

Prevalence of Anaerobic Ammonium-Oxidizing Bacteria in Contaminated Groundwater

Tara A. Moore,[†] Yangping Xing,[‡] Brent Lazenby,[†] Michael D. J. Lynch,[†] Sherry Schiff,[†] William D. Robertson,[†] Robert Timlin,[§] Sadia Lanza,^{||} M. Cathryn Ryan,[⊥] Ramon Aravena,[†] Danielle Fortin,[‡] Ian D. Clark,[‡] and Josh D. Neufeld^{*,†}

[†]University of Waterloo, 200 University Avenue West, Waterloo, Ontario N2L 3G1, Canada

[‡]University of Ottawa, 140 Louis Pasteur, Ottawa, Ontario K1N 6N5, Canada

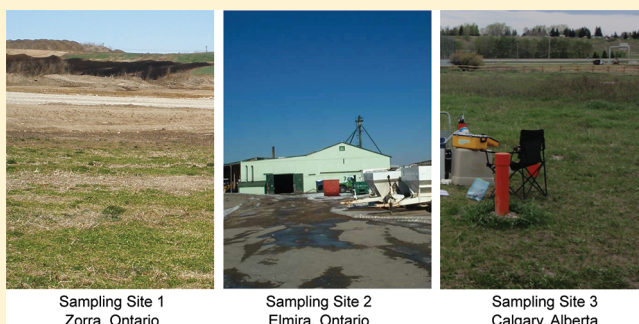
[§]SNC-Lavalin Environment, 20 Colonnade Road, Suite 110, Ottawa, Ontario K2E 7M6, Canada

^{||}Universidad Nacional de Honduras, Tegucigalpa, Honduras

[⊥]University of Calgary, 2500 University Drive N.W. Calgary, Alberta T2N 1N4, Canada

S Supporting Information

ABSTRACT: Anaerobic ammonium-oxidizing (anammox) bacteria perform an important step in the global nitrogen cycle: anaerobic oxidation of ammonium and reduction of nitrite to form dinitrogen gas (N₂). Anammox organisms appear to be widely distributed in natural and artificial environments. However, their roles in groundwater ammonium attenuation remain unclear and only limited biomarker-based data confirmed their presence prior to this study. We used complementary molecular and isotope-based methods to assess anammox diversity and activity occurring at three ammonium-contaminated groundwater sites: quantitative PCR, denaturing gradient gel electrophoresis, sequencing of 16S rRNA genes, and ¹⁵N-tracer incubations. Here we show that anammox performing organisms were abundant bacterial community members. Although all sites were dominated by *Candidatus* Brocadia-like sequences, the community at one site was particularly diverse, possessing four of five known genera of anammox bacteria. Isotope data showed that anammox produced up to 18 and 36% of N₂ at these sites. By combining molecular and isotopic results we have demonstrated the diversity, abundance, and activity of these autotrophic bacteria. Our results provide strong evidence for their important biogeochemical role in attenuating groundwater ammonium contamination.



Sampling Site 1
Zorra, Ontario

Sampling Site 2
Elmira, Ontario

Sampling Site 3
Calgary, Alberta

Here we show that anammox performing organisms were abundant bacterial community members. Although all sites were dominated by *Candidatus* Brocadia-like sequences, the community at one site was particularly diverse, possessing four of five known genera of anammox bacteria. Isotope data showed that anammox produced up to 18 and 36% of N₂ at these sites. By combining molecular and isotopic results we have demonstrated the diversity, abundance, and activity of these autotrophic bacteria. Our results provide strong evidence for their important biogeochemical role in attenuating groundwater ammonium contamination.

INTRODUCTION

For decades, microbiologists believed that the coupled processes of chemo lithoautotrophic ammonia oxidation (or nitrification; $\text{NH}_4^+ \rightarrow \text{NO}_3^-$) and chemo organoheterotrophic denitrification (or denitrification; $\text{NO}_3^- \rightarrow \text{N}_2$) were the sole pathways for returning fixed nitrogen to the atmosphere in the form of dinitrogen (N₂). In particular, ammonium (NH₄⁺) oxidation was attributed solely to aerobic nitrifying bacteria. In 1995, the discovery of anaerobic ammonium oxidation (anammox) revolutionized the nitrogen cycle by demonstrating that ammonium was also oxidized under anoxic conditions.^{1,2} The anammox reaction involves the coupled oxidation of ammonium and reduction of nitrite to produce N₂. Since their initial discovery in engineered environments, anammox bacteria have been implicated in substantial losses of fixed nitrogen from a variety of aquatic and terrestrial environments,³ and this process competes with denitrification for N₂ production.^{4,5}

Anammox bacteria have been identified in many naturally occurring anoxic environments including marine, freshwater, and estuarine sediments,^{4,6} oxygen minimum zones,⁷ soils,^{8–10}

wetlands,¹¹ and anoxic tropical freshwater lakes.¹² In addition to widespread distribution, the activity of anammox bacteria in the environment may also be substantial (e.g. ~67% fixed N-loss; ref 4), making anammox potentially more important for nitrogen loss than denitrification.³ Anammox appears to be widespread, and ammonium impacted groundwater has the potential to provide an ideal environment for the growth of anammox bacteria. That said, anammox bacterial communities have not been well studied in groundwater environments; their abundance and activity would be important given that fixed nitrogen represents a major threat to groundwater quality resulting from agriculture, waste disposal, and industrial activity.^{13–15}

