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Bacterial communities in grassland turfs respond to sulphonate addition while fungal communities remain largely unchanged

Achim Schmalenberger\textsuperscript{1,2} and Matthias Noll\textsuperscript{3}

\textsuperscript{1}Cell-Mineral Research Centre, Kroto Research Institute, University of Sheffield, Broad Lane, Sheffield, S3 7HQ, United Kingdom, \textsuperscript{2}University of Limerick, Department of Life Sciences, Limerick, Ireland

\textsuperscript{3}University of Applied Science and Arts, Department of Bioanalytics, Friedrich-Streib-Str. 2, 96450 Coburg, Germany

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*Corresponding author: Achim Schmalenberger, \textsuperscript{2}University of Limerick, Department of Life Sciences, Limerick, Ireland

Telephone: 00353 61233775

Fax: 00353 61 331490

E-mail: achim.schmalenberger@ul.ie
Highlights

- Sulphonate addition shifted bacterial and desulphonating communities significantly
- Sulphonate addition had no significant effect on the fungal communities
- A rhizosphere effect was detected in all microbial communities
- XANES results suggest that added toluenesulphonate was transformed.
- Presence of potentially endophytic fungi in the sulphonate treatments was detected
Abstract

Sulphonates are often the major form of sulphur in soils where sulphate usually represents less than 5% of the total sulphur. The use of sulphonates as a S source is limited to a functional bacterial guild. However, fungi may assist bacteria in sulphonate desulphurization. In this study, grassland turfs were watered periodically with a modified Hoagland’s solution that was i) sulphur free, ii) contained low molecular weight sulphonate iii) or high molecular weight sulphonate. DNA fingerprint analyses of fungal and bacterial communities revealed significant differences between the rhizosphere soil and the bulk soil. Sulphonate treatments had only significant effects on the bacterial and desulphonating bacterial communities and no significant effects on the fungal communities. However, sequencing of the fungal ITS region identified the presence of potentially endophytic fungi in sulphonate amended turfs. Analysis of the sulphur species in soil suggested that the added toluenesulphonate-sulphur was transformed despite the fact that the number of the desulphonating bacteria remained unchanged indicating no sulphonate limitation. The results showcase a robust fungal community in grassland turfs where only the bacterial community with its desulphonating bacterial guild is predominantly responding to the sulphonate amendment.
1. Introduction

Organically bound sulphur such as sulphate esters and sulphonates are the major sulphur (S) compounds in soil ecosystems where sulphate often represents less than 5% of the total S [1]. As plants almost entirely depend on sulphate as S source, they require microbial S-supply. Due to the vast reduction of air pollution, S has nowadays become a potential limiting factor of plant growth. It is therefore paramount to improve our understanding in organically bound S mobilization in order to implement a more sustainable land use approach, less dependent on inorganic fertilizers.

Organically bound S compounds arise in soils through deposition of S-containing biological material and are transformed through subsequent humification processes [2]. Animal-derived organic input in the form of sheep dung contains up to 80% of its S in carbon-bound form, largely as sulphonates [3]. Chemical and X-ray spectroscopic studies have shown that the dominant forms in a range of aerobic soils are sulphonates (30-70% of soil S) [4, 5]. Plants are unable to utilize these organic compounds as S-sources [6]. However, 80-90% of all plant species associate with mycorrhizal fungal partners [7, 8]. Their mycelial networks are several times longer than plant roots and often exceed 200 m per cm$^3$ [9]. These networks support plant hosts through the acquisition of limiting nutrients [10] acquired in partnership with other microorganisms including bacteria located in the mycorrhizosphere [11]. While some fungi are able to desulphurize sulphate-esters, the use of sulphonates as a S source seems to be limited to a defined functional bacterial guild [12]. Such soil bacteria can transform these compounds within weeks using a bacterial multi component monooxygenase system [12, 13]. Work with *Pseudomonas putida* has shown that the desulphonation of aromatic sulphonates resulted in a plant growth promotion effect [6]. The gene *asfA* is the key marker in this desulphonation process and this marker
has been used as a proxy in molecular approaches to study sulphonatase diversity [14-16]. Cultivation independent analysis of the asfA diversity allowed the identification of *Variovorax* and *Polaromonas* as the dominant sulphonate-metabolizing genera [14] in the rhizospheres of cereal crops and unmanaged *Agrostis* species [16, 17].

So far, a direct involvement of mycorrhizal and saprophytic fungi in desulphonation has not been observed. Treatments of artificial sulphonates with several fungi and their enzymes for transformation showed no sign of desulphonation activity [18, 19].

Brown and white rotters on minimal media plates with toluenesulphonate as the sole S source grew into larger colonies than on S free control media but growth of these saprophytic fungi on wood showed that sulphate esters and not sulphonates were used as the primary S source [20]. Soil isolates affiliated to the bacterial genera *Variovorax* and *Rhodococcus* are able to grow with 0.25 mM toluenesulphonate as sole S source with no apparent inhibition in growth [15, 21]. While the former isolate was cleaving S from the aromatic ring, resulting in the accumulation of para-cresol, the latter isolate was capable of breaking the aromatic ring, using it as a carbon source, leaving no aromatic product behind [15, 21].

