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AREA 7 • RISK ASSESSMENT OF LAS • RESEARCH ARTICLE

Influence of linear alkylbenzene sulfonate (LAS) on the structure of *Alphaproteobacteria*, *Actinobacteria*, and *Acidobacteria* communities in a soil microcosm

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Abstract
Background, aim, and scope Linear alkylbenzene sulfonate (LAS) is the most used anionic surfactant in a worldwide scale and is considered a high-priority pollutant. LAS is regarded as a readily biodegradable product under aerobic conditions in aqueous media and is mostly removed in wastewater treatment plants, but an important fraction (20–25%) is immobilized in sewage sludge and persists under anoxic conditions. Due to the application of the sludge as a fertilizer, LAS reaches agricultural soil, and therefore, microbial toxicity tests have been widely used to evaluate the influence of LAS on soil microbial ecology. However, molecular-based community-level analyses have been seldom applied in studies regarding the effects of LAS on natural or engineered systems, and, to our knowledge, there are no reports of their use for such appraisals in agricultural soil. In this study, a microcosm system is used to evaluate the effects of a commercial mixture of LAS on the community structure of *Alphaproteobacteria*, *Actinobacteria*, and *Acidobacteria* in an agricultural soil.
Material and methods The microcosms consisted of agricultural soil columns (800 g) fed with sterile water (8 ml h^{-1}) added of different concentration of LAS (10 or 50 mg l^{-1}) for periods of time up to 21 days. Sterile water was added to control columns for comparison. The structures of *Alphaproteobacteria*, *Actinobacteria*, and *Acidobacteria* communities were analyzed by a cultivation independent method (temperature gradient gel electrophoresis (TGGE) separation of polymerase chain reaction (PCR)-amplified partial 16S rRNA genes). Relevant populations were identified by subsequent reamplification, DNA sequencing, and database comparisons.
Results Cluster analysis of the TGGE fingerprints taking into consideration both the number of bands and their relative intensities revealed that the structure of the *Alphaproteobacteria* community was significantly changed in the presence of LAS, at both concentrations tested. The average number of bands was significantly lower in the microcosms receiving 50 mg l^{-1} LAS and in the lower portion of soil cores. The clear differentiation of the samples of the upper portion of the soil columns amended with LAS was specifically related to the presence and intensity of a distinctive major band (named band class 7). There was a statistically significant positive correlation between the concentrations of LAS detected in soil portions taken from LAS 10 mg l^{-1} and LAS 50 mg l^{-1} microcosms and the relative intensity of band class 7 in the corresponding TGGE profiles. Prevalent *Alphaproteobacteria* populations in the soil microcosms had close similarity (>99%) to cultivated species affiliated to genera of the *Rhizobiaceae*, *Methylocystaceae*, *Hyphomicrobiaceae*, *Rhodospirillaceae*, *Brucellaceae*, *Bradyrhizobiaceae*, and *Caulobacteraceae* families. The population represented by band class 7 was found closely related to the genus

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Phenylobacterium (*Caulobacteraceae*). According to cluster analysis of TGGE profiles, the structure of both *Actinobacteria* and *Acidobacteria* communities in the soil microcosms was remarkably stable in the presence of LAS at the two concentrations tested, as most bands were universally present in all samples and displayed fairly similar relative intensities.

Discussion Previous studies by others authors, based on biological and chemical tests, concluded that LAS toxicity was not an important microbial selection factor in sludge amended soil, while work based on the use of molecular fingerprinting to evaluate the impact of LAS in aqueous media and marine sediments showed that concentrations as low as 1 mg l^{-1} significantly influence the development of the bacterial community structure. Although TGGE is not a strictly quantitative method due to the bias introduced by the PCR reaction, changes of band intensity through experiments are a consequence of a change in the relative abundance of the corresponding populations in the community and can be used as a semiquantitative measure of bacterial diversity. Our results evidence that the *Phenylobacterium* population represented by band class 7 was favored by the presence of increasing concentrations of LAS in the soil and turned into a dominant population, suggesting its possible ability to use LAS in soil as a source of nutrients. As studies with pure cultures are required to confirm the ability of this population to degrade LAS, isolation strategies are currently under development in our laboratory. The weak effect of LAS on the structure of *Actinobacteria* and *Acidobacteria* communities is particularly interesting, as to our knowledge, there are no previous reports regarding the effects of LAS on these bacterial groups in soil.

Conclusions, recommendations, and perspectives The *Phenylobacterium*-related alphaproteobacterial population identified in this work was selectively enriched in LAS polluted soil and is a plausible candidate to play a relevant role in the biotransformation of the surfactant under the conditions tested. The surfactant had no remarkable effects on the *Actinobacteria* and *Acidobacteria* fingerprints in soil, even when present at concentrations widely exceeding those reached in soil immediately after sludge application. TGGE fingerprinting provides a reliable and low time-consuming method for the monitoring of the bacterial community structure and dynamics, and we recommend its integration with the biological and chemical analyses usually applied in risk assessment of LAS in the environment.

Keywords *Acidobacteria* · *Actinobacteria* · *Alphaproteobacteria* · LAS · Linear alkylbenzene sulfonate · *Phenylobacterium* · Soil microcosm · TGGE

1 Background, aim, and scope

Linear alkylbenzene sulfonate (LAS) is the most used anionic surfactant in household laundry/dishwashing detergents and multipurpose cleaning products (Jensen et al. 2007; Schowanek et al. 2007). Over 3×10^6 tons of LAS per year are currently consumed worldwide (HERA 2007), and the average daily use per individual in the European Union (EU) countries is estimated in the range of 1.5–4 g (Jensen et al. 2007). LAS is considered a high-priority pollutant and its toxicity to microorganisms, plants, terrestrial animals, and humans is well documented (HERA 2007). Studies on human and environmental risk assessment conclude, however, that commercial LAS is nonbioaccumulative and nonpersistent, and its major biodegradation intermediates, sulfophenyl carboxylates (SPCs), had lower persistence and toxicity than LAS itself (HERA 2007). LAS is regarded as a readily biodegradable product under aerobic conditions in aqueous media (EU Commission 1997). Wastewater treatment plants based on the conventional aerobic activated sludge processes efficiently remove up to 99.9% LAS in water, but LAS is persistent under anoxic conditions, easily adsorbs to organic matter, and precipitates with Ca^{2+} and Mg^{2+} salts (Elsgaard et al. 2003; Jensen et al. 2007; Mungray and Kumar 2008). These facts result in the immobilization in sewage sludge of an important fraction (20–25%) of the LAS discharged to wastewater (Schowanek et al. 2007). Commercial LAS reaches agricultural soil almost entirely by the application of the sewage sludge as a cheap fertilizer (Brandt et al. 2003; Schowanek et al. 2007). The concentrations of LAS in repeatedly sludge-amended soils are usually in the range of 0.7–20 mg kg^{-1} (Mortensen et al. 2001), and several studies reported half-lives of LAS in soils from different countries ranging from 3 to 33 days (HERA 2007), as degradation rates are influenced by the initial LAS concentration in the sludge and environmental parameters such as pH, soil moisture, or organic matter content (Kristiansen et al. 2003; Brandt et al. 2004).