Although anammox is amenable to reactor environments experiencing high N-loading, anammox organisms in natural

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Table 1. Field Metadata for Groundwater Samples Included in This Study^a

sample	[NH ₄ ⁺] mg-N/L	[NO ₃ ⁻] mg-N/L	EC (μS/cm)	pH	[DO] mg/L
Z103 (3.1 m)	2.5	3.2	1675	7.22	<1
Z103 (5.1 m)	1.5	4.0	1503	7.13	<1
Z103 (7.5 m)	8.3	0.2	1675	7.13	<1
Z106 (3.1 m)	1.6	4.6	2450	6.86	<1
Z106 (5.1 m)	1.5	2.6	1445	7.08	<1
Z106 (7.5 m)	1.3	2.3	1440	7.13	<1
Z86 (1.9 m)	ND	ND	ND	ND	ND
Z86 (2.3 m)	0.01	7.4	541	6.59	4.47
Z86 (3.1 m)	0.03	5.0	877	6.73	7.73
E1	16.1	28.0	ND	6.88	2.17
E2	67.1	83.3	ND	6.81	1.38
E3	313.5	189.7	ND	7.05	2.23
E4	175.3	186.7	ND	6.80	1.60
E5	354.4	177.5	ND	6.97	2.29
E6	47.0	42.7	ND	6.54	1.32
E7	313.92	238.8	ND	6.87	ND
E (composite)	ND	ND	ND	ND	ND
SDP (4.0 m)	31.4	86.2	2220	7.00	0.35
SDP (5.0 m)	34.9	32.1	2140	7.03	0.50
SDP (6.0 m)	69.9	29.0	2140	7.07	0.42

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

environments live under low nutrient conditions (NH₄⁺ below detection; refs 16 and 17) and have a high affinity for necessary substrates.^{18,19} Anammox organisms may live in low abundance but are capable of significant growth and metabolism when a shift in environmental parameters permits anammox communities to grow.²⁰ In addition, anammox organisms have also been detected in engineered reactors designed to favor denitrification over anammox.¹

Groundwater contamination by ammonium typically occurs because of anthropogenic surface activities including composting, manure stockpiling,²¹ fertilizer storage,¹⁴ and application.¹⁵ When dissolved ammonium enters groundwater, the positively charged ion competes for exchange sites on soil particle surfaces; nitrifiers in the unsaturated aerobic zone oxidize ammonium to nitrite (NO₂⁻) and nitrate (NO₃⁻). Groundwater carries the substrates and products of nitrification (NH₄⁺, N₂O, and NO₂⁻/NO₃⁻) through the aquifer where advection, dispersion, adsorption, and a range of possible degradation reactions can occur.¹⁴ Given the relatively long exposure time of groundwater to metabolically active microbial communities, we hypothesized that the anammox reaction is favored in contaminated groundwater when both NO₂⁻ and NH₄⁺ are present in areas of low oxygen concentrations. Anammox organisms have been identified in groundwater environments,^{8,22} and isotope evidence for the anammox reaction in NH₄⁺-impacted groundwater was shown at the site of a former fertilizer operation in Elmira, Ontario, Canada,²³ but the presence and activity of anammox organisms has yet to be confirmed with coupled ¹⁵N labeling incubations and biomarker-based evidence.

METHODS

Samples from multiple ammonium-impacted groundwater sites were collected (Zorra, Elmira, and Southland Dog Park, SDP; Table 1) and analyzed for total bacterial composition and

for anammox-specific molecular and isotopic signatures. For consistency, representative samples from each site were subjected to the same analyses (e.g., qPCR, denaturing gradient gel electrophoresis [DGGE], clone libraries), except for the Illumina sequencing (Zorra site only), and isotope incubations (Zorra and Elmira sites only).

Groundwater sites within Canada were chosen with the occurrence of both NH₄⁺ and NO₃⁻/NO₂⁻ contamination as a first criterion (Table 1). We collected samples from Zorra township, Ontario (Zorra site, Z), which is a manure composting facility where runoff from compost piles collects in a lagoon. The second site was in Elmira, Ontario (Elmira site, E), which is adjacent to a decommissioned fertilizer company. The third site was in Calgary, Alberta (Southland Dog Park, SDP), which is 2.5 km down gradient from a decommissioned plant involved in munitions production and fertilizer mixing from 1942 to 1992. For comparison to NH₄⁺-impacted groundwater sites, one “background” piezometer was sampled at three depths from the Zorra site (Z86). All three sites exhibit groundwater contamination, with groundwater NH₄⁺-N concentrations ranging between 2.5 and 350 mg L⁻¹ and NO₃⁻-N between 3.2 and 200 mg L⁻¹ (Table 1). For additional site details and sampling information, please see Methods in the Supporting Information.

DNA Sample Collection and Extraction. Between 240 mL and 1 L samples of groundwater were collected and filtered onto 0.22-μm Sterivex filters (Millipore). Filters were stored at -70 °C until DNA extraction. Nucleic acids were extracted from the filter surface using a lysis and purification technique described previously.²⁴

qPCR. Quantitative real-time PCR (qPCR) was performed using a C1000 thermal cycler with a CFX96 real-time system (Bio-Rad) and SYBR Green Supermix (Invitrogen). Anammox-specific qPCR was conducted similarly to a previously published protocol¹ using primers Amx368f (ref 25) and Amx820r (ref 26). General bacterial qPCR used primers 341f and 518r (ref 27).

