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Effects of Agronomic Treatments on Structure and Function of Ammonia-Oxidizing Communities

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The aim of this study was to determine the effects of different agricultural treatments and plant communities on the diversity of ammonia oxidizer populations in soil. Denaturing gradient gel electrophoresis (DGGE), coupled with specific oligonucleotide probing, was used to analyze 16S rRNA genes of ammonia oxidizers belonging to the β subgroup of the division *Proteobacteria* by use of DNA extracted from cultivated, successional, and native deciduous forest soils. Community profiles of the different soil types were compared with nitrification rates and most-probable-number (MPN) counts. Despite significant variation in measured nitrification rates among communities, there were no differences in the DGGE banding profiles of DNAs extracted from these soils. DGGE profiles of DNA extracted from samples of MPN incubations, cultivated at a range of ammonia concentrations, showed the presence of bands not amplified from directly extracted DNA. *Nitrosomonas*-like bands were seen in the MPN DNA but were not detected in the DNA extracted directly from soils. These bands were detected in some samples taken from MPN incubations carried out with medium containing 1,000 μg of NH_4^+-N ml^{-1}, to the exclusion of bands detected in the native DNA. Cell concentrations of ammonia oxidizers determined by MPN counts were between 10- and 100-fold lower than those determined by competitive PCR (cPCR). Although no differences were seen in ammonia oxidizer MPN counts from the different soil treatments, cPCR revealed higher numbers in fertilized soils. The use of a combination of traditional and molecular methods to investigate the activities and compositions of ammonia oxidizers in soil demonstrates differences in fine-scale compositions among treatments that may be associated with changes in population size and function.

Autotrophic ammonia-oxidizing bacteria carry out the first and rate-limiting step of nitrification, namely, the oxidation of ammonia to nitrite. Ammonia oxidation often involves the direct evolution of the greenhouse gas N_2O to the atmosphere (7) and indirectly leads to additional losses of N through denitrification of nitrate. The NO_3^- produced during nitrification is also a major cause of water pollution (45). Inhibition of ammonia oxidation occurs in many ecosystems (41), and the ability to achieve inhibition in agricultural systems could result in large financial savings in fertilizer costs while preventing much environmental pollution.

Understanding the effects of agricultural practices on the structure and function of microbial communities, in particular, the ammonia-oxidizing bacteria, may aid in the development of lower-input sustainable systems. Evaluation of the early system effects of management, for example, tillage, N inputs, and crop rotation, on parameters such as total microbial populations, bacterial/fungal ratios, and overall microbial activity is difficult. Naeem et al. (27), using experimental model systems, showed that soil community respiration and plant productivity were higher in more diverse plant communities. Organically based agricultural systems that include multiple crops often have higher yields under stress conditions, such as drought, than do fertilizer-based one- or two-crop systems (32). The functional composition and functional diversity of plant communities have been shown to be the principal factors controlling productivity and plant nitrogen uptake (15, 44). Findings such as these suggest that management practices that affect plant diversity and composition can have a profound effect on ecosystem processes. Assessment of the impact of changes in plant communities on soil community structure is made difficult by the high level of diversity of total bacterial communities in terrestrial environments (11, 24, 28, 29) but may be facilitated by investigation of specific groups of organisms. The oxidation of ammonia to nitrite therefore holds great promise as an indicator process in N cycling studies and in the study of soil microbial diversity relative to ecosystem disturbance.

Autotrophic ammonia oxidizers belong to two phylogenetic groups, one within the γ subdivision of the division *Proteobacteria* (γ-proteobacteria) and one within the β-proteobacteria. Representatives from the former have been isolated only from marine and brackish waters (47), whereas all soil ammonia oxidizers enriched or isolated to date belong to the β-proteobacteria (40, 46). Phylogenetic analysis of 16S rRNA genes amplified from extracted environmental DNA by PCR with primers selective for the β-proteobacterial ammonia oxidizers has indicated the existence of at least seven distinctive clusters, four belonging to the genus *Nitrosospira* and three belonging to the genus *Nitrosomonas* (40). The distribution of clone sequences among these clusters is related to the environments from which they were obtained (25, 34, 39, 40).

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TABLE 1. Management histories and fertilization applications for the soils from the LTER plots at the KBS[a]

Treatment	Vegetation	Nitrogen application	Variants
1	Corn, soybean, and wheat rotation	Ammonium nitrate at 124 kg of N ha^{-1} for corn and 84 kg of N ha^{-1} for wheat	Fertilized vs nonfertilized
2 (no tilling)	Corn, soybean, and wheat rotation	Ammonium nitrate at 124 kg of N ha^{-1} for corn and 84 kg of N ha^{-1} for wheat	Fertilized vs nonfertilized
5	Perennial *Populus* trees	None	None
7			
Fertilized	Successional since 1989	120 kg ha^{-1} annually	Tilled vs not tilled
Nonfertilized	Successional since 1989	None	Tilled vs not tilled
NDF	Native deciduous forest	None	None

[a] Treatments 1 and 2 reflect highly cultivated agricultural soils, while treatment 7 demonstrates a change in plant community to successional grassland following 40 years of intensive cultivation. Variants were drawn from microplots within the larger treatments which were left unfertilized (treatments 1 and 2) or were not tilled (treatment 7). Treatment 5 has a perennial cover crop of *Populus* trees. Additional information on LTER plots is available at http://www.lter.kbs.msu.edu.

Studies using molecular tools to characterize ammonia oxidizer communities in soils at the Long Term Ecological Research (LTER) experiment at the W. K. Kellogg Biological Station (KBS), Michigan State University, have demonstrated a reduced diversity of ammonia-oxidizing bacteria in cultivated soils. Cluster 3 *Nitrosospira* sp. 16S ribosomal DNA (rDNA) sequences were found in cultivated soils but not in noncultivated soils from the same area (5). Analysis of above-ground plant diversity in successional treatments demonstrated the replacement of initially dominant annual species by biennials and herbaceous perennials within 4 years (16). Although nitrogen addition significantly increased above-ground plant biomass, it had no significant effect on plant species diversity. Annual tillage of the nonseeded land produced low-diversity annual grassland. These results raise the question of which parameters, fertilization, tillage, or plant community, drive plant and microbial diversity shifts within these soils.

The objective of this study was to assess the relationship between the diversity of ammonia oxidizer populations and to assess differences in plant productivity and diversity brought about by different fertilizer N and tillage regimens. The LTER plots in southwestern Michigan enabled simultaneous measurement of the effects of tillage, fertilizer, and plant type on potential nitrification, nitrifier numbers, and diversity of ammonia oxidizers in cultivated and noncultivated soils.

MATERIALS AND METHODS

Soil sampling. Soil samples were collected in October 1996 and March 1997 from the LTER experiment at the KBS in southwestern Michigan. The site was established in 1988 from a field that had been under cultivation for over 100 years. Replicate plots of 0.9 hectare had six replicate plots of seven management treatments (http://www.lter.kbs.msu.edu). Molecular characterization of ammonia oxidizer communities was carried out with samples collected from cultivated and successional treatment plots. In addition, soil was investigated from a nearby native deciduous forest (NDF).

