Biol Fertil Soils (2017) 53:375–388
DOI 10.1007/s00374-017-1183-3

ORIGINAL PAPER

Long-term fertilisation regimes affect the composition of the alkaline phosphomonoesterase encoding microbial community of a vertisol and its derivative soil fractions

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Received: 12 December 2016 / Revised: 21 January 2017 / Accepted: 4 February 2017 / Published online: 25 February 2017

Abstract Alkaline phosphomonoesterase (ALP) mainly originates from soil microbial secretion and plays a crucial role in the turnover of soil phosphorus (P). To examine the response of ALP-encoding microbial communities (analysed for the biomarker of the ALP gene, *phoD*) of soils and derivative soil fractions to different fertilisation regimes, soil samples were collected from a long-term experimental field (over 35 years). The different organic P (Po) pools of soil fractions and the ALP activity of soil were also determined. Compared with chemical-only fertilised soils, the ALP activity was 232–815% higher in organic-amended soils, and the highest enzyme activity was observed in the organic-only fertilised treatment. The abundance of the *phoD* gene harbouring in soil fractions, determined by quantitative PCR (qPCR), was affected by different fertilisations. The highest abundance of *the phoD* gene was generally detected in the 2–63-μm-sized fraction (silt), but most *phoD*-encoding microbial species were associated to the 0.1–2-μm-sized fraction (clay) in the chemical-only fertilised soil. The contents of labile Po (LPo), moderately labile Po (MLPo) and fulvic acid-associated Po (FAPo) were significantly correlated with the *phoD* gene abundance, whereas only LPo content was significantly correlated with the ALP activity. The dominant *phoD*-encoding phylas were *Actinobacteria* and *Proteobacteria*, according to a high-throughput sequencing. *Bradyrhizobium*, a N_2-fixer identified as a *phoD*-encoding genus, showed the highest abundance in fertilised soils. The abundance of *Bradyrhizobium*, *Streptomyces*, *Modestobacter*, *Lysobacter*, *Frankia* and *Burkholderia* increased with the organic-only amendment and was significantly correlated with the ALP activity. According to structure equation models (SEM), pH and LPo content significantly and directly affected the ALP activity; the soil organic C (C_{org}) content was related to composition and abundances of *phoD*-harbouring microbial communities; since both microbial properties were correlated to the ALP activity, the C_{org} content was indirectly related to the ALP activity. In conclusion, soil management practices can be used to optimise the contents of soil available P and the organic P with regulation of soil ALP activity and the community composition of corresponding microbes.

Keywords Long-term fertilisation · Soil particle size fractions (PSFs) · Organic phosphorus fractions · Alkaline phosphomonoesterase activity · High-throughput sequencing · Structure equation model (SEM)

Electronic supplementary material The online version of this article (doi:10.1007/s00374-017-1183-3) contains supplementary material, which is available to authorized users.

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Introduction

Phosphorus (P) deficiencies can limit plant growth and crop yield (Welsh et al. 2009). Most of the P applied to agro-ecosystems is retained in the soil as mineral-associated P, which is often not available to plants (Waldrip et al. 2011), whereas when mobilised, it can reach waterways with

potential risks of P eutrophication (Withers and Haygarth 2007). To reduce these problems and to compensate for the insufficient resources of rock phosphate used for fertiliser production (Cordell et al. 2009), it is important to carefully consider the agronomical use of the "legacy phosphorus" or accumulated P, which includes both inorganic P (Pi) and organic P (Po).

The Po content of soil can range from 30 to 50% of the total soil P (Dalal 1978) and can be a P source for plants and microbes if it is mineralised to Pi by phosphatases (Tarafdar and Claassen 1988; Richardson 2001), which include phosphomonoesterases (hydrolysing phosphoric monoesters), phosphodiesterases (hydrolysing phosphoric diester), phosphoric triester hydrolases, polyphosphate hydrolases etc. Both acid (ACP) and alkaline phosphomonoesterase (ALP; commonly termed phosphatases) activities have been studied in soils to evaluate the organic P mineralisation to inorganic P (Nannipieri et al. 2011, 2012) since they are considered to play a main role in this process (Tarafdar and Jungk 1987). The primary producers of acid phosphomonoesterases are plant roots and soil microbes, whereas alkaline phosphomonoesterases only originate from soil microbes and fauna (Tarafdar and Claassen 1988; Spohn and Kuzyakov 2013a). However, the origin of these enzymes in soil is poorly known and understanding it may give insights on environmental factors and agricultural management, for example the type and rate of fertilisation, affecting the mineralisation of organic P to inorganic P. The ALP-encoding genes are grouped into three distinct families (*phoA*, *phoD* and *phoX*) based on their sequence similarities and substrate specificities (Kathuria and Martiny 2011). About 32% of soil prokaryotes contained at least one of these ALP-encoding genes (Zimmerman et al. 2013), which are classified as COG1785, COG3540 and COG3211, respectively, according to the Cluster of Orthologous Groups (COG) categorisation (Kageyama et al. 2011). Of these enzyme-encoding genes, the *phoD* gene is considered the key ALP gene in soil (Tan et al. 2013).

Long-term fertilisation influences soil nutrient cycling and nutrient availability by altering the physical, chemical and biological properties of soil, including effects on activity, biomass and composition of microbial communities responsible for soil functions. Agricultural management can affect both composition and abundances of *phoD* microbial community (Fraser et al. 2015a, b), but it is not known how the long-term fertilisation affects the distribution of the microbial communities across the soil particle size fractions (PSFs) and the relation of these communities with different soil Po fractions. This knowledge may get insight on the mechanisms responsible for the mineralisation of the different organic P fractions to plant available P.

In this study, we hypothesised that the different long-term fertilisation regimes would change the different soil organic P fractions, the abundance and composition of ALP-encoding microbial communities and ALP activity. Moreover, as changes in the distribution of organic matter in soil particle size fractions (PSFs) are well established under different fertilisation regimes (Nicolás et al. 2012; Yu et al. 2012; Ling et al. 2014), we also hypothesised that the abundance of *phoD*-harbouring microbes among the different PSFs may be affected by different fertilisation practises. The abundance and composition of *phoD*-harbouring microbial communities were determined by quantitative PCR (qPCR) and Illumina MiSeq sequencing. The *phoD* was chosen as the biomarker of ALP gene and multivariate statistics, e.g. redundancy analysis, structure equation models (SEM) etc., were employed to analyse the obtained data. Soil samples were collected from a long-term (started in 1981) field experiment with different fertilisation regimes.

Materials and methods

Site description and sample collection

The long-term field experiment cropped to corn–wheat rotation was established in 1981 in Suixi County, Anhui Province, China (116° 45′ E, 33° 37′ N). The mean annual temperature of this region is approximately 15.0 °C, and the mean annual precipitation is approximately 870 mm. The soils of this Province are vertisols developed in fluvial and lacustrine deposits and are also classified as a lime concretion black soil, according to the Chinese soil classification system (Zhang et al. 2014b). At the beginning of the experiment, the main soil physicochemical properties were pH 7.6; SOM, 10.22 g kg^{-1}; total N, 0.78 g kg^{-1}; total P, 0.47 g kg^{-1}; and available P, 2.5 mg kg^{-1}. This experiment included the following five treatments with four replicated plots (30 m^2 per plot) for each treatment: (1) CK, no fertiliser added; (2) NPK, only mineral NPK (N, 525 kg ha^{-1} $year^{-1}$; P_2O_5, 210 kg ha^{-1} $year^{-1}$; and K_2O, 210 kg ha^{-1} $year^{-1}$); (3) MNPK, mineral NPK (N, 262.5 kg ha^{-1} $year^{-1}$; P_2O_5, 105 kg ha^{-1} $year^{-1}$; and K_2O, 105 kg ha^{-1} $year^{-1}$) plus 3750 kg composted bean cake ha^{-1} $year^{-1}$: (4) HMNPK, mineral NPK (N, 420 kg ha^{-1} $year^{-1}$; P_2O_5, 168 kg ha^{-1} $year^{-1}$; and K_2O, 168 kg ha^{-1} $year^{-1}$) plus 6000 kg composted bean cake ha^{-1} $year^{-1}$; and (5) M, only composted bean cake (7500 kg ha^{-1} $year^{-1}$). Only three replicates of each treatment were considered in this study. The N application rates of the M, NPK and MNPK treatments were equal to 525 kg N ha^{-1} $year^{-1}$ by summing organic N and inorganic N inputs, whereas 840 kg N ha^{-1} $year^{-1}$ were applied in the HMNPK treatment with an organic N to inorganic N ratio of 1:1. The mineral NPK fertilisers were urea, superphosphate and potassium chloride, whereas composted bean cake contained 30–40% organic matter, 6–7% total N, 1–3% P_2O_5, 2–3% K_2O

and 10–15% water. Crop straw residues were removed after harvest each year.

