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Role of Anaerobic Ammonium Oxidation (Anammox) in Nitrogen Removal from a Freshwater Aquifer

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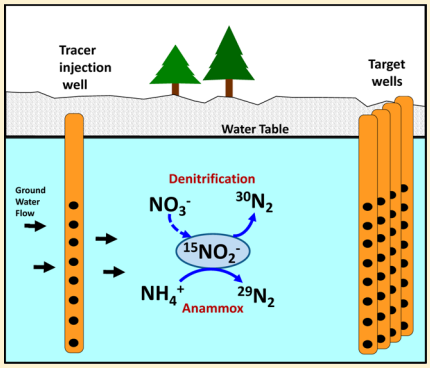
Supporting Information

ABSTRACT: Anaerobic ammonium oxidation (anammox) couples the oxidation of ammonium with the reduction of nitrite, producing N_2. The presence and activity of anammox bacteria in groundwater were investigated at multiple locations in an aquifer variably affected by a large, wastewater-derived contaminant plume. Anammox bacteria were detected at all locations tested using 16S rRNA gene sequencing and quantification of hydrazine oxidoreductase (*hzo*) gene transcripts. Anammox and denitrification activities were quantified by *in situ* $^{15}NO_2^-$ tracer tests along anoxic flow paths in areas of varying ammonium, nitrate, and organic carbon abundances. Rates of denitrification and anammox were determined by quantifying changes in $^{28}N_2$, $^{29}N_2$, $^{30}N_2$, $^{15}NO_3^-$, $^{15}NO_2^-$, and $^{15}NH_4^+$ with groundwater travel time. Anammox was present and active in all areas tested, including where ammonium and dissolved organic carbon concentrations were low, but decreased in proportion to denitrification when acetate was added to increase available electron supply. Anammox contributed 39–90% of potential N_2 production in this aquifer, with rates on the order of 10 nmol N_2–N L^{-1} day^{-1}. Although rates of both anammox and denitrification during the tracer tests were low, they were sufficient to reduce inorganic nitrogen concentrations substantially during the overall groundwater residence times in the aquifer. These results demonstrate that anammox activity in groundwater can rival that of denitrification and may need to be considered when assessing nitrogen mass transport and permanent loss of fixed nitrogen in aquifers.



INTRODUCTION

Humans have altered the global nitrogen (N) budget,[1,2] with impacts in virtually all aquatic and terrestrial environments. This includes freshwater aquifers, which receive an estimated 15 Tg/year or ~10% of all anthropogenic fixed N.[2] Denitrification is usually considered the key dissolved inorganic nitrogen (DIN) removal mechanism in groundwater,[2,3] but the discovery of anaerobic ammonium oxidation (anammox)[4] suggests that biogeochemical processes affecting the fate and transport of groundwater DIN, and thus its ultimate delivery to surface waters, may be far more complex than was previously thought. Relative rates of anammox and denitrification in groundwater are unknown, but could have far-reaching implications for N storage and water quality at local to global scales.

DIN (nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+)) arrive in groundwater from atmospheric deposition and weathering of rocks and soil organic matter and from a variety of local anthropogenic sources including landfill leachate, wastewater disposal, septic systems, and agriculture. N fate and transport in shallow aquifers depend on local geology, recharge conditions, hydraulic conductivity, and in-aquifer removal reactions. The protracted residence time of N in aquifers, relative to surface freshwater, causes aquifers to have both large inventories of anthropogenic N and extended contact times for in-aquifer reactions to potentially attenuate N loads during transport. NO_3^- is typically the dominant form of DIN in oxic aquifers, moving freely through the unsaturated and saturated zone. NH_4^+ tends to dominate the DIN pool in suboxic aquifers, but its transport is slower due to chemical sorption and exchange with aquifer sediments.[5] Coexistence of NO_3^- and NH_4^+, as well as O_2 and organic-C, can occur in groundwater due to differential advection rates, resulting in mixed redox zones of dissimilatory N-cycling processes. Such zones may be characterized by co-occurrence of N species of multiple redox states (e.g., NO_3^- and NH_4^+) and production/consumption of NO_2^- through both oxidizing and reducing reactions.[6] Denitrification has long been considered the primary N removal mechanism of fixed N and has been examined in a number of subsurface studies,[7−11] with an estimated 20% of the global extent of the process occurring in groundwater.[3]

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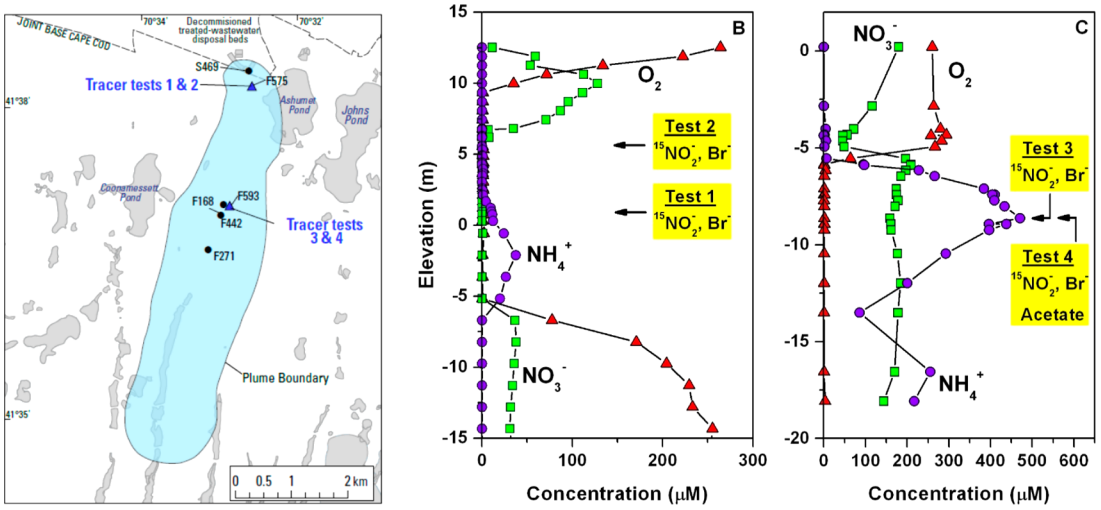