Cultivated plots (treatments 1 and 2) had been under corn-soybean rotation from 1989 to 1994, with wheat introduced as a rotation crop in 1995. At the time of sampling (October 1996), the crop was corn. Treatment 1 involved conventional tilling (annual mouldboard ploughing, disking, and cultivation), treatment with herbicides and insecticides, and fertilization with ammonium nitrate (124 kg of N ha^{-1} for corn and 84 kg of N ha^{-1} for wheat). Treatment 2 was like treatment 1, but a no-till practice was in place. Two perennial treatments were also sampled. Treatment 5 was a long-term perennial crop of *Populus* trees established in 1989. Successional grasslands (treatment 7) had been left to revert to native flora following establishment of the LTER plots (16). Within these treatments were microplots (5 by 5 m), established in 1989, amended or not amended with fertilizer. In addition, within treatment 7 there were microplots that had either tillage or no tillage (Table 1). This design enabled investigation of the effects of both tillage and fertilization in agricultural and successional treatments.

Ten composite samples (5-cm depth) were taken from the microplots of each of three replicate plots of each treatment. For the plots colonized by corn, samples were taken from between the rows of corn plants. Composite samples were pooled and sieved through a 1-cm sieve to remove large stones, twigs, and plant material, and subsamples were taken for moisture determination. Soil for DNA extraction was divided into aliquots and stored at −20°C, and the remaining soil was stored at 4°C for further analysis. Sterilized soil for competitive PCR (cPCR) calibrations was prepared by autoclaving three times at 121°C for 15 min each time.

Potential nitrification. Potential nitrification was determined by incubation at 25°C of 10 g of soil in a 250-ml Erlenmeyer flask containing 200 ml of phosphate buffer (1 mM; pH 7.2) and 1.5 mM $(NH_4)_2SO_4$ (13). After 0, 2, 4, 12, 22, and 24 h, soil particles were removed from 10-ml samples by centrifugation in a Sorvall CE25 centrifuge at 6,000 rpm for 10 min. The supernatant was decanted into glass scintillation vials and stored frozen at −20°C for nutrient analysis. Nitrate and ammonia concentrations were determined with a Lachet automated nutrient analyzer. Nitrification rates were determined from the linear regression of nitrite and nitrate concentrations versus time.

MPN counts. Most-probable-number (MPN) counts of ammonia oxidizers were determined with microtiter plates (36) using twofold dilution series and modified Skinner-Walker (38) growth medium (35) containing 5, 50, or 1,000 μg of NH_4^+-N ml^{-1}, giving a wide range of substrate concentrations to enumerate ammonia oxidizers with different substrate requirements. The microtiter plates were placed on top of a pad of water-saturated tissue, wrapped in plastic wrap to avoid evaporation, and incubated for 4 weeks in the dark at room temperature. Growth was assessed by color change from pink to yellow due to acid production. Ammonia oxidation was confirmed by measurement of nitrate and nitrite concentrations by the addition of diphenylamine reagent (0.2 g in 100 ml of concentrated sulfuric acid). MPN values were calculated using the tables of Rowe et al. (36). After MPN values were calculated, the contents of wells from the column with the highest dilution that showed growth in all eight replicates were harvested. Cells and soil in each sample were harvested by centrifugation in a microcentrifuge at 14,000 × *g* for 10 min. The supernatant was discarded, and the pellet was frozen at −20°C for DNA extraction and PCR amplification.

Direct microscopic counts. Soil bacteria were stained with 5(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF) (Sigma Chemical Co., St. Louis, Mo.) (2 mg in 10 ml of phosphate-buffered saline, consisting of 0.05 M Na_2HPO_4 in 0.85% [wt/vol] NaCl [pH 9.0]) for 30 min (31). Random images, obtained by epifluorescence microscopy with a charge-coupled device camera (Princeton Instruments, Trenton, N.J.), were transferred to a Power Macintosh 7100/66 computer for analysis with Adobe PhotoShop.

DNA extraction. DNA was extracted from 5 g of soil (48), and humic contaminants were removed by gel electrophoresis in a 1% (wt/vol) low-melting-point agarose gel (Gibco BRL, Gaithersburg, Md.) (40 V for 4 h). The DNA was excised from the gel, and the agarose was digested by β-agarose treatment (Boehringer Mannheim Corp., Indianapolis, Ind.) and concentrated through a Microcon 100 column (Amicon Inc., Beverly, Mass.). DNA quantity and purity were determined by measuring the absorbances at 260 and 280 nm on a model 8452A spectrophotometer (Hewlett Packard Co., Sunnyvale, Calif.).

PCR amplification. PCR was carried out in a total volume of 50 μl in 0.3-ml Eppendorf tubes on a GeneAmp PCR system 9600 (Perkin-Elmer, Foster City, Calif.). Reactions were carried out in a solution containing PCR buffer (Perkin-Elmer), 25 mM each deoxynucleoside triphosphate, 20 pmol of each primer, 1.5 mM $MgCl_2$, 400 ng of bovine serum albumin (20), and 1 U of *Taq* DNA polymerase (Perkin-Elmer). A nested PCR was carried out for all samples. Initial amplification was carried out using primers selective for β-proteobacterial ammonia oxidizers (AMO primers), βAMOf and βAMOr (26). Secondary amplification was carried out using ammonia oxidizer-specific CTO primers (19), which amplify a 426-bp fragment, including a 30-bp GC-rich domain, for denaturing gradient gel electrophoresis (DGGE) analysis. Conditions for each round of PCR with both AMO and CTO primers were initial denaturation at 95°C for 5 min; 94°C for 40 s, 55°C for 30 s, and 72°C for 2 min for 30 cycles; and 72°C for 5 min. PCR products were resolved by electrophoresis of 5 μl of the reaction mixture in a 1 or 1.5% (wt/vol) agarose minigel in Tris-acetate-EDTA (TAE) buffer for AMO or CTO primers, respectively.

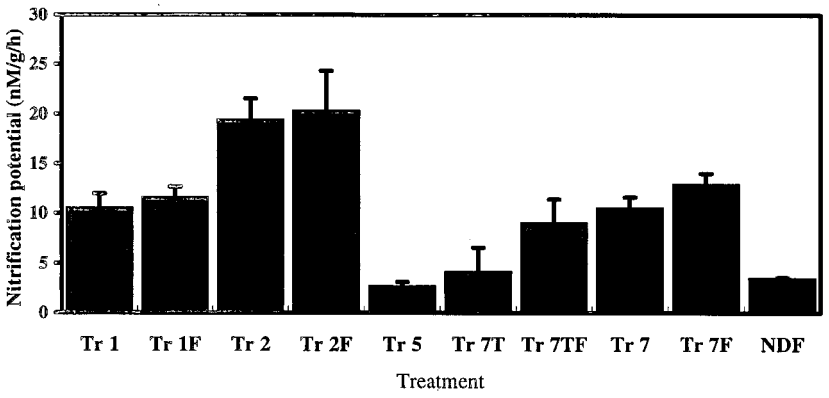

FIG. 1. Potential nitrification rates determined for LTER soils. Treatment (Tr) 1 is conventional tilling, treatment 2 is no tilling, treatment 5 has a *Populus* perennial cover crop, and treatment 7 was historically tilled (now in 7-year successional grassland). Error bars represent the standard error for six replicate samples of each treatment (for the NDF samples, $n = 3$). Suffixes T and F indicate tillage and fertilization, respectively, such that 7TF represents treatment 7, successional grassland, tilled and fertilized.

