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Reconstruction of the microbial phosphorus turnover in forest soils with different phosphorus stocks

Fabian Bergkemper

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Meiner Familie

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Zusammenfassung

Phosphor (P) ist ein essentieller Makronährstoff für alle Formen von zellulärem Leben auf unserem Planeten. Wesentliche Merkmale wie die Struktur, die Physiologie und die Reproduktion von Lebewesen basieren auf dem Vorhandensein von P, welcher in der Natur in keiner Weise ersetzt werden kann. Während der anthropogene relative P-Überschuss in vielen aquatischen Ökosystemen zu einer unerwünschten Wasserverschmutzung führt, wird im Gegensatz dazu die Leistungsfähigkeit von terrestrischen Ökosystemen häufig durch die P-Versorgung begrenzt. Bedingt durch den hohen Grad an chemischer Reaktivität ist besonders in Böden ohne externen Nährstoffeintrag die Bioverfügbarkeit von P ernsthaft limitiert, wodurch die Primärproduktion von Pflanzen entsprechend reduziert wird. Um dem immer massiveren Einsatz von P-haltigen Düngemitteln Einhalt zu gebieten und dabei gleichzeitig auch in Zukunft hohe Ernteerträge zu gewährleisten, ist die potentielle Rolle von Mikroorganismen bezüglich der Umsetzung und der Mobilisierung von P im Boden von großer Bedeutung. Es ist daher ein zentrales Ziel dieser Arbeit, sowohl die mikrobiellen Fähigkeiten als auch die entsprechenden mikrobiellen Hauptakteure, die an der Mobilisierung von P im Boden von naturnahen Waldökosystemen beteiligt sind, zu identifizieren. In diesem Zusammenhang wird auch der spezifische Einfluss des P-Gehaltes im Boden untersucht, bezogen auf den P-Gesamtgehalt und die Zusammensetzung der einzelnen P-Spezies und mit der entsprechenden Bedeutung von weiteren Bodennährstoffen verglichen.

Im Bereich des mineralischen Oberbodens von zwei unterschiedlichen Waldstandorten wurde das größte genetische Potential für mikrobielle P-Transporter sowie für regulatorische Systeme nachgewiesen, die an der Steuerung der Genexpression bei P-Mangel beteiligt sind. Dabei wurde ein höheres Potential für effiziente P-Transporter in einem P-armen Boden nachgewiesen, während die Fähigkeit zur Mobilisierung von anorganischem-P signifikant mit dem Gehalt an mineralischem- und Gesamt-P im Boden korrelierte. Im Gegensatz dazu war der hydrolytische Abbau von organischen P-Verbindungen grundsätzlich von geringerer Bedeutung. Dieses Muster änderte sich allerdings in Abhängigkeit des untersuchten Bodenhorizonts. In diesem Zusammenhang wurde speziell im organischen Auflagehorizont ein deutlich erhöhtes genetisches Potential für die Mineralisierung von Phosphormonoestern nachgewiesen, verglichen mit dem mineralischen Oberboden. Während die entsprechende Genabundanz im Bereich des mineralischen Oberbodens im zeitlichen Verlauf weitestgehend konstant blieb, wurde besonders im organischen Auflagehorizont ein hohes Maß an saisonaler Schwankung beobachtet. Insbesondere während der Wachstumsperiode von *Fagus sylvatica* L. beruhte die P-Versorgung von P-armen Waldstandorten vornehmlich auf dem organischen Auflagehorizont. Zusammengefasst deuten diese Ergebnisse eine Anpassung der mikrobiellen P-Ernährungsstrategie an die spezifischen P-Charakteristika eines Bodens an. Die Hauptakteure des mikrobiellen P-Umsatzes im Boden unterschieden sich abhängig vom untersuchten