Microorganisms and overall soil processes are not included in terrestrial risk assessments for LAS, as they are considered protected by the predicted no effect concentration value derived from the relative higher sensitivity of plants and invertebrates (Jensen et al. 2007; Schowanek et al. 2007; HERA 2007). A soil quality criterion of 5 mg LAS kg^{-1} soil was proposed by Jensen and Folker-Hansen (1995). Nevertheless, short-term effects of LAS on soil microbial parameters occur at concentrations slightly above this value (Elsgaard et al. 2001a, b; Sánchez-Peinado et al. 2009) and exposure to LAS at concentrations ranging 5–50 mg l^{-1} in laboratory cultures is reported harmful for soil bacteria, particularly ammonia oxidizers (Brandt et al. 2001, 2002; Sánchez-Peinado et al. 2008). Other studies, however, found no significant long-

term effects of either aqueous LAS or LAS-contaminated dry sewage sludge on the functional diversity of bacteria in soils (Vinther et al. 2003; Brandt et al. 2004).

Microbial toxicity tests used to evaluate the effects of LAS on soil microbial ecology often included single-species analysis, measurement of microbial numbers or biomass, respiration rates, C and N transformations, and soil specific enzymes (Elsgaard et al. 2001a, c; Sánchez-Peinado et al. 2008, 2009). Molecular-based fingerprinting methods for community analysis have been seldom used in studies regarding the effects of LAS on natural or engineered systems (Brandt et al. 2003; Lara-Martín et al. 2007; Duarte et al. 2008), and, to our knowledge, there are no reports of their use for such appraisals in agricultural soil. Polymerase chain reaction coupled to denaturing or temperature gradient gel electrophoresis (PCR-DGGE/TGGE) provides a sensitive tool to monitor changes of bacterial community structure and dynamics under the influence of environmental factors or anthropogenic factors and in addition enables the taxonomic identification of the prevalent populations (Kostanjšek et al. 2005; Lin et al. 2005; Aguirre de Cárcer et al. 2007; Molina-Muñoz et al. 2007; Vílchez et al. 2007; Moreno et al. 2009). The main goal of the present work was to evaluate the effects of LAS on the bacterial community of an agricultural soil in a microcosm system using the PCR-TGGE fingerprinting approach and specifically aiming for (a) the detection of significant changes on community structure of three major groups of soil bacteria (*Alphaproteobacteria*, *Actinobacteria*, and *Acidobacteria*) and (b) the identification of the prevailing populations of each soil group, particularly those shifting in the presence of LAS. *Alphaproteobacteria*, *Acidobacteria*, and *Actinobacteria* were selected as targets on the basis of previous clone library-based studies reporting them as dominant prokaryotic groups in soil, accounting respectively for 18.8%, 19.7%, and 12.7% on average (Janssen 2006).

2 Materials and methods

2.1 Linear alkylbenzene sulfonate

A commercial mixture of LAS was used in the experiments (69% water, 31% active matter). The active matter was composed of the linear alkyl chain homologs 5-phenyl C10 (0.8%), phenyl C10 (9.8%), phenyl C11 (33.9%), phenyl C12 (32.5%), phenyl C13 (22.6%), and phenyl C14 (0.3%). The product also contained 0.10% tetra-indole and 0.10% paraffin. Calculations of the amount of LAS added to soil were made according to the active matter of the product.

2.2 Soil samples

Soil was sampled from an agricultural field in Belicena, near Granada (southern Spain). The selected field was a fallow land uncultivated for over 7 years, with no previous exposure to LAS. Samples from the 20 cm upper layer of the field were collected in sterile plastic bags and shipped to the laboratory. The soil was air dried for 12 h and sieved through a 2-mm mesh screen to remove pebbles and vegetal residues. The soil type was a Xerofluvent with silt loam texture, containing 14% clay, 20% sand, and 65% silt. The chemical composition of the samples was pH (water) 7.8, organic C 1.39% (*w*/*w*), total N 0.14%, total P 25 mg kg^{-1} dry soil, and K 240 mg kg^{-1} dry soil.

2.3 Soil microcosms setup and sampling

The microcosm design and the conditions imposed throughout the study were used in previous work to estimate the response of soil enzymatic activities to LAS (Sánchez-Peinado et al. 2009). Briefly, each microcosm consisted of 800 g agricultural soil placed in a 1,000-ml glass column (6.0 cm diameter×35 cm length). Leachates from the bottom of the soil columns were collected in sterile glass bottles. The columns were closed at the bottom by a glass filter plate and a funnel, to avoid the infiltration of soil into the sampling bottles. Polyethylene tubing was used in the whole system.

The soil microcosms were amended from their top ends with either autoclaved bidistilled water (microcosms named W) or LAS solution at two different concentrations, 10 and 50 mg l^{-1} (microcosms named LAS10 and LAS50, respectively). LAS solutions were made in bidistilled water (MilliQ grade) and sterilized by filtration (0.22 µm, Millipore®). Three replicated columns were set up in each experiment. All soil microcosms were fed, either with sterile water or LAS solutions, at a rate of 8 ml h^{-1}, using a peristaltic pump. Accordingly, microcosms LAS10 received 2.4 mg LAS kg^{-1} dry soil day^{-1}, while the LAS50 microcosms received 12 mg LAS kg^{-1} dry soil day^{-1}. The concentrations of LAS applied to the microcosms were selected according to previous studies, which indicate that adverse effects for microorganisms in soils start in the range of 10–50 mg kg^{-1} soil (Elsgaard et al. 2001a; Sánchez-Peinado et al. 2008). Due to the different rates of LAS dosage, samplings of the LAS10 and LAS50 microcosms to evaluate changes in bacterial community structure were set at different times, based in the different mobility of LAS through the soil columns (Sánchez-Peinado et al. 2009). This mobility was calculated by setting up autoclaved and sealed versions of the LAS10 and LAS50 microcosms, and their leachates were tested daily for the presence of the LAS homolog of fastest mobility (C10). LAS leached after 21 days in the sterile

LAS10 microcosms and after 7 days in the sterile LAS50 microcosms. Glass columns corresponding to LAS10 microcosms were sampled by destruction of the column under aseptic conditions after 7, 14, and 21 days, while the LAS50 microcosms were sampled after 3, 7, and 21 days. The soil cores were divided in two halves, named upper (U) and lower (L). Portions of soil from both far ends of the column (ca. 2 cm height) were discarded. After soil from each half was thoroughly mixed, the upper and lower parts of the soil core were sampled separately, for further chemical and biological studies.

2.4 Detection of LAS in soil and leachate samples

The concentration of LAS in soil samples and leachates was analyzed as previously described (Nimer et al. 2007). LAS homologs C10, C11, C12, and C13 were detected. Sulfophenyl carboxylic acids were not measured, as the main purpose of the work was to evaluate changes in community structure and dynamics, rather than follow the dynamics of biodegradation patterns of LAS in the soil columns.

2.5 DNA extraction and PCR specific amplification of partial 16S rRNA genes from *Alphaproteobacteria*, *Actinobacteria*, and *Acidobacteria* communities

DNA was extracted from the microcosm soil samples (1 g) using the Ultra Clean Soil DNA Isolation kit (MoBio, USA), which involves a bead-beating step. For comparison, DNA was also extracted from the soil used to construct the microcosms, before starting the experiments. This sample was named C.

