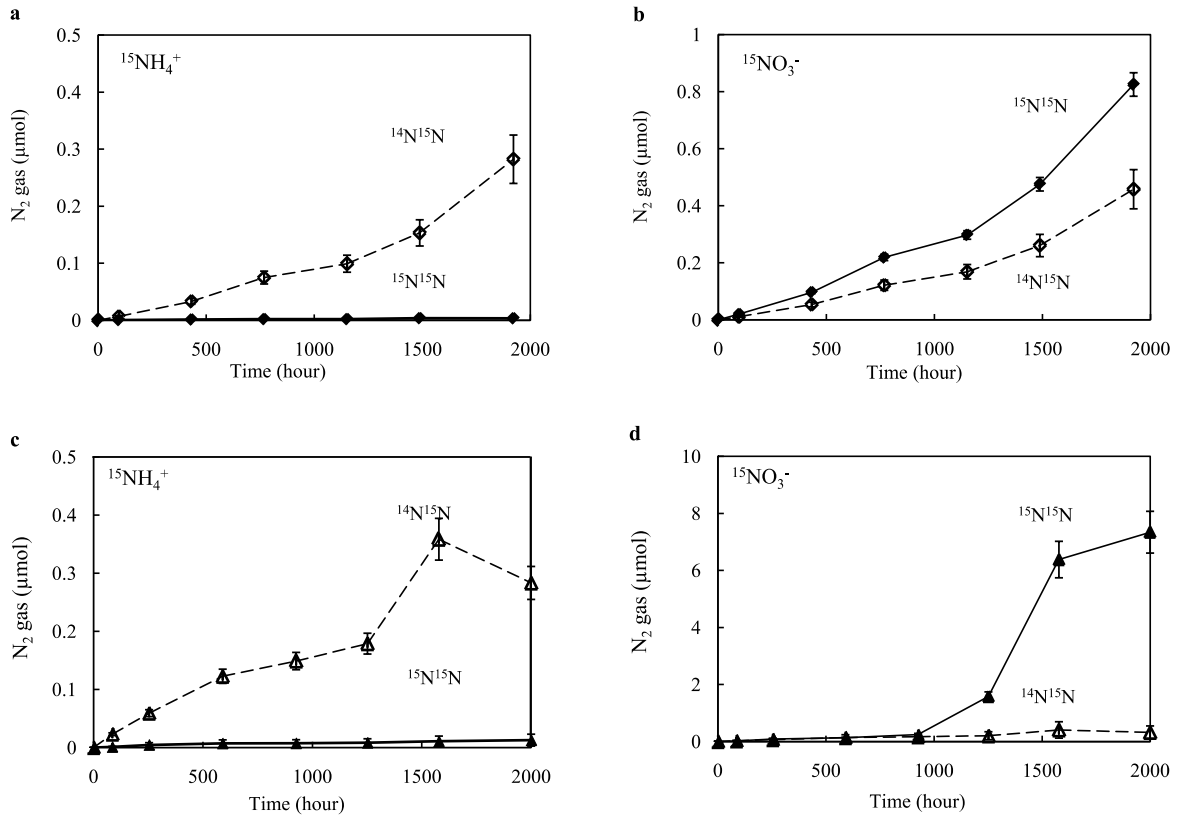

Figure 6: Isotope incubation data. a, **b**, Formation of $^{14}N^{15}N$ (open square) and $^{15}N^{15}N$ (solid square) in the incubation vial headspace with samples from the Elmira site. **c**, **d**, Formation of $^{14}N^{15}N$ (open triangle) and $^{15}N^{15}N$ (solid triangle) in the incubation vial headspace with samples from Zorra site.

is indicative of the anammox reaction. Piezometer Z86 (background piezometer) showed no production of $^{14}N^{15}N$ (data not shown). Production of $^{14}N^{15}N$ was detectable after 1 week but was very slow in groundwater samples from both sites, and a prolonged incubation time was required to determine the anammox reaction rate (approximately three months). At the end of the incubation time, the highest $\delta^{15}N$ was 14 300‰. At the Zorra site, the production of $^{14}N^{15}N$ showed an increasing trend through the three-month incubation (Figure 6c). At the Elmira site, $^{14}N^{15}N$ accumulated linearly and stably with time without a lag phase (Figure 6a). The rate of $^{14}N^{15}N$ production corresponded well with the anammox rate at both sites, as expected (Dalsgaard *et al.*, 2003; van de Graaf *et al.*, 1995; Thamdrup & Dalsgaard, 2002).

