



Earthworm activities change phosphorus mobilization and uptake strategies in deep soil layers

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ABSTRACT

The drilosphere is described as a microbial hotspot in soil, which is rich in labile nutrients. However, phosphorus (P) quality and availability change along the drilosphere from labile organically-bound P in upper layers to immobilized Ca-phosphate with increasing depth. In this study, we postulated (1) that microbial P turnover is generally accelerated in the drilosphere compared to bulk soil as a result of an increase in labile nutrients, and (2) that P mineralization is dominant in upper layers of the drilosphere and P solubilization in deeper layers of the drilosphere, respectively. Furthermore, we hypothesized (3) that the enhanced P mobilization in the drilosphere favors copiotrophic and earthworm gut derived bacteria like *Pseudomonadaceae*, which distinguishes the community composition from that of the bulk soil. We investigated the effect of earthworm activities on potential phosphatase activities, abundance and diversity of bacterial communities involved in P mobilization (*gcd*, *phoD*, *appA*, *phnX*, *phoN*) and uptake (*pstS*, *pitA*) along a soil depth gradient in a short rotation coppice in Northeast Germany. Potential phosphatase activities and gene abundances revealed increasing potentials for organic P mineralization and high-affinity P uptake via the Pst system in the drilosphere below 50 cm depth. Conversely, in the upper soil layers potential for bacterial P mobilization were similar between drilosphere and bulk soil. We also observed drilosphere-related changes in the composition of bacterial communities involved in P solubilization and low-affinity P uptake via the Pit system below 60 cm depth. In the drilosphere compared to bulk soil, *Pseudomonadaceae* were enriched and played a major role in solubilization and low-affinity uptake of P, while bacteria potentially involved in N turnover like *Bradyrhizobiaceae* and *Phyllobacteriaceae* were less abundant. Our study suggested that earthworms increased the potential enzyme activity and abundance of bacteria carrying the Pho-regulon genes in the drilosphere of the deeper soil layers. Furthermore, earthworm-derived bacteria were enriched in the drilosphere at the same soil depth, especially those involved in P solubilization and carrying the Pit system.

1. Introduction

Phosphorus (P) is an important macronutrient for all biota on Earth. However, its availability is low in soils because free bioavailable orthophosphate (P_i ; PO_4^{3-} , HPO_4^{2-} , $H_2PO_4^-$) is quickly immobilized into biomass or insoluble forms. Immobilization into microbial biomass is triggered via different transport systems. For example, well known microbial P transport systems are the inorganic phosphate transporter (Pit), which is driven by a proton motive force under sufficient P supply,

and the phosphate-specific transporter (Pst), which as part of the Pho regulon is induced under P_i limitation (Gebhard et al., 2009; Qi et al., 1997). Most microorganisms can increase the levels of P_i through mineralization of organic P (P_{org}) sources and solubilization of adsorbed P compounds (Richardson and Simpson, 2011). Microbial P mineralization is triggered by extracellular phosphatases including non-specific phosphatases, phytases, C—P lyases and phosphonatases (Lidbury et al., 2017; Rodríguez et al., 2006), while microbial P solubilization is mediated by the release of protons, organic and inorganic acids and

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siderophores (Alori et al., 2017; Park et al., 2009; Rawat et al., 2021). In soils, dominant P mobilization processes have been determined by the quantity and quality of available P sources (Bergkemper et al., 2016b; Dai et al., 2020; Grafe et al., 2018). In this regard, alkaline phosphatases had frequently been identified as the major extracellular enzyme that drives the mineralization of P_{org} (Eder et al., 1996; Kageyama et al., 2011). For P solubilization gluconic acid is well studied and is produced in cells via the oxidation pathway by microbial glucose dehydrogenase (An and Moe, 2016; Tripura et al., 2007).

In addition to P, microbial performance in soils is limited by the availability of carbon (C) and nitrogen (N) (Demoling et al., 2007; Wang et al., 2016). The external inputs of C sources (i.e., litter material, root exudates, earthworm deposits) can stimulate microbial growth and activity, forming microbial hotspots. Subsequently, the demand for N and P increases as microbes strive to maintain the stoichiometry of nutrients in microbial biomass (Cleveland and Liptzin, 2007; Heuck et al., 2015; Waring et al., 2014). The drilosphere is one of the microbial hotspots, defined as part of the soils influenced by activities of anecic and endogeic earthworm species (Brown et al., 2000). Earthworm burrowing and casting increase the amounts of labile C, mineral N, and extractable P in the drilosphere compared to the surrounding soil (Don et al., 2008; Ferlian et al., 2020; van Groenigen et al., 2019). Earthworm activities can also regulate soil pH through transportation of base cations from subsoil and the release of calciferous glands (Lemtiri et al., 2014), thereby altering the distribution of labile P fractions along the soil profile. For example, Bauke et al. (2017) reported larger proportions of labile P_{org} in the upper layers (up to 30 cm depth) and biologically cycled Ca-bound phosphate in the deeper layers (below 45 cm depth) of the drilosphere, when compared to the respective bulk soil. Unlike other microbial hotspots such as rhizosphere and detritusphere, the nutrient peaks in the drilosphere are observed occasionally and for a shorter period of time due to inconsistent nutrient input by earthworms (Kuzakov and Blagodatskaya, 2015).

Earthworms provide not only labile C but also a microbial inoculum deriving from their gut. Those microbes might be enriched in the drilosphere. In terms of P turnover, phosphate solubilizing bacteria (e.g., *Bacillus*, *Pseudomonas*, *Rhizobium*, *Actinomyces* and *Enterobacter*) have been often isolated from earthworm guts (Biswas et al., 2018; Hussain et al., 2016; Maheswari and Sudha, 2013). Wan and Wong (2004) observed that the introduction of earthworm-derived bacterial species increased the population of P solubilizing bacteria in sterile soils, suggesting that the gut microbiota can thrive in soil environments and enhance soil P mobilization. However, to the best of our knowledge, there is no published information on the impact of earthworm-derived microbiome on P turnover in the drilosphere beyond the above cited studies.

Therefore, our study aimed to assess the impact of earthworm activities on bacterial communities involved in P mobilization and uptake at different soil depths. We assumed (I) that microbial P turnover is generally higher in the drilosphere compared to bulk soil as a result of an increase in labile nutrients, (II) P acquisition strategies of bacteria change from P mineralization to P solubilization downward in the drilosphere due to different substrate availabilities. We also postulate (III) that bacterial community composition and abundance driving P mobilization and uptake differ between drilosphere and bulk soil, especially in deeper soil layers. Copiotrophic and potential earthworm gut microbiota dominate the drilosphere in subsoil because of lower competition with indigenous, oligotrophic microorganisms prevailing in subsoil (Uksa et al., 2015a).

To test these hypotheses, we collected drilosphere and bulk soil samples in a short rotation coppice in August 2018. The samples were taken in 10 cm steps down to 70 cm. This site was chosen because no tillage had been performed since 2014. Thus, a high density of earthworm population and intact, long lasting burrows were expected along soil profiles. We evaluated the absolute abundance and diversity of genes involved in bacterial P uptake (*pitA* and *pstS*), mineralization

(*phoN*, *phoD*, *phnX*, and *appA*) and solubilization (*gcd*) using quantitative real-time PCR (qPCR) and next-generation amplicon sequencing. These genes have been frequently detected in high abundance in previous studies (Bergkemper et al., 2016b; Dai et al., 2020; Grafe et al., 2018). Especially for the genes encoding alkaline phosphatases, we focused only on the *phoD* gene because previous metagenomic analyses revealed that it was generally more abundant in agricultural soils of the study region compared to the *phoA* and *phoX* genes (Grafe et al., 2018). These measurements were combined with the assessment of potential enzyme activities of P-hydrolyzing enzymes and abiotic soil properties.

2. Materials and methods

2.1. Site description, soil properties and soil sampling

Soil samples were collected from a short rotation coppice of willows in Rostock, Northeast Germany (54°03'41.2"N, 12°04'55.7"E) in August 2018. Mean annual temperature at the site is 8.1 °C and mean annual precipitation is 600 mm (Peine et al., 2019). The soil was classified as Stagnic Cambisol with a texture from loamy sand to sandy loam according to the World References Base for Soil Resources (IUSS Working Group WRB, 2015).

Tillage had not been performed since the field establishment in 2014, thus a high density of earthworm population was expected. Earthworm burrows covered 3 to 5 % of the surface of the soil profile. The earthworm population at the sampling site primarily consisted of anecic and endogeic earthworms, including *Allolobophora rosea* (32 %), *Aporrectodea* spp. (*caliginosa* and *nocturna*) (26 %), *Allolobophora chlorotica* (19 %), *Lumbricus terrestris* (17 %) (Saggau, 2011), based on morphologic characteristics under a stereo microscope (Askania, Rathenow, Germany) according to the identification guide by Sims and Gerard (1985). Total earthworm abundance ranged from 12 to 25 individuals per square meter (data not shown).

For soil sampling, a soil pit with a size of 1 m × 1 m × 1 m was excavated. Along the pit wall, three vertical earthworm burrows, preferentially with present earthworms or fresh worm traces, were selected at each soil depth (0 to 70 cm depth in 10 cm intervals) and treated as biological replicates. As a drilosphere sample, material from the first 2–3 mm surrounding the burrow (2 cm in diameter) were scrapped and collected, which resulted in approximately 10 g of fresh soil per burrow. As control, bulk soils in a 10 cm horizontal distance from the earthworm burrows, neither affected by earthworms nor plant roots, were collected by scratching the soil surface of the pit walls using a spatula. Both drilosphere and bulk soils were sieved at 2 mm, respectively. All sampling tools were cleaned with ethanol for each sample to avoid cross-contamination. A total of 42 samples (7 depth × 2 compartments × 3 biological replicates) were used for further analyses. Part of the soil samples were stored at 4 °C for soil chemical analyses and the assessment of potential enzyme activities and the remaining soil samples, approximately 2.0 g of fresh soil, were stored at −80 °C for molecular analyses.

2.2. Soil chemical analyses

Analyses of soil chemical properties were performed using composite samples of the three biological replicates but twice (technical duplicates) due to the limited amount of drilosphere soil. Soil pH was measured in a 0.01 M $CaCl_2$ solution (soil:solution ratio 2:5; w/V), without technical replication. Total contents of C and N in air-dried soil were determined in technical duplicates using a CNS Analyzer (Vario EL Fa. Foss Heraeus, Hanau, Germany). For the analysis of P content, 0.1 g of ground soil samples were digested with 5 mL of HNO_3 and 3 mL of H_2O_2 in a microwave (Mars Xpress, CEM, Kamp-Lintfort, Germany) and diluted with distilled H_2O to a total volume of 25 mL. Concentrations of P were determined twice with inductively coupled plasma-optical emission spectroscopy (ICP-OES, Optima 8300, Perkin Elmer,

Waltham, Massachusetts, USA) at wavelengths of 214.914 nm. The total contents of C, N, and P were transformed to a unit of g kg⁻¹ and used to calculate C:N, C:P, and N:P ratios. Water-extractable P was measured exceptionally using three biological replicates, according to van der Paauw (1971) with a modification of using only 0.1 g of dry soil due to low material availability in the drilosphere, and analyzed colorimetrically at a wavelength of 882 nm (Specord 50, Analytik Jena, Germany).

2.3. Potential soil enzyme activities

Potential enzyme activities were measured following the protocol by Tabatabai and Bremner (1969) for acid and alkaline phosphomonoesterases (ACP and ALP, respectively) and by Browman and Tabatabai (1978) for phosphodiesterases (PDE). Briefly, for each assay, 1.0 g of fresh soil was mixed with the respective buffer containing either *p*-nitrophenyl phosphate (for ACP and ALP) or bis-*p*-nitrophenyl phosphate (for PDE) and incubated for 1 h at 37 °C. The amount of *p*-nitrophenol released from phosphate hydrolysis was measured using a colorimetric assay at 400 nm. As suggested by Nannipieri et al. (1978), toluene was not added to the reaction mixture for this short incubation period.

