Microbial characterization of nitrification in a shallow, nitrogen-contaminated aquifer, Cape Cod, Massachusetts and detection of a novel cluster associated with nitrifying Betaproteobacteria

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Abstract

Groundwater nitrification is a poorly characterized process affecting the speciation and transport of nitrogen. Cores from two sites in a plume of contamination were examined using culture-based and molecular techniques targeting nitrification processes. The first site, located beneath a sewage effluent infiltration bed, received treated effluent containing O₂ (∼300 µM) and NH₄⁺ (51–800 µM). The second site was 2.5 km down-gradient near the leading edge of the ammonium zone within the contaminant plume and featured vertical gradients of O₂, NH₄⁺, and NO₃⁻ (0–300, 0–500, and 100–200 µM with depth, respectively). Ammonia- and nitrite-oxidizers enumerated by the culture-based MPN method were low in abundance at both sites (1.8 to 350 g⁻¹ and 33 to 35,000 g⁻¹, respectively). Potential nitrifying activity measured in core material in the laboratory was also very low, requiring several weeks for products to accumulate. Molecular analysis of aquifer DNA (nested PCR followed by cloning and 16S rDNA sequencing) detected primarily sequences associated with the Nitrosospira genus throughout the cores at the down-gradient site and a smaller proportion from the Nitrosomonas genus in the deeper anoxic, NH₄⁺ zone at the down-gradient site. Only a single Nitrosospira sequence was detected beneath the infiltration bed. Furthermore, the majority of Nitrosospira-associated sequences represent an unrecognized cluster. We conclude that an uncharacterized group associated with Nitrosospira dominate at the geochemically stable, down-gradient site, but found little evidence for Betaproteobacteria nitrifiers beneath the infiltration beds where geochemical conditions were more variable.

1. Introduction

Nitrogen contamination of shallow freshwater aquifers and soils by NH₄⁺ and NO₃⁻ is a serious problem and affects watersheds throughout the United States (Nolan et al., 1997) both in its potential health [i.e., methemoglobinemia due to NO₃⁻ (Bruning-Fann and Kaneene, 1993)] and environmental (i.e., eutrophication at discharge zones) consequences. Groundwater nitrogen contamination is commonly associated with septic wastes, landfill leachates, and agricultural activities (Barcelona and Naymik, 1984; Ptacek, 1998; Christensen et al., 2001). The movement of NH₄⁺ and NO₃⁻ is largely controlled by aquifer geochemistry and hydrology. Nitrate generally interacts very little with aquifer solids moving with groundwater flow, whereas the movement of NH₄⁺ is retarded due to cation exchange with the sediment, a common observation in multiple contaminated groundwater systems (Ceazan, 1987; Christensen et al., 2001; Buss et al., 2004). Models utilizing only geochemical and hydrological information have been developed to determine the transport and fate of NH₄⁺, but in some instances NH₄⁺ and NO₃⁻ concentrations do not strictly follow predictions (Barcelona and Naymik, 1984).

Microbial oxidation of NH₄⁺ to NO₃⁻ (nitrification) plays an important role in surface soils, wastewater treatment, and aquatic environments, but very little information is available...
Fig. 1. Study site on Cape Cod, Massachusetts depicting the geographic location of the wastewater plume and two vertical longitudinal sections through the wastewater plume near the source (A to A') and down-gradient (B to B') showing the concentration (µmol/L) of O₂, NO₃⁻, and NH₄⁺ in groundwater during 1996. The water table altitude is marked with an inverted triangle with flow going from left to right. Well site sampling depths are denoted by dots immediately below well site number. Cores for analysis were collected from sites S469 and F168.
about nitrification in the subsurface. In surface soils, nitrification affects the speciation, transport, and fate of NH₄⁺ and NO₃⁻ in watersheds (Hill and Shackleton, 1989; Fisk and Fahy, 1990). During nitrification, microorganisms convert NH₄⁺ in the presence of oxygen to NO₃⁻. In groundwater environments this conversion could potentially increase the impact and extent of nitrogen contamination due to the greater mobility of NO₃⁻ compared to NH₄⁺. Two groups of microorganisms within the Proteobacteria have historically been associated with the conversion of NH₄⁺ to NO₃⁻ in terrestrial systems. The first group, ammonia oxidizing bacteria (AOB), converts NH₄⁺ to NO₂⁻, Nitrosomonas and Nitrosospira, which are Betaproteobacteria, are the two predominant bacterial soil genera that carry out the first step in the nitrification reaction. Ammonia-oxidizing Gammaproteobacteria, such as Nitrosococcus, also have been isolated, but they are typically found in marine systems. The second group, nitrite oxidizing bacteria (NOB), oxidizes NO₂⁻ to NO₃⁻ and has representatives within the Alphaproteobacteria and Gammaproteobacteria (Teske et al., 1994). Nitrobacter, an Alphaproteobacteria, is thought to be the key genus that carries out the final step of nitrification reaction. Ammonia-oxidizing Gammaproteobacteria, such as Nitrosococcus, also have been isolated, but they are typically found in marine systems. The second group, nitrite oxidizing bacteria (NOB), oxidizes NO₂⁻ to NO₃⁻ and has representatives within the Alphaproteobacteria and Gammaproteobacteria (Teske et al., 1994). Nitrobacter, an Alphaproteobacteria, is thought to be the key genus that carries out the final step of nitrification reaction.

