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One-time phosphate fertilizer application to grassland columns modifies the soil microbiota and limits its role in ecosystem services

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HIGHLIGHTS

- Phosphate fertilization reduced arbuscular mycorrhizal colonization rates.
- Phosphate fertilization reduced the abundance of bacterial- and fungal-feeding nematodes.
- Phosphate fertilization shifted the bacterial, fungal and AM fungal community structures.
- Phosphate fertilization depressed abundance of *phoD* copy numbers.
- Rye grass dry matter yield did not significantly change with phosphate fertilization.

GRAPHICAL ABSTRACT

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ABSTRACT

This study evaluated the effect of one-time phosphate fertilization on the soil microbiota, its cycling of phosphorus (P) and grass growth. Soil columns were established in a greenhouse using a P-limited Irish soil (index 1), planted with *Lolium perenne* and fertilized with 0 (control), 5 (quarter), 10 (half) and 20 (full) kg P ha^{-1} as inorganic phosphate. Only traces of phosphate in soil solution were detected over the 14 week experiment, even after phosphate fertilization. Grass dry matter yield between treatments was not significantly different. Full phosphate fertilization significantly reduced the arbuscular mycorrhization (AM) rate, bacterial- and fungal-feeding nematode population, bacterial *phoD* gene abundance, but increased alkaline and acid phosphatase activities at the time of harvest. Full and half P treatments significantly shifted the bacterial, fungal and AM community structures compared to the control. Furthermore, the control had a significantly higher relative abundance of bacterial genera including *Bacillus*, *Bradyrhizobium*, *Paenibacillus*, *Nocardioides* and *Balneimonas*, that have been associated with P mobilization in the past, when compared to the full phosphate treatment. These results suggest that a positive effect of a single phosphate application on plant growth in a soil can be cancelled out by its negative effect on the soil microbiota and their ecosystem services.

1. Introduction

In order to feed the rising world population, the United Nations has predicted that food production needs to increase by 50% in 2020 (Nellemann et al., 2009). Grasslands play significant roles in food

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production as major source of feed for ruminants used for meat and milk production. About 70% of the world's agricultural land is occupied by grasslands making grass the world's most important crop (O'Mara, 2012). In Ireland, the main agricultural land use is grasslands with about 80% of the total land area devoted to permanent grasslands (Rath and Peel, 2005).

In conventional agriculture, high-yielding crop varieties, irrigation, pesticides and inorganic fertilizers are frequently used to attain higher crop yields (Matson et al., 1997) as well as to replace offtakes. However, decrease in agricultural soil quality and increased risks of environmental pollution are often associated with intensive use of inorganic fertilizer inputs (Tilman et al., 2002). Soil bacteria and fungi are important drivers of nutrient cycling in the soil. These microbes mediate the cycling of carbon (C), nitrogen (N), phosphorus (P), sulfur (S) and other elements thereby having great influence on crop productivity (Nannipieri et al., 2003; Kertesz and Mirleau, 2004). The development of sustainable agricultural systems is therefore premised on the understanding of the contribution of these microorganisms to plant nutrition and plant growth and their response to intensive application of inorganic fertilizers.

P is an essential macronutrient in plant nutrition and the second most important plant nutrient after N. It plays vital roles in most of the metabolic processes in plants including photosynthesis, energy transfer, signal transduction, biosynthesis of macromolecules and energy transduction (Khan et al., 2010). Unlike N with a large atmospheric pool, the world's source of high quality phosphate is finite making the development of more P efficient plant or agricultural systems essential (Cordell et al., 2009). Despite many soils containing a large amount of total P, only a small proportion (usually <1%; (George et al., 2011)) is available for plant uptake because most of the soil P is organically and inorganically bound. In order to provide plants with sufficient available P in agriculture, the principle of building up of P stocks via regular P fertilization is widely practiced (Voss, 1998). However, plant yield response to P fertilization can vary greatly. A recent study of Irish pastures has shown that in two out of eight soil series, no substantial increases in yield were identified within four years (Schulte and Herlihy, 2007). Microorganisms play important roles in the maintenance of plant available P pools because they mediate to a large extent the mineralization of organic P in soils (Macklon et al., 1997; Richardson et al., 2005). Soil microbes are important in the maintenance of pools of both inorganic and organic P in soil solution and biomass turnover represents an important potential source of plant P supply (Seeling and Zasoski, 1993; Oberson et al., 2005). The soil microbiota's ecosystem service in P cycling is an essential pathway for P movement and transport from various soil pools into plant available forms (Magid et al., 1996; Oberson et al., 2001). However, a paucity of studies have characterised the effects of inorganic P-based fertilizer management on soil microbial P cycling in P-limited soils until now.

This study aimed to provide a qualitative and quantitative understanding of how the soil microbiota and plants are affected by phosphate fertilization. The specific objectives were to: assess the microbial community (bacteria, fungi, AM fungi, nematodes) size, abundance, composition, and function in P cycling alongside the uptake of phosphate by the grassland plant *Lolium perenne* as affected by one-time application of different rates of phosphate fertilizer. We hypothesized that reduced phosphate application will benefit the abundance and affect the structure of soil microbiota that can contribute to P availability in grasslands.

2. Material and methods

2.1. Experimental setup, column harvest, plant and soil analyses

Soil columns were setup in a greenhouse using a P-limited soil (Irish soil P index 1) collected from Moorestown Cahir (County Tipperary, Ireland). In Ireland, Morgan's extractable P is used as the agronomic soil test for P and this has been classified as indices: 1 (deficient), 2 (low), 3 (optimum) and 4 (excessive) (Lalor and Coulter, 2008). The site had not been under cultivation for over 20 years (Cornelius Traas, personal communication, 2015). The soil type is a grey brown podzolic soil, with a sandy silt loam topsoil (10% clay) with a pH of 6.8 (FW; Lancrop Laboratories Ltd., York, UK). Soils were sampled from 0 to 20 cm and 20–40 cm of the profile, sieved through a 3.35 mm mesh to remove stones, mixed and repacked into the columns in layers of 0–20 and 20–40 cm as in the field. The columns consisted of 16 cm × 40 cm pipes and were planted with rye grass (*Lolium perenne* variety Trend). They were fertilized with 0, 5, 10 and 20 kg P ha^{-1} inorganic phosphate P treatments (potassiumphosphate in water, pH 7, applied on column surface) representing control, quarter, half and full rates respectively (full P and half P represents the recommended amount of P application for build-up rates at indices 1 and 2 respectively) alongside a full complement of other nutrients (125 kg ha^{-1} N, 150 kg ha^{-1} K, 20 kg ha^{-1} S and micronutrients). Each treatment was replicated six times and managed for 14 weeks. The columns were watered with 200 ml of rain water, three times a week. Weekly soil solution samples were collected in 10 cm height intervals using Rhizons (Rhizosphere Research Products, Wageningen, Netherlands) and analysed for phosphate, sulfate and other anions via ion chromatography using a Dionex ICS1100 with an AS23 column and a carbonate mobile phase following the manufacturer's recommendation (Dionex, Sunnyvale, CA).

