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Bacterial potentials for uptake, solubilization and mineralization of extracellular phosphorus in agricultural soils are highly stable under different fertilization regimes

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Summary

Phosphorous is one of the most important macronutrients for plants. In agriculture, amending fertilizer with phosphorus (P) is common practice. However, natural phosphorus sources are finite, making research for more sustainable management practices necessary. We postulated that the addition of carbon (C) and nitrogen (N) would stimulate phosphorus mobilization by bacteria because of their desire to maintain a stable intracellular C:N:P stoichiometry. Therefore, we chose a metagenomic approach to investigate two agricultural soils, which only received mineral N fertilizer or mineral N and organic fertilizer for more than 20 years. The most abundant genes involved in the acquisition of external P sources in our study were those involved in solubilization and subsequent uptake of inorganic phosphorus. Independent of site and season, the relative abundance of genes involved in P turnover was not significantly affected by the addition of fertilizers. However, the type of fertilization had a significant impact on the diversity pattern of bacterial families harbouring genes coding for the different P transformation processes. This gives rise to the possibility that fertilizers can substantially change phosphorus turnover efficiency by favouring different families. Additionally, none of the families involved in phosphorus turnover covered all investigated processes. Therefore, promoting bacteria which play an essential role specifically in mobilization of hardly accessible phosphorus could help to secure the phosphorus supply of plants in soils with low P input.

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Introduction

The availability of macronutrients in soil, especially nitrogen (N) and phosphorus (P), strongly influences crop yield and quality (Schachtman *et al.*, 1998; Marschner, 2011). Unlike N, P is a finite resource and P-fertilizers mainly originate from rock sources (Walker and Syers, 1976; Chadwick *et al.*, 1999). However, due to agricultural practices P has accumulated in soil (Stutter *et al.*, 2015), but mostly in inaccessible forms. Thus, its remobilization and future sustainable use is of major importance. In this regard bacteria play an important role. On the one hand, they are able to catalyze the release of P through solubilization of inorganic- and mineralization of organic- P, increasing the concentration of free orthophosphate in soil (Richardson and Simpson, 2011). On the other hand, bacteria possess efficient P uptake systems, like the high-affinity phosphate-specific transporter Pst and the low-affinity phosphate inorganic transporter Pit. These allow them to efficiently compete for available P sources (Willsky *et al.*, 1973; Wanner, 1993).

Recent publications (Mooshammer *et al.*, 2012; Spohn and Kuzyakov, 2013; Heuck *et al.*, 2015) demonstrated the importance of the C:N:P stoichiometry for many processes in soil. For example, Heuck *et al.* (2015) found that the release of P is rather a result of the bacterial need for C and N rather than the immediate need of P. We proposed, that the addition of mineral N will increase the bacterial demands for P and thus stimulate

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P mineralization from soil if the amount of easily degradable organic carbon is not limiting microbial activities.

In order to test this hypothesis, we compared the effects of mineral N fertilization (N_{min}), and mineral N fertilization combined with organic fertilization (N_{min}-organic) in two long-term field trials, located in Freising, Bavaria and Rostock, Mecklenburg Western-Pommerania, Germany. Here, none of the plots received any mineral P fertilizer. The samples were taken in spring and autumn to analyze how the presence and absence of crops influences P turnover. We used a metagenomics approach specifically targeting genes involved in P transformation as described for forest soils by Bergkemper *et al.* (2016) to (i) assess the relative abundance of genes catalyzing key processes in P turnover and (ii) to identify major bacterial families carrying the genes of interest. We focused on the bacterial potential to take up, solubilize and mineralize extracellular P and compared this to the actual incorporation of C, N and P in the microbial biomass.

Results and discussion

Microbial C, N and P pools

As several authors proposed stable C_{mic}:N_{mic}:P_{mic} ratios in the soil microbial biomass in the past (e.g., Cleveland and Liptzin, 2007), we investigated the role of different fertilizers as modulators of the stoichiometry of C:N:P on the microbiome (Table 1). Our data indicates both a high stability of C_{mic}, N_{mic} and P_{mic} even between treatments, where different fertilizers had been applied, and, in line with this observation, almost constant C_{mic}:N_{mic}:P_{mic} ratios in the microbial biomass. The mean ratios across all treatments were 36:1.2:1 for Rostock and 25:1.2:1 for Freising. Although the observed ratios were constant, they were lower as compared to the global values (60:7:1) described by Cleveland and Liptzin (2007). However, Cleveland and Liptzin (2007) only considered unfertilized forest and grassland sites, whilst here we investigated agricultural sites. Thus, a narrow C_{mic}:N_{mic}:P_{mic} ratio is not surprising. The same fertilization effect was observed by Heuck *et al.* (2015), where especially N_{mic}:P_{mic} were in the same range after fertilizer addition irrespective whether P or N fertilizers were added. In the frame of their study they could prove that this was due to a change in microbial community structure. Furthermore, in our study the microbial C_{mic}:N_{mic}:P_{mic} ratio nicely reflects the total C:N:P ratio of the soils, which is 20:2:1. This underlines the adaptation period of the microbial communities as a consequence of the long term experimental settings.