Nested PCR approaches were used for the specific amplification of the V3 hypervariable region of the 16S rRNA gene of selected bacterial groups. One microliter (2–5 ng) of DNA extracted from soil samples was used as template for the first PCR, using primers specific for the amplification of partial 16S rRNA genes of *Alphaproteobacteria*, *Acidobacteria*, and *Actinobacteria*. High performance liquid chromatography-purified oligonucleotides were purchased from Sigma, and AmpliTaq Gold hot-start polymerase (Applied Biosystems) was used for all PCRs, which were always performed with an initial denaturation step at 94°C for 7 min, and a final extension step at 72°C for 10 min. All PCR primers used were previously described in the literature. To amplify partial 16S rRNA genes of *Alphaproteobacteria*, primer F203alfa and the universal primer R-1378 were used (Heuer et al. 1997), and PCR amplification was achieved by conditions described elsewhere (Gomes et al. 2001). To amplify partial 16S rRNA genes of *Actinobacteria*, primer F243hgc (Heuer et al. 1997) and the universal rD1 primer (Weisburg et al. 1991) were used. The PCR conditions were kept as described by Heuer et al. (1997). Finally, partial amplification of 16S rRNA genes of *Acidobacteria* was achieved by using primer 31F (Barns et al. 1999) and rD1, under the conditions described by Barns et al. (1999).

One microliter of a 1/30 dilution of the first PCR product was used as a template for the nested PCR, using universal primers targeting the V3 region (Muyzer et al. 1993). Conditions for the nested PCR reaction were described elsewhere (Vílchez et al. 2007). Final PCR products were cleaned and/or concentrated (when required) using Microcon YM cartridges. Two to 5 µl (60–100 ng DNA) was loaded in each well for TGGE.

2.6 TGGE fingerprinting

TGGE separation of amplified V3 fragments was achieved using a TGGE Maxi system (Whatman-Biometra). Denaturing gels (6% polyacrylamide, 20% deionized formamide, 2% glycerol, and 8 M urea) were made and run with 2× Tris–acetate–ethylenediaminetetraacetic acid buffer. For the separation of the V3 amplicons of *Alphaproteobacteria* and *Acidobacteria*, the temperature gradient used was 43–63°C, previously optimized for efficient separation of eubacterial V3-region amplicons (Cortés-Lorenzo et al. 2006). The separation of V3 fragments of *Actinobacteria* required the optimization of the temperature gradient at 47–56°C, to improve resolution of high G+C DNA fragments. All gels were run at 125 V for 18 h. Bands were visualized by silver staining using the Gel Code Silver Staining kit (Pierce), as previously described (Vílchez et al. 2007). A six-species marker was also included to aid gel normalization (Vílchez et al. 2007). Different PCR reactions were tested and different TGGE gels were run, to check the reproducibility of the results. Cluster analysis was used to investigate the relationships between the TGGE profiles. Band patterns generated by TGGE were normalized, compared, and clustered using Gel Compar II v.5.10 (Applied Maths, Belgium). The profiles were compared using either the similarity index based on the Pearson correlation coefficient (curve based, independent of band assignment) or the Dice similarity coefficient (band based). A 1% position tolerance was applied in band assignment. Dendrograms relating band pattern similarities were calculated by the unweighted-pair group method using arithmetic averages (UPGMA). The significance of the clusters was estimated by calculation of the cophenetic correlation coefficient (Sokal and Rohlf 1962).

2.7 DNA sequencing, phylogenetic and molecular evolutionary analyses

Portions of prominent TGGE bands on silver-stained gels were picked up with sterile pipette tips, placed in 10 µl of

filtered and autoclaved water, freeze–thawed, and directly reamplified with the appropriate primers. PCR products were purified by gel extraction with the Quiaex-II kit (Quiagen). DNA recovered was sequenced using an ABI PRISM 3100 Avant Genetic Analyzer. The DNA sequences were analyzed using the online biocomputing tools of the European Bioinformatics Institute (http://www.ebi.ac.uk) and the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov). The BLASTn program (Altschul et al. 1997; Zhang et al. 2000) was used for sequencing similarity analysis. The ClustalX v. 2.0 software (Jeanmougin et al. 1998) was used for the aligning of the DNA sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 (Kumar et al. 2008). Gaps were excluded from the analysis. Bootstrap values below 50% are not shown in the trees.

2.8 Statistics

STATGRAPHICS 5.0 (STSC, Rockville, MD, USA) was used for calculation of Pearson's correlation coefficient among variables and for two-way analyses of variance (ANOVA). A significance level of 95% ($P<0.05$) was selected.

3 Results

3.1 Effect of LAS on community composition of *Alphaproteobacteria* in soil microcosms

The complexity of the TGGE profiles of *Alphaproteobacteria* communities in soil samples taken from the microcosms ranged from 11 to 21 bands. Two-way ANOVA analysis showed that the average number of bands on the TGGE profiles was significantly lower in the LAS50 microcosms and in the lower portion of soil cores (Table 1).

Cluster analysis of *Alphaproteobacteria* fingerprints based on the whole densitometric curve of the TGGE profiles (Pearson's product moment correlation coefficient) is displayed in Fig. 1a. All samples from the upper fraction of soil cores amended with LAS, at both concentrations tested, grouped in a single cluster, branching at 59% correlation. The rest of soil samples treated with LAS grouped in a separated cluster together with the W samples, although most of the samples from portion L of soil cores which received LAS were also differentiated in a consistent subcluster. Cluster analysis calculated using the Dice coefficient, based in the presence or absence of bands without taking into account band intensity (see Fig. 1b), recognized a total of 32 different band classes. The soil community profiles of microcosms amended with 50 mg l^{-1} LAS for 21 days significantly differentiate from the rest of samples, grouping separately at 60% correlation. The remaining profiles grouped together at 72.6% correlation and divide again into two consistent subclusters, one of which was entirely composed of the rest of samples amended with LAS at 50 mg l^{-1}.

Figure 1a evidences that the clear differentiation of the samples of portion U of the soil columns amended with LAS was specifically related to the presence and relative intensity of a distinctive major band (named band class 7). Using GelCompar II, the relative intensities of bands corresponding to class 7 were calculated, expressed as percentages of the total band intensity in each particular TGGE lane. ANOVA analysis of the influence of the treatment applied to soil (W, LAS10 or LAS50) and the soil portion analyzed (U or L) on the relative intensity of band class 7 in the TGGE profiles also demonstrated the significant influence of both factors and their interaction (Fig. 2a). Band class 7 had a very high contribution to the TGGE profile in samples from portion U of soils treated with LAS, particularly at the 50 mg l^{-1} concentration. There were also significant differences in the intensity of band class 7 between the upper and lower portions of the soil columns amended with LAS at both concentrations tested, but not in the case of the W microcosms.

Figure 2b shows the relative intensities of band class 7 in the fingerprints from all samples, together with the concentrations of LAS detected in each soil portion at the

Table 1 Summary of ANOVA analysis of the effect of LAS concentration applied to microcosms and portion of the soil cores analyzed on the average number of bands in TGGE profiles of the alphaproteobacterial community

Analysis of variance		Student's *t* test			
Main effects		LAS concentration (mg l^{-1})	No. of bands	Soil portion	No. of bands
(A) LAS concentration	$P<0.01$	0 (W)	17^{a}	Upper	17^{a}
(B) Soil portion	$P<0.05$	10 (LAS10)	18^{a}	Lower	15^{b}
(A) × (B) interaction	NS	50 (LAS50)	13^{b}	LSD ($P<0.05$)	2
		LSD ($P<0.05$)	2		

NS not significant

[a,b] Values marked with the same letter do not significantly differ (Student's T test, $p<0.05$)