The aim of this study was to find out whether addition of sulphonate S in form of the low molecular weight source toluenesulphonate (aromatic, TS) or the high molecular weight sulphonate lignosulphonate (aliphatic, LS) is impacting the structure and composition of the bacterial, the desulphonating bacterial guild, as well as the fungal community and the communities of the Ascomycetes, Basidiomycetes and Glomeromycota in grassland turfs. The hypotheses were that i.) the rhizosphere soil community interact differently to the sulphonate treatment than the bulk soil community; ii.) the sulphonate treatment is affecting the bacterial communities in particular the desulphonating bacterial guild and iii) bacterial desulphonation is
potentially mediated by the fungal community due to depolymerisation of large
sulphonate molecules and S transport in the mycorrhizosphere of *A. stolonifera*. 
2. Materials and methods

2.1 Sampling, incubation of turfs, isolation of microorganisms and culture conditions.

Turfs with *Agrostis stolonifera* were sampled from the field site at Butt Close, Woburn Experimental Farm [22], Bedfordshire, United Kingdom (0°36'W, 52°2'N) in early October 2008 (self-sown, unmanaged grassland). Turf-subsamples (15x20 cm) from three locations at Butt Close (approximately 10 m apart) were transported to the laboratory, and placed into plastic boxes with drainage holes (nine boxes in total). These mesocosms were incubated in a plant growth chamber (Conviron, Winnipeg, Canada) at 20 °C with a photon flux of approximately 230 mol m⁻² s⁻¹, 50-60% humidity, and were watered with deionised water three times a week. The turf pieces were then additionally supplemented (150 ml twice a week) over a period of five weeks with either 0.25 times i) sulphate-free modified Hoagland’s solution (SF) [23], or modified Hoagland’s solution, amended with ii) 0.1 mM tolenesulphonate (TS) or iii) 0.1 mM lignosulphonate-S (average M₆ 8,000; LS). In order to ensure, no inorganic sulphate was present in the LS solution, the LS was purified over the course of 48 h in ultra-pure water (replaced four times) using dialysis tubing (Float-A-Lyzer, Sigma-Aldrich, St. Louis, MO) at a cut off at 3.5 kDa. The nutrient concentrations in the modified Hoagland’s solution were as described previously [16].

Soil, not directly attached to the roots was harvested in triplicates per mesocosm as bulk soil (3 g fresh weight (FW)). For rhizosphere soil, loosely attached soil was shaken off the roots and the remaining roots with closely attached soil were sampled (3 g of root FW). Soil or roots were added to sterile phosphate buffered saline (PBS, 20 ml) and shaken on a Genie roto-shaker (Scientific Industries, Bohemia, NY) for 30 min at 4°C [21]. The obtained suspension was used directly for cultivation dependent
experiments and ion chromatographic analysis (see below). Bacteria able to utilize sulphonate were quantified by cultivation at 25 °C (most probable number, MPN in microtiter plates [14] with 10 replicates) in modified minimal medium MM according to Beil and colleagues but without vitamin solutions [24] and TS or LS as sole S source (0.25 mM S). In parallel, MPN was also carried out in liquid R2A medium [25] to enumerate the abundance of cultivable heterotrophic bacteria.

2.2 DNA extraction and PCR conditions

DNA was extracted from the bacterial suspensions using the FastDNA extraction kit for soil (MPBio, Irvine, CA) as described previously [21]. Amplification of asfAB fragments (1.3 kb asfA and 0.14 kb asfB) from environmental DNA for T-RFLP analysis was carried out in a Tgradient thermocycler (Biometra, Göttingen, Germany) using a touch-down protocol of 10 cycles and a starting annealing temperature of 65 °C (1 min) and further 30 cycles with a denaturating temperature of 94 °C (1 min), annealing temperature of 55 °C (1 min), extension temperature of 72 °C (3 min) and a final extension time of 10 min. The initial denaturation took place at 95 °C (4 min). The amount of template DNA added for each PCR reaction was within the range of 1 to 10 ng, and final concentrations of 5 % (v/v) DMSO, 0.5 U Kapa Robust HotStart Taq and 1x GC buffer (Kapa Biosystems, Woburn, MA), 2.5 mM MgCl₂, 0.5 μM primers and 200 μM dNTPs each were used for 25 μl reactions. All subsequent PCR applications used the Kapa Robust HotStart Taq system with the conditions as above but with Kapa Enhancer instead of DMSO and Kapa buffer A instead of GC as recommended by the manufacturer. PCR amplifications from environmental samples for 16S rRNA gene-based DGGE analysis were carried out in a PCR approach described previously [14] using the primers GC-341F and 518R [26]. The internal
transcribed spacer (ITS) region was amplified using a nested PCR approach. In the first reaction ITS1F was selected as forward primer \([27]\) and ITS4, ITS4A and ITS4B as reverse primers to amplify the higher fungi, the Ascomycetes and the Basidiomycetes, respectively \([27, 28]\). In the second PCR, the forward primer contained a GC clamp (ITS1F-GC) and ITS2 was chosen as the reverse primer to facilitate the ITS-DGGE analysis as described by Anderson and colleagues \([29]\). For the Glomeromycota a nested PCR was conducted with the primer pairs AM1 \([30]\) with NS31 \([31]\) at the start and Glo1 with NS31-GC \([32, 33]\) as the second PCR. PCR cycle conditions for 16S and ITS amplification are described in the supplementary table S1.

