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Effects of pH and Oxygen and Ammonium Concentrations on the Community Structure of Nitrifying Bacteria from Wastewater

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Shifts in nitrifying community structure and function in response to different ammonium concentrations (50, 500, 1,000, and 3,000 mg of N liter^{-1}), pH values (pH 6.0, 7.0, and 8.2), and oxygen concentrations (1, 7, and 21%) were studied in experimental reactors inoculated with nitrifying bacteria from a wastewater treatment plant. The abilities of the communities selected for these conditions to regain their original structures after conditions were returned to the original conditions were also determined. Changes in nitrifying community structure were determined by performing an amplified ribosomal DNA (rDNA) restriction analysis of PCR products obtained with ammonia oxidizer-specific rDNA primers, by phylogenetic probing, by small-subunit (SSU) rDNA sequencing, and by performing a cellular fatty acid analysis. Digestion of ammonia-oxidizer SSU rDNA with five restriction enzymes showed that a high ammonium level resulted in a great community structure change that was reversible once the ammonium concentration was returned to its original level. The smaller changes in community structure brought about by the two pH extremes, however, were irreversible. Sequence analysis revealed that the highest ammonium environment stimulated growth of a nitrifier strain that exhibited 92.6% similarity in a partial SSU rRNA sequence to its nearest relative, *Nitrosomonas eutropha* C-91, although the PCR product did not hybridize with a general phylogenetic probe for ammonia oxidizers belonging to the β subgroup of the class *Proteobacteria*. A principal-component analysis of fatty acid methyl ester data detected changes from the starter culture in all communities under the new selective conditions, but after the standard conditions were restored, all communities produced the original fatty acid profiles.

Autotrophic nitrifying bacteria that oxidize ammonium to nitrite and nitrate are found in soils, sediments, wastewaters, freshwater, and marine water and on building facades. They are essential components of the nitrogen (N) cycle, linking the most reduced and most oxidized forms of inorganic N. Nitrification occurs as a two-step process carried out by two distinct groups of bacteria; ammonia-oxidizing bacteria convert ammonia to nitrite, and then nitrite oxidizers convert nitrite to nitrate (22, 30). Environmental factors control the rate of nitrification. The most significant environmental factors are substrate concentration, pH, temperature, and oxygen availability (12, 23). Nitrifying bacteria exhibit different substrate concentration sensitivities (26). Media containing low substrate concentrations (10 mg of NH_4^+ liter^{-1}) can give larger most-probable-number counts of ammonia oxidizers than media containing higher NH_4^+ concentrations (6, 26). Also, ammonia oxidation is inhibited at high substrate concentrations. The growth rates of *Nitrosomonas* spp. cultures were reduced in the presence of 1,050 to 2,800 mg of NH_4^+-N liter^{-1} (16). Substrate inhibition of ammonia oxidation has also been observed in studies of wastewater systems (23). Natural environments, such as soil and water, usually contain 1 to 10 mg of NH_4^+-N liter^{-1} (22), yet liquid wastes from animal farms give rise to concentrations up to 1,600 or 5,600 mg of NH_4^+-N liter^{-1} (5, 17). Free ammonia (NH_3) rather than the total ammonium concentration inhibits ammonia oxidizers (1). As the ratio between the ionized form and the nonionized form depends on pH, the toxicity of ammonium also depends on the environmental pH.

The pH range for growth of pure cultures of ammonia oxidizers is 5.8 to 8.5, and the pH range for growth of nitrite oxidizers is 6.5 to 8.5 (30). Nitrification was inhibited at pH values below 5.8 in our preliminary experiments performed with an enriched culture of nitrifiers obtained from wastewater. Yet in natural environments, such as soil, nitrification has been reported to occur at pH values below 4.0 (7, 29).

Limiting amounts of dissolved oxygen (concentrations below 2 mg liter^{-1}) inhibit nitrification and cause nitrite accumulation or nitrous and nitric oxide production (9, 21). Ammonia-oxidizing bacteria are the key functional group in removing ammonium from wastewaters. Knowledge of the effect of oxygen on nitrification and nitrifying populations has economic importance since aeration of activated sludge is one of the most costly items in the operation of a wastewater treatment plant (21).

In environments with high inputs of ammonium, such as wastewaters, biooxidation of this substrate increases the oxygen uptake and lowers the pH. Such modifications of the environment not only affect the production of nitrite and nitrate but can also select a different nitrifying community that is perhaps specialized for these new conditions. Nitrification does occur in extreme environments that pure cultures of nitrifiers cannot tolerate (4). In this study we examined extreme environments in which nitrifying bacteria may be viable but have not been cultured thus far.

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TABLE 1. Experimental conditions and ammonium consumption rates in the reactors during incubation under the selective conditions and after restoration of the standard conditions

Treatment	Conditions			Ammonium consumption during incubation (mg of NH_4^+-N liter^{-1} day^{-1})[a]	
	NH_4^+ concn in reactor (mg of NH_4^+-N liter^{-1})	pH in reactor	% Oxygen in aeration gas mixture	Under selective conditions[b]	After return to standard conditions[c]
amm50	50	7.0–8.0	21	50	46
amm500	500	7.0–8.0	21	174	38
amm1000	1,000	7.0–8.0	21	163	47
amm3000	3,000	7.0–8.0	21	220	44
pH 7.0	200	7.0	21	118	74
pH 8.2	200	8.2	21	79	61
pH 6.0	200	6.0	21	7	41
ox 21%	200	7.0–8.0	21	140	50
ox 1%	200	7.0–8.0	1	3	75
ox 7%	200	7.0–8.0	7	16	37

[a] Includes ammonia oxidation and assimilation into biomass.
[b] Ammonium consumption rates were calculated between 48 and 58 days after inoculation under selective conditions.
[c] Ammonium consumption rates were calculated between 20 and 34 days after restoration of the original conditions.

