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Sulfate fertilization supports growth of ryegrass in soil columns but changes microbial community structures and reduces abundances of nematodes and arbuscular mycorrhiza

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ABSTRACT

The increased use of sulfate fertilizers to compensate for soil sulphur (S) limitation in agricultural soils may affect soil microbes and micro-fauna involved in S mobilization. Here, columns with podzolic soil material and ryegrass (*Lolium perenne*) were fertilized with 0, 5, 10 and 20 kg ha^{-1} (S0/S5/S10/S20) inorganic sulfate-S alongside a full complement of other nutrients. In the S10 and S20 columns, significantly higher amounts of sulfate were present in soil solution. After two grass cuts (14 weeks in total), there was a significant decrease in arylsulfatase activity, bacterial-feeding nematode abundances and mycorrhizal colonization in the S10 and S20 columns compared to the S0. Bacterial, fungal and AM community structures shifted significantly across the treatments. After final harvest, the S10 and S20 columns had significantly higher grass dry matter yield and uptake of S, N, K, Ca and Mg compared to the S0. While the overall bacterial diversity was reduced in the S20 treatment, abundance (*asfA*) and diversity (*ssuD* and *atsA*) of bacterial genes involved in S cycling were not significantly affected by one-time sulfate fertilization. These results indicate that short-term sulfate fertilization benefits to plant growth outweighed the negative feedback from parts of the soil biota. To improve nutrient use efficiencies in a sustainable manner, future studies should consider alternative S fertilizers which may be beneficial to both, the soil biota and plants in the long-term.

1. Introduction

Sulfur (S) is an important element for all living organisms. It is an essential macronutrient for plant nutrition as a component of proteins and enzyme co-factors, and is also a major constituent of the amino acids cysteine and methionine (Kertesz and Mirleau, 2004; Takahashi et al., 2011). Moreover, S is also vital in plants' response to pathogen attack as it is a major component in several plant defence compounds (Kertesz et al., 2007). Despite its important role, S cycling is often overlooked in nutrient cycling studies in comparison with nitrogen (N) and phosphorus (P).

Air pollution was the major source of S for plants prior to the enactment of the Clean Air Act (McGrath et al., 2003). However, reduction in atmospheric S deposition and a range of measures in agricultural intensification has resulted in widespread S deficiencies across Europe and other parts of the world (McGrath et al., 2003; Zhao et al., 2006). Plants depend almost entirely on inorganic sulfate for their S requirement (Kertesz et al., 2007). However, about 95% of S in agricultural soils is bound organically as sulfonates and sulfate-esters are not directly available for plant uptake (Kertesz et al., 2007; Kertesz and Mirleau, 2004; Scherer, 2001).

Previous research has shown that organo-S (sulfonates and sulfate-esters) is indirectly plant-bioavailable due to the inter-conversion into inorganic sulfate by soil microbes (Kertesz and Mirleau, 2004; Scherer, 2009; Schmalenberger and Noll, 2014). Microbial production of sulfatases, particularly arylsulfatases has been implicated in the mineralization of organo-S (Kertesz and Mirleau, 2004). In agricultural systems with reduced S inputs from fertilizer application or atmospheric deposition, the conversion of organo-S to inorganic sulfate is very vital for plants (Scherer, 2009). As part of the soil S cycle, S-containing compounds in soils undergo several transformations. These include microbial immobilization of inorganic sulfate and organo-S compounds into the microbial biomass, soil organic matter and enzymatic

mineralization of organo-S compounds via the production of sulfatases and sulfonatases (Kertesz and Frossard, 2015). Considering the high amount of aromatic sulfate-esters in soils (Freney, 1986), *atsA* genes encoding for arylsulfatases in bacteria play a crucial role in overall sulfate release in soils. Genes *ssuD* and *asfA* are part of a monooxygenase enzyme complex to cleave S from aliphatic and aromatic sulfonates and have been shown to be important for bacterial survival in soil (Mirleau et al., 2005). Moreover, recent studies have reported the significant role that arbuscular mycorrhizal (AM) symbiosis play in plant S supply including the actual transfer of S from AM fungi to plants, improving plant S uptake and reducing the requirement for inorganic sulfate fertilization (Gahan and Schmalenberger, 2014; Giovannetti et al., 2014; Sieh et al., 2013). In addition, microbial-feeding nematodes increase plant nutrient availability and uptake by excreting nutrients in excess of their metabolic requirement (Gebremikael et al., 2016; Irshad et al., 2011). The application of inorganic fertilizers aims to supply the plant with nutrients directly. However, this may have an adverse effect on the abundance and function of the soil biota involved in nutrient cycling. This was recently demonstrated for phosphate fertilization (Ikoyi et al., 2018) that resulted in greatly reduced abundances of arbuscular mycorrhiza and nematodes. It is therefore conceivable, that S fertilization could affect soil biota in a similar way to P fertilization. Despite the abovementioned roles of the soil microbiota in plant S supply and soil S cycling, studies on the effects of inorganic sulfate fertilizer application on soil microbiota are rare in the literature and absent in the case of soil nematodes.

Considering the importance of the soil microbiota in soil S cycling, our hypothesis was that sulfate fertilization is changing microbial community structures and functions as well as reducing abundances of AM fungi and nematodes, thus negating a plant fertilization effect. The aim of this study was to provide an understanding of the response of soil microbiota, nematodes, and plants to different rates of sulfate fertilizer application. Specifically, the

objectives were to: assess the effect of one-time sulfate fertilizer application on the abundance, composition, and function of the soil microbiota (bacteria, fungi, AM fungi) and micro-fauna (nematodes) alongside sulfate uptake by the grassland plant ryegrass (*Lolium perenne*). We hypothesized that sulfate fertilizer application will reduce *i*) nematodes and mycorrhiza abundances, *ii*) microbially mediated sulfate release from organo-S forms in soils thereby contributing less to S availability in grasslands and ultimately shaping community structures.

2. Material and methods

2.1 Experimental setup, column harvest, plant and soil analyses

Soil columns were setup in a greenhouse as described previously in a recent experiment on P cycling (Ikoyi et al., 2018) and maintained over a 14 week period in a glasshouse under natural light and ambient temperature. Briefly, pipes of 16 cm X 40 cm were used for the construction of the columns. Nylon mesh excluded root growth beyond the bottom of the pipes and allowed free drainage. The soil for the column experiments is a grey brown podzolic soil as described recently (Ikoyi et al., 2018) and was collected in 2016 from a site in Moorestown Cahir (52º22'4"N, 7º50'35"W; County Tipperary, Ireland). The soil is moderate in S, equivalent to the Wisconsin low S soil availability index (<30 ppm; Kelling et al., 1999) and neutral in pH (7.0). Further details of the soil properties are presented in the Supplementary Table S1. The site was not under cultivation for at least 20 years (oral communication, Cornelius Traas). Soils were sampled from the top 20 cm and at 20-40 cm depth, sieved to remove stones (3.35 mm mesh), then mixed and repacked into the columns in the same layers as they were sampled in the field (0-20 and 20-40 cm). Ryegrass (*Lolium perenne* variety Trend, Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Germany) was sown at a depth of 2 cm on the repacked soil. Columns were fertilized (week seven) with 0, 5, 10 and 20 kg S ha^{-1} inorganic sulfate S treatments (ammonium sulfate in water, applied on the surface of the columns) representing S0, S5, S10 and S20 treatments respectively (recommended amount of S application for grasslands was equivalent to the S20 treatment; Lalor and Coulter, 2008). Alongside, a full complement of other essential nutrients was applied (total amount of nutrients added: 125 kg ha^{-1} N, 150 kg ha^{-1} K, 20 kg ha^{-1} P and micronutrients). The ammonium addition by the fertilization with ammonium sulfate was compensated in the other treatments by using ammonium nitrate as a source of N. All treatments were replicated six times and managed for 14 weeks in total (representing two

grass cuts). The columns were watered three times a week with rain water (200 ml). Samples of soil solution were collected weekly using Rhizons (Rhizosphere Research Products, Wageningen, Netherlands) that were positioned in 10 cm height intervals in the columns and analysed for anions sulfate, nitrate, and phosphate through ion chromatography using a Dionex ICS1100 system with an AS23 column (2 mm system with guard and 400 mm main column), and a carbonate mobile phase according to the manufacturer's instructions (0.25 ml min^{-1}, 4.5 mM Na_2CO_3, 0.8 mM $NaHCO_3$ at 30°C, Dionex, Thermo Scientific, Sunnyvale, CA). In order to trace the transfer of the S fertilizer into the plant biomass in the columns more efficiently, a subset of the columns (three from each of the S5, S10 and S20 treatments) was spiked with stable isotope sulfate (^{34}S) while the remaining columns received same rate of ^{32}S sulfate at week 9 (2.1 kg/ha sulfate-S).