For the incubation of $^{15}NH_4^+$, $^{15}N^{15}N$ accumulation did not occur at either Zorra or Elmira site, which would have signified nitrification followed by denitrification or anammox. In control incubations without added tracer there was no production of ^{15}N-enriched N_2 from either site. In killed controls, no $^{29}N_2$ or $^{30}N_2$, and a very low concentration of $^{28}N_2$ were measured. The calculated anammox reaction was 31.3 ± 6.0 nmol L^{-1} h^{-1} at Elmira and 13.3 ± 1.6 nmol L^{-1} h^{-1} at Zorra. The $^{15}NO_3^-$ incubation produced more $^{15}N^{15}N$ than $^{14}N^{15}N$, indicating active and strong denitrification processes (Figure 6d). The application of Spott and Stange's comprehensive approach (which considers the contributions of anammox, denitrification, and atmosphere to an N_2 mixture based on a ^{15}N tracer technique; 2007) in our study showed that in the incubation of $^{15}NH_4^+$, anammox contributed 18.0 ± 6.5% of N_2 gas production in Zorra and 35.7 ± 13.6% in Elmira; these numbers agree with the results of the incubation of $^{15}NO_3^-$. In the incubation of $^{15}NO_3^-$, 21.4 ± 11.5% and 32.7 ± 15.6% of produced N_2 was from anammox at Zorra and Elmira sites, respectively (Figure 7). Hydroxylamine and hydrazine (anammox reaction intermediates) remained below detection limits throughout the incubations for samples

taken from both study sites, which is expected given that these intermediates are sequestered within the anammoxosome (Sinninghe Damsté *et al.*, 2002).

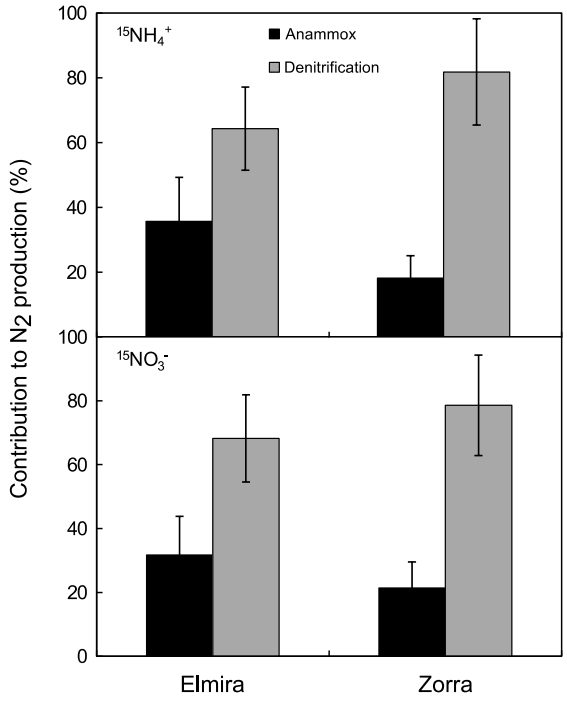

Figure 7. Calculated contributions of anammox and denitrification to microbiologically produced N_2 in $^{15}NH_4^+$ and $^{15}NO_3^-$ labeling experiments at the Elmira and Zorra sites. Error bars represent standard deviation.

