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Title: The role of sulfur and phosphorus mobilizing bacteria in biochar induced growth promotion of *Lolium perenne*

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This study investigated the abundance and diversity of sulfur (sulfonate) and phosphorus (calcium-phosphate, phosphate-ester, phosphonate) mobilizing bacteria in biochar amended soil and correlated the findings to plant growth promotion, bacterial diversity and abundance and diversity of soil nematodes. For the first time a link between biochar amendment, plant growth promotion and bacterial mobilization of sulfonate-S and phosphonate-P is suggested. These findings significantly advance the knowledge of bacterial ecology and function in biochar amended soils.
Abstract:

Plants rely on microorganisms to mobilize organic and inorganically bound sulfur (S) and phosphorus (P) which the plant can then readily utilise. The aim of this study was to investigate the role of S and P mobilizing bacteria in plant growth promotion in biochar amended soil, which has been rarely investigated so far. Pot experiments of *Lolium perenne* were established on S and P limited soil with 1 or 2% biochar (*Miscanthus x giganteus*) or without biochar (control) for a period of 126 days. Both biochar amendments resulted in significant plant growth promotion. Rhizobacteria capable of growing with a) S from aromatic sulfonates, b) P from phosphate-esters, c) P from phosphonates, and d) P from tri-calcium phosphates as sole source of S or P, respectively were significantly more abundant in the biochar treatments. 16S rRNA gene based rhizobacteria community analysis revealed a significant biochar treatment effect. Abundance of nematodes feeding on bacteria was also significantly increased in the biochar treatments. Diversity analysis of rhizospheric *asfA* and *phnJ* genes revealed broad sequence diversities in bacterial sulfonate and phosphonate mineralizing capabilities. These findings suggest that biochar amendment enhances microbially-mediated nutrient mobilisation of S and P resulting in improved plant growth.
Introduction:

Biochar is gaining attention as a method for long-term carbon sequestration in soil and as a soil amendment, having demonstrated many positive agronomic effects (Sohi et al., 2010; Verheijen et al., 2010). Biochar is produced from the thermal degradation of biomass in an oxygen-free environment, a process termed ‘pyrolysis’. It is highly porous, has a large surface area and often a negative surface charge resulting in an enhanced sorption ability (Mohan et al., 2006; Downie et al., 2009). The char influences the structure, texture, porosity, particle size distribution and density of soils to which it is added, resulting in plant growth promotion effects, increased soil water retention and cation exchange capacity (Atkinson et al., 2010). Consequently, biochar has the potential to significantly impact management of agro-ecosystems in the future (Verheijen et al., 2010).

The highly porous nature of biochar, its high internal surface area coupled with its capability to adsorb soluble organic matter and other inorganic nutrients is likely to provide a favourable habitat for microbes (Lehmann et al., 2011) when added to soil. Warnock and colleagues (Warnock et al., 2007) hypothesized that micropore structures in biochar reduce grazing on fungal hyphae and bacteria. An increased abundance of microorganisms including bacteria accessing plant unavailable sources of sulfur (S) and phosphorus (P) from the soil or the biochar directly could have a beneficial impact on plant nutrient availability and plant growth when these nutrients are in short supply.

S is essential for the formation of many proteins, enzymes and enzyme co-factors (Kertesz et al., 2007). Up to 95% of S present in agricultural soils is organically bound, largely as sulfate esters (-O-SO₃H) or sulfonates (C-SO₃H) which are unavailable to the plant and need conversion into inorganic forms via microbial desulfurization (Kertesz et al., 2007). While sulfate ester bonds can be broken by various microbes, sulfonates are desulfurized within the bacterial domain (Kertesz et al., 2007). Aromatic sulfonates are desulfurized by a functional bacterial guild equipped with the asf gene cluster (Schmalenberger et al., 2010) that is part of a multi enzyme complex responsible for cleaving the S-group from the aromatic ring (Vermeij et al., 1999).
Likewise, plants require soluble orthophosphate (Pi) as their main source of P, that represents less than 1% of the total P pool in soils (Sylvia et al., 2005). Bound P exists principally in two forms, organically bound and inorganically bound (Metclef & Wanner, 1991). A wide range of microbes are able to solubilise inorganic P (Whitelaw, 2000; Gyaneshwar et al., 2002; Bucher, 2007) via exudation of citrate, gluconate or oxalate to lower the soil pH, dissociating Ca$^{2+}$ bound P (Alikhani et al., 2006). Many soil microorganisms produce phosphatases to hydrolyse phosphate-ester bonds, releasing Pi into the soil solution (Eivazi & Tabatabai, 1977; Nannipieri et al., 2011). Up to 60% of the total organic P pool in soil may be hydrolysed that way (Bünemann, 2008). Phosphonates are commonplace in the remaining pool of organic P (Ternan et al., 1998). Its C-P bond is recalcitrant to chemical hydrolysis, thermal denaturation, and photolysis (Metclef & Wanner, 1991; Adams et al., 2008). The C-P lyase pathway is believed to involve a direct breakage of the C-P bond in a redox-dependant manner (Huang et al., 2005; He et al., 2009; He et al., 2011; Jochimsen et al., 2011). The genetic basis for the C-P lyase pathway is thought to be localised in the phn cluster of various bacteria (He et al., 2011).

