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Total and active microbial communities and *phoD* as affected by phosphate depletion and pH in soil

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Abstract

Background and Aims Soil microbial communities contribute to organic phosphorus cycling in a variety of ways, including secretion of the PhoD alkaline phosphatase. We sampled a long-term grassland fertilization trial in Switzerland characterized by a natural pH gradient. We examined the effects of phosphate depletion and pH on total and active microbial community structures and on the structure and composition of the total and active *phoD*-harboring community.

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Methods Archaeal, bacterial and fungal communities were investigated using T-RFLP and *phoD*-harboring members of these communities were identified by 454-sequencing.

Results Phosphate depletion decreased total, resin-extractable and organic phosphorus and changed the structure of all active microbial communities, and of the total archaeal and *phoD*-harboring communities. Organic carbon, nitrogen and phosphorus increased with pH, and the structures of all total and active microbial communities except the total fungal community differed between the two pH levels. *phoD*-harboring members were affiliated to *Actinomycetales*, *Bacilliales*, *Gloeobacterales*, *Planctomycetales* and *Rhizobiales*.

Conclusions Our results suggest that pH and associated soil factors are important determinants of microbial and *phoD*-harboring community structures. These associated factors include organic carbon and total nitrogen, and to a lesser degree phosphorus status, and active communities are more responsive than total communities. Key players in organic P mineralization are affiliated to phyla that are known to be important in organic matter decomposition.

Keywords 454-sequencing · Grassland · Alkaline phosphatase · DNA · RNA · T-RFLP

Abbreviations

ACP Potential acid phosphatase activity
ALP Potential alkaline phosphatase activity
MicC Microbial C
MicN Microbial N

MicP	Microbial P
P	Phosphorus
P _{org}	Organic P
P _{res}	Resin-extractable P
TN	Total N
TOC	Total organic C
TP	Total P

Introduction

Soils sustain complex and diverse microbial communities that are intimately associated with their habitat. Soil properties and vegetation fundamentally shape the microbial community by affecting its composition, diversity and functioning (Berg and Smalla 2009; Dequiedt et al. 2011). Within the soil microbial community, archaea, bacteria and fungi are influenced differently by soil properties and by the plants present. Among the soil properties, soil pH has repeatedly been shown to be the primary environmental driver influencing the total bacterial community structure studied at DNA level (Fierer and Jackson 2006; Griffiths et al. 2011; Lauber et al. 2008). The total fungal community has been reported to be less responsive to pH changes than the total bacterial community (Blagodatskaya and Anderson 1998; Wakelin et al. 2008), and only some specific groups of archaea such as ammonia oxidizers have been shown to change with pH (Nicol et al. 2008). Specific taxonomic groups such as the bacterial groups *Acidobacteria*, *Bacteroidetes* and *Firmicutes*, and the fungal *Helotiales*, *Hypocreales* and *Mitosporic Basidiomycetes* have been reported to be highly responsive to pH (Lauber et al. 2009; Rousk et al. 2010). However, the effect of pH on the active community of these microorganisms (studied at RNA level) remains unclear.

Soil pH also affects the availability of nutrients such as phosphorus (P) by modifying adsorption and desorption reactions (Frossard et al. 1995). This makes it difficult to distinguish the effect of P availability on microbial communities from that of pH. P is often a limiting nutrient for plants and microorganisms in soil (Vitousek et al. 2010) and phosphate fertilizer is therefore commonly applied to agricultural soils to maintain crop productivity. Phosphate addition can lead to a shift in the microbial community composition (Mander et al. 2012; Siciliano et al. 2014). Archaea and bacteria are

generally thought to be more responsive than fungi to water-soluble phosphate addition (Bissett et al. 2011; Cruz et al. 2009). Under low P availability, microorganisms up-regulate the genes of the *Pho* regulon, which includes genes encoding phosphatases, phosphate transporters and other systems for mobilizing soil P (Vershinina and Znamenskaya 2002). Phosphatases can potentially hydrolyze up to 89 % of the total organic P extracted from soil (Jarosch et al. 2015).

The *PhoD* alkaline phosphatase is found in a range of environments (Luo et al. 2009; Tan et al. 2013) and is mainly produced by bacteria (Ragot et al. 2015). It hydrolyzes phosphomonoesters and –diesters excluding inositol phosphates such as phytate (Kageyama et al. 2011), which represent an important fraction of organic P in soil (Condrón et al. 2005). The few studies addressing the diversity, total community structure and composition of the *phoD* gene report effects of organic and water-soluble phosphate fertilizers, crop management, vegetation and pH (Chhabra et al. 2012; Jorquera et al. 2014; Ragot et al. 2015; Sakurai et al. 2008; Tan et al. 2013; Wang et al. 2012a). Effects of soil pH on the total *phoD*-harboring community have been reported in studies in which organic C and available P increased together with pH (Cui et al. 2015; Wang et al. 2012b). Using pyro-sequencing, dominant *phoD*-harboring community members have been shown to be affiliated primarily to *Alpha*- and *Gamma-Proteobacteria* but also to *Acidobacteria* and *Cyanobacteria* (Tan et al. 2013; Fraser et al. 2015). However, these studies used a set of primers (ALPS primers; Sakurai et al. 2008) that has been shown to have an amplification bias towards *Alpha-Proteobacteria* and to cover the *phoD* diversity only partly (Tan et al. 2012; Ragot et al. 2015).

This study aimed to clarify the effect of phosphate depletion and soil pH on microbial communities in a long-term fertilization trial on a permanent grassland characterized by a pH gradient and low P availability (Bünemann et al. 2012). To evaluate the effect of phosphate depletion, we compared microbial communities in plots fertilized with N, P and K, which maintained soil P stocks, with plots fertilized with N and K only, which resulted in phosphate depletion (Liebisch et al. 2013). To evaluate the effect of pH, we sampled plots immediately adjacent to the fertilization trial, which had two different pH values. The total and active archaeal, bacterial, fungal and *phoD*-harboring community structures were examined, and related to soil and plant properties using multivariate analysis, allowing us to separate the

effects of phosphate depletion and pH. In addition, we analyzed the composition of the total and active *phoD*-harboring communities to identify the key microorganisms that harbor and/or express the *phoD* gene. It has been shown that the total and active microbial community can differ significantly during decomposition processes, as only some members of the total community (especially *Acidobacteria*, *Actinobacteria* and *Proteobacteria*) are metabolically active (Baldrian et al. 2012). We therefore expected stronger expression of the *phoD* gene by these taxonomic groups than by other *phoD*-harboring organisms, and the active community structures to differ more between treatments than the total community structures.