Figure 1. Map of study site on Cape Cod, MA, USA, showing groundwater contaminant plume, selected well locations, tracer test site locations, and depth profiles at the tracer test locations of groundwater concentrations of NO_3^-, NH_4^+, and O_2 in August 2011 (B) and June 2012 (C). Depths and primary constituents for each tracer test are indicated in yellow boxes. Elevation is relative to mean sea level.

Typically, excess N_2 production in aquifers is attributed exclusively to denitrification,[10,12] which is commonly organic-C driven, but can also utilize inorganic electron supplies, such as reduced iron and sulfur.[11,13,14]

Anammox couples NH_4^+ oxidation with NO_2^- reduction to produce nitrogen gas (N_2) under suboxic to anoxic conditions. Provided there is a source of NO_2^-, anammox can potentially remove large amounts of fixed N without requiring substantial amounts of organic-C, reduced iron, or mineral phase electron donors. Occurrence of anammox in environmental systems can have profound implications for DIN mass balance and natural attenuation. Anammox was first discovered in a wastewater treatment plant.[4] Since then, it has been found to be variably important in soils, in marine and fresh surface waters, and near sediment–water interfaces.[15−22] Its presence in groundwater has been inferred locally in contaminated settings from distributions of DIN constituents, natural abundance ^{15}N distributions, and biomarkers,[23−25] but has not been directly measured. In groundwater, anammox is not necessarily limited to narrow diffusional zones like it is in many surface water or soil environments; groundwater hydrology could create expanded zones suitable for anammox communities and widespread spatial distribution of anammox activity. Although the impact of anammox on the fate of DIN in aquifers could be substantial, there has not been a comprehensive study conducted to measure the rate and significance of anammox in the subsurface environment. Thus, we conducted *in situ* ^{15}N tracer experiments and molecular characterization of groundwater communities in a N-contaminated sand and gravel aquifer.

MATERIALS AND METHODS

Study Site. This study was conducted in different parts of an aquifer that was variably affected by a groundwater contaminant plume located on Cape Cod, MA, USA. The plume was created by land disposal of treated wastewater from ~1930 to 1995 onto a sand and gravel glacial outwash plain. It is contained within the unconfined, water table aquifer and measures >8 km long in the direction of groundwater flow, ~1 km wide, and ~30 m thick on a vertical axis. The plume is characterized by concentration gradients of organic and inorganic constituents that are typical of domestic wastewater, redox gradients, gradients of secondary, naturally occurring constituents, such as Fe and As, that have been mobilized by the altered geochemical environment, and gradients in microbial community composition. At essentially all locations, uncontaminated groundwater lies above and below the contaminant plume. The plume site has been intensively instrumented by the U.S. Geological Survey to conduct long-term investigations on the fate and transport of contaminants in an unconsolidated aquifer.[26]

Groundwater Sample Collection and Analyses. Well construction and sampling procedures are described elsewhere.[27] Groundwater was collected from multilevel sampling wells (MLSs) using a peristaltic pump. Specific conductance, pH, dissolved oxygen, and temperature were determined in the field immediately upon collection. N_2O samples were collected with a syringe and injected through a needle into stoppered, He-flushed serum bottles containing NaOH as a preservative.[7] Samples for N_2 analysis were pumped into 150 mL serum bottles, which were filled to overflowing for several bottle volumes at a slow pumping rate. Then, a pellet of KOH was added, and the bottles were stoppered under water using a syringe needle as vent for the displaced water and kept chilled at 4 °C.[7] All other samples for geochemical analysis were filtered with 0.45 μm capsule filters and preserved. Samples for nitrate, nitrite, acetate, and other anions were frozen. Samples for major cations (including ammonium) and dissolved total iron were acidified to pH <2 with concentrated H_2SO_4 or HNO_3, respectively.

Dissolved oxygen was determined colorimetrically and with a probe.[7] Anions, cations, and acetate were analyzed by ion chromatography.[28] N_2O was analyzed by gas chromatography.[29] Dissolved total iron was analyzed by inductively coupled plasma atomic emission spectroscopy.[30] Dissolved organic carbon (DOC) was analyzed with a C analyzer following acidification and persulfate oxidation.[31]

Tracer Tests. *In situ* tracer tests were conducted at sites F575 and F593 (Figure 1), which were instrumented with an array of MLSs located in rows down-gradient from an injection MLS (Figure S1). The injection tests were similar to previously described tests with NH_4^+.[5] Briefly, a 200 L gas-impermeable

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bladder was filled with ultrahigh-purity He and evacuated seven times to remove residual oxygen. Then, ~1 L of boiled, He-sparged DI water containing dissolved tracer salts [NaBr, $Na^{15}NO_2$ (98+ atom %), and with or without $NaC_2O_2H_3$] was pumped into the bladder, followed by 100 L of groundwater collected from the appropriate injection well port. Target final concentrations of Br^-, NO_2^-, and acetate were ~1100, 200, and 200 μM, respectively. During the filling process, the bladder was frequently agitated to ensure thorough mixing and was kept in a wading pool of water that was maintained at groundwater temperature using ice. After mixing, any gas remaining within the bladder was vented and the tracer solution pumped into the injection port, with two to three samples collected during the injection process. Caution was taken during the entire tracer preparation and injection process to avoid any contact of the solution with air. All tracer tests were conducted in anoxic zones (<5 μM O_2) within the aquifer (Tables S1 and S2).