DGGE. Following the nested PCR, CTO products were resolved on double-density DGGE gels (8) using a D-gene system (Bio-Rad Laboratories, Hertfordshire, United Kingdom). Polyacrylamide gels (6 to 12% polyacrylamide; 1.5 mm thick; TAE; 37:1 acrylamide-bisacrylamide; 35 to 50% denaturant; 20 by 20 cm) were poured using a gradient maker (Bio-Rad). A 5-ml stacking gel (8% acrylamide, 0% denaturant) was added to the top of the denaturing gel, and a 25-well comb was inserted, allowing between 5 and 15 μl of PCR product to be loaded onto each gel. Control clusters from the database of Stephen et al. (40) were included. The gels were run for 5.5 h at 200 V and 65°C. Migration patterns were visualized by staining with 1 mg of ethidium bromide ml^{-1} in TAE for 15 min followed by rinsing for 10 min in TAE or by silver staining.

Electroblotting of DGGE gels. Ethidium bromide-stained gels were electroblotted onto Hybond N^+ nylon membranes (Amersham International plc, Little Chalfont, Buckinghamshire, United Kingdom) using an electroblotter (HEP-1; Owl Scientific, Woburn, Mass.). Gels were trimmed to size and electroblotted for 1.5 h at 200 mA with TAE buffer according to the membrane manufacturer's instructions. Efficiency of transfer was checked by restaining the gels with ethidium bromide. Membranes were stored dry at 4°C prior to oligonucleotide probing.

Oligonucleotide probing. Membranes were probed with a selection of the ammonia oxidizer-specific probes of Stephen et al. (39). Probes β-AO233, Nsp436, and Nmo254, which recognize all ammonia oxidizer, all *Nitrosospira*, and all *Nitrosomonas* sequences, respectively, were used in conjunction with the cluster-specific probes (NspCL2_458, NspCL3_454, and NspCL4_446) for *Nitrosospira* clusters 2, 3, and 4, respectively (39). Each probe (20 pmol) was end labeled using T4 polynucleotide kinase (Promega) and 20 μCi of [γ-^{32}P]ATP (3,000 Ci $mmol^{-1}$; Amersham) in a 10-μl final volume.

Prehybridization of the membranes in Quickhyb solution (Stratagene Inc., Cambridge, United Kingdom) was carried out at 42°C for 30 min prior to the addition of the radiolabeled probe. Hybridization was carried out for 4 h or overnight at the hybridization temperature (39) in a Hybaid hybridization oven. Unbound probe was removed by washing with 2× SSC (1× SSC is 0.015 M sodium citrate plus 0.15 M NaCl)–0.1% sodium dodecyl sulfate (SDS) (Sigma, Dorset, United Kingdom) for 10 min at room temperature, followed by 0.1× SSC–0.1% SDS at 42°C for 30 min. Membranes were exposed to X-ray film overnight. Before being reprobed, membranes were stripped by two washes in a large volume of boiling 0.1× SSC–0.1% SDS. The membranes were checked for the complete removal of bound probe by ensuring that radioactive counts had returned to background levels. The membranes were rinsed in distilled water, air dried, and stored at 4°C until reprobed.

DGGE band sequencing. The middle portion of each selected DGGE band was excised for sequence analysis and placed in a 500-μl Eppendorf tube. The acrylamide was crushed using a sterile pipette tip, 10 μl of sterile MilliQ water was added to each tube, and the sample was incubated at 4°C overnight. Acrylamide was removed by centrifugation at 13,000 × *g* for 5 min, and PCR was carried out using the CTO primers as described previously. Products were cleaned and concentrated with Microcon 100 filter units (Amicon Inc., Bedford, Mass.) by rinsing several times with sterile MilliQ water. Products were quantified and checked for purity on 1% (wt/vol) agarose gels prepared in TAE buffer using a mass ladder (Life Technologies, Paisley, United Kingdom). Sequence analysis was carried out on both strands using the CTO forward primer (without the GC clamp) and the 537r internal 16S rDNA sequencing primer (10) with an automated sequencer. Sequence data were assembled and checked by using the Chromas 1.42 program (C. McCarthy, Griffith University, Brisbane, Queensland, Australia) before analysis using the Genetic Database Environment running in ARB. Phylogenetic analysis was carried out by aligning the partial 16S rDNA sequences from clones and the sequences of ammonia oxidizers and other β-proteobacteria contained in the ribosomal database project (22). Trees were generated from a 276-bp region of the 5′ region of the 16S rDNA using the Jukes-Cantor (18) correction and neighbor joining (37) with PHYLIP version 3.1 software (12) in ARB.

cPCR. cPCR was carried out on all of the soils using the COMP1 internal standard and conditions reported by Phillips et al. (33). The calibration curve was prepared by the addition of 10^7 *Nitrosomonas europaea* cells to 5 g of gamma-irradiated soil, and the DNA was extracted as described above. The calibration series was prepared from a 10-fold dilution series of DNA amplified with 7 pg of COMP1. Products were run on 2% (wt/vol) agarose gels in TAE buffer at 40 mV for 45 min. The gels then were stained in 1 mg of ethidium bromide solution ml^{-1} for 30 min at room temperature and destained in TAE for 10 min. UV gel images were captured by using the Imager System Amphigene, Illkirch, France) and were quantified by densitometry using Molecular Analyst software (Bio-Rad).

Nucleotide sequence accession numbers. All sequences were deposited in GenBank under accession numbers AF157707 to AF157741.

RESULTS

Potential nitrification. Potential nitrification rates were determined in October 1996 for all plots in treatments 1, 2, and 7. There was a marked tillage effect in cultivated plots, with potential nitrification values in treatment 2 (no tilling) almost twice those in treatment 1 ($P = 0.03$) (Fig. 1). However, in successional grasslands (treatment 7), there was no significant difference between tilled and nontilled plots. Fertilization did not affect the potential nitrification activities of cultivated soils ($P = 0.73$) or successional grasslands ($P = 0.15$) (Fig. 1). Samples from poplar plots (treatment 5) and NDF were taken in November 1996. Potential activities in these sites were lower than those in cultivated and successional soils; the lowest rate, 2.5 nM g of $soil^{-1}$ h^{-1}, was found in the poplar plots.