Melt curve analysis was performed at the end of the run to confirm that primer dimers did not interfere with signal detection and that primer binding was specific. All qPCR products were run on a 1% agarose gel along with a 1 kb Plus DNA ladder (Invitrogen) to confirm the size and quality of PCR products. The efficiency of general bacterial and anammox qPCRs were 88.9% and 84.6%, with R^2 values of 1.00 and 0.998, respectively.

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

DGGE. To compare community composition for all samples, DGGE was conducted using universal bacterial primers targeting the V3 region of the 16S rRNA gene,²⁷ and the technique was also adapted to target the 16S rRNA gene of anammox bacteria. General bacterial 16S rRNA gene profiles were generated using DGGE primers (GC-341f and 518r; ref 27), and PCR used the following thermal profile: initial denaturation of 5 min at 95 °C, then 30 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C, with a final extension step of 7 min at 72 °C. Anammox-specific 16S rRNA gene profiles were generated using a “nested” PCR technique: PCR products from amplification with An7f and An1388r (ref 28) were diluted (10^{-2}) in water, and products were used as template for PCR with bacterial DGGE primers, using only 20 cycles for the nested amplification. Thermal cycling conditions are listed in the cited references above.

DGGE used a 30% to 70% denaturing gradient in 10% acrylamide gels (CBS Scientific Company). Gels were run for 14 h at 85 V according to a previously published protocol.²⁹ Bands were cut from the gel and sequenced at The Center for Applied Genomics (TCAG; ABI 3730XL sequencer). DGGE band sequences were manually edited to correct base miscalls, and primer sequences were removed prior to analysis.

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

Illumina 16S rRNA Gene Sequencing. Illumina libraries were constructed according to a previously published protocol³⁰ for both 7.5-m samples from the Zorra site (Z103 and Z106) in order to obtain in-depth coverage of the bacterial communities at this site. In brief, the hypervariable region (V3) was amplified with modified 341f and 518r primers,^{27,30} quantified, and sent to Illumina (Hayward, CA) for 125-nucleotide paired-end multiplex sequencing. After sequencing, image analysis, base calling, and error estimation were performed using Illumina Analysis Pipeline (version 2.6). Sequencing and paired-end read assembly was conducted according to a previously described protocol (SI Methods; ref 30). All Illumina sequence data were submitted to the Short Reads Archive (SRA; NCBI) under accession number SRA030448.

16S rRNA Gene Sequencing. The An7f and An1388r PCR products used for cloning were run on a 1% agarose gel along with a 1 kb Plus DNA ladder (Invitrogen) to confirm the size and quality of PCR products. PCR products were cloned using a TOPO-TA cloning kit (Invitrogen) according to the manufacturer's instructions. DNA sequencing of plasmid inserts was performed at the University of Washington (ABI 3700 sequencer) and the University of Waterloo sequencing facility (Applied Biosystems 3130xl Genetic Analyzer). All DNA chromatograms

from clone libraries were manually edited to verify base accuracy and trimmed to ensure only high-quality reads were included in subsequent analyses. To provide potential taxonomy for experimental sequences in phylogenetic analysis the top 100 *blastn* results (ref 31) for each of 25 divergent experimental sequences were filtered to remove redundancy and added to the sequence data set. A Planctomycete outgroup sequence (EU703486) was also added for phylogenetic analysis. Sequences were aligned to a model of the bacterial 16S rRNA secondary structure using the program *ssu-align* v.0.1 (ref 32). The resulting alignment was trimmed to the majority consensus length of the experimental sequences and spanned 619 characters. The nucleotide model of sequence evolution used in phylogenetic analysis was determined by the AIC test implemented in *jModelTest* v.0.1.1 (ref 33). A Maximum Likelihood phylogeny was derived using the *PhyML* v.3.0.1 (refs 34 and 35), with the GTR model of sequence evolution³⁶ and estimated optimized parameters for the gamma distribution estimating rate variation (GTR+G). The approximate likelihood ratio test (aLRT) statistic in *PhyML* and 1000 Neighbor Joining bootstrap iterations using *PAUP* v.4b10 (ref 37) were used to provide support for the Maximum Likelihood topology. All DNA sequences were deposited in Genbank with the following accession numbers: clone libraries (Z, HQ595389-HQ595557; SDP, HQ595362-HQ595388; E, HQ595558-HQ595705) and DGGE bands (Z, HQ595721-HQ595722; SDP, HQ595706-HQ595708, HQ595718-HQ595720; E, HQ595709-HQ595717).

¹⁵N-Labeling Incubations. For N-isotope incubations, groundwater was collected from the Zorra and Elmira sites. Anammox rates were calculated using incubations of Elmira composite water and groundwater from piezometer Z92 at Zorra. Estimations of N_2 production represent an average among Elmira groundwater, wells 1, 3, 4, 5, 6, and 7, and an average among Zorra groundwater, wells Z92, Z95, Z122, Z124 (please refer to Methods in the Supporting Information for additional site details).