4.0 Discussion

Anammox bacterial communities are responsible for substantial attenuation of nitrogen from aquatic and terrestrial environments. Groundwater contamination by ammonium can lead to contamination of surface waters and receiving water bodies, or impact groundwater serving as a source of drinking water. In a previous study, Clark and colleagues (2008) suggested that anammox bacteria were responsible for attenuation of ammonium in heavily contaminated groundwater, based on a study of the native ^{15}N-isotope ratios—these observations were made at the Elmira field site, where our molecular data indicate that anammox diversity was highest (Figures 3, 5).

This study provides direct molecular evidence of the presence of a diverse assemblage of anammox performing organisms at the Elmira site and two additional contaminated groundwater locations, and marks the first combined isotope- and molecular-based confirmation of anammox communities in contaminated groundwater environments. Previous studies of anammox bacteria in contaminated aquifers have focused on single sites, and lacked the addition of isotope-based techniques to verify the activity of these organisms (Humbert *et al*., 2010; Smits *et al*., 2009).

There are five known genera of uncultured bacteria that perform anammox, and each appears to have its own niche for optimal growth in both natural and engineered environments. *Candidatus* Scalindua (Kuypers *et al*., 2003) has been observed in natural marine environments such as oxygen minimum zones, sediments and freshwater lakes (Dalsgaard *et al*., 2005; Penton *et al*., 2006; Schmid *et al*., 2007; Schubert *et al*., 2006; Woebken *et al*., 2008); other members of the same genus were found in wastewater treatment reactors and in laboratory

scale reactors (Li *et al*., 2009; Schmid *et al*., 2003). Three genera have been associated with both natural and reactor-type environments: *Candidatus* Jettenia (Dale *et al*., 2009; Quan *et al*., 2008), *Candidatus* Brocadia (Dale *et al*., 2009; Strous *et al*., 1999) and *Candidatus* Kuenenia (Amano *et al*., 2007; Dale *et al*., 2009; Kartal *et al*., 2006; Schmid *et al*., 2000). The remaining genus, *Candidatus* Anammoxoglobus, has thus far been identified only in a laboratory scale reactor (Kartal *et al*., 2007b).

Anammox organisms have slow growth rates and are recalcitrant to pure culture growth. As a result, molecular techniques help identify these organisms in mixed communities. PCR of environmental DNA extracts with general bacterial primers to generate clone libraries has been shown to underestimate the proportion of anammox organisms in the environment due to mismatches with "universal" primers; however amplification based-methods for detection of anammox organisms are easily applied, and can be more successful than FISH for identifying anammox in low abundance samples (Schmid *et al*., 2005). Thus, anammox population sizes may be greater than estimated due to known mismatches of anammox organisms with several primer sets.

Quantitative real-time PCR was performed with anammox specific (Amx368f and Amx820r) primers that were originally designed as FISH probes, and have been adapted for the detection of anammox organisms in environmental DNA extracts. Together these primers are designed to target anammox organisms belonging to *Candidatus* genera Brocadia and Kuenenia (Schmid *et al*., 2000), and were chosen for this study because clone library data suggest that the majority of anammox sequences retrieved from our three field sites are *Ca.* Brocadia-like sequences. The qPCR results suggest that maximum anammox 16S rRNA gene abundances ranged between 10.1 and 20.8% of the total bacterial 16S rRNA genes at all three

sites considered in this study. Anammox 16S rRNA gene copy numbers were highest at the Zorra site, and were comparable at the Elmira site (select wells only) and SDP site. There was a wide range in anammox abundance among the wells at the Elmira site. Samples collected from the Zorra site in 2009 showed the same consistently high proportion of anammox organisms in the total bacterial community, indicating prevalence and temporal stability of anammox bacteria within the groundwater community. All of these results may be underestimates of actual anammox bacterial relative abundance if the rRNA copy numbers in anammox bacteria (e.g. one copy in *Ca.* Kuenenia stuttgartiensis genome; Strous *et al*., 2006) are compared to the average copy number of other groundwater bacterial community members, which is likely higher than one.

