




Open Archive Toulouse Archive Ouverte (OATAO)

OATAO is an open access repository that collects the work of Toulouse researchers and makes it freely available over the web where possible

This is an author's version published in: <http://oatao.univ-toulouse.fr/23717>

Official URL: <https://doi.org/10.1016/j.apsoil.2012.09.004>

To cite this version:

İnceoğlu, Özgül and Sablayrolles, Caroline  and van Elsas, Jan Dirk and Falcão Salles, Joana *Shifts in soil bacterial communities associated with the potato rhizosphere in response to aromatic sulfonate amendments*. (2013) *Applied Soil Ecology*, 63. 78-87. ISSN 0929-1393

Any correspondence concerning this service should be sent to the repository administrator: tech-oatao@listes-diff.inp-toulouse.fr

Shifts in soil bacterial communities associated with the potato rhizosphere in response to aromatic sulfonate amendments

Özgül İnceoğlu^a, Caroline Sablayrolles^b, Jan Dirk van Elsas^a, Joana Falcão Salles^{a,*}

^a Department of Microbial Ecology, Centre for Life Sciences, Nijenborgh 7, 9747 AG Groningen, The Netherlands

^b Laboratoire de Chimie Agro-Industrielle (LCA), Ecole Nationale Supérieure des Ingénieurs en Arts Chimiques Et Technologiques (INP, ENSIACET), Université de Toulouse, 4 Allées Emile Monso, 31029 Toulouse, France

ARTICLE INFO

Keywords:

Rhizosphere
Aromatic sulfonate
asfA
Plant growth
LAS

ABSTRACT

Desulfonation is an important process in the sulfur cycle, through which organic sulfur compounds are mineralized, releasing S. Desulfonating bacteria are known to respond to inorganic S soil amendments. However, the extent to which these communities shift as a response to the addition of organic sulfur in the soil remains elusive. Here, we investigated how amendments of soil with inorganic or organic sulfur compounds influence the bacterial communities associated with potato, in a microcosm experiment. The soil was amended with two doses of linear alkylbenzene sulfonate (LAS), here used as a model aromatic sulfonate compound, or with sulfate. Degradation of LAS was observed already at the young plant stage, as in all treatments 10- to 50-fold reductions of the initial (background) LAS concentrations were noted. Quantitative PCR analyses showed no significant effects of treatment on the bacterial abundances, which tended to increase from the young plant to the flowering stages of plant development. The bacterial community structures, determined via PCR-DGGE, were strongly affected by the presence of plants. This rhizosphere effect became more apparent at the flowering stages. Both the bacterial and β -proteobacterial community structures were affected by the presence of LAS, but dose-related effects were not observed. LAS also caused significant changes in the community structures, as compared to those in inorganic sulfate amended soil. Sulfate did not influence the bacterial community structures and only affected the β -proteobacterial ones at the flowering stage. Surprisingly, the presence of LAS did not exert any significant effect on the abundance of the *Variovorax asfA* gene, although clone libraries revealed a dominance of *Variovorax* types in the rhizosphere, especially in the high-level LAS treatment. Our results suggest that rhizosphere communities are key players in LAS degradation in soils, and that desulfonator *Variovorax* spp. plays a minor role in the mineralization of aromatic sulfonates in soil cropped with potato.

1. Introduction

Sulfonates can constitute major components of the organo sulfur compounds in soil. Aeruginosin B is the only clearly identified natural aromatic sulfonate in soil (Autry and Fitzgerald, 1990), which might contain other as-yet-undefined forms of sulfonates. Additionally, sulfonated compounds like linear alkylbenzene sulfonate (LAS) may regularly enter soil (Brandt et al., 2001; Carlsen et al., 2002; Sanchez-Peinado et al., 2008; Vinther et al., 2003). In fact, LAS is the most commonly used anionic surfactant in household detergents and multipurpose cleaning products (Jensen et al., 2007). Even though LAS is biodegradable under aerobic conditions, it may – to some extent – persist in agricultural soils (Schowanek et al., 2007). Moreover, levels of 0.5–1 mg LAS/kg dry soil have been

found to be present in natural soils that remained uncultivated for 50–100 years (Carlsen et al., 2002).

The aromatic sulfonates in soil are inaccessible for plants. However, they can be important for plant sulfur nutrition in (otherwise) sulfur-depleted soils (Kertesz and Mirleau, 2004). In that case, microbial attack on aromatic sulfonates in soils is a key process, as it releases the sulfur-containing moiety from these, making it accessible to plants (Seegmüller and Rennenberg, 2002). The bacterial desulfonation of aromatic sulfonates has been elucidated in the sewage sludge isolate *Pseudomonas putida* S-313. The organism was shown to be able to decouple the sulfur moiety from several hundreds of different aromatic sulfonates (Cook et al., 1999). The cleavage of aromatic sulfonates requires the action of several enzymes, including a reductase/ferredoxin protein couple encoded by the *asfA/asfB* genes (Vermeij et al., 1999). Expression of the *asfA* and *asfB* genes is regulated by the internal sulfur level of the cell, being repressed in the presence of sulfate (Vermeij et al., 1999). *Pseudomonas putida* S-313, like many other *P. putida* strains, has

* Corresponding author. Tel.: +31 50 363 2162; fax: +31 50 363 2154.
E-mail address: j.falcao.salles@rug.nl (J. Falcão Salles).

been shown to exert a significant plant growth promotion (PGP) effect on tomato plants (Kertesz and Mirleau, 2004). This PGP effect is related to the desulfonation of arylsulfonates, as no effect was seen with a mutant with an inactivated *asfA* gene (Kertesz and Mirleau, 2004). That the desulfonation process is not limited to *P. putida* is indicated by recent studies which show members of other bacterial genera, i.e. *Variovorax*, *Polaromonas*, *Acidovorax* and *Rhodococcus*, also to be active in this process (Schmalenberger et al., 2008a,b, 2009). It is, however, not yet clear how the sulfur moiety released by the action of the *asfA* gene product is passed on to the plant (Schmalenberger and Kertesz, 2007).

Large fractions of the sulfonates in soil are commonly subjected to rapid flux (Cook et al., 1999), exemplifying the potential for desulfonation that is present in natural soil microbial communities (Ruff et al., 1999). Moreover, it has been shown that the cycling of sulfur between organic and inorganic forms is enhanced in the presence of crop plants (Castellano and Dick, 1991), suggesting that plant roots stimulate microbial desulfonators in the rhizosphere when sulfate levels are low. By using *asfA* as a functional marker for desulfonators, Schmalenberger et al. (2010) showed, in plant growth chambers, that there were differences between no-sulfate treatments and those in which sulfate was applied. This suggests that desulfonators are selected in relation to the fertilization regime and adapt to changing levels of sulfate. In a previous study, we revealed a rhizosphere as well as a potato cultivar effect on the abundance of potential desulfonators in a potato field. Specifically, the abundance of the *Variovorax*-specific *asfA* gene in the rhizosphere was higher than that in the corresponding bulk soil, indicating a stimulus of this organism by plant roots. However, the level of organic versus inorganic sulfur remained unaddressed in this study (Inceoglu et al., 2010).

Considering the close association between bacterial desulfonators – such as *Variovorax* – and plants, the aim of this study was to define the bacterial communities associated with potato roots that respond to the presence of aromatic sulfonates. Our hypothesis was that a bipartite association between such desulfonators and plants may occur on the basis of nutrients provided by the plant and that this selective process might be affected by the level of (organic and inorganic) sulfur in the soil. In a previous study, we showed that different potato cultivars can differ with respect to the abundance of desulfonators associated with their rhizospheres (Inceoglu et al., 2010). In the current paper, we describe a microcosm study which aimed to evaluate how the presence of two different levels of aromatic sulfonate versus sulfate in soil affects the bacterial communities associated with potato. For this experiment, LAS was used as model sulfonated aromatic compound. As we were interested in the putative differences between physiologically different cultivars, we used cultivar Aveka (high-starch tubers – slow growth) versus Premiere (low-starch tubers – fast growth). Thus, using quantitative PCR (qPCR) and PCR-DGGE, the bacterial communities were compared across treatments in both bulk and rhizosphere soils at different plant growth stages. Moreover, in the light of the relevance of a suite of β -proteobacteria for desulfonation (Schmalenberger and Kertesz, 2007), the structure of these communities as well as their diversities were evaluated. Finally, the abundance of the *Variovorax* class of desulfonators was assessed by qPCR targeting the *Variovorax asfA* gene (Schmalenberger et al., 2008a,b).