Grasses were cut back to 5 cm height after seven weeks of growth and entire grass shoots were harvested at the end of the experiment for dry matter determination. At the end of 14 weeks of grass growth, columns were deconstructed and grass shoot and root biomass were determined. Rhizosphere soil samples (soil shaken off roots) were collected for phosphatase activity determination, nematode analyses and other soil properties, while root samples were collected for mycorrhizal colonization analysis (see below).

For both cuts, grass shoots were dried at 55 °C for 72 h in a fan oven and dry weights were recorded. The elemental compositions of the dried shoot biomass were determined at Lancrop Laboratories Ltd. (employing atomic absorption spectroscopy, inductively coupled plasma spectrometry, titrations, and spectrophotometry, accredited to ISO/IEC 17025:2005).

Prior to P determination, soil samples were dried overnight at 40 °C and sieved using a 2 mm mesh. Morgan's extractable P (surrogate for plant available P) was determined by extracting soil with sodium hydroxide-acetic acid solution (pH 4.8) in a 1:5 soil to solution ratio (Peech and English, 1944). The extracts were filtered through Whatman No. 2 filter paper. P concentrations in the extracts were determined by colorimetry at 880 nm using a Camspec M500 UV–Visible Spectrophotometer (Camspec, UK) following the molybdate-ascorbic acid method (Murphy and Riley, 1962). The pH of the soil was measured potentiometrically using deionised water in a 1:2 soil solution ratio (McCormack, 2002).

2.2. Cultivation-dependent analysis of P mobilizing bacteria, potential phosphatase activity, mycorrhizal root colonization and nematode abundance determination

Bacteria were extracted from the rhizosphere soil in the top 10 cm of the column as follows. After the deconstruction of the columns, 3 g of roots with attached soils were added to 50 ml tubes containing 20 ml sterile saline (0.85% [w/v] NaCl) solution and rotated at 75 rpm on an Elmi Intelli-Mixer RM-2 (Elmi Tech Ltd., Latvia) for 30 min at 4 °C. Serial dilution was made by using 0.1 ml of the resultant suspension. The total heterotrophic bacteria (cultivated in Reasoner 2 media) and cultivable bacteria capable of utilizing P from phytate (phosphate-esters, MM2Phy) and phosphonoacetic acid (MM2PAA) as sole source of P, were quantified via 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

Table 1
Mass balance: mean total grass shoot dry weight (g per column; n = 6) ± standard error and mean grass nutrient content (mg per column). Different letters in the same row indicate significant difference ($P < 0.05$). P added as 0–20 kg ha^{-1} (P0–20).

	Control (P0)	Quarter P (P5)	Half P (P10)	Full P (P20)
Shoot dry weight	$26.4^{a} \pm 1.16$	$26.1^{a} \pm 0.63$	$26.8^{a} \pm 0.51$	$27.9^{a} \pm 0.42$
Calcium	125.37^{a}	126.40^{a}	129.65^{a}	130.22^{a}
Magnesium	48.25^{a}	48.66^{a}	50.67^{a}	50.56^{a}
Sulfur	47.55^{b}	49.38^{ab}	51.99^{a}	52.79^{a}
Nitrogen	172.62^{c}	219.57^{bc}	379.84^{a}	295.06^{ab}
Phosphorus	42.97^{a}	42.53^{a}	46.54^{a}	45.46^{a}
Potassium	518.68^{a}	501.19^{a}	559.74^{a}	552.44^{a}
Boron	0.19^{a}	0.19^{a}	0.20^{a}	0.20^{a}
Copper	0.15^{a}	0.16^{a}	0.15^{a}	0.15^{a}
Molybdenum	0.21^{a}	0.22^{a}	0.23^{a}	0.23^{a}
Iron	1.22^{a}	1.34^{a}	1.16^{a}	2.13^{a}
Zinc	0.24^{a}	0.25^{a}	0.25^{a}	0.26^{a}
Manganese	0.45^{a}	0.38^{a}	0.39^{a}	0.40^{a}

tri calcium phosphate (TCP) agar plates as indicated by a zone of clearance around the colonies (Rondon et al., 2007). After removal of the plant roots, the remaining rhizosphere suspension was centrifuged at 4500 rpm for 15 min at 4 °C and the pellet was stored at −20 °C for molecular studies.

Rhizosphere soil samples were analysed for potential acid phosphatase (ACP) and alkaline phosphatase (ALP) activities following the method of Tabatabai and Bremner (Tabatabai and Bremner, 1969). Assays were conducted by incubating 1 g of soil for 1 h at 37 °C at pH 6.5 (ACP) or 11.0 (ALP) using para-nitrophenyl phosphate (Sigma Aldrich, St. Louis, MO) as a substrate. Samples were filtered with Whatman 42 filter paper, diluted within the range of the standard curve, and the p-nitrophenol colour intensity measured using a spectrophotometer at 420 nm.

At the time of harvest, grass roots were examined for percentage root colonization by arbuscular mycorrhizal (AM) fungi using a modified version of the grid line intersect technique (McGonigle et al., 1990). Briefly, a representative root population were picked and cut into 1 cm segments. The roots were washed with dH_2O, bleached with alkaline H_2O_2 acidified in a 0.1 M HCl solution and stained with a lactoglycerol trypan blue stain (lactic acid: glycerol: H_2O in a 1:1:1 ratio, with 0.05% (w/v) trypan blue). The stained roots were de-stained in lactoglycerol de-stain (minus trypan blue) overnight prior to examination. The root segments were examined under the microscope for the presence of arbuscules and hyphae, one field of view at a time. For each column, 3 replicates of 100 intersections were used to calculate root AM fungal colonization.

Nematodes were extracted from 100 g fresh soil using the modified Baermann method (Whitehead and Hemming, 1965). The nematodes were counted and identified to the genus or family level using a light microscope based on morphological features and identification keys (Andrássy, 1984; Siddiqi, 1986; Bongers, 1988; Jairajpuri and Ahmad, 1992). Nematodes were assigned to feeding groups and abundances were calculated.