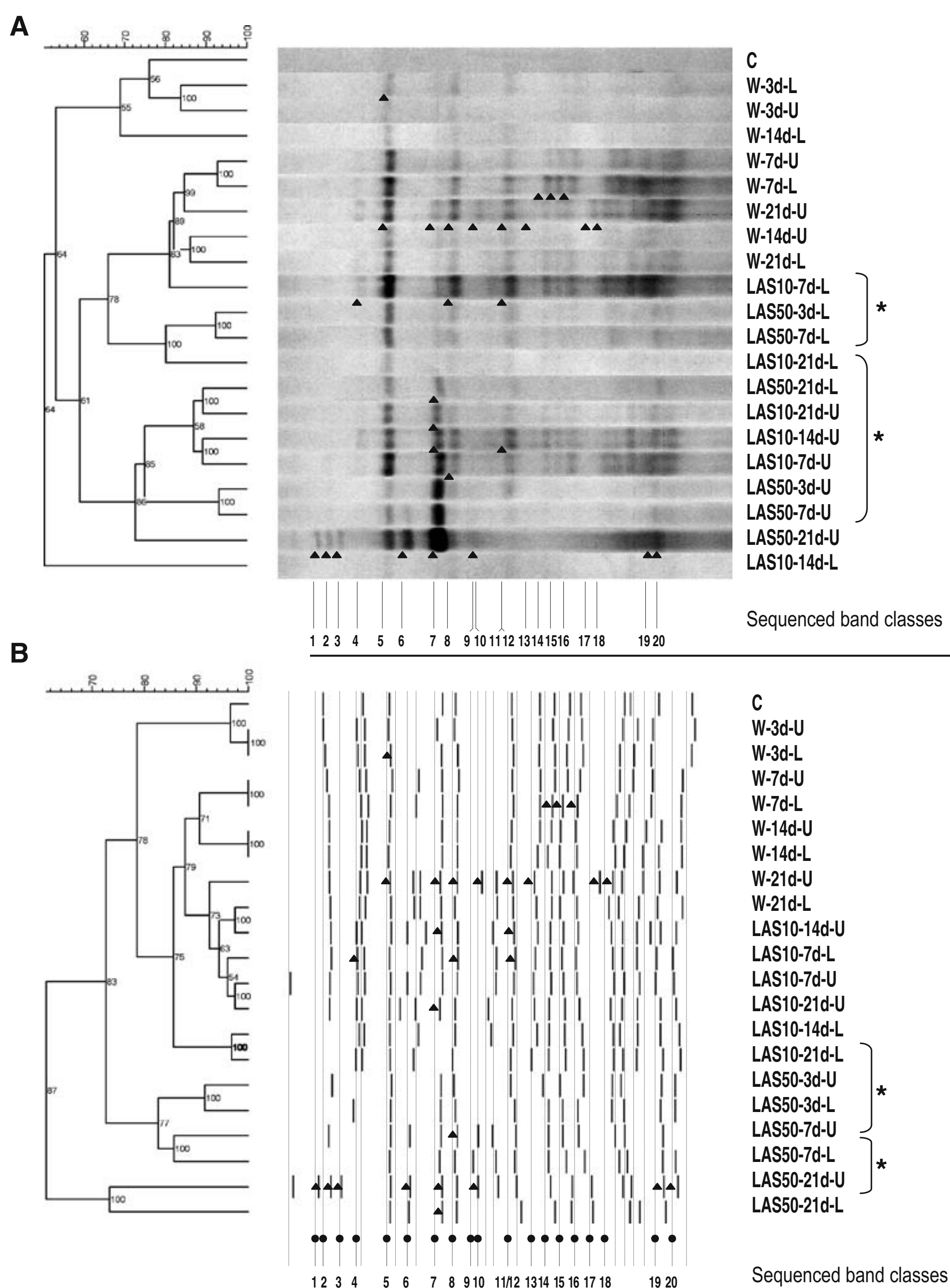

Fig. 1 Cluster analysis (UPGMA) of TGGE patterns (based on partial 16S rRNA gene sequences, ca. 150 bp) of the *Alphaproteobacteria* communities in soil samples from microcosms amended with water (*W*) or LAS solutions at 10 (*LAS10*) or 50 mg l^{-1} (*LAS50*) for 3 to 21 days (*3d*, *7d*, *14d*, *21d*). *U* upper portion of soil cores, *L* lower portion of soil cores, *C* control sample (original soil). **a** Curve-based analysis. **b** Band-based analysis. Scale bar: % correlation. *Numbers in nodes*: cophenetic correlation coefficient values. Band classes from which one or more representative bands were reamplified and sequenced are numbered (*1* to *20*). Bands picked up from gels are marked with *arrowheads*. Consistent clusters grouping LAS-treated soil samples are marked with an *asterisk*

time of sampling. LAS solutions were amended in continuous flow throughout the experiment, but the distribution of the surfactant was not homogeneous in the soil columns, and LAS concentrations were always higher in their upper portion. There was a statistically significant positive correlation (Pearson's correlation coefficient 0.806, $P<0.002$) between the concentrations of LAS detected in soil portions taken from LAS10 and LAS50 microcosms and the relative intensity of band class 7 in the corresponding TGGE profiles.

3.2 Identification of prevalent *Alphaproteobacteria* populations in the soil microcosms

Twenty-eight TGGE bands, representing 20 of the 32 band classes of *Alphaproteobacteria* detected in soil samples (see Fig. 1a), were successfully extracted from the gels, their DNA reamplified and sequenced. When complex bacterial communities are analyzed, there is a chance that TGGE profiles based on amplification with universal primers generate single bands composed of several overlapped

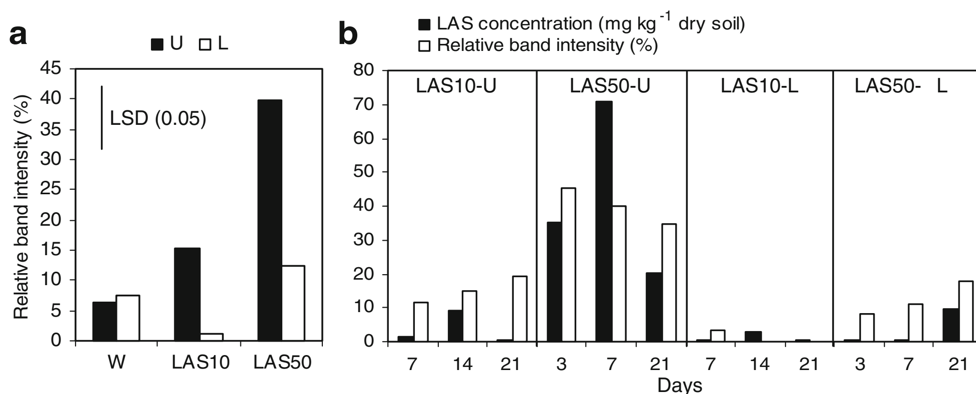

Fig. 2 **a** Effect of soil treatment (*W* water, *LAS10* LAS solution at 10 mg l^{-1}; *LAS50* LAS solution at 50 mg l^{-1}) and column depth (*U* upper portion of soil cores, *L* lower portion of soil cores) on the average relative intensities of band class 7 in TGGE patterns of soil microcosm samples. **b** Relative intensities of band class 7 and remaining concentrations of LAS in soil at sampling time. See Fig. 1 legend for the key to nomenclature of soil samples

sequences. For this reason, the identity of band class 7 was confirmed by the amplification and sequencing of five TGGE bands with the same electrophoretical behavior, taken from different samples chosen among the different treatments (LAS50-21s-U, LAS50-21d-L, LAS10-21dU, LAS10-14d-U, and W-21d-U), which yielded identical single sequences in all cases. Thus, the high intensity of this band detected in some samples is solely due to a high representation of this population on the analyzed soils.