2. Materials and methods

2.1. Bacterial growth on LAS in liquid culture

Growth experiments were carried out with *Variovorax paradoxi* DSM30034T in triplicate in sterile 100-mL Erlenmeyer flasks

containing 20 mL of newly inoculated minimal medium (MM) containing 0.3 g/L of yeast extract (Beil et al., 1995) and concentrations of 10, 20 and 100 mg/L LAS. Incubation was performed at 25 °C on a rotary shaker and samples were taken daily, for 4 days. Different set-ups were used, including a control with MgSO₄ without any carbon source. Furthermore, there were three set-ups with 20 mM succinate (as carbon source) with either 10 [0.29 mM S], 20 [0.58 mM S] or 100 mg LAS/L [2.90 mM S], or 0.12 g MgSO₄ [1 mM S], (as sulfur sources). The measurements were performed by determining OD₆₅₀ values in a Versamax microplate reader (Molecular Devices, Sunnyvale, USA).

2.2. Microcosm experiments

The soil used for the microcosm experiments (denoted soil B) was collected from root-free areas in a field used for potato cropping in Buinen, The Netherlands, in March 2010. The B soil was a loamy sand with 3.5% organic matter and a pH-KCl of about 5.5. Approximately twenty soil samples were collected with a scoop at depths of 0–20 cm and these were subsequently pooled to homogenization, in order to obtain one soil batch from which to establish the soil microcosms.

Two different potato cultivars, Aveka (A; high-starch tubers) and Premiere (P; low-starch tubers) were used in the experiments. Pot experiments with soil (hereafter defined as soil microcosms) were prepared in 5 L pots, each containing about 3 kg of soil. Before the experiment was started, the soil contained 1.05 ± 0.15 mg/kg dry soil sodium dodecylbenzene sulfonate. Then, LAS, at levels of 10 or 50 mg/kg dry soil, or calcium sulfate (10 mg/kg dry soil) were added, in separate, to six replicate pots (3 replicates per cultivar). In addition, replicate microcosms without any added sulfur source were established. Pots were then seeded with one potato tuber of either cultivar A or P. Besides, for each treatment one pot was left unseeded, serving as a reference for bulk soil. The LAS used had a purity of 80–85%, with a distribution of linear alkyl chains as follows: C10: ~5%, C11: ~45–50%, C12: ~35%, C13: ~10–15%, C14: ~<5% (Sigma Aldrich, Zwijndrecht, The Netherlands). The pots were randomly placed in trays, avoiding cross-contaminations, and placed in the greenhouse where artificial light was used to keep a 16/8 h day/night regimen (22/18 °C). Pots were watered daily with tap water from the trays in which they had been placed. Rhizosphere and bulk soil samples were collected at the young and flowering stages of plant development. For both cultivars, the young plant stages (EC30) occurred around 30 days post-planting (dpp), and the flowering stages (EC60) around 60 dpp (Hack et al., 1993).

2.3. Reagents and standards

All chemicals used were of analytical quality. Methanol and acetonitrile (HPLC grade) were purchased from Scharlau (Barcelona, Spain). Ammonium acetate was obtained by VWR Merck (Paris, France). Condea Chimie SARL supplied LAS, as Marlon ARL (a commercial powder containing 80% C10–C13 LAS). The LAS mixture used as the standard in chemical analyses (differing from the one used in the microcosm experiment) had the following mass distribution: C10: 14.3%, C11: 35.7%, C12: 30.8%, and C13: 19.2%. C8-LAS (Alfa Aesar, Schiltigheim, France) was used as an internal standard for the analyses, at 10 mg/L in each extract. Sodium dodecyl sulfate (SDS; 99% purity) was purchased from Acros Organics (France). It was added at a concentration of 2 mM. Syringe filters of polytetrafluoroethylene (PTFE) with a 0.45 μ m pore size (VWR Merck, France) were used to filter the extract before analysis.

2.4. Soil chemical analyses

Soil sulfate measurements were performed by Cyprio B.V. (Groningen, The Netherlands) using a LANGE Cuvette Test (Hach Lange, Tiel, The Netherlands). The analytical method used for LAS quantification (Sablayrolles et al., 2009) is explained briefly below. Soil samples were placed in plastic trays in a freezer at -20°C until analyses. Prior to extraction, samples were freeze-dried and ground. The solid/liquid extraction was carried out with a Soxtec System HT2 (Tecator, France). Two g of lyophilized soil were extracted with 100 mL of methanol for 45 min. Then, the extract was concentrated to 1 mL under a nitrogen stream with TurboVap[®] II (Caliper LifeSciences, France) and filtered through a $0.45\ \mu\text{m}$ filter. Then, $10\ \mu\text{L}$ of internal standard C8-LAS at 1 g/L and $20\ \mu\text{L}$ of SDS at 28.8 g/L were added to the extract. Recovery rates were measured on soil spiked with LAS, yielding a rate value of $91 \pm 6\%$.

Chromatographic analysis of LAS was performed on a high performance liquid chromatograph (Dionex, France) equipped with a SOR100 degasser, a P680 HPLC pump, an ASI 100 autosampler, a TCC 100 thermostated column compartment and a UVD340U ultraviolet diode array. A mass spectrometer (MSQ Surveyor, Thermo Finnigan, France) was connected on line. The system was computer-controlled with data acquisition and processing using Chromeleon (Dionex) software. LAS separation was carried out using a Lichrospher[®] 100 RP-18e ($250\ \text{mm} \times 4\ \text{mm}$ id, $5\ \mu\text{m}$) column protected by a Lichrospher[®] 100 RP-18e ($4\ \text{mm} \times 4\ \text{mm}$ id, $5\ \mu\text{m}$) guard column (VWR Merck, France). The injection volume was set at $30\ \mu\text{L}$, the column was kept at a temperature of 25°C and the flow rate was $0.8\ \text{mL}/\text{min}$. Elution was performed with a gradient composed of milliQ water (A) and a solution of acetonitrile/milliQ water (80/20; v/v) containing 2 mM of ammonium acetate (B). The gradient programme for the first 3 min was 30% B and it was increased linearly to 100% B over 10 min, then kept isocratic for 2 min and decreased linearly to 30% B over 2 min. Detection of LAS was carried out using a UV detector at 224 nm and a mass spectrometer equipped with an electrospray ionization (ESI) probe in negative mode. The ESI conditions were as follows: probe temperature 350°C , capillary tension 3 kV, ion fragmentation energy 80 V. The fragments (m/z) used for identification and quantification of LASs were: 297 (C10-LAS), 311 (C11-LAS), 325 (C12-LAS), 339 (C13-LAS), 270 (C8-LAS) and 183 as a confirmation fragment ion.

LASs were separated in 17 min, with retention times of 11.1, 11.9, 12.6 and 13.3 min for C10, C11, C12, C13-LAS, respectively. Calculations were made from the peak areas corrected with the C8-LAS peak area. The precision of the chromatographic measurements was evaluated by using a standard solution (LAS at 5 mg/L), which was analyzed tenfold. The results were used to calculate the relative standard deviation (%). The reproducibility of the chromatographic determination was found to be 7% for the sum of LASs. The limit of detection, determined as three standard deviations above the mean blank signal, was $0.005\ \mu\text{g}/\text{kg}$ for the sum of LASs. The limit of quantification was determined as 10 standard deviations above the blank signal and was equal to $0.015\ \mu\text{g}/\text{kg}$.

2.5. Soil DNA extraction

Following careful removal of plants from the pots, loosely adhering soil on the roots was shaken off, and the resulting roots with adhering (rhizosphere) soil were sampled per pot, followed by collection of the rhizosphere soil by brushing.

Three replicates of rhizosphere soil and corresponding bulk soil (from the separate pot) were used directly for DNA extraction. For this, the Powersoil DNA extraction kit (Mo Bio Laboratories Inc., NY, USA) was used in a modified form, as described previously (Inceoglu

et al., 2010). Assessment of DNA quantity and degree of purity was also done as described previously (Inceoglu et al., 2010).

2.6. Quantitative PCR

Quantification of bacterial 16S rRNA genes in the samples was performed with primers 341F and 518R according to Schmalenberger et al. (2008a,b), using an annealing temperature of 55°C and no added betaine. Standards (10^4 – 10^{10} molecules per reaction) were prepared using DNA from *V. paradoxus* DSM30034T.

Quantitative PCR (qPCR) of the *Variovorax* sp. *asfA* gene was performed using forward primer *asfA*.Varx_F1 (CTGTCGGGCATG-GAGTTCT) and reverse primer *asfA*.Varx_R1 (AGCGTCACCG-GAAAGTGCT), to yield 302-bp amplicons (Schmalenberger et al., 2008a,b). The reaction mixture contained $5\ \mu\text{L}$ of DyNamo capillary SYBR Green qPCR master mix (Finnzymes, Helsinki, Finland), 1 M betaine, 0.3 pmol of forward and reverse primers and 5 ng ($1\ \mu\text{L}$) of template DNA in a total volume of $10\ \mu\text{L}$. PCR conditions were 95°C for 10 min, followed by 40 cycles of 15 s 95°C , 20 s 60°C and 20 s 72°C . Standards (10^4 – 10^8 molecules per reaction) were prepared using DNA from *V. paradoxus* DSM30034T.