Functional profiles of microbes triggering P transformation

P-turnover was analyzed by comparing the relative abundances of 40 bacterial genes encoding proteins which are linked to regulation, mineralization, solubilization and uptake of extracellular P sources; genes that are involved in intracellular P turnover were excluded from further analysis (Supporting Information Table S1; Bergkemper *et al.*, 2016). Therefore, the filtered reads obtained by metagenomics sequencing were mapped against sequences of the KEGG database [using BLAST (Altschul *et al.*, 1990)] for gene annotation (for details see Supporting Information materials and methods). After subsequent filtering, we obtained 52,654,918 reads with an average read length of 296 bp after trimming. Of these, genes linked to P turnover made up

Table 1. Mean values of microbial biomass of two agricultural soils located in Freising and Rostock in 2015. Samples were taken with a soil auger of 3 cm diameter to a depth of 10 cm. Three cores were collected for each plot, pooled and subsequently homogenized on a 5mm sieve. For the assessment of C_{mic} and N_{mic} the chloroform- fumigation-extraction method was used. Average values and standard deviations (±) are calculated based on triplicates ($n = 3$). Underlined are parameters which were significantly influenced by season (single line), fertilizer (double lines) or interaction of both (dotted lines).

Season	Spring		Autumn	
Treatment	N_{min}	N_{min}-organic	N_{min}	N_{min}-organic
Freising				
C_{mic} ($\mu g\ g^{-1}$)	409.6 ± 27.3	371.9 ± 21.8	439.3 ± 16.2	433.9 ± 36.1
N_{mic} ($\mu g\ g^{-1}$)	22.1 ± 2.8	18.7 ± 1.7	16.9 ± 12.5	8.7 ± 2.8
P_{mic} ($\mu g\ g^{-1}$)	15.1 ± 12.1	11.8 ± 4.3	27.5 ± 6.9	145.4 ± 79.4
Rostock				
C_{mic}:P_{mic}	43.3 ± 33.9	34.1 ± 11.5	16.5 ± 3.5	4.0 ± 2.8
N_{mic}:P_{mic}	2.3 ± 1.7	1.7 ± 0.5	0.6 ± 0.3	0.1 ± 0.03
C_{mic} ($\mu g\ g^{-1}$)	304.2 ± 34.8	331.5 ± 37.5	304.1 ± 34.8	322.3 ± 23.2
N_{mic} ($\mu g\ g^{-1}$)	10.1 ± 2.1	14.9 ± 3.3	7.7 ± 1.7	13.8 ± 4.2
P_{mic} ($\mu g\ g^{-1}$)	10.7 ± 9.7	25.7 ± 4.8	7.4 ± 5.8	17.6 ± 16.9
C_{mic}:P_{mic}	20.7 ± 4.9[a]	13.4 ± 3.5	73.7 ± 67.5	37.8 ± 35.3
N_{mic}:P_{mic}	0.7 ± 0.1[a]	0.6 ± 0.2	2.0 ± 2.2	1.4 ± 1.1

[a]The mean of the C:P and N:P ratios is based on two field replicates only, as the P_{mic} value was below the detection limit for the third plot.

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approximately 0.34% at both sites. Rarefaction analysis (Supporting Information Fig. S2) revealed sufficient coverage in all samples. For further analysis, 27 genes were processed. The analysis of the relative abundances of the targeted genes indicated a strong similarity of the two sites in Rostock and Freising. Further the data indicated, that neither season nor the fertilization treatment had a significant effect on the relative abundance of the analyzed genes.

Bergkemper *et al.* (2016) proposed that solubilization processes are the main drivers for microbial P turnover in soils which are rich in mineral P. Therefore, not surprisingly, the most abundant gene of the 27 genes analyzed in this study was encoding for a glucose dehydrogenase (*gcd*). Gcd is a membrane bound enzyme which can oxidize aldose sugars (i.e., glucose to gluconic acid). Gluconic acid further solubilize inorganically bound fractions of P. Here a co-factor, namely pyrroloquinone (PQQ), is required to form the active enzyme. Together both genes form the PQQGDH holoenzyme (Goldstein, 1995). In our study, genes linked to the PQQ cofactor were lower in abundance than the genes coding for the glucose dehydrogenase. This suggests at least a partly PQQ independent activation of the GDH enzyme, which has also been described by Goldstein *et al.* (2003). However, it is important to mention that also other organic acids might be important for the solubilization of inorganic P such as malic-, lactic-, citric- and oxalic acids, being the most prominent (Sharma *et al.*, 2013). The aforementioned organic acids are also part of intracellular metabolic processes such as the tricarboxylic acid (TCA) cycle and fermentation, making the distinction between intracellular and extracellular usage impossible on the gene level.