Ammonia oxidizer population size. Cell concentrations of ammonia oxidizers from these different communities were estimated by two methods, conventional MPN counts and cPCR. MPN analysis was carried out at three ammonia concentrations to allow quantification of groups of ammonia oxidizers with different sensitivities to ammonia. In cultivated soils (treatments 1 and 2), MPN counts determined with medium containing the highest ammonium concentration, 1,000 μg of NH_4^+-N ml^{-1}, were between 1 and 2 orders of magnitude lower than those determined with 5 μg of NH_4^+-N ml^{-1}, while counts determined with 50 μg of NH_4^+-N ml^{-1} were intermediate (Fig. 2). MPN counts from successional grasslands (treatment 7) showed similar patterns, with the lowest numbers from counts determined with medium containing the highest ammo-

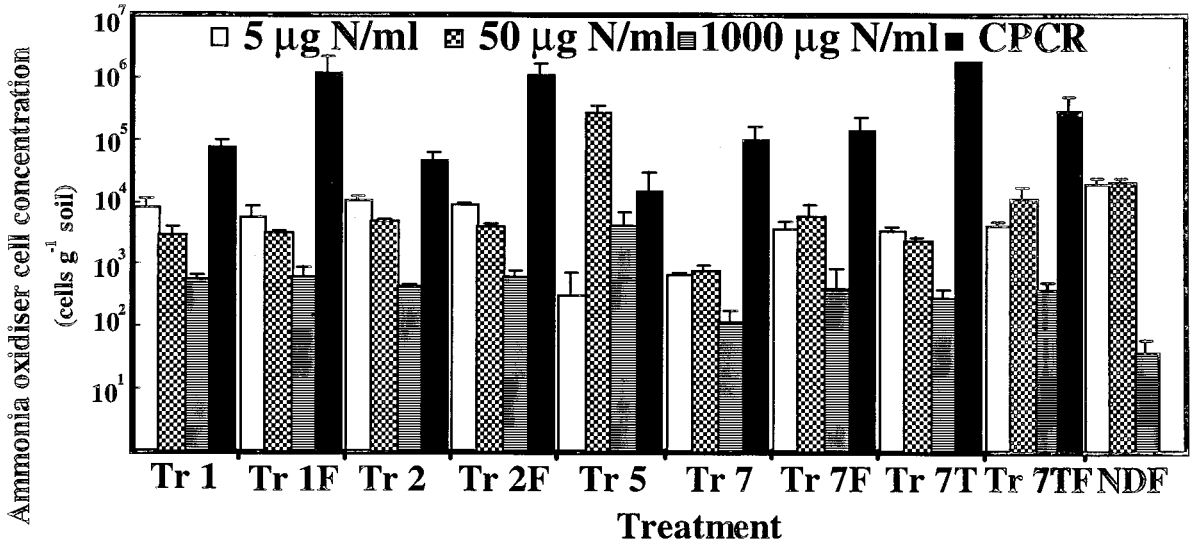

FIG. 2. Ammonia oxidizer cell concentrations in LTER soils, as determined by the MPN method with medium containing 5, 50, or 1,000 μg of NH_4^+-N ml^{-1} and by cPCR. Counts for treatments 1, 2, and 7 and for NDF were determined with samples collected in October 1996, and those for treatment 5 were determined with samples collected in March 1997. See the legend to Fig. 1 for explanations of designations.

nium concentration, although the differences between counts determined with 5 and 50 μg of NH_4^+-N ml^{-1} were less marked. The effect of ammonium concentration was greatest in NDF, which showed counts of 4 cells g of $soil^{-1}$ when the highest ammonium concentration was used and 21×10^4 and 20×10^4 cells g of $soil^{-1}$ when intermediate and low concentrations were used, respectively. Both tillage and fertilization increased MPN counts in successional soils ($P = 0.0175$ and $P = 0.062$, respectively). In contrast to the results obtained for the other experimental plots, the lowest counts were obtained from medium containing 5 μg of NH_4^+-N ml^{-1} for the poplar plots (treatment 5). There were no significant differences in MPN counts from cultivated and native soils at an intermediate ammonia concentration ($P = 0.431$), but counts obtained with the highest and lowest concentrations indicated higher numbers in cultivated soils ($P = 0.065$ and $P = 0.0005$, respectively).

Ammonia oxidizer cell concentrations were determined by cPCR by comparing the ratio of an internal standard (COMP1) to template DNA in PCR products, obtained from each of the LTER soils, with a calibration curve prepared from DNA extracted from sterile soil following the addition of known concentrations of *N. europaea*. An earlier study (33) demonstrated a linear relationship in this soil for concentrations in the range of 10^2 to 10^7 cells g of $soil^{-1}$. The numbers of ammonia oxidizers could not be determined for NDF soils, as nested PCR was required for the amplification of ammonia oxidizer DNA, probably due to the higher organic content in these soils. In all other situations, except for treatment 5, cPCR counts were significantly higher than MPN counts with medium containing 5 μg of NH_4^+-N ml^{-1}. Differences ranged from 0.3 to 2.5 orders of magnitude. For treatment 5, a single MPN count, determined with an intermediate ammonium concentration, was higher than the equivalent cPCR count. Both MPN and cPCR counts increased with tillage and fertilization in successional soils. In cultivated soils, cPCR counts, in contrast to MPN counts, increased significantly with fertilization but were not affected by tillage. The numbers of ammonia oxidizers determined by cPCR ranged from 1.5×10^4 to 1.1×10^7 cells g of $soil^{-1}$; in comparison, total bacterial cell numbers ranged from 5.8×10^9 to 8.5×10^9 cells g of $soil^{-1}$. Thus, ammonia oxidizers likely constitute a maximum of approximately 0.01% the total bacterial population in these soils.

DGGE analysis and oligonucleotide probing. The composition of ammonia oxidizer communities from the sites tested here was characterized by DGGE analysis of ammonia oxidizer 16S rDNA partial sequences amplified from extracted DNA using a nested PCR approach. Banding patterns of ethidium bromide-stained DGGE gels were similar for all plots, regardless of plant community or fertilization or tillage treatments (Fig. 3). Banding patterns were reproducible when gels were run on several occasions and from different sets of PCRs. The DGGE patterns from all sites included a group of slowly migrating bands (band C) that appeared to comigrate with the cluster 2 and cluster 3 *Nitrosospira* controls and a group of

