A survey of 16S rRNA and *amoA* genes related to autotrophic ammonia-oxidizing bacteria of the β-subdivision of the class proteobacteria in contaminated groundwater

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Abstract: In this study, we investigated the size and structure of autotrophic ammonia oxidizer (AAO) communities in the groundwater of a contamination plume originating from a mill-tailings disposal site. The site has high levels of dissolved N from anthropogenic sources, and exhibited wide variations in the concentrations of NO_3^- and NH_3 + NH_4^+. Community structures were examined by PCR-DGGE targeting 16S rDNA with band excision and sequence analysis, and by analysis of *amoA* fragment clone libraries. AAO population sizes were estimated by competitive PCR targeting the gene *amoA*, and correlated significantly with nitrate concentration. Most samples revealed novel diversity in AAO 16S rDNA and *amoA* gene sequences. Both 16S rDNA and *amoA* analyses suggested that all samples were dominated by *Nitrosomonas* sp., *Nitrosospira* sp. being detected in only 3 of 15 samples. This study indicated numerical dominance of *Nitrosomonas* over *Nitrosospira* in groundwater, and suggests that groundwater ammonia oxidizers are more similar to those dominating freshwater sediments than bulk soil.

Key words: *Nitrosomonas*, *Nitrosospira*, nitrification, groundwater, *amoA*, 16S.

Résumé : Dans cette étude, nous avons analysé la taille et la structure des communautés de bactéries autotrophes nitrificateurs (AAO) présentes dans les eaux souterraines provenant du panache de contamination d'un site d'enfouissement de résidus industriels. Le site contenait de forts niveaux d'azote dissoute provenant de sources anthropiques et affichait d'importantes variations dans les concentrations de NO_3^- et de NH_3 + NH_4^+. Les structures des communautés ont été analysées par PCR-DGGE en ciblant l'ADNr 16S avec excision de bandes et analyse de séquences, ainsi que par analyse des banques de clones de fragments *amoA*. La taille des populations AAO a été estimée par PCR compétitif en ciblant le gène *amoA*. Ces estimations corrélaient de façon significative avec la concentration en azote. La plupart des échantillons ont révélé une nouvelle diversité dans les séquences des ADNr 16S et des gènes *amoA* des AAO. Autant les analyses de l'ADN ribosomal que celle des gènes *amoA* ont indiqué que tous les échantillons étaient dominés par des espèces de *Nitrosomonas*, des espèces de *Nitrosospira* ayant été détectées dans seulement 3 des 15 échantillons. Cette étude a permis de souligner la supériorité numérique de *Nitrosomonas* sur *Nitrosospira* dans les eaux souterraines et suggère que les nitrificateurs autotrophes présents dans ces eaux ressemblent plus a ceux qui abondent dans les sédiments d'eau douce qu'à ceux retrouvés dans le sol.

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Mots clés : *Nitrosomonas*, *Nitrosospira*, fixateurs d'azote, eaux souterraines, *amoA*, 16S.

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Introduction

The oxidation of ammonia to nitrate by autotrophic nitrifiers is a key process in the global cycling of nitrogen (Prosser 1989). In terrestrial environments, the oxidation of ammonia to nitrite is primarily catalyzed by a monophyletic group of organisms in the β-subgroup of the class proteobacteria consisting of the genera *Nitrosomonas* and *Nitrosospira* (Head et al. 1993). Associations between the phylogeny and ecology of autotrophic ammonia-oxidizing bacteria (AAOs) in the β-subgroup of the class proteobacteria in soils have become apparent. Several studies have suggested that the genus *Nitrosospira* dominates over *Nitrosomonas* in bulk soil environments (Hiorns et al. 1995; Stephen et al. 1996; Kowalchuk et al. 2000*a*, 2000*b*, and references cited therein).

Due to the difficulties involved in culturing AAOs, the ecological studies on these organisms described above have relied on molecular biological methods to profile and enumerate AAO populations. The first target exploited in such studies was the gene encoding 16S rRNA, for which oligonucleotide primers have been developed for the selective and specific recovery of all currently known β-proteobacterial AA0 sequences from environmental samples (McCaig et al. 1994; Kowalchuk et al. 1997, respectively). Use of denaturing gradient gel electrophoresis (DGGE) subsequent to specific amplification by the polymerase chain reaction (PCR-DGGE; Kowalchuk et al. 1997) has been developed for this group, thus generating 16S rDNA fingerprints for AAO communities. Phylogenetic analysis of the community components is then achieved by excision of bands and sequence analysis or by hybridization with taxonomically useful probes (Stephen et al. 1998). This strategy is sufficiently sensitive to define at least 7 subgroups within the β-proteobacterial AAOs (Stephen et al. 1996). A second molecular marker, encoding ammonia monooxygenase subunit A (*amoA*), can also be specifically amplified from these organisms, and provides a finer level of resolution between species (Rotthauwe et al. 1997). As ecological and, by inference, physiological differences may be significant between even closely related species of AAOs (Whitby et al. 1999), the use of this marker has considerable potential in ecological studies in defining closely related ecotypes. A modification of this method has also been used to provide a quantitative estimation of β-proteobacterial AAO population sizes in environmental samples (Stephen et al. 1999; Kowalchuk et al. 1999).