Waldstandort und umfassten im Wesentlichen die abundantesten Taxa des jeweiligen Bodens. Folglich stammte in einem P-reichen Boden der überwiegende Anteil an Genen, die mit dem mikrobiellen P-Kreislauf assoziiert sind, von Vertretern der copiotrophen Alphaproteobakterien, während mit abnehmendem P-Gehalt die Bedeutung von eher oligotrophen Acidobakterien zunahm. Darüber hinaus war eine Vielzahl weiterer Taxa mit einer geringen Abundanz an den Prozessen des mikrobiellen P-Kreislaufs im Boden beteiligt, was die Komplexität der assoziierten mikrobiellen Gemeinschaft aufzeigt. Tatsächlich wurde jedoch sowohl die Zusammensetzung der am P-Kreislauf beteiligten mikrobiellen Gemeinschaft als auch die Zusammensetzung der gesamten mikrobiellen Gemeinschaft nur zu einem gewissen Teil vom P-Gehalt des Bodens beeinflusst. Im mineralischen Oberboden von fünf unterschiedlichen Waldstandorten hatte ausschließlich der pH-Wert einen signifikanten Einfluss auf die Zusammensetzung der bakteriellen Gemeinschaft, während die unterschiedlichen P-Gehalte lediglich eine Anpassung der jeweiligen Gemeinschaft an die spezifischen Standortbedingungen bewirkte. Diese Anpassung basierte im Wesentlichen auf einer Änderung der relativen Abundanz von Taxa und, nur zu einem geringeren Teil, auf dem einmaligen Auftreten von Spezies an einem bestimmten Standort. Diese Anpassung spiegelte sich zusätzlich durch eine Veränderung der elementaren Verhältnisse innerhalb der mikrobiellen Biomasse wieder, wobei eine P-Limitierung des Bodens durch ein steigendes Verhältnis von Kohlenstoff zu Phosphor innerhalb der mikrobiellen Biomasse gekennzeichnet war. Dessen ungeachtet, wurde ein bedeutendes bakterielles Kern-Mikrobiom in mehreren unterschiedlichen, räumlich getrennten Waldböden mit verschiedenen P-Gehalten und variierenden physikalischen und chemischen Bodenparametern nachgewiesen. Dieses bakterielle Kern-Mikrobiom wurde vornehmlich durch die Art des Ökosystems und die Hauptbaumart getriggert. Im Gegensatz zur bakteriellen Diversität korrelierte die bakterielle Abundanz stark mit dem P-Gehalt des Bodens. Abschließend betrachtet, liefert diese Arbeit entscheidende Einblicke in das mikrobielle Potential der P-Umsetzung im Boden von naturnahen Waldökosystemen. Darüber hinaus wird der spezifische Einfluss des P-Gehaltes im Boden sowohl auf die gesamte mikrobielle Gemeinschaft als auch auf die mit dem P-Kreislauf assoziierte mikrobielle Gemeinschaft im Boden offengelegt**.**

Summary

Phosphorus (P) is a crucial macronutrient for all kinds of cellular life on our planet. Major properties as the structure, the physiology and the reproduction of living things rely on the existence of P, which can be substituted in nature by no means. While the anthropogenic relative P surplus in many aquatic ecosystems causes adverse water pollution, the prosperity of terrestrial ecosystems is frequently limited by the P supply instead. As a result of the chemical reactivity, particularly in soils without external nutrient input, the bioavailability of P is severely restricted, whereby the primary production of plants is downscaled accordingly. To reduce the accelerating application of P fertilizers, but concurrently ensure high crop yields in the future, the potential role of microorganisms for the mobilization and the turnover of soil P is of great interest. Thus, it is the major goal of this study to identify the microbial traits as well as the microbial key players that perform the mobilization of soil P in close to nature forest ecosystems. In this respect, the specific impact of distinct soil P stocks, in terms of the total size and the species composition, is investigated and compared to further soil nutrient contents.

In the mineral topsoil of two different forest sites, the strongest genetic potential was proven for microbial P transporters and regulatory systems that are involved in P starvation-inducible gene expression. With respect to the soil P stock, the potential for efficient P transporters was higher in a P-depleted soil, while the ability for the solubilization of inorganic-P significantly correlated with the content of mineral- and total-P instead. As opposed to this, the hydrolytic breakdown of organic-P compounds was consistently of minor importance. This pattern, however, shifted with respect to the underlying soil horizon. Regarding the mineralization of soil phosphomonoesters, a distinctly increased genetic potential was detected in the organic layer, compared to the mineral topsoil. While the gene abundance in the latter horizon remained largely stable over time, an exceptionally high rate of seasonal fluctuation was observed in the soil organic layer. Particularly during the growth season of *Fagus sylvatica* L., the P supply of P-depleted sites strongly depended on the forest floor. Taken together, these findings indicate an adaptation of the microbial P nutrition strategy to the site specific soil P characteristics. The microbial key players of the soil P turnover differed with respect to the analyzed forest site and primarily comprised the most abundant taxa in the respective soil. Thus, the majority of P cycle associated genes was harbored by copiotrophic Alphaproteobacteria in a P-rich soil, while with decreasing P stocks the contribution of more oligotrophic Acidobacteria rose. In addition, however, a tremendous number of taxa contributed to the processes of the soil P turnover with low frequency, which underlines the complexity of the P cycle associated microbial community in forest soil. Certainly both, the P cycle associated as well as the total microbial community composition, were merely affected to some extent by the soil P stock itself. Indeed, exclusively soil pH significantly controlled the bacterial community structure in the mineral topsoil of five different