Ten soil cores (5 cm diameter) were collected from each plot at a depth of 0–20 cm in March 2016, carefully composited as a single sample, gently broken apart along the natural break points and then moist sieved (<2 mm) with removal of visible plant matter and organic debris. Each sample was then divided into two parts: one part was stored at 4 °C for biochemical and chemical analyses, and the other part was stored at −20 °C for DNA extraction.

Soil chemical and biochemical analyses

Soil properties were analysed as previously described (Chu and Grogan 2010; Ling et al. 2014). Soil pH was measured with a soil-water (1:2.5, *w*/*v*) slurry using a compound electrode (PE-10; Sartorious, Germany). Soil organic C (C_{org}) and total N (TN) were measured with an elemental analyser (Vario MAX; Elementar, Germany). Soil NO_3^--N and exchangeable NH_4^+-N were extracted with 0.01 M $CaCl_2$ and then detected on a continuous flow analytical system (Santt System; Skalar, Holland). Soil dissolved organic C (DOC) was extracted with 0.5 M K_2SO_4 and then determined with a TOC analyser (ELEMENTER, Germany). Soil Ca^{2+} was extracted with 1 M $Mg(NO_3)_2$ and determined by an inductively coupled plasma mass spectrometry (ICP-MS; Perkin Elmer Nexion 300×, USA). Available potassium (AK) was determined in 1 M ammonium acetate extracts with flame photometry (FP640: INASA). Soil total P was digested by HF-$HClO_4$ and ultimately determined via molybdenum-blue colorimetry (Xun et al. 2016). Soil available P (AP) was extracted with 0.5 M $NaHCO_3$ and determined using the ammonium molybdate ascorbic method (Zhong et al. 2010).

Fractionation procedure for soil particles

The size fractionation was carried out according to Stemmer et al. (1998). Briefly, fresh soils (140 g equivalent dry weight for each sample) were suspended in 400 ml of distilled water and the soil-water suspension was dispersed by low-energy sonication (output energy of 0.2 kJ/g). Subsequently, the suspensions were fractionated by a combination of wet sieving and repeated centrifugation (3900×*g*) to avoid disruption of micro-aggregates. The following four fractions were obtained for each sample: coarse sand fraction (200–2000 μm), fine-sand fraction (63–200 μm), silt fraction (2–63 μm) and clay fraction (0.1–2 μm). The fractions were freeze-dried, and then DNA was extracted from each fraction of the different fertilised soils (Ling et al. 2014). The average recovery of all fractions from the soil ranged from 92 to 95%.

Fractionation procedure for soil organic P

Soil organic P (Po) was fractionated according to the sequential extraction procedure by Bowman and Bowman and Cole (1978) and modified by Slazak et al. (2010). These were the obtained pools: (1) labile P pool (LPo), extracted from soil with 0.5 M $NaHCO_3$ solution (pH = 8.5); (2) moderately labile P pool (MLPo), extracted from the sediment of the first extraction with 1.0 M HCl; (3) more resistant Po pool (FAPo), extracted from the sediment of the second extraction with 0.5 M NaOH; and (4) highly resistant Po pool (HAPo), the P content of the residue remaining after all extractions. According to Slazak et al. (2010) and Tiecher et al. (2012), FAPo was primarily associated with fulvic acid and the HAPo was primarily associated with humic acids. The sum of the four fractions was the total Po (TPo).

Potential ALP activity assay

ALP activity was estimated by measuring the release of *p*-nitrophenol (PNP) from *p*-nitrophenyl phosphate (PNPP) as described by Tabatabai and Bremner (1969). One gramme of fresh soil was slightly shaken with 0.5 ml toluene for 15 min, and then incubated in modified universal buffer (pH 11.0) containing the *p*-nitrophenol phosphate (p-NPP; Sigma-Aldrich, USA) for 1 h at 37 °C. Reactions were stopped with 0.5 M NaOH, and solutions were filtered with Whatman 42 paper. The formation of *p*-nitrophenol was determined at 420 nm (Sakurai et al. 2008; Fraser et al. 2015a, b). The ALP activity was expressed as microgram per h per g of soil (Cui et al. 2015).

DNA extraction

Total genomic soil DNA was extracted from 0.25 g of soil (dry weight equivalent) using a Power Soil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA), according to the manufacturer's protocol. The DNA extracts were purified with a Wizard DNA Clean-Up System (Axygen Bio, USA), as recommended by the manufacturer. The DNA was stored at −80 °C until analysis.

Quantitative PCR analysis of *phoD* genes

The *phoD* gene was amplified with primers ALPS-F730 (5′-CAGTGGGACGACCACGAGGT-3′) and ALPS-1101 (5′ GAGGCCGATCGGC-ATGTCG-3′) (Sakurai et al. 2008; Acuña et al. 2016; Lagos et al. 2016), and the size of amplicon is 371 bp. Each sample involved three technical replicates for the *phoD* gene amplification, which was carried out in an ABI 7500 Cycle Real-time PCR System (Applied Biosystems, Germany); 25 μl of solution contained 12.5 μl of SYBR® Premix Ex Taq (2×; Tli RNaseH Plus), 0.5 μl of ROX

Reference Dye II (50×; TAKARA, BIO, INC, Japan), 0.5 μl of each primer and 1 μl of template and ddH_2O added to bring to a volume. Cycling conditions were as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and at 60 °C for 34 s. The plasmid for the standard curve was constructed according to Fraser et al. (2015a, b). The standard curve was prepared using serial 10-fold dilutions, and the number of gene copies was calculated by measuring the concentration of the plasmid and the number of base pairs. Amplification efficiencies ranged from 96 to 98% with R^2 values of 0.9992.

Illumina MiSeq high-throughput sequencing and data analysis

The diversity and composition of ALP-harbouring bacterial communities were assessed using high-throughput sequencing technique. Universal primers ALPS-F730 and ALPS-1101 were selected for PCR amplification of the *phoD* gene. Despite few biases, this primer can give accurate phylogenetic and taxonomic information (Tan et al. 2013; Ragot et al. 2015). Personal Biotechnology Co., Ltd. (Shanghai, China) conducted the sequencing using an Illumina MiSeq platform. Overlapping paired-end reads were assembled using PEAR software (Zhang et al. 2013). Primers and poor-quality sequences were removed using cut adapt software (Martin 2011). The sequences were converted to amino acid sequences using the Framebot tool in the RDP function gene pipeline (http://fungene.cme.msu.edu/FunGenePipeline/); sequences that did not correspond to the *phoD* gene or had a termination codon in the middle were removed. In total, 641,489 high-quality sequences were obtained from 15 samples, and the number of sequences per sample ranged from 25,295 to 70,381 (mean = 42,765). Each sample was rarefied to the identical number of reads (10,000) for downstream analyses. The UPARSE pipeline was used to cluster sequences into centroid operational taxonomic units (OTUs) (Edgar 2013). Sequences with 80% similarity were assigned to OTUs, and 941 OTUs were obtained; this resulted in reads that were mapped back to OTUs using USEARCH software. A representative sequence was selected from each OTU, and the RDP function gene database was used to assign taxonomic data to each representative sequence. MOTHUR software (Schloss et al. 2009) was used to calculate the percentages of shared OTUs between samples and to analyse α- and β-diversity; the similarity tree was visualised using the iTOL portal (Letunic and Bork 2006). All sequences were deposited in the NCBI Sequence Read Archive (SRA) database (accession number SRX2010902).