After seven weeks of growth, ryegrass was cut back to 5 cm height immediately before the main fertilization. Dry matter of the grass shoots was determined. After 14 weeks of grass growth, columns were destructively harvested, shoot and root biomasses were determined. Excess soil was gently shaken off roots. The soil retained on the roots up to this point was collected as rhizosphere soil for arylsulfatase activity determination, nematode studies and further analysis of soil properties. Root samples were collected to analyse mycorrhizal colonization (see below).

Dry shoot biomass for both cuts was recorded after oven drying at 55 °C for 72 hours. Elemental compositions were determined in the dried shoot biomass (second harvest) by Lancrop Laboratories Ltd. (using inductively coupled plasma spectrometry, atomic absorption spectroscopy, titrations, and spectrophotometry, according to ISO/IEC 17025:2005). The soil pH was determined potentiometrically in deionised water (1:2 soil solution ratio; (McCormack, 2002).

Amounts of ^{34}S in *L. perenne* shoots (second harvest) were determined using Elemental Analysis - Isotope Ratio Mass Spectrometry (EA-IRMS) at Iso-Analytical (Cheshire, UK) (Zhao et al., 2001). Analysis was based on monitoring the mass-to-charge ratio *m/z* at 48, 49 and 50 of SO^+ ions produced to ascertain the relative abundance of ^{32}S (48 *m/z*) to ^{34}S (50 *m/z*) and the total S content was calculated. Delta Units (δ) are expressed in molecules per thousand and are used to denote isotope ratios. For further details on the isotope ratio analysis, please refer to the supplementary information S1.

2.2 Cultivation-dependent analysis of aromatic sulfonate utilizing bacteria, potential arylsulfatase activity, mycorrhizal root colonization and nematode abundance determination

The rhizosphere soil from the top 10 cm of the column was used for bacterial extraction as described previously (Fox et al., 2014). In brief, 3 g of roots with attached soils were added to 50 ml tubes with 20 ml sterile saline solution and rotated at 75 rpm for 30 min at 4 °C on an Elmi Intelli-Mixer RM-2 (Elmi Tech Ltd, Latvia). Resultant suspensions were serially diluted. The total heterotrophic bacteria (growth in agar free R2 medium (Reasoner and Geldreich, 1985); R2A without agar) and cultivable bacteria able to utilize aromatic sulfonate (MM2TS, toluene sulfonate) as sole source of S, were quantified via a most probable number (MPN) approach in microtiter plates (Fox et al., 2014). Remaining rhizosphere suspensions (without plant roots) were centrifuged at 4500 rpm for 15 min at 4 °C and the obtained pellets were stored at -20 °C for molecular analysis.

Rhizosphere soil (0-20 cm and 20-40 cm) was analysed for potential arylsulfatase activity as described by Tabatabai and Bremner (Tabatabai and Bremner, 1970b). Soils (1 g) were incubated for 1 h at 37 °C with acetate buffer (pH 5.8) and para-nitrophenyl sulfate (Sigma Aldrich, St. Louis, MO) as the substrate. Samples were filtered (Whatman 2 filter paper) and

diluted to stay within the values of the standard curve. Intensity of the p-nitrophenol colour was measured with a spectrophotometer at 400 nm.

Grass roots from the top 20 cm were inspected for percentage root colonization by arbuscular mycorrhizal (AM) fungi following a modified version of the grid line intersect method (McGonigle et al., 1990). In brief, representative root samples were selected and then cut into segments of 1 cm. These segments were washed with dH_2O, bleached with alkaline H_2O_2, acidified with a 0.1 M HCl solution and then stained with lactoglycerol trypan blue (1:1:1 ratio of lactic acid:glycerol:H_2O including trypan blue (0.05%, w/v)). De-staining of the roots occurred overnight in lactoglycerol without trypan blue. The obtained root segments were assessed under the light microscope to account for arbuscules, vesicles and hyphae, one field of view at a time. For each column, root AM fungal colonization was calculated in 3 replicates of 100 intersections.

Nematodes were extracted from 100 g of fresh soil (0-20 cm) using a modified Baermann method (Whitehead and Hemming, 1965) as described previously (Ikoyi et al., 2018). Nematodes were quantified and identified to the family or genus level under a light microscope (600 times magnification) using morphological features and identification keys (Andrássy, 1984; Bongers, 1988; Jairajpuri and Ahmad, 1992; Siddiqi, 1986). Abundances were calculated after assigning all nematodes to feeding groups by counting the number of individual nematodes belonging to the respective feeding groups: bacterial-feeders, fungal-feeders, plant-parasites, omnivores and predators.

2.3 DNA extraction, community fingerprints and quantitative PCR

DNA was extracted from rhizosphere soil pellets (0.25 g) with the PowerSoil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA) following the instruction of the manufacturer.

Quantification of the obtained DNA was carried out with a Qubit Fluorometer (dsDNA HS Assay kit; Life Technologies, Carlsbad, CA).

PCR amplifications of the bacterial (16S rRNA), fungal (ITS) and AM fungal (18S rRNA) gene fragments and subsequent denaturing gradient gel electrophoresis (DGGE) were performed as previously described (Fox et al., 2014; Schmalenberger and Noll, 2014) and detailed in the supplementary information section S2. The phoretix 1D software (Totallab, Newcastle, UK) was used for DGGE image analysis by creating binary gel image matrices. The latter were analysed to create canonical correspondence analysis (CCA) biplots. Permutation testing (Monte Carlo; 9999 repeats) was carried out in CANOCO (Microcomputer Power Inc., Ithaca, NY). This allowed the identification of environmental properties that affected the community structures and significant differences between the treatments (Noll and Wellinger, 2008).

Assessment of the diversity of the rhizosphere bacterial *ssuD* and *atsA* genes was carried out via terminal restriction fragment length polymorphism (TRFLP) analysis using the primer sets ssuD_209F and ssuD_1001R, and atsA_F1 and atsA_CR_V9 (Guldner, 2015), respectively. The forward primers were labelled at the 5' end with the fluorescent dye 6-carboxyfluorescein (6-FAM, metabion). PCR and T-RFLP conditions are described under S3 in the supplementary information. After PCR amplification and restriction digestion, fragments of *atsA* and *ssuD* were analysed using an ABI 3130xl genetic analyser (Applied Biosystems). The data obtained on the abundance of the terminal restriction fragments were used for Principal Components Analysis (PCA).

Absolute quantification of *Variovorax asfA* genes was carried out from extracted DNA (see above) using primers asfA_Varx_F1 (5'-CTG TCG GGC ATG GAG TTC T-3') and asfA_Varx_R1 (5'- AGC GTC ACC GGA AAG TGC T- 3') (Schmalenberger et al., 2008). Quantification of *Burkholderia* and *Polaromonas asfA* genes was conducted with primer

pairs: asfA_Brk_F1 (5'- TGT GCG TTT CTC AGC AAC G -3') and asfA_Brk_R1 (5'- TGC ACG CTG CGA ATC ACC -3'), and asfA_Pol2_F1 (5' –GCT GCG CTT TCT TAA GCC G-3') and asfA_Pol2_R1 (5'-GCG ATC CAG GCT TTC ACC T -3'), respectively. All reactions ran in a LightCycler ® 96 Real-time PCR System (Roche, Basel, Switzerland), PCR conditions and primer design are detailed in the supplementary information S4.

