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IMPACT OF HERBICIDES ON THE ABUNDANCE AND STRUCTURE OF INDIGENOUS β-SUBGROUP AMMONIA-OXIDIZER COMMUNITIES IN SOIL MICROCOSMS

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Abstract—In this study, mixtures of five herbicide-formulated products (atrazine, dicamba, fluometuron, metolachlor, and sulfentrazone) were applied to soil microcosm columns in increasing concentrations. The toxic impact of herbicides on the indigenous β-subclass Proteobacteria autotrophic ammonia-oxidizer (β-AAO) community was assessed. The β-AAO population abundances were estimated by competitive polymerase chain reaction (PCR) targeting the gene *amoA*, encoding the α-subunit of ammonia monooxygenase. Community structure was examined by PCR and denaturing gradient gel electrophoresis targeting 16S rDNA with band excision and sequence analysis, and by analysis of *amoA* gene fragment clone libraries. The 16S rDNA analyses showed that a single ribotype of *Nitrosospira* cluster 3 was the dominant β-AAO in all treatments. At a finer scale, *amoA* clone library analysis suggested a shift in community structure corresponding to the 100-ppm application. Competitive PCR indicated significant differences between treatments. The control exhibited relatively stable population abundance over the time period examined. The 10-ppm treatment induced a population increase, but a significant decrease was induced by the 100-ppm application. At 1,000 ppm, the ammonia-oxidizer population dropped below the method detection limit by the first sampling point. An impact on ammonia oxidizers resulting from the application of herbicides was observed, both in abundance and community structure.

Keywords—Herbicide *Nitrosospira* 16S rDNA Population analysis Bioavailability

INTRODUCTION

The autotrophic ammonia-oxidizing bacteria are largely responsible for the oxidation of ammonia to nitrite and play a key role in the global cycling of nitrogen [1]. The first step in the oxidation of ammonia to nitrite is the oxidation of ammonia to hydroxylamine, catalyzed by ammonia monooxygenase [2]. Nitrifiers are found in most aerobic environments where organic matter is mineralized, and have been isolated from soil, freshwater, brackish water, seawater, and sewage disposal systems [3].

Difficulties in isolating and cultivating these slow-growing chemoautotrophs have favored the application of molecular biological methods to the study of their ecology in various environments [4,5]. Comparative 16S rRNA gene sequencing analysis revealed that ammonia oxidizers constitute two monophyletic assemblages [6]. The first assemblage is characterized by *Nitrosococcus oceani* and *Nitrosococcus halophilus* [7] in the γ-subclass of the Proteobacteria. The second group is a monophyletic assemblage within the β-subclass of the class Proteobacteria and comprises the majority of known isolates. This lineage is divided into two subgroups characterized by *Nitrosomonas* spp. (including *Nitrosococcus mobilis*) and *Nitrosospira* spp. (including strains formerly classified as *Nitrosolobus* and *Nitrosovibrio* spp.) [6]. Only autotrophic ammonia oxidizers of the β-subclass of the class Proteobacteria (β-AAOs) have been implicated in nitrification in terrestrial environments. Oligonucleotide primers targeting the 16S rDNA have been developed for the selective and specific recovery of all β-subdivision ammonia-oxidizer sequences from environmental samples [8,9]. Kowalchuk et al. [9] pioneered the use of denaturing gradient gel electrophoresis (DGGE) to analyze 16S rDNA for this group, thus generating 16S rDNA fingerprints for in situ ammonia-oxidizer communities. In addition to 16S rRNA, the functional ammonia monooxygenase gene (*amo*) has provided a molecular detection and fine-scale characterization of indigenous ammonia-oxidizing populations [10].

Because of their ecological importance, environmental studies on ammonia oxidizers have been well documented. As a ubiquitous and phylogenetically coherent group of organisms, ammonia oxidizers are a potential target group as indicator species of the bioavailability of anthropogenic chemicals. Changes in the community structure of ammonia oxidizers have been observed to occur in field studies in relation to soil pH and agricultural use, and in marine sediments as a result of contamination from fish cages [4,11–13]. Adaptation of ammonia oxidizers to hydrocarbon contamination in soil has also been observed [14], although whether this entailed changes in community size or structure has not been established. Furthermore, these organisms are also of potential interest to the field of bioremediation of metals in soils, Stephen et al. [15] recently demonstrated that a shift in β-ammonia-oxidizer community structure could be induced rapidly by metal contamination in agricultural soil microcosms.