2.3 Denaturing gradient gel electrophoresis (DGGE)

DGGE was carried out on 20 x 20 cm gels in a INGENYphorU electrophoresis chamber (Ingeny, Goes, The Netherlands). Gel electrophoresis of 16S and ITS fragments was conducted in 10% acrylamide gels with a gradient of 30-60% using urea and formamide as denaturing agents. Electrophoresis took place in 0.5 times TAE at 100 V for 18 h. Gels were stained for 30 min with SybrGold as recommended by the manufacturer (Invitrogen, Carlsbad, CA).

2.4 Cloning of ITS PCR products and genotyping

Amplified ITS fragments obtained with primers ITS1F and ITS4 from the bulk soil of the S free, TS and LS treatment were purified using the GeneJet PCR purification kit according to the manufacturer (Thermo Scientific, Waltham, MA), triplicates were pooled within each treatment and then ligated and transformed as described previously \([21]\) using a pGEM-T ligation kit from Promega (Madison, Wisconsin) and
competent cells of *E. coli* DH5alpha. Recombinant plasmids containing the expected insert were amplified with primers ITS1F and ITS4 for RFLP analysis. RFLP was carried out with 139 clones in total, as described elsewhere [21] with restriction enzyme *Hinfl* (Thermo Scientific).

2.5 **Terminal restriction fragment length polymorphism (T-RFLP) analysis**

Primers asfAF2 and asfBtoA [21] for the desulphonating bacteria were modified with fluorescent labels FAM and HEX (5’FAMasfAF2, 5’HEXasfBtoA) and used to amplify fragments of *asfAB* from bulk soil and rhizosphere extracts. PCR reactions were set up with only one fluorescent primer each and products were subsequently pooled. The amplification products were purified (GeneJet PCR purification kit) and approximately 200 ng DNA was digested with 10 U of AluI and RsaI (Thermo Scientific) at 37 °C for 12 h. Samples (1 µl) were mixed with formamide (9 µl) and a modified ROX500 size standard (Applied Biosystems, Warrington, UK) containing additional 585, 685 and 785 bp signals as described elsewhere [16] and analyzed on an automated sequencer (ABI Prism 3730, Applied Biosystems). Signals from the forward or the reverse labelled primers were detected on separate channels, and data analysis was carried out with the GeneMapper 3.7 software (Applied Biosystems). Peaks with heights of < 2% of the sum of all peak height intensities within one pattern were discarded from the analysis. Signals were treated as identical when the calculated size was equal or below a difference of two bases (statistical analysis described below).

2.6 **DNA sequence analysis**
Genotypes of ITS that were represented by two or more clones were sequenced, and sequence fragments were identified using BLAST [34]. Sequences were deposited in the EMBL Nucleotide Sequence Database (HG513094-HG513113).

2.7 Analytical methods

Measurement of sulphate was carried out by ion chromatography (IC). Rhizosphere soil from *A. stolonifera* roots or bulk soil (3 g) from the incubated turfs was detached in 20 ml of ultra-purified water (18 mΩ, Elga labwater, purelab ultra, High Wycombe, UK) using a vortex mixer for 1 min. Root material was removed and the soil suspension was centrifuged at 3500 rpm for 15 min. The supernatant was subjected to IC analysis using a Dionex DX-120 Ion Chromatograph with an AS14 column for anion separation coupled with an electro-conductivity detector as described previously [16].

2.8 S K-edge X-ray absorption near edge spectroscopy (XANES) analysis

S K-edge XANES analysis was performed at the XAS beamline (ANKA, Karlsruhe, Germany; [http://ankaweb.fzk.de](http://ankaweb.fzk.de)) as described previously [20] using a double crystal monochromator, an ionisation chamber to measure the primary flux and an energy dispersive detector (SDD - Silicon Drift Detector) to measure the fluorescence signal in reflexion mode. In order to identify the different oxidation states of S, the photon energy of the primary beam was varied around the S absorption edge at 2472 eV and scans were carried out at 2464 eV to 2510 eV in steps of 1 eV (2464-2468) and 0.2 eV (2468-2510) respectively. Exposure of the sample to the monochromatic beam was 10 s per step in triplicate measurements. Resulting spectra were loaded into Athena and after merging the replicate measurements, a Gaussian Peak Fit was conducted in
Athena as described by Salomon et al. [35] to describe the qualitative relationship of sulphur of different oxidation stages. S standards (reduced, intermediate and oxidized S species) were used to create Gaussian curves for the different types of S species. The reduced S species in the form of thiols was used to represent the first Gaussian peak (G1) at 2472.8 eV. The intermediate S species was represented by sulphoxides (G2) at 2475.8 eV and sulphonates (G3) at 2480.4 eV. The oxidized S species was represented by sulphate and sulphate-ester (G4) at 2481.8 eV [20].