Biochar addition to soil may also change the soil food web, which includes bacterial-feeding nematodes (Lehmann et al., 2011; Zhang et al., 2013), accounting for 60-80% of the total nematode community (Griffiths, 1989). Together with protozoa, they are the main grazers of soil bacteria and can alter bacterial community structures (Griffiths et al., 1999; Djigal et al., 2004), accelerate bacterial turnover (Griffiths, 1994; Alphei et al., 1996), and stimulate root growth (Griffiths, 1994; Alphei et al., 1996; Mao et al., 2006; Mao et al., 2007). However, evidence for a positive relationship between biochar and nematodes is still sparse (Zhang et al., 2013).

While numerous studies have demonstrated an impact of biochar on plant growth (Atkinson et al., 2010; Lehmann et al., 2011), rarely has the role of the nutrient mobilizing soil microbiota in biochar amended soil been examined in the context of plant growth promotion (Rondon et al., 2007). The aim of this study was to investigate the biochar’s impact on bacteria involved in the transformation
of S and P to foster plant nutrient supply and plant growth. The hypotheses were that biochar amendment in a pot experiment would i) lead to growth promotion of *Lolium perenne*; ii) shift bacterial community structures; iii) affect abundances and diversities of P and S mobilizing bacteria and iv) change abundances and community structure of soil nematodes.
Materials and methods:

Biochar production

Biochar from *Miscanthus x giganteus* biomass was prepared on a lab bench scale pyrolyser (1 dm³) at 600°C for 15 minutes. The pyrolyser consisted of an open ended quartz tube with a heating element wrapped around the top end. The quartz tube was flushed with N₂ gas to keep the O₂ levels to a minimum (Kwapinski et al., 2010). 15 g of dried (105°C for 12 h) biochar was ground to a fine powder using a mortar and pestle for elemental analysis. Samples were analysed using an Axios sequential X-ray fluorescence (XRF) spectrometer (University of Manchester, UK). Scanning electron microscopy (SEM) analysis of a biochar sample was conducted on a Hitachi SU 70 analytical SEM (Oxford Instruments, Abingdon, UK) at 5-10 keV.

Pot experiments

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) in spring 2012. The soil type is a poorly drained gley soil, with loamy topsoil (18% clay) and a pH of 6. The experiment was laid down in 1968, when the field site was ploughed and sown with *Lolium perenne*. Predominant grassland species on the site were *Lolium perenne*, *Dactylis glomerata* and various meadow grass species. Soil obtained for this study has not received any P or S fertiliser since 1968 and has not been ploughed since 1970 (Tunney et al., 2010). The agricultural managements were described in detail in previous studies (Culleton *et al*., 2002; King-Salter, 2008; Griffiths *et al*., 2012).

The soil was sieved (2 mm) and mixed 1:2 with distilled H₂O washed sand. Two biochar treatments 1% (w/w) and 2% (w/w) and a biochar free control were established. Pots were given a specific soil profile with a biochar free top and bottom layer of 300 g. The 300 g middle layer (M) contained 3
and 6% biochar (w/w) resulting in a biochar concentration for the whole pots of 1 and 2%. Middle layers of the control experiment were kept biochar free. Pot experiments were set up in replicates of eight with *Lolium perenne var.* Malambo (Deutsche Saatveredelung AG, Lippstadt, Germany) as the plant host (270 seeds per pot) and incubated for a period of 126 days in a greenhouse.

At harvest, all above ground plant material (shoots) was dried in a fan oven at 55°C for 72 h before dry weights (DW) were recorded. Plant shoot heights were measured by measuring 5 random shoots per pot. Dried shoot biomass was pooled per treatment (7 g respectively) and subjected to elemental analysis at Lancrop Laboratories (York, UK; employing atomic absorption spectroscopy, inductively coupled plasma spectrometry, titrations and spectrophotometry). The pH of the soil was potentiometrically measured by rotating 5 g of air-dried, sieved (2 mm) M layer soil at 70 rpm for 5 min in 20 ml of CaCl$_2$ solution (0.01 M) on a RM2 mixer (Elmi, Riga, Latvia). This solution was allowed to settle for 2 h and the pH was measured in the supernatant.

