

Effect of Soil Ammonium Concentration on N₂O Release and on the Community Structure of Ammonia Oxidizers and Denitrifiers

Sharon Avrahami, Ralf Conrad, and Gesche Braker*

Max-Planck-Institute for Terrestrial Microbiology, Karl-von-Frisch-Strasse, 35043 Marburg, Germany

Received 30 May 2002/Accepted 6 August 2002

The effect of ammonium addition (6.5, 58, and 395 μg of $\text{NH}_4^+\text{-N}$ g [dry weight] of soil⁻¹) on soil microbial communities was explored. For medium and high ammonium concentrations, increased N₂O release rates and a shift toward a higher contribution of nitrification to N₂O release occurred after incubation for 5 days at 4°C. Communities of ammonia oxidizers were assayed after 4 weeks of incubation by denaturant gradient gel electrophoresis (DGGE) of the *amoA* gene coding for the small subunit of ammonia monooxygenase. The DGGE fingerprints were invariably the same whether the soil was untreated or incubated with low, medium, or high ammonium concentrations. Phylogenetic analysis of cloned PCR products from excised DGGE bands detected *amoA* sequences which probably belonged to *Nitrosospira* 16S rRNA clusters 3 and 4. Additional clones clustered with *Nitrosospira* sp. strains Ka3 and Ka4 and within an *amoA* cluster from unknown species. A *Nitrosomonas*-like *amoA* gene was detected in only one clone. In agreement with the *amoA* results, community profiles of total bacteria analyzed by terminal restriction fragment length polymorphism (T-RFLP) showed only minor differences. However, a community shift occurred for denitrifier populations based on T-RFLP analysis of *nirK* genes encoding copper-containing nitrite reductase with incubation at medium and high ammonia concentrations. Major terminal restriction fragments observed in environmental samples were further described by correspondence to cloned *nirK* genes from the same soil. Phylogenetic analysis grouped these clones into clusters of soil *nirK* genes. However, some clones were also closely related to genes from known denitrifiers. The shift in the denitrifier community was probably the consequence of the increased supply of oxidized nitrogen through nitrification. Nitrification activity increased upon addition of ammonium, but the community structure of ammonium oxidizers did not change.

Nitrous oxide (N₂O) is produced as a by-product during nitrification and occurs as an intermediate during denitrification (9). The production and emission of N₂O in the environment is of major importance for global warming (11), as well as for the destruction of the stratospheric ozone layer (10). Microbial processes in soils contribute about 70% of the atmospheric budget of N₂O (9). N₂O emissions from soils have greatly increased with increasing N inputs by fertilization of agricultural soils (37). Laboratory (36) and field (25) studies observed an increased contribution of nitrification to total N₂O production in correlation with increasing ammonium concentrations, indicating an increase in nitrifier activity after fertilization with ammonium. However, it remained unclear whether fertilization also results in a change in the community structure, as such a change was only observed for nitrifiers when wastewater was applied instead of mineral fertilizer (28). Although field studies clearly indicate that the community structures of ammonia oxidizers can be different in different soils (see below), few experimental studies exist which address the in situ dynamics of nitrifier populations (23, 29).

The aerobic ammonia-oxidizing nitrifiers in soils have mainly been studied by targeting the 16S rRNA genes. The known ammonia oxidizers that exist in pure culture comprise two monophyletic groups (31). One group belongs to the γ subdivision of the class *Proteobacteria*, with *Nitrosococcus oceani*

and *Nitrosococcus halophilus* as the only known species. The other group belongs to the β subdivision of the class *Proteobacteria* and includes two genera: *Nitrosomonas* and *Nitrosospira*. Ammonia-oxidizing bacteria and clones belonging to the β -*Proteobacteria* were divided into at least seven clusters based on 16S rRNA gene sequences (31, 39). Soils were dominated by *Nitrosospira* species of clusters 2, 3, and 4 (8, 17, 29, 38). The community patterns of ammonia oxidizer 16S rRNA clones were found to depend on the soil acidity, with *Nitrosospira* cluster 2 being established at low soil pH (21, 38). Another selective factor was the concentration of ammonium, with *Nitrosospira* cluster 3 dominating in early successional soils with relatively high ammonium concentrations while *Nitrosospira* clusters 2 and 4 dominated in old successional soils with low ammonium concentrations (8, 20, 21). Fields to which manure or compost had been added contained in addition a significant population of *Nitrosomonas* species (14, 18). Management of grassland soil also affected the ammonia oxidizer populations, which were more diverse in unimproved than in improved soil (40). Soil improved by the addition of N fertilizer was dominated by *Nitrosospira* cluster 3 and *Nitrosomonas* cluster 7 (40).

The key enzyme for all aerobic ammonia oxidizers is ammonia monooxygenase. The gene coding for a subunit of this enzyme (*amoA*) can serve as a useful target for environmental studies, since it reflects the phylogeny of the ammonia oxidizers very well (2, 31, 33). Although the *amoA* gene is both specific and universal for all ammonia oxidizers, so far it has not been used to the same extent as the 16S rRNA gene in studying soil ammonia oxidizers (19).

For comparison, we also investigated the structure of the

* Corresponding author. Mailing address: Max-Planck-Institute for Terrestrial Microbiology, Karl-von-Frisch-Strasse, D-35043 Marburg, Germany. Phone: 06421/178 810. Fax: 06421/178 999. E-mail: braker@mail.uni-marburg.de.

denitrifier community and that of the total bacterial community by targeting the *nirK* genes (5) and the 16S rRNA genes, respectively. *nirK* encodes the copper-containing nitrite reductase. NirK and the cytochrome *cd₁*-containing nitrite reductase (NirS), which are functionally equivalent although structurally different, are the key enzymes of the denitrification process. Nitrite reductase genes are functional marker genes of denitrifying bacteria, since this physiological group is widespread among phylogenetically unrelated groups. Both genes have been used to detect denitrifiers in environmental samples. In a recent study (30), PCR-based assays detected *nirK* more readily in soils whereas *nirS* was preferentially found in marine sediments (6). Community structures of denitrifiers were analyzed by cloning of both genes (6, 30) and by terminal restriction fragment length polymorphism (T-RFLP) analysis of *nirS* genes (4) from environmental samples. In this study, we introduce community analysis by T-RFLP based on *nirK* genes.