2.3. DNA extraction, community fingerprinting and phoD abundance

DNA was extracted from 0.25 g of the frozen rhizosphere soil pellets using the PowerSoil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA) according to manufacturer's instruction. The extracted DNA was quantified using a Qubit dsDNA HS Assay kit (Life Technologies, Carlsbad, CA) and a Qubit Fluorometer (Life Technologies). PCR amplifications of the bacterial 16S rRNA gene fragments for denaturing gradient gel electrophoresis (DGGE) were performed with GC-341F/518R primer pair targeting the V3 region (Muyzer et al., 1993). Each 25 μl reaction contained 1 × buffer (2 mM $MgCl_2$), 0.2 mM dNTP mix, 0.4 mmol of each primer, 0.5 U of DreamTaq polymerase (Fisher Scientific, Waltham, MA) and approximately 10 ng template DNA. A touchdown PCR protocol was used with the following cycling conditions: 20 cycles of 94 °C denaturation (45 s), 65–55 °C touchdown (45 s), 72 °C extension (45 s), plus 18 further cycles with an annealing temperature at 55 °C. DGGE was carried out on 200 × 200 × 1 mm gels in a TV400 DGGE apparatus (Scie-Plas, Cambridge, UK). Gels of 10% (w/v) acrylamide/bisacrylamide were prepared and run as described previously (Fox et al., 2014) using a linear 35–65% gradient in 1 × TAE buffer (60 °C) for 16.5 h at 63 V. After completion, gels were stained in 10,000 times diluted SYBR Gold (Invitrogen, Carlsbad, CA) for 30 min and the image taken on a G:Box (Syngene, Cambridge, UK).

DNA fingerprinting of the fungal community was undertaken using the fungal specific primer ITS-1F (Gardes and Bruns, 1993) and ITS-4 (White et al., 1990). This product was tenfold diluted and used as template in a nested PCR using an ITS-1FGC primer with a 40 base GC clamp to the 50 end of the primer (Bougoure and Cairney, 2005) and ITS-2 reverse primer (White et al., 1990). Both PCRs were undertaken in 25 μl reactions as described previously (Gahan and Schmalenberger, 2015). DGGE was run as before with a gradient of 20–55%.

AM fingerprinting was carried out using the AM specific primer AM1 (Helgason et al., 1998) alongside the universal eukaryotic primer NS31 (Simon et al., 1992) targeting the 18S rRNA gene. The PCR product was diluted tenfold and used as template for a nested PCR using the primer set Glo1 (Kowalchuk et al., 2002) and NS31-GC (Cornejo et al., 2004). Both PCR conditions were as described previously (Schmalenberger and Noll, 2014). DGGE was run as before with a gradient of 25–50%.

DGGE images were analysed using the phoretix 1D software (Totallab, Newcastle, UK). The binary gel image matrices generated were used for canonical correspondence analysis (CCA) biplots. Permutation tests (Monte Carlo) were conducted (9999 repeats) in CANOCO (Microcomputer Power Inc., Ithaca, NY) to identify environmental properties affecting the community structures as well as significant

Table 2
MPN values of phytate mobilizing (MM2Phy) and phosphonoacetic acid mobilizing (MM2PAA) bacteria, total heterotrophic bacteria (R2A), CFU values of tri calcium phosphate solubilizing (TCP) bacteria, mean abundances of bacterial-feeding (BF) and fungal-feeding nematodes (FF), arbuscular (AC) and hyphal colonization (HC) rates of rye grass roots, alkaline (ALP) and acid phosphatase (ACP) activities as well as the bacterial *phoD* gene copy number per gram of soil (phoD) normalized for DNA extraction determined via qPCR. Different letters in the same row indicate significant differences ($P < 0.05$); ±represents standard error; replicates of six after 98 days of incubation; P added as 0–20 kg ha^{-1} (P0–20).

	Control (P0)	Quarter P (P5)	Half P (P10)	Full P (P20)
MM2Phy	$5.9 \times 10^{6a} \pm 2.2 \times 10^{6}$	$5.8 \times 10^{6a} \pm 1.2 \times 10^{6}$	$5.7 \times 10^{6a} \pm 1.2 \times 10^{6}$	$4.6 \times 10^{6a} \pm 6.1 \times 10^{5}$
MM2PAA	$1.0 \times 10^{6a} \pm 3.7 \times 10^{5}$	$9.8 \times 10^{5a} \pm 2.9 \times 10^{5}$	$9.5 \times 10^{5a} \pm 2.7 \times 10^{5}$	$1.1 \times 10^{6a} \pm 2.4 \times 10^{5}$
TCP	$4.2 \times 10^{5a} \pm 1.7 \times 10^{5}$	$3.7 \times 10^{5a} \pm 4.5 \times 10^{4}$	$3.4 \times 10^{5a} \pm 3.6 \times 10^{4}$	$4.3 \times 10^{5a} \pm 6.1 \times 10^{4}$
R2A	$2.1 \times 10^{7a} \pm 5.0 \times 10^{6}$	$2.3 \times 10^{7a} \pm 7.5 \times 10^{6}$	$1.3 \times 10^{7a} \pm 2.2 \times 10^{6}$	$1.9 \times 10^{7a} \pm 2.2 \times 10^{6}$
BF (No./100 g soil)	$426^{a} \pm 47$	$278^{b} \pm 20$	$179^{b} \pm 27$	$214^{b} \pm 28$
FF (No./100 g soil)	$78^{a} \pm 16$	$64^{ab} \pm 8$	$36^{b} \pm 6$	$32^{b} \pm 4$
AC (%)	$57.5^{a} \pm 3.4$	$49.4^{ab} \pm 8.5$	$40.6^{ab} \pm 7.6$	$26.4^{b} \pm 3.2^{b}$
HC (%)	$61.4^{a} \pm 5.5$	$58.6^{a} \pm 7.7$	$46.4^{ab} \pm 6.4$	$27.5^{b} \pm 2.9$
ALP (μgPNP/g soil/h)	$324.3^{b} \pm 38.4$	$305.5^{b} \pm 26.5$	$432.4^{ab} \pm 40.8$	$495.9^{a} \pm 35.3$
ACP(μgPNP/g soil/h)	$346.6^{b} \pm 18.2$	$344.1^{b} \pm 9.9$	$378.9^{b} \pm 10.8$	$452.3^{a} \pm 12$
phoD	$5.5 \times 10^{4a} \pm 7.9 \times 10^{3}$	$4.6 \times 10^{4ab} \pm 3 \times 10^{3}$	$4.0 \times 10^{4ab} \pm 6.3 \times 10^{3}$	$2.5 \times 10^{4b} \pm 3.4 \times 10^{3}$

differences between the samples as described by Noll and Wellinger (Noll and Wellinger, 2008).