2.4. DNA extraction

Genomic DNA was extracted from 0.30 g bulk or drilosphere soil

$$\text{Gene copies g}^{-1} \text{ dry soil} = \frac{\text{gene copies } \mu\text{L}^{-1} \times \text{dilution factor} \times \text{volume of total DNA extracts (50 } \mu\text{L)}}{\text{amount of dry soil used for DNA extraction}}$$

using a FastDNA® Spin Kit for Soil (MP Biomedicals, Eschwege, Germany) following the manufacture's instruction. To improve DNA extraction efficiency, an additional incubation step at 55 °C for 5 min was performed before centrifuging the final eluate. As a negative extraction control (blank), extractions without soils were processed alongside the samples. The quality of the DNA extracts was assessed by measuring A260 nm/A280 nm and A260 nm/A230 nm ratios on a NanoDrop 1000 spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany). DNA extracts were quantified using a Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific, Darmstadt, Germany) and stored at -20 °C until further use. As an indicator for microbial biomass, DNA concentration per gram of dry soil was calculated.

$$\text{DNA concentration ng}^{-1} \text{ g dry soil} = \frac{\text{extracted DNA concentration ng } \mu\text{L}^{-1} \times \text{volume of total DNA extracts (50 } \mu\text{L)}}{\text{amount of dry soil used for DNA extraction}}$$

The respective data are presented in Supplementary Table S1.

2.5. Quantitative real-time PCR assays

Copy numbers of the 16S rRNA gene and genes encoding P_i transporters or enzymes that drive different P mobilization processes were quantified on a 7300 Real-time PCR System (Thermo Fisher Scientific, Darmstadt, Germany). The primer pairs and bacterial strains used for the absolute quantification are listed in Supplementary Table S2. To reduce PCR inhibition, a pre-experiment with 2-fold serial dilutions of DNA extracts was performed and revealed an optimal dilution of 1:128 for the 16S rRNA gene and of 1:32 for the genes involved in P mobilization and

uptake, respectively (data not shown).

For the 16S rRNA gene, a reaction mixture contained 12.5 μL of Sybr Green PCR Master Mix (Thermo Fisher Scientific, Illkirch Cedex, France), 1 μL of each primer (10 pmol μL⁻¹), 2 μL of DNA templates, and was filled up to 25 μL with DEPC-treated water. Amplification reactions were initiated by a denaturation step at 95 °C for 10 min, followed by 35 cycles of 95 °C for 20 s, 60 °C for 1 min and 72 °C for 30 s. A melting curve analysis was performed at the end of cycling at 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s.

For the genes involved in P mobilization and uptake, a reaction mixture containing 12.5 μL of Sybr Green, 0.8 μL of each primer (except 0.5 μL for *pitA* and 0.2 μL for *phnX*, 10 pmol μL⁻¹ each), 0.5 μL of bovine serum albumin (BSA), 2 μL of DNA templates was used, and filled up to 25 μL with DEPC-treated water. Amplification reactions were initiated with a denaturation step at 95 °C for 10 min, followed by 45 cycles of 95 °C for 20 s, 60 °C for 1 min, 72 °C for 30 s, and 81 °C for 1 min, and a final melting curve analysis. Amplification efficiency was calculated using the formula:

$$\text{Amplification efficiency} = [10^{(-1/\text{Slope})} - 1] \times 100$$

It exceeded 80 % for all reactions. The coefficient of correlation obtained for standard curves (R²) exceeded 0.99 for the 16S rRNA, *pitA*, *gcd*, *phoD*, and *pstS* genes. The copy numbers of the genes were calculated on a soil dry weight base according to the formula:

The copy numbers of the *phoN*, *appA*, and *phnX* genes were below the detection limit (20 gene copies per reaction) and were not included into further analysis.

2.6. Amplicon sequencing

To characterize community composition of bacteria mediating major P mobilization processes and uptake and compare with overall bacterial community composition, amplicon sequencing was performed on an Illumina Miseq platform (Illumina Inc., San Diego, USA) targeting the 16S rRNA, *phoD*, *gcd*, *pitA*, and *pstS* genes. Two depths, 0–10 (topsoil) and 60–70 cm depth (subsoil), were chosen because the difference be-

tween drilosphere and bulk soil based on qPCR data was the biggest. Sequencing libraries were prepared following the “16S Metagenomic Sequencing Library Preparation” protocol (Illumina Inc., San Diego, USA) and quality guidelines recommended by Schöler et al. (2017). This includes processing of extraction blanks and PCR negative controls alongside the sample preparation. Amplification programs of the targeted genes are summarized in Supplementary Table S3. Each PCR amplification was performed in triplicates.

The primer pair S-D-Bact-0008-a-S-16 and S-D-Bact-0343-a-A-15 (Klindworth et al., 2013) with Illumina-overhangs was used for the assessment of overall bacterial community composition. This primer pair amplifies the V1-V2 region of the 16S rRNA gene (Wear et al., 2018). A reaction mixture contained 1.0 μL of DNA templates, 0.5 μL of

each primer (10 pmol μL^{-1}), 1.5 μL of 3 % BSA, 12.5 μL of NEBNext High-Fidelity 2X PCR Master Mix (New England Biolabs, Frankfurt am Main, Germany), and 9 μL of DEPC-treated water. As a negative control, reaction mixtures with 1.0 μL of extraction blank or nuclease-free water were processed. Success of the PCR was checked on a 1.0 % agarose gel. The PCR triplicates were combined and purified using Agencourt AMPure beads (AMPure/PCR product ratio = 0.8; Beckman Coulter, Krefeld, Germany). The length and quantity of the amplicon fragments were determined using a Fragment Analyzer (DNF-473 NGS Fragment kit, Agilent Technologies Inc., Santa Clara, USA).

For bacterial communities involved in major processes of P mobilization and uptake, libraries of the *phoD*, *gcd*, *pitA*, and *pstS* genes were prepared following a nested PCR procedure as described by Bergkemper et al. (2016a), with a modification of the reaction mixture composition. In the first PCR, the targeted genes were amplified using the primer pairs that were used for the qPCR assays without Illumina adapters. A reaction mixture contained DNA templates (25 ng for *phoD* and *pitA*, 50 ng for *gcd*, and 2.0 μL of 10-fold diluted DNA templates for *pstS*), 0.5 μL of each primer (10 pmol μL^{-1}), 1.5 μL of 3 % BSA, 1.0 μL of 50 mM MgSO_4 (Thermo Fisher Scientific, Darmstadt, Germany), 0.5 μL of 10 mM dNTP, 2.5 μL of 10 × High Fidelity PCR Buffer (Thermo Fisher Scientific, Darmstadt, Germany), and 0.2 μL of Platinum® Taq DNA Polymerase High Fidelity (5 U μL^{-1} , Thermo Fisher Scientific, Darmstadt, Germany), and was filled up to 25 μL with DEPC-treated water. In the second PCR, products from the first PCR (2.0 μL for *phoD* and *gcd*, 1.0 μL for *pitA*, and 3.0 μL for *pstS*) were used as templates and amplified using the same amplification programs as in the first step but with primer sequences containing Illumina overhang adapters and only 10 amplification cycles. Success of each PCR step was checked on a 1.0 % agarose gel. Thereafter the PCR triplicates were combined, purified and quantified.

Indexing PCR was performed with a reaction mixture consisting of the purified PCR products (10 ng for the 16S rRNA, *phoD*, and *pitA*, 5.0 ng for *gcd* and *pstS*), 2.5 μL of each indexing primer (Nextera® XT Index Kit v2 set A; Illumina Inc., San Diego, USA), and 12.5 μL NEBNext High-Fidelity 2X PCR Master Mix, and filled up to 25 μL with DEPC-treated water. All indexed amplicons were purified and quantified as described above. Each library was diluted to 4.0 nM and sequenced with the MiSeq Reagent kit v3 (600 cycles) (Illumina Inc., San Diego, USA) for paired-end sequencing. PhiX was used as a spike-in. One sample for the *pstS*-library was re-sequenced due to the insufficient reads.

Sequences were deposited in the NCBI Sequence Read Archive and are available under the Bioproject PRJNA706689.

2.7. Bioinformatic analysis

Sequence data were processed using Quantitative Insights into Microbial Ecology 2 (QIIME2) software package version v2018.8.0 (Bolyen et al., 2019). Raw sequences were separated from adapters using AdapterRemoval v2.1.7, and reads were trimmed with a minimum read length of 50 bp (Schubert et al., 2016). For quality control, 10 bp from the N-terminus were truncated and the reads were removed at certain positions from the C-terminus depending on the read quality (Supplementary Table S4) using the qiime dada2 denoise-paired command of the QIIME 2 plugin DADA2 v1.3.4 (Callahan et al., 2016). This was followed by merging paired reads and removing chimeric sequences and PhiX contamination with the default filtering parameters. For the 16S rRNA amplicon library, the generated unique amplicon sequence variants (ASVs) were used for taxonomic assignment using the SILVA database (release 132; Quast et al., 2013) trained with a Naïve Bayes classifier (Bokulich et al., 2018). The *pstS* libraries from two different sequencing runs were merged after chimera and contamination removal, using qiime feature-table merge command in QIIME2. Taxonomy of the genes encoding P_i transporters or enzymes that drive different P mobilization processes were assigned as described by Bergkemper et al. (2016a). Briefly, open reading frames (ORFs) were predicted from the ASVs using FragGeneScan version 1.19 (Rho et al., 2010) and

subsequently functionally annotated using HMMER version 3.1b2 (<http://hmmerr.org>) against the TIGRFAMs database (version 15) (Haft et al., 2012) and the Pfam database (version 30) (Finn et al., 2014). Overlapping motifs were removed and results were quality filtered (e-value threshold = 10^{-5} , Supplementary Table S5). The corresponding sequences were extracted from the original datasets and used for taxonomical annotation against the non-redundant NCBI database (January 2018) (Tatusova et al., 2014) using Kaiju v1.4.4 (Menzel et al., 2016).

Further bioinformatic analyses were carried out using the statistical software R v3.5.3 (R Core Team, 2018) and Rstudio v1.1.463 (RStudio Team, 2016). Rarefaction curves of each library were generated using the command “rarecurve” in the vegan package (Oksanen et al., 2019). To exclude potential contamination, ASVs occurring in two of the blank extraction or PCR negative controls were removed from the libraries, which accounted for 2.64 % (16 ASVs), 0.22 % (14 ASVs), 0 %, 2.24 % (16 ASVs) and 0 % of the obtained reads per sample in the amplicon libraries of 16S rRNA, *phoD*, *gcd*, *pitA*, *pstS* genes, respectively. Sequences assigned to Eukaryota and Archaea in the *phoD*-, *pitA*-, and *pstS*-harboring communities (<0.032 % of the obtained reads), plastid reads from the 16S rRNA gene libraries (<0.25 %), and singletons (<0.01 %) were removed using the “phyloseq” package (McMurdie and Holmes, 2013). Subsequently, the datasets were rarefied to the lowest number of obtained reads, using the “rarefy even depth” command in the vegan package (Supplementary Table S6). After rarefying remaining reads still reached saturation (Supplementary Fig. S1). The relative abundance of each ASV was calculated by dividing its read count by the rarefied count.

2.8. Data visualization and statistical analysis

Data visualization and statistical analysis were conducted using the statistical software R v3.5.3 and Rstudio v1.1.463. To visualize variations in bacterial communities among the samples, Bray-Curtis dissimilarities of generated ASVs were calculated for all investigated genes using the command “vegdist” in the vegan package and displayed in principal coordinates analysis (PCoA) plots. To test effects of soil depth and compartment (drilosphere vs. bulk) on the dissimilarities of bacterial communities, a two-way permutational analysis of variance (PERMANOVA; Anderson and Walsh (2013)) was performed using the command “adonis” in the vegan package. Alpha-diversity of soil bacterial communities at ASV level was calculated as Shannon-Weaver diversity index, Pielou's evenness, and the number of observed ASVs (richness) using the command “diversity” in the vegan package. For better visualization of the bacterial communities, ASVs were summarized at family level. When ASVs were not assigned at family level, a higher level of identification was given to the ASVs (e.g. unassigned Proteobacteria). Only families found at least two out of three biological replicates were included in the following analyses. Dominant families, whose relative abundance is >1 % of the overall bacterial communities and 2 % of the bacterial communities involved in P mobilization and uptake, were visualized in a stacked bar plot and a heatmap, respectively, using the ggplot2 package (Wickham, 2016). In the stacked bar plot, low-abundant families (< 1 % of relative abundance) were grouped into “< 1 %”. In the heatmap, low-abundant families (< 2 % of relative abundance) were cut off and the relative abundance of the dominant families were transformed to a logarithmic scale. Venn diagrams were created using the “ggvenn” command in the ggvenn package (Yan, 2021), to sort families occurring in drilosphere (unique drilosphere), bulk soil (unique bulk) or both of them (shared drilosphere/bulk). The community composition of the unique/shared families were plotted in a stacked bar using the ggplot2 package, and low-abundant families (< 2 % of relative abundance) were grouped into “< 2 %”. We performed robust *t*-test, one-way ANOVA, and two-way ANOVA on trimmed means using the commands “yuen”, “t1way”, and “t2way” in the WRS2 package (Mair and Wilcox, 2020), to determine significant soil depth, compartment, and the interaction effects. We considered differences as significant if *p*-values were < 0.05.