The problem of environmental contamination due to a worldwide application of agricultural herbicides is increasing. Since World War II, the use of fertilizers, and later herbicides, has been rising to increase crop yield. Expansion of industrial fertilizers and herbicides in modern agriculture contribute substantially to both soil and groundwater contamination. The

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environmental chemistry, fate, and toxicology of herbicides already have been studied in great detail [16–18], and their applications were reported to decrease the bacterial diversity in soils and alter the functional ability of soil microbial communities [19–21].

However, little is known about the impact of herbicides and fertilizers on indigenous soil microbial community structures. The autotrophic ammonia-oxidizing bacteria of the β-subgroup of the Proteobacteria may make a useful indicator group in this respect. Many herbicides in common use are urea-based (e.g., chlorsulfuron, fluometuron, isoproturon, linuron, metobromuron, and monuron), and therefore may enhance growth of ureolytic ammonia-oxidizer species. Conversely, herbicides also target the carbon-fixing Calvin cycle, common to all autotrophic organisms, and therefore herbicide use may select for resistant ammonia-oxidizing strains (both atrazine and fluometuron are inhibitors of the Calvin cycle, as is sulfentrazone, to a less extent). A negative impact on soil nitrification previously was associated with herbicides [22]. In this microcosm study we sought to profile β-ammonia-oxidizer populations associated with herbicide application of various concentrations, over a time course. We targeted ammonia-oxidizer 16S rDNA by a polymerase chain reaction (PCR) and DGGE analysis [9] and the β-subunit of ammonia monooxygenase (*amoA*) [10] to monitor the community structure. Competitive PCR of the *amoA* gene was used to monitor population size changes [15]. Aims of this study were to test the hypotheses that indigenous β-AAO populations would change in terms of the diversity of dominant β-AAO 16S and *amoA* genes with herbicide contamination; and that β-AAO populations would respond to herbicide exposure via changed population size.

MATERIALS AND METHODS

Soil microcosms

A Whitwell loam soil containing 41% sand, 39% silt, and 20% clay was collected from The University of Tennessee Agricultural Experiment Station, Plant and Soil Science Unit (Knoxville, TN, USA) and air-dried. The soil was sieved through a 6.35-mm (0.25-inch) screen, then through a 3.2-mm (0.125-inch) screen. The soil was mixed with the formulated product of five herbicides (atrazine 4L, dicamba 4 emulsifiable concentrate, fluometuron 4L, metolachlor 7.8EC, and sulfentrazone 75 dilution factor [75DF]) to achieve final concentrations of active ingredient of 0, 10, 100, and 1,000 ppm for each herbicide.

Column design and packing. A 10-cm-diameter by 25-cm-tall thin-walled polyvinyl chloride pipe fitted with two polypropylene barbs was used as the column housing. The hose barbs were placed at the bottom and top of the column, with hoses attached to the barbs and a water supply bottle. Columns were packed with 2.77 to 2.88 kg of soil to yield a uniform bulk density in the range of 1.34 to 1.4 g/cm^3. When active, the water was fed through the siphon tube to the bottom fitting. The water was gravity fed, and once the water level reached the top fitting it entered the vacuum tube, which prevented any more water from entering the column.

Soil columns were placed randomly in dark environmental chambers with a continual airflow of ≈3.8 m/s and ambient temperature (25°C). Columns were alternated in a 5-d flood–dry cycle. At 30, 60, and 90 d, columns of each concentration were sacrificed and a portion of the contents frozen at −80°C until biological analysis. Another portion was dried and used for soil chemical analysis.

Chemical analysis

All chemical analyses were conducted with dry soil. The soil was crushed and homogenized with a mortar and pestle before analysis. Soil pH was measured with a Corning pH meter 440 (Corning, NY, USA) on a 1:2 soil:water paste (v/v). Soil was extracted with 1 M KCl and the extracts were assayed for ammonium and nitrate by the indophenol blue method adapted for microtiter plates by Sims et al. [23]. Color intensity was measured with a 7250 microplate reader (Cambridge Technology, Watertown, MA, USA) with a 650-nm filter.

