

Microbial ecology of ammonia oxidation in the Grand River

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

The Grand River is the largest catchment in Southern Ontario and is heavily impacted by the results of human activities, including wastewater effluent and agricultural and urban runoff. Ammonia oxidation is an important biogeochemical process for maintaining ecosystem health in impacted rivers because high ammonium concentrations are toxic to aquatic life and affect drinking water quality. In this thesis, I focus on the microorganisms involved in aerobic and anaerobic ammonia oxidation within a freshwater context. Aerobic ammonia oxidizing bacteria (AOB) and archaea (AOA) oxidize ammonium to nitrite under oxic conditions, whereas anaerobic ammonia-oxidizing (anammox) bacteria oxidize ammonium and reduce nitrite to produce N_2 gas under anoxic conditions. Anammox bacteria play an important role in removing fixed N from both engineered and natural ecosystems, yet broad scale distributions of anammox bacterial have not yet been summarized. Chapter 2 investigates global distributions and diversity of anammox bacteria and explores factors that influence their biogeography. Combined bioinformatics and multivariate analyses demonstrates that an important factor influencing anammox bacterial distributions was salinity, in addition to selection based on natural and engineered ecosystems. In Chapter 3, I address a limitation of anammox surveys, which is the specificity of primers used to study environmental distributions of anammox bacteria. The published primers commonly used in anammox surveys were verified for their specificity and tested by multiple molecular approaches, including denaturing gradient gel electrophoresis (DGGE), quantitative PCR (qPCR), and cloning. The A438f/A684r primer set was specific for anammox bacterial detection in freshwater environments. Because anammox bacteria are not the only microorganisms capable of ammonia oxidation, Chapter 4 investigates the oxidation of ammonium to nitrite by AOB and AOA under different environmental conditions. Both sediment and water column samples were studied to assess the impact of anthropogenic inputs on in-river microbial communities, identifying key players removing ammonium from the Grand River. DGGE demonstrated that wastewater effluent impacted the in-river microbial community downstream. Together, qPCR and RT-qPCR indicated that AOB and anammox are important within river sediments, reflecting a possible nitrification-anammox coupled process. However, only AOB were implicated in water column ammonia oxidation. This study also demonstrates the importance of combined molecular and activity-based studies for disentangling molecular signatures of wastewater effluent from autochthonous prokaryotic communities. In order to confirm that molecular signals corresponded to metabolic activity, the differential nitrification inhibitors (ATU and PTIO) were used in Chapter 5 to

confirm AOB activity within the Grand River, for both sediment and water column samples. Urea hydrolysis was tested in parallel to nitrification activity, examining this alternative source of ammonium for fuelling ammonia oxidation within the river. The results confirmed the dominant activity of AOB in both sediment and water column samples collected downstream in waters receiving wastewater effluent. Water column AOB likely hydrolyzed urea and used the resulting ammonium as an energy source. In Chapter 6, the full length of the Grand River was sampled to identify the composition of bacterial taxa, as revealed by next-generation sequencing and bioinformatics. The major bacterial taxa detected along the river were *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. The wastewater effluents harbored unique taxa, including TM6 and GN02; these two were poorly represented in the river itself. Distance-specific relationships, from the head to the mouth of the river, including hydrodynamics (i.e., lake and dam effects), were key factors correlating with measured in-river microbial communities. Water chemistry (i.e., pH, DOC, NO_3^-) showed weak correlations with in-river bacterial distributions. Together, my research demonstrates the biogeography of anammox bacteria and niche partitioning of AOB, AOA, and anammox bacteria within the heterogeneous microbial community background of the Grand River. This thesis represents an important step forward toward understanding the roles of microbial nitrogen cycling within aquatic habitats, especially those impacted by anthropogenic activities.

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List of Genes and Abbreviations

aLRT	approximate likelihood ratio test
AMO	ammonia monooxygenase
<i>amoA</i>	ammonia monooxygenase
<i>amtB</i>	ammonium transport
anamnox	anaerobic ammonia-oxidizing bacteria
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
AOP	ammonia-oxidizing prokaryotes
ATU	allylthiourea
AXIOME	Automation, eXtension, and Integration Of Microbial Ecology
BLAST	basic local alignment search tool
BSA	bovine serum albumin
CCA	canonical correspondence analysis
CD-HIT	cluster database at high identity with tolerance
DCD	dicyandiamide
DGGE	denaturing gradient gel electrophoresis
DIC	dissolved inorganic carbon
DMPP	3,4-dimethylpyrazole phosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DNRA	dissimilatory nitrate reduction to ammonia
DO	dissolved oxygen
DOC	dissolved organic carbon
FISH	fluorescence <i>in situ</i> hybridization
<i>focA</i>	formate/nitrite transport
FWM	inorganic freshwater medium
GTR	general time-reversible
<i>hao</i>	hydroxylamine oxydoreductase
<i>hzo</i>	hydrazine oxydoreductase
<i>hzs</i>	hydrazine synthase

MRPP	multi-response permutation procedure
<i>nirS</i> , <i>nirK</i>	nitrite reductases
NMDS	non-metric multidimensional scaling
NOB	nitrite oxidizing bacteria
NxOR	nitroxyl oxidoreductase
OMZ	oxygen minimum zone
ORF	open reading frame
OTU	operational taxonomic unit
PC	principal coordinate
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
PD	phylogenetic diversity
PTIO	2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide
QIIME	quantitative insights into microbial ecology
qPCR	quantitative real-time PCR
RDA	redundancy analysis
rRNA	ribosomal ribonucleic acid
RT-qPCR	reverse transcriptase qPCR
SDZ	sulfadiazine
SIP	stable-isotope probing
TAN	total ammonium nitrogen
TN	total nitrogen
T-RFLP	terminal restriction fragment length polymorphism
WWTP	wastewater treatment plant
UPGMA	unweighted pair group method with arithmetic mean

Chapter 1

Introduction

1.1 Literature review

1.1.1 The river ecosystem

River ecosystems are characterized by interconnected hydrological and limnological features. River flows are impacted by rain, snow, groundwater, and human activities. Flow path is influenced by geology, slope, and vegetation (Allan and Castillo 2007). All these features, including location and climate, are factors that make each river unique. River ecosystem can be linked to terrestrial and marine ecosystems by nutrient transport. Nutrients can be exchanged between river ecosystem and other connected ecosystems, such as terrestrial, ocean, and groundwater. Rivers receive, transport, and export nutrients to downstream water. Headwaters are usually deep and potentially export both natural and anthropogenic inputs to the lower section of the river, including receiving lakes and oceans (Allan and Castillo 2007). Nutrients can be exchanged between rivers and the adjacent drainages, land uses, and floodplains. The surface of soils or sediments can temporarily store the nutrients by sorption-desorption reactions, delaying downstream transport. Consequently, the alteration of nutrient dynamics in river ecosystem can impact biogeochemical cycles at local to global scales. The biogeochemical cycles in river ecosystem are complex because several factors must be considered simultaneously, including terrestrial-aquatic linkages, hydrologic exchanges between the channel and hyporheic zone, floodplain/riparian complex, subsurface waters, and interactions between coupled biogeochemical cycles (Helton et al., 2010).

The Grand River in Southwestern Ontario flows in a north to south direction, spanning 280 km from Dundalk in the north to Port Maitland on Lake Erie. The Grand and its tributaries, including the Nith, the Conestogo, the Speed, and Eramosa rivers cover an area of approximately 7,000 km² (GRCA website). There are the large and diverse wetlands in the northernmost of the Grand River. The northern and southern regions of the watershed are heavily impacted by agriculture and farming. The central region, including Kitchener, Waterloo, Guelph, Cambridge, and Brantford, is the most populated area (GRCA website). The main sampling location in this thesis was the central portion of the Grand River (Chapters 4 and 5). More than 100 dams are constructed along the Grand River (GRCA website). These irrigation systems impact the river flow. In summer, water levels and river flows are low due to low rainfall and hot temperature. Consequently, the river flows depend on the

discharges from dams and groundwater. The dams help maintain minimum flows and water levels to support human uses (i.e. drinking water and agriculture). The minimum level in the river receiving wastewater effluent is required to meet provincial environmental standards (GRCA website). Together with agriculture and farming, 29 wastewater treatment plants (WWTPs) and 221 wetlands along the Grand River watershed release high nitrogen discharges into the water column. Nitrogenous substances can be temporarily stored in the river, exported to downstream water, or permanently removed through microbial processes. This thesis focuses on the impact of nitrogen on in-river microorganisms.

1.1.2 The biogeochemical nitrogen cycle

Nitrogen (N) is an important nutrient for living organisms because it is a building block for amino acids, proteins, and DNA. Although N can limit primary producers in freshwater ecosystems, excessive nitrogen is potentially toxic to ecosystem health and living organisms. In this thesis, ecosystem health means the quality of water for both people and aquatic life. The increase in N-rich fertilizer use, wastewater discharge, fossil fuel burning, and livestock waste from farming lead to an imbalance between N inputs and outputs within ecosystems (Rosswall 1981). For example, high nutrient loads (i.e., NH_4^+ and NO_3^-) in aquatic ecosystems promote the growth of primary producers. Algae and cyanobacterial blooms deplete dissolved oxygen (DO) and subsequently affect aquatic life. Consequently, N transformation is important to balance N inputs and outputs within ecosystems.

The biogeochemical N cycle is complex because N can be oxidized or reduced into various forms (i.e., NH_4^+ , NO_2^- , NO_3^-) due to valence states ranging from -3 to +5. The N cycle is composed of a combination of oxidation and reduction processes. Some processes occur in both oxic and anoxic conditions, whereas others occur strictly in either oxic or anoxic conditions (Figure 1.1). Each process is mediated by a specific group of microorganisms (Table 1.1). There are two main oxidation processes in the transformation of nitrogenous compounds: ammonia oxidation and nitrite oxidation, (Figure 1.1). Another oxidation process recently reported is anaerobic phototrophic nitrite oxidation (Schott et al., 2010). The three main reduction processes are denitrification, N_2 fixation, and dissimilatory nitrite reduction to ammonium (DNRA; Figure 1.1). The other two processes, combining the two main reactions, involved in the N cycle are anaerobic ammonia oxidation (anammox) and methane denitrification. Anammox involves anaerobic ammonia oxidation coupled with nitrite reduction. Denitrification can involve anaerobic methane oxidation coupled with nitrite reduction; this process was discovered in a continuous stirred tank reactor, inoculated with sludge

from the anaerobic treatment process (Islas-Lima et al., 2004). Other processes involved the transformation of organic N are mineralization of N-containing organic matter and assimilation of ammonium. Nitrite and nitrate assimilation can be performed by *Bacillus subtilis* (Ogawa et al., 1995) and several species of algae (Grant and Turner 1969). Amino acid assimilation also exists; this process is carried out by *Cyanobacteria* (Michelou et al., 2007). However, this thesis focuses mainly on aerobic ammonia oxidation and anammox processes.

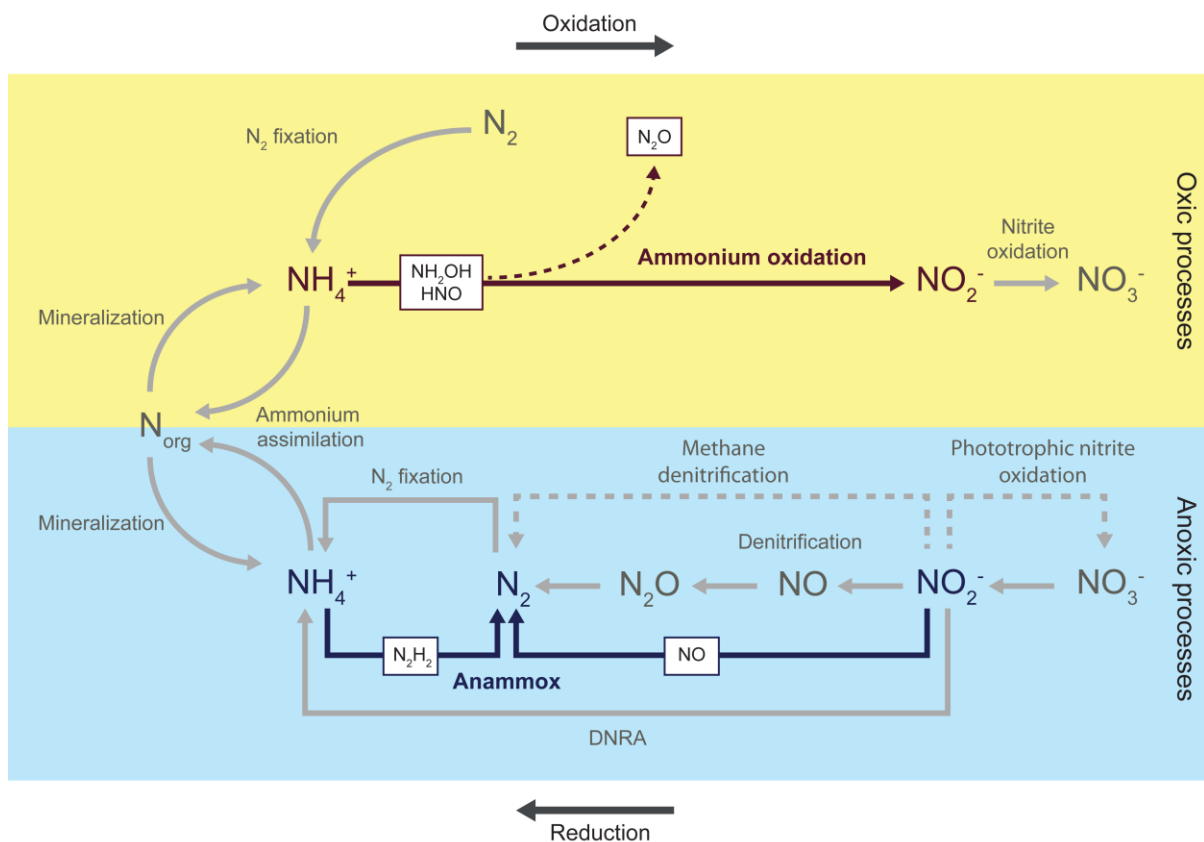


Figure 1.1 Schematic diagram of the N cycle (modified from Thamdrup 2012). Intermediates are shown in boxes. Gray dashed lines represent newly discovered N transformation processes: methane-based denitrification (Islas-Lima et al., 2004) and phototrophic nitrite oxidation (Schott et al., 2010).

Table 1.1 Example microorganisms involved in the N cycle.

Processes	Example microorganisms
ammonia oxidation	<i>Nitrosomonas</i> , <i>Nitrosopumilus</i>
nitrite oxidation	<i>Nitrobactor</i>
mineralization	<i>Bacillus</i> , <i>Clostridium</i> , many others
anaerobic phototrophic nitrite oxidation	<i>Thiocapsa</i> , <i>Rhodopseudomonas</i>
denitrification	<i>Sulfurimonas denitrificans</i>
N ₂ fixation	<i>Cyanobacteria</i>
ammonium assimilation	<i>Rhizobium</i> spp., many others
DNRA	<i>Fusarium oxysporum</i>
anammox	<i>Candidatus Brocadia</i>
methane-based denitrification	<i>Candidatus Methylospirillum oxyfera</i>

1.1.3 Microorganisms involved in aerobic ammonia oxidation

Ammonia oxidation was believed to be mediated solely by a group of chemolithoautotrophic ammonia-oxidizing bacteria (AOB). In 2004, it was first revealed that autotrophic ammonia oxidation was possibly not restricted to the domain *Bacteria*, but could also include members of the domain *Archaea* (Venter et al., 2004). Molecular evidence demonstrated the presence of ammonia monooxygenase (*amoA*) genes on an archaeal scaffold and indicated a potential role for archaea in marine nitrification. Subsequently, Treusch et al. (2005) discovered genes that potentially encoded *amo* genes from a soil sample metagenomic library. Confirmation of ammonia-oxidizing archaea (AOA) activity was achieved by the first cultivation and isolation of an autotrophic ammonia-oxidizing marine archaeon named *Nitrosopumilus maritimus* (Könneke et al., 2005). Like AOB, AOA grow chemolithoautotrophically by oxidizing ammonia to nitrite under typically mesophilic conditions. In addition, both of these groups contain putative genes for all three subunits (*amoA*, *amoB*, and *amoC*) of the ammonia monooxygenase (AMO); *amoA* genes are widely used as functional marker genes for both organisms.

Metagenomes of *N. maritimus*, *Nitrososphaera viennensis*, *Candidatus Cenarchaeum symbiosum*, *Candidatus Nitrosoarchaeum limnia*, *Candidatus Nitrosocaldus yellowstonii*, *Candidatus Nitrosoarchaeum koreensis*, and *Candidatus Nitrososphaera gargensis* revealed that the arrangement

of genes coding for the archaeal AMO is different from that of bacterial AMO (as reviewed by Bartossek et al., 2012 and Spang et al., 2012). The orientation of bacterial AMO subunit genes is *amoB*, *amoA*, and *amoC*, respectively, whereas an archaeal AMO arrangement differs across known representatives (Figure 1.2). Archaeal *amoA* is consistently associated with a gene for a hypothetical protein (*amoX*), and an additional open reading frame (ORF) is found between archaeal *amo* subunits (i.e., *Ca. C. symbiosum* and *N. viennensis*).

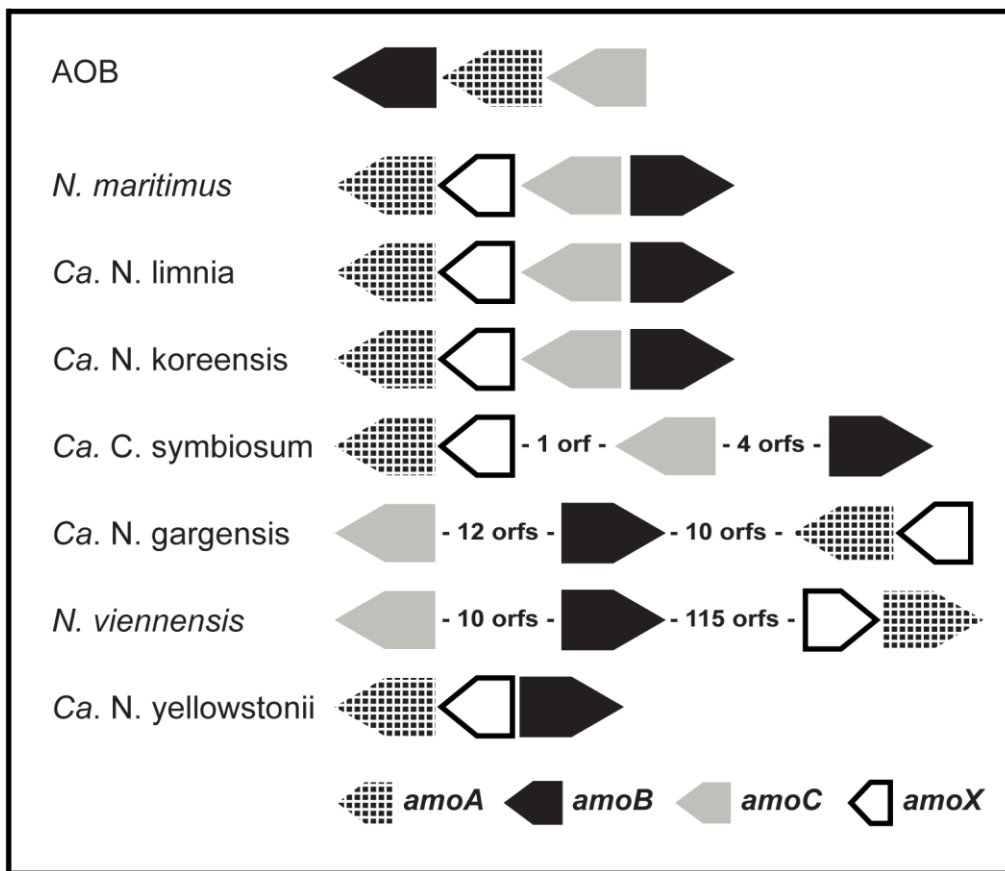


Figure 1.2 Orientations of bacterial and archaeal ammonia monooxygenase subunit genes (modified from Bartossek et al., 2012 and Spang et al., 2012). AOA = *N. maritimus*, *Ca. N. limnia*, *Ca. N. koreensis*, *Ca. C. symbiosum*, *Ca. N. gargensis*, *N. viennensis*, *Ca. N. yellowstonii*. Open reading frame (orf) is a nucleotide region that contains no termination codons and is potentially translated to a protein sequence.

The ammonia oxidation pathway of AOB differs from that of AOA (as reviewed by Hatzenpichler 2012). AOB oxidize ammonia to hydroxylamine (NH_2OH) by the bacterial AMO, and hydroxylamine is subsequently oxidized to nitrite by a hydroxylamine oxidoreductase (HAO). Four electrons are released from the latter process; two electrons are transferred to an electron transport chain while the other two are delivered back to the bacterial AMO to activate the enzyme (Arp et al., 2002; Klotz and Stein 2008). However, the HAO has not been identified in archaeal genomes so far (Hallam et al., 2006; Walker et al., 2010; Blainey et al., 2011; Kim et al., 2011; Spang et al., 2012). Consequently, AOA might have either other unique intermediates or a distinct enzyme system for oxidizing hydroxylamine to nitrite (Walker et al., 2010). AOA may oxidize ammonia to nitroxyl (HNO) by the archaeal AMO, with nitroxyl subsequently oxidized to nitrite by a nitroxyl oxidoreductase (NxOR). The latter process releases only two electrons, instead of the four electrons generated by AOB; these two electrons are transferred to an archaeal electron transport chain. As with the bacterial AMO, the archaeal AMO needs to be activated. AOA overcome the lack of extra electrons by recycling nitric oxide (NO) being produced from the reduction of nitrite by a nitrite reductase (*nirK*) to activate the archaeal AMO (Schleper and Nicol 2010). Another predicted archaeal ammonia oxidation pathway is that AOA generate hydroxylamine but use a different enzyme system to generate nitrite. Although the *N. maritimus* genome contains no HAO, the production of hydroxylamine as the intermediate from ammonia oxidation by *N. maritimus* was experimentally verified (Vajjala et al., 2012). *N. maritimus* likely possesses a unique enzyme system responsible for oxidizing hydroxylamine to nitrite.

Other than the differences in the ammonia oxidation pathway, AOB and AOA rely on a different electron transport system (Schleper and Nicol 2010; Walker et al., 2010). AOB possess an iron-based electron transfer (i.e., heme-rich HAO and cytochromes), whereas AOA rely on a copper-based electron transfer (i.e., multicopper oxidases and copper-containing proteins). Another difference between bacterial and archaeal AMO enzymes is substrate affinity. The half-saturation constant (K_s) for ammonia is higher for AOB. The K_s of AOB species are 30-61, 14-43, and 19-46 μM for *Nitrosomonas europaea*, *Nitrosomonas communis*, and *Nitrosomonas nitrosa*, respectively (as reviewed by Koops et al., 2006). The K_s of AOA species are 0.13 and 0.69 μM for *N. maritimus* (Marten-Habbena et al., 2009) and *Ca. Nitrosoarchaeum koreensis* (Jung et al., 2011), respectively. Although the K_s of only some AOB and AOA species have been reported, growth kinetic experiments have revealed that a thermophilic enrichment culture from a hot spring, *Ca. Nitrososphaera gargensis*, was active at both 0.14 and 0.79 mM NH_4^+ , whereas ammonium concentrations of 3.08 mM were

enough to inhibit cell growth (Hatzenpichler et al., 2008). In contrast to AOA, the most ammonium-sensitive AOB strain known to date is *Nitrosomoas oligotropha*; growth is inhibited by ammonium concentrations of 21.4 mM (Suwa et al., 1994). Although AOB and AOA oxidize ammonia to nitrite, their AMO enzyme structures, ammonia oxidation pathways, and enzyme substrate affinities are different. All these potentially reflect niche differentiation of AOB and AOA in environments.

Although many publications have demonstrated an abundance of AOA in both natural environments (e.g., freshwater and marine sediments, thermal springs and soils) and engineered systems (e.g., activated sludge bioreactors, aquarium filters, lab-scale drinking water treatment plant) (Francis et al., 2005; Hatzenpichler et al., 2008; Herrmann et al., 2008; Park et al., 2006; Tourna et al., 2008; Urakawa et al., 2008; Well et al., 2009; Zhang et al., 2009; Sauder et al., 2011 and 2012), the role of AOA in nitrification and potential for niche differentiation of AOA and AOB are still unclear. Erguder et al. (2009) summarized the current knowledge on the environmental factors that may influence the ecological niche of AOA, which include ammonium levels, organic carbon, temperature, salinity, oxygen levels, pH, sulfide, and phosphate. Importantly, AOA play a key role cycling N in low-ammonium environments (Erguder et al., 2009). Molecular techniques revealed that AOA were more abundant than AOB in oligotrophic environments, such as unfertilized soil (He et al., 2007; Leininger et al., 2006; Shen et al., 2008), deep ocean (Agogue et al., 2008; Mincer et al., 2007; Wuchter et al., 2006) and freshwater sediment (Herrmann et al., 2008). Soil samples were incubated with 0, 20 and 200 $\mu\text{g NH}_4^+$ per g of soil; the AOA:AOB ratios indicated that AOA outnumbered AOB in low to intermediate ammonium concentrations, whereas AOB were predominant in the highest ammonium concentrations (Verhamme et al., 2011). Stable-isotope probing (SIP) with ^{18}O -water revealed that increasing ammonium inputs in soil stimulated the growth rate of AOB, but not AOA, and the mortality rate of AOA increased with the addition of ammonium (Adair and Schwartz 2011). SIP with $^{13}\text{CO}_2$ also supported that only AOB were dominant and active in stream biofilms enriched with high ammonium concentrations (Avrahami et al., 2011).

1.1.4 Microorganisms involved in anaerobic ammonia oxidation

Aside from aerobic ammonia oxidizing processes, anaerobic ammonia oxidizing processes are driven by anaerobic ammonia-oxidizing bacteria (anammox) belonging to the phylum *Planctomycetes*. These anammox bacteria are associated with five *Candidatus* genera, including *Brocadia*, *Kuenenia*, *Scalindua*, *Anammoxoglobus*, and *Jettenia*. Anammox bacteria have the ability to transform ammonium to N_2 gas by using nitrite as an electron acceptor under anoxic conditions

without producing nitrous oxide (N₂O), a potent greenhouse gas. Anammox bacteria are slow-growing microorganisms with a doubling time of 11 – 20 days (Jetten et al., 2009). Consequently, the cultivation and isolation of anammox bacteria is difficult. Anammox bacterial cells were physically isolated by density gradient centrifugation for the first time (Strous et al., 1999b); however, there is no pure culture of anammox bacteria available so far. A molecular mechanism of the anammox process has been proposed based on genome assemblies of *Ca. Kuenenia stuttgartiensis* (Kartal et al., 2011), *Ca. Jettenia asiatica* (Hu et al., 2012), *Ca. Brocadia fulgida* (Gori et al., 2011), and *Ca. Scalindua profunda* (van de Vossenberg et al., 2013). There are three important steps to the anammox reaction. The first step is the reduction of nitrite to nitric oxide. The key enzyme for the first step is a nitrite reductase (either NirS or NirK). The NirS enzyme was identified in the genomes of *Ca. Kuenenia stuttgartiensis* (Kartal et al., 2011) and *Ca. Scalindua profunda* (van de Vossenberg et al., 2013), whereas the NirK enzyme was present in the genome of *Ca. Jettenia asiatica* (Hu et al., 2012). These findings demonstrate that nitrite reductase is not conserved across all anammox bacterial species. The second step involves combining nitric oxide and ammonium to produce hydrazine (N₂H₂) as an intermediate by a hydrazine synthase (HZS). The last step is the production of N₂ gas from hydrazine by a hydrazine oxidoreductase (HZO). The HZS and HZO were found in all four available anammox genomes (Kartal et al., 2011; Gori et al., 2011; Hu et al., 2012; van de Vossenberg et al., 2013). Nitric oxide and hydrazine were experimentally verified as the intermediates generated by *Ca. Kuenenia stuttgartiensis* enrichment (Kartal et al., 2011).

1.1.5 Global distributions of anammox bacteria

Anammox bacteria were first discovered in a pilot plant reactor (Mulder et al., 1995). Since then, anammox bacteria were subsequently investigated in natural environments, including marine (e.g., Kuypers et al., 2003; Woebken et al., 2008), estuary (e.g., Dale et al., 2009; Hou et al., 2013), freshwater (i.e., Hu et al., 2012b; Sonthiphand and Neufeld 2013), and terrestrial (e.g., Zhu et al., 2011a; Shen et al., 2013a) habitats. The contribution of anammox bacteria to the biogeochemical N loss was more than 50% in marine environments (Dalsgaard et al., 2005), 18-36% in groundwater (Moore et al., 2011), 4-37% in paddy soil (Zhu et al., 2011a), 9-13% in lake (Schubert et al., 2006), and 1-8% in estuary (Trimmer et al., 2003). This indicated that anammox bacteria play a key role in the global N cycle. Anammox bacteria can also play a role in removing fixed N from wastewater treatment plants (WWTPs). Many studies focused on the application of anammox bacteria in wastewater treatment because the anammox process is cost-effective and environmentally friendly

because of less requirement for aeration, no need for extra organic carbon, and less production of greenhouse gases (i.e., N₂O and CO₂), compared to conventional denitrification (Jetten et al., 1997; van Dongen et al., 2001). Although anammox bacteria are widespread and important in the biogeochemical N cycle, no study has summarized global anammox bacterial distributions.

Environmental factors influencing anammox bacterial distributions, abundance, and activity have been identified across habitats in various studies. Nitrite and ammonium concentrations have been found to affect anammox bacterial abundances in freshwater sediment (Sun et al., 2014) and their diversity in soil (Shen et al., 2013a), respectively. Organic carbon impacted the distribution of anammox bacteria in freshwater sediment (Hu et al., 2012b). Temperature influenced the activity of anammox bacteria in tropical freshwater (Schubert et al., 2006) and an estuary (Hou et al., 2013). Salinity correlated with anammox bacterial contributions to N loss and affected the distribution and diversity of anammox bacteria in estuaries (Dale et al., 2009; Li et al., 2011c; Hou et al., 2013). Seasonality influenced the diversity of anammox bacteria in an estuary (Li et al., 2011c). Depth affected the anammox bacterial diversity in marine sediment (Shu and Jiao 2008) and their abundance in soil (Shen et al., 2012). However, all these findings were investigated in individual studies within specific habitats. In this thesis, I explored distributions of environmental anammox bacteria and identified key factors that impacted their global distributions (Chapter 2).

1.1.6 Molecular detection of anammox bacteria

Molecular detection using DNA-based and 16S rRNA-based methods has been the main approach to reveal the diversity, abundance, and activity of anammox bacteria. Due to the biased coverage and specificity of the primers targeting anammox 16S rRNA genes, sequencing results from clone libraries with anammox-specific primers are problematic; only 40% of the amplified sequences affiliate with anammox clades (Schmid et al., 2000). Fluorescence *in situ* hybridization (FISH) probes were first used to quantify anammox cells in bioreactors and WWTPs whose samples contained dominant anammox strains affiliated with *Ca. Brocadia* and *Ca. Kuenenia* (Helmer-Madhok et al., 2002; Schmid et al., 2000). Other than artificial environments, many researchers have examined the diversity and composition of anammox bacteria in marine environments using 16S rRNA gene based techniques. The results demonstrated that anammox bacterial diversity was very low in most samples and most retrieved sequences were related to the *Ca. Scalindua* lineage (Dong et al., 2009; Kirkpatrick et al., 2006; Penton et al., 2006; Schmid et al., 2003; Woebken et al., 2008). Based on the analysis of environmental samples, the implication is that specific primer sets or probes must be

designed for specific ecosystems. No primer sets targeting freshwater anammox bacteria were available; the widely used anammox primers were designed to amplify anammox from WWTPs and anaerobic reactors. In the past decade, several studies focused on the abundance of anammox bacteria using qPCR methods based on 16S rRNA genes (Dale et al., 2009; Lam et al., 2007; Tsushima et al., 2007) or a functional gene such as *hzs*, the gene encoding hydrazine oxidoreductase (Junier et al., 2010; Li et al., 2010b; Schmid et al., 2008). Primers targeting all five known anammox genera are still needed. In Chapter 3 of this thesis, specific anammox bacterial primer sets were tested for multiple molecular approaches, including denaturing gradient gel electrophoresis (DGGE), clone library analysis, and quantitative real-time PCR (qPCR).

1.1.7 Ammonia oxidation in the Grand River

The Grand River watershed is the largest catchment in Southwestern Ontario and is a major source of drinking water for more than 500,000 people. However, there are 29 WWTPs within the watershed and 70% of the land is occupied by active farming activity (Dale and Emerson 2008, Statistics Canada 2012). Water quality in the Grand River is important for human health, aquatic life, and natural resources. Water quality can reflect land usage, land management practices, and urban growth. A rapidly increasing population and intensive agricultural production affect the overall health of the water bodies of the river. Both wastewater effluent discharges and agricultural runoff activities can release N in the form of ammonia (NH_3 and NH_4^+) into the Grand River; this ammonia is oxidized by microorganisms to less toxic forms (e.g. NO_3^- or N_2). In the absence of oxidation, levels of ammonia in an impacted river can exceed toxicity thresholds for aquatic organisms and drinking water standards.

Although ammonium assimilation occurs broadly, the AOB, AOA, and anammox bacteria were specifically studied in this thesis as key microorganisms involved with ammonium removal from freshwater habitats. AOB in freshwater have been investigated in both oligotrophic and eutrophic conditions. The common AOB lineage found in oligotrophic freshwater is *N. oligotropha* (Speksnijder et al., 1998; Koops and Pommerening-Röser 2001). However the *N. europaea*/*Nitrosococcus mobilis* and the *Nitrosomonas marina* lineages were also detected in oligotrophic freshwater (Speksnijder et al., 1998; Bollmann and Laanbroek 2001). In eutrophic freshwater environments, *N. europaea*, *Nitrosomonas eutropha*, and *N. nitrosa* lineages were present (Koops and Pommerening-Röser 2001). A molecular survey of AOB in the wastewater-impacted Seine River revealed that wastewater AOB existed in the downstream river, and their growth might

be favored by ammonia pollution in the river (Cébron et al., 2004). Since AOA were first isolated and an ability to oxidize ammonia was confirmed in 2005 (Könneke et al., 2005), molecular surveys have been conducted to reveal the co-occurrence of both AOB and AOA in freshwater environments. The abundance and diversity of AOB and AOA in the surface sediments of a eutrophic lake, Lake Taihu, showed that AOA outnumbered AOB in most sampling sites, and AOA retrieved from this study were associated with soil (I.1b) and marine (I.1a) lineages, whereas AOB fell into the *Nitrosomonas* lineage (Wu et al., 2010). The dominance of AOA in the sediments of Lake Taihu was confirmed by the comparison of bacterial and archaeal *amoA* gene copy numbers, and the resulting diversity showed that AOB and AOA diversities correlated with depth (Zhao et al., 2013). In contrast to the higher abundance of AOA in eutrophic freshwater sediments, the ratio of AOA to AOB in water samples from the Dongjiang River in Hong Kong was the highest in the sampling site with the lowest ammonium concentration (Liu et al., 2011). A cultivation-based approach was conducted to compare the growth rates of AOB and AOA being enriched from freshwater (French et al., 2012). Three strains of AOA from freshwater sediment were enriched and showed 81%, 85%, and 91% purity. One of them was highly related to *Ca. Nitrosoarchaeum koreensis*, while the other two strains had potential to represent a novel AOA genus; AOB were affiliated with *N. oligotropha* lineage (French et al., 2012). The results demonstrated that ammonium concentrations enhanced the growth rates of AOB, whereas increasing ammonium concentrations decreased the growth rates of AOA.

Although many studies focused on the occurrence of anammox bacteria in marine sediments (Dalsgaard and Thamdrup 2002; Penton et al., 2006; Schmid et al., 2007; Tal et al., 2005) and marine anoxic water columns (Dalsgaard et al., 2003; Kuypers et al., 2003 and 2005; Lam et al., 2007), anammox bacteria were first reported in Lake Tanganyika and their activity was confirmed by ¹⁵N measurements (Schubert et al., 2006). Since then the diversity and abundance of anammox bacteria were explored in freshwater sediments from the Xinyi River in China (Zhang et al., 2007), Lake Rassnitzer in Germany (Hamersley et al., 2009), Lake Kitaura in Japan (Yoshinaga et al., 2011), the Qiantang River in China (Hu et al., 2012b), Lake Taihu in China (Wu et al., 2012), and the Dongjiang River in Hong Kong (Sun et al., 2014). Because not many studies have investigated anammox in river or lake habitats, little is known about the diversity, abundance, activity, and specific-niche of these organisms in freshwater environments.

The interaction and co-existence of ammonia-oxidizing prokaryotes (AOP; AOB, AOA and anammox bacteria) have been reported in the Black Sea (Lam et al., 2007), Peruvian oxygen minimum zone (OMZ; Lam et al. 2009), and mangrove sediments (Li et al., 2010a). It is proposed

that AOB and AOA could provide NO_2^- by aerobic ammonia oxidation; anammox can use this intermediate to form a nitrification-anammox coupling process (Lam et al., 2007 and 2009; Meyer et al., 2005). However, little is known about the relative contributions of each microorganism in N cycling; molecular approaches must be developed to reveal the activities of both aerobic and anaerobic ammonia oxidizers. No study has yet investigated the occurrence, abundance, and activities of these three microbial groups in water columns and river sediments, particularly those impacted by treated wastewater effluent exposed to high, moderate, and low ammonia inputs. Consequently, the Grand River is an ideal model for studying the effects of ammonia concentration on AOB, AOA, and anammox bacteria in a natural environment. Moreover, oxygen gradients within sediments are useful for investigating both aerobic and anaerobic ammonia-oxidizing communities. That said, AOB and AOA can grow and perform aerobic ammonia oxidation on sediment surfaces and generate nitrite for anammox bacteria deeper within the sediment. I hypothesize that the Grand River relies on ammonia-oxidizing prokaryotes (AOP) to complete ammonia oxidation in the river and they may contribute to N removal depending on their specific-niche partitioning. I also predict that these aerobic and anaerobic ammonia oxidation processes are separated spatially and temporally within the Grand River to represent coupled nitrification-anammox processes (Chapter 4).

1.1.8 Differential nitrification inhibitors

As previously described, the bacterial and archaeal *amoA* genes are divergent (Walker et al., 2010). Differential nitrification inhibitors can be used to differentiate the relative contributions of AOB and AOA to ammonia oxidation because mechanisms, inhibition thresholds, and responses of AOB and AOA to nitrification inhibitors are different. Nitrification inhibitors were used to determine the relative contributions of AOB and AOA in different soil types (Di et al., 2009; O'Callaghan et al., 2010; Taylor et al., 2010; Kleineidam et al., 2011; Di and Cameron 2011; Lehtovirta-Morley et al., 2013). Dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP) inhibit nitrification and are used frequently to prevent NO_3^- leaching to groundwater and drinking water (Di et al., 2009; Kleineidam et al., 2011). DCD deactivates the bacterial AMO enzyme by binding to the active site (Amberger 1989). However, there is no report on the mechanism of DCD on archaeal AMO enzyme activity. *Nitrosospira multiformis* was much more sensitive to DCD than *Ca. Nitrososphaera viennensis*, which means DCD at low concentration likely inhibits only AOB (Shen et al., 2013b). It has been reported that DCD decreased AOB abundance, but slightly affected AOA abundance in grassland soils (Di et al., 2009). DMPP likely affected only the first step of nitrification (Li et al.,

2008), but the mode of action of DMPP remains unclear. DMPP evidently inhibits AOB, but not AOA, in the rhizosphere and bulk soil (Kleineidam et al., 2011). Sulfadiazine (SDZ) is another inhibitor that has been used to examine the activity of AOB and AOA in soils. SDZ is used in veterinary medicine (Burkhardt et al., 2005) and it impacts the folic acid pathway of bacteria (Brown 1962). AOB were more susceptible to SDZ than AOA in pig manure (Ollivier et al., 2010) and agricultural soils (Schauss et al., 2009), perhaps because of differing permeability of SDZ in bacterial and archaeal cell envelopes (Schauss et al., 2009). Due to limited information about AOA genomes, an effect of SDZ on archaeal folic acid metabolism cannot be ruled out. Allylthiourea (ATU) is commonly used to inhibit nitrification in environmental samples (Lam et al., 2009; Taylor et al., 2010; Santoro and Casciotti 2011; Oishi et al., 2012). By chelating copper from the AMO active site (Bédard and Knowles 1989), the first step of nitrification is inhibited. AOB and AOA respond differently to ATU due to differences in inhibition thresholds and amino acids in the active site of the AMO enzyme (Lehtovirta-Morley et al., 2013; Shen et al., 2013b). AOB appear to be more sensitive to ATU than AOA in soil (Taylor et al., 2010) and marine (Santoro and Casciotti 2011) environments. *Ca. Nitrosoarchaeum koreensis* (AOA enrichment from agricultural soil) was not inhibited by ATU, whereas ATU at low concentrations inhibited *N. europaea* (Jung et al., 2011). At the same concentration, *N. multiformis* was more sensitive to ATU than *N. viennensis* (Shen et al., 2013b). PTIO (2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide) inhibits ammonia oxidation by acting as a nitric oxide (NO) scavenger because NO is a likely intermediate of AOA ammonia-oxidation, but not in AOB ammonia oxidation (Walker et al. 2010). PTIO can effectively inhibit *N. viennensis* without affecting *N. multiformis* (Shen et al., 2013b). Except for PTIO, AOB are more sensitive than AOA to most known nitrification inhibitors. In this thesis, differential inhibitors were used to distinguish the activity of AOB and AOA in sediment and water samples collected from upstream and downstream of wastewater effluent (Chapter 5).

1.1.9 Microbial biogeography of the Grand River

Other biogeochemical processes in addition to the nitrogen cycle (e.g., mineralization of organic matter) are important in controlling water quality in impacted freshwater environments. These biogeochemical processes are mediated by microorganisms and understanding bacterial diversity and function is important for ensuring sustainable water management. Most freshwater bacterial taxa are difficult to culture, so information on their physiology, function, and ecology are still insufficient. Culture-independent methods are important for investigating freshwater bacterial biogeography.

DNA-based approaches have generally been used to reveal the bacterial community composition in freshwater (i.e., Zwart et al., 2002; Ibekwe et al., 2012; Winter et al., 2007). Five bacterial taxa commonly found in freshwater are *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Verrucomicrobia* (Zwart et al., 2002; Eiler and Bertilsson 2004). However, it is unclear whether specific bacterial community members are cosmopolitan or distinctly distributed across freshwater habitats. Some studies reported a “typical” bacterial community in freshwater environments (Glöckner et al., 2000; Zwart et al., 2002; Lindström et al., 2005). Other studies suggest that bacterial community composition among lakes was different (Lindström, et al., 2000; Yannarell and Triplett 2005). Recently, it has been reported that *Betaproteobacteria* were dominant in Scandinavian freshwater lakes, whereas they were less abundant in Antarctic lakes (Logares et al., 2013). More investigations of freshwater bacterial taxa from various climatic zones would help understand their distribution and composition. Another argument is that water chemistry (i.e., dissolved organic carbon) or physical conditions (i.e., water flow, dam construction, water retention time) correlate better to freshwater bacterial distributions. It has been reported that pH is an important factor impacting freshwater bacterial communities (Stepanauskas et al., 2003; Lindström et al., 2005; Yannarell and Triplett 2005). Temperature was also found to be another key factor influencing the composition of freshwater bacteria (i.e., Jardillier et al., 2004; Lindström et al., 2005). The bacterial composition within a humic lake shifted according to the DOC gradient (Eiler et al., 2003). Other than water chemistry, hydrological retention time affected bacterial communities in lakes (Lindström et al., 2005 and 2006; Stepanauskas et al., 2003). Lakes with short retention times were characterized by allochthonous bacterial from adjacent drainages, but communities in lakes with high retention time had distinct communities that developed in the lakes (Lindström et al., 2005 and 2006). In this thesis, a comprehensive survey of the microbial community within the entire Grand River watershed was conducted by combining next-generation sequencing and bioinformatics (Chapter 6). Large sequence datasets reveal taxonomic compositions of Bacteria within the Grand River, from phylum to genus levels. The higher taxonomic resolution helps identify both major and minor bacterial assemblages, including unique lineages that can be associated with specific conditions within a sampled river. Key factors affecting the distributions and composition of river bacteria were determined by multivariate statistical analyses.

1.2 Thesis summary

Five linked projects are contained within this thesis, represented by Chapters 2 to 6. My conclusions and several future considerations are included in Chapter 7. Chapter 2 provides a comprehensive overview of global distributions and diversity of anammox bacteria and a better understanding of the key factors influencing their biogeography. Over 6,000 anammox 16S rRNA gene sequences from a public database were analyzed. Data ordinations indicated that salinity was an important factor governing anammox bacterial distribution, with distinct populations inhabiting natural and engineered ecosystems. Gene phylogenies and rarefaction analyses demonstrated that freshwater environments and the marine water column harbored the highest and the lowest diversity of anammox bacteria, respectively. Co-occurrence network analysis indicated that *Ca. Scalindua* strongly connected with other *Ca. Scalindua* taxa, whereas *Ca. Brocadia* co-occurred with both known and unknown anammox clusters.

Understanding the diversity and abundance of anammox bacteria requires reliable molecular tools that were not well established at the start of this project. Thus, an initial stage of this work involved developing these tools. Chapter 3 shows validations of PCR primers for the detection of anammox bacteria within freshwater ecosystems. The objectives of this study were to identify a suitable anammox-DGGE fingerprint method by using GC-clamp modifications to existing primers, and to verify the specificity of anammox-specific primers used for these DGGE, cloning, and qPCR methods. The primer set A438f/A684r was highly specific to anammox bacteria, provided reliable DGGE fingerprints and generated a high proportion of anammox-related clones. A second primer set (Amx368f/Amx820r) was anammox specific, based on clone library analysis, but PCR products from different candidate species of anammox bacteria resolved poorly using DGGE analysis. Together, the results demonstrate that although Amx368f/Amx820r was useful for anammox-specific qPCR and clone library analysis, A438f/A684r was the most suitable primer set for multiple molecular assessments of anammox bacteria in the freshwater environments.

