Miscanthus biochar promotes growth of spring barley and shifts bacterial community structures including phosphorus and sulfur mobilizing bacteria

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Highlights:

- Biochar soil amendment significantly promoted growth of spring barley
- Biochar soil amendment significantly altered bacterial community structures
- S and P mobilizing bacterial abundances significantly increased with biochar addition
- Stepwise regression attributed 74 % of plant growth to P and S mobilizing bacteria
- Pyrolysis of *Miscanthus* grass produced biochar with sulfate ester as main S source

Keywords:

Hordeum vulgare / DGGE / quantitative PCR / S K-edge XANES / next generation sequencing

Abstract

Biochar has shown great potentials in plant growth promotion but its effect on soil bacteria that potentially support plant growth is less well understood. In this study, the effect of biochar soil amendment was investigated on the growth of spring barley, its rhizobacteria diversity and the abundance of S and P mobilizing bacteria. Furthermore, S species compositions were identified in soil, feedstock and biochar. Soil amendment with biochar made from *Miscanthus x giganteus* resulted in significantly increased growth of barley and grain formation four to seven fold. Further significant increases were found for the soil pH and abundances of rhizosphere soil bacteria capable of growing with tri-calcium phosphate, phosphate-esters, phosphonates or aromatic sulfonates as sole source of S or P. A stepwise regression model explained 74 % of the plant growth with the abundances of P and S mobilizing bacteria. 16S rRNA gene based fingerprint analysis revealed a significant shift in the bacterial community structure upon biochar amendment that correlated significantly with the above mentioned changes (pH, plant growth, bacterial abundances). Under biochar amendment, up to 100 times increases in genera *Brevundimonas* known for P cycling and *Arthrobacter* and *Cupriavidus* previously involved in sulfonate desulfurization were identified via quantitative PCR. S k-edge XANES confirmed that the key S source sulfonate that was largely absent in the applied biochar, thus bacterial mobilization of sulfonate-S may have originated from the soil.

1. Introduction

Amendment of soils with biochar is often practised to sustainably improve soil fertility or to increase soil carbon stocks (Atkinson et al., 2010; Lehmann et al., 2006). Physical and chemical attributes of buried biochars are principally thought to be responsible for potential beneficial effects to soils (Jeffery et al., 2011), including greater surface area, porosity, higher water retention and lower bulk density (Downie et al., 2009; Sohi et al., 2010). Pyrolysis increases pore spaces in the parent material by over a thousand fold and these pores provide a habitat for microorganisms as well as offering protection from grazing predators (Thies et al., 2015; Warnock et al., 2007b). Changes in physicochemical soil properties upon biochar amendment are very likely to shift microbial community structures (Anderson et al., 2011) and these changes were observed in the rhizosphere of ryegrass (Fox et al., 2014).

Despite the recalcitrant nature of many biochars (Lehmann et al., 2006), they may serve as a source of nutrients such as phosphorus (P) or act as a soil conditioner, improving soil nutrient access (Amonette and Joseph, 2009). Biochar P content may vary substantially depending on feedstock and pyrolysis ranging from 2.7 to 480 g kg^{-1} (Yin Chan and Xu, 2009). While very little to no P will be lost to the gas phase during pyrolysis, parts of the sulfur (S) in the feedstock may be lost to the atmosphere, depending on the pyrolysis conditions and feedstock type chosen (Di Blasi et al., 1999; Lang et al., 2005). Unlike nitrogen (N), macro-nutrients P and S are largely absent in the atmosphere and only found in smallest amounts in atmospheric depositions. Plants largely access P and S in the form of orthophosphate and sulfate from soil solution even though approximately 99 and 95 % of P and S in soils are organically or inorganically bound (Autry and Fitzgerald, 1990; Randriamanantsoa et al., 2013). Thus, the role of soil microbes mobilizing P and S is of utmost importance in the adequate supply of these nutrients to support plant growth.