In agreement with the high abundance of genes encoding for enzymes involved in catalyzing P solubilization, those encoding for proteins involved in the uptake of inorganic P (Fig. 1), such as the multimeric ABC-type phosphorus specific transporter (*pst*) and the inorganic phosphorus transporter (*pit*), were the second most abundant genes in our datasets. Of the two, Pit is a constitutively expressed low affinity transporter, which is able to transport metallic cations complexed with phosphate (Willsky *et al.*, 1973; Wanner, 1993). The Pst transporter is induced under P starvation through tight regulation and is additionally involved in regulation and P-signaling. It has been reported to transport inorganic P and likely phosphonates (Yuan *et al.*, 2006). Genes involved in the mineralization of organic P sources were also found in our datasets; however, they were far less

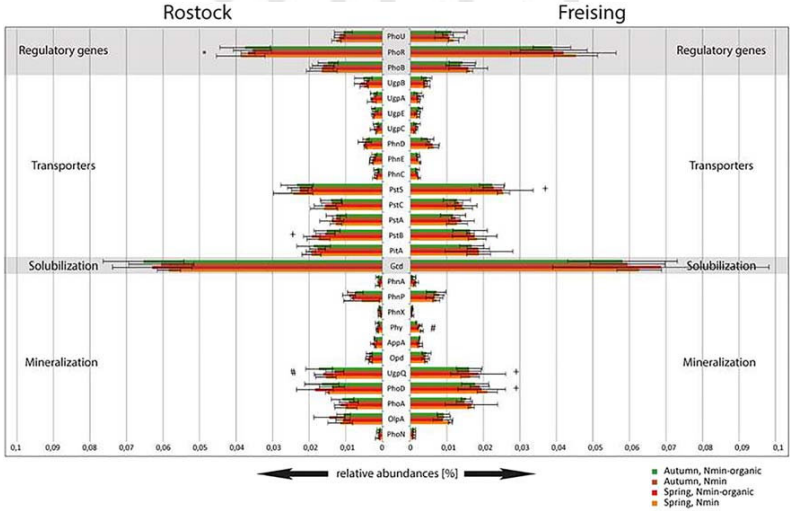

Fig. 1. Barplot showing relative abundances of genes for proteins, which are involved in P turnover in Freising and Rostock. Metagenomic data presented is of both test sites, over the change of the season, as well as two treatments. The quality filtered reads were blasted against the KEGG database and assigned with a K0 number by means of MEGAN. Significant differences in the number of annotated reads among both were calculated by two-way ANOVA ($n = 3$, $P < 0.05$). Error bars represent standard deviations. Significant influence is symbolized for season (+), organic fertilizer (#) or interaction (*) respectively.

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abundant. Their low abundance could be surprising, considering that organic fertilizers contain large amounts of organically bound P.

It has to be taken into account that the addition of organic fertilizer had been performed two years before sampling. However, our focus was on the long-term effects of a triennial application of organic matter rather than short term fertilizer effects. The low abundances of genes encoding for mineralizing enzymes could therefore be the result of the depletion of the organically bound P in the organic fertilizer over time. Interestingly the amount SOM did not differ between the different treatments at both sides. However, most likely the quality of SOM is affected by the different fertilizer quality. Differences in SOM quality might be the reason for shifts in the major bacterial families triggering the investigated processes related to P transformation. Constant SOM contents in contrast might explain that no differences in the overall abundance of genes coding for a particular transformation step were found.

The third most abundant genes in our dataset were the alkaline phosphatase genes (ALP) *phoA*, *phoD* and a class C acid phosphatase *olpA*. The OlpA acid phosphatase belongs to a group classified as nonspecific acid phosphohydrolases (NSAPs). These enzymes hydrolyze a broad array of structurally unrelated substrates. They are known to exhibit optimal catalytic activity at acidic to neutral pH values (Rossolini *et al.*, 1998). Nannipieri *et al.* (2011) described ALPs as being typical for neutral to alkaline environments, which is in agreement with our findings considering the pH of the soil at the two sites.