2.9 Statistical analysis

Cultivable bacteria were quantified via the MPN method [36] as described previously [14] employing the U.S. Food and Drug Administration (FDA) Biological Analytical Manual MPN table (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm). MPN values were considered as significantly different when the difference of the log (MPN) was higher than 0.516 (95% confidence interval). Standard deviations were calculated for sulphate and a one sided ANOVA was carried out (SPSS, IBM, Armonk, NY).

Binary data sets of the DGGE image were obtained employing the Phoretix advanced 1D software (Nonlinear Dynamics, Newcastle upon Tyne, UK). Binary data sets of T-RFLP fingerprints comprising signals from the forward and the reverse labelled primer were combined and processed as described earlier [14] using GeneMapper 3.7 (Life Technologies, Carlsbad, CA). Binary DGGE and T-RFLP data sets were subjected to correspondence analysis (CA) using Canoco 4.5 (Microcomputer Power, Ithaca, NY). Results were summarized in ordination diagrams where replicates were displayed as means with standard deviations and visualized as error bars. To test the
effect of experimental variables (application of Hoagland’s solution with or without TS and LS) on the bacterial, the desulphonating bacteria and the fungal (higher fungi, Ascomycetes, Basidiomycetes, Glomeromycota) community structures, permutation tests were performed available in CANOCO using 9999 replicates as described elsewhere [16, 37].

3. Results

3.1 Effect of sulphonate treatments on soil sulphate concentration and cultivable desulphonating bacteria

Grassland turfs were taken from Woburn experimental farm, UK [22], dominated by A. stolonifera to examine the effect of sulphonate addition on the microbial communities in rhizosphere soil and bulk soil. Measurement of sulphate in the rhizosphere soil and bulk soil revealed levels of 17-25 ppm per gram soil or root (FW) (Table 1) but no significant changes in the sulphate concentration among the different S treatments or sample origin were detected (P > 0.05 for all). Concentrations of sulphate before the start of incubation were only marginally higher (22-27 ppm). All MPN measurements with sulphonate as sole S source were in the range of 2.3-7.7 $10^6$ g$^{-1}$ root or bulk soil (FW) while the MPN measurements for the heterotrophic growth was in the range of 1.7-3.0 $10^7$ g$^{-1}$ root or bulk soil (FW). No significant differences were determined among the treatments (P > 0.05) except between bulk soil samples from the SF and LS treatment when using LS as sole S source in the medium (Table 2). The combination of MPN data from the total heterotrophs and the LS and TS utillizers allowed deducing a percentage of sulphonate utilizers among the cultivable heterotrophs ranging from 8 to 45 %. The highest proportion of sulphonate utilizing bacteria was found in the SF treatment (29-45 %) while the lowest proportion
of sulphonate utilizing bacteria was found in the LS treatment (8-29 %) and the TS treatment (14-19 %).

3.2 Effect of sulphonate treatments on bacterial community structures.

Bacterial community structures were profiled using amplified 16S rRNA gene fragments and DGGE (supplementary Fig. S1A). Community profiles derived from bulk soil and rhizosphere soil samples were significantly different to each other (P < 0.05; Fig. 1). While rhizosphere soil samples were only significantly different between the TS treatment and the SF and LS treatments, bulk soil samples were significantly different in all three treatments (P < 0.05; Fig. 1).

3.3 Effect of sulphonate treatments on the asfA gene based T-RFLP patterns of desulphonating bacteria

The number of T-RF peaks as indicator for operational taxonomical units (OTU) richness included in the subsequent analysis for each sample was in the range of 11 to 25 in the bulk soil and 11 to 22 in the rhizosphere soil samples. AsfA gene based T-RFLP patterns in bulk soil and rhizosphere soil samples were significantly different (P < 0.05; Fig. 2). However, TS, LS and SF treatments in rhizosphere soil samples were not significantly different (P > 0.05, Fig. 2). In contrast, the TS treatment in bulk soil samples was significantly different to all other treatments (P < 0.05; Fig. 2).

