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ORIGINAL PAPER

The effect of pharmaceutical waste-fungal biomass, treated to degrade DNA, on the composition of eubacterial and ammonia oxidizing populations of soil

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Abstract The aim of this work was to study variations in the composition of eubacteria and ammonia-oxidizing populations of soil, both determined by denaturing gradient gel electrophoresis (DGGE), after the addition of a pharmaceutical fungal biomass, treated to degrade its DNA. This waste can be used as an amendment. The fungal biomass waste was added at three rates: 0.05, 0.1, and 1% per dry weight of soil. Control soil, without any amendment, was also investigated. Total DNA was extracted, purified, and amplified by using either universal (eubacteria) or specific (*amo*A) primers. Amplicons were separated by DGGE. Sequencing was also carried out to better assess the diversity of ammonia oxidizing bacteria. Changes in the composition of eubacterial community were detected after 3 days only in the soil treated with the highest dose, while the ammonia oxidizing population responded more promptly (after 1 day) with evident modifications at level of *Nitrosolobus* like sequences.

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Introduction

Antibiotics are produced by pharmaceutical industry in large quantities through the fermentation of nutrient solutions by specific microbial species. The wastes originated from the use of fungal species have been used as organic fertilizers with beneficial effects on biological, chemical, and physical properties of soil (Haselwandter et al. 1988; Aescht and Foissner 1992). However, it has not been considered that these fertilizers can contain large amounts of DNA, and the massive application of these wastes rich in antibiotics encoding genes to soil can be risky because these genes can be incorporated by horizontal gene transfer into the genome of soil microflora (Andersen et al. 2001). For this reason, our laboratory and the ACS DOBFAR company have established a procedure to degrade DNA to low molecular weight fragments, and this procedure (described in Materials and methods) is now an Italian (IT MI20032129) and an European patent (EP 1 529 766 B1).

Amendment of soil with organic residues can affect the indigenous microbial communities and cycles of plant nutrients such as the N cycle (Hastings et al. 1997; Ceccherini et al. 2001). No studies have been carried out on the effect of fungal waste from antibiotic production on the composition of soil microbial communities by molecular techniques, which can determine unculturable and culturable microorganisms (Torsvik et al. 1998). Usually, these procedures are based on polymerase chain reaction (PCR) amplification of the small subunit of the rDNA extracted from soil. Among genetic fingerprinting methods,

denaturing gradient gel electrophoresis (DGGE) is useful for the rapid screening of the composition of soil microbial communities of multiple samples (Kowalchuk et al. 1997; Torsvik et al. 1998; Nannipieri et al. 2003). This technique can determine dominating populations (Øvreås et al. 1998; Smalla et al. 2001; Fromin et al. 2002), and by using specific primers, it is possible to assess the composition of specific subsets of the entire community (Sakano and Kerkhof 1998; Boon et al. 2002; Rowan et al. 2003; Agnelli et al. 2004; Zhou et al. 2004) or to determine particular microbial functional groups, such as ammonia oxidizing bacteria (AOB). These bacteria play a crucial role in the N cycle by oxidizing ammonium to nitrite and are very sensitive to environmental stresses (Hastings et al. 1997; Burton and Prosser 2001; Webster et al. 2002).

The objectives of this study were to study the effects of fungal biomass wastes, derived from the industrial production of antibiotics and treated to degrade fungal DNA, on (1) the composition of eubacterial communities and (2) the composition of AOB after DNA extraction from soil and amplification by specific primers followed by DGGE. Since DNA bands with identical electrophoretic mobility after DGGE may indicate equal DNA sequence, band sequencing was also carried out to better assess diversity of AOB at the genus level. Autotrophic nitrification, unlike most of microbial processes occurring in soil, is only carried out by AOB, and thus, they are considered key-stone species (Nannipieri et al. 2003). Since both N and P mineralization rates of this pharmaceutical waste are not known, we have preferred to use a broad range of application rate (0.05, 0.1, and 1.0% per dry weight of soil).

Materials and methods

Soil characteristics

The soil was sampled from the 0–15-cm soil layer at Romola, near Florence, in May 2004 and sieved at 2 mm. It had the following characteristics: pH 7.2; sand 81.9%; silt 6.7%; clay 11.4%; total organic C 0.7%; total organic N 0.07%.