Two-way analysis of variance (ANOVA) and Tukey's post hoc test were used to compare the target gene numbers between different treatments, cultivars and growth stages using the R package (R Development Core Team, 2012).

2.7. PCR amplifications for denaturing gradient gel electrophoresis (DGGE) community fingerprinting

On the basis of DNA extracted from the rhizosphere and bulk soils, PCR amplifications targeting the 16S ribosomal RNA (rRNA) genes of total bacteria as well as β -proteobacteria were run. For the ensuing bacterial PCR-DGGE, primers GC-341 and 518R (35 cycles) were used, as described previously (Inceoglu et al., 2010). A nested PCR approach was used for amplification of the β -proteobacterial communities. More specifically, the initial amplification was carried out with primers 27F and 865R (Lane, 1991) in a touchdown protocol (30 cycles), as described previously (Cunliffe and Kertesz, 2006). A second PCR reaction was then performed, in which $1\ \mu\text{L}$ (5 ng) of primary PCR product was used as a template in a $25\text{-}\mu\text{L}$ mix with DGGE primers GC-341 and 518R, following the protocol used for total bacteria, except that the number of cycles was reduced to 25 (Schmalenberger et al., 2008a,b).

2.8. DGGE community fingerprinting and analyses

All DGGE profiles were generated in the Ingeny Phor-U system (Ingeny International, Goes, the Netherlands). The amplicons obtained from the soil DNAs, at estimated concentrations of 200 ng, were loaded onto polyacrylamide gels [6% (w/v) acrylamide in $0.5 \times$ Tris-acetate-EDTA (TAE) buffer (2.42 g Tris-base, 0.82 g sodium acetate, 0.185 g EDTA, H_2O 1 L)]. The bacterial amplicons were run on 35–65% denaturant gradient gels at 100 V for 16 h at 60°C . All gels were SYBR gold stained, after which they were digitized for further analyses.

The profiles of the different DGGE gels were stored as TIFF files. Images were normalized using the markers and the patterns were subsequently compared by using clustering methods. Similarity matrices were generated using Pearson's correlation coefficient (r). Subsequently, the patterns were clustered using the unweighted pair group method with arithmetic averages (UPGMA) in the GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium). In addition, transformed (fourth square) data based on relative band intensities and positions of bands were used for analysis of similarity (Euclidean distance based) using one- and two-way ANOSIM, implemented in PRIMER 6 (Primer v6, Plymouth, UK).

Table 1

Measurements of LAS and sulfate (average \pm S.E.) in the rhizosphere and bulk soil samples, at young plant and flowering stages.

Samples		Young plant LAS ($\mu\text{g}/\text{kg}$ dry soil)	Sulfate (mg/kg dry soil)	Flowering LAS ($\mu\text{g}/\text{kg}$ dry soil)	Sulfate (mg/kg dry soil)
CA	Rhizosphere	34.75 \pm 9.6	11.9	73.26 \pm 11.88	70.6
CP	Rhizosphere	nd	94.4	31.2 \pm 12.07	128.8
C	Bulk	49.86 \pm 1.34	nd	55.68 \pm 0.97	nd
SO4A	Rhizosphere	19.96 \pm 8.3	148.9	44.7 \pm 31.45	315
SO4P	Rhizosphere	27.4 \pm 17.19	117.5	45.47 \pm 31.06	210
SO4	Bulk	45.2 \pm 1.67	nd	44.98 \pm 0.6	nd
L10A	Rhizosphere	97.7 \pm 24.9	136.3	25.41 \pm 21.77	88.8
L10P	Rhizosphere	43.26 \pm 14.29	103.8	28.73 \pm 18.45	371.9
L10	Bulk	36.18 \pm 0.78	nd	50.17 \pm 0.78	nd
L50A	Rhizosphere	38.1 \pm 6.38	95.6	57.96 \pm 40.1	206.9
L50P	Rhizosphere	108.55 \pm 18.05	142.6	27.24 \pm 1.9	101.3
L50	Bulk	68.86 \pm 6.43	nd	33.64 \pm 0.6	nd

Initial (background) concentration was 1052 \pm 150 μg LAS/kg dry soil and 21.1 mg SO₄/kg dry soil.

C, control; SO₄, sulfate treated; L10, 10 mg LAS/kg dry soil treated; L50, 50 mg LAS/kg dry soil treated; A, Aveka; P, Premiere; and nd, not determined.

Samples were grouped per rhizosphere/bulk and treatment. One-way analyses with replicates were done with 999 permutations. The global *R* value, varying between -1 and 1 , was used. $R=0$ indicated completely random groupings while $R=1$ indicated that samples within a soil were more similar to each other than to samples from the other soil. A significant global *R* indicated the presence of differences between soil types somewhere in the analyses.

2.9. Cloning and sequencing of β -proteobacterial 16S rRNA gene amplicons from selected samples

Eight clone libraries of β -proteobacterial specific 16S rRNA gene amplicons were generated (primers 27f and 865r), using duplicates, to cross-compare treatments at the flowering stage. The selected treatments were: initial bulk soil (before starting the experiment), control (no treatment), sulfate-treated and LAS-50-treated cultivar P rhizosphere and flowering stage. This selection was based on the fact that the communities in the flowering cultivar P rhizospheres were most responsive to the treatments, as evidenced by the qPCR and DGGE analysis.

The amplicons were ligated into the pGEM[®]-T easy vector (pGEM[®]242-T Vector System II, Promega, Madison, WI, USA), followed by introduction into competent *Escherichia coli* JM109 cells by transformation according to the manufacturer's instructions. White colonies were picked and diluted in 10 μL sterile water, after which they were used as templates for PCR using the universal M13f/M13r vector primers. The inserts were sequenced by LGC (Berlin, Germany).

2.10. Analysis of β -proteobacterial 16S rRNA gene sequence diversity

Prior to the analyses of the sequences, these were checked for chimera formation using Bellerophon v.3 (<http://greengenes.lbl.gov>). The remaining sequences were then classified using the Ribosomal Database Project II (RDP) classifier with a confidence threshold of 80% (<http://simo.marisci.uga.edu>). To determine the closest phylogenetic relatives of the sequences, BLAST-N was used against the non-redundant NCBI database. Sequence alignments and tree building were carried out using the Molecular Evolutionary Genetics Analysis (MEGA) software package (Tamura et al., 2007) using the Kimura two-parameter algorithm with bootstrap tests of inferred phylogeny with 1000 replications (Kimura, 1980). Pairwise sequence similarities were calculated with the programme DNADIST (<http://cmgm.stanford.edu/phylip/dnadist.html>) using the Kimura

two-parameter algorithm. On the basis of the generated similarity matrix, the sequences were assigned to operational taxonomic units (OTUs) using DOTUR (Schloss and Handelsman, 2005). The frequency data assigned to an OTU at the "species" (97% similarity criterion) level was used to yield rarefaction curves, and CHAO1 richness estimates. Sequences were also subjected to library shuffling analysis using LIBSHUFF to determine if the clone libraries were significantly different from each other. The sequences generated in this study were deposited in Genbank under numbers JF809023–JF809566.

3. Results

3.1. Growth rates of *V. paradoxus* DSM30034 in liquid batch cultures

V. paradoxus DSM30034T grew well in minimal medium (MM; Beil et al., 1995) supplemented with 20 mM succinate and MgSO₄. It also grew in MM containing 10 and 20 mg/L LAS when this was used as the sole carbon source (Fig. S1), although at much lower rates than with succinate. The presence of succinate in addition to the LAS treatments enhanced the growth rates observed with LAS alone, but these remained inferior to the growth rates observed with succinate only. This indicated a toxicity effect of LAS. Indeed, no growth was observed for up to 10 days in MM in the presence of 100 mg/L LAS, with or without succinate, indicating the growth-inhibiting nature of LAS at the higher levels.

