Phosphorus depletion in forest soils shapes bacterial communities towards phosphorus recycling systems

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Summary

Phosphorus (P) is an important macronutrient for all biota on earth but similarly a finite resource. Microorganisms play on both sides of the fence as they effectively mineralize organic and solubilize precipitated forms of soil phosphorus, but conversely also take up and immobilize P. Therefore, we analyzed the role of microbes in two beech forest soils with high and low P content by direct sequencing of metagenomic DNA. For inorganic P solubilization, a significantly higher microbial potential was detected in the P-rich soil. This trait especially referred to *Candidatus* Solibacter usiatus, likewise one of the dominating species in the datasets. A higher microbial potential for efficient phosphate uptake systems (*pstSCAB*) was detected in the P-depleted soil. Genes involved in P starvation response regulation (*phoB*, *phoR*) were prevalent in both soils. This underlines the importance of effective phosphate (Pho) regulon control for microorganisms to use alternative P sources during phosphate limitation. Predicted genes were primarily harbored by Rhizobiales, Actinomycetales and Acidobacteriales.

Introduction

Phosphorus (P) is an important macronutrient for all biota on earth as it is an essential component of the energy metabolism, the genetic backup and stable cell structures. Next to nitrogen, P is the second major growth limiting macronutrient for plants thus affecting plant health and crop yields (Schachtman *et al*., 1998). Unlike nitrogen, in developing terrestrial ecosystems the phosphorus supply mainly depends on weathering of the parent material since the amounts introduced into soil by atmospheric P deposition are low (Walker & Syers, 1976; Chadwick *et al*., 1999). Over time in the initial phase of ecosystem development the amount of mineral phosphate constantly decreases, whereas the proportion of labile-, plant-, occluded- and soil organic-P increases (Vitousek *et al*., 2010). Losses of P from soils developed on phosphorus poor parent material cannot be replenished without external input (Walker & Syers, 1976). Therefrom plants are only able to take up free orthophosphate, which is available in the range of 1 ppm or less (Holford, 1997; Rodriguez & Fraga, 1999). In this regard especially microorganisms play an important role in maintaining the P status of soils. On the one hand microorganisms enhance plant available P through i) mycorrhizal growth or phytostimulation ii) microbial population dynamics, which lead to increased levels of orthophosphate in the soil solution and iii) direct mineralization and solubilization of soil P by the release of hydrolytic enzymes and organic anions (Richardson & Simpson, 2011). Depending on the substrate, microbial enzymes releasing P from organic compounds can be classified into three distinct groups: 1) Nonspecific Phosphatases (Phosphohydrolases), 2) Phytases and 3) Phosphonatases and C-P Lyases (Rodriguez *et al*., 2006). Moreover plant growth promoting bacteria (PGPB) are also effective in solubilizing precipitated and adsorbed forms of inorganic P (Gyaneshwar *et al*., 2002). On the other hand microorganisms also compete for the available P with other biota, as they have efficient P uptake systems. Most prominent are the high affinity Phosphate-specific transporter Pst and the low affinity Phosphate inorganic transporter Pit (Willsky *et al*., 1973; Wanner, 1993).

Overall our knowledge about P mineralizing and solubilizing enzymes is mostly restricted to the characterization of isolates or the effect of PGPB like *Pseudomonas*, *Burkholderia, Rhizobium* and *Bacillus* strains under controlled conditions (Rodriguez & Fraga, 1999). However the interplay of the different functional groups of microbes driving P turnover in natural ecosystems mainly in relation to the actual phosphorus status is still unclear. We hypothesize that in soils, with large amounts of mineral and total P, microbial solubilization processes of inorganic P prevail. In contrast in P-depleted soils, mineralization of organic phosphorus will be the main driver of the microbial phosphorus turnover. A higher potential for efficient microbial phosphate transporters is further expected in these soils. To test this hypothesis we investigated two contrasting beech forest soils: One of them with high P stocks and a large proportion of P bound to soil minerals and the other one with low P content and a large proportion bound to soil organic matter. Since none of the two forest sites received any fertilizer input, the soils represent the natural and undistorted state of P turnover. To provide an unbiased view into the actual soil microbial community structure and uncover major processes of the soil P turnover, a metagenomic sequencing approach was applied and data was analyzed on a taxonomic and functional level.

Results

Soil microbial biomass

Soil microbial biomass carbon, nitrogen and phosphorus (Cmic, Nmic, Pmic) data are summarized in Table 1. The P-rich soil (BBR) revealed more than ten times higher Pmic values (105 µg P g^{-1}) compared to the P-depleted soil (LUE) (10 µg P g^{-1}). The values for Cmic and Nmic were approximately eight times, respectively seven times, higher in BBR. The ratio of microbial carbon and nitrogen was higher in LUE (33), compared to BBR (15). The ratios of Cmic:Pmic and Nmic:Pmic were 11 and 0.8 in samples from BBR and 19 and 1 in samples from LUE, respectively.

"Preferred Position Table 1"

77

78 Phylogenetic annotation of metagenomic datasets

79 Six soil samples from two German forest soils were used for metagenomic sequencing. 388 MB of data
80 were generated in total using 454 technology. This corresponded to 1,122,938 filtered sequences with
81 an average read length of 344 bp. All details of the sequencing run are summarized in Supporting
82 Information Table S1. Subsampled datasets were phylogenetically analyzed using blastn (Camacho *et al*.,
83 2009) against the SILVA SSU database (Pruesse *et al*., 2007) and MEGAN (Huson *et al*., 2011). The
84 majority of assigned sequences referred to Bacteria (91.08%), followed by Eukaryotes (8.22%) and
85 Archaea (0.70%). As only a small proportion of all reads (0.05%) could be aligned to the ribosomal
86 database, analysis focused on phylum level exclusively. Both forest soils were dominated by
87 Proteobacteria, Acidobacteria and Actinobacteria (Supporting Information Fig. S1).
88 For a broader characterization of the microbial communities of the two soils, subsampled datasets were
89 aligned against the NCBI Non-redundant protein sequences (nr) database
90 (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz; October 2014). In total 464,438 sequences could be
91 assigned. To detect global differences within the community structures of the samples a principal
92 component analysis (PCA) was performed (Supporting Information Fig. S2). Depending on the soil type, a
93 clear separation of the two forest sites was detected on order level. PC 1 explained about 85% of the
94 total variance within the metagenomic datasets. To estimate the coverage of microbial diversity,
95 rarefaction analysis was performed based on subsampled, phylogenetically annotated reads. In
96 Supporting Information Fig. S3 the number of annotated reads on order level is plotted against the
97 amount of sequenced reads. The rarefaction curves showed a sufficient coverage of the microbial
98 diversity for all six samples. Curves depicting biological replicates were comparable; overall a slightly
99 higher microbial richness was detected in LUE. Phylogenetic annotation of sequencing reads highlighted
100 Proteobacteria as the dominating phylum in both soils, accounting for 44.9% of all assigned reads (Fig.