Application of molecular tools has yielded unique insights into the diversity, abundance, and potential activities of nitrifiers in many ecosystems (Kowalchuk and Stephen, 2001). In broadest terms, molecular studies indicate that Betaproteobacteria nitrifiers are more important than Gammaproteobacteria nitrifiers in soil and wastewater processing environments. However, notably low abundances of Betaproteobacteria and Gammaproteobacteria nitrifiers or their limited metabolic capabilities (i.e., oxygen dependence) helped to identify new groups of nitrifiers. One major finding is the description of numerous Archaea in ocean, estuary, and soil ecosystems which are capable of nitrification and may be the predominant nitrifiers in many ecosystems (Beman and Francis, 2006; Leininger et al., 2006; Wuchter et al., 2006; Francis et al., 2007). Another important discovery is a group of microorganisms that utilize anaerobic ammonium oxidation (anammox). These microorganisms couple NH₃ oxidation and NO₂⁻ reduction (Jetten et al., 1998; Strous et al., 1999) and are found widely distributed in anoxic aquatic environments (Penton et al., 2006).

There are very few studies that have specifically examined nitrification in the subsurface. Barcelona and Naymik (1984) developed a solute transport model that generally described the movement and fate of nitrogen in a fertilizer contaminant plume, but found that actual NH₄⁺ concentrations were lower and NO₃⁻ concentrations were higher than the model predicted. Hypothesizing that nitrification may have been responsible for this result, they measured a transitory increase in AOB abundance, and concluded that biological nitrogen transformations were involved. A more recent model incorporating nitrification and denitrification was better able to predict the concentrations of NH₄⁺, NO₂⁻, NO₃⁻, and dissolved O₂ in shallow groundwater down-gradient from an Australian cattle feedlot (Lee et al., 2006). This model also predicted numbers of AOB and NOB within the aquifer but did not present any actual field measurements of AOB and NOB for verification. Likewise, concentrations of inorganic nitrogen species in groundwater, including nitrous oxide, have been used to infer that nitrification was active at various study sites (Bjerg et al., 1995; DeSimone and Howes, 1998; Hiscock et al., 2003; Buss et al., 2004) and culture-based enumerations detected substantial populations of nitrifying bacteria in the Chalk aquifer in England (Whitelaw and Rees, 1980). Two recent studies at the Ashumet Valley field site on Cape Cod, Massachusetts examined nitrification as a process and concluded that low-level nitrifying activity occurred on top of an NH₄⁺ subplume, down-gradient from the plume wastewater source (where O₂ and NH₄⁺ coexist). This conclusion was based upon the distribution of stable isotopes in the groundwater, the short-term in situ response of the microbial community to the injection of nitrification substrates (O₂ and NH₄⁺) into the aquifer, and the use of ¹⁵N-enriched NH₄⁺ in natural gradient tracer tests (Böhleke et al., 2006; Smith et al., 2006).

The objective of the present study is to complement the earlier process-level studies on nitrification within the Cape Cod aquifer by characterizing the abundance and diversity of nitrifying Betaproteobacteria. The approach taken was to compare the nitrifier communities at opposing ends of the groundwater NH₄⁺ plume using traditional laboratory-based assays and enumerations along with molecular analysis of nitrifier DNA extracted from the aquifer. The results suggest that the nitrifier populations differ substantially between the 2 sites. At present the reason for the differences is not clear, but could have important implications to the ultimate fate of ammonium within the aquifer.