**DNA extraction and cultivation dependent quantification of bacteria**

3 g of rhizosphere associated soil was randomly picked from the M layer of each pot, added to 20 ml of sterile NaCl (0.85% [wt vol$^{-1}$]) solution and rotated at 75 rpm (RM-2 mixer) for 30 min at 4°C. After mixing, suspensions without roots and large soil particles were transferred into centrifuge tubes. While 0.1 ml suspension was subjected to a serial dilution in saline (10 fold) for cultivation based analyses (see below), the remainder was centrifuged at 4500 rpm for 15 min at 8°C. The clear supernatant was discarded and the pellet was subjected to DNA extraction (rhizospheric soil) using the protocol of the ULTRA CLEAN™ soil DNA kit (MO BIO Laboratories, Cupertino, CA) as per instructions by the manufacturer.

A most probable number (MPN) analysis in microtitre plates was established on liquid R2 medium (Reasoner & Geldreich, 1985) as previously described (Schmalenberger *et al.*, 2008). Furthermore, MPNs were established in minimal media to grow bacteria capable of mobilising a) S from
toluenesulfonate (MM2TS), b) P from phytate (MM2Phy), and c) P from phosphonoacetic acid (MM2PAA) as sole source of S or P, respectively (supplementary Table S1 A, B, C). The microtitre plates were incubated for 14 days at 25°C and 75 rpm. Growth of bacteria was identified using an ELX 808 microtitre plate reader at 590 nm (BioTek, Winooski, VT). A colony forming unit analysis (CFU) was conducted to quantify bacteria capable of solubilizing P from tri-calcium phosphate on tri-calcium phosphate agar plates (modified from Subba Rao, 1982). After an incubation period of 7 days at 25°C, the colonies which had formed a clear halo around their colony were counted.

Isolation and identification of functional bacteria

A loop full of bacterial suspension was removed from random microtitre wells of the highest growth dilutions of each treatment from the MM2TS and MM2PAA minimal media and spread on solid R2A plates to obtain sulfonate and phosphonate utilizing bacterial isolates. DNA from pure cultures was extracted using a quick lysis protocol (Schmalenberger et al., 2001). The 16S rRNA gene was subsequently amplified using the universal primers 27F and 1492R (Lane, 1991). Total reaction volume of 25 µL contained 1X buffer with 2 mM MgCl₂, 0.2mM dNTP-mix, 0.5 µM primers and 0.5 U Taq polymerase (all Thermo Scientific, Waltham, MA); 0.5 µL of template DNA was added per reaction. PCR cycling conditions: initial denaturation at 94°C for 4 min, followed by 32 cycles of 94°C denaturation for 45 s, 50°C annealing for 45 s and 72°C extension for 2 min and a final extension step at 72°C for 5 min. All PCR work was performed on a G-Storm thermo-cycler (Somerset, UK). PCR amplicons were purified according to the manufacturer using the GenElute PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MO) and quantified spectrophotometrically (ng µL⁻¹) using a Nano Drop ND-1000 (Thermo Scientific). Amplicons were subjected to sequence identification (GATC Biotech, Konstanz, Germany).
Community analysis of the bacteria was conducted from extracted DNA employing PCR and subsequently a denaturing gradient gel electrophoresis (DGGE). PCR reactions in a volume of 25 µL contained final concentrations as outlined above for 16S rRNA gene amplification, but with primers GC-341F and 518R (Muyzer et al., 1993) and additional 1 M betaine (Sigma Aldrich). PCR cycling conditions were as follows: an initial denaturation step of 95°C for 5 min, 20 cycles of denaturation at 94°C for 45 s, annealing at 65-55°C (touch down, -1°C per cycle) for 45 s and an extension at 72°C for 1 min. This was followed by a further 20 cycles with an annealing temperature of 55°C for 1 min with a final elongation step of 72°C for 10 min. PCR products were investigated for product yield and quality via agarose gel electrophoresis.

DGGE was performed in a TV-400 DGGE system (Scie-plas, Cambridge, UK) with 200 x 200 x 1 mm gels. The polyacrylamide gel (10% [vol vol⁻¹]) in 1x TAE had a denaturing gradient of 30-70% (100% denaturing gradient was 7 M urea and 40% formamide). Electrophoresis was carried out in 1 x TAE buffer at 63 V for 16 h at 60°C. Gels were stained with SYBR Gold (Invitrogen, Carlsbad, CA) 10,000 diluted in 1x TAE for 30 min in the dark. After rinsing in distilled H₂O, gels were visualized using a gel image analysis system with a UV transilluminator (G:Box, Syngene, Cambridge, UK). DGGE profiles were analysed using the Phoretix advanced 1D software (Nonlinear Dynamics, Newcastle, UK). The binary gel image matrix was used to establish detrended correspondence analysis (DCA) biplots based on Decorana as published earlier (Schmalenberger et al., 2008). Further statistical analyses were performed in CANOCO (version 4.5; Microcomputer Power Inc., Ithaca, NY) to test the effect of environmental variables (soil pH, plant height, plant weight, MPN and CFU values) on the bacterial 16S rRNA gene community structure. Canonical correspondence analysis (CCA) and permutation tests (Monte-Carlo) were carried out using forward selection and 9,999 replicates.