In contrast, the abundance of enzymes that specifically target substrates, which are more difficult to access or lower in concentration, such as phosphonate or phytate respectively, were rare in our study. This underlines the fact that P pools in arable soils are dominated by inorganic orthophosphate (Stutter *et al.*, 2015) despite the organic fertilization two years before the sampling for this study. The majority of the aforementioned genes are tightly regulated by means of a two-component system, namely *phoR*, *phoB*, *phoU*. These are involved of several phosphate starvation inducible genes (PSI) of the phosphate (Pho) regulon, specifically detecting P starvation (Hsieh and Wanner, 2010). The relatively high abundance of P signaling genes in our datasets emphasized the importance of effective PSI gene regulation for microbial communities, to efficiently use alternative phosphorus sources in times of P starvation. Taken into account the high conservation of gene abundances across seasons and sites, which was also confirmed by by two-factorial ANOVA, a regulation on transcription level seems to be the mode of action rather than a shift in community composition.

This is consistent with the theory about functional redundancy and underlines the importance of these genes in agricultural ecosystems (Allison and Martiny, 2008). These findings are based on the relative abundances of genes. However, at our sites microbial biomass (as indicated by C_{mic} values) was not significantly affected by any of the analyzed factors. Therefore, absolute abundance pattern between the sites might not be differing compared to the relative abundance values described in this study.

Structure of bacterial communities triggering P transformation

Both sites showed a strong similarity in the functional profiles of P turnover. These findings are in line with the findings described by Allison and Martiny (2008). They argued that important ecosystem functions are preserved even if the microbial community structure changes. For this reason, we compared microbial communities involved in P transformation at both sites and linked our data to total bacterial community structure, which was comparable between both sites at the family level as indicated by NMDS analysis (Supporting Information Fig. S1A). Therefore the extracted sequences of genes involved in P turnover were blasted against the NCBI-nr database (http://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (June 2011) (Kanehisa and Goto, 2000) using DIAMOND (0.5.2.32) on sensitive settings (Buchfink *et al.*, 2015). Subsequently taxonomic and functional assignment was performed by using MEGAN5 v 5.10.6 (Huson *et al.*, 2011). For further details, we refer to Supporting Information materials and methods.

Overall, 295 bacterial families were identified, of which 292 families were present at both sites. The most abundant families were Acidobacteriaceae, Bradyrhizobiaceae, Verrucomicrobia subd. 3, Planctomycetaceae and Chitinophagaceae. This is not surprising because they are known to be widely spread throughout terrestrial ecosystems (Rosenberg, 2014). The effect of season was much stronger than that of the fertilizer addition as indicated by means of a two-way blocked ANOVA. Of the 295 families, 36 were influenced by season, 14 by fertilizer and 18 by the interaction of both (Supporting Information Table S2).

Of the 295 total families, 258 harboured genes encoding for proteins involved in extracellular P turnover. Of these, 85 families comprised a core microbiome (as defined by Shade and Handelsman, 2012), which was present at both sites, independent from seasons and treatments. 91% of all filtered reads could be assigned to these families (Supporting Information Fig. S2). Interestingly, NMDS analysis on the distribution of genes

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involved in P turnover amongst bacterial families (Supporting Information Fig. S1B) indicated a stronger influence of the fertilizer addition as compared to the total bacterial community structure (Supporting Information Fig. S1A). Moreover, the effect of season was strongly connected to the fertilizer effect. Subsequent ANOVA analysis revealed that 7 of the families involved in P turnover were influenced by season, 38 by fertilizer and 37 by the interaction of season and fertilizer (Supporting Information Table S3).

To summarize, the similarity of total bacterial communities at both sites concurs with observations that the diversity and richness of bacterial communities are shaped primarily by ecosystem type rather than location (Fierer and Jackson, 2006; Cao *et al.*, 2016). According to Fierer and Jackson (2006) and Fierer (2017), the major determining factor for the community structure is pH, which was similar at both sites. In contrast, fertilizer application has a minor role in shaping the total soil microbiome in the above mentioned study. In our experimental setting fertilizer had a strong influence on how the genes are distributed amongst the families. This again, coincides with the theory of functional redundancy as suggested by Allison and Martiny (2008). However, metagenomics only gives information on microbial potential, and not their actual activity. The fact that genes necessary for P turnover were harboured by different families gives rise to the possibility that these could perform the same function with a different efficiency. Indeed, it has been shown by Ragot *et al.* (2016) that for some taxa there are significant differences between the presence and expression of genes involved in P turnover. Therefore, the observed influence of fertilizer on the gene distribution pattern in different bacterial families could cause substantial changes in activity pattern. This hypothesis could be strengthened by the high abundance of regulatory genes that could influence the efficiency of P turnover.