2. Field site and sampling

2.1. Site description

The study site (Fig. 1) is a shallow sand and gravel aquifer characterized by an extensive contamination plume (~1 km wide, 25 m deep, and ~6 km long) that developed over 60 years as a result of continuous discharge of dilute, treated wastewater (LeBlanc, 1984). Increased boron concentrations in the groundwater roughly delineate the extent of the contaminant plume, which is suboxic to anoxic and dominated by substantial concentrations of both NO₃⁻ and NH₄⁺. Since 1983, a large number of multilevel sampler (MLS) wells and observation wells have been installed at this site. Multilevel sampler wells consist of 15 individual polyethylene sample tubes bundled together with screened ports open to the aquifer over a vertical profile, whereas observation wells are constructed with 2-inch-diameter polyvinylchloride pipes screened over a 2-foot length at defined depths in the aquifer. Observation and MLS well construction and installation are described in more detail elsewhere (Savoie and LeBlanc, 1998). On-going studies at this field site have documented denitrification within the anoxic core of the plume (Smith and Duff, 1988; Smith et al., 2001) and indicated that narrow, overlapping vertical gradients of NO₃⁻, O₂, and NH₄⁺ found within the contamination plume are suitable environments for nitrifying microorganisms (Ceazan, 1987; Ceazan et al., 1989). Two well sites, S469 and F168, were selected for this study because of their differing geochemistry and history. Well site S469 is located within one of the wastewater infiltration beds and consists of a MLS well with evenly spaced ports spanning a depth of +15 to ~15 m relative to mean sea level. Well site F168 is located roughly 2.5 km down-gradient from the infiltration beds and consists of two
potential (not in situ) to nitri...selected, since it ensures an excess of substrates are available (MPN) technique in autotrophic media containing either NH$_4$ or NO$_3$.

2.2. Sample collection

A peristaltic pump was used to collect groundwater samples from the MLSs (after purging each port). The samples were analyzed immediately for O$_2$ and pH or preserved and analyzed later for NH$_4$, NO$_3$, dissolved organic carbon (DOC), and dissolved inorganic carbon (DIC) as described by Savoie and LeBlanc (1998). Five cores were collected at well site S469 spanning an elevation of +13.64 to −5.20 m relative to mean sea level, and six cores were collected at well site F168 spanning an elevation of −5.85 to −9.25 m relative to mean sea level in June 1997. At the time of sampling, groundwater surface elevation was +15.1 m and +11.8 m relative to mean sea level for sites S469 and F168, respectively. Cores were collected using a wire-line piston core barrel through hollow stem augers (Zapico et al., 1987), shipped overnight on ice to the laboratory, and processed immediately.

3. Laboratory incubations and molecular analyses

3.1. Potential nitrification activity and culture-based nitrifier enumeration

In the laboratory, sediment from each core was mixed with a sterile spatula and sub-divided into three portions—the first for potential nitrification incubations, the second for most probable number-based nitrifier enumeration, and the third for DNA extraction and molecular analyses. Earlier incubations with subsurface material indicated very low potential microbial activity. Two approaches, column/intact core incubations and slurry incubations, were considered to assess potential nitrifier activity. The slurry approach was selected, since it ensures an excess of substrates are available to nitrifiers and generally provides the largest measure of potential (not in situ) activity. Triplicate samples from each core were slurried with groundwater collected from the MLS port at the corresponding depth in the aquifer that had been amended with NH$_4$Cl (final conc. 0.14 mM). Core slurries from well site S469 also were amended with sodium chloride (10 mM final conc.) to inhibit the conversion of NO$_2$ to NO$_3$ whereas the core slurries from well site F168 were not amended with chloride. Core slurries were incubated at 15 °C, and water samples were collected over a 2-week period, filtered through 0.45-µm Metricel membrane filters (Gelman Sciences, Ann Arbor, Michigan), and frozen. After all samples had been collected, NO$_2$ and NO$_3$ were analyzed by ion chromatography as previously described (Smith et al., 2006).