For ¹⁵N-labeling experiments, the protocol was modified from a previous publication.⁷ Triplicate samples were collected for ¹⁵N-labeling experiments. The ¹⁵N-labeling experiments were begun immediately after return to the laboratory (less than 2 h). After return to the lab, 3 mL of headspace was created by injecting high purity helium gas; each sample was flushed with high purity helium for at least 15 min to remove background N_2 and dissolved O_2 and N_2 . ¹⁵N-Enriched compounds were added by syringe to a final concentration of 100 μmol in 10 mL as ¹⁵ NH_4Cl and $\text{Na}^{15}\text{NO}_3$ (all >99% ¹⁵N; Sigma-Aldrich). Although the final concentration of enriched ¹⁵N varied in previous studies (ranging from 40 $\mu\text{mol L}^{-1}$ to 10 mmol L^{-1} ; refs 7,16, and 38), the concentration in the current study was higher because background ¹⁴N mixing ratios in the collected samples can reach up to 20 mmol L^{-1} . A series of killed controls (autoclaved samples) were spiked with ¹⁵ NH_4^+ and were monitored for three days to confirm that produced N_2 gas was of microbiological origin. The ¹⁵N-labeling experiments were performed in November 2009 and were incubated in the dark at 15 °C, which was an approximation of the *in situ* groundwater temperature (data not shown). ¹⁴ N^{15}N :¹⁴ N^{14}N and ¹⁵ N^{15}N :¹⁴ N^{14}N ratios were determined by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) and calculated as excess above their natural abundances³⁹ (GG Hatch isotope laboratory, University of Ottawa). Samples were incubated with labeled isotopes for three months, during which time the N_2 headspace gas in the Exetainer was measured approximately every two weeks.

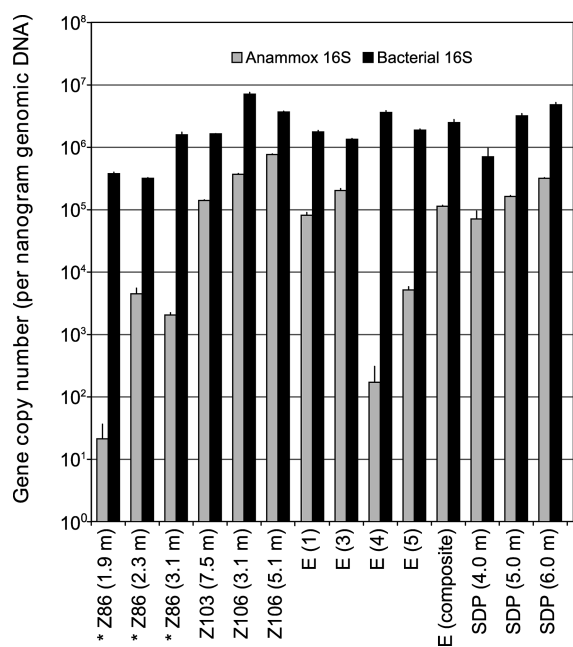


Figure 1. Comparison of anammox and bacterial 16S rRNA gene copies per nanogram genomic DNA at all three sites. Z = Zorra; E = Elmira; SDP = Calgary; * = “background” well located outside of ammonia plume. Error bars represent standard deviation.

The concentration of NH_4^+ was measured before and after incubation by a salicylate colorimetric method on a spectrophotometer at 690 nm.⁴⁰ The concentrations of NO_3^- and NO_2^- were determined before and after incubation by ion chromatography (DX100 IC, Dionex). The intermediate products of anammox reaction, hydroxylamine and hydrazine, were determined before and after incubation according to the methods of Frear and Burrell⁴¹ and Watt,⁴² respectively. The detection limits for hydroxylamine and hydrazine are approximately 0.25 and 0.05 mg L^{-1} by these colorimetric methods, respectively. The molar fractions of ^{15}N label in NH_4^+ and NO_3^- (Fx) were calculated by comparing the initial concentrations of each nitrogen species and the increase in concentration subsequent to the addition of labeled ^{15}N .

The rates of anammox were extrapolated from linear regression of $^{14}\text{N}^{15}\text{N}$ as a function of time in the incubation with $^{15}\text{NH}_4^+$. Rates of denitrification were determined from the slope of linear regression of $^{15}\text{N}^{15}\text{N}$ over time in the incubation with $^{15}\text{NO}_3^-$. The anammox contribution to total N_2 production was calculated by assuming that the $^{15}\text{NH}_4^+$ pool turns over at the same rate as the ambient $^{14}\text{NH}_4^+$ pool. In addition to the above, a mathematical methodology for calculating the contribution of anammox, denitrification, and atmosphere to an N_2 mixture was applied to $^{15}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$ incubation experiments.⁴³

Please see Supplemental Information for an unabridged version of the Methods for additional details.

RESULTS AND DISCUSSION

Quantitative PCR using anammox-specific primers (Amx368f and Amx820r) was used to estimate the relative abundance of anammox bacteria across the samples included in this study (Figure 1). These primers were originally designed as FISH probes and have been adapted for the detection of anammox organisms in environmental DNA extracts. They target

anammox organisms belonging to *Candidatus* genera *Brocadia* and *Kuenenia*²⁶ and were chosen for this study because clone library data suggest that the majority of anammox sequences retrieved from our three field sites are *Can. Brocadia*-like sequences. Despite possible biases that are associated with PCR amplification, we demonstrate that anammox 16S rRNA gene copies represented 5.2–20.8% of the bacterial 16S rRNA gene copy numbers at the Zorra site, 5.1–10.1% at the SDP site, and 4.6% in the Elmira composite sample (with values ranging between <0.00% and 15% among the other wells at Elmira). Subsequent sampling of wells Z103 and 106 in 2009 showed similar abundances of anammox targets (data not shown), indicating temporal stability of anammox bacteria within the groundwater community. Groundwater from a background piezometer (Z86) at the Zorra site, which was not influenced by the manure lagoon, had between 1 and 5 orders of magnitude fewer anammox gene copies than for the ammonium-impacted wells. Clone library analysis of qPCR products demonstrated high assay specificity: >97% of anammox amplification was specific to anammox bacterial 16S rRNA genes in the qPCR amplification products from these sites (data not shown).