Our analysis revealed that none of the families covered all processes. Therefore, we pooled genes involved in P turnover based on their functions into regulation (3 genes), mineralization (23 genes), solubilization (1 gene), organic P uptake (7 genes) and inorganic P uptake (6 genes), and assigned their taxonomic affiliation for a more detailed analysis of the distribution of functional traits (Fig. 2). In fact, certain families exhibited a more 'universal' spectrum of functions, while others were considered as specialists for single processes. The 'universal' group included Verrucomicrobia subdivision 3, Sphingomonadaceae, Anaerolinaceae, Planctomycetaceae, Chitinophagaceae, Acidobacteriaceae and Bradyrhizobiaceae harboring genes coding for enzymes of four of the five investigated processes.

The 'specific' group included families which harboured genes only for one of the investigated process. Some of them appeared to be involved only in organic and inorganic P uptake, pursuing a rather copiotrophic lifestyle by favouring the use of easily available nutrients instead of investing energy into P solubilization, mineralization or regulation. These entail Rhodocyclaceae, Chlorobiaceae,

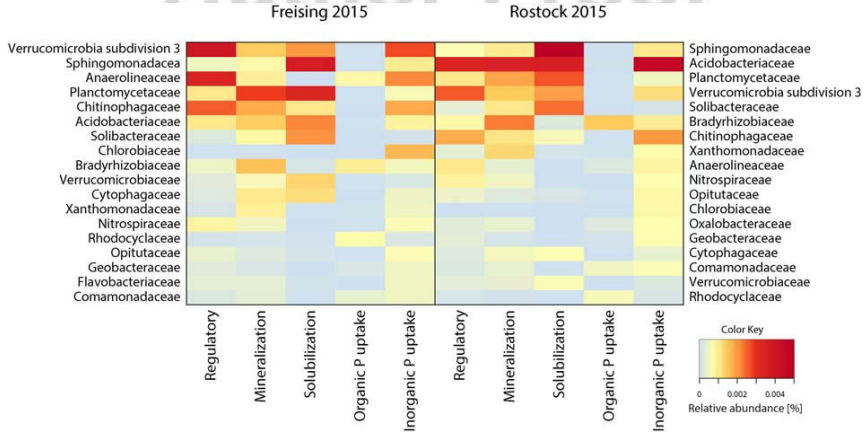

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Fig. 2. Visualization of mean relative abundances of genes harboured by different families in Freising and Rostock independent of season and fertilizer, by means of a heatmap. Genes involved in P turnover were selected by their K0 numbers and grouped depending on their function. These were then assigned to taxa by means of MEGAN.

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Geobacteraceae, Flavobacteriaceae and Opitutaceae. Others, such as Verrucomicrobiaceae and Solibacteraceae, exhibited preferences either for mineralization or solubilization of P. Intriguingly, we did not detect genes necessary for P uptake from organic or inorganic sources in these families. Thus it is possible that these genes were present only in a very few taxa and relative abundance was too low to be detected in the analyzed metagenomes. A further explanation could be the existence of so far unknown alternative uptake mechanisms, which makes the detection in the metagenomes impossible, especially because a slightly divergent organic phosphate transporter might show strong similarities to sulfate transporters, or even carboxylate transporters, depending on the organic backbone. Nevertheless, it is possible that these microbes release P as a byproduct during C turnover, which coincides with the theory proposed by Heuck *et al.* (2015).

In conclusion, although no mineral P fertilizer was added to the investigated fields, C_{mic}:N_{mic}:P_{mic} ratios were narrow and the most abundant gene triggering P transformation was affiliated to the solubilization of inorganic P, both indicate that sufficient amounts of P are stored in the analyzed soils and that the addition of mineral N fertilizer and organic fertilizer stimulated P acquisition. While gene abundance patterns were not affected by season, fertilization or site, the bacterial families involved in the different processes differed. The consequences might be that (i) shifts in microbial community structure strongly influence the potential to use extracellular P sources and (ii) the regulation of P acquisition rather takes place on the expression level. Accordingly, future work should address the conditions under which the different P acquiring strategies are prevalent and under which conditions the genetic potential for P transformation is activated by implementing metatranscriptomic analysis.

Overall our study focused on bacteria and their role in P transformation processes in soil, although it is well accepted that fungi also play an important role in plant P nutrition, including the transport of P by mycorrhizal fungi. However most of the public data bases so far lack of sequences belonging to fungi. Thus most reads linked to fungal sequences are considered as unknowns and excluded from further analysis. Therefore current metagenomics approach do not allow a sound analysis of functional traits of fungi, which hopefully changes when the data from the analysis of 1000 genomes has been finalized (https://genome.jgi.doe.gov/programs/fungi/1000fungalgenomes.jsf).