In this study, the above methods were combined to profile β-proteobacterial AAO populations in groundwater extracted from the subsurface of a contamination plume resulting from the disposal of tailings from a Uranium mill in Shiprock, N. Mex. This site is currently the subject of research through the Uranium Mill Tailings Remedial Action (UMTRA) program (Anonymous 1996). Groundwater contamination at the site is derived mainly from the leaching of mill tailings that were continuously placed in disposal ponds during mill operations. The tailings contained U metal in solution, as well as sulfate, and ammonia from the milling process. According to mill design and records, neither nitrate nor nitric acid were used in the milling process. However, nitrate is now a major contaminant in the groundwater, presumably generated via ammonia oxidation either during milling, in disposal ponds, or in the subsurface. Processes involved in nitrogen transformation are important to the fate of uranium at this site. In this study we seek to identify the microbial species involved in subsurface ammonia oxidation at this contaminated site. These organisms are of potential interest to the field of metal-bioremediation in soils for a variety of reasons. Primarily, the activity of these obligate aerobes is usually the rate-limiting step in the conversion of ammonia to nitrate (nitrification; Prosser 1989). (Brown and Sherriff 1999; Lovley and Coates 1997; Stephen and Macnaughton 1999). Furthermore, uranium bioremediation in situ is based on manipulation of redox states; in the reduced form (U^{4+}), uranium ions are relatively insoluble, and therefore less mobile, toxic, and bioavailable than when oxidized (U^{6+}). Thus the activity of β-proteobacterial AAO may affect the redox balance of subsurface uranium species both by consuming oxygen, and by generating highly soluble nitrate (Prosser et al. 1989). Transport of nitrate from surface to subsurface soils provides a more energetically favorable and bioavailable electron-acceptor than oxidized uranium or sulfate. Consequently, the growth of metal-reducing species, which includes sulfate reducers, may be inhibited through competition for nutrients and electron donors. Further, microbial denitrification may induce metal oxidation (Benz et al. 1998), although this has not as yet been demonstrated for uranium.

The persistence of high levels of inorganic N at Shiprock may be linked to a failure in N-cycling due to U-toxicity. The aims of this study were to characterize the AAO populations at this site in terms of the diversity of dominant AAO 16S and *amoA* genes, and to determine whether the groundwater AAO population size was linked to the dissolved nitrate concentration, thereby supporting the hypothesis that these organisms are critical to the oxidation of surface and subsurface ammonia and ammonium.

Materials and methods

Site description

The Shiprock UMTRA Project site is on Navajo Nation land in San Juan County, New Mexico. Approximately 1 200 000 m^3 of contaminated materials on 53 ha were stabilized in a 29-ha disposal cell in the same location as the former milling operations. Remedial action was completed in September 1986. The site is arid, averaging 15 cm of precipitation and 10.4 cm of snowfall annually.

The site is along the south side of the San Juan River on an elevated terrace about 21 m above the river (samples 728, 813, 826, 828, and 830). This wash is ephemeral, except for the lower 200 m that received a constant discharge of about 200 $L \cdot min^{-1}$ from a potable water artesian well west of the wash. This water has created wetlands within Bob Lee Wash and at the mouth of the wash where it discharges into the floodplain of the river (samples 602, 603, 608, 610, 614, 615, 617, 619, 630, 736, 853, 856, and 857). In addition, seeps (sample 425) flow from the base of the escarpment

below the disposal cell into the floodplain of the river. These seeps flow at an estimated rate of 1 to 4 $L \cdot min^{-1}$. A canal and ditches in the floodplain contain water year-round. However, all samples on the floodplain proper were subsurface samples. Other surface water and small wetland areas are in the San Juan River floodplain below the disposal cell.

Terrace samples were collected from the top 3.0 m of the water table, averaging a minimal depth of 4.9 m below the soil surface. Floodplain samples were taken from the top 1.3 m of the water table, averaging a minimal depth of 2.1 m below the soil surface.

The Shiprock disposal cell is on unconsolidated alluvial terrace deposits underlain by Mancos Shale. Ground water occurs at the contact between the terrace alluvium and the upper portion of the Mancos Shale, where it has been weathered. Uranium milling and processing activities have resulted in ground water contamination in the alluvium and upper Mancos Shale on the terrace and in the floodplain alluvium. The contaminated ground water in the river terrace alluvium and upper Mancos Shale beneath the site and in the floodplain alluvium along the river have exceeded the maximum concentration limits for nitrate and Uranium (Anonymous 1993*a*). The volume of contaminated ground water is estimated to be 610 000 m^3 (160 million gal).

Sample collection

All samples were collected in January 1999. Prior to sample collection, all glassware used was washed in a 10% (v/v) Micro cleaning solution (VWR Scientific), rinsed 10 times in tap water then 10 times in deionized water. The glassware was then heated at 450°C for 4 h in a muffle furnace prior to use. Groundwater samples (varying between 600 and 2200 mL) were collected using a downhole peristaltic or impeller pump. A minimum of 3 well volumes was purged from the well before sampling. Between each well sampling, the pump and associated tubing were decontaminated using a dilute cleaning solution followed by deionized water. Samples were collected after purging was complete. Samples were filtered at the Shiprock Dineh College (approximately 5 km from the site) through sterile (methanol rinsed) Anodisc filters (Whatman International Ltd., Maidstone, England), 47 mm diameter, 0.2 μm pore size. All glassware used during filtration was rinsed with methanol between samples. Filters were stored in muffle-sterilized glass Petri dishes, preserved on dry ice and shipped overnight to the University of Tennessee, Knoxville.