While some advancements have been made in biochar research when it comes to plant N supply and microbial cycling of N (Thies et al., 2015), advances in P and S cycling research in biochar amended soils are scarce. Soil P pools are dominated by monophosphate esters and inorganically bound P such as calcium phosphates (Shen et al., 2011; Turner et al., 2005). P availability in biochar amended soils can increase with the alterations in soil pH, enzyme efficiencies, organo-mineral complex formation and changes in the soil microbial community (DeLuca et al., 2015). Microbial mobilization of these bound forms is largely achieved through ester hydrolysis by a variety of esterase enzymes or through exudation of acids to solubilize inorganically bound P (Alikhani et al., 2006; Lim et al., 2007). Abundances of phosphate solubilizing, phytate and phosphonate utilizing bacteria may be increased (Anderson et al., 2011; Fox et al., 2014) and phosphatase enzyme activity may be enhanced under biochar amendment (Du et al., 2014; Ventura et al., 2014). Soil S pools are dominated by sulfonates while sulfate-esters are commonly the second most abundant form of S (Autry and Fitzgerald, 1990; Kertesz and Mirleau, 2004). Aromatic sulfonates appear to be particularly important and are mobilized by a functional bacterial guild (Schmalenberger et al., 2008). Recent biochar soil amendments identified higher abundances of sulfonate utilizing bacteria alongside ryegrass growth promotion in pots (Fox et al., 2014) and increased sulfatase activities in the field (Ventura et al., 2014).

This study aimed to identify correlations between biochar based growth promotion of barley, bacterial community structures, bacterial utilization of calciumphosphate, phytate, phosphonate and sulfonate and abundances of selected bacterial genera. The hypothesis was that in a biochar based barley growth promotion scenario, bacterial community structures will be shifted and that these changes in the bacterial community includes in particular P and S mobilizing bacteria.

2. Materials and methods:

2.1 Pot experiment:

Biochar from *Miscanthus x giganteus* biomass was prepared as described previously and contained 2.44 mg P g^{-1} DW and 0.96 mg S g^{-1} DW (Fox et al., 2014). For the pot trial, soil was taken from the long-term cowlands experiment conducted at Johnstown Castle research centre (Teagasc) in the south east of Ireland (52° 16'N,06° 30'W), free of P fertiliser since 1968 (Tunney et al., 2010). The soil type is a poorly drained gley soil, with a loamy topsoil (18 % clay) with a pH of 6 (Mollic Histic Stagnosol; WRB, 2006), 46.1 g kg^{-1} carbon (Griffiths et al. 2012), 2.8 g kg^{-1} nitrogen, 0.34 g kg^{-1} S and 0.32 g kg^{-1} P (FW; Lancrop Laboratories Ltd., York, UK). Total nutrient content water extractable phosphate and sulfate (biochar, soil) was tested via ion chromatography in a Dionex ICS1100 with an AS23 column and a carbonate mobile phase as recommended by the manufacturer (Sunnyvale, CA).

Pots were established with the soil mixed 1:1 with distilled H_2O washed sand (900 g per pot, replicates of eight). A top layer (300 g) was kept biochar-free to exclude an effect of the biochar on seed germination. The middle layer (300 g, M layer) was amended with 3 or 6 % biochar (1 % and 2 % per pot; biochar particles < 1 cm in diameter; referred to as 1 and 2 % amendment from hereon), while the bottom layer was again biochar free (300 g) as published recently (Fox et al., 2014). Each pot was planted with four seedlings of spring barley (*Hordeum vulgare* var. SY Taberna) and thinned to two plant shoots after a period of two weeks. After a growth period of 80 days in a greenhouse at ambient temperatures (Irish summer) and natural lighting, plants had fully developed heads that lost their green colour. Pots were harvested and plant shoot heights, weights and soil pH were recorded as described previously (Fox et al., 2014). Barley shoots were analysed for elemental composition at Lancrop Laboratories Ltd. (employing atomic absorption spectroscopy, inductively coupled plasma spectrometry, titrations, and spectrophotometry). Seed numbers and weights (DW)

were recorded. Total seed N was quantified in a Vario EL Cube elemental analyser (Elementar, Goettingen, Germany) and crude protein subsequently calculated. Starch content was measured in the seeds using a Megazyme (Bray, Ireland) total starch assay kit as recommended by the manufacturer. Where replicate measures were taken, results were subjected to univariate analysis (Tukey HSD in SPSS 20, IBM, Armonk, NY).