1a). Further dominating phyla were Actinobacteria (21.3%), Acidobacteria (20.6%), Planctomycetes (3.8%) and Verrucomicrobia (2.0%). On order level Rhizobiales, Actinomycetales and Acidobacteriales were most abundant (Fig. 1b). While Rhizobiales were clearly dominating in BBR, Actinomycetales and Acidobacteriales showed the highest abundance in LUE. Eukaryotic sequences assigned to Ascomycota (0.6%) and Basidiomycota (0.2%) were found in all six datasets. Most abundant fungal orders referring to Eurotiales, Agaricales and Hypocreales were dominating in LUE. See Supporting Information Table S2 for absolute number of sequences annotated to the most abundant microbial phyla, respectively orders, in the datasets. To detect significant differences within the microbial communities of BBR and LUE the abundance of all annotated taxa was statistically compared. Supporting Information Table S3 comprises all taxa that differed significantly ($P<0.05$) in the number of phylogenetically annotated reads. To be more stringent only taxa with an abundance of at least 0.05% (referred to the total number of assigned reads) were included. On phylum level 9 taxa were found to fulfill these criteria. Among them were Proteobacteria, showing significantly more annotated reads in the P-rich soil, and Acidobacteria, having a significantly higher abundance in the P-depleted soil. On class level 11 taxa differed significantly in the number of assigned reads. Among them 2 classes of fungi were detected. Both Eurotiomycetes and Agaricomycetes showed a significantly higher abundance in the P-depleted soil.

"Preferred Position Figure1"

Functional annotation of metagenomic datasets

Functional annotation of metagenomic datasets was performed against the KEGG database (Kanehisa & Goto, 2000). Based on subsampled data, 266,415 sequences were assigned and further analyzed using MEGAN (Huson *et al*., 2011). Genes encoding pathways for two−component systems, ABC transporters and purine metabolism were most abundant in both soils (Supporting Information Fig. S4).

Further analysis exclusively focused on genes coding for proteins involved in the microbial turnover of soil P. This included enzymes performing the solubilization of inorganic as well as the mineralization of organic bound soil phosphorus, microbial P transporter and uptake systems, phosphate-starvation inducible genes and their crucial regulation systems. Genes coding for intracellular phosphatases (and further enzymes hydrolyzing phosphoester bonds) which are involved in metabolic processes were disregarded, since they are not directly contributing to the turnover of soil P. Supporting Information Table S4 comprises all enzymes, corresponding genes and KEGG KO numbers that were included in the analysis. In total 0.82% of all functionally assigned sequences referred to genes coding for proteins of the soil microbial P cycle. All genes with curated KEGG KO numbers that were detected in the subsampled datasets are shown in Supporting Information Fig. S5. However statistical analysis focused on genes encoding enzymes which are directly involved in cleavage and release of P or having crucial functions for the cellular P uptake, while auxiliary and enzymatic upstream reactions were omitted.

Most abundant genes in the datasets referred to microbial phosphate uptake and regulation systems (Fig. 2). In total 469 sequences were assigned to genes coding for subunits of the highly efficient phosphate-specific transporter. Genes coding for all components (*pstSCAB*) showed a higher abundance in the P-depleted soil (LUE) compared to the soil rich in P (BBR). In addition genes coding for the low-affinity phosphate-inorganic transporter (*pit*) were more abundant in LUE. Compared to the Pst system, sequences referring to glycerol-3-phosphate transporter (*ugpBAEC*) and genes coding for phosphonate transporter (p*hnCDE*) were less abundant by seven or ten times, respectively. Most of their components showed a higher abundance in the P-rich soil. Genes coding for the subunits of a two-component system involved in regulation of phosphate starvation inducible genes were frequently detected. Genes encoding the sensor kinase (*phoR*) were significantly more abundant in the P-rich soil based on the number of reads, while genes coding for the response regulator (*phoB*) and the negative regulator protein (*phoU*) showed a slightly higher abundance in the P-depleted soil.

In addition to microbial uptake and regulation systems, also genes coding for P mineralizing and solubilizing enzymes were detected (Fig. 2). Most abundant were genes coding for the quinoprotein glucose dehydrogenase (PQQGDH), which performs the solubilization of inorganic bound P. In total 257 sequences referring to this gene (*gcd*) were detected in the six datasets. Significantly more reads were assigned in the P-rich soil (BBR). Interestingly, both soils showed a higher abundance of genes coding for alkaline phosphatases (ALP) compared to acid phosphatases. Altogether 82 genes coding for ALP were detected, showing more assigned reads in the P-rich soil. Overall genes coding for the alkaline phosphatase PhoD were three times more abundant compared to PhoA independent from the soil investigated. 54 sequences could be assigned to genes coding for acid phosphatases. Significantly more reads coding for acid phosphatases (K01078) were found in the P-rich soil. As KEGG orthology number K01078 does not represent a specific class of acid phosphatase the assigned sequences might correspond to one of the classes A, B or C. In addition genes coding for two types of enzymes degrading specific forms of organic phosphodiesters were detected. Genes encoding the glycerophosphoryl diester phosphodiesterase (*ugpQ*) and the phosphoribosyl 1,2-cyclic phosphate phosphodiesterase (*phnP*) had a frequency of 54 and 49 sequencing reads in the six datasets, respectively. The latter one is part of the C-P lyase multienzyme complex performing the degradation of multiple organophosphonates. Sequences referring to phosphotriesterases and phytases were detected with a higher abundance in the P-rich soil. In contrast, significantly more sequences were assigned to phosphonatases in the P-depleted soil. Further all remaining genes coding for enzymes contributing to the C-P lyase core reaction were detected (*phnG*, *phnH*, *phnI*, *phnJ*, *phnL*, *phnM*). Most genes had a relatively low abundance of 4 reads or less. To further confirm these results derived from the KEGG database, a second approach for the functional annotation of sequencing data was applied. Based on subsampled datasets, open-reading frames were predicted and subsequently scanned for a set of Hidden Markov Models (HMM), comprising conserved domains of investigated proteins (Supporting Information Table S4). Basically both

172 approaches led to similar results concerning the relative abundance of genes, with respect to the
173 different soil types (Supporting Information Fig. S6). However the absolute numbers of predicted genes
174 varied slightly. Sole exception was *phoR*, where a relative decrease in abundance related to *phoB* was
175 detected at both sites.