Chapter 4 contains the observations of freshwater N cycling within the impacted Grand River, focusing on sediment and water columns, both inside and outside a WWTP plume. The diversity, relative abundance (DNA) and activity (rRNA) of AOB, AOA, and anammox bacteria were investigated by DGGE, qPCR, and RT-qPCR, targeting both 16S rRNA and functional genes. The analysis of bacterial 16S rRNA fingerprints showed that the WWTP effluent strongly affected autochthonous river microbial communities, but not sediment patterns. Thus, WWTP effluent has the

potential to influence other measurements of autochthonous river microbial communities, including AOB, AOA, and anammox bacteria. Careful interpretation of the resulting abundant and active ammonia-oxidizing communities revealed that both anammox bacteria and AOB were abundant and active in sediment, reflecting a potential nitrification-anammox coupled process independent from effluent input. Unlike sediment, only AOB dominated in water columns along the river, although water column AOB abundance and patterns were difficult to discern from allochthonous effluent inputs. AOA were less dominant compared to anammox bacteria and AOB in both water and sediment samples, but were more diverse, based on DGGE fingerprint profiles, and the profiles were completely distinct from effluent patterns.

Follow-up experiments were conducted to reveal the relative contributions of AOB and AOA to ammonia oxidation in the impacted Grand River. Assessment of urea hydrolysis was also conducted in parallel with ammonia oxidation experiments to test for an alternative NH_4^+ source for AOB and AOA. Chapter 5 includes study of both nitrification and urea hydrolysis in sediment and water column samples in the Grand River. Nitrification inhibitors (i.e., ATU and PTIO) helped identify the relative contributions of AOA and AOB to ammonia oxidation. Despite the presence of AOA, the results implicated AOB as dominant contributors to ammonia oxidation, both directly and in association with urea hydrolysis.

Because wastewater effluent impacted in-river microbial fingerprints, Chapter 6 investigated the distribution of river microbial communities over time and space within the entire length of the Grand River during a single day, identifying factors that correlate with microbial community distributions. In September 2013, water samples were collected from 23 locations along the entire 300 km length of the Grand River, including three wastewater effluents before discharge into the river. The results demonstrated that in-river microbial biogeography was apparently influenced by increasing distance from the headwater to the mouth of the Grand River, and the impounding reservoir affected the microbial composition.

Chapter 2¹

Global distributions of anammox bacteria

2.1 Introduction

The anaerobic ammonia oxidation (anammox) process converts ammonia to N₂ gas by using nitrite as electron acceptor under anoxic conditions (van de Graaf et al., 1995). This process is important for removing fixed N from both engineered and natural systems. The anammox process can be applied to wastewater treatment, replacing conventional treatment systems. Anammox is cost effective and environmentally friendly because it does not require aeration, does not require organic carbon inputs, and reduces the production of greenhouse gases (i.e., N₂O and CO₂) compared to conventional denitrification (Jetten et al., 1997; van Dongen et al., 2001). This process was developed and implemented initially in full scale wastewater treatment plants (WWTPs) in Rotterdam, Netherlands (van Dongen et al., 2001; van der Star et al., 2007; Abma et al., 2007). Although anammox bacteria were first discovered in WWTPs and their applications have been studied worldwide, they may account for more than 50% of N loss from marine environments (Arrigo 2005; Francis et al., 2007). The contributions of anammox bacteria to the biogeochemical N cycle were 18-36% in groundwater (Moore et al., 2011), 4-37% in paddy soils (Zhu et al., 2011a), 9-13% in lakes (Schubert et al., 2006), and 1-8% in estuaries (Trimmer et al., 2003). These results indicate that anammox bacteria play a key role in the global N cycle.

Anammox bacteria branch deeply within the *Planctomycetes* phylum. There are five known anammox genera, with 16 species proposed to date. The first discovered anammox bacterium was *Ca. Brocadia anammoxidans*, enriched from a denitrifying fluidized bed reactor (Mulder et al., 1995; Kuenen and Jetten, 2001). The three characterized species within the *Ca. Brocadia* genus are *Ca. Brocadia fulgida* (Kartal et al., 2008), *Ca. Brocadia sinica* (Oshiki et al., 2011), and *Ca. Brocadia caroliniensis* (Rothrock et al., 2011); all of these were enriched in anammox bioreactors. The only species reported within the *Ca. Kuenenia* genus is *Ca. Kuenenia stuttgartiensis*, which was isolated from a trickling filter biofilm (Schmid et al., 2000). The *Ca. Scalindua* genus consists of nine

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proposed species, six of which were discovered in marine environments (Kuypers et al., 2003; Woebken et al., 2008; Hong et al., 2011a; Fuchsman et al., 2012; Dang et al., 2013; van de Vossenberg et al., 2013). *Ca. Scalindua sorokinii* was the first anammox species found in a natural environment (the Black Sea; Kuypers et al., 2003). *Ca. Scalindua richardsii* was also recovered from the Black Sea (Fuchsman et al., 2012). Although these two species originated from the Black Sea, they dominated in different zones. A cluster associated with *Ca. Scalindua sorokinii* was detected in the lower suboxic zone where ammonium concentration was high, but nitrite concentration was low, whereas a cluster associated with *Ca. Scalindua richardsii* was found in the upper suboxic zone where ammonium concentration was low, but nitrite concentration was high (Fuchsman et al., 2012). *Ca. Scalindua brodae* and *Ca. Scalindua wagneri* were both identified in WWTPs (Schmid et al., 2003). *Ca. Scalindua arabica* originated in the Arabian Sea and the Peruvian oxygen minimum zone (OMZ; Woebken et al., 2008). *Ca. Scalindua pacifica* (Dang et al., 2013) and *Ca. Scalindua profunda* (van de Vossenberg et al., 2013) were retrieved from the Bohai Sea and a marine sediment of a Swedish fjord, respectively. Two additional species names were tentatively proposed from molecular surveys: *Ca. Scalindua sinooilfield* from a high temperature petroleum reservoir (Li et al., 2010) and *Ca. Scalindua zhenghei* from marine sediments (the South China Sea; Hong et al., 2011a). The only known species affiliated with the *Ca. Anammoxoglobus* genus was *Ca. Anammoxoglobus propionicus*, being enriched from an anammox reactor (Kartal et al., 2007). *Ca. Jettenia asiatica* was retrieved from a granular sludge anammox reactor (Quan et al., 2008). Notably, known anammox bacteria species have mostly been discovered in engineered environments, but they have commonly been detected in various natural ecosystems and are more widespread than previously thought. However, it should be noted that *Ca. Scalindua sinooilfield* and *Ca. Scalindua zhenghei* are not in the category *Candidatus* on the list of prokaryotic names with standing in the nomenclature (LPSN) website. The classification and nomenclature of anammox *Ca.* species need to be better clarified and standardized in the future.

Observations of anammox bacterial diversity demonstrated that *Ca. Brocadia*, *Ca. Kuenenia*, and *Ca. Anammoxoglobus* were commonly found in non-saline environments (e.g., Egli et al., 2001; Moore et al., 2011; Hu et al., 2013), whereas *Ca. Scalindua* dominated in saline environments (e.g., Woebken et al., 2008; Hong et al., 2011a; Villanueva et al., 2014). However, because all previous molecular surveys of the anammox 16S rRNA genes were from individual studies of specific habitats, an overall understanding of global anammox bacterial diversities, distributions, and co-occurrences among lineages remains unclear.

Factors affecting anammox bacterial diversity and distribution have been investigated across individual habitat-specific studies. For example, organic carbon influenced anammox diversity in freshwater sediment (Hu et al., 2012b), soil (Shen et al., 2013a), and an estuary (Hou et al., 2013). Ammonium and nitrite concentrations correlated with anammox diversity in mangrove sediment (Li et al., 2011a). Temperature impacted anammox communities in freshwater sediment (Osaka et al., 2012) and an estuary (Hou et al., 2013). Depth affected anammox diversity in marine sediment (Li et al., 2013). However, no comprehensive study has explored factors that govern global anammox distributions thus far.

The main objectives of this study were to investigate global anammox bacterial distributions and identify factors influencing anammox bacterial distributions and diversity. Over 6,000 anammox 16S rRNA gene sequences from Genbank were collected and analyzed by phylogenetics and multivariate statistics. An anammox-based 16S rRNA-based phylogenetic tree revealed anammox distributions across habitats, including marine sediment, marine water column, estuary, mangrove sediment, soil, freshwater, freshwater sediment, groundwater, reactor, WWTP, marine sponge, biofilter, fish gut, shrimp pond, and oil field. Co-occurrence analysis demonstrated strong relationships among dominant anammox phylotypes. Global distributions of anammox bacteria revealed factors that influence anammox bacterial distributions, with salinity being the most important environmental variable. This study provides a better understanding of the prevalence of anammox bacterial 16S rRNA genes across habitats and the key factors impacting their distribution patterns.

2.2 Materials and Methods

2.2.1 Data collection and preparation

All anammox 16S rRNA gene sequences available in Genbank were extracted on October 25th, 2013. In total, 14,790 potential anammox-related sequences were collected using the following keyword searches: “*uncultured planctomycete 16S ribosomal RNA gene*”, “*anammox bacterium 16S ribosomal RNA gene*”, “*anaerobic ammonium-oxidizing bacterium 16S ribosomal RNA gene*”, “*Candidatus Brocadia 16S ribosomal RNA gene*”, “*Candidatus Scalindua 16S ribosomal RNA gene*”, “*Candidatus Kuenenia 16S ribosomal RNA gene*”, “*Candidatus Anammoxoglobus 16S ribosomal RNA gene*”, and “*Candidatus Jettenia 16S ribosomal RNA gene*”. Most anammox bacterial 16S rRNA gene sequences were deposited in the Genbank with the definition “*uncultured planctomycete 16S*

ribosomal RNA gene” (data not shown). However, this keyword-based search retrieved both anammox and non-anammox sequences. All collected sequences were searched by BLAST against known anammox species in Genbank database and aligned by QIIME v1.7 (Caporaso et al., 2010) using Infernal (Nawrocki and Eddy 2013) against the Greengenes database (May 2013 revision; DeSantis et al., 2006) to screen for anammox-related sequences. After removing non-anammox, low quality, and insufficient length sequences, over 6,000 sequences from >200 isolation sources were included in the analysis. All anammox sequences from across many specific “Isolation source” Genbank designations were assigned to 15 general habitats: marine sediment, marine water column, estuary, freshwater sediment, freshwater, groundwater, soil, mangrove sediment, wastewater treatment plant (WWTP), reactor, marine sponge, biofilter, fish gut, oil field, and shrimp pond. These 15 habitat keywords were included in most of isolation sources indicated in Genbank, so these key words helped for habitat assignment. For example, “Bohai sea sediments”, “South China Sea sediment”, and “surface sediment of equatorial Pacific” were categorized into “marine sediment”. “paddy soil”, “garden soil”, and “agricultural soil” were categorized into “soil”. I also categorized anammox sequences from wetlands into “soil”. For those Genbank isolation sources with no 15 habitat keywords indicated, I double checked with their original publications to see where they were from. For example, “marine OMZ”, “Namibian upwelling system”, and “eastern south Pacific” were from “marine water column”.

Limitations of this analysis included metadata inconsistencies and missing environmental parameters across multiple studies. Consequently, metadata were qualitatively grouped into three broad categories: salinity (saline, mixed, and non-saline environments), ecosystem (natural and engineered) and habitat (listed above). Another limitation was that it was not possible to consistently determine relative abundances of anammox sequences within each study due to inconsistencies with reporting, sampling efforts, and methodologies. To address this shortcoming, all anammox 16S rRNA gene sequences were clustered into operational taxonomic units (OTUs) at 97% identity with cd-hit-est v4.5.4 (Fu et al., 2012) and the abundance of each anammox OTU was only counted as present or absent for each study.

2.2.2 Statistical and multivariate analyses

Individual studies that contributed anammox 16S rRNA gene sequences were usually associated with unique Genbank isolation sources. Because of this, the numbers of anammox 16S rRNA gene sequences contributed per study and/or unique isolation source were broad, ranging from

1-623 sequences. In order to ensure that dissimilarity matrices were generated from datasets derived from the same number of sequences from each study, multiple rarefied datasets were generated that varied in the number of sequences derived from each study/isolation source. In cases where multiple studies represented compatible isolation sources, yet with relatively low numbers of sequences, these sequence data were pooled whenever possible into additional isolation source categories to maximize habitat representation in the rarefied analyses. Subsequently, I tested datasets rarefied to 10, 40, or 100 sequences from each isolation source category.

After clustering the sequences at 97% identity, all sequences were aligned and trimmed in order to consider a single homologous spanning region of the 16S rRNA gene. Any sequences with less than 100 bases after trimming were discarded from the analysis. Consequently, the sequences from some isolation sources within five minor habitats (marine sponge, biofilter, fish gut, oil field, and shrimp pond) were below the threshold for rarefied datasets of 10 and 40 sequences. These five minor habitats were removed. The minimum sequence threshold remained at 10, 40, and 100 after being trimmed. These five minor habitats were also excluded from the rarefied dataset of 100 sequences because the numbers of sequences were below the threshold. Consequently, 10 major habitats (marine sediment, marine water column, estuary, freshwater sediment, freshwater, groundwater, soil, mangrove sediment, WWTP, reactor) were considered in this analysis.

Principal coordinates analysis (PCoA) ordinations were generated from unweighted UniFrac distance matrices (Lozupone and Knight 2005) through QIIME (Caporaso et al., 2010). Non-metric multidimensional scaling (NMDS) ordinations were calculated based on a Jaccard dissimilarity matrix, using the AXIOME pipeline (Lynch et al., 2013). To test treatment effects and within group agreement, multi-response permutation procedures (MRPP) were tested on 999 permutations, using the R library *vegan* (Oksanen et al., 2008) from within AXIOME.

2.2.3 Rarefaction curve and diversity indices

Rarefaction curves, observed species, phylogenetic diversity (PD), Chao1, and Shannon indices were generated by QIIME (Caporaso et al., 2010). Rarefaction curves and diversity indices were calculated by randomly subsampling the OTU table at the various plotted sequence depths. The random subsampling was repeated several times at each depth to generate confidence intervals. The only data actually used was the OTU table based on 97% clustering. The Wilcoxon signed-rank test was performed by the R function *wilcox.test* (R Core Team, 2013). The null hypothesis was that the number of OTUs between habitats was the same. If p was ≤ 0.05 , the null hypothesis was rejected.

2.2.4 Phylogenetic construction

Representative sequences for each OTU from each habitat were selected for phylogenetic analysis. The most abundant sequence in each cluster was chosen as the representative sequence. A total of 505 OTU sequences from across all 15 habitats included all known anammox *Candidatus* species. Outgroups included cultured non-anammox species of *Planctomycetales*, including *Planctomyces maris* (X62910), *Isophaera* sp. (X81958), *Gemmata obscuriglobus* (X85248), *Blastopirellula marina* (HE861893), *Rhodopirellula baltica* (FJ624346), and *Pirellula* sp. (X81942). Sequences were aligned using MUSCLE (Edgar 2004) and trimmed to a final homologous length of ~310 bases. A maximum likelihood tree was constructed with the PhyML v.3.0.1, using the GTR model (Guindon and Gascuel 2003). The tree topology was optimized at five random starts. The approximate likelihood ratio test (aLRT) was conducted to provide tree topology support. The phylogenetic tree was visualized by SEAVIEW (Galtier et al., 1996).

2.2.5 Co-occurrence network analysis

Anammox sequences were sorted by habitat, and an OTU table was generated by AXIOME. Co-occurrence was assessed using a previously described method (Barberán et al., 2012). All singletons were discarded, and OTUs having a Spearman's correlation ≥ 0.8 were considered to have a strong co-occurrence relationship. The result was visualized with Gephi (Bastian et al., 2009).

2.3 Results

2.3.1 Distributions of anammox bacteria across habitats

Anammox sequences were collected from multiple studies and isolation sources. The number of sequences was considerably different from one isolation source to another. Three rarefied sequence collections were generated to compare distribution patterns. Set 10 (i.e., 10 sequences per environmental source) showed poor groupings with low correlations (data not shown). Set 40 and set 100 showed similar distribution patterns with high correlations (Figure 2.2). Because the broad range of analyzed sequences (10 – 623 sequences) affected dissimilarity measurements, I chose to analyze set 40 in more detail to include as many isolation sources as possible in this analysis while maximizing sequence sample size.

All anammox sequences from 10 habitats were visualized within an ordination plot based on phylogenetic distances by using an unweighted Unifrac distance matrix (Figure 2.1). The first two

PCoA principal coordinates (PC1 and PC2) explained of the 46% variability among all samples. The ordination demonstrated that anammox sequences clustered significantly by habitat (Figure 2.1B), which was supported by MRPP ($T = -7.6$, $A = 0.14$, $p < 0.001$; Figure 2.2A). All anammox sequences clustered separately into two main groups (Figure 2.1B). Marine sediment, marine water column, estuary, and mangrove sediment grouped together and were dominated by the *Ca. Scalindua* cluster (Figures 2.1A and 2.1B). The WWTP, reactor, soil, freshwater, freshwater sediment, and groundwater grouped together and were dominated by *Ca. Brocadia*, *Ca. Jettenia*, and the unknown clusters. Three samples, one each from freshwater, soil, and WWTP, were present in both groups.

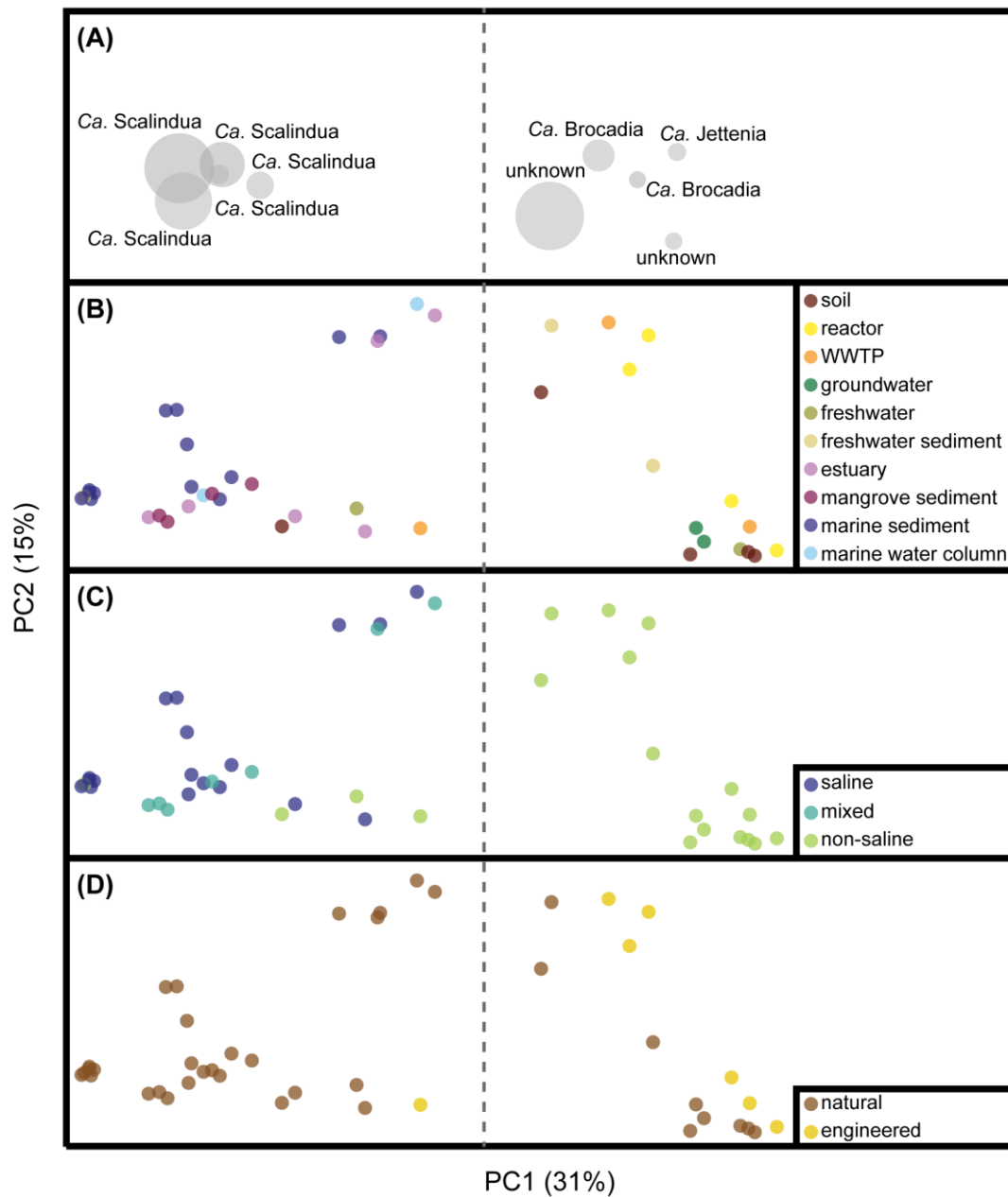


Figure 2.1 PCoA ordination based on an unweighted UniFrac distance matrix of anammox bacterial 16S rRNA gene profiles. The taxonomic biplot information for all panels is represented in Panel A. Panels B, C, and D show distributions of anammox isolation source representation (points) coloured by habitat, salinity, and ecosystem, respectively. A dotted line separates two main groups within the ordination space. The proportion of the variation explained is indicated on the axes.

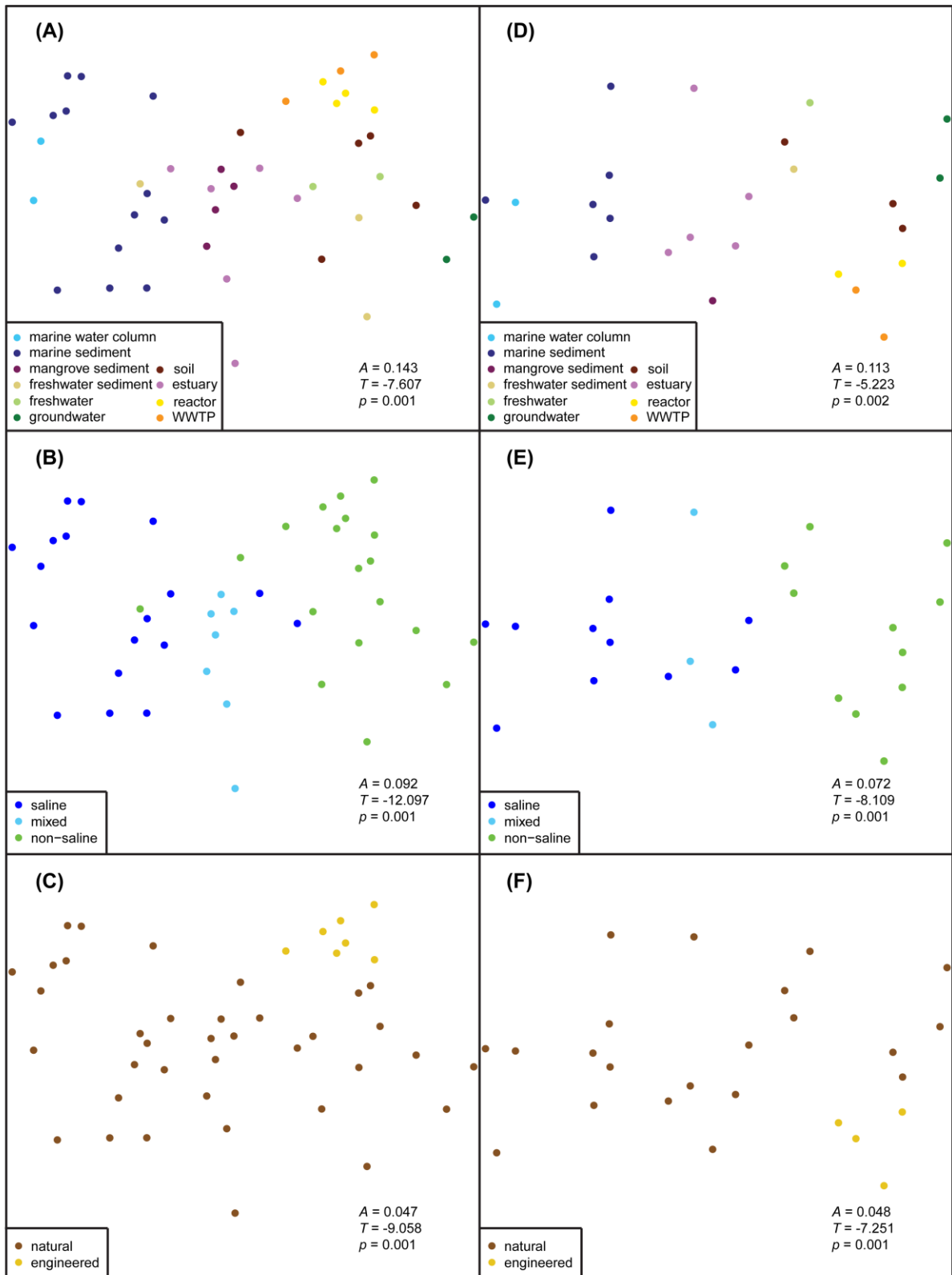


Figure 2.2 NMDS plots of anammox 16S rRNA gene sequences. The correlations of habitat, salinity, and ecosystem were calculated by a Jaccard dissimilarity matrix. The first column (A-C) shows the rarefied dataset 40 sequences; all sequences were from 44 isolation sources. The second column (D-F) shows the rarefied dataset 100 sequences; all sequences were from 25 isolation sources. The significance of group separations (A , T , and p) is indicated for each test.

2.3.2 Key factors affecting global anammox bacterial distribution

The strongest separation of anammox bacterial sequences was linked to sample salinity (Figure 2.1C), which I assigned qualitatively as saline, “mixed”, and non-saline environments. The mixed environments were generally river-marine transitional zones, mostly from mangrove and estuary habitats. Although the “salinity” category was qualitatively assigned, assigning this characteristic in this way was unlikely to affect the results. Saline and mixed environments clustered together and differed significantly from non-saline environments (Figures 2.1C and 2.2B; $T = -12.1$, $A = 0.09$, $p < 0.001$).

However, a few non-saline samples grouped with saline and mixed samples. The *Ca. Scalindua* cluster was clearly dominant in saline environments but almost never detected in non-saline environments (Figures 2.1A and 2.1C). The major complement of anammox bacteria found in non-saline environments was *Ca. Brocadia*, *Ca. Jettenia*, and the unknown clusters. Salinity was the key factor governing global distribution and diversity of anammox bacteria.

2.3.3 Distinct anammox bacteria in natural and engineered ecosystems

Another factor that showed a significant relationship with the anammox bacterial distribution was ecosystem type. Although most anammox sequences were from natural ecosystems, those from engineered ecosystems grouped together (Figures 2.1D and 2.2C; $T = -9.1$, $A = 0.05$, $p < 0.001$). However, one sample from a WWTP grouped separately from other samples of engineered ecosystems (Figure 2.1D). The phylogenetic tree was constructed by including all anammox sequences from this WWTP, all known anammox *Ca.* genera, and outgroups. The result showed that this WWTP sample contained very few anammox sequences (1/64 sequences) associated with the *Ca. Scalindua* cluster (data not shown). However, these WWTP anammox sequences were from an unpublished article and additional system details are unavailable. More robust group separation was visualized by the NMDS generated from an OTU-based Jaccard distance matrix (Figures 2.2C and

2.2F). This observation demonstrated environmental selection of anammox bacteria in natural and engineered ecosystems.

2.3.4 Diversity richness of anammox bacteria

Rarefaction curves and diversity indices showed that freshwater possessed the highest anammox bacterial diversity whereas the marine water column was associated with the lowest diversity (Figure 2.3 and Table 2.1). The richness of anammox bacteria in freshwater and marine water column differed significantly ($p = 0.01$). Rarefaction curves of freshwater showed no saturation although only 170 sequences were analyzed. The majority of freshwater anammox sequences were from unpublished data; only a few publications have reported on anammox bacterial 16S rRNA gene sequences from freshwater (Schubert et al., 2006; Hamersley et al., 2009; Pollet et al., 2011; Han and Gu 2013; Sonthiphand and Neufeld 2013). Consequently, more research on anammox bacteria in freshwater would be required to support this finding. The diversities of anammox bacteria in freshwater and freshwater sediment were not significantly different ($p = 0.22$). The results imply that novel anammox clusters remain undiscovered within freshwater habitats.

The diversity of anammox bacteria in marine sediments was higher than in the marine water columns ($p = 0.02$; Figure 2.3 and Table 2.1). The reason for this observation might be higher physical and biogeochemical heterogeneity in marine sediments, associated with a greater overall microbial diversity (Table 2.1). The richness among other isolation source samples, including freshwater sediment, estuary, mangrove sediment, soil, and marine sediment, showed no significant differences (Figure 2.3 and Table 2.1). The diversity of anammox bacteria in engineered ecosystems, including WWTPs and reactors, were not significantly different ($p = 0.15$), consistent with the observation that anammox bacteria from engineered ecosystems grouped together (Figures 2.1D, 2.2C, and 2.2F).

Although groundwater, freshwater, and freshwater sediment were non-saline isolation sources, the diversity of groundwater was low and significantly different from freshwater ($p = 0.01$) and freshwater sediment ($p = 0.01$). However, the interpretation of this observation must be cautious because only a few publications have surveyed anammox bacterial 16S rRNA gene sequences in groundwater (Moore et al., 2011; Hirsch et al., 2011; Sonthiphand and Neufeld 2013). Only 126 sequences were included in this analysis; however, 472 anammox sequences were collected from Genbank database (Table 2.1). The majority of groundwater anammox sequences were from contaminated groundwater in Canada (Moore et al., 2011), and most sequences were excluded due to

the region of analyzed 16S rRNA genes being outside of the region used to generate a phylogenetic tree, which was the basis of this analysis.

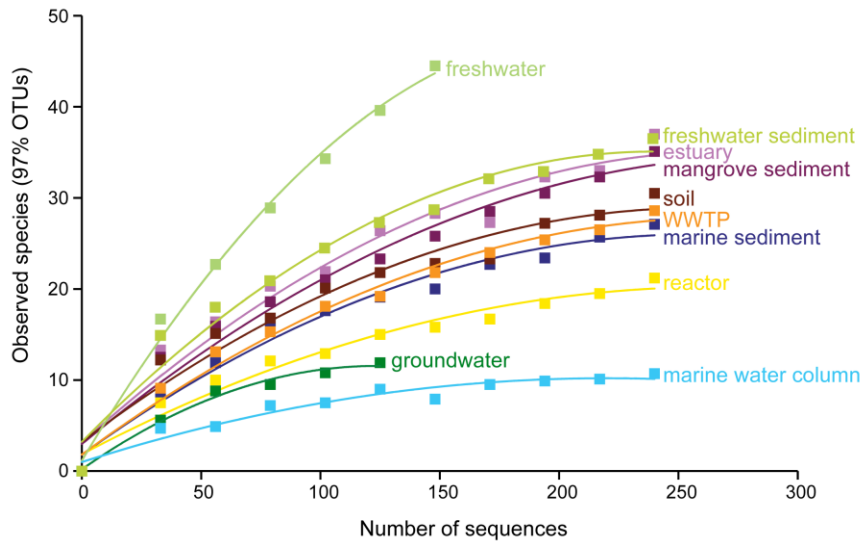


Figure 2.3 Rarefaction curves of the anammox bacterial 16S rRNA gene diversity among the 10 habitats. OTUs were generated at 97% identity.

Table 2.1 The number of anammox sequences and diversity indices of each habitat.

Habitat	Total collected sequences	Total analyzed sequences	Diversity indices		
			PD	Chao1	Shannon
marine sediment	2046	1921	1.11	35.2	2.66
marine water column	325	324	0.74	10.3	1.07
estuary	1365	1347	0.99	53.3	3.66
freshwater sediment	479	473	1.64	35.5	3.59
freshwater	170	170	2.26	103.5	3.93
groundwater	472	126	0.55	13.7	2.06
soil	816	815	0.78	28.0	3.33
mangrove sediment	366	339	1.22	42.4	3.30
WWTP	288	249	1.30	36.3	2.77
reactor	420	355	1.10	22.9	2.51

Table 2.2 The *p* values of diversity richness among 10 habitats calculated by the Wilcoxon signed-rank test.

	estuary	freshwater	freshwater sediment	groundwater	mangrove sediment	marine sediment	marine water column	reactor	soil
freshwater	0.10	-	-	-	-	-	-	-	-
freshwater sediment	0.69	0.22	-	-	-	-	-	-	-
groundwater	0.01	0.01	0.01	-	-	-	-	-	-
mangrove sediment	0.69	0.10	0.55	0.01	-	-	-	-	-
marine sediment	0.15	0.03	0.10	0.12	0.31	-	-	-	-
marine water column	0.01	0.01	0.01	0.10	0.01	0.02	-	-	-
reactor	0.02	0.01	0.02	0.22	0.03	0.31	0.03	-	-
soil	0.42	0.06	0.31	0.01	0.69	0.42	0.01	0.03	-
WWTP	0.15	0.03	0.15	0.06	0.31	0.84	0.01	0.15	0.55

2.3.5 Phylogeny and co-occurrence of anammox bacteria

The dominant anammox OTU phylotypes recovered from across all isolation sources were *Ca. Scalindua* and *Ca. Brocadia*, in addition to lower abundance anammox OTU phylotypes, including *Ca. Kuenenia*, *Ca. Anammoxoglobus*, and *Ca. Jettenia* (Figure 2.4A). The unknown cluster comprised 76 OTUs; however, the average sequences per OTU were only 1.78 sequences, reflecting that the unknown anammox clusters were likely low abundance and high diversity anammox bacteria, possibly representing part of the rare biosphere of these isolation sources.

Approximately 70% of total *Ca. Scalindua* OTU sequences were from saline-related environments, including marine sediment, marine water column, estuary, and mangrove sediment (Figure 2.4B). *Ca. Scalindua* was also detectable in soil and freshwater environments, representing 13% and 8% of all anammox OTUs from those isolation sources, respectively.

Ca. Brocadia was most commonly retrieved from non-saline environments, including freshwater sediment, freshwater, groundwater, and soil (Figure 2.4B). All freshwater-related environments and soil accounted for 38% and 24% of *Ca. Brocadia* OTU sequences, respectively. Engineered ecosystems, including WWTP and reactor, accounted for 15% of *Ca. Brocadia* OTU sequences. Although 16% of *Ca. Brocadia* OTU sequences were recovered from estuary isolation sources, only 1% of these OTUs were associated with marine sediment (Figure 2.4B). *Ca. Brocadia* was not detected in marine water column.

Ca. Kuenenia was the third most abundant cluster found across all isolation sources (Figure 2.4A). This cluster was detected across nine of the main habitats, but not the marine water column (Figure 2.4B). *Ca. Kuenenia* was also found in all five minor habitats, including marine sponge, biofilter, fish gut, shrimp pond, and oil field. Although *Ca. Kuenenia* was present in almost all habitats, a few OTUs (1-3 OTUs) per habitat were discovered. This observation indicated that *Ca. Kuenenia* cluster was not ubiquitous, but still widespread across habitats.

The *Ca. Anammoxoglobus* cluster was distributed similarly to the *Ca. Brocadia* cluster across isolation sources. For example, soil and freshwater-related environments accounted for 32% and 28% *Ca. Anammoxoglobus* OTU sequences (Figure 2.4B), respectively (compare to 24% and 38% for *Ca. Brocadia*, respectively). Estuary, WWTP, and reactor equally accounted for 14% of total *Ca. Anammoxoglobus* OTUs. OTU sequences from marine sediment or marine water column were not affiliated with the *Ca. Anammoxoglobus* cluster.

The least abundant of the known anammox bacterial genera was *Ca. Jettenia* which comprised of only eight OTUs (Figure 2.4A). Although *Ca. Jettenia* was not commonly detected within most isolation sources, the majority of this cluster was retrieved from engineered ecosystems, including WWTPs and reactors (Figure 2.4B). These engineered isolation sources accounted for 51% of all recovered *Ca. Jettenia* OTUs. Freshwater sediment, groundwater, and soil equally accounted for 13% of total *Ca. Jettenia* OTUs. None of the *Ca. Jettenia* OTUs were associated with saline-related environments (Figure 2.4B).

The distributions of anammox bacterial OTUs sequences of the unknown cluster were relatively similar to those of the *Ca. Scalindua* cluster. The majority of sequences found in this cluster was from saline-related environments, including marine sediment, marine water column, estuary, and mangrove sediment; they accounted for 57% of unknown OTU sequences (Figure 2.4B). Freshwater, freshwater sediment, soil, and WWTPs accounted for 12%, 9%, 7%, and 5% of unknown OTU sequences, respectively. As with the *Ca. Scalindua* cluster, the unknown cluster was present across nine of the main habitats, but not found in groundwater.

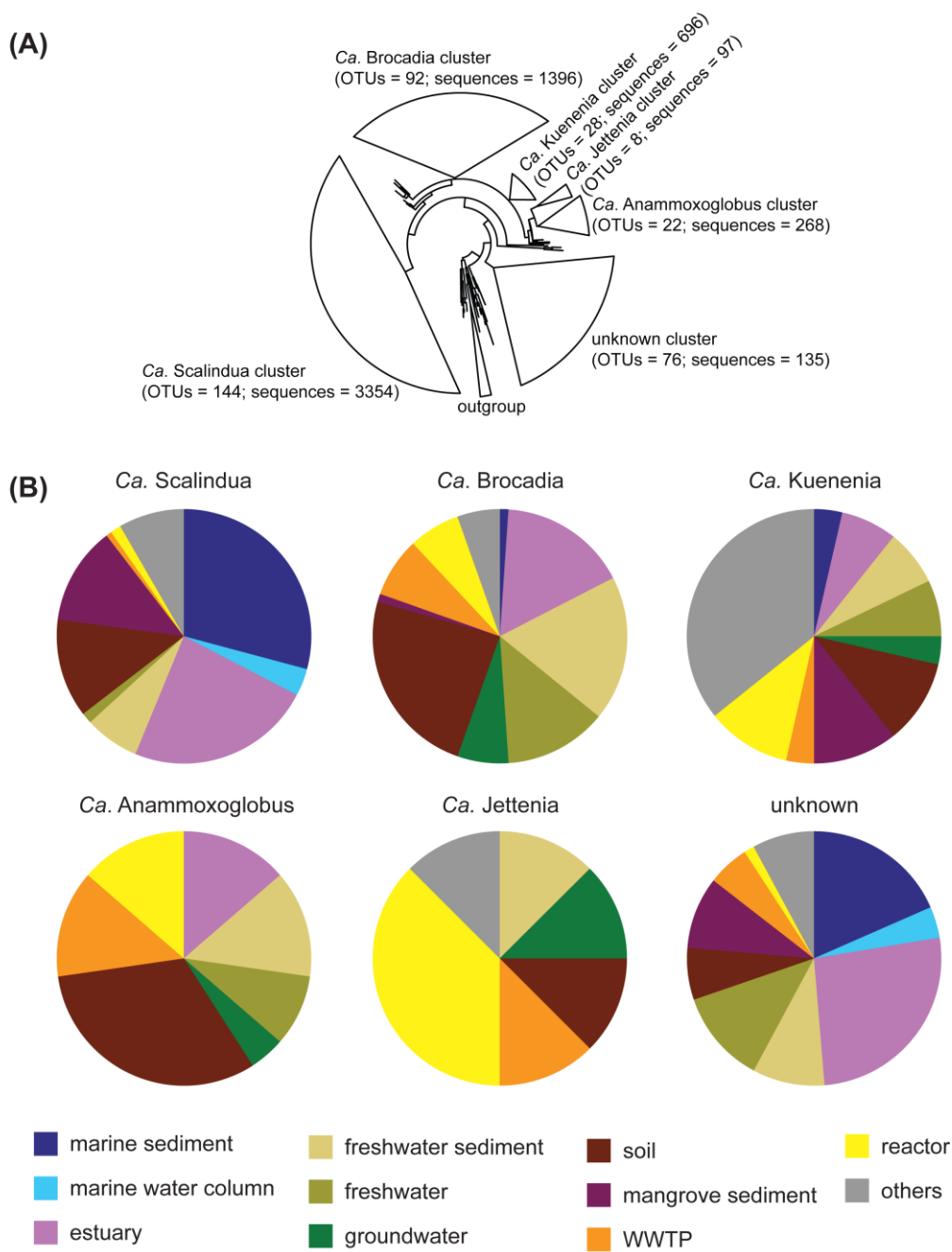


Figure 2.4 A) A 16S rRNA based-phylogenetic tree of representative anammox OTU sequences from 15 habitats. The numbers of OTUs and anammox sequences are shown in the bracket of each cluster. B) Six anammox clusters with % composition of OTU sequences according to annotated habitats. Others represent five minor habitats, including marine sponge, biofilter, fish gut, shrimp pond, and oil field.

Generating a network co-occurrence plot revealed an overview of the relationships among anammox phylotypes (Figure 2.5). The results show that *Ca. Scalindua* OTUs correlated very well with other *Ca. Scalindua* OTUs, associated additionally with several unknown clusters (Figure 2.5). In some cases, *Ca. Scalindua* was found together with *Ca. Brocadia*, *Ca. Kuenenia*, and additional unknown clusters. However, strong co-occurrences of *Ca. Scalindua* with *Ca. Anammoxoglobus* and *Ca. Jettenia* were not observed. *Ca. Brocadia* OTUs within the co-occurrence network were correlated with OTUs spanning all known and unknown anammox clusters (Figure 2.5). *Ca. Anammoxoglobus* correlated consistently with *Ca. Brocadia*, indicating a close relationship between OTUs of these two genera. Although eight OTUs of *Ca. Jettenia* were reported (Figure 2.4A), singleton OTUs were removed from this network analysis. Only one common *Ca. Jettenia* OTU formed part of a co-occurrence network (Figure 2.5). A *Ca. Jettenia* OTU correlated with a *Ca. Anammoxoglobus* OTU, and these linked to a *Ca. Brocadia* OTU. Overall, the resulting network revealed the close relationships among OTUs of *Ca. Jettenia*, *Ca. Anammoxoglobus*, and *Ca. Brocadia* clusters. The most neighboring cluster of *Ca. Kuenenia* was *Ca. Brocadia* (Figure 2.5). The co-occurrence of *Ca. Kuenenia* with *Ca. Scalindua* and one specific unknown cluster was also observed.

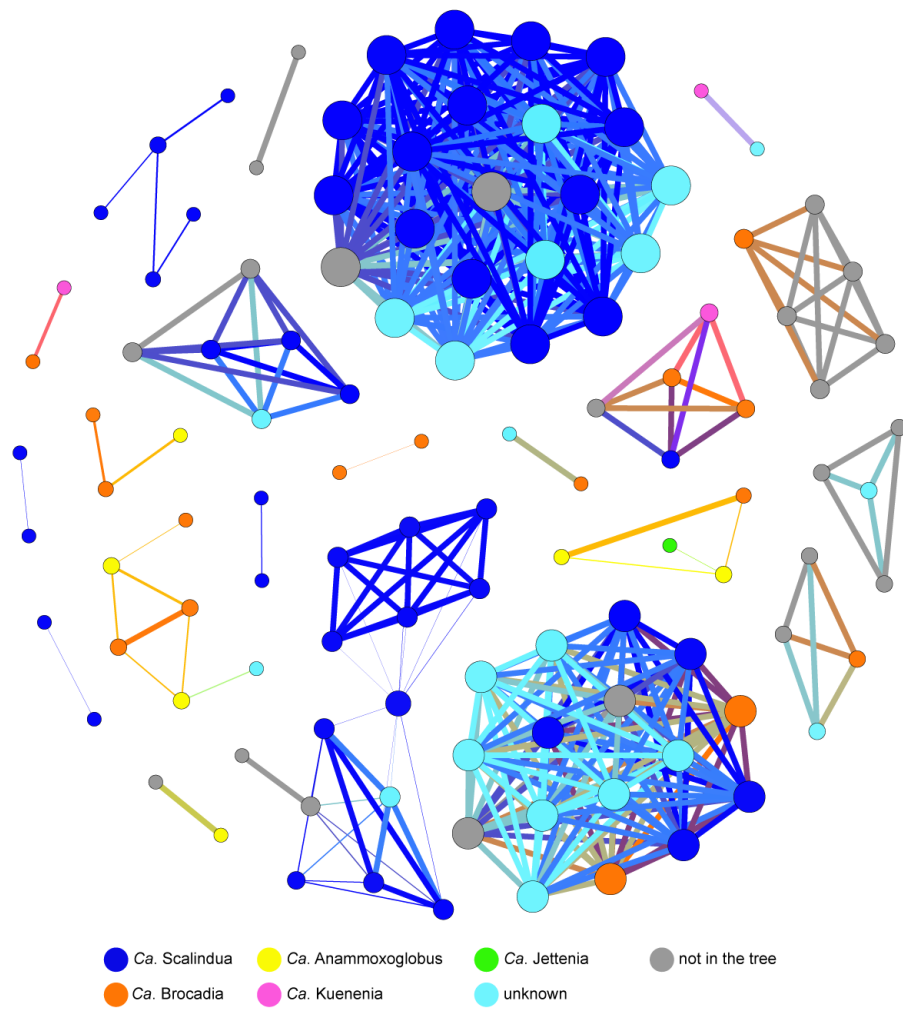


Figure 2.5 Co-occurrence network of 97% OTU identity sequences from 15 habitats. A connection represents a strong correlation (Spearman's $\rho \geq 0.8$). Singleton OTU sequences were removed. Nodes are colored by anammox cluster according to a phylogenetic tree (Figure 2.4A). Some of the OTUs were excluded from the network prior to the analysis because of differing 16S rRNA gene regions contained within the analysis. Node sizes represent the number of connections.