Nitrifying microorganisms were enumerated in the second portion of the core material by the most probable number (MPN) technique in autotrophic media containing either NH$_4$ or NO$_2$ and HCO$_3$ (Schmidt and Belser, 1994). Ten-fold serial dilutions of aquifer sediment in 1 mM potassium phosphate buffer (pH 7) were inoculated (5 reps/dilution) into both NH$_4$ and NO$_2$ media and incubated 3 months at 22 °C in the dark. Growth of AOB was determined on the basis of acid accumulation and NO$_2$ or NO$_3$ accumulation. Growth of NOB was determined by NO$_2$ loss and NO$_3$ accumulation. Enumerations of nitrifiers by MPN were also conducted on two groundwater samples from site F168 collected at −5.9 moxic/anoxic transition zone. The upper and lower ranges for the 95% confidence interval would be respectively 3.3 and 0.303 times the calculated MPN value based upon the dilutions and number of replicates.

3.2. DNA extraction and molecular techniques

Several DNA extraction and purification methods were tested in order to determine the best protocol that yielded the greatest amount of DNA. Although a bead mill homogenization method was evaluated (Miller et al., 1999), the low bacterial biomass contained in 0.5-g samples proved too limiting. To increase the amount of microbial biomass, the extraction method of Smith and Tiedje (1992) with sodium dodecyl sulfate (SDS) substituted for lauryl sulfate followed by sepaharose column purification (Miller, 2001) was utilized and found to produce a sufficient amount of highly purified DNA. Briefly, 50 g of core material was added to 50 ml centrifuge tubes containing 2.5 ml of 0.5 M NaH$_2$PO$_4$ (pH 8), 7.5 ml of lysis solution [3 M NaCl; 0.2 M Tris HCl; 0.2 M EDTA (pH 8)], and 1.25 ml of 12% SDS. The extract was then incubated at 70 °C for 45 min with occasional mixing. After cooling to room temperature, 15 ml of chloroform/isoamyl alcohol (24:1) was added, shaken well, and centrifuged (200 x g) to separate the phases. The upper aqueous phase was collected, concentrated to roughly 7.5 ml volume through a series of butanol extractions (Sambrook et al., 1989), and dialyzed overnight in Tris–EDTA buffer (10 mM Tris, 1 mM EDTA; pH 8) to remove excess salts and SDS. After dialysis, the extract was concentrated again by butanol extraction to 0.5 ml and purified by Sepharose 4B column chromatography (Miller, 2001). DNA was then precipitated in the purified extract by adding 1/10 volume 3 M sodium acetate and 2.2 volumes ethanol. After centrifugation, the DNA pellet was resuspended in 50 µl TE buffer (1 µl equivalent to 1 g wet sediment). To ensure that PCR inhibitors had been removed (and any negative PCR results were due to the absence of target DNA and not due to PCR inhibition), the purified DNA extracts were serial diluted (10-fold) in sterile H$_2$O and challenged by PCR amplification using general eubacterial 16s rDNA primers P0mod and PC5 (Wilson et al., 1990).

The AOB 16s rDNA was amplified from serial dilutions (10-fold) using a nested approach with two sets of primers to amplify nitrifying bacteria belonging to the Betaproteobacteria. A nested PCR approach has been used extensively to amplify nitrifier sequences from environmental samples (Hoorns et al., 1995; Hastings et al., 1998; Phillips et al., 1999; Whitby et al., 1999; Ward et al., 2000; Cébron et al., 2004) and was utilized because of the very low abundance of nitrifiers in our sediment samples (single PCR amplification yielded little to no product). The results obtained using a nested approach, however, need to be interpreted very cautiously because different combinations of nitrifier primers in single PCR and nested PCR can greatly affect the diversity of nitrifiers observed in environmental samples (Mamood et al., 2006). In the first amplification, primers jAMOf (5′-TGCGGRTAAACCCGCAAAAC; R=A or G, Y=C or T) and jAMOr (5′-AGACTCGATCCGACTAGC) (McCaig et al., 1994) were used to initially amplify predominantly 16s rDNA sequences from nitrifiers (Purkhold et al., 2000). In the second amplification, internal primers Nso190R (5′-
Determined using the Library Compare tool in the Ribosomal Database II (release 9) (Cole et al., 2005). In order to simplify the production of phylogenetic trees, sequences having >99% sequence identity with one another were determined using MatGAT version 2.02 (Campanella et al., 2003) and placed into a single operational taxonomic unit (OTU). Consensus sequences for each OTU were generated using BioEdit Sequence Alignment Editor version 7.0.5.3 (Hall, 1999). A phylogenetic tree was constructed using MEGA version 3.1 (Kumar et al., 2004). Neighbor-joining with bootstrap replication (2500 replications) was used to place sequences onto phylogenetic trees.