Fungal biomass characteristics

Cephalosporium acremonium (synonym *Acremonium chrysogenum*) is used to produce Cephalosporin C, a β-lactam antibiotic (Adinarayana et al. 2003). The exhausted fungal biomass from an industrial bioreactor (ACS DOBFAR S.p.A., Tribiano, Milano, Italy) was treated with 0.1 M H_3PO_4 to reach pH 1, heated at 90°C for 60 min to degrade DNA and neutralized with 0.2 M $Ca(OH)_2$; in this way, the fungal waste can be used as soil amendment. After the treatment, the fungal biomass had the following characteristics: 40% organic C, 7% humic C, 7% total N, 6.5% N – NH_4^+, <0.5% N–NO_3, 1.5% P_2O_5, 0.5% K_2O; the amount of the residual Cephalosporin C was under the detection limit (<0.1 ppm) by high performance liquid chromatography (HPLC; data supplied by ACS DOBFAR). The efficacy of the procedure established to degrade DNA of the fungal biomass (including the antibiotic encoding genes) was monitored by a specific nested 18S rDNA-PCR using fungal primers NS1f/NS8r (1st round PCR) and NS1+gc/NS2+10r (2nd round PCR) (Kowalchuk 1999), respectively. The absence of products after both rounds of PCR, as judged by electrophoresis on agarose gel, confirmed the efficacy of the DNA-degradation procedure; since the obtained DNA fragments were inferior to 560 bp (2nd round PCR), the presence of complete genes encoding the antibiotic Cephalosporin C could be excluded (Gutiérrez et al. 1992). The procedure to degrade DNA in the pharmaceutical waste obtained the Italian patent IT MI20032129 "BIOCO" and the European patent EP 1 529 766 B1.

Microcosm preparation

Each microcosm was constituted of two Plexiglas cylinders of 10 cm height and 15 cm of diameter, laid one on top of the other (Fig. 1a). The base of the upper cylinder was perforated, and two layers of glass spheres of different diameter were put at the bottom to permit aeration and water flow. Then, this cylinder was filled with 1 kg of moist and sieved soil, whereas the lower cylinder was used as a reservoir collecting any loss.

The soil was amended with sterile suspensions containing 0, 0.05, 0.1, and 1% of fungal biomass per dry weight of soil, and soil moisture was adjusted to 50% of the water holding capacity of soil in all treatments. The solutions were added covering the soil surface uniformly. The first sampling was carried out 50 min after spreading fungal biomass (0 days), and the other were carried out at 1, 3, 7, 15, 60, and 90 days.

Each microcosm was replicated twice, and three soil samples were collected per microcosm, so to have six independent replicates for each (four) treatment and for each (seven) sampling time. We chose to use two replicates per microcosm because the large dimensions of the Plexiglas cylinders permitted picking up the soil core samples without any sample overlapping during all the period of the experiment. Soil cores (3 cm high with a diameter of 1.2 cm) were sampled with a plastic cylinder in a different site respect to the previous samplings by using a perforated wheel (Fig. 1b), so that cores were unique and undisturbed at each sampling.

Extraction and quantification of DNA from soil

Soil (0.5 g) DNA was extracted by the bead-beating method using FastDNA SPIN Kit and the FastPrep instrument (Bio

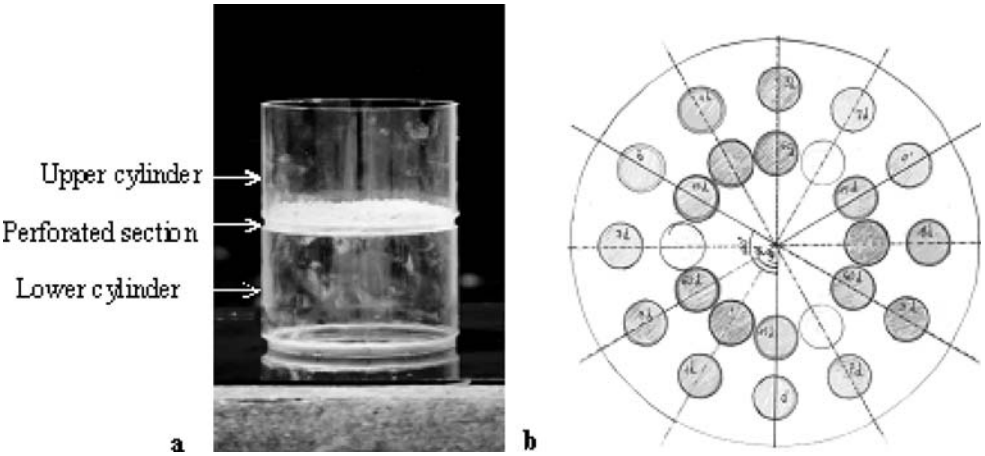

Fig. 1 **a** shows the empty cylinders used in the microcosm experiment; **b** shows the perforated wheel used to sample soil cores