3.2. Soil analyses and plant performance

The levels of SO₄ and LAS were assessed across all bulk and rhizosphere soil samples from the microcosm experiment (Table 1). The initial concentration of sulfate in the B soil was 20 mg/kg dry soil. This value increased in all samples during the experiment, indicating mineralization. Besides, the initial (background) concentration of LAS in the B soil was 1 mg/kg dry soil. The addition of different levels of LAS increased this concentration by about 10- and 50-fold, respectively. Thus, LAS levels of 10 and 50 mg/kg dry soil were established. Measurements at both the young and flowering plant growth stages revealed that, in all treatments, the LAS levels had dropped to roughly 0.02–0.1 mg per kg dry soil (Table 1). The slight increase in the LAS levels in soil from the young plant to flowering stage, as observed in a few treatments, might be attributed to the release of LAS that had been previously adsorbed soil particles. The analyses of the individual LAS components are shown in supplementary material (Tables S1 and S2). Plant development and

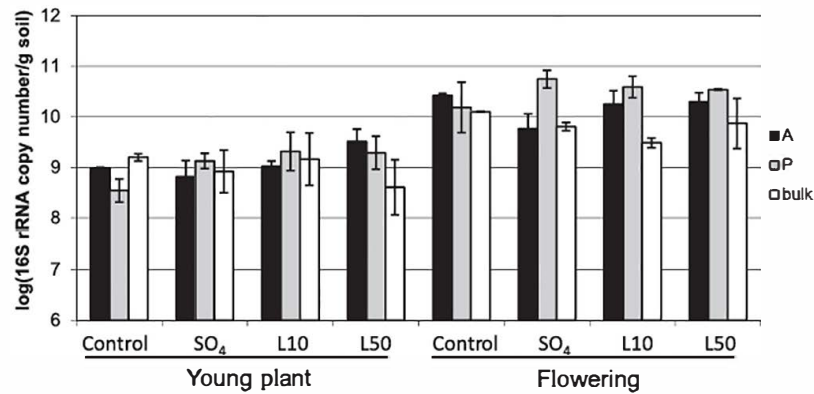


Fig. 1. Abundance of bacterial 16S rRNA genes in rhizosphere and bulk soil per growth stage for different treatment. Error bars indicate standard deviation. Two-way analyses of variance (ANOVA) showed a significant effect of plant growth on bacterial abundance ($F = 66.91$; $P < 0.0001$) whereas cultivar and sulfur addition (organic and inorganic), as well as all possible interactions, were not significant ($P \geq 0.141$). Black bars – cultivar A, gray bars – cultivar P, white bars – bulk soil. C, control; SO₄, sulfate treated; L10, 10mg LAS treated; and L50, 50mg LAS treated.

biomass production was similar for both the fertilized and unfertilized microcosms (data not shown).

3.3. Dynamics of total bacterial and *Variovorax* abundances in bulk and rhizosphere soils as assessed by qPCR

The abundances of the bacterial populations in the different treatments at the initial and young plant and flowering stages were estimated on the basis of bacterial 16S rRNA gene abundance measurements using qPCR. To determine whether the soil background would affect the quantification, DNA of *V. paradoxus* strain DSM30034T was added, in varying concentrations, to the bulk and rhizosphere soil DNA extracts and subsequently quantified. At the onset of the experiments, the B soil had 4.6×10^8 16S rRNA gene copies per g dry soil. At the young plant stage, bacterial 16S rRNA gene abundance measurements revealed variable target gene numbers in the bulk soils, ranging from 4.0×10^8 to 1.6×10^9 gene copies/g dry soil (Fig. 1). Besides, slight positive, yet insignificant, rhizosphere effects were observed for cultivars A and P across the treatments.

At flowering stage, the 16S rRNA gene copy numbers showed significant increases in all rhizosphere soil samples as compared to those in the young plant stage ($P < 0.05$). Surprisingly, an increase was noted for bulk soil as well. The measurements in the bulk soils, at this stage, ranged between 3.0×10^9 and 1.3×10^{10} gene copies/g dry soil, whereas those in the rhizospheres were slightly raised (significant only in the sulfate-

and LAS-10-treated cultivar P rhizospheres [$P < 0.05$]). We did not detect any significant treatment effect on the bacterial 16S rRNA gene copy numbers at this stage. Two-way analyses of variance (ANOVA) showed a significant effect of plant growth on bacterial abundance ($F = 66.91$; $P < 0.0001$), whereas effects of cultivar and sulfur addition (organic and inorganic), as well as all possible interactions, were not significant ($P \geq 0.141$).

In the light of the presumed importance of *Variovorax* spp in the desulfonation process in soil, the abundance of the *Variovorax* specific *asfA* genes was measured by qPCR to assess the putative effects of rhizosphere, cultivar and/or treatment. The bulk soil, before any treatment, contained 3.4×10^4 *asfA* gene copies/g dry soil (Fig. 2). Then, in the young plant stage, the bulk soils revealed gene copy numbers in the range 2.9×10^4 to 1.5×10^5 /g dry soil. Remarkably, at this stage there were significant increases in the *Variovorax* like *asfA* gene numbers for the control and sulfate-treated bulk soil samples, whereas the gene copy numbers in the LAS-10 and LAS-50 treated samples were similar to those in the initial bulk soil. Significant rhizosphere effects were not noted other than for the LAS-50-treated cultivar P rhizosphere.

At flowering stage, the copy numbers of the *Variovorax* like *asfA* gene in all bulk soil samples were significantly higher than those in the initial bulk soil, ranging from 1.0×10^5 to 4.63×10^5 /g dry soil. Interestingly, there was a negative rhizosphere effect in the control samples, whereas no effect was found in any of the LAS- or sulfate-amended soils (Fig. 2). Two-way analyses of variance (ANOVA) confirmed the occurrence of a significant effect of plant growth on

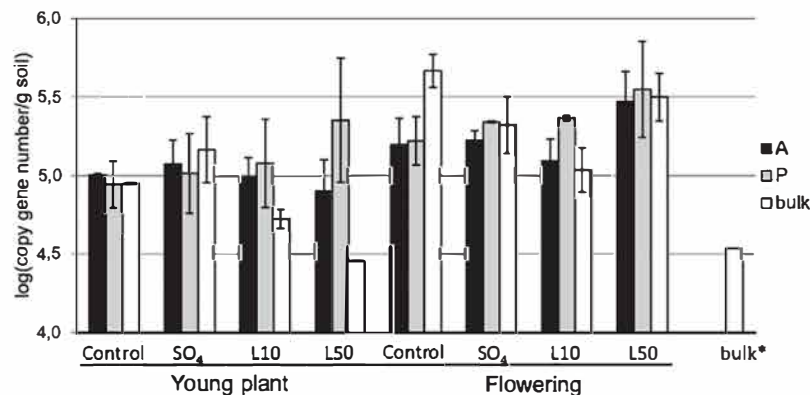


Fig. 2. Abundance of bacterial *Variovorax asfA* gene in rhizosphere and bulk soil per growth stage for different treatment. Error bars indicate standard deviation. Two-way analyses of variance (ANOVA) confirmed a significant effect of plant growth on *Variovorax asfA* gene abundance ($F = 23.52$; $P < 0.0001$), whereas the other treatments and their interactions were not significant ($P \geq 0.118$). Black bars – cultivar A, gray bars – cultivar P, white bars – bulk soil. C, control; SO₄, sulfate treated; L10, 10 mg LAS treated; and L50, 50 mg LAS treated. Bulk* indicates the values observed in the bulk soil at the onset of the experiment.

the *Variovorax asfA* gene abundance ($F = 23.52$; $P < 0.0001$), whereas the other treatments and their interactions did not exert significant effects ($P \geq 0.118$).

3.4. Bacterial diversity and community composition in bulk and rhizosphere soils as assessed by PCR-DGGE

Clustering of all bacterial and β -proteobacterial PCR-DGGE profiles revealed clear treatment and rhizosphere effects, albeit to different extents (Figs. 3 and 4). First, no difference was observed between the profiles at the two potato cultivars. Although the clustering was not tight, a trend could be discerned, as follows. In the young plant stage, the bacterial community profiles showed three major clusters, one containing most bulk soils, a second one encompassing the control and sulfate treatment (mostly rhizospheres) and a third one harboring mainly LAS-treated samples (Fig. 3A). Since the profiles derived from the rhizosphere soils grouped apart from those of their corresponding bulk soils, rhizosphere effects were evident, as confirmed by ANOSIM ($P < 0.05$). These ANOSIM analyses also indicated that the profiles of the control and sulfate-treated samples were significantly different from those of the 10- and 50-mg LAS-treated ones (Table 2). In the flowering stage, the joint clustering of the profiles from the rhizospheres became more pronounced, whereas those from all bulk soils grouped apart (Fig. 3B, $P < 0.05$). The profiles from the control and sulfate-treated bulk soil samples grouped together, whereas those from LAS-50-treated soils formed a separate cluster. Profiles from the LAS-10-treated samples showed high variability and were spread over the dendrogram. Again, (one-way) ANOSIM analysis showed that the profiles from the control and sulfate-treated soils were significantly different from those from the LAS-10 and LAS-50 treated ones (Table 2). Besides, two-way ANOSIM analysis based on the interaction cultivar \times treatment showed that there was no significant difference between cultivars at the young plant stage ($R = 0.07$, $P > 0.05$), whereas a significant cultivar effect was observed at the flowering stage ($R = 0.30$, $P < 0.05$).