2.4 Next generation sequencing and sequence analysis

Sequencing was carried out at the Novogene Bioinformatics Technology Co., Ltd. Briefly, DNA was amplified using the 341F/806R primer set (341F: 5′- CCT AYG GGR BGC ASC AG -3′, 806R: 5′-GGA CTA CNN GGG TAT CTA AT -3′), targeting the V3-V4 region of the bacterial 16S rDNA (Glaring et al., 2015). PCR reaction was performed using phusion high-fidelity PCR Mastermix (New England Biolabs, Ipswich, MA). PCR products were purified using a Gel Extraction Kit (Qiagen, Dusseldorf, Germany). NEBNext® Ultra^{TM} DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA) was used for generating sequencing libraries following the manufacturer's instructions. Qubit Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system were used to assess library quality. The library was then sequenced on an Illumina HiSeq 2500 platform and 250 bp paired-end reads were generated. Quality filtering and reads assignment at OTU level are described in the supplementary information S5.

Alpha and beta diversities were calculated with QIIME (Version 1.7.0). PCoA analysis was conducted with the R software (Version 2.15.3) using the WGCNA, stat and ggplot2 packages. R was also used to create heat maps to display the abundance distribution of dominant taxa among the treatments. Nucleic acid sequences were deposited in the Nucleotide Archive (Project PRJEB31225; ERS3140271 - ERS3140294).

2.5 Statistical analyses

In order to capture dry matter yield and mass balances, data on grass elemental composition were first converted to unit per column basis. The grass dry matter yield, elemental composition, $\delta^{34}S_{V\text{-}CDT}$ values, MPN, arylsulfatase activity, percentage root mycorrhizal colonization, abundance of nematode feeding groups, and alpha diversity indices were analysed using a one-way ANOVA in R software (Version 3.0.1) (R Development Core Team, 2013). Normality and homogeneity of variance of the data was checked using Shapiro-Wilk's and Levene's tests, respectively. Where normality and homogeneity of variance was confirmed, a Tukey's HSD post-hoc test was applied. Some data were logarithmically transformed, analysed by ANOVA and the back transformed values to the original scale were reported in order to conform to the assumptions above. Where homogeneity of variance was not achieved through transformation, the Games-Howell test was used. When both assumptions of ANOVA were not fulfilled, the Kruskal-Wallis test was performed instead.

3. Results

3.1 Soil solution chemistry, dry grass elemental composition and yield

The concentration of sulfate in soil solution was measured weekly at four different soil depths for the duration of 14 weeks. Higher amount of sulfate was detected in the bottom 20 cm of the columns than in the top 20 cm (upper two and lower two measures were combined respectively in Figure 1 and Supplementary Figure S1). For the top 20 cm, the sulfate concentration was similar until the seventh week (Figure 1). After the fertilizer application event in week 7 and until the end of the experiment, the S10 and S20 treatments had significantly higher sulfate concentration ($P<0.05$) than the S0 and S5 treatments (Figure 1). The soil solution pH ranged between 7.15 and 8.55. The grass growth was similar ($P>0.05$) in all columns before fertilization as seen in the dry matter yield of the first harvest (Table 1). The grass dry matter yield (second harvest) increased significantly with increasing S fertilizer application rates ($P<0.01$, Table 1). The elemental composition of the grass dry matter showed that the S10 and S20 columns had a significantly higher ($P<0.05$) uptake of S, N, K, Ca and Mg (Table 1). For the non-spiked samples, grass uptake of ^{34}S was similar among the treatments (Table 1). For the samples spiked with ^{34}S, the $\delta^{34}S$ values were significantly higher ($P<0.05$) in the S10 and S20 treatments when compared to the S5 treatment. This was despite the fact that all three treatments were spiked with the same amount of ^{34}S, hence higher initial S fertilizer application resulted in higher ^{34}S uptake. The S5 treatment had a significantly higher pH compared to the S20 treatment while the other treatments were not different statistically (Table 2).

3.2 Effect of sulfate fertilization on cultivable bacteria abundances, arylsulfatase activity, mycorrhizal root colonization and abundance of nematodes

Although there was a trend of higher cultivable total heterotrophic bacterial abundances, including sulfonate utilizing bacteria in the S0 than in the S20 treatment (Table 2), this was not statistically significant ($P>0.05$). Abundances of the cultivable heterotrophic and sulfonate utilizing bacteria ranged from 3.5-5.3 x 10^7 MPN g^{-1} ($P=0.63$) and 1.3-1.9 x 10^7 MPN g^{-1} ($P=0.41$), respectively.

The arylsulfatase activity decreased with increasing rates of S fertilizer application. At 0-20 cm of the column, arylsulfatase activities were statistically similar in the S0 and S10 treatments. However, the S20 treatment had significantly lower activity compared to the S0 ($P<0.05$) representing a 19 % decrease (Table 2). There was no significant difference in the arylsulfatase activity between the treatments for the bottom (20-40 cm) of the column. Soil from the top 20 cm of all columns had significantly higher arylsulfatase activity compared to the bottom 20 cm.

Lolium perenne roots of the S10 and S20 treatments had significantly lower percentages of mycorrhizal arbuscules, hyphae and vesicles ($P<0.05$; Table 2) than the S0 treatment. The S0 treatment had over a third more arbuscules, almost three times more vesicles, and a third more hyphae than the S10 and S20 treatments. Like the mycorrhizal hyphae, bacterial-feeding nematodes were significantly more abundant in the S0 than in the S10 and S20 treatments ($P<0.05$) with the bacterial feeders being about 42 & 45 % more abundant in the S0 than in the S10 and S20 treatments, respectively (Table 2).

3.3 Bacterial, fungal and AM fungal community fingerprints, ssuD *and* atsA *gene diversity, and abundance of* Variovorax, Burkholderia *and* Polaromonas asfA *genes*

The bacterial community structure was initially analysed via 16S rRNA gene fragment fingerprinting using DGGE. The extracted binary matrix was subjected to canonical

correspondence analysis (CCA). The obtained biplot revealed that S0 and S5 treatments were distinguishable on the first axis (CCA1, Figure 2a). S10 and S20 were also clearly separated from S0 on the first axis. However, S5, S10 and S20 partially overlapped on the first axis. Nevertheless, S10 and S20 were partially separated on the second axis (CCA2; Figure 2a). Permutation testing of the DGGE fingerprints confirmed that S0 and all S treatments were significantly different ($P<0.05$). Significant differences were also found between the S5, S10 and S20 treatments. Permutation testing and CCA of the bacterial fingerprints revealed a highly significant correlation of shoot dry matter (Shoot DM), root biomass (Root BM), grass S content (Grass S), arylsulfatase activity of the top 20 cm of the column (SULFD20), grass N content (Grass N) and the bacterial-feeding nematode abundance (BF) as indicated by the arrows in Figure 2a.

DGGE profiles of the ITS region revealed the higher fungi community structure. A clear separation of the low S (S0 and S5) and the high S (S10 and S20) treatments was visible on the first axis of the biplot (CCA1, Figure 2b). S0 and S5 as well as S10 and S20 overlapped, respectively on the first axis. No separations by treatment were visible on the second axis (CCA2, Figure 2b). Permutation tests confirmed the high significance of a general shift in the fungal community structure upon S fertilizer application ($P<0.01$). This fungal shift was significantly correlated ($P<0.05$) with the following environmental factors (indicated as arrows in Figure 2b): grass S and N contents, shoot dry matter, root biomass, percentage of mycorrhizal arbuscules (AC), vesicles (VS) and hyphae (HC), total nematode abundance and abundance of fungal-feeding nematodes (FF).

The AM fungi community structure, based on 18S rRNA gene fragments separated by DGGE, shifted significantly ($P<0.05$) upon S fertilization (CCA biplot, Figure 2c). On the first axis (CCA1) a clear separation was detected between low S (S0/S5) and and high S (S10/S20) treatments (Figure 2c). No separation was detected on the second axis (CCA2).