2.4 Discussion

Based on ordination analysis and a non-parametric analysis of the distance matrix, I conclude that salinity is the dominant factor governing the global distribution of anammox bacteria (Figures 2.1A and 2.1C). These results are not surprising given that within-study correlation analyses have previously demonstrated that salinity influenced the geographical distribution of anammox bacteria in estuary and marsh sediments (Dale et al., 2009; Hu et al., 2012a; Hou et al., 2013). *Ca. Scalindua* dominated saline-related environments, including marine sediment, marine water column, estuary, and mangrove sediment. The comprehensive phylogenetic analysis also supported that ~70% of *Ca. Scalindua* were from saline-related environments (Figures 2.4A and 2.4B).

Salinity levels also affected the diversity of anammox bacteria. In a lab-scale bioreactor, the population of *Ca. Kuenenia* shifted toward *Ca. Scalindua* after being enriched in high salt concentrations for 360 days (Kartal et al., 2006). Although the *Ca. Scalindua* cluster was the most abundant among the co-occurrence networks (Figure 2.5), this cluster showed low connection to other known anammox clusters. This implied that other known anammox clusters possibly became less dominant, whereas the *Ca. Scalindua* cluster became more dominant because this cluster can survive better in high salinity environments. It has been reported that salinity showed negative correlations with *Ca. Scalindua* diversity in the Bohai Sea sediment (Dang et al., 2013).

Although there is no pure anammox culture available so far, comparative metagenomic studies of *Ca. Kuenenia* (Strous et al., 2006; Speth et al., 2012), *Ca. Brocadia* (Gori et al., 2011), *Ca. Jettenia* (Hu et al., 2012), and *Ca. Scalindua* (van de Vossenberg et al., 2013; Villanueva et al., 2014) revealed that *Ca. Scalindua* has unique characteristics, favoring this cluster being ubiquitous in marine environments. *Ca. Scalindua* has high-affinity ammonium transport (*amtB*) and formate/nitrite transport (*focA*) proteins; both genes are highly expressed compared to those present in other anammox species (van de Vossenberg et al., 2013). These characteristics help *Ca. Scalindua* adapt to marine environments where ammonium and nitrite may be limited (Lam and Kuypers 2011). So far, only *Ca. Scalindua* is known to contain genes involved in dipeptide and oligopeptide transport with moderate expression (van de Vossenberg et al., 2013). Consequently, *Ca. Scalindua* has an alternative ammonium source from degraded and mineralized organic matter that sinks into the ocean. *Ca. Scalindua* also has a relatively versatile metabolism. *Ca. Scalindua* can use NO_2^- , NO_3^- , and metal oxides as alternative e^- acceptors (van de Vossenberg et al., 2008 and 2013). In the presence of organic acids (i.e., propionate, acetate, formate), *Ca. Scalindua* can perform dissimilatory nitrate

reduction to ammonia (DNRA; Jensen et al., 2011). Lipid assay demonstrated that ladderane lipids with three cyclobutane rings and one cyclohexane ring may be specific to *Ca. Scalindua* (van de Vossenberg et al., 2008; Kuypers et al., 2003 and 2005). However, this unique lipid structure may or may not facilitate *Ca. Scalindua* being dominant in marine environments. The specific function of this lipid needs further biochemical assay to verify.

Salinity impacted not only the distribution patterns and diversity of anammox bacteria but also the abundance and activity of anammox bacteria. The abundance of anammox bacteria increased with the salinity gradients in Cape Fear River estuary (Dale et al., 2009) and Yangtze estuary (Hou et al., 2013). In contrast to abundance, the activity of anammox bacteria negatively correlated with salinity (Trimmer et al., 2003; Rich et al., 2008; Koop-Jakobsen and Giblin 2009). However salinity can be linked with other factors such as NO_3^- , NH_4^+ , vegetation zones, and relative contribution to denitrifiers, so it was difficult to rule out the independent effect of salinity on anammox activity. Salinity might not be the factor that directly influenced anammox activity. Inhibitory effects of salinity on nitrification and denitrification rates were observed in estuary sediment (Rysgaard et al., 1999).

The influence of salinity on other microorganisms involved in the N cycle was reported previously. The abundance and diversity of ammonia oxidizing bacteria (AOB) and archaea (AOA) were affected by salinity (Francis et al., 2003; Santoro et al., 2008). One of the important factors affecting aquatic AOA diversity was salinity (Biller et al., 2012). The diversity of denitrifying bacteria in WWTP was also impacted by salinity (Yoshie et al., 2004). Not only does salinity affect the distributions of specific groups of microorganisms, salinity also impacts fingerprints and species richness metrics of Bacteria, Archaea, and Eukaryotes within a solar saltern in Spain (Casamayor et al., 2002). The bacterial community composition along an estuary shifted due to a salinity gradient (Crump et al., 2004). Statistical and multivariate approaches indicated that salinity was the key factor driving the global distribution patterns of Bacteria (Lozupone and Knight 2007) and Archaea (Auguet et al., 2010).

Ordinations, including PCoA (Figure 2.1D) and NMDS (Figures 2.2C and 2.2F), showed that anammox bacteria from natural ecosystems formed clusters apart from those of engineered ecosystems. This observation reflects environmental selection of anammox bacteria in natural and engineered ecosystems. A possible reason for this finding is the difference in physiological properties of anammox species, including specific growth rate (μ_{\max}), affinity for ammonia and nitrite (K_s),

optimum growth temperature, and pH. The physiological properties of *Ca. Kuenenia stuttgartiensis* (Egli et al., 2001; van der Star et al., 2008a and 2008b), *Ca. Brocadia anammoxidans* (Jetten et al., 2005; Strous et al., 1998 and 1999a), and *Ca. Brocadia sinica* (Oshiki et al., 2011) are characterized. The physiological properties demonstrate that *Ca. Brocadia sinica* adapts better to engineered ecosystems because of a lower affinity for ammonia and nitrite, higher tolerance to O₂, and higher growth rate (Oshiki et al., 2011). Engineered ecosystems are typically associated with high ammonia and nitrite loads. Wastewater treatment technologies apply O₂ to facilitate AOB activity so that the coexistence of anammox bacteria and AOB transforms fixed N to N₂ gas (van Dongen et al., 2001; Third et al., 2001).

After being enriched in fluctuating nitrite concentrations, a *Ca. Brocadia* dominated community shifted to a *Ca. Kuenenia* dominated community due to differences in affinity for NO₂⁻ (van der Star et al., 2008a). *Ca. Scalindua* from marine environment changed to *Ca. Brocadia* and *Ca. Kuenenia* after being enriched in a bioreactor (Nakajima et al., 2008). Either *Ca. Brocadia* or *Ca. Kuenenia* was commonly dominant in lab-scale bioreactors (Egli et al., 2001; Hu et al., 2010; Park et al., 2010). In this study, network co-occurrence analysis showed that *Ca. Brocadia* and *Ca. Kuenenia* OTUs are correlated with one other (Figure 2.5). However, more research on physiological properties, including kinetic and biochemical analyses, of other anammox species are needed to better understand niche differentiation of anammox bacteria in different ecosystems.

Although diversity richness of anammox bacteria in marine water column and marine sediment was significantly different (Figure 2.3 and Table 2.2), marine environments harbored a low diversity of anammox bacteria, mostly restricted to *Ca. Scalindua* (i.e., Schmid et al., 2007; Woebken et al., 2008; Hong et al., 2011a and 2011b). A microdiversity within *Ca. Scalindua* was previously discovered in marine oxygen minimum zones (OMZs); *Ca. Scalindua* comprised of several subclusters (Woebken et al., 2008). The microdiversity of *Ca. Scalindua* was also found in other marine environments, including the South China Sea (Hong et al., 2011a; Han and Gu 2013), Jiaozhou Bay, China (Dang et al., 2010), and the Bohai Sea, China (Dang et al., 2013). The novel subcluster, *Ca. Scalindua Zhenghei*, was tentatively proposed after being identified in the South China Sea (Hong et al., 2011a). *Ca. Scalindua* showed strong connections within its cluster but relatively low connectivity to other known anammox clusters (Figure 2.5). This observation reflected the microdiversity within *Ca. Scalindua* cluster. However co-occurrence of *Ca. Scalindua* and the

unknown clusters was high and consistent, reflecting the close relationship between the two. The unknown cluster might be a second dominant cluster found in marine environments.

In contrast to marine environments, freshwater environments showed high diversity of anammox bacteria. Coexistence of *Ca. Brocadia* with known and unknown anammox clusters was generally found in previously reported freshwater habitats (Zhang et al., 2007; Hamersley et al., 2009; Hirsch et al., 2010; Yoshinaga et al., 2011; Hu et al., 2012b; Sonthiphand and Neufeld 2013). However, one dominant anammox phylotype, *Ca. Brocadia*, was detected in the sediments of the Dongjiang River, Hong Kong (Sun et al., 2013), Lake Taihu, China (Wu et al., 2012), and the Grand River, Canada (Sonthiphand et al., 2013). Network analysis also showed that *Ca. Brocadia* clusters connected to all known and unknown anammox clusters (Figure 2.5). *Ca. Scalindua*, a major marine anammox cluster, was solely detected in Lake Tanganyika, which is meromictic with a sharp chemocline and thermohaline stratification (Schubert et al., 2006). Overall, *Ca. Brocadia* clusters were found in all previously reported freshwater habitats, except Lake Tanganyika.

As with other freshwater environments, *Ca. Brocadia* was the major anammox phylotype detected in ammonium contaminated groundwater; although, *Ca. Kuenenia*, *Ca. Jettenia*, *Ca. Scalindua*, and the unknown clusters were also present (Moore et al., 2011). However, most of sequences from Moore and colleagues were removed from this current analysis, resulting in low diversity richness and underestimation of anammox phylotypes in groundwater. However, there is insufficient information based on a paucity of anammox surveys of groundwater thus far. I recommend further surveys of ammonia-rich groundwater isolation sources to obtain a better understanding of anammox bacterial diversity in groundwater.

The transitional zone between freshwater and marine environments, including estuary and mangrove sediment, is typically a dynamic habitat. River-sea interactions (i.e., river runoff, ocean tides, and inflow/outflow) possibly enhance the diversity of anammox bacteria. The mixture of known and unknown anammox clusters was evident in estuary habitats (Dale et al., 2009; Hirsch et al., 2010; Hu et al., 2012a; Hou et al., 2013) and mangrove sediment (Han and Gu 2013; Li and Gu 2013; Wang et al., 2013).

The combination of anammox clusters associated with *Ca. Brocadia*, *Ca. Kuenenia*, *Ca. Anammoxoglobus*, and *Ca. Jettenia* was also found in various soil types, including peat soil (Hu et al., 2011), fertilized paddy soil (Zhu et al., 2011a), a flooded paddy soil (Hu et al., 2013), and an agricultural soil (Shen et al., 2013a). However, a single anammox phylotype was reported in some

other soil types. *Ca. Jettenia* was recovered from manure pond soil (Sher et al., 2012) and permafrost soil (Humbert et al., 2010). *Ca. Kuenenia* was detected in rhizosphere soil (Humbert et al., 2010). Interestingly, a rice paddy soil was dominated by *Ca. Scalindua* (Wang and Gu 2013). The difference in soil properties (i.e., nutrients, O₂, and pH) and depth reflected a microniche of anammox bacteria within terrestrial habitats (Zhu et al., 2011a; Sher et al., 2012).

One limitation of this study was that different methodologies were used to obtain anammox sequences across individual studies. Some anammox clusters might be underrepresented due to methodological bias (i.e., DNA extraction, primer design, and PCR conditions). According to available information in Genbank, several different primer sets used to capture anammox bacteria from different environments. The major primer sets were Amx368f/Amx820r and A438f/A684r, whereas an example of minor primer sets was Pla46f/Amx820r, Brod541f/Brod1260r, and An7f/1388r. Although not all individual studies indicated the primers used to retrieve anammox sequences, a multiple sequence alignment showed that most anammox primers generated amplicons covering a common homologous region of the 16S rRNA gene.

Chapter 3²

Anammox Primers

3.1 Introduction

Even before the discovery of anaerobic ammonia oxidizing (anammox) bacteria as Planctomycetes (Strous et al., 1999b) and elucidation of their metabolic pathways, physiology and morphology (Jetten et al., 2003; Schmid et al., 2005), the anammox process was recognized by nutrient profiles and thermodynamic calculations (Broda 1977; Richards 1965; Helmer et al., 1999). Anammox bacteria have the ability to transform ammonium into nitrogen gas by using nitrite as an electron acceptor under anoxic conditions. Anammox bacteria were first discovered in a laboratory-scale reactor in 1995 (Mulder et al., 1995). Since then, many reports have demonstrated the widespread occurrence and contribution of anammox bacteria in a variety of natural ecosystems (Li et al., 2010a; Dang et al., 2010; Hong et al., 2011a; Hirsch et al., 2011; Mohamed et al., 2010). Anammox bacteria are important in the global nitrogen cycle, and responsible for high nitrogen losses: ~50% in marine sediments (Thamdrup and Dalsgaard 2002; Engstrom et al., 2009; Brandsma et al., 2011; Trimmer and Nicholls 2009), ~40% in contaminated groundwater (Moore et al., 2011) and 4 – 37% in terrestrial habitats (Zhu et al., 2011a). These findings demonstrate the important role of anammox bacteria in natural environments.

There are still no pure culture isolates of anammox bacteria due to their extremely slow growth rates, relatively low biomass yields and inactivation by low concentrations of oxygen and nitrite (Strous et al., 1998; Manz et al., 1992; Schmid et al., 2001). Culture-independent methods such as 16S rRNA gene-based analysis (Penton et al., 2006; Dale et al., 2009; Humbert et al., 2010) and fluorescence *in situ* hybridization (FISH; Schmid et al., 2000 and 2003) are the common methods used for anammox bacterial community analysis. The 16S rRNA genes of known anammox bacteria show several phylogenetically distinct *Candidatus* genera including *Ca. Brocadia*, *Ca. Scalindua*, *Ca. Kuenenia*, *Ca. Anammoxoglobus* and *Ca. Jettenia*. The average sequence similarity between *Ca.*

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Scalindua and *Ca. Brocadia* or *Ca. Kuenenia* clusters is only 85% (Jetten et al., 2003; Schmid et al., 2003). Thus, it is very challenging to design primer sets that target all known anammox genera.

Although several anammox-specific primers have been used for 16S rRNA gene amplification, reported problems include low recovery efficiencies of anammox-related clones, non-specific amplification and an inability to target all anammox bacterial clusters (Li et al., 2010b; Penton et al., 2006; Humbert et al., 2010; Amano et al., 2007; Rich et al., 2008; Zhang et al., 2007; Tal et al., 2005 and 2006; Kirkpatrick et al., 2006). Anammox bacteria from groundwater have recently been profiled by comparing bacteria-specific denaturing gradient gel electrophoresis (DGGE) patterns (341f-GC/518r) with “anammox specific” patterns from a nested PCR protocol (An7f/An1388r followed by 341f-GC/518r; Moore et al., 2011). The intense bands that appeared in DGGE fingerprints were confirmed to be related to anammox bacteria.

Since identifying the contribution of anammox bacteria to fixed nitrogen losses in natural ecosystems (Kuypers et al., 2003), much research has focused on studying anammox bacteria in marine environments (Dalsgaard et al., 2005). Since anammox bacteria were first reported in freshwater environments (Schubert et al., 2006), there have been only five known studies characterizing anammox bacterial communities in freshwater habitats (Moore et al., 2011; Penton et al., 2006; Zhang et al., 2007; Schubert et al., 2006; Yoshinaga et al., 2011). Thus, information on the diversity, abundance and activity of anammox bacteria in freshwater ecosystems is still scarce. In this study, I focused on PCR primer-based detection methods for anammox bacteria within freshwater environments, including samples taken from the Grand River and from a previously studied groundwater site.

The primer set, A438f and A684r, successfully quantified anammox bacteria 16S rRNA gene copies in wetland soils (Humbert et al., 2012) but was not tested for DGGE prior to this study. Another primer set, Amx368f and Amx820r, was commonly used to detect anammox bacteria in various environments. Both primers were originally designed for FISH probes and were then applied as forward and reverse PCR primers for detecting anammox bacteria by clone library analysis in freshwater, terrestrial and marine ecosystems, such as Lake Kitaura (Yoshinaga et al., 2011), groundwater (Moore et al., 2011), fertilized paddy soil (Zhu et al., 2011a), constructed wetland (Zhu et al., 2011b), peat soil (Hu et al., 2011), Cape Fear River estuary (Dale et al., 2009), coastal marine sediment (Amano et al., 2007), the Jiaojiang estuary (Hu et al., 2012a), the South China Sea (Hong et al., 2011a), and a high-temperature petroleum reservoir (Li et al., 2010). In this study, these existing

PCR primers were modified with GC clamps and tested for the ability to generate anammox bacterial 16S rRNA gene DGGE fingerprints. The other two primer sets investigated here (An7f/An1388r and Pla46/1392r) amplified a large amplicon (~1,400 bp). The primers An7f and An1388r were designed originally to target anammox bacteria in freshwater and marine sediments (Penton et al., 2006). The Planctomycetes-specific FISH probe, Pla46, has been used as a forward primer with the reverse universal primer 1392R to obtain PCR products that were subsequently used for nested PCR templates in both natural (Mohamed et al., 2010; Rich et al., 2008; Tal et al., 2005; Woebken et al., 2007) and artificial (van der Star et al., 2008a; Bae et al., 2010) environments.

The main objectives of this study were (i) to identify suitable PCR primer combinations for DGGE assessment of anammox bacterial communities and (ii) to compare the efficiency and specificity of the existing primer sets for DGGE, clone library and qPCR assays. The results provide important experimental validation for using specific primer sets for investigating the diversity and abundance of anammox bacteria within freshwater environments.

3.2 Materials and Methods

3.2.1 Sampling site description and sample collection

The Grand River is located in southwestern Ontario, Canada. This large river and its tributaries receive high nitrogen inputs, mainly from agricultural runoff and wastewater discharge. Two sampling sites along the Grand River (Bridgeport and Blair), located in the city of Waterloo, were chosen as representative freshwater environmental sites. Sediment (SedBr and SedBl), epilithic biofilm (EpBr and EpBl) and water (WaBr and WaBl) samples were collected from each sampling site in June 2010. In addition, groundwater samples were collected from the Zorra township, Ontario (site details and sampling information were previously described in Moore et al., 2011). Both groundwater (GW) and groundwater sediment core (GS) samples were collected at 7.5-m depth in July 2009. Water samples of approximately 300 ml were filtered onsite onto 0.22- μ m Sterivex filters (Millipore, USA). All samples were stored on dry ice during transportation and kept at -80°C until DNA extraction. The environmental chemistry analyses from each site are shown in Table 3.1.

Table 3.1 Water chemistry for each sampling site.

Sampling site	NH ₄ ⁺ -N (mg NH ₄ ⁺ L ⁻¹)	NO ₂ ⁻ -N (mg NO ₂ ⁻ L ⁻¹)	NO ₃ ⁻ -N (mg NO ₃ ⁻ L ⁻¹)	DO (mg L ⁻¹)	pH
Bridgeport	0.05	0.04	2.06	7.9	8.14
Blair	0.47	0.46	1.80	7.3	7.91
Zorra*	ND	NA	10	1.97	7.04

NA = Not available; ND = Not detected; * Samples from Zorra site were collected in July, 2010 but all parameters reported were measured in August 2010.

3.2.2 DNA extraction

Genomic DNA was extracted from Grand River sediment and epilithic biofilm samples using the MoBio PowerSoil DNA kit (MoBio Laboratories, USA), following the manufacturer's protocol. Nucleic acids from all Sterivex filters were extracted according to a previously published protocol (Neufeld et al., 2007). The quality and quantity of extracted DNA were measured by agarose gel electrophoresis and spectrophotometry (NanoDrop Spectrophotometer ND-100; Thermo Fisher Scientific, USA), respectively. Extracted DNA was then diluted to 5 - 10 ng μl^{-1} for use as PCR template.

3.2.3 Denaturing gradient gel electrophoresis (DGGE)

All samples were PCR amplified with bacteria-specific primers (341f-GC/518r), targeting the bacterial 16S rRNA gene. This PCR, in addition to a nested PCR approach for detecting anammox bacteria, followed previously published protocols (Moore et al., 2011). Briefly, for the nested PCR, template was amplified with anammox bacteria-specific primers An7f/An1388r, followed by amplification by the bacteria-specific DGGE primers 341f-GC/518r. Additional published primers were also modified with GC-clamps for DGGE assessment (Green et al., 2010). These anammox-specific nested PCR amplifications involved either An7f/An1388r or Pla46/1392r for the first round of PCR, followed by anammox-specific A438f-GC/A684r or Amx368f-GC/Amx820r for a second reaction. The PCR components contained 2.5 μl of 10X ThermoPol Reaction Buffer, 0.05 μl of forward and reverse primer (100 μM stocks), 0.05 μl of dNTPs (100 mM stock), 0.1 μl of Taq DNA polymerase (5 U μl^{-1} stock), 1.5 μl of bovine serum albumin (10 mg ml^{-1} stock) and 1 μl of DNA template (representing 5 - 10 ng of genomic DNA) in a total reaction volume of 25 μl . All PCR amplifications were carried out with an initial denaturation at 95°C for 5 min, followed by primer-set-specific thermal cycling conditions (Table 3.2) with a total of 30 – 35 cycles for the first round PCR

and a final extension of 72°C for 10 min to complete the reaction. The first round PCR products were diluted 100-fold to serve as template for the nested PCR. Nested PCR conditions and thermal cycle profiles of each primer set were the same as previously described, except for the number of PCR cycles. All nested PCR were run for a total of 20 – 25 cycles. After each amplification, PCR products were verified by agarose gel electrophoresis to confirm amplicon size. The 341f-GC/518r and A438f-GC/A684r PCR products were run on 10% acrylamide gels, with 30%-70% denaturing gradients. The Amx368f-GC/Amx820r PCR products were profiled on 8% acrylamide gels, with 30%-70% denaturing gradients. All DGGE gels were run for 15 h at 85 V and at 60°C, using a DGGEK-2401 (CBS Scientific Company, USA). The DGGE gels were stained with SYBR green (Invitrogen, USA) and scanned with a Pharos FXTM Plus Molecular Imager (Bio-Rad, USA). Representative bands were excised and sequenced by the corresponding anammox-specific primers at Beckman Coulter Genomics using an ABI 3730XL sequencer. “Representative bands” were selected based on a band position on the DGGE gel. Nonetheless, all bands being generated by the same primer set that migrated to the same position were selected for sequencing.

3.2.4 Clone library analysis

Three representative samples (SedBr, SedBl and GW) were selected to generate clone libraries with three anammox primer sets (An7f/An1388r, A438f/A684r and Amx368f/Amx820r) to compare the efficiency and specificity of each primer set. The PCR conditions for cloning were the same as those described for DGGE (Table 3.2). The PCR products were ligated and transformed using a TOPO TA Cloning kit and One Shot TOP10 Chemically Competent cells (Invitrogen, USA), respectively, according to manufacturer’s protocols. Between 30-70 white colonies were selected from each library and screened for the presence of inserts by each anammox-specific PCR primer set prior to being sequenced at Beckman Coulter Genomics, USA, as previously mentioned in the DGGE section.

3.2.5 Quantitative real-time PCR (qPCR)

Anammox bacterial 16S rRNA genes were quantified by two specific primer sets (A438f/A684r and Amx368f/Amx820r) for comparison. Total bacterial abundance was also investigated by primers 341f/518r as a reference. The qPCR master mix contained 5 µl of SsoAdvanced SYBR Green Supermix (Bio-Rad, USA), 0.03 µl of each primer (100 µM stocks), 0.02 µl of bovine serum albumin (10 mg ml⁻¹ stock) and 1 µl of genomic DNA template (5 - 10 ng stock) in a total volume of 10 µl. All qPCR amplifications were performed in duplicate on a CFX96 real-

time system (Bio-Rad, USA). Although specific annealing temperatures were used (Table 3.2), qPCR thermal programs were common for all three primer sets. An initial denaturation at 98°C for 2 min was followed by 35 cycles of 98°C for 5 sec, annealing at the primer-specific annealing temperature for 30 sec and 72°C for 30 sec, with a plate read after each cycle. Following PCR, melt curves were generated between 65°C - 95°C in 0.5°C increments to ensure PCR specificity. Reference freshwater samples with high anammox abundance were amplified by each specific primer set, pooled by primer set, then purified to serve as anammox bacterial standard templates for qPCR. For general bacterial qPCR, the standard curves were constructed from *Escherichia coli* genomic DNA. Each PCR product was purified using a MinElute kit (Qiagen, USA) and quantified by the NanoDrop Spectrophotometer ND-100. Ten-fold serial dilutions were applied to the standard DNA PCR product template to create the qPCR standard curves, which was linear between $10^1 - 10^7$ copies, with efficiencies of 84 – 93% and coefficients of determination (R^2) ≥ 0.996 for all standard curves. The specificity of qPCR amplicon size was confirmed by melting curve analysis and agarose gel electrophoresis of all products after each run.

3.2.6 Statistical and phylogenetic analysis

All analyzed sequences from clone libraries were clustered into operational taxonomic units (OTUs), based on 1% and 3% dissimilarity cut-off settings for 16S rRNA gene nucleotide similarity by AXIOME version 1.5.0 (Lynch et al., 2013). All clone sequences showing 97% and 99% identical sequences were grouped together before constructing two phylogenetic trees, based on differing distance levels in nucleotide sequences, to compare anammox bacterial clusters and tree topologies. All sequences from DGGE and clone library analysis were compared to the Genbank non-redundant database with the Basic Local Alignment Search Tool (BLAST) to identify related sequences. All DGGE bands and representative clones from each library were aligned with selected uncultured anammox bacteria sequences and reference *Ca.* anammox bacteria species using MUSCLE (Edgar 2004). A phylogenetic tree was constructed in PhyML v.3.0.1 (Guindon et al., 2003; Galtier et al., 1996), with the GTR model. The approximate likelihood ratio test (aLRT) statistic was used to calculate branch support values. Phylogenetic trees were run with five random starts to optimise the tree topology.

3.2.7 Nucleotide accession number

All anammox bacterial 16S rRNA gene sequences were deposited in GenBank with accession numbers JX392915-JX392948.

Table 3.2 Summary of the PCR primers and conditions used in this study.

Primer ¹	Specificity	<i>E. coli</i> position	PCR conditions			Reference
			Denaturation	Annealing	Extension	
A438f ²	All anammox bacteria	438-455	95°C, 30 sec	55°C, 30 sec	72°C, 30 sec	Humbert et al., 2012
A684r ²		667-684				
Amx368f	All anammox bacteria	368-385	95°C, 45 sec	59°C, 45 sec	72°C, 45 sec	Schmid et al., 2003
Amx820r	<i>Ca. Kuenenia</i> , <i>Ca. Brocadia</i>	820-841				Schmid et al., 2000
An7f	<i>Ca. Kuenenia</i> , <i>Ca. Brocadia</i> , <i>Ca. Scalindua</i>	7-26	95°C, 45 sec	63°C, 1 min	72°C, 1 min	Penton et al., 2006
An1388r		1372-1388				
Pla46	Planctomycetes	46-63	95°C, 45 sec	59°C, 1 min	72°C, 1 min	Neef et al., 1998
1392r	Universal bacteria	1392-1406				Ferris et al., 1996
341f	Universal bacteria	341-357	95°C, 30 sec	55°C, 30 sec	72°C, 30 sec	Muyzer et al., 1993
518r		518-534				

¹For DGGE, a GC-clamp was attached to the forward primers for PCR (A348f-GC, Amx368f-GC and 341f-GC).

²The qPCR conditions were exactly the same (primer concentrations, annealing temperature and without additional BSA) as the original condition (Humbert et al., 2012)

3.3 Results

3.3.1 Anammox bacterial primers for DGGE

In a previous study, DGGE, qPCR and Illumina 16S rRNA gene data indicated that anammox bacteria were numerically important community members of an ammonium-contaminated groundwater site (Zorra, Ontario). The initial benchmarking experiment for this study was to repeat the DGGE protocol with DNA extracts from this groundwater at a 7.5-m depth, and include additional sediment, epilithic biofilm and water samples from two representative sites within the Grand River, which are not as strongly dominated by anammox bacteria (Sonthiphand et al., 2013). As positive controls, I used plasmids carrying anammox 16S rRNA genes associated with *Ca. Jettenia*, *Ca. Brocadia* and *Ca. Scalindua*; genomic DNA from *E. coli* was used as a negative control. The results demonstrated that in all cases, patterns generated by bacteria-specific primers (341f-GC/518r) were distinct from those generated by the nested anammox PCR protocol for DGGE (Figure 3.1).

Of the 20 bands selected for sequencing, all three analyzed bands from SedBr, one band from SedBl and three bands from GW were affiliated with anammox bacteria (Figure 3.1), demonstrating that this nested PCR design resulted in the enrichment of anammox bacterial amplicons. Genbank BLAST analysis results showed that the sequences were 97% – 100% identical to previously reported sequences recovered from sediment in wetlands (JQ762203) and ammonium-contaminated groundwater from the same site analyzed in this study (HQ595700 and HQ595667). Phylogenetic analysis revealed that all six bands indicated by a yellow triangle were associated with Unknown 1 cluster, while another band indicated by a purple triangle fell into Unknown 2 cluster (Figures 3.1 and 3.2). Importantly, non-specific amplification of the *E. coli* 16S rRNA gene was observed (Figure 3.1), which was a non-specific amplification problem also seen in most of the freshwater samples included in this study. Bands indicated by black triangles were not related to anammox bacteria. These bands were 97% – 100% identical to *Actinobacteria*, *Chloroflexi* and *Cyanobacteria*. These results demonstrate that a coupling of an anammox-specific amplification using An7f/An1388r with bacteria-specific PCR for DGGE (341f-GC/518r) was not suitable for targeting anammox bacteria in the environmental samples at the expense of all other bacteria, especially if anammox bacterial abundances were relatively low, such as in the Grand River epilithic biofilm and water column samples. *In silico* analysis (Table 3.3) revealed that An7f showed two mismatches at the 5' end and one, none and three mismatches at 3' end against *Actinobacteria*, *Chloroflexi*, and *E. coli* 16S rRNA

gene sequences, respectively. Although, An1388r showed many mismatches for *Actinobacteria*, *Chloroflexi*, and *E. coli*, it was highly specific for both anammox and non-anammox of Planctomycetales 16 rRNA gene sequences. This analysis supported that An7f/An1388r could amplify non-anammox bacterial sequences. Note that band positions on a DGGE gel were different for the three anammox *Ca.* genera (Figure 3.1), which is useful for distinguishing different anammox populations if non-specific amplification is not a concern.

Many bands from the previously published nested DGGE approach were not affiliated with anammox bacteria in the Grand River epilithic biofilm and water samples (Figure 3.1). Therefore, alternative published primer sets were combined to generate DGGE profiles from the selected freshwater environments. The two tested primer sets were A438f-GC/A684r and Amx368f-GC/Amx820r; neither primer set had been tested with GC clamps for DGGE prior to this study. A nested PCR technique was also included in this comparison to identify whether this approach, useful for samples with low target abundance, could increase the sensitivity of anammox bacterial 16S rRNA gene signals without altering the profiles generated. I used primers An7f/An1388r, which targets a near full-length (~1,400 bp) region of the anammox bacterial 16S rRNA gene associated with *Ca. Scalindua*, *Ca. Brocadia* and *Ca. Kuenenia* genera (Penton et al., 2006). Primers Pla46/1392r, targeting all bacterial 16S rRNA genes within the *Planctomycetes* phylum (~1,400 bp), were also used for the first PCR amplification for comparison. In all nested PCR assays, after generating a larger amplicon from the initial PCR, a shorter fragment was amplified by more specific anammox bacterial primers with the GC-clamp. All freshwater samples and both positive and negative controls were amplified by direct PCR using the two main primer sets (Figures 3.3A and 3.3D) and the four additional combinations for nested PCR approaches (Figures 3.3B, 3.3C, 3.3E, and 3.3F) to compare the DGGE patterns, anammox-specific bands and diversity of anammox bacteria detected by each set.

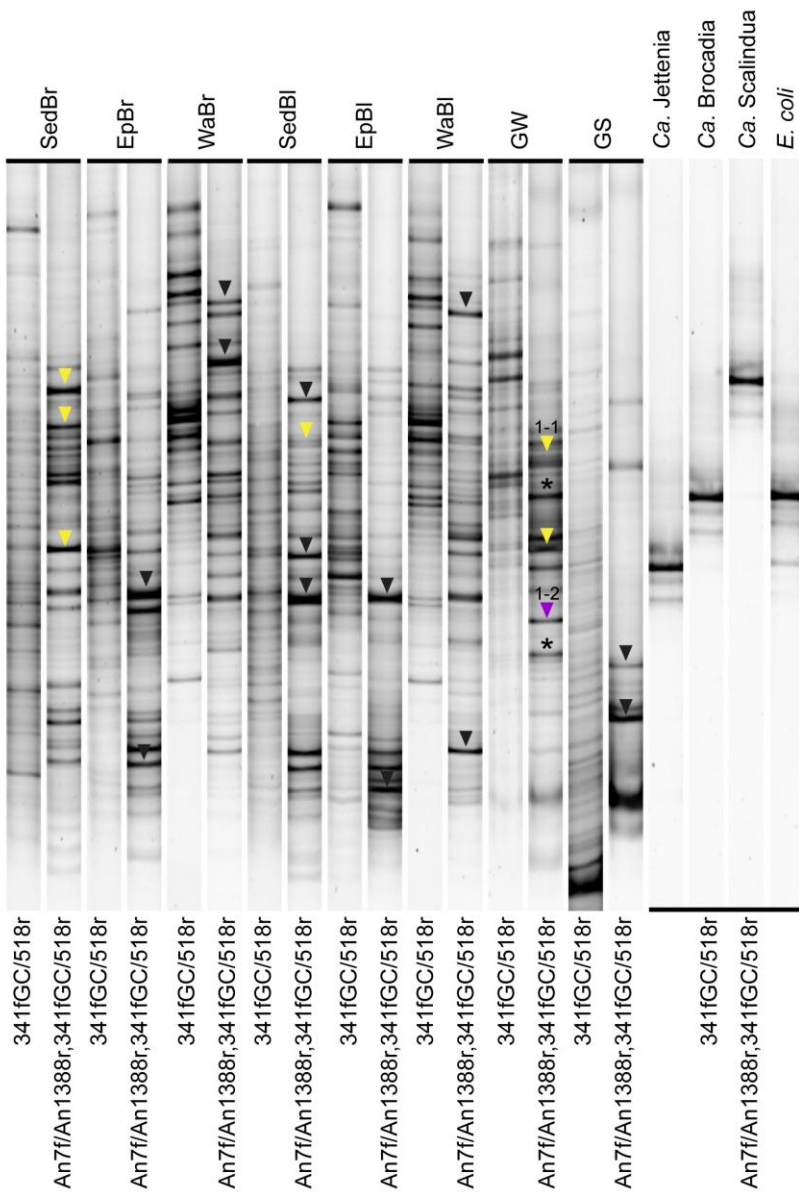


Figure 3.1 DGGE profiles of bacterial and anammox bacterial 16S rRNA genes in comparison. Together with eight environmental samples, positive control template (*Ca. Jettenia*, *Ca. Brocadia* and *Ca. Scalindua*) and a negative control (*E. coli*) were included. Triangles indicate sequenced bands. Band 1-1, indicated by a yellow triangle, was associated with Unknown anammox cluster 1 (Moore et al., 2011) and band 1-2, indicated by a purple triangle, was affiliated with Unknown anammox cluster 2 (Moore et al., 2011). Bands indicated by black triangles were not affiliated with anammox bacteria. A star indicates a band with low quality sequence, which was excluded from subsequent phylogenetic analysis.

Table 3.3 Alignment of anammox primer sequences against known anammox *Ca.* species, non-anammox species of Planctomycetales and non-Planctomycetales¹. The direction of all sequences is 5'-3'.

Taxonomic affiliations (16S rRNA gene sequences)	Genbank Accession	An7f	An1388r	Pla46	1392r
		GGCATGCAAGTCGAACGAGG	CACACCGCCCGTCAAGC	GGATTAGGCATCGAAGTC	GTACACACCGCCCGT
Known anammox <i>Candidatus</i> species					
<i>Ca. Brocadia</i> sp. 40	AM285341
<i>Ca. Brocadia fulgida</i>	DQ459989----
<i>Ca. Brocadia anammoxidans</i>	AF375994
<i>Ca. Brocadia sinica</i>	AB565477	.CT...T.....GTT.T.AAT.C.CCG..A
<i>Ca. Brocadia caroliniensis</i>	JF487828	---.....AT.C.CCG..A
<i>Ca. Scalindua sorokinii</i>	AY257181----
<i>Ca. Scalindua brodae</i>	AY254883
<i>Ca. Scalindua wagneri</i>	AY254882
<i>Ca. Scalindua arabica</i>	EU478624
<i>Ca. Scalindua sinooilfield</i>	HM208769	-----	-----	-----	-----
<i>Ca. Scalindua zhenghei</i>	GQ331167	-----	-----	-----	-----
<i>Ca. Scalindua marina</i> clone 12C	EF602039N.N.....
<i>Ca. Kuenenia stuttgartiensis</i>	AF375995
<i>Ca. Jettenia asiatica</i>	DQ301513	TAC....T....GAGT
<i>Ca. Anammoxoglobus propionicus</i>	DQ317601
Cultured non-anammox species of Planctomycetales					
<i>Pirellula</i> sp.	X81942G.....TG..C.AGCG....
<i>Pirellula staleyi</i>	M34126TG..C.AGC....
<i>Rhodopirellula</i> sp. SM34	FJ624371T....AGC....
<i>Rhodopirellula baltica</i>	FJ624346C....AGC....
<i>Blastopirellula marina</i>	HE861893CG...TAGC....
<i>Blastopirellula</i> sp. LHWP2	JF748733G....AGC....
<i>Gemmata</i> sp. IIL30	JX088244GGG..ATGC....
<i>Gemmata obscuriglobus</i>	X85248GGG..ATGC....
<i>Isophaera</i> sp.	X81958G.CGC....
<i>Schlesneria paludicola</i>	AM162407GG..AGC....
<i>Singulisphaera</i> sp. Io4-3	GQ889443	-.....-.....G.CG..G..A.CC.CG
<i>Singulisphaera rosea</i>	FN391026T..G.CGC....
<i>Planctomyces limnophilus</i> DSM 3776	NR_029225GG..AGC....
<i>Planctomyces maris</i>	X62910	-----	-----
Other bacteria (non Planctomycetales)					
<i>Actinobacterium</i> YJF2-33	FJ405887	CA.....G..	T.GCTAA.G.A.T...TAT.C.CTG.TA
<i>Chloroflexi bacterium</i>	HQ675640	TA.....	A.GCAAA.G.G.T...TAA.C.CCG.TA	AAGTTG.....T.G
<i>Escherichia coli</i>	J01695	CA.....GTA	G.GCTAA.G.G.T...TAT.C.CTG.TA	..C...G...TAAAC

Taxonomic affiliations (16S rRNA gene sequences)	Genbank Accession	Amx368f	Amx820r	A438f	A684r
		TTCGCAATGCCCGAAAGG	GGGCACTAAGTAGAGGGTTTT	GTCRGGAGTTADGAAATG	GAGAGTGGAACTTCTGGT
Known anammox <i>Candidatus</i> species					
<i>Ca. Brocadia</i> sp. 40	AM285341
<i>Ca. Brocadia fulgida</i>	DQ459989G.....
<i>Ca. Brocadia anammoxidans</i>	AF375994C..
<i>Ca. Brocadia sinica</i>	AB565477T..
<i>Ca. Brocadia caroliniensis</i>	JF487828G..
<i>Ca. Scalindua sorokinii</i>	AY257181AA..AAA..
<i>Ca. Scalindua brodae</i>	AY254883A..AG.....A.....
<i>Ca. Scalindua wagneri</i>	AY254882AA..AA.G.....A.....
<i>Ca. Scalindua arabica</i>	EU478624A..AA.....
<i>Ca. Scalindua sinooilfield</i>	HM208769	-----	-----	-----	-----
<i>Ca. Scalindua zhenghei</i>	GQ331167	-----	-----	-----A.....
<i>Ca. Scalindua marina</i> clone 12C	EF602039AA..AG..A.....C.....
<i>Ca. Kuenenia stuttgartiensis</i>	AF375995
<i>Ca. Jettenia asiatica</i>	DQ301513G.....CG.....G..
<i>Ca. Anammoxoglobus propionicus</i>	DQ317601C.....CG.....G..
Cultured non-anammox species of Planctomycetales					
<i>Pirellula</i> sp.	X81942	.CG.....GA.....T	.A.....GTCT.G...ACCC	TGGT...CGG.....	.TAT.C....AGA....
<i>Pirellula staleyii</i>	M34126	.CG.....GG.....C	.A.....GGACC.GA.CTC.GC	TGGT...CGG.....	.TA..C....GA....
<i>Rhodopirellula</i> sp. SM34	FJ624371	.G.....GG.....C	.A.....GG..T....AC..	TGGT...CGG.....	.T..TG.....A....
<i>Rhodopirellula baltica</i>	FJ624346	.CG.....GA.....T	.A.....GGCT....AAC..	TGGT...CGG.....	.T..TG.....A....
<i>Blastopirellula marina</i>	HE861893	.G.....GG..C...C	.A.....GGA.C....AC.C	TGGT...CGG.....	.T...C....GA....
<i>Blastopirellula</i> sp. LHWP2	JF748733	.CG.....GA..C...T	.A.....GGA.C....ACC.C	TGGT...CGG.....	.T...C....GA....
<i>Gemmata</i> sp. IIL30	JX088244	CCGT...GC.A.....	...T...GA...TA..C.AGA	AGGT...CGG.....	.CAT.C....GTA...
<i>Gemmata obscuriglobus</i>	X85248	.CG.....GGG.C..CC	...T...GA...TA.ACCAGA	AGGT...CGG.....	.CAT.....GTA...
<i>Isosphaera</i> sp.	X81958	.CG.....GG..C...C	.A.....GG...G...C.CGC	CGGT...CGG.....	.G.TC.....C....
<i>Isosphaera pallida</i> ATCC 43644	NR_028892	.CG.....GG..C...CTG.C..T..T.CG.	CGGT...CGG.....	.GACTC.....C...
<i>Schlesneria paludicola</i>	AM162407	.CG.....GA.....T	.AA.....GGAGGA....GC..	CGGT...CGG.....	.T.T.....C....
<i>Singulisphaera</i> sp. Io4-3	GQ889443	.CG.....GG..C...C	...T...GGA...G...C.CGC	CGGT...CGG.....	.GACC.....C....
<i>Singulisphaera rosea</i>	FN391026	.CG.....GG..C...CGG...G...C.CGC	CGGT...CGG.....	.GAGC.....C....
<i>Planctomyces limnophilus</i> DSM 3776	NR_029225	.CG.....GG.....C	.A.....GG.GGAG..ACGGG.	CGGT...CGG.....	.T.TAC.....C...
<i>Planctomyces maris</i>	X62910	.CG.....GA..C...T	.A.T...GT..GGA..AGC..	CGGT...CGG.....	.T.TAC.....C....
Other bacteria (non Planctomycetales)					
<i>Actinobacterium</i> YJF2-33	FJ405887	G.G..CCAA...T....G..GTG...A..A	TGGT.T..CGG.....	..TCAC....T.C....
<i>Chloroflexi bacterium</i>	HQ675640	.G.A.....GG.....C	CTT.GGGTC...A..C.C....	CGGT.T...GG.....	.GAG.....T.C.C...
<i>Escherichia coli</i>	J01695	G.....GA..AA.G...	AACTTT...A.T..A.A...G	AGGT.T..CGG.....	.G.G..A...T.C.A...

[†]Perfect matches for 16S rRNA gene primers and analyzed sequences are represented by dots (.) and gaps are represented by dashes (-). Capital letters represent mismatches with primer sequences.

The primer set A438f-GC/A684r generated reproducible patterns that were unique for the different samples included in this study. DGGE fingerprints generated directly by primers A438f-GC/A684r (Figure 3.3A) were similar to those from the nested PCR techniques (Figures 3.3B and 3.3C). The DGGE fingerprints produced by both nested PCR amplifications were highly similar to each other. Seven of eight samples showed positive anammox signals from direct PCR amplification with A438f-GC/A684r (Figure 3.3A). Only one sample, GS, was not amplified by direct PCR, but a nested PCR generated an anammox band for this sample. Intense DGGE bands from each sample were sequenced and included in a phylogenetic analysis (Figure 3.2). All analyzed bands from primer set A438f-GC/A684r were related to anammox sequences. The BLAST results indicated them to be 97% – 100% identical to uncultured anammox bacteria recovered from range of freshwater habitats.

Phylogenetic analysis demonstrated that bands identified as 2-1 were associated with *Ca. Scalindua*-like sequences (Figure 3.2). They were 96% and 94% identical to *Ca. Scalindua brodae* and *Ca. Scalindua wagneri*, respectively. These two identical bands, found in samples EpBr and WaBr, were at the same position as *Ca. Scalindua* control plasmid (Figure 3.3A). Bands labeled as 2-2 fell into the Unknown 1 cluster, which was an anammox cluster previously reported (Moore et al., 2011). All of the anammox bands from GW and GS were related to this Unknown 1 cluster. The majority of anammox bands found across the samples, corresponding to bands labeled 2-3, were closely related to *Ca. Brocadia*-like sequences (Figure 3.2). They were 98% identical to *Ca. Brocadia caroliniensis* and *Ca. Brocadia fulgida*.