101, USA). The amount of extracted dsDNA was quantified by fluorimeter (Hoefer DyNA Quant 200) using bisbenzimide-dye (Hoechst H 33258); the quality was checked by electrophoresis on 1% agarose gels.

Determination of soil eubacterial community

The eubacterial community structure was determined by amplifying the 16S rDNA sequences, using the primer set GC-968f (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TA-3′) and 1401r (5′-GCG TGT GTA CAA GAC CC-3′; Felske and Akkermans 1998). The gc-16S amplified fragments were 473 bp long. Soil template DNA (80 ng) was amplified with a mix containing 2.5 U Polytaq polymerase (from Polymed-Firenze), 12.5 pM of primers, 12.5 mM deoxyribonucleotide triphosphates, 7.5 mM $MgCl_2$, 500 μg/ml bovine serum albumin and 50× reaction buffer in a final reaction volume of 50 μl. The PCR was then performed with a Perkin-Elmer 2400 thermalcycler with the following temperature cycle: 94°C denaturation for 90 s, 56°C annealing for 30 s, and 72°C extension for 45 s, followed by 33 cycles at 95°C for 20 s, 56°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 7 min. PCR products were checked on 1% agarose gel by electrophoresis.

Determination of soil ammonia oxidizing population

The ammonia oxidizing population was determined by amplifying the ammonia-monooxygenase gene with primers *amo*A: gc-1F CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCAC GGG GGT TTC TAC TGG TGG T and 2R CCC CTC KGS AAA GCC TTC TTC (Avrahami and Conrad 2003; Rotthauwe et al. 1997). Soil DNA (80 ng) was amplified according to the following cycle: 94°C 5 min; 35 cycles of 94° 45 s, 50°C 30 s, 72°C 1 min, and a final extension at 72°C for 7 min. The gc-*amo*A amplified fragments were 506 bp long. The composition of the PCR mix was that described above for the eubacterial amplification.

DGGE of the amplified eubacterial 16S rDNA and the *amo*A sequences were performed on the DCode System following the protocol for casting the gels (Universal Mutation Detection System, Biorad). DNA (300 ng), amplified by eubacterial primers, was loaded onto a 20× 20 cm 6% (*w*/*v*) polyacrylamide gel (acrylamide/bisacrylamide 37.5:1, Biorad) containing a denaturant gradient of 46 to 56% made of urea and formamide (100% denaturant contains 7 M urea and 40% formamide); gels were run at 60°C and 75 V voltage for 16 h. DNA (300 ng), amplified by primers *amo*A, was loaded on 16×16 cm 6% polyacrylamide gel with a denaturant gradient of 45–65%. Gels were run at 60°C and 70 V voltage for 18 h. Amplicons of *Nitrosomonas europaea* (ATCC 25978) and *Nitrosolobus multiformis* (ATCC 25196), generating known band profiles, were loaded on the gel to facilitate the interpretation of the DGGE patterns and to allow the direct comparison among the various gels.

All the gels were stained with SybrGreen I 1:10,000 (FMC Bio Products, Rockland, ME, USA) in Tris-acetate-ethylenediamine tetraacetic acid buffer (TAE) for 1 h. DNA bands were detected by UV transillumination of the gel (254/497 nm), photographed (Polaroid Gel Cam, Elect; Polaroid Type 667 Film ISO 3000) and analyzed for finding bands in the profiles by the DNAscan software (Scanalytics, Fairfax). Where bands were not well defined, they were detected manually.

The similarity index (SI) was calculated as $S = 2c/(a + b)$, where *a* and *b* are the number of bands in two samples, and *c* is the number of bands shared between the two samples. When *S* is 0, the two samples are completely different, whereas when *S* is 1, the two samples are identical. The calculation was based on pair wise comparisons between each treatment and the control (Dabo et al. 1997; Turpeinen et al. 2004).