Diversity of phnJ (phosphonate mobilization) and asfA (sulfonate desulfurization)
DNA extracted from the rhizosphere of the three treatments cultivated under *Lolium perenne* was amplified using primers PhnJoc1 and PhnJoc2 (Karl, 2007). The PCR was conducted using a Kapa 2G Robust PCR kit (Kapa Biosystems, Woburn, MA) in volumes of 25 µL containing, 1x buffer A, 1x enhancer, 5% DMSO (Sigma-Aldrich), 2 mM MgCl₂, 0.2 mM dNTP, 0.5 µM primer each and 0.5 U Kapa Robust polymerase (Kapa Biosystems, Wilmington, MA). Cycling conditions were as follows: Initial denaturation 95°C for 4 min, 10 cycles of denaturation at 94°C for 45 s, touch-down annealing 65-55°C (-1°C per cycle) for 45 s and extension at 72°C for 1 min. This was followed by 30 further cycles at 55°C annealing and a concluding step of 72°C for 10 min.

Amplification of a fragment of the *asfA* was conducted using primers asfAF2 and asfBtoA (Schmalenberger & Kertesz, 2007). Polymerase and chemicals were used as described for the amplification of *phnJ* above. PCR cycling conditions were identical to the one for *phnJ* with the exception that the annealing temperature was 1°C higher, annealing time was shortened to 30 s and the extension time was increased to 90 s.

Amplicons of *phnJ* and *asfA* were cloned after PCR purification using a pGEM-T vector (Promega, Madison, WI). The ligation and transformation steps were undertaken as per manual instructions using competent *E. coli* DH5α cells. Selective cultivation of clones was carried out on LB plates with ampicillin and X-Gal as recommended by the manufacturer. Separate ligations were carried out for the control, the 1 and 2% biochar treatment. Screening of transformants was conducted by selecting clones with inserts (blue-white screening) at random for cell lysis (Schmalenberger et al., 2001) and subsequent PCR of *phnJ* and *asfA* as described above. 40 clones with the correct insert were selected from each clone library for restriction fragment length polymorphism (RFLP) analysis of *phnJ* using restriction enzyme *Hae*III and for *asfA* using restriction enzymes *Alu*I and *Rsa*I (all Thermo-Scientific) at 37°C for 4 hours with 5 U of each restriction enzyme and 1 times buffer as recommended by the manufacturer. RFLP pattern were visualised for *phnJ* on 10% non-denaturing acrylamide gels or for *asfA* on 2% agarose gels. Restriction patterns obtained from the RFLP analysis of *phnJ* and *asfA* were
screened for operational taxonomical units (OTU) using the Phoretix advanced 1D software (Nonlinear Dynamics). OTUs of more than one representative were selected for sequencing at GATC Biotech. Clone library coverage was calculated as described previously (Schmalenberger et al., 2007).

The asfA sequence fragments obtained were imported into an asfA database which had been generated previously (Schmalenberger & Kertesz, 2007; Schmalenberger et al., 2008) using the ARB software package (Ludwig et al., 2004). Trees for the N-terminally truncated peptide sequences (AsfA) were calculated with the randomised accelerated maximum likelihood (and Dayhoff model) method (Stamatakis, 2006) using a positioning filter to restrict data analysis to the sequenced regions obtained in this study. N- and C-terminally truncated protein sequences from PhnJ obtained from this study together with available PhnJ sequences mined from the NCBI database were used to create a tree with the same model as described above using ARB. 16S rRNA, asfA and phnJ gene sequences from isolates and clones were deposited into Genbank under the accession numbers KJ666647-KJ666686.

Soil nematodes

Nematodes were extracted from ca. 100 g fresh weight of soil by decanting and sieving through consecutive 250, 150, 75 and 53 µm sieves followed by a Baermann funnel extraction (Brown & Boag, 1988). The extracted nematodes were left to settle overnight at 4°C, the majority of the supernatant aspirated off and the nematodes were killed by heating at 65°C for 4 min and fixed in 1% (final concentration) formaldehyde. Total numbers were counted under low power microscopy and a representative sub-sample mounted on glass slides for identification at high magnification. The slides were scanned in a regular fashion and the first 50 nematodes, adult or juvenile, on the slide identified (Bongers & Bongers, 1998) as they were encountered.
Statistical analyses

Data obtained from CFU, MPN and nematodes were analysed using SPSS 20 (IBM, Armonk, NY).