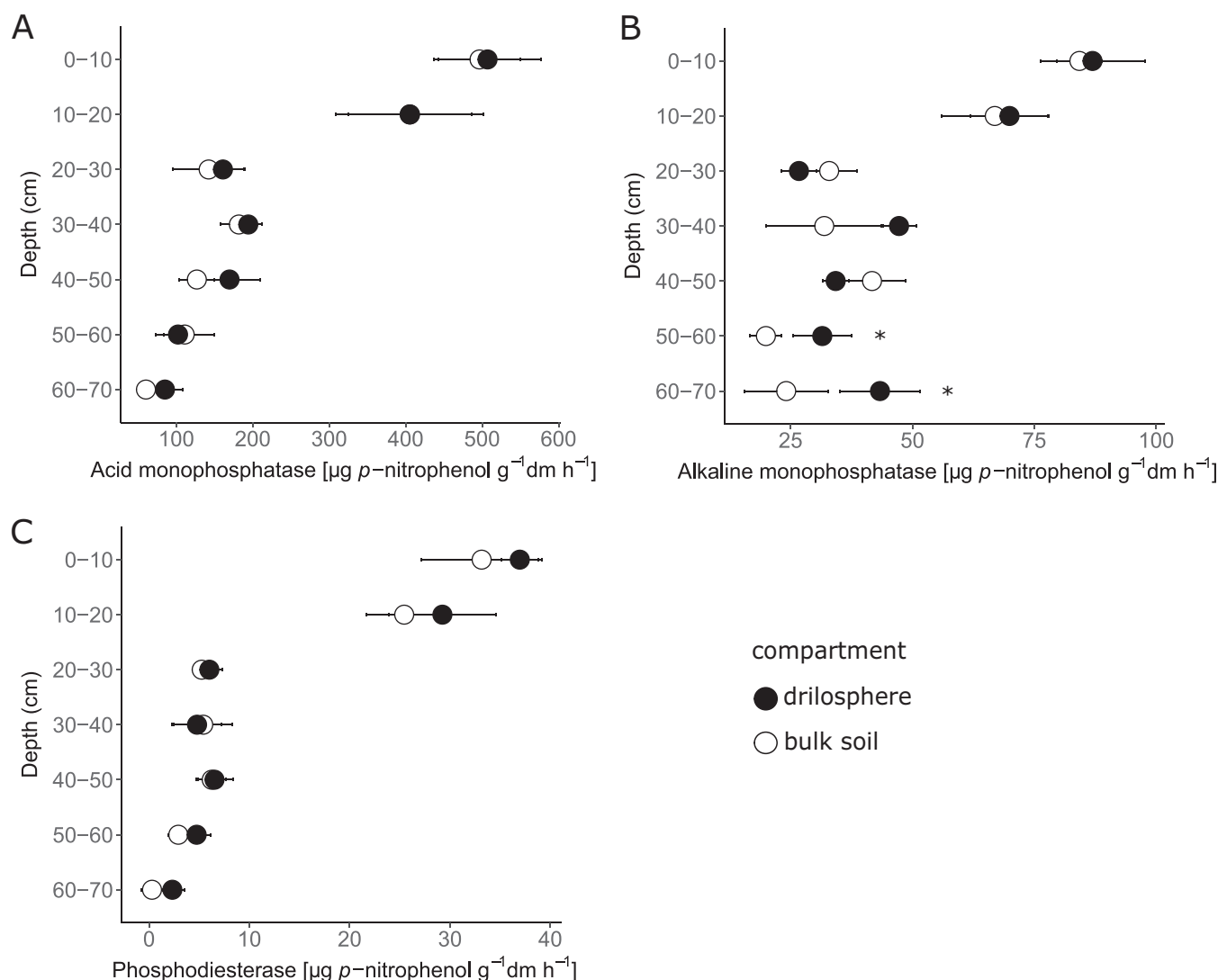


Fig. 1. Mean potential activities and standard deviation of (A) acid (ACP) and (B) alkaline (ALP) phosphomonoesterases, and (C) phosphodiesterase (PDE) in drilosphere (in black) and bulk soils (in white) from 0 to 70 cm depth ($n = 3$). Asterisks indicate significant differences between drilosphere and bulk soil at the respective depths (p -value < 0.05) according to a robust t -test.

3. Results

3.1. Potential enzyme activities and soil properties

All measured potential phosphatase activities were significantly influenced by soil depth, irrespective of the analyzed soil compartment (Fig. 1 and Supplementary Table S7; a robust one-way ANOVA p -value < 0.05). Highest values were measured at 0–10 cm depth with a significant decrease below 20–30 cm depth. Overall decreases between top- and subsoil ranged from 50 % (ALP) to 99 % (PDE). Only the potential activities of ALP were significantly higher in the drilosphere compared to the bulk soil below 50 cm depth (a robust t -test p -value < 0.05).

Water extractable P content was higher in the drilosphere throughout the soil profile, except at 10–40 cm depth (Supplementary Table S1). In the drilosphere it peaked at 20–30 cm depth and in bulk soil at 30–40 cm depth, respectively. Thereafter, water extractable P content gradually decreased with increasing soil depth and the lowest content was observed at 50–60 cm depth for both drilosphere and bulk soil. The ratios of C:P and N:P in the soil samples increased down to 40 cm depth in the drilosphere samples and steadily decreased below that depth. In the bulk soil, except for a slight decrease from 0 to 20 cm depth, the patterns were similar. For each of the compartments, the

ratios were the lowest at 60–70 cm depth. Overall the C:P and N:P ratios were higher in the bulk soil than in the drilosphere at 20–40 and 20–70 cm depth, respectively. For all other depths, the ratios were higher in the drilosphere than in the bulk soil. Soil pH was not affected by soil compartment and ranged between 5.22 and 5.67.

3.2. Abundance of total bacteria and key players involved in major processes of P mobilization and uptake

A robust one-way ANOVA on the total bacterial abundance and bacteria involved in P mobilization and uptake did not reveal any significant differences along the depth profile in the drilosphere. In contrast, the abundance of all targeted bacterial groups significantly decreased with increasing soil depth in the bulk soil (Fig. 2 and Supplementary Table S7; a robust one-way ANOVA p -value < 0.05). The total bacterial abundance dropped by 93 % at 60–70 cm depth compared to 0–10 cm depth. Similarly, *phoD*- and *pstS*-harboring bacteria decreased in abundance by 83 % and 99 % along the bulk soil profile, respectively. The different patterns in abundance along the soil profile resulted in lower abundance in the bulk soil compared to the drilosphere for all targeted bacterial groups and reached significance for the *pstS*-harboring bacterial abundance at 60–70 cm depth.

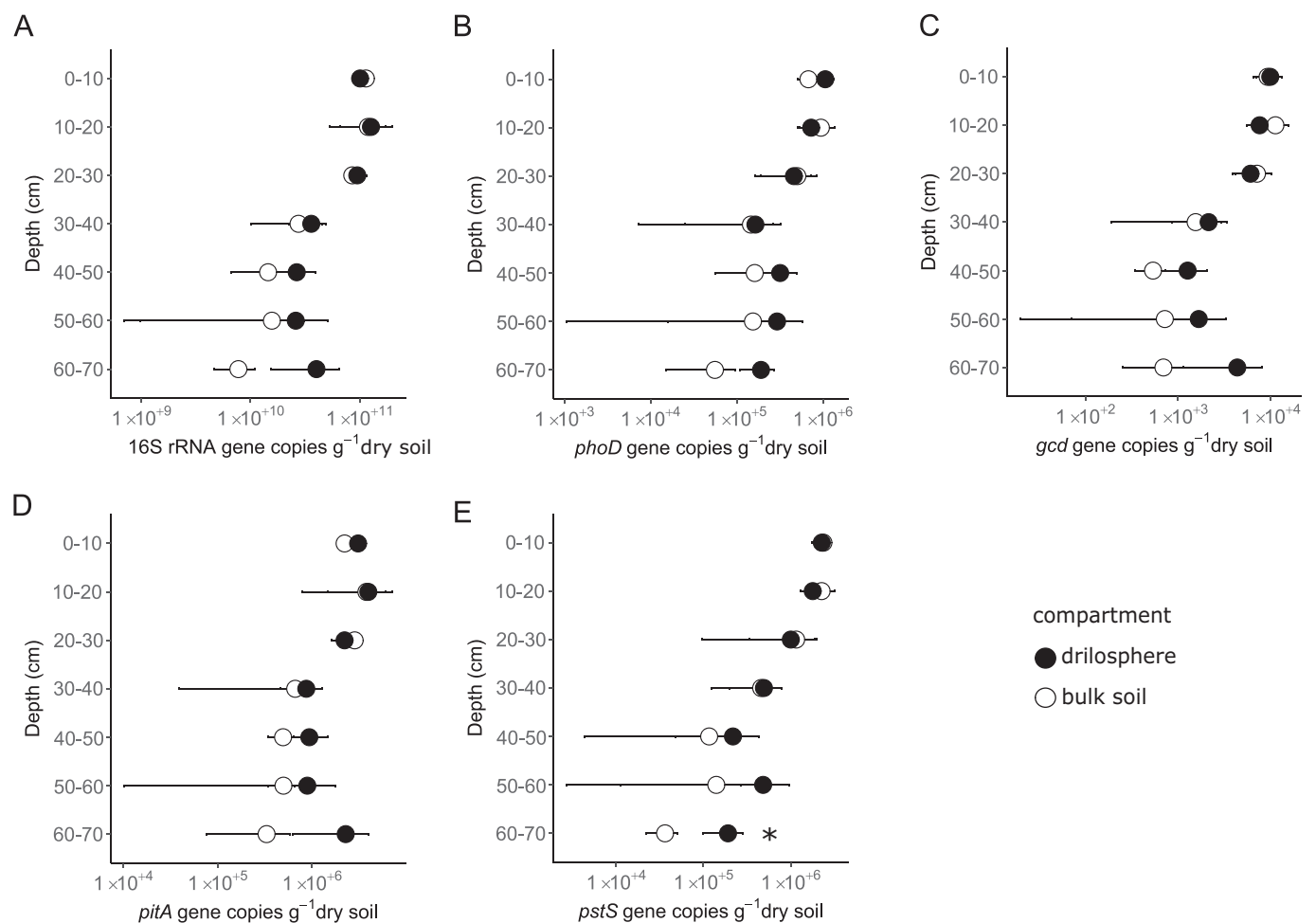


Fig. 2. Mean abundances and standard deviations of the (A) 16S rRNA, (B) *phoD*, (C) *gcd*, (D) *pitA*, and (E) *pstS* genes in drilosphere (in black) and bulk soils (in white) from 0 to 70 cm depth (n = 3). Asterisks indicate significant differences between drilosphere and bulk soils at the respective depths (p-value < 0.05) according to a robust t-test.

Table 1
 α -diversity indices (Shannon–Weaver diversity index, amplicon sequence variants (ASVs), and Pielou's evenness) of bacterial communities carrying the 16S rRNA, *phoD*, *gcd*, *pitA*, and *pstS* genes in drilosphere and bulk soil at 0–10 cm depth (topsoil) and 60–70 cm depth (subsoil). Values represent the mean and standard deviations (SD.) of three biological replicates. Different letters represent significantly different pairs ($p < 0.05$) between topsoil and subsoil according to a robust two-way ANOVA.