In this study I developed a method for assessing anammox community distribution using DGGE because previously published protocols (Sànchez-Melsió *et al*., 2009) were unsuccessful at retrieving anammox bands, or extant primers were not anammox specific (Pla46f; Innerebner *et al*., 2007). A "nested" PCR approach was used—where anammox 16S rRNA genes were amplified before applying a GC-clamp using general bacterial 16S rRNA gene primers. This permitted successful retrieval of anammox bands from DGGE gels. In addition, bands from general bacterial 16S rRNA gene profiles were directly comparable to anammox profiles because bands migrated to the same location (relative to DGGE ladder bands) for both gels (Figure 3); it was possible to see the location of anammox bands in the general bacterial profiles. It is important to note that some caution should be taken when using this approach. In samples with low relative anammox DNA, primers An7f and An1388r have been known to amplify DNA from other organisms (Dale *et al*., 2009; Junier *et al*., 2010; Li *et*

al., 2010). For these samples, high anammox sequence retrieval was confirmed by cloning and sequencing the PCR products from amplification with An7f and An1388r (Table 4).

Known anammox reference sequences have only one mismatch with 341f and no mismatches with 518r (data not shown). The Illumina library and qPCR data estimated that anammox organisms represented 6.8 or 8.5% of the total bacterial community at the Zorra site (Z103, 7.5 m), respectively, and qPCR data suggested that anammox bacteria represented between 4.6 and 20.8% of the bacterial community across all ammonium-impacted sites included in this study, excluding two samples (E 4 and E 5) with <0.25% anammox.

The diversity of anammox bacteria in aquatic environments is determined by the surrounding environment; anammox communities shift in response to salt concentration (Kartal *et al*., 2006) and available carbon substrates (Kartal *et al*., 2007b). There appear to be niches typically occupied by different genera of anammox bacteria, and several studies have concluded that the anammox populations acting in a single environment have very low diversity (Kartal *et al*., 2006; Kartal *et al*., 2007b). High diversity of anammox organisms (like that seen at the Elmira site) was observed once in the Cape Fear estuary (Dale *et al*., 2009). *Ca*. Brocadia and *Ca*. Kuenenia were present in the freshwater reaches of the estuary, while *Ca.* Scalindua organisms dominated in the saline end of the estuary; a correlation existed between the distribution of anammox organisms and salt concentration. Groundwater is not saline at these sites (EC <2450 μS cm^{-1}; Table 1), although the Elmira site has had historically high concentrations of K^+ and Cl^- which could contribute to higher EC values at this site (Clark *et al*., 2008). The dominant organisms at all three sites belonged to the *Ca.* Brocadia genus, which primarily represent freshwater organisms. *Ca.* Scalindua is commonly associated with marine environments (Woebken *et al*., 2008), however these organisms have been detected in

freshwater environments as well (Schubert *et al*., 2006). A small proportion of anammox clones grouped with the *Ca.* Scalindua genus at Zorra and Elmira sites (Figure 5). *Ca.* Jettenia and *Ca.* Kuenenia clones were identified at only one field site (Elmira). *Ca.* Kuenenia is also recognized as a freshwater genus of anammox bacteria (Kartal *et al*., 2006). No clone library data exist for the distribution of anammox genera among individual wells at the Elmira field site, however sequenced DGGE bands revealed *Ca.* Brocadia in the composite sample, *Ca.* Kuenenia in well 5 and *Ca.* Scalindua in well 1. In combination with DGGE results, these observations suggest the possibility of site-specific heterogeneity in anammox bacterial distributions.

High anammox diversity at the Elmira site may be explained by location-specific characteristics. The heterogeneous Elmira field site contained the only phylotypes not associated with known or proposed taxa (Figure 5), which suggests that diversity of anammox sequences may be high in other similar sites. Investigations of such locations may identify other novel lineages of anammox bacteria. Groundwater contamination at Elmira has potentially been present for over 100 years, and the sampling wells cover an area within the plume of ~ 5100 m^2. The concentrations of NH_4^+ and NO_3^- at the site are derived from two different high-strength sources, which are variably diluted as they mix with local groundwater to form gradients and pockets of high and/or low substrate. The age of the plume, combined with the large area of coverage and the heterogeneous substrate availability likely produces many small niche environments that may meet the needs of the different genera of anammox organisms present on site. The possibility of anammox organisms growing in niches of natural environments is supported by the literature (Kartal *et al*., 2006). A large, long-lasting plume of ammonium-contaminated groundwater may provide these niches for anammox organisms.