While the total number of nematodes, bacterial and fungal feeding nematodes were normally distributed, other data sets were not normally distributed and transformed to Log_{10} to achieve normality. Nematode taxa and nematode omnivore normalization attempts failed and consequently a non-parametric test was conducted (Kruskal-Wallis with manual posthoc). All other data were tested via univariate analysis (Tukey HSD). A P value of ≤0.05 or ≤0.01 was deemed to be statistically significant or highly significant.
Results

Plant growth promotion effect, soil pH, biochar and shoot analysis

The mean plant shoot weight at harvest for *Lolium perenne* was highest (3.96 g) for the biochar 2% treatment. Significantly lower values were recorded for the biochar 1% treatment (3.12 g) and for the control (1.61 g). Both biochar treatments had a highly significant higher shoot dry weight when compared to the control (Table 1). The mean height of *Lolium* shoots from both biochar treatments was significantly greater than the control. There was also a significant increase in shoot height in the 2% biochar treatment over the 1% treatment. Likewise, a highly significant neutralization effect on the soil solution pH was observed between the biochar treatments and the control and between both biochar inclusions (all P ≤ 0.001; Table 1). XRF analysis revealed that the char was mainly composed of carbon (90.78%) but also retained P (0.244%) and S (0.096%) (Table 2A). SEM images clearly displayed the porosity of the char as well as retention of the carbon skeletal structures of the original plant material (Fig. 1). While potassium (K) and P content in the shoot biomass increased with the biochar amendments, this was not the case for nitrogen (N), S, calcium (Ca) and magnesium (Mg), where reductions were recorded (Table 2B). However, mass balance analysis for the shoot biomass revealed that the total amount of all major nutrients from above increased per pot, including N, S, Ca and Mg (Table 2C).

Abundance of cultivated rhizosphere bacteria

MPN and CFU analysis revealed a highly significant increase in the abundance of heterotrophic, sulfonate desulfurizing, tri-calcium phosphate solubilizing, phosphonacetic acid and phytate utilizing bacteria upon biochar amendment against the control (all P < 0.001, Table 3). A significant increase was detected in the abundance of heterotrophic (P = 0.005), desulfonating (P = 0.006) and tri-calcium
phosphate solubilising bacteria (P=0.031) in the 2% biochar treatment over the 1% treatment (Table 3). However, no significant differences in the abundance of phosphonoacetic acid (P=0.666) or phytate (P=0.086) utilizing bacteria were detected upon comparison of the 1 and 2% treatment (Table 3).

Increases in the abundance of bacterial heterotrophs of over a log scale was recorded with MPN ml$^{-1}$ of 2.5 10$^4$ in the controls increasing to 3.0 10$^5$ and 8.6 10$^5$ in the biochar amendments which represented a 12 and 34 fold increase. Increases were found for the S and P mobilizing bacteria that were highest for the phosphonate utilizers where biochar amendment resulted in an 87 and 121 fold increase in abundance. Tri-calcium phosphate mobilizing bacteria increased 3 and 28 fold, while the increase of desulfonating bacteria was 19 to 103 fold. Sulfonate utilizing bacteria represented 9% of the heterotrophs in the controls and this increased to 15% in the 1% biochar treatment and peaked in the 2% biochar treatment at 28%. Likewise, phosphonate utilizing bacteria increased with biochar addition from 3% in the controls to 24 and 12% for the 1 and 2% biochar amendment, respectively. In contrast, tri-calcium phosphate mobilizing bacteria that represented 17% in the control were down to 4% in the 1% biochar treatment and recovered again to 14% in the 2% biochar amendment.

Functional bacterial isolates

Bacteria related to Caulobacter segnis, Arcicella rosea, Chitinimonas taiwanensis, Kocuria rhizophila, Stenotrophomonas rhizophila, Acidovorax delafieldii and Cuprividus basilensis were isolated from MM2PAA MPNs capable of growing with phosphonoacetic acid as sole source of P (Table 4). Bacterial isolates associated to Stenotrophomonas rhizophila, Pseudomonas brassicacearium, Acidovorax delafieldii and various species of the genera Arthrobacter were the main bacterial genera recovered from the rhizosphere of Lolium perenne on MM2TS media (Table 4) capable of growing with toluenesulfonate as sole source of S. Although, Isolates associated to genera of
Stenotrophomonas and Acidovorax were isolated from MM2TS and MM2PAA media, the isolates represented different strains.