DNA extraction and PCR

The DNA was extracted directly from soil samples by mechanical disruption [15]. Briefly, soil (0.5 g), sodium phosphate buffer (425 μl; 0.12 M, pH 8.0), chaotropic reagent (175 μl; CRSR, BIO-101, Vista, CA, USA), and 0.17-mm glass beads (0.5 g) were agitated in a 1.5-ml microcentrifuge tube with a high-speed (6.5-m/s) BIO 101 FastPrep (FP120, BIO-101, Vista) for 45 s. The sample mixture was centrifuged at 13,000 *g* for 5 min and the supernatant was collected. Chloroform (300 μl) was added to the soil pellet, mixed thoroughly, and centrifuged at 13,000 *g* for 5 min. The aqueous supernatant was collected and combined with the first supernatant fraction. The DNA was precipitated from the aqueous phase with an equal volume of isopropanol in an ice bath for 30 min. The DNA was pelleted by centrifugation at 13,000 *g* at 4°C for 15 min, washed with 80% ethanol (1 ml) twice, air-dried, and redissolved in Tris-ethylenediaminetetraacetic acid (Tris-EDTA) buffer (200 μl; pH 8.0). The DNA extract was purified by extracting twice with an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1, v/v/v), followed by a glass milk DNA purification protocol with a GeneClean kit (BIO-101, Vista) as described by the manufacturer.

Construction and sequence analysis of amoA *gene fragment libraries*

The *amoA* fragments were amplified with the primers and PCR conditions described by Rotthauwe et al. [10], as modified by Stephen et al. [15]. Amplification products were gel purified and extracted with a GeneClean kit. Purified fragments were cloned with the vector PCR2.1 TOPO® and *Escherichia coli* TOP10F′® competent cells according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The *amoA* products from duplicate samples were mixed and gel purified before cloning. Representative plasmids from each clone library were selected randomly across control and the 10-ppm and 100-ppm treatment soil for sequencing (four clones from control; average of three clones each from 10 ppm at 30, 60, and 90 d, respectively; average of four clones each from 100 ppm at 30, 60, and 90 d; more clones were picked up at 100 ppm to ensure a better reflection of the community). The cloned inserts were reamplified with the vector primers M13 reverse and T7 (Invitrogen; 30 cycles of 94°C, 30 s; 55°C, 30 s; and 72°C, 45 s). The M13 reverse/T7 amplification product was gel purified, extracted with a GeneClean kit, and subjected to double-strand sequencing with the same primers on an Applied Biosystems automated sequencer (model 310, Foster City, CA, USA) with prism big-dye terminators.

Quantitative PCR

Competitive PCR analysis of *amoA* template numbers was performed as described in Stephen et al. [15], with the ex-

Table 1. Quantitative measurements of data relevant to nitrification processes

Samples[a]	Nitrate (ppm)	Ammonium (ppm)	pH	Herbicide level (ppm each herbicide)
Control-0	22.9 ± 1.07	8.42 ± 0.25	5.31 ± 0.11	0
Control-30	11.82 ± 1.99	6.09 ± 2.96	5.48 ± 0.09	0
Control-60	8.79 ± 2.01	10.88 ± 4.66	5.63 ± 0.15	0
Control-90	13.31 ± 2.20	7.8 ± 3.65	5.48 ± 0.03	0
10-30	10.91 ± 0.72	14.26 ± 1.85	5.60 ± 0.08	10
10-60	9.8 ± 0.98	7.39 ± 1.25	5.58 ± 0.03	10
10-90	9.05 ± 2.59	5.12 ± 1.19	5.58 ± 0.13	10
100-30	9.31 ± 1.71	31.72 ± 0.74	5.84 ± 0.08	100
100-60	8.94 ± 1.93	33.62 ± 2.77	5.84 ± 0.08	100
100-90	6.68 ± 1.14	35.14 ± 4.70	5.85 ± 0.10	100
1,000-30	13.81 ± 1.81	30.07 ± 13.45	5.38 ± 0.06	1,000
1,000-60	12.72 ± 0.87	24.19 ± 10.65	5.50 ± 0.06	1,000
1,000-90	9.68 ± 1.15	38.39 ± 12.94	5.45 ± 0.09	1,000