3.4 Effect of sulphonate treatments on fungal community structures.

Fungal community structures were profiled using amplicons of the ITS region and DGGE (supplementary Fig. S1B). Community profiles from bulk soil and rhizosphere soil samples were significantly different to each other (P < 0.05; Fig. 3). Although CA
biplots separated the sulphonate treatments (TS and LS) from the SF controls, permutation tests revealed no significant differences between the sulphonate treatments and the SF treatment in bulk soil and rhizosphere soil samples (P > 0.05; Fig. 3). To phylogenetic characterize abundant community members, ITS PCR products from bulk soils of the TS, LS and SF treatment, respectively, were cloned. 46 clones from the SF, 46 clones from the LS and 47 clones from the TS treatment were screened, respectively. Separation of the clones according to their RFLP patterns identified in total 63 OTUs (32, 31 and 27 OTUs in the SF, TS and LS treatment, respectively). The overall coverage of the clone libraries [38] reached 69 % (48, 51 and 52 % in the SF, TS and LS treatment, respectively). 20 OTUs with more than one representative were sequenced. The two dominating OTUs were associated with the Pezizomycotina (Penicillium and Cystodendron) and were present in all clone libraries. OTUs that only appeared in the sulphonate treatments were affiliated to members of the genera Exophiala and Paecylomyces (both Ascomycota) (Table 3). In order to investigate if a single fungal phylum may be strongly affected by the sulphonate treatments, DGGE fingerprints were conducted targeting Ascomycota, Basidiomycota and Glomeromycota, respectively. While the resulting DGGE profiles showed diverse fungal community patterns of all three selected phyla, none of the fungal community patterns differed significantly in the sulphonate treatments to each other (P > 0.05; supplementary Fig. S2A, B and C).

3.5 Sulphur oxidation state analysis using S K-edge XANES

The XANES spectrum from bulk soil of SF and TS treatments revealed the presence of four types of S species with different oxidation stages, but no major differences were detected between the treatments. No reduced S species in the form of sulphide
(2472.0 eV) was detected in the analysed soils. The resulting peak fitting with G1-4 (Fig. 4) revealed the relative abundance of thiols, sulphone sulphonate and sulphate/sulphate-ester with percentages of 15, 5, 39 and 41 % for the TS and 19, 6, 39 and 36 % for the SF treatment, respectively. While intermediate S appeared to be the same in the TS and SF treatment, slight reductions were estimated for the thiols and slight increases were estimated for the oxidised S species in the TS treatment.

4. Discussion

In this study, the effect of sulphonate addition to microbial communities in *A. stolonifera* dominated grassland turfs was investigated. Bacterial, desulphonating bacterial and fungal communities of rhizosphere soil were significantly different to the respective bulk soil communities. Moreover, significant shifts were detected in the bacterial communities and the diversity of the desulphonating bacteria in the sulphonate treatments. This was not the case for the fungal communities although sequences were exclusively identified in the sulphonate treatments associated to endophytic fungi. Quantitative cultivation of desulphonating bacteria and XANES analysis suggested that although the grassland turfs were not sulphonate limited, added TS was desulphurized.

Unimproved grasslands dominated by bentgrass are commonly associated with low levels of production and high content of soil organic matter and fungal biomass [39-41]. This low productivity was associated to the limited amount of available nutrients. S is an essential macronutrient and is required for growth of all organisms [12] in order to form functional proteins, enzymes and enzyme co-factors. While application of inorganic fertilizer to grasslands increases the plant productivity significantly, it also shifts the microbial community from fungal dominance to bacterial dominance.
and a loss of soil organic carbon is usually associated with this shift [39, 41]. Recent investigations in sulphate additions to grassland turfs identified a significant increase in cultivable heterotrophic bacteria and a significant shift in the bacterial communities when compared to a sulphate free treatment [16]. These findings confirmed that microbial shifts in grassland soils can also be caused by sulphate addition alone in soil that was highlighted as S deficient in the past [42]. However, very little is known about potential microbial community shifts when organically bound S is introduced to grassland systems in the form of sulphonates.

In this study, incubation of grassland turfs with additional sulphonates resulted into marginally higher levels of cultivable heterotrophic soil bacteria but this increase was not significant, suggesting that sulphonate S was not a limiting factor for bacteria in the grassland turfs in this study. Likewise, İnceoğlu and colleagues found no significant increases of the bacterial abundance when linear alkylbenzene sulphonate (LAS) was added to soil microcosms [43]. Since in many soils, sulphonates are the dominant source of S [1], the form of the added sulphonate and the ease of accessibility by the microbes were important to study. TS has been used successfully for cultivation dependent studies and been regarded as an aromatic sulphonate that can be readily accessed by recently isolated desulphonating bacteria [14, 16, 21]. Until now it was unclear whether soil bacteria are capable of utilizing polymeric sulphonates such as ligonosulphonate as a S source. Since it is believed that sulphonate desulphurization is taking place intracellular, the polymeric sulphonate would need to either be imported or degraded into smaller molecules before transport [13]. The MPN results with minimal medium and lignosulphonate as sole S source suggested the presence of bacteria capable of utilizing lignosulphonate as sole S source. The cultivation dependent approaches as conducted in this study have their
limitations due to low levels of cultivability of soil bacteria [44]. Likewise, the cultivation independent study of functional groups of microbes can be challenging due to limitations of PCR primers hybridizing with all relevant targets [45]. In this instance, the combination of cultivation dependent and independent analysis has the potential to cancel out some of the respective limitations.

S K-edge XANES analysis in this study confirmed that a large proportion of the total soil S was indeed attributed to sulphonates (Fig. 4). Nevertheless, significant shifts in the bacterial community structure were observed, both in the bulk soil and in the rhizosphere although in the rhizosphere only the TS treatment resulted in a significant shift. The application of LAS to soil microcosms by İnceoğlu and colleagues also influenced the bacterial community structure but significance was not determined in this case [43].