Groundwater samples were collected from the injection port and appropriate ports in the tracer MLS array with time as detailed above. Arrival of the tracer cloud at down-gradient MLS ports was determined in the field by monitoring specific conductance.

Detection and Phylogenetic Analysis of Anammox Bacteria. Groundwater sampling was conducted at four wells (S469, F575, F168, and F442) within the groundwater contamination plume (Figure 1). Water samples (4 L) from each well were filtered through 0.22 μm Millipore Sterivex filters (Millipore Corp., Billerica, MA, USA) to concentrate microbial biomass using a peristaltic pump and immediately frozen. Genomic DNA was extracted from the filters using a Gentra Puregene Genomic DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN, USA) as described by Oates and Song.[32] Anammox bacterial 16S rRNA genes in the extracted DNA were amplified, cloned, and sequenced as described in the Supporting Information.

Quantification of *hzo* and *nosZ* Transcripts. The gene transcripts in anammox (hydrazine oxidoreductase, *hzo*) and denitrification (nitrous oxide reductase, *nosZ*) were analyzed in conjunction with the tracer tests. For tests 1 and 2, groundwater samples (1 L) were pumped through 0.22 μm Sterivex filters, whereas the samples from tracer tests 3 and 4 were filtered through 5 μm membranes to remove sediment particles before the cells were concentrated on 0.22 μm Supor filters (Pall Life Sciences). All filters were immediately frozen in liquid nitrogen. Total RNA was extracted using a mirVAN miRNA isolation kit (Ambion) and treated with a TURBO DNA-free kit (Ambion) to remove genomic DNA. The treated RNA was used for cDNA synthesis using an iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR of *hzo* and *nosZ* transcripts was conducted as described by Lisa et al.,[19] whereas 16S rRNA was quantified using the primers 341F and 685R. The ratio of *hzo* or *nosZ* transcript copies to 16S rRNA copies was calculated and used to compare different levels of gene expression in each tracer experiment.

Isotopic Analyses. Isotopic analyses of NO_3^- and NO_2^- were done by modified versions of the bacterial denitrifier method, in which compounds of interest are converted to N_2O for isotope-ratio mass spectrometry (IRMS). *Pseudomonas aureofaciens* was used to convert NO_3^- + NO_2^- to N_2O,[33] and *Stenotrophomonas nitritireducens* was used to convert NO_2^- to N_2O.[34] Calibrations were done by analyzing laboratory standards as samples with ^{15}N mole fractions ($x^{15}N$) ranging from 0.0037 to 0.982. NH_4^+ was separated from groundwater by diffusion into $NaHSO_4$ traps and analyzed for $\delta^{15}N$ after reaction with O_2 to produce N_2 for continuous-flow IRMS.[35] Isotopologues of dissolved N_2 ($^{14}N^{14}N$, $^{14}N^{15}N$, and $^{15}N^{15}N$) in groundwater were analyzed by multiple techniques. First, sample headspace gas was reacted with CuO + Cu_2O and CaO in sealed tubes to remove O_2, H_2O, and CO_2 from N_2 for dual-inlet IRMS.[5] Alternatively, headspace gas was passed unreacted through a mole-sieve gas chromatograph, and the N_2 peak was analyzed in continuous-flow mode.[36] For both methods, IRMS responses at m/z 28, 29, and 30 were calibrated against air and air-saturated water samples and adjusted for NO artifacts at m/z 30.

For each sample, measured N_2 isotopologue ratios were converted to estimated concentrations of background N_2, N_2 produced by anammox during the experiment, and N_2 produced by denitrification during the experiment, using equations similar to those derived previously and presented as a "comprehensive approach" for three-component isotopologue attribution.[37] Application of those equations included the following modifications and assumptions: (1) Measured isotopologue ratios of *in situ* background N_2 (rather than atmospheric N_2) were used as the nontracer baseline values. (2) Although unlabeled NO_3^- at F593 (tests 3 and 4) had $\delta^{15}N$ of +15 to 19‰, the NO_3^- was not considered to contribute unlabeled N to N_2 production directly, but only indirectly through isotope dilution of the ^{15}N-labeled NO_2^- pool, which was measured in each sample and included in the N_2 production equations. (3) NO_2^- contributing to N_2 produced by both anammox and denitrification in each sample during the tracer experiment was assumed to have had a time-integrated $x^{15}N$ equal to the midpoint of the measured $x^{15}NO_2^-$ value at time zero and the measured value at the sample time (important mainly for test 4, in which $x^{15}NO_2^-$ decreased substantially). Measured variations and uncertainties of $\delta^{15}N$ values of NH_4^+ were incorporated in the equations, although they had negligible effects on the calculated contributions of anammox and denitrification to N_2. Reported values of anammox N_2 outnumber reported values of denitrification N_2 because some dual-inlet N_2 isotopic analyses were affected by N_2O that was converted to N_2 by reaction with CuO + Cu_2O during off-line sample preparation for dual-inlet IRMS. For tests 1, 2, and 3, detection limits for accumulated N_2 produced by anammox and denitrification were approximately 2−5 nmol L^{-1}, corresponding to accumulation rates of the order of approximately 0.5 nmol L^{-1} day^{-1} over the length of the experiments. For test 4, detection limits were similar, but estimated rates may have larger uncertainties, as $x^{15}N$ of NO_2^- was changing rapidly, and possibly not linearly as assumed.