Among the S fertilizer application rates, permutation tests showed that the S0 and S5 treatments differed significantly (P<0.05) from the S10 and S20 treatments. Moreover, permutation test revealed that the shift was significantly (P<0.05) correlated with the environmental factors: shoot dry matter, percentage of mycorrhizal arbuscules (AC) and hyphae (HC) (indicated as arrows in Figure 2c).

The PCA biplots of the diversity of *ssuD* (Supplementary Figure S2) and *atsA* (Supplementary Figure S3) genes showed that there were no clear differences in their diversities between the sulfate treatments although some samples (five out of 24 for *atsA* and seven out of 24 for *ssuD*) failed to amplify with the primers modified for TRFLP (a minimum of four replicates was obtained for each treatment). Similar results were obtained with detrended correspondence analysis (DCA, data not shown). Permutation tests confirmed the absence of significant shifts based on *atsA* or *ssuD* diversity.

The *asfA* gene copy numbers of *Variovorax*, *Burkholderia* and *Polaromonas* were not significantly affected by the sulfate treatment. The abundance of *Burkholderia asfA* was the highest while *Polaromonas asfA* was the lowest in all treatments (Table 2).

3.4.4 Next generation sequencing-based bacterial diversity and community composition

The sequences obtained consisted of 1,885,215 raw tags (merged paired-end raw reads), 1,577,583 clean tags (after filtering) and 1,374,987 effective tags (after chimera removal). Alpha and beta diversities of the S treatments were estimated based on sequences of the 16S rRNA gene (amplicons) using QIIME. The alpha diversities ranged between an average of 9.81 and 9.88 (Shannon) or 3052 and 3142 (chao1), respectively (Supplementary Table S2). While the ACE, Chao1 and goods coverage were similar among the treatments, the Shannon

and Simpson indices were significantly higher ($P<0.05$) in the S0 and S5 treatments compared to the S10 and S20 treatments (Supplementary Table S2).

Principal component analysis (PCA) for genera with significant differences among treatment groups (MetaStat Analysis) identified a clear separation of the S0 treatment from the S20 columns on the first axis (beta-diversity; Figure 3). However, the separation between the S5 and the S10 treatment from the other treatments was less pronounced. Moreover, permutational multivariate analysis of variance using distance matrix (ADONIS) and analysis of similarities (ANOSIM) confirmed a highly significant separation ($P<0.01$) between the S0 and S20 treatment. Furthermore, the S20 treatment contained significantly higher ($P<0.05$) abundances of *Bacillus*, *Mycobacterium*, *Bradyrhizobium*, *Candidatus Xiphinematobacter, Roseiflexus, Streptomyces, Sporosarcina, Rhizobium, Defluviicoccus, Microvirga, Nitrosospira* and *Microlunatus* compared to the S0 treatment (Figure 4a and b). Most of these genera have been previously identified to mobilize N and/or P in soils (Wang et al., 2017; Yolcu et al., 2011).

4. Discussion

S is considered as a requirement for the growth of all living organisms (Kertesz et al., 2007) and is an essential macronutrient element similar to N and P. In this study, the application of 10 and 20kg ha^{-1} sulfate S (S10 and S20 treatments) resulted in a significant increase in the levels of sulfate concentration in the soil solution and in ryegrass shoot dry matter. The nutrient balance analysis indicated the causal relationship between sulfate in soil solution and shoot growth, as S uptake by ryegrass was significantly lower in the S0 and S5 treatments compared to the S10 and S20 treatments. This implies that the S10 and S20 treatments with higher sulfate fertilization resulted in higher amount of sulfate in soil solution for grass uptake which ultimately supported higher grass growth. Similar findings were reported previously in a long-term field trial (Broadbalk, Rothamsted Research, UK (Schmalenberger et al., 2008)), where plots without sulfate fertilization had only half of the soil sulfate concentrations when compared with plots that received sulfate. In the present study, S10 and S20 treatments also resulted in a significantly higher total uptake of N, K Ca and Mg despite the fact that all columns received the same amount of these nutrients. The application of S fertilizer has been reported to improve N use efficiency by increasing N uptake in wheat (Salvagiotti et al., 2009). The present study confirmed this observation, reporting an increase in N uptake with increasing sulfate fertilizer application. On balance, sulfate fertilization had a beneficial effect on plant growth, despite any subsequent negative effects that the fertilizer may have on members of the soil biota.

In the present study, the spiking of the columns with $^{34}S\text{-}SO_4$ identified an increased uptake of S by the plants in the S10 and S20 treatments over S5. This further increase in S uptake took place when sulfate was already present at over 50 mg l^{-1} in soil solution. At the outset of this study, we anticipated that the S5 treatment would take up more ^{34}S than the S10 and S20 treatments due to putative S limitations and subsequent higher S needs after the main

fertilizer event. Indeed, the shoot dry weight to shoot S ratio was lower in the S5 than the S10 or S20 treatments (251 vs. 232 and 208 g g^{-1}, respectively), and the S5 treatment showed lower grass growth performance. The uptake of ^{34}S was highest in the S10 to S20 treatments. This suggests that plants of the S10 and S20 treatment benefitted more from the S spiking than S5 despite the fact that S5/10/20 received identical amounts of S in week 9. Further research is needed to find out why ^{34}S uptake was higher in the S10/20 treatments and whether this is directly related to plant transporter activities not being suppressed or if the lack of S in S5 is limiting S transporter activity. In contrast, the expression of bacterial genes involved in S acquisition through organo-S mineralization is down-regulated when sufficient amounts of sulfate is present (Schmalenberger and Kertesz, 2007).

Arylsulfatase is the enzyme that acts on aromatic sulfates by cleaving the O-S bond thereby releasing sulfate into the soil solution. In this study, significantly lower arylsulfatase activity was observed in the S20 treatment compared to the S0. Reduced levels of arylsulfatase activities have been reported to be linked to higher sulfate and phosphate levels (Fitzgerald and Strickland, 1987; Tabatabai and Bremner, 1970a). However, the higher sulfatase activity was not enough to supply the plant roots with amounts of S that were at levels similar to the S10 and S20 fertilizer. Arylsulfatase activity has been reported to decrease with soil profile depth, which is attributed to a decrease in soil organic matter content (Tabatabai and Bremner, 1970b). Indeed, arylsulfatase activity in this study was significantly higher in the top 0-20 cm of the columns than in the bottom half (20-40 cm) of the column (Table 2).

A significant reduction in AM fungal *colonization* of ryegrass roots in the S10 and S20 treatments was observed in this study. While there are several studies reporting on the effect of P fertilizer on AM fungi colonization of plant roots (Baum and Makeschin, 2000; Ikoyi et al., 2018; Nayyar et al., 2008), this is the first report on the effect of sulfate fertilizer application on AM fungal colonization. The reduction in the AM colonization with increasing

S fertilizer application might be due to the high concentrations of sulfate in soil solution, hence, no need for the plant to invest heavily in mycorrhizal symbioses. Mycorrhizal-plant association have been reported to enhance plant S uptake. For instance, the presence of AM fungi enhanced S uptake in maize, clover (Gray and Gerdemann, 1973), tomato (Cavagnaro et al., 2006) and carrot (Allen and Shachar-Hill, 2009). This enhanced S uptake has been attributed to specific plant sulfate transporters that are expressed in arbusculated cells which become up-regulated in response to AM fungal symbiosis especially under conditions of S limitation (Giovannetti et al., 2014). Another reported mechanism is the ability of AM fungi to recruit a diverse hypospheric bacterial community involved in the mineralization of organic S forms (Gahan and Schmalenberger, 2015; Johansson et al., 2004). The transfer of S from AM fungi to plants has been demonstrated previously (Sieh et al., 2013). The putative beneficial effect of the mycorrhization on the plant S supply in this study may have alleviated some of the S deficiencies in the S0 and S5 treatment. Without AM fungal colonization, the plants of S0/S5 would be most likely further reduced in growth.