The prevalence of anammox bacteria was confirmed by the presence of anammox bands in both the general bacterial and anammox-specific 16S rRNA gene profiles. The anammox-specific DGGE method was developed for this study, and anammox DGGE fingerprints were produced for each sample and compared to general bacterial profiles (Figure 2). Of 25 sequenced DGGE bands, 18 sequences grouped closely with putative anammox sequences or with known anammox reference sequences. Anammox bands were detected in general bacterial DGGE profiles for samples SDP (4.0 m), Z103 (5.1 m), and Elmira well 3. DGGE profiles suggest that the bacterial and anammox communities at the Elmira site are more heterogeneous than either the SDP or Zorra sites. These DGGE data suggest that anammox organisms were prevalent members of the microbial community at all three sites because bands with anammox identity (Table S3, Supporting Information) were clearly visible in general bacterial 16S rRNA gene profiles (Figure 2).

In addition to qPCR and DGGE of all samples (see above), we used primers with coverage of most bacteria (341f and 518r) to amplify 16S rRNA genes from selected samples. This approach generated approximately 100,000 ~200-base sequences from each of the two 7.5-m depths in the Zorra site (Z103 and Z106; Tables S1 and S2, Supporting Information). Both of these Illumina libraries were set up as local BLAST databases. These BLAST databases were queried with anammox clone sequences (retrieved from the Zorra site using anammox-specific primers An7f and An1388r; see below) to determine the proportion of anammox sequences in the bacterial Illumina library. Anammox sequences represented 6.8% (7342 of 107,777) and 6.7% (6427 of 95,873) of the total number of sequences in Illumina libraries from Z103 (7.5 m) and Z106 (7.5 m), respectively. Anammox bacterial identity was associated with the fourth most abundant bacterial sequence in both groundwater Illumina libraries. These proportions may be underestimates of anammox bacterial relative abundance if the 16S rRNA copy numbers in anammox bacteria (e.g., one copy in *Candidatus* *Kuenenia stuttgartiensis* genome)⁴⁴ are compared to the average copy number of other groundwater bacterial community members.⁴⁵

The Illumina 16S rRNA gene libraries also suggested the presence of denitrifying bacteria (Tables S1 and S2, Supporting Information). In the Z103 and Z106 Illumina libraries, the most

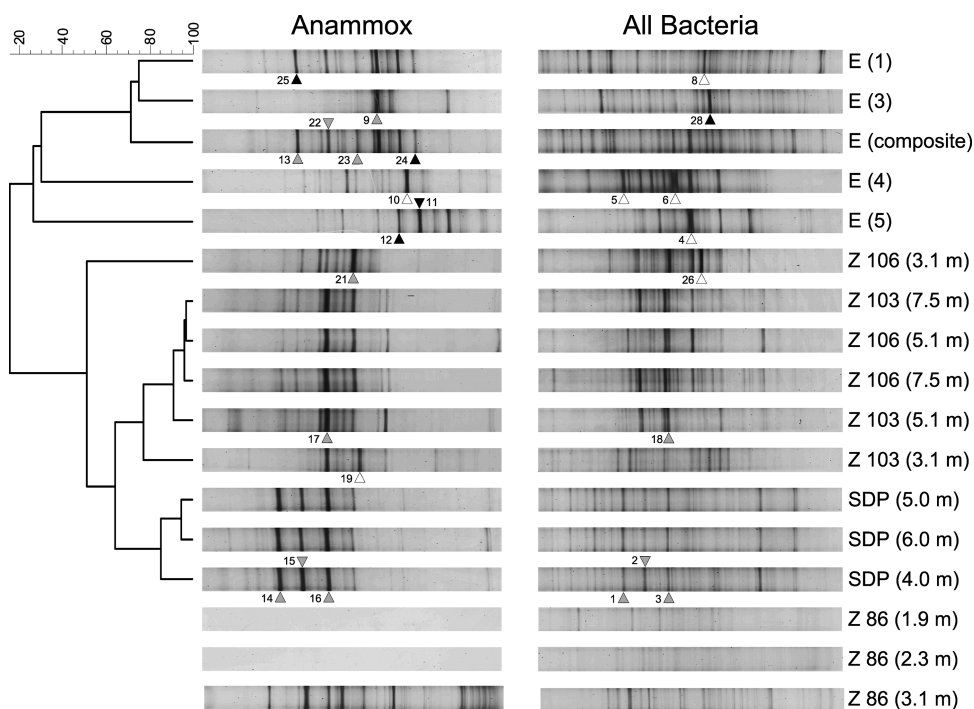


Figure 2. Anammox and general bacterial 16S rRNA gene fingerprints from all three field sites, obtained by denaturing gradient gel electrophoresis (DGGE). Black triangles indicate sequenced bands clustering within 95% similarity to known anammox reference sequences. Gray triangles indicate bands with putative anammox identity (i.e., closest BLAST search result of “uncultured anaerobic ammonium oxidizing clone” or similar). White triangles indicate bands that yielded sequences that did not affiliate with anammox bacteria. See Table S3, Supporting Information for band identities. Z = Zorra; SDP = Calgary; E = Elmira.