Because of the difficulty of obtaining nitrifier isolates, nucleic acid-based methods have greatly aided studies of the diversity of nitrifiers (11, 20, 27, 28). Recent molecular investigations have provided valuable information concerning the diversity of ammonia oxidizers in natural environments (5, 15, 20, 25). However, no previous study has focused on the structural or compositional responses of nitrifying communities to perturbations in the environment. In the present laboratory study we examined the effects of high ammonium concentrations, different pH values, and different oxygen concentrations on nitrification and on the community structure of nitrifying bacteria from wastewater. To test the abilities of the communities to regain their original structures, growth of nitrifying communities under the new conditions was followed by incubation under the original conditions.

MATERIALS AND METHODS

Nitrifying culture and mineral medium. An enrichment culture of nitrifying bacteria was prepared by inoculating an aerated, continuous flow of fresh basal mineral medium containing 100 to 500 mg of NH_4^+-N liter^{-1} with municipal wastewater. After a few months of growth, there was enough biomass to harvest the culture from the column. The basal mineral medium for nitrifying bacteria (30) was used, except that no phenol red was added and higher ammonium concentrations and modified buffer (0.1 M phosphate buffer) were used (17). Ammonium-N was added to the basal medium as $(NH_4)_2SO_4$.

Experimental design. The enriched nitrifying bacterial culture was used as a starter culture for 10 continuously fed reactors, each containing 100 ml of basal mineral medium supplemented with ammonium-N. The reactors were made from 25-cm-high glass cylinders having a diameter of 4 cm. Each reactor had a side arm with an opening that enabled outflow and maintenance of a constant volume (100 ml) of the reaction mixture. The reactor medium in each cylinder was aerated from the bottom by using coiled Teflon tubing with tiny holes. The reactors were sparged with preset gas mixtures, and the ammonium concentrations in the reactors were maintained at the levels shown in Table 1. Incubation for 74 days was conducted in the dark at 25°C. The dissolved oxygen content was monitored, and the pH was adjusted with 1 M Na_2CO_3. The reactors were not operated as chemostats; instead, the solution concentrations of ammonium and oxygen and the pH were kept approximately constant. To do this, the flow rate of medium was increased with time from 0.8 to 2.5 ml h^{-1} as biomass accumulated. Most of the biomass was retained in the reactor since the organisms grew as flocks. The ammonium concentration was measured initially at 3- to 5-day intervals and later daily to determine the amount of ammonium supplement needed to maintain the ammonium concentration within 15% of the starting concentration. Nitrate and nitrite concentrations were measured periodically to confirm that nitrifying activity was occurring. Samples of biomass were collected for molecular analysis after 14, 25, 38, 50, 60, and 74 days of incubation. The reactor cultures were briefly stirred prior to sampling.

To test the abilities of the nitrifying communities to regain their original structures after the selective conditions were eliminated, we harvested the microbial biomass (planktonic biomass plus attached biomass) from each reactor. After all parts of the reactors were carefully cleaned, the reactors were filled with fresh mineral medium and reinoculated with small portions of the nitrifying communities obtained from each selective condition (0.3 mg of cell protein equivalents for each reactor). All of the reactors were then incubated for 34 days under the standard conditions (i.e., 200 mg of NH_4^+-N liter^{-1}, 21% oxygen, pH 7.0 to 8.0). Reactor maintenance, biomass sampling (on days 8, 14, 23, and 34), and community structure analysis were carried out as described above.

Analytical methods. Nitrification was confirmed by measuring the nitrite and nitrate contents and the ammonium consumption in the reactors. Ammonium concentrations were determined colorimetrically with indophenol blue (14). Nitrite and nitrate contents were measured by high-performance liquid chromatography. Biomass protein contents were estimated by the biuret method (10). The contents of the heterotroph populations in the enrichment cultures were determined on R2A agar plates (Difco Laboratories, Detroit, Mich.).

Nucleic acid extraction and SSU rDNA amplification. Genomic DNAs were extracted from three pure cultures of ammonia oxidizers, from the starter culture, and from the nitrifying communities obtained from all of the reactors. Biomass subsamples were freeze-thawed three times and then processed by the DNA extraction procedure of Ausubel et al. (2). The concentration and purity of DNA in each sample were estimated by determining the ratio of absorbance at 260 nm to absorbance at 280 nm. DNAs from pure cultures of *Nitrosomonas europaea* ATCC 25928, *Nitrosolobus multiformis* ATCC 5976, and *Nitrosospira* strain NpAV were used as positive controls in PCR, as reference DNA in hybridization tests, and for restriction analyses.

PCRs were carried out by using group-specific primers βAMOf and βAMOr for small-subunit (SSU) rRNA genes (rDNA) of ammonia oxidizers belonging to the β subgroup of the class *Proteobacteria* (β-proteobacteria) (20), 100 ng of template DNA, and a model 9600 GeneAmp PCR system (Perkin-Elmer, Foster City, Calif.). Positive controls contained DNA from the three pure cultures of ammonia oxidizers. Negative controls contained either no template DNA or genomic DNA of five selected heterotrophs isolated from the reactor communities and/or genomic DNA of *Pseudomonas* strain G179 and *Achromobacter cycloclastes* ATCC 21921. The PCR conditions were as follows: initial denaturation at 94°C for 120 s; 35 cycles consisting of 92°C for 30 s, 68°C for 60 s, and 72°C for 120 s; and final extension at 72°C for 7 min. Amplification specificity was checked on 1% agarose gels.