Community profiling of the marker gene asfA in this study revealed a significant change in the desulphonating bacterial soil community structure when TS was applied to the mesocosms but this change was not significant for the LS treatment. Since LS is an aliphatic sulphonate, the asf gene cluster is not necessary for its desulphonation [46] and therefore, no such shift was expected in this particular case. No significant change in the desulphonating community was observed in the rhizosphere according to permutation tests (Fig 2). In contrast, sulphate addition to grassland turfs resulted in significant changes in asfA based rhizosphere community profiles before [16]. These findings suggest that the amount of sulphonates was not sufficient to change the desulphonating bacterial community in the rhizosphere but influenced other groups of rhizobacteria instead, presumably bacteria that were capable of utilizing the sulphonates as a carbon source.

A closer inspection of the asfA gene based T-RFLP patterns revealed that only one out
of four T-RFs indicative for *Variovorax* like OTUs was present in bulk soil and none was present in the rhizosphere soil, suggesting that *Variovorax* did not play a pivotal role in this study. Instead, several T-RFs were identified that were associated with *asfA* previously associated with *Polaromonas* like OTUs from environments including the Damma glacier forefield, spring barley and wheat rhizosphere [14, 17, 21]. The primers chosen to hybridize the *asf* gene cluster may not detect all types of *asfA* effectively, thus overlooking unspecified types of desulphonating bacteria. Indeed, recent modifications of the minimal media resulted in the isolation of bacteria capable of utilizing TS as S source where current *asf* primers are inefficient to amplify *asfA* (unpublished data and [47]).

The spectroscopic S analysis (XANES) of the bulk soils from the TS treatment and the control did not identify an increase in the sulphonate fraction. However, TS can be quickly converted into para-cresol and sulphite by desulphonating bacteria [46] and would therefore no longer be detected as intermediate S. Indeed, XANES analysis estimated a slight increase in oxidized S in the TS treatment, suggesting that desulphonating bacteria were indeed oxidizing the added sulphonate-S.

CA suggested that the application of both, LS and TS treatments had an effect on the fungal community structures in bulk soil and to a lower extend in the rhizosphere soil samples. However, permutation test could not confirm the significance of these findings (Fig. 3) where only differences between bulk soil and rhizosphere soil communities were deemed significant. In contrast, bacterial communities were significantly affected by the sulfonate treatments (Fig. 1 and 2). The recent advent of next generation sequencing platforms has increased the amount of sequence information received from an environmental sample considerably [48]. However, comparisons of 16S rRNA gene based DGGE fingerprints and pyrosequencing data
demonstrated that differences in bacterial community structures were observed by both techniques [49, 50] thus confirming the efficacy of the longer established fingerprinting methods in microbial ecology.

Sequences associated to the genera *Exophiala* and *Paecilomyces* were only picked up in the bulk soil samples of TS and LS treatments (7 out of 96 identified clones). Members of both genera are mainly fungal endophytes and are able to produce phytohormones to protect their host plants from environmental stress [51, 52]. Members of the genus *Exophiala* have been picked up before in soils [53] and associated to orchid roots [54] while members of the genus *Paecilomyces* were found previously in grassland soil [55] and as root endophyte [56] for instance to protect the host plant from parasitic nematodes [57]. It has been shown in subalpine grassland ecosystems that mowing caused shifts in the plant symbiotic interaction from fungal endophytic to arbuscular mycorrhiza (AM) [58]. However, their role in plant S metabolism is to date unknown.

AM fungal communities are distinctive to the respective grass species such as *A. stolonifera* and maintain even harsh environmental shifts [59]. For instance, after addition of limestone and nitrogen [59] or phosphorus to soil [60] the AM fungi diversity remained stable and distinctive to the respective plant species. These findings are in line with the present findings of unchanged fungal and Glomeromycota communities in response to sulphonate treatments (Fig. 3; supplementary Fig. S2C).

Future in depth and less complex studies of fungal cultures including species of *Exophiala* and *Paecilomyces*, desulphonating bacteria and *A. stolonifera* are needed to improve our understanding of plant-microbe interactions in function to the plant derived S cycle.

This study aimed to analyse the implication of the rhizosphere and bulk soil bacterial
and fungal communities on the desulphonation of low molecular and high molecular weight sulphonates. While significant changes were identified in the bacterial communities on the 16S and the functional gene level (\textit{asfA}), the implication of saprophytic and mycorrhizal fungi in the sulphonate-S cycle was not confirmed after five weeks of sulphonate addition. Sequencing results suggest an implication of putative endophytic fungi instead.

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References


[47] J. Gahan, A. Schmalenberger, Microbial community dynamics and desulfonylating bacterial abundance in the mycorrhizosphere of bi-compartmental microcosms
respond to inoculation with arbuscular mycorrhizal fungi, Society for General Microbiology Conference, Manchester, March 24th-28th, 2013.