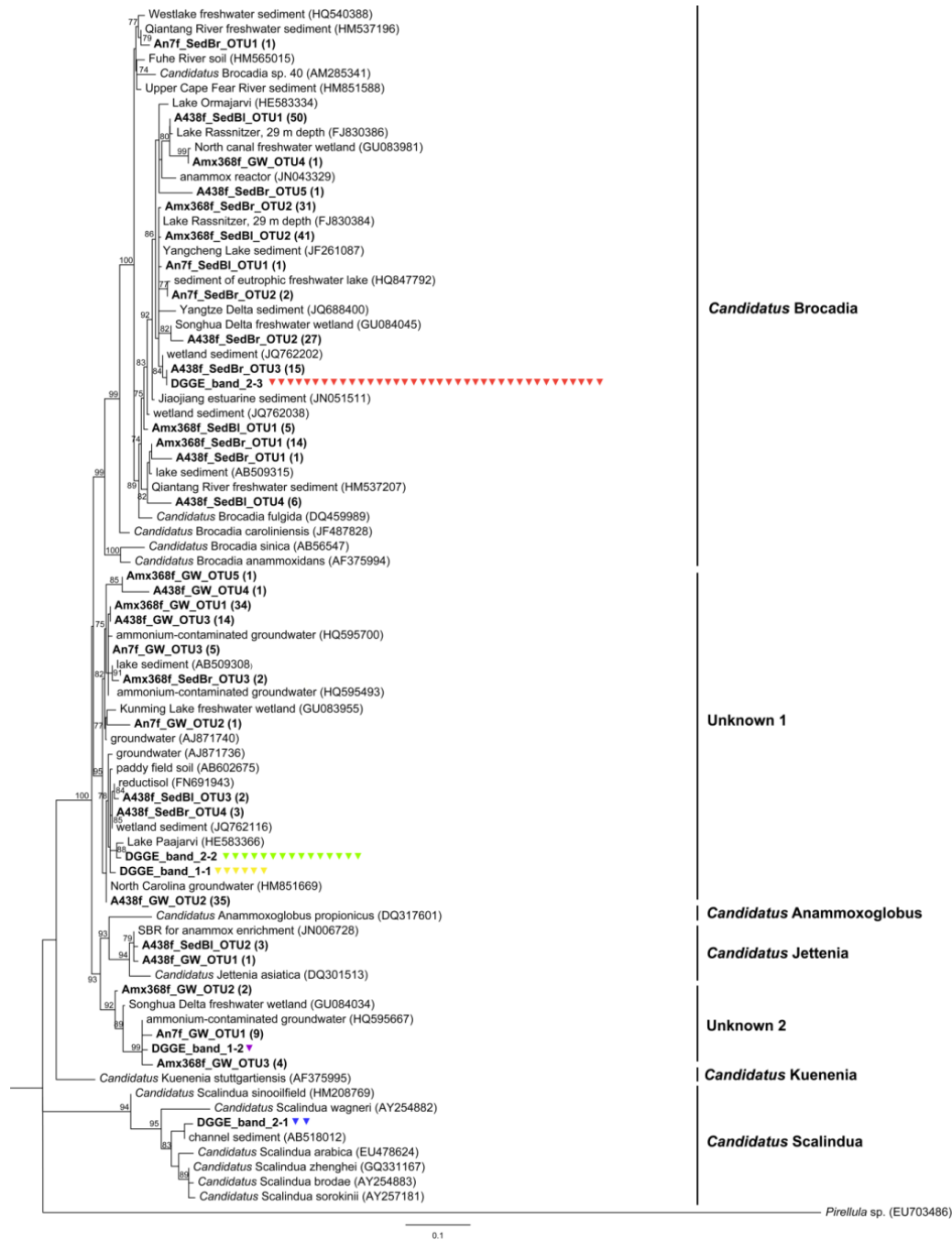


Figure 3.2 Phylogenetic tree of anammox bacterial 16S rRNA genes retrieved from both DGGE and clone library methods (shown in bold). The number of individual DGGE bands is indicated by the triangles with corresponding colour. Clone sequences with 97% identity from each library were grouped together; the representative clones from each operational taxonomic unit (OTU) were included in the analysis. The number of sequences belonging to each OTU is indicated in parentheses.

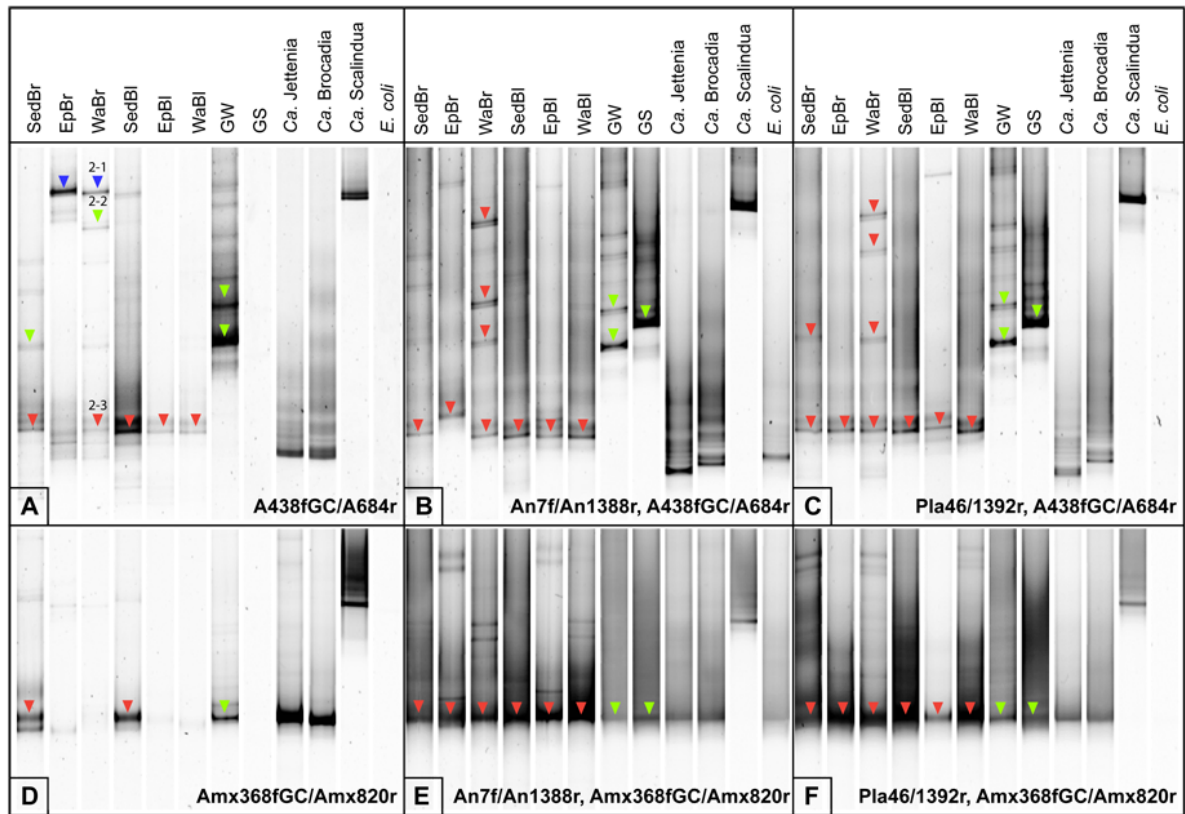


Figure 3.3 DGGE profiles of anammox bacterial 16S rRNA genes. The two main primer sets were used to generate the anammox-fingerprints (A and D) by direct PCR amplification. The additional four patterns (B, C, E and F) were produced by the nested PCR assay, using other two primer sets and followed by the two main sets. Triangles indicate a total of 55 bands, associated with anammox bacteria. Only three representative bands were shown in the phylogenetic tree. Bands 2-1, 2-2 and 2-3 were indicated by the blue, green and red triangles, respectively. Each coloured triangle indicates exactly the same phylogeny shown in Figure 3.2.

All retrieved anammox bands from the Grand River samples from nested PCR DGGE fingerprints fell into the *Ca. Brocadia* cluster. This demonstrates the potential for the direct PCR method to generate higher anammox diversity representation than that detected by either nested PCR approach for the Grand River samples (Figures 3.3A, 3.3B and 3.3C). All three bands detected in WaBr (bands 2-1, 2-2, and 2-3) fell into *Ca. Scalindua*, Unknown 1 and *Ca. Brocadia* clusters, respectively, whereas all four bands from the same samples, generated by both nested PCR amplifications, were all *Ca. Brocadia*-like phylotypes. The nested PCR approach, due to probable PCR bias, underrepresented anammox bacterial diversity in the freshwater samples but can nonetheless increase amplification of anammox template from samples with low anammox bacterial abundance.

Apart from the *Ca. Scalindua* plasmid template, all PCR amplicons, with or without a nested PCR design, migrated similarly on the DGGE gel for primer set Amx368f-GC/Amx820r (Figures 3.3D, 3.3E, and 3.3F). In addition, only three samples, SedBr, SedBl and GW, could amplify product by direct PCR with primers Amx368f-GC/Amx820 (Figure 3.3D); anammox bands from all samples can be captured by both nested PCR conditions (Figures 3.3E and 3.3F). The DGGE patterns from this primer set were clearly different from those from the previous set, A438f-GC/A684r. All sequenced bands were indicated by the triangles. They were 99% – 100% identical to the previously reported anammox-like sequences retrieved from various freshwater environments. Phylogenetic analysis revealed that anammox sequences from GW and GS still grouped together and fell into Unknown 1 cluster; all sequenced bands from the Grand River samples were affiliated with a *Ca. Brocadia*-like phylotype (Figure 3.2). All positive controls were amplified by the two primer sets A438f-GC/A684r and Amx368f-GC/Amx820, with or without nested PCR. The results were quite similar for both sets; *Ca. Brocadia* and *Ca. Jettenia* controls were close to each other on DGGE gels, but further from the *Ca. Scalindua* control. The negative control, *E. coli*, showed no signal for all primer combinations, except sets An7f/An1388r nested by both A438f-GC/A684r and Amx368f-GC/Amx820 (Figures 3.3B and 3.3E).

In addition to the two main primer sets, A438f-GC/A684r and Amx368f-GC/Amx820r, two additional primer combinations, A438f-GC/Amx820r and Amx368f-GC/A684r were tested with and without nested PCR amplifications, following the same pattern as previously described (Figure 3.4). The gradient PCR program was run at annealing temperatures between 51-60°C; the optimum temperature (sharp and bright band on agarose gel) was 55°C for both primer sets (data not shown).

The primer set A438f-GC/Amx820r generated a dominant band across all samples, although Amx368f-GC/A684r produced additional DGGE bands (Figure 3.4). Several representative bands from each position were sequenced and included in phylogenetic analysis (data not shown). All analyzed bands were anammox-related sequences and fell into *Ca. Brocadia*, Unknown 1 and Unknown 2 groups.

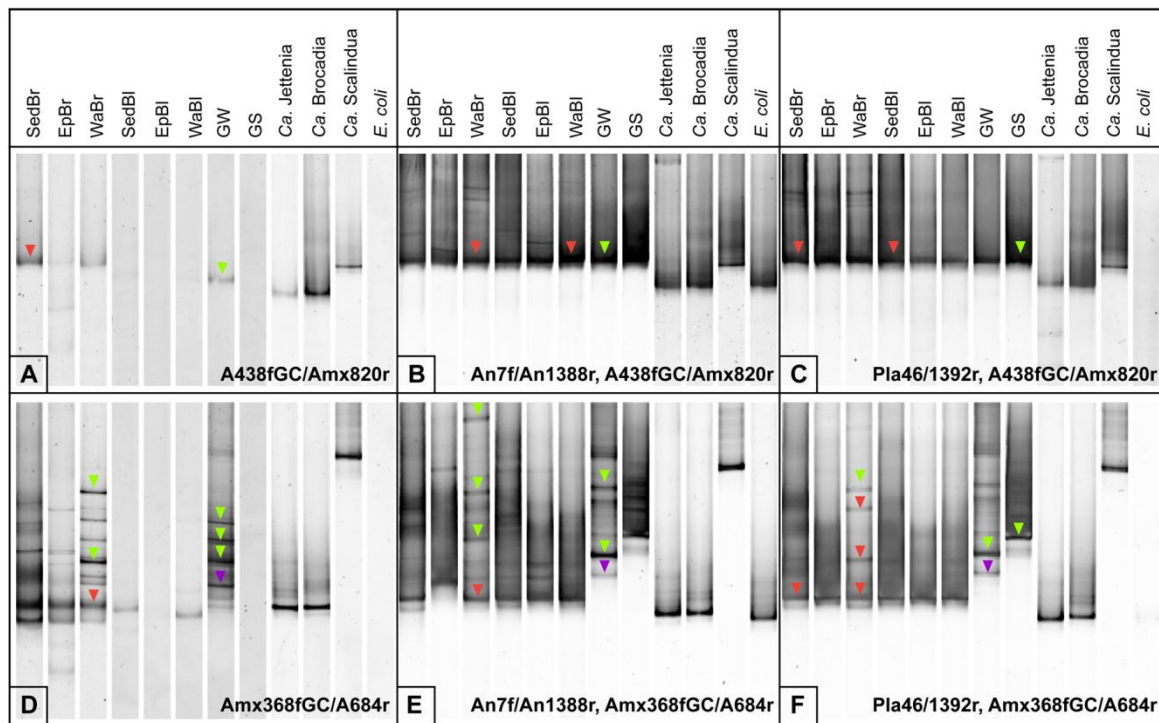


Figure 3.4 DGGE profiles of anammox bacterial 16S rRNA genes. The two optional primer sets (A438f-GC/Amx820r and Amx368f-GC/A684r) were used to generate the anammox-fingerprints (A and D) by direct PCR amplification. The additional four patterns (B, C, E and F) were produced by the nested PCR assay, using primers An7f/An1388r or Pla46/1392r, followed by these two optional sets. Triangles indicate representative bands that were associated with anammox bacteria. Triangle colours correspond to the phylogeny shown in Figure 3.2.

3.3.2 Diversity of anammox bacteria within freshwater environments

To confirm the specificity of An7f/An1388r, A438f/A684r and Amx368f/Amx820, three representative samples were selected to construct clone libraries from each pair. Based on DGGE profiles, two sediments from the Grand River (SedBr and SedBl) and Zorra groundwater (GW) samples were included in the clone library analysis. The cloning results showed that the ratio between the numbers of colonies containing inserts and the total number of selected colonies was lower for primers An7f/An1388r (Table 3.4). Using this primer set, the ratio was ~0.6 for the SedBr and SedBl libraries, but it increased to 0.9 for the GW library. The other two primer sets produced higher insert ratios, with >90% of screened colonies containing inserts (Table 3.4). The sequencing results revealed that only 3 out of 37 and 1 out of 36 analyzed sequences from SedBr and SedBl, constructed by primers An7f/An1388r, were anammox-related sequences, respectively. The GW library, constructed by the same primers, yielded a better result because 15 out of 25 were closely related to anammox bacteria (Table 3.4). The other primer sets (A438f/A684r and Amx368f/Amx820) revealed 100% specificity; all analyzed clones from all libraries were associated with anammox-related sequences. The BLAST results indicated that all clone sequences were 92% – 100% identical to previously reported anammox bacteria found in various ecosystems. The number of OTUs and anammox bacterial sequences were similar for A438f/A684r and Amx368f/Amx820 libraries (Table 3.4). The clone sequences showing as 99% and 97% identical from each library grouped together. Representative clones from each OTU were included in the phylogenetic analysis (Figure 3.2). Note that phylogenetic trees based on both 1% and 3% cut-off nucleotide sequences exhibited the same anammox grouping and tree topology (data not shown). The one percent cut-off was also analyzed to confirm the tree topology at a higher taxonomic resolution. The resulting phylogeny of the clone libraries constructed by primers An7f/An1388r revealed that *Ca. Brocadia*-like sequences made up the majority of anammox bacteria found in SedBr and SedBl; GW contained anammox bacterial sequences associated with Unknown 1 and 2 clusters (Figure 3.2). For primers A438f/A684r, both Grand River sediment and GW were dominated by *Ca. Brocadia* and Unknown 1 clusters, respectively (Figure 3.2). Although *Ca. Jettenia*-like sequences are targeted by this primer set (Table 3.3), only a few clones from SedBl and GW samples fell into this cluster. Fewer clones recovered from SedBr and SedBl were closely related to Unknown 1 cluster, compared to the GW library. The expected diversity of anammox bacteria was supported by Amx368f/Amx820r libraries because the majority of anammox bacteria found in SedBr and SedBl were related to *Ca. Brocadia*-like

sequences, whereas Unknown 1 cluster dominated the GW library (Figure 3.2). Other minor clone library OTUs detected from GW were related to Unknown 2 and *Ca. Brocadia* clusters.

To test spatial and temporal changes of anammox bacterial diversity, representative sediment samples from Bridgeport (SedBr) were collected at three time points, including Summer 2010, Fall 2010 and Summer 2012. All samples were amplified by primer set A438f-GC/A684r and profiled by DGGE (Figure 3.5). Overall, anammox patterns were consistent across the three time points. One additional band was apparent in the pattern from Summer 2012 and its sequence clustered with *Ca. Brocadia* phylotypes.

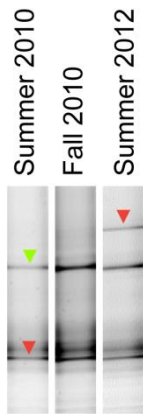


Figure 3.5 Anammox bacterial DGGE profiles of SedBr, collected in Summer 2010, Fall 2010 and Summer 2012, using primer set A438f-GC/A684r. Triangles represent sequenced bands that were included in phylogenetic analysis.

Table 3.4 Summary of the cloning results using three primer sets and three sample sites.

Primer set	Site	Total screened clone (A)	Total inserted clone (B)	B/A ratio	Anammox sequence (% recovery)	Number of OTUs		Cluster ¹
						1%	3%	
An7f/An1388r	SedBr	62	38	0.61	3/37 (8)	3	2	<i>Ca. Brocadia</i> (3/3)
	SedBl	62	39	0.63	1/36 (3)	1	1	<i>Ca. Brocadia</i> (1/1)
	GW	30	27	0.90	15/25 (60)	6	3	Unknown 1 (6/15), Unknown 2 (9/15)
A438f/A684r	SedBr	55	53	0.96	47/47 (100)	13	5	<i>Ca. Brocadia</i> (44/47), Unknown 1 (3/47)
	SedBl	70	66	0.94	61/61 (100)	11	4	<i>Ca. Brocadia</i> (56/61), <i>Ca. Jettenia</i> (3/61), Unknown 1 (2/61)
	GW	58	54	0.93	51/51 (100)	10	4	<i>Ca. Jettenia</i> (1/51), Unknown 1 (50/51)
Amx368f/Amx820r	SedBr	52	48	0.92	47/47 (100)	10	3	<i>Ca. Brocadia</i> (45/47), Unknown 1 (2/47)
	SedBl	51	48	0.94	46/46 (100)	4	2	<i>Ca. Brocadia</i> (46/46)
	GW	52	48	0.92	42/42 (100)	12	5	<i>Ca. Brocadia</i> (1/42), Unknown 1 (35/42), Unknown 2 (6/42)

¹The number of clones out of the total number of sequences affiliated with each cluster is listed in parentheses.

3.3.3 Abundance of anammox bacteria in freshwater environments

DGGE and cloning results demonstrated that the primer sets A438f/A684r and Amx368f/Amx820 were specific for detecting anammox bacteria within freshwater environments. I used these existing two primer sets to assess the qPCR method. Total bacterial 16S rRNA gene copies were also quantified for comparison. The results showed that the measured bacterial 16S rRNA gene copies were consistent for all analyzed samples (Figure 3.6). The abundance of anammox bacterial 16S rRNA genes in GW was the highest ($\sim 10^3$ - 10^4 copies per ng of genomic DNA), whereas those in SedBr and SedBl were lower and similar to each other ($\sim 10^2$ - 10^3 copies per ng of genomic DNA). The qPCR with Amx368f/Amx820r generated bacterial 16S rRNA gene abundance estimates that were approximately four times higher than for A438f/A684r in all analyzed samples (Figure 3.6). Consequently, caution must be taken in using Amx368f/Amx820r to quantify anammox abundance due to possible overestimation. The qPCR efficiencies were low, in some cases, possibly due to the presence of qPCR inhibitors (i.e., humic acids) in the environmental samples. This could be tested by comparisons between a spiked standard and a pure standard.

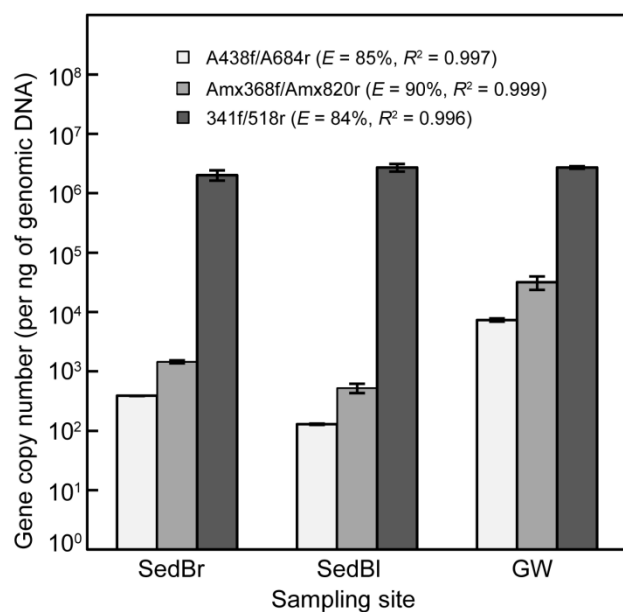


Figure 3.6 Abundance of anammox bacterial 16S rRNA genes quantified by two anammox specific primer sets, and general bacterial qPCR data for comparison. The qPCR efficiency (E) and coefficient of determination (R^2) of each primer set are shown in parentheses. Standard deviation (SD) was calculated from technical replicates.

3.4 Discussion

The anammox-DGGE method developed by Moore and colleagues (Moore et al., 2011), using a nested amplification beginning with An7f/An1388r, was able to generate DGGE patterns with confirmed anammox bands for only three samples in this study (SedBr, SedBl and GW). Of 20 bands sequenced, 7 sequences were associated with anammox bacteria (Figure 3.1). The remaining bands showed no relationship with any reported anammox bacterial sequences in the Genbank database. However, this method provided a reliable result for all previously analyzed groundwater samples (Moore et al., 2011), obtained from the same site as GW. Improved specificity for anammox bacterial template may be explained by a high sample-specific relative abundance of anammox bacteria, which is supported by anammox bacterial bands appearing in both bacterial and anammox-specific DGGE profiles from the GW site. The qPCR results demonstrated that most Grand River samples contained anammox bacterial 16S rRNA genes at $\leq 10^2$ copies per ng of genomic DNA (data not shown), except for GW ($\sim 10^4$ gene copies; Figure 3.6). Clone library analysis was consistent with this finding because lower recovery frequencies of anammox-related clones were obtained from the SedBr (8%) and SedBl (3%) libraries, yet higher for GW (60%; Table 3.4). Consistent with this observation, a previous marine sediment clone library constructed by primers An7f/An1388r showed 12% anammox-related sequences (Li et al., 2010b). The previous groundwater libraries showed a high proportion of anammox clones with this primer set, in the range of 86% – 100% (Moore et al., 2011). Overall results suggest that primers An7f/An1388r may be specific only for anammox samples with high proportions of anammox bacteria. Note that the frequency of inserts (screened clone/inserted clone ratio) may be related to the cloning reaction; however, An7f/An1388r consistently showed the lowest ratio, compared to A438f/A684r and Amx368f/Amx820 sample by sample (Table 3.4).

To improve the detection efficiency of anammox bacteria present in low abundance, different primer combinations, with or without a nested PCR step, were tested to enhance the specificity of DGGE (Figure 3.3). In this study, primer sets A438f-GC/A684r and Amx368f-GC/Amx820 were confirmed as specific for detecting anammox bacteria within freshwater environments. The A438f-GC/A684r primer pair was superior for DGGE based on well-resolved bands for both samples and controls. The resulting phylogeny revealed that direct PCR amplification by A438f-GC/A684r primers captured the most diverse anammox bacterial groups. *Ca. Scalindua*-like sequences were retrieved from picked bands only by these primers in this study. Although this cluster was normally found in marine and estuary environments and proposed to be a marine anammox-specific cluster

(Dang et al., 2010; Hong et al., 2011a and 2011b; Lam et al., 2007; Schmid et al., 2007; Ward et al., 2009; Woebken et al., 2008), the *Ca. Scalindua* genus has been associated with freshwater habitats such as Lake Tanganyika (Schubert et al., 2006), Wintergreen Lake (Penton et al., 2006) and Lake Rassnitzer (Hamersley et al., 2009). This anammox cluster was also retrieved previously from ammonium-contaminated groundwater samples (Moore et al., 2011).

Both the A438f/A684r and Amx368f/Amx820r primer sets were highly specific based on clone library analysis; all clones were affiliated with anammox bacterial sequences (Table 3.4). The Amx368f/Amx820r primer pair has been used for cloning in many previous studies. Reported recoveries of anammox-related clones with this primer set vary depending on the sampling sites. Clone libraries constructed from coastal marine sediment and eutrophic freshwater lake yielded 98% and 90% – 100% anammox bacterial sequences, respectively (Amano et al., 2007; Yoshinaga et al., 2011). However, low recovery frequencies of 12% – 59% were previously reported from deep-sea subsurface sediment libraries (Hong et al., 2011a). There is no prior information on the specificity of anammox bacterial community libraries generated by the primer set A438f/A684r. *In silico* analysis revealed that these four existing primers (Amx368f, Amx820r, A438f and A684r) showed high specificity and were specific for known anammox *Ca.* species; however, some anammox *Ca.* species sequences possessed mismatches to these primers and may be missed (Table 3.3). In this study, the anammox bacterial diversity recovered from cloning was in general agreement with that of the DGGE method.

The majority of anammox bacterial 16S rRNA genes found in the Grand River samples were similar to those of previously studied environments in identifying that *Ca. Brocadia*-like phylotypes were dominant in freshwater ecosystems (Moore et al., 2011; Zhang et al., 2007; Yoshinaga et al., 2011). The dominant anammox bacteria found in groundwater-related samples were Unknown 1 and 2 clusters. This result agreed with previous findings, showing that these unknown and uncultured groups have a potential to be a specific anammox cluster present in groundwater sites (Moore et al., 2011). Sequences from a reductisol and ammonium contaminated aquifer also fell into a distinct group without any affiliation to known anammox clusters, being named “cluster II” in the study of Humbert and colleagues (2010). These sequences were related to the Unknown 1 cluster in this study. *Ca. Jettenia* cluster was a minority population found in both the Grand River sediment and groundwater samples. The *Ca. Jettenia* lineage does not normally dominate in any specific habitat previously reported but was detectable in various terrestrial habitats (Humbert et al., 2010), peat soil

(Hu et al., 2011), paddy soil (Zhu et al., 2011a), estuarine sediments (Hu et al., 2012a) and groundwater (Hirsch et al., 2011; Moore et al., 2011). Due to limited studies on the distribution of anammox bacteria in freshwater environments, future research should include more freshwater aquifers to explore their anammox bacterial communities.

The abundance of anammox bacterial 16S rRNA genes in GW was in the same range as in groundwater samples from the Zorra site previously reported (Moore et al., 2011). In the case of SedBr and SedBl, anammox bacterial 16S rRNA genes were present in the range of $10^2 - 10^3$ copies per ng of genomic DNA. River estuary sediments also contained anammox bacterial 16S rRNA in this range (Dale et al., 2009). The qPCR results revealed that Amx368f/Amx684r captured more anammox bacterial 16S rRNA genes in all analyzed samples. Primer pair A438f/A684r could provide more accurate results than Amx368f/Amx820 because of potential false positive amplification by Amx368f/Amx820 in samples with low anammox bacterial abundance (Humbert et al., 2012). These findings are obtained mainly from the anammox bacterial 16S rRNA genes within freshwater environments; future research should include a broad range of environmental samples such as marine, terrestrial and engineered systems to evaluate the efficiency and specificity of primers for targeting anammox bacteria. Other than anammox 16S rRNA genes, functional genes such as the hydrazine oxidoreductase (*hzo*) gene have been used for anammox bacterial detection in marine sediment (Li et al., 2010b; Dang et al., 2010; Hong et al., 2011b), aquatic ecosystems (Hirsch et al., 2011) and mangrove sediment (Li et al., 2011a). Another functional gene marker is the nitrite reductase (*nirS*) gene, which has been used to detect anammox bacteria in the ocean (Li et al., 2011b; Lam et al., 2009). The hydrazine synthase (*hzs*) gene has also been tested as a unique biomarker for detecting anammox bacteria in both natural and built environments (Harhangi et al., 2012). However, primers targeting these functional genes are still limited. The results demonstrate that primer sets should be evaluated in a range of environments and with a careful selection of positive and negative controls to avoid false positive amplification, as seen here with An7f/An1388r. I recommend primers A438f/A684r or Amx368f/Amx820 for clone library or qPCR analyses, but only A438f-GC/A684r for DGGE-based analyses of freshwater anammox communities.

Although DGGE is rapid, reliable, and suitable for screening anammox bacteria in freshwater environments, there are limitations of DGGE that will apply to all environmental studies. For example, only dominant species can be detected by DGGE (i.e., greater than ~1%) and co-migration of PCR amplicons from different species can result in underestimation of microbial community

(Green et al., 2010). Although one band is thought to represent a single OTU in theory (Muyzer et al., 1993), multiple 16S rRNA genes or co-migration of amplicons from different species can occur (Green et al., 2010). Consequently, the DGGE method must be used cautiously for estimations of species richness and diversity.

Chapter 4³

Ammonia oxidation within the Grand River

4.1 Introduction

The Grand River is the largest watershed in Southwestern Ontario, draining into Lake Erie, and is impacted by ammonia (NH_3 and NH_4^+) sources that directly affect water quality. The oxidation of anthropogenic ammonia to less toxic forms (e.g., NO_3^- or N_2) by ammonia-oxidizing prokaryotes (AOP) helps to prevent ammonia from exceeding toxicity thresholds for aquatic organisms and limits for drinking water. AOP include aerobic ammonia-oxidizing bacteria (AOB), archaea (AOA), and anaerobic ammonia-oxidizing (anammox) bacteria.

Nitrification is composed of two biological processes: ammonia and nitrite oxidation. Ammonia oxidation is considered the rate-limiting step in nitrification (Kowalchuk and Stephen 2001) and was believed for many decades to be mediated solely by a group of chemolithoautotrophic AOB (Prosser 1989). In 2005, AOA were also discovered (Könneke et al., 2005). Although studies have confirmed the presence and abundance of AOA in both natural (e.g., Herrmann et al., 2008; Tourna et al., 2008; Verhamme et al., 2011) and engineered (e.g., Limpiyakorn et al., 2011; Sauder et al., 2011 and 2012) environments, relative contributions to ammonia oxidation in these environments are difficult to assess. Although AOA, rather than AOB, were active in soil microcosms and acid soils due to low ammonia availability and their kinetic constant (K_s) for ammonia (He et al., 2012), AOB showed evidence of ammonia-oxidizing activity in an agricultural soil (Jia and Conrad 2009). More information on the contribution of AOA to ammonia oxidation and the potential for niche differentiation for AOA and AOB is still needed, especially in freshwater environments. Environmental factors with potential influence on the ecological niche of AOA in freshwater are ammonium, organic carbon, temperature, oxygen, pH, sulfide, and phosphate (Erguder et al., 2009; Liu et al., 2013). Several studies have confirmed that AOA predominate in low-ammonia environments, including freshwater (Verhamme et al., 2011; Sauder et al., 2011 and 2012; Herrmann et al., 2011; Sonthiphand and Limpiyakorn 2011). However, a recent study demonstrated high AOA

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abundance in high-ammonia freshwater sediment (Liu et al., 2013). Because ammonia concentration in freshwater has the potential to govern AOA:AOB ratios, rivers receiving wastewater effluent, with associated ammonia input and concentration gradients, are valuable systems for studying the influence of ammonia concentration as a controlling factor.

Ammonia oxidation under anoxic conditions is catalyzed by anammox bacteria belonging to the phylum *Planctomycetes*. Anammox bacteria have the ability to transform ammonia into N₂ gas by using nitrite (NO₂⁻) as an electron acceptor. Anammox bacteria were first discovered in a pilot plant reactor (Mulder et al., 1995) and have since been characterized in both marine and freshwater habitats (Lam et al., 2007; Moore et al., 2011), often associated with marine oxygen minimum zones (OMZs) (Thamdrup et al., 2006; Hamersley et al., 2007). Lab-scale experiments confirmed that anammox activity is reversibly inhibited by oxygen (Jetten et al., 1997; Strous et al., 1997). Consequently, in-river diel changes in O₂ levels are predicted to directly impact anammox activity; river O₂ levels are generally high during the day and become low during the night due to photosynthesis and respiration, respectively. In impacted rivers, aerobic nitrification rates are higher during the day than at night, while denitrification rates are greater at night (Gammons et al., 2011; Rosamond et al., 2011). Thus, diel and spatial oxygen gradients within sediments are ideal for investigating both aerobic and anaerobic ammonia-oxidizing communities. Only a few studies on anammox bacteria in freshwater have been published so far (Moore et al., 2011; Schubert et al., 2006; Zhang et al., 2007; Yoshinaga et al., 2011). Although the co-occurrence of AOP in the ocean have been reported (Lam et al., 2007 and 2009), no study has yet investigated the abundance, activity, and diversity of these three microbial groups in both freshwater and sediment environments, particularly those impacted by treated wastewater effluent. Due to the difference in biogeochemical properties of water columns and sediments, I hypothesize niche partitioning of AOP reflecting mixing ratios of both oxygen and ammonia.

The overall goal of my research is to study the ecology of AOP communities and investigate the effects of anthropogenic inputs on in-river N-cycling communities. The specific objective of this study was to identify the key contributors to ammonia oxidation within water column and sediment samples that varied in impact from effluent ammonia of a municipal wastewater treatment plant (WWTP). The AOP assessments were conducted with a multi-pronged approach combining the polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), clone libraries, quantitative real-time PCR (qPCR), reverse transcriptase qPCR (RT-qPCR), sequencing, and an

activity-based assessment of sediment nitrification. In addition, general bacterial 16S rRNA gene fingerprints were generated for all sample sites in both sediment and water samples to help assess the effects of effluent input on autochthonous in-river microbial communities.

4.2 Materials and Methods

4.2.1 Sampling site description and Sample collection

The Grand River is located in Southwestern Ontario, Canada (43°28'36"N, 80°28'44"W), flowing 280 km south from Dundalk to its mouth on Lake Erie. The central part of the river is associated with heavily populated areas (grey area in Figure 4.1) and the entire watershed is impacted by agricultural activities. All sampling sites were located in the central region of the Grand River in the Waterloo-Kitchener-Cambridge areas. The WWTP of the City of Waterloo, Ontario, is relatively large with the effective rated capacity and projected flow in 2010 of 56,050 and 45,994 m³/d, respectively (Region of Waterloo 2012). This WWTP treats wastewater from approximately 130,000 people using conventional primary and secondary treatments before releasing effluent directly into the Grand River (Region of Waterloo 2012). Below the WWTP, 12 sample sites were chosen to represent high, moderate and low ammonia concentrations (Figure 4.1). The plume location remains similar even when the absolute ammonia concentration of the river changes due to the diel, seasonal, and river discharge variations. There were two sampling sites (upstream1 and upstream2), located 960 m upstream of the WWTP plume, followed by eight sampling sites (downstream1 to downstream8), a total span of 1,050 m, within the WWTP plume and the other two sites (opposite1 and opposite2), located on another side of the river, were outside of the plume. Wastewater effluent was discharged from a pipeline into receiving water 50 m above site downstream1 (Figure 4.1). The effluent plume was not well mixed within the river over the sampled range, instead remaining close to the riverbank. Thus, sites of the same distance downstream from the plume but on opposite sides of the river differed substantially in ammonia (and effluent) concentrations. In addition to these twelve transect sites, five additional sampling sites (R1, R3, R4, R5, and R6) were selected to be additional “background” sites along the central region of the Grand River in Waterloo-Kitchener-Cambridge, spanning a total length of 72 km (Figure 4.1).

Conductivity, dissolved oxygen (DO), and pH were measured onsite. Water samples were stored at 4°C until water chemistry analysis. Total ammoniacal nitrogen (NH₃ and NH₄⁺; TAN) and NO₂⁻ were measured by colorimetric methods using an AutoAnalyzer II (Technicon Instruments,

USA). Sediment and water samples were collected from the 12 ammonia transect sites on October 21st, 2010 and from the five additional locations along the river on November 11th, 2010. Wastewater effluent directly from the pipeline outlet, 50 m above site downstream1, was also collected on October 21st, 2010. Triplicate sediment samples were collected using a plastic core tube from each site. Approximately 100 – 300 mL of water was filtered onsite onto 0.22- μ m Sterivex filters (Millipore, USA). Both sediment samples and all filters were kept on dry ice during transportation and stored at -80°C until DNA extraction. Additional sediment samples were collected upstream and within-plume downstream on July 25th, 2012 for assessing nitrification rates.

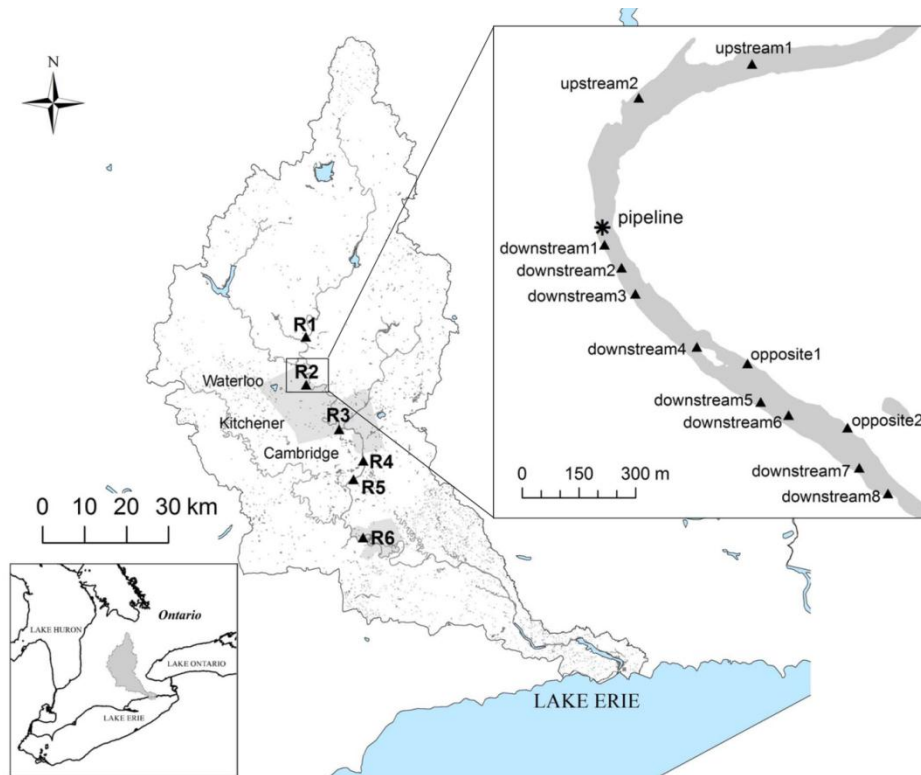


Figure 4.1 Sampling site map. Shown are six sampling sites (R1 to R6) within the central part of the Grand River in the Waterloo-Kitchener-Cambridge areas. Inset: Twelve effluent transect sites.

4.2.2 DNA extraction and quantitative real-time PCR

DNA was extracted from sediment samples using the MoBio PowerSoil DNA kit (Mo Bio Laboratories, USA) following the manufacturer's protocol. Triplicate samples from each site were independently extracted and maintained as separate biological replicates. Nucleic acids from the Sterivex filters were extracted following a protocol published previously (Neufeld et al., 2007). Due to high homogeneity of the water samples, there were no replicate DNA extractions included. All extracted DNA concentrations were estimated using a NanoDrop spectrophotometer ND-100 (Thermo Fisher Scientific, USA) and a Qubit 2.0 fluorometer (Invitrogen, USA), with a dsDNA-specific dye. Extracts were then diluted to 5 – 10 ng μl^{-1} in preparation for qPCR. The qPCR amplifications were performed on six targeted genes with primer sets and conditions published previously (Table 4.1). The qPCR mix for each reaction contained 5 μl of 2x iQ SYBR Green Supermix (Bio-Rad, USA), 0.3 μM of each primer, 0.3 μM of bovine serum albumin and 5-10 ng of DNA template (1 μl) in total volume of 10 μl . All amplifications were conducted in duplicate on a CFX96 real-time system (Bio-Rad, USA). PCR products generated by individual primers were used as qPCR standard templates. Genomic DNA from *Escherichia coli* was amplified to generate bacterial 16S rRNA standard template. Plasmids with inserts of amplified DNA from an aquarium biofilter (FW27; Sauder et al., 2011) served as template to generate PCR amplicons of AOA 16S rRNA, AOA amoA, and AOB amoA genes for qPCR standards. Environmental samples with high abundance of anammox bacteria and AOB were amplified and pooled to generate anammox bacterial 16S rRNA and AOB 16S rRNA gene standard templates. Each PCR product was purified using a MinElute kit (Qiagen, USA) and quantified by the NanoDrop spectrophotometer ND-100. Ten-fold serial dilutions were performed in a range of 10^1 - 10^7 copies to create a standard curve for each gene. All standard curves were linear with an efficiency of 80 – 98% and R^2 values > 0.995. The specificity of qPCR was confirmed by melt-curve analysis and agarose gel electrophoresis after each run.

Table 4.1 Summary of primers and qPCR conditions used in this study¹.

Primer	Target gene	Amplicon size (bp)	PCR conditions	Reference
341F 518R	Bacterial 16S rRNA	177	5 min at 95°C, follow by 95°C 30 s; 55°C 30 s; 72°C 30 s; plate read at 72°C (x 35)	Muyzer et al., 1993
771F 957R	AOA 16S rRNA	220	5 min at 95°C, follow by 95°C 30 s; 55°C 30 s; 72°C 30 s; plate read at 80°C (x 35)	Ochsenreiter et al., 2003
Amx368F Amx820R	Anammox bacterial 16S rRNA	437	5 min at 95°C, follow by 95°C 45 s; 59°C 45 s; 72°C 45 s; plate read at 72°C (x 35)	Schmid et al., 2003 Schmid et al., 2000
NitA CTO654r	AOB 16S rRNA	518	5 min at 95°C, follow by 95°C 45 s; 57°C 45 s; 72°C 45 s; plate read at 81°C (x 35)	Voytek and Ward 1995 Kowalchuk et al., 1997
amoA1F amoA2R	AOB <i>amoA</i>	491	5 min at 95°C, follow by 95°C 45 s; 55°C 45 s; 72°C 45 s; plate read at 80°C (x 35)	Rotthauwe et al., 1997
crenamoA23F crenamoA166R	AOA <i>amoA</i>	624	5 min at 95°C, follow by 95°C 45 s; 53°C 45 s; 72°C 45 s; plate read at 80°C (x 35)	Tourna et al., 2008

¹Each PCR amplification was completed by a 10 min elongation step at 72°.

4.2.3 RNA extraction, cDNA synthesis and RT-qPCR

RNA extractions from sediment and water samples were conducted using the RNA PowerSoil Total RNA Isolation Kit (Mo Bio Laboratories, USA), following the manufacturer's protocol and the PowerWater Sterivex DNA Isolation Kit (Mo Bio Laboratories, USA) with a minor modification to the manufacturer's protocol to recover extracted RNA. In brief, the extracted Sterivex filters were incubated at 70°C, instead of 90°C, for 5 minutes. Both 1.5 ml of ST4 (kit solution) and 1.5 ml of ethanol, instead of only 3 ml of ST4, were applied to optimize the salt concentration for selective binding of RNA onto the filter membrane, according to steps 8 and 25 of the manual, respectively. Total RNA was eluted with nuclease-free water instead of ST7 (kit solution) in the final step. The quantity and quality of extracted RNA was verified using 1% agarose gel electrophoresis and the Qubit 2.0 fluorometer, with a RNA-specific dye. Reverse transcription and cDNA synthesis were performed following a previously published protocol (Nicol and Prosser 2011). All RT-qPCR amplifications were carried out using the same primer sets and conditions as described in the qPCR section.

4.2.4 DGGE, cloning and sequencing

DGGE of bacterial 16S rRNA, anammox bacterial 16S rRNA, AOB 16S rRNA, and AOA 16S rRNA genes used primer sets and conditions as shown in Table 4.1 except that GC-clamps were attached to 341F-GC, Amx368F-GC, NitA-GC and 957R-GC primers (Muyzer et al., 1993). PCR mixtures contained 2.5 µl of 10x ThermoPol Reaction Buffer, 0.2 µM of each primer, 200 µM of dNTPs, 0.5 U of Taq DNA polymerase, and 5-10 ng of extracted DNA in a total reaction volume of 25 µl. The DGGE gels were run on a DGGEK-2401 (CBS Scientific Company, USA). Bacterial 16S rRNA gene profiles were generated according to a protocol published elsewhere (Moore et al., 2011). All AOP 16S rRNA gene profiles were run on 8% acrylamide gels for 15 h at 85 V and at 60°C, but using different denaturing gradients for each gene. I used 30%-70%, 30%-60% and 35%-70% denaturing gradients for anammox bacteria, AOB and AOA, respectively. The primer set for AOB DGGE (NitA/CTO654r) was originally used for cloning and qPCR (Jin et al., 2011), and the primer set for AOA DGGE (771F/957R) was originally used for qPCR (Ochsenreiter et al., 2003). This study modified the primers by attaching GC-clamps to one primer of each pair to generate AOB and AOA 16S rRNA gene profiles. The same annealing temperature was used for each gene as the original publications; however, the DGGE gradients were optimized for each gene. The DGGE gels were stained with SYBR green (Invitrogen, USA) and scanned with a Pharos FX Plus Molecular Imager

(Bio-Rad, USA). Representative bands were excised, amplified, verified by DGGE, then sequenced at The Center for Applied Genomics (TCAG; ABI 3730XL sequencer).