SSU rDNA ARDRA. Amplified SSU rDNAs (19) of the pure cultures and nitrifying communities were digested individually with the following five restriction enzymes: *Rsa*I and *Sau*3A, obtained from Gibco BRL, Life Technologies, Gaithersburg, Md.; and *Hae*III, *Hin*PI1, and *Bst*U1, obtained from New England Biolabs, Ltd. The digested fragments were separated by electrophoresis on 3.5% MetaPhor agarose gels (FMC Bioproducts, Rockland, Maine) in Tris-acetate-EDTA buffer for 4 h at 4°C. Bands were visualized by UV excitation of ethidium bromide-stained gels and photographed. Individual amplified rDNA restriction analysis (ARDRA) patterns were compared by eye, and a similarity index was determined for each treatment by comparing its pattern with the pattern of the starter culture. The similarity index was the ratio of the number of common ARDRA bands after digestion with all five restriction enzymes to the total number of bands in both of the samples analyzed. Very faint ARDRA bands were counted as half bands.

Southern blotting and SSU rDNA probe hybridization procedures. Restricted SSU rDNAs from nitrifying communities were hybridized with the following two phylogenetic probes: Ammo_Cl_2/3/4/6, which hybridizes with all known terrestrial β-proteobacterial ammonia oxidizers; and All_Spira, which hybridizes with all known representatives of the *Nitrosospira* group (6). Pure-culture DNAs of three ammonia oxidizers were used as positive controls for hybridization. The

restricted SSU rDNAs were transferred to a Hybond N+ membrane (Amersham Life Sciences Inc., Cleveland, Ohio) (2) and were cross-linked with UV light. The probes were end labeled with [^{32}P]ATP by using T4 polynucleotide kinase (DuPont NEN Biotechnology Division, Wilmington, Del.). After prehybridization the membranes were hybridized with the ^{32}P-labeled probes at 42°C for 6 to 18 h and then washed at 42°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate and placed in film cassettes for exposure to autoradiogram film (Kodak, Inc., Rochester, N.Y.).

Sequencing of SSU rDNA and phylogenetic analysis. The PCR product (10 μl) from the community maintained in the presence of the highest ammonium concentration was cloned by using the manufacturer's recommended procedure (TA cloning kit; Invitrogen, San Diego, Calif.). The clones were screened for different inserts by digesting reamplified fragments with *Hae*III, *Hin*PI1, and *Bst*U1. Five different cloned SSU rDNA fragments (lengths, ca. 1,140 to 1,180 bp) were PCR amplified by using the primers and conditions described above and were sequenced by performing automated fluorescent *Taq* cycle sequencing with a model 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). Approximately 340 unambiguous nucleotide positions between positions 140 and 508 (*Escherichia coli* numbering) were used for comparison. Sequences from the nearest relatives were identified and obtained from the Ribosome Database Project (RDP) by using the SIMILARITY_RANK and SUBALIGNMENT programs of the RDP (18). Sequences were aligned manually by using both primary and secondary structures and the GDE editor obtained from the RDP. The levels of similarity of aligned sequences were determined by using the AE2 program obtained from the RDP. Phylogenetic relationships were inferred by the distance matrix method of De Soete (8) by using evolutionary distances estimated by the method of Jukes and Cantor (13).

FAME analysis. The total fatty acid contents of samples were determined by using the protocol developed by MIDI, Newark, Del. Fatty acids were quantified by comparison with known standard fatty acids by using peak width and area. The fatty acid methyl ester (FAME) data were normalized, and the major fatty acids were examined by using the principal-component analysis (PCA) portion of the statistical package S-PLUS (StatSci, Division of MathSoft, Inc., Seattle, Wash.) to find the similarities and differences in fatty acid composition among the experimental cultures.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank database under accession no. AF043136, AF043137, AF043138, AF043139, and AF043140.

RESULTS

Growth of nitrifying populations under new selective conditions. The effects of ammonium concentration, pH, and oxygen concentration were determined in continuously fed reactors (Table 1). Measurements of ammonium consumption (Table 1) and nitrite and nitrate production during incubation (data not shown) confirmed that nitrifying activity and growth occurred in all reactors. Nitrification, as determined by activity measurements, became established within 3 days after inoculation under all conditions. At the end of the 74-day incubation period the community in the reactor containing 3,000 mg of NH_4^+-N liter^{-1} had the highest biomass concentration (0.259 mg of protein/ml). The low-pH reactor and the reactor containing the lowest oxygen concentration exhibited the slowest nitrification, and their biomasses were 3.5- to 6.2-fold lower than the biomass of the fastest-growing community. Dissolved oxygen measurements confirmed that the oxygen concentrations were maintained near 0.43, 3, or 9 mg liter^{-1} (i.e., 1, 7, or 21% O_2 in the aeration mixtures). Heterotrophic bacteria were present in all of the reactors; the concentrations of heterotrophic bacteria ranged from 1.5×10^7 CFU ml^{-1} in the low-pH reactor to 6.8×10^7 CFU ml^{-1} in the reactor containing 1,000 mg of NH_4^+-N liter^{-1}. Colony morphologies indicated that the different enrichment cultures were dominated by different heterotrophs.