Material and Methods

Site description and sampling

Soils were sampled in September 2012 from a long-term fertilization trial (1992–2014) at the Agroscope research station near Watt in the Swiss lowland (47°26'44"N, 8°29'32"E, 500 m asl) (Huguenin-Elie et al. 2006). The vegetation consists of a permanent grassland of *Arrhenatherion elatioris* association with 30 to 40 angiosperm species (Liebisch et al. 2013), which is harvested as hay three times per year with a late first harvest (after 15th June) to preserve plant diversity. The mesic grassland grows on a medium-deep (50–70 cm) Cambisol (WRB 2014) with a medium base saturation. The soil in the upper 10 cm consists of 220, 340 and 440 g kg⁻¹ clay, silt and sand, respectively (Philipp et al. 2004).

The fertilization trial has a randomized block design with four replicates and a plot size of 2 m × 5 m (Fig. 1). The treatments represent different combinations of mineral N, P and K additions. In this study, we selected two treatments: one fertilized with N, P and K (NPK) and one fertilized with N and K only (NK). The amounts added were 45 kg N ha⁻¹ yr⁻¹ as ammonium nitrate, 17 kg P ha⁻¹ yr⁻¹ as single superphosphate and 83 kg K ha⁻¹ yr⁻¹ as potassium chloride. The phosphate input in NPK plots was adequate to reach a zero P balance, while phosphate depletion via the negative P balance of -10 kg P ha⁻¹ yr⁻¹ in NK decreased P availability (Liebisch et al. 2013).

Previous work indicated the presence of a pH gradient along the field that affects mainly one NPK plot

(Southwest corner plot in block IV; Fig. 1), which has a higher pH than the other NK and NPK plots (Liebisch et al. 2013). In order to assess the effect of pH, an additional set of samples was taken in the border strip next to the original field trial at both the lower (pH 5.0) and the higher (pH 6.1) pH end of the trial by extending each block by one plot on each side as indicated in Fig. 1. This border strip has not been fertilized since 1992, but the vegetation has been cut and removed along with harvests in the trial. Five soil cores (0–5 cm) were randomly collected in each plot, and were combined and homogenized by sieving (8 mm sieve) directly after sampling. A subsample of soil was immediately frozen in liquid nitrogen in the field and stored at -80 °C until molecular analysis. Further subsamples were stored at -20 °C for later enzymatic analysis, or dried and finely ground in a ball mill for determination of total nutrient contents. The remaining soil was stored at 4 °C for 2 weeks until analysis. All results are converted to soil dry weight equivalents.

Soil and vegetation analyses

Total C, N and P, and pH

Total organic C (TOC) and total N (TN) were measured on finely ground soil using a CNS analyzer (ThermoFisher Flash EA 1112). Total P (TP) in soil was determined by wet digestion with H₂O₂/H₂SO₄ (Anderson and Ingram 1993) and measured with malachite green at 610 nm using a UV-1800 Shimadzu Spectrometer (Ohno and Zibilske 1991). Soil pH was measured in a soil suspension in 0.01 M CaCl₂ (1 to 2.5 soil mass to volume ratio, shaken for 1 h) using a Benchtop pH 720 A (Orion Research Inc., Jacksonville, FL).

NaOH-EDTA-extractable organic P

Organic P was determined using alkaline extraction after Bowman and Moir (1993). Briefly, 2.5 g of finely ground soil was extracted with 0.25 M NaOH - 0.05 M EDTA in a 1 to 10 (w/v) ratio for 16 h on a horizontal shaker. Soil extracts were then centrifuged and filtered (Whatman No. 1). The concentration of inorganic P in the extract was measured colorimetrically using malachite green (Ohno and Zibilske 1991). The concentration of total P in the extract was measured colorimetrically using malachite green on neutralized extracts after

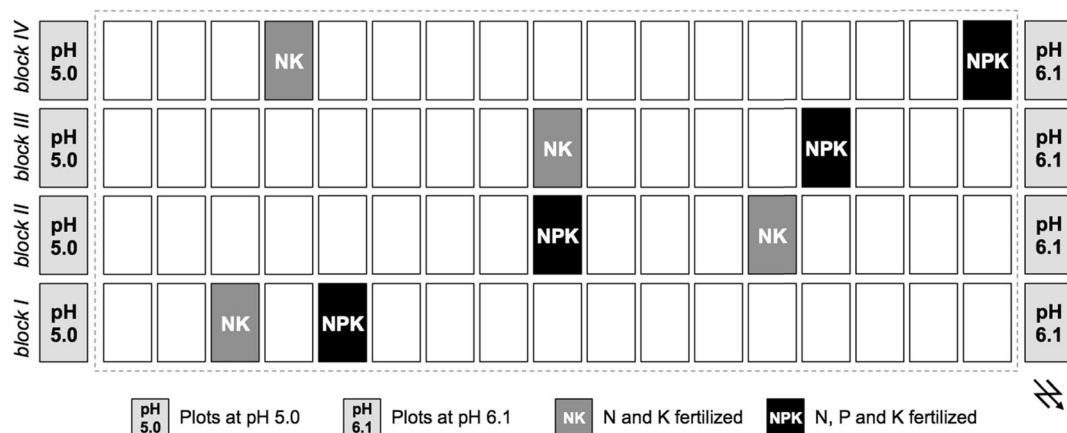


Fig. 1 Layout of the long-term field trial in Switzerland showing the selected plots in the fertilization trial (NK and NPK treatments) and the additional pH levels

digestion with ammonium persulphate in an autoclave (20 min, 121 °C). Organic P was calculated by subtracting inorganic P from total P in the extract.

Microbial C, N and P and resin-extractable P

Microbial C and N analysis was done using chloroform fumigation-extraction on 25 g of moist soil (Vance et al. 1987). Total organic C and total N in the extracts were measured using a Formacs^{SERIES} TOC/TN analyzer (Skalar, The Netherlands). Concentrations in non-fumigated subsamples were subtracted from those in fumigated ones to calculate chloroform-labile C (MicC) and N (MicN). No conversion factor was applied since this is soil-specific and has not been determined for the soils in this study.

Microbial and resin-extractable P were determined on moist samples by fumigation-extraction using anion-exchange resin membranes (BDH laboratory supplies #55,164 2S, England) charged with CO_3^{2-} and 1-hexanol as fumigant (Kouno et al. 1995) as modified by Bünemann et al. (2007). Microbial P (MicP) was calculated as the difference between fumigated and non-fumigated subsamples (resin-extractable P, P_{res}) and corrected for the incomplete recovery of a P spike, which ranged between 88 % and 99 %. No conversion factor was applied, for the same reason as above.

Potential acid and alkaline phosphatase activity

Potential acid (ACP) and alkaline (ALP) phosphatase activity were determined following Marx et al. (2001) as modified by Poll et al. (2006). Briefly, one gram of

moist soil was dispersed in 100 mL of autoclaved ddH₂O using an ultrasonic probe Labsonic U (Braun Melsungen, Germany) at 50 J s⁻¹ for 120 s. The assay contained 50 µL aliquots of the soil suspension in a microplate with 6 analytical replicates, using 1 mM 4-methylumbelliferylphosphate as substrate and either 0.1 M MES buffer (pH 6.1) or Modified Universal Buffer (pH 11) (Alef et al. 1995) as buffer for ACP and ALP activity, respectively. Microplates were incubated on a horizontal shaker for 3 h at 30 °C. The linear increase in fluorescence over time was measured, and the enzymatic activity ($\mu\text{mol h}^{-1} \text{g soil}^{-1}$) was calculated by normalization to a 4-methylumbelliferone standard added to the soil suspension.