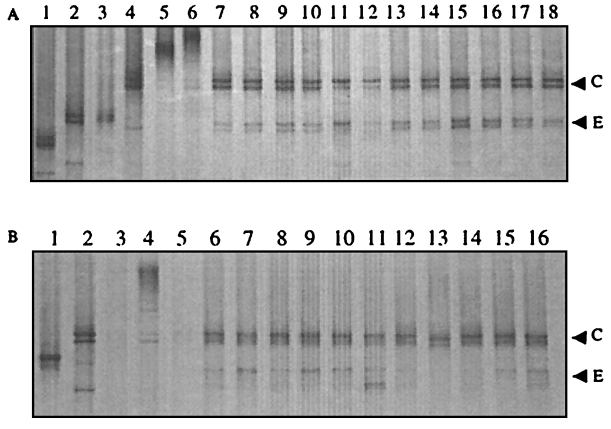

FIG. 3. Silver-stained DGGE analysis of PCR products of β-subgroup ammonia-oxidizing bacteria from LTER soils. (A) Cultivated and successional soils for the fertilization effect. Lanes 1 to 6, control clones pH7B_8 (*Nitrosospira* cluster 3), pH7C_24 (*Nitrosospira* cluster 2), pH7B_C3 (*Nitrosospira* cluster 4), pH4.2A_D2 (*Nitrosospira* cluster 3), pH7C_37 (*Nitrosomonas* cluster 6), and EnvC1-19 (*Nitrosomonas* cluster 6), respectively; lanes 7 to 9, treatment 1F; lanes 10 to 12, treatment 2F; lanes 13 to 15, treatment 7F; lanes 16 to 18, treatment 7. (B) Cultivated and successional soils for the tillage effect. Control clones were pH7B_C3 (*Nitrosospira* cluster 4), pH4.2A_D2 (*Nitrosospira* cluster 2), pH7C_53 (*Nitrosospira* cluster 3), and EnvC1-19 (*Nitrosomonas* cluster 6) (lanes 1 to 4, respectively). Other lanes are as in panel A. See the text for explanations of bands C and E. See the legend to Fig. 1 for explanations of designations.

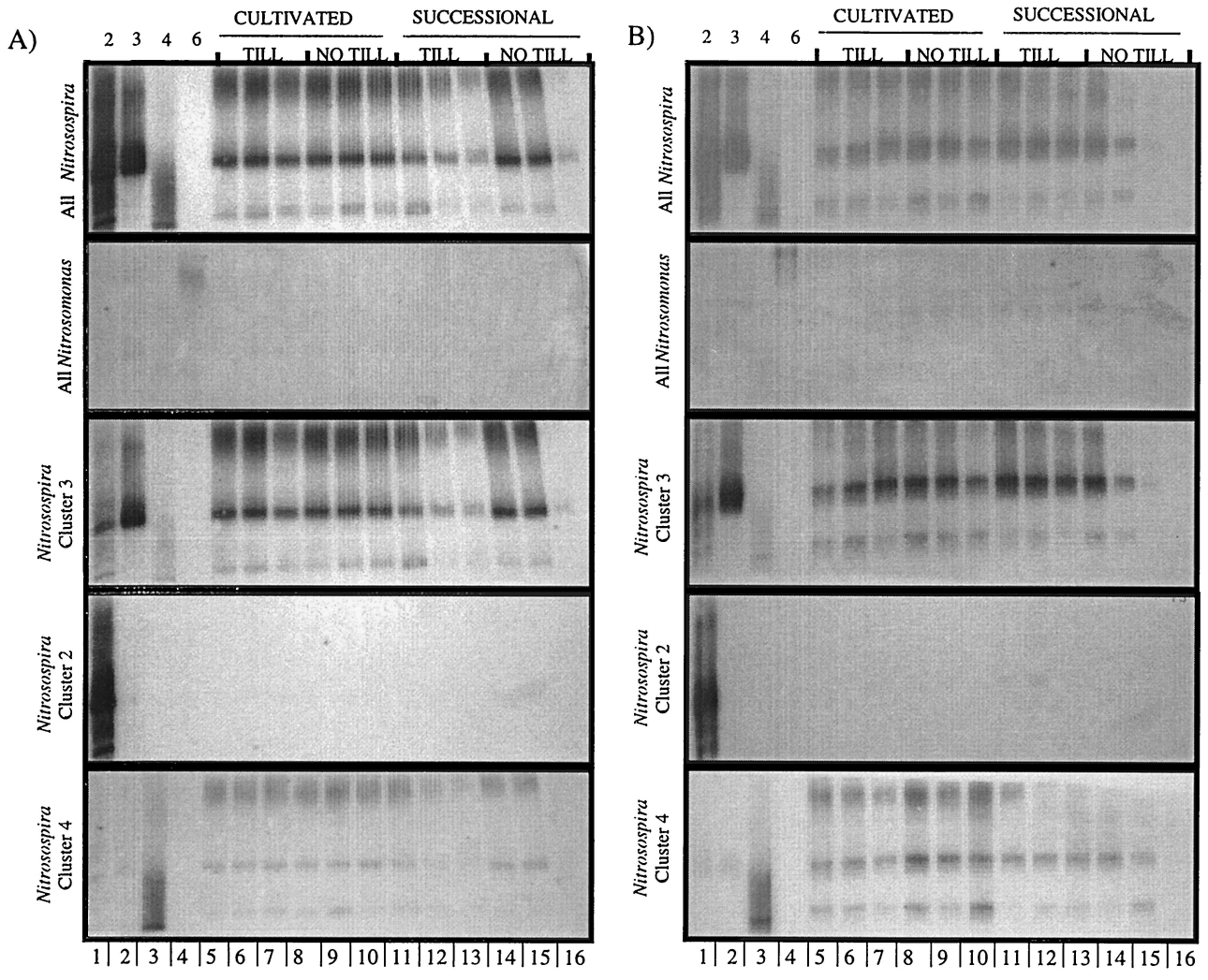

FIG. 4. DGGE analysis and Southern oligonucleotide hybridization following PCR amplification of 16S rDNA of β-subgroup ammonia-oxidizing bacteria from LTER soils. (A) Cultivated and successional soils for the tillage effect. Lanes 1 to 4, controls for *Nitrosospira* cluster 2 (pH4.2A/27), cluster 3 (pH4.2A/4) and cluster 4 (pH7B/C3) and *Nitrosomonas* cluster 6 (EnvC1-19), respectively; lanes 5 to 7, treatment 1; lanes 8 to 10, treatment 2; lanes 11 to 13, treatment 7T; lanes 14 to 16, treatment 7. (B) Cultivated and successional soils for the fertilization effect. Lanes 5 to 7, treatment 1F; lanes 8 to 10, treatment 2F; lanes 11 to 13, treatment 7F; lanes 14 to 16, treatment 7TF. Controls were as in panel A. Oligonucleotide hybridizations were done with probe Nsp436 (all nitrosospiras), probe Nmo254a (all nitrosomonads), probe NspCl2_458 (cluster 2 *Nitrosospira*), probe NspCl3_454 (cluster 3 *Nitrosospira*), and probe NspCl4_446 (cluster 4 *Nitrosospira*). See the legend to Fig. 1 for explanations of designations.

faster migrating bands (band E) that comigrated with the controls for cluster 4 *Nitrosospira*. Between two and three bands appeared in each group, due to small variations in the denaturing gradients. DGGE banding patterns of PCR products from these soils did not include any indication of representatives of *Nitrosomonas*.