Changes in community structures of nitrifying populations. The ARDRA revealed that the community in the reactor receiving 3,000 mg of NH_4^+-N liter^{-1} was most different from the starter culture and that the communities grown at low and high pH values were somewhat different (Fig. 1). We observed new fragments at approximately 900 and 200 bp in the restriction pattern of the community receiving a high concentration of ammonium, while the two fragments at 530 and 370 bp disappeared. The change in the restriction patterns was evident on day 38 of incubation and was greater on subsequent sampling days. The new faint fragment at 200 bp was also present in the patterns of the communities grown at low and high pH values.

Structural changes in the nitrifying communities under different selective conditions were evaluated by summarizing the ARDRA data by using the similarity index based on the starter culture data (Fig. 2). Restriction digests of the community incubated in the presence of 3,000 mg of NH_4^+-N liter^{-1} revealed that only 9 of 23 fragments matched fragments in the starter culture restriction pattern; i.e., the similarity index was 0.39. However, this ammonium concentration affected the community structure but not the nitrifying activity. Other substrate concentrations ranging from 50 to 1,000 mg of NH_4^+-N liter^{-1} did not induce structural shifts; all 24 fragments matched fragments in the starter culture band pattern. Cultures grown at pH 6.0 and 8.2 had similarity indices of 0.89 and 0.83, respectively. In the pH 8.2 environment the nitrification rate remained the same as the nitrification rate at pH 7.0, but the nitrification rate in the pH 6.0 reactor was very low. In most other nitrifying communities the similarity index was not altered (1.0 to 0.96), and the nitrifying activity was not affected; the only exception was the reactor which had 1% oxygen in the aeration gas mixture, in which the nitrification rate was retarded.

Ability of the communities to regain their structures. The three communities that showed structural changes under selective conditions (3,000 mg of NH_4^+-N liter^{-1}, pH 8.2, and pH 6.0) produced 60 to 70% less (NO_2^- + NO_3^-)-N than the other communities in the first week after they were returned to the standard conditions (data not shown). The amounts of ammonium consumed after 8 days were almost equal in all reactors, ranging from 37 to 75 mg of NH_4^+-N liter^{-1} day^{-1} (Table 1).

The nitrifying community with the greatest structural change resulting from a selective condition (high ammonium concentration) regained its original structure after the selective condition was eliminated (Fig. 2 and 3). Restoration of the original ARDRA pattern was first noticed with restriction enzymes *Hae*III, *Bst*U1, and *Sau*3A after 8 days of incubation and was completed after 15 days, as determined with all five restriction enzymes used (data not shown). In contrast, the communities from reactors in which the selective conditions were pH 6.0 and pH 8.2 did not recover their original structures. Digestion of these two communities under the original conditions with restriction enzymes *Hae*III, *Hin*PI1, and *Bst*U1 resulted in a band pattern which was the same as the band pattern obtained when the cultures were incubated under selective conditions. In addition, digestion with *Sau*3A resulted in a band at the same position as a band produced by the community grown in the presence of the selective high ammonium concentration, indicating that additional changes in the communities occurred. Also, the community grown in the presence of 500 mg of NH_4^+-N liter^{-1} displayed minor additional changes in its ARDRA pattern even when it was grown under the original conditions.

SSU rDNA probe hybridization. The All_Spira probe, which was designed to detect ammonia oxidizers belonging to the *Nitrosospira* group (including the genus *Nitrosolobus*), hybridized with pure-culture DNA of *Nitrosospira* strain NpAV and *Nitrosolobus multiformis* ATCC 5976 and not with the restriction products of the cultures from the 10 experimental treatments. The general probe Ammo_Cl_2/3/4/6, which was designed to hybridize with all known terrestrial ammonia oxidizers belonging to the β-proteobacteria, produced strong