176 "Preferred Position Figure 2"

177

178 Taxonomic assignment of investigated genes

179 The taxonomic assignment of investigated genes was based on KEGG database results. Sequencing reads
180 were aligned against the NCBI Non-redundant protein sequences (nr) database using DIAMOND
181 (Buchfink *et al*., 2015) and further analyzed employing MEGAN (Huson *et al*., 2011). Subunits of P
182 transporters and the C-P lyase multienzyme complex as well as different classes of acid and alkaline
183 phosphatases were pooled, respectively. Results are shown on phylum level (Supporting Information
184 Fig. S7a+b) and reflected the overall abundance of taxa in the metagenomic datasets. Most of the
185 predicted genes were harbored by Proteobacteria (50%), Acidobacteria (24.8%), Actinobacteria (14.4%),
186 Planctomycetes (2.6%), Firmicutes (1.9%) and Verrucomicrobia (1.9%). While the phylum Proteobacteria
187 covered all groups of predicted genes, Acidobacteria especially harbored genes coding for the PQQGDH
188 and the Pst transporter. On order level (Fig. 3a+b) Rhizobiales (25.5%), Actinomycetales (17%),
189 Acidobacteriales (12.2%), Burkholderiales (5.6%) and Rhodospirillales (4.3%) were among the most
190 abundant taxa. Especially in the P-rich soil (BBR), a substantial amount of genes referring to acid and
191 alkaline phosphatases, phosphodiesterases, C-P lyases, PQQGDH, Pst-, Pit-, phosphonate- and glycerol-
192 3-phosphate transporters were harbored by Rhizobiales. By contrast in LUE different orders, including
193 Actinomycetales, Acidobacteriales, Burkholderiales and Rhodospirillales, contributed to the soil
194 microbial P cycle, whereas Rhizobiales played a subsidiary role. Also Solibacterales were a rich source for

P cycle associated genes (8.3%), although this order was generally not very abundant in the six datasets (3.4%).

"Preferred Position Figure 3"

Discussion

Microbial phosphate uptake systems and Pho regulon control

Functional annotation of metagenomic datasets underlined the importance of microbial phosphorus uptake systems in our study. Especially in P-depleted soils efficient P transporters are of great relevance, as they allow microorganisms to compete with plants in the struggle for bioavailable P (Raghothama, 2000; Yuan *et al*., 2006). Subunits of the highly-efficient Pst transporter were among the most abundant P cycle associated genes in the datasets. All components (*pstSCAB*) were detected more frequently in the LUE samples. While the constitutively expressed Pit system mainly transports metallic cations in complex with P, the Pst transporter is also involved in P signaling and gene regulation (Wanner, 1993; Wanner, 1996). Jointly with genes of a two-component system (*phoR*, *phoB, phoU*), likewise frequently detected in both soils, several phosphate starvation inducible genes (PSI) of the phosphate (Pho) regulon are controlled depending on the extracellular P supply (Hsieh & Wanner, 2010). The high abundance of P signaling and Pho regulation genes in the datasets emphasized the significance of effective PSI gene regulation for microbial communities, to efficiently use alternative phosphorus sources in times of P starvation.

Microbial inorganic phosphorus solubilization

Microbial solubilization of calcium and mineral phosphates is attributed to acidification of the periplasmic space (Goldstein, 1995). The direct oxidation pathway of glucose (via the PQQGDH) and other aldose sugars sets the metabolic basis for this mineral phosphate solubilizing (Mps) phenotype in

Gram-negative bacteria (Goldstein, 1995). We hypothesized that in soils rich in mineral-P, solubilization processes of inorganic phosphates are key drivers of the microbial P turnover. The significantly higher abundance of genes coding for the PGGGDH in BBR corroborates our hypothesis. This enzyme is an indicator for the mineral-P solubilizing potential of a microbial community. However the bacterial Mps phenotype depends on formation of the PQQGDH holoenzyme, comprising glucose dehydrogenase (GDH) and cofactor pyrroloquinoline quinone (PQQ) (Goldstein, 1994). Due to the limited amount of sequencing reads in the datasets co-occurrence studies regarding GDH (*gcd*) and PQQ biosynthesis genes (*pqqABCDEF*) were not performed. However pyrroloquinoline quinone is a crucial cofactor for several quinoproteins in Gram-negative bacteria. It is known to be produced by a variety of different microorganisms (Duine, 1999; Igarashi & Sode, 2003). Goldstein *et al*. (2003) reported induction of PQQGDH activity through novel DNA fragments with no homology to known PQQ genes. The authors proposed an alternative pathway for PQQ biosynthesis in *Escherichia coli*. In some microorganisms the GDH apoenzyme is produced in a constitutively manner. This allows direct oxidation of glucose upon availability of exogenous PQQ, although biosynthesis genes are lacking in the genome (Goldstein, 1994). Therefore we assume that PQQ availability in the soils does not limit the Mps efficiency of the microbial communities. Consequently the higher abundance of PQQGDH genes in BBR may serve as an indicator for an increased microbial potential of mineral-P solubilization. Still this process might not directly enhance the P bioavailability in soils since microorganisms could primarily meet their own demands. Plants rather profit from higher P turnover rates in the microbial biomass (Richardson & Simpson, 2011).