Elution and PCR amplification of DNA fragments from polyacrylamide gel

Bands from the DGGE gels were excised, placed in Eppendorf tubes containing 160-µl sterile ddH_2O, and stored at −70°C. Before PCR amplification, the samples were thawed for 1 h at room temperature, frozen again at −70° C for 1 h, and finally thawed at 8°C overnight to elute the DNA fragments (Bäckman et al. 2003). The eluted DNA (5 µl) was used as template in PCR amplification with the *amo*A primers (without the GC-clamp) by using the thermocycling programs already described above.

Sequencing and phylogenetic analysis of *amo*A fragments separated on polyacrylamide gels

Bands at 49% denaturant for which the sequence has been determined are shown in Fig. 3 by arrows.

Before sequencing, 45 µl of each PCR product was purified with the PCR montage columns supplied by Millipore. The sequencing reactions were performed with their respective oligonucleotide primers at the Austrian Research Council (ARC) Seibersdorf Research GmbH Center.

Sequences were aligned using Clustal X (Version 1.81), and dissimilarities were converted to evolutionary distances according to Jukes and Cantor (1969). The construction of neighboring joining trees and bootstrap analysis of 1,000 resamplings were performed using the software package TREECON for Windows version 1.3 b (Van de Peer and De Wachter 1997).

The similarity of each of the sequenced *amo*A bands with those of the reference strains *Nitrosolobus multiformis* ATCC 25196 and *Nitrosomonas europaea* ATCC 25978 was determined by basic local alignment search tool (BLAST) analysis. A stand-alone executable program for aligning two given sequences (bl2seq) can be retrieved from Tatusova and Madden (1999; http://www.ncbi.nlm.nih.gov).

Results and discussion

Eubacterial community

At time zero, control and treated soils showed the same band profiles (Fig. 2). After 1 day of incubation, a new band appeared corresponding to 49% of the denaturant gradient (Fig. 2) in both treated and control soils and probably, it represented an eubacterial species whose growth was favored by soil moisture at 50% of the water holding capacity. This variation, however, was transient because the band was not detected after a week of incubation.

From 3 to 90 days, four new bands, located in the 51–54% denaturant range (Fig. 2), were present in the profile of soil treated with the highest dose of fungal biomass (1%). These new dominant eubacterial species persisted for about 90 days of incubation as shown by the value (0.52) of the SI when the DGGE profile of 1% biomass treated soil was compared with that of the control (or the other treated soils), during the 3–90 days of incubation period (Table 1). This means that some of the less abundant microbial species can grow in this soil upon addition of the treated pharmaceutical fungal biomass applied at the highest dose; in this way, they become detectable.

Ammonia oxidizing population and phylogenetic analysis of fragments separated on polyacrylamide gels

At 0 days, no variations in the composition of AOB were detected among control and treated samples; the dominant