Figure legends

**Figure 1** Ordination plot (correspondence analysis) of DGGE profiles of bacterial 16S rRNA gene fragments amplified from bulk soil (open symbol) and rhizosphere soil (closed symbol) samples after incubation with sulphate free Hoagland’s solution (triangle), Hoagland’s solution with lignosulphonate (square), Hoagland’s solution with toluenesulphonate (circle). Error bars indicate the standard deviation of the mean of triplicate measurements. Letters indicate significant differences ($P \leq 0.05$, permutation test) between sample origin and the treatments. Eigenvalues ($\lambda$) of each axis are indicated.

**Figure 2** Ordination plot (correspondence analysis) of TRFLP profiles of the desulphonating community based on *asfA* gene fragments amplified from bulk soil (open symbol) and rhizosphere soil (closed symbol) samples after incubation with sulphate free Hoagland’s solution (triangle), Hoagland’s solution with lignosulphonate (square), Hoagland’s solution with toluenesulphonate (circle). Error bars indicate the standard deviation of the mean of triplicate measurements. Letters indicate significant differences ($P \leq 0.05$, permutation test) between sample origin and the treatments. Eigenvalues ($\lambda$) of each axis are indicated.

**Figure 3** Ordination plot (correspondence analysis) of DGGE profiles of fungal communities based on ITS fragments amplified from bulk soil (open symbol) and rhizosphere soil (closed symbol) samples after incubation with sulphate free Hoagland’s solution (triangle), Hoagland’s solution with lignosulphonate (square), Hoagland’s solution with toluenesulphonate (circle). Error bars indicate the standard deviation of the mean of triplicate measurements. Letters indicate significant differences ($P \leq 0.05$, permutation test) between sample origin and the treatments. Eigenvalues ($\lambda$) of each axis are indicated.

**Figure 4** Normalised S K-Edge XANES spectra from bulk soil samples after 35 days of incubation with sulphate free Hoagland’s solution (SF) or Hoagland’s solution with toluenesulphonate (TS). Gaussian (G) curves were generated based on the data.
collected from standard spectra for reduced (G1, dark grey), intermediate (G2, light grey; G3, dotted dark grey) and oxidised sulphur species (G4, dotted light grey). A Gaussian fit of G1-G4 was conducted for SF and TS to estimate the presence of reduced, intermediate and oxidised sulphur species.

Table 1

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>Sulphate [ppm]</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-T₀</td>
<td>22.76</td>
<td>2.45</td>
</tr>
<tr>
<td>R-T₀</td>
<td>26.66</td>
<td>2.39</td>
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<tr>
<td>SF-S</td>
<td>21.21</td>
<td>1.30</td>
</tr>
<tr>
<td>SF-R</td>
<td>23.53</td>
<td>4.11</td>
</tr>
<tr>
<td>LS-S</td>
<td>23.28</td>
<td>9.31</td>
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<tr>
<td>LS-R</td>
<td>21.25</td>
<td>1.95</td>
</tr>
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<td>TS-S</td>
<td>17.75</td>
<td>0.32</td>
</tr>
<tr>
<td>TS-R</td>
<td>24.96</td>
<td>5.28</td>
</tr>
</tbody>
</table>

SD = standard deviation of three replicate measurements
Table 2
Most probable number (MPN) of desulphonating and heterotrophic bacteria from bulk soil (S) and rhizosphere soil (RS) samples after 35 days of incubation with sulphur free (SF), lignosulphonate (LS), or toluenesulphonate (TS) containing Hoagland’s solution.

<table>
<thead>
<tr>
<th>2A</th>
<th>Mesocosm</th>
<th>Confidence (95%)</th>
<th>Type</th>
<th>MPN $10^6$ g$^{-1}$</th>
<th>Lower</th>
<th>Upper</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>LS</td>
<td>5.0</td>
<td>2.2</td>
<td>12</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>SF</td>
<td>6.7</td>
<td>2.9</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TS</td>
<td>2.7</td>
<td>1.2</td>
<td>6.2</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>2B</th>
<th>RS MPN in minimal media with LS as sulphur source</th>
<th>Confidence (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type</td>
<td>MPN $10^6$ g$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>LS</td>
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<tr>
<td></td>
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<td>6.7</td>
</tr>
<tr>
<td></td>
<td>TS</td>
<td>3.3</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>2C</th>
<th>S MPN in minimal media with TS as sulphur source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type</td>
</tr>
<tr>
<td></td>
<td>LS</td>
</tr>
<tr>
<td></td>
<td>SF</td>
</tr>
<tr>
<td></td>
<td>TS</td>
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<table>
<thead>
<tr>
<th>2D</th>
<th>S MPN in minimal media with LS as sulphur source</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Type</td>
</tr>
<tr>
<td></td>
<td>LS</td>
</tr>
<tr>
<td></td>
<td>SF</td>
</tr>
<tr>
<td></td>
<td>TS</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>2E</th>
<th>RS MPN in complex R2A medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type</td>
</tr>
<tr>
<td></td>
<td>LS</td>
</tr>
<tr>
<td></td>
<td>SF</td>
</tr>
<tr>
<td></td>
<td>TS</td>
</tr>
</tbody>
</table>
2F  S MPN in complex R2A medium