4. Results

4.1. Groundwater chemistry

The two sites sampled for this study were selected based upon existing groundwater chemistry and distance from the wastewater infiltration beds (Fig. 1). At the first site, a vertical transect along a groundwater flow line extending from the infiltration beds to 500 m down-gradient shows the groundwater concentrations of NO₃⁻, NH₄⁺ and O₂ in June 1996 (Fig. 1, A–A' transect). The contaminant plume in the vicinity of S469 consisted of a central, anoxic ammonium-containing zone surrounded by oxy to suboxic nitrate-containing zones. The upper nitrate-containing zone increased in concentration (>1400 µM) along the flow-path, whereas the deeper nitrate-containing zone had lower concentrations (300 to 600 µM). At the time of the core collection in 1997, the wastewater disposal practice had been discontinued for about 1.5 years. Total DOC and inorganic nitrogen loads had decreased precipitously at S469 in response to the cessation of the discharge (Fig. 2). Vertically, NH₄⁺ was detected only from ~5 to ~12 m altitude at this site (Fig. 3A). Oxygen also was present in this zone along with increased NO₃⁻ concentrations and increased acidity (Fig. 3B). DOC and DIC varied with depth, with DOC highest near the discharge source and DIC peaking in the anoxic core of the plume. Increased oxygen concentrations (Fig. 3B) and low specific conductance water (data not shown) below ~10 m altitude at S469 indicate the lower margin of the contaminant plume underneath the infiltration beds. Sediment cores were collected throughout most of the vertical interval at site S469 within which it was deemed that nitrification may have occurred in the past or was occurring at the time of sample collection (Fig. 3A).

At F168, there were stable zones of vertical chemical gradients that were consistent both up-gradient and down-gradient of the site (Fig. 1, B–B' transect), and had been observed at the site for nearly 5 years (Fig. 2). Based on the movement of the concentration gradients within the ammonium plume (30 m yr⁻¹) (Böhlke et al., 2006), it is estimated that ammonium had been present at the site prior to 1997 for a minimum of 18 years. In June 1997, the chemical gradients at F168 included a vertical interval ~0.5 m wide at about ~6.3 m altitude which contained both O₂ and NH₄⁺ (24 and 37 µM, respectively), relatively high concentrations of NO₃⁻ (260 µM) and increased acidity (Fig. 3C, D). Compared to S469, DOC and DIC at F168 were quite low and not as variable with depth. Sediment cores were collected at F168 across the juxtaposing zones of O₂ and NH₄⁺ and deeper through the anoxic core of the NH₄⁺ plume.
4.2. Sediment core incubations

Both AOB and NOB were present throughout the core profile at both sites (Fig. 4). The abundance of NOB, based upon MPN, was consistently higher than the abundance of AOB, but the overall abundance of either group was quite low, ranging from 1.8 to 350 AOB/g sediment and 33 to 35,000 NOB/g sediment. The abundance of AOB was greater in the two upper S469 cores (A and B) compared to the three deeper S469 cores (C, D, and E) \( (P<0.05) \). Similarly, NOB abundance was generally greater in shallower cores compared to deeper cores at S469; cores C and E were lower in NOB abundance compared to cores A, B, and D \( (P<0.05) \). In F168 cores, AOB and NOB were distributed uniformly from top to bottom with no statistical differences in abundance with increasing depth detected. In the two groundwater samples from the oxic/anoxic boundary at F168, non-attached AOB abundance was quite low \( 0.2 \) and \( 0.45/ml \), whereas non-attached NOB were
more abundant (13 and 130/ml). As a proportion in the wet sediment sample [aquifer porosity=0.39 (Böhlke et al., 2006)], non-attached AOB and NOB comprised 0.3% and 32%, respectively, of the estimated population at the oxic/anoxic boundary at site F168. Total bacterial abundance (direct microscopic counts) associated with sands in this Cape Cod aquifer range from $0.4 \times 10^7$ to $1.0 \times 10^7$ microorganisms g$^{-1}$ of wet sediment based upon porosity and a density of 2.6 g cm$^{-3}$ for quartz/feldspar (Harvey et al., 1984). Thus, together MPN estimates of AOB and NOB were only a small fraction (<0.44%) of the total microbial community with NOB comprising the bulk of the nitrifiers.