DGGE profiles of the communities at each well at the Elmira site were distinct, with a few shared bands, suggesting that the bacterial community at each well location is diverse and different from other wells at Elmira. As mentioned above, identification of three *Candidatus* genera (Brocadia, Kuenenia, and Scalindua) in three separate wells at Elmira further suggests the possibility of niche development in the heterogeneous site.

In addition to biomarker-based evidence for anammox in ammonium contaminated groundwater, the production of only $^{14}N^{15}N$ in the incubation of $^{15}NH_4^+$ (rather than $^{15}N^{15}N$) provides stoichiometric evidence for N_2 production by the anammox reaction (van de Graaf *et al*., 1995; Jetten *et al*., 2001). Production of $^{15}N^{15}N$ would signify nitrification coupled with either denitrification or anammox. Isotope data suggest that anammox organisms are responsible for 32.7 ~ 35.7% of N_2 production at the Elmira field site, and 18.0 ~ 21.4% at the Zorra field site, with anammox rates of 31.4 ± 6.0 and 13.3 ± 1.6 nmol L^{-1} h^{-1}, respectively. Dalsgaard and colleagues (2003) reported anammox reaction rates between 3 and 18 nmol L^{-1} h^{-1} in the anoxic water column of Golfo Dulce, which are comparable to the reaction rates observed in these contaminated groundwater sites. Many lower rates have been found in oxygen-deficient water such as in the eastern South Pacific (≤0.7 nmol L^{-1} h^{-1}; Thamdrup *et al*., 2006) and in the Black Sea (~7 nmol d^{-1}; Kuypers *et al*., 2003). Our results are within the reported reaction rates in freshwater lakes, ranging from 6 to 504 nmol N_2 L^{-1} d^{-1} (Hamersley *et al*., 2009). At Elmira, $^{14}N^{15}N$ accumulated linearly and stably with time without a lag phase, which indicates that anammox was the active process and no intermediates were involved in the reaction (Galán *et al*., 2009). Low substrate concentrations in Zorra samples (e.g. NH_4^+; Table 1) might explain the suppression of anammox activity with time. These relatively high N_2 production rates by anammox suggest that anammox bacteria are important members of the

microbiological community at ammonium contaminated groundwater sites, and are important players in the nitrogen cycle of groundwater systems. To date, the reported relative contributions of anammox to N_2 production have been variable; the highest value was reported in marine sediments in Denmark (67%; Dalsgaard & Thamdrup, 2002).

At the Zorra site, isotope evidence suggests that the production of N_2 was due to the activity of both anammox and denitrifying organisms. The concurrent growth of denitrifiers and anammox is commonly observed (Song & Tobias, 2011) because both kinds of organisms require similar environmental conditions for growth (e.g. low oxygen and NO_2^-/NO_3^- substrates). Results from Illumina libraries support this observation because the 16S rRNA genes of putative denitrifying organisms were among the most dominant sequences retrieved. Some organic carbon sources inhibit the growth of anammox organisms, while many denitrifiers require an organic carbon source as a reductant. Unfortunately, DOC was not measured during these experiments, so we were not able to correlate the anammox reaction rate to organic carbon present in the groundwater. Further research into the environmental factors affecting differential contributions of denitrifiers and anammox bacteria in groundwater should include measurements of DOC values in addition to the abundance and expression of denitrification functional genes.

Anammox bacterial communities are implicated in losses of nitrogen from aquatic and terrestrial environments worldwide. Groundwater contamination by ammonium most often occurs by anthropogenic activities, and has the potential to negatively impact surface water and aquifers which supply drinking water. This study is the first to present joint molecular and isotopic data to identify diverse and active anammox bacterial communities in ammonium contaminated groundwater. Previous studies of anammox communities in groundwater yielded

very few anammox sequences, and did not involve activity measurements (Humbert *et al*., 2010; Smits *et al*., 2009).