Gene	Depth	Compartment	Shannon–Weaver Diversity Index		Number of ASVs		Pielou's Evenness	
			Mean	SD.	Mean	SD.	Mean	SD.
16S rRNA	Topsoil	drilosphere	7.37	0.09	2530	236	0.94a	0.00
		bulk	7.26	0.26	2351	701	0.94a	0.00
	Subsoil	drilosphere	7.25	0.10	2547	385	0.93b	0.01
		bulk	7.11	0.17	2349	569	0.92b	0.02
<i>phoD</i>	Topsoil	drilosphere	4.70a	0.16	190a	34	0.90a	0.00
		bulk	4.61a	0.53	187a	93	0.90a	0.01
	Subsoil	drilosphere	3.62b	0.24	88b	4	0.81b	0.05
		bulk	3.93b	0.23	101b	23	0.85b	0.01
<i>gcd</i>	Topsoil	drilosphere	5.13	0.13	268	37	0.92	0.00
		bulk	4.64	0.26	168	32	0.91	0.02
	Subsoil	drilosphere	4.40	0.53	158	69	0.88	0.01
		bulk	4.38	0.59	149	78	0.89	0.04
<i>pitA</i>	Topsoil	drilosphere	4.41	0.26	146a	33	0.89	0.01
		bulk	4.19	0.30	115a	21	0.89	0.03
	Subsoil	drilosphere	3.98	0.34	85b	30	0.91	0.00
		bulk	3.99	0.26	88b	17	0.89	0.02
<i>pstS</i>	Topsoil	drilosphere	4.64	0.19	217	73	0.87a	0.02
		bulk	4.51	0.15	167	26	0.88a	0.01
	Subsoil	drilosphere	4.86	0.24	180	38	0.94b	0.01
		bulk	4.60	0.19	140	16	0.93b	0.02

3.3. Diversity of bacteria mediating major processes of P mobilization and uptake

A total of 2,077,560, 1,815,495, 661,562, 1,879,737, and 1,347,040 raw reads were obtained for the 16S rRNA, *phoD*, *gcd*, *pitA*, and *pstS* gene libraries, respectively. The number of reads obtained from the sequencing runs before and after filtering processes are summarized in Supplementary Table S6. The datasets were rarefied to the lowest number of reads for each library, and a rarefaction analysis based on the rarefied number of ASVs confirmed sufficient coverages of bacterial diversity for all libraries (Supplementary Fig. S1).

The α -diversity indices are summarized in Table 1. The impact of soil depth on the indices differed among the analyzed functional

communities. While no depth-related changes were observed for the *gcd*-harboring communities, the strongest impact was observed for the *phoD*-harboring communities, where all three indices significantly dropped in both bulk soil and drilosphere with increasing soil depth (a robust two-way ANOVA p-value < 0.05). Richness or evenness decreased with soil depth for the overall bacterial communities (16S rRNA gene) and the *pitA*-harboring communities, respectively. The evenness of the *pstS*-harboring bacteria was the only index that increased with increasing soil depth in both compartments, from 0.87 to 0.94 for the drilosphere and from 0.88 to 0.93 for the bulk soil. No significant differences between bulk soil and drilosphere were determined irrespective of soil depth.

The β -diversity based on PCoA (Supplementary Fig. S2) and

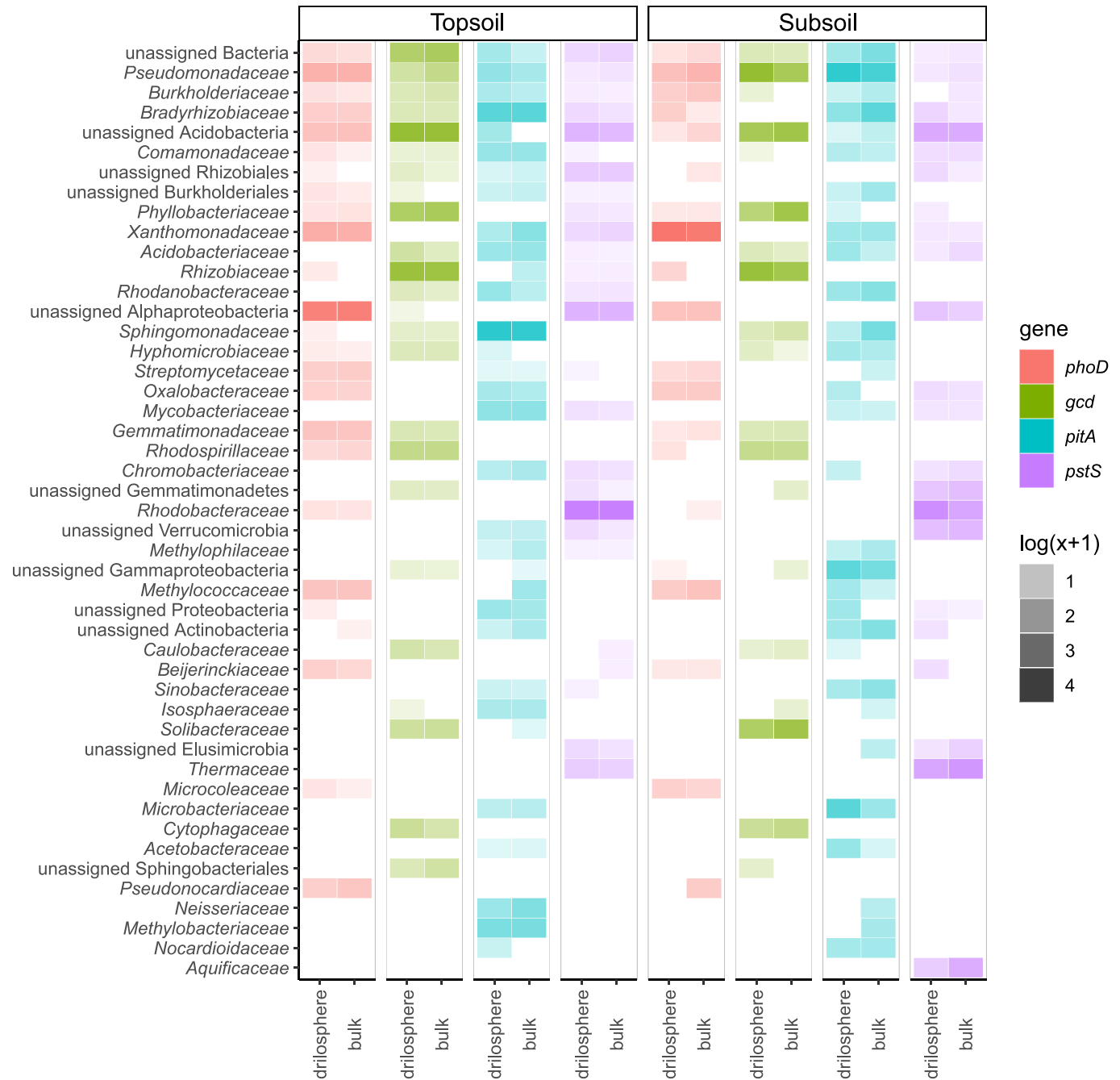


Fig. 3. Mean relative abundance of bacterial families carrying the *phoD*, *gcd*, *pitA*, and *pstS* genes in drilosphere and bulk soil of topsoil (0–10 cm depth) and subsoil (60–70 cm depth). Only dominant families found in at least two out of three replicates and with >2 % relative abundance are presented. Colors stand for different phosphate mobilizing genes. Density of the colors present the relative abundance on a logarithmic scale $\log(x + 1)$.

The relative abundances of major bacterial families are displayed in Fig. 3 and Supplementary Fig. S3, and the number of the detected bacterial phylogenetic groups is summarized in Supplementary Table S9. In general, the composition of the different functional groups did not resemble the overall bacterial community composition as abundant families differed. The *phoD*-harboring communities were dominated by unassigned Alphaproteobacteria and *Xanthomonadaceae*, the *gcd*-harboring communities by unassigned Acidobacteria, *Rhizobiaceae*, *Pseudomonadaceae* and *Phyllobacteriaceae*, the *pitA*-harboring communities by *Bradyrhizobiaceae*, *Sphingomonadaceae* and *Pseudomonadaceae* and the *pstS*-harboring communities by *Rhodobacteraceae*, *Thermaceae* and unassigned Acidobacteria. Among those families, only unassigned Acidobacteria, *Rhizobiaceae*, *Xanthobacteraceae*, and *Pseudomonadaceae* reached relative abundance of >1 % in the overall bacterial communities based on the 16S rRNA sequences.

Overall, the number of families unique in the drilosphere decreased

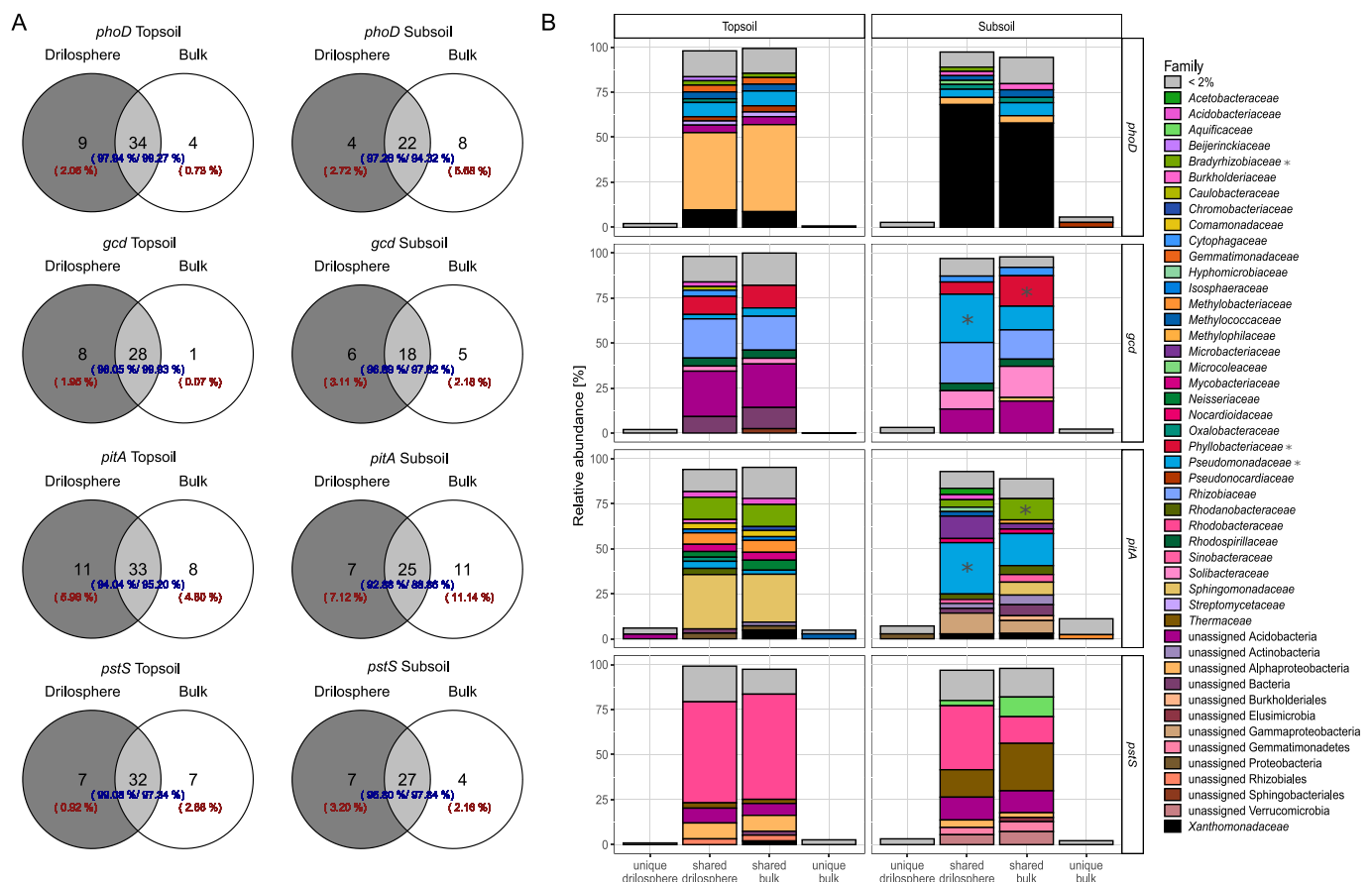


Fig. 4. (A) Venn diagram showing the distribution of bacterial families carrying the *phoD*, *gcd*, *pitA*, and *pstS* genes in topsoil (0–10 cm depth) and subsoil (60–70 cm depth). Only families found in at least two out of three replicates are included. Numbers indicate the number of families shared between the compartments as well as the number of families unique to each compartment. Percentages indicate the relative abundances of the shared (in blue) and unique families (in red) in each community. (B) Taxonomic assignment of the *phoD*, *gcd*, *pitA*, and *pstS* genes. Mean relative abundances of families in drilosphere and bulk soil of topsoil (0–10 cm depth) and subsoil (60–70 cm depth) are displayed. Families with <2 % of relative abundance are grouped into “<2 %”. Asterisks indicate significant differences (*p*-value < 0.05) between drilosphere and bulk soils at the respective depths according to a robust t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with increasing soil depth, while the relative abundance of the remaining drilosphere-specific families increased, as in the case of *Rhizobiaceae* in the *phoD*-harboring communities and *Hymenobacteraceae* in the *gcd*-harboring communities. Additionally, *Methylocystaceae*, *Oxalobacteraceae*, and *Beijerinckiaceae* were unique to the drilosphere only in the subsoil, for the *gcd*-, *pitA*-, and *pstS*-harboring communities, respectively.