Microbial organic phosphorus mineralization

Regarding organic P mineralization a significantly higher abundance of nonspecific acid phosphatases was detected in BBR compared to LUE. This group of enzymes hydrolyzes a broad range of organic phosphomonoester and -anhydride bonds. Extracellular soluble and membrane-bound forms might act

as phosphoester scavengers (Rossolini *et al*., 1998). Thereby organic high molecular-weight compounds are sequentially degraded until orthophosphate and by-products are absorbed. In Enterobacteriaceae, acid phosphatases are commonly regulated in a P irrepressible manner (Rodriguez & Fraga, 1999). Thus, higher gene abundance in BBR does not necessarily imply a greater potential for supplying microorganisms with P, when it becomes limiting. These enzymes rather continuously provide essential nutrients, including phosphorus, to cells. In contrast, microbial alkaline phosphatases (ALP) presumably are regulated in a P repressible manner. In *Escherichia coli* and *Bacillus subtilis* corresponding genes (*phoA*, *phoD*) are under control of the Pho regulon (Wanner, 1993; Eder *et al*., 1996). Unlike acid phosphatases, these enzymes reflect the actual potential of providing orthophosphate to microorganisms under P starvation. Interestingly a higher abundance of alkaline phosphatase genes (compared to acid phosphatases) was detected in the datasets, although both soils are rather acidic. Primarily ALP activity prevails in neutral and alkaline environments (Nannipieri *et al*., 2011). However minor levels of activity were also detected in acid mineral topsoils of Norway spruce and beech dominated forests (Zimmermann & Frey, 2002). Incidentally, DNA based sequencing approaches merely reveal the genetic potential of microbial communities, rather than reflecting actual levels of gene expression or enzymatic activity. Data from previous studies on comparable forest sites certainly suggests also for BBR and LUE the predominance of acid phosphatase activity (Zimmermann & Frey, 2002). Especially forest litter and organic layers are hotspots of microbial phosphatase activity, whereas a decline was observed in mineral soils (Pang & Kolenko, 1986). Presumably microbial phosphatase gene abundance reaches maximum in the uppermost forest floors rather than in the sampled Ah-horizons. Since plants are incapable of producing alkaline phosphatases (Nakas *et al*., 1987) the high ALP potential in both soils might be explained by an ecological niche, allowing microbes to profit against plants in P limited environments. Microorganisms could benefit from soil heterogeneity, generating pH neutral microsites within a rather acidic environment (Šimek & Cooper, 2002). In contrast to acid phosphatases

267 microbial genes coding for ALP are upregulated during phosphate starvation, thereby enabling usage of
268 alternative P sources. The high abundance of Pho regulated ALP encoding genes underlines their
269 importance for microbes in the struggle for P. Alkaline phosphatase PhoD was found to be three times
270 more abundant in the datasets compared to PhoA. This is in accordance with previous studies, since
271 PhoD is the most frequently found ALP in metagenomic datasets derived from soil and water samples
272 (Luo *et al*., 2009; Tan *et al*., 2013). While enzymes of the PhoA family predominantly dephosphorylate
273 monoester bonds, PhoD also shows phosphodiesterase activity against cell wall teichoic acids and
274 phospholipids (Rodriguez *et al*., 2014). The broader substrate specificity allows usage of various P
275 sources and might be one reason for the higher gene abundance in the datasets. However taking into
276 account that the investigated soils are classified as extremely acid according to the Soil Survey Manual
277 (Soil Survey Division Staff, 1993), the expression of the related genes, that we have identified in our
278 metagenomics library needs to be confirmed in future studies focusing on gene expression.
279 Phytate (myo-Inositol-1,2,3,4,5,6-hexakisphosphate, IP_6) degrading enzymes were rarely detected,
280 although the substrate makes up a major fraction of organic P in many soils (Turner, 2007). In terrestrial
281 ecosystems IP_6 is mainly derived from storage compounds of plants, especially seeds (Turner *et al*.
282 2002). Inherently phytate tends to accumulate in top horizons due to the formation of insoluble
283 complexes with metallic cations or adsorption to clay minerals (Bowman *et al*., 1967; Turner *et al*.,
284 2002). Especially in soils classified as extremely acid (Soil Survey Division Staff, 1993) phytate is stabilized
285 effectively, leading to increased absolute and relative phytate levels (as a fraction of soil organic P)
286 (Turner & Blackwell, 2013). This might explain the low abundance of phytase genes in the present study,
287 since soil samples were derived from the Ah-horizon exclusively. A higher potential for phytase
288 mineralization can be expected in the organic or litter layer.
289
290 Microbial community involved in turnover of soil phosphorus

Taxonomic assignment of predicted genes emphasized the importance of Rhizobiales, Actinomycetales, Acidobacteriales and Solibacterales for the soil microbial P turnover. Interestingly Rhizobiales contributed to P cycling predominantly in the P-rich soil. This also reflected the total abundance of taxa in the datasets. While Actinomycetales and Acidobacteriales were dominating in LUE, Rhizobiales were significantly more abundant in BBR. Members of the latter order are known as effective plant growth promoting bacteria (Rodriguez & Fraga, 1999). Isolates producing acid and alkaline phosphatases or exhibiting Mps traits were detected (Halder *et al*., 1990; Abd-Alla, 1994). Generally Rhizobia perform well under commonly found soil P concentrations and are known to be important in forest litter and humus layers (Baldrian *et al*., 2012). However growth might be restricted in severely P-depleted soils (Smart *et al*., 1984). The limited availability of soil P in LUE might restrain rhizobial growth, simultaneously favoring oligotrophic microorganisms. Ratios of Cmic:Pmic indicated a higher P content in the BBR biomass compared to LUE, while the soil seemed to be relatively limited by the nitrogen (N) content. Generally Rhizobiales are famous for their N-fixing potential, although only few families are truly capable (Spaink, 2000). In our datasets the majority of rhizobial sequences (40%; data not shown) was assigned to the N-fixing genus of *Bradyrhizobium*. However symbiontic N fixation requires root nodulation of legumes. Since rhizospheric and root material were excluded from our sequencing run we propose, that Rhizobiales are predominantly contributing to the turnover of soil P in BBR and LUE whereas N fixation is more important in symbiontic interactions. Consequently the high abundance of Rhizobiales led to a significant domination of Alphaproteobacteria in BBR. The LUE soil in contrast was characterized by a stronger contribution of Actinomycetales and Acidobacteriales to microbial phosphorus cycling. Generally Acidobacteria are classified as oligotrophic bacteria. High substrate affinities and efficient sugar-transporters favor growth under resource limitation (Ward *et al*., 2009). Fierer *et al*. (2007) proposed soil carbon availability as the crucial factor in this respect. Generally, microbial growth in LUE was restricted due to the low nutrient availability, since biomass carbon was