Confirmation of the identity of banding patterns may be achieved by probing with genus- and cluster-specific probes and is necessary for the differentiation of clusters 2 and 3, due to their similar migration characteristics. Figure 4 shows DGGE gels prepared from cultivated and successional soils hybridized with probes specific for all nitrosospiras, for all nitrosomonads, and for clusters 2, 3, and 4. Sequences representative of cluster 2 *Nitrosospira* and of *Nitrosomonas* were either absent from these soils or below the limit of detection. Both cluster 3 and cluster 4 *Nitrosospira* probes showed nonspecific hybridization, as indicated by the faint hybridization with control clusters as well as with the slow- and fast-migrating bands of the soil samples. DGGE analysis did not, therefore, conclusively distinguish between the presence and the absence of cluster 3 and cluster 4. Evidence that all bands belonged to cluster 3 *Nitrosospira* was obtained by comparing the ratios of the intensity of each band when hybridized with either the cluster 3 or the cluster 4 probe to that of its respective control with the *Nitrosospira* probe. This analysis demonstrated that the cluster 3 probe hybridized to a greater extent to all the bands, suggesting that the sequences were cluster 3 and not cluster 4, despite the slower bands migrating in a manner similar to that of the cluster 4 control. This result was further confirmed by sequence analysis.

DGGE analysis was also carried out on β-proteobacterial ammonia oxidizer 16S rDNA partial sequences amplified from DNA extracted from the highest dilutions showing positive results for MPN counts. PCR amplification and DGGE analysis were not successful for all MPN samples obtained. The inability to obtain PCR products was not related to soil treat-

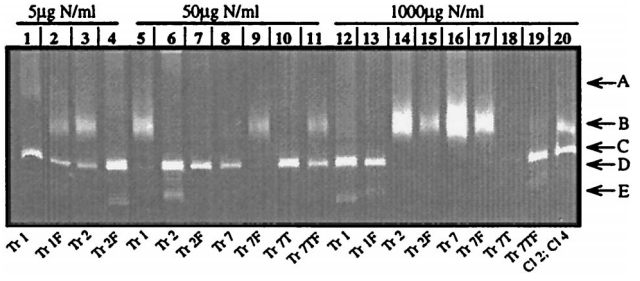

FIG. 5. DGGE analysis following PCR amplification of 16S rDNA of β-subgroup ammonia-oxidizing bacteria from the MPN dilution tubes of LTER soils incubated with 5, 50, and 1,000 μg of NH_4^+-N ml^{-1}. DNA was extracted from the tube with the highest dilution that showed growth in all eight replicates. Lanes 1 to 4, treatments 1, 1F, 2, and 2F, respectively, with 5 μg of NH_4^+-N ml^{-1}; lanes 5 to 11, treatments 1, 2, 2F, 7, 7F, 7T, and 7TF, respectively, with 50 μg of NH_4^+-N ml^{-1}; lanes 12 to 19, treatments 1, 1F, 2, 2F, 7, 7F, 7T, and 7TF, respectively, with 1,000 μg of NH_4^+-N ml^{-1}; lane 20, control for clusters (Cl) 2 (pH4.2A/27) and 4 (pH7B/C3). Band migration distances are noted as A to E and refer to the bands excised for sequence analysis in Fig. 6 and 7. See the legend to Fig. 1 for explanations of designations.

ments and might have resulted from difficulties in removing sufficient material from the wells of microtiter plates, particularly where evaporation was significant. Although no sequence representative of the *Nitrosomonas* clade was detected in the DNA extracted directly from soil samples, banding patterns typical of *Nitrosomonas* were detected in DGGE gels of DNA amplified from the MPN samples after incubation for 1 month. Representative DGGE banding profiles from MPN samples of cultivated and successional soils with 5, 50, and 1,000 μg of NH_4^+-N ml^{-1} are illustrated in Fig. 5. In many samples, banding patterns were similar to those obtained from DNA extracted directly from the soil, but in several samples, a band typical of *Nitrosomonas* was observed (for example, Fig. 5, lanes 2, 3, 5, 9, and 11). This result was particularly evident for samples from cultures obtained with medium containing 1,000 μg of NH_4^+-N ml^{-1}, where a *Nitrosomonas* band frequently appeared to the exclusion of the *Nitrosospira* bands (Fig. 5, lanes 14, 15, 16, and 17).

Sequence analysis. The presence in soil and MPN cultures of particular clusters of β-proteobacterial ammonia-oxidizing bacteria was confirmed by sequencing of bands excised randomly from DGGE gels. Phylogenetic analysis (Fig. 6 and 7)