[a] Samples indicate the herbicide concentration followed by the time point (d) of sampling.

ception that the competitive standard was prepared by amplification of clone p428-NAB_8_23 with the vector-specific primers M13 reverse and T7. The product was purified with a Geneclean Spin-Column (BIO-101, VISTA) to provide the *amoA* deletion fragment flanked by 70 base pairs of vector sequence on each end. Reactions were analyzed by electrophoresis on 2% (w/v) agarose TEA (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) gels containing ethidium bromide at 0.2 μg/ml. Band density was quantified by means of UVP Image Analysis Software (Ultra-Violet Products, Upland, CA, USA).

PCR-DGGE analysis

With DNA extracted from soils as template, PCR was performed with universal bacterial primers 27F and 1492R, amplifying nearly the full length of the 16S fragment [24]. A nested PCR was then performed with the CTO189f-GC and CTO654r primers, which are designed to specifically target 16S rDNA β-subgroup ammonia oxidizers, with the addition of a 40-base-pair clamp to facilitate DGGE analyses [9]. Reactions used 1.0 ng of template DNA in a total volume of 25 μl and the PCR conditions described by Kowalchuk et al. [9].

Amplification product concentrations were estimated by comparison to known standards after agarose gel electrophoresis (1.2% agarose, 1 × TAE, 40 mM Tris-acetate, 1.0 mM EDTA, pH 8.0) and ethidium bromide staining. Approximately 200 ng of PCR product per sample was subjected to DGGE, according to the protocol of Muyzer et al. [25] as adapted for the study of ammonia-oxidizing bacteria [9] (8% polyacrylamide, 1.5-mm thickness, 0.5 × TAE; 37:1 acrylamide:bisacrylamide [v/v], 38–50% denaturant; 200 × 200 mm, 85 V for 16 h at 60°C). The DNA bands to be sequenced were excised from the gel, placed into sterile Eppendorf tubes containing 36 μl of sterilized water, and incubated for 2 d at −20°C with occasional thawing. A 5.0-μl volume of the DNA diffused in water served as a template for PCR amplification. Excess primers and unincorporated nucleotides were removed from the PCR products with GeneClean Spin-Columns.

Sequence analysis

Reamplified PCR-DGGE products of 16S rDNA fragments from gel slices were sequenced with the primer 519r [24]. The *amoA* sequences were obtained after reamplification of cloned DNA with the vector-specific primers M13 reverse and T7, and purification through Geneclean Spin-Columns. These products were then sequenced in both directions with the same vector primers as described above. Sequences were assembled with SeqPup Version 0.6 [26]. Reference sequences were retrieved from GenBank via the National Institute for Biotechnology Information [NCIB; *http://www.ncbi.nlm.nih.gov*] Internet node with the Entrez facility [27]. Phylogenetic algorithms (DNA-DIST, NEIGHBOR, and SEQBOOT) also operated within the ARB software environment [28]. Ribosomal sequences were screened for possible chimeric origin by use of the RDP CHECK CHIMERA program [29], and *amoA* sequences were checked for chimeric origin by independent neighbor-joining analysis of the 5′ and 3′ halves of sequences within an alignment of all published *amoA* sequences.

Nucleotide accession numbers

Partial 16S sequences recovered from DGGE gels were submitted to GenBank with the accession numbers AY010070 to AY010076 and partial cloned *amoA* sequences were submitted with the accession numbers AY010077 to AY010102.

Statistical analysis

Chi-square and Student's *t* tests were performed with a SigmaPlot® spreadsheet (Jandel Scientific, San Rafael, CA, USA). Pearson product-moment correlation was performed with the Statistic software package (Statsoft®, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Impact of herbicides on nitrification

Crop producers are most interested in two major forms of N: ammonium (NH_4^+) and nitrate (NO_3^-). When ammonium-N is added to the soil, it is subject to nitrification whereby ammonia oxidizers convert the ammonium to nitrate. If ammonium-N is not immediately taken up by plants, it is converted to nitrate under aerobic soil conditions. The use of a nitrification inhibitor will stop or slow the conversion of ammonium to nitrate. Nitrification by ammonia-oxidizing bacteria is known to be susceptible to inhibition by several environmental stress conditions such as a wide range of compounds, light, pH, and even temperature shifts [30,31]. In this study, the pH of all samples was in the range of 5.3 to 5.8 (Table 1), which is suitable for the growth and activity of ammonia-oxidizing bacteria [32].