Nitification potential was low, but observable, in all sediment slurries spiked with NH$_4^+$ and incubated aerobically (Fig. 4B, D). Typically, no NO$_2^-$ accumulated in the slurries during the first week of incubation, and NO$_3^-$ only accumulated after 2 weeks in the F168 incubations (no NO$_3^-$ accumulated in S469 cores due to the presence of chlorate). Given sufficient time (1 to 2 months), microbial nitrification converted the NH$_4^+$ into NO$_3^-$ in all slurry incubations, but there

Fig. 4. Abundance of nitrifying organisms (Panels A and C) and potential nitrification activity (Panels B and D) in sediment cores collected from sites S469 and F168, respectively. Nitrifier abundance determined using the culture-based MPN method. Error bars for MPN represent a 95% confidence interval. Potential nitrification activity determined by NO$_3^-$ accumulation on day 13 of the incubation. Error bars for potential nitrification represent the standard error ($n=3$).
was poor correlation between accumulated NO₂ and AOB abundance (R=0.167). Similarly, there was no obvious relationship between the accumulation of NO₂ and the vertical position of sediment cores (oxic, anoxic, and nitrifying zones).

### 4.3. Molecular analysis of extracted DNA

PCR-amplification with general eubacterial 16S rDNA primers produced a 1.5 kb product of the expected size from all sediment cores (data not shown) indicating that the DNA extraction and purification method was adequate to yield DNA from indigenous microorganisms and to remove PCR inhibitors. Single PCR amplification with ammonium-oxidizer primers βAMOF and βAMOr produced a small amount of PCR product from several sediment cores collected at sites S469 and F168. However, reamplification of a small portion of the PCR with internal primers NITB and Nso190r in a nested PCR yielded product from all sediment cores collected at both sites. In order to identify which PCR products originated from AOB, the nested PCR products generated from each core were cloned and sequenced. According to the Classifier program available at the Ribosomal Database Project web site, all non-chimeric sequences belonged to the class Betaproteobacteria, but significant differences were detected between the two libraries (Table 1). At site F168, the largest group of sequences fell within the family Nitrosomonadaceae (47 of 65), with most of the remaining sequences (15 of 17) grouping within the order Burkholderiales. Five of the F168 Nitrosomonadaceae sequences were classified within the genus *Nitrosomonas*, whereas 41 were classified within the genus *Nitrosospira*. Notably, the *Nitrosomonas* sequences all originated in two of the three deeper cores (Core D and F), which were anoxic and contained higher concentrations of ammonium than cores collected nearer the surface. The largest group of sequences at S469 was within the order Burkholderiales (57 of 70), and only one sequence at S469 was classified within the family Nitrosomonadaceae. Ten sequences at S469 and three sequences at F168 were fairly divergent and could not be classified (>80% confidence) with any particular order within the Betaproteobacteria.

The phylogenetic tree obtained from nitrifier 16S rDNA sequences at F168 and S469 confirmed the results obtained using the Classifier program. Most of the sequences obtained from F168 grouped within the *Nitrososphaera* genus rather than in the *Nitrosomonas* genus (Fig. 5). Only a single *Nitrososphaera* clone (469A1) was obtained from S469. *Nitrososphaera*-associated sequences grouped predominantly into two groups, one within Clusters 2 and 3, and another cluster of 33 sequences not closely associated with the previously recognized clusters, yet confidently placed within the *Nitrososphaera* genus (100% confidence threshold for 28 sequences). The *Nitrosomonas* sequences were associated with nitrifiers belonging to clusters 5 and 6.

## 5. Discussion

A plume-wide consideration of the geochemistry at the Cape Cod study site suggests potential zones where nitrification may occur or had recently occurred as the result of discharged wastewater. Historical records from 1974 to 1995 document that the wastewater contained high concentrations of NH₄⁺ (51 to 800 μM), NO₃⁻ (259 to 1568 μM), and O₂ (>300 μM) (Repert et al., 2006). Thus, one potential site where nitrification might have been occurring is the discharge locale (S469), where highest NO₃⁻ concentrations (>1400 μM) were found near the water table surface and extended downgradient roughly 500 m (Fig. 1B). After 1996, when the treated discharge source was removed, contour plots of NH₄⁺ extended to clusters directly impacted by wastewater.

#### Table 1

A hierarchical comparison of the clone libraries (n=70 and 65) generated from the wastewater infiltration site (S469) and the down-gradient site (F168), respectively.*

<table>
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<th>Number of clones</th>
<th>Site S469</th>
<th>Site F168</th>
<th>P₀</th>
<th>Pᵢ</th>
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* Differences in the composition of the two libraries were determined using the Library Compare tool in the Ribosomal Database II (release 9) (Cole et al., 2005).