Vegetation

All vascular plant species were identified and their relative abundance at heading stage of the dominant grass species was visually estimated in May 2013 following Dietl (1995). Plant samples for analysis of P and N content were taken in June 2013, shortly before the first cut of the year. The vegetation was cut at 4 cm above the ground using electric scissors in a randomly selected 50 × 50 cm square with at least 0.5 m distance to the border of the plot. The material was sorted into grasses, legumes and forbs, and the three groups were dried at 60 °C for 3 days and milled. Plant N content was measured by a CNS analyzer. To determine plant P content, 250 mg of plant material was ashed (6 h, 550 °C) and dissolved in 3 mL 10.3 M HNO₃ (Westerman 1990). Samples were diluted and

neutralized before colorimetric P determination using malachite green.

Molecular analysis

Nucleic acid extraction and reverse transcription

Nucleic acids were extracted in analytical duplicates from 2 g of snap-frozen soil using the RNA Powersoil® Total RNA isolation kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's instructions, with an additional homogenizing step (2.8 mm zirconium beads for 1 min at 5 m s^{-1}) using the Omni Bead Ruptor Homogenizer (Omni International, Kennesaw, GA) prior to isolation. RNA was eluted from the RNA/DNA Capture Column, precipitated and resuspended following the manufacturer's instructions. Subsequently, DNA was eluted using 4 mL of DNA elution solution (1 M NaCl, 50 mM MOPS, 15 % (v/v) isopropanol, pH 7). DNA was precipitated with isopropanol, washed, dried, and redissolved in DEPC-treated H₂O. RNA samples were treated with Turbo DNase (Thermo Fisher Scientific Inc., Waltham, MA) to remove DNA contamination. RNA concentrations were measured using a Qubit® 2.0 Fluorometer (Life Technologies, Inc., Grand Islands, NY). Complementary DNA (cDNA) was synthesized from 200 ng of total RNA, using 500 ng random hexamers (Thermo Fisher Scientific Inc.) and 160 Units GoScript™ Reverse Transcriptase (Promega, Madison, WI) according to the manufacturer's recommendations.

Analysis of the archaeal, bacterial and fungal community structures using T-RFLP

Archaeal, bacterial and fungal communities were studied using regions of the 16S rRNA gene for archaea and bacteria, and the ITS region for fungi, as detailed below. All PCRs (25 µL) contained 0.6 Units GoTaq (Promega), 1X reaction GoTaq Buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM of each primer, and 1–2 ng template DNA. Amplification of archaeal 16S DNA and cDNA was carried out with Ar109F/FAM-Ar915R primers (Lueders and Friedrich 2000). Bacterial 16S DNA and cDNA were amplified using 1064R/FAM-356F primers (Winsley et al. 2012). The fungal community was investigated with FAM-ITS1-F (Gardes and Bruns 1993)/ITS4-R (White et al. 1990) primers. After initial denaturation for 5 min at 95 °C, amplification was done for 35 cycles comprising a denaturation step of 30 s at

95 °C, an annealing step of 30 s at 53 °C (archaea), 60 s at 52 °C (bacteria) or 30 s at 60 °C (fungi), and an extension step of 60 s at 72 °C. A final extension step of 5 min at 72 °C was carried out to complete the reaction. Results from DNA- and cDNA-analysis are referred to as measurements of the total and active communities, respectively.

Restriction enzyme digestion reaction contained 10 µL PCR product, 5 U of MspI (Promega), 2 µg acetylated bovine serum albumin and water to a final volume of 20 µL, and was carried out for 3 h at 37 °C. Digested products were precipitated by adding 3 µL of 3 M CH₃COONa (pH 5.2), 3 µL of 100 mM Na₂EDTA pH 8.0, 400 µg of glycogen and 60 µL 95 % (v/v) ice-cold ethanol. Products were centrifuged, washed with 70 % (v/v) ice-cold ethanol and redissolved in 15 µL DEPC-treated H₂O. The purified products (3 µL) were analyzed on an ABI 3130XL sequencer using Liz500 as standard (Life Technologies, Inc.). The T-RFLP electropherograms of the archaeal, bacterial and fungal communities were analyzed in GeneMapper v3.7 (Applied Biosystems, Foster City, CA) with a fragment length range of 50–500 base pairs. T-RFLP profiles were processed and analyzed with the T-REX program (Culman et al. 2009), using the peak area to identify operational taxonomic units (OTUs) and a clustering threshold of 0.5 bp.

*Analysis of total and active *phoD*-harboring community structure and composition using 454-sequencing*

The *phoD* alkaline phosphatase gene was amplified by PCR from DNA and cDNA using *phoD*-F733/R1083 primers (Ragot et al. 2015) in a reaction mixture (25 µL) containing 0.6 Units GoTaq (Promega) 1X GoTaq buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM of each primer, and 1–2 ng DNA or cDNA as template. Amplification included an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of a denaturation step of 30 s at 95 °C, an annealing step of 30 s at 58 °C and an extension step of 60 s at 72 °C. The final extension step was carried out for 10 min at 72 °C. Field replicates of DNA and cDNA extracts of each plot were diluted to 20 ng µL⁻¹, pooled and analyzed by 454-sequencing using a GS-FLX+ platform (Roche 454 Life Sciences, Branford, CT), at Research and Testing Laboratory (Lubbock, TX). The Standard Flowgram Format (SFF) files were submitted to the European

Nucleotide Archive (ENA) under the accession number ERP010357.

Reads resulting from the 454-sequencing were analyzed using MOTHUR (Schloss et al. 2009) as described in Ragot et al. (2015). Briefly, resulting reads were trimmed at 150 bp and 450 bp as minimum and maximum length, respectively, and aligned using the Needleman-Wunsch global alignment algorithm as implemented in MOTHUR, using 6-mers searching and aligned reference sequences as template as in Ragot et al. (2015). The pairwise distance matrix was calculated from the alignment and sequences clustered using the k-furthest method as implemented in MOTHUR with a similarity cutoff at 75 % to define the operational taxonomic units (OTUs) as calculated by Tan et al. (2013). OTU matrices were then normalized to the smallest library size using the *normalized.shared* command as implemented in MOTHUR to allow comparison between plots (Supplementary Table S4). Taxonomy was assigned using BLAST+ (Camacho et al. 2009) with a minimum e-value of $1e^{-8}$ to retrieve NCBI sequence identifiers (gi accession number), and in-house Perl scripts were then used to obtain the taxonomy for each read (scripts written by Stefan Zoller, Genetic Diversity Centre, ETH Zurich) (Supplementary Table S5).