Experimental procedures

Samples were taken from two long term field trials in Germany. The first site is located in Freising, Bavaria (48°24′13.38″ N, 11°41′32.93″ E), having a split plot design where two treatments were analyzed in the frame of this study: (i) N fertilization (2015) 150 kg ha^{-1}, no additional organic fertilization (N_{min}); (ii) N fertilization (2015) 150 kg ha^{-1}, organic fertilization with straw and catch crops as green manure (N_{min}-organic). The second site is located in Rostock, Mecklenburg Western-Pomerania (54°03′42.44″ N, 12°05′07.32″ E) and has a split plot design with four replicates, where two treatments were analyzed in the frame of this study: (i) N fertilization 120 kg ha^{-1}, no additional organic fertilization (N_{min}); (ii) N fertilization 120 kg ha^{-1}, organic fertilization with biowaste compost (30 t ha^{-1}) (N_{min}-organic).

Samples were taken from both sites in April and September of 2015 using a soil auger of 3 cm diameter to a depth of 10 cm. As suggested by Vestergaard *et al.* (2017), three cores were collected for each plot, pooled and subsequently homogenized on a 5 mm sieve. A total of twenty-four (24) samples were taken (6 per site, in three replicates, per season). Despite the different fertilization regimes, no significant differences in SOM contents were found at the time of sampling and only differences between the two different sites were found. For Freising, TOC ranged between 11.61 and 12.30 g kg^{-1}, TN between 1.36 and 1.42 g kg^{-1} and TP between 0.61 und 0.69 g kg^{-1}; for Rostock overall values were lower (TOC 7.15 – 10.40 g kg^{-1}, TN 0.82 – 1.05 g kg^{-1}; TP 0.54 – 0.62 g kg^{-1})

For the assessment of C_{mic} and N_{mic}, the chloroform-fumigation-extraction method as described by Vance *et al.* (1987) was used. Analysis of microbial P (P_{mic}) was performed also using the chloroform-fumigation-extraction method but as described by Brookes *et al.* (1982). For nucleic acid extraction and subsequent sequencing, a phenol-chloroform based protocol modified according to Lueders *et al.* (2004) and Töwe *et al.* (2011) was used to extract total genomic DNA, which was then prepared for sequencing using NEBNextR Ultra™ DNA Library Prep Kit (New England Biolabs®, Inc.). Sequencing was performed on an Illumina® Miseq® (Illumina®, USA) sequencing machine and using the MiSeq® Reagent Kit v3 (600 cycles) (Illumina®) for paired end sequencing. The raw sequencing data is available at the sequencing read archive (SRA) under the BioProject ID PRJNA385596 (SAMN06894543-SAMN06894566). Taxonomic and functional annotations of the sequences were accomplished by blasting against the NCBI-nr database (http://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (June 2011) (Kanehisa and Goto, 2000) using DIAMOND (0.5.2.32). Taxonomic and functional assignment was performed by using MEGAN5 (5.10.6). Statistical analyses and visualization was performed using Rstudio [Rstudio Team (2015)],

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based upon the relative abundance of reads. A two-way blocked analysis of variance (ANOVA) was used to analyze the effects of season, fertilizer and interaction of both, on taxonomic as well as the functional profiles of the samples. For more detailed descriptions, please refer to the Supporting Information (Text S1: Materials and Methods).

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References

Allison, S.D, and Martiny, J.B. (2008) Resistance, resilience, and redundancy in microbial communities. *Proc Natl Acad Sci U S A* **105**: 11512–11519.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W, and Lipman, D.J. (1990) Basic Local Alignment Search Tool. *J Mol Biol* **215**: 403–410.

Bergkemper, F., Schöler, A., Engel, M., Lang, F., Krüger, J., Schloter, M, and Schulz, S. (2016) Phosphorus depletion in forest soils shapes bacterial communities towards phosphorus recycling systems. *Environ Microbiol.*

Brookes, P., Powlson, D, and Jenkinson, D. (1982) Measurement of microbial biomass phosphorus in soil. *Soil Biol Biochem* **14**: 319–329.

Buchfink, B., Xie, C, and Huson, D.H. (2015) Fast and sensitive protein alignment using DIAMOND. *Nat Methods* **12**: 59–60.

Bünemann, E.K., Prusisz, B., Ehlers, K. (2011) Characterization of phosphorus forms in soil microorganisms. In *Phosphorus in Action. Soil Biology.* Bünemann, E., Oberson, A., Frossard, E. (eds), Berlin, Heidelberg: Springer, pp. 37–57.