Bacterial community analysis

DCA revealed a separation between the biochar 2% treatment against both the biochar 1% and the control treatment (supplementary Fig. S1) that was statistically significant between the biochar treatments and the control (P=0.027 and P=0.004, respectively) and as well between the two biochar treatments (P=0.011). Permutation analysis of environmental variables revealed that soil pH (P=0.008), plant heights (P=0.005) and weights (P=0.003) had a significant effect on the 16S rRNA gene bacterial community composition as visualised via CCA (Fig. 2). This was also the case with the data from the cultivation of heterotrophs (P<0.001), desulfonating (P=0.005), phytate mobilizing (P=0.014), phosphonate mobilizing (P<0.001) and TCP solubilizing (P<0.001) bacteria, exhibiting an influence on the rhizospheric bacterial community structure (Fig. 2).

Diversity of PhnJ and AsfA

PhnJ protein sequences from the NCBI database were mined and a tree was constructed using the mined sequences and partially N- and C-terminally truncated and translated PhnJ from this study (clone library coverage 55%) using a maximum likelihood method. The largest subsection of obtained PhnJ OTUs (38 clones; 27%) clustered together with Bradyrhizobium (Rhizobiales 1 clade) at a sequence similarity of approx. 93% and above that represented the control (17 clones), 1 (11 clones) and 2% (10 clones) biochar treatment. A further cluster of PhnJ from this study (16 clones; 14%) was clustering with Acidophilium multivorum (approx. 70% sequence similarity) (Rhodospirillales/Burkholderiales clade), that represented the control (10 clones), 1 (1 clones) and 2% (5 clones) biochar treatment (Fig. 3, supplementary table S2). A further OTU (L8) clustered with
Rhizobiales 2 clade, separated from all other OTUs containing 2 clones from the 1% biochar treatment (Fig. 3). In total, 27 clones from the controls were represented in the maximum likelihood tree of PhnJ while only 14 and 19 clones represented the 1 and 2% biochar treatment, respectively. Clones found exclusively in the control (18) was almost half the number of clones occurred exclusively in the biochar treatments (28 for 1%; 29 for 2% biochar). The number of clones (19) that were recovered from all three clone libraries was higher than the number of clones found in the control and 1% biochar (3), control and 2% biochar (15) or in both biochar treatments (8).

Protein AsfA sequences of abundant OTUs from this study (clone library coverage 50%) were integrated into an existing AsfA tree (Schmalenberger et al., 2010). A total of 41% of the OTUs obtained in this study were associated (~85% similarity) to the type strain of Variovorax paradoxus (DSM30034) and isolates of Polaromonas (84-90% similarity for clade 1; 77-80% similarity for clade 2) and Hydrogenophaga (87% similarity) which have been shown to desulfurize a variety of arylsulfonates as a S source ([Schmalenberger & Kertesz, 2007; Schmalenberger et al., 2008 and unpublished data] (Fig. 4, supplementary table S3). This represented the most abundant clustering of obtained AsfA sequences (80%) with 12, 18 and 19 clones originating from the control, biochar 1 and 2% clone libraries, respectively. Only one OTU clustered closely with the Variovorax clade (>90% similarity) containing one clone from the control and two clones from the 1% biochar (L12). OTU L11 with one clone from the control and 2% biochar respectively was found closely associated to Cupriavidus metallidurans (>98 % similarity). A further OTU branched distantly with the Polaromonas 2 clade (86% similarity) with 2 clones from the biochar treatment (Fig. 4, supplementary table S3).

Nematode abundances

The total abundance of nematodes did not respond in a linear manner to the addition of biochar. Higher numbers were identified in the 2% biochar (1396 ± 646 100 g⁻¹) than either the 1% biochar (491 ± 304 100 g⁻¹) or the control (856 ± 287 100 g⁻¹) with only the difference between 1 and 2%
biochar being significant (P≤0.05). There was, however, a consistent shift in nematode community structure with highly significant increases (P≤0.01) in bacterial-feeding nematodes at 2% biochar over the control (19.4, 55.6 and 83.1% for control, 1% biochar and 2% biochar respectively) and significant decreases in omnivores (54, 26 and 2% for control, 1% biochar and 2% biochar respectively, P=0.02). A decrease in fungal-feeding nematodes (10.4, 1.5 and 2.9% for control, 1% biochar and 2% biochar respectively) was not significant (P>0.05). Increases in bacterial-feeding *Acrobeloides* and decreases in omnivorous Dorylaimidae (Table 5) were the main drivers of these changes upon addition of biochar.
Discussion

Overwhelming evidence of the plant growth promoting potential of biochar can be found in the literature (Atkinson et al., 2010; Graber et al., 2010; Kookana et al., 2011; Jones et al., 2012; Rajkovich et al., 2012). This study confirmed that biochar treatment of 1 and 2% had a highly significant effect on above ground biomass heights and weights of *Lolium perenne*. The causality of biochar derived plant growth promotion was often attributed to a number of physico-chemical factors and more recently also to soil biota (Atkinson et al., 2010; Lehmann et al., 2011).