β Probability that the frequency of a given taxon is the same for the two libraries. Probabilities were not calculated for unclassified taxa.

Given wastewater geochemistry and insights from other wastewater-impacted sites, one would expect a similar nitrifier community dominated by *Nitrosomonas* (Oved et al., 2001; Urakawa et al., 2006), which is in part attributable to the dominance of *Nitrosomonas* in wastewater treatment systems (Otawa et al., 2006; Siripong and Rittmann, 2007). Given wastewater geochemistry and insights from other wastewater-impacted sites, one would expect a similar nitrifier community dominated by *Nitrosomonas* at the S469 site. In our study, nitrifying activity was present in cores collected from the shallower portion of the aquifer, with substantial numbers of AOB and NOB present in those cores. It is very surprising, then, that the S469 clone libraries were essentially devoid of Betaproteobacteria nitrifiers, including *Nitrosomonas*. Certainly DNA extraction or PCR difficulties could be involved in a negative result, but the positive results (large numbers of clones associated with nitrifying
Betaproteobacteria) obtained from the F168 samples that were prepared and analyzed simultaneously with the S469 samples indicates that methodology was not the root cause. A possible explanation is that nitrifying Archaea are responsible for the nitrifying potential in observed slurries and MPN tubes, and since the primers used in this study would not anneal to the binding sites in the Archeal 16S rDNA, the absence of PCR product is consistent with our results. Archeal nitrifiers were enriched from estuary sediment and aquarium gravel using autotrophic media (Könneke et al., 2005) very similar to the MPN media used in this study. Future molecular analyses will need to focus on this possibility in order to determine if these organisms are the predominant nitrifiers at the wastewater infiltration site.

Another striking feature of the wastewater plume is the high concentration NH$_4^+$ subplume that extends from 750 m to 3000 m down-gradient of the discharge source. In an earlier report (Böhlke et al., 2006), the bulk of the NH$_4^+$ subplume was attributed to discharge during the earliest years of operation beginning in 1936. That conclusion was based upon the age of groundwater within the NH$_4^+$ subplume and the retardation factor of NH$_4^+$ of 0.28 times the rate of groundwater flow. The main portion of the ammonium subplume is anoxic (Figs. 1 and 3), thus precluding oxygen-dependent nitrification at the time this study was conducted. However, a source of O$_2$ to sustain contemporary nitrification would be the diffusion and (or) dispersion of oxygen from groundwater above the NH$_4^+$ subplume. A recent research report concluded that low-level nitrification on top of the NH$_4^+$ plume near the leading edge of the NH$_4^+$ subplume (where O$_2$ and NH$_4^+$ coexist) is likely responsible for the distribution of N isotopes in the NH$_4^+$ (Böhlke et al., 2006). Indeed, injection of nitrification substrates (O$_2$ and NH$_4^+$) at a well field adjacent to one of the two sites in this study (F168) produced strong evidence for nitrification within the upper boundary of the NH$_4^+$ subplume and some evidence for nitrification potential deeper within the NH$_4^+$ subplume (Böhlke et al., 2006; Smith et al., 2006). It is likely that nitrification occurs throughout the entire depth interval of the NH$_4^+$ subplume as the subplume first arrives at a particular well site. For example, in 1996 as the leading edge of the NH$_4^+$ subplume arrived at well site F350, O$_2$ and NH$_4^+$ coexisted at the same altitude as the anoxic center of the NH$_4^+$ subplume at F168 (Fig. 1). But as O$_2$ is subsequently depleted, active nitrifying communities likely become dormant, or must switch to an alternate metabolism. For both AOB and NOB, the latter could perhaps be accomplished by switching to denitrification (Bock et al., 1988; Bock et al., 1995; Casciotti and Ward, 2001).