Four composite cDNA libraries, based on samples pooled by ammonia concentration, were generated to verify the specificity of NitA and CTO654r primers, targeting the AOB 16S rRNA gene. The first library was from five sampling locations along the river where ammonia concentrations were in the range of 0.02 – 0.07 mg TAN L⁻¹, the second library was from the low ammonia concentration sites (0.01 – 0.02 mg TAN L⁻¹), followed by moderate (0.15 – 5.29 mg TAN L⁻¹), and high (8.39 – 9.94 mg TAN L⁻¹) ammonia concentration sites, respectively. The RT-qPCR products were pooled and purified using a MinElute PCR Purification kit (Qiagen, USA). The 3' A-overhangs were subsequently added to the purified RT-qPCR products before ligation and transformation using a TOPO TA Cloning kit and One Shot TOP10 Chemically Competent *Escherichia coli* (Invitrogen, USA), according to manufacturer's protocols. Five white colonies were selected from each library to be sequenced at The Center for Applied Genomics.

4.2.5 Phylogenetic and statistical analysis

All analyzed sequences were searched against GenBank database with BLAST to obtain closely related reference sequences including environmental, isolated, and enriched sequences. All collected sequences were aligned using MUSCLE (Edgar 2004). Phylogenetic trees were constructed using the MEGA package, version 5 (Tamura et al., 2011). Neighbor-joining trees were generated using maximum a composite likelihood model with 1000 bootstrap replications for the 16S rRNA gene based phylogenetic trees of AOP. Bacterial fingerprints within the Grand River were analyzed by Gelcompar II version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) to show the allochthonous and autochthonous river microbial signatures in both sediment and water samples. All bacterial 16S rRNA gene PCR products were randomized on DGGE gels for sediment and water samples. The UPGMA dendrograms were generated using Pearson correlations of background-corrected and normalized densitometric curves. All Spearman ranked correlations were performed using InStat 3 (GraphPad Inc., USA) to show the correlations between DNA and RNA trends for each gene, as well as respective gene abundances and ammonia concentrations.

4.2.6 Nitrification rates

The *in vitro* nitrification incubations were conducted according to a modified protocol (Oishi et al., 2012) with sediment samples from upstream (site upstream2) and downstream (between sites

downstream1 and 2) sites (ammonia concentrations of <0.07 and 8.73 mg TAN L⁻¹, respectively). Three g of sediment were dispensed into 30 ml of sterilized water and mixed, then 200 µl of sediment suspension was transferred into 10 ml of an inorganic freshwater medium (Tourna et al., 2011). The concentration of ammonia (NH₃ + NH₄⁺) was 200 µM and all cultures were set up in triplicate and incubated for 14 days in the dark at room temperature (~25°C). One ml of each sample was collected on days 0, 3, 7, 11, and 14 for chemistry measurements. Ammonia and NO₂⁻/NO₃⁻ analyses were conducted according to established protocols (Poulin and Pelletier 2007; Miranda et al., 2001), using a FilterMax F5 multi-mode microplate reader (Molecular Devices, Canada). Triplicates of original sediment and post-incubation (Day 14) samples were pooled and DNA from one ml of each composite was extracted using the MoBio PowerSoil DNA kit (Mo Bio Laboratories, USA). All downstream qPCR analyses were performed according to the previously described protocol (section 4.4.2).

4.2.7 Nucleotide sequence accession numbers

Nucleotide sequences reported in this study were deposited in GenBank under accession numbers JX025367 – JX025378 for anammox bacterial 16S rRNA genes, JX025402 – JX025431 for AOB 16S rRNA genes, and JX025379 – JX025401 for AOA 16S rRNA genes.

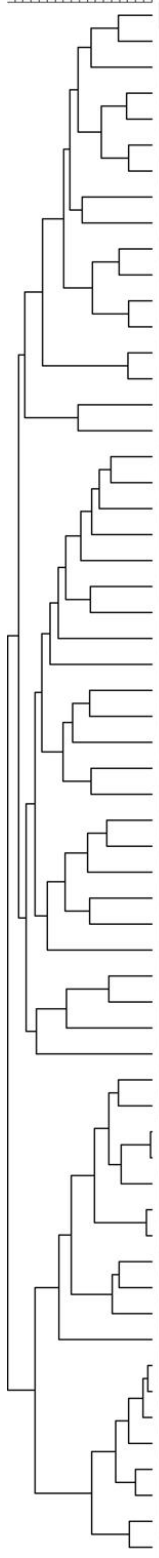
4.3 Results and Discussion

4.3.1 Bacterial communities within the Grand River

Bacterial DGGE fingerprints were generated for all sites and Pearson correlation coefficients among densitometric curves demonstrated that sediment fingerprints clustered distinctly from water sample patterns (Figure 4.2). Fingerprints associated with water samples fell into two clusters, with effluent-impacted water samples clustering distinctly from samples located outside of the effluent plume. Importantly, impacted water column fingerprints clustered with the pattern generated from effluent DNA (“water_x1_pipeline_31.83*”). Conversely, sediment sample fingerprints were dissimilar to those from effluent (Figure 4.2). Nonetheless, sediment bacterial community fingerprints clustered into two main clusters, with most of the sediment samples from effluent-impacted sites grouping together in the first sediment cluster, with the exception of the triplicate site downstream1 samples. Most fingerprints of sediment samples taken from outside of the plume and all background location samples grouped in the second cluster. Sediment sample fingerprints were diverse and replicates grouped poorly overall, suggesting high sample complexity and heterogeneity, respectively.

An important observation from the DGGE data is that patterns of water column bacterial communities can be overwhelmed by molecular signatures from treated effluent itself. Wastewater effluent may also affect the environmental features (i.e., nutrients and pH), providing suitable conditions for specific microbial populations, resulting in a similar communities across impacted sites.

100 90 80 70 60 50 40 30 20 10 0



sediment_x2_downstream8_1.23
sediment_x3_downstream8_1.23
sediment_x3_downstream4_0.15
sediment_x3_downstream2_9.94
sediment_x3_downstream3_8.39
sediment_x2_downstream7_3.89
sediment_x3_downstream7_3.89
sediment_x3_downstream5_5.29
sediment_x3_downstream6_3.63
sediment_x2_downstream5_5.29
sediment_x2_downstream6_3.63
sediment_x2_downstream2_9.94
sediment_x2_downstream3_8.39
sediment_x1_downstream2_9.94
sediment_x1_downstream3_8.39
sediment_x1_downstream8_1.23
sediment_x1_downstream7_3.89
sediment_x1_opposite2_0.02
sediment_x1_opposite1_0.02
sediment_x2_opposite1_0.02
sediment_x3_opposite1_0.02
sediment_x1_downstream4_0.15
sediment_x1_downstream6_3.63
sediment_x3_upstream1_0.01
sediment_x1_R3_0.07
sediment_x1_R4_0.06
sediment_x2_upstream2_0.02
sediment_x2_downstream1_8.86
sediment_x1_upstream2_0.02
sediment_x1_downstream1_8.86
sediment_x3_downstream1_8.86
sediment_x2_opposite2_0.02
sediment_x3_opposite2_0.02
sediment_x1_upstream1_0.01
sediment_x2_upstream1_0.01
sediment_x2_downstream4_0.15
sediment_x3_upstream2_0.02
sediment_x1_R2_0.02
sediment_x1_R1_0.03
sediment_x1_R5_0.03
sediment_x1_R6_0.03
water_x1_R3_0.07
water_x1_R2_0.02
water_x1_upstream1_0.01
water_x1_upstream2_0.02
water_x1_downstream4_0.15
water_x1_opposite2_0.02
water_x1_opposite1_0.02
water_x1_R6_0.03
water_x1_R4_0.06
water_x1_R5_0.03
water_x1_R1_0.03
water_x1_downstream2_9.94
water_x1_downstream3_8.39
water_x1_downstream1_8.86
water_x1_downstream5_5.29
water_x1_downstream6_3.63
water_x1_downstream7_3.89
water_x1_downstream8_1.23
water_x1_pipeline_31.83*

Figure 4.2 DGGE profiles of bacterial communities detected in sediments and water samples. Note that triplicate patterns were generated from replicate sediment samples associated with ammonia transect sites (upstream1 and 2, opposite1 and 2, and downstream1 to 8). The scale bar indicates difference (%) based on Pearson correlations of densitometric curves compared in this unweighted paired group method with arithmetic mean (UPGMA) dendrogram. Sample names are denoted at the right of the figure, beginning with the sample type, followed by the sample replicate number, sampling site and ammonia concentration. For example, sediment_x2_downstream8_1.23 represents the second sediment replicate sample from site downstream8 with a water column ammonia concentration of 1.23 mg TAN L⁻¹. * The ammonia concentration of the pipeline was provided by the Region of Waterloo; it was measured on October 22nd, 2010.

Such a strong effluent “signature” provides both a caution for the interpretation of molecular survey data from wastewater-impacted freshwater environments and the potential for source tracking with molecular “signatures” as a powerful approach for watershed management. Several previous studies used molecular (DNA/RNA based) analyses to examine bacterial communities associated with river environments, including the Danube River, Europe (Winter et al., 2007), Changjiang River, China (Sekiguchi et al., 2002), the Seine River, France (Cébron et al., 2004), Cértima River, Portugal (de Figueiredo et al., 2012), Santa Ana River, USA (Ibekwe et al., 2012), Paraná River, Brazil (Lemke et al., 2009), and Anacostia River, USA (Bushaw-Newton et al., 2011). To my knowledge, no study has yet used these techniques to directly address the impact of wastewater treatment systems on perceived riverine bacterial communities as I have done here.

Bacterial 16S rRNA genes were quantified for each DNA extract to ensure that sample composition (e.g. bacterial versus eukaryotic DNA) did not account for any subsequently reported differences in relative gene abundance for AOP communities. The results demonstrated that bacterial 16S rRNA gene copies in sediment and water samples were consistent when reported as copies per ng DNA (Table 4.2). In order to complement DNA-based techniques that represent the abundance of AOP DNA, an RNA-based approach was also used to reflect the abundance of viable cells. As with the DNA-based approach, total bacterial 16S rRNA copies per ng RNA were relatively constant across all sampling sites (Table 4.2). Note that biases from DNA/RNA quantifications may affect the calculations for nucleic acids per ng of extracted DNA/RNA. This may account for a 10-fold difference of bacterial 16S rRNA gene abundances in sediment and water samples.

Table 4.2 Abundance of bacterial DNA and RNA based on qPCR of bacterial 16S rRNA genes.

site ID	Abundance of bacterial DNA (copies per ng of extracted DNA)		Abundance of bacterial RNA (copies per ng of extracted RNA)	
	sediment	water	sediment	water
R1	$(5.8 \pm 0.8) \times 10^5$	$(5.1 \pm 0.1) \times 10^7$	$(5.4 \pm 0.2) \times 10^7$	$(8.1 \pm 0.2) \times 10^7$
R3	$(5.8 \pm 0.7) \times 10^5$	$(5.4 \pm 0.2) \times 10^7$	$(9.8 \pm 1.3) \times 10^7$	$(1.2 \pm 0.3) \times 10^8$
R4	$(3.9 \pm 0.9) \times 10^5$	$(6.8 \pm 0.6) \times 10^7$	$(2.0 \pm 0.2) \times 10^7$	$(1.3 \pm 0.2) \times 10^8$
R5	$(4.6 \pm 0.3) \times 10^5$	$(5.1 \pm 0.1) \times 10^7$	$(5.1 \pm 0.0) \times 10^7$	$(1.0 \pm 0.0) \times 10^8$
R6	$(6.3 \pm 0.9) \times 10^5$	$(5.1 \pm 0.1) \times 10^7$	$(7.4 \pm 1.1) \times 10^7$	$(9.1 \pm 0.4) \times 10^7$
upstream1	$(5.0 \pm 0.1) \times 10^5$	$(5.5 \pm 0.9) \times 10^7$	$(1.9 \pm 0.1) \times 10^7$	$(8.3 \pm 1.3) \times 10^7$
upstream2	$(9.2 \pm 1.2) \times 10^5$	$(5.1 \pm 0.2) \times 10^7$	$(7.1 \pm 1.0) \times 10^7$	$(4.9 \pm 1.8) \times 10^7$
opposite2	$(6.1 \pm 0.3) \times 10^5$	$(3.8 \pm 0.1) \times 10^7$	$(3.7 \pm 0.4) \times 10^7$	$(9.2 \pm 0.1) \times 10^7$
opposite1	$(6.2 \pm 0.7) \times 10^5$	$(6.2 \pm 0.2) \times 10^7$	$(4.6 \pm 0.2) \times 10^7$	$(7.0 \pm 0.9) \times 10^7$
downstream4	$(6.2 \pm 0.8) \times 10^5$	$(5.0 \pm 0.5) \times 10^7$	$(4.8 \pm 0.4) \times 10^7$	$(8.4 \pm 0.4) \times 10^7$
downstream8	$(5.8 \pm 0.5) \times 10^5$	$(7.0 \pm 0.3) \times 10^7$	$(4.9 \pm 0.1) \times 10^7$	$(5.4 \pm 0.5) \times 10^7$
downstream6	$(7.0 \pm 0.3) \times 10^5$	$(1.9 \pm 0.0) \times 10^8$	$(3.0 \pm 1.3) \times 10^7$	$(6.8 \pm 3.4) \times 10^7$
downstream7	$(5.9 \pm 0.6) \times 10^5$	$(1.4 \pm 0.2) \times 10^8$	$(3.0 \pm 0.4) \times 10^7$	$(7.0 \pm 0.3) \times 10^7$
downstream5	$(7.4 \pm 0.9) \times 10^5$	$(1.1 \pm 0.0) \times 10^8$	$(4.0 \pm 0.5) \times 10^7$	$(8.0 \pm 0.5) \times 10^7$
downstream3	$(6.8 \pm 2.4) \times 10^5$	$(8.4 \pm 0.0) \times 10^8$	$(5.9 \pm 0.0) \times 10^6$	$(4.8 \pm 1.6) \times 10^7$
downstream1	$(7.9 \pm 2.9) \times 10^5$	$(1.1 \pm 0.0) \times 10^8$	$(1.0 \pm 0.3) \times 10^7$	$(6.4 \pm 0.2) \times 10^7$
downstream2	$(7.0 \pm 1.2) \times 10^5$	$(2.8 \pm 0.2) \times 10^8$	$(5.5 \pm 0.5) \times 10^6$	$(3.9 \pm 0.2) \times 10^7$
pipeline	-	$(2.7 \pm 0.2) \times 10^8$	-	$(9.6 \pm 1.8) \times 10^7$

4.3.2 Relative abundance and activities of AOP in river sediment

The abundance of AOP was quantified in river sediment extracts by targeting all AOP 16S rRNA, and AOB and AOA *amoA* genes. All AOP 16S rRNA (RNA) and 16S rRNA gene (DNA) qPCR signals showed a positive correlation (AOA: $r = 0.81$, $P < 0.001$; AOB: $r = 0.83$, $P < 0.001$; anammox: $r = 0.54$, $P = 0.02$). Both AOB *amoA* and 16S rRNA gene qPCR signals correlated significantly across all sampling sites ($r = 0.85$, $P < 0.001$). Based on AOB 16S rRNA gene abundances, sediment AOB increased within the effluent plume, correlating with ammonia concentrations (Figure 4.3; $r = 0.89$, $P < 0.001$). Both AOA *amoA* and 16S rRNA genes showed a positive correlation ($r = 0.63$, $P = 0.007$). In contrast to AOB, the abundance of sediment AOA decreased in effluent-impacted samples of high ammonia concentration (Figure 4.3), with a significant negative correlation between sediment AOA 16S rRNA gene abundance and ammonia concentration ($r = -0.66$, $P = 0.004$).

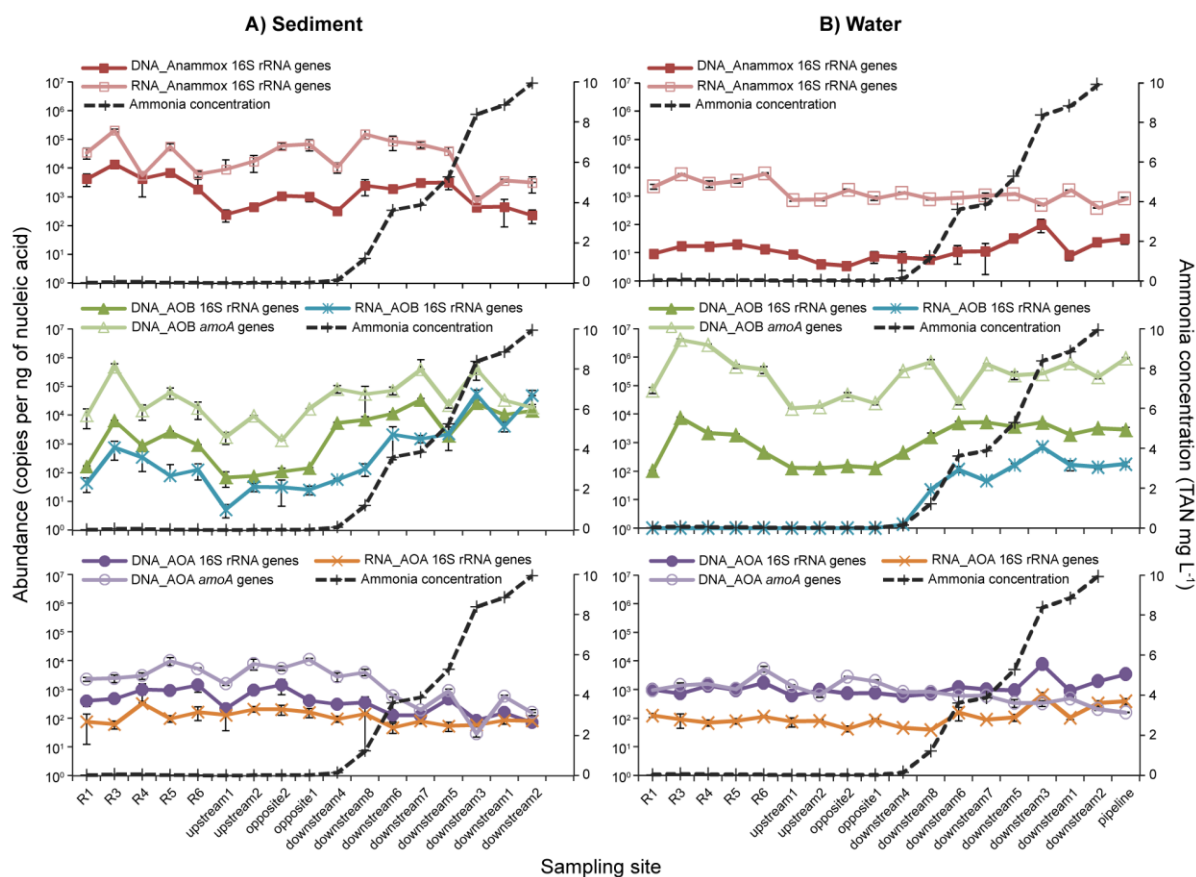


Figure 4.3 Abundance of AOP in both DNA and RNA extracted from sediment (A) and water samples (B) across an ammonia gradient along the Grand River. The ammonia concentration of the effluent pipeline was provided by the Region of Waterloo; the ammonia concentration measured on October 22nd, 2010 was 31.83 mg TAN L⁻¹ and the average of the month was 36.13 mg TAN L⁻¹.

Importantly, converting sediment 16S rRNA gene abundances of AOP DNA to relative abundances (Figure 4.4A) showed that anammox bacteria and AOA were dominant across the sample sites with low ammonia concentrations (< 0.07 mg TAN L⁻¹) and the proportion of both AOA and anammox bacterial genes decreased across the sampling sites within the WWTP plume proportional to AOB. These results from temperate river sediments are consistent with recent research demonstrating a positive correlation between anammox bacteria and AOA in mangrove sediments, with abundances affected by ammonia input (Li et al., 2010a). Two available AOA pure cultures showed a low half-saturation constant (K_s) for ammonia uptake, which supports the predominance of

AOA in oligotrophic environments (Hatzenpichler et al., 2008; Martens-Habbena et al., 2009). The AOB relative abundances increased in sediment samples taken from within the WWTP plume (>0.15 mg TAN L⁻¹), reaching more than 94% of AOP genes detected at sites downstream1, 2, and 3, with ammonia concentrations of $\sim 8 - 10$ mg TAN L⁻¹. These findings agree with previous reports suggesting that high ammonia concentrations favor the growth of AOB rather than AOA in terrestrial (Verhamme et al., 2011; Adair and Schwartz 2011), aquatic (Herrmann et al., 2011), and engineered (Sauder et al., 2011 and 2012; Sonthiphand and Limpiyakorn 2011) environments.

Analyzing rRNA reflects viable cells and suggests potential contributions to ammonia oxidation from each microbial group. However, care should be taken when interpreting 16S rRNA gene data. The concentration of rRNA and growth rate are not always linear, the relationship of rRNA concentration and growth rate are different among taxa, and dormant cells can contain high rRNA concentrations (Blazewicz et al., 2013). The overall qPCR gene abundances from sediment DNA and RNA of the five reference sites were similar to sites located upstream and on the opposite side of the river, distinct from three sites (downstream1 to 3) directly within the effluent plume. The proportions of AOA rRNA in sediments were relatively low, with a maximum of $\sim 23\%$ at upstream site, and they were extremely low (always less than 3%) across sampling sites within the WWTP plume (Figure 4.4C). Conversely, anammox bacteria and AOB were proportionally abundant across sediment sample sites in this study. Anammox bacteria and AOB accounted for more than 77% of AOP rRNA at all sites. Especially at sites within the effluent plume (sites downstream1 to 8), the proportions of anammox bacteria and AOB rRNA were more than 97% of AOP rRNA (Figure 4.4C). The coexistence of anammox bacteria and AOB has been reported previously for the Black Sea (Lam et al., 2007), the Peruvian OMZ (Lam et al., 2009), the Namibian OMZ (Woebken et al., 2007), a fertilized paddy soil (Zhu et al., 2011a), and a constructed wetland (Zhu et al., 2011b). Others have suggested that aerobic ammonia oxidation can provide NO₂⁻ for the anammox reaction at the oxic-anoxic interface. Anammox bacteria may then use this intermediate metabolite to form a nitrification-anammox coupled process (Lam et al., 2007 and 2009; Meyer et al., 2005).

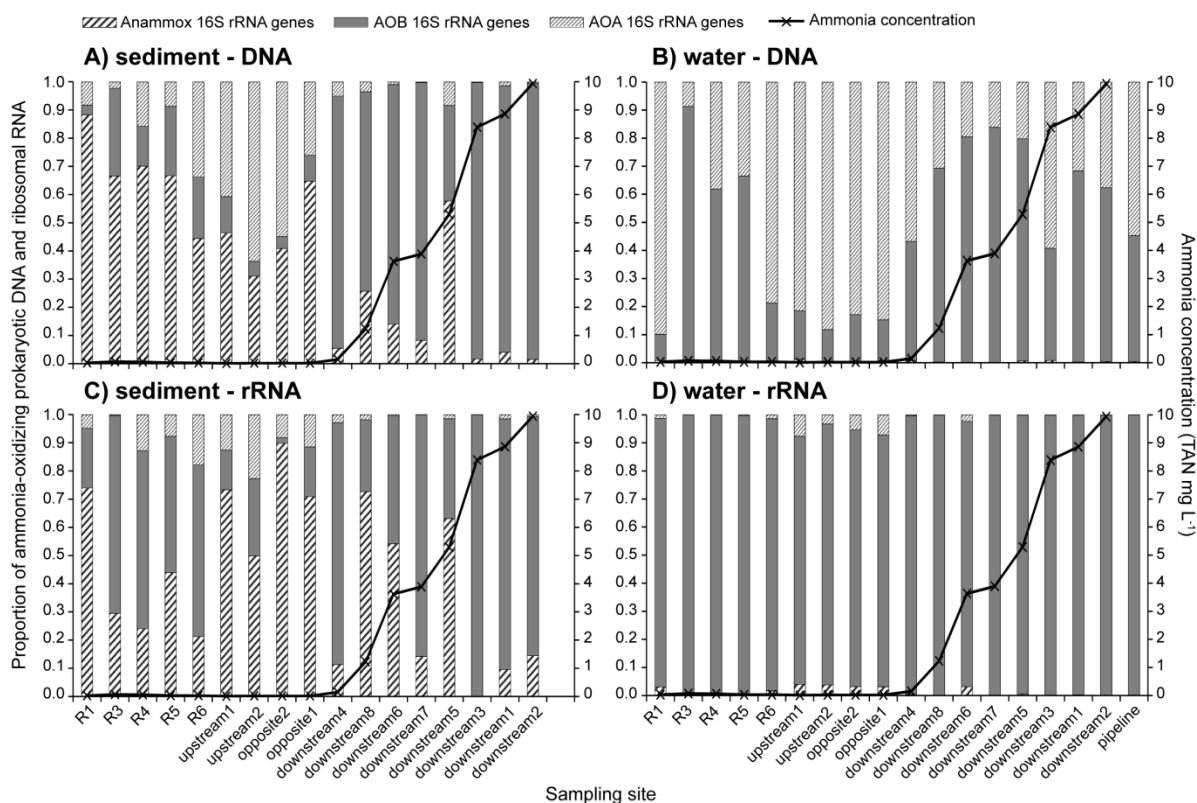


Figure 4.4 Proportion of AOP 16S rRNA genes for each sampling site arranged by increasing ammonia concentration from 0.01 to 9.94 mg TAN L⁻¹. The ammonia concentration of the effluent pipeline was provided by the Region of Waterloo; the ammonia concentration measured on 22nd October 2010 was 31.8 mg TAN L⁻¹ and the average of the month was 36.1 mg TAN L⁻¹. (A) DNA ratios of AOP in sediments, (B) DNA ratios of AOP in water samples and effluent pipeline, (C) rRNA ratios of AOP in sediments, (D) rRNA ratios of AOP in water samples and effluent pipeline.

The follow-up sediment samples were collected for nitrification rate experiments to demonstrate potential AOP activities in both upstream and downstream sediments. The results showed ammonia depletion and NO₂⁻+NO₃⁻ accumulation, indicating ammonia-oxidizing activity within upstream sediment (Figure 4.5A). Ammonia-oxidation was higher for downstream sediment samples: ammonia decreased from day 0 to day 7 and was depleted below detection limits by day 14 (Figure 4.5B). The NO₂⁻+NO₃⁻ concentrations increased until day 7 and were stable subsequently. No NO₂⁻ accumulation was observed, possibly due to the presence of nitrite oxidizing bacteria (NOB) in downstream sediments. The co-occurrence of AOB and NOB was reported in the wastewater

effluents and they colonized biofilm in the receiving waters (Mußmann et al., 2013). The *in vitro* incubations used here differ from in-river conditions and, as a result, only reflect potential ammonia oxidation rates. Nonetheless, I expect that the strong response of AOB to high ammonia incubations will be very similar to the response of sediment communities impacted by ammonia-rich wastewater effluent.

Sediment samples from upstream and downstream sites, together with 14-day-post-incubation samples were analyzed by qPCR to measure the relative abundances of AOB and AOA 16S rRNA genes before and after incubation. The resulting qPCR data indicated that although AOA dominated upstream sediment (Figure 4.5C), AOB 16S rRNA genes became dominant for upstream sediment enrichments at 200 μ M ammonia. For downstream sediment with higher initial activity, AOB outnumbered AOA in both the original sediment and 14-day-post-incubation DNA extracts (Figure 4.5C). Activity assays, together with DNA and rRNA data indicated that AOB were the dominant ammonia oxidizers in downstream sediment.

This current study represents the first discovery of the coexistence and metabolic activity of AOP within freshwater sediment. I hypothesize that AOB and AOA inhabit the surface of the sediment and exposed biofilm layers whereas anammox bacteria likely exist at the anoxic interface or deeper within the sediment biofilm, depending on sediment oxygen penetration. Alternatively, diel O₂ cycles might affect microbial N transformations in the same sediment regions, separated in their activities by time. Variable oxygen gradients within sediments possibly contribute to the cohabitation of multiple populations with complementary metabolism.

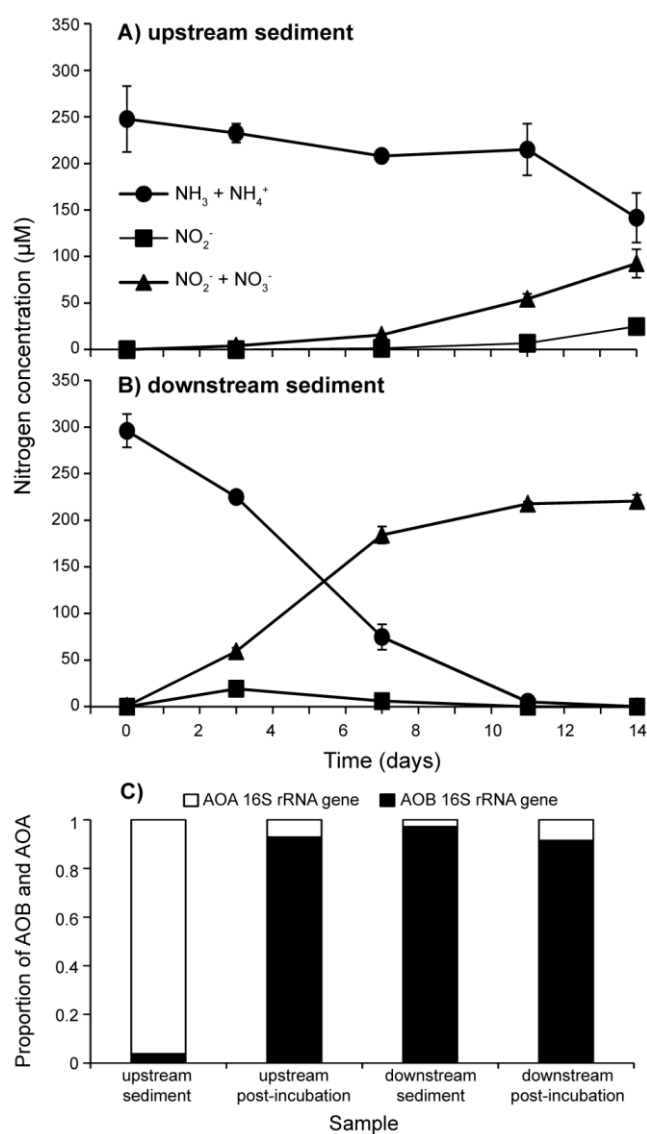


Figure 4.5 Nitrification activity of aerobic ammonia oxidizers in upstream (A) and downstream (B) sediment. Standard deviation (SD) was calculated from triplicate samples. The relative AOB and AOA 16S rRNA gene abundances are shown for the original sediment and 14-day-post-incubation composite samples (C).

4.3.3 Relative abundance and activities of AOP in the water column

In addition to five reference locations and twelve ammonia-transect sites, wastewater effluent (“pipeline”) discharged from the Waterloo WWTP was included in an analysis of water column DNA extracts. As mentioned earlier, the DNA and rRNA levels of total bacterial 16S rRNA genes were quantified and relatively stable across the water sample nucleic-acid extracts (Table 4.2). Similar to the sediment analysis, there was a strong positive correlation between the DNA and rRNA levels of anammox bacteria and AOB ($r = 0.82$, $P < 0.001$). The AOB 16S rRNA gene abundance and ammonia concentrations showed a positive correlation ($r = 0.73$, $P = 0.001$). Although both AOB *amoA* and 16S rRNA genes agreed significantly with each other across all samples ($r = 0.59$, $P = 0.01$), AOB *amoA* genes were below the detection limit (< 2 copies per ng of genomic DNA) at all sites with low ammonia concentration (< 0.07 mg TAN L⁻¹). Melt curves and agarose gel electrophoresis confirmed that qPCR signals were below the detection limit. Surprisingly, there was a negative correlation between AOA DNA and rRNA (Figure 4.3; $r = -0.41$, $P = 0.09$). The DNA levels of AOA 16S rRNA genes and ammonia concentrations were not significant ($r = 0.37$, $P = 0.14$), while there was a strongly negative correlation between the rRNA levels of AOA 16S rRNA genes and ammonia concentrations ($r = -0.80$, $P < 0.001$; Figure 4.3). The results imply that AOA were less active when water column effluent and ammonia concentrations increased, despite their DNA being abundant across the sampling sites within the WWTP plume. The DNA levels of both AOA *amoA* and 16S rRNA genes were consistent ($r = 0.75$, $P < 0.001$), indicating that rRNA analysis was essential for revealing a discrepancy between DNA-based abundance and corresponding metabolic activity.

The proportions of AOP DNA showed very low numerical abundance of anammox bacteria in water column samples compared to the corresponding sediment samples. Anammox bacteria accounted for less than 2% of AOP genes across all water samples (Figure 4.4B). Dissolved oxygen (DO) concentrations in the range of 7.13 – 12.51 mg L⁻¹ was associated with fewer anammox genes across the sampling sites (Table 4.3). Anammox bacteria are commonly associated within marine OMZs (Lam et al., 2007; Thamdrup et al., 2006; Hamersley et al., 2007) and enrichment culture studies confirm reversible inhibition of anammox activity by oxygen (Jetten et al., 1997; Strous et al., 1997). Anammox bacteria detected in the water columns probably originated in sediment and would potentially be active only under specific conditions, such as during diel oxygen minima. Overall, the results suggest that anammox bacteria are unlikely to contribute substantially to ammonia oxidation in the water column of the Grand River.

Table 4.3 Chemistry of water samples collected for this study.

Site ID	Conductivity (μS)	$\text{NH}_3 + \text{NH}_4^+$ (mg TAN L^{-1})	NO_2^- ($\mu\text{g N-NO}_2^- \text{L}^{-1}$)	DO (mg L^{-1})	pH
upstream1	464	0.01	14	9.56	8.13
upstream2	493	0.02	13	9.81	8.61
opposite1	472	NA	NA	12.51	8.79
opposite2	472	0.02	13	12	8.81
downstream1	981	8.86	302	8.77	8.01
downstream2	988	9.94	344	8.64	8.14
downstream3	943	8.39	336	8.64	8.15
downstream4	554	0.15	25	9.16	8.16
downstream5	849	5.29	306	8.72	8.14
downstream6	850	3.63	355	7.13	7.89
downstream7	853	3.89	351	7.52	8.02
downstream8	650	1.23	189	10.42	8.49

NA = Not Available

Unlike anammox bacteria, AOB and AOA genes were well represented in DNA extracts from water samples. Together, AOB and AOA represented more than 98% of AOP DNA across the water samples (Figure 4.4B). AOA represented more than 80% of the AOP DNA of water samples from upstream (sites upstream1 and 2) and outside of the WWTP plume (sites opposite1 and 2), representing ammonia concentrations below $0.02 \text{ mg TAN L}^{-1}$. AOA gene relative abundances decreased within the WWTP plume compared to those of AOB, likely corresponding to WWTP effluent input DNA and cells. Even though both AOB and AOA were dominant in the AOP DNA level, the majority of AOP rRNA detected across water samples was from AOB, which accounted for more than 88% (Figure 4.4D). Although AOA were dominant in DNA extracts from low ammonia concentration sites, AOA represented a substantially lower contribution to AOP rRNA (sites upstream1 and 2, and opposite1 and 2). Five reference sites along the river showed results that were consistent overall with the other transect samples, with respect to gene abundances associated with both DNA and RNA extracts.

Interestingly, the abundance of AOB and AOA were almost equal in the WWTP effluent based on 16S rRNA (Figure 4.4B). However, only AOB were dominant in the rRNA (Figure 4.4D). Chlorination treatment of the WWTP effluent is intended to destroy microorganisms prior to discharge of treated wastewater effluent. The results suggest that AOB were either still viable or their RNA escaped degradation by chlorine treatment.

Together, the quantitative DNA and RNA data indicate that AOB were the dominant prokaryotes catalyzing ammonia oxidation within the Grand River water column, but these results were difficult to interpret due to the strong influence of wastewater effluent on bacterial community DGGE patterns and the apparent contribution of effluent AOB DNA and rRNA to water column AOB nucleic acids (Figures 4.4B and 4.4D). Although the detection of AOB rRNA in WWTP effluent suggests metabolically active cells, I cannot rule out rRNA persisting despite chlorination. Regardless, previous reports suggest that AOB are most active in high ammonia terrestrial environments (Jia and Conrad 2009) and marine sediments (Cao et al., 2012; Schippers and Neretin 2006). This study provides initial evidence for active AOB within the water column of a river both upstream and downstream of a WWTP plume.

4.3.4 Communities of AOB within the Grand River

Anammox bacterial genes were abundant in sediment samples, based on qPCR and RT-qPCR results. Consequently, only sediment samples were investigated for communities of anammox bacteria using PCR, DGGE, and sequencing. The anammox bacterial DGGE profiles showed a single dominant band with different band intensity across sediment samples (Figure 4.6); the modified DGGE primer with a GC-clamp may affect amplification efficiency. Twelve representative bands were selected to sequence. All twelve band sequences were identical to uncultured anammox bacterial 16S rRNA gene sequences recovered from freshwater environments from the Yangtze Delta (GQ504007) and Songhua Delta (GU084030). Phylogenetic analysis revealed that all anammox bacterial sequences retrieved from Grand River sediments were *Candidatus* Brocadia-like phylotypes (Figure 4.7). The prevalence of Brocadia-like sequences in this study is consistent with other freshwater environments such as river sediment (Zhang et al., 2007), lake (Yoshinaga et al., 2011), and groundwater (Moore et al., 2011; Robertson et al., 2011). Although anammox communities in the water column were not assessed explicitly in this study, communities in water column and sediment samples were analyzed previously (Sonthiphand and Neufeld 2013). Because the majority of anammox bacteria detected in the water column cluster within the *Ca.* Brocadia genus, there were likely no distinctive water-column anammox bacteria associated with the Grand River sites.

The DGGE fingerprints of AOB communities showed that AOB patterns from sediment samples were more diverse than those in water samples (Figure 4.6). All analyzed sequences obtained from DGGE bands fell into AOB clades associated with *Betaproteobacteria* (Figure 4.8). All AOB sequences showed 98 – 99% identity to previously published AOB sequences recovered from

environmental samples. In sediments, AOB were related to *Nitrosomonas oligotropha*, *Nitrosospira* spp., *Nitrosomonas communis*, and *Nitrosococcus mobilis* clusters (Figure 4.8). The DGGE profiles of AOB from the water samples and effluent showed only one major band, related to the *Nitrosomonas oligotropha* cluster (Figure 4.6). Consequently, this single band could not be used to differentiate between autochthonous and allochthonous in-river microbial signatures. Within this experimental context, I consider an autochthonous community to be native river microorganisms that are not a result of anthropogenic input. Upstream sites (upstream1 and 2), opposite side of the river (opposite1 and 2), and five sites along the river (R1, R3, R4, R5, and R6) were treated as primarily autochthonous river signatures (Figure 4.1). I consider the strong molecular signatures from downstream sites (downstream1 to 8), impacted by wastewater effluent, as representing an allochthonous community.

In addition to DGGE band sequences, composite cDNA clone libraries of AOB 16S rRNA genes were generated to confirm primer specificity. The RT-qPCR products were pooled based on ammonia concentration to construct four libraries (L1 – L4). Although only a small number of sequences were obtained, DGGE and cDNA sequences were consistent (Figure 4.8). Previous research suggests that *N. oligotropha*, *N. communis*, and *Nitrosospira* spp. clusters associate with low ammonia systems, while the *N. mobilis* cluster is generally associated with high ammonia conditions (Sonthiphand and Limpiyakorn 2011). All detected AOB clusters have also been found in other freshwater habitats (Speksnijder et al., 1998; Koops and Pommerening-Röser 2001). The *Nitrosospira* cluster dominates in soils (Koops and Pommerening-Röser 2001) and one sediment AOB sequence was affiliated with this group. Furthermore, BLAST results indicated that all AOB sequences in the water columns and some associated with sediment samples showed 98 – 100% identity to AOB sequences recovered from heavy fractions of a stable-isotope probing (SIP) experiment (Avrahami et al., 2011). Overall, these AOB sequencing results complement RT-qPCR results, which potentially indicate important AOB contributions to ammonia oxidation in both subsurface sediments and water columns.

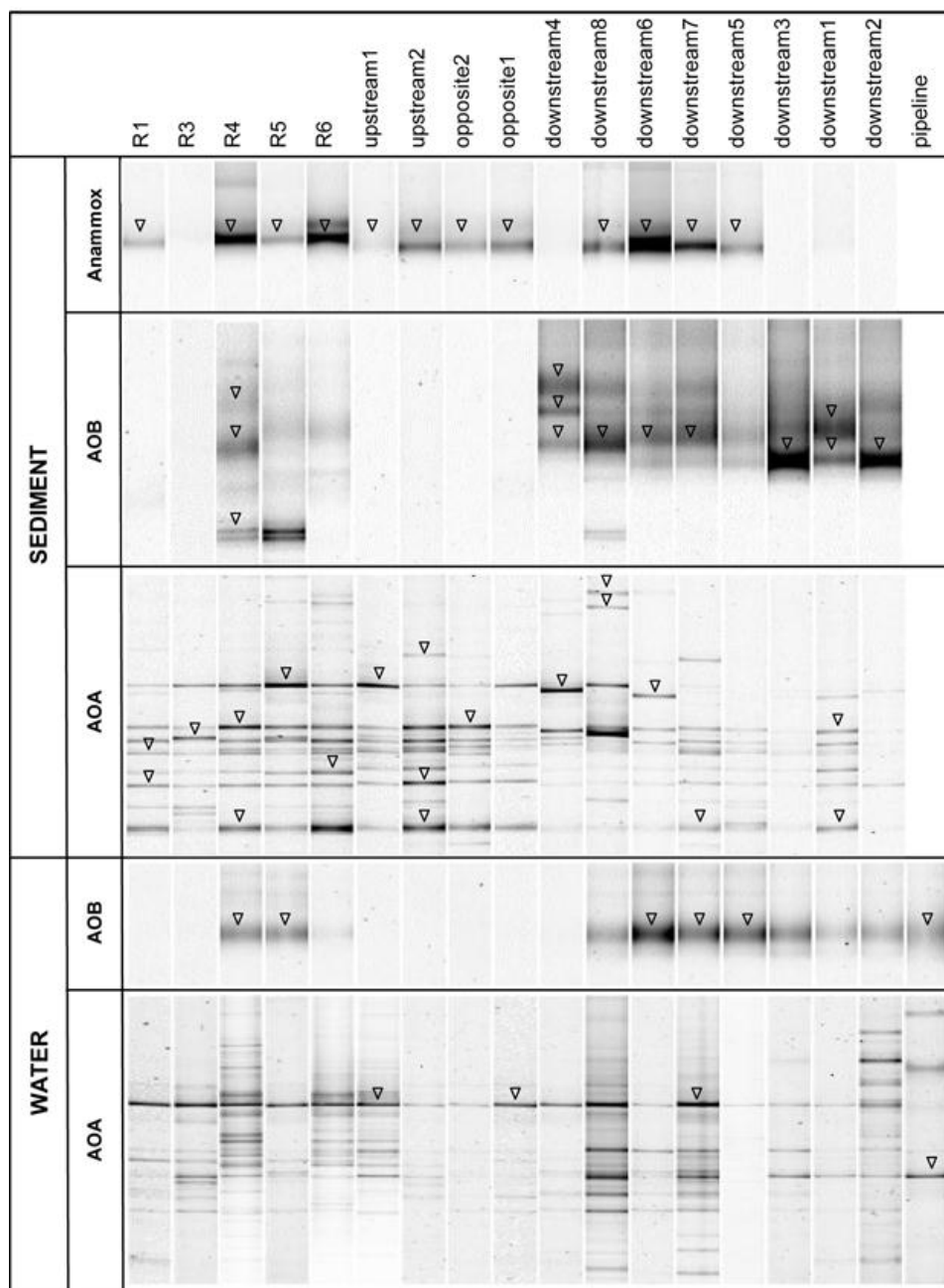


Figure 4.6 DGGE profiles of AOP in sediment and water samples. Triangles indicated analyzed sequences shown in phylogenetic trees (Figures 4.7, 4.8 and 4.9). For those samples with more than one band, the number of bands was ordered numerically from top to bottom, respectively, and was illustrated at the end of sample names in each tree.

The AOA 16S rRNA gene diversity in sediment and water samples was profiled by DGGE. The results demonstrated that AOA patterns from sediment were different than those in the water, based on assigned external and internal DGGE bands in the Gelcompar program (Figure 4.6), and the effluent pipeline patterns differed from all other patterns. Representative bands were selected from the sediment and water samples. The AOA sequences were broadly distributed throughout the phylogenetic tree with other representative AOA 16S rRNA sequences (Figure 4.9) belonging to I.1a, I.1b, and ThAOA clusters. AOA recovered from the sediments and pipeline showed 99 – 100% identity to previously published sequences recovered from various environments such as soil, marine, freshwater, and an anaerobic reactor. Two bands, sediment_R4_1 and sediment_opposite2_1, were at the same position on the DGGE gel, but fell into different AOA groups (Figure 4.9). BLAST results supported this finding because the two band sequences were identical to previous environmental sequences retrieved from cropland soil and a lake, respectively. On the other hand, the DGGE bands of sediment_R4_2, sediment_upstream2_3, sediment_downstream7_1, and sediment_downstream1_2 were at the same position and they grouped together in the tree (Figure 4.9). Consequently, band position and sequence identity were somewhat independent for the AOA 16S rRNA DGGE data. Although many bands were associated with sediment sample fingerprints, one major AOA band appeared across all water samples yet was not detected in patterns from the pipeline effluent (Figure 4.6). All three representative bands from the water samples fell into group I.1a and were identical to previously reported sequences retrieved from marine environments. However, the pipeline band was identical to sequences retrieved from an anaerobic reactor, and was phylogenetically distinct from native AOA found in the Grand River (Figure 4.9). This pipeline band appeared across the downstream sites (downstream1 to 3) located within the WWTP plume (Figure 4.6). Unlike AOB, the water AOA profiles distinguished between autochthonous and allochthonous AOA communities within this impacted river site. Many studies on AOA diversity in freshwater have found that freshwater AOA are also affiliated with group I.1a (Auguet and Casamayor 2008). In this study, AOA recovered from subsurface river water and sediments did not fall within a single unique cluster. Previous reports have also suggested that archaeal communities in freshwater habitats are more diverse than those in soil and marine environments (Galand et al., 2006; Auguet et al., 2010).