Statistical analyses

Statistical analyses were performed using the IMB SPSS statistical software package version 20 (IBM Corporation, New York, USA). Data were analysed with one-way ANOVA, and Fisher's least significant difference ($p < 0.05$) was used to compare the mean for each variable. Shannon diversity and Chao richness were calculated considering OTU tables, and redundancy analysis (RDA) was performed by the R statistical software package (version 2.15.0) using vegan packages to explore the relationships between the composition of ALP-encoding microbial community and soil properties. The phylogenetic tree was generated using the neighbour-joining method based on representative ALP partial nucleotide sequence matches using MEGA7.0 software. The abundance of the identified genus (*A*) was calculated as follows:

$$A = QA\backslash\text{timesRA} \quad (1)$$

where *QA* is the total abundance of the *phoD* gene measured by qPCR and *RA* is the relative abundance of each identified genus.

SEM were constructed to test for indirect or direct relationships among abundance, diversity and composition of the *phoD*-harbouring communities, potential explanatory variables and ALP activities in a multivariate approach using AMOS software (IBM SPSS AMOS 20.0.0). SEM are better than other statistical analyses, e.g. multiple regressions, because directions can be assigned to several relationships yielding multiple explanatory as well as multiple response variables in one model (Grace 2006). The RDA first axis scores of samples were used as indicators of the community composition of *phoD*-harbouring microbes. The fit of a model to the data was tested using the maximum likelihood (χ^2) goodness-of-fit test, *p* value and the root mean square error of approximation (RMSEA).

Results

Effects of different fertilisation treatments on soil properties

The soil physicochemical properties were significantly affected by fertilisation regimes. Soil pH values increased in M and HMNPK treatments but deceased in the NPK treatment, compared with the CK and MNPK. Soil EC values increased in all treatments compared with the CK. The percentage of fine-sand fraction increased significantly in soils treated with organic fertiliser compared with the NPK and CK treatments, whereas soils only treated with chemical fertilisers contained the highest percentage of coarse sand. Little variation in the percentages of silt and clay fractions was found among treatments (Table 1). The concentrations of NO_3^--N, exchangeable NH_4^+-N and available K increased with fertilisation. The highest concentration of exchangeable NH_4^+-N (9.56 mg/kg) was in the NPK-treated soil whereas that of NO_3^--N was in

Table 1 Soil properties of different fertilisation treatments

Soil attributes	CK	M	NPK	MNPK	HMNPK
N-NH_4^+ (mg/kg)	1.19 ± 0.06d	1.33 ± 0.165d	9.56 ± 0.58a	2.52 ± 0.23c	4.56 ± 1.20b
N-NO_3^- (mg/kg)	4.66 ± 1.13b	26.39 ± 6.29a	21.54 ± 6.78a	24.65 ± 7.82a	29.61 ± 8.24a
DOC (mg/kg)	25.92 ± 1.22c	79.50 ± 5.93a	67.12 ± 4.05b	62.41 ± 3.75b	86.44 ± 6.59a
C_{org} (g/kg)	15.76 ± 0.49d	26.24 ± 0.65b	22.32 ± 1.87c	23.36 ± 1.28c	29.17 ± 0.57a
TN (g/kg)	1.15 ± 0.01d	1.84 ± 0.04b	1.63 ± 0.11c	1.78 ± 0.06b	2.07 ± 0.07a
AK (mg/kg)	79.12 ± 2.57d	144.42 ± 6.00b	123.08 ± 7.23c	126.66 ± 2.17c	161.64 ± 6.86a
pH (H_2O)	7.25 ± 0.03b	7.79 ± 0.03a	5.86 ± 0.05d	7.22 ± 0.03b	7.09 ± 0.10c
EC (μs/cm)	48.82 ± 0.27e	85.58 ± 0.89b	57.62 ± 0.59d	70.34 ± 0.64c	103.9 ± 0.57a
Soil texture (M%)					
Clay	24.86 ± 0.61a	21.01 ± 0.59c	24.22 ± 1.35ab	24.02 ± 1.53ab	22.19 ± 0.46b
Silt	56.55 ± 2.01a	56.97 ± 2.08a	58.28 ± 3.69a	56.04 ± 2.09a	56.78 ± 1.28a
Fine sand	16.14 ± 1.30b	19.59 ± 1.81a	14.06 ± 1.07c	17.36 ± 0.62ab	18.22 ± 1.44a
Coarse sand	2.45 ± 0.20c	2.43 ± 0.15c	3.44 ± 0.50a	2.58 ± 0.31c	2.81 ± 0.20b
Ca^{2+} (g/kg)	5.75 ± 0.34a	5.38 ± 0.08b	4.29 ± 0.18c	4.61 ± 0.16c	4.44 ± 0.09c

Each value represents the average ± standard error

CK no fertiliser application, *M* application of single organic fertiliser, *NPK* application of single chemical fertiliser, *MNPK* mixed application of organic and chemical fertiliser, *HMNPK* mixed application of organic and chemical fertiliser with higher N rate, *DOC* dissolved organic carbon, C_{org} soil organic carbon, *TN* total nitrogen, *TK* total potassium

the HMNPK-treated soil (29.61 mg/kg). Compared with other treatments, the concentrations of C_{org}, TN and DOC were relatively higher in HMNPK-treated soils (29.17 g/kg, 2.07 g/kg and 86.44 mg/kg, respectively), follow by M-treated soils (26.24 g/kg, 1.84 g/kg and 79.50 mg/kg, respectively). The contents of Ca^{2+} in the M and CK soils (5.38 and 5.75 g/kg, respectively) were much higher than those in the other treated soils (Table 1).

Effects of different fertilisation treatments on soil ALP activity

The highest ALP activity (54.57 μg PNP g^{-1} soil h^{-1}) was in the M soil, followed by that of the CK soil (47.39 μg PNP g^{-1} soil h^{-1}). The NPK soil had the lowest ALP activity (5.96 μg PNP g^{-1} soil h^{-1}), which was almost 9.2-fold lower than that in the M soil. The ALP activity of the MNPK and HMNPK soils decreased by 11% and 27%, respectively, compared with that of the CK soil (Fig. 1).