The 28 sequences were compared to those filed in the European Molecular Biology Laboratory database, and a phylogenetic tree was generated (Fig. 3). All sequences had close similarity (>99%) to cultivated species. Populations identified by this approach were affiliated to genera of the *Rhizobiaceae*, *Methylocystaceae*, *Hyphomicrobiaceae*, *Rhodospirillaceae*, *Brucellaceae*, *Bradyrhizobiaceae*, and *Caulobacteraceae* families, all described as common members of the soil bacterial community (Sait et al. 2002; Brenner et al. 2005). The population represented by band class 7 was found closely related to the genus *Phenylobacterium* (*Caulobacteraceae*). Presently, there are published descriptions of nine *Phenylobacterium* species, seven of them validated (*Phenylobacterium immobile*, *Phenylobacterium lituiforme*, *Phenylobacterium koreense*, *Phenylobacterium falsum*, *Phenylobacterium composti*, *Phenylobacterium conjunctum*, *Phenylobacterium haematophilum*) and two remaining nonvalidated (*Phenylobacterium mobile*, *Phenylobacterium zucineum*; Ke et al. 2003; Kanso and Patel 2004; Aslam et al. 2005; Eberspächer 2005; Tiago et al. 2005; Zhang et al. 2007; Abraham et al. 2008; Weon et al. 2008). The sequence of the partial 16S rRNA gene V3 region corresponding to band class 7 was phylogenetically closer to the *P. koreense* cluster (see Fig. 3). The sequence corresponding to band class 5 is also related to *Phenylobacterium*, but it did not suffer significant changes of its relative intensity in connection with the addition of LAS to soil (calculated by ANOVA, data not shown), and its sequence was found more related to the *P. immobile* cluster. Hence, the effect of LAS on *Phenylobacterium* diversity under the experimental conditions tested seemed limited to a particular population of this genus.

The reamplified DNA sequences of most of the bands retrieved from the LAS50-21d-U TGGE fingerprint (band classes 1, 2, 3, 6, 10, 19, and 20) yielded also partial sequences identical to that of band class 7. Several of these band classes were exclusively detected in this sample and were probably PCR artifacts, generated due to the presence of a very high concentration of the DNA representative of band class 7 in the PCR templates.

As previously reported, soils treated with LAS, particularly those receiving LAS at 50 mg l^{-1} during 21 days, displayed a significantly lower number of band classes (see Fig. 1). Only two of the absent band classes (4 and 13) were successfully identified in this study (see Fig. 3).

3.3 Impact of LAS on community composition of *Actinobacteria* and *Acidobacteria* in soil

According to cluster analysis of TGGE profiles, the structure of both *Actinobacteria* and *Acidobacteria* communities in the soil microcosms was remarkably stable in the presence of LAS at the two concentrations tested, as most bands were universally present in all samples and displayed fairly similar relative intensities (Fig. 4).

Dominant band classes of both bacterial groups were identified by reamplification, sequencing, and database comparison. Most *Actinobacteria* sequences identified (75%) were very closely related and phylogenetically affiliated to the genus *Streptomyces*. The rest of *Actinobacteria* sequences retrieved were evolutively close to the *Geodermatophilaceae* (three band classes) and *Nocardiaceae*/*Frankiaceae* (one band class) families. Due to the complexity of *Acidobacteria* profiles, only seven band classes were successfully reamplified and yielded useful sequence data. None of the sequences was close to any of the five validly described genera (*Acidobacterium*, *Holophaga*, *Geotrix*, *Terriglobus*, and *Edaphobacter*), and all the bands displayed 94–100% identity with sequences of uncultured clones filed in the nucleotide databases. All the

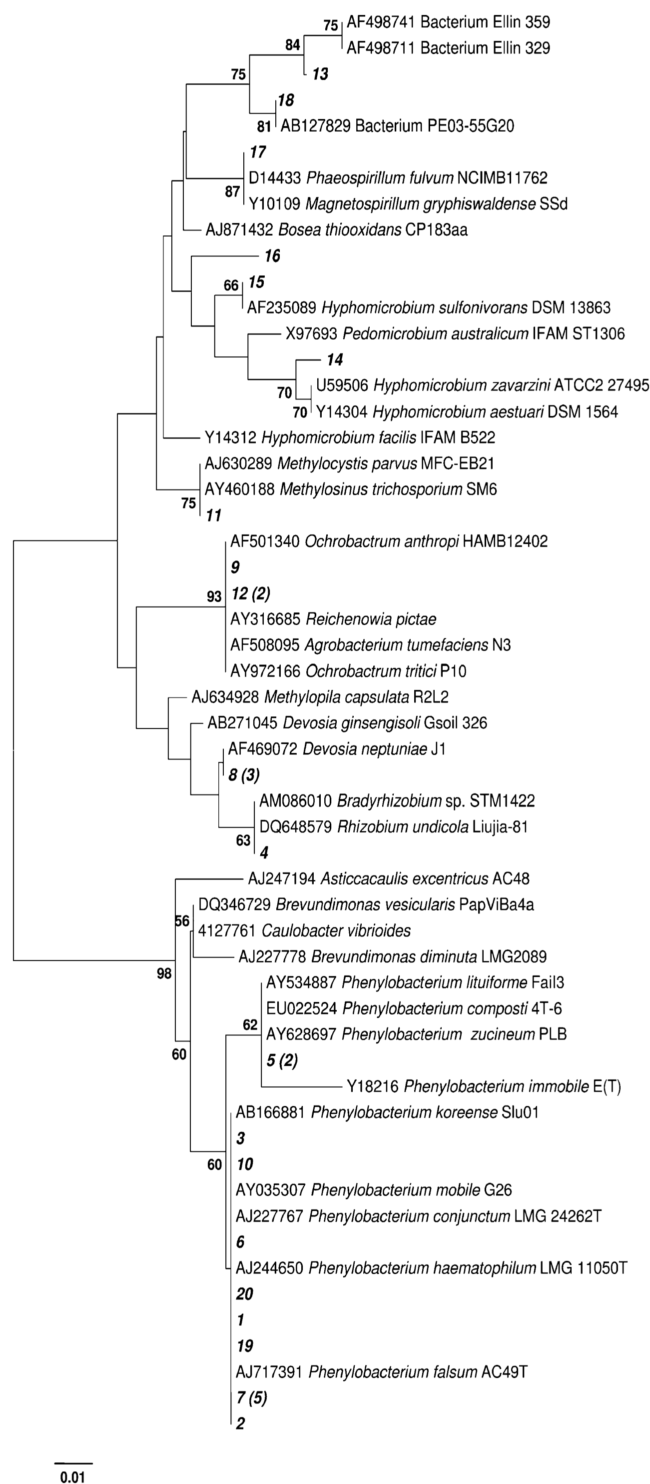

Fig. 3 Unrooted neighbor-joining phylogenetic tree, showing the relationships between the sequences of the V3 hypervariable region of the 16S rRNA gene (ca. 150 bp) of bands reamplified from the alphaproteobacterial TGGE gels and the most similar sequences of cultivable relatives retrieved from the DNA databases. Band classes are numbered as in Fig. 1. The total number of bands which were sequenced of each particular band class is shown in *brackets*. Scale bar—1% divergence. Bootstrap values <50% are not shown

identified band classes were related to group IV (six band classes) and group VI (one band class), according to the grouping of Hugenholtz et al. (1998).

