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 N₂. 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 15NO₂⁻ tracer tests along anoxic flowpaths in areas of varying ammonium, nitrate, and organic carbon abundances. Rates of denitrification and anammox were determined by quantifying changes in ²⁸N₂, ²⁹N₂, ³⁰N₂, ¹⁵NO₂⁻, ¹⁵NO₃⁻, and ¹⁵NH₄⁺ 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 N₂ production in this aquifer, with rates on the order of 10 nmol N₂−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, 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. Denitrification is usually considered the key dissolved inorganic nitrogen (DIN) removal mechanism in groundwater, but the discovery of anaerobic ammonium oxidation (anammox) 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 (NO₃⁻), nitrite (NO₂⁻), and ammonium (NH₄⁺)) 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. NO₃⁻ is typically the dominant form of DIN in oxic aquifers, moving freely through the unsaturated and saturated zone. NH₄⁺ tends to dominate the DIN pool in suboxic aquifers, but its transport is slower due to chemical sorption and exchange with aquifer sediments. Coexistence of NO₃⁻ and NH₄⁺, as well as O₂ and organic-C, can occur in groundwater due to differential advection rates, resulting in mixed redox zones of dissipatory N-cycling processes. Such zones may be characterized by co-occurrence of N species of multiple redox states (e.g., NO₃⁻ and NH₄⁺) and production/consumption of NO₃⁻ through both oxidizing and reducing reactions. Denitrification has long been considered the primary N removal mechanism of fixed N and has been examined in a number of subsurface studies, with an estimated 20% of the global extent of the process occurring in groundwater.

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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.$^4$

Anammox couples NH$_4^+$ oxidation with NO$_3^-$ reduction to produce nitrogen gas (N$_2$) under suboxic to anoxic conditions. Provided there is a source of NO$_3^-$, 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.$^1$ 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,$^{23,24}$ 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.

**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.

**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 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.$^{20}$

**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$_2$O 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$_2$SO$_4$ 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$_2$O was analyzed by gas chromatography.$^{28}$ Dissolved total iron was analyzed by inductively coupled plasma atomic emission spectroscopy.$^{26}$ 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 FS575 and FS93 (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$_4$.$^3$ Briefly, a 200 L gas-impermeable
by diffusion into NaHSO₄ traps and analyzed for δ¹⁵N after reaction with O₂ to produce N₂ for continuous-flow IRMS. Isotopologues of dissolved N₂ (³⁰N²⁰N, ³¹N²⁰N, and ³²N²⁰N) in groundwater were analyzed by multiple techniques. First, sample headspace gas was reacted with CuO + Cu₂O and CaO in sealed tubes to remove O₂, H₂O, and CO₂ from N₂ for dual-inlet IRMS. Alternatively, headspace gas was passed unreacted through a mole-sieve gas chromatograph, and the N₂ peak was analyzed in continuous-flow mode. 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 N₂ isotopologue ratios were converted to estimated concentrations of background N₂, N₂ produced by anammox during the experiment, and N₂ produced by denitrification during the experiment, using equations similar to those derived previously and presented as a “comprehensive approach” for three-component isotopologue attribution. Application of those equations included the following modifications and assumptions: (1) Measured isotopologue ratios of in situ background N₂ (rather than atmospheric N₂) were used as the nontracer baseline values. (2) Although unlabeled NO₃⁻ at F593 (tests 3 and 4) had δ¹⁵N of +15 to 19‰, the NO₃⁻ was not considered to contribute unlabeled N to N₂ production directly, but only indirectly through isotope dilution of the ¹⁵N-labeled NO₃⁻ pool, which was measured in each sample and included in the N₂ production equations. (3) NO₂⁻ contributing to N₂ produced by both anammox and denitrification in each sample during the tracer experiment was assumed to have had a time-integrated x¹⁵N equal to the midpoint of the measured x¹⁵NO₂⁻ value at time zero and the measured value at the sample time (important mainly for test 4, in which x¹⁵NO₂⁻ decreased substantially). Measured variations and uncertainties of δ¹⁵N values of NH₄⁺ were incorporated in the equations, although they had negligible effects on the calculated contributions of anammox and denitrification to N₂. Reported values of anammox N₂, outnumber reported values of denitrification N₂ because some dual-inlet N₂ isotopic analyses were affected by N₂O that was converted to N₂ by reaction with CuO + Cu₂O during off-line sample preparation for dual-inlet IRMS. For tests 1, 2, and 3, detection limits for accumulated N₂ 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 x¹⁵N of NO₂⁻ was changing rapidly, and possibly not linearly as assumed.