abundant ten sequences in both libraries included members of the genera *Herbaspirillum*, *Pseudomonas*, and *Methylophilus*. Sequences from these genera were previously associated with denitrifying reactors or have known denitrifying activity.^{46–48} It is important to note that these 16S rRNA gene sequences are unable to assign putative denitrification metabolism to these abundant organisms, and functional gene analyses (e.g., *nirS* or *nirK*) would provide stronger support. Nonetheless, the groundwater chemistry provides additional support for the possibility of denitrification. Organic carbon was quite high at these sites. Samples from the Zorra site have high DOC (~3 ppm to 30 ppm). Even samples in Elmira sites had DOC concentrations that ranged from ~5 to 20 ppm. As described later, we also detected active denitrification in all samples. The concurrent growth of denitrifiers and anammox is commonly observed⁴⁹ because both kinds of organisms require similar environmental conditions for growth (e.g., low oxygen and $\text{NO}_2^-/\text{NO}_3^-$ substrates). Further research into the environmental factors affecting differential contributions of denitrifiers and anammox bacteria in groundwater will include measurements of DOC values in addition to the abundance and expression of denitrification functional genes. Also note that the redox conditions of our groundwater sites (Table 1) were not favorable for aerobic reactions, and these additional processes (e.g., involving ammonia-oxidizing bacteria and archaea) were not studied here.

To better characterize the diversity of anammox bacterial communities, clone libraries were constructed with anammox-specific 16S rRNA gene primers.²⁸ The retrieved 16S rRNA gene sequences were aligned to known anammox sequences (Figure 3; Table S4, Supporting Information). The community composition of anammox bacteria differed between these three contaminated

groundwater sites. At the SDP field site, the clone library was dominated by *Candidatus Brocadia*-like phylotypes. The dominance of *Can. Brocadia* was also evident at the Zorra site, where the vast majority of clones (164 of 171) grouped with known *Can. Brocadia* reference sequences; the five remaining clones grouped with *Can. Scalindua* anammox reference sequences. The diversity of anammox sequences was highest in the Elmira composite library, with four of the five known genera of anammox sequences represented. Nonetheless, *Can. Brocadia*-like clones outnumbered all other anammox genera sequences combined in the Elmira library. Combined with the finding that *Can. Brocadia* have been detected in reactor environments not designed to operate in anammox-mode,¹ our data suggest a broad metabolic and environmental niche of these particular anammox bacteria. Furthermore, the Elmira clone library contained three separate phylotypes that showed no phylogenetic affinity to known anammox genera (Figure 3). These phylotypes likely represent novel anammox diversity and suggest that further study at such sites is warranted.

The diversity of anammox bacteria in aquatic environments is governed by environmental factors; anammox communities shift in response to salt concentration⁵⁰ and available carbon substrates.⁵¹ Measured NO_2^- concentrations are positively correlated with anammox bacterial abundance and activity,^{16,17,52} and anammox activity is negatively correlated with sediment organic content.⁴ Niche adaptation by anammox bacteria appears to be common, and several studies have concluded that the anammox populations acting in a single environment have very low diversity.^{50,51} High diversity of anammox organisms (like that seen at the Elmira site) was observed previously in the Cape Fear estuary.⁶ *Can. Brocadia* and *Can. Kuenenia* were present in the freshwater reaches of the estuary, while *Can. Scalindua* organisms