In the bulk soil, both the relative abundance and the number of unique families increased from topsoil to subsoil, only for the *pstS*-harboring communities the opposite pattern was observed. Unlike in the drilosphere, there were no overlaps of bulk soil-specific families between topsoil and subsoil for the respective functional communities.

4. Discussion

4.1. The potential for bacterial P turnover is stable in the drilosphere

Our results emphasize that bacterial communities regulating P uptake and mineralization upon P scarcity were favored in deep drilosphere layers. This conclusion is based on significantly higher potential ALP activity and *pstS* gene abundance in the drilosphere of deep soil layers. On the one hand, a similar trend in the 16S rRNA gene abundance (Fig. 2A) suggests a positive correlation between microbial biomass and ALP as a potential reason, which was also previously described by Lipiec et al. (2016). On the other hand, we found no correlation between the *phoD* gene abundance and ALP activity. This is in contrast to previous studies showing a positive correlation between ALP and *phoD* gene abundance (Chen et al., 2019; Fraser et al., 2015b). There may be several reasons for these contrasting results. First, ALP has been also detected in earthworm coelomic fluid and the gastrointestinal system (Pramanik, 2010; Sanchez-Hernandez et al., 2018). Thus, the enzyme can be released into soils and remain in fresh cast deposits (Le Bayon and Binet, 2006; Tao et al., 2009) and may have contributed to the increased ALP potential in the drilosphere. Second, a large fraction of the soil microorganisms in environments are dormant (Gao and DeLuca, 2018), and thus the gene abundance could remain high, even when potential activities are reduced. It is also important to note that bacterial genes (i. e. *phoA* and *phoX*), which we did not investigate in this study, also contribute to the production of ALP (Fraser et al., 2015b; Luo et al., 2009). A third explanation could be the different availability of substrate fractions between soil compartments. ALP catalyzes only the degradation of monophosphate ester bonds of P_{org} , whereas the *phoD* gene encodes monomeric enzymes that hydrolyze both alkaline phosphomonoesters and phosphodiester (Eder et al., 1996; Kageyama et al., 2011). Additionally, the stable PDE activity in bulk soil and drilosphere observed in our study (Fig. 1C) may have obscured the significant difference in the *phoD* abundance. PDE is produced by earthworms (Park et al., 1992), microorganisms (Konno et al., 2018) and plant roots (Rejmánková et al., 2011) to mediate the degradation of phospholipids and nucleic acids derived from cell debris and dead cells (Cosgrove, 1967). Therefore, multiple complex and distinct parameters influence the *phoD* gene abundance and ALP activity. A more comprehensive understanding of these parameters may help to better assess environmental P turnover potential in future studies. In contrast to ALP, earthworm activities did not affect potential ACP activity, which is in line with other studies (Hoang et al., 2016; Lipiec et al., 2016). A reason for that might be that the major source of ACP in soils are plant roots (Colvan et al., 2001; Dinkelaker and Marschner, 1992; Tarafdar and Claassen, 1988).

Similar to ALP, also the *pstS* gene abundance remained stable across the soil profile in the drilosphere (Fig. 2E) and was comparable to previous studies done in agricultural (Grafe et al., 2021) and forest sites (Kurth et al., 2021). This gene encodes the periplasmic binding protein of the phosphate-specific transporter (Pst), which is for example upregulated by the two-component system PhoRB under P scarcity in *Bacillus subtilis* (Qi et al., 1997). In the same system, also alkaline phosphatase

expression is induced (Jiang et al., 1995; Kasahara et al., 1991). Thus, the increase in both ALP activity and *pstS* gene abundance observed in this study suggest at least temporally occurring P limitation in the drilosphere, which is further supported by an increasing C:P ratio in the drilosphere, which indicates unfavorable nutrient stoichiometry.

Although our study supports the hypothesis that P mobilization is higher in drilosphere compared to bulk soil, other studies observed that effect already at shallower depths. For example, Lipiec et al. (2016) reported significantly higher phosphatase activities in the drilosphere than in the bulk soil at 10 cm depth, while Uksa et al. (2015b) reported the same observation at 45 cm soil depth. A possible explanation on the various observations is different substrate availability in differently textured soils. Stagnic Cambisol investigated in this study contains lower clay content in the subsoil horizons compared to Haplic Luvisols and Haplic Stagnosols that have been the focus of the earlier studies. As clay minerals stabilize and protect soil organic C (SOC), the lower clay content in this Stagnic Cambisol likely led to a larger proportion of microbially available SOC compared to the other soils (Torres-Sallan et al., 2018). In addition, high root density homogenize the substrate in the topsoil horizons, resulting in smaller variation of microbial communities and activities between soil compartments in the topsoil soil layers (Uksa et al., 2015a). At our field site, visual inspection of the topsoil revealed great homogeneity from highest fine root biomass of short rotation coppice willows at 10 to 20 cm depth, which was in line with Heinsoo et al. (2009) and Steele et al. (1997). Moreover, the inhabiting endogeic earthworms (i.e. *Allolobophora chlorotica*), which actively create horizontal burrows at a depth range of 0–30 cm (Bastardie et al., 2003), might have contributed to soil homogenization as well.

In our study, the abundance of the *pitA* and *gcd* genes did not significantly differ between the drilosphere and bulk soil, which is against our initial assumption that P acquisition strategies of bacteria change from P mineralization to P solubilization downwards in the drilosphere due to different substrate quality and availability. However, the regulation of these genes is independent of P scarcity. For example, proton-motive force drives P uptake through the Pit transporter (Jackson et al., 2008; Yin et al., 2019), and the *pitA* gene is constitutively expressed in several bacterial strains such as *Escherichia coli* (Harris et al., 2001) and *Mycobacterium smegmatis* (Gebhard et al., 2009). Greater abundance of the *gcd* gene has been frequently observed in P-rich environments (Bergkemper et al., 2016b; Dai et al., 2020; Zhi et al., 2023). In our study, water-extractable P was slightly accumulated at 60–70 cm depth possibly due to P leaching, however its concentration was even less than that in P-depleted environments (approximately 10 mg P g⁻¹) from the previous studies by Bergkemper et al. (2016b) and Dai et al. (2020).

4.2. Bacterial communities involved in P mobilization and uptake in the drilosphere

As expected, α -diversity decreased with increasing soil depth for all analyzed functional microbial groups in this study, most likely due to strong filtering of soil microorganisms as a result of limited availability of water, nutrient and O₂ in subsoils (Angst et al., 2018; Chabbi et al., 2009). However, *pstS*-harboring communities were an exception as evenness significantly increased in the subsoil compared to the topsoil. According to Coleman and Chisholm (2010), beneficial nutrient-scavenging genes including the *pstS* gene have been more frequently and diversely found in bacteria inhabiting a P-scarce ocean region, which allows certain bacteria to survive in extreme environments (Miroshnichenko and Bonch-Osmolovskaya, 2006). Therefore, the high diversity of the *pstS*-harboring communities in the subsoil underlines P limited conditions in the subsoil in our study.

As already observed for the abundance also the diversity and composition of the *phoD*-harboring bacterial communities were only affected by soil depth but not by compartment. In previous studies

dynamical shifts in the *phoD*-harboring communities have been rather observed in long-term organically managed fields (Fraser et al., 2015a; Luo et al., 2017; Tian et al., 2021), suggesting SOC as important factor determining the composition of *phoD*-harboring communities. In our study, the irregular and small C input by earthworms as well as no litter layer at the surface of the site during the sampling campaign might be an explanation for no differences in the *phoD*-harboring communities between drilosphere and bulk soil. These stable *phoD*-harboring communities between drilosphere and bulk soil suggest the importance of the entire *phoD*-harboring communities for P mineralization at this site. However, it is also possible that the intensity of interaction within the communities changes between compartments (Liu et al., 2021). The *phoD*-harboring communities in this study were dominated by *Xanthomonadaceae*, especially in the subsoil. This family has been described as keystone whose relative abundance is positively correlated with the *phoD* gene abundance in different terrestrial environments (Campos et al., 2021; Lang et al., 2020; Zhao et al., 2022), and may therefore benefit from the ability to mineralize P_{org} in a wide range of terrestrial environments including the drilosphere.

In contrast, the communities carrying *gcd* and *pitA* genes showed more dynamic changes, which seem to be related to their preferential nutrient acquisition strategy. For example, the family *Pseudomonadaceae* significantly increased in their relative abundance in the drilosphere, following the trend observed on the basis of 16S rRNA sequencing. *Pseudomonadaceae* are a well-known copiotrophic family, and its active degradation of C compounds in the drilosphere has been reported previously (Dallinger and Horn, 2014). *Pseudomonas*, found as the major genus belonging to the family *Pseudomonadaceae* in our study, has often been isolated from the gut of earthworms in previous studies (Biswas et al., 2018; Hussain et al., 2016; Maheswari and Sudha, 2013). This suggests that earthworm-derived microorganisms could settle and drive P solubilization and uptake in the drilosphere. It is important to note that this family was also detected in the *phoD*- and *pstS*-harboring communities in our study but with lower relative abundance and no difference between the compartments. This is surprising, as the *pstS* and *phoD* genes are a major regulatory element of the Pho regulon and are conserved in *Pseudomonas* species (Monds et al., 2006). However, incompatible changes between 16S rRNA and functional communities could occur due to the niche specialization of specific ecotypes (Chase et al., 2018). In addition, horizontal gene transfer (Jha et al., 2018) and less records in the databases relative to 16S rRNA database (e.g. Avşar and Aras, 2020) could also explain the difference in the drilosphere effect on the overall and functional communities.

In contrast to *Pseudomonadaceae*, the relative abundance of *Phyllobacteriaceae* and *Bradyrhizobiaceae* within the *gcd*- and *pitA*-harboring communities dropped in the drilosphere at 60–70 cm depth in our study. It was demonstrated previously that *Pseudomonas* competed with *Phyllobacterium* for root exudates in the rhizosphere of wheat (Breitkreuz et al., 2019). Thus, *Pseudomonadaceae* might have outcompeted *Phyllobacteriaceae* and *Bradyrhizobiaceae* in the drilosphere subsoil. Alternatively, species from *Phyllobacteriaceae* and *Bradyrhizobiaceae* have been characterized for their N turnover capabilities, such as denitrification and N fixation (Hara et al., 2019; Long et al., 2018; Rojas et al., 2001). In the drilosphere, where both N and P were limited relative to the bulk soil, these families might have prioritized N acquisition over P acquisition due to niche partitioning (Hartman and Richardson, 2013).

4.3. Comparison of P turnover in drilosphere with other microbial hotspots

Our study confirms that bacterial community abundance and potential activity in the drilosphere are similar to rhizosphere and detritusphere, while community composition seems to be more habitat specific. We observed high potential ALP activity and *pstS* gene abundance in the drilosphere. Similar results have been reported in the rhizosphere (Enebe and Babalola, 2021) and detritusphere (Bai et al.,

2021). Due to continuous nutrient inputs from plant roots in the rhizosphere and during litter decomposition processes in the detritusphere high microbial P turnover has been observed for weeks to months (Kuz'yakov and Blagodatskaya, 2015). In the drilosphere the moment of high nutrient input and turnover would last only for a shorter period of time (e.g., for a few days to weeks) and occurs occasionally due to irregular C input by earthworms (Brown et al., 2000). That still allows for microbial enrichment but has implications for prevalent taxa. In the rhizosphere and detritusphere more different phosphate-mineralizing bacteria are enriched compared to drilosphere (Hu et al., 2020; Jorquera et al., 2008; Purahong et al., 2016). This includes, for example, *Bradyrhizobium*, *Rhizobium*, *Streptomyces*, and *Enterobacter* in addition to the *Pseudomonas* detected as dominant genus in our study. On the one hand, it might be due to the availability of different and more complex substrates. On the other hand, this could also be attributed to the short “hot” moment in the drilosphere, which might foster quicker community turnover processes, where only very prominent responders remain detectable.