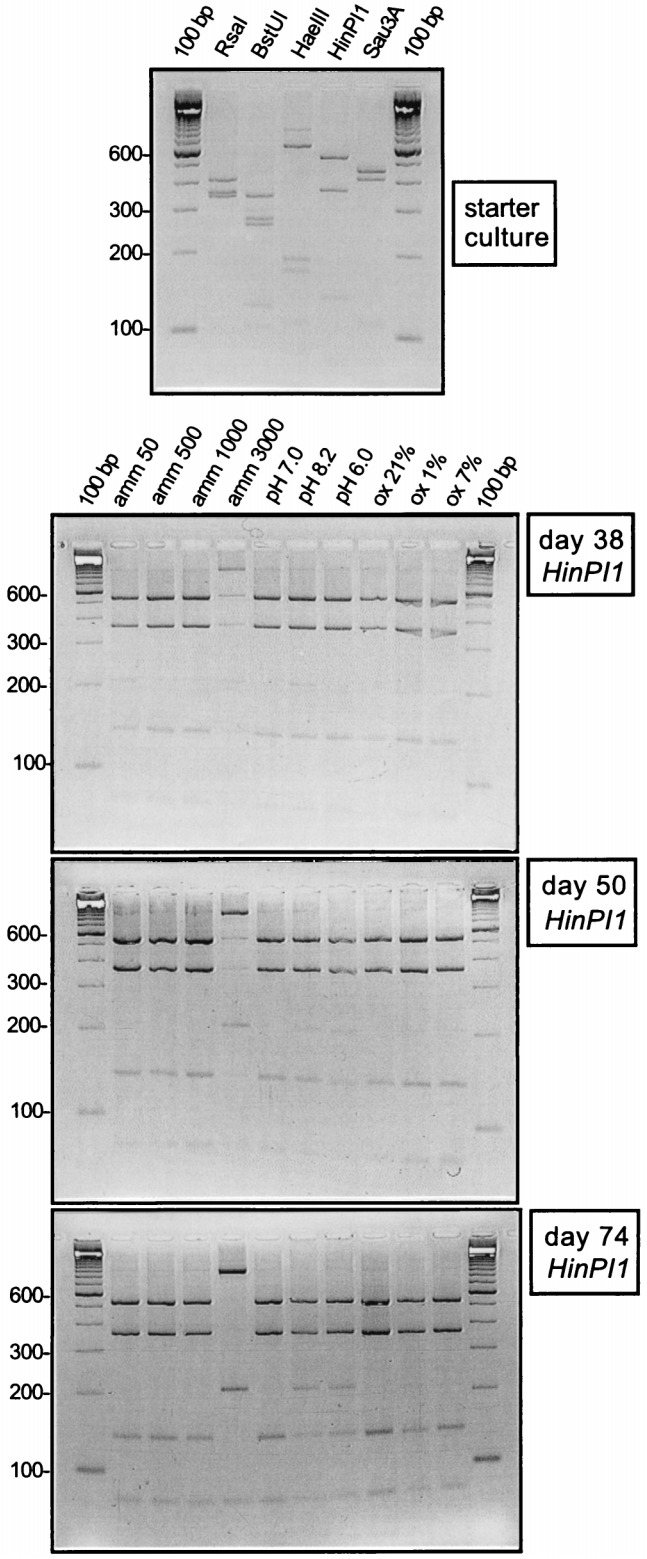

FIG. 1. Agarose gel electrophoresis (3% MetaPhor) of restriction digests of the starter culture and the resulting communities (restriction enzyme *Hin*PI1) after 38, 50, and 74 days of incubation under selective conditions. Lanes 100 bp contained the molecular weight standard. For an explanation of the abbreviations see Table 1.

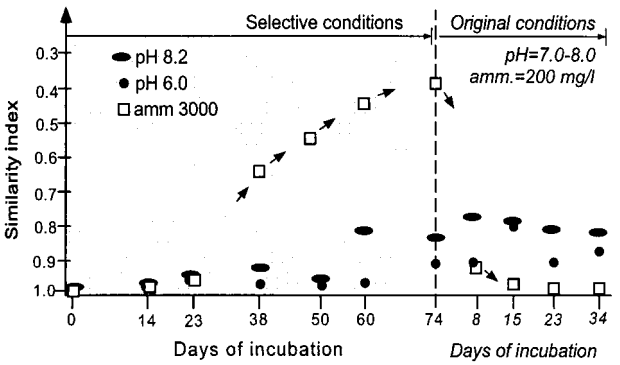

FIG. 2. Similarity indices of nitrifying communities after incubation under selective conditions, as determined by comparison with the starter culture, which had a similarity index of 1.0. The ARDRA similarity index is the ratio of the number of common electrophoretic bands after digestion with five restriction enzymes (*Rsa*I, *Hae*III, *Hin*PI1, *Bst*U1, and *Sau*3A) to the total number of bands.

hybridization signals with the restriction fragments from all of the pure cultures tested and from cultures subjected to all of the experimental treatments except 3,000 mg of NH_4^+-N liter^{-1}. The PCR primers used in this study could have generated amplification products from nonammonia oxidizer DNA, or this community contained one or more ammonia oxidizers that are different from the ammonia oxidizers already known. On the basis of the results of the two hybridization tests (All_Spira and Ammo_Cl_2/3/4/6), we estimated that the predominant members of all of the reactor communities except the community grown in the presence of 3,000 mg of NH_4^+-N liter^{-1} were ammonia oxidizers belonging to the *Nitrosomonas* group.

Cloning and sequencing of cloned SSU rDNA PCR fragments. The restriction patterns of 87 cloned SSU rDNA fragments obtained from the community grown in the presence of 3,000 mg of NH_4^+-N liter^{-1} revealed five different clones of ammonia oxidizers. The predominant pattern, pattern Al-7K, accounted for 74% of the clones. A clone that was representative of each of the five ARDRA patterns was partially sequenced. All of the sequences clustered in the *Nitrosomonas* group (Fig. 4). The sequence that was most dissimilar to the database among these five clones was Al-7K; this sequence was 92.6% similar to the sequence of *Nitrosomonas eutropha* C-91,

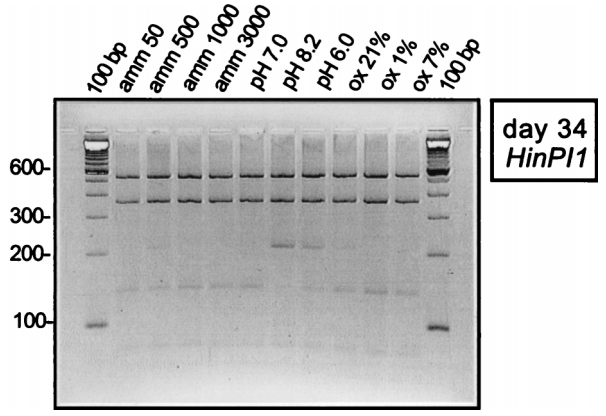

FIG. 3. Agarose gel electrophoresis (3% MetaPhor) of restriction digests of the communities (restriction enzyme *Hin*PI1) after 34 days of incubation under the original conditions. Lanes 100 bp contained the molecular weight standard. For an explanation of the abbreviations see Table 1.