Similar to the AM fungal response, the abundance of bacterial-feeding nematodes was significantly reduced in the S10 and S20 treatments. It has been reported previously that soil nematodes are sensitive to salt toxicity and negative effects of high phosphate application on soil nematodes (attributed to salt toxicity) has been previously reported (Ikoyi et al., 2018; Zhao et al., 2014). It is very likely that the application of sulfate resulted in a similar response from the bacterial-feeding nematodes. Particularly, the application of 20 kg/ha sulfate resulted in soil sulfate concentrations above 60 mg/L for up to seven weeks after application. Soil nematodes (particularly bacterial- and fungal-feeding nematodes) play significant roles in biological cycling of nutrients due to their feeding activities. For instance, increased nitrate and P availability have been linked to nematode grazing on rhizosphere bacteria (Gebremikael et al., 2016; Irshad et al., 2011). The reduction in abundance of bacterial-

feeding nematodes in the S10 and S20 treatments most likely limited their contribution to S mineralization in these columns. Similarly to the mycorrhization effect discussed above, bacterial feeding nematodes may have partially alleviated the lack of S availabilities in the S0/S5 columns. Since the ^{34}S application occurred two weeks after the main fertilizer event, reductions in grazing nematodes at S10 and S20 treatments may have contributed to a reduction in S mobilization from the soil biomass (largely as ^{32}S). Consequently, ^{34}S concentrations in the soil solution in the S10 and S20 columns might have been higher than in the S5 treatment due to reduced nematode activity.

Sequences of the bacterial 16S rRNA and the corresponding fingerprints (DGGE) showed a clear separation between the S0 and the S20 treatment. While in-depth OTU analysis showed that the relative abundances of the bacterial phyla was not affected by the sulfate fertilizer application rates, certain genera were however, more abundant in the S20 treatment than in the S0 treatment. Particularly, *Bacillus*, *Bradyrhizobium*, *Mycobacterium*, *Candidatus_Xiphinematobacter*, *Roseiflexus*, *Streptomyces*, *Sporosarcina*, *Rhizobium*, *Defluviicoccus*, *Nakamurella*, *Microvirga*, *Nitrosospira* and *Microlunatus* were significantly more abundant in the S20 treatment than in the S0. Most of these genera are regarded as plant growth promoters playing vital roles in N and P cycling (Wang et al., 2017). *Bacillus* and *Bradyrhizobium* have been reported to increase plant uptake of N, P and S (Adesemoye et al., 2008; Fox et al., 2014; Mander et al., 2012; Rojas et al., 2001; Yolcu et al., 2011). *Microlunatus* has been reported to accumulate polyphosphate thereby playing vital roles in P cycling (Kawakoshi et al., 2012). *Rhizobium* and *Microvirga* have been reported to fix N (Ardley et al., 2012), *Sporosarcina* is involved in cycling of urea (Gruninger and Goldman, 1988) and *Nitrosospira* is recognized as nitrite-oxidizing bacteria (Hayatsu et al., 2008; Longa et al., 2017). It is likely that the higher abundance of bacterial genera involved in N

cycling in the S20 treatment contributed to the enhanced N use efficiency and hence the higher N uptake in the S20 treatment.

The abundance of several bacterial genera previously identified to be involved in the desulfurization of organo-S forms in the soil (Fox et al., 2014; Schmalenberger et al., 2008; Schmalenberger et al., 2009; Schmalenberger and Kertesz, 2007) were not affected by sulfate fertilizer application rates. These included the genera *Variovorax*, *Polaromonas*, *Burkholderia*, *Hydrogenophaga*, *Cupriavidus*, *Acidovorax*, *Rhodococcus*, *Stenotrophomonas* and *Williamsia.* The only exception was *Pseudomonas,* which was significantly higher in the S0 than in the S20 treatment (Figure 4b). However, in addition to *Pseudomonas*, other genera such as *Sphingomonas*, *Gemmatimonas*, *Lysobacter*, *Phenylobacterium*, *Arenimonas* and *Rhizomicrobium* were significantly more abundant in the S0 than in the S20 treatment. Apart from *Pseudomonas putida* S-313 which is documented to promote plant growth through S mobilization (Kertesz and Mirleau, 2004), whether these other genera have any role to play in soil sulfur cycling is not yet known.

The copies of *Variovorax*, *Burkholderia* and *Polaromonas asfA* genes obtained via qPCR were statistically similar in all treatments. This is in contrast to a previous study (Schmalenberger et al., 2010) where repeated application of sulfate fertilizer to *Agrostis stolonifera* was reported to affect the desulfonating bacterial community leading to the dominance of *Polaromonas* in the sulfate limited-treatment. Of the genus specific abundance of *asfA*, copies of *Burkholderia asfA* gene were the most abundant in all treatments while *Polaromonas asfA* gene copies were the least abundant in all treatments. These results confirm that under this experimental condition, sulfonate cycling in the rhizosphere by *Burkholderia* may be more prominent than *Variovorax* and *Polaromonas*. In addition, the *ssuD* and *atsA* gene diversity was not affected by the one-time application of the sulfate fertilizer. Unlike the changes observed for the AM fungi and the nematodes, the changes

observed in the bacterial community structures may not have had a negative impact on soil S cycling and plant uptake. The putative resilience of the S cycling bacteria in the present study seems to support this conclusion for short-term fertilizer events.

The present study analysed the effects of different levels of sulfate fertilization on various soil biota, plant growth and the sulfate levels in soil solution. While these findings are limited to a single soil type and were obtained under controlled conditions of soil columns, it allows for the development of a conceptual model (Figure 5) as well as to propose some basic mechanisms. Our results demonstrate that under the presented conditions, a one-time sulfate application is beneficial to increase plant shoot biomass. This is as a result of the enhanced uptake of essential nutrients including S (Figure 5). In contrast to our predictions, no reduction of plant S uptake was evident when sulfate concentration in soil solution remained high. Although abundance of AM fungi and bacterial-feeding nematodes and arylsulfatase activity was affected negatively by the S fertilization event (Figure 5), their putative beneficial effects in the control (S0) were not able to fully substitute S-fertilization at 10-20 kg S ha^{-1}. This is in contrast to a previous study on phosphorus, where the phosphate application effect on the soil biota cancelled out the P fertilizer input to the plant (Ikoyi et al., 2018). Unlike the phosphate fertilizer that was undetectable in soil solution within days after application, sulfate remained present in soil solution at elevated levels and plant roots appeared to have greatly benefited from the sustained uptake of sulfate. While the present study found evidence of changes in the fungal and bacterial community structures (Figure 5), a shift in the organo-S mobilizing microbial community was not detected. This was evident in the lack of change in the diversity or abundance of *ssuD, atsA* and *asfA* as well as the similar abundance of cultivable bacteria utilizing aromatic sulfonates as sole source of S. It is very likely that most organo-S mobilizing bacteria play multiple roles in soil and rhizosphere. Therefore, a one-time application of sulfate as applied in the present study may constitute as a

too small disadvantage for these microbes. We theorise that at least in the short-term, organo-S mobilizing microbes were not negatively affected unlike the nematodes and AM fungi. This may be fundamentally different under long-term scenarios as indicated previously in the world's oldest ongoing field trial experiment in Rothamsted, UK (Schmalenberger et al., 2008).

Currently a paucity of studies investigated the effect of sulfate fertilization on soil microbiota, nematodes and plant growth. Future studies need to widen the scope of the presented one in order to identify common principles of S cycling in different soil types and agricultural systems. An enhanced contribution of soil biota towards plant nutrient supply (including S) may allow the reduction of fertilizer input in agriculture and therefore contribute to a more sustainable form of agriculture. This could be achieved by replacing sulfate S in fertilizers with forms of S that have fewer negative impacts on soil biota and can be made plant available.

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Fig. 1.

Mean sulfate concentrations in soil solution of the top 20 cm of the column. S added as 0 - 20 kg ha^{-1} (red squares = S0, light blue diamonds = S5, green circles = S10 and blue triangles = S20), error bars = standard error of means, n = 6.