5. Conclusion

In this study, we demonstrate that earthworm activities influence bacterial P mobilization and uptake in two different manners. For some processes, gene abundance and enzyme activities increased in the drilosphere compared to the bulk soil, while maintaining a stable community composition. For other processes, major shifts in community composition occurred in response to earthworm activities but gene abundance was not affected.

Regarding the first mechanism, our analyses revealed an increased potential for P mineralization and high-affinity uptake via the Pst system below 50 cm depth of the drilosphere compared to the bulk soil. In addition to our chemical data, the fact that these dominant processes are regulated by the Pho regulon suggest that changing nutrient stoichiometry by earthworm activities is most likely driving P limitation in the drilosphere subsoil. The stability of the *phoD* and *pstS*-harboring community composition underlines the importance of the entire bacterial members carrying these genes. However, in order to evaluate which bacteria caused the increase in potential enzyme activity, studies at the gene expression level are needed.

Regarding the second mechanisms, the amplicon sequencing approach showed a clear drilosphere-effect on communities involved in P solubilization and uptake via the Pit system in the subsoil. This was demonstrated by significantly higher relative abundance of *Pseudomonadaceae* that are most likely derived from earthworm gut and often involved in C turnover in the drilosphere. Concomitantly, we observed significantly lower relative abundance of bacteria potentially involved in N turnover, such as *Phyllobacteriaceae* and *Bradyrhizobiaceae*, in the drilosphere subsoil. Those results emphasize that earthworm activities alter bacterial community composition involved in P mobilization and uptake in deeper soil layers. Our study captured a moment of fluctuating P turnover in the drilosphere by taking samples from fresh earthworm channels at one sampling date. Observations at different sampling times might differ due to seasonal changes in the field. For example, our sampling campaign was conducted in August when there was no litter on the field surface. Litterfall could enhance the incorporation of nutrients into soil by earthworms, which subsequently increases microbial activity in the drilosphere even at shallower soil depths (Hoang et al., 2020). These variations may therefore mainly affect the topsoil.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.apsoil.2023.105168>.

References

- Alori, E.T., Glick, B.R., Babalola, O.O., 2017. Microbial phosphorus solubilization and its potential for use in sustainable agriculture. *Front. Microbiol.* 8, 971. <https://doi.org/10.3389/fmicb.2017.00971>.
- An, R., Moe, L.A., 2016. Regulation of PQQ-dependent glucose dehydrogenase activity in the model rhizosphere dwelling bacterium *Pseudomonas putida* KT2440. *Appl. Environ. Microbiol.* 82, 4955–4964. <https://doi.org/10.1128/AEM.00813-16>.
- Anderson, M.J., Walsh, D.C.I., 2013. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? *Ecol. Monogr.* 83, 557–574. <https://doi.org/10.1890/12-2010.1>.
- Angst, G., Messinger, J., Greiner, M., Häusler, W., Hertel, D., Kirfel, K., Kögel-Knabner, I., Leuschner, C., Rethemeyer, J., Mueller, C.W., 2018. Soil organic carbon stocks in topsoil and subsoil controlled by parent material, carbon input in the rhizosphere, and microbial-derived compounds. *Soil Biol. Biochem.* 122, 19–30. <https://doi.org/10.1016/j.soilbio.2018.03.026>.
- Avşar, C., Aras, E.S., 2020. Quantification of denitrifier genes population size and its relationship with environmental factors. *Arch. Microbiol.* 202, 1181–1192. <https://doi.org/10.1007/s00203-020-01826-x>.
- Bai, X., Dippold, M.A., An, S., Wang, B., Zhang, H., Loepmann, S., 2021. Extracellular enzyme activity and stoichiometry: the effect of soil microbial element limitation during leaf litter decomposition. *Ecol. Indic.* 121, 107200. <https://doi.org/10.1016/j.ecolind.2020.107200>.
- Bastardie, F., Capowiez, Y., de Dreuz, J., Cluzeau, D., 2003. X-ray tomographic and hydraulic characterization of burrowing by three earthworm species in repacked soil cores. *Appl. Soil Ecol.* 24, 3–16. [https://doi.org/10.1016/S0929-1393\(03\)00071-4](https://doi.org/10.1016/S0929-1393(03)00071-4).
- Bauke, S.L., von Sperber, C., Siebers, N., Tamburini, F., Amelung, W., 2017. Biopore effects on phosphorus biogeochemistry in subsoils. *Soil Biol. Biochem.* 111, 157–165. <https://doi.org/10.1016/j.soilbio.2017.04.012>.
- Bergkemper, F., Kublik, S., Lang, F., Krüger, J., Vestergaard, G., Schlöter, M., Schulz, S., 2016a. Novel oligonucleotide primers reveal a high diversity of microbes which drive phosphorus turnover in soil. *J. Microbiol. Methods* 125, 91–97. <https://doi.org/10.1016/j.mimet.2016.04.011>.
- Bergkemper, F., Schöler, A., Engel, M., Lang, F., Krüger, J., Schlöter, M., Schulz, S., 2016b. Phosphorus depletion in forest soils shapes bacterial communities towards phosphorus recycling systems. *Environ. Microbiol.* 18, 1988–2000. <https://doi.org/10.1111/1462-2920.13188>.
- Biswas, J.K., Banerjee, A., Rai, M., Naidu, R., Biswas, B., Vithanage, M., Dash, M.C., Sarkar, S.K., Meers, E., 2018. Potential application of selected metal resistant phosphate solubilizing bacteria isolated from the gut of earthworm (*Metaphire postuma*) in plant growth promotion. *Geoderma* 330, 117–124. <https://doi.org/10.1016/j.geoderma.2018.05.034>.
- Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G.A., Gregory Caporaso, J., 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* 6, 90. <https://doi.org/10.1186/s40168-018-0470-z>.
- Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., D'Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwards, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K. Bin, Keefe, C.R.,
- Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciulek, T., Kreps, J., Langille, M.G. I., Lee, J., Ley, R., Liu, Y., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimy, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Priesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. <https://doi.org/10.1038/s41587-019-0209-9>.
- Breitkreuz, C., Buscot, F., Tarkka, M., Reitz, T., 2019. Shifts between and among populations of wheat rhizosphere *Pseudomonas*, *Streptomyces* and *Phyllobacterium* suggest consistent phosphate mobilization at different wheat growth stages under abiotic stress. *Front. Microbiol.* 10, 3109. <https://doi.org/10.3389/fmicb.2019.03109>.
- Browman, M.G., Tabatabai, M.A., 1978. Phosphodiesterase activity of soils. *Soil Sci. Soc. Am. J.* 42, 284–290. <https://doi.org/10.2136/sssaj1978.03615995004200020016x>.
- Brown, G.G., Barois, I., Lavelle, P., 2000. Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. *Eur. J. Soil Biol.* 36, 177–198. [https://doi.org/10.1016/S1164-5563\(00\)01062-1](https://doi.org/10.1016/S1164-5563(00)01062-1).
- Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. <https://doi.org/10.1038/nmeth.3869>.
- Campos, M., Rilling, J.I., Acuña, J.J., Valenzuela, T., Larama, G., Peña-Cortés, F., Ogram, A., Jaishi, D.P., Jorquera, M.A., 2021. Spatiotemporal variations and relationships of phosphorus, phosphomonoesterases, and bacterial communities in sediments from two Chilean rivers. *Sci. Total Environ.* 776, 145782. <https://doi.org/10.1016/j.scitotenv.2021.145782>.
- Chabbi, A., Kögel-Knabner, I., Rumpel, C., 2009. Stabilised carbon in subsoil horizons is located in spatially distinct parts of the soil profile. *Soil Biol. Biochem.* 41, 256–261. <https://doi.org/10.1016/j.soilbio.2008.10.033>.
- Chase, A.B., Gomez-Lunar, Z., Lopez, A.E., Li, J., Allison, S.D., Martiny, A.C., Martiny, J. B.H., 2018. Emergence of soil bacterial ecotypes along a climate gradient. *Environ. Microbiol.* 20, 4112–4126. <https://doi.org/10.1111/1462-2920.14405>.
- Chen, X., Jiang, N., Condron, L.M., Dunfield, K.E., Chen, Z., Wang, J., Chen, L., 2019. Soil alkaline phosphatase activity and bacterial *phoD* gene abundance and diversity under long-term nitrogen and manure inputs. *Geoderma* 349, 36–44. <https://doi.org/10.1016/j.geoderma.2019.04.039>.
- Cleveland, C.C., Lipitzin, D., 2007. C:N:P stoichiometry in soil: is there a “Redfield ratio” for the microbial biomass? *Biogeochemistry* 85, 235–252. <https://doi.org/10.1007/s10533-007-9132-0>.
- Coleman, M.L., Chisholm, S.W., 2010. Ecosystem-specific selection pressures revealed through comparative population genomics. *Proc. Natl. Acad. Sci. U. S. A.* 107, 18634–18639. <https://doi.org/10.1073/pnas.1009480107>.
- Colvan, S., Syers, J., O'Donnell, A., 2001. Effect of long-term fertiliser use on acid and alkaline phosphomonoesterase and phosphodiesterase activities in managed grassland. *Biol. Fertil. Soils* 34, 258–263. <https://doi.org/10.1007/s003740100411>.
- Cosgrove, D., 1967. Metabolism of organic phosphates in soil. In: McLaren, A., Peterson, G. (Eds.), *Soil Biochemistry*. Marcel Dekker, New York, U.S.A., pp. 216–228.
- Dai, Z., Liu, G., Chen, H., Chen, C., Wang, J., Ai, S., Wei, D., Li, D., Ma, B., Tang, C., Brookes, P.C., Xu, J., 2020. Long-term nutrient inputs shift soil microbial functional profiles of phosphorus cycling in diverse agroecosystems. *ISME J.* 14, 757–770. <https://doi.org/10.1038/s41396-019-0567-9>.
- Dallinger, A., Horn, M.A., 2014. Agricultural soil and drilosphere as reservoirs of new and unusual assimilators of 2,4-dichlorophenol carbon. *Environ. Microbiol.* 16, 84–100. <https://doi.org/10.1111/1462-2920.12209>.
- Demoling, F., Figueroa, D., Bååth, E., 2007. Comparison of factors limiting bacterial growth in different soils. *Soil Biol. Biochem.* 39, 2485–2495. <https://doi.org/10.1016/j.soilbio.2007.05.002>.
- Dinkelaker, B., Marschner, H., 1992. In vivo demonstration of acid phosphatase activity in the rhizosphere of soil-grown plants. *Plant Soil* 144, 199–205. <https://doi.org/10.1007/BF00012876>.
- Don, A., Steinberg, B., Schöning, I., Pritsch, K., Joschko, M., Gleixner, G., Schulze, E., 2008. Organic carbon sequestration in earthworm burrows. *Soil Biol. Biochem.* 40, 1803–1812. <https://doi.org/10.1016/j.soilbio.2008.03.003>.
- Eder, S., Shi, L., Jensen, K., Yamane, K., Hulett, F.M., 1996. A *Bacillus subtilis* secreted phosphodiesterase/alkaline phosphatase is the product of a *pho* regulon gene, *phoD*. *Microbiology* 142, 2041–2047. <https://doi.org/10.1099/13500872-142-8-2041>.
- Enebe, M.C., Babalola, O.O., 2021. The influence of soil fertilization on the distribution and diversity of phosphorus cycling genes and microbes community of maize rhizosphere using shotgun metagenomics. *Genes* 12, 1022. <https://doi.org/10.3390/genes12071022>.
- Ferlian, O., Thakur, M.P., Castañeda González, A., San Emeterio, L.M., Marr, S., da Silva Rocha, B., Eisenhauer, N., 2020. Soil chemistry turned upside down: a meta-analysis of invasive earthworm effects on soil chemical properties. *Ecology* 101, e02936. <https://doi.org/10.1002/ecy.2936>.
- Finn, R.D., Bateman, A., Clements, J., Coghill, P., Eberhardt, R.Y., Eddy, S.R., Heger, A., Hetherington, K., Holm, L., Misty, J., Sonnhammer, E.L.L., Tate, J., Punta, M., 2014. Pfam: the protein families database. *Nucleic Acids Res.* 42, D222–D230. <https://doi.org/10.1093/nar/gkt1223>.
- Fraser, T., Lynch, D.H., Bent, E., Entz, M.H., Dunfield, K.E., 2015a. Soil bacterial *phoD* gene abundance and expression in response to applied phosphorus and long-term