Our objective was to test how a change in soil ammonium concentration affects the activity and structure of the ammonia oxidizers in comparison to other soil bacteria, denitrifiers in particular.

MATERIALS AND METHODS

Soil samples. Samples from the upper 10 cm of the silt loam soil (50°28.933'N, E8°45.930') used by Gödde and Conrad (13) were taken at Eberstadt, Germany, in April 2000. The soil was dried under ambient air to 12.5% gravimetric water content, sieved to <2-mm aggregate size, and stored at 4°C. Ammonium (16) and nitrate (34) concentrations were measured colorimetrically. The pHs determined in 0.01 M CaCl₂ and water were 5.8 and 6.1, respectively. The soil gravimetric water content, as well as the maximal water-holding capacity, was determined by standard protocols (34). At the beginning of each experiment, the soil water content was adjusted to 60% of the water-holding capacity.

Experimental setup for N₂O release. The experiment described by Gödde and Conrad (13) was slightly modified. Ammonium was added to the soil, since the original ammonium concentration was very low (0.19 µg of NH₄⁺-N g [dry weight] of soil⁻¹). To investigate the effect of the addition of ammonium to the soil, the ammonium content was adjusted to three different concentrations, i.e., 6.5, 58, and 395 µg of NH₄⁺-N g (dry weight) of soil⁻¹, followed by incubation at 4°C. The ammonium concentrations were measured after 5 days of incubation. The release of N₂O was measured using a Carlo Erba 8000 gas chromatograph equipped with an electron capture detector as described previously (13). The contribution of nitrification to N₂O release was measured after preincubation with and without 10 Pa of acetylene as described previously (13).

Molecular analysis. Samples were incubated for 4 weeks at 4°C at low, medium, and high ammonium (LA, MA, and HA, respectively) concentrations. The 120-ml serum bottles containing 5 g of soil were opened for aeration every 3 days for 10 min each time to maintain aerobic conditions and stable water content. Approximately 500 mg (wet weight) of soil was transferred from each treatment to a 2-ml screw-cap tube. In addition, samples (ZT) were taken before incubation as a reference.

DNA extraction. DNA was extracted from these samples following the protocol of Lüdemann et al. (22) with slight modifications. Each sample was mixed with 750 µl of Na-phosphate buffer (120 mM; pH 8) and 187 µl of sodium dodecyl sulfate solution (10% sodium dodecyl sulfate, 0.5 M Tris-HCl [pH 8], 0.1 M NaCl). The samples were resuspended homogeneously by vortexing them. After incubation of the solution for 10 min at 60°C, 0.5 g of glass beads (0.17- to 0.18-mm diameter) was added and the suspension was shaken for 1 min at maximum speed in a bead beater (Dismembrator-S; B. Braun Biotech, Melsungen, Germany). After centrifugation (10 min; 20,817 × g at 4°C), the supernatant was collected and extracted three times with phenol (pH 8), phenol-chloroform-isoamyl alcohol (25:24:1; pH 8) and chloroform-isoamyl alcohol (25:24). The addition of ammonium acetate (to a final concentration of 2.5 M) and an equal volume of cold isopropanol followed by 30 min at -20°C and centrifugation (20 min; 20,817 × g at 4°C) allowed the precipitation of DNA. Subsequently, the DNA pellet was washed with 80% ethanol, centrifuged (5 min; 20,817 × g at 4°C), and dried under vacuum. Finally, the DNA was resuspended with 100 µl of TE buffer (10 mM Tris base, 1 mM EDTA, pH 8). DNA was cleaned from humic

acids by using polyvinylpyrrolidone columns as described by Henckel et al. (15). The purity of the DNA was high as judged by the ratios of absorption at 260 and 280 nm of 1.49 to 1.82 ($n = 6$).

PCR amplification of *amoA*. The primers used for PCR amplification were an *amoA* set which was described previously by Rotthauwe et al. (33). For denaturant gradient gel electrophoresis (DGGE) analysis, a guanosine-cytosine clamp, as described by Muyzer et al. (26), was added to the 5' end of the forward primer. Amplification was performed by using 0.5 µM each primer, 1 U of AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany), and 25 µl of MasterAmp 2× PCR premix F containing 100 mM Tris-HCl (pH 8.3), 100 mM KCl, 7 mM MgCl₂, 400 µM each deoxynucleoside triphosphate, and the PCR enhancer betaine (Epicentre Technologies, Madison, Wis.). The DNA was diluted 10 times, due to humic acid inhibition, and 1 µl was added to a final volume of 50 µl. Amplifications were always started by placing the PCR tubes into the preheated (94°C) thermal block of a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). The thermal profile which was used for amplification was modified after that of Rotthauwe et al. (33) and included 5 min at 94°C followed by 35 cycles of 45 s at 94°C, 30 s at 60°C, and 1 min at 72°C, and 6 min at 72°C for the last cycle.

DGGE and cloning of *amoA*. DGGE was performed as described previously with slight modifications (26). PCR products were separated on polyacrylamide gels, using a gradient of 45% (6% [wt/vol] acrylamide-bisacrylamide [37.5:1; Bio-Rad Laboratories GmbH, Munich, Germany], 18% deionized formamide, 3.1 M urea) to 65% (6% [wt/vol] acrylamide-bisacrylamide [37.5:1; Bio-Rad], 26% deionized formamide, 4.5 M urea). The gels were electrophoresed by using the D GENE system (Bio-Rad) with 0.5× Tris-acetate-EDTA at 60°C and 100 V for 17 h. The gels were stained with 1:50,000 (vol/vol) SYBR Green (Biozym, Hessisch-Oldendorf, Germany) and scanned with a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Bands were excised from DGGE gels using a Dark Reader transilluminator (Clare Chemical Research, Ross on Wye, United Kingdom). The excised bands were resuspended in 200 µl of PCR water, reamplified, and then separated on DGGE again. Due to the appearance of multiple bands in each sample, the reamplified PCR products from the excised bands were ligated to pGEM T-Easy vectors, which were used to transform *Escherichia coli* JM109 competent cells (Promega, Madison, Wis.) according to the manufacturer's instructions. Clones containing a correct insert were reamplified using DGGE-specific primers and screened by DGGE by comparing the clones to environmental and experimental samples. Different clone types reamplified with *amoA* primers were sequenced as described below.