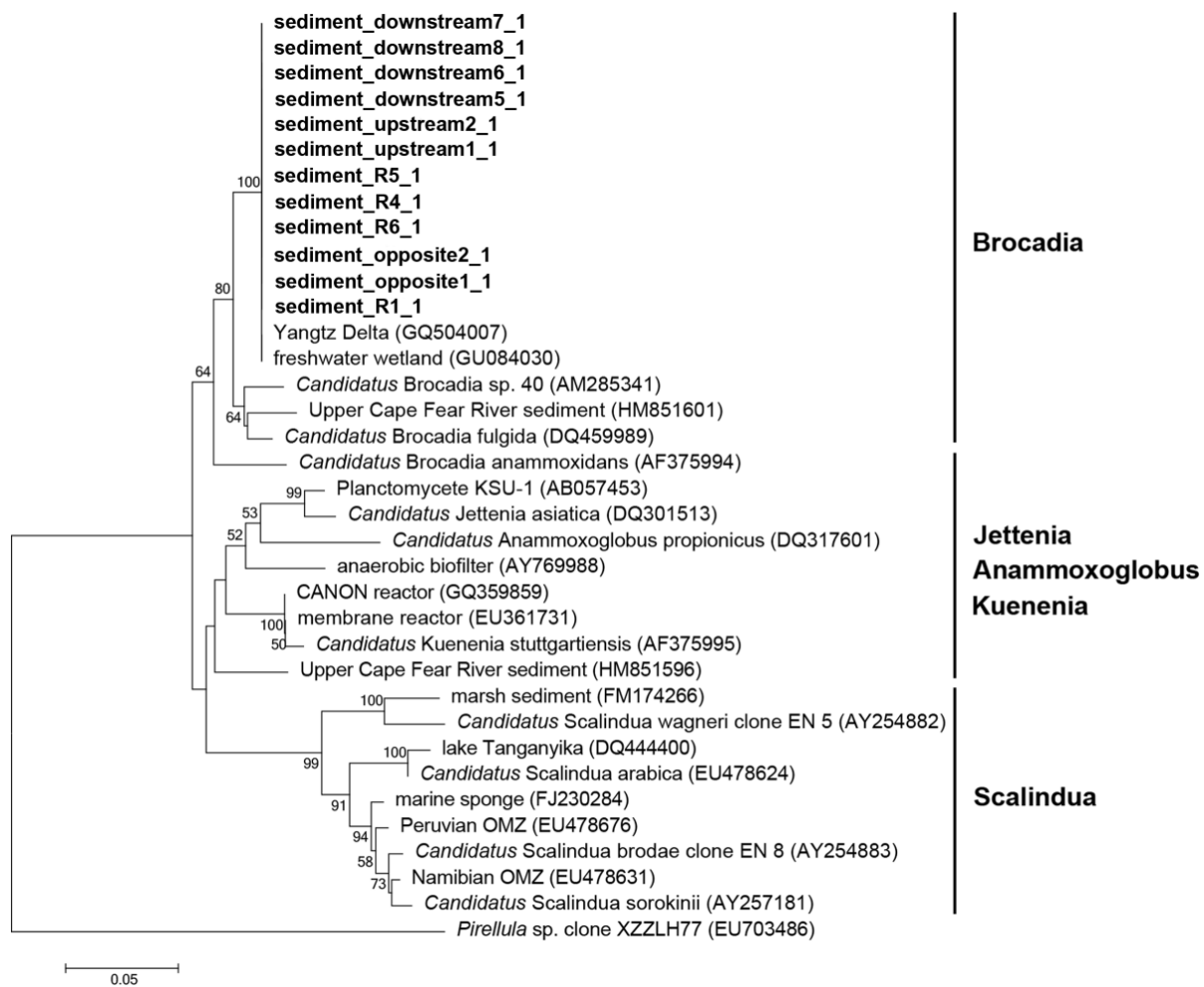


Figure 4.7 Phylogenetic analysis of anammox bacterial 16S rRNA genes obtained from sediment samples.

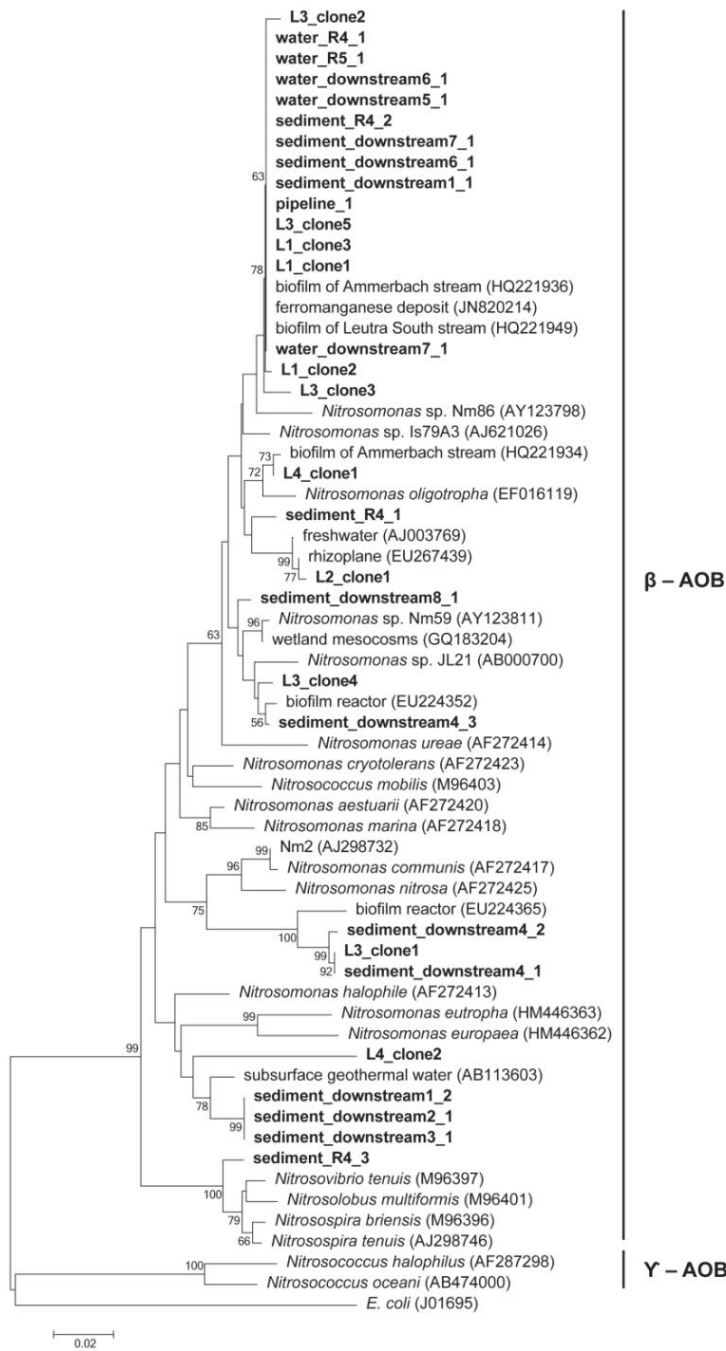


Figure 4.8 Phylogenetic analysis of AOB 16S rRNA genes retrieved from sediment and water samples. Sequences associated with this study are shown in bold. L1, L2, L3 and L4 were composite clone libraries, constructed from five reference sampling sites, sites upstream1, 2 and opposite1, 2, sites downstream4 to 8, and sites downstream1 to 3 (including pipeline effluent), respectively.



Figure 4.9 Phylogenetic analysis of AOA 16S rRNA genes retrieved from sediment and water samples.

4.4 Conclusions

Anthropogenic input such as WWTP effluent can complicate molecular approaches used for investigating microbial communities in natural environments, yet combined molecular approaches can help disentangle allochthonous from autochthonous microorganisms, revealing abundant and active autotrophic prokaryotes involved in oxidizing ammonia. This finding suggests cautious interpretations of microbial community data are required for both general assessments of beta diversity (e.g., bacterial DGGE) and for focused analyses of specific population abundances (e.g., qPCR of AOP) for effluent-impacted river environments. Despite the potential for effluent-influenced community profiles, there are potential advantages to the observation of strong effluent signatures. For example, tracking inputs represents a potential advantage for a form of “modified source tracking” for disentangling molecular signatures of both autochthonous and allochthonous microbial communities within impacted rivers. Future research should investigate additional WWTP plumes within the Grand River to better assess the influence of upstream inputs on investigations of in-river microbial communities. The resulting abundance and activity of AOP suggest that AOP contribute to N removal depending on their specific-niche partitioning, which was observed primarily as a sediment-water column partitioning in this study. Both aerobic and anaerobic ammonia oxidation are likely important processes for ammonia removal within the Grand River sediment, reflecting possible nitrification-anammox coupled processes. Unlike sediment samples, aerobic ammonia oxidation is the key process within the water column. AOB are likely the dominant ammonia oxidizers in water columns along the river, including upstream and downstream of the WWTP plume even though water AOB signatures from both allochthonous and autochthonous microbial communities are the same. AOA were the most diverse AOP in this freshwater environment yet their activity may have been inhibited by effluent based on differential DNA and RNA signals. Water column AOA fingerprints of both the pipeline effluent and “background” samples could be distinguished. Additional information on AOP activities within impacted rivers is essential to confirm their relative contributions to ammonia oxidation.

Chapter 5⁴

Nitrification and urea hydrolysis

5.1 Introduction

Nitrification is composed of two oxidative processes. The first step is ammonia oxidation to nitrite under oxic conditions, which is mediated by ammonia-oxidizing bacteria (AOB) and archaea (AOA). This process is followed by nitrite oxidation to nitrate, which is mediated by aerobic nitrite-oxidizing bacteria (NOB). High ammonia loads in impacted rivers adversely affect drinking water quality, aquatic life, and ecosystem health. Ammonia oxidation is an important process, removing ammonia from impacted freshwater environments (Sonthiphand et al., 2013). For many decades, ammonia-oxidizing bacteria (AOB) were believed to be the sole microorganisms responsible for ammonia oxidation, until the discovery and isolation of ammonia-oxidizing archaea (AOA; Könneke et al., 2005). Although both AOB and AOA have an ability to oxidize ammonia, each has unique physiological properties (i.e., enzyme structure, intermediates, and substrate affinity; Zhalnina et al., 2012), evidenced by differential sensitivities to nitrification inhibitors. Distinct ammoniacal N sources might also differentially affect the relative environmental contributions of AOB and AOA. Previous studies demonstrated AOB to be more abundant and active than AOA in soils amended with inorganic fertilizer (Jia and Conrad 2009; Xia et al., 2011). Systems treated with organic fertilizer (i.e., urea and animal urine substrate) were also dominated by AOB (Di et al., 2009; O'Callaghan et al., 2010; Di and Cameron 2011). However, AOA utilized ammonia from the mineralization of organic substances and outnumbered AOB in soil microcosms (Gubry-Rangin et al., 2010; Zhang et al., 2010).

Ammonia monooxygenase (AMO) is a key enzyme for ammonia oxidation by both AOA and AOB. However, urease represents an optional enzyme for ammonia oxidizers to hydrolyze urea as an alternative N energy source, discovered in some AOB and AOA species. Urease genes have been reported for *Nitrosomonas ureae*, *Nitrosomonas nitrosa*, *Nitrosomonas oligotropha*, *Nitrosomonas marina*, and *Nitrosomonas aestuarii* (Pommerening-Röser and Koops 2005). Although only two

⁴ A version of this chapter has been published as:
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AOA pure cultures are available, genome analysis revealed the presence of a urease gene within *Candidatus* Cenarchaeum symbiosum (Hallam et al., 2006), *Nitrososphaera viennensis* (Tourna et al., 2011), *Candidatus* Nitrososphaera gargensis (Spang et al., 2012), and *Candidatus* Nitrosopumilus salaria (Mosier et al., 2012). Consequently, particular AOB and AOA species may generate ammonia for aerobic respiration from urea hydrolysis *in situ*.

Many studies have investigated the relative ammonia oxidation activities of AOB and AOA using differential inhibitors in terrestrial environments (e.g., Di et al., 2009; Taylor et al., 2010). However, no study has reported on nitrification rates with differential inhibitors in freshwater environments. By chelating copper from the AMO active site (Bédard and Knowles 1989), the first step of nitrification is inhibited by ATU. AOB and AOA differently respond to ATU due to differences in inhibition thresholds and amino acids in the active site of the AMO enzyme (Lehtovirta-Morley et al. 2013; Shen et al. 2013b). AOB appear to be more sensitive to ATU than AOA in soil (Taylor et al. 2010) and marine (Santoro and Casciotti 2011) environments. An AOA enrichment from agricultural soil, *Ca. Nitrosoarchaeum koreensis*, was not inhibited by ATU, whereas ATU at low concentrations inhibited AOB (*Nitrosomonas europaea*; Jung et al. 2011). At the same concentration, AOB (*Nitrospira multiformis*) were more sensitive to ATU than AOA (*N. viennensis*; Shen et al. 2013b). PTIO (2-Phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide) inhibits ammonia oxidation by acting as a nitric oxide (NO) scavenger. Because NO is a likely intermediate of AOA ammonia-oxidation, but not in AOB ammonia oxidation, PTIO can effectively inhibit AOA without affecting AOB (Walker et al., 2010). PTIO can effectively inhibit AOA (*N. viennensis*) without affecting AOB (*N. multiformis*; Shen et al. 2013b). This study represents the first application of PTIO to environmental sample incubations.

The objectives of this study were to assess *in vitro* ammonia oxidation and urea hydrolysis to reveal ammonia oxidizer dynamics within the largest watershed in Southern Ontario, the Grand River. This activity-based study follows on my initial molecular assessment of AOA, AOB and anaerobic ammonia-oxidizing (anammox) bacteria in Grand River sediments and water column, and observation of a strong enrichment of AOB within WWTP effluent plumes (Sontheiphand et al., 2013). I tested the hypothesis that AOB dominate ammonia oxidation within the Grand River, which would be consistent with my initial observation that AOB became enriched following prolonged high-ammonia incubations. This is the first study to combine nitrification rates and urea hydrolysis measurements with inhibitors to differentiate between AOB and AOA activities within a freshwater environment.

5.2 Materials and Methods

5.2.1 Sample collection

The Grand River watershed is the largest catchment in Southern Ontario and much impacted by human activities. In this study, the central portion of the Grand River was used as an example of a watershed impacted by wastewater effluent. Downstream and upstream sampling sites represent sampling sites with high and low impact by wastewater effluent, respectively. Sediment and water samples were collected approximately 180 m downstream from an ammonia-rich wastewater discharge pipeline from a municipal wastewater treatment plant in Waterloo, Ontario, Canada on June 11th, 2013. Due to high spatial heterogeneity, sediment samples were collected at five random locations, using a plastic core tube, and pooled on site. Water samples were well mixed at the sampling site and were collected using a 500-ml plastic container. All the samples were kept on ice during transport to the lab. Conductivity, pH, and dissolved oxygen (DO) were measured on site (Table 5.1). Follow-up samples were taken on July 31st, 2013. Not only downstream samples, but also upstream samples were included in the follow-up study for comparison. Upstream sediment and water samples were collected approximately 390 m above the pipeline effluent, with the same methods described above.

Table 5.1 Downstream and upstream water chemistry data.

	Mid June		Late July
	Downstream water	Upstream water	Downstream water
Conductivity (μS)	1031	480	1019
pH	7.5	8.0*	7.9*
DO (mg L^{-1})	8.23	NA	NA
N- $[\text{NH}_3 + \text{NH}_4^+]$ (μM)	560	BDL	572
N- $[\text{NO}_2^- + \text{NO}_3^-]$ (μM)	147	151	167
N- $[\text{NO}_2^-]$ (μM)	BDL	BDL	20.6
N-Urea (μM)	BDL	240	BDL

NA = Not available; BDL = Below detection limit; * analyzed in the lab

5.2.2 *In vitro* nitrification activity

For each sediment treatment replicate, one gram of the pooled downstream sediment was added to 10 ml of modified inorganic freshwater medium (FWM; Tourna et al. 2011). The modified FWM, without HEPES buffer, was adjusted to pH 7.5. The initial total ammonia ($\text{NH}_3 + \text{NH}_4^+$) concentration was 600 μM and ATU (10 and 100 μM) and PTIO (100 μM) were added to a subset of samples. In addition to sediment slurries, 10 ml of downstream water samples were added to test tubes, without FWM. Ammonia, ATU, and PTIO were added to each tube at the same concentrations as those for the sediment set. Negative controls were conducted with FWM without sample inoculation for all conditions. All treatments and negative controls were conducted in triplicate and statically incubated in the dark at room temperature ($\sim 25^\circ\text{C}$) for 14 days. The incubations were gently shaken manually once a day. Upstream samples both sediment and water column were conducted the same as downstream samples described above. One-ml of each sample was collected for water chemistry on days 0, 1, 3, 5, 7, 11, and 14. Nitrification rates were estimated based on linear regression of $\text{NO}_2^- + \text{NO}_3^-$ concentrations vs. time between days 3 and 7. A significant difference of $\text{NO}_2^- + \text{NO}_3^-$ production between any two conditions was supported by *t*-tests using Excel software (Microsoft Corp., USA). The null hypothesis was that $\text{NO}_2^- + \text{NO}_3^-$ productions between two conditions were the same. If *p* value was ≤ 0.05 , the null hypothesis was rejected.

5.2.3 *In vitro* urea hydrolysis

Urea hydrolysis was tested in parallel to *in vitro* nitrification rate incubations. Both sediment and water samples from downstream and upstream of wastewater effluent, including negative controls, were incubated with differential inhibitors, using the same conditions described in the nitrification activity section (5.2.2). Urea-N (600 μM) was added to the modified FWM instead of ammonia. All samples were monitored at the same seven time points.

5.2.4 Chemistry analyses

Ammonia concentrations were measured by a fluorometric technique according to an established protocol (Poulin and Pelletier 2007). Nitrite and nitrate measurements were conducted by a colorimetric assay (Miranda et al., 2001). Urea was measured by a previously published protocol (Zawada et al., 2009), with analysis at 450 nm instead of 430 nm. All ammonia, urea, and N-oxide analyses were performed on a FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices, Canada).

5.2.5 DNA extraction and quantitative real-time PCR

DNA was extracted from sediment and water samples, using MoBio PowerSoil DNA kit (Mo Bio Laboratories, USA) and PowerWater Sterivex DNA Isolation Kit (Mo Bio Laboratories, USA), respectively, following the manufacturer's protocols. All extracts were analyzed for DNA concentration using a NanoDrop spectrophotometer ND-100 (Thermo Fisher Scientific, USA) before being diluted to $5 \text{ ng } \mu\text{l}^{-1}$ to serve as template for qPCR. AOB and AOA 16S rRNA genes were quantified using primers NitA (Voytek and Ward 1995)/CTO654r (Kowalchuck et al., 1997) and primers 771F/957R (Ochsenreiter et al., 2003), respectively. The qPCR master mix contained $5 \mu\text{l}$ of SsoAdvanced SYBR Green Supermix (Bio-Rad, USA), $0.03 \mu\text{l}$ of each primer ($100 \mu\text{M}$), $0.02 \mu\text{l}$ of bovine serum albumin (10 mg ml^{-1}) and $1 \mu\text{l}$ of genomic DNA template ($5 \text{ ng } \mu\text{l}^{-1}$) in a total volume of $10 \mu\text{l}$. All amplifications were conducted in duplicate on a CFX96 real-time system (Bio-Rad, USA). A qPCR thermal cycle started with an initial denaturation at 98°C for 2 min, followed by 35 cycles of 98°C for 5 sec, annealing at 57°C and 55°C for AOB and AOA 16S rRNA genes, respectively, for 30 sec and 72°C for 30 sec. After each cycle, plate reads were added at 81°C and 80°C for AOB and AOA 16S rRNA genes, respectively. Plasmids containing AOB and AOA 16S rRNA gene fragments were amplified, purified by a MinElute kit (Qiagen, USA) and quantified by the NanoDrop Spectrophotometer ND-100 before being diluted for qPCR standards. Ten-fold serial dilutions were performed in a range of $10^1 - 10^7$ copies to create a standard curve of each gene. The standard curves showed an efficiency of 80.8% ($R^2 = 0.99$) and 82.6% ($R^2 = 0.99$) for AOB and AOA 16S rRNA genes, respectively. The specificity of amplification was verified by melt curve analysis and agarose gel electrophoresis.

5.3 Results

5.3.1 Activity of aerobic ammonia oxidizers within impacted river

Downstream sediment and water samples were collected in mid-June. Follow-up samples were collected in late-July. Differential inhibitors were used to distinguish between AOB and AOA activities. ATU and PTIO at specific concentrations were used to selectively inhibit AOB and AOA, respectively. For mid-June downstream sample sediment incubations, ammonia concentrations decreased to below detection limits by day 10, and $\text{NO}_2^- + \text{NO}_3^-$ production increased to $\sim 600 \mu\text{M}$ by day 14, indicating active ammonia oxidation (Figure 5.1A). NO_2^- did not accumulate, presumably due to active nitrite-oxidizing bacteria (NOB) in the downstream pipeline effluent (Mußmann et al. 2013).

Ammonia concentrations decreased gradually in downstream sediment incubations with PTIO (depleted by day 10) and $\text{NO}_2^- + \text{NO}_3^-$ concentrations accumulated between days 5 to 14 (Figure 5.1B). $\text{NO}_2^- + \text{NO}_3^-$ productions between unamended and PTIO-treated conditions were not significantly different ($p > 0.05$). Nitrification rates of unamended and PTIO-treated conditions were 83.3 ($R^2 = 0.8$) and 101.7 ($R^2 = 0.8$) $\mu\text{M N-}[\text{NO}_2^- + \text{NO}_3^-] \text{ g}^{-1} \text{ sediment day}^{-1}$, respectively. In contrast, 100 μM ATU inhibited all $\text{NO}_2^- + \text{NO}_3^-$ production and ammonia depletion within downstream sediments (Figure 5.1C).

The initial day 0 ammonia concentration for downstream water samples was higher than for sediment sample incubations because there were two ammonia sources for the water samples: natural ammonia in the river water (Table 5.1) and experimental ammonia addition. Ammonia was completely depleted by day 7 in downstream water samples without inhibitor and with PTIO; $\text{NO}_2^- + \text{NO}_3^-$ production suddenly increased between days 5 to 7, then was relatively unchanged until day 14 (Figures 5.1D and 5.1E). $\text{NO}_2^- + \text{NO}_3^-$ production between these two conditions was not significantly different ($p > 0.05$). NO_2^- concentrations were depleted to below the detection limits for incubations without inhibitor (Figure 5.1D) but remained present in incubations with PTIO (Figure 5.1E). This observation corresponded with other downstream water column samples; NO_2^- concentrations eventually decreased to below the detection limit (Figures 5.2D, 5.6D, and 5.7D), but they remained undepleted in all PTIO-treated downstream water samples (Figures 5.2E, 5.6E, and 5.7E), implying that PTIO inhibited *in vitro* NO_2^- oxidation. Downstream water samples amended with 100 μM ATU showed very little evidence for ammonia oxidation (Figure 5.1F). Nitrification rates of downstream water samples with no inhibitor, with PTIO, and with ATU additions were 32.3 ($R^2 = 0.9$), 36.2 ($R^2 = 0.7$), 1.8 ($R^2 = 0.06$) $\mu\text{M N-}[\text{NO}_2^- + \text{NO}_3^-] \text{ ml}^{-1} \text{ water day}^{-1}$, respectively.

Late-July downstream sediment samples depleted ammonia more slowly than June samples, only depleting added ammonia by the last incubation day (day 14; Figures 5.2A and 5.2B) in comparison to nearly complete depletion by day 10 for mid-June samples (Figures 5.1A and 5.1B). The increasing ammonia during the first two to five days from downstream sediments (Figures 5.2A to 5.2C) was possibly from mineralization of organic matter and/or dissimilatory nitrate reduction to ammonium (DNRA). Ammonia gradually decreased throughout the incubation period, although I observed very little $\text{NO}_2^- + \text{NO}_3^-$ accumulation in both unamended and PTIO-supplemented treatments. Possible reasons for this observation might be that other N transformation processes occurred within an oxic-anoxic interface within these static sediment incubations. Anammox bacteria

oxidize ammonia by using nitrite as an electron acceptor to produce N_2 gas and I observed molecular evidence for anammox bacteria in these same sites previously (Sonthiphand et al., 2013). Denitrifiers transform NO_3^- to N_2 gas and other N-oxide intermediates (Thamdrup 2012). Other than microbial processes, sediments might adsorb ammonia from FWM as an exchangeable ion on sediment surfaces (Simon and Kennedy 1987; Rysgaard et al., 1999). Nitrification rates of unamended and PTIO-treated conditions were 43.0 ($R^2 = 0.9$) and 74.7 ($R^2 = 0.8$) $\mu\text{M N-}[\text{NO}_2^- + \text{NO}_3^-] \text{ g}^{-1} \text{ sediment day}^{-1}$, respectively. Consistent with the mid-June samples, downstream sediment samples with ATU showed no nitrification activity (Figure 5.2C).

For late-July downstream water samples, ammonia concentrations were undetectable by day 10 (Figures 5.2D and 5.2E), which represents a longer period than for the mid-June samples (Figures 5.1D and 5.1E). Total $NO_2^- + NO_3^-$ production increased from days 3 to 7, then NO_2^- remained stable subsequently, only in the presence of PTIO (Figures 5.2D and 5.2E). $NO_2^- + NO_3^-$ productions for these two conditions showed no significant difference ($p \leq 0.05$). As with mid-June samples, late-July downstream water with ATU showed very little ammonia depletion and $NO_2^- + NO_3^-$ production (Figure 5.2F). Nitrification rates of unamended, PTIO-treated, and ATU-treated downstream water samples were 25.1 ($R^2 = 1$), 26.19 ($R^2 = 1$), and 5.8 ($R^2 = 0.9$) $\mu\text{M N-}[\text{NO}_2^- + \text{NO}_3^-] \text{ ml}^{-1} \text{ water day}^{-1}$, respectively.

Upstream samples were included in this investigation as a background site where the ammonia concentration was below the detection limit (Table 5.1). Upstream samples, both sediment and water, were analyzed only in the follow-up study (Figure 5.3). Nitrification rates of upstream sediment samples with no inhibitor and with PTIO were 21.3 ($R^2 = 0.9$; Figure 5.3A) and 17.1 ($R^2 = 0.9$; Figure 5.3B) $\mu\text{M N-}[\text{NO}_2^- + \text{NO}_3^-] \text{ g}^{-1} \text{ sediment day}^{-1}$, respectively. As with downstream sediments, ATU completely inhibited nitrification activity (Figure 5.3C). Upstream water showed no nitrification activity in all conditions (Figures 5.3D to 5.3F). All negative controls showed no microbial activity (Figures 5.4A to 5.4C).

Two ATU concentrations ($10 \mu\text{M}$ and $100 \mu\text{M}$) were used in the follow-up experiment for comparison. The results of ATU-treated samples under both conditions (10 and $100 \mu\text{M}$) were similar. The results of $10 \mu\text{M}$ ATU-treated samples for both upstream and downstream sediment and water samples, including negative control, showed no nitrification activity (Figure 5.5).

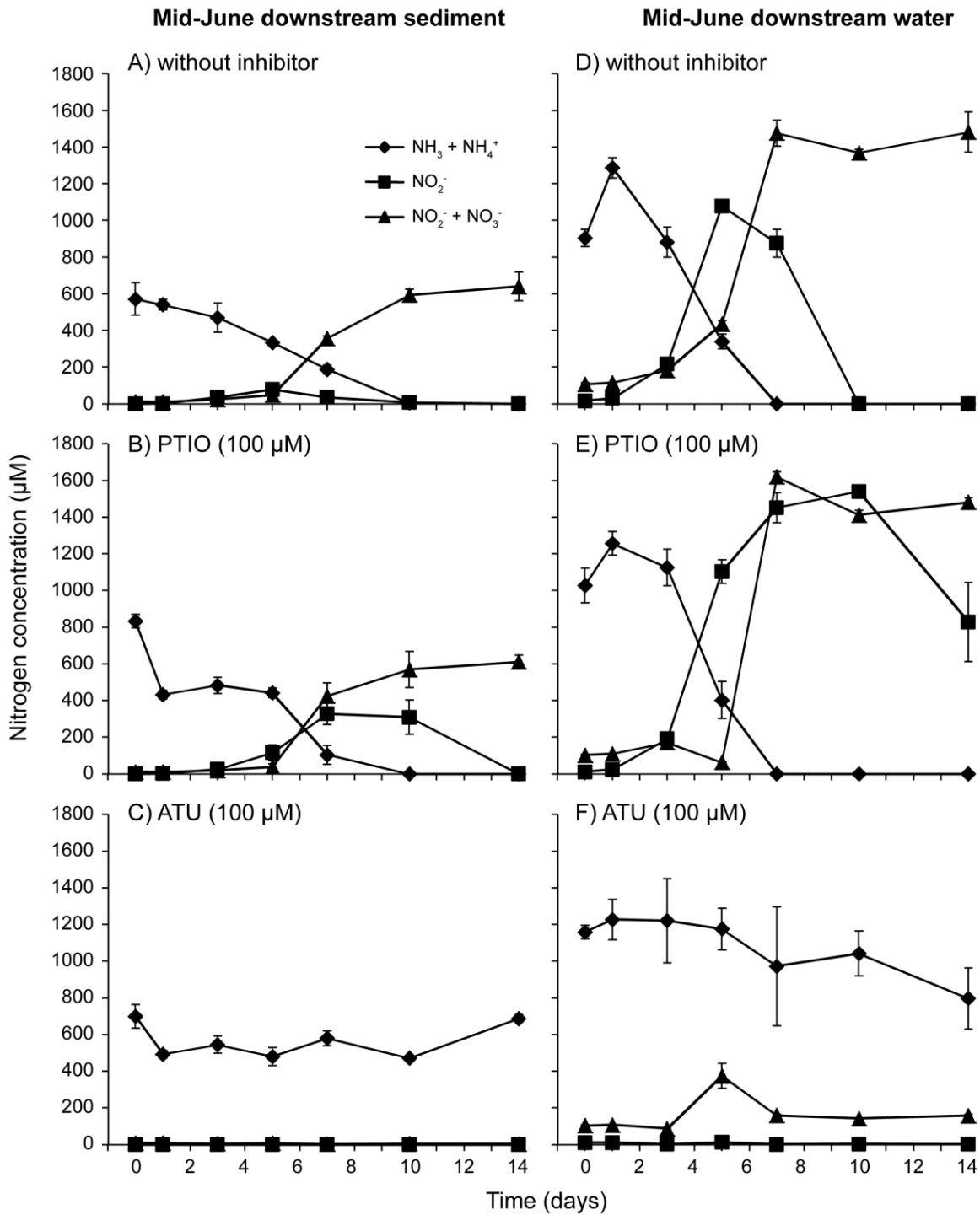


Figure 5.1 Nitrification activity of mid-June downstream sediment and water samples with three conditions, including without inhibitor, with PTIO, and ATU additions. Error bars represent standard deviation of biological triplicates.

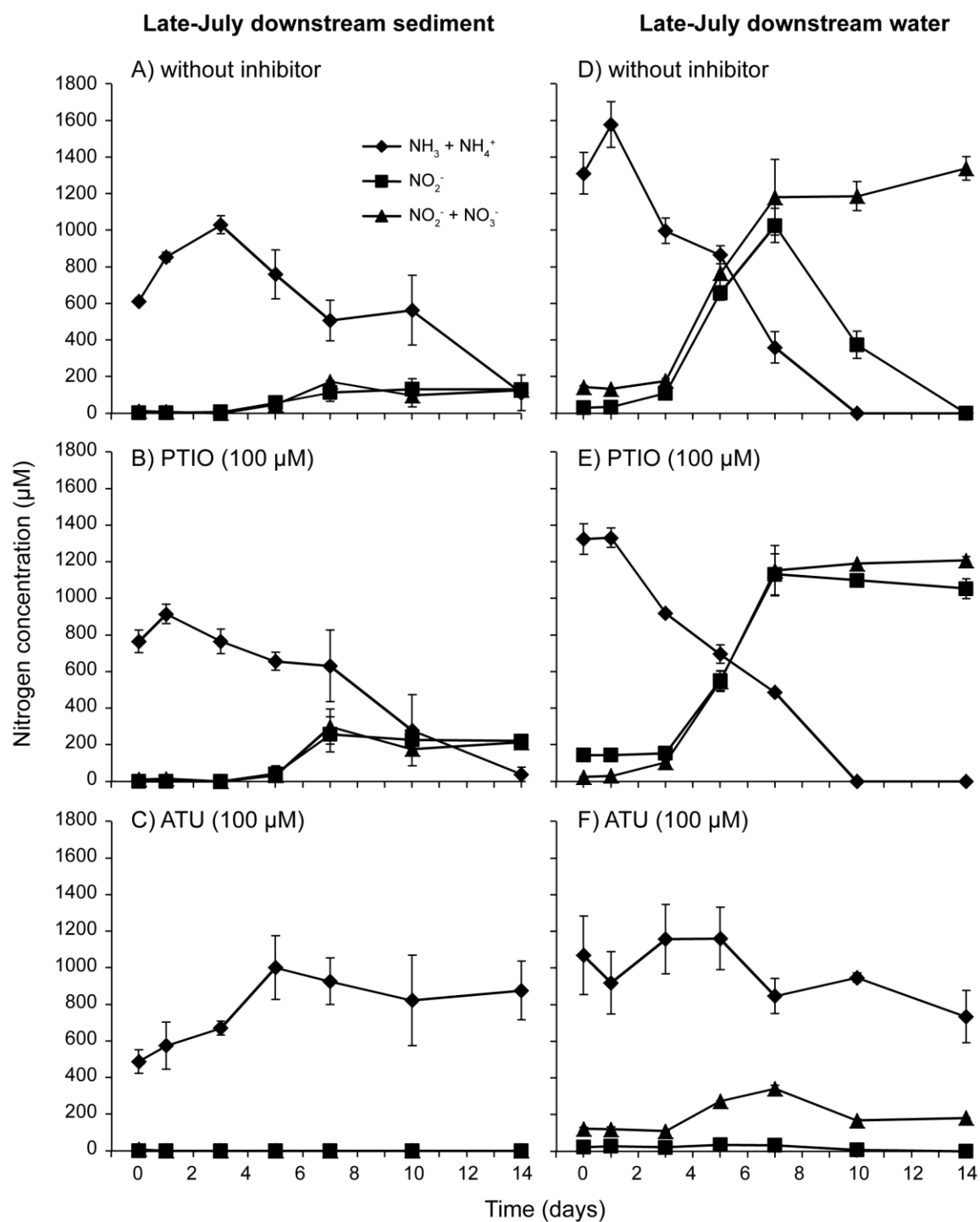


Figure 5.2 Nitrification activity of late-July downstream sediment and water samples with three conditions, including without inhibitor, with PTIO, and ATU additions. Error bars represent standard deviation of biological triplicates.

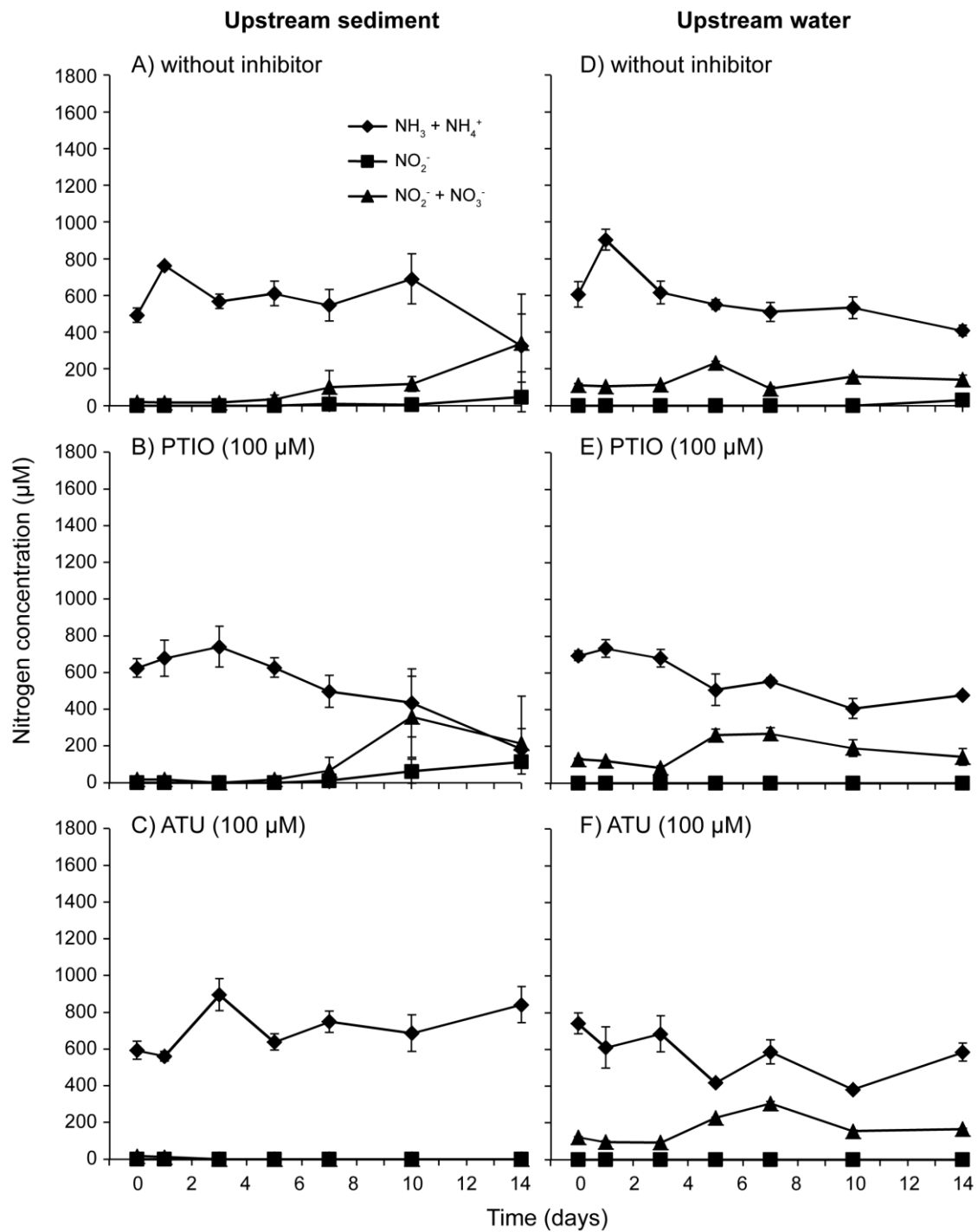


Figure 5.3 Nitrification activity of late-July upstream sediment and water samples with three conditions, including without inhibitor, with PTIO, and ATU additions. Error bars represent standard deviation of biological triplicates.

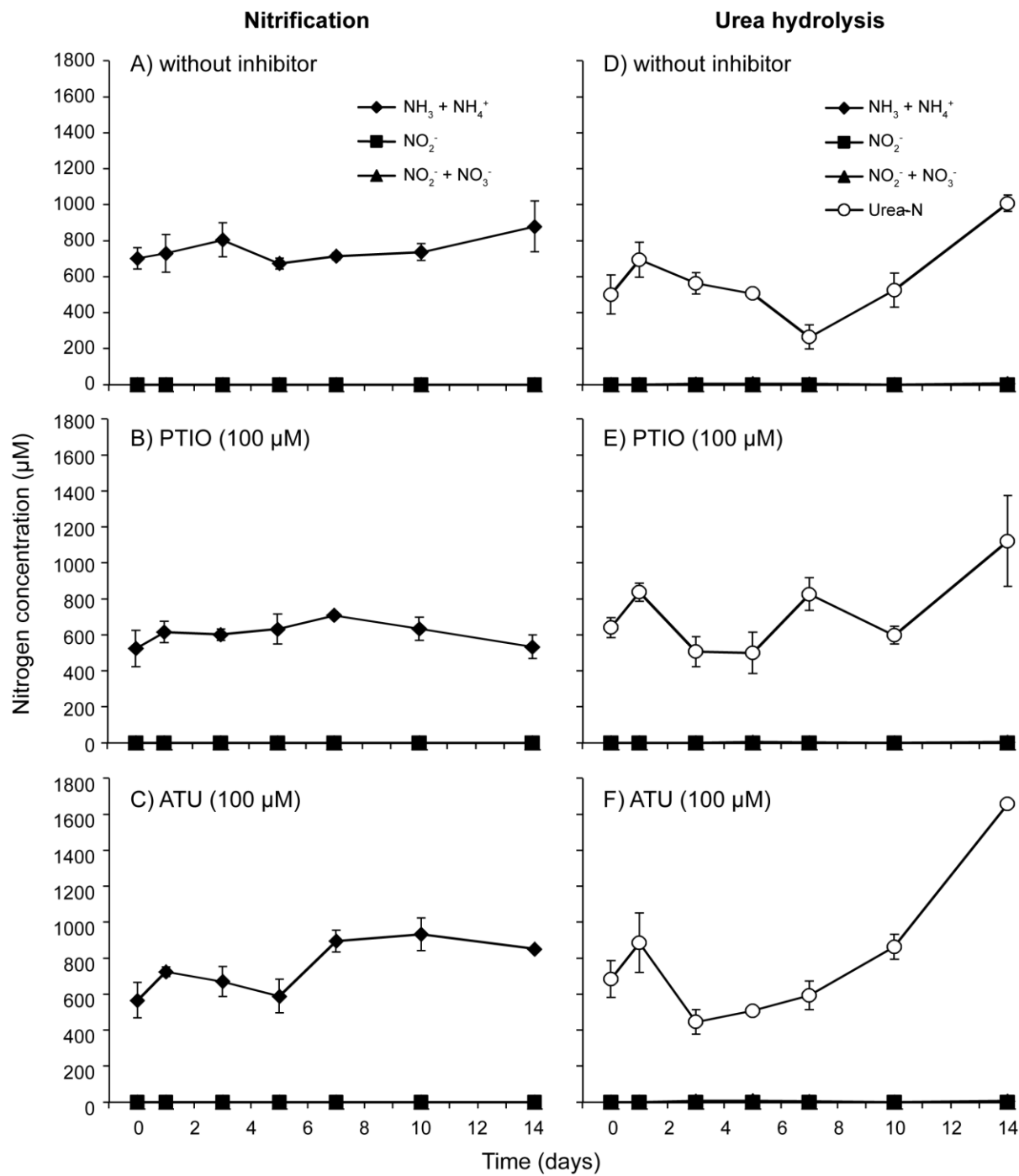


Figure 5.4 Negative controls of nitrification and urea hydrolysis experiments with three conditions, including without inhibitor, with PTIO and ATU additions.

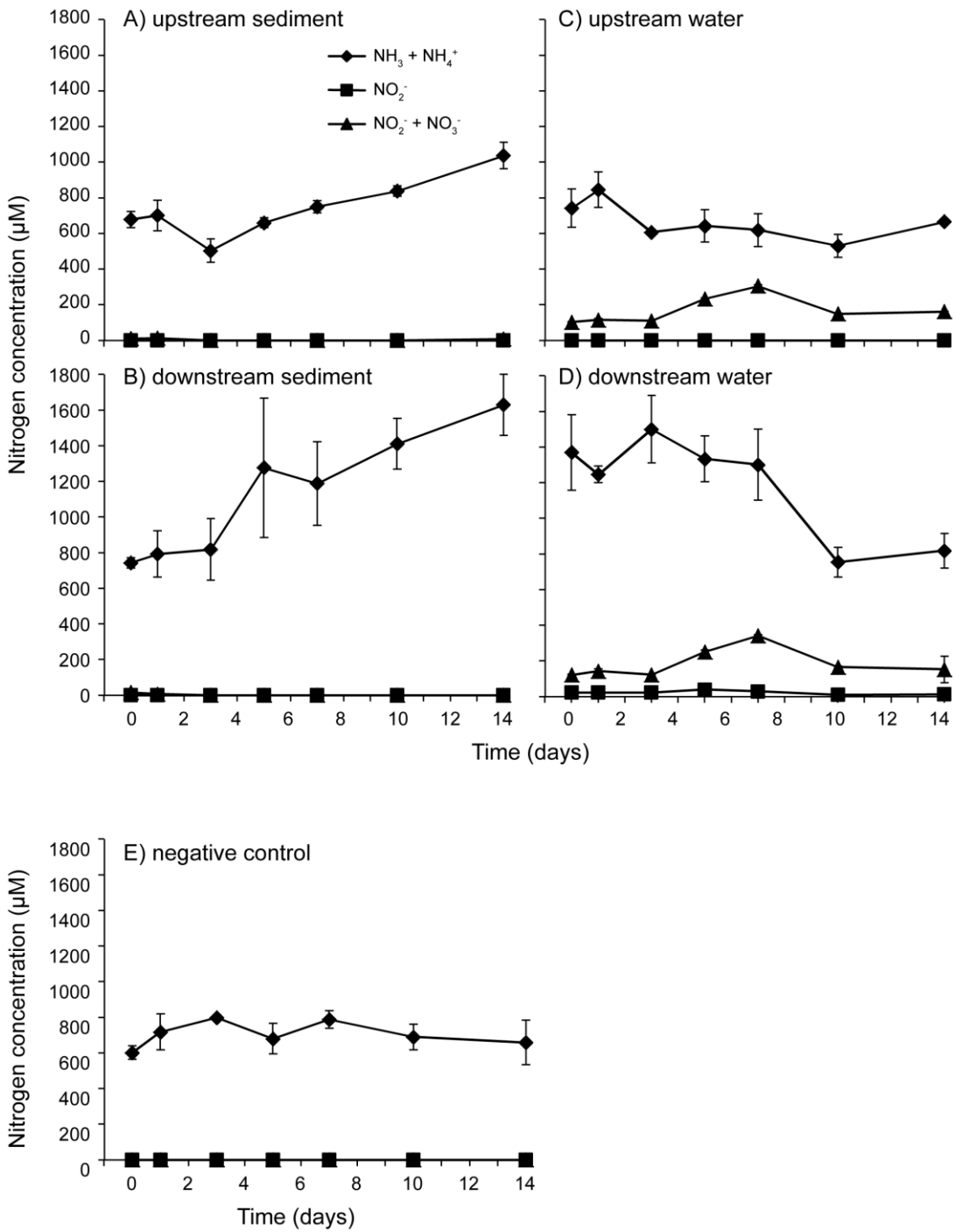


Figure 5.5 Nitrification activity of late-July upstream sediment (A), downstream sediment (B), upstream water (C), and downstream water (D) samples with 10 μM ATU addition, including the negative control (E). Error bars represent standard deviation of biological triplicates.