Cao, H., Chen, R., Wang, L., Jiang, L., Yang, F., Zheng, S. *et al.* (2016) Soil pH, total phosphorus, climate and distance are the major factors influencing microbial activity at a regional spatial scale. *Sci Rep-UK* **6**: 25815.

Chadwick, O.A., Derry, L.A., Vitousek, P.M., Huebert, B.J, and Hedin, L.O. (1999) Changing sources of nutrients during four million years of ecosystem development. *Nature* **397**: 491–497.

Cleveland, C.C, and Liptzin, D. (2007) C: N: P stoichiometry in soil: is there a "Redfield ratio" for the microbial biomass? *Biogeochemistry* **85**: 235–252.

Cordell, D., Drangert, J.-O, and White, S. (2009) The story of phosphorus: Global food security and food for thought. *Global. Environ Chang* **19**: 292–305.

de Vries, M., Schöler, A., Ertl, J., Xu, Z, and Schloter, M. (2015) Metagenomic analyses reveal no differences in genes involved in cellulose degradation under different tillage treatments. *FEMS Microbiol Ecol* **91**: fiv069.

Fierer, N. (2017) Embracing the unknown: disentangling the complexities of the soil microbiome. *Nat Rev Microbiol* **15**: 579–590.

Fierer, N, and Jackson, R.B. (2006) The diversity and biogeography of soil bacterial communities. *Proc Natl Acad Sci U S A* **103**: 626–631.

Goldstein, A., Lester, T, and Brown, J. (2003) Research on the metabolic engineering of the direct oxidation pathway for extraction of phosphate from ore has generated preliminary evidence for PQQ biosynthesis in Escherichia coli as well as a possible role for the highly conserved region of quinoprotein dehydrogenases. *BBA-Proteins Proteom* **1647**: 266–271.

Goldstein, A.H. (1995) Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by gram negative bacteria. *Biol Agric Hortic* **12**: 185–193.

Heuck, C., Weig, A, and Spohn, M. (2015) Soil microbial biomass C: N: P stoichiometry and microbial use of organic phosphorus. *Soil. Biol Biochem* **85**: 119–129.

Holford, I.C.R. (1997) Soil phosphorus: its measurement, and its uptake by plants. *Soil Res* **35**: 227–240.

Hsieh, Y.-J, and Wanner, B.L. (2010) Global regulation by the seven-component P i signaling system. *Curr Opin Microbiol* **13**: 198–203.

Huson, D.H., Mitra, S., Ruscheweyh, H.-J., Weber, N, and Schuster, S.C. (2011) Integrative analysis of environmental sequences using MEGAN4. *Genome Res* **21**: 1552–1560.

Kanehisa, M, and Goto, S. (2000) KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res* **28**: 27–30.

Lueders, T., Manefield, M, and Friedrich, M.W. (2004) Enhanced sensitivity of DNA-and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* **6**: 73–78.

Marschner, H. (2011) *Marschner's Mineral Nutrition of Higher Plants.* Cambridge, MA: Academic Press.

Mooshammer, M., Wanek, W., Schnecker, J., Wild, B., Leitner, S., Hofhansl, F. *et al.* (2012) Stoichiometric controls of nitrogen and phosphorus cycling in decomposing beech leaf litter. *Ecology* **93**: 770–782.

Nannipieri, P., Giagnoni, L., Landi, L., Renella, G. (2011) Role of phosphatase enzymes in soil. In *Phosphorus in Action. Soil Biology.* Bünemann, E., Oberson, A., Frossard, E. (eds), Berlin, Heidelberg: Springer, pp.215–243.

Opheusden, A., Burgt, G.-J.H., Rietberg, P. (2012) *Decomposition rate of organic fertilizers: effect on yield, nitrogen availability and nitrogen stock in the soil.* The Netherlands: Louis Bolk Institute.

Ragot, S.A., Huguenin-Elie, O., Kertesz, M.A., Frossard, E, and Bünemann, E.K. (2016) Total and active microbial communities and phoD as affected by phosphate depletion and pH in soil. *Plant Soil* **408**: 15–30.

Redfield, A.C. (1958) The biological control of chemical factors in the environment. *Am Sci* **46**: 230A–2221.

Richardson, A.E, and Simpson, R.J. (2011) Soil microorganisms mediating phosphorus availability update on microbial phosphorus. *Plant Physiol* **156**: 989–996.