Statistical analysis

Since the plots with different pH levels were not part of the randomized block design of the fertilization trial, soil properties and plant data from the fertilization trial and from the two pH levels were subjected to independent statistical analysis. Redundancy analysis (RDA) was then used to evaluate the effect of soil and plant properties on microbial community structures across the whole dataset.

Two-tailed *t*-test was used to examine differences differed between fertilization treatments, and between pH levels, respectively. Analysis of dissimilarity (*anosim*) in the *vegan* package (*vegan*: Community Ecology Package) in R v.2.15.0 (R Core Team, <http://www.R-project.org/>, 2014) was used to test whether the vegetation composition was significantly different between fertilization treatments and between pH levels, respectively. Linear correlations between soil properties were tested in a Pearson's correlation matrix (Supplementary Table S1).

T-RFs of the archaeal, bacteria and fungal communities were normalized using Hellinger distance (Legendre

and Gallagher 2001) and T-RFs were further converted into a binary matrix. Species richness was represented by the number of OTUs found in the T-RF profiles in each community (Supplementary Table S3). Analysis of dissimilarity was used to evaluate whether the T-RF profiles of the total and active microbial communities were significantly different between fertilization treatments and between pH levels.

Rarefaction curves based on *phoD* reads were calculated and extrapolated to an average library size of 5099 reads to standardize the samples using EstimateS (Version 9, <http://purl.oclc.org/estimates>). Species richness of the *phoD*-harboring community was calculated using the Chao1 index (Chao and Shen 2003) (Supplementary Table S3). Structures of the *phoD*-harboring community within the fertilization treatments and within the pH levels were compared using pairwise *Libshuff* analysis with 1000 iterations as implemented in MOTHUR (Schloss et al. 2004).

Relationships between the microbial and *phoD*-harboring community structures and soil and plant properties standardized by the z-score method (Ramette 2007) were analyzed using RDA on T-RFLP data for the archaeal, bacterial and fungal community and on 454-sequencing data for the *phoD*-harboring community. Plant species were only included in the analysis if they had an abundance of more than 2 % and differed significantly in abundance ($p < 0.05$) in either fertilization treatments or pH levels (Supplementary Table S2).

Results

General soil physicochemical and biological properties

Soil pH was similar in the two fertilization treatments, although one NPK replicate had a pH of 5.9, while pH in all other NK and NPK plots varied between 4.9 and 5.3 (Table 1). Phosphate depletion in NK decreased TP, P_{org} , P_{res} , MicP and ACP activity compared to NPK. The pH levels differed significantly by one pH unit. At higher pH values, concentrations of TOC, TN and TP were greater than at lower pH.

Potential ACP and ALP activities were strongly correlated with pH, with ALP activity being five times greater at pH 6.1 than at pH 5.0, whereas ACP activity showed the opposite trend (Supplementary Table S1). Among the other soil properties, pH was linearly

Table 1 General soil physicochemical and biological properties (means of four field replicates \pm standard deviation). Letters indicate significant differences within the fertilization treatments and within the pH levels, respectively ($p < 0.05$)

Plots	pH (CaCl ₂)	TOC (g kg ⁻¹)	TN	TP (mg kg ⁻¹)	P _{org}	P _{res}	MicC	MicN	MicP	ACP (nmol substrate g ⁻¹ h ⁻¹)	ALP (nmol substrate g ⁻¹ h ⁻¹)
NK	5.1 \pm 0.3	31.2 \pm 1.1	2.88 \pm 0.17	610 \pm 35 ^a	336 \pm 18 ^a	0.3 \pm 0.1 ^a	432 \pm 13	86.8 \pm 4.5	37.4 \pm 6.8 ^a	5113 \pm 543 ^a	196 \pm 50
NPK	5.3 \pm 0.3	30.7 \pm 2.4	2.85 \pm 0.16	720 \pm 48 ^b	372 \pm 13 ^b	2.5 \pm 1.2 ^b	510 \pm 75	96.6 \pm 3.8	50.2 \pm 4.1 ^b	3434 \pm 647 ^b	221 \pm 73
pH 5.0	5.0 \pm 0.1 ^a	27.5 \pm 0.3 ^a	2.65 \pm 0.09 ^a	612 \pm 32 ^a	331 \pm 17	0.3 \pm 0.1	398 \pm 14	91.3 \pm 8.2	39.7 \pm 2.9 ^a	4108 \pm 643 ^a	225 \pm 61 ^a
pH 6.1	6.1 \pm 0.3 ^b	34.5 \pm 2.1 ^b	3.41 \pm 0.31 ^b	703 \pm 31 ^b	320 \pm 20	0.2 \pm 0.1	383 \pm 23	103.9 \pm 12.5	54.8 \pm 3.8 ^b	3037 \pm 305 ^b	1150 \pm 325 ^b

correlated with TOC, TN, TP, MicC, MicN and MicP (Supplementary Table S1).

Plant productivity and composition

Phosphate depletion led to a 1.8-fold decrease in total plant yield, though different plant groups were differently affected, with a 2.7-fold decrease in grass biomass contrasting with a 2-fold increase in forb biomass (Table 2). Phosphate depletion also decreased plant N and P content by factors of two and four, respectively. In total, 40 plant species were observed in the fertilization treatments (Supplementary Table S2). The vegetation composition was significantly different between the fertilization treatments, while the plant richness remained similar (p -value = 0.03). Among the eight plant species differing in abundance between NK and NPK treatments, the legumes *Trifolium pratense* and *Lotus corniculatus* were more abundant in NK than NPK, while the grasses *Arrhenatherum elatius* and *Holcus lanatus* were more abundant in NPK than NK.

Grass, legume and forb yields were similar at both pH levels (Table 2). Lower pH led to a higher plant N content, associated with a slightly reduced species richness. A total of 42 plant species was observed at the two pH levels (Supplementary Table S2). Vegetation composition was significantly different between the pH levels (p -value = 0.03). Acidomesophilic plant species such as *Anthoxanthum odoratum*, *Cynosurus cristatus* and *Rumex acetosa* were more abundant at pH 5.0, while alkalomesophilic plant species such as *Knautia arvensis* and *Galium album* were more abundant at pH 6.1 (Hill et al. 1999). This shows that one unit difference in pH can significantly influence the vegetation composition.

Effect of phosphate depletion and soil pH on total and active archaeal, bacterial, fungal and *phoD*-harboring community structures

Phosphate depletion led to a significant shift in both total and active archaeal and *phoD*-harboring community structures (Table 3). For bacteria and fungi, an effect of phosphate depletion on active but not on total community structures was observed.