Measurement of uranium, nitrate, nitrite, and ammonia/ammonium

Uranium (soluble, toxic form, U(VI)) concentrations were determined using a kinetic phosphorescence analyzer (Model KPA-11, Chemchek Instruments, Inc.) according to McKinley et al. (1995). The detection limit for uranium was 0.3 $\mu g \cdot L^{-1}$, with quantitation within 0.05 $\mu g \cdot L^{-1}$. Quantitation was against NIST-traceable standards over the standard concentration range for uranium of 0.25–50 $mg \cdot L^{-1}$ in eleven steps. Samples were treated only by the addition of a phosphorescent complexant, and were run in batch using an autosampler. When necessary, samples were diluted before analysis. Nitrate was determined using ion chromatography (Dionex Model DX-300; AS-4a column, chemical suppression, and conductivity detection) according to McKinley et al. (1997). Ammonia/ammonium and nitrite were measured using 25 mL samples, both with a minimum detection limit of 200 $\mu g \cdot L^{-1}$ by Wastren Inc., Grand Junction, Colo., according to Anonymous (1993*b*).

DNA extraction from filters, PCR, DGGE, and competitive PCR

Anodisk filters were broken into shards by hand using solvent sterilized forceps and placed into 2 mL screw-capped microcentrifuge tubes. DNA, extracted directly from filters by mechanical disruption (Stephen et al. 1999), was used as template for PCR with the CTO189f-GC and CTO654r primers designed to target specifically 16S rDNA β-subgroup ammonia oxidizers (Kowalchuk et al. 1997). Reactions used 1.0 ng of template DNA in a total volume of 25 μL using the PCR conditions described by Kowalchuk et al. (1997). PCR product concentrations were estimated by comparison to known standards after agarose gel electrophoresis (1.5% agarose, 1 × TAE (40 mM tris-acetate, 1.0 mM EDTA, pH 8.0)) and ethidium bromide staining. Approximately 200 ng of PCR product per sample was subjected to denaturing gradient gel electrophoresis (DGGE), as described by Kowalchuk et al. 1997.

Amplification of *amoA* gene fragments and competitive PCR

Fragments of *amoA* were amplified using the primers and PCR conditions described by Rotthauwe et al. 1997, as modified by Stephen et al. (1999). *amoA* clone libraries were constructed and screened by *Msp*I digestion as described in Stephen et al. (1999). Competitive PCR analysis of *amoA* template numbers was performed as described in Stephen et al. (1999) with modification. The modification was that the competitive standard was prepared by amplification of clone p428-NAB_8_23 with the vector primers M13 Reverse and SP6 (Invitrogen, Carlsbad, Calif.) followed by purification using a Geneclean Spin-Column (Bio101, Vista, Calif.) to provide the *amoA* deletion fragment flanked by 70 bp of vector sequence on each end. Reactions were performed in triplicate and contained between 10 and 10^5 competitor molecules. Amplification products were analysed by electrophoresis on 2% (w/v) agarose TAE (40 mM tris-acetate, 1 mM EDTA, pH 8.0) gels followed by ethidium bromide staining.

Cloning of *amoA* and 16S rDNA amplification products

Cloning of PCR products was conducted for *amoA* gene fragments. Gene fragments derived from 16S rDNA were also cloned if sequence analysis of bands excised from DGGE gels generated mixed (and therefore illegible) sequences. PCR products were gel purified and extracted using a Gene-Clean kit (BIO-101; Vista, Calif.). Purified fragments were cloned using the vector PCR2.1 TOPO and *E. coli* TOP10F′ competent cells according to manufacturer's instructions (Invitrogen, Carlsbad, Calif.). From each library, 40 white *amoA* or 12 white 16S rDNA colonies were randomly selected and the cloned inserts re-amplified using the vector primers M13 reverse and T7 (30 cycles of 94°C (30 s), 55°C (30 s) and 72°C (45 s)). A portion (5 μL) of the resulting amplification product was digested with the restriction endonuclease *Msp*I as per manufacturer's instructions (Boehringer Mannheim, Indianapolis, Ind.) and analysed by separation of fragments on a 2% (w/v) agarose TAE gel. Representative plasmids from each digestion pattern were selected for sequencing.

Sequence analysis

Extraction of DNA from DGGE gels, re-amplification and sequencing were as described by Kowalchuk et al. (1997). The *amoA* sequences were recovered from recombinant plasmids as described in Stephen et al. (1999). Sequencing was performed using an ABI PRISM Dye Terminator Cycle Sequence Ready Reaction Kit (Perkin Elmer, Foster City, Calif.), and analysed using an Applied Biosystems sequencer Model 373 (San Jose, Calif.). Sequences were assembled using Seqpup v. 0.6 (Gilbert 1996). Reference sequences were retrieved from GenBank via the National Institute for Biotechnology Information (NCIB) internet node using the Entrez facility (Schuler et al. 1996). Ambiguous bases and regions that could not be unambiguously aligned were deleted from phylogenetic analysis by use of the Genetic Data Environment 2.2 mask function operated within ARB (Strunk and Ludwig 1997). Following masking, alignments consisted of 448 bp and 287 bp for *amoA*

and 16S rDNA respectively. Phylogenetic algorithms (DNA-DIST, NEIGHBOR and SEQBOOT) also operated within the ARB software environment, and the resulting tree is shown in Fig. 1. Ribosomal sequences were screened for possible chimeric origin by use of the RDP CHECK CHIMERA program (Maidak et al. 1999). The *amoA* sequences were checked for chimeric origin by independent neighbor-joining analysis of the 5′ and 3′ halves of sequences within an alignment of all published *amoA* sequences (Fig. 2).