2.2 Quantification of P and S mobilizing bacteria:

Bacteria were extracted from rhizosphere associated soil from the M layer of each pot (replicates of eight). After destructive disassembly of the plant pots, roots of 3 g with loosely attached soil (and biochar therein for the 1 and 2 % treatment) were added to 50 ml conical tubes with 20 ml of sterile saline (NaCl 0.85 % [wt vol^{-1}]) solution and rotated at 75 rpm (RM-2 mixer) for 30 min at 4°C. 0.1 ml of the obtained suspension was used for serial dilution and subsequent cultivation. The remainder without plant roots was centrifuged at 4500 rpm for 15 min at 8 °C and the pellet was stored at -20 °C. Cultivable bacteria capable of mobilizing P from phytate (phosphate-esters, MM2Phy) and phosphonoacetic acid (MM2PAA) and S from toulenesulfonate (MM2TS) as sole source of P or S respectively, were quantified through a most probable number (MPN) approach in microtiter plates (Fox et al., 2014). Colony forming units (CFU) were established to determine the cultivable bacteria solubilizing P from tri-calcium phosphate agar plates as indicated by a zone of clearance around the colonies (Rondon et al., 2007). Both CFU and MPN data were normalized (log_{10}) for univariate analysis (Tukey HSD in SPSS 20).

2.3 Soil DNA extraction, amplification and analysis

DNA was extracted using the ULTRA CLEAN™ soil DNA kit (MO BIO Laboratories, Cupertino, CA) and subjected to amplification of 16S rRNA gene fragments (replicates of

eight). Amplicons were used individually for denaturing gradient gel electrophoresis (DGGE). PCR was performed with primers GC-341F and 518R (Muyzer et al., 1993) and a touch-down protocol as described in the supplementary materials (SM1). Gel electrophoresis was performed in a TV-400 DGGE system (Scie-Plas, Cambridge, UK) with 200 x 200 x 1 mm gels, with a polyacrylamide gel strength of 10 % (vol vol^{-1}) in 1x TAE and a denaturing gradient of 30-70 % made from urea and formamide at 63 V for 16 h at 60 °C. Gels were stained with SYBR Gold as recommended by the manufacturer (Invitrogen, Carlsbad, CA). DGGE profiles were translated into a binary gel image matrix (Phoretix advanced 1D; Nonlinear Dynamics, Newcastle, UK) for canonical correspondence analysis (CCA) biplots and permutation tests (Monte-Carlo) using forward selection and 9,999 replicates (CANOCO 4.5; Microcomputer Power Inc., Ithaca, NY).

PCR reactions for next generation sequencing (NGS) were conducted with the universal primer pair 16SF and 16SR to target the V3 and V4 region of the 16S rRNA gene (Klindworth et al., 2013) as described in the supplementary materials (SM2). The PCR products were purified using the GeneElute PCR purification kit (Sigma-Aldrich, St. Louis, MO) and pooled according to treatments (biochar 1 %, biochar 2 %, control). An indexing PCR was then undertaken to attach the dual indices and Illumina sequencing adaptors using the Nextera XT index kit (Illumina, San Diego, CA) as per the manufacturer's instructions, purified and quantified using a Qubit dsDNA HS Assay kit (Life Technologies, Carlsbad, CA) and a Qubit Fluorometer (Life Technologies). DNA concentrations for each sample was adjusted to 4 nM in 10 mM tris (pH 8.5) and sequenced on an Illumina MiSeq NGS platform as per manufacturer's instructions (Illumina) at the Teagasc Next Gen Sequencing Centre, Moorepark (Ireland). Illumina pair-end sequence read (2 X 250) analysis was conducted as described in the supplementary materials (SM3) to obtain OTUs (97 % similarity). Next generation sequences were deposited in the European Nucleotide Archive (ERP014455).

NGS data were used to select targets for quantitative PCR analysis. Genus specific primers were designed using ARB (Ludwig et al., 2004) and the latest 16S rRNA gene database from SILVA (release 123). Rhizosphere DNA was quantified using the dsDNA HS assay kit and a Qubit Fluorometer (Life Technologies) as described above. Soil DNA was diluted to 5 ng μl^{-1} and 1 µl was used in 10 µl qPCR reactions (SybrGreen) in a Roche 480 II cycler (Basel, Switzerland) for the genera *Arthrobacter* (218F:GTG GTT TTG GAT GGA CTC GC; 662R:CAT CAC TCT AGT CTG CCC GT), *Brevundimonas* (728F:GAA GGC GAC ATA CTG GCT CA; 1242R:GGA TTA ACC CTC TGT AGT CG) and *Cupriavidus* (728F:AAG GCA GCC CCC TGG GAC GW; 1261R:GGC AAC CCT CTG TAC GCA CC) as outlined in the supplementary materials (SM4). Where normality (Shapiro-Wilk test) of the data was reached, analysis of variance was conducted (SPSS; Tukey HSD for data with equal variance, Games-Howell for unequal variance). Where normality was not reached, a Kruskal-Wallis test was performed instead.