Note that for tracer test results denitrification and anammox are defined as the net reduction of NO_2^- through N_2O to produce N_2 (all N atoms in N_2 from NO_2^-) and net anaerobic NH_4^+ oxidation coupled with NO_2^- reduction to produce N_2 (one atom each from NH_4^+ and NO_2^-), respectively. Production of $^{30}N_2$ from $^{15}NO_2^-$ could result from any combination of classical denitrification,[38] chemodenitrification,[39] or anammox "disguised" as denitrification[40,41] and thus may differ, *sensu stricto*, from the definition of anammox and denitrification as interpreted from the phylogenetic analysis and gene transcript data.

RESULTS AND DISCUSSION

Groundwater Geochemistry. This study investigated groundwater anammox in a large (>8 km long) wastewater

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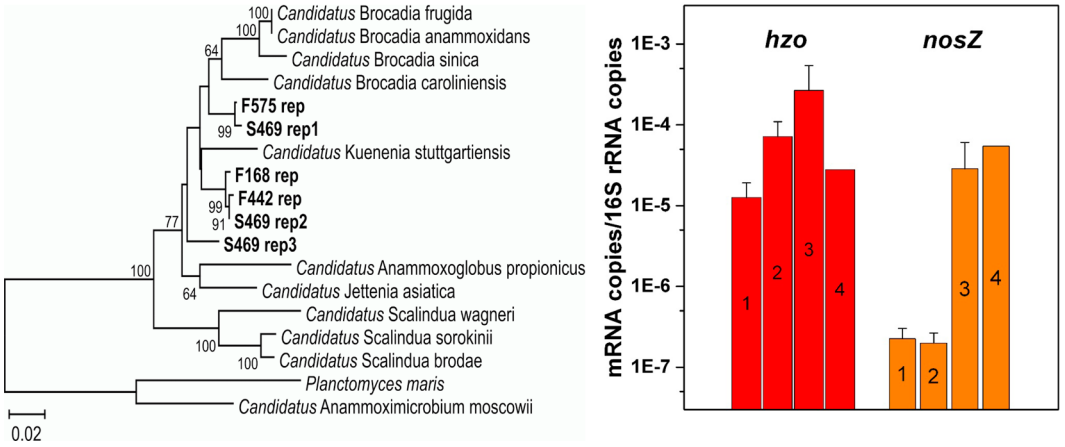

Figure 2. (Left) Phylogenetic tree of anammox bacterial 16S rRNA genes detected in groundwater samples collected from well locations S469, F575, F168, and F442. Bootstrap values are indicated at branch points. (Right) Comparison of transcriptional expression of hydrazine oxidoreductase (*hzo*) and nitrous oxide reductase (*nosZ*) genes as transcript copies normalized to total 16S rRNA copies in groundwater communities collected during the tracer tests (1−4). Error bars are ± standard deviation of replicate samples.

contaminant plume located in an unconfined sand and gravel aquifer used for drinking water (Figure 1A). The plume was characterized by vertical and longitudinal concentration gradients of dissolved and sorbed constituents, including NO_3^-, NH_4^+, dissolved O_2, and DOC. Typical vertical profiles of groundwater chemistry for the upper and middle regions of the plume are shown in Figure 1B,C. The long axis of the plume center can be divided into three regions. The up-gradient third, closest to the source, had anoxic zones of NO_3^-, Fe(III), and Mn(IV) reduction, and NO_3^- and NH_4^+ did not coexist in the same water. The middle third of the plume had anoxic water that contained both NO_3^- and NH_4^+. The down-gradient third contained O_2 and NO_3^- but little or no NH_4^+, which was less mobile because of ion exchange in the aquifer.

Sixty years of wastewater discharge contributed an estimated organic carbon load to the sand and gravel aquifer of 600 t.[31] A portion of that carbon was sorbed onto aquifer solids,[42,43] and a portion remained as DOC, which was subsequently transported down-gradient. DOC decreased in concentration during transport by continued sorption, degradation, and dispersion.[42,44,45] After 3 km of transport in the vicinity of well site F593 (see map Figure 1), DOC concentrations had decreased by approximately 80% and were primarily composed of recalcitrant compounds.[45−47] The number of free-living bacteria also decreased within 3 km of the source by about 10-fold,[45] reflecting the limited supply of reactive electron donors in the existing geochemical conditions present at F593, the well site for tracer tests 3 and 4.