The difference in pH of about one unit between pH levels affected the structure of both total and active communities of archaea, bacteria and *phoD* (Table 3). The total fungal community structure was similar at both

Table 2 Plant productivity, plant N and P content of the first cut in 2013 (means of four field replicates \pm standard deviation) and plant species richness. Letters indicate significant differences between fertilization treatments and pH levels ($p < 0.05$)

Plots	Grass yield (T ha ⁻¹)	Legume yield (T ha ⁻¹)	Forb yield (T ha ⁻¹)	Total yield (T ha ⁻¹)	Plant N content (kg ha ⁻¹)	Plant P content (kg ha ⁻¹)	Plant species richness
NK	1.8 \pm 0.2 ^a	0.2 \pm 0.2	1.0 \pm 0.5	3.0 \pm 0.3 ^a	38.2 \pm 1.5 ^a	2.46 \pm 0.53 ^a	33 \pm 3
NPK	4.9 \pm 0.8 ^b	0.2 \pm 0.1	0.4 \pm 0.1	5.5 \pm 0.9 ^b	65.4 \pm 7.3 ^b	8.71 \pm 0.39 ^b	33 \pm 3
pH 5.0	1.7 \pm 0.5	0.3 \pm 0.2	1.1 \pm 0.5	3.1 \pm 0.6	56.5 \pm 9.8 ^a	2.83 \pm 0.58	28 \pm 3 ^a
pH 6.1	1.1 \pm 0.2	0.2 \pm 0.1	1.2 \pm 0.1	2.5 \pm 0.4	36.6 \pm 7.3 ^b	2.12 \pm 0.54	34 \pm 2 ^b

pH values, whereas the active fungal community structure differed.

Correlations between the total and active archaeal, bacterial, fungal and *phoD*-harboring community structures and environmental factors

Based on the first and second component of the RDA, the total archaeal community structure in the NPK treatment was positively correlated to P_{res} , plant P content, grass yield and total plant yield, and negatively correlated to forb yield (Fig. 2a and b, Table 4). In addition to pH, the total archaeal community structure at pH 6.1 was also positively correlated to TOC, TN and the abundances of *Galium album* and *Knautia arvensis*, while the community structure in NK and at pH 5.0 was negatively related to these variables. The abundance of *Anthoxanthum odoratum* decreased with pH and was thus negatively related to the communities at pH 6.1. The active archaeal community structure was correlated to the same plant species as the total archaeal community, and was mainly affected by pH, TOC and TN.

Table 3 Analysis of similarity comparing total and active archaeal, bacterial, fungal and *phoD*-harboring communities between fertilization treatments and pH levels. Significant differences are indicated by * (p -value < 0.05)

Community		Fertilization treatments	pH levels
Archaea	Total	0.03*	0.03*
	Active	0.04*	0.02*
Bacteria	Total	0.07	0.02*
	Active	0.02*	0.02*
Fungi	Total	0.40	0.46
	Active	0.03*	0.02*
<i>phoD</i>	Total	0.02*	0.01*
	Active	0.01*	0.01*

Both total and active bacterial community structures showed a strong separation between the two pH levels and to a lesser degree between the fertilization treatments, which was mostly correlated with pH, TOC, TN and the abundances of *Galium album* and *Knautia arvensis* (Fig. 2c and d, Table 4). In contrast, the total fungal community structure was not correlated to any of the soil and plant properties (Fig. 2e, Table 4). The active fungal community structure, however, was related to soil pH as well as to plant N and P content and grass yield, which were all significantly higher in NPK than in NK treatment (Fig. 2f, Table 4).

The total *phoD*-harboring community structure was correlated with pH, TOC and TN, whereas the active *phoD*-harboring community structure was only correlated with pH and TOC. In both cases, these soil properties were mainly associated with the first component, which explained 19.9 and 17.9 % of the variability in the total and active *phoD*-harboring community structures, respectively (Fig. 3a and b, Table 4). Additionally, the active *phoD*-harboring community structure of the NK and NPK treatments showed tighter clustering than for the total *phoD*-harboring community structure, suggesting a more similar community structure of the active than of the total *phoD*-harboring members (Fig. 3a and b). The distribution of the total and active *phoD*-harboring community structures was similar at both pH levels (Fig. 3a and b).

Taxonomic composition of the total and active *phoD*-harboring community as related to soil and plant properties

The taxonomic composition of the total and active *phoD*-harboring community was investigated using 454-sequencing and BLAST+ to elucidate the key *phoD*-harboring members in the studied soils. Taxonomy was assigned to the majority of OTUs, which were all affiliated

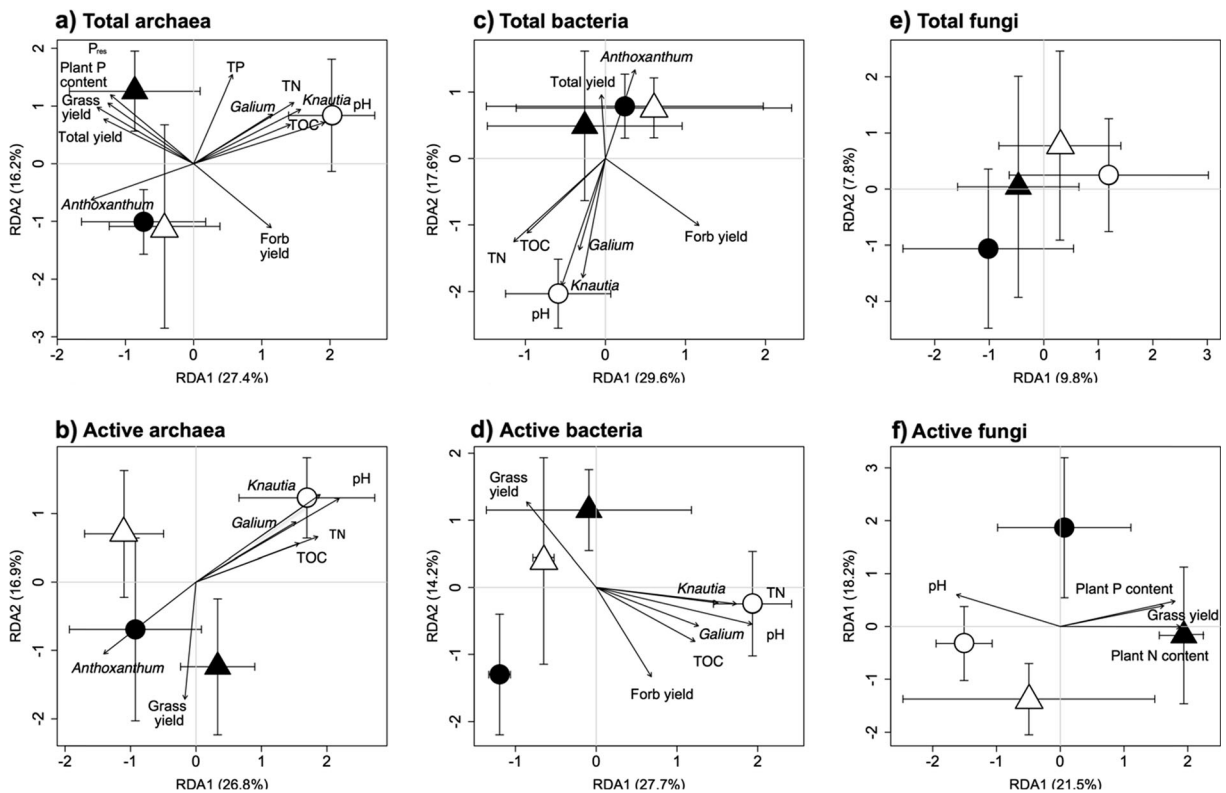


Fig. 2 Redundancy analysis (RDA) of the community structures of archaea (a, b), bacteria (c, d) and fungi (e, f) based on the T-RF profiles in NK (●) and NPK (▲) and at pH 5.0 (Δ) and 6.0 (○)