Ammonium levels in microcosms exposed to 100 ppm of

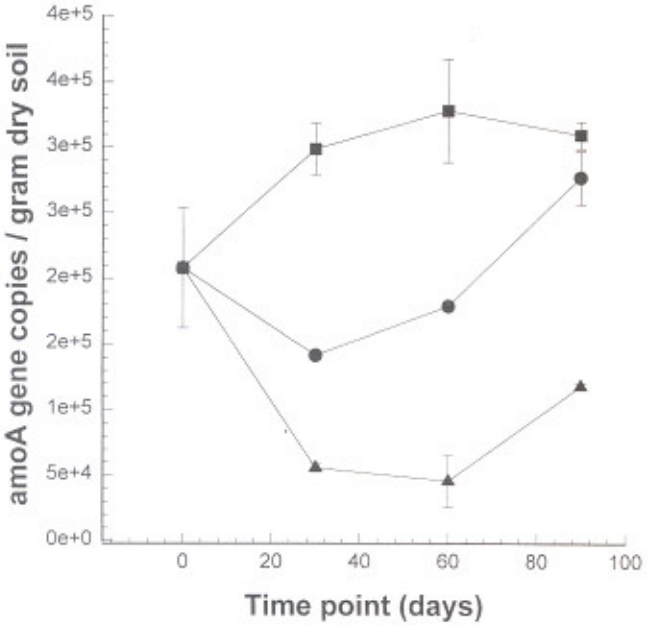

Fig. 1. Quantification of *amoA* target sequences by competitive polymerase chain reaction. Error bars indicate standard deviation. The symbols represent soil samples with 100-ppm (▲), 10-ppm (■), and 0-ppm (●) herbicide treatments.

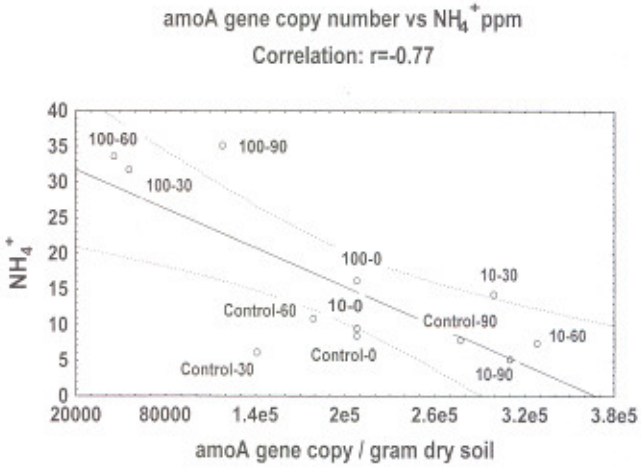

Fig. 2. Scatter-plot of *amo*A gene copy per gram of dry soil versus NH_4^+ (ppm). Dotted lines indicate 95% confidence limits.

each herbicide or above increased significantly, whereas nitrate was relatively stable and was not affected (Table 1). This is likely attributed to the application of herbicides, which all contain an amino group with the potential of releasing NH^{4+} into the microcosm soil, particularly fluometuron, which is a urea-based herbicide. The increasing ammonium levels suggested a drop in soil nitrification, possibly due to inhibition of β-AAO activity or cell death. In agreement with our observation, herbicide treatment has been reported to inhibit nitrification after two weeks in both sandy loam soil and organic soils [22].