forest sites, while the distinct P stocks solely caused an adaption of the respective communities to the site specific conditions. The latter was attributed to changes in the relative abundance of taxa and, to a lesser extent, to the occurrence of unique species. This adaption was moreover reflected by shifts regarding the elemental ratios of the microbial biomass, whereby soil P limitation was characterized by increasing ratios of biomass carbon to phosphorus. At the same time, however, a considerable bacterial core microbiome was detected in spatially separated forest soils with contrasting P stocks and physicochemical parameters that was primarily triggered by the ecosystem type and the main tree species. In contrast to the bacterial diversity, the overall bacterial abundance was strongly correlated to the soil P content. In conclusion, this study provides fundamental insights into the microbial potential for the soil P turnover in close to nature forest ecosystems, and moreover reveales the specific impact of the soil P stock on the total and the P cycle associated microbial community.

I. Introduction

1. The indispensable role of P for life on earth

The story of Phosphorus (P) began in the year 1669 with its accidental discovery from urine by the German alchemist Henning Brand. In early years, P was mainly used for suspect medical purposes. In the late 18th century, bones were revealed as a more efficient source of P. This set the basis for the commercial application of P in frame of the mass production of phosphorus matches. Alongside with this beneficial feature, P also gained notoriety as the "Devil's element" due to its poisonous virtue in military applications (Ashley et al., 2011). In the year 1840, Liebig initially postulated the importance of P for plant growth in the context of his "mineral theory" and thereby revolutionized the agricultural management practices of the Western world (Liebig, 1840; Ashley et al., 2011). Meanwhile, the crucial role of P for all kinds of cellular life on our planet is well-known. De facto, P is ubiquitous in living cells. Of particular importance is the functional role of P regarding the self-organization of membrane lipid-bilayers. Moreover, P acts as a driving force for cellular bioenergetics and represents the key element for storage and procession of the genetic backup (Elser, 2012). Regarding the latter, the majority of cellular P is associated to ribosomal RNA (rRNA). According to the "Growth Rate Hypothesis" (GRH), the growth-related demand for the build-up of rRNA induces the distinct P content of living organisms (Elser, 2012). The concept of the GRH is most suitable for microorganisms and vascular plants, since in vertebrates bone is the primary pool of body P, instead of rRNA. However, with reference to the former organisms the GRH constitutes a fundamental principle of P in nature: High cellular growth rates in combination with high productivity come along with an extraordinary elevated P demand that is required for the construction of ribosomes (Elser, 2012). With reference to terrestrial ecosystems, the optimal growth of food plants is frequently limited by an insufficient supply of bioavailable-P, together with nitrogen (N) and potassium (K) (White and Brown, 2010). This in turn directly affects the food supply of the entire mankind. Consequently, the preservation of high crop yields severely depends on the sufficient P supply of plants (Cordell et al., 2009). In the end, there is no substitute for P in nature (Elser, 2012).

2. The biogeochemical P cycle – a broken cycle?

The biogeochemical cycle of P might be retitled as the "human P cycle" these days. After all, the human demand for P has drastically accelerated the global rate of P mobilization in the past century (Childers et al., 2011). Most recently, more than twenty-three million tons of P were mined from rock phosphate annually (Elser and Bennett, 2011). The vast majority thereof was used for the production of agricultural fertilizers, while merely a small portion made the way into livestock feed supplements,