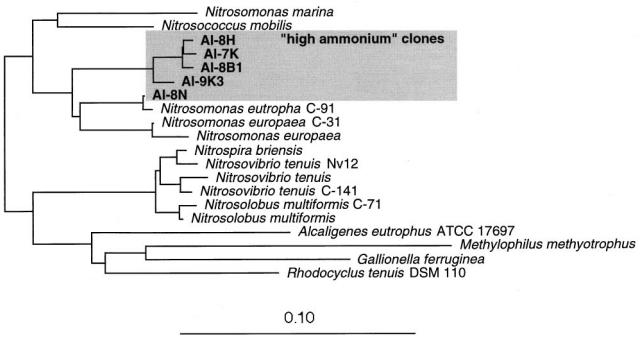

FIG. 4. Phylogenetic tree of the clones from the nitrifying community incubated in the presence of 3,000 mg of NH_4-N liter^{-1} and their nearest relatives. The predominant clone in this community was Al-7K.

the closest relative in the database. Thus, the phylogenetic analysis revealed a previously unknown sequence type produced by a member of the β-proteobacterial ammonia oxidizers that has not been cultured. The neighboring clones, Al-8H and Al-8B1, as well as Al-9K3, differed by only 0.5 to 1.5% from the dominant clone Al-7K. The rDNA sequence of minor clone Al-8N was very similar to the rDNA sequence of *Nitrosomonas eutropha* C-91 (98.5% similarity).

Fatty acid analysis of the nitrifying enrichment cultures. The fatty acid compositions of the starter culture and reactor communities incubated under selective conditions, as well as under standard conditions, varied with respect to the levels of three fatty acids that are commonly found in nitrifiers, 16:0, 16:1ω7c, and 18:1 (data not shown) (3). Some reactor communities grown under the selective conditions contained small amounts of up to nine other fatty acids as well. We used multivariate PCA to expose differences in major fatty acid fractions among the experimental cultures (Fig. 5). This analysis showed that the nitrifying communities of the reactors under selective conditions produced different FAME profiles than the starter culture (the data for the pH 6.0 treatment is not included in the PCA because the sample was lost during extraction). The profiles that diverged the most were obtained for the community grown at a high pH and for the community grown in the presence of 500 mg of NH_4^+-N liter^{-1}. As expected, the community grown at pH 7.0 had a FAME profile similar to the FAME profile of the starter culture. The PCA also showed that all of the reactor communities returned to the starter culture FAME profile after the original growth conditions were restored.

DISCUSSION

Ammonium concentration, oxygen concentration, and pH are thought to be the environmental parameters most important to the nitrification rate and also likely to determine the nitrifier community selected. We found that ammonium at a very high concentration (3,000 mg of NH_4^+-N liter^{-1}) selected a novel nitrifier population and that the pH extremes tested, pH 6.0 and 8.2, selected a somewhat altered community, but the other conditions did not result in community shifts detectable by ARDRA. The community shift caused by a high ammonium concentration occurred gradually, increasing at each sampling time up to 74 days (Fig. 2). Apparently, a nitrifier population better adapted to the high ammonium concentration slowly outgrew the original members of the community. The shift in population composition was not apparent from the nitrification rates since the ammonium consumption rates were rapid and equal before and after the structural change. This shows that the original, probably more conventional nitrifiers were also quite active in the presence of a high ammonium concentration. Nonetheless, they were eventually replaced by a more adapted strain.

The structural changes brought about by the pH extremes took longer to develop. The nitrification rate in the low-pH environment was retarded, but the nitrification rate in the high-pH environment was not retarded. This suggests that in the former environment the shift was delayed by the generally unfavorable conditions even for the newly dominant nitrifiers, while in the latter environment the selective advantage of the newly dominant group must have been minor.

The ammonia oxidizer population selected in the reactor containing the high ammonium concentration seemed to be substantially different from the nitrifiers in the other reactors and from the nitrifiers described previously since the shift was detected with each of the five restriction enzymes used. This difference in the populations was confirmed by the lack of hybridization to the general ammonia oxidizer rDNA probe and by the finding that the rDNA sequence similarity between the dominant operational taxonomic unit (OTU) and all other ammonia oxidizers in the database was only 92.6%. The sequence of this clone and the sequences of clones belonging to three other minor OTUs varied by 0.4 to 1.5%. Together, these clones appear to represent a cluster of organisms specialized for very high ammonium concentrations (Fig. 4). Clone Al-7K of the dominant OTU might be a member of a new species since strains with SSU rRNA evolutionary distances that differ by more than 2.5 to 3.0% have been found to be members of different species (24). The evidence that clone Al-7K is actually a nitrifier rests on the facts that it branches within the family of ammonia oxidizers in the β-proteobacteria and that it was the dominant clone recovered by primers for this family from a highly active nitrifying community. No nonnitrifiers have been found yet in this family. The high ammonium concentration which we used (3,000 mg of NH_4^+-N liter^{-1}), although unusual for natural environments, can be found in animal wastewaters (5). Biological treatment of such waste streams is an important practical problem. Hence, finding nitrifier strains adapted to high ammonium concentrations may have some value in treatment of high-strength ammonium wastes.