2.4 S K-edge X-ray Absorption Near Edge Spectroscopy (XANES)

Powders of *Miscanthus* grass, biochar and soil were extracted in 0.01M $CaCl_2$ to remove any absorbed inorganic sulfate. Powdered samples were mounted on a sample holder for the S K-edge for the XANES analysis (microfocus spectroscopy I18 beam line, using an unfocused beam at Diamond Light Source). The monochromator of the beamline was operated in a step by step mode using a Si111 crystal. An ionization chamber was used to measure the primary flux and a four element Si drift detector was used to measure the fluorescence signal in a helium atmosphere. The X-ray energy was calibrated to elemental S at 2472 eV and scans were carried out from 2464 to 2510 eV in steps of 1 eV (2464-2468) and 0.2 eV (2468-2510) (1 s per step for standards and 4 s for samples) in order to identify the different oxidation states of S. XANES spectroscopy resolved the presence of four tested S-oxidation states;

reduced (thiols), intermediate (sulphoxides and sulphonates) and highly oxidised (sulphates / sulphate-esters). Each sample was measured in triplicates. Recorded spectra were normalised and peak fitting using Gaussian curves and arctangent steps were carried out using the Athena software package to estimate the relative abundance of each oxidation state present (Schmalenberger et al., 2011) and white line intensities were corrected (Prietzel et al., 2011).

2.5 Modelling relationship between plant growth and environmental factors

In order to model the relationship between plant growth promotion (shoot dry weight) and the measured environmental variables, a multiple linear regression analysis was conducted in R (R Core Team, 2015). The following predictors were used in the regression analyses: soil pH, calcium phosphate solubilizing bacteria (CFU), phytate utilizing bacteria (MPN), phosphonate utilizing bacteria (MPN), 16S rRNA gene copies of *Bevundimonas* sp. and toluenesulfonate utilizing bacteria (MPN). Prior to the analysis, the bacterial abundance data were log-transformed to ensure normality. All predictors were checked for collinearity based on the variance inflation factor (VIF). The regression was done in a stepwise manner to select the best predictors based on the Akaike Information Criterion (AIC). As a result of collinearity among the predictors, a second regression analyses which excluded the soil pH and 16S rRNA gene copies of *Bevundimonas* sp was carried out. This exclusion was based on the recommendations of Zuur et al. (2010).

3. Results

3.1 Plant growth promotion and elemental analysis

The highest mean plant shoot dry weight at harvest for *Hordeum vulgare* was recorded for the biochar 2 % treatment (3.44 g per pot). Significantly lower values were observed for both the biochar 1 % treatment (2.9 g) and the control treatment (0.71 g) ($P < 0.05$; Table 1). Thus biochar amendment resulted in a four to nearly five times increase in shoot biomass. A highly significant neutralization effect on soil pH was recorded between both biochar treatments and the control (all $P \leq 0.001$; Table 1). Of the major elements, only potassium increased in the shoot biomass upon biochar amendment (Table 2A), other elements stayed largely unchanged in their concentration (magnesium, phosphorus) or were reduced upon biochar addition to soil (calcium, nitrogen, sulfur). However, shoot element balance per pot revealed that the addition of biochar resulted in an increased removal of major elements from the soil biochar matrix into the shoots by two to seven times (Table 2B). Water extractable phosphate in the applied biochar was at 0.1 mg/g P (4 % of the total P), while water extractable sulfate from biochar was detected at 0.04 mg/g S (4 % of the total S). Water extractable phosphate from the soil represented 0.004 mg/g P (1 % of the total P), while water extractable sulfate from soil was measured at 0.001 mg/g S (0.5 % of the total S). Consequently, the soil contained 2 or 4 times more phosphate-P and 1.25 or 2.5 times more sulfate-S than the added biochar (1 and 2 % w/w added), respectively.

Seed numbers per head were significantly higher for the 1 and 2 % biochar treatment over the control (15.25; 15.94 and 3.13, respectively). Likewise, average seed weights were significantly higher for the biochar amendments (51 and 52 mg) than for the control (36 mg). As a consequence, biochar amendment resulted in an up to seven times increased seed yield. Total starch content was highest for a composite seed sample from the 2 % treatment (60.9 %), followed by the 1 % treatment (51.9 %) and the control (38.5 %). In contrast, total N

content in the composite sample was lowest for the 1 and 2 % biochar treatment (1.24 and 1.36 %) and higher in the control seeds (1.83 %). Crude protein content was consequently lowest in the 1 % treatment (7.76 %), followed by the 2 % treatment (8.52 %) and highest in the control (11.46 %).