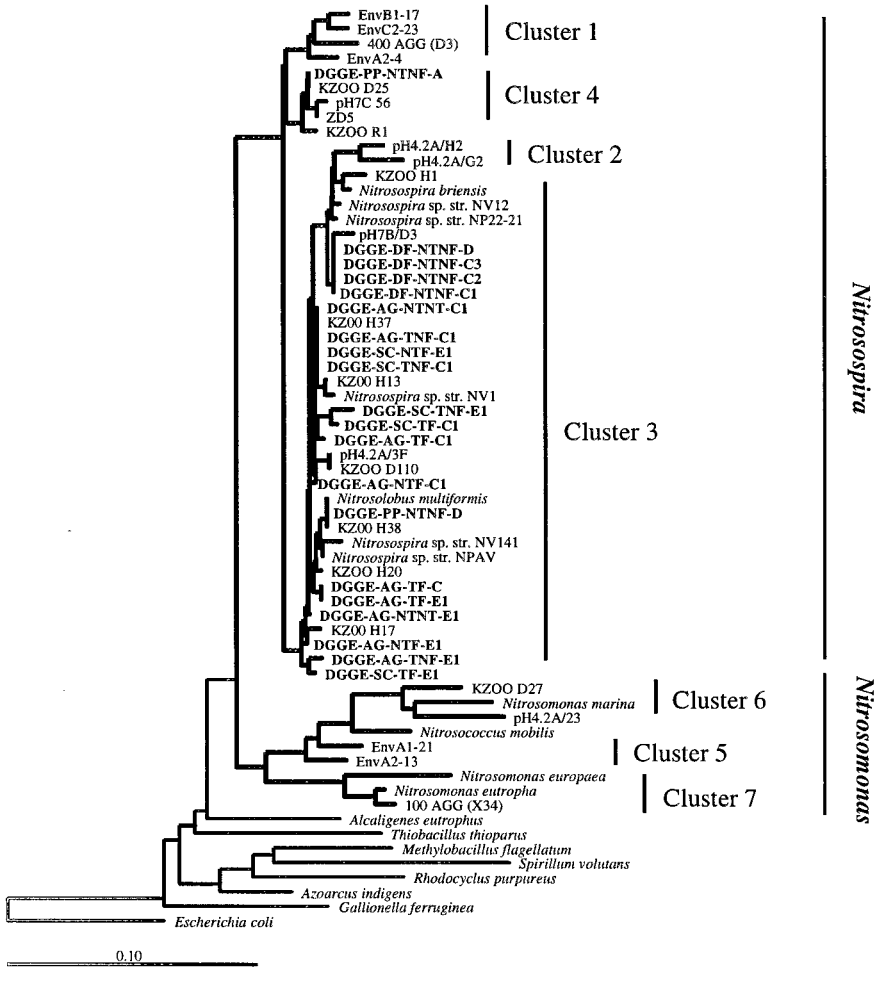

FIG. 6. Neighbor-joining tree showing the relationship of the sequences obtained from bands excised from DGGE gels after PCR amplification of β-subgroup ammonia-oxidizing bacteria from DNA extracted directly from LTER soils. The tree was based on an analysis of 294 bases of aligned 16S rDNA sequences. Bands excised from the gels (shown in bold) have the nomenclature DGGE, to distinguish them from clone sequences and pure-culture sequences, followed by the treatments AG (cultivated), SC (successional grassland), DF (deciduous forest), and PP (*Populus* trees). The treatment variables—tillage (T), no tillage (NT), fertilization (F), and no fertilization (NF)—are followed by the migration distances of the bands (A to E) (see the legends to Fig. 3 and 5). Scale bar, 0.1 substitution per nucleotide.

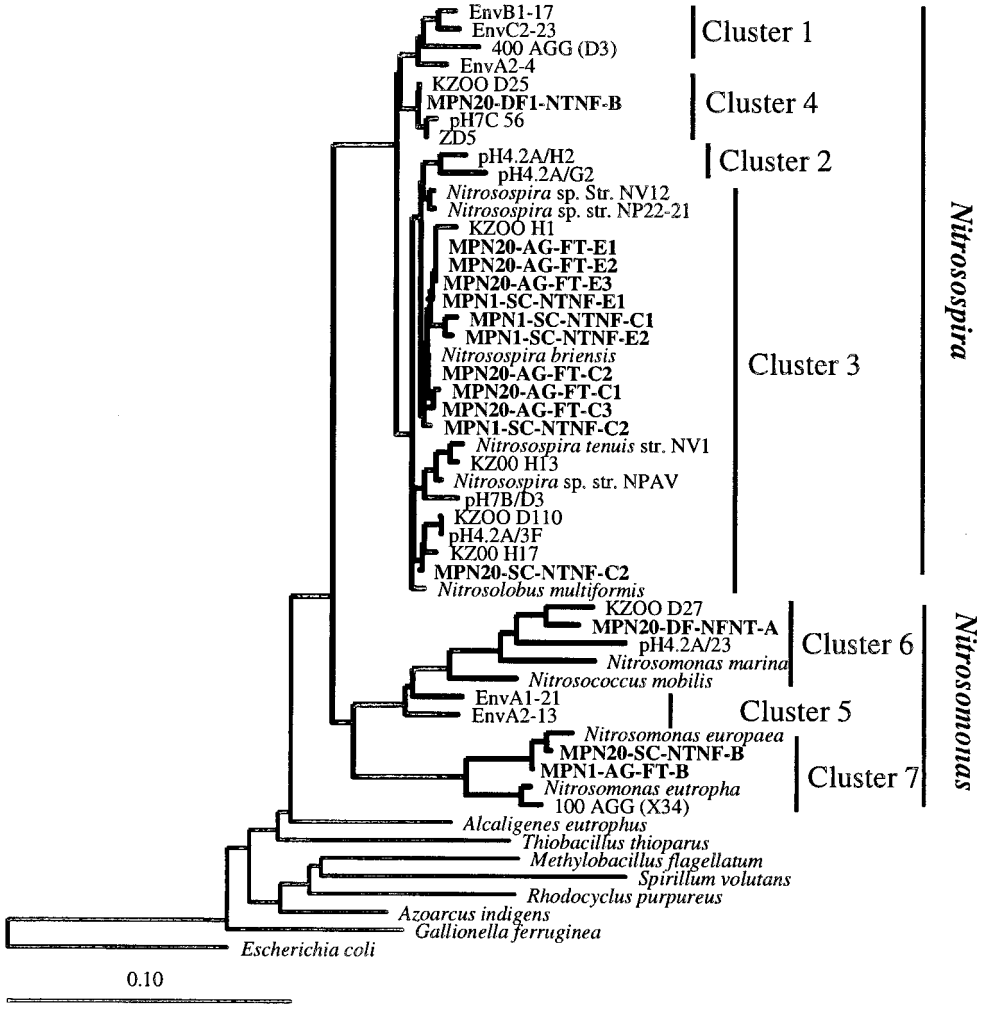

FIG. 7. Neighbor-joining tree showing the relationship of bands excised from DGGE gels after PCR amplification of DNA extracted from MPN dilution tubes of LTER soils incubated with 5, 50, and 1,000 μg of NH_4^+-N ml^{-1}. Sequences excised from the gel (shown in bold) have the nomenclature MPN1 or MPN20 to indicate incubation with 50 or 1,000 μg of NH_4^+-N ml^{-1}, respectively, followed by the treatments DF (deciduous forest), AG (cultivated), SC (successional grassland), and PP (*Populus* trees). The treatment variables—tillage (T), no tillage (NT), fertilization (F), and no fertilization (NF)—are followed by the migration distances of the bands (A to E) (see the legends to Fig. 3 and 5). The scale bar is as described in the legend to Fig. 6.

showed that all of the bands fell within the known β-proteobacterial ammonia oxidizer groupings described by Stephen et al. (40). All bands sequenced that were representatives of the *Nitrosospira* grouping belonged to cluster 3. However, bands excised from unusual banding profiles obtained from MPN cultures of samples from the NDF and from poplar plots (treatment 5) were representative of cluster 4 *Nitrosospira.* The *Nitrosomonas* bands detected in the MPN samples were closely related to *N. europaea*. One sequence from the MPN samples was found to lie within cluster 6 and was closely related to another sequence, KZOO_D27, that was also isolated from this location (5). Within the cluster 3 grouping, sequences from the NDF samples clustered together, suggesting that there might be a treatment effect within cluster 3. Some sequences from the slowly (band C) and quickly (band E) migrating bands were different by only 1 bp over the 290 bp used for phylogenetic analysis; however, despite this fact, they consistently migrated at different rates in gels. This mismatch was in the middle of the sequence, but single mismatches within the primer region due to an ambiguous base led to closely migrating bands (19).

DISCUSSION

Abundance estimates. This study used conventional and molecular techniques to assess the relationship among the abundance, activity, and diversity of ammonia oxidizer populations in soils. The communities reflected treatments ranging from intensive cultivation to NDF. Estimated concentrations of ammonia-oxidizing bacteria were dependent on the enumeration method and protocol. With the exception of the poplar plot, the use of higher concentrations of ammonia in the growth media significantly reduced MPN counts. Similar results have been reported by other workers (3, 41) and may result from growth inhibition of the ammonia oxidizers at high ammonia concentrations (41, 42). Ammonia oxidizer cell concentrations obtained by cPCR were 10- to 1,000-fold higher than MPN counts at these sites, with the exception of the poplar soil. The anomalous results found for this treatment may have been due to differences in the cover crop and potential consequent changes in the activities of different groups of ammonia oxidizers in this soil. DeGrange and Bardin (9) also found that the

numbers of bacteria calculated by MPN-PCR counts were 100 times higher than those calculated by traditional MPN counts in a sandy calcareous soil, whereas the difference was only 10-fold in a sandy loam soil.