- management. *Soil Biol. Biochem.* 88, 137–147. <https://doi.org/10.1016/j.soilbio.2015.04.014>.
- Fraser, T., Lynch, D.H., Entz, M.H., Dunfield, K.E., 2015b. Linking alkaline phosphatase activity with bacterial *phoD* gene abundance in soil from a long-term management trial. *Geoderma* 257–258, 115–122. <https://doi.org/10.1016/j.geoderma.2014.10.016>.
- Gao, S., DeLuca, T.H., 2018. Wood biochar impacts soil phosphorus dynamics and microbial communities in organically-managed croplands. *Soil Biol. Biochem.* 126, 144–150. <https://doi.org/10.1016/j.soilbio.2018.09.002>.
- Gebhard, S., Ekanayaka, N., Cook, G.M., 2009. The low-affinity phosphate transporter PitA is dispensable for in vitro growth of *Mycobacterium smegmatis*. *BMC Microbiol.* 9, 254. <https://doi.org/10.1186/1471-2180-9-254>.
- Grafe, M., Goers, M., von Tucher, S., Baum, C., Zimmer, D., Leinweber, P., Vestergaard, G., Kublik, S., Schlöter, M., Schulz, S., 2018. Bacterial potentials for uptake, solubilization and mineralization of extracellular phosphorus in agricultural soils are highly stable under different fertilization regimes. *Environ. Microbiol. Rep.* 10, 320–327. <https://doi.org/10.1111/1758-2229.12651>.
- Grafe, M., Kurth, J.K., Panten, K., Raj, A.D., Baum, C., Zimmer, D., Leinweber, P., Schlöter, M., Schulz, S., 2021. Effects of different innovative bone char based P fertilizers on bacteria catalyzing P turnover in agricultural soils. *Agric. Ecosyst. Environ.* 314, 107419. <https://doi.org/10.1016/j.agee.2021.107419>.
- van Groenigen, J.W., van Groenigen, K.J., Koopmans, G.F., Stokkermans, L., Vos, H.M.J., Lubbers, I.M., 2019. How fertile are earthworm casts? A meta-analysis. *Geoderma* 338, 525–535. <https://doi.org/10.1016/j.geoderma.2018.11.001>.
- Haft, D.H., Selengut, J.D., Richter, R.A., Harkins, D., Basu, M.K., Beck, E., 2012. TIGRFAMs and genome properties in 2013. *Nucleic Acids Res.* 41, D387–D395. <https://doi.org/10.1093/nar/gks1234>.
- Hara, S., Morikawa, T., Wasai, S., Kasahara, Y., Koshiba, T., Yamazaki, K., Fujiwara, T., Tokunaga, T., Minamisawa, K., 2019. Identification of nitrogen-fixing *Bradyrhizobium* associated with roots of field-grown sorghum by metagenome and proteome analyses. *Front. Microbiol.* 10, 407. <https://doi.org/10.3389/fmicb.2019.00407>.
- Harris, R.M., Webb, D.C., Howitt, S.M., Cox, G.B., 2001. Characterization of PitA and PitB from *Escherichia coli*. *J. Bacteriol.* 183, 5008–5014. <https://doi.org/10.1128/JB.183.17.5008-5014.2001>.
- Hartman, W.H., Richardson, C.J., 2013. Differential nutrient limitation of soil microbial biomass and metabolic quotients (qCO₂): is there a biological stoichiometry of soil microbes? *PLoS One* 8, e57127. <https://doi.org/10.1371/journal.pone.0057127>.
- Heinsoo, K., Merilo, E., Petrovits, M., Koppel, A., 2009. Fine root biomass and production in a *Salix viminalis* and *Salix dasycarpa* plantation. *Est. J. Ecol.* 58, 27–37. <https://doi.org/10.3176/eco.2009.1.03>.
- Heuck, C., Weig, A., Spohn, M., 2015. Soil microbial biomass C:N:P stoichiometry and microbial use of organic phosphorus. *Soil Biol. Biochem.* 85, 119–129. <https://doi.org/10.1016/j.soilbio.2015.02.029>.
- Hoang, D.T.T., Pausch, J., Razavi, B.S., Kuzyakova, I., Banfield, C.C., Kuzyakov, Y., 2016. Hotspots of microbial activity induced by earthworm burrows, old root channels, and their combination in subsoil. *Biol. Fertil. Soils* 52, 1105–1119. <https://doi.org/10.1007/s00374-016-1148-y>.
- Hoang, D.T.T., Maranguit, D., Kuzyakov, Y., Razavi, B.S., 2020. Accelerated microbial activity, turnover and efficiency in the rhizosphere is depth dependent. *Soil Biol. Biochem.* 147, 107852. <https://doi.org/10.1016/j.soilbio.2020.107852>.
- Hu, M., Peñuelas, J., Sardans, J., Tong, C., Chang, C.T., Cao, W., 2020. Dynamics of phosphorus speciation and the *phoD* phosphatase gene community in the rhizosphere and bulk soil along an estuarine freshwater-oligohaline gradient. *Geoderma* 365, 114236. <https://doi.org/10.1016/j.geoderma.2020.114236>.
- Hussain, N., Singh, A., Saha, S., Kumar, M.V.S., Bhattacharyya, P., Bhattacharya, S.S., 2016. Excellent N-fixing and P-solubilizing traits in earthworm gut-isolated bacteria: a vermicompost based assessment with vegetable market waste and rice straw feed mixtures. *Bioresour. Technol.* 222, 165–174. <https://doi.org/10.1016/j.biortech.2016.09.115>.
- IUSS Working Group WRB, 2015. World Reference Base for soil resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps. In: *World Soil Resour. Reports No. 106* Rome: FAO.
- Jackson, R.J., Binet, M.R.B., Lee, L.J., Ma, R., Graham, A.I., McLeod, C.W., Poole, R.K., 2008. Expression of the PitA phosphate/metal transporter of *Escherichia coli* is responsive to zinc and inorganic phosphate levels. *FEMS Microbiol. Lett.* 289, 219–224. <https://doi.org/10.1111/j.1574-6968.2008.01386.x>.
- Jha, V., Tikariha, H., Dafale, N.A., Purohit, H.J., 2018. Exploring the rearrangement of sensory intelligence in proteobacteria: insight of Pho regulon. *World J. Microbiol. Biotechnol.* 34, 172. <https://doi.org/10.1007/s11274-018-2551-3>.
- Jiang, W., Metcalf, W.W., Lee, K.S., Wanner, B.L., 1995. Molecular cloning, mapping, and regulation of Pho regulon genes for phosphonate breakdown by the phosphonate pathway of *Salmonella typhimurium* LT2. *J. Bacteriol.* 177, 6411–6421. <https://doi.org/10.1128/jb.177.22.6411-6421.1995>.
- Jorquera, M.A., Hernández, M.T., Rengel, Z., Marschner, P., de la Luz Mora, M., 2008. Isolation of culturable phosphobacteria with both phytate-mineralization and phosphate-solubilization activity from the rhizosphere of plants grown in a volcanic soil. *Biol. Fertil. Soils* 44, 1025. <https://doi.org/10.1007/s00374-008-0288-0>.
- Kageyama, H., Tripathi, K., Rai, A.K., Cha-um, S., Waditee-Sirisattha, R., Takabe, T., 2011. An alkaline phosphatase/phosphodiesterase, PhoD, induced by salt stress and secreted out of the cells of *Aphanethece halophytica*, a halotolerant cyanobacterium. *Appl. Environ. Microbiol.* 77, 5178–5183. <https://doi.org/10.1128/AEM.00667-11>.
- Kasahara, M., Nakata, A., Shinagawa, H., 1991. Molecular analysis of the *Salmonella typhimurium phoN* gene, which encodes nonspecific acid phosphatase. *J. Bacteriol.* 173, 6760–6765. <https://doi.org/10.1128/jb.173.21.6760-6765.1991>.
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41, e1. <https://doi.org/10.1093/nar/gks808>.
- Konno, H., Yoshida, Y., Nagano, K., Takebe, J., Hasegawa, Y., 2018. Biological and biochemical roles of two distinct cyclic dimeric adenosine 3',5'-monophosphate-associated phosphodiesterases in *Streptococcus mutans*. *Front. Microbiol.* 9, 2347. <https://doi.org/10.3389/fmicb.2018.02347>.
- Kurth, J.K., Albrecht, M., Karsten, U., Glaser, K., Schlöter, M., Schulz, S., 2021. Correlation of the abundance of bacteria catalyzing phosphorus and nitrogen turnover in biological soil crusts of temperate forests of Germany. *Biol. Fertil. Soils* 57, 179–192. <https://doi.org/10.1007/s00374-020-01515-3>.
- Kuzyakov, Y., Blagodatskaya, E., 2015. Microbial hotspots and hot moments in soil: concept & review. *Soil Biol. Biochem.* 83, 184–199. <https://doi.org/10.1016/j.soilbio.2015.01.025>.
- Lang, M., Zou, W., Chen, Xiuxiu, Zou, C., Zhang, W., Deng, Y., Zhu, F., Yu, P., Chen, X., 2020. Soil microbial composition and *phoD* gene abundance are sensitive to phosphorus level in a long-term wheat-maize crop system. *Front. Microbiol.* 11, 605955. <https://doi.org/10.3389/fmicb.2020.605955>.
- Le Bayon, R.C., Binet, F., 2006. Earthworms change the distribution and availability of phosphorus in organic substrates. *Soil Biol. Biochem.* 38, 235–246. <https://doi.org/10.1016/j.soilbio.2005.05.013>.
- Lemtiri, A., Colinet, G., Alabi, T., Cluzeau, D., Zirbes, L., Haubruge, É., Francis, F., 2014. Impacts of earthworms on soil components and dynamics. A review. *Biotechnol. Agron. Soc. Environ.* 18, 121–133.
- Lidbury, I.D.E.A., Murphy, A.R.J., Fraser, T.D., Bending, G.D., Jones, A.M.E., Moore, J. D., Goodall, A., Tibbett, M., Hammond, J.P., Scanlan, D.J., Wellington, E.M.H., 2017. Identification of extracellular glycerophosphodiesterases in *Pseudomonas* and their role in soil organic phosphorus remineralisation. *Sci. Rep.* 7, 2179. <https://doi.org/10.1038/s41598-017-02327-6>.
- Lipiec, J., Frac, M., Brzezinska, M., Turski, M., Oszust, K., 2016. Linking microbial enzymatic activities and functional diversity of soil around earthworm burrows and casts. *Front. Microbiol.* 7, 1361. <https://doi.org/10.3389/fmicb.2016.01361>.
- Liu, S., Zhang, X., Dungait, J.A.J., Quine, T.A., Razavi, B.S., 2021. Rare microbial taxa rather than *phoD* gene abundance determine hotspots of alkaline phosphomonoesterase activity in the karst rhizosphere soil. *Biol. Fertil. Soils* 57, 257–268. <https://doi.org/10.1007/s00374-020-01522-4>.
- Long, X., Huang, Y., Chi, H., Li, Y., Ahmad, N., Yao, H., 2018. Nitrous oxide flux, ammonia oxidizer and denitrifier abundance and activity across three different landfill cover soils in Ningbo, China. *J. Clean. Prod.* 170, 288–297. <https://doi.org/10.1016/j.jclepro.2017.09.173>.
- Luo, H., Benner, R., Long, R.A., Hu, J., 2009. Subcellular localization of marine bacterial alkaline phosphatases. *Proc. Natl. Acad. Sci.* 106, 21219–21223. <https://doi.org/10.1073/pnas.0907586106>.
- Luo, G., Ling, N., Nannipieri, P., Chen, H., Raza, W., Wang, M., Guo, S., Shen, Q., 2017. Long-term fertilisation regimes affect the composition of the alkaline phosphomonoesterase encoding microbial community of a vertisol and its derivative soil fractions. *Biol. Fertil. Soils* 53, 375–388. <https://doi.org/10.1007/s00374-017-1183-3>.
- Maheswari, N.U., Sudha, S., 2013. Enumeration and detection of phosphate solubilizing bacteria from the gut of earthworm varieties. *J. Chem. Pharm. Res.* 5, 264–267.
- Mair, P., Wilcox, R., 2020. Robust statistical methods in R using the WRS2 package. *Behav. Res. Methods* 52, 464–488. <https://doi.org/10.3758/s13428-019-01246-w>.
- McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8, e61217. <https://doi.org/10.1371/journal.pone.0061217>.
- Menzel, P., Ng, K.L., Krogh, A., 2016. Fast and sensitive taxonomic classification for metagenomics with Kaiju. *Nat. Commun.* 7, 11257. <https://doi.org/10.1038/ncomms11257>.
- Miroshnichenko, M.L., Bonch-Osmolovskaya, E.A., 2006. Recent developments in the thermophilic microbiology of deep-sea hydrothermal vents. *Extremophiles* 10, 85–96. <https://doi.org/10.1007/s00792-005-0489-5>.
- Monds, R.D., Newell, P.D., Schwartzman, J.A., O'Toole, G.A., 2006. Conservation of the Pho regulon in *Pseudomonas fluorescens* Pf0-1. *Appl. Environ. Microbiol.* 72, 1910–1924. <https://doi.org/10.1128/AEM.72.3.1910-1924.2006>.
- Nannipieri, P., Johnson, R.L., Paul, E.A., 1978. Criteria for measurement of microbial growth and activity in soil. *Soil Biol. Biochem.* 10, 223–229. [https://doi.org/10.1016/0038-0717\(78\)90100-1](https://doi.org/10.1016/0038-0717(78)90100-1).
- Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagne, H., 2019. Vegan: community ecology package. URL. <https://cran.r-project.org/package=vegan>.
- van der Pauw, F., 1971. An effective water extraction method for the determination of plant-available soil phosphorus. *Plant Soil* 34, 467–481. <https://doi.org/10.1007/BF01372799>.
- Park, S.C., Smith, T.J., Bisesi, M.S., 1992. Activities of phosphomonoesterase and phosphodiesterase from *Lumbricus terrestris*. *Soil Biol. Biochem.* 24, 873–876. [https://doi.org/10.1016/0038-0717\(92\)90008-L](https://doi.org/10.1016/0038-0717(92)90008-L).
- Park, K., Lee, C., Son, H., 2009. Mechanism of insoluble phosphate solubilization by *Pseudomonas fluorescens* RAF15 isolated from ginseng rhizosphere and its plant growth-promoting activities. *Lett. Appl. Microbiol.* 49, 222–228. <https://doi.org/10.1111/j.1472-765X.2009.02642.x>.
- Peine, M., Vitov, N., Grafe, M., Baum, C., Zicker, T., Eichler-Löbermann, B., Schulz, S., Schlöter, M., Leinweber, P., 2019. Effect of triple superphosphate and biowaste compost on mycorrhizal colonization and enzymatic P mobilization under maize in a