In the β -proteobacterial analyses, at young plant stage, the profiles did not reveal any tight clustering, except for those from the LAS-50 treated bulk and rhizosphere soils, which grouped together and separate from the others (Fig. 4A). However, one-way ANOSIM analysis indicated no statistical difference between any of the treatments at this stage (Table 2). At flowering stage, both treatment and rhizosphere effects became apparent (Table 2, Fig. 4B). However, treatment did not affect the β -proteobacterial communities in the bulk soils, since the relevant profiles all clustered together regardless of treatment, and apart from the rhizosphere ones. The profiles from the control, sulfate- and LAS-50 treated rhizosphere soil samples grouped within each other, whereas those from the LAS-10 treated samples were distributed between the sulfate- and LAS-50 treated rhizosphere soil samples. ANOSIM analyses indicated a clear treatment effect among the rhizosphere communities (Table 2). Specifically, large differences were observed between the rhizospheric β -proteobacterial profiles in the control and all other treatments, except for the LAS-10-treated soils (Table 2). Moreover, two-way ANOSIM analysis based on the interaction cultivar \times treatment showed no significant differences between the cultivars at the young plant and flowering stages ($P > 0.05$).

3.5. Analysis of β -proteobacterial 16S rRNA gene clone libraries

Four duplicated libraries were successfully produced. After quality and chimera checks, shuffling of the remaining sequences (Singleton et al., 2001) showed that all duplicate libraries were similar to each other per treatment. Hence, these were pooled per treatment for further analyses. Totals of 111, 156, 164 and 158 sequences were thus obtained for the libraries from bulk soil

and unamended (control), sulfate- and LAS-50-treated rhizosphere soils, respectively (flowering stage). Rarefaction curves were then generated to estimate the completeness of sampling and the richness within the libraries. For this, 97% cut-off criteria were used to group the OTUs at "species" level. None of the rarefaction curves reached the plateau level (Fig. S2). Furthermore, no substantial rhizosphere or treatment effect on the predicted richness of the β -proteobacterial communities was found, since values on the same order were estimated for all libraries. However, the CHAO1 estimator did reveal a decrease of richness, in the order sulfate-treated (242), LAS-50-treated (228), control rhizosphere (172) and bulk soil (161). Only the sulfate treatment library was found to be significantly different from that of the bulk soil. Libshuff analyses showed that all four libraries were significantly different from each other.

The majority of the sequences found (56–89% across all libraries) was affiliated with recognized classes of the β -proteobacteria (Cole et al., 2005), whereas the remainder in all libraries was affiliated with unclassified β -proteobacteria, as determined by the RDP library comparison (confidence threshold of 80%). There were significant differences in the community structures, with respect to the prevalence of particular groups within the β -proteobacteria, between the rhizosphere and bulk soils and also between treatments. Leaving aside the unclassified groups, the control and LAS-50-treated rhizosphere soil samples contained the highest number of genera, respectively, 13 and 12, whereas the control bulk soil had the lowest number (9; Table 3). On the other hand, the prevalence of unclassified β -proteobacterial sequences was lowest in the control rhizosphere soil sample (11%) and highest in the initial bulk soil (44%).

Comamonadaceae and *Burkholderiaceae* were the most dominant families found in all libraries (Table 3). Sequences assigned to the family *Oxalobacteriaceae* were only observed in the three rhizosphere samples, whereas those of the family *Alcaligenaceae* were only found in the sulfate-treated and control rhizosphere soils. Members of the *Comamonadaceae* accounted for 25.6%, 14.0% and 13.7% of the clones from the rhizospheres of control, sulfate- and LAS-50-treated samples, respectively, whereas this group made up only 9.9% of the amplicons generated from the bulk soil (Table 3). The composition of the community of *Comamonadaceae* was also different between the different samples. At the genus level, overall, *Burkholderia* was found to be the most abundant group, being about equally represented in all samples (Table 3). Considering the genera containing known desulfonating species, *Polaromonas* and *Acidovorax* were found, but these did not seem to be specifically selected in the rhizosphere, whereas *Ramlibacter* was abundant only in rhizospheres. Within the *Comamonadaceae*, *Variovorax* was found to be the most abundant genus in the LAS-50-treated samples (54%), while it encompassed only 18% of this family in the bulk soil, 20% in the control sample and 9% in the sulfate-treated rhizosphere soil.

4. Discussion

Microbial desulfonation of organic sulfur compounds in soil is a process of great importance for the sulfur nutrition of plants, since plants are thought to be unable to utilize such organic sulfur directly (Kertesz and Mirleau, 2004). We increasingly understand that the mineralization of aromatic sulfonate compounds in soil is largely mediated by desulfonating bacterial communities, among which several members of the β -proteobacteria (Schmalenberger et al., 2008a,b, 2010). Both the β -proteobacterial community structure and the diversity of bacteria with the capacity to desulfonate may respond to different levels of inorganic sulfate, as found in both field and microcosm experiments (Schmalenberger et al., 2008a,b, 2010). However, the extent to which these communities respond to the addition of organic sulfur in soil has remained elusive. Thus, in

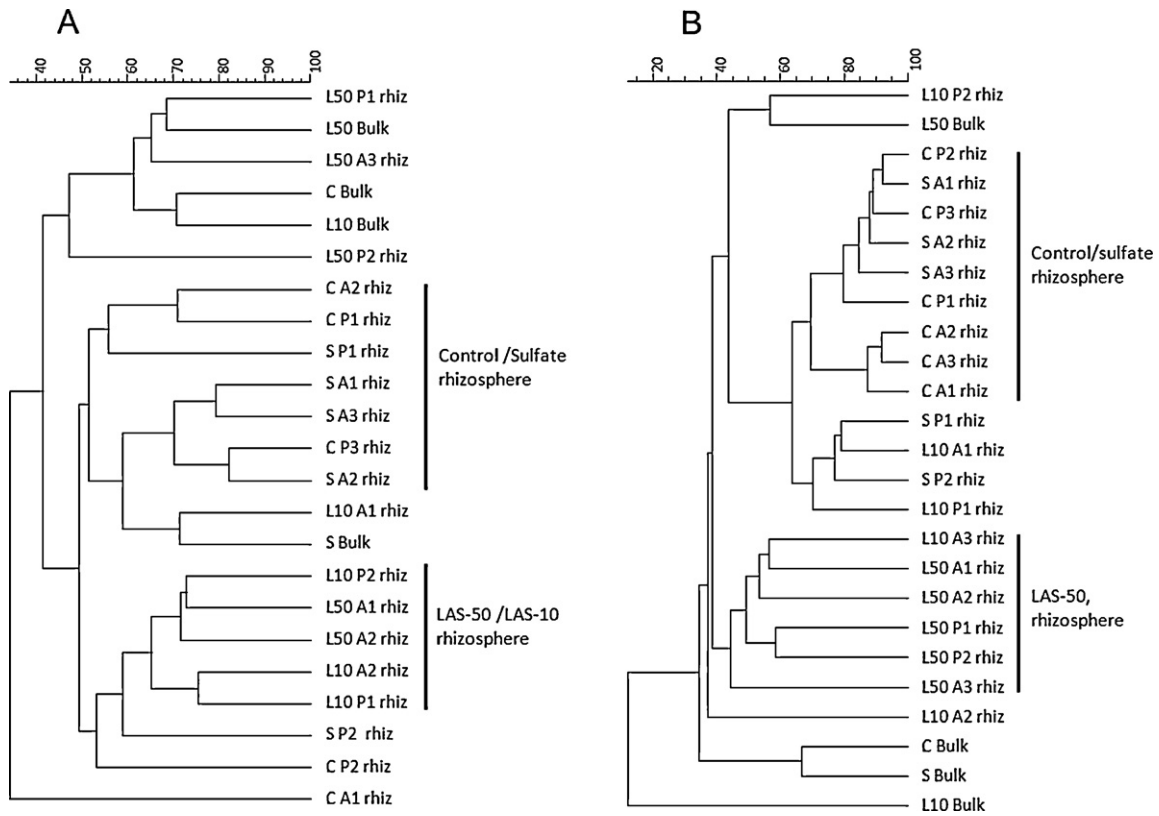


Fig. 3. UPGMA dendrograms representing the similarity of PCR-DGGE profiles of bacterial communities associated with bulk and rhizosphere soil from potato plants (A) at the young plant and (B) at flowering stage. B, bulk soil; yp, young plant; f, flowering; A, cultivar Aveka; P, cultivar Premiere; C, control; S, sulfate treated; L10, 10 mg LAS treated; and L50, 50 mg LAS treated.