food preservatives as well as detergents and cleaning agents (Elser and Bennett, 2011). The rapid exploitation of deposits worldwide raises concern about a “peak phosphorus” coming soon. In the end, the access and affordability of P fertilizers might directly affect food quality and quantity (Childers et al., 2011). This prospective is of particular brisance, since merely a fraction of the fertilized P ends up in the human nourishment (Figure 1). As assumed by the United Nations Food and Agriculture Organization, crops generally incorporate fifteen to thirty percent of the fertilized P into their biomass (Childers et al., 2011). The low P use efficiency is mainly attributed to the excessive application of fertilizers, which often exceeds the P demand of plants. Consequently, the surplus P is fixed to the soil matrix where it potentially serves as a resource for future harvests. In practice, however, the majority of mined P is actually lost from the human P cycle at some point (80%). This refers to losses from farmlands via soil leaching and erosion, together with inefficient processing along the way from the initial P fertilization up to the ultimate food consumption (Childers et al., 2011). To minimize this steady loss, special attention is paid to the recycling of P from human excretes. To date, up to fifty percent of P are recovered back thereof globally and are subsequently reused for agricultural production. Still, a substantial amount of the mined P enters landfills in forms of food waste or is released from wastewater treatment plants (Childers et al., 2011). Depending on the applied technology and the effort, different levels of P are recovered from sewage (Blackall et al., 2002). However, the remainder as well as the additional P inputs that arise from surface drainage of agricultural production facilities, erosion or soil leaching ultimately enter rivers or other waterbodies and are irretrievably lost from the human P cycle. This paves the way for the paradox dual role of P in nature, being simultaneously a crucial macronutrient for cellular life and a growth limiting factor in terrestrial ecosystems, and at the same time a pollutant in marine and freshwater ecosystems. Particularly the latter systems and the coastal zones of oceans suffer from hypoxia or anoxia events due to enhanced P input, whereby the water quality is severely affected (Childers et al., 2011). As the sediments of oceans and waterbodies serve as the ultimate sink for P, the human P cycle can be characterized as “open” or “broken” (Elser and Bennett, 2011). In conclusion, the anthropogenic usage of P can rather be described as an unidirectional flow of P from ancient deposits, to agricultural production facilities, to waterways and oceans (Elser and Bennett, 2011). However, in geological timeframes, the sedimented P in the seafloor forms P-rich phosphorites again (Childers et al., 2011).

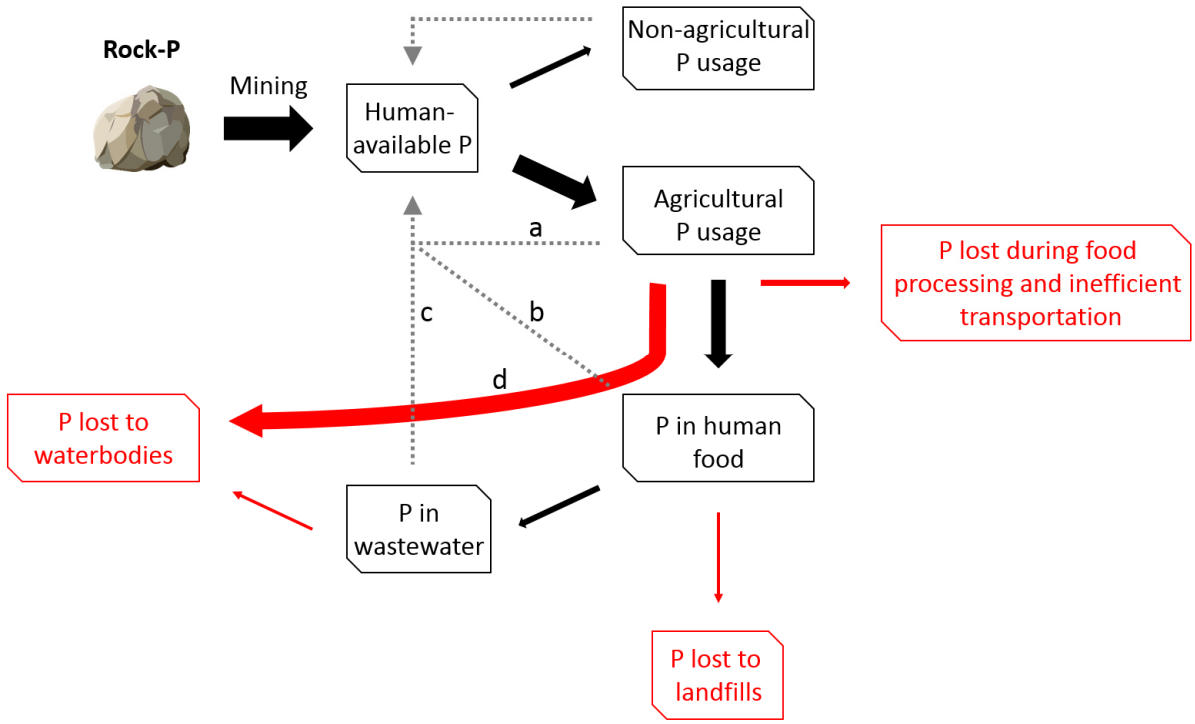

Figure 1 Conceptual model of the human P cycle (adapted from Childers et al, 2011).