Community analysis of bacteria and denitrifiers. For amplification of 16S ribosomal DNA (rDNA), DNA from soil samples was extracted according to the modified protocol of Lüdemann et al. (22) as described previously for *amoA*. DNA (1 µl) from a 1:10-diluted extract was used for PCRs (50 µl) as described elsewhere (4) with the bacterial 16S rDNA primers 8-27F [5'-AGAGTTTGAT C(A/C)TGGCTCAG-3'] and 1392-1407R (5'-ACGGGCGGTGTGACA-3') described by Amann et al. (3) and modified by C. L. Moyer (School of Ocean and Earth Science and Technology, University of Hawaii) (unpublished results). For *nirK*, DNA was extracted using the FastDNA Soil Spin kit (BIO101, Carlsbad, Calif.). PCRs (50 µl) were performed with 1 µl of a 1:2-diluted DNA extract as described elsewhere (6). For T-RFLP, the bacterial forward 16S rDNA primer and the reverse *nirK* primer were 5'-end labeled with 6-carboxyfluorescein, and a 2 mM concentration of the labeled *nirK* primer was used. Three PCRs each were pooled and purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). The purified PCR products (100 ng) were cleaved with 3 U of *Hha*I (Promega, Mannheim, Germany) for 16S rDNA and with *Hae*III (New England Biolabs, Frankfurt, Germany) for *nirK* for 3 h at 37°C. The restriction fragments were separated on an ABI 377 automated sequencer for 15 h with limits of 2,500 V and 40 mA. After electrophoresis, the lengths of the fluorescently labeled terminal restriction fragments (T-RFs) were determined by comparison to the internal standards Genescan 2500-ROX (Applied Biosystems, Warrington, United Kingdom) for terminal 16S rDNA fragments and Genescan 1000-ROX (Applied Biosystems) for terminal *nirK* fragments by using GeneScan 2.1 software (Applied Biosystems). Peaks with a fluorescence of 100 U over the background fluorescence and larger than 50 bp were analyzed by peak height. The relative abundances of T-RFs were determined by calculating the ratio between the height of each peak and the total peak height of all peaks within one sample. Ratios were converted to percentages, and the results are displayed as histograms.

Cloning of *nirK* genes from soil. Amplified *nirK* genes from sample MA were cloned using the TOPO TA cloning kit (Invitrogen, Paisley, United Kingdom) according to the manufacturer's instructions. White colonies picked at random were screened for inserts of the proper size by PCR amplification of the inserts

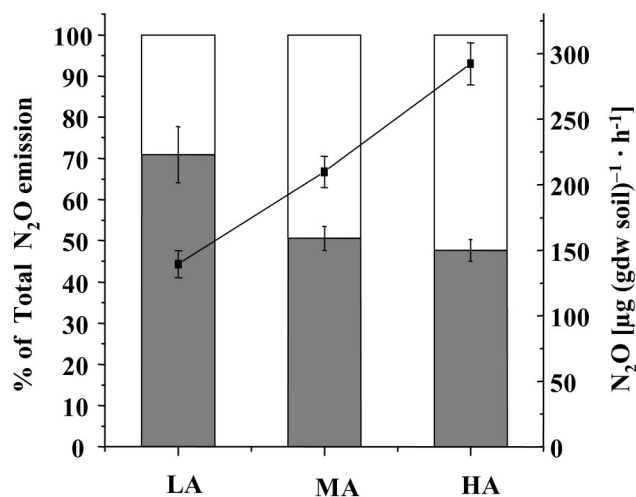


FIG. 1. Effect of incubation at LA, MA, and HA concentrations on ammonium transformation. The stacked bars indicate percent contributions of nitrification (open) and denitrification (shaded) to total N₂O emission, and the small squares indicate the rates of total N₂O emission. Means \pm standard errors are shown ($n = 3$).

with *nirK* primers used for T-RFLP under the conditions described previously. The clones were also screened by T-RFLP, and those showing correspondence to T-RFs from the environmental DNA extract were chosen for sequencing. The inserts were PCR amplified using the T7 promoter primer and the M13 reverse primer as described elsewhere (6), except that the annealing temperature was lowered to 55°C.

Sequencing. PCR products from clones were purified with the QIAquick PCR purification kit (Qiagen). Both strands were sequenced directly from 70 ng of PCR product with vector-specific primers and the ABI Big Dye terminator kit version 2.0 (Applied Biosystems) according to the manufacturer's instructions. Subsequently, excess primers and dye terminators were removed with Autoseq G-50 columns (Amersham-Pharmacia Biotech, Freiburg, Germany), and cycle-sequencing reactions were analyzed with an ABI 377 DNA sequencer.

Phylogenetic analysis. The sequences were aligned to sequences from the EMBL database using the ARB Fast aligner feature (<http://www.arb-home.de>). For phylogenetic analysis, 150 deduced amino acid positions for *amoA* and 102 for *nirK* were included, omitting insertions or deletions due to ambiguous positional homology. Phylogenetic analyses were performed using ARB and the PHYLIP software package version 3.6a2.1 (12). Trees were reconstructed using the distance matrix-based methods neighbor joining (ARB and PHYLIP) and FITCH (PHYLIP), parsimony (PROTPARS [PHYLIP]), and maximum likelihood (MOLPHY [Institute Pasteur, Paris, France; <http://bioweb.pasteur.fr/sequalnal/interfaces/molphy.html>]).

Nucleotide sequence accession numbers. The *amoA* and *nirK* gene sequences have been deposited in the EMBL nucleotide sequence database under accession no. AY098867 through AY098890 and AJ487545 through AJ487555, respectively.