Evaluation of nitrifying bacteria at the F168 site by standard incubation (culture-based) and culture-independent molecular methods indicates that nitrifying Betaproteobacteria were present at this site. One would expect nitrifiers to be particularly abundant within a narrow zone at F168 where chemical gradients overlap. Somewhat surprisingly, the incubation (culture)-based approaches indicate that AOB and NOB abundances were fairly uniform with depth through the NH$_4^+$ subplume and not particularly enriched within the upper boundary of the NH$_4^+$ subplume where oxygen was present (Fig. 4). In contrast to the wastewater discharge site (S469), Betaproteobacteria sequences closely associated with nitrifiers were readily amplified from all depths at F168 within the aquifer material. If the clone libraries roughly approximate the distribution of indigenous nitrifiers, then this community appears to be dominated by microorganisms belonging to the Nitrosospira group. However, the phylogenetic affiliation of the majority of sequences represents an unrecognized cluster very closely associated with Nitrosospira. Future research effort should focus on isolating and characterizing microorganisms from this novel cluster to determine whether they are indeed nitrifiers and to better understand their biology and ecology. Nitrifiers belonging to the Nitrosomonas group represented only 11% of the nitrifier clones obtained from F168 and were found exclusively in the deeper anoxic, ammonium-enriched zones. These nitrifiers were most closely affiliated with Clusters 5 and 6 of the nitrifying Betaproteobacteria. A similar physical environment, coastal sand dunes, also contained a nitrifying population dominated by Nitrosospira (Kowalchuk et al., 1997). However, Nitrosomonas dominated at a uranium-contaminated groundwater site (Ivanova et al., 2000), indicating that differing geochemical conditions, and not simply a subsurface environment, select for particular nitrifier communities in subsurface environments. The presence of nitrifiers throughout the ammonium subplume at F168 suggests long-term survival capacity in the subsurface under anoxic conditions, as has been observed in other anoxic environments (Abeliovich, 1987).

Why would the nitrifier communities at the wastewater infiltration site (S469) and the down-gradient site (F168) be so different? One possible explanation is that the infiltration site experiences a much more dynamic geochemical history. An excellent example is seen in the cessation of discharge in 1996, where the mass of nitrogen and DOC at S469 changed markedly over a few years, as opposed to groundwater at F168, which was comparatively stable geochemically (Fig. 2). Past geochemical changes at S469 were likely even greater than what is represented in Fig. 2 because discharge rates and constituent concentrations changed with seasonal loads to the wastewater treatment facility and as human populations and constituents at S469. Far down-gradient at F168, those variations would be moderated by

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**Fig. 5.** Neighbor-joining phylogenetic tree of 16S rRNA clones from the wastewater infiltration and down-gradient sites. The tree was constructed from the aligned 16S rRNA genes from aquifer clones (bold), previously described Nitrosospira sequences (clusters 0 through 4) and Nitrosomonas sequences (clusters 5 through 8 and cryotolerans) from isolates and environmental samples (Purkhold et al., 2000), and closely related non-nitrifying Betaproteobacteria. Numbers at branching points are bootstrap values (percentages) estimated from 2500 re-sampling replicates. The nomenclature of sequences originates from the core site (F168) site; 469=S469), the core (A to F, see Fig. 3), and the clone number from that site and core (ranges from 1 to 13). Groups of sequences that share >99% sequence identity with one another are considered an operational taxonomic unit. Group 1 includes 168A1, 168A4, 168B1, 168B2, 168B6, 168B9, 168B10, 168B11, 168B3, and 168D7; group 2 includes 168A12, 168B5, 168B12, and 168E4; group 3 includes 168B14, 168C4, and 168CS.
mixing and dispersion. Thus, the narrow, extremely stable zone for nitrification activity within the upper boundary of the NH₄⁺ subplume at F168 would be a very different environment compared to the variability at S469.

6. Conclusions

The geochemical and microbial evidence support one another and indicate the presence of a nitrifying community in the contamination plume in this aquifer. However, there is a compelling argument that the nitrifying communities at the two widely separated areas of the aquifer differ due to the absence of Betaproteobacteria nitrifiers at S469 and their dominance at F168. Molecular analyses of sequences at F168 indicate that a novel grouping closely-related to nitrifiers of the *Nitrosospira* genus, yet with no known cultivated representatives are also present. This observation will require further investigation to determine their identity as nitrifiers and whether the subsurface is a preferred environment for members of this cluster. Additionally, important questions remain regarding the possible contribution of nitrifying members of this cluster. Additionally, important questions remain regarding the possible contribution of nitrifying Archaea within the wastewater plume, particularly at the wastewater infiltration site (S469), the long-term survival and function of AOB and NOB within anoxic portions of the contaminant plume, and the overall role that nitrification will have in the ultimate fate and transport of the ammonium subplume within this contaminant plume.

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