Confirming the presence and activity of anammox bacteria opens up the possibility of modifying the designs of engineered systems that treat nitrogen-rich sources such as septic systems, manure handling facilities and groundwater permeable reactive barriers to enhance the anammox reaction (Aravena & Robertson, 1998). The importance of such an engineered design would be to help protect surface waters from NH_4^+-impacted groundwater in a one-step remediation approach instead of through the combined activities of nitrifiers and denitrifiers.

5.0 Conclusions and Future Research

5.1 Conclusions

The research conducted in this thesis involved the characterization of anammox-performing bacteria at three ammonium-contaminated groundwater sites in Canada. The source of ammonium contamination varied between the sites (Zorra: compost; Elmira: chemical and fertilizer plant, Southland Dog Park: fertilizer plant), but the sample sites provided the substrates ($NH_4^+/NO_2^-/NO_3^-$) and anoxic conditions required for anammox metabolism. The methods used to assess these communities (sequencing, DGGE, qPCR, isotope-based incubations) were combined to minimize bias and strengthen the interpretation of data resulting from this study. This work builds on initial isotopic evidence obtained for the anammox reaction in NH_4^+-impacted groundwater at the site of a former fertilizer operation in Elmira, Ontario, Canada (Clark *et al*., 2008). This research is the first to show diverse and active groundwater anammox organisms via coupled ^{15}N labeling incubations and biomarker-based evidence.

My original hypotheses were that ammonium contaminated groundwater sites would harbour anammox performing communities related to freshwater genera. The hypothesis was confirmed because all three contaminated groundwater sites examined revealed anammox organisms which were detectable with traditional molecular methods. *Candidatus* Brocadia-like sequences were present at all field sites (Figure 4). The dominance of *Ca.* Brocadia was expected because Brocadia-like sequences are often identified in freshwater environments and were identified in groundwater previously (Humbert *et al*., 2010; Smits *et al*., 2009) but with lower diversity than at the Elmira site. Scalindua-like sequences were also present at three of

the sites and have been detected in freshwater previously (Hamersley *et al*., 2009; Schubert *et al*., 2006), despite the tendency of *Ca.* Scalindua to inhabit marine environments (Woebken *et al*., 2008).

I also hypothesized that anammox organisms would make up an appreciable proportion of the microbial community in ammonium contaminated groundwater. Results generated by three complementary methods confirmed this hypothesis. Illumina sequencing results indicated that anammox-like sequences accounted for approximately 7% of the bacterial community at Zorra (see Appendix A), and qPCR revealed that anammox-like sequences make up as much as 20% of the bacterial populations across the three sites studied with this technique (Figure 2). In addition, a visual comparison of general bacterial and anammox specific DGGE profiles shows that anammox bands are visible and often dominant in the general bacterial 16S rRNA profile (Figure 3). These data all suggest that anammox organisms are prevalent community members in ammonium-contaminated groundwater.

This research was performed in collaboration with Dr. Ian D. Clark and Dr. Yangping Xing at the University of Ottawa, who conducted ^{15}N-isotope incubations to determine the activity of anammox bacteria in groundwater incubations. These techniques permitted differentiation between anammox- and denitrification-generated N_2, and the results suggested that anammox organisms were active and potentially responsible for ~18 and 35% of N_2 production at Elmira and Zorra sites, respectively. These results agreed with biomarker-based evidence, and confirmed the presence and activity of anammox bacteria in contaminated groundwater. Future research will assess the generality of these findings to additional contaminated sites, and include multivariate statistics to help identify controls on anammox bacterial community diversity and composition.