Fig. 2.

CCA biplots of the DGGE profiles showing the effects of S treatments on a) 16S rRNA gene b) Fungal ITS and c) AM fungal 18S rRNA with bacterial-feeding nematodes abundance (BF), Arylsulfatase activity (SULFD20), total nematode (totnema), fungal-feeding nematodes abundance (FF), total grass shoot dry weight (Shoot DM), grass S and N contents, root biomass (Root BM) and root mycorrhizal arbuscular and hyphal colonization rates (AC, HC) defined as environmental factors. Red squares = S0, light blue diamonds = S5, green circles = S10 and blue triangles = S20 (n=6; S added as 0 - 20 kg ha-1=S0-20). Arrows for each variable tested denote significant correlation ($p \leq 0.05$, permutation test) of environmental factors on shift of the bacterial, fungal and AM fungal community structures upon sulfate fertilization.

Fig. 3.

Principal components analysis (PCA) of the bacterial community sequences based on 16S rRNA genes. PCA was calculated using the genera OTUs (n=6; S added as 0 - 20 kg ha-1=S0-20; red squares = S0, light blue diamonds = S5, green circles = S10 and blue triangles = S20).

Fig. 4.

a) Cluster heatmap of families based on the 16S rRNA gene sequence analyses; and b) t test bar plots of differences in relative abundances of genera (S0 = light orange, S20 = dark blue). Asterisks (*) indicate significant differences in relative genera abundance between S0 and S20 (n=6; S added as 0 - 20 kg ha-1=S0-20).

Fig. 5.

Conceptual model of sulfur (S) cycling in grasslands showing the effects of sulfate fertilizer application on the soil biota, plant host and environment. Green arrows (+) indicate beneficial effects, while red arrows (-) indicate negative effects.

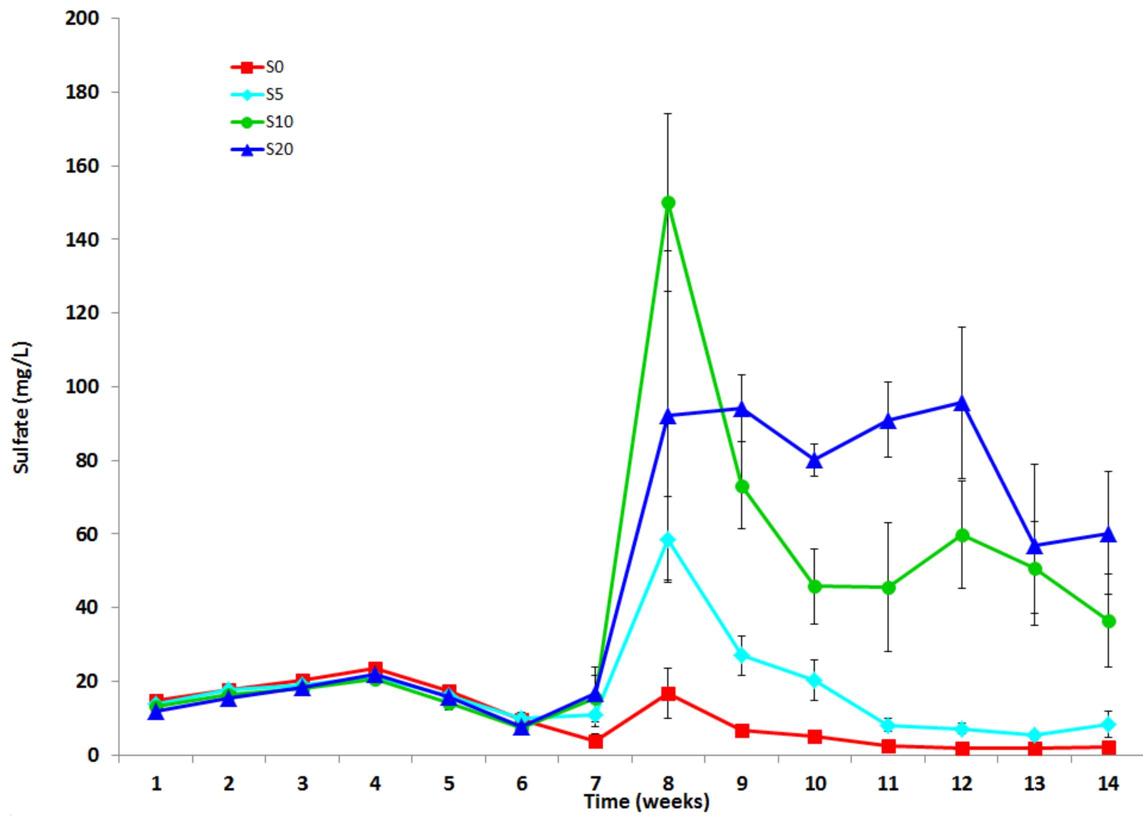

Fig. 1.

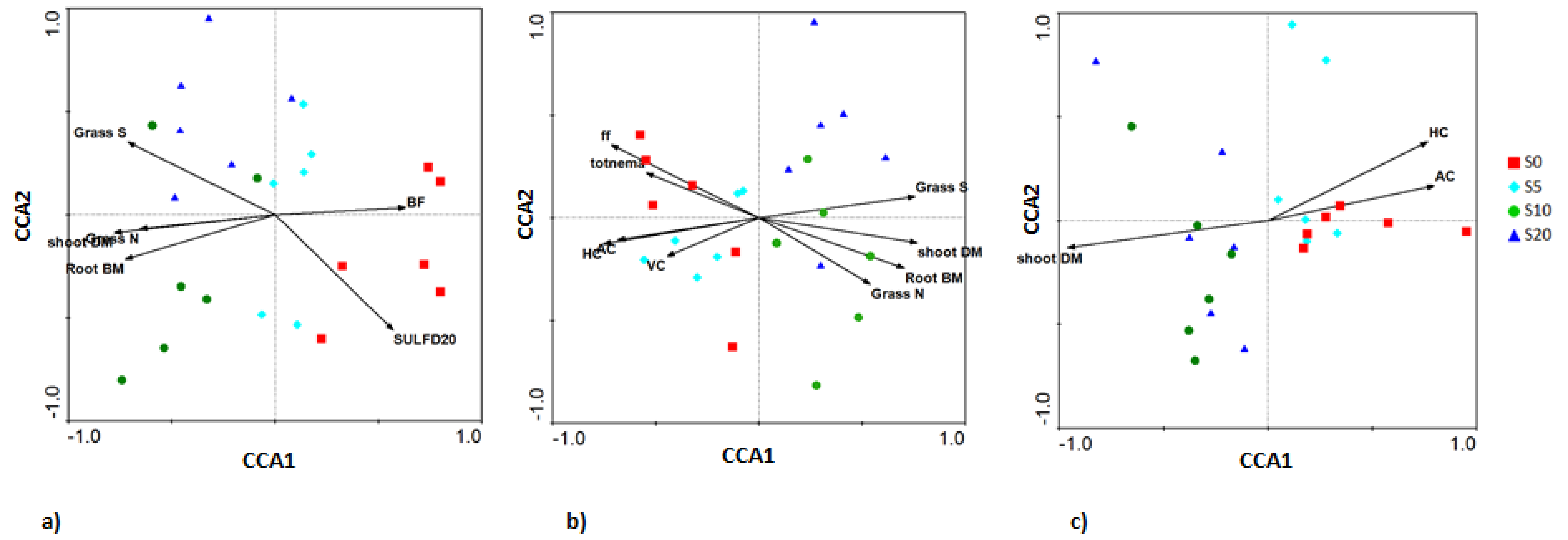

Fig. 2.