several magnitudes lower compared to BBR. Apparently LUE microbial biomass was mainly limited in P, since the Cmic:Pmic and Nmic:Pmic ratios exceeded the BBR values by twice. This assumption was supported by considerably higher ratios of soil total C:P and N:P in LUE. Fierer et al. (2009) proposed a significant correlation between rising soil C/N ratios and the fungal to bacterial community composition. Given the high ratio of microbial C/N in LUE, an increasing predominance of fungal biomass can be expected at this forest site. This assumption is underlined by a distinctly (10 fold) higher abundance of fungal sequences detected in the LUE datasets compared to BBR (SILVA SSU database). Inherently the LUE soil promoted occurrence of oligotrophic taxa, due to its relatively low content of P and other nutrients. However soil nutrient availability strongly depends on soil texture. Since LUE predominantly consists of sandy material, the texture itself potentially has an influence on microbial community composition. Thus a significantly higher abundance of Acidobacteria was detected in LUE. In addition microbial community structures are strongly influenced by soil pH (Rousk *et al.,* 2010). Lauber *et al*. (2009) reported a severe domination of Acidobacteria in soils classified as extremely acid (Soil Survey Division Staff, 1993), representing 63% of assigned sequences. By exclusion of further environmental factors shaping microbial communities Rousk *et al.* (2010) confirmed Acidobacteria as the dominating bacterial group in extremely acid soils while an increasing abundance of Proteobacteria was coupled to rising pH (very strongly acid and strongly acid soils). However this was not confirmed for BBR and LUE although both soils are classified as extremely acid (Soil Survey Division Staff, 1993). Since Acidobacteria merely accounted for 20.6% of all assigned sequences in our datasets the exceptionally high abundance of Proteobacteria (44.9%) and Actinobacteria (21.2%) was outstanding. In case of the underlying samples soil pH probably was not the main factor shaping microbial community compositions. Instead it seems that the effect of pH was overruled by the soil phosphorus and nutrient availability or other factors, respectively. A surprisingly high portion of predicted genes was harbored by members of Solibacterales, contributing almost exclusively to inorganic-P mineralization. This hitherto poorly

characterized order comprises merely one single family and genus, respectively. *Candidatus* Solibacter usitatus virtually represents the only cultured and sequenced isolate. In our datasets the latter one was detected as one of the dominating species accounting for 7.9% of assigned sequences. This finding is in accordance with previous work on soil derived databases (Pearce *et al*., 2012). Although metabolic profiling is scarce, genome sequencing revealed the tremendous genetic potential of this species. Different metabolic, defensive and regulatory traits enable growth under unfavorable environmental conditions (Challacombe *et al*., 2011). Ward *et al*. (2009) proposed a considerable participation of Acidobacteria like *Candidatus* Solibacter usitatus in cycling of plant, fungi and insect derived organic matter. Our results further suggest an important contribution of this species to the soil microbial P turnover and the phosphorus availability in soils.

Fungal contribution to the microbial turnover of soil phosphorus

Besides bacteria particularly mycorrhizal fungi are known to be effective in both, mineralization and solubilization of soil phosphorus (Bolan, 1991; Habib *et al*., 2013). However in our datasets solely Ascomycota harbored few alkaline and acid phosphatase genes. As a general rule, DNA extraction method greatly impacts downstream analysis of microbial community composition. Especially soil homogenization is a critical step for the recovery of microbial (particularly fungal) DNA. Duration and intensity of the homogenization step are decisive factors in this respect (Plassart *et al*., 2012). The applied DNA extraction protocol is likely to be unsuitable for recovery of the entire fungal diversity. Moreover O'Brien *et al*. (2005) detected highest fungal richness in forest organic horizons with a consistently decrease in deeper soil layers. Baldrian *et al*. (2012) reported a decline of the fungal to bacterial rDNA copy number ratio from 1.1 (litter layer) to 0.3 (organic horizon) in a spruce forest. Exclusion of rhizosphere material, litter and organic soil layers might explain the low fungal abundance in the present study to some extent. Furthermore accurate annotation of metagenomic datasets

strongly depends on reliable databases. Sufficient coverage and taxonomic diversity of curated organisms are decisive factors. Public available databases generally are biased towards culturable organisms (Nilsson *et al*., 2006; Wooley *et al*., 2010). Since eukaryotic genes furthermore contain intronic regions, longer sequencing reads were required for accurate annotation. As a consequence the fungal contribution to soil P cycling might be underestimated to some extent in our datasets.

Conclusions

In conclusion ecosystem P supply strongly influences soil microbial community structures and nutrient cycling processes. As expected, a significantly higher potential for microbial inorganic phosphorus solubilization was observed in a P-rich soil, while efficient phosphate uptake systems prevailed in a P-poor soil. Surprisingly, a tremendous potential for P cycling processes was observed within poorly characterized orders like Solibacterales, Acidobacterales and Actinomycetales. Taking into account their high abundance in natural and nutrient poor soils, members of these orders might strongly affect the soil microbial P cycle. The underlying study focused on two rather unique and contrasting ecosystems, having either very high or low contents of soil total P. Therefore our results should serve as a starting point, setting the stage for further in-depth characterizations of the P cycling microbial community. Based on our recent findings future work should include soils from different kinds of forest and also non-forest ecosystems to expand our view on this crucial nutrient cycle. Quantification of seasonal and spatial distribution patterns of the active P cycling community can help to unravel microbial hotspots and hot moments of P turnover and uptake.