Absolute quantification of bacterial *phoD* genes was carried out from extracted DNA (see above) by amplifying with primers ALPS-F730 (5'-CAGTGGGACGACCACGA GGT-3′) and ALPSR1101 (5'-GAGGCCGATCG GCATG TCG-3′) (Sakurai et al., 2008) on a LightCycler 480 Real-time PCR System (Roche, Basel, Switzerland). The PCR reactions were prepared with 5 μl of KAPA SYBR FAST qPCR Universal Master Mix (Kapa Biosystems, Boston, MA), 3 pmol of each primer, and 1 μl template (10 ng/μl) in a 10 μl reaction. The cycling conditions used were: enzyme activation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 3 s, annealing at 65–57 °C for 20 s (touchdown, −1 °C per cycle) and an extension at 72 °C for 20 s. Samples, standards (10^2–10^4 molecules per reaction) and non-template controls were run in triplicates on 96-well microtiter plates. To check the specificity of the reaction, a melt curve analysis was conducted immediately following the run.

2.4. Next generation sequencing (NGS), bioinformatics pipeline and sequence data analysis

Sequencing was performed at the Novogene Bioinformatics Technology Co., Ltd. Briefly, DNA was amplified using the 341F/806R primer set (341F: 5′- CCTAYGGGRBGCASCAG -3′, 806R: 5′-GGACTACNNGGGT ATCTAAT -3′), which targets the V3-V4 region of the bacterial 16S rDNA. 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). Sequencing libraries were generated using a TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an IlluminaHiSeq2500 platform and 250 bp paired-end reads were generated.

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (Magoč and Salzberg, 2011). Quality filtering on the raw tags was performed under specific filtering conditions to obtain high-quality clean tags (Bokulich et al., 2013) following the QIIME (Caporaso et al., 2010b) quality controlled process.

Tags were compared with the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using the UCHIME algorithm (Edgar et al., 2011). Sequence analyses were performed by Uparse software (Edgar, 2013). Sequences with ≥97% similarity were assigned to the same OTUs. The GreenGene Database (DeSantis et al., 2006) was used for OTU screening based on RDP classifier (Wang et al., 2007) algorithm. Multiple sequence alignments were conducted using the PyNAST software (Caporaso et al., 2010a) to study phylogenetic relationship of different OTUs.

Alpha and beta diversities were calculated with QIIME (Version 1.7.0). Principal coordinates analysis (PCoA) was performed to get principal coordinates. PCoA analysis was displayed with the WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3). Heat maps were created in R to display the abundance distribution of dominant taxa among the treatments. Nucleic acid sequences were deposited in the Nucleotide Archive (Project: PRJEB20744, ERS1802324-ERS1802347).

2.5. Statistical analyses

Prior to statistical analyses, data on grass elemental composition were converted to unit per column basis in order to capture the total dry matter yield and mass balances. The grass dry matter yield and elemental composition, Morgan's P, soil pH, MPN, CFU, phosphatase activity, percentage root mycorrhizal colonization, abundance of nematode feeding groups, *phoD* gene abundance, and alpha diversity indices were analysed using a one-way ANOVA in R software (Version 2.15.3). Statistical significance was tested at $P < 0.05$. Shapiro-Wilk's and

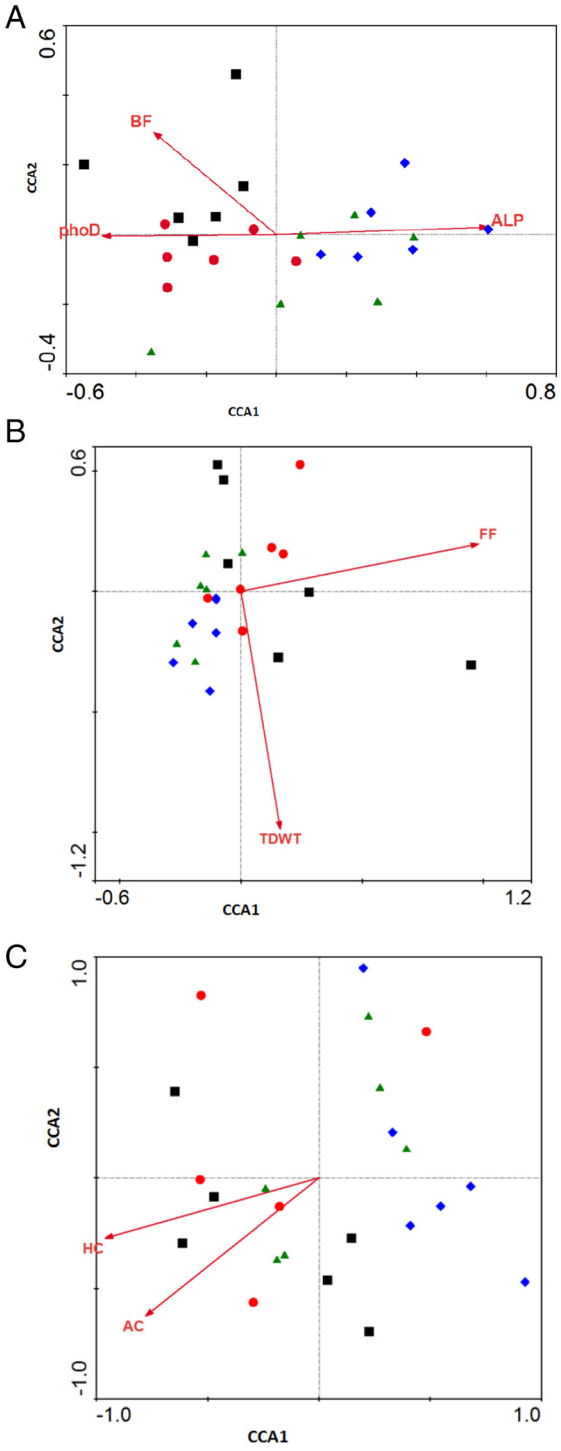

Fig. 1. CCA biplots showing the effects of P treatments on A) 16S rRNA gene B) Fungal ITS and C) AM fungal 18S rRNA with bacterial-feeding nematodes abundance (BF), bacterial phoD gene abundance (phoD), alkaline phosphatase (ALP), fungal-feeding nematodes abundance (FF), total grass shoot dry weight (TDWT), and root mycorrhizal arbuscular and hyphal colonization rates (AC, HC) defined as environmental factors. Black squares = control, red circles = quarter P (P5), green triangles = half P (P10) and blue diamonds = full P (P20) ($n = 6$; P added as 0–20 kg ha^{-1} = P0–20). 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 phosphate fertilization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Levene's tests were used to check data for normality and homogeneity of variance respectively. Tukey's HSD post-hoc test was applied for pairwise comparisons to assess any significant differences ($P < 0.05$) between treatment means, when normality and homogeneity of variance was confirmed. Data violating the model assumptions were logarithmically transformed, analysed by ANOVA and the back transformed values to the original scale were reported. Where homogeneity of variance was not achieved, the Games-Howell test was used and where both assumptions of ANOVA were not satisfied, the Kruskal-Wallis test was performed instead.