(means of four field replicates with standard deviation). Displayed vectors represent the soil and plant properties that are significantly correlated to the community structures ($p \leq 0.05$)

with the bacterial kingdom. A remainder of 692 reads could not be identified and represented 0.8–3.1 % of the reads before normalization (Supplementary Table S4 and S5). The *phoD* gene was found in 28 bacterial orders (Fig. 4). The dominant bacterial orders across all samples were *Actinomycetales* (15–32 %), *Bacilliales* (5–15 %), *Gloeobacterales* (6–23 %), *Planctomycetales* (17 %–30 %) and *Rhizobiales* (5 %–26 %).

The dominant bacterial orders that harbor and express the *phoD* gene were similar in all plots. Nonetheless, some differences between treatments and/or between the total and active community were observed. For example, *Rhizobiales* had a low relative abundance in the NK treatment, while they were dominant in the NPK treatment (Fig. 4). *Xanthomonadales* had a low relative abundance in the total community, but a high relative abundance in the active community of the NK treatment. Finally, the relative abundance of some groups such as *Gloeobacterales* was not affected by the treatments.

Furthermore, the relative abundance of several *phoD*-harboring orders was strongly correlated to individual soil

properties (Fig. 4). The abundance of *Caulobacteriales* and *Pseudomonadales* decreased with increasing P_{org} and P_{res} , while that of *Planctomycetales* increased with P_{org} and P_{res} . Additionally, the abundance of *Actinomycetales* and *Gemmatimonadales* increased together with pH, TN, TOC and TP, while the abundance of *Burkholderiales* decreased with increasing pH, TN and TP, and the abundance of *Gloeobacterales* decreased with increasing pH, TOC and TN. All correlated soil properties were in turn affected by either phosphate depletion or pH.

Discussion

Effect of phosphate depletion on the total and active archaeal, bacterial, fungal and *phoD*-harboring community structures

In this work, we studied the effects of phosphate depletion and soil pH on the archaeal, bacterial, fungal and *phoD*-harboring community structures in a long-term

Table 4 Correlation between microbial community structures and soil and plant properties. *p*-values were retrieved from the RDA on the community structures

	Archaea		Bacteria		Fungi		<i>phoD</i>	
	Total	Active	Total	Active	Total	Active	Total	Active
pH	0.002	0.001	0.001	0.001	n.s.	0.027	0.083	0.094
TN	0.006	0.009	0.001	0.028	n.s.	n.s.	0.097	n.s.
TOC	0.023	0.045	0.016	0.043	n.s.	n.s.	0.093	0.100
TP	0.023	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Porg	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Pres	0.005	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Grass yield	0.003	0.044	n.s.	0.026	n.s.	0.022	n.s.	n.s.
Legume yield	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Forb yield	0.032	n.s.	0.011	0.039	n.s.	n.s.	n.s.	n.s.
Total yield	0.023	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Plant N	n.s.	n.s.	n.s.	n.s.	n.s.	0.014	n.s.	n.s.
Plant P	0.005	n.s.	n.s.	n.s.	n.s.	0.046	n.s.	n.s.
Plant richness	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Anthoxanthum odoratum</i>	0.015	0.031	0.049	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Arrhetherum elatius</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Cynosurus cristatus</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Dactylis glomerata aggr.</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Galium album</i>	0.047	0.035	0.034	0.07	n.s.	n.s.	n.s.	n.s.
<i>Knautia arvensis</i>	0.001	0.001	0.001	0.002	n.s.	n.s.	n.s.	n.s.
<i>Lotus corniculatus</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Ranunculus acris</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<i>Trifolium pratense</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

n.s.: non-significant

fertilization trial characterized by a pH gradient on site. Our results show that the active archaeal, bacterial and fungal community structures are more responsive to phosphate depletion than their corresponding total community structures, except for the total archaeal community structure, which was affected by phosphate depletion and correlated to phosphate availability (P_{res}). Indicators of phosphate depletion (TP, P_{org} and P_{res}) were otherwise not significant drivers of the communities in the first and second components of the RDA (Fig. 2, Table 4). The active community structures of archaea, bacteria and fungi were all correlated with pH and grass yield, which is an indicator of productivity (Fig. 2, Table 4).

Our results are in agreement with those of He et al. (2007) who showed a significant shift in total archaeal community structure between NK and NPK treatments in a wheat-maize rotation cropping system. In their

study, phosphate addition specifically promoted ammonia-oxidizing *Crenarchaeota*. Our results on the total bacterial community, however, contrast with several studies that reported a significant effect of phosphate addition on the total bacterial community structure in grassland, cropped and forest soils (Beauregard et al. 2009; Liu et al. 2012; Tan et al. 2013; Zhong and Cai 2007). The total fungal community has also been reported to be shaped primarily by phosphate availability (Lauber et al. 2008). The fact that in our study only the active bacterial and fungal community responded to phosphate depletion may be related to the level of phosphate fertilization. Previous studies in the same long-term trial revealed that microorganisms were P limited in the NK treatment (Bünemann et al. 2012) and that the P balance equaled zero in NPK treatment, suggesting that phosphate addition was adequate to maintain plant productivity but