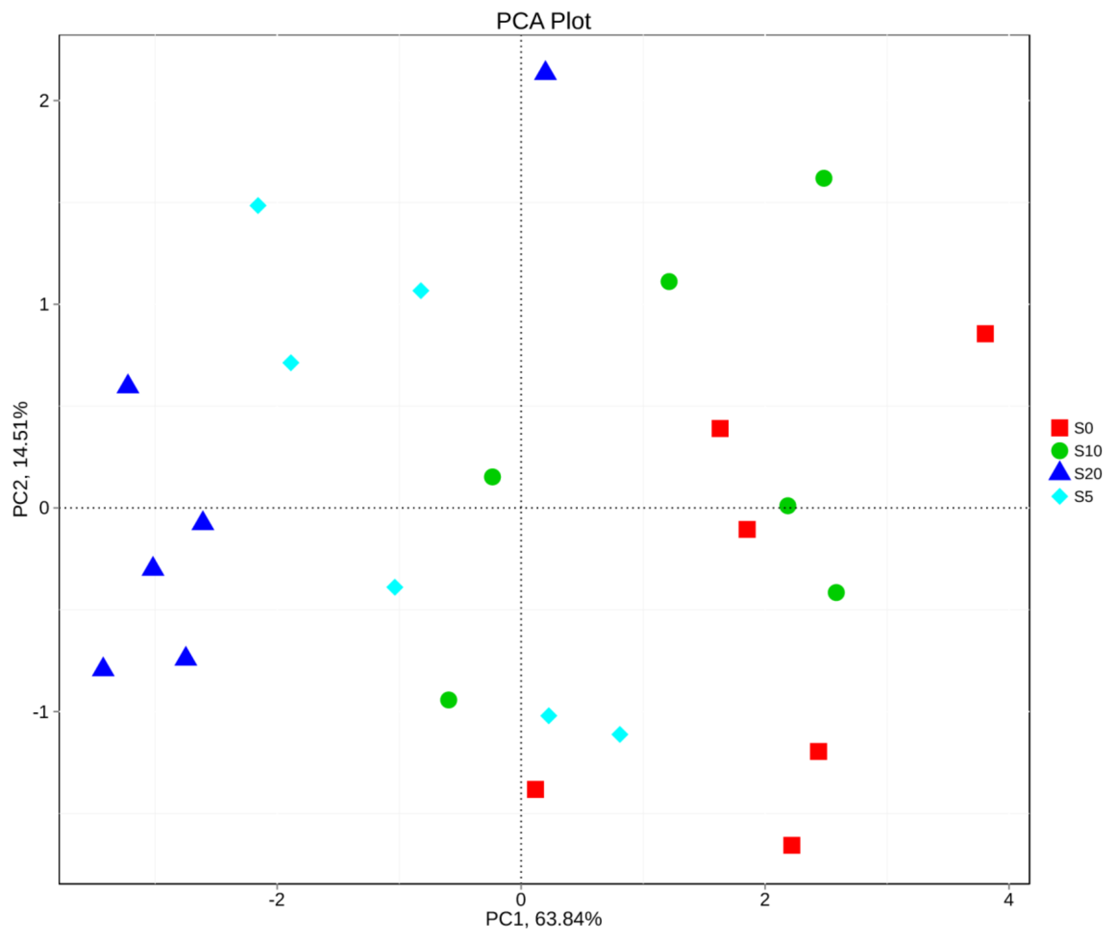

Fig. 3.

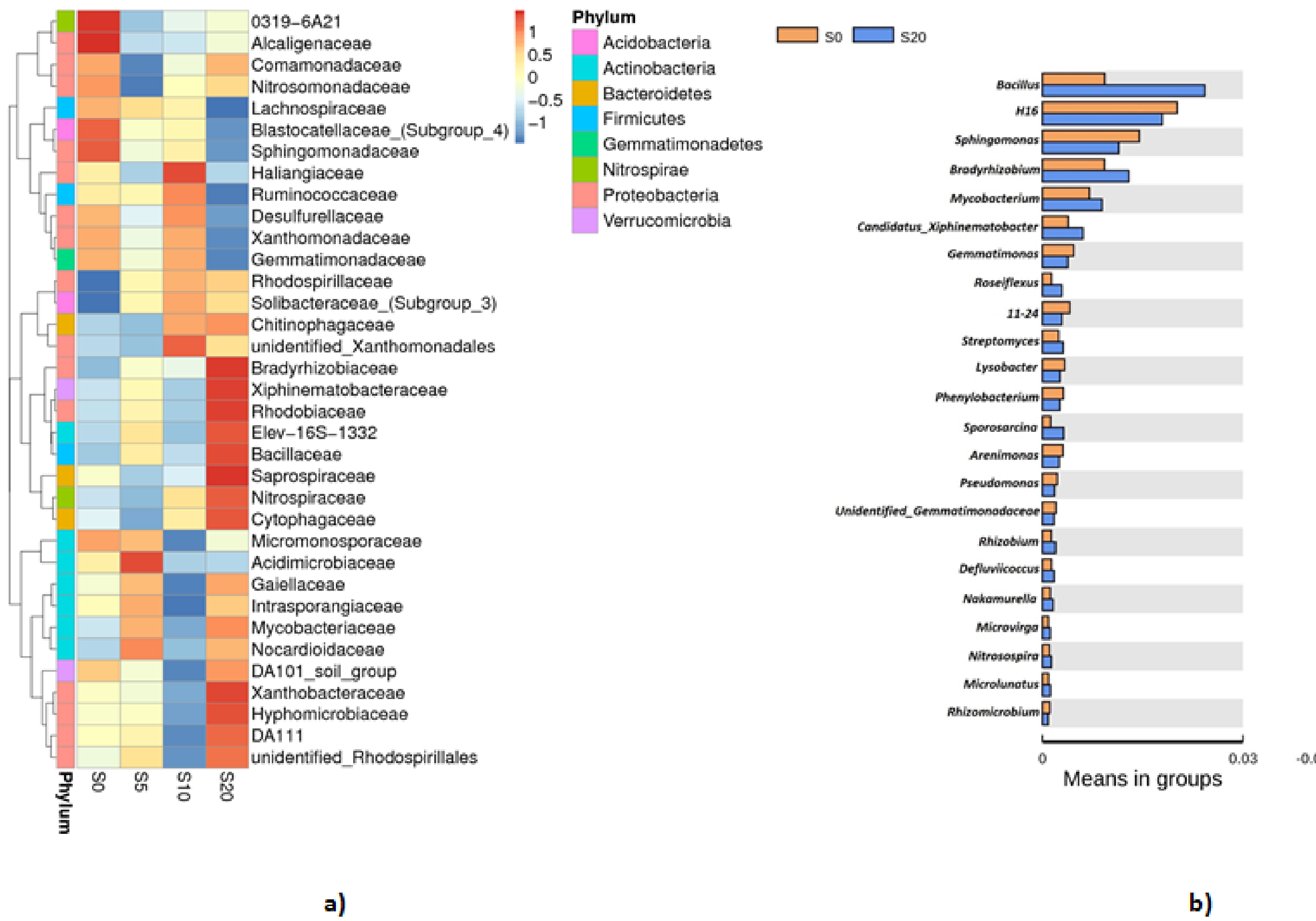

Fig. 4.