The molecular techniques applied in these experiments relied on the detection of bacterial 16S rRNA genes in contaminated groundwater environments. The 16S rRNA gene is a commonly used target for detection and quantification of bacteria and archaea in environmental samples. The 16S rRNA genes of anammox organisms are known to have mismatches with commonly used "universal" bacterial primers (as seen with primer pairs 27f, 1492r, 341f and 518r used in this study). In addition, the 16S rRNA genes within anammox *Candidatus* genera vary (~85% similarity between *Ca.* Brocadia and *Ca.* Kuenenia), and primers targeting all known anammox genera lack specificity when applied to environmental samples (Jetten *et al*., 2003). The primers Amx368f and Amx820r are used heavily for the detection of anammox-like sequences in environmental samples (Amano *et al*., 2007; Dale *et al*., 2009; Li *et al*., 2010; Penton *et al*., 2006) and were used in qPCR detection of anammox sequences in this study. Despite collecting target sequences closely related to known genera, unknown anammox genera may remain undetected due to lack of primer specificity. Newly designed anammox-specific 16S rRNA gene primers, in an attempt to capture all anammox diversity, may also target non-anammox sequences when applied to environmental samples (Dale *et al*., 2009; Li *et al*., 2010; Penton *et al*., 2006). Despite bias in using the 16S rRNA gene to detect anammox bacteria, these primers continue to be useful for environmental surveys of anammox bacteria and future research will decrease bias by continuing to modify primers as new sequences are retrieved.

This study could have been improved by including additional samples from within one or more sites representing many more depths and geochemical conditions (e.g. NO_2^-/NO_3^-, NH_4^+, DO, and pH), followed by molecular and isotopic analyses as conducted in this study. Such a comprehensive survey would provide more detailed information about the distribution

and activity of anammox bacteria across the sites. Ideally, the final goal would be to map anammox community distribution, abundance, and activity within and along the plume, and study changes in the communities over multiple years. The objective of the current study was limited to identifying the presence and diversity of anammox bacteria and even with the number of samples obtained, coordination of sampling for concurrent chemical, molecular, and isotopic analyses was challenging. Future work should include additional samples, and sites, and make use of multivariate statistics so that the relationship between site-specific characteristics (e.g. salinity, N-loading) and phylogeny can be tested explicitly. The aim would be to identify controls on anammox bacterial community diversity and composition.

5.2 Considerations for future investigations

This study of 16S rRNA genes to detect the presence of anammox organisms in groundwater was conducted in parallel with ^{15}N stable-isotope work to determine anammox activity (Yangping Xing, University of Ottawa). When used in tandem, these techniques confirmed the presence of anammox-like sequences, and provided isotopic evidence for the anammox reaction. This study would have yielded more information if I had compared anammox abundance and diversity, as well as activity, along the length of the contaminant plume. To assess the feasibility of such an approach, a current study in the Neufeld lab has begun to examine soil-associated anammox bacterial diversity and activity in an agricultural field with varying drainage practices, and will apply DGGE and qPCR techniques described in this thesis, as well as ^{15}N stable-isotope techniques and soil physicochemical measurements. By a combination of coordinated sampling efforts and NO_2^-/NO_3^-, NH_4^+, DO, and pH measurements, we are beginning to assess the factors controlling anammox abundance and

diversity. Anammox activity can also be detected by measuring the production of functional gene products by RT-qPCR.

Using molecular methods alone, it is also possible to detect active anammox organisms using FISH, and to quantify their activity using RT-qPCR. FISH is often used to monitor enrichment in anammox reactors, but this technique suffers from a high signal threshold, and the fluorescent signal is often quenched when FISH is applied to environmental samples. Indeed, FISH was attempted using groundwater samples collected from the Zorra site, but image quality was very low due to high levels of background signal (work performed by Elif Tekin, University of Ottawa). Perhaps identifying alternative sample preparation methods would improve this technique; organic matter is the suspected source of high levels of background signal on filters prepared for FISH (personal communications with Danielle Fortin and Elif Tekin).