Experimental Procedures

Site description and soil sampling

386 Soil samples were taken from two beech (*Fagus sylvatica*) dominated German forest soils. Both sites are
387 ICP Level II forests (International Co-operative Programme on Assessment and Monitoring of Air
388 Pollution Effects on Forests) namely Bad Brueckenau (BBR) and Luess (LUE). The stands possess an
389 average age of 120 years and have been intensively monitored since 1995 and 1990, respectively. Both
390 soils have been spared from chemical fertilizer input. The forest site near Bad Brueckenau (BBR) is
391 located in the Bavarian Rhoen Mountains (50°21'7.26" N, 9°55'44.53" E) and reaches up to 850 m above
392 sea level. The mean annual temperature and precipitation are 5.8 °C and 1031 mm, respectively.
393 According to the World Reference Base for Soil Resources (WRB) the soil is classified as Dystric Skeletic
394 Cambisol with Mull and alkaline igneous rock/metamorphite as the substrate. The soil (Ah-horizon) has
395 a pH_{H2O} of 3.84 and consists of sand (8%), silt (55%) and clay (37%). It is characterized by a total carbon
396 content of 174.8 mg g^{-1}, a total nitrogen content of 11.2 mg g^{-1}, a total phosphorus content of 2965.8 mg
397 kg^{-1}, an N:P ratio of 3.76 and a C:P ratio of 58.9.
398 In contrast the forest stand near Unterluess (LUE) has a soil (Ah-horizon) N:P ratio of 19.2, a C:P ratio of
399 492.8, a total carbon content of 96.5 mg g^{-1}, a total nitrogen content of 3.8 mg g^{-1} and a total
400 phosphorus content of 195.8 mg kg^{-1}. The soil has a pH_{H2O} of 3.52 and consists of sand (75%), silt (19%)
401 and clay (6%). According to the WRB it is classified as Hyperdystric Folic Cambisol with Moder and poor
402 pleistocene sands as substrate. The mean annual temperature and precipitation respectively are 8 °C
403 and 730 mm. The forest stand has an elevation of 150 m above sea level and is situated in the Lower
404 Saxon Plain (52°50'21.77" N, 10°16'2.37" E).
405 Soil samples from the Ah-horizon were taken in October 2013 using a soil auger with a diameter of 8 cm
406 up to a depth of 20 cm. At both forest sites three biological replicates, each pooled from five contiguous
407 soil cores, were sampled. Samples were taken in the direct surroundings of the Level II plots. After
408 pooling, aliquots of the three replicates were immediately deep frozen on dry ice for nucleic acid
409 extraction. The remaining soil was stored at 4 °C for further analysis.

410

411 Microbial biomass C, N and P

412 The extraction of soil samples for microbial biomass carbon (Cmic), nitrogen (Nmic) and phosphorus
413 (Pmic) was done as described in Brankatschk *et al*. (2011). Cmic and Nmic were determined using the
414 chloroform fumigation-extraction method after Vance *et al*. (1987), and modified after Joergensen
415 (1996) (k_{EC} 0.45) and Joergensen & Müller (1996) (k_{EN} 0.54). Microbial biomass phosphorus (Pmic) was
416 determined by chloroform fumigation-extraction referring to Brookes *et al*. (1982) (k_{EP} 0.4). To allow a
417 direct comparison of Cmic, Nmic and Pmic from one extract, 0.01 M $CaCl_2$ was used instead of 0.5 M
418 $NaHCO_3$ for the extraction of inorganic P. The concentration of orthophosphate was measured as
419 molybdenum blue using NANOCOLOR tube tests "NANOCOLOR ortho- and total-Phosphate 1"
420 (Macherey-Nagel, Germany).

421

422 Nucleic acid isolation

423 Total nucleic acids were co-extracted from frozen soil samples as described by Töwe *et al*. (2011). To
424 enhance the DNA yield two aliquots (0.5 g) of each sample were homogenized separately, using
425 Precellys 24 (Bertin Technologies, France) and Lysing Matrix E tubes (MP Biomedicals, France). Extracted
426 DNA was photometrically quantified (Nanodrop ND-1000; Thermo Fischer Scientific, USA) and stored at
427 -20 °C.

428

429 Pyrosequencing

430 Total genomic DNA of six soil samples was sequenced. Pyrosequencing was performed on a Genome
431 Sequencer FLX+ instrument (454 Life Sciences, Roche, USA). Library preparation was accomplished
432 according to the Roche protocol "Rapid Library Preparation Method Manual" using Roche MID Adaptors.
433 As different sequencing depths were applied, libraries of replicates were pooled in a 2:1:1 ratio.

Subsequent emulsion PCR was carried out as described in the manual "emPCR Amplification Method Manual – Lib-L LV". The GS FLX Titanium Kit XL+ was used for sequencing. Image- and signal-processing was accomplished by the software "GS Run Processor v2.9". Sequences are stored in SRA under the accession number: PRJNA288276.

Analysis of sequencing data

Roche SFF files were separated based on the applied MID Adaptors. Sequencing reads were trimmed using a modified Dynamic Trim (Cox *et al*., 2010) as supplied by MG-Rast (Meyer *et al*., 2008). The following parameters were applied: h=15, n=5 and I=50. Remaining Adaptor sequences and duplicated sequences were removed using Biopieces (www.biopieces.org) and cd-hit (Fu *et al*., 2012). For taxonomic annotation filtered sequencing reads were blasted against the SILVA SSU-database (version 108) (Pruesse *et al*., 2007) using blastn with an expect value of 10^{-4} (BLAST+ suite version 2.2.27+) (Camacho *et al*., 2009). Additionally sequences were aligned against the NCBI Non-redundant protein sequences (nr) database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz; October 2014) using DIAMOND with default parameters (version 0.5.2) Buchfink *et al*., 2015). For functional annotation filtered sequencing reads were aligned against the KEGG database (Kyoto Encyclopedia of Genes and Genomes) (June 2011) (Kanehisa & Goto, 2000) using DIAMOND with default settings. Taxonomic and functional assignment was performed using MEGAN (version 5.6.5) (Huson *et al*., 2011) and current mapping files (October 2014). The following parameters were applied: Min Score: 50, Max Expected: 10^{-5}, Top Percent: 10, Min Support Percent: 0.0, Min Support: 1, LCA Percent: 100, Min Complexity: 0.0. See Supporting Information Table S4 for all enzymes associated with the soil microbial P turnover that were investigated in this study. Corresponding KEGG orthology (KO) numbers were searched within the functionally annotated datasets. Intracellular phosphatases involved in metabolic processes (e.g. Glucose-6-phosphatase) were omitted from the analysis since they are not part of the soil P turnover. To

further confirm KEGG database results with a second approach, open-reading frames were predicted based on filtered sequencing reads using FragGeneScan (version 1.18) (Rho *et al*., 2010) and subsequently scanned for Profile Hidden Markov Models (HMM) of investigated proteins (Supporting Information Table S4) using hmmscan (HMMER 3.0) (www.hmmer.org). See Supporting Information Experimental Procedures for detailed information.