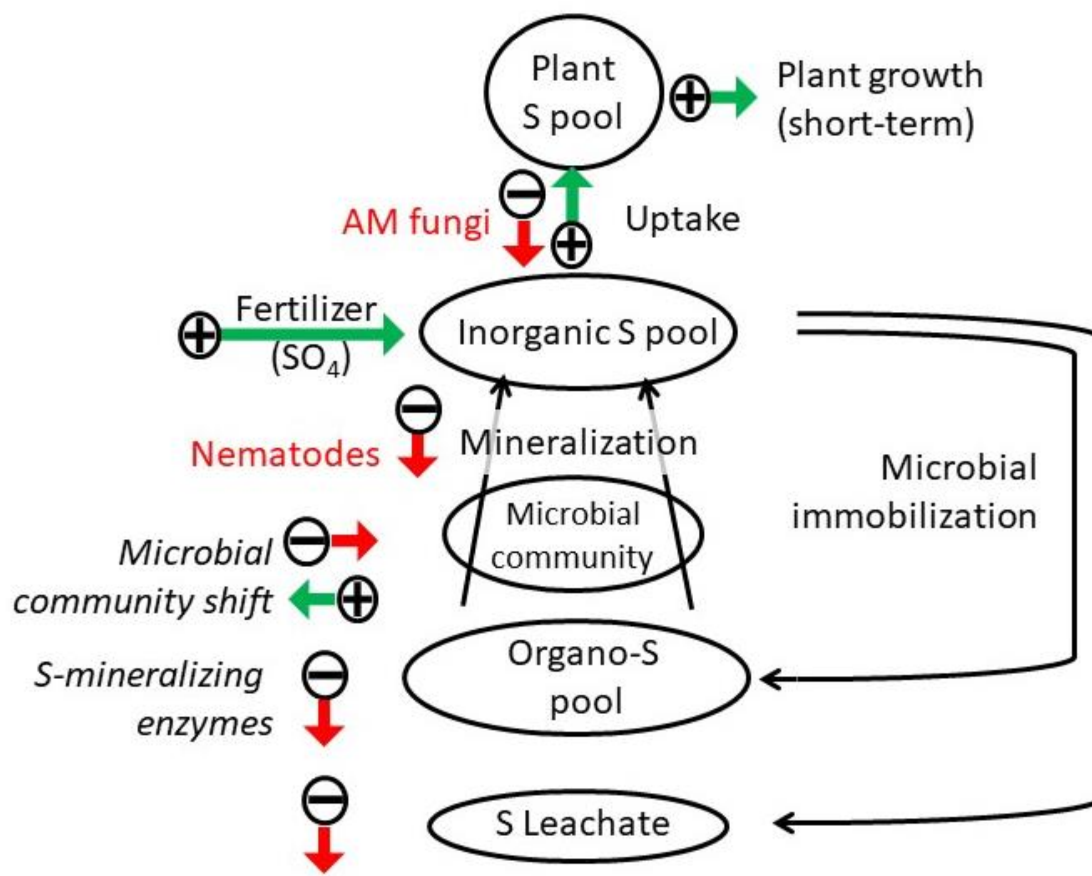

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Fig. 5.

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Table 1

Mass balance: mean grass shoot dry weights (g/column; n=6) ± standard error and mean grass nutrient content (mg/column, n=6), $\delta^{34}S$ (‰, n=3). Different letters within the same row represent significantly different means ($P \leq 0.05$). S added as 0 - 20 kg ha^{-1} (S0-20).
15 NA= not applied. * First harvest carried out directly before fertilization event. All nutrient concentrations relate to second harvest shoot samples.

	S0	S5	S10	S20
Shoot dry weight (first harvest*)	6.4[a] ± 0.17	6.5[a] ± 0.17	7.3[a] ± 0.26	6.8[a] ± 0.36
Shoot dry weight (second harvest)	17.2[b] ± 0.74	18.5[b] ± 0.70	20.9[a] ± 0.95	20.0[a] ± 0.38
Total shoot dry weight	23.3[b] ± 0.70	24.2[b] ± 0.50	27.6[a] ± 0.46	26.9[a] ± 0.50
Calcium	103.16[b]	109.98[ab]	129.64[a]	117.48[ab]
Magnesium	41.97[b]	44.13[b]	51.96[a]	47.99[ab]
Sulphur	62.80[b]	73.58[b]	90.00[a]	96.31[a]
Nitrogen	125.75[b]	137.24[b]	165.56[a]	153.75[a]
Phosphorus	31.04[a]	31.68[a]	35.23[a]	36.55[a]
Potassium	420.47[b]	470.31[ab]	565.05[a]	526.74[ab]
Boron	0.17[a]	0.16[a]	0.17[a]	0.17[a]
Copper	0.09[b]	0.09[b]	0.11[a]	0.10[ab]
Molybdenum	0.10[a]	0.08[b]	0.08[b]	0.07[b]
Iron	1.38[a]	1.79[a]	3.36[a]	1.22[a]
Zinc	0.23[ab]	0.21[b]	0.26[a]	0.24[ab]
Manganese	0.21[a]	0.22[a]	0.26[a]	0.23[a]
$\delta^{34}S$ (non-spiked)	NA	5.94[a]	6.06[a]	6.32[a]
$\delta^{34}S$ (spiked)	NA	44.47[b]	90.67[a]	81.33[a]

Table 2

Mean MPN values of sulfonate mobilizing (MM2TS) bacteria, total heterotrophic bacteria (R2A), abundances of bacterial-feeding (BF) and fungal-feeding (FF) nematodes, arbuscular (AC) and hyphal colonization (HC) rates of ryegrass roots, arylsulfatase (ASULF) activity (ug PNP/g soil/hr), bacterial *asfA* gene copies obtained via qPCR (copy number per gram of soil normalized for DNA extraction). Different letters within the same row represent significantly different means ($P \leq 0.05$); $n=6$; ± denotes standard error; S added as 0 - 20 kg ha^{-1} (S0-20).

	S0	S5	S10	S20
MM2TS	$1.9 \times 10^{7a} \pm 9.0 \times 10^{6}$	$1.3 \times 10^{7a} \pm 2.7 \times 10^{6}$	$1.8 \times 10^{7a} \pm 2.5 \times 10^{6}$	$1.7 \times 10^{7a} \pm 6.1 \times 10^{5}$
R2A	$5.3 \times 10^{7a} \pm 1.8 \times 10^{7}$	$3.5 \times 10^{7a} \pm 6.9 \times 10^{6}$	$4.5 \times 10^{7a} \pm 6.6 \times 10^{6}$	$5.1 \times 10^{7a} \pm 1.2 \times 10^{7}$
BF (No./100g soil)	$185^{a} \pm 20$	$119^{ab} \pm 15$	$107^{b} \pm 19$	$102^{b} \pm 23$
FF (No./100g soil)	$25^{a} \pm 10$	$20^{a} \pm 10$	$12^{a} \pm 8$	$13^{a} \pm 9$
AC (%)	$75.6^{a} \pm 3.4$	$76.4^{a} \pm 8.5$	$48.6^{b} \pm 7.6$	$45.6^{b} \pm 3.2$
VC (%)	$29.4^{a} \pm 5.5$	$20.8^{ab} \pm 7.7$	$13.9^{bc} \pm 6.4$	$9.7^{bc} \pm 2.9$
HC (%)	$85.0^{a} \pm 5.5$	$83.1^{a} \pm 7.7$	$53.3^{b} \pm 6.4$	$51.7^{b} \pm 2.9$
Soil pH	$7.90^{ab} \pm 0.03$	$8.10^{a} \pm 0.08$	$7.78^{ab} \pm 0.11$	$7.77^{b} \pm 0.10$
$ASULF_{0-20cm}$	$286^{a} \pm 11$	$251^{ab} \pm 10$	$243^{ab} \pm 15$	$231^{b} \pm 5$
$ASULF_{21-40cm}$	$132^{a} \pm 12$	$109^{a} \pm 16$	$141^{a} \pm 12$	$142^{a} \pm 16$
asfA_Variovorax	$4.2 \times 10^{4a} \pm 2.1 \times 10^{4}$	$4.8 \times 10^{4a} \pm 1.8 \times 10^{4}$	$3.8 \times 10^{4a} \pm 7.7 \times 10^{3}$	$4.9 \times 10^{4a} \pm 1.5 \times 10^{4}$
asfA_Burkholderia	$8.3 \times 10^{4a} \pm 3.3 \times 10^{4}$	$1.1 \times 10^{5a} \pm 3.8 \times 10^{4}$	$7.7 \times 10^{4a} \pm 1.6 \times 10^{4}$	$8.2 \times 10^{4a} \pm 1.6 \times 10^{4}$
asfA_Polaromonas	$1.2 \times 10^{4a} \pm 3.0 \times 10^{3}$	$7.8 \times 10^{3a} \pm 1.9 \times 10^{3}$	$1.1 \times 10^{4a} \pm 1.1 \times 10^{3}$	$2.0 \times 10^{4a} \pm 5.4 \times 10^{3}$