3.2 Abundance of cultivated rhizosphere bacteria

Both MPN and CFU revealed a highly significant increase in the abundance of sulfonate desulfurizing, tri-calcium phosphate solubilizing, and phytate utilizing bacteria upon both biochar amendments against the control (all $P < 0.001$, Table 3). A significant increase in abundance of phosphonoacetic acid mobilizing bacteria was also observed upon biochar 2 % inclusion over the control ($P = 0.011$). Abundances of phytate and phosphonoacetic acid mobilizing bacteria increased six and five fold upon biochar 1 % amendment, and increased seven and nine fold upon biochar 2 % amendment. Tri-calcium phosphate mobilizing bacteria increased three and 33 fold, while desulfonating bacteria increased four and six fold.

3.3 Bacterial community analysis

CCA of DGGE fingerprints and permutation tests revealed a significant separation between the biochar 2 % treatment against both the biochar 1 % and control treatment (Figure 1). Furthermore, pH, shoot weight, shoot height and the identified quantities of tri-calcium phosphate mobilizing and aromatic sulfonate and phytate mobilizing bacteria were significantly correlated with the observed changes in the bacterial community structure ($P < 0.01$). Next generation sequencing (NGS) generated over 380,000 reads per composite sample (1.3 Mio reads in total). Alpha diversity ranged from 8.3 to 9.0 (Shannon index) across the three amplicon libraries. The phyla Planctomycetes, Proteobacteria, Verrucomicrobia, Acidobacteria and Actinobacteria appeared to dominate the bacterial

communities, contributing to over 80 % of the 16S sequences assigned at the phylum level (Supplementary figure S1). On the genus level, 1058 taxa were assigned in total of which 670 taxa were assigned to uncultured clades. Taxa that could be assigned to a cultivated genus made up about 15 % of all 16S sequences. Genera *Acidothermus, Bacillus, Isosphaera, Planctomyces, Bradyrhizobium, Rhodobium, Pseudolabrys* and *Rhodanobacter* appeared to be most abundant (Supplementary figure S2). NGS of composite samples suggested that several genera increased or decreased in abundance upon biochar amendment.

Increasing abundances upon biochar amendment were confirmed to be significant for the genera *Arthrobacter*, *Brevundimonas* and *Cupriavidus* through qPCR of all replicate samples. The 2 % biochar treatment increased the abundance of these three genera significantly over the control, exceeding a 100 times increase for *Brevundimonas*, a 20 times increase for *Cupriavidus* and a 1.6 times increase for *Arthrobacter* (Table 4). Likewise, significant increases over the control were also reported for *Brevundimonas* and *Cupriavidus* in the 1 % biochar amendment, albeit abundances were significantly lower when compared to the 2 % treatment (four and two times, respectively; Table 4).

3.4 XANES analysis

The analysis of the *Miscanthus* biomass, the biochar and the pot soil identified four major oxidation states, namely thiols (2472 eV), sulfoxides (2476 eV), sulfonates (2781 eV) and sulfate-esters (2482 eV) (Supplementary figure S3). While the *Miscanthus* biomass and the tested soil contained significant amounts of all these four S oxidation stages, the pyrolysed biomass (biochar) contained mostly sulphate-esters, very little thiols and no detectable sulfonates. Subsequent Gaussian peak fitting was conducted to estimate the respective peak areas. Two arctangents were introduced to compensate for the background at the positions 2472.6 and 2479.8 eV. Resulting estimates for peak areas after white lines correction showed

that in the *Miscanthus* biomass, thiols were most abundant, closely followed by the sulfonates and sulfate-esters (Table 5). For soil, sulfonates were most abundant, followed by thiols and sulfate esters. In contrast, pyrolysis of *Miscanthus* biomass resulted in the oxidation or loss of most reduced and intermediate S species resulting in the accumulation of S in the form of sulfate-esters. Incubation of the biochar in soil for over 80 days resulted in only minor changes in the present S species (data not shown).