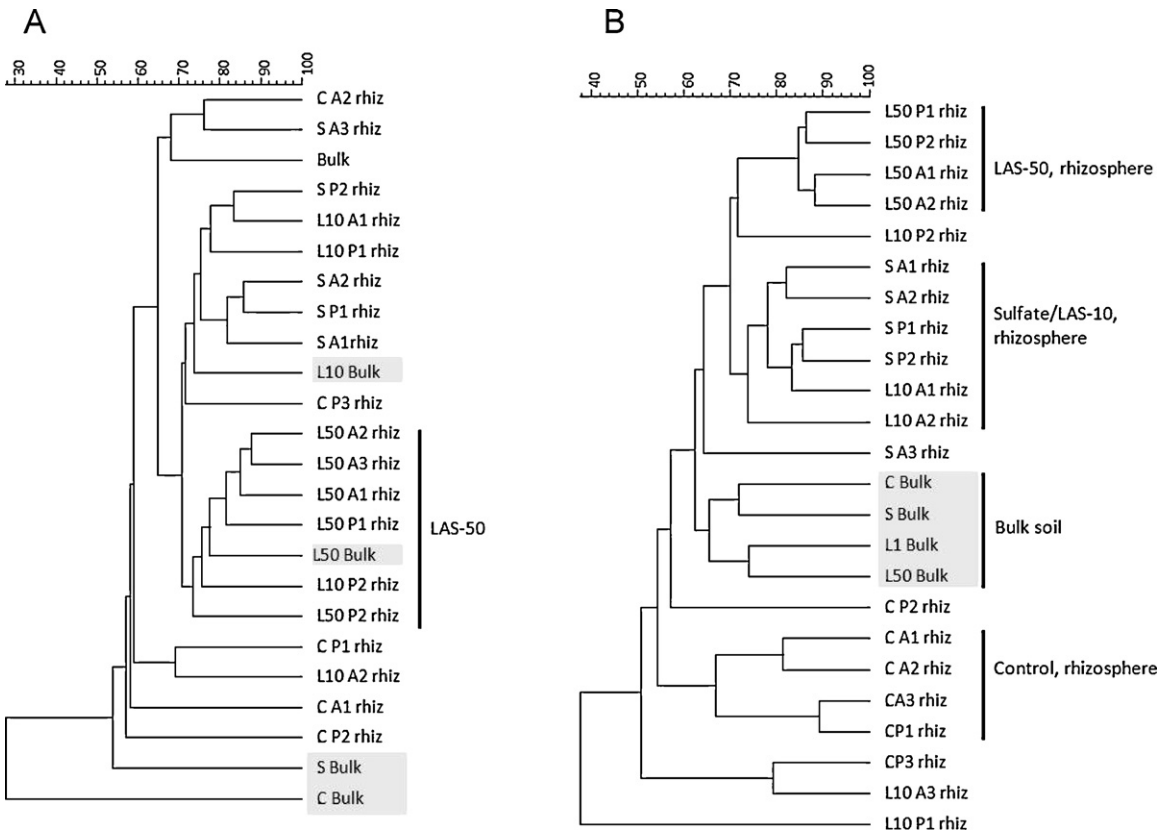


Fig. 4. UPGMA dendrograms representing the similarity of PCR-DGGE profiles of β -proteobacterial communities associated with bulk and rhizosphere soil from potato plants (A) at the young plant (B) at flowering stage. B, bulk soil; yp, young plant; f, flowering; A, cultivar Aveka; P, cultivar Premiere; C, control; S, sulfate treated; L10, 10 mg LAS treated; and L50, 50 mg LAS treated.

Table 2

One-way ANOSIM analysis evaluating treatment effect on the bacterial and β -proteobacterial community make-up, at different growth stages. Significant relationships are shown in bold.

Samples	Bacterial				β -Proteobacterial			
	young plant		flowering		young plant		flowering	
	R	p	R	p	R	p	R	p
Control-sulfate	0	0.650	0.057	0.260	0.019	0.110	0.089	0.005
Control-LAS 10	0.128	0.070	0.292	0.004	0	0.360	0.650	0.100
Control-LAS 50	0.270	0.040	0.290	0.020	0.089	0.600	0.155	0.050
Sulfate-LAS 10	0.145	0.050	0.172	0.050	0.005	0.390	0.208	0.040
Sulfate-LAS 50	0.222	0.010	0.220	0.030	0.356	0.060	0.254	0.040
LAS 10-LAS 50	0	0.560	0.061	0.200	0.109	0.180	0.035	0.140

this study, we investigated how amendments of soil with inorganic or organic sulfur compounds influence the size and structure of bacterial communities associated with potato. Moreover, we used two physiologically different cultivars to assess whether plant physiology affects these communities. In the light of the relevance of a suite of β -proteobacteria for the desulfonation process (Schmalenberger and Kertesz, 2007), we also measured the community structure and diversity of this bacterial class, as well as the abundance of the *Variovorax* like *asfA* gene, a key gene associated with desulfonation (Schmalenberger and Kertesz, 2007).

Table 3

Comparison of clone libraries, at family and genus level, based on 16S rRNA gene fragment specific for β -proteobacterial class, obtained from bulk or rhizosphere soil from the potato cultivar Premiere. Values represent the percentage of the total.

	Bulk	Control	Sulfate	LAS 50
Family^a				
<i>Rhodocyclaceae</i>	5.4	1.3	0.6	1.9
<i>Nitrosomonadaceae</i>	0.9	0.6	0.6	3.1
<i>Comamonadaceae</i>	9.9	25.6	14	13.7
<i>Burkholderiaceae</i>	15.3	24.4	19.5	18
<i>Burkholderiales incertae sedis</i>	16.2	15.4	18.9	18.6
<i>Oxalobacteraceae</i>		5.1	0.6	1.2
<i>Alcaligenaceae</i>		3.2	0.6	
<i>Unclassified Burkholderiales</i>	11.7	12.8	12.8	10.6
<i>Unclassified Betaproteobacteria</i>	44.1	11.5	32.3	32.9
Genera^a				
<i>Variovorax</i>	2	5	1	7
<i>Nitrospira</i>		1	1	3
<i>Pelomonas</i>	2	9	3	1
<i>Acidovorax</i>	1.8	2.6	1.2	
<i>Ramlibacter</i>		1.9	0.6	1.9
<i>Dechloromonas</i>	0.9	1.3		0.6
<i>Herbaspirillum</i>		2.6		0.6
<i>Ideonella</i>				0.6
<i>Ottowia</i>				0.6
<i>Polaromonas</i>	1.8			0.6
<i>Achromobacter</i>		2.6		
<i>Aquabacterium</i>	0.9			
<i>Chitinimonas</i>	0.9			
<i>Methylibium</i>	5.4	7.7	9.8	10.6
<i>Burkholderia</i>	12.6	16.1	17.7	17.4
<i>Cupriavidus</i>		3.9		
<i>Azohydromonas</i>		0.6	0.6	
<i>Caenimonas</i>			0.6	
<i>Massilia</i>			0.6	
<i>Leptothrix</i>				0.6
<i>Wautersia</i>		0.6		
<i>Unc. Comamonadaceae</i>	3	7	7	2
<i>Unc Burkholderiaceae</i>	1	4	2	1
<i>Unc Oxalobacteraceae</i>		3		1
<i>Unc Burkholderiales incertae sedis</i>	7.2	5.8	9.1	7.5
<i>Unc Rhodocyclaceae</i>	4.5		0.6	1.2
<i>Unc Burkholderiales</i>	11.7	12.8	12.8	10.6
<i>Unc β-proteobacteria</i>	44.1	11.5	32.3	32.9

^a Classification based on RDP analysis. Unc, uncultivated.

In the experiments, we used LAS as the model aromatic sulfonated compound. It has been previously shown that LAS is readily degradable by the soil microbiota, with mineralization rapidly occurring in soil with no history of prior exposure to LAS (Branner et al., 1999). Consistent with this, in our experiment LAS was found to quickly vanish in all LAS-treated microcosms. The LAS levels in the unamended or sulfate-amended soils also decreased from the start to the first sampling time (30 days after planting). Thus, the collective data suggest that a LAS degrading community had been readily activated following the LAS treatments. The LAS that was still detected in the flowering stage may not have been in sufficient contact with the active LAS degrading microbial communities to incite further degradation. Adsorption of LAS to soil particles may have played a role, however the extent of such a process was difficult to estimate. Overall, the results thus indicated that a strong LAS degrading activity took place in the early stages of the experiment (0–30 days), i.e. the period from the start of the experiment up to the young plant stage. Further degradation of LAS was not detectable throughout the experiment. Moreover, we did not observe any toxic effect of LAS on the growth of either of the two potato cultivars.