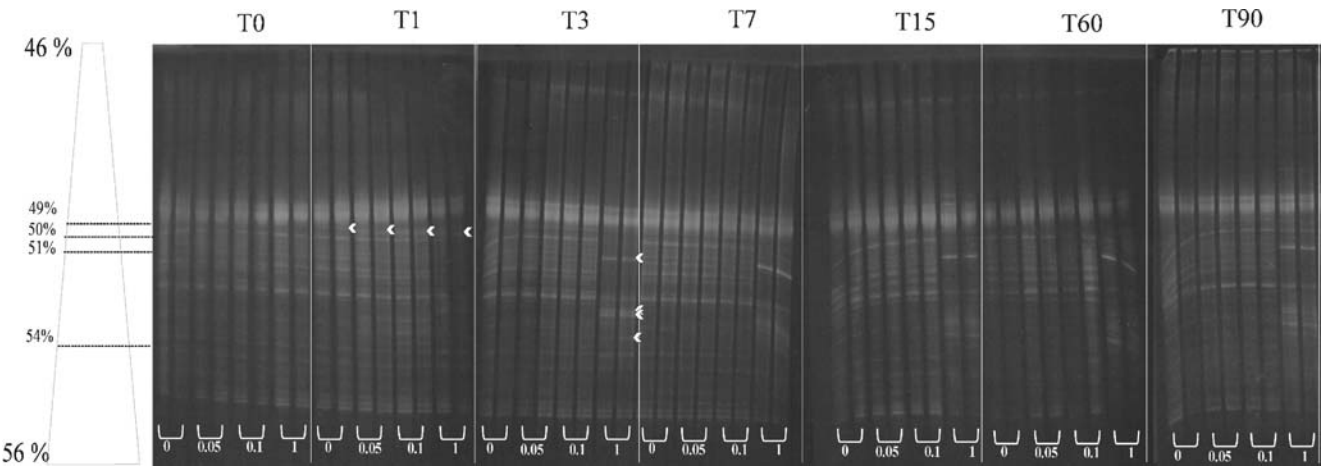

Fig. 2 DGGE of the soil eubacterial community by 16S rDNA amplification. T_0–T_{90}, time of incubation after the treatment with the inactivated fungal biomass; *0*, *0.05*, *0.1*, *1%*: amount of biomass added to microcosms. Two of the six replicates for each treatment are reported

Table 1 Similarity Index values of the eubacterial profiles after 0, 1, 3, 7, 15, 60 and 90 days incubation (T_0–T_{90})

	Fungal biomass amendment (%)	T_0	T_1	T_3	T_7	T_{15}	T_{60}	T_{90}
Eubacteria	0	1	1	1	1	1	1	1
	0.05	1	0.96	0.96	0.96	0.96	0.96	0.96
	0.1	1	0.96	0.96	0.96	0.96	0.96	0.96
	1	1	0.96	0.52	0.52	0.52	0.52	0.52

bands showed a distance of migration similar to that of *Nitrosolobus multiformis* rather than that of *Nitrosomonas europaea*, both chosen as reference strains (Fig. 3).

Major differences between AOB of control and treated soils were detected after 1 day. In fact, at this time, a band migrating at 53% of the gradient and already present in the profiles at 0 day became more evident. This band was not present in the profile of the reference strains, and it may be due to the growth of a different ammonia oxidizer species. Seven days after, the 53% migrating band only persisted in the profile of the control soil, indicating that the corresponding ammonia oxidizing species found the suitable conditions to persist without any addition of nutrients. A new band in the higher part of the gel (corresponding to about 49% denaturant) was detected in all treated soils but not in the control. This band, well-defined and sharp, migrated correspondingly to that of the *Nitrosomonas europaea* (Fig. 3) and was not present after 3 days, suggesting that the fungal biomass added to soil markedly favored the growth of some ammonia oxidizing species within a few days. Generally, the genus *Nitrosolobus* (identified also as *Nitrosospira*) prevails over the genus *Nitrosomonas*, unless the content of soil nutrients is increased after adding organic fertilizers to soil (Belser and Schmidt 1978; Hastings et al. 1997; Ceccherini et al. 1998; Phillips et al. 2000). In DGGE analysis, it is very difficult to infer phylogenetic relatedness from band mobility. Distantly related sequences always displayed very different melting behavior and, consequently, different mobility in a DGGE gel, but on the other hand, identical mobility did not imply sequence identity (Kowalchuk et al. 1997; Muyzer et al. 1993). Thus, as we were interested in monitoring the shift in the ammonia oxidizers, it seemed interesting to sequence the bands at 49% denaturant to characterize them phylogenetically at least at the genus level, and this was done for all the treated replicates.

Alignment of the sequences showed that of the total 18 different sequences, 16 (sequences of the bands of two replicates, one treated with 0.1%, and the other with 1% biomass, resulted incomplete) were β-subgroup AOB-like sequences phylogenetically closer to *Nitrosolobus* than to *Nitrosomonas* species. The neighbor-joining tree inferred from comparative analysis of partial *amo*A gene sequences corroborated the higher similarity of the unknown bands with the *amo*A sequence of *Nitrosolobus multiformis* than that of *Nitrosomonas europaea*, both chosen as reference strains (Fig. 4). As the aim of the research was to monitor the modification in the composition of ammonia oxidizing population at the genus level, we conducted phylogenetic analysis and BLAST2 alignment by only comparing the unknown sequences to the reference strains (Table 2). The fact that all the bands sequenced appeared to be genetically related indicates that the treated fungal biomass, when applied in the range of 0.05 to 1%, selected for a particular *Nitrosospira*- rather than a *Nitrosomonas*-like AOB microflora, but this ammonia oxidizer was again under the detection limit of amplification after 3 days.