Nucleotide accession numbers

Partial 16S sequences recovered from DGGE gels were submitted to GenBank with the accession numbers AF196813 – AF196832 and partial cloned *amoA* sequences with the accession numbers AF196783 – AF196812.

Results and discussion

Not all sample wells were successfully examined for all three measurements of population size and sequence diversity. A total of 18 sites were used for competitive PCR, 14 of which were successful in 16S rDNA PCR-DGGE profiling, and a subset of 8 of these were selected for *amoA* profiling. A further sample was taken from a natural seep (sample 425, not a sampling well), and was used only in 16S rDNA PCR-DGGE analysis. See Table 1 for details.

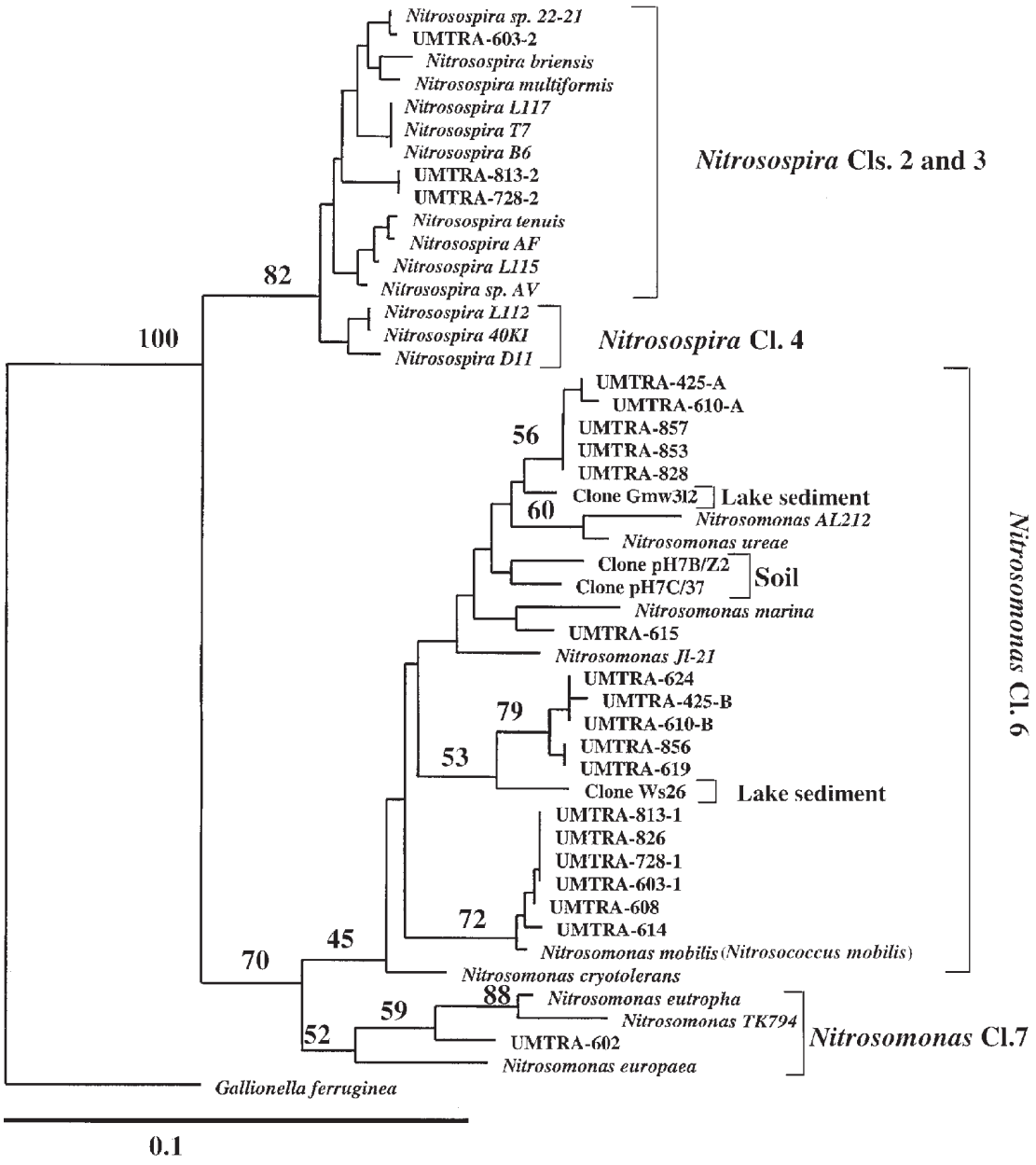
Phylogenetic diversity of β-proteobacterial AAOs in Shiprock groundwater