Most of the methods used in this research rely on molecular detection of nucleic acids present within the environment. The approaches do not directly demonstrate whether detected populations are directly involved in the measured activities. Alternative methods such as DNA stable-isotope probing (DNA-SIP) provide a culture-independent and probe-independent tool to enrich and detect autotrophic anammox activity in mixed communities. This technique is used to enrich the DNA of active organisms involved in assimilating a fully labelled ^{13}C substrate, and uses density gradient ultracentrifugation to separate heavy DNA from light DNA. When used in conjunction with DGGE, one could detect whether anammox cells were actively assimilating CO_2 (while oxidizing ammonium to N_2), as well as monitor their enrichment over time. Future research could include DNA-SIP using $^{13}CO_2$ to identify active anammox populations from contaminated groundwater at the sites studied in this thesis

research. For example, this method could identify the anammox genera that assimilate the most CO_2 at the Elmira site, under varying experimental conditions (e.g. inorganic N concentrations, temperature, presence/absence of DOC). The feasibility of using DNA-SIP to identify anammox bacteria has not yet been demonstrated, however this approach has been applied to the study of active AOA and AOB communities (Pratscher *et al*., 2011; Tourna *et al*., 2010; Zhang *et al*., 2011), and is a promising approach for detection and quantification of anammox bacteria in natural environments (Li & Gu 2011).

Anammox has been confirmed at three groundwater contaminated sites, and this activity in contaminated groundwater suggests implications for modifying the designs of engineered systems that treat nitrogen-rich sources such as septic systems, manure handling facilities and groundwater permeable reactive barriers (Robertson & Cherry, 1995; Vlaeminck *et al*., 2009). Indeed, Lucas Carson (an MSc student in Earth and Environmental Sciences at the University of Waterloo) is currently working at the Zorra site, and is attempting to enrich anammox bacteria in an underground reactor fed with local groundwater and exposed to a range of environmental conditions like changes in temperature and groundwater flow. He is monitoring N isotopic ratios, and concentrations of NO_2^-/NO_3^- and NH_4^+, and has collected water samples at one-month time intervals for analysis by qPCR. The goal of his project is to monitor the enrichment of anammox organisms over time and learn how to enhance this reaction and lead to natural attenuation of ammonium. On a larger scale, the anammox reaction is applied in full scale wastewater treatment in the Netherlands (van Dongen *et al*., 2001), and can be applied in combination with other bacterial groups to treat wastewater using the OLAND (Vlaeminck *et al*., 2009), SHARON (van Dongen *et al*., 2001), and CANON (Third *et*

al., 2001) treatment methods. The anammox process can provide a less costly and more efficient method for removing nitrogen from a variety of effluent sources.

Over the last 15 years, the discovery and study of anammox bacteria has revolutionized our understanding of the nitrogen cycle, and new techniques have been developed to exploit their natural capacity for N-attenuation. Their presence in a wide variety of aquatic and terrestrial environments demonstrates the adaptability and prevalence of anammox bacteria worldwide. Groundwater can be contaminated by anthropogenic nitrogen from a variety of sources (Böhlke *et al*., 2006; Minnesota Pollution Control Agency, 2001; Rudolph *et al*., 1998), and this study clearly demonstrated that anammox bacteria have adapted to the habitat provided by contaminated groundwater. In contrast to previous studies which provided limited biomarker based evidence of anammox bacteria in groundwater (Humbert *et al*., 2010; Smits *et al*., 2009), I have also demonstrated that anammox bacteria are prevalent and active bacterial community members in contaminated groundwater, and that these communities can be highly diverse within a single site. All sites were dominated by *Ca*. Brocadia, an anammox genus which is most commonly detected in other freshwater environments. Anammox bacteria were detected at all study sites, which suggests that anammox bacteria may be active at other similar locations, and are already involved in natural attenuation of ammonium. This research suggests the possibility of leveraging the natural abundance of anammox bacteria in contaminated groundwater to engineer reactive-barrier based solutions for remediating the impacts of groundwater anthropogenic N-loading.

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Appendix A: Illumina bacterial 16S rRNA gene results

Please refer to the enclosed CD for Tables A1 and A2: Illumina bacterial 16S rRNA gene libraries from Zorra wells 103 and 106, 7.5 m depth. This data is also available electronically from the Department of Biology upon request.