Effects of different fertilisation treatments on *phoD* gene abundance of soil and relative fractions (PSFs)

The trend of *phoD* gene abundance was similar to that of soil ALP activity (Fig. 1), being the highest in the M soil (8.94×10^7 copies g^{-1} soil) and the lowest was in the NPK soil (19.44-fold lower than that in M) (Fig. 2a). The distribution of the *phoD* gene in soil fractions was also affected by the different long-term fertilisation regimes. The highest abundance of the *phoD* gene was detected in the soil silt fraction (1.12×10^7–4.77×10^7 copies g^{-1} soil) and the lowest in the soil coarse sand fraction (2.39×10^5–2.96×10^6 copies g^{-1} soil; Fig. 2b). In all soils, excluding the NPK soil, most of the *phoD*-harbouring microbes commonly colonised the silt fraction, accounting for 62–71% of the total abundance in bulk soil (Fig. 2c). In the NPK soil, the percentage of *phoD* abundance associated to the

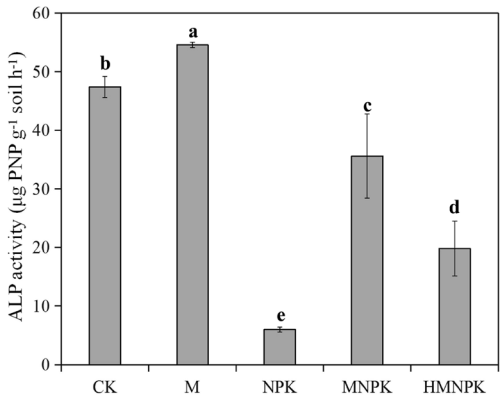

Fig. 1 Potential alkaline phosphatase (*ALP*) activity measured in soils amended with different long-term fertilisation treatments. Data points represent treatment means ($n = 3$), and *different letters* indicate significant differences ($p < 0.05$). *CK* no fertiliser application, *M* application of single organic fertiliser, *NPK* application of single chemical fertiliser, *MNPK* mixed application of organic and chemical fertilisers, *HMNPK* mixed application of organic and chemical fertilisers with higher N rate

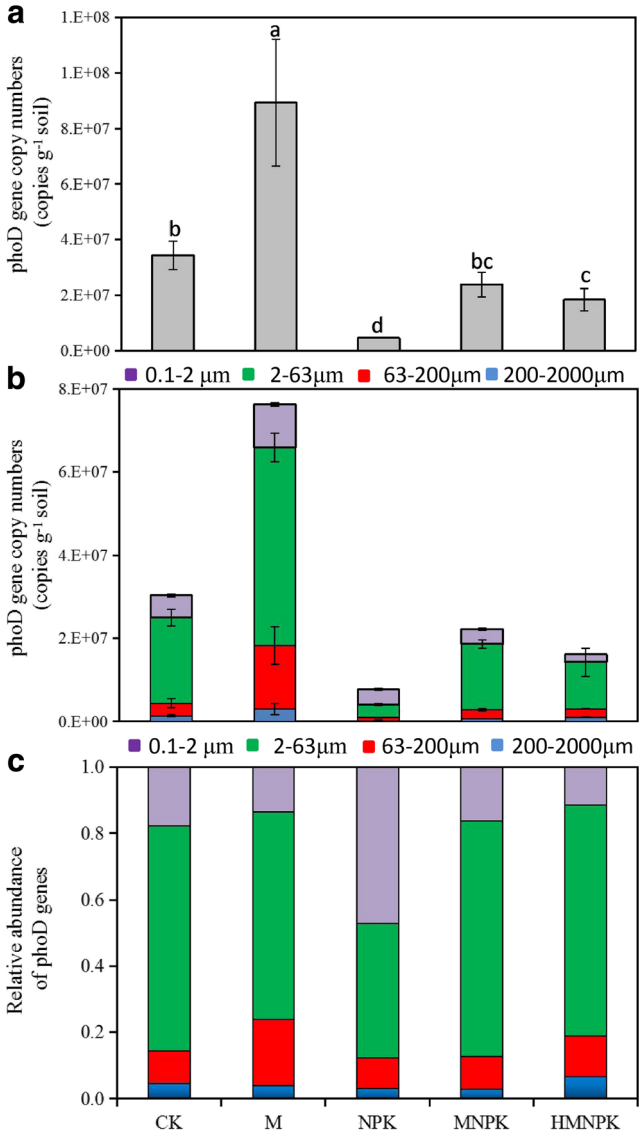

Fig. 2 Abundances of the bacterial *phoD* gene estimated by gene copy numbers, as quantified by qPCR. **a** Total *phoD* gene copy numbers in each treatment, **b** *phoD* gene copy numbers in different soil fractions of each treatment and **c** relative abundance of *phoD* genes summing copy numbers of the different soil fractions. Data points represent treatment means (n = 3), and *different letters* indicate significant differences ($p < 0.05$) between treatments: 0.1–2 μm, clay; 2–63 μm, silt; 63–200 μm, fine sand; and 200–2000 μm, coarse sand. *CK* no fertiliser application, *M* application of single organic fertiliser, *NPK* application of single chemical fertiliser, *MNPK* mixed application of organic and chemical fertilisers, *HMNPK* mixed application of organic and chemical fertilisers with higher N rate

clay fraction (47%) was higher than that associated to the silt fraction (40%) (Fig. 2c).

Effects of different fertilisation treatments on soil P fractions

The content of AP was the highest in the chemical fertiliser-treated soils (NPK, MNPK and HMNPK) (Fig. 3a), and the total P (TP) content was higher in all fertilised soils than in the control (CK), with the highest content (0.74 g/kg) in the HMNPK soil. The contents of soil AP and TP in the organic-only fertilised soil were intermediate between the CK and chemically fertilised soils. Moreover, the percentages of inorganic P and organic P of soils depended on the different fertilisation regimes (Fig. 3b). The percentage of organic P contents were 81% and 73% in the CK and M soils, respectively, being higher than those (32–49%) of the NPK-, MNPK- and HMNPK-treated soils (Fig. 3b).

Compared with the CK treatment, the contents of MLPo, FAPo and HAPo (59.88–81.54 mg/kg, 88.20–123.62 mg/kg and 45.62–59.91 mg/kg, respectively) significantly increased in organic amended treatments (M, MNPK and HMNPK). Notably, the content of LPo was not significantly different between CK (80.24 mg/kg) and M (83.19 mg/kg) treatments, but it was higher than those of the chemical-fertilised (NPK, MNPK and HMNPK) soils. The lowest content of MLPo was in the NPK treated soil, whereas the lowest contents of FAPo and HAPo were observed in the CK soil. Both MLPo and FAPo contents increased by the only-organic amendment, compared with the chemical-only fertilisation (Fig. 3). The content of HAPo (54.96 mg/kg) was significantly higher (Fig. 3f) in the NPK than the CK soil. The highest percentage soil total Po present as LPo occurred in the CK treatment (32.05%), but the highest percentages of total Po present as FAPo and HAPo were displayed in the NPK soil and that present as MLPo in the MNPK soil. The fertilised soils (M, NPK, MNPK and HMNPK) have higher percentage of FAPo than other Po fractions (35.5%, 40.35%, 30.36% and 42.64%, respectively). The percentage soil total Po present as HAPo was the lowest in the CK and M soils (Fig. S1).

Relationship of soil P status with *phoD* gene abundance and ALP activity

Significant positive correlation was observed between the *phoD* gene abundance and ALP activity (Fig. S2a; $p < 0.01$, $r^2 = 0.856$). The *phoD* gene abundance was significantly correlated with the LPo (Fig. S3a; $p < 0.01$, $r^2 = 0.551$), MLPo (Fig. S3b; $p < 0.01$, $r^2 = 0.403$) and FAPo (Fig. S3c; $p < 0.05$, $r^2 = 0.272$) contents but not with the HAPo content (Fig. S3d; $p = 0.875$, $r^2 = 0.002$). The ALP activity was significantly correlated with the LPo content (Fig. S3e; $p < 0.01$, $r^2 = 0.840$) but not with MLPo (Fig. S3f), FAPo (Fig. S3g) or HAPo (Fig. S3h) contents. In addition, a negative relationship was observed between the *phoD* gene abundance and available P content (Fig. S2b; $p < 0.05$, $r^2 = 0.272$) and ALP activity with both AP (Fig. S2c; $p = 0.01$, $r^2 = 0.526$) and total P (Fig. S2d; $p = 0.01$, $r^2 = 0.398$) contents.