Note that for tracer tests results denitrification and anammox are defined as the net reduction of NO₃⁻ through N₂O to produce N₂ (all N atoms in N₂ from NO₃⁻) and net anaerobic NH₄⁺ oxidation coupled with NO₃⁻ reduction to produce N₂ (one atom each from NH₄⁺ and NO₃⁻), respectively. Production of ³²N₂ from ¹⁵NO₂⁻ could result from any combination of classical denitrification, chemodenitrification, or anammox “disguised” as denitrification 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
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. A portion of that carbon was sorbed onto aquifer solids, and a portion remained as DOC, which was subsequently transported down-gradient. DOC decreased in concentration during transport by continued sorption, degradation, and dispersion. 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. The number of free-living bacteria also decreased within 3 km of the source by about 10-fold, 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, 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. 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.

**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. 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. 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$_2$O reductase (nosZ), indicated that the reduction capacity for N$_2$O 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 $^{15}$O$_2$ 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 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.
the wastewater source, representing groundwater travel times of the order of 2 and 20 years, respectively. 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 NO₃⁻ and NH₄⁺ 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 NO₃⁻ to NO₂⁻ and then produced N₂ from NO₂⁻ and NH₄⁺. NO₂⁻ 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, NO₃⁻ and NH₄⁺ coexisted in the absence of O₂ 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 NH₄⁺ 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 ²⁹N₂ (±²³N₁ depending on x¹⁵NO₂⁻; Figures 3 and 4), including test 2 in a zone in which no detectable dissolved NH₄⁺ was present. Rates of anammox N₂ 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 ³⁰N₂ (±³¹N₂ depending on x¹⁵NO₂⁻) and as nitrous oxide (N₂O). 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 NO₃⁻ reduction to NO₂⁻ was high, the ¹⁵N mole fraction in the NO₂⁻ tracer generally remained high with time and travel distance (Figures 3 and 4), indicating little or no detectable ¹⁴NO₂⁻ production. δ¹⁵NH₄⁺ values in representative tracer breakthrough peak samples from tests 3 and 4 were higher than background values by no more than 1 and 10%o, respectively, indicating the rate of NH₄⁺ production by dissimilatory nitrate reduction to ammonium (DNRA) was not more than approximately 2 and 1% of the total N₂ production rate for tests 3 and 4. For test 1, δ¹⁵NH₄⁺ measurements were affected by cross-contamination and not useful for assessing DNRA, but nonetheless confirmed that ¹⁵N-enriched NH₄⁺ significant source of error in the calculation of N₂ components. Thus, anammox disguised as denitrification (0.4%) was not a significant source of ⁵⁸⁸N₂ at least in tests 1, 3 and 4. NH₄⁺ isotope ratios
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$_2$O was also produced by denitrification in tests 1 and 2. N$_2$O 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$_2$O 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$_2$O production in that test was due to chemodenitrification. 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$_3^-$ 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
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, NO3− was the primary oxidant and NO2− production and consumption rates were nearly balanced, resulting in decreasing 15NO3− mole fraction due to 14NO3− reduction to 14NO2−; 100% of the NO3− decrease was recovered as N2O and N2. The anammox stimulation in test 4 relative to test 3 indicated enhanced anaerobic NH4+ oxidation in the presence of the organic-C, in essence representing a multiplier effect for total N2 production beyond what might be predicted stoichiometrically from oxidation of organic-C by denitrification alone.

The timing of the anammox and denitrification N2 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 NH4+ was present but N oxides were not and likely had not been for many years (Figure S2), the highest rates of NH4+ oxidation to N2 were evident within the leading edge of the tracer cloud, whereas denitrification took longer to respond and the highest concentration of N2 from denitrification occurred at the end of the tracer breakthrough peak. This suggests that anaerobic NH4+ oxidation was active in this zone and quickly responded to the NO2− tracer, whereas denitrifying activity required time for induction.

Tracer test 2 was conducted in a transient zone in which vertical gradients of NO3−, NH4+, 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 N2O net accumulation. This appears to be a zone primed for removing fixed N, with a low but steady production of electrons and NH4+ 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 NO3− and NH4+ (Figure S3 and Table S2). Anammox was clearly active and ready to respond rapidly to the tracers at this site. In test 3, N2 accumulation was nearly linear with time, suggesting a zero-order response to the NO2− 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 15NH4+ 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 15NO3−, 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 O2 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, 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, in groundwater recharge areas beneath wetlands or lakes, 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 N2 production in freshwater aquifers. Anammox appears to be favored when the ratio of NO3− 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), NO3− and NH4+ 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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