3.5 Modelling relationship between plant growth and environmental factors

The stepwise multiple linear regression analysis in R with all predictors regardless of VIF resulted in a final model which identified the soil pH as the only significant predictor of the observed plant growth promotion effect ($R^2 = 0.784$), thus was able to explain about 78% of the variation observed in the shoot dry weight gain. However, in the initial model that was used for the multiple linear regression, both the soil pH and 16S rRNA gene copies of *Bevundimonas* sp. had a VIF of above five, indicating their collinearity with other predictors. When the stepwise regression analysis was repeated excluding these two predictors, a significant model ($p < 0.001$) was obtained which included phytate utilizing bacteria, calcium phosphate solubilizing bacteria and toluenesulfonate utilizing bacteria as the best predictors. This model explained over 74 % of the variation in shoot dry weight gain. The coefficients of the phytate utilizing bacteria (1.45) and calcium phosphate solubilizing bacteria (0.57) were significant ($P \leq 0.01$ and $P \leq 0.05$ respectively). Thus, abundances of these P mobilizing bacteria could be used to predict promotion of plant growth.

4. Discussion

Biochar soil amendment to promote plant growth has received increasing attention in recent years (Jeffery et al., 2011). Furthermore, recent findings indicate a beneficial impact of many biochars on the soil N cycle and the facilitating soil microbes (Thies et al., 2015). However, much less information is available on the cycling of the remaining macro-nutrients P and S under biochar soil amendment, where the limited amount of information is largely based on soil enzymatic assays of sulfatase and phosphatase (Schmalenberger and Fox, 2016). This study revealed an increase in bacterial abundance of P and S mobilizing bacteria alongside a shift in bacterial community structure and enhanced plant growth upon biochar amendment.

Neutralization of soil pH as observed in this study was reported on numerous occasions (Atkinson et al., 2010) and often attributed to the surface functionality of the applied biochars including basic properties (Amonette and Joseph, 2009). This neutralization effect in acidic soils may improve P availability (Warnock et al., 2007a) and thus could be linked to improved plant growth. Indeed, soil pH, plant growth, abundance of calcium-phosphate, phytate and phosphonate utilizing bacteria were increased in the study. Likewise, changes in soil pH were also correlated with the changes in the bacterial community structure. A similar outcome was detected in a related experiment, where ryegrass was cultivated under biochar amendments in pots (Fox et al., 2014). However, increases in soil pH were also attributed to microbial soil activities in the past (Rillig et al., 2010).

The increased number of pores found in many biochars is believed to create a favourable habitat for bacteria (Lehmann et al., 2011; O'Neill et al., 2009). Consequently, bacterial abundances should be increased in biochar amended soils (Chen et al., 2015; Fox et al., 2014) (Han et al., 2013; Sun et al., 2012). In this study, biochar soil amendment and cultivation of spring barley significantly increased abundances of tri-calciumphosphate solubilizing, phytate, phosphonate and aromatic sulphonate utilizing bacteria. Unfortunately, biochar

345 particles inhibited representative measurements of phosphatase and sulfatase enzymatic activity (data not shown). There is currently a lack of molecular tools available to study sulfatase activity and diversity which would allow circumnavigating the challenges experienced with the soil enzymatic assays. However, the cultivation based findings are in accord with previous reports where similar increases in P and S mobilizing bacteria in the 350 rhizosphere of ryegrass and tomato were identified under biochar amendment (Fox et al., 2014; Schmalenberger and Fox, 2016).

In this study, a stepwise regression analysis revealed that soil pH had a significant effect on the observed growth promotion (four times) of barley, as it explained 78 % of the plant growth variabilities. Similar findings were confirmed by conducting a multiple regression of 355 the same predictors in SPSS and a Distance based Linear Model in Primer-E (data not shown). Increased pH alongside other physicochemical changes in soils upon biochar amendment were previously identified to substantially contribute to plant growth promotion (Jeffery et al., 2011). However, a high degree of collinearity (VIF > 5) of the predictor soil pH was also found which indicated a potential overestimation of the tested pH effect. After 360 removal of pH from the model, abundances of P and S mobilizing bacteria alone were able to explain 74 % of the plant growth variabilities (VIF < 3). Biochar amendment in the presented study resulted in a complex response of abiotic and biotic factors that were the main drivers of the reported plant growth promotion. The biochar and its induced physicochemical changes in the soil significantly altered the bacterial communities, including P and S 365 mobilizing bacteria, that in-turn explained most of the plant growth promotion effect (74 %). A pH change on its own is unlikely to be solely responsible for the increased level of plant growth. Vaughn and colleagues (2015) acidified biochar in their experiments to exclude a pH effect in their study and still reported a plant growth promotion effect at pH 6. Likewise,

Jones and colleagues (2012) identified a plant growth promotion effect three years after soil amendment with biochar when a soil pH change effect was already lost.