In this study, a significant increase in soil pH from below 6 to around 7 was observed with the application of biochar. Soil neutralization effects through biochar application were reported previously (Liang et al., 2006; Atkinson et al., 2010), possibly related to the biochar’s surface functionality. Basic properties were identified as a result of the thermo-chemical pyrolysis conditions (Amonette & Joseph, 2009). Increases in soil pH may improve bioavailability of soil phosphorus (Warnock et al., 2007) and as a consequence promote plant growth directly. In this study, pH increases may not have been the main driver for increased plant growth since similar improvements of soil pH in soils of the same location and type that has been used in this study using lime resulted in only very modest grassland growth promotion effects of around 3% (Sheil et al., 2014). Increases in soil pH have also been attributed to microbial biomass and microbial activity in the literature (Aciego-Piety & Brookes, 2008; Rillig et al., 2010). However, a pot experiment, similar to this study, with commercial biochar and *L. perenne* found only a significant increase in soil pH, but no significant plant growth promotion or increased abundances of bacteria (Carr & Schmalenberger, unpublished results). Therefore, other factors may have substantially contributed to the observed plant growth promotion in this study including the activity of soil microbiota (Lehmann et al., 2011) driving soil biogeochemical cycling (Anderson et al., 2011). Elemental analysis of the shoots from this study (Table 2B) revealed adequate amounts of S and Mg in the biomass for all treatments, but
concentrations of P and in particular N was low. Chan and colleagues (Chan et al., 2007) found that their plant growth promotion efforts with biochar amendment was held back by N limitations in the soils. These findings suggest that addition of N to this study’s pot experiment would have increased the plant growth promotion effect further.

Biochar’s porous structure as confirmed for the biochar applied in this study (Fig. 1) has been described previously as a favourable habitat for mycorrhizal fungi (Saito, 1990; Ishii & Kadoga, 1994; Matsubara et al., 2002; Yamato et al., 2006; Warnock et al., 2007) and bacteria (O’Neill et al., 2009; Lehmann et al., 2011). Recent studies have identified potential changes in bacterial biochemical cycling of P and N including N-fixation when biochar was introduced to soils (Rondon et al., 2007; Anderson et al., 2011; Lehmann et al., 2011). This study reports for the first time highly significant increases in MPN and CFU values of aromatic sulphonate desulfurizing, phosphonate mobilizing, phosphate-ester mineralizing and tri-calcium phosphate solubilising rhizospheric bacteria. These increases in abundance for all but tri-calcium phosphate mobilizers were proportionally higher than the ones observed for bacterial heterotrophs, suggesting that biochar amendment enhanced bacterial S and P mobilization leading to plant growth promotion. These findings support the hypothesis that soil bacteria in biochar amended soils actively promote plant growth and contrasts a meta-analysis by Jeffery and colleagues (Jeffery et al., 2011). There, plant growth promotion effects of biochar were attributed to alteration of physio-chemical properties in the soil with such effects as cation exchange capacity (CEC) and associated nutrient retention, increased pH and base saturation, a rise in the level of available P and greater amounts of plant available water. In this reasoning, the biological parameters were considered consequences of the altered physio-chemical state, rather than being the primary drivers of growth promotion (Jeffery et al., 2011).

Leaving biochar aside, S and P mobilizing microorganisms were identified as the main driver in plant growth promotion before. Growth of tomato was significantly increased with the addition of the arylsulfonate desulfurizing bacterium Pseudomonas putida S-313 (Kertesz & Mirleau, 2004).
Likewise, tri-calcium phosphate solubilising and organo-phosphoros mineralizing microorganisms have been linked to plant growth promotion (Rodriguez & Fraga, 1999; Whitelaw, 2000; Igual et al., 2001; Konietzny & Greiner, 2004; Rodriguez et al., 2006; Jorquera et al., 2008). Taken this into consideration, promotion of these types of bacteria as identified in this study would support the idea of a synergistic effect between the physio-chemical and bacteriological parameters in the growth promotion effect.