3. Results

3.1. Soil solution chemistry, grass dry matter yield and elemental composition, Morgan's P and soil pH

The weekly measurements of phosphate in soil solution at four different soil depths for the duration of 14 weeks showed no significant differences between the treatments ($P > 0.05$) and stayed between 2 ppm and the detection limit of the ion chromatography system (0.5 ppm). The soil solution pH ranged between 6.92 and 8.34. The grass dry matter yield tended to increase with increasing P fertilization, albeit not at statistically significant rates ($P > 0.05$, Table 1). The analyses of the elemental composition of the grass dry matter revealed that while all treatments had comparably similar uptake of P (not significantly different, $P > 0.05$) that represented 6.9–7.5% of the total P in the soil columns (42–47 mg P). The half and full P treatments had a significantly higher ($P < 0.05$) uptake of N and S compared to the control (see mass balances in Table 1).

After 14 weeks of cultivation (destructive harvest) the mean concentrations of the available P as extracted by the Morgan's solution in the 0–20 cm depth of the columns were 2.62, 2.71, 2.64 and 2.69 mg L^{-1} for the control, quarter, half and full P treatments respectively. In the 20–40 cm depth of the columns, the Morgan's extractable P concentrations were 2.87, 2.89, 2.93 and 3.09 mg L^{-1} for the control, quarter, half and full P treatments respectively. There were no significant differences in these concentrations between the various treatments. The phosphate fertilization had no significant effect on the soil pH ($P > 0.05$) at time of harvest. The soil pH values were just slightly above neutral ranging between 7.17 and 7.26.

3.2. Effect of phosphate treatments on abundance of cultivable bacteria, phosphatase activity, mycorrhizal root colonization rates and abundance of nematodes

The alkaline phosphatase (ALP) and acid phosphatase (ACP) activities increased alongside the levels of P fertilization. While the control to half P treatments had statistically similar ALP and ACP activities, the full P treatment had significantly higher activities compared to the control ($P < 0.05$) representing a 50% increase for the ALP and a 30% increase for the ACP (Table 2). The abundances of cultivable heterotrophic, calcium-phosphate-, phytate- and phosphonate utilizing bacteria ranged from $1.3–2.3 \times 10^7$ MPN g^{-1}; $3.4–4.3 \times 10^5$ CFU g^{-1}; $4.6–5.9 \times 10^6$ MPN g^{-1} and $9.5 \times 10^5–1.1 \times 10^6$ MPN g^{-1} respectively. There was no statistically significant difference ($P > 0.05$) in the abundances of cultivable heterotrophic, calcium-phosphate-, phytate- and phosphonate-utilizing bacteria upon P fertilization (Table 2).

Lolium perenne roots of the full P treatment had significantly lower hyphal and arbuscular mycorrhizal colonization rates ($P < 0.05$; Table 2) than the control. Colonization rates were 61.4% for control whereas for full P treatment, the colonization rate was 27.5%. Likewise, the percentage of arbuscules found in the roots was greatly reduced from 57.5% in the controls down to 26.4% in the full P columns.

Similarly to the mycorrhizal hyphae, the abundance of bacterial- and fungal-feeding nematodes were significantly higher in the control compared to the P treatments ($P < 0.05$) with both nematode groups being twice as abundant in the controls over the full P treatment (Table 2). The higher abundance of bacterial-feeding nematodes in the control was as a result of higher prevalence of bacterial-feeding nematodes belonging to the Cephalobidae, Plectidae and Rhabditidae families in these samples (Supplementary table S1).

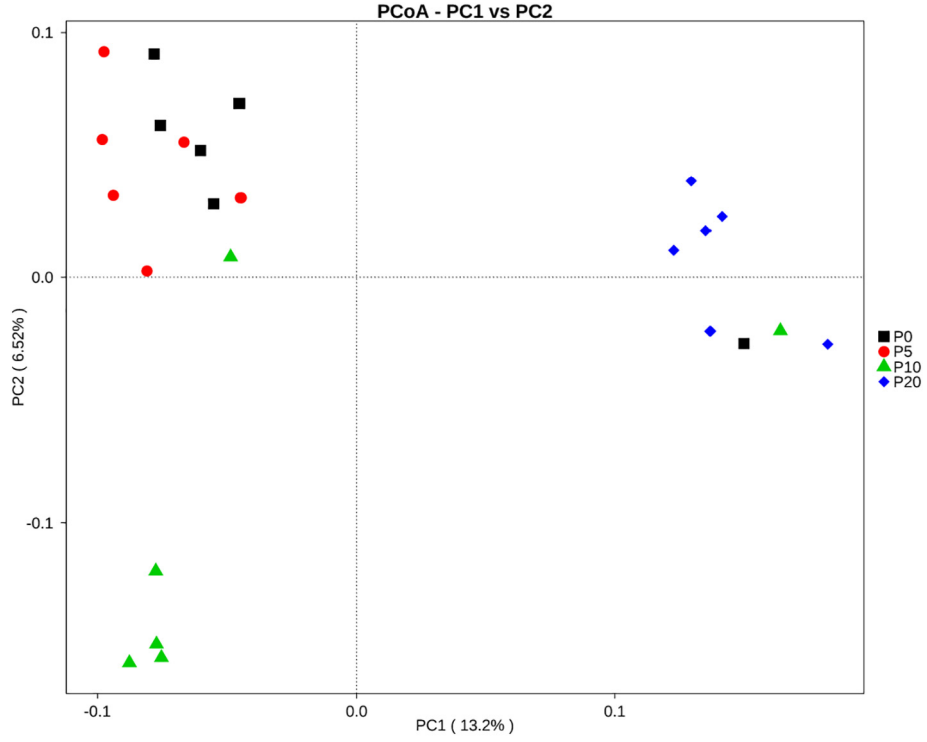

Fig. 2. Principal coordinates analysis (PCoA) of the bacterial community sequences based on 16S rRNA. PCoA was calculated using the unweighted Unifrac distance matrix (n = 6; P added as 0–20 kg ha^{-1} = P0–20).