In 1995, wastewater disposal was discontinued and the contaminant plume was allowed to dissipate by natural attenuation. Since that time, the concentrations and depth-integrated masses of NO_3^-, NH_4^+, and DOC have substantially decreased within the 0.5 km interval down-gradient of the wastewater disposal beds,[5,48] which includes F575, the well site for tracer tests 1 and 2. In contrast, the changes in concentrations at F593 during this same period have been relatively small, particularly for ammonium (Figure S3), as have changes in the integrated mass of DOC.[48] Available electron supply from degradation of reactive organic compounds continued to be limiting at the time of the tracer tests at F593 and was in the process of decreasing at F575. Degradation and mineralization of organic compounds sorbed onto aquifer solids underneath the former disposal beds continued to consume O_2 coming from up-gradient groundwater sources and were serving as a source of the continued supply of inorganic nitrogen arriving at F575.[42]

Microbial Evidence for Anammox and Denitrification. Anammox bacteria were detected by targeting 16S rRNA genes in groundwater obtained from the entire length of the NH_4^+-containing zone within the contaminant plume, which extended 3.3 km to well F271 in 2013 (Figure 1A). Anammox community composition was related to sampling location. Three different operational taxonomic units (OTUs) were present at the former wastewater disposal beds that were closely related to *Candidatus* Kuenenia and Brocadia spp. Kuenenia- and Brocadia-like sequences and have been reported in aquifers, wetlands, lakes, and terrestrial environments and likely represent anammox species that occur naturally in freshwater.[20,22,25,49] One of the anammox OTUs (S469 rep3) was unique to the disposal bed location; one (S469 rep2) clustered with other anammox bacteria collected 0.3 km down-gradient; and the third (S469 rep1) clustered with other anammox bacteria in the middle region of the contaminant plume at 2.5 and 2.7 km down-gradient (Figure 2A).

Transcriptional expression of the genes involved in anammox and denitrification was detected at both tracer locations during all four tracer experiments. A key anammox gene, *hzo*, was uniformly expressed at both locations, but was highest for test 3 (Figure 2B). This showed uncoupling of *hzo* gene expression and activities of anammox bacteria, as previously reported.[50] On the basis of 16S rRNA gene sequences, Brocadia-like bacteria were present during tests 1 and 2, whereas bacteria associated with "Ca. Kuenenia spp." were expressed during tests 3 and 4. The gene expression for the final step of the denitrification pathway, N_2O reductase (*nosZ*), indicated that the reduction capacity for N_2O was more prevalent at the down-gradient location (tests 3 and 4).

Rates of Anammox and Denitrification from *in Situ* Tracer Tests. Natural gradient tracer tests were conducted within the aquifer using $^{15}NO_2^-$ and Br^- as tracers to demonstrate *in situ* activity and to investigate the competition between anammox and denitrification for electron acceptor supply and their relative contribution to overall N_2 production. Tests were conducted at plume locations 0.3 and 2.5 km from

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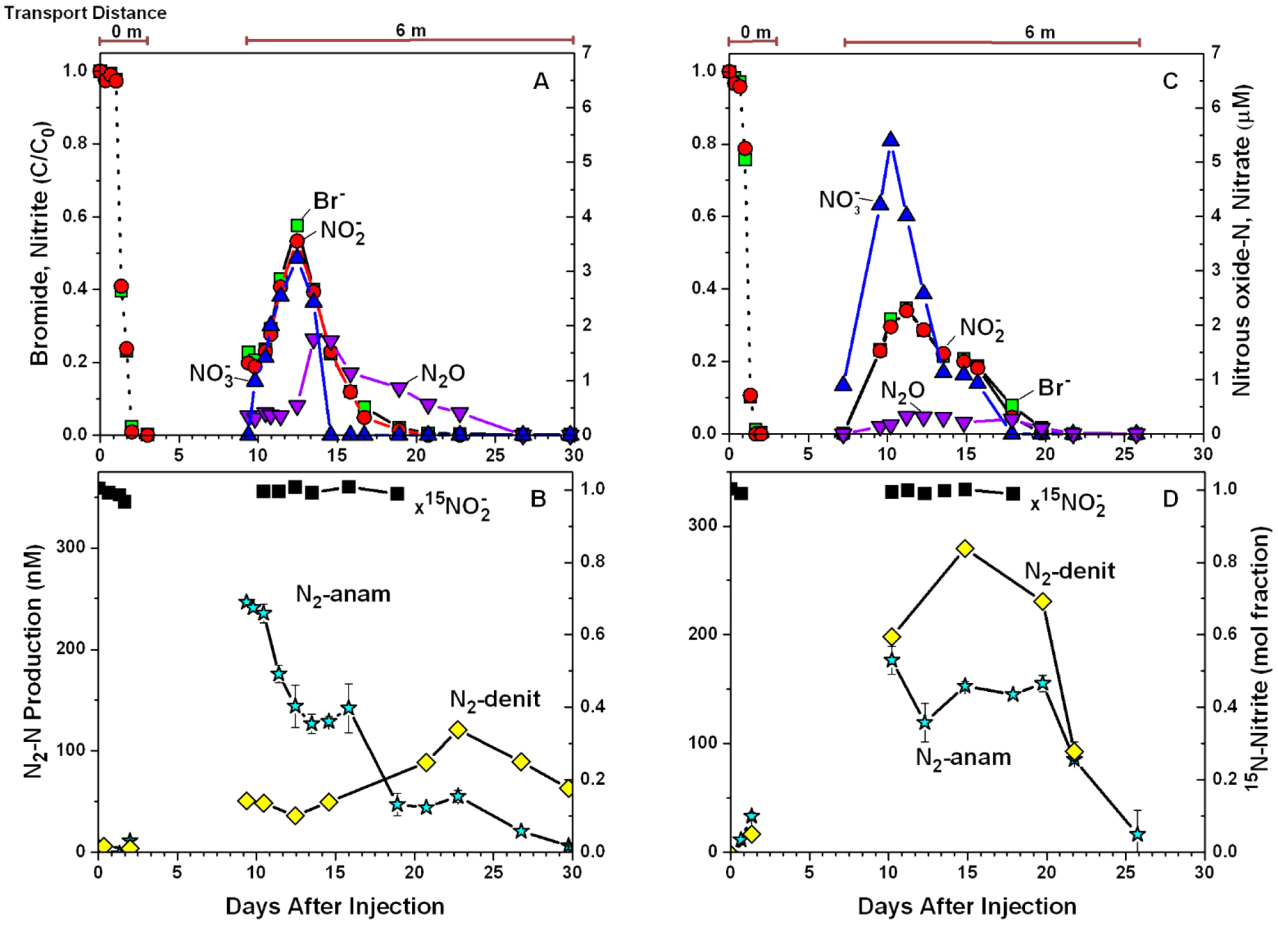