RESULTS

To explore the effects of ammonium addition, rates of N₂O release were measured after 5 days of incubation at 4°C as a function of the initial ammonium concentration, i.e., LA, MA, and HA (6.5, 58, and 395 μg of NH₄⁺-N g [dry weight] of soil⁻¹, respectively). The N₂O release rates increased with the initial ammonium concentration (Fig. 1). The final nitrate concentrations in the LA, MA, and HA treatments were 18, 20, and 21 μg of NO₃⁻-N g (dry weight) of soil⁻¹ and were significantly different only between the LA and HA treatments ($P < 0.05$). The contribution of nitrification to the total N₂O

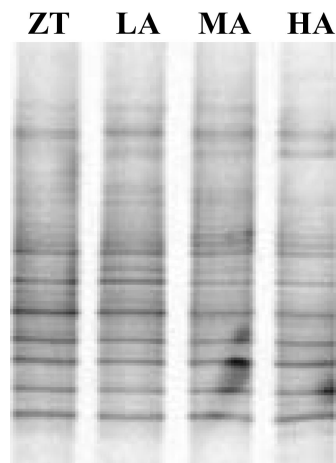


FIG. 2. DGGE analysis of *amoA* fragments from soil samples taken before treatment (ZT) and after incubation with LA, MA, and HA concentrations at 4°C for 4 weeks.

release shifted from 25% at LA to 50 and 52% in the treatments with MA and HA, respectively (Fig. 1).

The diversity of the ammonia oxidizer community was analyzed after incubation of soil at LA, MA, and HA concentrations for 4 weeks at 4°C. The DGGE fingerprints of the amplified *amoA* populations showed no difference between the samples with different ammonium concentrations and the sample (ZT) that was taken from the original soil at the beginning of incubation (Fig. 2). Apparently, the nitrifier community had not changed significantly during 4 weeks of incubation at 4°C.

Representative bands from the different DGGE lanes were excised, and the PCR products were cloned and sequenced. The sequences were used to reconstruct phylogenetic trees including other environmental *amoA* clones from the database (Fig. 3). Most of the clones (23 clones) branched with species of the genus *Nitrosospira*, and only one clone (Agb11) was affiliated with the genus *Nitrosomonas*, most closely related to *Nitrosomonas* sp. strain Nm103 (Fig. 3). Clones closely related to *Nitrosospira* clustered with *amoA* genes from species that belong to clusters 3 and 4 defined by 16S rRNA phylogeny. Additionally, clones clustered with *amoA* genes from *Nitrosospira* sp. strains Ka3 and Ka4 (cluster 1) and within an *amoA* cluster from unknown species (cluster 8b). The majority of clones occurred within two clusters, 1 and 3b. The clones of cluster 1 originated from each of the samples (ZT, LA, MA, and HA), while the clones of cluster 3b originated from samples LA, MA, and HA. Collectively, these results give no indication of the selection of specific *amoA* populations by these different incubation conditions.

For comparison, we also analyzed the diversity of denitrifiers containing the *nirK* gene (Fig. 4) and that of the total bacterial flora based on 16S rRNA genes (data not shown) by T-RFLP fingerprinting. We focused on *nirK* genes, since amplification of *nirS* genes according to the protocol described by Braker et al. (4) was not successful. Community profiles of bacterial 16S rRNA were almost identical for incubations at LA, MA, and HA concentrations with minor differences, in agreement with the results from DGGE community analysis of *amoA* genes. For *nirK*, community profiles were similar for the untreated