Abundance of β-AAO determined by quantitative PCR

The effect of herbicide application on soil indigenous ammonia-oxidizer population size was estimated by competitive PCR. Changes in *amoA* target numbers per gram of dry soil were plotted over the experimental period (Fig. 1). The number of *amoA* gene copies detected in all four herbicide-treated soils were significantly different ($p < 0.05$) and ranged from 10^4 to 10^5 gene copies per gram of dry (lyophilized) soil. However, considering loss of DNA during extraction, and the finding that β-AAOs generally carry two or three copies of *amoA* per genome [33], these numbers cannot be extrapolated directly to infer cell numbers. Note that this form of analysis generally suggests higher cell numbers than culture-based most probable number analysis [34]. The control soil contained about 2×10^5 *amoA* gene copies per gram of dry soil and remained relatively stable over the experimental time, with some minor fluctuations (±1–10%). At 10-ppm application of the herbicide, the *amoA* gene copies showed a significant increase compared with the control soil, whereas the number decreased significantly by an order of magnitude (to about 5×10^4 copies per gram of dry soil) with 100-ppm application. However, some recovery at the end of the experiment was observed. No *amoA* genes were detectable in the soil treated with 1,000-ppm herbicides by the first postexposure sampling date (day 30). The low concentration (10 ppm) of herbicides may have served as a nutrient source to indigenous β-AAOs, most probably because of release of growth substrates from fluometuron, which is a urea-based herbicide. At higher levels, a toxic impact of herbicides on the abundance of the β-AAO community became obvious (100 ppm) and the *amoA* gene was no longer detectable shortly after exposure to 1,000-ppm herbicide. These results were in agreement with the elevated ammonium levels seen in microcosms exposed to higher herbicide levels. A negative correlation ($R = -0.77$; $p \leq 0.05$) was found between soil $[NH^{4+}]$ and *amoA* gene copy numbers per gram of dry soil. A scatter plot of this data is shown in Figure 2.

Phylogenetic diversity of β-AAOs determined by 16S rDNA

The phylogenetic diversity of the AAO populations in herbicide-treated microcosm samples was examined by 16S rDNA PCR-DGGE and band-excision as described by Kowalchuk et al. [9]. Primary amplification of 16S rDNA fragments from β-subgroup ammonia-oxidizer DNA from DNA extracts was unsuccessful in all samples. Initial amplification of nearly full-length 16S rDNA fragments from all bacteria, followed by reamplification of the recovered product with ammonia-oxidizer 16S rDNA primers generated strong products from all control and 10-ppm and 100-ppm treated soil samples. No or weak products were generated from 1,000-ppm treated soil samples from the first postexposure sampling date onward. The DGGE analysis revealed the presence of a dominant doublette band comigrating with standards representing cluster 2 and 3 *Nitrosospira* [9] from all successful amplifications (data not shown). Amplification products generated and analyzed in this way typically produce doublette bands during DGGE analysis because of the presence of an ambiguous base in the reverse (unclamped) PCR primer [9]. This dominant band was recovered from all control and 10-ppm and 100-ppm application soils. Successful amplification was achieved from only one 1,000-ppm application at day 30; all others failed. Sequence analysis of reamplified material derived from these bands demonstrated that a single *Nitrosospira*-like ribotype, identical to the corresponding region of the 16S rDNA gene of *Nitrosospira* L115 (a ureolytic strain originally isolated from peatbog, Finland [35]) dominated all analyzed communities. Other bands visible in the single successful 1,000-ppm amplification were derived from β-subclass proteobacteria out