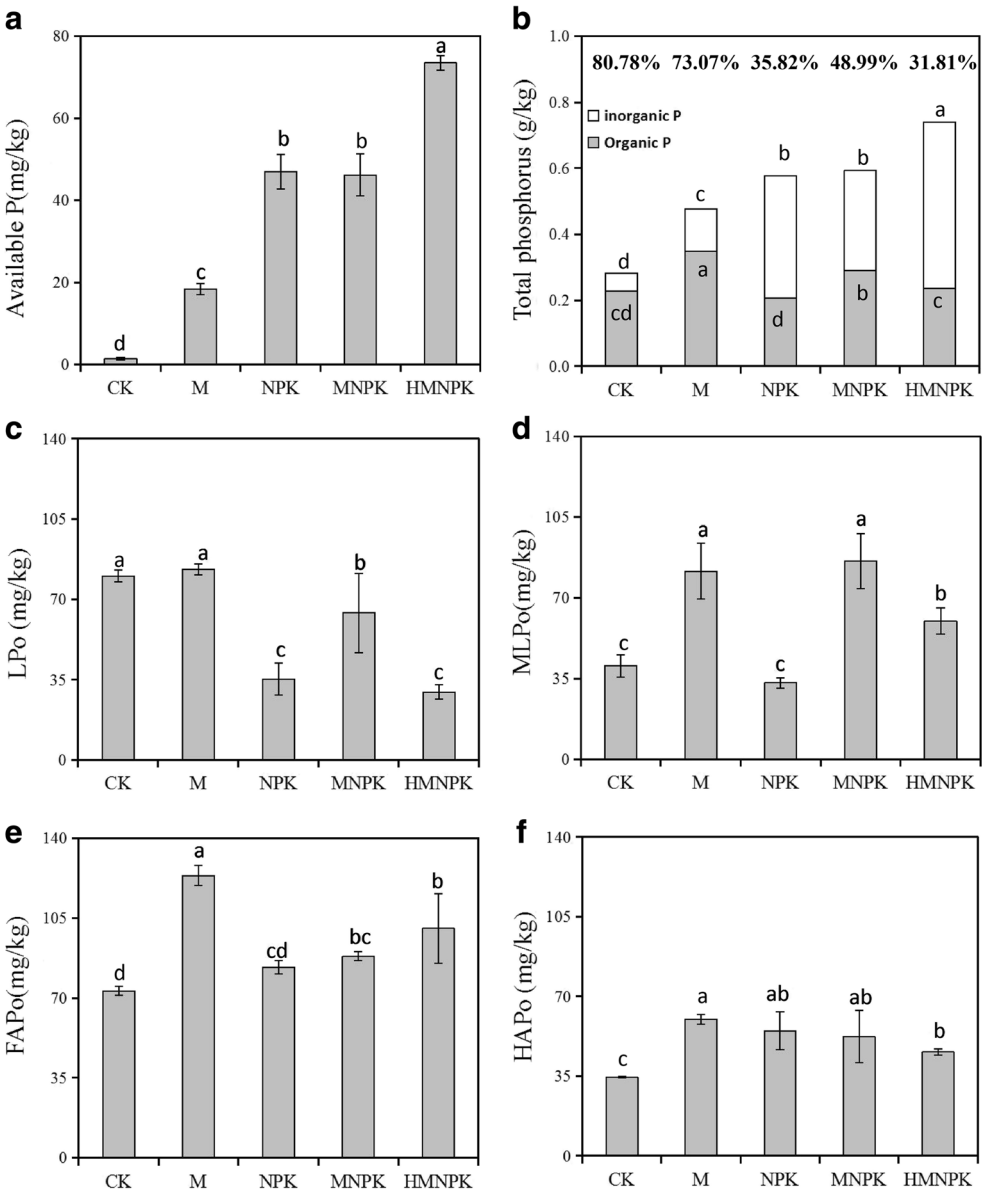

Fig. 3 Contents of the different soil P fractions: **a** content of soil $NaHCO_3$-extractable available P in original soil; **b** concentrations of organic P, inorganic P and total P of original soil; **c** content of labile organic P (*LPo*); **d** content of moderately labile organic P (*MLPo*); **e** content of fulvic acid-associated organic P (*FAPo*); and **f** content of humic acid-associated organic P (*HAPo*). *CK* no fertiliser application, *M* application of single organic fertiliser, *NPK* application of single chemical fertiliser, *MNPK* mixed application of organic and chemical fertilisers, *HMNPK* mixed application of organic and chemical fertilisers with higher N rate. *Numbers on top of* (**b**) represent the percentage of total P present as organic P. Data points represent treatment means ($n = 3$), and *different letters* indicate significant differences ($p < 0.05$)

Effects of different fertilisation treatments on the composition of microbial community harbouring the *phoD* gene

The rarefaction curves of all treated soils nearly reached a plateau, and all samples rarefied to 10,000 sequences; this suggests that the read number per sample was sufficient to capture the overall richness. The species richness and alpha diversity of M and CK soils were lower than those of the other fertilised soils (NPK, MNPK and HMNPK), as shown by the rarefaction curves, Shannon index and Chao index (Fig. S4; Table 2). The first two axes of redundancy analysis (RDA) explained 43.56% and 17.45% of the total variation. The *phoD* gene communities of CK and M soils were separated from the other treated soils (NPK, MNPK and HMNPK) along the first axis. The gene community compositions of NPK and CK soils were separated from those of the other

Table 2 Shannon indexes and Chao indexes in different fertilisation treatments

Treatment	Shannon index	Chao index
CK	4.26 ± 0.02b	493.65 ± 20.90c
M	3.82 ± 0.02c	498.67 ± 25.73c
NPK	4.66 ± 0.01a	573.98 ± 24.40b
MNPK	4.40 ± 0.01ab	592.03 ± 19.13b
HMNPK	4.64 ± 0.02a	670.36 ± 24.09a

Each value represents the mean ± standard error and different letters indicate significant differences ($p < 0.05$)

organic-fertilised soils (M, MNPK and HMNPK) along the second axis (Fig. 4). According to the Monte Carlo permutation test (permutation = 9999), the ALP activity, pH, EC and AP; LPo; TP; Ca^{2+}; C_{org}; DOC; and TN contents were represented with long arrows and were significantly correlated with the composition of the *phoD* microbial communities (Table S1).

Effects of different fertilisation treatments on the dominant *phoD*-harbouring microbial communities

Most of the OTUs were affiliated with the bacterial kingdom. Whereas, only a few OTUs were affiliated with the fungal kingdom. The dominant *phoD*-harbouring bacterial phyla in all treatments were *Actinobacteria* and *Proteobacteria,* and the dominant genera were *Actinoplanes*, *Micromonospora*, *Streptomyces*, *Bradyrhizobium*, *Burkholderia* and *Lysobacter* (>1%) (Fig. S5). *Bradyrhizobium* was the dominant phoD-harbouring genus in the different fertilised soils, particularly in the M, CK and MNPK soils (accounting for 57%, 53% and 51% of the total, respectively). The relative abundance of *Streptomyces* was higher in the organic-fertilised soils (range, 15–20%) than in the NPK and CK soils. The *Sphingomonas* and *Thauera* relative abundances were the highest in the NPK (12% of the total) and in the HMNPK soil (10%), respectively (Fig. S5).

The dominant species (OTUs; >10%) were used to construct the phylogenetic tree (Fig. 5). The OTU24880, OTU16861 and OTU22270 were closely related to *Bradyrhizobium*, and the OTU5587 was closely related to *Mesorhizobium*. The OTU4187 and OTU3540 were closely related to *Sphingomonas* and *Actinomyces*, respectively. The OTU26581 and OTU31653 were closely related to *Frankia*, and OTU7961 and OTU9863 to *Streptomyces*. The other OTUs were not closely related to a species but were located in either the *Proteobacteria* or the *Actinobacteria* cluster (Fig. 5).