The nitrifier populations in all of the other reactors, including the starter culture, appeared to consist of *Nitrosomonas-*

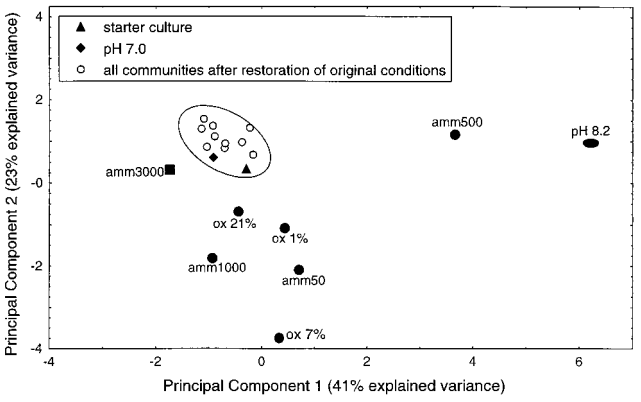

FIG. 5. PCA of FAMEs from the starter culture and from nitrifying communities incubated under selective conditions and after restoration of the standard conditions. The ellipse indicates the 95% confidence limit of selected data points. For an explanation of the abbreviations see Table 1.

like nitrifiers since their rDNA hybridized strongly to the general ammonia oxidizer family probe but not to the *Nitrosospira* family probe. This was expected since *Nitrosomonas* strains are the most common type of ammonia oxidizers found in wastewaters (28, 30). Seven phylogenetic clusters of ammonia oxidizers belonging to the β-proteobacteria are currently recognized (25), and many new ammonia oxidizer sequences from different environments have recently been described (6, 11, 15, 25). These investigations showed that *Nitrosospira* types are the most common ammonia oxidizers in soil and freshwater, not *Nitrosomonas* types, as was previously thought based on culture-based studies.

The evidence of Suwa et al. (26) suggests that there is some correspondence between ammonia oxidizer sensitivity or tolerance to ammonia and phylogeny, a result also noted in this study at least for very high ammonium concentrations (26). So far, there is little evidence for a similar correspondence between ammonia oxidizer type and pH or oxygen status. In a recent study researchers found closely related *Nitrosospira* sequences in both neutral and acid soils, although some sequences might have been more common in one soil type than in the other (25). In contrast, Kowalchuk et al. found different nitrifier sequence types in acid and alkaline Dutch dune sites (15).

An important finding of this study is that the community which exhibited the greatest structural shift (similarity index, 0.39) was able to reacquire its original structure after the selection conditions were eliminated. The time needed for recovery (8 to 15 days) was relatively short. The speed of recovery was probably aided by the fact that we cleaned and reinoculated the reactors when the conditions were changed, which reduced the residual biomass of the community grown in the presence of the high ammonium concentration. Recovery depended on the selection conditions, however, since the communities selected at pH 6.0 and 8.2 did not return to their original structures when the original conditions were reestablished. Restoration of the original community structure is not usually expected in microbial ecology because the high diversity in most habitats usually leads to many community structures with virtually the same functions. In this case, however, we were dealing with a community from wastewater that was already highly selected before the experiment was started. Hence, the probability of restoring the original structure of a simpler community is higher.

In addition to the nucleic acid-based methods used in this study, we also used a biochemical method to analyze the nitrifying communities. The three major fatty acids which we found are common but not unique to nitrifiers; 16:0 and 16:1 are found in nitrite oxidizers, and 18:1 is found in ammonia oxidizers (31). The PCA of these three fatty acids, as well as the eight major fatty acids, showed that the communities in all of the reactors incubated under selective conditions diverged from a starter culture but that all of the communities, including the communities in the reactors containing high ammonium concentrations and the reactors at extreme pH values, returned to their original states (Fig. 5). The FAME analysis, however, encompassed the entire community, including the nitrite oxidizers and heterotrophs. Since the numbers of heterotrophs in all of the communities were similar (10^7 CFU ml^{-1}), the FAME profiles for the treatments which resulted in low biomasses (e.g., 1% oxygen and 50 mg of NH_4^+-N $liter^{-1}$) could have resulted from relatively high proportions of heterotrophs. The FAME analysis appeared to be much more sensitive than ARDRA for revealing the community shifts since the shifts were detected by the former method under all treatment conditions. This is consistent with the finer level of resolution (e.g., species level resolution) of microbial taxa provided by FAME analysis. The fact that ARDRA did not detect recovery in the two reactors incubated at the pH extremes but FAME analysis did could be due either to physiological adaptation to pH by the existing populations or to the fact that the FAME analysis reflected the fatty acids of the entire community, including nitrite oxidizers and heterotrophs, and that these organisms and not the ammonium oxidizers returned to the original composition.