The amendments of soil with either LAS or sulfate did not affect the abundance of total bacteria to a great extent. This indicated that the soil's carrying capacity for bacteria, reflecting the levels of available nutrients versus potential in situ toxicity, was not strongly affected by any of the treatments. On the other hand, the community structures of both total bacteria and β -proteobacteria were clearly affected by the treatments. As evidenced by the 16S rRNA gene based analyses, the main drivers of the bacterial community structures may have been both the presence of a plant (rhizosphere effect) and of LAS. In this respect, no differences were observed between the two LAS levels on the one hand, and between the presence of added sulfate or its absence (unamended soil) on the other hand. The latter finding could be explained by the fact that sulfate may already have been available in the soil, at least to a certain extent. The soil amendments clearly affected the β -proteobacterial communities at the flowering stages of the potato plants, with clustering patterns being similar to those found for total bacteria. The exception was the presence of inorganic sulfur, which led to a change in the community structure of β -proteobacteria. This is consistent with the contention that the β -proteobacterial community is sensitive to changes in the amount of inorganic S (Schmalenberger et al., 2008a,b, 2010). Regarding the soils amended with organic S, it is important to note that the changes in both the bacterial and β -proteobacterial communities might have been related not only to desulfonation, but also to carbon mineralization. Indeed, LAS can be used as a source of energy and carbon, in addition to sulfur (Elsgaard et al., 2003). Further studies correlating the degradation of LAS in soil with the expression of genes involved in desulfonation should provide more evidence as to what extent to LAS can be used as a sulfur or carbon source by the local community.

It was recently shown that slight changes in the community structures of *Alphaproteobacteria* and *Actinobacteria* may occur in soil due to the addition of LAS (Sanchez-Peinado et al., 2010). In contrast, other studies could not discern any effect of added LAS on the functional bacterial diversity in soil (Brandt et al., 2001; Sanchez-Peinado et al., 2010). Specifically, the community-level physiological profiles (CLPP) measured were rather insensitive to different LAS levels. However, the studies in which LAS did not lead to changes in bacterial communities were carried out in the absence of plants (Brandt et al., 2001; Sanchez-Peinado et al., 2010; Vinther et al., 2003). Considering that metabolic activity increases in the rhizosphere (Curl and Truelove, 1986) and that we here observed a clear effect of LAS on both total bacterial and β -proteobacterial communities in the potato rhizosphere, we would like to posit that the extrusion of carbon compounds by plant roots might activate the desulfonation process. According to Mortensen et al. (2001), the growth of plant roots can stimulate the biodegradation of LAS in soil, which is possibly due to the fact that several bacterial guilds that are responsible for an attack on sulfonates are often associated with plants (Schmalenberger et al., 2010; Inceoglu et al., 2010). The clear separation of bacterial and β -proteobacterial communities between bulk and rhizosphere soils, and the absence of treatment effects in bulk soils, especially at the flowering stage, indicate that the putative treatment effects indeed become more robust in the presence of plants. The extent to which the positive effect of plants on LAS biodegradation was completely due to desulfonation (and use of the cleaved-off moiety as a sulfur source) or mineralization (use of LAS as a C source) remains unsolved. However, given the growth of strain DSM30034T in the presence of LAS and succinate and the easily degradable organic compounds that are often available in the rhizosphere, stimulation of LAS mineralization by *V. paradoxus* DSM30034T like organisms can be predicted (see further).

Within the β -proteobacteria, members of the family *Comamonadaceae* are of great relevance for desulfonation, which is particularly dominant in the rhizosphere of plants. In previous work, molecular analyses based on the *asfA* gene revealed that the genus *Variovorax* plays an important role as a desulfonating rhizobacterium (Schmalenberger and Kertesz, 2007). However, other members of the *Comamonadaceae*, such as *Polaromonas* and *Acidovorax* species, may also be important for the process (Schmalenberger et al., 2008a,b). Our clone library data, based on β -proteobacteria-specific 16S rRNA gene sequences, revealed that *Comamonadaceae* were indeed enriched in the rhizosphere as compared to bulk soil, confirming previous findings (Inceoglu et al., 2010). However, a clear correlation with the presence of LAS was missing. Nevertheless, LAS-treated soils incited an increase in the diversity within the *Comamonadaceae*, being dominated by *Variovorax*, which is consistent with the putative role of this organism in desulfonation. Regarding other desulfonating species from the *Comamonadaceae*, we did not find clear evidence for their relevance in desulfonation in the potato rhizosphere. For instance, our results revealed *Acidovorax* types to be absent from the library generated from the LAS-50-treated rhizosphere soils. In contrast, such types were found in bulk and control soils as well as in the sulfate-treated rhizosphere soils. It is worth noting that the status of *Acidovorax* spp. as important desulfonators in the rhizosphere has been questioned recently, since *Acidovorax asfA* genes were found in very low quantities in this habitat (Schmalenberger and Kertesz, 2007). In a similar fashion, our results did not show evidence for the selection of *Polaromonas* – like organisms in the potato rhizosphere in LAS-50-treated soils. Particular *Polaromonas* types have been previously suggested as important desulfonators in the rhizosphere of monocotyledonous plants (Schmalenberger et al., 2010; Schmalenberger and Noll, 2010). As most of the previous studies have focused on the diversity of desulfonating bacteria in the rhizospheres of cereals

(Schmalenberger et al., 2010), we hypothesize that the differences observed are due to the use of different plants. Consistent with this hypothesis, it has been shown that desulfonating *Polaromonas* types are more important in the barley than in the wheat rhizosphere (Schmalenberger et al., 2008a,b). Similarly, whereas the genus *Variovorax* does not appear to dominate the desulfonating communities associated with cereals (Schmalenberger et al., 2008a,b), it does represent up to 10% of the β -proteobacterial communities associated with the potato rhizosphere (Inceoglu et al., 2010), confirming its association with potato. Given the tangible transformation of LAS observed in this study, our results indicate that, in conjunction with *Variovorax* species, other bacterial types may have been responsible for the degradation of LAS.

Considering the relevance of members of the genus *Variovorax* in our settings, we quantified the *V. paradoxus* specific *asfA* gene, whose product is involved in the mineralization of aromatic sulfonates. Despite the fact that *Variovorax* types were highly dominant in the 16S rRNA gene based clone library of the potato rhizosphere in LAS-50 treated soils, the qPCR analyses indicated that the soil amendments had no substantial effects on the *asfA* gene abundance in the rhizosphere soil. However, at the young plant stage, there was a significant increase in the abundance of *Variovorax* specific *asfA* genes in the rhizosphere soils of the LAS-treated microcosms. This was especially true for the LAS-50 soil, when compared to the baseline set by the bulk soils. These results reinforce our hypothesis that root exudation stimulates desulfonation and highlight the key role of rhizospheric bacterial communities, and more specifically *Variovorax* species, in LAS degradation in this setting. In this context, it is worth noting that in the LAS-treated soils we observed no increase in the abundance of the *Variovorax* specific *asfA* gene in bulk soil as compared to values detected at the onset of the experiment. Although we discerned differential trends in the abundance development across the treatments (LAS, sulfate or control) in bulk soils, a range of putative conclusions might be drawn. This is partially due to the uncertainties about the whereabouts of the added LAS (adsorbed or freely available in the soil liquid) and partially to technical reasons (see paragraph below). In order to check whether the added LAS levels had negative effects on *Variovorax* species, we studied the population dynamics of *V. paradoxus* DSM30034T in MM at varying LAS concentrations (0.01–0.1 mg/cm³). We thus clearly found that LAS, at the level of 0.01 and 0.02 mg/cm³ could serve as a carbon/sulfur source for strain DSM30034T, although growth was stimulated by the addition of another carbon source (succinate). LAS at higher concentrations (0.1 mg/cm³) was prohibitive to growth, even in the presence of additional carbon sources. Considering the bulk density of a sandy soil to be approximately 1.6 g/cm³, we estimate that the soil LAS amendments corresponded to 0.02 and 0.08 mg/cm³, for LAS-10 and LAS-50, respectively. However, the fact that only a small part of the added LAS might be readily available to microbial communities (the remaining being adsorbed or inaccessible) would make these LAS concentrations suitable, and not prohibitive, for bacterial growth.

Another possible explanation for our findings lies in the specificity of the primers used for quantification. We performed in silico (BLAST-N) analyses of the *Variovorax asfA* primers used in the experiments. As expected, these analyses revealed that several, as-yet-unclassified, *Comamonadaceae* may also represent potential target organisms (data not shown). Thus, a lack of complete specificity of the primers might have blurred the analyses and the possible amplification of genes from bacterial types other than *Variovorax* might underlie the discrepancies between the data from the clone library and qPCR results. In a previous study, we showed that the abundance of *Variovorax*-like *asfA* gene copies in soil follows complex patterns. For instance, its enrichment in the rhizosphere appeared stronger in soils with a higher organic

matter content. Moreover, in a given soil, its response to potato cultivars was growth-stage-dependent (Inceoglu et al., 2010). Thus, our finding of similar levels of abundance of *Variovorax*-like *asfA* gene copies in control and amended soils may have multiple and complex underpinnings.

Overall, the results presented in this study provide evidence for the contention that (1) LAS is a biodegradable compound, which is indeed rapidly degraded in soil; (2) LAS does not strongly affect the bacterial abundances, but it exerts remarkable effects on the structures of the general bacterial and β -proteobacterial communities in soil, especially at 50 mg/kg dry soil; (3) the communities capable of mineralizing LAS are stimulated by the presence of plants, highlighting the key role of rhizospheric communities in the desulfonation process and (4) *Variovorax* spp. are positively selected by potato roots in initially high LAS conditions, indicating their possible role in LAS transformations in this setting.