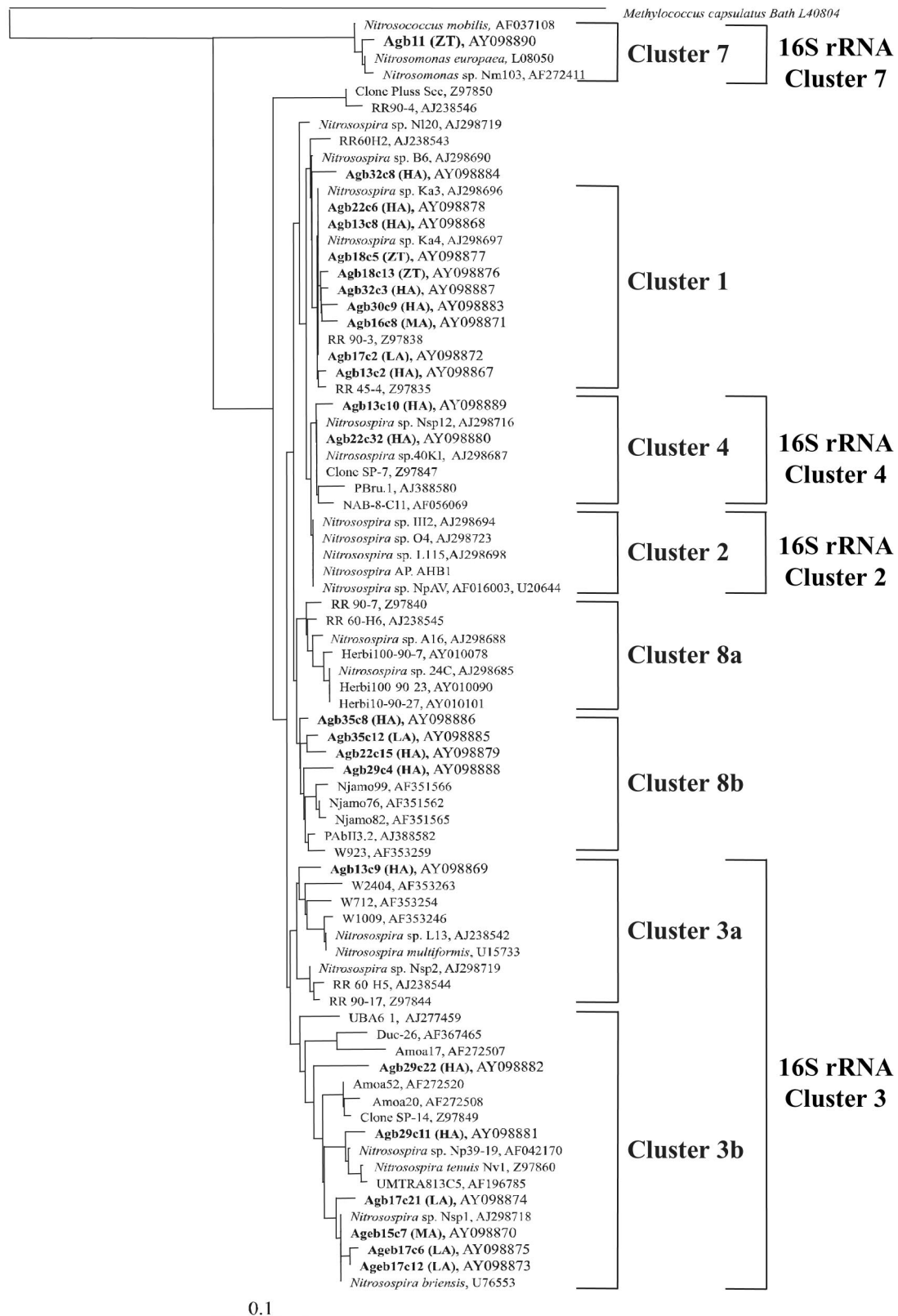


FIG. 3. Phylogenetic Fitch-Margoliash tree (using global rearrangement and randomized input order [three jumbles]) based on partial *amoA* sequences (150 amino acids). Clones obtained from this experiment are shown in boldface. The sources of sequences were soil samples taken before treatment (ZT) and after incubation with LA, MA, and HA concentrations at 4°C for 4 weeks. The scale bar indicates 10 mutations per 100 sequence positions.

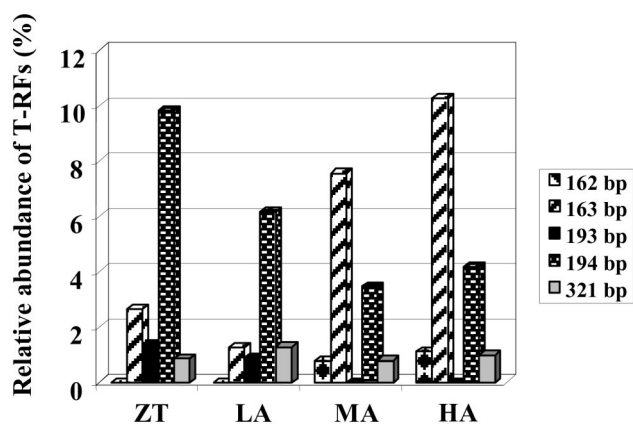


FIG. 4. T-RFs of amplified *nirK* fragments (514 bp) with major differences in relative abundance according to soil treatment. Soil samples were taken before treatment (ZT) and after incubation with LA, MA, and HA concentrations at 4°C for 4 weeks. The key indicates the lengths of the T-RFs.

soil and at LA concentration and similar for MA and HA concentrations, respectively (Fig. 4). The main differences were detected for the T-RF of 162 bp, which was present only at MA and HA concentrations, and for the T-RF of 193 bp, which was lacking in those two treatments but was present in the untreated soil and at LA concentrations. Based on the relative abundances of T-RFs, shifts were most obvious for the T-RF of 163 bp, whose relative abundance increased at MA and HA concentrations, and T-RFs of 194 and 321 bp, which comprised a larger fraction in the untreated soil and at LA concentrations. These shifts were confirmed from replicate DNA extractions of soil samples with LA and MA concentrations.

Screening cloned *nirK* genes from soil sample MA by T-RFLP showed correspondence of the T-RFs mostly to major T-RFs from the environmental sample. Clones were found corresponding to T-RFs of 59 or 61, 69, 131, 154, and 189 bp, respectively, considering a possible deviation of up to 2 bp in the sizes of the T-RFs due to the nature of gel separation. Sequences from clones representing major T-RFs were also analyzed in silico with respect to *Hae*III restriction sites, and the calculated T-RFs showed the expected results (Fig. 5). Phylogenetic analysis placed soil sequences mainly within soil clusters I and III. In addition, genes were found which were closely related to *Ochrobactrum* spp., and two clones branched off within a *Hyphomicrobium-Rhizobium* cluster (Fig. 5).

DISCUSSION

Our study showed that the N₂O release activity of the soil nitrifier community clearly responded to an increased ammonium supply, resulting in a higher total N₂O release rate. With MA and HA concentrations, we also observed a shift toward a greater contribution of nitrification to N₂O production compared to soil without incubation or after the addition of a small amount of ammonium. These results are in agreement with those of other studies (25, 36, 37).

On the other hand, our study showed that the soil nitrifier

population represented by DGGE patterns of the *amoA* gene did not change significantly during 4 weeks of incubation at 4°C, even when the soil was amended with HA concentrations. Therefore, the response of the activity was probably not the result of a major change in the ammonia-oxidizing population but of a physiological shift. Mendum et al. (23) arrived at a similar conclusion after studying ammonia-oxidizing populations in fertilized and unfertilized soil over a period of 6 weeks. This conclusion was further supported by analysis of the phylogenetic structure of *amoA* sequences retrieved from the soil ammonia-oxidizing community, which did not reveal any clustering of genes with respect to ammonium treatment. In contrast, our analysis revealed an amazingly high diversity of *amoA* sequences from the different ammonium treatments, affiliated with five *Nitrosospira* clusters and one *Nitrosomonas* cluster. No clones were affiliated with *Nitrosospira* sp. strain AP. AHB1 from cluster 2, which was isolated from an acidic soil (21, 38). It should also be noted that all *amoA* sequences had a similarity of >85% (amino acid sequence level) to those of known ammonia oxidizer species. Hence, all the diversity was displayed within a relatively narrow range of species. Generally, the diversity of ammonia oxidizers in tilled agricultural soils is expected to be much lower than in native soils (8). However, our soil had been treated briefly with composted organic material before sampling, which may have led to an input of organisms to the native ammonia oxidizer populations. Although *amoA* exists in two or three copies in the genome of ammonia oxidizers (7, 27), it is unlikely that part of the diversity is due to multiple copies of *amoA*. Direct sequencing of *amoA* genes from 31 pure cultures indicated that partial sequences obtained with the primer set used in our study were unambiguous and thus were not contributing to diversity (1).