Figure 3. Breakthrough curves of natural gradient tracer tests 1 (A, B) and 2 (C, D) at the injection port and a down-gradient sampling port: added tracer constituent concentrations normalized to injection concentrations (C/C_0); other reactant and product concentrations; accumulated N_2 production attributed to anammox and denitrification; and ^{15}N mole fraction ($x^{15}N$) of recovered NO_2^-. Travel distances for individual curves are indicated by horizontal bars above the figure panels; dotted lines represent 0 m, and solid lines represent 6 m. See Figure 1 and Figure S1 for maps of tracer test wells and tracer cloud travel paths. Error bars are ± one standard deviation of replicate samples.

the wastewater source, representing groundwater travel times of the order of 2 and 20 years, respectively.[51,52] These locations differed in labile C supply and exhibited two different anammox OTUs (F575 rep and F168 rep; Figure 2A). At the up-gradient location (F575) where more reactive C was available, parts of the vertical profile containing NO_3^- and NH_4^+ did not overlap and were separated by an anoxic, DIN-free zone (Figure 1B). We hypothesized that the DIN-free zone was due to coupled denitrification/anammox activity that reduced NO_3^- to NO_2^- and then produced N_2 from NO_2^- and NH_4^+. NO_2^- is naturally present in groundwater just above the DIN-free zone (6.2−8.7 m elevation; Table S1). At the down-gradient location (F593), where reactive C was less abundant in the water or aquifer sediment, NO_3^- and NH_4^+ coexisted in the absence of O_2 throughout the vertical profile below about −5 m elevation (Figure 1C).

Four tracer tests were conducted. Tests 1 and 2 at the up-gradient location compared activity within the NH_4^+ zone and DIN-free zone, respectively. Tests 3 and 4 were performed in a single zone at the down-gradient location and compared the absence (test 3) and presence (test 4) of added acetate as an additional electron supply. Groundwater tracer travel distances ranged from 0 to 11 m (Figure S1), and travel times ranged from 0 to 40 days.

Anammox activity was detected in all four tests as $^{29}N_2$ ($\pm^{28}N_2$ depending on $x^{15}NO_2^-$; Figures 3 and 4), including test 2 in a zone in which no detectable dissolved NH_4^+ was present. Rates of anammox N_2 production were similar for tests 1−3, ranging from 9.1 to 12.2 nmol N L^{-1} day^{-1} (Table 1), and 50-fold higher in test 4. Denitrification activity was detected in all four tests as $^{30}N_2$ ($\pm^{29}N_2$ depending on $x^{15}NO_2^-$) and as nitrous oxide (N_2O). It was lowest in test 3, where labile C was scarce, and was stimulated 400−600-fold by acetate in test 4. With the exception of tracer test 4, where the rate of NO_3^- reduction to NO_2^- was high, the ^{15}N mole fraction in the NO_2^- tracer generally remained high with time and travel distance (Figures 3 and 4), indicating little or no detectable $^{14}NO_2^-$ production. $\delta^{15}NH_4^+$ values in representative tracer breakthrough peak samples from tests 3 and 4 were higher than background values by no more than 1 and 10‰, respectively, indicating the rate of NH_4^+ production by dissimilatory nitrate reduction to ammonium (DNRA) was not more than approximately 2 and 1% of the total N_2 production rate for tests 3 and 4. For test 1, $\delta^{15}NH_4^+$ measurements were affected by cross-contamination and not useful for assessing DNRA, but nonetheless confirmed that ^{15}N-enriched NH_4^+ significant source of error in the calculation of N_2 components. Thus, anammox disguised as denitrification[40,41] was not a significant source of $^{30}N_2$, at least in tests 1, 3 and 4. NH_4^+ isotope ratios