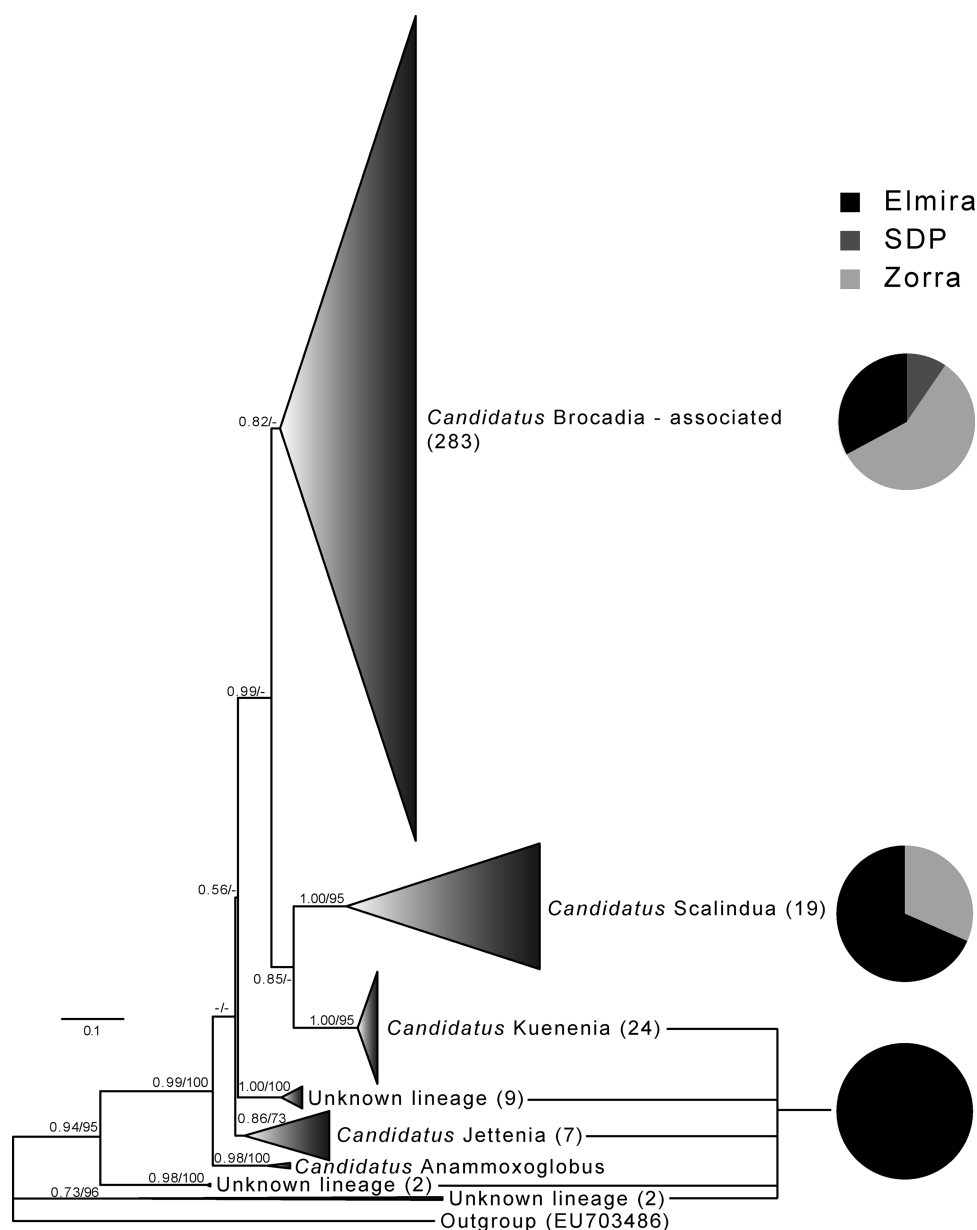


Figure 3. Maximum Likelihood phylogeny of groundwater anammox sequences from all three sites aligned with known anammox reference sequences acquired from GenBank. Numbers in brackets represent the number of clones identifying with each cluster. Pie chart insets represent the number of sequences within the corresponding clade that were obtained from each field site. Node support values correspond to aLRT and neighbor joining bootstrap values, respectively, with dashes representing values <0.5 (aLRT) or 50 (bootstrap). Scale bar represents the number of nucleotide substitutions per site. Asterisks represent unknown lineages of exclusively experimental sequences. The full tree file is available in Supplemental Information.

dominated in the saline end of the estuary; a correlation existed between the distribution of anammox organisms and salt concentration. Groundwater is not saline at our sites ($EC < 2450 \mu S \text{ cm}^{-1}$; Table 1), and the dominant organisms at all three sites were similar to the *Can. Brocadia* genus (Figure 3), which is primarily affiliated with freshwater environments. *Can. Scalindua* is commonly associated with marine environments;⁵³ however, these organisms have been detected in freshwater environments as well.¹² *Can. Kuenenia* is also recognized as a freshwater genus of anammox bacteria.⁵⁰ A small proportion of anammox clones grouped with the *Can. Scalindua* genus at the Zorra and Elmira sites (Figure 3). *Can. Jettenia* and *Can. Kuenenia* clones were identified at only one field site (Elmira).

These results suggest the possibility of site-specific heterogeneity in anammox bacterial distributions. Similarly, as the heterogeneous Elmira field site contained the only phylotypes not associated with known or proposed taxa, diversity of anammox sequences may be high in other similar sites. Investigations of such locations may identify other novel lineages of anammox bacteria. This initial study reports on the discovery of abundant and diverse anammox populations and future work will include additional samples and sites in order that multivariate statistics can test the relationship between site-specific characteristics (e.g., salinity, N-loading) and phylogeny.

This study coupled both molecular and isotopic approaches to characterize groundwater anammox bacterial community

presence/abundance (molecular) and activity (isotope) because the presence of anammox biomarkers is not necessarily associated with *in situ* activity.²⁸ N-Isotope incubation experiments were performed for Zorra and Elmira sites. Incubation of impacted groundwater samples from both sites with $^{15}\text{NH}_4^+$ yielded $^{14}\text{N}^{15}\text{N}$ (Figure S1, Supporting Information), which is indicative of the anammox reaction. Groundwater from piezometer Z86 (background piezometer) showed no production of $^{14}\text{N}^{15}\text{N}$. Production of $^{14}\text{N}^{15}\text{N}$ was detectable after one week but was very slow, and a prolonged incubation time (approximately three months) was required to determine the anammox reaction rates. At the end of the incubation time, the highest $\delta^{15}\text{N}/^{14}\text{N}$ increase was 14,300‰. The rate of $^{14}\text{N}^{15}\text{N}$ production corresponded well with the anammox rate at both sites.^{2,4,7}