5.3.2 Urea hydrolysis coupled with ammonia oxidation

Urea can be degraded to two ammonia molecules by microorganisms expressing urease genes, including AOB and AOA. Consequently, urea is a possible organic ammoniacal N source for AOB and AOA. The results for the mid-June sediment samples demonstrated that added urea was depleted within five days (Figure 5.6A). Ammonia concentrations increased transiently, then decreased as ammonia was oxidized, corresponding with $\text{NO}_2^- + \text{NO}_3^-$ accumulation (Figure 5.6A). PTIO-treated downstream sediments also showed that ammonia was generated from urea hydrolysis and sequentially oxidized (Figure 5.6B). $\text{NO}_2^- + \text{NO}_3^-$ productions from these two conditions were not significantly different ($p > 0.05$). In the ATU treatment, urea persisted at a consistent level from days 0 to 10, and then dropped to $\sim 480 \mu\text{M}$ by day 14 (Figure 5.6C). Ammonia concentrations increased over time; however, no $\text{NO}_2^- + \text{NO}_3^-$ production was observed, indicating no ammonia oxidation. Potential sources of ammonia include urea hydrolysis and mineralization of sediment-associated cell debris.

The ammonia concentration in downstream water was $\sim 560 \mu\text{M}$ (Table 5.1), whereas the initial ammonia concentration of sediment samples was below the detection limit, due to no ammonia addition to the FWM. In downstream water, the urea and ammonia were depleted by days 3 and 7, respectively (Figure 5.6D). NO_2^- production increased rapidly from days 3 to 5, then NO_2^- subsequently dropped to below detection limits by day 10. $\text{NO}_2^- + \text{NO}_3^-$ accumulation was observed between days 3 to 7, then remained constant thereafter as NO_2^- was presumably oxidized to NO_3^- (Figure 5.6D). PTIO-treated downstream water showed the same trends as the set without inhibitor, except for the absence of NO_2^- depletion by day 10 (Figure 5.6E). $\text{NO}_2^- + \text{NO}_3^-$ productions from these two conditions were not significantly different ($p > 0.05$). Nitrification rates of unamended and PTIO-treated downstream water samples were 30.5 ($R^2 = 0.9$) and 31.8 ($R^2 = 0.9$) $\mu\text{M N} - [\text{NO}_2^- + \text{NO}_3^-] \text{ ml}^{-1} \text{ water day}^{-1}$, respectively. Neither urea hydrolysis nor ammonia oxidation was observed in the ATU treatment, implying that $100 \mu\text{M}$ ATU had the potential to inhibit both urea hydrolysis and ammonia oxidation by AOB (Figure 5.6F).

As with the ammonia oxidation study (section 5.3.1), the urea hydrolysis follow-up studies were conducted in late-July. Ammonia produced from urea remained in all downstream sediment conditions (Figures 5.7A to 5.7C). Urea was completely depleted by day 3 in the unamended and PTIO-treated conditions, implying that PTIO had no effect on urea hydrolysis. Nevertheless, urea disappeared by day 7 in the ATU-treated samples. The overall results for the late-July and mid-June

downstream waters were consistent. However, mid-June samples depleted urea and ammonia faster than the follow-up samples. Late-July downstream waters in the unamended and PTIO-treated samples showed that urea and ammonia were depleted by days 5 and 10, respectively (Figures 5.7D and 5.7E). $\text{NO}_2^- + \text{NO}_3^-$ accumulated over time for both conditions. $\text{NO}_2^- + \text{NO}_3^-$ concentration between these two conditions were not significantly different ($p > 0.05$). Nitrification rates of late-July unamended and PTIO-treated water samples were 11.5 ($R^2 = 0.9$) and 21.9 ($R^2 = 1$) $\mu\text{M N}[\text{NO}_2^- + \text{NO}_3^-] \text{ ml}^{-1} \text{ water day}^{-1}$, respectively. ATU inhibited both urea hydrolysis and ammonia oxidation in downstream water (Figure 5.7F).

Upstream sediments without inhibitor and with PTIO addition showed urea depletion, together with increasing ammonia (Figures 5.8A and 5.8B). The condition treated with 100 μM ATU was somewhat complicated because a urea spike was observed earlier before reducing to the expected concentrations (~800 to ~1000 μM). Urea was relatively constant from days 3 to 14, whereas ammonia gradually increased and remained consistent subsequently (Figure 5.8C). However, the results of upstream waters in all conditions showed neither urea hydrolysis nor ammonia oxidation (Figures 5.8 D to 5.8F). $\text{NO}_2^- + \text{NO}_3^-$ concentrations of upstream water (Table 5.1) remained in all treatments and were not significantly different among these three conditions ($p > 0.05$). Urea-N concentration was higher in the upstream water, but undetectable in the downstream water (Table 5.1), suggesting that urease-positive microorganisms may have transformed urea into ammonia for AOB in downstream samples. All negative controls showed no microbial activities (Figures 5.4D to 5.4F).

As with nitrification experiments, two concentrations of ATU (10 and 100 μM) were used in the late-July samples. The results between two ATU concentrations were consistent. The results of 10 μM ATU-treated samples for upstream and downstream sediments showed urea depletion and ammonia production (Figures 5.9A and 5.9B), whereas upstream and downstream water samples, including negative control showed neither urea depletion nor ammonia production (Figures 5.9C to 5.9E).

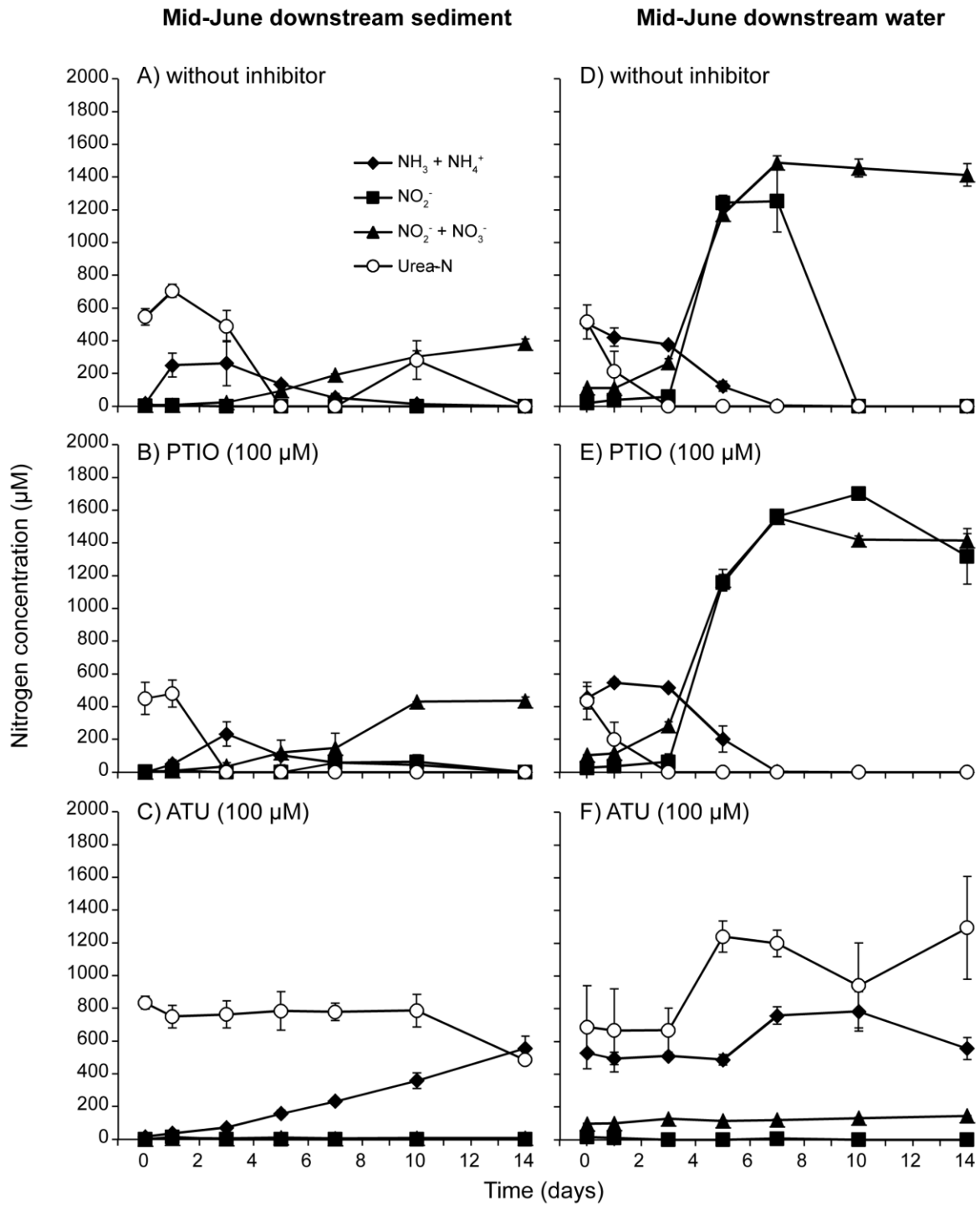


Figure 5.6 Urea hydrolysis and nitrification activities of mid-June downstream sediment and water samples with three conditions, including without inhibitor, with PTIO, and ATU additions. Error bars represent standard deviation of biological triplicates.

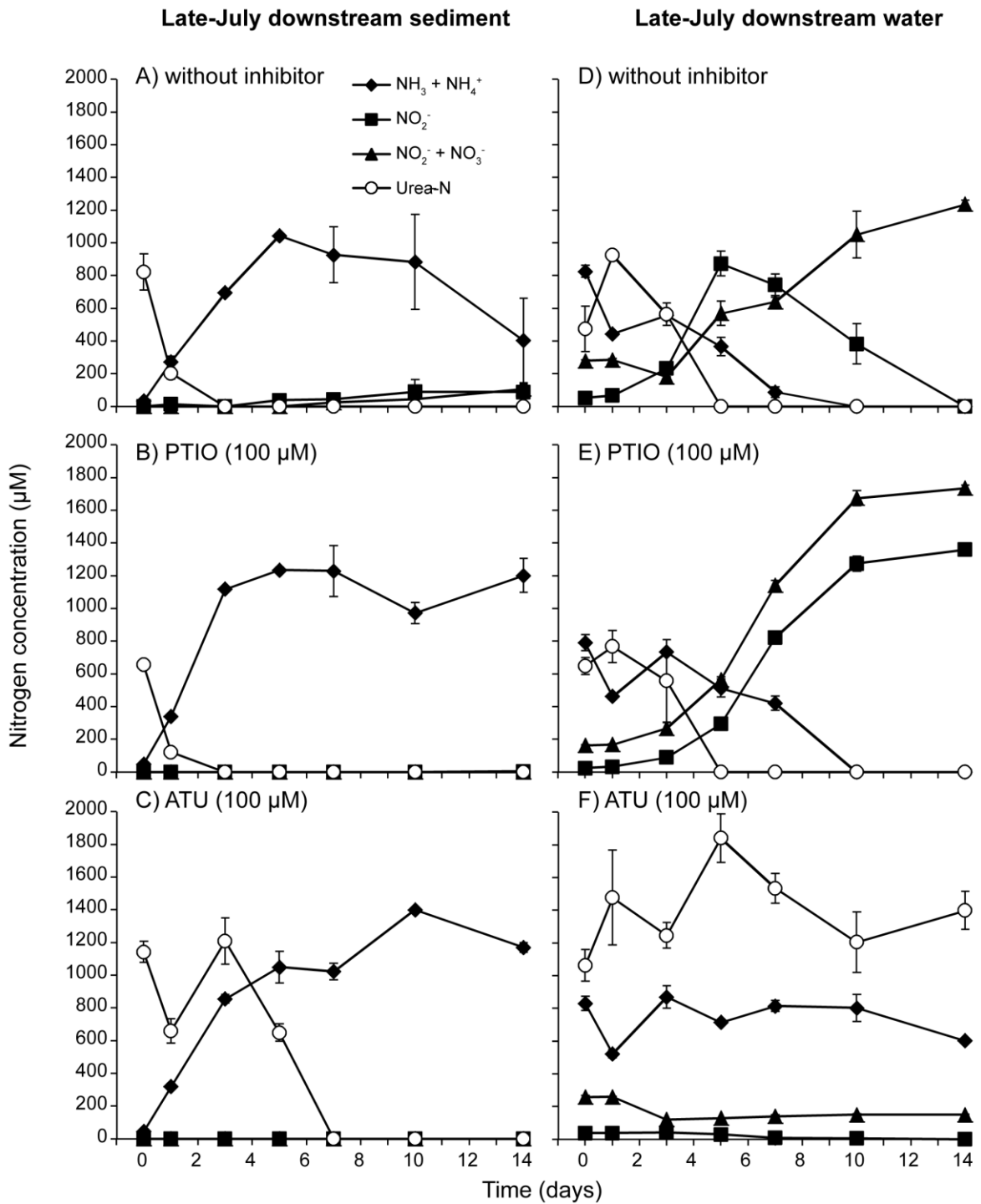


Figure 5.7 Urea hydrolysis and nitrification activities of late-July downstream sediment and water samples with three conditions, including without inhibitor, with PTIO, and ATU additions. Error bars represent standard deviation of biological triplicates.

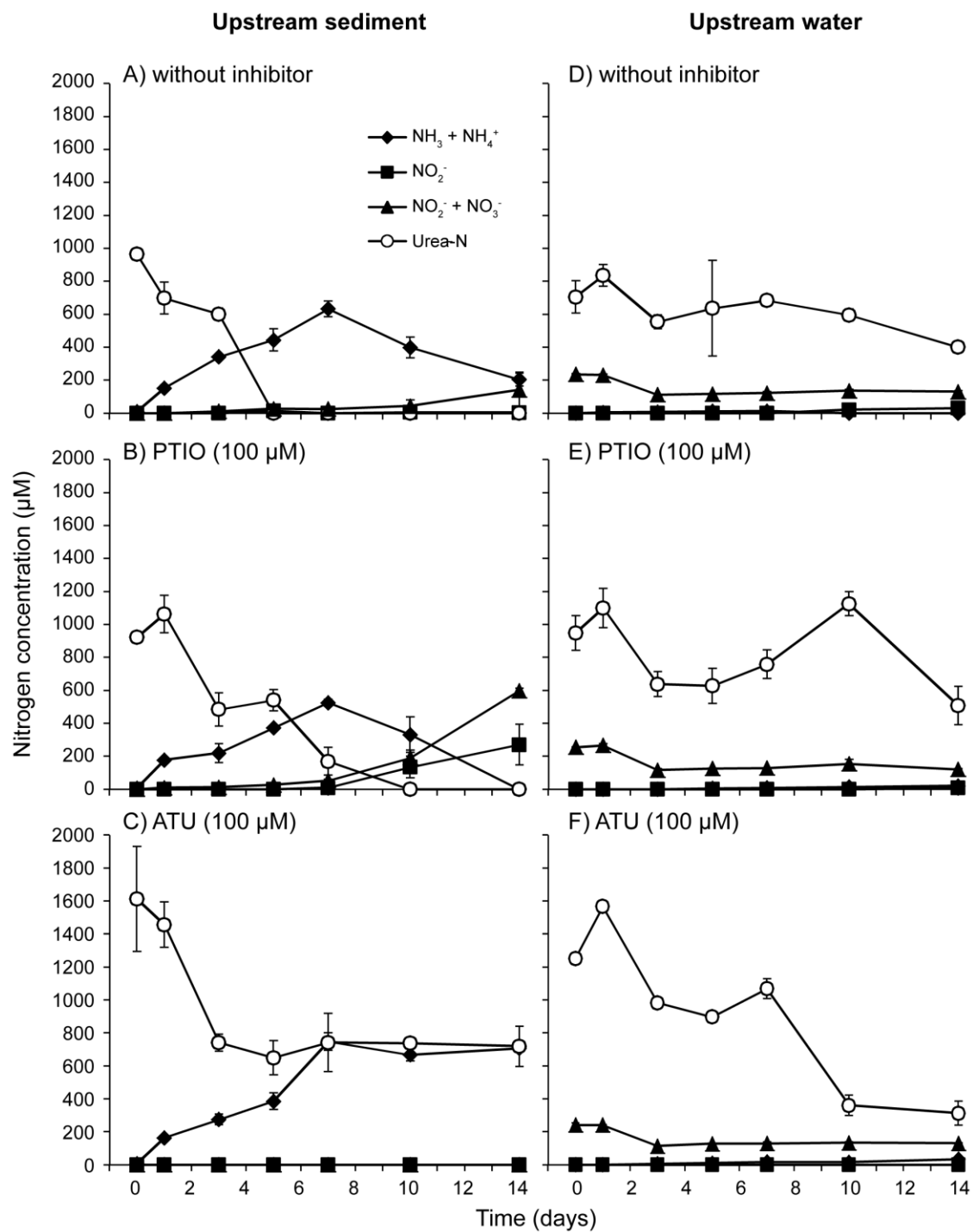


Figure 5.8 Urea hydrolysis and nitrification activities of late-July upstream sediment and water samples with three conditions, including without inhibitor, with PTIO, and ATU additions. Error bars represent standard deviation of biological triplicates.

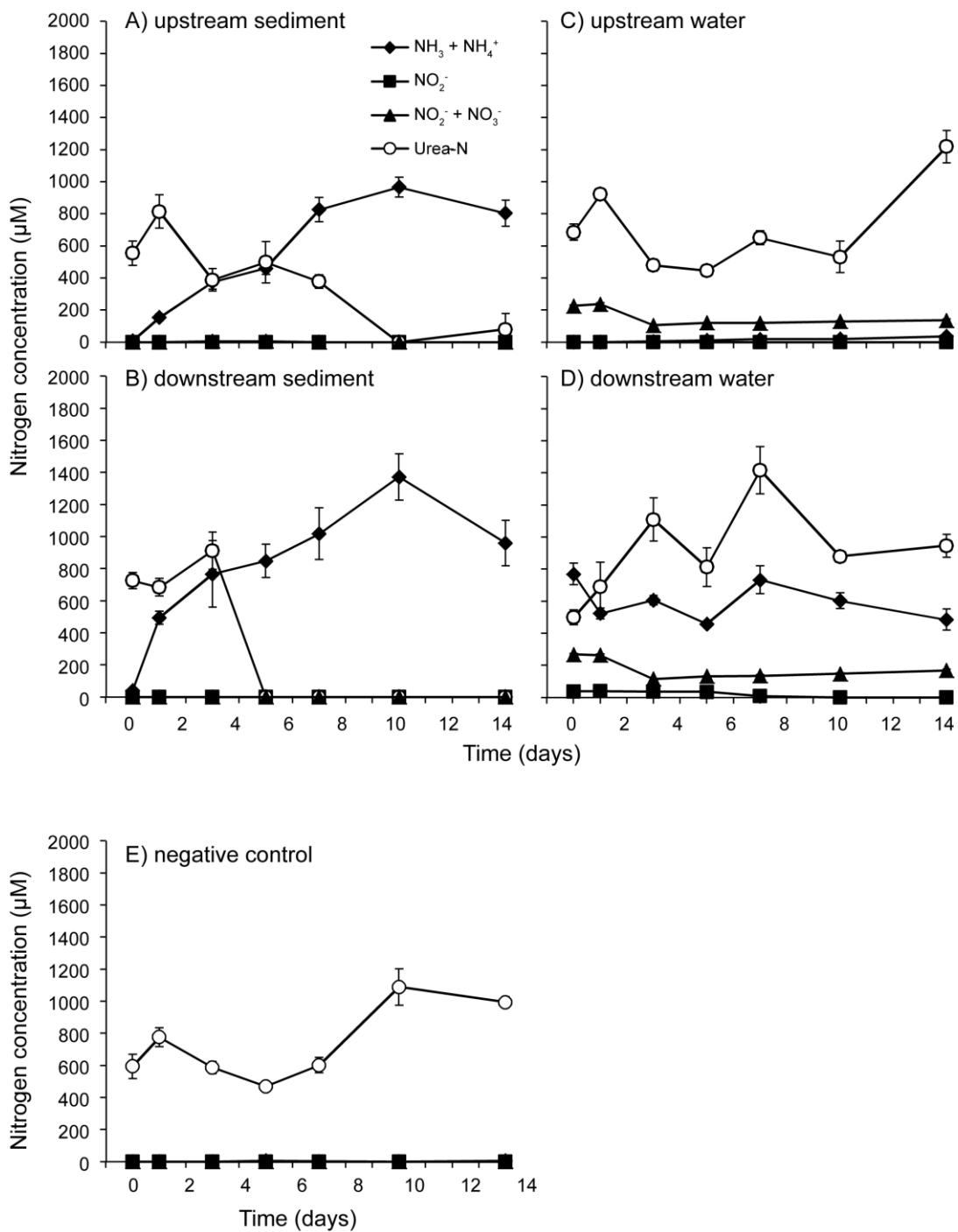


Figure 5.9 Urea hydrolysis of late-July upstream sediment (A), downstream sediment (B), upstream water (C), and downstream water (D) samples with 10 μM ATU addition, including the negative control (E). Error bars represent standard deviation of biological triplicates.

5.3.3 Relative abundance of AOB and AOA

Sediment and water samples from mid-June and late-July were quantified for AOB and AOA 16S rRNA gene abundances. The results showed that although AOA were more abundant than AOB in sediments, AOB dominated water samples (Figure 5.10). Although nitrification activity and urea hydrolysis implicated AOB activity within sediment samples, AOA accounted for 80% and 57% of ammonia oxidizer 16S rRNA genes in mid-June and late-July sediments, respectively. In contrast to a dominance of AOA genes in sediment samples, AOB accounted for ~90% of ammonia oxidizer 16S rRNA genes in both water samples (Figure 5.10).

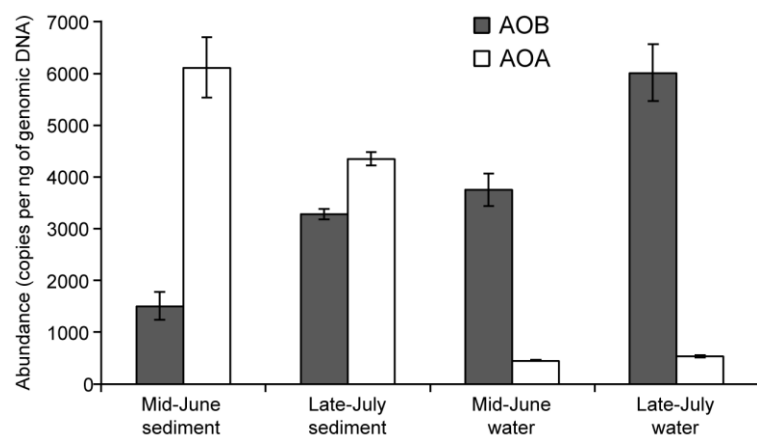


Figure 5.10 Relative abundance of AOB and AOA 16S rRNA genes for the sediment and water samples used for assessing nitrification and urea hydrolysis activity. Error bars represent standard deviation of duplicate qPCR amplifications.

5.4 Discussion

The effective ATU concentration for inhibiting environmental AOB was unclear. Consequently, two concentrations (100 and 10 μM) of ATU were applied to both sediment and water samples. It has been reported that 100 μM ATU inhibited nitrification rates by 80% and 85% in marine and soil samples, respectively (Jäntti et al., 2013; Lehtovirta-Morley et al., 2013). ATU at 100 μM affected both bacterial and archaeal ammonia oxidation in manure compost (Oishi et al., 2012). However, some AOB cultures were highly sensitive to ATU. AOB (*N. europaea*) were potentially inhibited by < 10 μM ATU (Jung et al., 2011), and the nitrification activity of AOB (*N. multiformis*)

was significantly reduced by 0.4 μM ATU (Shen et al., 2013b). These current findings demonstrate that ATU at both concentrations effectively inhibited in-river AOB, with less effect on AOA present in the Grand River.

As for PTIO, two-hundred μM PTIO inhibited AOA, without disturbing AOB, in a low oxygen lab-scale reactor (Yan et al., 2012). PTIO was tested on both AOB (*N. multiformis*) and AOA (*N. viennensis*) cultures (Shen et al., 2013b). The results showed that 50 μM PTIO completely inhibited NO_2^- production of AOA (*N. viennensis*), whereas 200 μM PTIO did not affect AOB (*N. multiformis*). According to the current findings, 100 μM PTIO had no effect on AOB and was likely sufficient for inhibiting AOA in the impacted river. Interestingly, PTIO likely had the potential to inhibit NOB in downstream water in this study. Although PTIO has been reported to inhibit AOA (Yan et al., 2012) and anammox bacteria (Kartal et al. 2011), there has been no information on the effect of PTIO on NOB so far. Future research needs to investigate the potential modes of action and mechanism of PTIO on nitrite oxidation.

Nitrification rates for unamended and PTIO-treated downstream sediment and water samples were higher than those for ATU-treated samples. In all cases of downstream sediment and water samples, $\text{NO}_2^- + \text{NO}_3^-$ production rates from unamended and PTIO-treated conditions were significantly higher than those from ATU-treated conditions ($p \leq 0.05$). Overall, the results suggested AOB oxidized most ammonia from both sediment and water columns impacted by wastewater effluent discharge. High abundances and activities of AOB have been reported previously in ammonia-rich environments, including freshwater (Sonthiphand et al., 2013), marine (Bouskill et al., 2012), and soil (Di et al., 2009). Although the activity of terrestrial AOB has been investigated in many studies, few studies have considered AOB activity in aquatic environments. AOB activity has been examined in a freshwater ecosystem by using stable isotope probing (SIP; Avrahami et al., 2011). This current study is the first study using *in vitro* nitrification rates, with differential inhibitors to confirm AOB dominance in an impacted aquatic environment. Although upstream water showed no ammonia-oxidizing activity in all conditions, the results suggest that AOB also oxidized ammonia within upstream sediment. Less activity within the upstream site samples was possibly due to lower biomass and less *in situ* substrate for microbial activity. Also, the wastewater microbial community showed less impact on in-river upstream microbial community, possibly resulting in less nitrification activity within upstream samples (Sonthiphand et al., 2013). Potentially viable and active nitrifiers

from wastewater effluent might enhance the nitrification activity within downstream samples, whereas upstream samples were less impacted by wastewater nitrifiers.

Although the late-July samples showed lower nitrification activity than the mid-June samples, differential nitrification inhibitors implied that the ammonia oxidation process was driven primarily by AOB. Nitrification activity is impacted by various environmental factors such as O₂ concentration (Triska et al., 1990), temperature (Fdz-Polanco et al., 1994), and organic carbon (Strauss and Dodds 1997; Strauss and Lamberti 2002). However, environmental parameters analyzed in this study were insufficient to correlate with nitrification activity. Consequently, I cannot identify the reason for lower nitrification activity for late-July samples.

Urea hydrolysis was observed in both downstream and upstream sediments; however, ammonia oxidation was inconsistent between mid-June and late-July samples. Consequently, it is complicated to relate the co-occurrence between these two processes within sediment samples. However, the results for downstream water for both time periods suggest that AOB were likely capable of an ammonia oxidation-urea hydrolysis coupled process, reinforcing that AOB can gain energy from ammonia oxidation to uptake urea for subsequent hydrolysis. Indeed, *N. oligotropha* showed a high urea hydrolysis rate in ammonia-supplemented media (Pommerening-Röser and Koops 2005). Although AOB were implicated in ammonia oxidation, and possibly also in urea hydrolysis, I cannot rule out an important role for additional microorganisms involved in urea hydrolysis that may also be inhibited by ATU. In summary, urea hydrolysis within the effluent-impacted region of the Grand River may be driven by AOB with urease genes, non-nitrifiers with urease genes, or both. Non-nitrifiers with urease genes detected in freshwater associated with genera *Hydrogenophaga*, *Acidovorax*, *Janthinobacterium*, and *Arthrobacter* (Gresham et al., 2007). The results suggest that 100 µM ATU likely slowed and inhibited urea hydrolysis in the downstream sediment and water samples, respectively. However, the mode of action and direct or indirect mechanism of ATU on urease activity needs further investigation. It should be noted that other uncertainties (i.e., the degradation of inhibitor and sample type) related to inhibitor studies need to be considered.

The relative abundance of AOA was higher than that of AOB in downstream sediment samples. A higher relative abundance of freshwater AOA was reported in eutrophic Lake Taihu sediment (China) and showed a negative correlation with organic material (Wu et al., 2010). Although AOA abundance was high, their activity might be inhibited by an accumulation of organic matter within downstream WWTP sediment, just as the growth of *Nitrosopumilus maritimus* was

inhibited by low concentration of organic compounds (Könneke et al., 2005). My experimental conditions (i.e., high substrate and neutral pH) likely inhibited AOA growth and activity given that AOA play a key role in ammonia oxidation in soils with low nutrient concentrations and a low pH (Gubry-Rangin et al., 2010; Zhang et al., 2012). Also, AOA might use other alternative substrates (e.g., cyanate) for their energy source (Spang et al., 2012). AOA found in petroleum refinery WWTP are not chemolithoautotrophs; they possibly use hydrocarbons or other unknown substances for their energy and carbon sources (Mußmann et al., 2011). Another possible reason for high abundance but less activity might be an accumulation of AOA cell debris in sediment samples that is detected by DNA-based analyses. In contrast to downstream sediment samples, AOB 16S rRNA genes were higher than AOA 16S rRNA genes in downstream water samples. These results are consistent with a strong dominance of AOB activity in the downstream water column and with previous research showing that wastewater effluent can enhance nitrification activity and AOB abundance in receiving waters (Mußmann et al., 2013).

It should be noted that the incubation length could affect the results. In this study, the incubation period was limited to 14 days in order to monitor nitrification activity and urea hydrolysis without the degradation effects of inhibitors used in this study. To measure nitrification activity, ATU has been used previously in various incubation periods. For example, manure compost, soil, and archaeal marine enrichment were incubated with ATU for 14 days (Oishi et al., 2012), 30 days (Lehtovirta-Morley et al., 2013), and 40 days (Santoro and Casciotti 2011). They did not report the degradation of ATU over time. Both *Ca. Nitrososphaera viennensis* and *Nitrosospira multiformis* were incubated with PTIO for 12 days (Shen et al., 2013). Although our incubation period was two days longer, the results for no inhibitor and PTIO treated samples were consistent in most cases.

5.5 Conclusions

AOB are important microorganisms for in-river biogeochemical cycling, and are implicated in ammonia oxidation in effluent-impacted Grand River samples. PTIO had no effect on in-river ammonia oxidation. Compared to the negative controls, ATU completely inhibited bacterial ammonia oxidation in this incubation condition. AOB within downstream water likely have the ability to oxidize ammonia and hydrolyze urea, indicating that ammonia produced from urea is an alternative N source for AOB. Modes of actions and direct or indirect mechanisms of PTIO and ATU on urease and nitrite oxidizing activities require further investigation. Molecular analysis confirmed the activity of

AOB within this WWTP effluent impacted river. These findings implicate AOB as dominant microbial players for both ammonia oxidation and urea hydrolysis within the Grand River.

Chapter 6

Microbial biogeography of the Grand River

6.1 Introduction

Understanding the composition of bacterial communities in aquatic environments is important because these microorganisms drive key biogeochemical cycles (i.e., carbon and nitrogen cycles) that affect water quality. A unique microbial community could possibly be used as an indicator to evaluate healthy or impacted rivers and could be linked to pollutant sources. Freshwater microbial communities have been studied in the Changjiang River (China; Sekiguchi et al., 2002), the Danube River (Europe; Winter et al., 2007), the Paraná River (Brazil; Lemke et al., 2009), the Santa Ana River (USA; Ibekwe et al., 2012), Lake Gossenköllesee (Austria; Glöckner et al., 2000), and Lake Erken (Sweden; Heinrich et al., 2013). Molecular surveys of the Changjiang River showed that bacterial diversity decreased from upstream to downstream, based on DGGE profiles (Sekiguchi et al., 2002). The bacterial communities in the Changjiang River were different from those in two adjacent lakes, even though the river and lake waters mix. Bacterial communities of the Danube River and its four main tributaries were analyzed by PCR-DGGE (Winter et al., 2007). The results showed that bacterial communities from the Danube River and its three tributaries were similar, but were different from those in another tributary, owing to temperature differences. Combining molecular approaches and multivariate analyses revealed that bacterial communities in a low-flowing river were in distinct clusters, according to pollutant sources (Ibekwe et al., 2012). Recently, a high-throughput pyrosequencing technology has been used to explore a freshwater microbial community (Eiler et al., 2012; Heinrich et al., 2013; Logares et al., 2013). Modern sequencing technologies generate larger sequencing datasets with increased taxonomic information and coverage, in comparison to more traditional DNA fingerprinting approaches.

The Grand River watershed is the largest catchment in Southern Ontario and is impacted by different land uses and human activities. The upstream northern portion of the river is influenced primarily by agricultural activities, whereas the central region receives a higher proportion of wastewater effluent and urban runoff. A previous study, discussed in Chapter 4, focused only on the central part of the Grand River; I found that wastewater effluent affected in-river microbial communities downstream. Consequently, a complete survey of the Grand River was conducted,

covering the northern to the southern parts of the watershed. I hypothesized that land usages and river conditions would influence bacterial communities detected along the Grand River.

The objectives of this survey were to investigate the distribution of in-river microbial communities over time and space within an entire watershed and to identify factors that correlate with microbial community distributions. Next-generation sequencing and bioinformatics were used to address the microbial distributions of the Grand River. Statistical and multivariate analyses demonstrated correlations between environmental parameters and in-river bacterial distributions. This study provides a comprehensive baseline for a “Day in the Life of the Grand” from a bacterial perspective, and provides a powerful baseline dataset for better understanding factors that influence the distributions of microbial assemblages within an impacted river.

6.2 Materials and Methods

6.2.1 Sample collection

Water samples were collected from 23 sampling sites along the Grand River, in a total span of ~300 km (Figure 6.1), including three wastewater effluents from Waterloo, Kitchener, and Guelph wastewater treatment plants (WWTPs) on September 10th, 2013. All samples from 23 sites were collected at two time points: before sunrise (T1; 3 am - 6 am) and after solar noon (T2; 12 pm - 3 pm). The samples from seven sampling sites (sites 1, 4, 8, 12, 16, 20, and 23) were also collected for an additional time point: mid-morning (T3; ~10 am). Wastewater effluents were collected at one time point (9 am - 2 pm). Water samples of approximately 120 ml were filtered onsite onto 0.22-mm Sterivex filters (Millipore, USA). The filters were kept on ice during transportation to the lab and transferred to -20°C until DNA extraction.

N-NO₃⁻, SO₄²⁻, and Cl⁻ were measured by ion chromatography, using a Dionex model (Thermo Scientific, Canada). N-NH₄⁺ and N-NO₂⁻ were measured by colorimetric methods, using an AutoAnalyzer II (Technicon Instruments, USA). TN and DOC were measured by an acid combustion method, using an Apollo 9000 Total Organic Carbon Analyzer (Teledyne Tekmar, USA) and a Total Organic Carbon Analyzer TOC-L Series (Shimadzu, Canada), respectively. DO and N₂O were measured following previously published protocols (Rosamond et al., 2012).

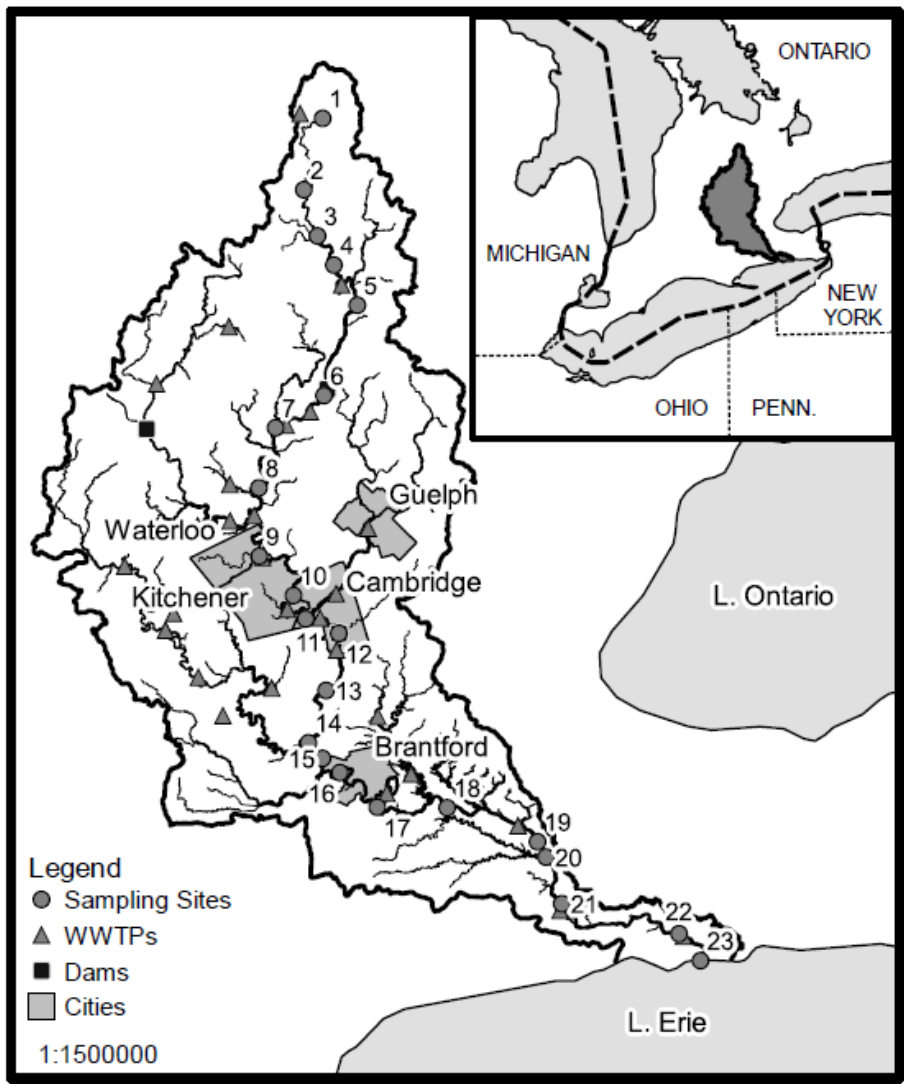


Figure 6.1 Map of 23 sampling sites within the Grand River watershed. The original figure is in Venkiteswaran et al., 2014 (shown here with permission).

Table 6.1 Water chemistry of all sampling sites¹.

Sampling site	Distance	Time	Temp	DO	pH	Conductivity	NH ₄ ⁺ -N	NO ₂ ⁻ -N	NO ₃ ⁻ -N	TN	DOC	Cl ⁻	SO ₄ ²⁻	N ₂ O
	km		°C	mg l ⁻¹	μS		mg l ⁻¹	mg l ⁻¹	mg l ⁻¹	mgN l ⁻¹	mgC l ⁻¹	mg l ⁻¹	mg l ⁻¹	nmol l ⁻¹
Site 1	2.93	T1	16.00	7.05	7.80	614.50	0.05	0.02	1.32	1.64	9.87	23.28	15.23	48.40
Site 2	21.43	T1	17.10	8.40	7.75	546.00	0.00	0.00	0.68	1.42	13.82	28.61	9.50	16.51
Site 3	33.18	T1	17.00	8.00	7.85	508.00	0.04	0.01	0.57	0.93	12.01	21.64	11.01	15.25
Site4	40.45	T1	17.20	8.05	7.97	492.50	0.02	0.00	0.47	0.92	11.48	18.85	11.07	15.13
Site 5	53.11	T1	17.50	7.15	7.94	529.50	0.05	0.08	0.75	1.15	11.07	20.41	14.58	13.25
Site 6	71.01	T1	17.50	6.30	7.81	347.00	0.04	0.11	0.13	0.69	8.74	12.50	8.81	10.13
Site 7	83.91	T1	19.00	8.15	7.97	414.00	0.04	0.06	0.67	1.06	8.64	20.48	12.57	9.95
Site 8	98.05	T1	18.00	9.40	7.91	457.00	0.07	0.07	0.88	1.21	8.78	21.99	13.89	11.46
Site 9	119.24	T1	19.20	8.70	8.04	479.50	0.10	0.07	1.32	1.66	6.52	26.50	22.50	12.26
Site 10	135.00	T1	19.10	6.15	7.76	594.50	0.19	NA	1.95	2.44	6.67	52.01	25.09	33.01
Site 11	145.82	T1	19.80	7.70	7.80	691.50	0.05	0.68	2.97	3.92	6.75	76.64	31.48	126.58
Site 12	153.07	T1	19.00	9.65	8.14	662.50	0.14	0.21	2.56	3.07	5.96	76.26	32.21	26.92
Site 13	164.13	T1	18.00	7.50	7.86	705.50	0.08	0.17	2.91	3.75	5.80	83.07	35.48	32.90
Site 14	175.45	T1	18.00	6.90	7.90	678.00	0.06	0.10	2.54	3.02	6.32	74.76	38.53	14.17
Site 15	181.76	T1	19.00	7.00	7.99	681.00	0.08	0.08	2.38	2.78	5.44	71.72	50.22	13.86
Site 16	187.89	T1	19.00	6.75	8.00	705.50	0.06	0.08	2.37	2.78	4.68	67.11	71.07	15.45
Site 17	216.64	T1	19.00	10.40	8.06	769.00	0.15	0.13	2.69	3.28	4.94	86.76	67.04	18.71
Site 18	232.29	T1	20.00	8.80	7.97	802.00	0.08	0.14	2.78	3.53	5.10	95.10	68.26	44.43
Site 19	250.60	T1	20.00	7.70	7.82	794.00	0.10	0.02	2.30	2.88	5.23	86.99	82.41	38.08
Site 20	253.60	T1	19.00	7.40	7.84	788.50	0.08	0.03	2.28	2.87	5.49	86.56	80.96	27.12
Site 21	263.05	T1	21.00	8.15	7.90	791.00	0.08	0.04	2.21	2.68	4.99	85.45	84.08	30.37
Site 22	288.12	T1	19.00	9.70	8.07	824.00	0.05	0.07	1.77	2.13	4.93	88.49	108.19	60.04
Site 23	295.66	T1	21.00	8.70	7.97	535.00	0.09	0.05	0.82	1.01	3.33	50.11	60.57	15.55

Sampling site	Distance	Time	Temp	DO	pH	Conductivity	NH ₄ ⁺ -N	NO ₂ ⁻ -N	NO ₃ ⁻ -N	TN	DOC	Cl ⁻	SO ₄ ²⁻	N ₂ O
	km		°C	mg l ⁻¹		µS	mg l ⁻¹	mg l ⁻¹	mg l ⁻¹	mgN l ⁻¹	mgC l ⁻¹	mg l ⁻¹	mg l ⁻¹	nmol l ⁻¹
Site 1	2.93	T2	22.00	11.65	7.56	608.00	0.04	0.05	1.24	1.64	10.38	22.75	15.13	31.79
Site 2	21.43	T2	21.00	10.40	7.89	538.00	0.06	0.03	0.62	1.16	13.34	27.19	9.77	15.61
Site 3	33.18	T2	20.00	11.00	8.00	490.50	0.00	0.00	0.67	1.08	12.11	21.28	11.24	13.26
Site 4	40.45	T2	19.80	10.35	7.99	490.00	0.08	0.06	0.60	1.01	11.95	19.89	10.99	13.52
Site 5	53.11	T2	19.80	10.40	8.10	506.50	0.08	0.14	0.58	1.06	11.41	19.64	13.88	11.31
Site 6	71.01	T2	21.00	9.25	7.95	416.00	0.09	0.08	0.09	0.60	9.25	12.18	8.39	9.75
Site 7	83.91	T2	20.50	9.70	8.11	410.00	0.08	0.09	0.61	1.06	8.67	19.65	12.06	9.87
Site 8	98.05	T2	22.80	10.25	8.30	422.50	0.06	0.07	0.74	1.07	7.60	21.22	13.49	14.62
Site 9	119.24	T2	21.20	10.80	8.10	478.50	0.04	0.04	1.34	1.63	6.62	25.07	21.85	13.00
Site 10	135.00	T2	23.10	11.35	8.12	566.00	0.13	0.67	1.76	2.35	6.63	48.12	24.35	40.41
Site 11	145.82	T2	23.00	13.30	8.20	716.50	0.09	0.44	2.97	4.13	6.94	84.00	32.28	79.14
Site 12	153.07	T2	22.80	12.50	7.88	694.00	0.08	0.39	2.58	3.34	6.55	74.40	32.27	44.11
Site 13	164.13	T2	21.00	18.25	8.20	702.50	0.08	0.17	2.77	3.60	5.70	82.53	33.88	17.62
Site 14	175.45	T2	20.90	9.80	8.18	662.50	0.06	0.13	2.53	2.98	5.60	73.11	35.16	13.18
Site 15	181.76	T2	23.20	11.55	8.31	672.00	0.05	0.11	2.35	2.81	5.40	70.28	49.77	15.61
Site 16	187.89	T2	22.10	11.50	8.22	706.50	0.05	0.09	2.37	2.83	4.66	66.73	70.11	14.04
Site 17	216.64	T2	20.00	9.35	8.10	722.50	0.16	0.13	2.50	2.89	4.93	75.91	63.78	21.83
Site 18	232.29	T2	22.00	7.25	7.98	785.50	0.13	0.13	2.66	3.41	4.98	87.55	61.88	51.48
Site 19	250.60	T2	24.00	9.65	8.05	782.50	0.10	0.10	2.42	2.86	4.88	84.83	81.59	40.95
Site 20	253.60	T2	22.00	9.65	8.07	781.00	0.06	0.04	2.41	2.88	4.83	85.55	82.54	30.54
Site 21	263.05	T2	21.00	7.15	7.88	800.00	0.10	0.10	2.29	2.84	4.93	85.98	85.01	34.35
Site 22	288.12	T2	21.00	8.90	8.16	825.00	0.03	0.10	1.89	2.16	5.05	88.72	109.50	36.90
Site 23	295.66	T2	21.00	8.05	8.16	690.00	0.13	0.10	1.31	1.47	4.36	71.26	84.87	32.40

Sampling site	Distance	Time	Temp	DO	pH	Conductivity	NH ₄ ⁺ -N	NO ₂ ⁻ -N	NO ₃ ⁻ -N	TN	DOC	Cl ⁻	SO ₄ ²⁻	N ₂ O
	km		°C	mg l ⁻¹		µS	mg l ⁻¹	mg l ⁻¹	mg l ⁻¹	mgN l ⁻¹	mgC l ⁻¹	mg l ⁻¹	mg l ⁻¹	nmol l ⁻¹
Site 1	2.93	T3	17.10	8.40	7.64	606.50	0.09	0.00	1.26	1.68	10.31	22.19	14.55	38.03
Site 4	40.45	T3	18.00	8.65	7.98	497.00	0.05	0.07	0.48	1.06	12.19	19.33	10.37	15.05
Site 8	98.05	T3	18.90	8.60	7.96	448.00	0.06	0.08	0.73	1.15	7.94	20.80	13.18	11.00
Site 12	153.07	T3	21.00	8.20	7.97	685.00	0.14	0.30	2.61	3.27	6.31	77.65	32.74	32.70
Site 16	187.89	T3	19.00	7.90	7.89	714.50	0.07	0.09	2.43	2.84	4.70	67.21	70.27	16.20
Site 20	253.60	T3	20.00	8.05	7.91	792.00	0.06	0.07	2.37	2.83	5.06	85.84	81.61	30.20
Site 23	295.66	T3	21.00	9.00	8.04	632.00	0.08	0.08	1.17	1.41	4.63	67.76	80.14	33.70
GTP	NA	NA	NA	8.05	7.64	2215.00	0.05	0.02	NA	NA	NA	NA	NA	NA
WTP	119.47	NA	NA	7.00	7.63	2325.00	37.87	0.87	NA	NA	NA	NA	NA	NA
KTP	140.29	NA	NA	5.80	7.31	2400.00	0.14	0.67	NA	NA	NA	NA	NA	NA

¹All parameters were provided by Schiff lab, Department of Earth and Environmental Sciences, University of Waterloo.