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Rodríguez, H., and Fraga, R. (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol Adv* **17**: 319–339.
Rosenberg, E. (2014) *The Prokaryotes*. Berlin, Heidelberg: Springer.
Rossolini, G., Schippa, S., Riccio, M., Berlutti, F., Macaskie, L, and Thaller, M. (1998) Bacterial nonspecific acid phosphohydrolases: physiology, evolution and use as tools in microbial biotechnology. *Cell Mol Life Sci* **54**: 833–850.
Rstudio Team (2015). *RStudio: Integrated Development for R*. RStudio, Inc., Boston, MA. URL http://www.rstudio.com/.
Schachtman, D.P., Reid, R.J, and Ayling, S.M. (1998) Phosphorus uptake by plants: from soil to cell. *Plant Physiol* **116**: 447–453.
Shade, A, and Handelsman, J. (2012) Beyond the Venn diagram: the hunt for a core microbiome. *Environ Microbiol* **14**: 4–12.
Sharma, S.B., Sayyed, R.Z., Trivedi, M.H, and Gobi, T.A. (2013) Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus* **2**: 587.
Spohn, M, and Kuzyakov, Y. (2013) Phosphorus mineralization can be driven by microbial need for carbon. *Soil Biol Biochem* **61**: 69–75.
Stutter, M.I., Shand, C.A., George, T.S., Blackwell, M.S.A., Dixon, L., Bol, R. *et al.* (2015) Land use and soil factors affecting accumulation of phosphorus species in temperate soils. *Geoderma* **257–258**: 29–39.
Töwe, S., Wallisch, S., Bannert, A., Fischer, D., Hai, B., Haesler, F. *et al.* (2011) Improved protocol for the simultaneous extraction and column-based separation of DNA and RNA from different soils. *J Microbiol Method* **84**: 406–412.
Uksa, M., Schloter, M., Endesfelder, D., Kublik, S., Engel, M., Kautz, T. *et al.* (2015) Prokaryotes in subsoil—evidence for a strong spatial separation of different phyla by analysing co-occurrence networks. *Front Microbiol* **6**: 1269.
Vance, E., Brookes, P, and Jenkinson, D. (1987) An extraction method for measuring soil microbial biomass C. *Soil Biol Biochem* **19**: 703–707.
Vestergaard, G., Schulz, S., Schöler, A, and Schloter, M. (2017) Making big data smart—how to use metagenomics to understand soil quality. *Biol Fert Soils* **53**: 479–484.
Walker, T.W, and Syers, J.K. (1976) The fate of phosphorus during pedogenesis. *Geoderma* **15**: 1–19.
Wanner, B. (1993) Gene regulation by phosphate in enteric bacteria. *J Cell Biochem* **51**: 47–54.
Willsky, G.R., Bennett, R.L, and Malamy, M.H. (1973) Inorganic phosphate transport in *Escherichia coli*: involvement of two genes which play a role in alkaline phosphatase regulation. *J Bacteriol* **113**: 529–539.
Yuan, Z.-C., Zaheer, R, and Finan, T.M. (2006) Regulation and properties of PstSCAB, a high-affinity, high-velocity phosphate transport system of Sinorhizobium meliloti. *J Bacteriol* **188**: 1089–1102.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Text S1. Materials and Methods

Table S1. Detailed information of sequencing run. Summarized are the number of reads and average length of reads for metagenomics datasets obtained from Freising and Rostock, before and after quality filtering. Samples were taken from plots under different fertilization regimes, in spring and autumn.

Table S2. All investigated enzymes related to the microbial turnover of soil P with KO numbers corresponding genes and references.

Table S3. Bacterial families whose relative abundances and involvement in P turnover were significantly affected by season, fertilizer or interaction of both factors. Significant differences between the treatments were determined by a two-way ANOVA ($n = 3$, $p < 0.05$)

Table S4. R packages used for data visualization.

Fig. S1. NMDS ordination plots depicting taxonomic profiles on the family level for bacteria found at both sites. Shown in (A) is the overall community, and depicted in (B) distribution of genes involved in P turnover among bacterial families ($n = 24$). 95% confidence level is depicted as ellipses for each of the triplicates. Taxonomic assignment was performed against the National Center for Biotechnology Information Non-Redundant (NCBI-NR) protein sequences database. Functional genes were derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database.

Fig. S2. Rarefaction curves of metagenomic datasets from samples taken in spring and autumn from Freising and Rostock. Depicted are the numbers of assigned genes involved in P turnover as a function of sequencing depth. Genes were assigned using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database. 'S' and 'A' at the beginning of the sample names stand for 'spring' and 'autumn'. The following '–' and '+' stand for 'N_{min}' and 'N_{min}-organic'. The numbers represent the replicate number, 1–3 respectively.

Fig. S3. Core microbiome for the comparison of the two fertilization treatments N_{min} fertilizer and N_{min}-organic fertilizer by means of stacked barplots. Depicted are all genes involved in P turnover. Percentages describe the total relative abundance of reads assigned to P turnover.