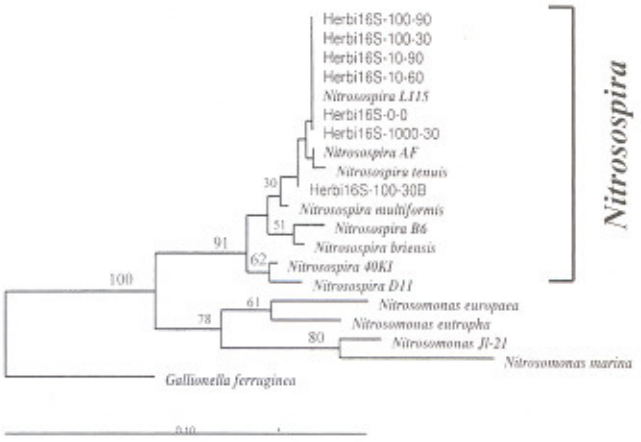

Fig. 3. Neighbor-joining analysis of 16S rDNA fragments recovered from polmerase chain reaction–denaturing gradient get electrophoresis bands. The relationship is shown of herbicide microcosm partial 16S rDNA sequences to partial sequences from reference ammonia oxidizers. Bootstrap values (%) on 100 replicates are given at nodes when they exceed 30% of replicates. The scale equals 10% estimated substitutions. Sequences labeled Herbi16S were generated in this study. The prefix Herbi16S is followed by the herbicide concentration and the time point (d) of the sample from which the sequence was recovered; B indicates the minor band. Reference sequences were selected as the closest matching cultured organisms or cloned sequences where no closely matching cultures were available: *Nitrosospira* B6, *N.* AF, *N.* 4OKI, *N.* D11, *N.* L115 [35]; *N. briensis, N. tenuis, N. multiformis, Nitrosomonas europaea, N. eutropha* [6]; *N. marina* [37]; and *N.* JL-21 [38].

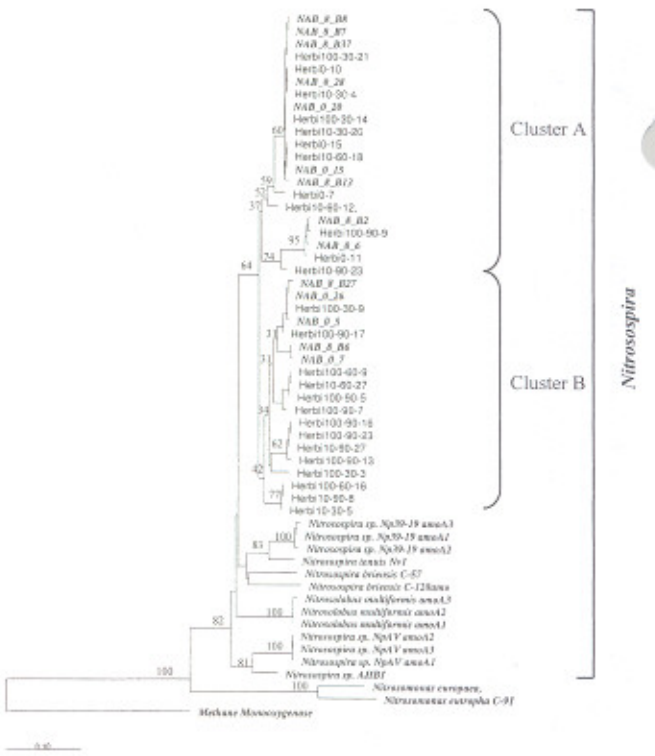

Fig. 4. Neighbor-joining analysis of *amoA* fragments randomly selected from clone libraries. Sequences prefixed Herbi were generated during this study. The prefix is followed by the herbicide concentration and time point (d). Other sequences: *N. briensis* str. C57, *N. tenuis* NV1, *N. eutropha* C-91 [10]; *N.* AHB1 [39]; *Nitrosolobus multiformis,* copies *amo*A1, *amo*A2, *amo*A3 (accession nos. U91603, U15733, and U89833); *N. briensis* C128 (accession no. U76553); *N.* sp. 39-19, copies *amo*A1, *amo*A2, *amo*A3 (accession nos. AF 042170, AF 016002, and AF006692); *N. europaea* [40]; *N.* AV, copies *amo*A1, *amo*A2, *amo*A3 (accession nos. AF032438, AF016003, and U92432). Sequences prefixed NAB are cloned *amo*A fragments recovered from a geographically similar soil [15]. Numbers on tree refer to bootstrap values (%) on 100 replicates; only values above 30 are given. Scale bar represents 10% estimated change.

of ammonia-oxidizer radiation (data not shown). Such occasional nonspecific amplification with these primers has been reported previously [13]. A faint band common to all samples was also recovered from one sample, sequencing of which demonstrated the presence of a novel, minor *Nitrosospira*-like ribotype (Herbi16S-100-30B in Fig. 3). Sequence clusters detected are presented in Figure 3, a neighbor-joining tree pruned from a larger one containing a selection of proteobacterium reference sequences.