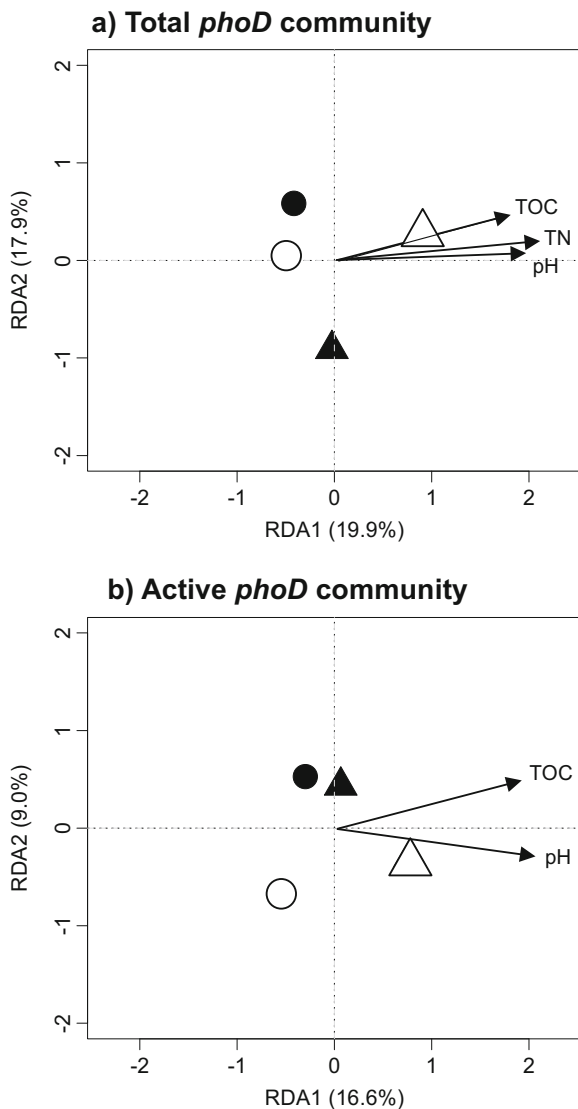


Fig. 3 Redundancy analysis (RDA) of total and active *phoD*-harboring community structures based on 454-sequencing data in NK (●) and NPK (▲) and at pH 5.0 (△) and 6.0 (○). Displayed vectors represent the soil and plant properties that are significantly correlated to the community structures ($p \leq 0.1$)

did not lead to a net increase in available and total P (Liebisch et al. 2013). Our results suggest that phosphate depletion over time impacts only the active bacteria and fungi, but does not change the structure of the greater total community. Our data also suggest that archaea are more sensitive to phosphate depletion than bacteria and fungi.

Since *phoD* is part of the *Pho* regulon (Vershina and Znamenskaya 2002), we expected available P (P_{res})

to be correlated with the active *phoD*-harboring community structure. Moreover, low available P (P_{res}) was expected to result in an increase of ACP/ALP activity in NK compared to NPK. Our results showed that phosphate depletion led to a significant shift in the total and active *phoD*-harboring community structure and increased ACP activity (Fig. 3, Table 3). However, this shift in the total and active *phoD*-harboring community structure was not correlated to any of the phosphate depletion indicators (TP, P_{org} and P_{res}) (Fig. 3, Table 4). Based on the first and second RDA components, our results show that the total and active *phoD*-harboring community structures were most strongly correlated to pH and TOC, suggesting that the effect of phosphate depletion may be masked by the strong effect of pH and TOC in the RDA. Previous DNA-based studies on *phoD* have shown contrasting results of phosphate addition. Diversity of *phoD* has been reported to increase, decrease and not to be affected by phosphatase addition (Chhabra et al. 2012; Jorquera et al. 2014; Tan et al. 2013). In a long-term trial including non-fertilized soils, soil fertilized with composted manure or water-soluble phosphate, and prairie soils, Fraser et al. (2015) observed a general positive correlation between *phoD* gene abundance and potential ALP activity in soil, suggesting that PhoD contributes significantly to the total ALP activity. However, comparing the non-fertilized and the water-soluble fertilized soils, they did not observe a consistent response to phosphate addition in ALP activity and *phoD* gene abundance over 2 years of the experiment, suggesting that water-soluble phosphate addition does not always decrease potential ALP activity. Our results are in agreement with the outcome of their study and showed that phosphate depletion did not increase ALP activity nor change the total *phoD*-harboring community structure as hypothesized. However, our results suggest that phosphate depletion affects significantly the active *phoD*-harboring community composition, even though phosphate depletion indicators (TP, P_{org} and P_{res}) were not significant drivers in the first and second components of the RDA (Fig. 3, Table 4).

Effect of soil pH on the total and active archaeal, bacterial, fungal and *phoD*-harboring community structures

One unit difference in pH value affected all investigated total and active microbial community structures except

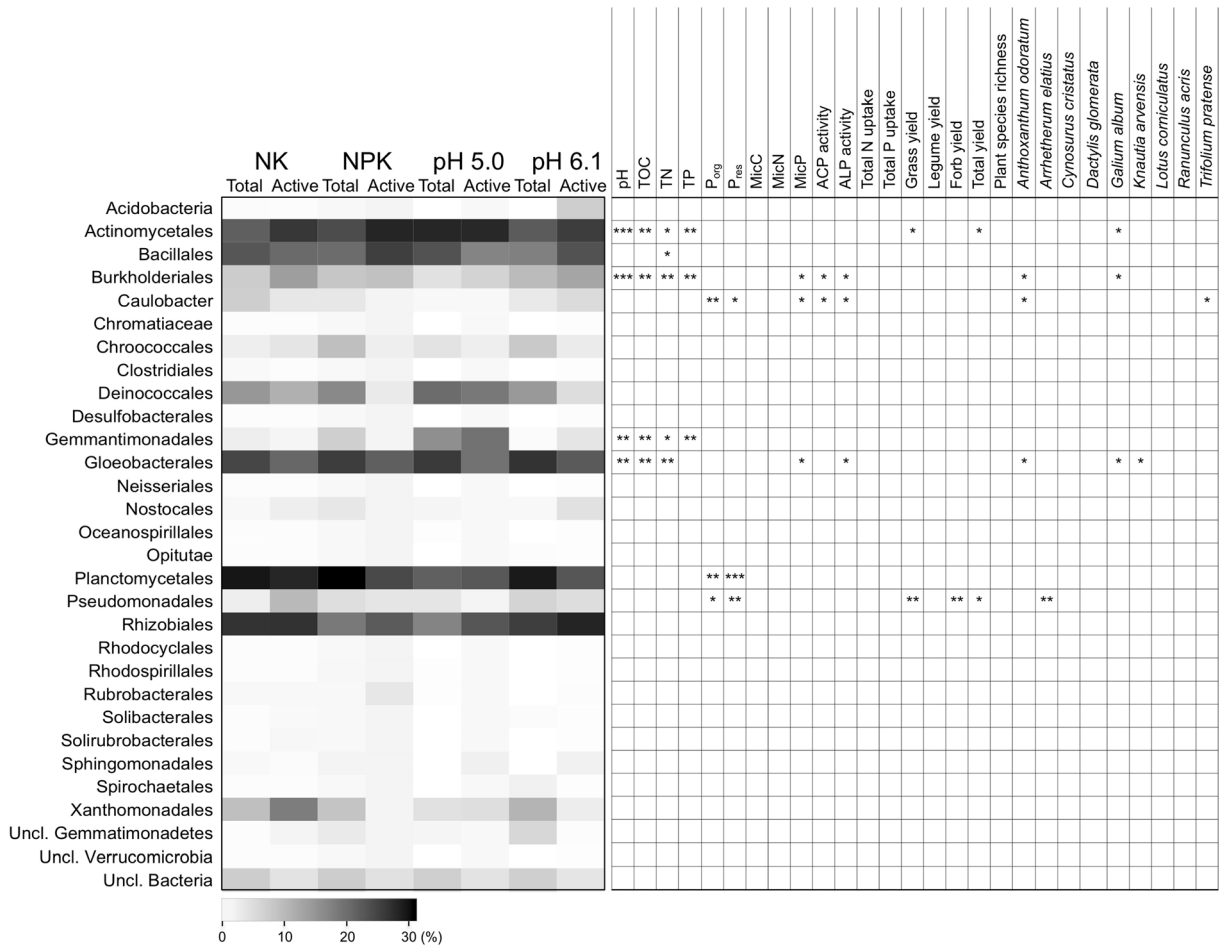


Fig. 4 Heatmap representing the relative abundances of the total and active *phoD*-harboring orders. The table shows the significant correlations between the relative abundance of each order with soil