Hence, in the light of the putative nutritive or otherwise stimulatory effect of the potato rhizosphere on the desulfonation process, novel studies are needed to discern the importance of desulfonation in agricultural soil and understand the intricate mechanism of the combined effect of raised LAS levels and the rhizosphere effect.

Acknowledgements

The soil used for the microcosm experiment was taken from a field laid out by Avebe (Foxhol, NL). We gratefully acknowledge the help by Paul Heeres. We also thank Eelco Hoogwout for his help with the soil sampling. We kindly acknowledge Sander Heijs from Cyprio B.V., Groningen for the sulfate measurements. This work was supported by a grant from the NWO-ERGO Program awarded to JDvE.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.apsoil.2012.09.004>.

References

- Autry, A., Fitzgerald, J., 1990. Sulfonate S – a major form of forest soil organic sulfur. *Bio. Fertil. Soils* 10, 50–56.
- Beil, S., Kehrli, H., James, P., Staudenmann, W., Cook, A.M., Leisinger, T., 1995. Purification and characterization of the arylsulfatase synthesized by *Pseudomonas aeruginosa* PAO during growth in sulfate-free medium and cloning of the arylsulfatase gene (*atsA*). *Eur. J. Biochem.* 229, 385–394.
- Brandt, K.K., Hesselsoe, M., Roslev, P., Henriksen, K., Sorensen, J., 2001. Toxic effects of linear alkylbenzene sulfonate on metabolic activity, growth rate, and microcolony formation of *Nitrosomonas* and *Nitrospira* strains. *Appl. Environ. Microbiol.* 67, 2489–2498.
- Branner, U., Mygind, M., Jørgensen, C., 1999. Degradation of linear alkylbenzene sulfonate in soil columns. *Environ. Toxicol. Chem.* 18, 1772–1778.
- Carlsen, L., Metzton, M., Kjelsmark, J., 2002. Linear alkylbenzene sulfonates (LAS) in the terrestrial environment. *Sci. Total Environ.* 290, 225–230.
- Castellano, S.D., Dick, R.P., 1991. Cropping and sulfur fertilization influence on sulfur transformations in soil. *Soil Sci. Soc. Am. J.* 55, 114–121.
- Cook, A.M., Laue, H., Junker, F., 1999. Microbial desulfonation. *FEMS Microbiol. Rev.* 22, 399–419.
- Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McFarrell, D.M., Garrity, G.M., Tiedje, J.M., 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res.* 33, D294–D296.
- Cunliffe, M., Kertesz, M., 2006. Effect of *Spingobium yanoikuyae* B1 inoculation on bacterial community dynamics and polycyclic aromatic hydrocarbon degradation in aged and freshly PAH-contaminated soils. *Environ. Pollut.* 144, 228–237.
- Curl, E., Truelove, B., 1986. *The Rhizosphere*. Springer-Verlag, Heidelberg, Germany.
- Elsgaard, L., Pojana, G., Mirraval, T., Eriksen, J., Marcomini, A., 2003. Biodegradation of linear alkylbenzene sulfonates in sulfate-leached soil mesocosms. *Chemosphere* 50, 929–937.
- Hack, H., Gall, H., Klemke, T., Klose, R., Meier, U., Stauss, R., Witzemberger, A., 1993. Phänologische entwicklungsstadien der Kartoffel (*Solanum tuberosum* L.). *Nachrichtenbl Deut Pflanzenschutz* 45, 11–19.
- Inceoglu, Ö., Salles, J.F., Van Overbeek, L., Van Elsas, J.D., 2010. Effect of plant genotype and growth stage on the β -proteobacterial community associated with different potato cultivars in two fields. *Appl. Environ. Microbiol.* 76, 3675–3684.
- Jensen, J., Smith, S.R., Krogh, P.H., Versteeg, D.J., Temara, A., 2007. European risk assessment of LAS in agricultural soil revisited: Species sensitivity distribution and risk estimates. *Chemosphere* 69, 880–892.
- Kertesz, M., Mirleau, P., 2004. The role of soil microbes in plant sulfur nutrition. *J. Exp. Bot.* 55, 1939–1945.
- Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide-sequences. *J. Mol. Evol.* 16, 111–120.
- Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), *Nucleic Acid Techniques in Bacterial Systematics*. Wiley, New York, USA, pp. 147–155.
- Mortensen, G., Elsgaard, K.H., Ambus, P., Jensen, E.S., Gron, C., 2001. Influence of plant growth on degradation of linear alkylbenzene sulfonate in sludge-amended soil. *J. Environ. Qual.* 30, 1266–1270.
- R Development Core Team, 2012. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0 <http://www.R-project.org/>
- Ruff, J., Hitzler, T., Rein, U., Ritter, A., Cook, A.M., 1999. Bioavailability of water-polluting sulfonate aromatic compounds. *Appl. Microbiol. Biotechnol.* 52, 446–450.
- Sablajrolles, C., Montrejeaud-Vignoles, M., Silvestre, J., Treilhou, M., 2009. Trace determination of linear alkylbenzene sulfonates: application in artificially polluted soil-carrots system. *Int. J. Anal. Chem.* 2009, 1–6.
- Sanchez-Peinado, M., Rodelas, B., Martinez-Toledo, M., Gonzelaz-Lopez, L., Pozo, C., 2008. Effect of linear alkylbenzene sulfonates on the growth of aerobic heterotrophic cultivable bacteria isolated from an agricultural soil. *Ecotoxicology* 17, 549–557.
- Sanchez-Peinado, M., Gonzelaz-Lopez, L., Martinez-Toledo, M., Pozo, C., Rodelas, B., 2010. Influence of LAS on the structure of Alphaproteobacteria Actinobacteria and Acidobacteria communities in soil microcosm. *Environ. Sci. Pollut. Res.* 17, 779–790.
- Schloss, P., Handelsman, J., 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl. Environ. Microbiol.* 71, 1501–1506.
- Schmalenberger, A., Kertesz, M., 2007. Desulfurization of aromatic sulfonates by rhizosphere bacteria: high diversity of the *asfA* gene. *Environ. Microbiol.* 9, 535–545.
- Schmalenberger, A., Noll, M., 2010. Shifts in desulfonating bacterial communities along a soil chronosequence in the forefield of a receding glacier. *FEMS Microbiol. Ecol.* 71, 208–217.
- Schmalenberger, A., Hodge, S., Bryant, A., Hawkesford, M., Singh, M., Kertesz, M., 2008a. The role of *Variovorax* and other Comamonadaceae in sulfur transformations by microbial wheat rhizosphere communities exposed to different fertilization regimes. *Environ. Microbiol.* 10, 1486–1500.
- Schmalenberger, A., Hodge, S., Bryant, A., Hawkesford, M., Singh, B., Kertesz, M.A., 2008b. The role of *Variovorax* sp. and other Comamonadaceae in desulfonation of aromatic sulfonates in the rhizosphere of wheat exposed to different types of sulfur fertilization. *Environ. Microbiol.* 10, 1486–1500.
- Schmalenberger, A., Hodge, S., Hawkesford, M.J., Kertesz, M.A., 2009. Sulfonate desulfurization in *Rhodococcus* from wheat rhizosphere communities. *FEMS Microbiol. Ecol.* 67, 140–150.
- Schmalenberger, A., Telford, A., Kertesz, M.A., 2010. Sulfate treatment affects desulfonating bacterial community structures in *Agrostis* rhizospheres as revealed by functional gene analysis based on *asfA*. *Eur. J. Soil Biol.* 46, 248–254.
- Schowaneck, D., David, H., Francaviglia, R., Hall, J., Kirchmann, H., Krogh, P.H., Schraepen, N., Smith, S., Wildemann, T., 2007. Probabilistic risk assessment for linear alkylbenzene sulfonate (LAS) in sewage sludge used on agricultural soil. *Regul. Toxicol. Pharmacol.* 49, 245–259.
- Seegmüller, S., Rennenberg, H., 2002. Transport of organic sulfur and nitrogen in the roots of young mycorrhizal pedunculate oak trees (*Quercus robur* L.). *Plant Soil* 242, 291–297.
- Singleton, D., Furlong, M.A., Rathbun, S.A., Whitman, W.B., 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. *Appl. Environ. Microbiol.* 67, 4374–4376.
- Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599.
- Vermeij, P., Wietek, C., Kahnert, A., Wüest, T., Kertesz, M., 1999. Genetic organization of sulfur-controlled aryl desulfonation in *Pseudomonas putida* S-313. *Mol. Microbiol.* 32, 913–926.
- Vinther, F., Mortensen, G., Elsgaard, L., 2003. Effects of linear alkylbenzene sulfonates on functional diversity of microbial communities. *Environ. Toxicol. Chem.* 22, 35–39.