- long-term field experiment. *J. Plant Nutr. Soil Sci.* 182, 167–174. <https://doi.org/10.1002/jpln.201800499>.
- Pramanik, P., 2010. Quantification of hydrolytic and proteolytic enzymes in the excreta of three epigeic earthworms and detection of thiocarbamic acid by GC-MS-MS. *Environmentalist* 30, 212–215. <https://doi.org/10.1007/s10669-010-9264-y>.
- Purahong, W., Wubet, T., Lentendu, G., Schlöter, M., Pecyna, M.J., Kapturska, D., Hofrichter, M., Krüger, D., Buscot, F., 2016. Life in leaf litter: novel insights into community dynamics of bacteria and fungi during litter decomposition. *Mol. Ecol.* 25, 4059–4074. <https://doi.org/10.1111/mec.13739>.
- Qi, Y., Kobayashi, Y., Hulett, F.M., 1997. The *pst* operon of *Bacillus subtilis* has a phosphate-regulated promoter and is involved in phosphate transport but not in regulation of the *pho* regulon. *J. Bacteriol.* 179, 2534–2539. <https://doi.org/10.1128/jb.179.8.2534-2539.1997>.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, 590–596. <https://doi.org/10.1093/nar/gks1219>.
- R Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. URL: <https://www.r-project.org/>.
- Rawat, P., Das, S., Shankhdhar, D., Shankhdhar, S.C., 2021. Phosphate-solubilizing microorganisms: mechanism and their role in phosphate solubilization and uptake. *J. Soil Sci. Plant Nutr.* 21, 49–68. <https://doi.org/10.1007/s42729-020-00342-7>.
- Rejmánková, E., Sirová, D., Carlson, E., 2011. Patterns of activities of root phosphomonoesterase and phosphodiesterase in wetland plants as a function of macrophyte species and ambient phosphorus regime. *New Phytol.* 190, 968–976. <https://doi.org/10.1111/j.1469-8137.2011.03652.x>.
- Rho, M., Tang, H., Ye, Y., 2010. FragGeneScan: predicting genes in short and error-prone reads. *Nucleic Acids Res.* 38, e191 <https://doi.org/10.1093/nar/gkq747>.
- Richardson, A.E., Simpson, R.J., 2011. Soil microorganisms mediating phosphorus availability update on microbial phosphorus. *Plant Physiol.* 156, 989–996. <https://doi.org/10.1104/pp.111.175448>.
- Rodríguez, H., Fraga, R., Gonzalez, T., Bashan, Y., 2006. Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. *Plant Soil* 287, 15–21. <https://doi.org/10.1007/s11104-006-9056-9>.
- Rojas, A., Holguin, G., Glick, B.R., Bashan, Y., 2001. Synergism between *Phyllobacterium* sp. (N₂-fixer) and *Bacillus licheniformis* (P-solubilizer), both from a semiarid mangrove rhizosphere. *FEMS Microbiol. Ecol.* 35, 181–187. <https://doi.org/10.1111/j.1574-6941.2001.tb00802.x>.
- RStudio Team, 2016. RStudio: Integrated Development for R. URL: <http://www.rstudio.com/>.
- Saggau, P., 2011. Investigation of Abundance, Biomass and Diversity of Earthworms Under Short Rotation Coppice and Arable Soils. [Master's Thesis, University of Rostock] Unpublished Results.
- Sanchez-Hernandez, J.C., Ríos, J.M., Attademo, A.M., 2018. Response of digestive enzymes and esterases of ecotoxicological concern in earthworms exposed to chlorpyrifos-treated soils. *Ecotoxicology* 27, 890–899. <https://doi.org/10.1007/s10646-018-1914-8>.
- Schöler, A., Jacquioud, S., Vestergaard, G., Schulz, S., Schlöter, M., 2017. Analysis of soil microbial communities based on amplicon sequencing of marker genes. *Biol. Fertil. Soils* 53, 485–489. <https://doi.org/10.1007/s00374-017-1205-1>.
- Schubert, M., Lindgreen, S., Orlando, L., 2016. AdapterRemoval v2: rapid adapter trimming, identification, and read merging. *BMC. Res. Notes* 9, 88. <https://doi.org/10.1186/s13104-016-1900-2>.
- Sims, R.W., Gerard, B.M., 1985. *Earthworms: Keys and Notes for the Identification and Study of the Species*. Linnean Society, London.
- Steele, S.J., Gower, S.T., Vogel, J.G., Norman, J.M., 1997. Root mass, net primary production and turnover in aspen, jack pine and black spruce forests in Saskatchewan and Manitoba, Canada. *Tree Physiol.* 17, 577–587. <https://doi.org/10.1093/treephys/17.8-9.577>.
- Tabatabai, M.A., Bremner, J.M., 1969. Use of *p*-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biol. Biochem.* 1, 301–307. [https://doi.org/10.1016/0038-0717\(69\)90012-1](https://doi.org/10.1016/0038-0717(69)90012-1).
- Tao, J., Griffiths, B., Zhang, S., Chen, X., Liu, M., Hu, F., Li, H., 2009. Effects of earthworms on soil enzyme activity in an organic residue amended rice-wheat rotation agro-ecosystem. *Appl. Soil Ecol.* 42, 221–226. <https://doi.org/10.1016/j.apsoil.2009.04.003>.
- Tarafdar, J.C., Claassen, N., 1988. Organic phosphorus compounds as a phosphorus source for higher plants through the activity of phosphatases produced by plant roots and microorganisms. *Biol. Fertil. Soils* 5, 308–312. <https://doi.org/10.1007/BF00262137>.
- Tatusova, T., Ciufu, S., Fedorov, B., O'Neill, K., Tolstoy, I., 2014. RefSeq microbial genomes database: new representation and annotation strategy. *Nucleic Acids Res.* 42, D553–D559. <https://doi.org/10.1093/nar/gkt1274>.
- Tian, J., Kuang, X., Tang, M., Chen, X., Huang, F., Cai, Y., Cai, K., 2021. Biochar application under low phosphorus input promotes soil organic phosphorus mineralization by shifting bacterial *phoD* gene community composition. *Sci. Total Environ.* 779, 146556 <https://doi.org/10.1016/j.scitotenv.2021.146556>.
- Torres-Sallan, G., Creamer, R.E., Lanigan, G.J., Reidy, B., Byrne, K.A., 2018. Effects of soil type and depth on carbon distribution within soil macroaggregates from temperate grassland systems. *Geoderma* 313, 52–56. <https://doi.org/10.1016/j.geoderma.2017.10.012>.
- Tripura, C., Sudhakar Reddy, P., Reddy, M.K., Sashidhar, B., Podile, A.R., 2007. Glucose dehydrogenase of a rhizobacterial strain of *Enterobacter asburiae* involved in mineral phosphate solubilization shares properties and sequence homology with other members of enterobacteriaceae. *Indian J. Microbiol.* 47, 126–131. <https://doi.org/10.1007/s12088-007-0025-7>.
- Uksa, M., Schlöter, M., Endesfelder, D., Kublik, S., Engel, M., Kautz, T., Kopke, U., Fischer, D., 2015a. Prokaryotes in subsoil-evidence for a strong spatial separation of different phyla by analysing co-occurrence networks. *Front. Microbiol.* 6, 1269. <https://doi.org/10.3389/fmicb.2015.01269>.
- Uksa, M., Schlöter, M., Kautz, T., Athmann, M., Köpke, U., Fischer, D., 2015b. Spatial variability of hydrolytic and oxidative potential enzyme activities in different subsoil compartments. *Biol. Fertil. Soils* 51, 517–521. <https://doi.org/10.1007/s00374-015-0992-5>.
- Wan, J.H.C., Wong, M.H., 2004. Effects of earthworm activity and P-solubilizing bacteria on P availability in soil. *J. Plant Nutr. Soil Sci.* 167, 209–213. <https://doi.org/10.1002/jpln.200321252>.
- Wang, J., Wu, Y., Zhou, J., Bing, H., Sun, H., 2016. Carbon demand drives microbial mineralization of organic phosphorus during the early stage of soil development. *Biol. Fertil. Soils* 52, 825–839. <https://doi.org/10.1007/s00374-016-1123-7>.
- Waring, B.G., Weintraub, S.R., Sinsabaugh, R.L., 2014. Ecoenzymatic stoichiometry of microbial nutrient acquisition in tropical soils. *Biogeochemistry* 117, 101–113. <https://doi.org/10.1007/s10533-013-9849-x>.
- Wear, E.K., Wilbanks, E.G., Nelson, C.E., Carlson, C.A., 2018. Primer selection impacts specific population abundances but not community dynamics in a monthly time-series 16S rRNA gene amplicon analysis of coastal marine bacterioplankton. *Environ. Microbiol.* 20, 2709–2726. <https://doi.org/10.1111/1462-2920.14091>.
- Wickham, H., 2016. *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag, New York.
- Yan, L., 2021. ggvenn: Draw Venn diagram by “ggplot2”.
- Yin, X., Wu Orr, M., Wang, H., Hobbs, E.C., Shabalina, S.A., Storz, G., 2019. The small protein MgtS and small RNA MgrR modulate the PitA phosphate symporter to boost intracellular magnesium levels. *Mol. Microbiol.* 111, 131–144. <https://doi.org/10.1111/mmi.14143>.
- Zhao, X., Zhang, Y., Cui, Z., Peng, L., Cao, C., 2022. Dynamics of *phoD*- and *gcd*-harboring microbial communities across an age sequence of biological soil crusts under sand-fixation plantation. *Front. Microbiol.* 13, 831888 <https://doi.org/10.3389/fmicb.2022.831888>.
- Zhi, R., Deng, J., Xu, Y., Xu, M., Zhang, S., Han, X., Yang, G., Ren, C., 2023. Altered microbial P cycling genes drive P availability in soil after afforestation. *J. Environ. Manag.* 328, 116998 <https://doi.org/10.1016/j.jenvman.2022.116998>.