GTP = Guelph WWTP; WTP = Waterloo WWTP; KTP = Kitchener WWTP.

6.2.2 DNA extractions

DNA was extracted from the Sterivex filters using the PowerWater Sterivex DNA isolation kit (Mo Bio Laboratories, USA), following the manufacturer's protocol. The quantity and quality of extracted DNA were verified by a NanoDrop spectrophotometer ND-100 (Thermo Fisher Scientific, USA) and agarose gel electrophoresis, respectively. All DNA extracts were diluted to 1 – 5 ng μl^{-1} for a PCR template to subsequently generate a paired-end 16S rRNA gene sequence library.

6.2.3 Illumina library preparation

All samples were amplified following a previously published protocol (Bartram et al., 2011) with minor modifications of the PCR mixture and reaction condition. The sequences of V3 modified forward primer and V4 index primer are AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNCCTACGGGAGGCAGCAG and CAAGCAGAAGACGCATACGAGATXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT, respectively. "XXXXXX" represents an index sequence. All index sequences have been previously published (Bartram et al., 2011). The PCR mixture comprised of 0.05 μl V3 modified forward primer (100 μM), 0.5 μl V4 index primer (10 μM), 0.05 μl dNTPs (100 mM), 0.125 μl *Taq* polymerase (New England Biolabs, Canada), 2.5 μl 10X ThermoPol reaction buffer, 1.5 μL BSA (10 mg/mL), 1 μl DNA template (1 – 5 ng μl^{-1}) in a total volume of 25 μl . The PCR thermal profile was: initial denaturation at 95°C for 30 sec, followed by 30 cycles of 95°C for 15 sec, 50°C for 30 sec, and 68°C for 30 sec, and the final extension at 68°C for 5 min. Each sample was amplified in triplicate and each triplicate was pooled before estimating DNA concentration by gel quantification, using an AlphaImager with Alpha Innotech Software version 3.0.3.0. All samples at equal DNA amount (ng) were pooled into one tube and run on an agarose gel before being purified by a QIAquick Gel Extraction Kit (Qiagen, Canada). A final purified PCR product was prepared for sequencing with a MiSeq Reagent Kit V3, 600 cycles (2x300 bases; Illumina, Canada), following the manufacturer's protocol.

6.2.4 Bioinformatics, statistical, and multivariate analyses

PANDAsseq paired-end assembly, with quality threshold 0.9, generated overlapping regions within paired-end reads (Masella et al., 2012). Sequencing data were clustered at 97% identity using CD-HIT (version 4.5.4; Fu et al. 2012). All chimera sequences were removed using UCHIME *de novo* (version 6.1.544; Edgar et al., 2011). All sequences were assigned taxonomic affiliations using RDP version 2.2 with GreenGenes database (May 2013 revision; DeSantis et al., 2006), with a 0.8

confidence threshold. An OTU table was calculated by the QIIME package v1.7.0 (Caporaso et al., 2010). All sequences were aligned, using Infernal (Nawrocki and Eddy 2013) through QIIME, before phylogenetic tree construction using FastTree (version 2.1.3; Price et al., 2009). Principal coordinates analysis (PCoA) was calculated based on a weighted UniFrac distance matrix (Lozupone and Knight 2005) and a Bray-Curtis dissimilarity matrix (Bray and Curtis 1957) through QIIME (Caporaso et al., 2010), using the AXIOME pipeline (Lynch et al., 2013).

6.3 Results

6.3.1 The composition of bacterial taxa along the Grand River

Illumina paired-end sequencing generated an average of 61,286 sequences per sample. The majority of river-associated bacterial taxa were affiliated with the *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* (Figure 6.2). The relative OTU abundances of bacterial taxa for each location at different time points were consistent. *Proteobacteria* accounted for ~40 – 60% of all OTU abundance. *Bacteroidetes* and *Actinobacteria* accounted for ~10 – 45% and ~10 – 37%, respectively. *Verrucomicrobia* was a minor phylum detected at all sites, in the range of 0.3 – 2.4% of the total taxa. Organelles from eukaryotic *Stramenopiles* were detected at all sampled locations in the Grand River; however, their proportion increased within the southern portions of the watershed, starting from sites 18 to 23 (Figure 6.2). Detecting *Stramenopiles* across the Southern region likely reflected an algal bloom.

In wastewater effluent communities, *Proteobacteria* accounted for ~60 – 70% of all OTU abundance (Figure 6.2). Although *Actinobacteria* were dominant across sampling sites along the river, they only occurred at ~10%, ~3%, and less than 1% in Kitchener, Waterloo, and Guelph WWTPs, respectively. *Firmicutes* were more highly represented in wastewater effluent samples (Figures 6.2 and 6.3). *Firmicutes* accounted for 2 – 11% of all OTU abundance in wastewater samples, but were present in low abundance along the Grand River, in the range of 0.1 – 1.4%. TM7 were also highly represented in wastewater effluent samples, accounting for 1 – 4% in wastewater samples, but below 0.3% in all sampling sites along the river (Figure 6.2). GN02 were high only in Waterloo wastewater effluent, accounting for 3% of all OTU abundance, while they represented less than 0.3% of all taxa in the rest of samples. TM6 were highly represented only in Guelph wastewater effluent and accounted for ~6% of total OTU abundance, while they were below 0.3% of other samples (Figure 6.2).

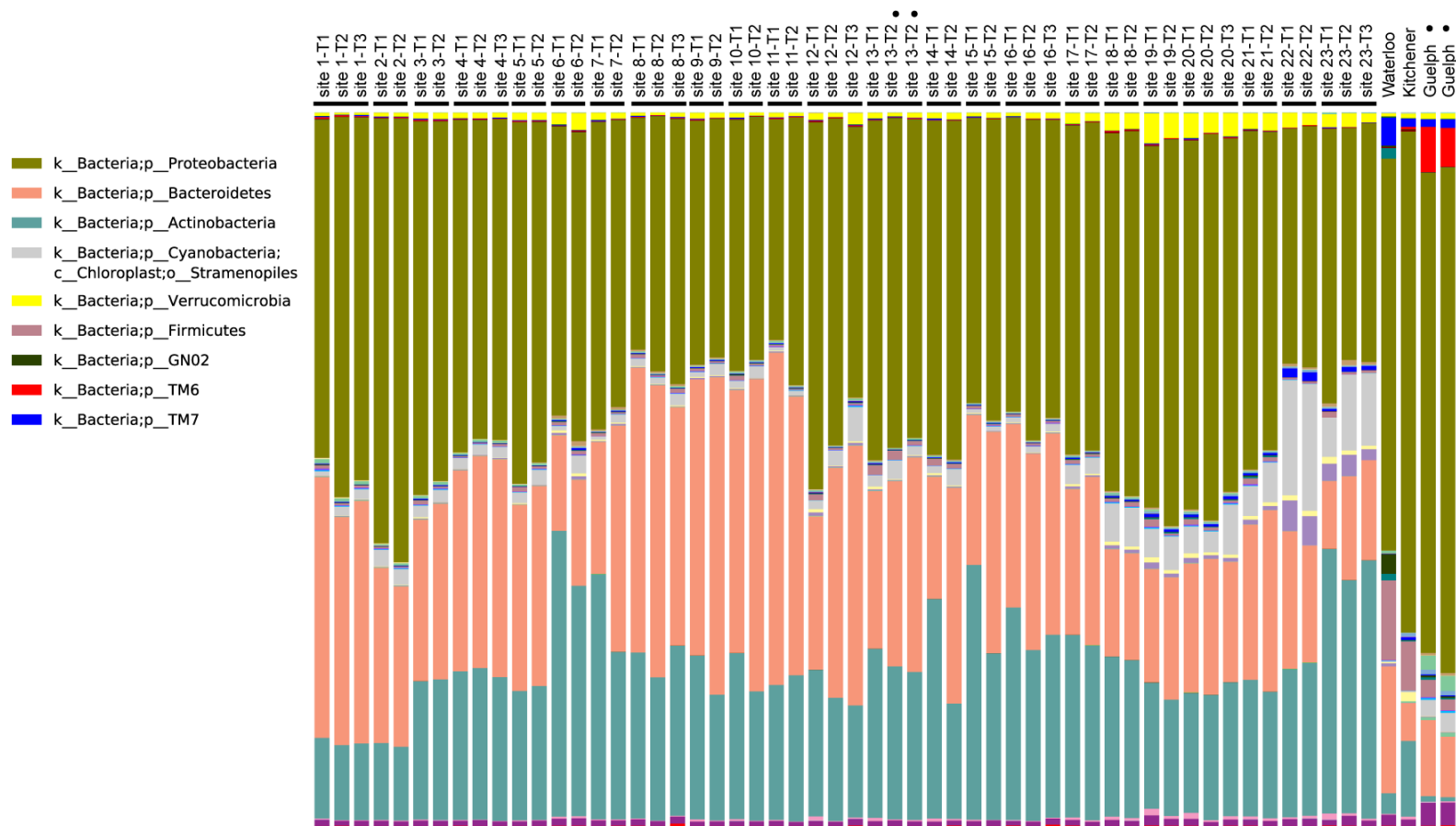


Figure 6.2 Taxonomic composition at the phylum level of all sampling sites (sites 1 to 23), all time points (T1, T2, and T3), including wastewater effluents from Waterloo, Kitchener, and Guelph WWTPs. The y-axis represents the proportion of OTU abundance. Black dots indicate single-sample replicate DNA extractions.

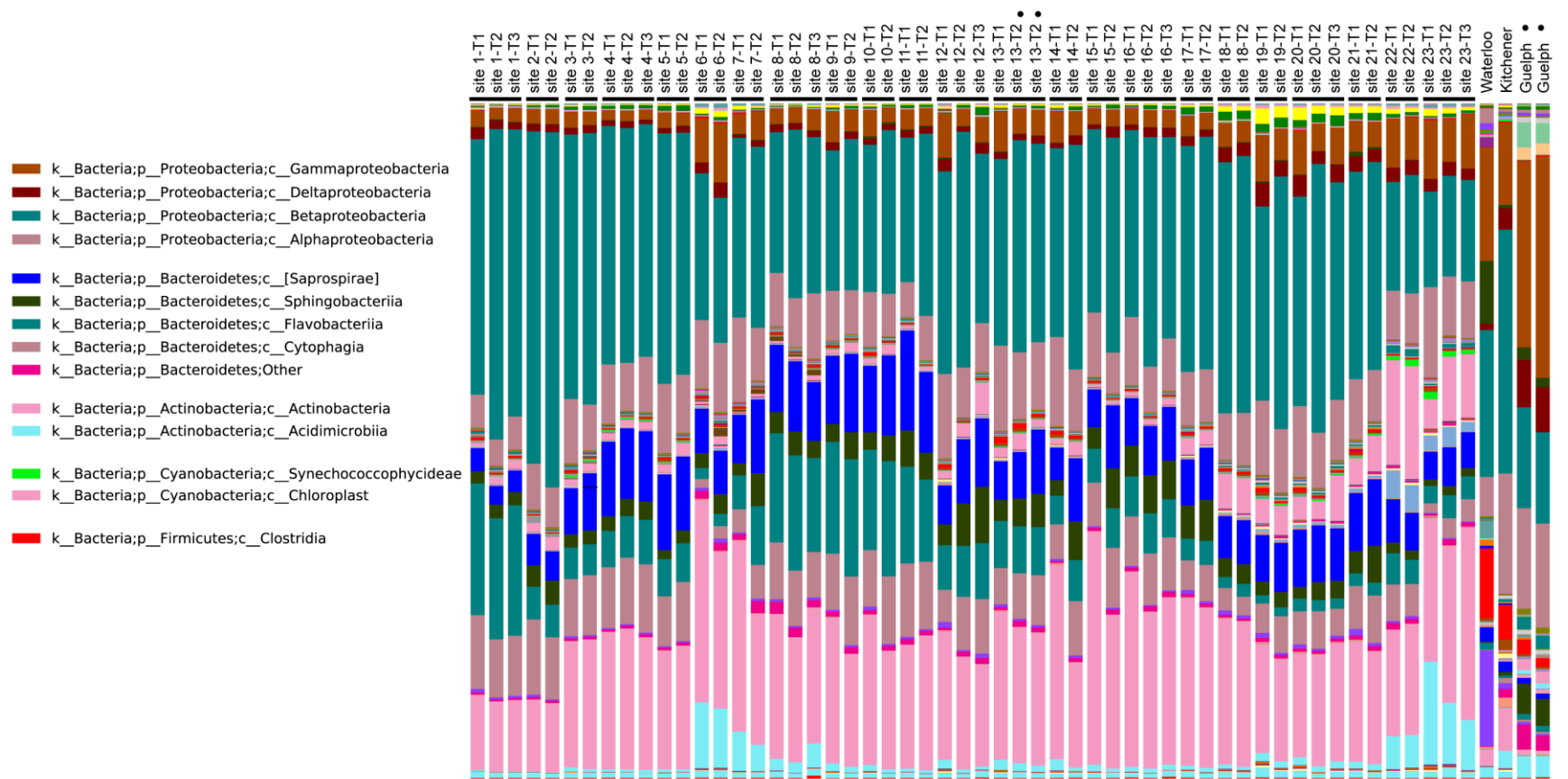


Figure 6.3 Taxonomic composition at the class level of all sampling sites (sites 1 to 23), all time points (T1, T2, and T3), including wastewater effluents from Waterloo, Kitchener, and Guelph WWTPs. The y-axis represents the proportion of OTU abundance. Black dots indicate single-sample replicate DNA extractions.

6.3.2 The factors impacting in-river bacterial distribution

A PCoA ordination based on a weighted UniFrac distance matrix demonstrated that the major bacterial taxa detected along the Grand River were Proteobacteria, Bacteroidetes, and Actinobacteria (Figures 6.4A). These results agreed with the overall taxonomic composition of samples that were previously observed (Figure 6.2). The bacterial communities in wastewater effluents from Waterloo, Kitchener, and Guelph WWTPs were distinct from those in the Grand River (Figures 6.2 and 6.3). Consequently, all three samples from wastewater effluents were removed prior to further analysis. Bacterial community changes, as reflected in the ordination space, were related to distance from the headwater to the mouth of the Grand River (Figure 6.4B). Following site 1 samples, those from sites 2 to 5 clustered together. The samples from site 6 were apart from those of the previous sampling sites (Figure 6.4B). Samples from sites 7 to 11 grouped together and were followed by those from sites 12 to 21. Samples from sites 22 and 23 clustered together, and they were close to those from site 6 (Figure 6.4B). Similarly, a PCoA-based ordination based on a Bray-Curtis dissimilarity metric also showed the gradual shift in microbial communities from sites 1 to 23 (Figure 6.5). However, samples from site 6 were apart from site 5, and samples from sites 22 and 23 grouped further away from site 21 (Figure 6.5). Overall, the results showed a clear relationship with increasing distance (Figures 6.4B and 6.5). An interesting exception to this trend was that the samples from sites 6, 22, and 23 were close to one another, but they differed from other sampling sites. The possible reason for this finding was different geography at sites 6, 22, and 23. There is a dam above site 6, and sites 22 and 23 are located close to Lake Erie (Figure 6.1), reflecting an impounding reservoir effect. The taxonomic compositions of sites 6, 22, and 23 were different from those of other sampling sites along the Grand River. Although the community shift was not clear at a phylum level (Figure 6.2), a class level demonstrated that the composition within Actinobacteria changed in sites 6, 22, and 23 (Figure 6.3). The class Acidimicrobiia was higher in sites 6, 22, and 23, representing 5 – 15% of all OTU abundance. LD12 are a freshwater sister clade of marine SAR11; they were proposed to be the most widely distributed freshwater lake cluster of the *Alphaproteobacteria* (Logares et al., 2009; Newton et al., 2010). In this study, LD12 were represented by the family *Pelagibacteraceae* in the taxonomic compositions at the family and genus levels; they accounted for ~1 – 4 % of all OTU abundance in sites 6, 22, and 23, but they were less than 1% of OTU abundance in all other samples (data not shown). The samples from the same sampling site at multiple time points (T1, T2, and T3) were highly similar to one another (Figures 6.4B and 6.5).

Biplot vectors within the PCoA-based ordination based on a Bray-Curtis dissimilarity metric demonstrated all potential factors influencing in-river microbial biogeography (Figure 6.5). The strong environmental variables were DOC, TN, NO₃⁻, conductivity, Cl⁻, SO₄²⁻, and distance. DOC showed an opposite direction to the other strong variables on the ordination space (Figure 6.5). NH₄⁺, N₂O, pH, temperature, DO, and NO₂⁻ showed a lower magnitude associated with microbial biogeography. NH₄⁺, N₂O, pH, and temperature influenced the microbial distributions in a similar direction, but they differed from DO and NO₂⁻ on the ordination space. The samples from sites 6, 22, and 23 were near one another at the right corner on the ordination space. These results indicated that the samples from sites 6, 22, and 23 have comparable microbial taxa composition.

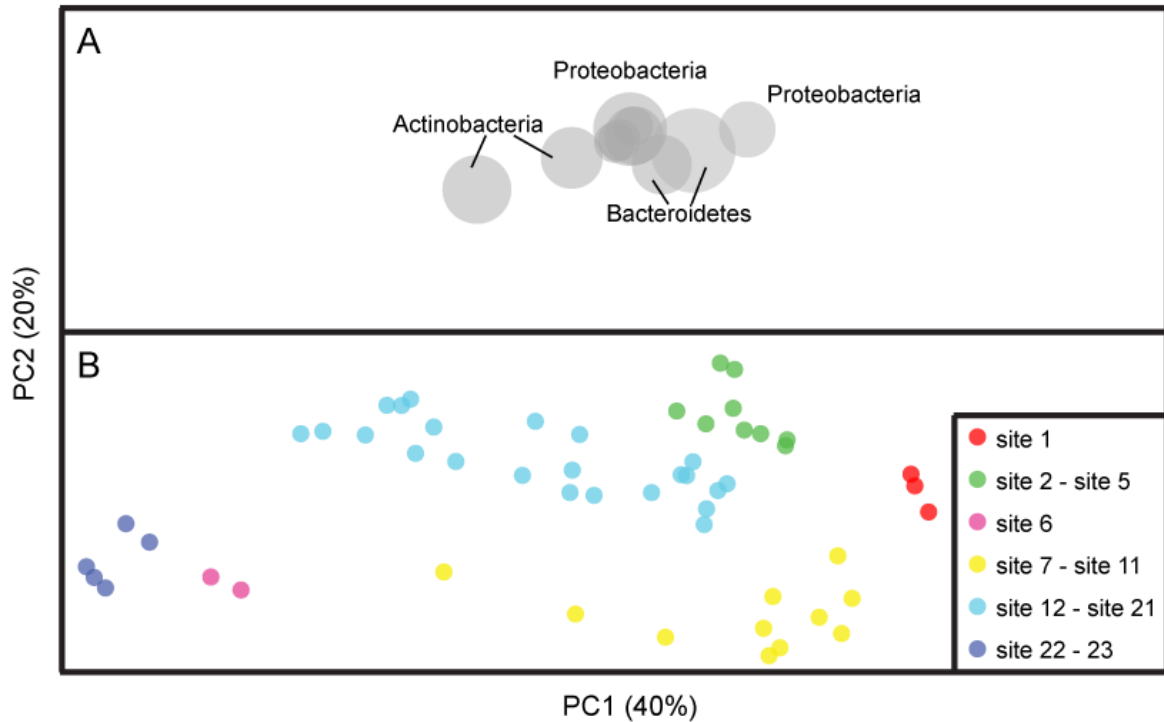


Figure 6.4 **PCoA ordination plot based on a weighted UniFrac distance matrix with all river samples** collected for the Day in the Life of the Grand analysis. A) The majority of bacterial phyla detected across all sampling sites are shown in relation to the ordination shown in panel B). B) The distributions of microbial composition from each sampling site and all time points. Each colored symbol represents samples from different time points, for example, a red symbol represents samples from site 1 at three time points (T1, T2, and T3). The proportion of the variation explained by each axis is indicated.

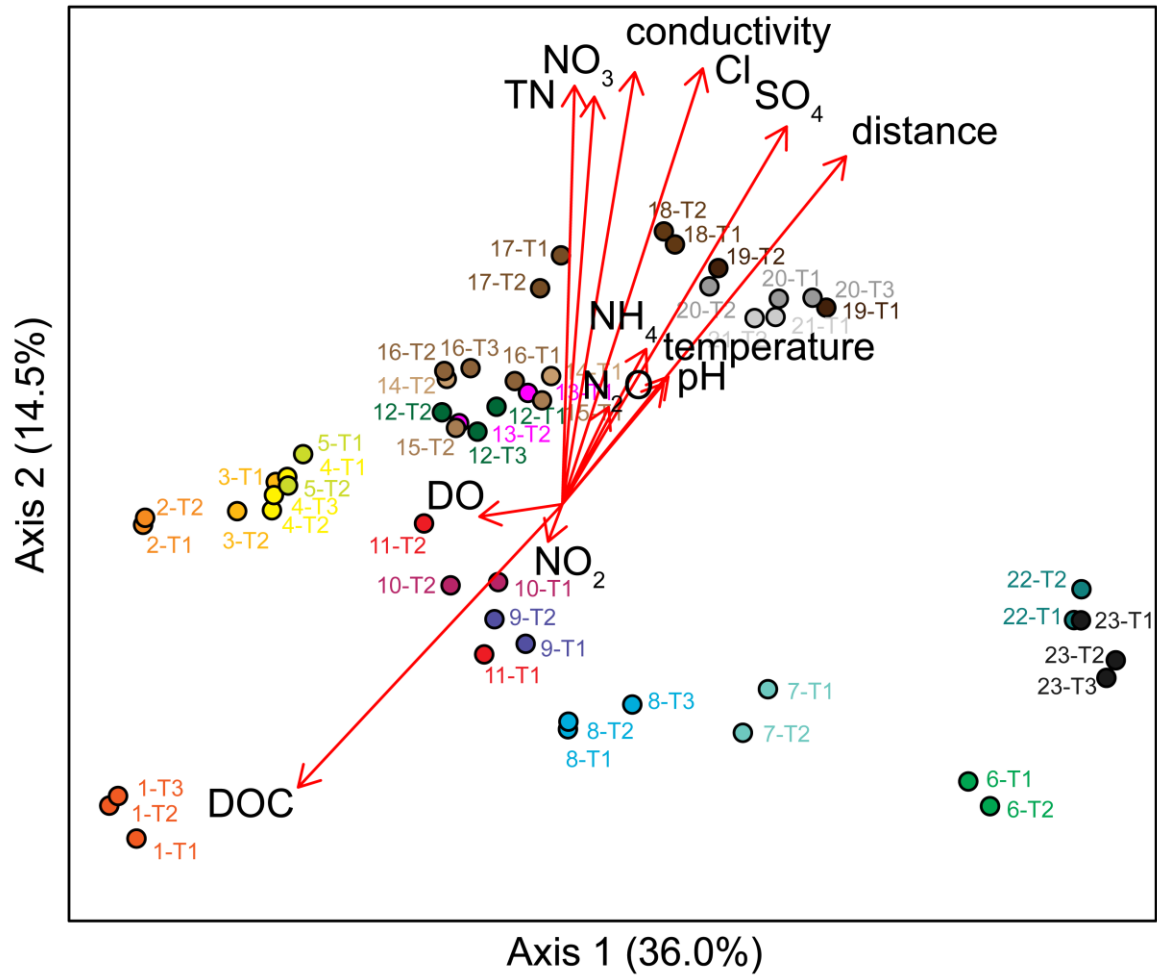


Figure 6.5 A PCoA ordination based on a Bray-Curtis distance matrix. Biplot vectors show the direction of environmental variables as they correlate within the ordination space. Colored symbols represent samples from different sampling sites (sites 1 – 23) at different time points (T1, T2, and T3). The proportion of the variation explained is indicated on the axes.

6.4 Discussion

Proteobacteria, Bacteroidetes, and Actinobacteria were the major bacterial taxa detected in the Grand River, suggesting that they were an important component of in-river microbial communities. These three phyla were also detected in other freshwater environments such as the Danube River (Winter et al., 2007), the Santa Ana River, California USA (Ibekwe et al., 2012), Parana River, Brazil (Lemke et al., 2009), Lake Taihu, China (Wu et al., 2007) and Lake Erken, Sweden (Eiler et al., 2012). Verrucomicrobia were present in low abundance across all sampling sites. Verrucomicrobia were a minor bacterial taxa detected in other rivers and lakes (Sekiguchi et al., 2002; Zwart et al., 2002). Proteobacteria, Bacteroidetes, Actinobacteria, and Verrucomicrobia were present in four Swedish lakes where cyanobacterial blooms were observed (Eiler and Bertilsson 2004). The proportion of Stramenopiles increased across the southern portion of the Grand River; those 16S rRNA gene sequences were classified as chloroplasts, possibly indicating an algal bloom. For all sampling sites, the microbial communities from multiple time points were consistent, indicating that the bacterial communities at each sampling location were stable throughout the day. However, additional samples from different seasons and flow magnitudes are needed to reveal potential seasonal effects and long-term successions of bacterial communities throughout the year.

Site 1, located near the headwater of the Grand River, was highly impacted by DOC (Figures 6.1 and 6.5). Due to the topography of headwater, sediments and organic carbon are delivered from headwater to downstream water (Allan and Castillo 2007). The concentration of DOC was high in the sites located close to the headwater and it gradually decreased across the downstream sites until reached the lowest concentration in site 23 (Table 6.1). Sites 1 to 5 possibly received DOC from the wetlands and agriculture in the northern portion of the Grand River. Another strong variable was TN (Figure 6.5). The concentration of TN was high ($\sim 3 - 4 \text{ mg N l}^{-1}$) across sites 11 to 21 (Table 6.1). The possible sources of TN were from wastewater effluent, urban runoff, and agriculture.

The ordinations demonstrated that the effects of environmental variables on in-river microbial communities were not as clear as the effect of increasing distance from the headwater to the mouth of the Grand River (Figures 6.4B and 6.5). Geographical distance was reported to influence the bacterioplankton communities in tundra lakes (Crump et al., 2007). Within connected lakes, the bacterioplankton communities shifted with distance and lakes separated the most along the catchment were the most dissimilar. Bacterial communities in high-mountain lakes located closer were more similar than those located in lakes separated by a greater distance, whereas water chemistry (i.e. DOC and chlorophyll-a) had no effect on the composition of bacteria (Reche et al., 2005). The

bacterioplankton communities in lakes within the same drainage area were more similar to those from the different drainage areas (Lindström and Bergström 2005). However, geographical location and stream flow were unrelated to the bacterial distribution in stratified lakes (Konopka et al., 1999). Geographical distance showed a weak relationship to the bacterioplankton communities between coastal lakes, but the lakes with similar salinity levels tended to have similar bacterioplankton compositions (Logares et al., 2013).

The microbial communities of sites 6, 22, and 23 did not follow the downstream trend (Figures 6.4B and 6.5). Dam construction affected the water flow of site 6. Sites 22 and 23 were located close to and connected to Lake Erie (Figure 6.1). An impounding reservoir impacted the Actinobacteria composition in sites 6, 22, and 23. The impounding reservoir reduces water flow and increases hydrological retention time. These enhance an accumulation of nutrients, which affect the availability of electron donors and acceptors for microorganisms in the impacted sampling sites. The Grand River travel time from site 6 to 23 was 78-93 hours and 43 hours in low flow and high flow, respectively (Mark Anderson, GRCA, via Madeline Rosamond, personal communication). The low flow and high flow were calculated based on an increase or decrease in reservoir discharge. The Grand River travel time was estimated based on hydraulic modeling; however, the model is still developed (Mark Anderson, GRCA, via Madeline Rosamond, personal communication). In further analysis, the Spring samples which represent high water flow will be analyzed for comparison. The effect of a lake was also observed in the Changjiang River, China (Sekiguchi et al., 2002). The bacterial communities in lakes and rivers were different because the water flow in lakes is slower than that in rivers. A reservoir also affected bacterial and archaeal diversities in the Sinnamary River, France (Dumestre et al., 2002). Certain bacterial and archaeal assemblages were detected in the reservoir, but they were not recovered from upstream of the dam. Also, minor bacterial assemblages became dominant in the reservoir (Dumestre et al., 2002). Hydrological retention time showed high correlation with the bacterial distributions in lakes (Lindström et al., 2005).

In contrast to these current findings, environmental variables influenced bacterial communities, as determined by DGGE, while geographical factors had no effect on bacteria diversity in the Danube River (Winter et al., 2007). Chlorophyll-a and P-PO₄ concentrations affected the number of bacterial DGGE bands. The difference in temperature between the Danube River and its tributary impacted bacterial communities; however, large impounding reservoirs had no effect on bacterial communities (Winter et al., 2007). The explanation for these contrasting findings may be

associated with the limitations of the DGGE approach (i.e., detection limit and resolution), compared to next-generation sequencing, which was used in this current study. DGGE detects only the predominant taxa, but Illumina sequencing generates datasets with greater resolution, covering various taxonomic levels. The effect of impounding reservoirs on bacterial community composition was clearer at the class level (Figure 6.3) than at the phylum level (Figure 6.2); these findings would not be detectable by DGGE or other fingerprinting approaches. Consequently, the methodology employed may strongly influence the conclusions of studies. However, there are some limitations of Illumina sequencing method. Short sequence reads by a typical Illumina sequencing reduced the taxonomic resolution (Claesson et al., 2010). The modified paired-end Illumina reads used in this study generated two-fold coverage of PCR amplicons which help improve the accuracy and quality of sequence data (Bartram et al., 2011). The sequences of modified forward and reverse primers are longer than those used for a regular PCR. These long primer sequences potentially introduce methodological bias for the amplification. In this study, triplicate PCR amplifications were performed and pooled to reduce methodological bias. The effect of pooling PCR amplicons, template concentration, and sample preparation on the reproducible results of Illumina paired-end reads were evaluated previously (Kennedy et al., 2014). The results demonstrated that PCR template concentration significantly affects sample profile variability, especially for a high-diversity soil sample. However, the samples analyzed in this study were homogeneous; the impact of methodological bias on 16S rRNA gene surveys might be less than for highly heterogeneous samples.

Chapter 7

Conclusions

This thesis provides further understanding of N cycle related microbial communities in freshwater environments. My work focused on specific microorganisms, including AOB, AOA and anammox bacteria. All of these microbial groups were implicated in N transformations within the Grand River and were potentially impacted by wastewater effluent. In particular, anammox bacteria in freshwater habitats were overlooked for many decades due to the limitation of existing molecular tools and the recalcitrance of these bacteria to traditional methods for laboratory cultivation. Consequently, knowledge of anammox bacterial distributions and activity in freshwater environments were particularly limited prior to my project. This thesis explored the biogeography of anammox bacteria, and provided information that will support future research on anammox bacteria. For example, this thesis identified appropriate molecular tools that can be used to target anammox bacteria in freshwater environments. The discovery of AOA represented a major change in the understanding the N cycle and has raised many questions for environmental microbiologists, including their relative contributions of AOA, potential for functional redundancy with AOB, and possible niche partitioning between AOB and AOA. This thesis helped address the possible relationship and niche partitioning of AOB, AOA, and anammox bacteria within the sediments and water columns of the Grand River. Together with molecular approaches, activity-based experiments with nitrification inhibitors helped confirm the overall relative contributions of AOB and AOA to ammonia oxidation within the Grand River. Finally, because wastewater microbial signatures were evident in water column samples downstream of a WWTP, in-river microbial communities within the entire Grand River, from the headwater to the mouth, were explored to increase the sampling of river microorganisms to the bacterial community level. This final biogeography represents the first comprehensive baseline for bacterial biogeography within river ecosystem and contributes to a better understanding of the geographical and environmental factors that affect in-river bacterial communities. The conclusions, significance, and future considerations of each project are summarized below.

7.1 Global distributions of anammox bacteria

Anammox bacteria have been detected in natural ecosystems, including marine sediment (Penton et al., 2006; Hong et al., 2011a; Han and Gu 2013), marine water column (Woebken et al., 2007 and 2008; Galán et al., 2009), estuary (Dale et al., 2009; Hou et al., 2013), mangrove sediment (Li and Gu 2013; Wang et al., 2013; Han and Gu 2013), soil (Zhu et al., 2011a; Hu et al., 2013; Shen et al., 2013a), freshwater (Hamersley et al., 2009; Han and Gu 2013; Sonthiphand and Neufeld 2013), freshwater sediment (Sonthiphand et al., 2013; Sun et al., 2014; Wu et al., 2012), groundwater (Hirsch et al., 2011; Moore et al., 2011; Sonthiphand and Neufeld 2013), reactors (Egli et al., 2001; Hu et al., 2010; Park et al., 2010), and WWTPs (Araujo et al., 2011; Bae et al., 2010). Anammox bacteria have also been detected in marine sponge (Hoffmann et al., 2009; Mohamed et al., 2010), fish gut (unpublished), oil fields (Li et al., 2010; Shartau et al., 2010), biofilters (van Kessel et al., 2010) and a shrimp pond (Amano et al., 2010). In Chapter 2, all anammox 16S rRNA gene sequences from multiple isolation sources were assigned to 15 habitats and analyzed by bioinformatics, statistical, and multivariate analyses. The primary finding was that the global distribution pattern of anammox bacteria is controlled primarily by salinity (Figures 2.1 and 2.2). Distinct partitioning of anammox bacterial communities among natural and engineered ecosystems was also observed in this sequence survey. Insufficient information on anammox genomes and physiological properties is available to draw detailed conclusions on how environmental factors (i.e., NH_4^+ and NO_2^-) affect possible anammox bacterial distributions. Additional metagenomic studies of other anammox species will help compare and contrast the specific genes and their functions that significantly differ among anammox species to better understand the specific distribution and co-occurrence of anammox clusters. Further investigations of kinetic and biochemical properties of more anammox enrichments, or ideally isolates, are needed to better understand the metabolism and ecological niches of specific anammox bacteria.

In particular, this survey shed light on anammox bacteria in freshwater environments, where the diversity is known to be high (i.e., Moore et al., 2011; Sonthiphand and Neufeld 2013). Anammox bacteria in groundwater need more attention to better understand their diversity. I propose freshwater as a promising target habitat for the discovery and characterization of additional phylogenetically novel anammox species because anammox bacteria in freshwater environments were the most diverse among sampled environments (Figure 2.3). Overall, research on anammox bacteria is still an active area with both applied and fundamental research considerations. Multidisciplinary approaches,

including cultivation, metagenomics, and molecular approaches, are needed to fill in missing knowledge gaps. This research helps understand the nature of anammox bacterial species across various habitats, and raises questions about why some of anammox species are dominant in specific habitats, whereas others are not abundant but widespread.

7.2 Anammox primers

Anammox community surveys are highly dependent on molecular tools because anammox bacteria grow slowly, and there is no pure anammox culture available thus far (Jetten et al., 2009). Both 16S rRNA and functional genes are used as biomarkers to capture anammox bacteria from environments. The limitations of using functional genes are the reduced availability of primers and smaller database than when using 16S rRNA genes. The nitrite reductase (*nirS*) gene was used to detect anammox bacteria in the ocean (Lam et al., 2009; Li et al., 2011b); however, metagenomic studies revealed that *Ca. Jettenia asiatica* (Speth et al., 2012) and *Ca. Brocadia fulgida* (Gori et al., 2011) contain *nirK* instead of *nirS*. Consequently, some species could be missed if some specific functional genes are used as biomarkers. In contrast, anammox bacterial 16S rRNA genes are commonly used as targets for molecular surveys. However, non-specific amplification is still problematic. The general problems of using specific primers for targeting anammox 16S rRNA genes are the low recovery efficiencies of anammox-related clones, non-specific amplification, and inability to target all anammox clusters (Li et al., 2010b; Penton et al., 2006; Humbert et al., 2010; Amano et al., 2007; Rich et al., 2008; Zhang et al., 2007; Tal et al., 2005 and 2006). In Chapter 3, published primers commonly used in anammox research were validated for investigating the diversity and abundance of anammox bacteria within freshwater environments. The results demonstrated that A438f/A684r or Amx368f/Amx820 are appropriate for clone library analysis or qPCR analyses, but only A438f-GC/A684r was suitable for DGGE-based analyses of anammox bacteria (Figure 3.3). Because DGGE is cost-effective, rapid, and reproducible, my research also is the first to validate DGGE fingerprinting for screening anammox bacteria and these findings directly benefit researchers who are working on anammox molecular surveys. In addition, selecting a specific primer is one of the most important steps in molecular surveys. A reliable primer set helps reduce biases and increase the accuracy of results. My research indicates that A438f and A684r can target all known anammox clusters and this primer pair is suitable for multiple molecular approaches. However, my results were obtained mainly from the anammox bacterial 16S rRNA genes within freshwater environments. Future research should include a broad range of environmental samples such as marine, terrestrial,

and engineered systems to evaluate the efficiency and specificity of primers for targeting anammox bacteria.

7.3 Ammonia oxidation within the Grand River

The Grand River was studied as an example of a large watershed impacted by ammonia inputs from WWTP discharge and agricultural runoff. Samples were collected from sites along an ammonia transect, covering upstream and downstream of a discharge from the Waterloo WWTP. In addition to the ammonia transect, other samples were collected from sampling sites located in the Waterloo-Kitchener-Cambridge area. Chapter 4 demonstrated the coexistence of AOB and anammox bacteria, implicating a possible link between nitrification and anammox activities in freshwater sediments. Only AOB played an important role in river water in my results (Figure 4.4), AOA activity was less important along the Grand River as assessed by the incubation conditions in this study. The coexistence of anammox bacteria and AOB has been reported previously for the Black Sea (Lam et al., 2007), the Peruvian OMZ (Lam et al., 2009), the Namibian OMZ (Woebken et al., 2007), a fertilized paddy soil (Zhu et al., 2011a) and a constructed wetland (Zhu et al., 2011b). Only a few studies on communities of anammox bacteria, AOB, and AOA in freshwater systems have been available so far. I suggest that ongoing metagenomic studies should be conducted on both local and global scales to gain further insight into their communities, which can be linked to the controlling factors and niche specificity of each microbial group. Future research should investigate additional WWTP plumes within the Grand River to reveal wastewater effluent impacts on in-river microbial communities. Long-term observation will be required to understand the effect of seasonal changes and the temporal changes in N cycling throughout the year and a diel basis. Multidisciplinary approaches such as stable-isotope probing (SIP), differential enzyme inhibitors, and isotope-pairing techniques should be applied in the future to gain better insight into the relative bacterial contributions to ammonia oxidation.

7.4 Nitrification and urea hydrolysis

Although nitrification assays with differential inhibitors have been conducted to reveal the relative contributions of AOB and AOA in soil (Di et al., 2009; Taylor et al., 2010; Schauss et al., 2009) and marine (Santoro et al., 2011) environments, little is known about the relative contribution of AOB and AOA to ammonia oxidation within freshwater environments. In Chapter 5, sediment and water samples from the Grand River were incubated in freshwater media with PTIO or ATU

additions. PTIO (100 μM) was used to inhibit AOA, whereas ATU (10 and 100 μM) is thought not to inhibit AOA. However, PTIO also apparently affected nitrite-oxidizing bacteria (NOB) in downstream water samples. There has been no information on the effect of PTIO on NOB thus far. An alternative ammonium source, urea hydrolysis, was also investigated in parallel with nitrification. These findings confirmed that AOB are the key microorganisms removing ammonium from the Grand River. AOB within downstream water also have the ability to hydrolyze urea, indicating that ammonia produced by urea is an alternative N source for AOB. Future research needs to investigate the modes of action and direct or indirect mechanisms by which PTIO and ATU may affect urease and nitrite oxidizing activities. Other than environmental samples containing the mixture of AOB and AOA in nature, AOB and AOA enrichments should be included as controls in future studies. Other nitrification inhibitors with different modes of action will be included to confirm the relative activity of AOB and AOA. Nitrification inhibitors with different concentrations will be applied to identify the inhibition thresholds of AOB and AOA in environmental samples. These would help select inhibitors with an appropriate concentration to inhibit one microorganism with minimal effect on another.

7.5 Microbial biogeography of the Grand River

The microbial community within freshwater has been studied worldwide by fingerprinting approaches such as PCR-DGGE (Lindström et al., 2006; Winter et al., 2007; Dumestre et al., 2002) and terminal restriction fragment length polymorphism (T-RFLP; Szabó et al., 2007; Ibekwe et al., 2012). However, the sensitivity and detection limits of DNA fingerprinting methods are insufficient to capture various bacterial taxa, especially those occurring at low relative abundance. In this study, combining next-generation sequencing, bioinformatics, statistical, and multivariate approaches generated a comprehensive dataset at different taxonomic levels of the microbial community within an entire watershed. The relationship with distance from the headwater to the mouth of the river, including hydrodynamics (i.e., lake and dam effects), was the key factor affecting in-river microbial community. However, research on this project is ongoing. More chemistry data (i.e., %C, %N, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, CH_4 , CO_2 , DIC) will be included in future analyses. Statistical supports (i.e., RDA, CCA, Mantel test) will be conducted to reveal the relationship between multiple environmental variables and in-river bacterial distributions. All chemistry data will be plotted against distance from source to reveal the trend, which can be linked to bacterial taxa composition. Also, a recent Spring sampling, collected on April 11th, 2014, will be analyzed for comparison with my Fall 2013 baseline data. The major difference between the first (Fall 2013) and the second (Spring 2014) sampling events is the

water flow, which peaks in Spring. In a broad perspective, it would be interesting to compare and contrast the freshwater bacterial composition in different climatic zones (i.e., tropical, temperate, and boreal zones).

7.6 Summary

My research has furthered the understanding of the N cycle in freshwater environments, especially those impacted by anthropogenic input in the form of wastewater effluent. Prior to this work, only the combined contributions of nitrification and denitrification were considered in removing fixed N from impacted freshwater environments. This thesis work helps identify overlooked N-cycling processes and microorganisms and implicates possible nitrification-anammox coupling processes for N loss within freshwater environments. A complete picture of the global N cycle and the relationship of microbial N transformations are needed to better predict the effects of human activities on the biogeochemical N cycle within freshwater environments. An understanding of the N cycle can also be linked to other nutrient cycles (i.e., carbon, phosphorus, and sulfur) to better develop nutrient management strategies for impacted freshwater environments. In addition, this research provides new insight into in-river microbial communities, representing various taxonomic levels. Prior to this study, a comprehensive view of microbial biogeography within an entire watershed had not been explored. This study is the first step toward understanding the co-occurrence patterns of both abundant and rare taxa within an impacted river watershed, which could be further developed as a microbial source tracking tool to differentiate between autochthonous and allochthonous microbial signatures.

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