The phylogenetic diversity of the AAO populations in Shiprock groundwater samples was examined by 16S rDNA PCR-DGGE and band-excision as described by Kowalchuk et al. (1997). DNA from a total of 15 sites generated PCR products of sufficient strength after primary amplification to permit analysis. All of these revealed a band that co-migrated with standards representing *Nitrosomonas* sp., and three samples generated additional clusters of bands indicative of *Nitrosospira* sequences. Sequence analysis of re-amplified material derived from these bands demonstrated that the *Nitrosomonas* populations consisted of a number of diverse ribotypes forming distinct lineages associated with clusters 6 and 7 of this genus (as designated in Stephen et al. 1996; Fig. 1). Of the three *Nitrosospira* populations detected, one was affiliated with cluster 3 (well 603), and two could not be placed within any of the 4 sequence clusters previously defined within this genus, although this may well be due to the generally poor stability of phylogenies based on this short rDNA segment (wells 728 and 813; Stephen et al. 1996; Kowalchuk et al. 1997). Sequence clusters detected are summarized in Table 1 and a neighbor-joining analysis is presented in Fig. 1. Excised bands from sites Samples 425 and 610 appeared each to contain single *Nitrosomonas*-like ribotypes, based upon DGGE patterns. However, sequences derived from these DGGE bands produced partially illegible sequence, suggesting the presence of multiple sequences. These products were cloned in *E. coli* and representative transformants selected for sequencing, confirming the presence of two lineages of *Nitrosomonas* at each of these sites in approximately equal abundance. These results suggested a dominance by no more than two ribotypes of AAO at each of the 15 sites examined. A single site (602) generated a sequence similar to that of *Nitrosomonas europaea*, a lineage that has only previously been detected in natural environments by PCR following the application of manure to soil (Hastings et al. 1997) and in oligotrophic lake sediments during summer months (Whitby et al. 1999).

Diversity of *amoA* sequences recovered from Shiprock groundwaters

Analysis of AAO population structure based on 16S rDNA sequence data alone is open to criticism for a number of reasons. Chiefly, these are the possibility that biases may be inherent in the use of any one set of PCR primers and, secondly, the slow rate of mutation of ribosomal genes may disguise ecological differences between species that are closely related on this basis alone. In so much as two independent sets of PCR primers are unlikely to suffer identical biases (Suzuki and Giovannoni 1996; Chang et al. 2000), targeting a second marker, *amoA*, to some extent counteracts both of these drawbacks in studies of β-proteobacterial AAOs (Rotthauwe et al. 1997). Further, as the coverage of the *amoA* primer set is less well established than that of the 16S-directed PCR primers (Utaker and Nes 1998), consistency between the populations described by 16S rDNA and *amoA* clone library sequencing was sought as a qualitative validation of the *amoA*-directed quantitative PCR approach. Initial screening of *amoA* clone libraries for commonly occurring sequences was by restriction-digestion analysis. Representatives of commonly occurring pattern-types were subjected to DNA sequence analysis, providing an assessment of the diversity of *amoA* sequences present in these samples, and affording a finer-scale of differentiation between population components than was possible by 16S analysis (Rotthauwe et al. 1997). A total of 12 *Msp* I restriction-digestion pattern classes were identified from 320 clones (40 clones per sample, 8 samples selected to represent the variation in chemistry of the samples taken). Representatives of patterns that occurred more than 3 times in any one library were selected for sequence analysis of two or more clones. All of the recovered sequences appeared to be *amoA* fragments as judged by database searching of their primary and predicted amino acid sequences. A comparison of the *amoA*-like sequences recovered from Shiprock groundwaters with reference sequences from cultured AAOs and environmental *amoA* clones is given in Fig 2. This analysis demonstrates a generally good agreement with the population structures suggested by 16S rDNA analysis, in so much as a predominance of *Nitrosomonas*-like clones were recovered overall. Sites 603, 728, and 813, from which *Nitrosospira*-like 16S rDNA sequences were recovered, also generated *Nitrosospira*-like *amoA* clones. At a finer scale, congruence between the *Nitrosomonas*-like populations described by the two approaches is harder to assess. A 16S rDNA sequence associated with the *Nitrosomonas europaea*-lineage was recovered only from site 602, from which *N. europaea*-like *amoA* clones were also recovered. However, *amoA* sequences clustering with *N. europaea* were also recovered from sites 603, 614, 615, 610, 728, and 853. As was the case in the 16S rDNA analysis, the high level of diversity precluded any association with the environmental parameters measured. Five cloned sequences were discarded as probable chimeric artifacts. All of these were recovered from sites containing both *Nitrosomonas* sp., and *Nitrosospira* sp.

Fig. 1. Neighbor-joining analysis of 16S rDNA fragments recovered from PCR-DGGE bands. Sequences labeled UMTRA were generated in this study. Nomenclature: The prefix UMTRA is followed by the well number from which the sequence was recovered. Suffixes 1 and 2 indicate that two distinct products were visible following DGGE analysis, 1 indicates the upper band, 2 the lower. Suffixes A and B indicate that the sequence recovered from a single DGGE band was not legible directly and was cloned and sequenced following restriction digestion screening. Reference sequences were selected as the closest matching cultured organisms or cloned sequences where no closely matching cultures were available: *Nitrosospira B6, N. L117, N. T7, N. AF, N. L112, N. 4OKI, N. D11* (Utaker et al. 1995); *N. briensis, N. tenuis, N. multiformis, N. mobilis, Nitrosomonas europaea, N. eutropha* (Head et al. 1993). *N. AV,* (McCaig et al. 1994) Clone Gmw312, Clone Ws26, (Lakewater, Speksnijder et al. 1998); Clones pH7B/Z2 and pH7C/37 (Soil; Stephen et al. 1996); *N. ureae, N. cryotolerans, N. marina,* (Pommerening-Roeser et al. 1996); *N. JL-21, N. AL212,* (Suwa et al. 1997); *N. TK794* (Takashi et al. 1992). *Gallionella ferruginaea* (Hallbeck et al. 1993). Numbers on tree refer to bootstrap values on 100 replicates, only values above 50 are given. AAO cluster designations follow (Stephen et al. 1996). Scale bar represents 10% estimated change.