The abundance of each dominant genus, which was calculated considering the relative abundance and qPCR data, was significantly shifted in soils by different fertilisation regimes (Fig. S6). All 12 dominant genera, except *Sphingomonas*, had lower abundance in the NPK soil than in the other fertilised soils. *Micromonospora, Modestobacter, Streptomyces, Lysobacter* and *Bradyrhizobium* were dominant genera in the M soil (1.82×10^6 copies g^{-1} soil, 5.90×10^6 copies g^{-1} soil, 1.39×10^7 copies g^{-1} soil, 6.56×10^6 copies g^{-1} soil and 4.83×10^7 copies g^{-1} soil, respectively). Notably, the highest abundance of *Frankia* occurred in the CK soil (3.34×10^6 copies g^{-1} soil), and the abundance of *Burkholderia* was higher in CK (3.52×10^6 copies g^{-1} soil) and M (2.83×10^6 copies g^{-1} soil) soil than in the other treated soils (Fig. S6).

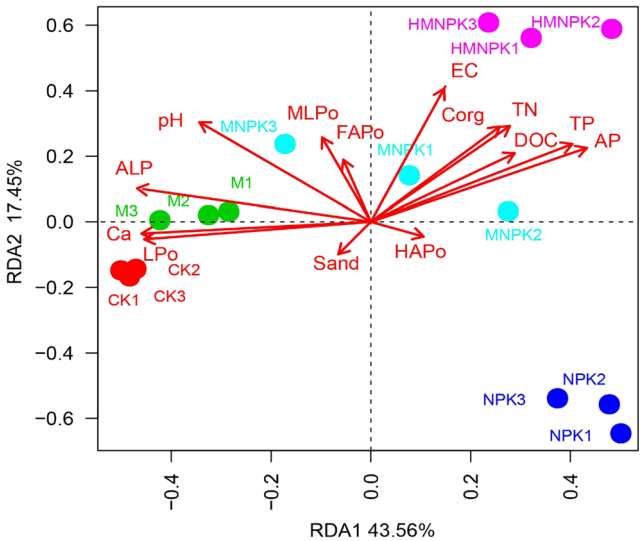

Fig. 4 Ordination plots by the redundancy analysis (*RDA*) used to explore the relationships between the ALP-encoding microbial community and selected soil properties. The *arrows* indicate the lengths and angles between explanatory and response variables and reflect their correlations. Samples from different long-term fertilisation treatments were marked with *different colours*. Symbols of fertiliser regimes are as described in Table 1. *CK* no fertiliser application, *M* application of single organic fertiliser, *NPK* application of single chemical fertiliser, *MNPK* mixed application of organic and chemical fertilisers, *HMNPK* mixed application of organic and chemical fertilisers with higher N rate. *Numbers behind the treatments* indicate the replications

Correlations of dominant genera abundances with ALP activity and soil properties

A significantly positive relationship was observed between ALP activity and abundances of *Bradyrhizobium* ($p < 0.01$, $r^2 = 0.939$), *Modestobacter* ($p < 0.05$, $r^2 = 0.360$), *Frankia* ($p < 0.05$, $r^2 = 0.462$), *Streptomyces* ($p < 0.01$, $r^2 = 0.645$), *Lysobacter* ($p < 0.01$, $r^2 = 0.913$) and *Burkholderia* ($p < 0.01$, $r^2 = 0.747$), and the abundances of these genera were the highest in the M or CK soils (Figs. S6 and S7).

According to the Pearson's correlations (Table S2), the abundances of all dominant genera were significantly correlated with pH, except for those of *Thauera* and *Frankia*. The abundances of *Modestobacter*, *Frankia*, *Bradyrhizobium*, *Burkholderia* and *Lysobacter* were positively correlated with the LPo content. And the abundances of *Frankia*, *Bradyrhizobium*, *Burkholderia* and *Lysobacter* were positively correlated with the Ca^{2+} content but negatively correlated with the AP content. The abundances of *Frankia* were negatively correlated with TN, DOC and C_{org} contents, and these soil properties were positively correlated with *Rhodanobacter* abundance. Among all investigated soil properties, the abundance of *Micromonospora*, *Streptomyces* and *Sphingomonas* was only significantly correlated with soil pH (Table S2).

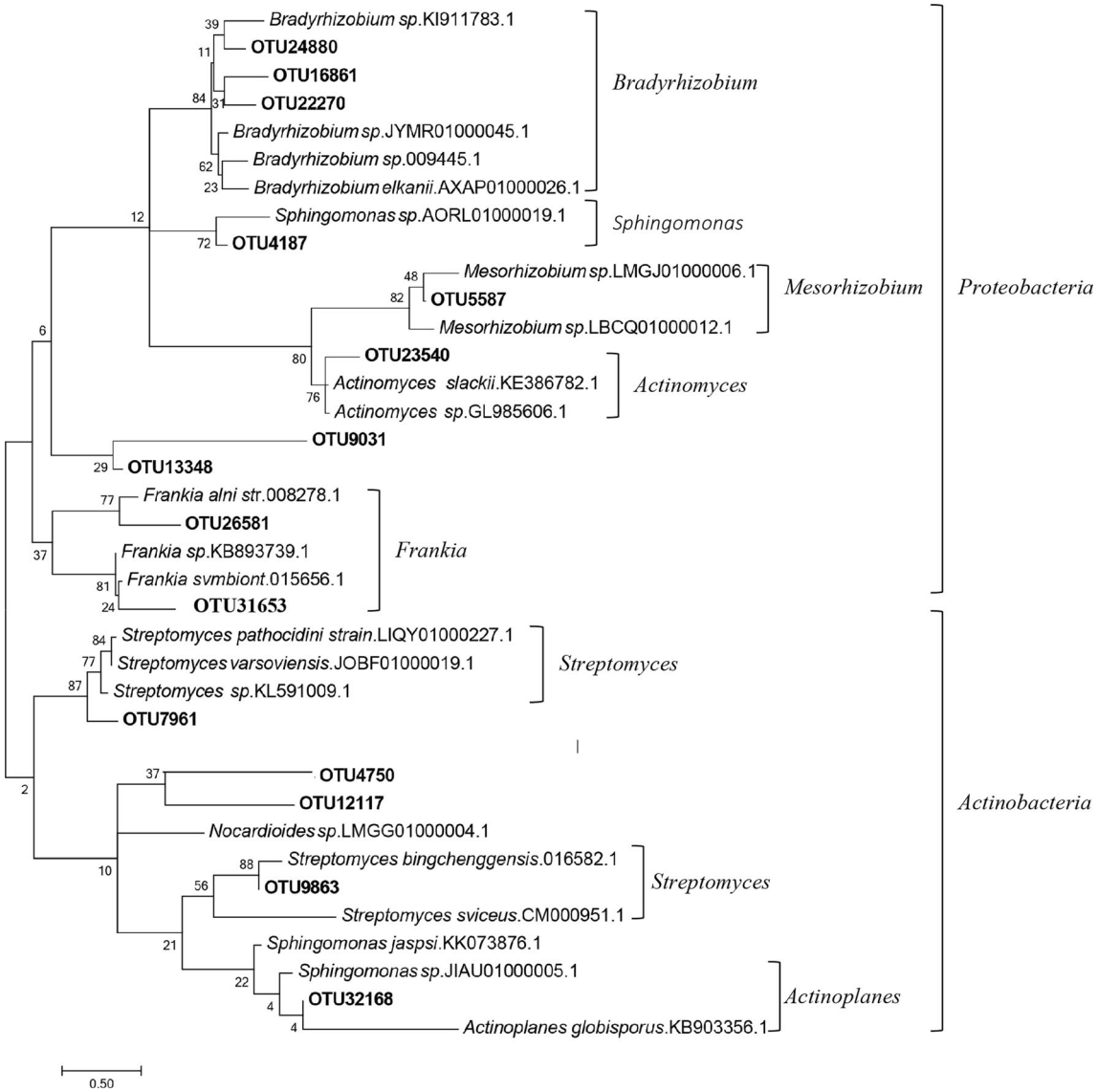

Fig. 5 Neighbour-joining phylogenetic tree that represents the phylogenetic relationships of the 15 OTUs (>10%) to the most closely related identified sequences obtained from the NCBI was inferred using the maximum likelihood method based on the Tamura-Nei model for a neighbour-joining tree. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A bootstrap analysis was performed with 1000 trials. The *bar* indicates an estimated sequence divergence