4 Discussion

Previous studies (reviewed by Jensen et al. 2007) concluded that LAS toxicity was not an important microbial selection factor in sludge amended soil, as no significant induction of LAS tolerance by the soil community was observed in long-term trials. However, the use of molecular fingerprinting (PCR-DGGE) to test the impact of LAS on community composition of bacteria in aquatic microcosms revealed that concentrations of LAS as low as 1 mg l^{-1} significantly influence the development of the bacterial community structure, and that the effects were visible after 23 or 43 days, for concentrations of LAS of 10 and 100 mg l^{-1}, respectively (Brandt et al. 2004). Similar conclusions were reached under anoxic conditions in coastal sediment microcosms amended with LAS (50 mg kg^{-1}) and analyzed by PCR-DGGE after 160 days (Lara-Martín et al. 2007). In the present study, exposure to LAS was the main factor determining the evolvement of community structure of *Alphaproteobacteria* in the soil microcosms. The continuous addition of LAS produced significant changes in community composition of this group, particularly involving the shifting of a specific population (band class 7), found dominant in the upper part of soil columns only in the microcosms amended with LAS, and positively correlated in its relative intensity to the concentration of LAS remaining in the soil at the time of sampling.

LAS accumulated in the upper portion of the soil microcosms throughout the experiment at both concentrations tested (see Fig. 2b). The poor downward transport of LAS in soil and sediments has been often reported in the previous literature (León et al. 2001; Jacobsen et al. 2004). The maximal concentration of the surfactant was measured in the upper fractions of the LAS50 microcosms after 7 days and widely exceeded those reached in soil after sludge amendment (Jensen et al. 2007). However, the concentration of LAS was drastically lowered after 21 days of uninterrupted application, indicating that, even under this worst-case scenario conditions, soil microbiota was able to degrade LAS. Observed changes of community structure are thus likely related to the selection of LAS degrading populations after continuous exposure to the surfactant in the microcosms.

Although TGGE is not a strictly quantitative method due to the bias introduced by the PCR reaction, changes of band intensity through experiments are a consequence of a change in the relative abundance of the corresponding populations in the community and can be used as a

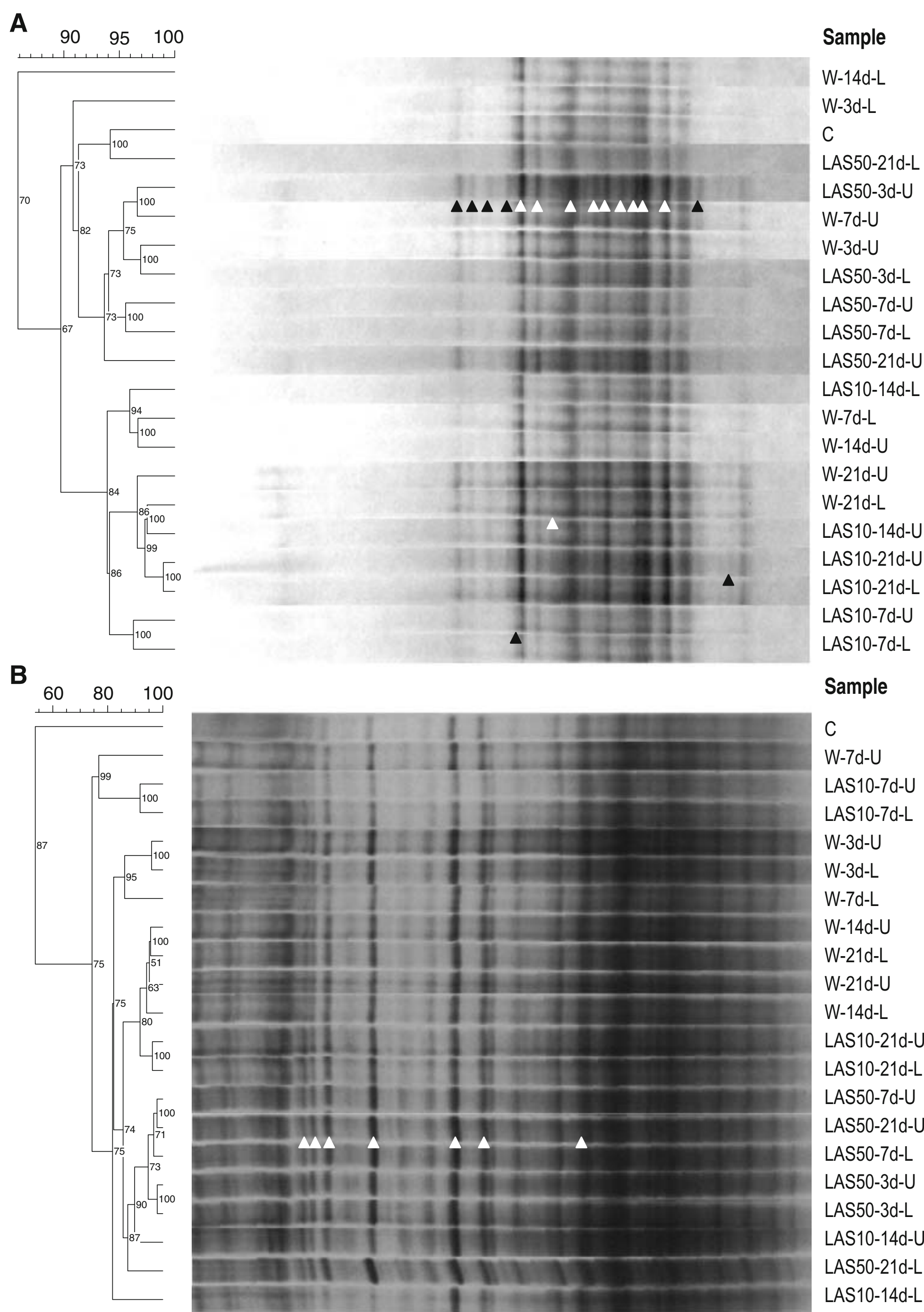

Fig. 4 Dendrograms generated by UPGMA clustering of 16S-rRNA based TGGE patterns of the community of **a** *Actinobacteria* and **b** *Acidobacteria* in soil microcosm samples, either amended with water or LAS solutions (see Fig. 1 legend for the key to nomenclature of soil samples). All profiles are based on the amplification and separation of the V3 hypervariable region of the 16S rRNA gene. Both analyses are curve-based. Scale bar—% correlation. Bands which were successfully reamplified and sequenced are marked with *arrowheads*

semiquantitative measure of bacterial diversity (Lin et al. 2005; Brüggemann et al. 2000; Moreno et al. 2009). In the already mentioned work by Lara-Martín et al. (2007), the PCR-DGGE approach allowed the identification of a *Sedimentibacter* population which appeared as a band of high intensity only in anoxic coastal sediment microcosms where effective biodegradation of LAS was taking place, and hence, this population was pointed as possibly involved in the degradation of LAS. Accordingly, our results evidence that the *Phenylobacterium* population represented by band class 7 was favored by the presence of increasing concentrations of LAS in the soil and turned into a dominant population, suggesting its possible ability to use LAS in soil as a source of nutrients. This is also supported by the fact that one of the closest relatives to the sequence of band class 7 was *P. mobile*, a nonvalidated species described following characterization of a strain isolated from soil (strain G26), whose ability to use LAS as sole C

source is reported in a publication in Chinese (Ke et al. 2003). Hence, the *Phenylobacterium* population represented by band class 7 is a plausible candidate to play a relevant role in the biotransformation of LAS in soil under the conditions tested in the study. We made attempts to isolate *Phenylobacterium* spp. from the upper fraction of soil in the LAS-treated microcosms, using the mineral media described by Eberspächer (2005) amended with 50 mg l^{-1} of LAS and solidified with 1.2% agarose (molecular grade purity), but none of the colonies obtained was found phylogenetically close to *Phenylobacterium* spp. after partial 16S rRNA gene sequence comparison (data not shown). As studies with pure cultures are required to confirm the ability of this population to degrade LAS, new isolation strategies are currently under development in our laboratory.