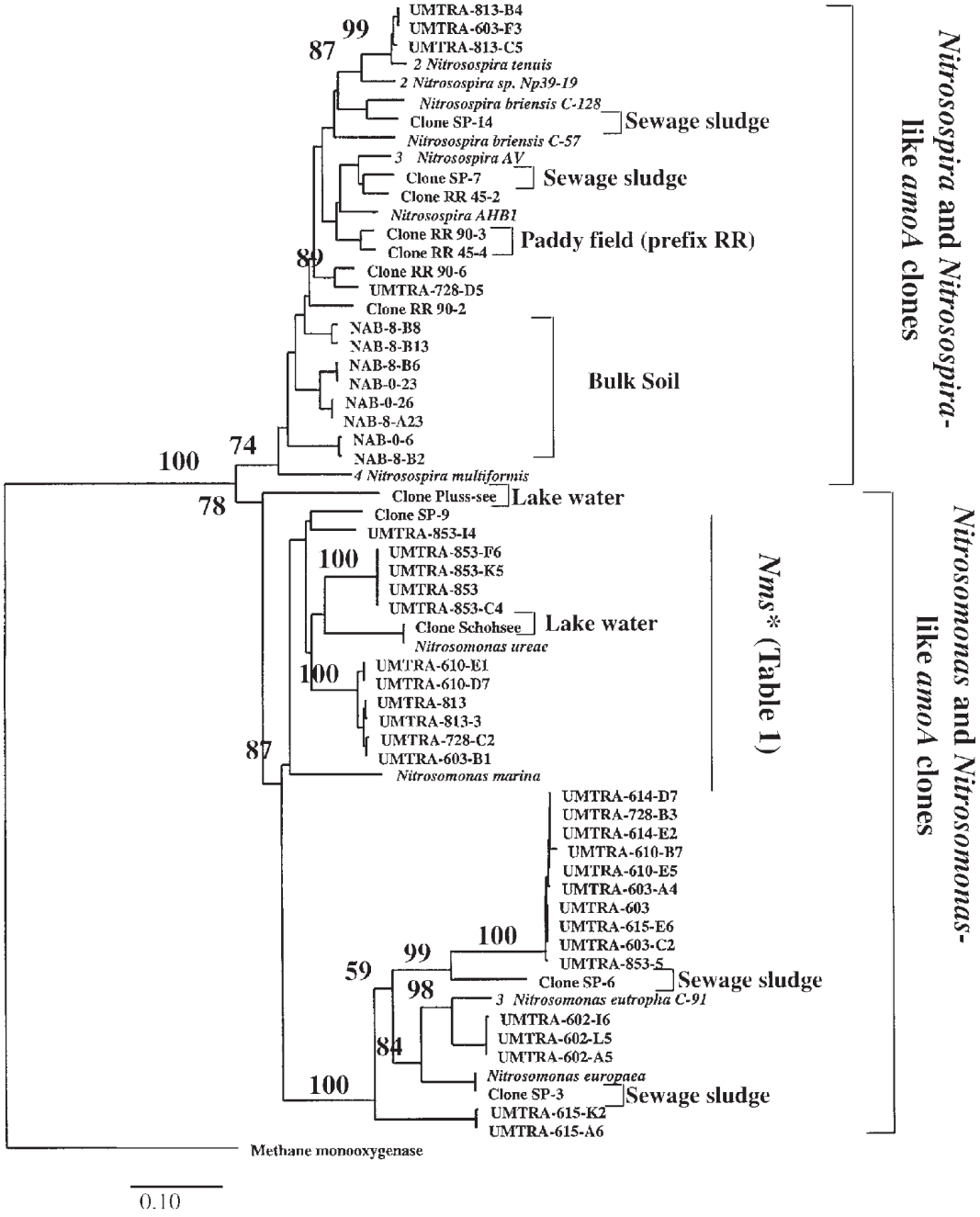


as dominant AAOs and were clearly inter-genus hybrids between other sequences recovered from the same site. Some intra-genus chimeras may also be presented in Fig. 2, but could not unequivocally be detected by treeing analysis. The basic similarity between the two community-structural analyses presented provided confidence that the use of *amoA* as a target for competitive PCR with primers based on those of Rotthauwe et al. (1997) was a valid approach to monitor the population sizes of the dominant AAOs in Shiprock groundwaters.