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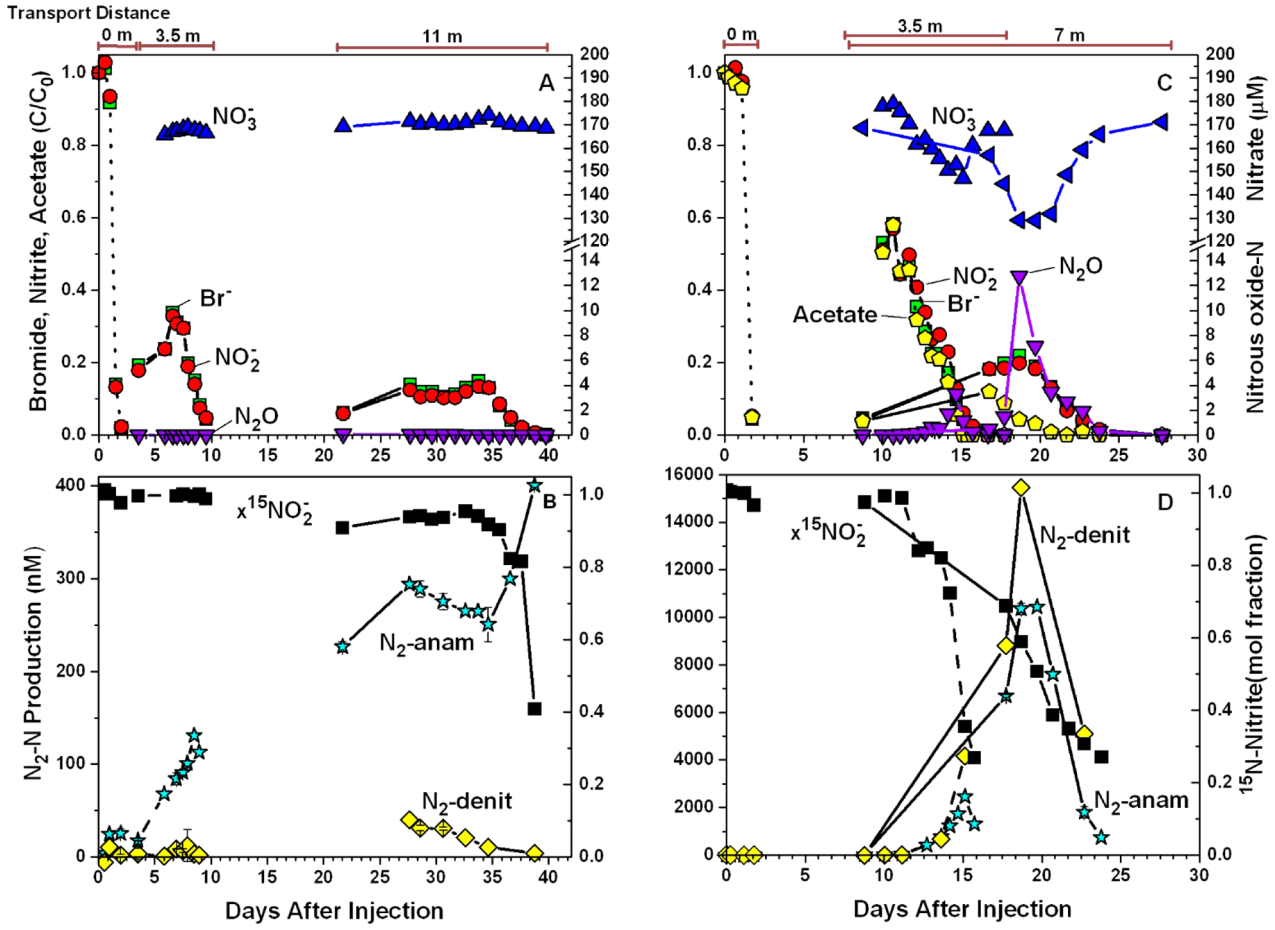

Figure 4. Breakthrough curves of natural gradient tracer tests 3 (A, B) and 4 (C, D) at the injection port and at down-gradient sampling ports: added tracer constituent concentrations normalized to injection concentrations (C/C_0); other reactant and product concentrations; accumulated N_2 production attributed to anammox and denitrification; and ^{15}N mole fraction ($x^{15}N$) of recovered NO_2^-. Travel distances for individual curves are indicated by horizontal bars above the figure panels; dotted lines represent 0 m, dashed lines represent 3.5 m, and solid lines represent 7 or 11 m. See Figure 1 and Figure S1 for maps of tracer test wells and tracer cloud travel paths. Error bars are ± one standard deviation of replicate samples.

Table 1. Gas Production Rates by Anammox and Denitrification from *in Situ* Groundwater Tracer Tests Using $^{15}NO_2^-$[a]

	nitrogen gas (nmol N L^{-1} day^{-1})		nitrous oxide (nmol N L^{-1} day^{-1})	anammox contribution (%)	
tracer test	anammox	denitrification	(net)	N_2 produced	N_2 + N_2O produced
1	12.2 (8.9)	3.9 (1.2)	107 (30.8)	75.7	9.9
2	10.7 (3.8)	16.6 (4.3)	20.4 (5.9)	39.0	22.3
3	9.1 (1.3)	1.0 (0.3)	1.6 (1.1)	89.7	77.8
4[b]	458 (98.7)	662 (233)	324 (266)	40.9	31.7

[a]Rates were calculated from the average accumulated concentrations at all time points within the tracer breakthrough curves with the longest travel distance (see Figures 3 and 4) assuming constant accumulation with time during transport; values in parentheses are ± one standard deviation. Rates do not include compensation for dispersion. [b]Sodium acetate was included with other tracers in test 4.

could not be measured in test 2 samples, which had undetectable NH_4^+ concentrations. At the up-gradient site, N_2 production via denitrification slightly exceeded N_2 production by anammox in the shallower test near the NO_3^- zone (test 2) but was less than N_2 production by anammox in the deeper NH_4^+ zone (test 1; Table 1). However, N_2O was also produced by denitrification in tests 1 and 2. N_2O production rates differed between the two zones but in both cases exceeded N_2 production. If denitrification is considered as the sum of N_2 and N_2O production, then the relative proportion of anammox activity was lower (Table 1). There were small amounts of dissolved Fe(II) in the tracer test 1 zone (Table S1); thus, it is possible that some portion of the N_2O production in that test was due to chemodenitrification.[39] The largest relative contribution of anammox to total N_2 production was approximately 90% in test 3 at the down-gradient location where the NH_4^+ concentration was high and labile organic-C was scarce.