No obvious $^{15}\text{N}^{15}\text{N}$ accumulation occurred for incubations from either the Zorra or Elmira sites from $^{15}\text{NH}_4^+$ incubations, which would have signified nitrification followed by denitrification or coupled with anammox. In killed controls, no $^{14}\text{N}^{15}\text{N}$ or $^{15}\text{N}^{15}\text{N}$ and a very low concentration of $^{14}\text{N}^{14}\text{N}$ were measured. The limited $^{14}\text{N}^{14}\text{N}$ is likely derived from entrapped air in the sampling syringe during the gas sampling operation. The comprehensive mathematical approach used in this study⁴³ takes atmospheric N_2 and side reactions (such as DNRA) into account when calculating the contributions of anammox and denitrification. The calculated anammox reaction rate was $31.3 \pm 6.0 \text{ nmol L}^{-1} \text{ h}^{-1}$ at Elmira and $13.3 \pm 1.6 \text{ nmol L}^{-1} \text{ h}^{-1}$ at Zorra. The $^{15}\text{NO}_3^-$ incubation produced more $^{15}\text{N}^{15}\text{N}$ than $^{14}\text{N}^{15}\text{N}$, indicating active and strong denitrification processes (Figure S1, Supporting Information). The $^{15}\text{NH}_4^+$ production in $^{15}\text{NO}_3^-$ incubations accounted for <1% of total N-NH_4^+ , indicating that DNRA was not substantial. The application of the comprehensive approach⁴³ in our study showed that in the incubation of $^{15}\text{NH}_4^+$, anammox contributed $18.0 \pm 6.5\%$ of N_2 gas production in Zorra and $35.7 \pm 13.6\%$ in Elmira, which is close to the results of the incubation of $^{15}\text{NO}_3^-$ ($21.4 \pm 11.5\%$ and $32.7 \pm 15.6\%$, respectively; Figure 4). Hydroxylamine and hydrazine (anammox reaction intermediates) remained below detection limits throughout the incubations for samples taken from both study sites, which is expected given that these intermediates are sequestered within the anammoxosome.⁵⁴

Dalsgaard and colleagues⁷ reported anammox reaction rates between 3 and $18 \text{ nmol L}^{-1} \text{ h}^{-1}$ in the anoxic water column of Golfo Dulce, which are comparable to the reaction rates observed in this study. Lower rates have been found elsewhere, such as in the eastern South Pacific ($\leq 0.7 \text{ nmol L}^{-1} \text{ h}^{-1}$; ref 16) and in the Black Sea ($\sim 7 \text{ nmol d}^{-1}$; ref 55). Our results are within the reported reaction rates in freshwater lakes, ranging from 6 to $504 \text{ nmol N}_2 \text{ L}^{-1} \text{ d}^{-1}$ (ref 56). At Elmira, $^{14}\text{N}^{15}\text{N}$ accumulated linearly and stably with time without a lag phase, which indicates that anammox was the active process, and no intermediates were involved in the reaction.³⁸ Low substrate concentrations in Zorra samples (e.g., NH_4^+ ; Table 1) might explain the suppression of anammox activity with time. Given that the concentrations of dissolved organic matter, NH_4^+ and NO_3^- and anammox activity were high at the Elmira site, and in particular in well #7 (unpublished data), we speculate that these chemical controls were an important influence on anammox community composition. These relatively high N_2 production rates by anammox suggest that anammox bacteria are important members of the microbiological community at ammonium contaminated groundwater

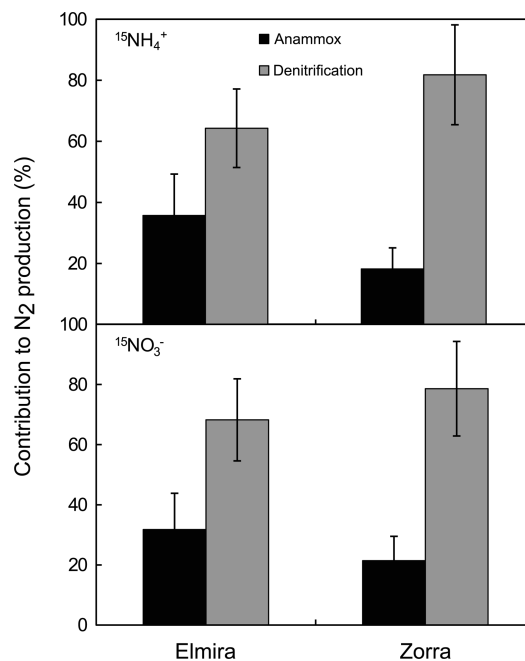


Figure 4. Calculated contributions of anammox and denitrification to microbially produced N_2 in $^{15}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$ labeling experiments at the Elmira and Zorra sites. Error bars represent standard deviation.

sites and are important players in the nitrogen cycle of groundwater systems.

Anammox bacterial communities are responsible for substantial attenuation of nitrogen from aquatic and terrestrial environments. Groundwater contamination by ammonium can lead to contamination of surface waters and receiving water bodies or impact groundwater serving as a source of drinking water. This study provides direct molecular evidence of the presence of a diverse and abundant assemblage of anammox performing organisms at three contaminated groundwater locations and represents a combined isotope and molecular confirmation of anammox communities in contaminated groundwater environments. Future research will assess the generality of these findings to additional contaminated sites and include multivariate statistics to help identify controls on anammox bacterial community diversity and composition. Previous studies of anammox bacteria in aquifers have focused on single sites and lacked the addition of isotope-based techniques to verify the activity of these organisms.^{8,22} Confirming the presence and activity of anammox bacteria opens up the possibility of modifying the designs of engineered systems that treat nitrogen-rich sources such as septic systems, manure handling facilities, and groundwater permeable reactive barriers to enhance the anammox reaction.⁵⁷ The importance of such an engineered design would be to help protect surface waters from NH_4^+ -impacted groundwater in a one-step remediation approach instead of through the combined activities of nitrifiers and denitrifiers.

■ ASSOCIATED CONTENT

S Supporting Information. Materials and Methods, Tables S1–S4, Figure S1, and a phylogenetic tree file. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*Phone: 519-888-4567. Fax: 519-746-0614. E-mail: jneufeld@uwaterloo.ca.

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