Black arrows represent the major flows of P, while the size indicates the estimated dimension of the individual P fractions. Dashed grey arrows constitute recycling processes that recover P for the human usage. Red arrows depict processes, where P is irretrievably lost from the human P cycle. a) P is recycled for agricultural production at farm sites; b) P is recovered from food for human usage through composting; c) P is recovered from wastewater treatment plants for agricultural production; d) P is lost from farm sites via soil erosion and soil leaching.

3. P pools in terrestrial ecosystems

Much of the conceptual framework regarding P nutrition of terrestrial ecosystems is derived from the groundbreaking work of Walker and Syers (1976). The authors assumed, that the P content of terrestrial ecosystems primarily depends on weathering of the parent material. Accordingly, developing ecosystems start with a fixed amount of P, while with ongoing pedogenesis the content of total-P and the species composition are altered (Figure 2). In this regard, the bedrock material, in terms of the mineral composition, is of importance. After all, the median P content of distinct rock types varies by several magnitudes, ranging from 120 parts per million (ppm) in ultramafic rocks up to 3000 ppm and higher in alkali basalts (Porder and Ramachandran, 2013). Moreover, the form and the stability of the predominant minerals affect the progression of pedogenesis and the release rate of P from the bedrock material (Walker and Syers, 1976). Further decisive factors in terms of

pedogenesis are the interactions of climate, relief and biological activity as a function of time (Huggett, 1998). In the initial state of ecosystem development, the biogeochemical P cycle is based on primary mineral-P as the sole P source (Figure 2). During ecosystem maturation, the fractions of soil organic- and labile-P (non-occluded P) strongly increase, while the pool of plant-P is moderately enlarged. As a function of time, particularly the content of soil organic-P increases, while plant-P still plays a subsidiary role related to the ecosystem's total-P content. At a certain point of pedogenesis, the initial content of primary mineral-P of the parent material is entirely depleted. While the majority thereof is converted into different P species, the total-P content of the ecosystem is also distinctly reduced. Over time, the quantity of labile-P slowly declines, while an increasing fraction of soil P is converted into occluded-P forms (Walker and Syers, 1976; Vitousek et al., 2010). With ongoing ecosystem maturation, the relevance of occluded-P is progressively increased, while for the rest merely the fractions of soil organic-P and plant-P are of relevance. At very old sites, more than ninety percent of the initial P content of the parent material might be lost from the ecosystem (Chadwick et al., 1999). This particularly refers to the leaching of dissolved organic-P (Hedin et al., 2003). Thus, in highly weathered soils even the low rates of atmospheric P deposition are of importance, since they might compensate the P losses from the ecosystem to some extent (Chadwick et al., 1999). As proposed by Walker and Syers (1976), mature ecosystems eventually reach a "terminal steady state" of P depletion, where the biological activity is limited accordingly. In this regard, ecosystem P limitation might be attributed to several mechanisms as reviewed by Vitousek and colleagues (2010): Most evident **i)** is the depletion driven P limitation of ecosystems, which is caused by exploitation of soil mineral-P and leaching of dissolved organic- and inorganic-P as previously discussed. A second mechanism **ii)** is attributed to the formation of soil barriers, which physically prevent roots from tapping additional P sources. Moreover **iii)** the P content and the texture of the parent material as well as **iv)** the release rate of P from the parent material in relation to further nutrient contents might provoke ecosystem P limitation. **v)** The existence of P sinks, which inhibit further circulation of the element through the ecosystem, in terms of an accumulation of occluded-P forms, restrains the P availability in soils. **vi)** Anthropogenic P limitation is a result of the enhanced atmospheric N deposition and the subsequent stimulation of biological P mobilization, which is, however, insufficient to balance the excess nitrogen input. Naturally, the abovementioned effects and processes might occur individually or in various combinations.