Spatial/Temporal Controls of Anammox and Denitrification Rates. When NO_2^- was supplied as a tracer, N_2 production by anammox was detectable even within a few hours at the injection wells. If relatively abundant degradable C was present (tests 1, 2, and 4), denitrification also was detected immediately. But when denitrification was limited by more restricted electron supply (test 3), 90% of the N_2 production was attributable to anammox; this is higher than the relative

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anammox contribution reported in almost all other environments studied to date. The addition of an exogenous electron supply, in the form of acetate (test 4; Figure 4C,D), substantially stimulated both anammox and denitrification. Anammox bacteria can utilize short-chain organic acids to enhance growth.[41] In the presence of acetate, NO_3^- was the primary oxidant and NO_2^- production and consumption rates were nearly balanced, resulting in decreasing $^{15}NO_2^-$ mole fraction due to $^{14}NO_3^-$ reduction to $^{14}NO_2^-$; 100% of the NO_3^- decrease was recovered as N_2O and N_2. The anammox stimulation in test 4 relative to test 3 indicated enhanced anaerobic NH_4^+ oxidation in the presence of the organic-C, in essence representing a multiplier effect for total N_2 production beyond what might be predicted stoichiometrically from oxidation of organic-C by denitrification alone.

The timing of the anammox and denitrification N_2 product peaks relative to the arrival of the conservative tracer demonstrated interesting interactions between the two processes. During test 1 at the up-gradient location in the zone in which NH_4^+ was present but N oxides were not and likely had not been for many years (Figure S2), the highest rates of NH_4^+ oxidation to N_2 were evident within the leading edge of the tracer cloud, whereas denitrification took longer to respond and the highest concentration of N_2 from denitrification occurred at the end of the tracer breakthrough peak. This suggests that anaerobic NH_4^+ oxidation was active in this zone and quickly responded to the NO_2^- tracer, whereas denitrifying activity required time for induction.

Tracer test 2 was conducted in a transient zone in which vertical gradients of NO_3^-, NH_4^+, and DOC concentrations were changing at interannual and possibly shorter time scales (Figure S2). These changes were driven by a combination of fluctuations in the water table elevation (and associated fluctuations in gradient direction) and variations in up-gradient sources of groundwater constituents. This likely resulted in a subsurface microbial population capable of responding to the fluctuating N speciation. Anammox and denitrification occurred concurrently and consistently during test 2, at approximately equivalent rates, with relatively little N_2O net accumulation. This appears to be a zone primed for removing fixed N, with a low but steady production of electrons and NH_4^+ via degradation of the organic pool.

In contrast, tests 3 and 4 were conducted in a C-depleted zone that contained high concentrations of both NO_3^- and NH_4^+ (Figure S3 and Table S2). Anammox was clearly active and ready to respond rapidly to the tracers at this site. In test 3, N_2 accumulation was nearly linear with time, suggesting a zero-order response to the NO_2^- addition, even at relatively low concentrations (<10 μM) in the trailing edge of the tracer cloud (Figure 4B). A previous tracer test in this zone with $^{15}NH_4^+$ determined that anammox activity was below an estimated detection limit of 27 nmol N L^{-1} day^{-1}.[5] This is consistent with the anammox rate of 9.1 nmol N L^{-1} day^{-1} in the current study using $^{15}NO_2^-$, which permitted quantification with an improved detection limit. Denitrification at this location was undetectable until an electron supply was added, after which both anammox and denitrification activity responded vigorously, in a short time interval (days) relative to the contaminant travel time to this site (decades).

Implications for Anammox in Aquifers. This study is one of only a few giving evidence for anammox potential in aquifers and the first to quantify its rate and relative importance with respect to denitrification. If anammox is found to be common in freshwater aquifers, it could have a substantial effect on local, regional, and global assessments of DIN fluxes and the return of fixed N to the atmosphere. Anammox was clearly present and active in Cape Cod groundwater under a variety of geochemical conditions. The process was not necessarily limited to narrow zones by diffusive flux, but rather was found in situations where fixed N persisted, whether oxidized or reduced, and dissolved O_2 was depleted. These are conditions commonly found in the subsurface and suggest that anammox can be an important geochemical mediator in that environment. This could be especially true where groundwater has been affected by wastewater,[23−25] whether from domestic, agricultural, or industrial sources, including hydrocarbon extraction, in heterogeneous aquifers where flow velocities and redox reaction rates vary locally and reaction zones overlap,[53] in groundwater recharge areas beneath wetlands or lakes,[20,54,55] and in groundwater discharge areas (riparian zones) where oxic and suboxic groundwater flow paths converge and encounter various lithologies.[11,56] This study indicates that although anammox contribution can range widely, depending on geochemistry, it can be a significant contributor to *in situ* DIN removal and total N_2 production in freshwater aquifers. Anammox appears to be favored when the ratio of NO_3^- to degradable DOC is high.[57−59] Thus, whereas subsurface rates of activity may be substantially lower than those at the surface water−land interface, groundwater residence times commonly are much longer than in surface freshwater, and the volume of affected water may be greater, resulting in potentially equivalent or even greater total N removal in the subsurface.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b02488.

PCR amplification method, maps of the tracer test sites (Figure S1), NO_3^- and NH_4^+ concentration histories at sites F575 (Figure S2) and F168 (Figure S3), and groundwater geochemistry at the time of the tracer tests at F575 (Table S1) and F168 (Table S2) (PDF)

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Notes

The authors declare no competing financial interest.

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