Fifteen days after, the profiles of the treated soils were similar to that of the control because both bands at 49 and 53% of denaturant were not detected, showing a recovery

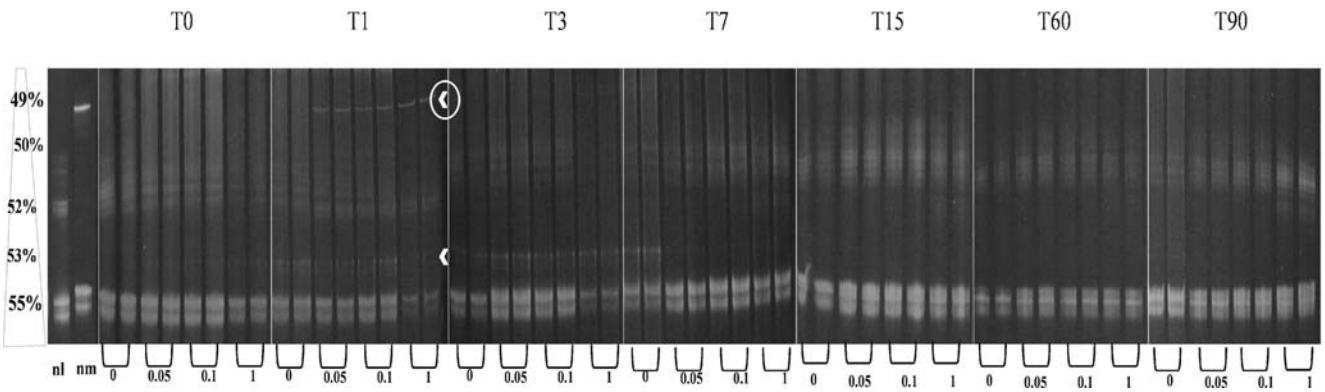

Fig. 3 DGGE of the ammonia oxidizing bacteria by amoA gene amplification. T_0–T_{90}, time of incubation after the treatment with inactivated fungal biomass; *0*, *0.05*, *0.1*, *1%*: amount of biomass added to the microcosms. Two of the six replicates for each treatment are reported. The *circled arrow* showed the sequenced bands

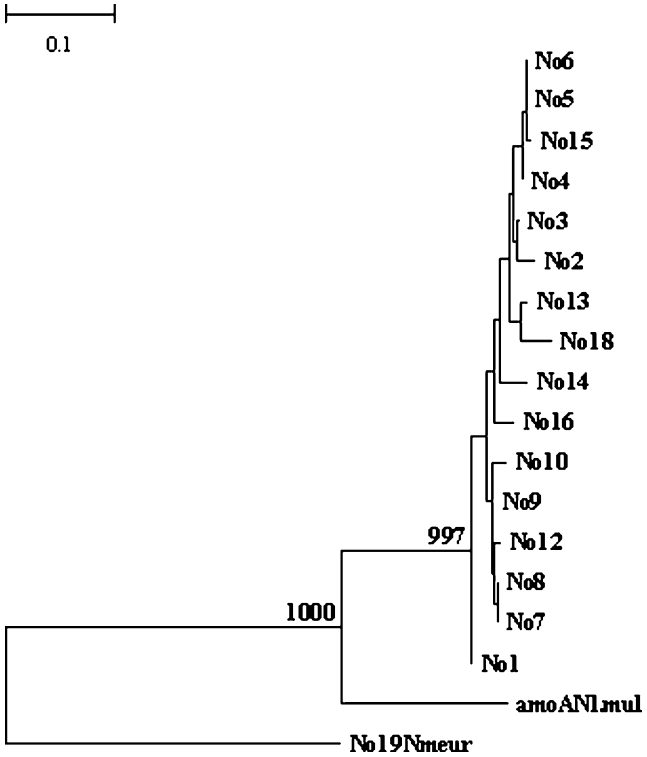

Fig. 4 Phylogenetic tree constructed by considering *amo*A sequences of the 49% denaturant DGGE bands at time T_1 (*Nos. 1 to 18*), and sequences of the reference strains *Nitrosolobus multiformis* ATCC 25196 (*amo*ANl.mul) and *Nitrosomonas europaea* ATCC 25978 (No19Nmeur). Bootstrap proportions of confidence (1,000 bootstrap repetitions) of 80% or more are indicated at the branch points as real values. The *bar* represents 0.1 substitution per nucleotide position

of the initial composition of the sequences belonging to ammonia oxidizers.