3.3. Bacterial, fungal and AM fungal community fingerprints

The bacterial community structure was analysed via DGGE fingerprints of the 16S rRNA gene fragments. After canonical correspondence analysis (CCA) of the binary matrix, the resulting biplot revealed that the control and the quarter P treatments were indistinguishable on the first axis. However, on the second axis, both treatments only partially overlapped (Fig. 1a). In contrast, the full P treatment was clearly

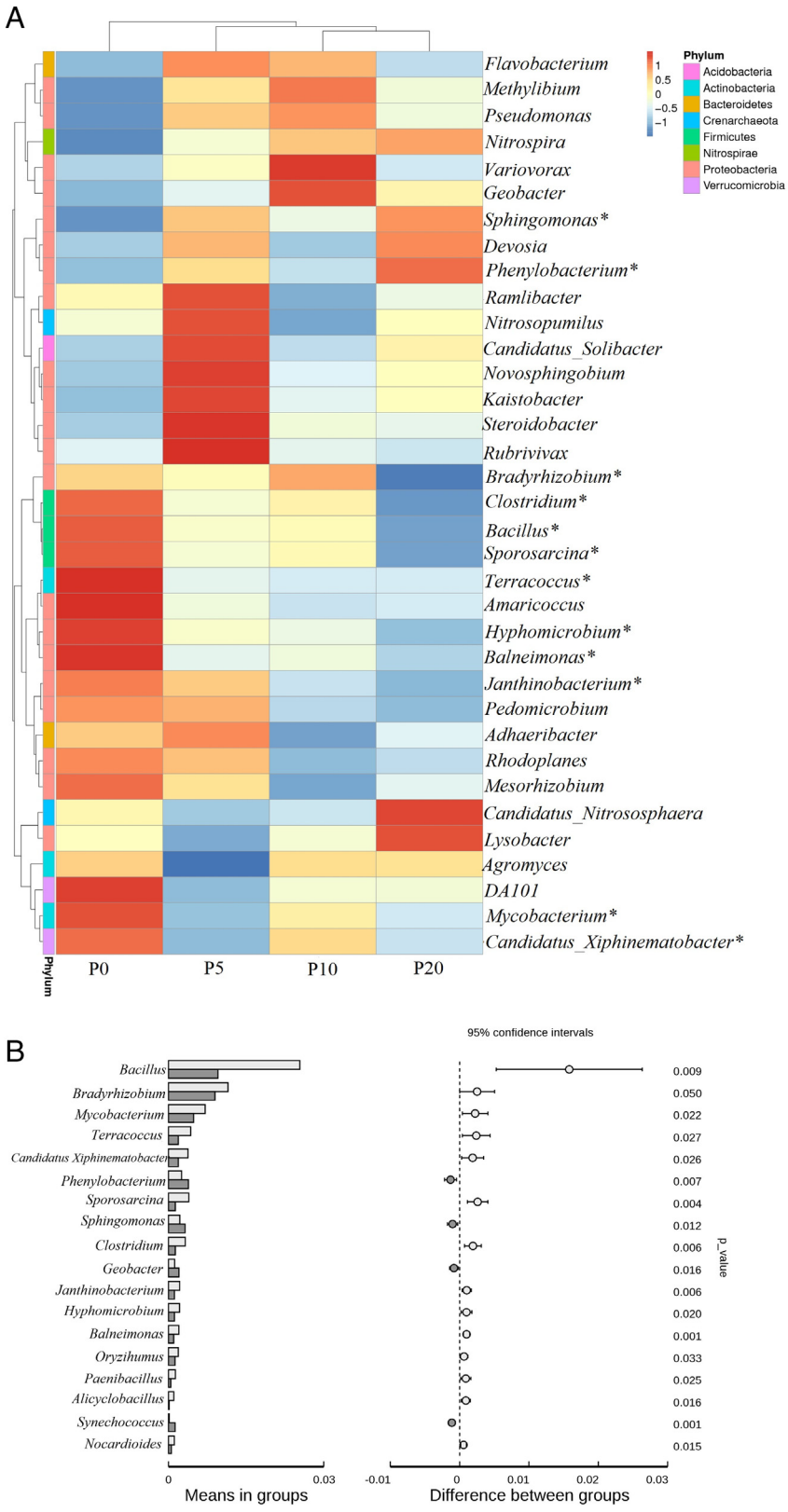

Fig. 3. a) Cluster heatmap of genera based on the 16S rRNA gene sequence analyses; and b) *t*-test bar plots of differences in relative abundances of genera between P0 (control, light grey) and P20 (full P, dark grey). Asterisks (*) indicate significant differences in relative genera abundance between P0 and P20 (n = 6; P added as 0–20 kg ha^{-1} = P0–20).

separated from the control and quarter P treatment on the first axis, while the half P treatment partially overlapped with the quarter and full P treatments (Fig. 1a). Permutation tests of the bacterial DGGE community profile confirmed a significant separation between the control and all P treatments ($P < 0.05$). There were also significant differences among the quarter, half and full P treatments. CCA and permutation analysis of the bacterial community profiles revealed a significant correlation of ALP activity, abundance of bacterial-feeding nematodes (BF) and *phoD* copy numbers (phoD) with the bacterial community structure (see Section 3.4 below).

The community structure of the higher fungi was analysed via DGGE fingerprints of the ITS region. The resulting CCA biplot revealed a clear separation between the control and the P treatments on the first axis (Fig. 1b). Permutation tests showed a highly significant shift in the fungal community structure upon P fertilization ($P < 0.01$). This shift was significantly associated with the abundance of fungal-feeding nematodes (FF) and total shoot dry weight (TDWT) ($P < 0.05$). Significant differences were also detected between the P treatments.

The community structure of the AM fungi analysed using DGGE fingerprints targeting the 18S rRNA gene revealed a significant shift ($P < 0.05$) in the AM fungal community structure upon P fertilization as visualised via the CCA biplot (Fig. 1c). This shift was similar to those obtained in the 16S and ITS profiles. Unlike the higher fungal community structure, the control was less well separated from the quarter and half P treatments. In contrast, control and full P treatments were well separated on the first axis. This shift was significantly ($P < 0.05$) correlated with the mycorrhizal arbuscular (AC) and hyphal colonization (HC) rates as revealed by permutation tests.