The observed invariability of the ammonia oxidizer community upon incubation at 4°C does not support the hypothesis that the pattern of N₂O release, which had been observed in earlier studies upon adaptation to particular temperatures (13), might be the result of changes in the community structure. However, other bacterial populations, such as denitrifiers, contribute to N₂O release from soils and presumably are affected by the addition of ammonium to soils. Enhanced nitrification rates yield an increase in soil nitrate, which can then be reduced via denitrification. To determine whether changes in the total bacterial community or in the denitrifying population had an impact on the physiological status of the soil, we also explored changes in the community of bacteria and denitrifiers by T-RFLP based on 16S rDNA and *nirK* genes, respectively. *nirS* genes were not detected in our soil. However, Priemé et al. (30) successfully amplified *nirS* genes from a mixed deciduous marsh in Michigan, using the *nirS* primer sets we tried to use in our study. Similar to the results for the ammonia-oxidizing population, only minor changes were detected for the bacterial community from the untreated soil and after treatment with different ammonium additions. Ammonia oxidizers and denitrifiers comprise only a subfraction of the total soil community. Consequently, in this approach, changes within these groups are supposedly hidden by an overall stable total bacterial community. However, a shift was detected for the denitrifying population based on *nirK*. Consistent with the shift toward a higher contribution of nitrification to the overall release of N₂O upon the addition of medium and large

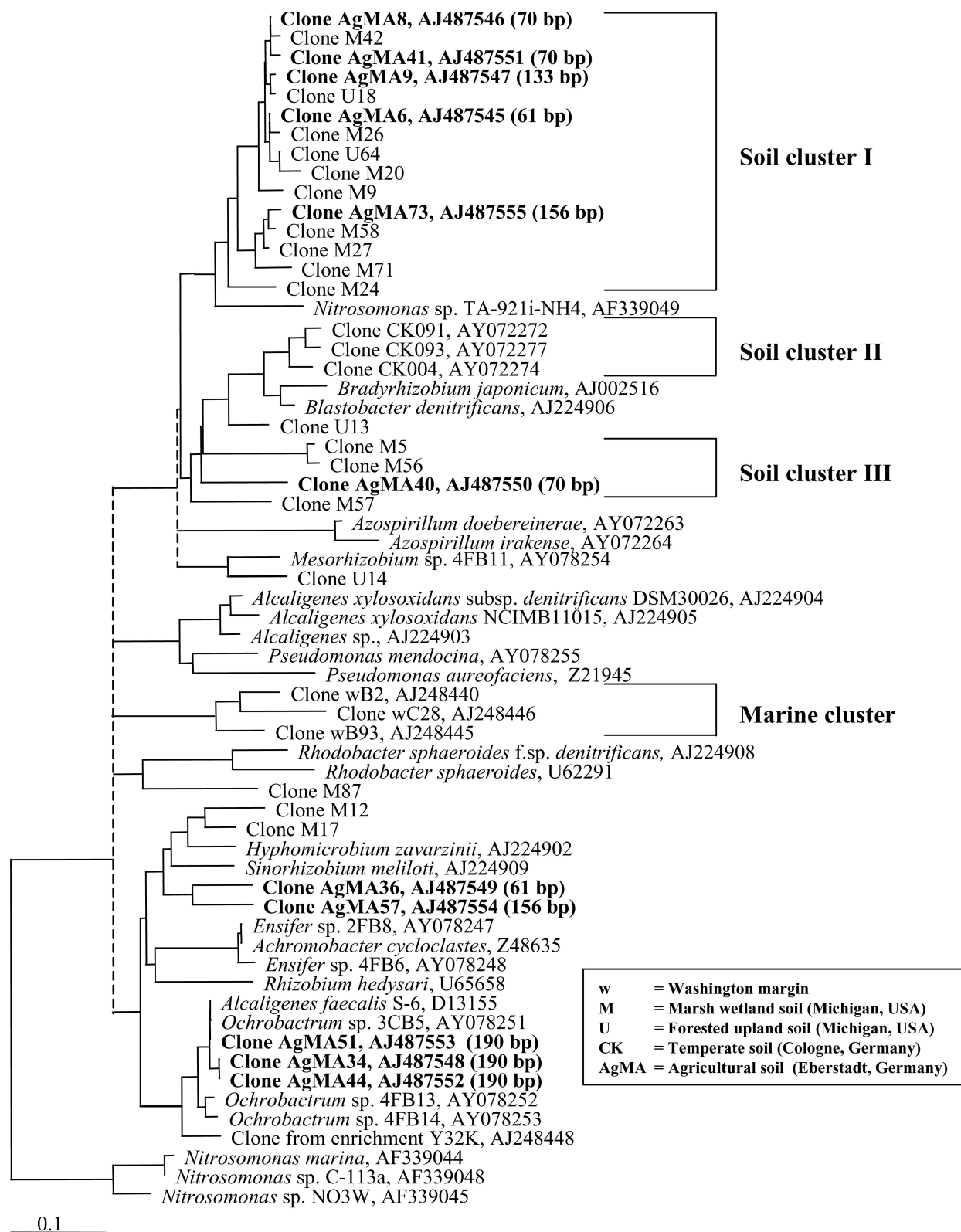


FIG. 5. Neighbor-joining tree of partial *nirK* genes based on 102 amino acids. The consensus tree was reconstructed based on neighbor-joining, parsimony, FITCH, and maximum-likelihood (MOLPHY) analyses. Unresolved nodes are displayed as multifurcations and are indicated by dashed lines. Clones obtained from this experiment are shown in boldface, and the calculated sizes of T-RFs are indicated in parentheses. The scale bar represents 10 mutations per 100 sequence positions.

amounts of ammonium, a shift was observed for T-RFLP. Patterns were similar for these two samples but differed from those for the untreated soil and soil treated with a small amount of ammonium. Although T-RFLP has a slightly higher level of resolution than DGGE (24), community shifts for our incubation conditions generally are probably more likely to occur for denitrifiers than for ammonia oxidizers, since the latter are slow-growing organisms (35). Consequently, an adaptation period of 4 weeks might be too limited.