SEM pathways of fertilisation regimes indicate effects on soil ALP activity via soil properties

The SEMs explained 98% of the variance in soil ALP activities (Fig. 6), which depended on pH and contents of AP, DOC, LPo and C_{org} as affected by the different fertilisation regimes. Particularly, ALP activity was directly mediated by both soil pH (path coefficient = 0.480) and LPo content (path coefficient = 0.42) and probably indirectly by AP, C_{org} and DOC contents (Fig. 6). The abundance of the *phoD* gene was directly affected by the AP content, which affected the ALP activity as reported above. Probably, soil C_{org} content affected ALP activity indirectly via direct effect on composition of the *phoD* gene community or AP content. Finally, soil DOC content directly affected total bacterial abundance (total bacterial abundance is shown in Fig. S8) and then mediated the abundance and composition of the *phoD* gene microbial community; probably, this resulted in the ultimate regulation of ALP activity. Although 72% of the total variance in the α-diversity of *phoD*-harbouring microbes was explained by soil pH and DOC content, these soil properties did not play a significant role in the regulation of ALP activity. Nevertheless, biotic factors, including composition and abundance of the *phoD* gene communities directly affected ALP activity, with the path coefficients of −0.18 and 0.13, respectively (Fig. 6).

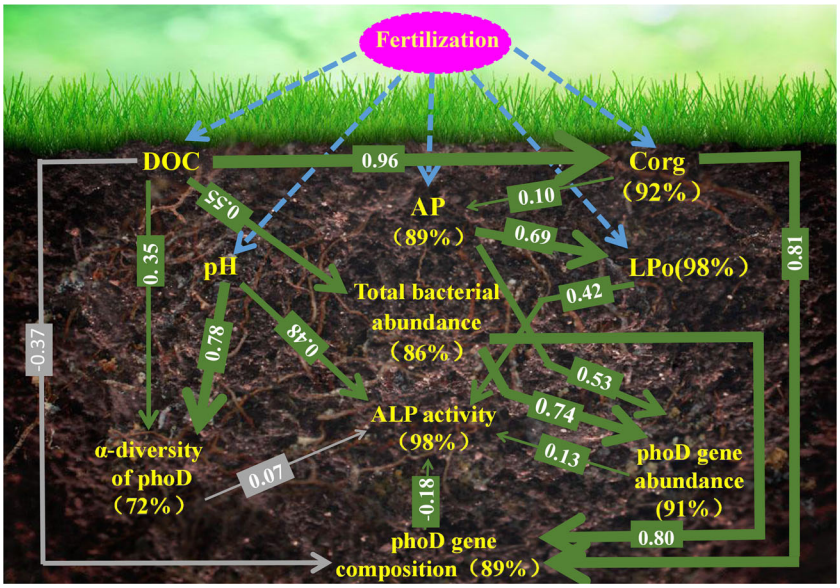

Fig. 6 Structural equation model (SEM) of the influences of *phoD* gene composition and abundance, pH and labile organic P (*LPo*) content on ALP activity, as mediated by total bacterial abundance, dissolved organic C (*DOC*), C_{org}, and available P (*AP*) in the soil. *Numbers following the included variables* show the explained percentage of its variance by its predictors. *Numbers on arrows* are standardised path coefficients. *Green* and *grey arrows* indicate significant and unsignificant effects, respectively, with the thickness representing the extent of influence. The models fit the data well. Model fits are given in Table S3, with the significance levels provided in Table S4. The total bacterial population is shown in Fig. S8

Discussion

The abundance of the *phoD* gene increased significantly by organic-only fertilisation but decreased by chemical-only fertilisation when compared with the control. The organic fertiliser, rich in C substrates but low in available P, probably favoured the growth of some *phoD*-harbouring species, thus increasing the abundance of the *phoD* genes and ALP activity. However, the organic fertilisation decreased the diversity of the *phoD* genes in soil. Fraser et al. (2015b) also found fewer OTUs and higher ALP activity in organically managed soils than in conventionally managed soils. In addition, it is well established that low available P favour the synthesis of phosphatases (Nannipieri et al. 2011). On the other hand, the long-term, chemical-only fertilisation might have developed a low C resource environment, which might have favoured antagonistic bacteria (Bakker et al. 2013) with the increase in the competitive interactions of microbes for C substrate. As previously demonstrated, greater proportions of antagonistic bacteria are positively correlated with bacterial diversity (Schlatter et al. 2015). Therefore, although the soil had high *phoD* gene diversity, the ALP activity remained low, probably regulated by available P content and also associated with thew low *phoD*-harbouring microbial abundance.

Significant differences in the soil *phoD* bacterial community composition were observed with different fertilisation regimes. Our results confirmed that changes in the composition of *phoD* microbial communities can occur under organic fertilisation (Sakurai et al. 2008; Tan et al. 2013). The soil pH was the primary driver of the composition of *phoD*-harbouring communities in arable and grassland soils (Ragot et al. 2015; Wang et al. 2012); in addition, Ca^{2+}, as a cofactor for the activity of ALP synthesised by the *phoD* gene (Kageyama et al. 2011), may affect indirectly the composition of *phoD*-harbouring microbial communities (Wu et al. 2007). Indeed, the Ca^{2+} content of soil with the contents of Po, C_{org}, DOC and pH had significant effects on the *phoD* microbial community composition, based on the Monte Carlo test (Table S1). However, this was not observed by the SEM model probably because only the first RDA axis score was considered to represent the community composition and Ca^{2+} content might affect community composition via the other axes.

The dominant *phoD*-harbouring microbes were *Actinobacteria* and *Proteobacteria* (Lagos et al. 2016). Both phyla together to *Cyanobacteria* have been already reported to be the dominant *phoD*-harbouring in soils subjected to different phosphate fertilisation regimes (Tan et al. 2013; Chaudhry et al. 2012; Chhabra et al. 2012). In all studied soils, the dominant genera of *phoD*-harbouring microbes were *Bradyrhizobium* and *Streptomyces,* thus conferming that the dominance of the *phoD*-harbouring genera does not change in soils by changing environmental conditions (Ragot et al. 2015). Particularly, the abundances of *Actinoplanes*, *Micromonospora,* Nocardioides, *Rhodanobacter*, *Streptomyces* and *Thauera* increased in organic fertilised soils. The abundances of *Modestobacter*, *Frankia*, *Bradyrhizobium*, *Burkholderia* and *Lysobacter* were significantly and

positively affected by the LPo content, negatively affected by the AP content and positively correlated with the Ca^{2+} content, with the exception of *Modestobacter*. The abundances of *Micromonospora*, *Streptomyces* and *Sphingomonas* were only sensitive to the variation in soil pH. All of these results obviously suggest that the particular taxa within a phylum differ in their ecological characteristics (Spohn et al. 2015).