<table>
<thead>
<tr>
<th>Type</th>
<th>MPN $10^7 \text{g}^{-1}$</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS</td>
<td>3.0</td>
<td>1.3</td>
<td>6.0</td>
</tr>
<tr>
<td>SF</td>
<td>1.7</td>
<td>0.7</td>
<td>4.0</td>
</tr>
<tr>
<td>TS</td>
<td>2.3</td>
<td>1.0</td>
<td>5.4</td>
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Table 3
Assignment of sequences of representative fungal RFLP genotypes derived from bulk soil samples after 35 days of incubation with sulphate free (SF), lignosulphonate (LS), or toluenesulphonate (TS) containing Hoagland’s solution.

<table>
<thead>
<tr>
<th>Phylogenetic affiliation</th>
<th>Phylum</th>
<th>Subphylum</th>
<th>OTU</th>
<th>Closest relative phylogenetic assignment</th>
<th>Similarity to closest relative [%]</th>
<th>Presence /OTU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascomycota</td>
<td>Pezizomycotina</td>
<td>1</td>
<td><em>Penicillium canescens</em></td>
<td>99</td>
<td>SF, TS, LS</td>
<td>19</td>
</tr>
<tr>
<td>Ascomycota</td>
<td>Pezizomycotina</td>
<td>2</td>
<td>Uncultured <em>Cystodendron</em></td>
<td>99</td>
<td>SF, TS, LS</td>
<td>24</td>
</tr>
<tr>
<td>Basal fungal lineages</td>
<td>Mucoromycotina</td>
<td>3</td>
<td>Uncultured Mortierellaceae</td>
<td>&lt;90</td>
<td>SF, TS, LS</td>
<td>5</td>
</tr>
<tr>
<td>Chytridiomycota</td>
<td>Chytridiomycetes</td>
<td>4</td>
<td>Uncultured fungus</td>
<td>&lt;90</td>
<td>SF, LS</td>
<td>4</td>
</tr>
<tr>
<td>Glomeromycota</td>
<td>Glomeromycetes</td>
<td>5</td>
<td>Uncultured <em>Arcaeoaspora</em></td>
<td>94</td>
<td>SF, TS</td>
<td>3</td>
</tr>
<tr>
<td>Basidiomycota</td>
<td>Agaricomycotina</td>
<td>6</td>
<td>Uncultured <em>Cryptococcus</em></td>
<td>94</td>
<td>SF, LS</td>
<td>3</td>
</tr>
<tr>
<td>Ascomycota</td>
<td>Pezizomycotina</td>
<td>7</td>
<td>Uncultured Coniochaetales</td>
<td>&lt;90</td>
<td>SF, TS</td>
<td>3</td>
</tr>
<tr>
<td>Ascomycota</td>
<td>Pezizomycotina</td>
<td>8</td>
<td>Uncultured <em>Exophiala</em></td>
<td>99</td>
<td>TS, LS</td>
<td>3</td>
</tr>
<tr>
<td>Ascomycota</td>
<td>Pezizomycotina</td>
<td>9</td>
<td>Uncultured fungus</td>
<td>&lt;90</td>
<td>LS</td>
<td>4</td>
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<tr>
<td>Ascomycota</td>
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<td>Uncultured fungus</td>
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<td>SF</td>
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<tr>
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<td>Uncultured <em>Cryptococcus</em></td>
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<td>SF, TS</td>
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<td>Uncultured soil fungus</td>
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<td>SF</td>
<td>4</td>
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<td><em>Clonostachys</em></td>
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<td>SF</td>
<td>2</td>
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<td>Ascomycota</td>
<td>Pezizomycotina</td>
<td>15</td>
<td>Uncultured Ascomycota</td>
<td>90</td>
<td>TS</td>
<td>3</td>
</tr>
<tr>
<td>Basal fungal lineages</td>
<td>Mucoromycotina</td>
<td>22</td>
<td><em>Mortierella</em></td>
<td>98</td>
<td>SF, TS</td>
<td>2</td>
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<tr>
<td>Glomeromycota</td>
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<td>Uncultured glomeromycetes</td>
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<td>SF, TS</td>
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<td>Uncultured Coniochaetales</td>
<td>&lt;90</td>
<td>SF, LS</td>
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<tr>
<td>Ascomycota</td>
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<td>Dactylella like clone</td>
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<tr>
<td>Ascomycota</td>
<td>Pezizomycotina</td>
<td>51</td>
<td>Uncultured Ascomycota</td>
<td>&lt;90</td>
<td>TS, LS</td>
<td>2</td>
</tr>
</tbody>
</table>

The genus level is indicated if the environmental clone sequences exhibited overall similarity values of at least 95% to the 16S rRNA gene sequences of taxonomically described members of the respective genus. Otherwise, higher taxonomic levels are given.