To confirm the specificities of T-RFs, amplified *nirK* genes from soil sample MA were cloned and screened by T-RFLP, and representative clones were sequenced. Screening revealed that the clones corresponded to major T-RFs, but not all T-RFs detected in the T-RFLP of the soil sample were found in the clone library. The screening results were confirmed by in silico analysis of restriction sites. Generally, the sizes of T-RFs determined by gel electrophoresis seemed to be below the calculated size by 1 or 2 bp. Thus, clones AgMA6 and AgMA36 would represent the T-RF of 59 bp rather than that of 61 bp, although the relative abundance of the 61-bp T-RF exceeded that of the 59-bp T-RF by severalfold and thus it was expected to be cloned preferentially. However, cloning is known to be a factor that possibly introduces bias. Phylogenetic analysis placed *nirK* genes into soil cluster I, consisting of *nirK* genes obtained from marsh wetland soil and forested upland soil in Michigan (30). One clone clustered with clones from soil cluster III, consisting of other marsh wetland clones. The origins of these genes remain obscure, since isolates with this genotype are lacking. Thus, the genes seem to be derived from novel, presumably yet-uncultivated denitrifiers present in temperate world soils. Similar to ammonia oxidizers, no *nirK* clones closely related to those found in a highly acidic soil were found (32), suggesting that pH is indeed a selective factor. However, in their study, Rösch et al. used a different set of *nirK* primers (32). Thus, soil cluster II was either not detected in our soil due to bias introduced by our PCR primers or it was indeed absent. Surprisingly, in our soil sample, *nirK* genes were found which show close relationship to genes from denitrifying strains of *Ochrobactrum* sp. and *Alcaligenes faecalis*. Genes were also found within the *Hyphomicrobium-Rhizobium* radiation, although they were not as closely related. Strains of *Ochrobactrum* spp., *Hyphomicrobium* spp., and *Rhizobium* spp. are readily isolated from soils. This suggests that these cloned *nirK* genes which cluster with the soil bacteria originate from similar soil organisms. However, the *nirK* genes also might be derived from organisms with a different phylotype, since the phylogeny of *nirK* is not consistent with the phylogeny of these organisms generally, e.g., *Ochrobactrum* spp. and *A. faecalis*, belonging to the α - and β -*Proteobacteria*, respectively.

Our results show that rates of N₂O emission from soil are positively correlated to soil ammonium concentrations. The contribution of ammonia oxidation to N₂O release increases and thus provides the electron acceptor for denitrification. Consequently, both processes lead to higher overall emission rates. Community shifts were negligible for ammonia oxidizers and the total bacterial community but were detected on the level of the faster-growing denitrifiers. Thus, adaptation occurs on the level of physiological status rather than by community shifts.

ACKNOWLEDGMENTS

We acknowledge Sonja Fleissner for excellent technical assistance. This study was supported financially by the German Federal Ministry for Education and Research within the BIOLOG Biodiversity Program (01LC0021).

REFERENCES

1. Aakra, A., J. B. Utaker, and I. F. Nes. 2001. Comparative phylogeny of the ammonia monooxygenase subunit A and 16S rRNA genes of ammonia-oxidizing bacteria. *FEMS Microbiol. Lett.* **205**:237–242.
2. Aakra, A., J. B. Utaker, A. Pommerening-Roser, H. P. Koops, and I. F. Nes. 2001. Detailed phylogeny of ammonia-oxidizing bacteria determined by rDNA sequences and DNA homology values. *Int. J. Syst. Evol. Microbiol.* **51**:2021–2030.
3. Amann, R. L., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**:143–169.
4. Braker, G., H. L. Ayala-del-Rio, A. H. Devol, A. Fesefeldt, and J. M. Tiedje. 2001. Community structure of denitrifiers, *Bacteria*, and *Archaea* along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nirS*) and 16S rRNA genes. *Appl. Environ. Microbiol.* **67**:1893–1901.
5. Braker, G., A. Fesefeldt, and K. P. Witzel. 1998. Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Appl. Environ. Microbiol.* **64**:3769–3775.
6. Braker, G., J. Zhou, L. Wu, A. H. Devol, and J. M. Tiedje. 2000. Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment communities. *Appl. Environ. Microbiol.* **66**:2096–2104.
7. Bruns, M. A., M. R. Fries, J. M. Tiedje, and E. A. Paul. 1998. Functional gene hybridization patterns of terrestrial ammonia-oxidizing bacteria. *Microb. Ecol.* **36**:293–302.
8. Bruns, M. A., J. R. Stephen, G. A. Kowalchuk, J. I. Prosser, and E. A. Paul. 1999. Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. *Appl. Environ. Microbiol.* **65**:2994–3000.
9. Conrad, R. 1996. Soil microorganisms as controllers of atmospheric trace gases (H₂, CO, CH₄, OCS, N₂O, and NO). *Microbiol. Rev.* **60**:609–640.
10. Crutzen, P. J. 1970. Influence of nitrogen oxides on atmospheric ozone content. *Q. J. R. Meteorol. Soc.* **96**:320.
11. Dickinson, R. E., and R. J. Cicerone. 1986. Future global warming from atmospheric trace gases. *Nature* **319**:109–115.
12. Felsenstein, J. 1993. PHYLIP: phylogeny inference package (version 3.5c). University of Washington, Seattle.
13. Gödde, M., and R. Conrad. 1999. Immediate and adaptational temperature effects on nitric oxide production and nitrous oxide release from nitrification and denitrification in two soils. *Biol. Fertil. Soils* **30**:33–40.
14. Hastings, R. C., M. T. Ceccherini, N. Miclaus, J. R. Saunders, M. Bazzicalupo, and A. J. McCarthy. 1997. Direct molecular biological analysis of ammonia oxidizing bacteria population in cultivated soil plots treated with swine manure. *FEMS Microbiol. Ecol.* **23**:45–54.
15. Henckel, T., U. Jäkel, S. Schnell, and R. Conrad. 2000. Molecular analyses of novel methanotrophic communities in forest soil that oxidize atmospheric methane. *Appl. Environ. Microbiol.* **66**:1801–1808.
16. Kandeler, E., and H. Gerber. 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. *Biol. Fertil. Soils* **6**:68–72.
17. Kowalchuk, G. A., P. L. E. Bodlier, G. H. Heilig, J. R. Stephen, and H. J. Laanbroek. 1998. Community analysis of ammonia-oxidizing bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridization. *FEMS Microbiol. Ecol.* **27**:339–350.
18. Kowalchuk, G. A., Z. S. Naoumenko, P. J. Derikx, A. Felske, J. R. Stephen, and I. A. Arkhipchenko. 1999. Molecular analysis of ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in compost and composted materials. *Appl. Environ. Microbiol.* **65**:396–403.
19. Kowalchuk, G. A., and J. R. Stephen. 2001. Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annu. Rev. Microbiol.* **55**:485–529.
20. Kowalchuk, G. A., A. W. Stienstra, G. H. Heilig, J. R. Stephen, and J. W. Woldendorp. 2000. Changes in the community structure of ammonia-oxidizing bacteria during secondary succession of calcareous grasslands. *Environ. Microbiol.* **2**:99–110.
21. Kowalchuk, G. A., A. W. Stienstra, G. H. Heilig, J. R. Stephen, and J. W. Woldendorp. 2000. Molecular analysis of ammonia-oxidizing bacteria in soil of successional grasslands of the Drentsche A (The Netherlands). *FEMS Microbiol. Ecol.* **31**:207–215.
22. Lüdemann, H., I. Arth, and W. Liesack. 2000. Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy soil cores. *Appl. Environ. Microbiol.* **66**:754–762.
23. Mendum, T. A., R. E. Sockett, and P. R. Hirsch. 1999. Use of molecular and isotopic techniques to monitor the response of autotrophic ammonia-oxidiz-