Bradyrhizobium, a free-living and symbiotic N_2-fixer, had the highest relative abundance in all treated soils, suggesting that this genus might play an important role in coupling soil N and P cycle. Some *alpha-Proteobacteria* (e.g. *Bradyrhizobium*) respond to P stress by increasing ALP activity and P transport rates (Smart et al. 1984; Alniemi et al. 1997; Sakurai et al. 2008). Moreover, Dick et al. (1988) found that ALP is highly correlated with total N content. Therefore, future work should clarify the coupling between N cycling and P turnover under various conditions (e.g. AP-limiting) as mediated by *Bradyrhizobium*, which might not only contribute to the increases in ALP activity and P transport rates but also to modify soil N pools.

Because of differences in mineralogical composition and organic matter content, soil fractions provide different surface properties and microenvironments, which may affect the adsorption of chemicals and select for distinct microbial communities (Hemkemeyer et al. 2015). The highest abundance of the *phoD* gene was generally detected in the soil silt fraction, but the fertilisation regimes affected the abundance of the *phoD*-harbouring microbes within the different soil fractions (Fig. 2). The most abundant *phoD*-encoding microbes were harboured in the soil clay fraction in the chemical-only fertilisation treatment, which was unexpected because SOM in the soil clay fraction is suggested to be more recalcitrant than in the other soil fractions (Huang et al. 2010; Chung et al. 2008). However, phosphorylation of soil organic compounds is one of the strategies for increasing the recalcitrance of organic compounds by microbes (Yagil and Beacham 1975). Generally, plant phosphatases only function to mineralise organic P, whereas microbial phosphatases and other microbial enzymes are involved in organic C mineralisation to make both P and C available to microbial cells from Po (Steenbergh et al. 2011; Spohn and Kuzyakov 2013b; Heuck et al. 2015). Probably, microbes of long-term, chemical-only fertilised soils are under C-limited conditions and might release phosphomonoesterase not only to mineralise organic P but also to render organic-phosphorylated compounds available as a C source after dephosphorylation. If this hypothesis is true, enriched *phoD*-harbouring microbes in soil clay fraction under long-term chemical-only fertilisation may accelerate the dephosphorylation of SOM allocated to clay particles thus increasing degradation of SOM protected in clay, and soil ALP activity can play a role in regulating the stabilisation of soil C by clay particles. Further studies are required to understand the potential links between composition and function of *phoD*-harbouring microbial communities associated with different soil fractions under the influence of long-term fertilisation (Hemkemeyer et al. 2015) and the contributions of phosphomonoesterase activities to soil C stabilisation in different soil fractions.

Soil ALP activity was negatively correlated with AP (Acuña et al. 2016) but positively correlated with Po, thus conforming that available P represses the synthesis of phosphomonoesterases (Sakurai et al. 2008; Nannipieri et al. 2011; Zhang et al. 2012; Fraser et al. 2015a, b; Wei et al. 2014; Cui et al. 2015). In our study, organic amendment increased the contents of soil MLPo, FAPo and HAPo, and that of LPo significantly increased in organic-only amended soil. Whereas, the chemical fertiliser inputs decreased the LPo and MLPo concentration (Fig. 3). Probably, the content of AP were sufficient to microorganisms in the chemical-fertilised soil, and thus they did not need to decompose the stable Po (FAPo and HAPo) whose content increased with the fertilisation. Cui et al. (2015) found that soil LPo and MLPo contents showed a significant relationship with the *phoD* gene community composition, whereas in this study, the *phoD* gene abundance was significantly correlated with LPo, MLPo and FAPo contents, whereas the ALP activity was only positively correlated with the LPo content (Fig. S3) probably because LPo is the Po fraction more easily hydrolysed by the enzyme than the other Po fractions. The most recalcitrant Po fraction, HAPo, likely had no role in affecting either the abundance of *phoD*-harbouring microbes or ALP activity. Further research should also investigate the link between other phosphatases (i.e. acid phosphomonoesterase, phytase and phosphodiesterase) and the MLPo, FAPo and HAPo fractions.

Both acid and alkaline phosphomonoesterase activities can be used as indicators of the potential organic P mineralisation in soil (Chen 2003). Soil ALP activity was significantly affected by the different long-term fertilisation regimes, being increased by the addition of organic matter as already reported (Saha et al. 2008; Sakurai et al. 2008; Nannipieri et al. 2011; Zhang et al. 2014a), but decreased by the addition of mineral P (Saha et al. 2008; Spohn et al. 2015; Marklein and Houlton 2012). However, contradictory reports concerning the relationships between ALP activity and some soil properties have been reported. Dick et al. (1988) and Saha et al. (2008) found a positive correlation between phosphomonoesterase activity and SOM content, whereas others showed no relationship (Brockett et al. 2012; Fraser et al. 2015a, b; Senwo et al. 2007). By using the SEM model, we have observed that both soil pH (path coefficient = 0.480) and LPo (path coefficient = 0.42; Fig. 6) contents directly mediated ALP activity. Other studies showed that pH and LPo content were the primary driving factors affecting ALP activity; it is well established that ALP activity prevails over acid phosphomonoesterase activity at alkaline pH values and the opposite occurs at acid pH values (Dick et al. 2000; Nannipieri et al.

2011; Wei et al. 2014; Cui et al. 2015). In addition, we also observed indirect effects of soil C_{org}, DOC and AP contents on ALP activity via regulation of other biotic and abiotic variables caused by the different fertilisation regimes (Fig. 6).

According to the SEM, ALP activity was directly regulated by the *phoD* microbial abundance and composition. It has been known already that the *phoD* gene abundances and ALP activity are significantly correlated in soil under different management systems (Fraser et al. 2015b), and that the variation in the composition of the *phoD* gene induced by fertilisation regimes can affect ALP activity (Tan et al. 2013; Chaudhry et al. 2012; Chhabra et al. 2012). Here, we have observed that the abundances of *Bradyrhizobium*, *Streptomyces*, *Modestobacter*, *Lysobacter*, *Frankia* and *Burkholderia* were higher in organic-treated than in chemical-only fertilised soils, and they were significantly correlated with soil ALP activity. Probably, these genera may be more efficiently involved in the synthesis of the enzyme upon organic fertilisation than others (Figs. S6 and S7). To verify this, hypothesis research on expression of the relative enzyme-encoding genes is needed.

In conclusion, different fertilisation regimes notably changed the abundance and composition of ALP gene (*phoD*) harbouring microbial communities and ALP activities mainly by affecting soil properties as pH and contents of AP, DOC, LPo and C_{org}. The SEM showed that the changes in soil pH and LPo content induced by the different fertilisations were the primary driving forces affecting the ALP activity directly, whereas the other abiotic factors probably affected the ALP activity indirectly by altering the composition and abundance of *phoD*-harbouring microbes. Soil organic P fractions played an important role in shaping the composition of ALP-encoding microbial community and ALP activity. The highest number of the *phoD* gene was commonly associated to the soil silt fraction. It is hypothesised that the *phoD* microbial communities of the clay particles of the chemical-only fertilised soil may contribute to the degradation of the recalcitrant organic P compounds associated with clay particles by dephosphorylating these compounds. The obtained data confirmed the hypothesis that the abundance *phoD* gene differed in soil fractions of the different fertilised soils. *Actinobacteria* and *Proteobacteria* were identified as the dominant *phoD*-encoding phyla. The *Bradyrhizobium*, as a N_2-fixer, was identified as an abundant *phoD*-encoding bacteria in all treated soils. Further studies should address the temporal variability and the expression of *phoD*-encoding microbial communities, and both determinations of mRNA and synthesised proteins are needed to confirm the relationship between *phoD* microbial communities and ALP activity.

Acknowledgements This work was financially supported by the China Science and Technology Ministry (2015CB150500, 2013CB127403), Youth Innovation Fund of Anhui Academy of Agricultural Sciences (15B0204) and Jiangsu Science and Technology Department (BK20150059).

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