Sequences of predicted genes, as obtained from the KEGG database, were phylogenetically assigned using DIAMOND against the NCBI Non-redundant protein sequences (nr) database and MEGAN (parameters as previously described). Sequencing data was visualized using the R software package (R Core Team, 2015).

Statistical analysis of sequencing data

Statistical analysis of sequencing data was performed on subsampled metagenomic datasets. Subsampling using Biopieces (www.biopieces.org) corresponded to the lowest quantity of filtered sequences achieved in one of the datasets (133,179 reads). Significant differences between the metagenomes of two different forest soils were ascertained by unpaired t-test statistics. *P*-values were adjusted using Bonferroni correction (R Core Team, 2015). Differences were counted as significant if the adjusted *P*-value was below 5% ($P<0.05$). To be more stringent only taxa, with an abundance of at least 0.05% of all assigned reads in one of the datasets, were included in the analysis.

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683 **Figure 1** Relative abundance of microbial phyla (a) and orders (b) in metagenomic datasets of two forest
684 soils. Sequences were assigned using DIAMOND against the NCBI Non-redundant protein sequences (nr)
685 database and MEGAN. Shown are the 20 most abundant taxa. Significant differences in the amount of
686 annotated reads among both sites are shown (n=3).
687 *P<0.05
688
689 **Figure 2** Relative abundance of microbial genes coding for enzymes involved in soil phosphorus
690 mineralization and solubilization as well as P uptake and P starvation response regulation. Metagenomic
691 datasets of two forest soils were aligned against the KEGG database using DIAMOND. Significant
692 differences in the amount of annotated reads among both soils are shown (n=3).
693 *P<0.05
694
695 **Figure 3** Taxonomic assignment of microbial genes coding for enzymes involved in the turnover of soil P.
696 Metagenomic datasets of two forest soils were assigned on functional level using DIAMOND against the
697 KEGG database. Sequences coding for microbial phosphate uptake systems (pooled subunits) (a) and
698 enzymes performing mineralization and solubilization of soil P (b) were taxonomically assigned
699 (DIAMOND against NCBI Non-redundant protein sequences (nr) database). Shown are absolute numbers
700 of assigned sequences; (*Glycerophosphoryl Phosphodiesterase).
701

Table 1 Microbial biomass carbon, nitrogen and phosphorus (including corresponding ratios) of two different forest soils. Shown are means and standard deviations (SD) of three biological replicates (n=3).

	Bad Brueckenau		Luess	
Microbial Biomass	Mean	SD	Mean	SD
Cmic (µg C g^{-1})	1203.49	447.26	144.96	92.06
Nmic (µg N g^{-1})	79.46	23.05	11.42	8.28
Pmic (µg P g^{-1})	104.75	35.05	9.85	4.10
Cmic:Nmic	14.90	2.56	33.42	46.84
Cmic:Pmic	11.36	0.67	19.14	18.67
Nmic:Pmic	0.77	0.09	1.02	0.50