Effect of sample chemistry on AAO population size

Competitive PCR targeting *amoA* was conducted for groundwater samples taken from 18 wells chosen to be rep-

Fig. 2. Neighbor-joining analysis of *amoA* fragments selected from clone libraries as frequently occurring *Msp* I digestion patterns. Sequences prefixed UMTRA were generated during this study. Nomenclature: The prefix is followed by the well number and a laboratory designation respectively. Clones were selected from libraries on the basis of *Msp* I restriction patterns to provide a preliminary survey of the most commonly recovered *amoA* sequences from each sample well. Other sequences: sequence Schohsee, Pluss-see (lakewater) sequences prefixed RR (rice root) and SP (sewage sludge) are environmental clones; *N. briensis* str. C57, *N. tenuis* NV1, *N. eutropha* C-91 are pure cultures. All above from (Rotthauwe et al. 1997); *N. AHB1* (Rotthauwe et al. 1995); *N. eutropha* copies A1 and A2 (Ac. No. U51630 and U72670); *N. multiformis* copies A1, A2 and A3 (Ac. No. U91603, U15733, and U89833 respectively); *N. briensis* C128 (Ac. No. U76553); *N. tenuis* NV12 (Ac. No. U76552); *N.* sp. 39–19 copies A2 and A3 (Ac. No. AF016002 and AF006692, respectively). *N. europaea* (McTavish et al. 1993); *N. AV,* (Sayavedra-Soto et al. 1998). *N. marina,* (Ac. No. AJ388586); *N. ureae* (Ac. No. AJ388584). Sequences prefixed NAB (bulk soil) were selected from Stephen et al. (1999). Numbers prefixing a species name indicate the number of gene copies recovered from that species and have been collapsed to a single branch using the Group function of ARB (Strunk and Ludwig 1997). Numbers on tree refer to bootstrap values on 100 replicates, only values above 50 are given. Scale bar represents 10% estimated change.

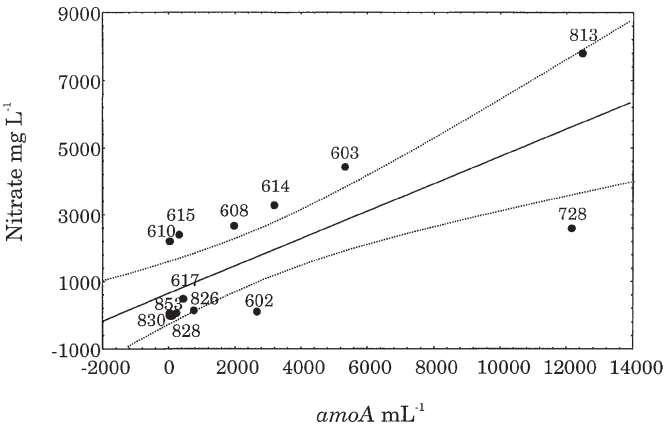


resentative of the contamination plume and the groundwater entering the site. Chemical data and *amoA* template numbers per mL groundwater are summarized in Table 1. The lack of detectable nitrite in most samples indicated that ammonia oxidation was likely to be the rate-limiting step of nitrification, as is generally held to be the case in soil (Prosser

Table 1. Quantitative measurements of [U(VI)] and data relevant to nitrification processes and summary of β-subdivision Proteobacteria ammonia oxidizers detected in Shiprock groundwaters. Standard deviations on *amoA* quantitation data ranged from 2–7% of the value given. Abbreviations. *Nms*. = *Nitrosomonas*. *Nsp*. = *Nitrosospira*. * Indicates membership of the sequence lineage affiliated with environmental clones Schohsee and SP-9 (Rotthauwe et al. 1997), and cultures *N. ureae* and *N. marina,* which are distinct from *amoA* sequences recovered from other cultured *Nitrosomonas* strains. See Fig. 2. The annotation (×2) indicates that two distinct sequences within the same cluster were recovered. ND indicates not done. Failed indicates that no amplification product was detected. Cl is an abbreviation of cluster, and designates groupings defined in Stephen et al. (1996).

Well No.	Nitrate ($mg \cdot L^{-1}$)	Nitrite ($mg \cdot L^{-1}$)	Ammonia ($mg \cdot L^{-1}$)	Uranium ($mg \cdot L^{-1}$)	Sequence types, 16S rDNA	Sequence types, *amoA*	amoA$\cdot mL^{-1}$
425*	ND	ND	ND	ND	*Nms.* Cl. 6 (×2)	ND	ND
602	122.0	<0.5	630.0	0.805	*Nms.* Cl.7	*Nms.*	2628
603	4450.0	<5.0	ND	0.011	*Nsp.* Cl. 3 and *Nms.* Cl. 6	*Nms*, *Nms**,and *Nsp.*	5320
608	2687.0	<0.5	ND	1.600	*Nms.* Cl. 6	ND	1967
610	2220.0	<0.5	94.1	1.450	*Nms.* Cl. 6 (×2)	*Nms.*, and *Nms.**	22
614	3290.0	<0.5	22.5	2.220	*Nms.* Cl. 6	*Nms.*	3168
615	2430.0	<0.5	68.1	2.150	*Nms.* Cl. 6	*Nms.* (×2)	294
617	477.0	<0.5	42.0	0.456	Failed	ND	436
619	328.0	<0.5	7.8	1.640	*Nms.* Cl. 6	ND	2.5
630	10.9	<0.1	<0.001	0.116	Failed	ND	54
728	2600.0	<0.1	181.0	0.429	*Nsp.*, and *Nms.* Cl. 6	*Nms.*, *Nms.** and *Nsp.*	12150
736	2.9	<0.5	0.009	0.625	Failed	ND	4
813	7820.0	<5.0	67.1	0.157	*Nsp.*, and *Nms.* Cl. 6	*Nsm**., and *Nsp.*	12450
826	169.0	<0.5	43.8	2.860	*Nms.* Cl. 6	ND	744
828	83.9	<0.5	1.8	0.306	*Nms.* Cl. 6	ND	226
830	86.2	<0.1	14.1	<0.001	Failed	ND	25
853	0.1	<0.1	16.0	0.166	*Nms.* Cl. 6	*Nms* and *Nms.**	86
856	0.2	<0.1	0.009	0.140	*Nms.* Cl. 6	ND	15
857	0.1	<0.1	16.3	0.174	*Nms.* Cl. 6	ND	19