3.4. Gene abundance of phoD

The full P treatment had significantly lower copy numbers of *phoD* compared to the control which were about half of that of the control ($P < 0.05$; Table 2). However, the control, quarter and half P treatments had no statistically different gene copy numbers of *phoD* ($P > 0.05$) ranging from 4 to 5.5 × 10^4 copies per gram of dry soil. No relationship was observed between *phoD* gene abundance and Morgan's extractable soil P ($P = 0.98$, $r = 0.007$).

3.5. Bacterial diversity and community composition based on next generation sequencing

Sequences of the 16S rRNA gene (amplicons) allowed the estimation of alpha and beta diversities of the treatments using QIIME. The alpha diversity in the experiment ranged from an average of 9.90 to 10.09 (Shannon) or 3190 to 3834 (chao1), respectively (Supplementary table S2). In contrast, the Simpson diversity index was very similar for all treatments (0.998). Most of the alpha diversity indices were not statistically different among the various treatments except for the Shannon index which was significantly higher in the full P treatment compared to the quarter and half P treatments.

Principal coordinate analysis (PCoA, unweighted Unifrac distance matrix) revealed a clear separation between the full and half P treatment from the control and quater P columns (beta-diversity; Fig. 2). However, the separation between the control and the quarter P treatment was less pronounced. Moreover, linear discriminant analyses (LDA) revealed that bacteria belonging to *Actinobacteria, Firmicutes* and *Proteobacteria* phyla were more abundant in the control P treatments while the half and full P treatments members of the *Acidobacteria* phylum were more abundant (Supplementary fig. S1). Furthermore, the control treatment had a significantly higher ($P < 0.05$) abundance of genera belonging to *Bacillus*, *Bradyrhizobium*, *Mycobacterium*, *Terracoccus, Candidatus Xiphinematobacter, Sporosarcina, Clostridium, Janthinobacterium, Hyphomicrobium, Balneimonas, Oryzihumus, Paenibacillus, Alicyclobacillus* and *Nocardioides* than in the full P treatment (Fig. 3a and b). The abundances of genera such as *Bacillus*, *Bradyrhizobium*, *Balneimonas, Terracoccus, Paenibacillus,* and *Nocardioides* that have been identified to mobilize P in soils were significantly lower in the full P treatment compared to the control.

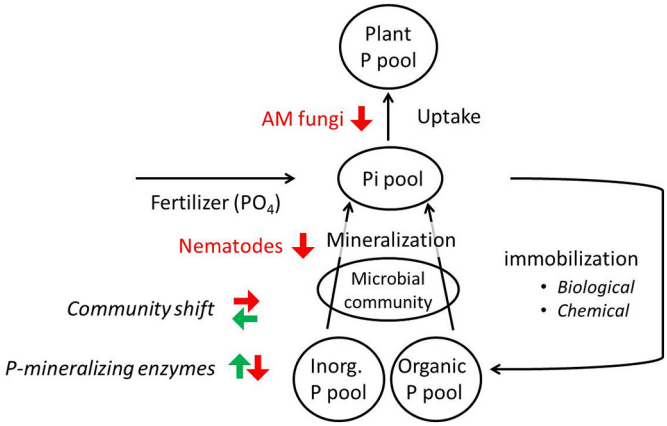

Fig. 4. Conceptual model of P cycling in the soil columns.

4. Discussion

The influence of one-time phosphate fertilization on soil biota involved in P cycling and rye grass yield was investigated in this study. At the outset we hypothesized that without P or with reduced P fertilizer inputs, limitation of P stimulates the microbial mobilization of P otherwise not directly accessible to plants. Consequently, a lack of an increased plant yield after phosphate fertilizer applications may not only be linked to the soil chemistry but also to the soil biology. This is aligned with the emerging hypothesis in the scientific community that soil microbes play important roles in plant P availability by tapping into the P reserves of soils with low amounts of labile inorganic P. For instance, Mander et al. (2012) using culture-dependent studies showed that low P soils induce selective pressure that may lead to selection of bacteria involved in P mineralization. Also, Jorquera et al. (2013) demonstrated that addition of phytate to soil induced changes in the abundance and expression of P cycling genes in *Bacillus*. Statistically equivalent plant growth, yield and nutrient (particular N and P) uptake have been reported previously when 75% of recommended fertilizer rates were supplemented with plant growth promoting rhizobacteria and AM fungi (Adesemoye et al., 2009). Here, the one-time application of 20 kg ha^{-1} P as phosphate failed to result in a statistically significant plant growth response. Instead a significant response by the soil microbiota was detected.

In the current study, the abundances of bacterial- and fungal-feeding nematodes were significantly lower in the full P treatment compared to the control. A paucity of studies looked into the effect of mineral P fertilization on free-living soil nematodes until now. High mineral P addition to soil can harm soil nematodes as a result of sensitivity to salt toxicity, thus negative effects of P addition on the soil nematode community have been reported (Zhao et al., 2014). The influence of soil nematodes on biological cycling of nutrients is linked to their feeding habits (Ritz and Trudgill, 1999). Both bacterial- and fungal-feeding nematodes have been reported to increase P availability for plants through the mineralization of organic P in bacterial and fungal biomass (Perez-Moreno and Read, 2001; Irshad et al., 2011; Gebremikael et al., 2016).

Similar to the nematode response, phosphate addition has been reported to supress mycorrhizal symbiosis (Bentivenga and Hetrick, 1992; Baum and Makeschin, 2000). Higher AM colonization rates have been reported to compensate for reduced soil P fertility so that dry matter was not affected by P fertilization rates (Nayyar et al., 2008). In this study, significantly higher root arbuscular and hyphal colonization rates were found in the control compared to the full P treatment. This could be the result of the ongoing plant P limitation leading to increased

formations of mycorrhizal associations. AM fungi are greatly involved in the mobilization and supply of nutrients such as P and N to their plant hosts, where the mycorrhizal symbiont receives plant derived photosynthesized carbon in return (Smith and Read, 2008; Jansa et al., 2011). The mycorrhizal hyphal network extends the root surface area and the fungal hyphae penetrate into microsites within the soil matrix, inaccessible to the plant roots (Smith and Read, 2008). Hyphae-colonizing bacteria have been shown to contribute to S mobilization (Gahan and Schmalenberger, 2015) and thus hyphosphere bacteria may also contribute to the mobilization of P.