Figure 1

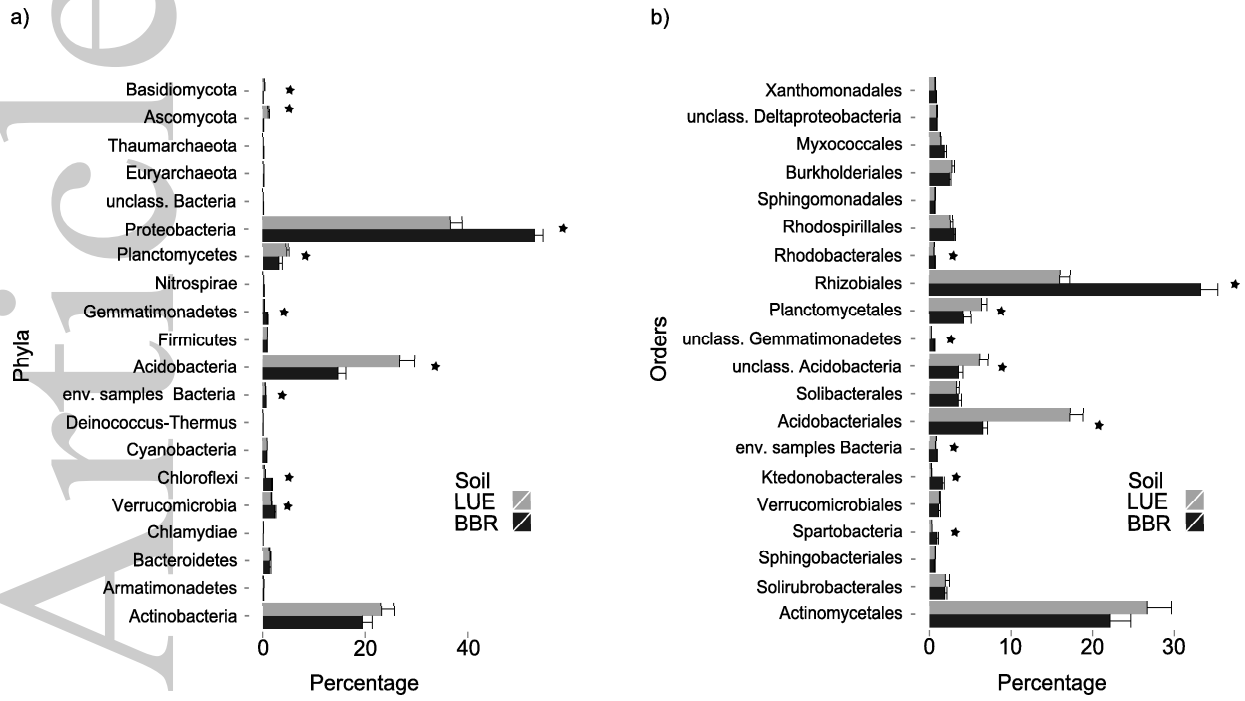
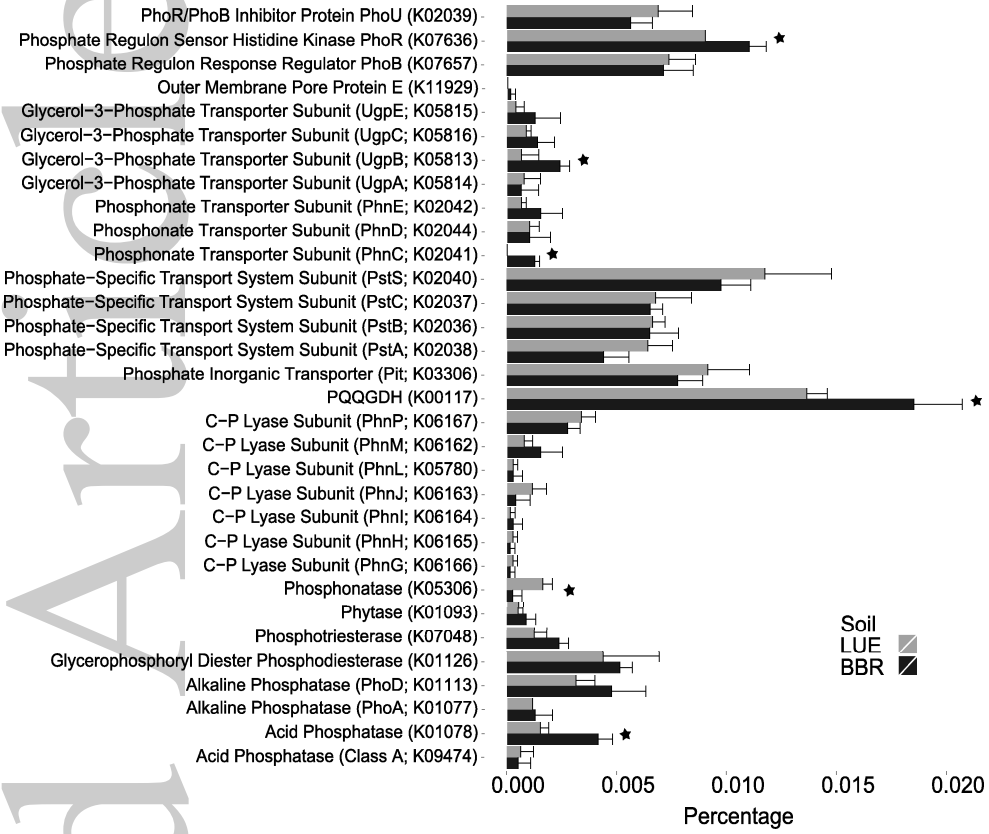
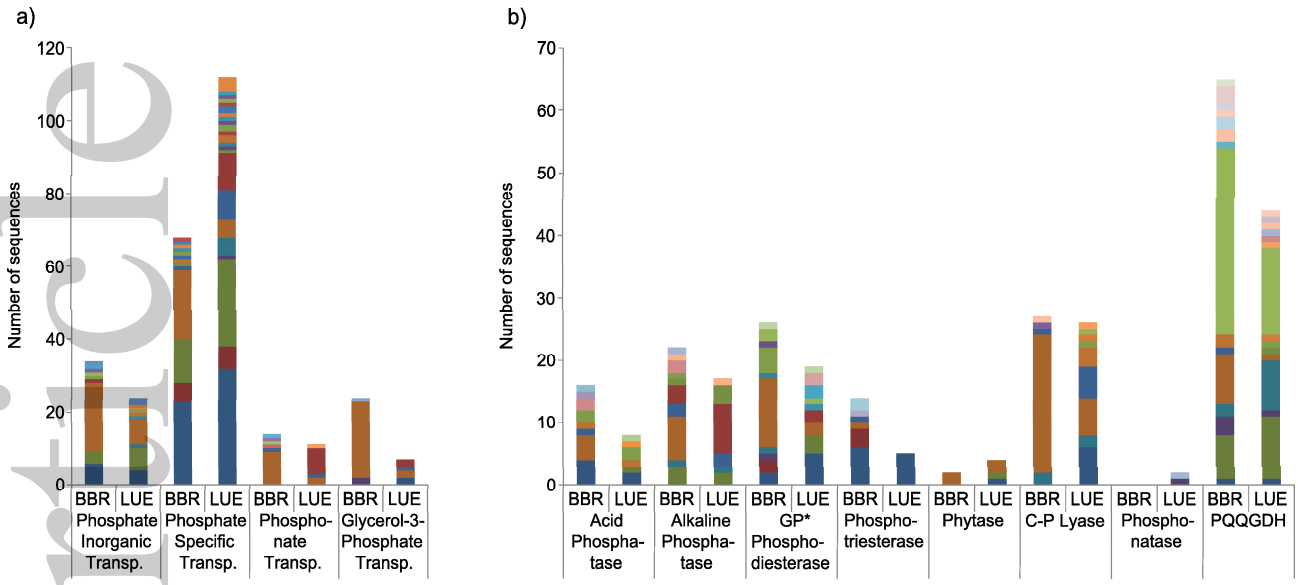


Figure 2





- Actinomycetales
- Planctomycetales
- Xanthomonadales
- Ktedonobacterales
- Clostridiales
- Chromatiales
- Firmicutes
- Myxococcales
- unclass. Chloroflexi
- Actinobacteria
- Hypocreales
- Xanthomonadales
- Betaproteobacteria
- unclass. Gemmatimonadetes
- unclass. Thaumarchaeota
- Solirubrobacterales
- Rhizobiales
- Acidimicrobiales
- Chrysiogenales
- Rhodobacterales
- Methanomicrobiales
- unclass. Alphaproteobacteria
- Legionellales
- Alteromonadales
- Spartobacteria
- leotiomyceta
- Flavobacteriales
- unclass. Deltaproteobacteria
- Oceanospirillales
- Acidobacteriales
- Rhodospirillales
- Bacteroidetes
- unclass. Acidobacteria
- Desulfobacterales
- Nitrososphaerales
- Solibacterales
- Pseudomonadales
- unclass. Gammaproteobacteria
- Neisseriales
- Caulobacterales
- env. samples Archaea
- Gammaproteobacteria
- unclass. Gammaproteobacteria
- Bacillales
- Burkholderiales
- Verrucomicrobiales
- Lactobacillales
- Desulfuromonadales
- Sphingobacteriales
- Gemmatimonadetes (class)
- unclass. Betaproteobacteria
- Cytophagales
- Piroplasmida
- Myxococcales
- Sphingomonadales
- env. samples Acidobacteria
- Poribacteria