With regard to other effects of LAS on the alphaproteobacterial community in soil, several band classes disappeared from fingerprints of samples receiving LAS at 50 mg l^{-1}, especially after 21 days (see Fig. 1). This fact reflects a reduction in the number of individuals of the corresponding populations, either due to a inhibitory effect of LAS, or just their outcompeting by other populations able to use LAS and/or its degradation products as a source of C or other nutrients (i.e., S). Disappearance of bands from TGGE profiles must not be interpreted as a complete loss of the corresponding population in soil, as TGGE is a PCR-based method, which mainly reflects only the dominant members of a community (Muyzer 1999).

The results of this work demonstrated that, under the experimental conditions tested, the *Actinobacteria* and *Acidobacteria* communities remained largely undisturbed under the two concentrations of LAS tested, in comparison to the water-amended microcosms, even at the highest concentration applied. These results are coherent with the previously known high resistance of members of these phyla to xenobiotics of different nature, such as pesticides, hydrocarbons, or heavy metals (Gremion et al. 2003; Saul et al. 2005; Paul et al. 2006; Barns et al. 2007). This finding is particularly interesting, as to our knowledge, there are no previous reports regarding the effects of LAS on these bacterial groups in soil. Although a low number of cultivable strains of *Acidobacteria* are described to date, molecular studies have revealed the complex diversity and coherence of the phylum and its wide distribution in aquatic and terrestrial habitats (Barns et al. 1999, 2007; Quaiser et al. 2007). The divergence of the 16S rRNA gene inside the group is comparable to that of the *Proteobacteria* (Hugenholtz et al. 1998), suggesting an equivalent range of yet unknown metabolic capacities and ecological roles.

In view of our results, we recommend the integration of TGGE fingerprinting with the biological and chemical analyses usually applied in risk assessment of LAS in the environment, as a reliable and low time-consuming method for the monitoring of the bacterial community structure and dynamics.

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References

Abraham WR, Macedo AJ, Lünsdorf H, Fischer R, Pawelczyk S, Smit J, Vancanneyt M (2008) Phylogeny by a polyphasic approach of the order *Caulobacterales*, proposal of *Caulobacter mirabilis* sp. nov., *Phenylobacterium haematophilum* sp. nov. and *Phenylobacterium conjunctum* sp. nov., and emendation of the genus *Phenylobacterium*. Int J Syst Evol Microbiol 58:1939–1949

Aguirre de Cárcer DA, Martín M, Mackova M, Macek T, Karlson U, Rivilla R (2007) The introduction of genetically modified microorganisms designed for rhizoremediation induces changes on native bacteria in the rhizosphere but not in the surrounding soil. ISME J 1:215–223

Altschul SF, Madden TL, Schaeffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acid Res 25:3389–3402

Aslam Z, Im WT, Ten LN, Lee ST (2005) *Phenylobacterium koreense* sp. nov. isolated from South Korea. Int J Sys Evol Microbiol 55:2001–2005

Barns SM, Takala SL, Kuske CR (1999) Wide distribution and diversity of members of the bacterial kingdom Acidobacterium in the environment. Appl Environ Microbiol 65:1731–1737

Barns SM, Cain EC, Sommerville L, Kuske CR (2007) Acidobacteria phylum sequences in uranium-contaminated subsurface sediments greatly expand the known diversity within the phylum. Appl Environ Microbiol 73:3113–3116

Brandt KK, Heselsøe M, Roslev P, Henriksen K, Sørensen J (2001) Toxic effects of linear alkylbenzene sulfonate on metabolic activity growth rate and microcolony formation of *Nitrosomonas* and *Nitrosospira* strains. Appl Environ Microbiol 67:2489–2498

Brandt KK, Pedersen A, Sorensen J (2002) Solid-phase contact assay that uses a *lux*-marked *Nitrosomonas europea* reporter strain to estimate toxicity of bioavailable linear alkylbenzene sulfonate in soil. Appl Environ Microbiol 68:3502–3508

Brandt KK, Krogh PH, Sørensen J (2003) Activity and population dynamics of heterotrophic and ammonia-oxidizing microorganisms in soil surrounding sludge bands spiked with linear alkylbenzene sulfonate: a field study. Environ Toxicol Chem 22:821–829

Brandt KK, Jørgensen NOG, Nielsen TH, Winding A (2004) Microbial community-level toxicity testing of linear alkylbenzene sulfonates in aquatic microcosms. FEMS Microbiol Ecol 49:229–241

Brenner DJ, Krieg NR, Staley JT (eds) (2005) Bergey's manual of systematic bacteriology, 2nd edn, vol II, The Proteobacteria—part C: the Alpha- Beta- Delta- and Epsilonproteobacteria. Springer, New York

Brüggemann J, Stephen JR, Chang YJ, Macnaughton SJ, Kowalchuk GA, Kline E, White DC (2000) Competitive PCR-DGGE analysis of bacterial mixture: an internal standard and a appraisal

of template enumeration accuracy. Microbiol Methods 49:111–123

Cortés-Lorenzo C, Molina-Muñoz M, Gómez-Villalba B, Vílchez R, Ramos A, Rodelas B, Hontoria E, González-López J (2006) Analysis of community composition of biofilms in a submerged filter system for the removal of ammonia and phenol from industrial wastewater. Biochem Soc Trans 34:161–168

Duarte IC, Oliveira LL, Saavedra NK, Fantinatti-Garboggini F, Oliveira VM, Varesche MB (2008) Evaluation of the microbial diversity in a horizontal-flow anaerobic immobilized biomass reactor treating linear alkylbenzene sulfonate. Biodegradation 19:375–385

Eberspächer J (2005) Genus IV *Phenylobacterium* Lingens, Blecher, Blecher, Blobel, Eberspächer, Fröhner, Görisch and Layh, 1985 38VP. In: Garrity GM (ed) Bergey's manual of systematic bacteriology, vol 2, part C, 2nd edn. Springer, New York, pp 316–325

Elsgaard L, Petersen S, Debozs K (2001a) Effects and risk assessment of linear alkylbenzene sulfonates in agricultural soil. 1. Short-term effects on soil microbiology. Environ Toxicol Chem 20:1656–1663

Elsgaard L, Petersen S, Debozs K, Kristiansen IB (2001b) Effects of linear alkylbenzene sulfonates (LAS) on soil microbial ecology. Tenside Surf Det 38:94–97

Elsgaard L, Petersen S, Debosz K (2001c) Effects and risk assessment of linear alkylbenzene sulfonates in agricultural soil. 2. Effects on soil microbiology as influenced by sewage sludge and incubation time. Environ Toxicol Chem 20:1664–1672

Elsgaard L, Pojana G, Miraval T, Eriksen J, Marcomini A (2003) Biodegradation of linear alkylbencene sulfonates in sulfate-leached soil mesocosms. Chemosphere 50:929–937

EU Commission DGIII (1997) Study on the possible problems for the aquatic environment related to surfactants in detergents. WRc, EC4294

Gomes NCM, Heuer H, Schönfeld J, Costa R, Mendonca-Hagler L, Smalla K (2001) Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant Soil 232:167–180