Functional marker genes, such as \( \text{asfA} \) and \( \text{phnJ} \), allow evaluation of the functional bacterial structures in connection to \( S \) (sulfonate-S) and \( P \) (phosphonate-P) mobilization (Schmalenberger & Kertesz, 2007). After translation into AsfA protein sequences, nine of the 12 dominating OTUs were associated to a clade bordering with clades of \( \text{Variovorax} \), \( \text{Polaromonas} \) 1 and \( \text{Hydrogenophaga} \). These genera have been previously identified as desulfonating bacteria in crop- and grassland soil and rhizosphere (Schmalenberger & Kertesz, 2007; Schmalenberger et al., 2008; Dodd et al., 2009; Peralta et al., 2010; Schmalenberger & Noll, 2010; Schmalenberger et al., 2010). Interestingly, \( \text{Variovorax} \) was not isolated through cultivation in this study (Table 4). The AsfA clade that dominated the \( \text{Lolium} \) rhizosphere was possibly too dissimilar (approx. 85% similarity) in order to be recognised as \( \text{Variovorax} \) and thus most likely represent a not yet identified type of beta-proteobacterium. Bacteria associated to \( \text{Acidovorax} \) and \( \text{Pseudomonas} \) were isolated in this study but sequences of \( \text{asfA} \) were not retrieved from these isolates with the PCR primers available. However, both genera have been associated with sulfonate desulfurization before (Zürrer et al., 1987; Schmalenberger & Kertesz, 2007). \( \text{Stenotrophomonas} \) and \( \text{Arthrobacter} \) represent newly isolated genera with aromatic sulfonate utilization activity. Unfortunately, attempts to recover the sequence of \( \text{asfA} \) from these isolates have failed too, even after extensive primer re-design. The lack of an amplicon suggests that these isolates either have an entirely different desulfonation pathway or primer binding sites were substantially different to the so far identified \( \text{asfA} \) genes.
The *phn* gene cluster is structurally and compositionally varied among different bacterial genera but presence of *phnJ* appears to be highly common (Huang *et al.*, 2005). Saxton and colleagues (Saxton *et al.*, 2011) investigated the effect of the phosphonate herbicide glyphosate on the phytoplankton community structure through phylogenetic analysis of the *phnJ* gene using primers different to the ones selected in this study. The capacity of soil bacteria to cleave the C-P bond is often strain specific and possibly acquired via lateral gene transfer (Huang *et al.*, 2005). After translation into PhnJ protein sequences, all abundant OTUs from this study clustered within the Rhizobiales and the Rhodospirillales/Burkholderiales clades. While no shift upon biochar amendment was identified, a trend towards a higher bacterial diversity was recognised. No OTUs clustered with any gamma proteobacteria, Actinobacteria or Bacteroides although these phylogenetic groups were also isolated as phosphonoacetic acid utilizing bacteria in this study (Table 4). Deficiencies in PCR primer coverage, or the presence of other phosphonate degrading pathways not involving the *phn* cluster could have been the reason for this omission. *Stenotrophomonas* and *Acidovorax* were isolated independently on MM2TS and MMPAA, suggesting that both genera have the capacity to utilize arylsulfonates and phosphonates as S and P source, respectively.

Permutation tests of the bacterial community structures based on DGGE of 16S rRNA gene fragments revealed a significant shift in the composition of the soil bacterial community upon biochar inclusion against the control treatment in this study. Similar shifts in microbial community composition upon biochar amendment have been reported previously (Steinbeiss *et al.*, 2009; Khodadad *et al.*, 2011; Kolton *et al.*, 2011; Watzinger *et al.*, 2014; Wei *et al.*, 2014). Higher abundances of S and P mobilizing bacteria have significantly contributed to shifts in the bacterial community structure with biochar amendment and changes in the diversity of respective functional bacterial guilds may have had a similar impact. Further contributing factors to such changes are discussed in the literature and are thought to be based on biochar-associated volatile organic compounds (Spokas, 2010; Kolton *et al.*, 2011).
In this study, significant increases in bacterial-feeding nematodes were identified, indicating an enhanced bacterial activity in response to the addition of biochar. The nematodes to benefit from the addition of biochar were mainly *Acrobeloides* spp. which are of coloniser-persister (c-p) group 2 (Table 5) (Yeates *et al.*, 1993). Nematodes from c-p group 1 are fast-growing opportunists that require large concentrations of bacterial substrate (Georgieva *et al.*, 2005). Therefore, the increase here in *Acrobeloides* would imply a more gradual and sustained increase in substrate akin to an increase in plant growth over the duration of the experiment, rather than a sudden huge increase in substrate. The increased grazing pressure, particularly as it appears to be limited to a single nematode taxa, will alter bacterial community structure (Griffiths *et al.*, 1999) as identified in this study, and is likely to favour plant growth hormone producing bacteria (Mao *et al.*, 2007; Cheng *et al.*, 2011). This would provide a positive feedback mechanism further improving plant growth above the here reported effects of the biochar.

The potential benefits of biochar colonizing bacteria are among the most understudied aspects of soil biochar amendments to date (Lehmann *et al.*, 2011). In this study, the application of biochar to temperate soil shifted bacterial community structures, increased numbers of S and P mobilizing bacteria that may have enhanced nutrient mobilization leading to plant growth promotion. Future biochar applications to temperate soils may allow sustained crop nutrient supply at reduced inorganic fertilizer application rates, exploiting more efficiently the natural sources of S and P present in soil and in the added biochar.

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