Sixty and ninety days after, the lowest bands of the profiles of the 0.1 and 1% treated soils were less evident and defined than the respective bands of the control soil. The population of the soil treated with the highest doses of fungal biomass was the most dissimilar respect to that of the control.

In our study, DGGE analysis of *N. multiformis* and *N. europaea amo*A amplicons produced multiple bands. This could be due to both the degeneracy of the primer and the multiple copies of the *amo*A gene. The *amo*A primer pair used in this work only targets the β-subclass AOB. However, it is reasonable to hypothesize that the used primers are suitable to characterize the composition of the ammonia oxidizer population of soil since only two AOB species belonging to the γ-subclass of Proteobacteria have been described, and both are from marine environments (Purkhold et al. 2000). As single base mutations may be detected by DGGE, even similar gene sequences from a given organism may give rise to multiple bands in a DGGE gel, and also degenerated primers can produce multiple band patterns through their degenerated sequence (Hornek et al. 2006). In particular, the use of the degenerated reverse primer can improve the detection of β-proteobacterial ammonia oxidizers, since functional genes like *amo*A are characterized by sequence (e.g., 3rd base) degeneracy (Hornek et al. 2006). It is well established that the *amo*A gene is present in two copies in *N. europaea* (McTavish et al. 1993) and in three copies in *Nitrosospira* sp. NpAV

Table 2 Sequence affiliations

		Nitrosomonas europea (ATCC 25978)	*Nitrosolobus multiformis* (ATCC 25196)	
Treatment	Bands	Identities	Identities	bp sequenced
	1	210/275 (76%)[a]	277/311 (89%)	366 bp
	2	199/262 (75%)	271/309 (87%)	354 bp
0.05%	3	212/277 (76%)	326/376 (86%)	427 bp
	4	213/277 (76%)	319/370 (86%)	432 bp
	5	218/284 (76%)	324/375 (86%)	429 bp
	6	218/284 (76%)	324/375 (86%)	429 bp
	7	216/281 (76%)	330/375 (88%)	429 bp
	8	216/281 (76%)	330/375 (88%)	430 bp
0.1%	9	215/281 (76%)	326/372 (87%)	423 bp
	10	215/281 (76%)	330/376 (87%)	427 bp
	12	214/281 (76%)	333/376 (88%)	430 bp
	13	211/277 (76%)	327/376 (86%)	384 bp
	14	202/264 (76%)	264/303 (87%)	326 bp
1%	15	219/284 (77%)	326/376 (86%)	423 bp
	16	209/274 (76%)	275/311 (88%)	330 bp
	18	211/277 (76%)	301/347 (86%)	387 bp

[a] 210 bp of the retrieved *amo*A sequenced was compared to 275 (data base entry) and a similarity of 76% was obtained

(Norton et al. 1996), and these copies are supposed to be originated from gene duplication and not from horizontal gene transfer, as there is a higher similarity between different gene copies in each investigated strain than between genes from different strains (Klotz and Norton 1998). It is also possible that our strains had a different number of gene copies than those investigated by Norton et al. (1996) and McTavish et al. (1993).

Conclusions

The eubacterial 16S rDNA DGGE analysis revealed very complex band patterns, reflecting a high bacterial diversity of the soils with most of the bands found in all samples. However, the application of the pharmaceutical fungal biomass at the highest dose changed the eubacterial community composition 3 days after the treatment, and this change persisted until monitoring was discontinued 90 days after amendment.

The ammonia oxidizing population of soil was sensitive to the application of the pharmaceutical fungal biomass since changes in the composition were shown 1 day after the treatments in all soils. The detected sequences belonged to the genus *Nitrosolobus* (*Nitrosospira* like AOB microflora). These results contradict what was previously observed by Hastings et al. (1997) and Ceccherini et al. (1998), who argued that usually in soil the genus *Nitrosolobus* prevails over the genus *Nitrosomonas* and the opposite occurs when swine manure is added to soil. Probably, the different behavior observed in the present study may be due to differences between the composition of microbial communities of the fungal biomass, derived from an industrial bioreactor and successively heated and chemically treated to degrade genomic DNA, and the composition of microbial communities of the swine manure.

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