- ing populations of the β subdivision of the class *Proteobacteria* in arable soils to nitrogen fertilizer. *Appl. Environ. Microbiol.* **65**:4155–4162.
24. Moeseneder, M. M., J. M. Arrieta, G. Muyzer, C. Winter, and G. J. Herndl. 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **65**:3518–3525.
 25. Müller, C., R. R. Sherlock, and P. H. Williams. 1998. Field method to determine N₂O emission from nitrification and denitrification. *Biol. Fertil. Soils* **28**:51–55.
 26. Muyzer, G., T. Brinkhoff, U. Nübel, C. Santegoeds, H. Schafer, and C. Wawer. 1997. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, p. 1–27. *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.
 27. Norton, J. M., J. M. Low, and M. G. Klotz. 1996. The gene encoding ammonia monooxygenase subunit A exists in three nearly identical copies in *Nitrosospira* sp. NpAV. *FEMS Microbiol. Lett.* **139**:181–188.
 28. Oved, T., A. Shaviv, T. Goldrath, R. T. Mandelbaum, and D. Minz. 2001. Influence of effluent irrigation on community composition and function of ammonia-oxidizing bacteria in soil. *Appl. Environ. Microbiol.* **67**:3426–3433.
 29. Phillips, C. J., D. Harris, S. L. Dollhopf, K. L. Gross, J. I. Prosser, and E. A. Paul. 2000. Effects of agronomic treatments on structure and function of ammonia-oxidizing communities. *Appl. Environ. Microbiol.* **66**:5410–5418.
 30. Priemé, A., G. Braker, and J. M. Tiedje. 2002. Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils. *Appl. Environ. Microbiol.* **68**:1893–1900.
 31. Purkhold, U., A. Pommerening-Roser, S. Juretschko, M. C. Schmid, H. P. Koops, and M. Wagner. 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. *Appl. Environ. Microbiol.* **66**:5368–5382.
 32. Rösch, C., A. Mergel, and H. Bothe. 2002. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. *Appl. Environ. Microbiol.* **68**:3818–3829.
 33. Rothauwe, J. H., K. P. Witzel, and W. Liesack. 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* **63**:4704–4712.
 34. Schlichting, E., and H. P. Blume. 1966. *Bodenkundliches Praktikum*. Paul Parey, Hamburg, Germany.
 35. Schmidt, E. L. 1982. Nitrification in soil. *Agronomy* **22**:253–288.
 36. Schuster, M., and R. Conrad. 1992. Metabolism of nitric-oxide and nitrous-oxide during nitrification and denitrification in soil at different incubation conditions. *FEMS Microbiol. Ecol.* **101**:133–143.
 37. Skiba, U., and K. A. Smith. 2000. The control of nitrous oxide emissions from agricultural and natural soils. *Glob. Change Sci.* **2**:379–386.
 38. Stephen, J. R., G. A. Kowalchuk, M. A. V. Bruns, A. E. McCaig, C. J. Phillips, T. M. Embley, and J. I. Prosser. 1998. Analysis of β -subgroup proteobacterial ammonia oxidizer populations in soil by denaturing gradient gel electrophoresis analysis and hierarchical phylogenetic probing. *Appl. Environ. Microbiol.* **64**:2958–2965.
 39. Stephen, J. R., A. E. McCaig, Z. Smith, J. I. Prosser, and T. M. Embley. 1996. Molecular diversity of soil and marine 16S rRNA gene sequences related to beta-subgroup ammonia-oxidizing bacteria. *Appl. Environ. Microbiol.* **62**:4147–4154.
 40. Webster, G., T. M. Embley, and J. I. Prosser. 2002. Grassland management regimes reduce small-scale heterogeneity and species diversity of β -proteobacterial ammonia oxidizer populations. *Appl. Environ. Microbiol.* **68**:20–30.