and plant properties with *p*-values of <0.05, <0.01 and <0.001 indicated by *, ** and ***, respectively

for the total fungal community (Fig. 2, Fig. 3, Table 4). Soil pH also changed some soil and plant properties such as TOC and TN, which in turn were important drivers of the microbial community structures. While bacterial community structure is widely known to be strongly correlated with soil pH (Fierer and Jackson 2006; Griffiths et al. 2011; Lauber et al. 2008; Nacke et al. 2011), less information is available for archaea and fungi. Studies of *Crenarchaeota* (Lehtovirta et al. 2009) and crenarchaeal ammonium-oxidizing archaea (Nicol et al. 2008) showed that pH can select for particular archaeal groups. Lauber et al. (2008) reported that the total fungal community structure is stable over pH values ranging from 3.6 to 4.7, though it is possible that only specific fungal taxa such as *Helotiales* and *Hypocreales* are responsive to pH changes (Rousk

et al. 2010). Our results suggest that the total fungal community is also stable over pH values ranging from 5.0 to 6.0, while one unit difference in pH value significantly affected the active fungal community. This suggests that the active fungal community is more sensitive to pH differences than the greater total fungal community. More importantly, we could not distinguish the effect of pH from that of TOC and total N, which are both commonly correlated with pH in soil (Drenovsky et al. 2004; Lauber et al. 2008; Siciliano et al. 2014). Previous studies have reported pH as the main driver of the total *phoD*-harboring community in arable and grassland soils (Cui et al. 2015; Ragot et al. 2015; Wang et al. 2012b). This suggests that pH rather than TOC and TN led to a shift in the structure of the total and active *phoD*-harboring community.

Key *phoD*-harboring microorganisms

The *phoD* gene was only found in bacteria in this study, although it is also present in archaea (e.g. *Euryarcheota*) and fungi (e.g. *Ascomycetes*) (see Integrated Microbial Genomes database, <https://img.jgi.doe.gov/>). Our results showed that despite differences in soil and plant properties, the dominant bacterial orders that harbor and express the *phoD* gene were similar in all plots, suggesting that the composition of the total and active *phoD*-harboring community is rather stable. Nonetheless, differences between treatments in relative abundances of both the total and active communities were also observed. For example, *phoD*-harboring *Gemmatimonadales* were dominant at pH 5.0 in both the total and active communities, whereas they were almost absent at pH 6.1 (Fig. 4). Furthermore, differences between total and active *phoD*-harboring community compositions within one treatment indicate that several orders are more active than others under certain soil conditions. For example, the relative abundance of *phoD*-harboring *Xanthomonadales* was higher in the active than in the total community of the NK treatment, while it was lower in the active than in the total community of the NPK treatment. Similarly, the active *phoD*-harboring *Planctomycetes* and *Pseudomonadales* had a lower relative abundance in the NPK than in the NK treatment, while they had a similar relative abundance in the total community. Additionally, the relative abundance of the total and the active *phoD*-harboring *Bacilliales* at pH 5.0 and 6.1 suggests that higher pH strongly up-regulates the expression of the *phoD* gene in this taxonomic group. Additionally, strong correlations were observed between soil pH, TOC, TP and TN and the relative abundance of some bacterial orders such as *Actinomycetales* (Fig. 4).

In contrast to our results, Tan et al. (2013) observed that water-soluble phosphate addition decreased the relative abundance of *phoD*-harboring *Acidobacteria* and *Pseudomonas fluorescens*. They reported that the dominant *phoD*-harboring phyla were the same at all studied phosphate fertilization regimes (0, 15 and 30 kg P ha⁻¹) and included mainly *Proteobacteria* (predominantly *Alphaproteobacteria*), *Actinobacteria* and *Cyanobacteria*. In our study, the key *phoD*-harboring phyla were *Actinobacteria*, *Cyanobacteria*, *Firmicutes* and *Planctomycetes*. The differences in the *phoD*-harboring community composition between our results and

those of Tan et al. (2013) may be attributed to the fact that Tan et al. (2013) used primers designed by Sakurai et al. (2008), which have been shown to have an amplification bias, amplifying only a limited number of microbial groups and resulting in an over-representation of *Alphaproteobacteria* in the *phoD*-harboring community (Ragot et al. 2015; Tan et al. 2013). Nonetheless, our findings show that the regulation of *phoD* expression in *Bacilliales*, *Burkholderiales*, *Chroococcales*, *Gloeobacterales*, *Gemmatimonadales*, *Planctomycetales*, *Rhizobiales* and *Xanthomonadales* may be linked to phosphate depletion and pH.

In support of our findings, some of the key *phoD*-harboring taxonomic groups identified in our study are known to play an important role in other nutrient cycles. For example, *Proteobacteria* (e.g. *Rhizobiales*) are known to be important players in C cycling as decomposers (Štursová et al. 2012) and in N cycling as N₂-fixing bacteria (Hayatsu et al. 2008). *Actinobacteria* (e.g. *Actinomycetales*) and *Cyanobacteria* (e.g. *Gloeobacterales*) also play a relevant role in N cycling as N₂-fixing and nitrifiers (Fierer et al. 2007; Hayatsu et al. 2008). Moreover, although their ecological role is not clear, *Deinococcus-Thermus* and *Planctomycetales* have been reported to grow well in high organic matter soils (Buckley et al. 2007; Štursová et al. 2012), suggesting that they also play a role as decomposers. Our results show that these taxonomic groups play an additional ecological function as organic P mineralizers in soil.

Conclusions

Our results showed that the active communities are generally more responsive to pH and phosphate depletion and, thus, may reflect better the response of microbial communities to their environment. Additionally, our results suggest that the fungal community is less responsive to pH and phosphate depletion than the archaeal, bacterial and *phoD*-harboring communities. Soil pH, C_{org} and TN were more important drivers than P status (TP, P_{org} and P_{res}) which did not appear in the first and second components of the RDA. Despite differences in soil and plant properties, the dominant *phoD*-harboring members were overall the same in all plots (*Actinomycetales*, *Rhizobiales*, *Gloeobacterales* and *Planctomycetales*). This suggests that the potential

key player in organic P mineralization remain similar despite differences in certain soil conditions.

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