Gremion F, Chatzinotas A, Harms H (2003) Actinobacteria might be a dominant part of the metabolically active bacteria in heavy-metal contaminated bulk and rhizosphere soil. Environ Microbiol 5:896–907

HERA (2007) Human and environmental risk assessment on ingredients of European household cleaning products. Linear alkylbenzene sulphonate. http://heraproject.com

Heuer H, Krsek M, Baker P, Smalla K (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microbiol 63:3233–3241

Hugenholtz P, Goebel BM, Pace NR (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 180:4765–4774

Jacobsen AM, Mortensen GK, Hansen HCB (2004) Degradation and mobility of linear alkylbenzene sulfonate and nonylphenol in sludge-amended soil. J Environ Quality 33:232–240

Janssen PH (2006) Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl Environ Microbiol 72:1719–1728

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Clustal X. Trends Biochem Sci 23:403–405

Jensen J, Folker-Hansen P (1995) Soil quality criteria for selected organic compounds. Arbejdsrapport N 47 fra Miljøstyrelsen, Copenhagen, DK EPA

Jensen J, Smith RS, Krogh PH, Versteeg DJ, Temara A (2007) European risk assessment of LAS in agricultural soil revisited: species sensitivity distribution and risk estimates. Chemosphere 69:880–892

Kanso S, Patel BKC (2004) Phenylobacterium lituiforme sp. nov. a moderately thermophilic bacterium from a subsurface aquifer and emended description of the genus *Phenylobacterium*. Int J Sys Evol Microbiol 54:2141–2146

Ke N, Xiao C, Ying Q, Ji S (2003) A new species of the genus *Phenylobacterium* for the degradation of LAS (linear alkylbenzene sulfonate). Wei Sheng Wu Xue Bao 43:1–7 (in Chinese)

Kostanjšek R, Lapanje A, Drobne D, Perović S, Perović A, Zidar P, Štrus J, Hollert H, Karaman G (2005) Bacterial community structure analyses to assess pollution of water and sediments in the Lake Shkodra/Skadar, Balkan Peninsula. Environ Sci Pollut Res 12:361–368

Kristiansen IB, de Jonge H, Nørnberg P, Mather-Christensen O, Elsgaard L (2003) Sorption of linear alkylbenzene sulfonate to soil components and effects on microbial iron reduction. Environ Toxicol Chem 22:1221–1228

Kumar S, Dudley J, Nei M, Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9:299–306

Lara-Martín PA, Gómez-Parra A, Köchling T, Sanz JL, Amils R, González-Mazo E (2007) Anaerobic degradation of linear alkylbenzene sulphonates in coastal marine sediments. Environ Sci Tech 41:3573–3579

León VM, González-Mazo E, Forja-Pajares JM, Gómez-Para A (2001) Vertical distribution profiles of linear alkylbenzene sulfonates and their long-chain intermediate degradation products in coastal marine sediments. Environ Tox Chem 20:2171–2178

Lin B, Braster M, van Breukelen BM, van Verseveld HW, Westerhoff HV, Röhling W (2005) *Geobacteraceae* community composition is related to hydrochemistry and biodegradation in an iron-reducing aquifer polluted by a neighboring landfill. Appl Environ Microbiol 71:5983–5991

Molina-Muñoz M, Poyatos JM, Vilchez R, Hontoria E, Rodelas B, González-López J (2007) Effect of the concentration of suspended solids on the enzymatic activities and biodiversity of a SMB for aerobic treatment of domestic wastewater. Appl Microbiol Biotechnol 73:1441–1451

Moreno B, Vivas A, Nogales R, Macci C, Masciandaro G, Benitez E (2009) Restoring biochemical activity and bacterial diversity in a trichloroethylene-contaminated soil: the reclamation effect of vermicomposted olive wastes. Environ Sci Pollut Res 16(3):253–264

Mortensen GK, Elsgaard H, Ambus P, Jensen ES, Groen C (2001) Influence of plant growth on the degradation of LAS in sludge-amended soil. J Environ Quality 30:1266–1270

Mungray AK, Kumar P (2008) Occurrence of anionic surfactants in treated sewage: risk assessment to aquatic environment. J Haz Mat 160:362–370

Muyzer G (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. Curr Op Microbiol 2:317–322

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Nimer M, Ballesteros O, Navalón G, Crovetto C, Verge C, López I, Berna JL, Vílchez JL (2007) New simple treatment for determination of linear alkylbenzene sulfonate (LAS) in agricultural soil by liquid chromatography with fluorescence detection. Anal Bioanal Chem 387:175–2184

Paul D, Pandey G, Meier C, van de Meer JR, Jain RK (2006) Bacterial community structure of a pesticide-contaminated site and assessment of changes induced in community structure during bioremediation. FEMS Microbiol Ecol 57:116–127

Quaiser A, López-García P, ZIvanovic Y, Henn M, Rodríguez-Valera F, Moreira D (2007) Comparative analysis of genome fragments of Acidobacteria from deep Mediterranean plankton. Environ Microbiol 10:2704–2717

Sait M, Hugenholz P, Janssen PH (2002) Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. Environ Microbiol 4:654–666

Sánchez-Peinado MM, González-López J, Rodelas B, Galera V, Pozo C, Martínez-Toledo MV (2008) Effect of linear alkylbenzene sulfonates (LAS) on the growth of aerobic heterotrophic cultivable bacteria isolated from an agricultural soil. Ecotoxicology 17:549–557

Sánchez-Peinado MM, Rodelas B, Martínez-Toledo MV, González-López J, Pozo C (2009) Response of soil enzymes to linear alkylbenzene sulfonates (LAS) addition in soil microcosms. Soil Biol Biochem 41:69–76

Saul DJ, Aislabie JM, Brown CE, Harris L, Foght JM (2005) Hydrocarbon contamination changes the bacterial diversity of soil from around Scott base Antarctica. FEMS Microbiol Ecol 53:141–155

Schowanek D, David H, Francaviglia R, Hall J, Kirchman H, Krogh PH, Schraepen N, Smith S, Wildemann T (2007) Probabilistic risk assessment for linear alkylbenzene sulphonate (LAS) in sewage sludge used on agricultural soil. Regul Toxicol Pharm 49:245–259

Sokal RR, Rohlf FJ (1962) The comparison of dendrograms by objective methods. Taxon 11:33–40

Tiago I, Mendes V, Pires C, Morais PV, Veríssimo A (2005) *Phenylobacterium falsum* sp. nov., an Alphaproteobacterium isolated from a nonsaline alkaline groundwater, and emended description of the genus *Phenylobacterium*. Sys Appl Microbiol 28:295–302

Vílchez R, Pozo C, Gómez MA, Rodelas B, González-López J (2007) Dominance of sphingomonads in a copper-exposed biofilm community for groundwater treatment. Microbiology 153:325–337

Vinther FP, Mortensen G, Elsgaard L (2003) Effects of linear alkylbenzene sulfonates on functional diversity of microbial communities in soil. Environ Toxicol Chem 22:35–39

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703

Weon HY, Kim BY, Kwon SW, Go SJ, Koo BS, Stackebrandt E (2008) *Phenylobacterium composti* sp. nov., isolated from cotton waste compost in Korea. Int J Syst Evol Microbiol 58:2301–2304

Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol 7:203–214

Zhang K, Han W, Zhang R, Xu X, Pan Q, Hu X (2007) *Phenylobacterium zucineum* sp nov a facultative intracellular bacterium isolated from a human erytroleukemia cell line K562. Sys Appl Microbiol 30:207–212