The biochar amendment based physicochemical changes also contribute to shifts in the bacterial community structure as observed by Anderson and colleagues where wood based biochar addition shifted bacterial communities towards the *Bradyrhizobiaceae*, *Hyphomicrobiaceae* and *Streptosporangineae* (Anderson et al., 2011). Shifts in bacterial community structures upon biochar soil amendments were also confirmed elsewhere (Chen et al., 2015; Hu et al., 2014). Changes in bacterial community structure were observed in this study based on DGGE fingerprint analysis (Fig. 1), where permutation testing confirmed these shifts to be significant and correlated to bacterial abundances of phytate, tri-calciumphosphate and sulfonate utilizing bacteria. NGS data revealed that a large proportion of 16S sequences were assigned to phyla, typically found in soils. Interestingly, abundances of Verrucomicrobia appeared to be higher than for the Proteobacteria in the composite samples of both biochar treatments and the control. A previous study of grassland and forest soils recorded much lower abundances of Verrucomicrobia (Nacke et al., 2011), while in tillage soil with and without biochar application, abundances of the Verrucomicrobia where around half of the amount that were recorded in this study (Nielsen et al., 2014). However, developing soils under restoration have been found to contain similar high abundances of Verrucomicrobia (Schmalenberger et al., 2013). The abundances of Planctomyces, Acidobacteria and Actinobacteria found in this study were similar to abundances of the same phyla found in other agricultural soils amended with biochar or traditional fertilizers (Nielsen et al., 2014).

Significantly higher abundances of sequences associated to the genera *Arthrobacter* and *Cupriavidus* were detected in the biochar amendments. Both genera have been recently associated to aromatic sulfonate desulfurization in various rhizospheres including ones under

biochar amendment (Fox et al., 2014; Gahan and Schmalenberger, 2015; Schmalenberger et al., 2008; Schmalenberger and Kertesz, 2007). Likewise, significantly higher abundances of the genus *Brevundimonas* were reported in this study in both biochar amendments that have been previously reported to be organophosphorus hydrolase active (Gorla et al., 2009). Although abundances of these bacterial genera were only confirmed on the level of their 16S rRNA gene, the above mounting evidence from the literature indicates that a substantial proportion of these bacterial genera are capable of S or P mobilization, respectively. Together, the cultivation based, community based and qPCR based information strongly support this study's hypothesis that biochar amendment resulted not only in a general bacterial community shift but also a shift in P and S mobilizing bacteria. Nutrients in direct plant available form (sulfate, phosphate) supplied through the biochar soil deposition may have contributed to the enhanced plant growth, however, the total amount of phosphate and sulfate potentially supplied by the soil was higher. At least for the plant S uptake, the nutrient balance revealed that most of the plant S must have come from initially plant unavailable forms. In contrast, a substantial proportion of the plant P could have been taken up directly as plant available phosphate from both the soil and the biochar. Thus in this study, biochar deposition most likely acted as a fertilizer and as a soil conditioner. However, a second growing season of spring barley conducted by reusing the same soil (quadruplicates) revealed a 2.8 times higher shoot biomass in the 2 % biochar amendment over the control (data not shown). These findings suggest that the applied biochar had a lasting soil conditioning effect and not only short-term fertilizing effect.

S K-edge XANES analysis of soil, *Miscanthus* grass and biochar revealed that the oxidation of S during pyrolysis resulted in the loss or transformation of sulfonates in the biochar, although the abundance of sulfonate utilizing bacteria increased upon biochar soil amendment. Consequently, any mobilization of bound S directly from the deposited biochar

would have predominantly come from the ester fraction. Future investigations are necessary to study the importance of these ester groups in plant S supply and microbial mobilization. Further investigations based on the application of stable isotope in feedstock may shed further light on the origin of plant S under biochar amendment.

In conclusion, barley growth promotion through *Miscanthus* biochar soil amendment is linked to a soil conditioning effect that includes *i*) physicochemical changes such as soil pH and P supply and *ii*) a shift of the bacterial community structure towards a higher number of bacteria capable of mobilizing P and S from plant unavailable sources.

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