Diversity of amoA *sequences recovered*

Because unknown biases may be inherent in the use of any one set of PCR primers [36] and the slow rate of mutation of ribosomal genes may disguise ecological differences between species, a second AAO-specific marker, *amoA,* was recovered [10]. Representative plasmids from each clone library were randomly selected and subjected to DNA sequence analysis, providing an assessment of the diversity of *amoA* sequences present in these samples, and affording a finer scale of differentiation between population components than was possible by 16S analysis [10]. A total of 26 plasmids was sequenced. All of the recovered sequences appeared to be *amoA* fragments as judged by database searching of their primary and predicted amino acid sequences. A comparison of the *amoA*-like sequences recovered with reference sequences from cultured AAOs and environmental *amoA* clones (Fig. 4) demonstrated a generally good agreement with the population structures suggested by 16S rDNA analysis, in so much as a predominance of *Nitrosospira*-like clones was recovered. The *amoA* sequence of *Nitrosospira* L115 has not been established.

The clones recovered fell into two clusters, designated A and B (Figs. 4 and 5). In control soil, all *amoA* sequences detected belonged to cluster A. Cluster B sequences emerged when 10-ppm herbicides were applied and were dominant in clone libraries from microcosms exposed to 100 ppm of each herbicide. This suggests that a community shift may have been induced by herbicides, providing a selective advantage for the source organisms of cluster B *amoA* sequences over cluster A. These major subclusters could be further subdivided into

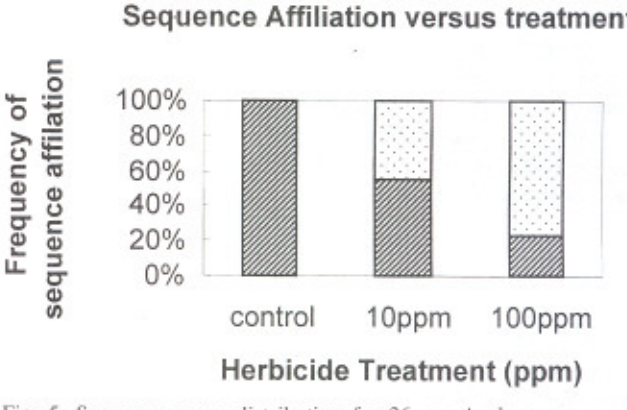

Fig. 5. Sequence group distribution for 26 *amo*A clone sequences obtained from microcosms with different herbicide concentration applied. At 1,000-ppm application, *amo*A sequences were below detection limits. ▨, cluster A; ⬚ cluster B.

groupings indicative of multiple *Nitrosospira*-like species. Most β-AAO strains contain two to four nearly identical copies of the *amoA* gene (see *Nitrosospira* sp. 39-19, NpAV, and *Nitrosospira* [*Nitrosolobus*] *multiformis* in Fig. 4) [33]. The depth of branching of *amoA* sequences derived from a single organism provides a measure of the level of variation that might be expected from a single source organism. The depth of branching between clusters of environmental *amoA* genes seen in Figure 4 indicates that *amoA* analysis detected at least eight distinct source organisms in these microcosms, whereas 16S rDNA analysis detected no more than two. This fully agrees with the concept that *amoA* provides a finer-scale environmental marker than does 16S rDNA for the β-AAO group. However, the survey-level sampling of *amoA* sequences applied here only provides weak evidence that herbicide treatment affected the subpopulations of ammonia oxidizers differentially.

CONCLUSIONS

The β-AOO communities in this soil are dominated by organisms closely related to *Nitrosospira* L115, a ureolytic strain. Herbicide application at low levels (10 ppm) induced an increase in β-AAO numbers, possibly because of the provision of growth substrates from the urea-based herbicide fluometuron. This dominant β-AAO population could be subdivided further into at least two groupings, based on *amoA* clone sequence analysis, designated clusters A and B. The source organisms of cluster A *amoA* genes dominated in the control soils, but herbicide application seemed to favor the source organisms of cluster B. Herbicide application at 10 ppm did not seem to have any toxic effects on the β-AAO population size or nitrification, but higher levels resulted in a rapid drop in β-AAO numbers and a correlating accumulation of ammonium. These results indicated that indigenous soil β-AAO populations may be suitable indicators of soil damage induced by excessive herbicide application, or recovery of soil from such damage.

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