Bacterial 16S rRNA sequences as well as the corresponding fingerprints revealed a separation of the half and full P treatments from the control and quarter P treatments. In depth analysis of the OTUs via LDA revealed that the phyla *Actinobacteria*, *Firmicutes* and *Proteobacteria* were distinctly more abundant in the control and quarter P treatments. These bacterial phyla have been reported previously to contain *phoD* genes (Tan et al., 2013; Ragot et al., 2015). Specifically, there were higher abundances of *Bacillus*, *Bradyrhizobium*, *Mycobacterium*, *Terracoccus, Candidatus Xiphinematobacter, Sporosarcina, Clostridium, Janthinobacterium, Hyphomicrobium, Balneimonas, Oryzihumus, Paenibacillus, Alicyclobacillus* and *Nocardioides* genera in the control than in the full P treatment.

Bacillus and *Paenibacillus* have been reported to carry β-propeller phytase encoding genes with the ability to degrade phytate thereby playing significant roles in rhizospheric organic P mobilization (Jorquera et al., 2011; Mander et al., 2012; Sharma et al., 2013). Likewise, strains belonging to *Bacillus* have been named as powerful phosphate solubilizers (Rodriguez and Fraga, 1999). *Balneimonas* and *Bradyrhizobium* belonging to *Bradyrhizobiaceae* produce organic acids and/or phosphatases giving them the ability to solubilize organic P compounds thereby releasing phosphate into soil solution for plant uptake (Mander et al., 2012; Sharma et al., 2013; Fraser et al., 2015). In addition, several *Bradyrhizobium* strains appear to have the capacity to cleave phosphorus directly from carbon via the C-P lyase pathway (Fox et al., 2014). *Nocardioides* and *Terracoccus* have been shown to accumulate phosphate in their cell wall polymers which may improve the availability of phosphate to plants upon cell death (Prauser et al., 1997; Mander et al., 2012).

In the present study, P fertilization significantly reduced the abundance of *phoD* per gram of soil. These findings concur with the changes in the bacterial community structures. Indeed, P fertilization has been reported to reduce the diversity of the *phoD* gene in barley rhizosphere soil (Chhabra et al., 2013). In contrast, no significant changes in *phoD* abundance and expression was measured in a pot experiment with ryegrass when mineral P fertilizer was added (Fraser et al., 2015). Absolute quantification of the acid phosphatase gene *phoC* in this study returned only low copy numbers close to the qPCR detection limit without any significant differences among the treatments (data not shown).

In this study, increased potential alkaline and acid phosphatase enzyme activities (ALP, ACP) were measured under full P fertilization at the time of harvest. There seems to be some inconsistences in the literature on the effect of phosphate fertilization on potential phosphatase activities. While it is reported in some studies that application of mineral P inhibits phosphatase activities (Saha et al., 2008), some others have reported increased ALP activity (Kanchikerimath and Singh, 2001) or no response of ALP activity with increasing P fertilization (Fraser et al., 2015). The inability of the phosphate fertilization to inhibit phosphatase activity may be due to the fact that the enzyme assay measures extracellular phosphatase stabilized on soil particles or phosphatase released constitutively by microbial cells rather than measuring phosphatase activity due to P starvation alone (Nannipieri et al., 2011; Fraser et al., 2015). In soil sulfur (S) cycling, recently formed organo sulfur is quicker re-mineralized than more distantly formed bound S (Ghani et al., 1993). The results from this study suggest that this may be also the case for P. Therefore, the phosphate fertilization may have resulted in higher immobilization rates immediately after the fertilizer application event. This was then followed by higher re-mineralization rates of the recently formed organo phosphorus towards the end of the experiment resulting in higher phosphatase activity being measured in the full P treatment at the time of harvest. A continuous measurement of ALP and ACP for the duration of the column experiments to monitor the dynamics of these enzyme activities would have had the potential to resolve this question. Indeed, the authors of this study attempted to answer this by conducting microcosm (so called rhizobox) experiments where the front panel was removable for repeated sampling. However, in these microcosms the increase in enzymatic activity over the course of the microcosm experiment was overriding any differences between the treatments (data not shown).

In the current study, the soil solution did not differ in phosphate concentration among the treatments. It is likely that a large proportion of the added phosphate was adsorbed to soil particles, fixed into less available P fractions, immediately taken up by grass roots and/or immobilized by soil microbes. Microbial activity in the control may have released phosphorus into soil solution over the course of the experiment that is equivalent to the applied P fertilizer as the amount of P been taken out of the columns via plant biomass is equivalent to the total P application in the full P treatment. Indeed, the arbuscular mycorrhizal colonization rate, abundance of bacterial- and fungal-feeding nematodes, *phoD* gene abundance and abundance of certain genera of P cycling bacteria were significantly higher in the control than in the full P treatment.

Unlike many previous investigations, this study reports on the effects of single phosphate fertilization on the prokaryotic and eukaryotic microbes as well as nematodes (abundances, community structure, activity and function) in low P index grassland soils (Fig. 4). In conclusion, this study has shown that one-time application of phosphate fertilizer affects directly and indirectly the abundance, diversity and taxonomic composition of soil microbiota (significant changes: bacteria, fungi, AM fungi, nematodes) which has an impact on the ecosystem services of P cycling. This in turn resulted in similar grass growth between the control and P application rate of 20 kg ha^{-1}. The data reported here support the hypothesis that limited P application to soils leads to a better functioning soil microbiome in relation to P mobilization. In this study, a single phosphate application was not beneficial for plant growth as the abundance of bacterial and fungal feeding nematodes and arbuscular mycorrhization dropped. At the same time, a build-up of P from repeated fertilization was not established in the soil columns that the plants could have accessed. While higher amounts of phosphate may have been taken up by the roots within seven days of application, the reduced mycorrhization may have resulted in reduced P uptake for the remainder of the study. A reduction in bacterial and fungal feeding nematodes may have reduced the amount of microbial P being mineralized. A shift in the bacterial and fungal community structure may have been the result of the phosphate application directly in the form of altered P mineralization as well as the reduced predation and loss of habitat e.g. hyphosphere (Fig. 4). Consequently, a positive effect of a single inorganic P fertilizer application on plant growth in a soil was largely cancelled out by its negative effect on the soil microbiota as outlined in Fig. 4. These findings support the hypothesis that soil microbiota provide an important ecosystem service in plant P supply in low P index soils. Future research should investigate the response of the soil microbiome to long-term build-up of P reserves in soils via repeated fertilizer applications to find out if the biota will recover and adapt to fertilization events.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.02.263.

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