Detection limits for cPCR methods were observed to be between 10 and 1,000 times lower than those for standard dilution plating methods when a genetically modified strain of the fungus *Trichoderma virens* in soil was investigated (2). The differences might also reflect limitations of laboratory growth media and incubation conditions, which do not support the growth of all culturable organisms within natural populations and which will not detect nonculturable cells. Populations with lag periods longer than the incubation period also will not be detected, and Matulewich et al. (23) found increasing MPN counts of nitrifying bacteria even after incubation for 90 days. Belser and Schmidt (3) showed that the use of different media for MPN enumeration of ammonia oxidizers produced different results in an actively nitrifying soil. They also found dominance by *Nitrosomonas* in media inoculated with lower sample dilutions and by *Nitrosospira* at higher dilutions. In our study, comparison of 16S rDNA partial sequences amplified from DNA extracted directly from the soil and from positive MPN cultures indicated a similar shift in composition. Samples from the MPN cultures were dominated by sequences representative of *Nitrosomonas*, which were not detected in soil DNA extracts, while *Nitrosospira*-like sequences, which dominated in soil DNA extracts, were less frequent in MPN cultures and sometimes were not detected. Selection for *Nitrosomonas* was greatest in MPN counts when 1,000 μg of NH_4^+-N ml^{-1} was used. Hiorns et al. (14) have detected *Nitrosomonas* DNA in lake water and sediment enrichments but not in extracted DNA, supporting the belief that *Nitrosomonas*-like organisms are better adapted to growth on laboratory media (3).

Compositional differences in ammonia oxidizers. Despite the significant differences in potential nitrification rates among these communities, ammonia oxidizers were found to constitute a relatively small proportion of the total bacterial population detected by microscopic DTAF staining. MPN estimates were 6 to 8 orders of magnitude lower than total cell counts. cPCR may provide a more accurate estimate of total cell counts; in this study, cPCR indicated that β-proteobacterial ammonia oxidizers constituted a maximum of 0.01% of the total population. This low relative abundance in soil may explain the lack of detection of ammonia oxidizer sequences in clone libraries generated by amplification of 16S rDNA using eubacterial primers (17, 21, 25, 28). Borneman et al. (4) found that the majority of the β-proteobacterial clones from a Wisconsin soil showed 80% homology to the ammonia oxidizers. Our data indicate that the characterization of several thousand eubacterial clones would be necessary for the detection of ammonia oxidizers, even in agricultural soils, and that detection by DGGE analysis of eubacterial PCR products would be unlikely.

Potential nitrification rates were higher in cultivated soils than in native soils and successional grassland soils. This result may have been due to increased aeration of these soils through repeated crop regimens. The types of plant community and N fertilization dictate the amount of available ammonia for oxidation by microbes, as was particularly evident in the poplar plots. Soils that were not tilled (treatment 2) had significantly higher nitrification rates than their nontilled equivalents for both fertilized and nonfertilized plots. Soils under no-till practice maintain pore structure and continuity, leading to significantly greater hydraulic conductivity and infiltration rates than are found in conventionally tilled soils (1). This information might mean that ammonia oxidizer communities in nontilled soils would be more stable and therefore more active than the communities in tilled soils. Treatment effects were not detectable by MPN counts, but cPCR data indicated that fertilization led to larger populations. This result might reflect the ability of molecular methods to detect nonculturable organisms in environmental samples. There was no correlation between observed nitrification rates and the numbers of ammonia oxidizers present, calculated by either traditional MPN counts or cPCR.

Although nitrification rates and ammonia oxidizer cell concentrations varied with different treatment regimens, there were no detectable differences in the compositions of the ammonia oxidizer communities, as determined by DGGE analysis of 16S rDNA partial sequences obtained by PCR amplification of extracted DNA using primers specific for the β-proteobacterial ammonia oxidizers. Soils from all sites were dominated by members of *Nitrosospira* cluster 3, which are commonly found in soil (5, 40) and which contain the majority of cultured representatives of the genus *Nitrosospira*. Bruns et al. (5) did not detect *Nitrosospira* cluster 3 in native and unfertilized soils, but we found no effects of fertilization or cultivation on community structure, and *Nitrosospira* cluster 3 dominated in all soils sampled. Sequence analysis of DGGE bands indicated that for different soils, there was a clustering of sequences within cluster 3. This result was particularly evident for deciduous forest soils, although the conclusions drawn must be considered tentative given the small number of sequences analyzed. The stability of other components of the microbial community in these soils has been reported by Buckley et al. (6), who found no differences in *Crenarchaeota* sequences in cultivated and native soils. However, significant differences were seen in a comparison of two Norwegian agricultural soils for total bacterial diversity (29).

Relating structure and function. There are several explanations for the lack of correlation between β-proteobacterial ammonia oxidizer population structure and nitrification rates. The treatments imposed, i.e., tillage and fertilizer, may not drive ammonia oxidizer community structure, which may be more dependent on soil properties, which were initially the same for all treatments. The already established populations survived in systems that lowered the available NH_4^+ substrate levels, and substrate additions would be required to bring in new populations. On a phylogenetic level, it has been suggested that two sequences showing up to a 0.3% difference in sequence homology in the 16S rDNA gene could represent two species with different ecological functions (43). Pankhurst et al. (30) suggested that there does not need to be great taxonomic diversity for there to be functional diversity in soils. In this study, differences seen in the sequences of cluster 3 may mean that, although the organisms are very closely related phylogenetically, they are in fact physiologically different, leading to the differences in the nitrification rates observed between treatments.

The AMO primers are not completely specific for β-proteobacterial ammonia oxidizers but, in combination with CTO primers, amplify all known sequences representative of this group. Although primer bias cannot be dismissed, similar findings have been reported with either set of primers for amplification of 16S rDNA sequences from the same soils and marine sediments (25, 39, 40). The possibility that ammonia oxidizers in natural communities have sequences that are not amplified by these primers cannot be excluded.

This study has demonstrated that the structures of β-proteobacterial ammonia oxidizer populations were quite similar in soils collected from a wide range of communities under different soil cultivation conditions, which resulted in significant changes in potential rates of nitrification and in the sizes of ammonia oxidizer populations. Community structure was

assessed at the level of precision provided by analysis of clusters characterized by 16S rDNA sequences and indicated dominance by *Nitrosospira* cluster 3. Further studies are required to determine whether subtle changes occur within this cluster or whether stability under a variety of environmental conditions is due to physiological and functional diversity within the populations.

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