Fig. 3. Scatter-plot of *amoA* number mL^{-1} vs. NO_3^-. For clarity, labels corresponding to sites 619, 630, 736, 853, and 856 have been omitted. These clustered with sample 826. Dotted lines indicate 95% confidence limits. The equation relating *amoA* to nitrate in Shiprock groundwaters was: Nitrate ($mg \cdot L^{-1}$) = 639.0 + 0.4. * *amoA* copies$\cdot mL^{-1}$. $R = 0.76$; $P < 0.00$.



1989). However, accumulation of nitrite was detected in two of the sites exhibiting high nitrate levels. It is unlikely that nitrite accumulation in these sites was due to toxic effects of U on autotrophic nitrite oxidizers, as one of these had the lowest [U(VI)] of any sample tested (well No. 603). A correlation ($R = 0.76$; $P = < 0.001$) was found between groundwater nitrate concentrations and ammonia *amoA* template numbers per mL groundwater. A scatter plot of these data is shown in Fig. 3. No correlations were observed between *amoA*$\cdot mL^{-1}$ and any of the other chemical parameters of the groundwater measured (data not shown). Considering loss of DNA during extraction, and the finding that β-proteobacterial AAOs generally carry 2–3 copies of *amoA*$\cdot$ genome^{-1}, (Norton et al. 1996; Klotz and Norton 1998), these data cannot be extrapolated directly to infer cell numbers. However, it should be noted that this form of analysis generally suggests higher cell numbers than culture based MPN analysis (Kowalchuk et al. 1999, 2000*a*, 2000*b*; Phillips et al. 2000).

Conclusions

Several studies have suggested that the genus *Nitrosospira* dominates over *Nitrosomonas* in bulk soil (Stephen et al. 1996, 1998; Bruns et al. 1999; Hiorns et al. 1995; Hastings et al. 1997; Hastings et al. 2000; Kowalchuk et al. 2000*a*, 2000*b*). We were therefore surprised to find that the groundwaters studied here showed the reverse relationship, irrespective of sample chemistry. In this respect, groundwater appears to select for ammonia-oxidizing bacteria similar to those detected in freshwater sediments using similar technical approaches (Speksnijder et al. 1998). It is likely that most nitrification is conducted by soil-associated ammonia oxidizers rather than the groundwater-dwelling organisms detected here. However, the lack of detectable nitrite in most samples, combined with the correlation between nitrate and *amoA* templates mL^{-1} groundwater supports the hypothesis that nitrification of the contaminant ammonia/ammonium at this site is mediated by AAOs. A high degree of AAO diver-

sity was found between sites, but the survey-level sampling scheme used here was insufficiently detailed to assess any link between AAO numbers or species composition and sample chemistry, other than a link between numbers and standing nitrate. For example, elevated ammonia/ammonium levels may have been expected to select for *Nitrosomonas* sp. over *Nitrosospira* sp., yet *Nitrosospira* sp. were not detected in samples with the lowest ammonia / ammonium, but were detected in sample 728, containing the second highest levels of these growth substrates (181 $mg \cdot L^{-1}$). However, the only site in which *Nitrosomonas* Cl. 7 appeared as a dominant nitrifier, also had the highest ammonia / ammonium levels (Site 602; 630 $mg \cdot L^{-1}$), in agreement with the findings of Suwa et al. (1997) that high levels of ammonium select for this branch of the genus *Nitrosomonas* over Cl. 6 organisms, which dominated all other groundwaters (as low as 1.6 $mg \cdot L^{-1}$). Further, Hastings et al. (2000) have observed that levels of *Nitrosomonas* Cluster 7 sp. increase in soil following wetting, while Kowalchuk et al. (2000*b*) made similar observations on Cluster 6 organisms in wetter areas of the Drentsche A. Stephen et al. (1996, 1998) demonstrated that, while the relative abundances of the 3 soil-related *Nitrosospira* groupings altered dramatically in soils of varying pH but identical water content, the relative abundance of *Nitrosomonas* to *Nitrosospira* did not change. Taken together with the findings presented here, these data may form the basis for the testable hypothesis of ecological partitioning between the genera *Nitrosospira* and *Nitrosomonas* between soil and groundwater. This work did not provide evidence of a link between β-proteobacterial AAO numbers or ecology and metal speciation in these groundwaters, but may add to our appreciation of the ecology and diversity of the genus *Nitrosomonas*. The failure of the Shiprock site to remediate anthropogenic N since its decommission is unlikely to be linked to toxic effects of uranium on autotrophic nitrification, and is more likely to be linked to factors effecting heterotrophic denitrification, such as availability of organic carbon or other electron donors.

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