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REFERENCES

1. **Anthonisen, A. C., R. C. Loehr, T. B. S. Prakasam, and E. D. Srinath.** 1976. Inhibition of nitrification by ammonia and nitrous acid. J. Water Pollut. Control Fed. **48:**835–852.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York, N.Y.
3. **Blumer, M., T. Chase, and S. W. Watson.** 1969. Fatty acids in the lipids of marine and terrestrial nitrifying bacteria. J. Bacteriol. **99:**366–370.
4. **Bock, E., H.-P. Koops, and H. Harms.** 1986. Nitrifying bacteria, p. 81–96. *In* H. G. Schlegel and B. Bowien (ed.), Autotrophic bacteria. Springer-Verlag, Berlin, Germany.
5. **Brione, E., G. Martin, and J. Morvan.** 1994. Non-destructive technique for elimination of nutrients from pig manure, p. 33–37. *In* N. J. Horan, P. Lowe, and E. I. Stentiford (ed.), Nutrient removal from wastewaters. Techonomic Publishing Co., Inc., Lancaster, Pa.
6. **Bruns, M. A.** 1996. Nucleic acid analysis of autotrophic ammonia-oxidizing bacteria in soils. Ph.D. thesis. Michigan State University, East Lansing.
7. **De Boer, W., P. J. A. Klein Gunnewiek, S. R. Troelstra, and H. J. Laanbroek.** 1989. Two types of chemolithotrophic nitrification in acid heathland humus. Plant Soil **119:**229–235.
8. **De Soete, G.** 1983. On construction of "optimal" phylogenetic trees. Z. Naturforsch. Sect. C Biosci. **38:**156–158.
9. **Goreau, T. J., W. A. Kaplan, S. C. Wofsy, M. B. McElroy, F. W. Valois, and S. W. Watson.** 1980. Production of NO_2^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen. Appl. Environ. Microbiol. **40:**526–532.
10. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 328–364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
11. **Hiorns, W. D., R. C. Hastings, I. M. Head, A. J. McCarthy, J. R. Saunders, R. W. Pickup, and G. H. Hall.** 1995. Amplification of SSU ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of nitrosospiras in the environment. Microbiology **141:**2793–2800.
12. **Jones, R. D., and M. A. Hood.** 1980. Effects of temperature, pH, salinity, and inorganic nitrogen on the rate of ammonium oxidation by nitrifiers isolated from wetland environments. Microb. Ecol. **6:**339–347.
13. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism, vol. 3. Academic Press, New York, N.Y.
14. **Kenney, D. R., and D. W. Nelson.** 1982. Nitrogen—inorganic forms, p. 643–698. *In* A. L. Page (ed.), Methods of soil analysis, part 2. Chemical and microbiological properties. Agronomy monograph no. 9, 2nd ed. American Society for Agronomy and Soil Science Society of America, Madison, Wis.
15. **Kowalchuk, G. A., J. R. Stephen, W. De Boer, J. I. Prosser, T. M. Embley, and J. W. Woldendorp.** 1997. Analysis of ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl. Environ. Microbiol. **63:**1489–1497.
16. **Lozinov, A. B., and V. A. Ermachenko.** 1959. NH_4^+ oxidation by nitrite bacteria as a function of certain factors of the medium. I. The effect of $(NH_4)_2SO_4$ concentration. Microbiology (Washington, D.C.) **28:**674–679.
17. **Mahne, I., A. Prinčič, and F. Megušar.** 1996. Nitrification/denitrification in nitrogen high-strength liquid wastes. Water Res. **30:**2107–2111.
18. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K.**

Fogel, J. Blandy, and C. R. Woese. 1994. The Ribosomal Database Project. Nucleic Acids Res. **22:**3485–3487.
19. **Massol-Deya, A. A., D. A. Odelson, R. F. Hickey, and J. M. Tiedje.** 1995. Bacterial community fingerprinting of amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA), p. 3.3.2/1–8. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
20. **McCaig, A. E., T. M. Embley, and J. I. Prosser.** 1994. Molecular analysis of enrichment cultures of marine ammonia oxidizers. FEMS Microbiol. Lett. **120:**363–368.
21. **Painter, H. A.** 1986. Nitrification in the treatment of sewage and waste waters, p. 185–211. *In* J. I. Prosser (ed.), Nitrification. Society for General Microbiology, Oxford IRL Press, Washington, D.C.
22. **Prosser, J. I.** 1989. Autotrophic nitrification in bacteria. Adv. Microb. Physiol. **30:**125–181.
23. **Sharma, B., and R. C. Ahlert.** 1977. Nitrification and nitrogen removal. Water Res. **11:**897–925.
24. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and SSU rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
25. **Stephen, J. R., A. E. McCaig, Z. Smith, J. I. Prosser, and T. M. Embley.** 1996. Molecular diversity of soil and marine SSU rRNA gene sequences related to β-subgroup ammonia-oxidizing bacteria. Appl. Environ. Microbiol. **62:**4147–4154.
26. **Suwa, Y., Y. Imamura, T. Suzuki, T. Tashiro, and Y. Urushigawa.** 1994. Ammonium-oxidizing bacteria with different sensitivies to $(NH_4)_2SO_4$ in activated sludges. Water Res. **28:**1523–1532.
27. **Voytek, M. A., and B. B. Ward.** 1995. Detection of ammonium-oxidizing bacteria of the beta subclass of the class *Proteobacteria* in aquatic samples with the PCR. Appl. Environ. Microbiol. **61:**1444–1450.
28. **Wagner, M., G. Rath, R. Amann, H.-P. Koops, and K.-H. Schleifer.** 1995. *In situ* identification of ammonia oxidizing bacteria. Syst. Appl. Microbiol. **18:**251–264.
29. **Walker, N., and K. N. Wickramasinghe.** 1979. Nitrifications and autotrophic nitrifying bacteria in acid tea soils. Soil Biol. Biochem. **11:**231–236.
30. **Watson, S. W., E. Bock, H. Harms, H.-P. Koops, and A. B. Hooper.** 1989. Nitrifying bacteria, p. 1808–1834. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. The Williams and Wilkins Co., Baltimore, Md.
31. **Wilkinson, S. G.** 1988. Gram-negative bacteria, p. 299–461. *In* C. Ratledge and S. G. Wilkinson (ed.), Microbial lipids. Academic Press Ltd., London, United Kingdom.