In contrast to P, the effect of ecosystem maturation is contrary regarding the contents of nitrogen (N) and carbon (C). Since N is virtually absent from the majority of bedrock materials, developing ecosystems are almost devoid of this element. However, N as well as C are progressively introduced into ecosystems by biological fixation and atmospheric deposition (Vitousek et al., 2010; Berg, 2011).

Consequently a chronological pattern of carbon and nitrogen, respectively, phosphorus limitation can be expected in natural, undistorted terrestrial ecosystems (Vitousek et al., 2010).

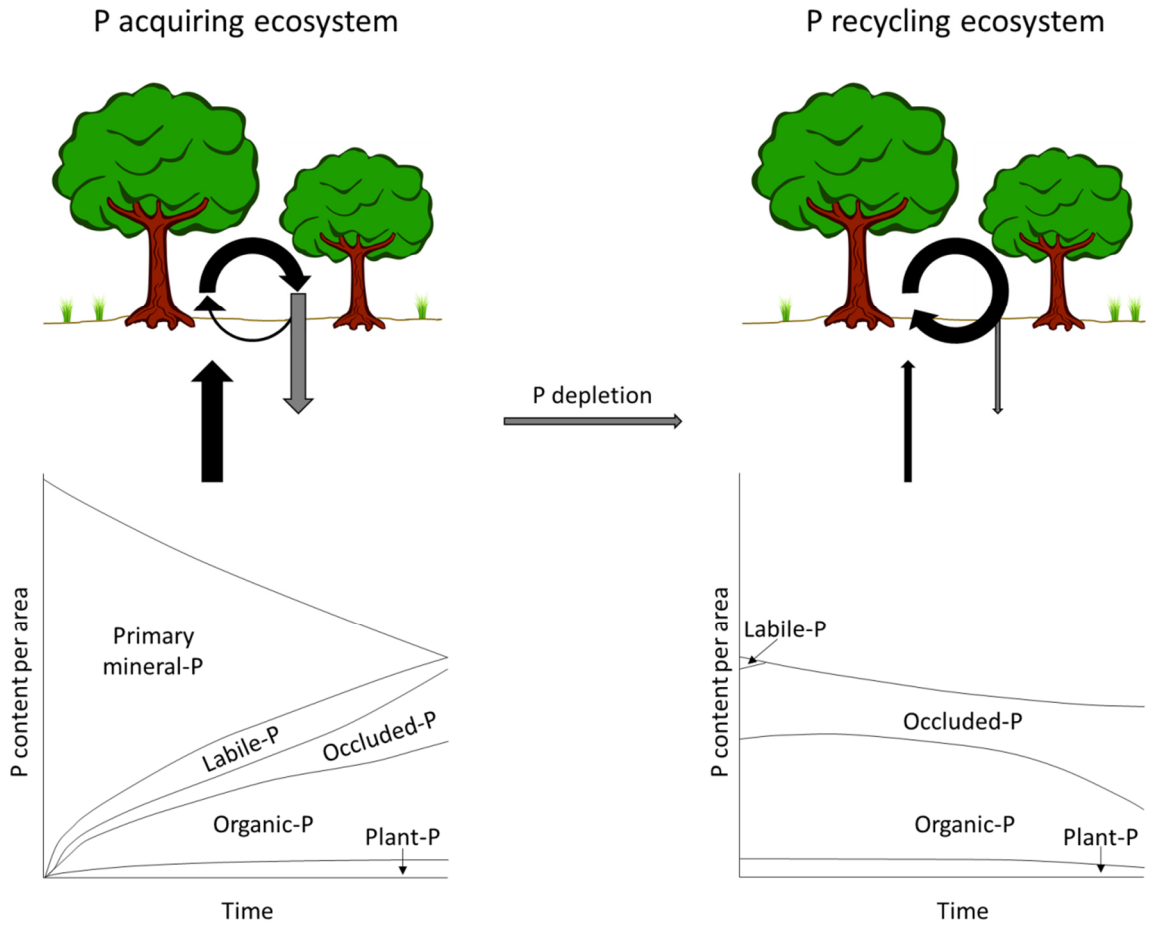

Figure 2 The P nutrition strategy of forest ecosystems as a result of the soil P status (adapted from Walker & Syers, 1976 and Lang et al., 2016).

At mineral-P rich sites, the plant and microbial communities introduce P from primary mineral-P into the biogeochemical P cycle (P acquiring system). With ongoing P depletion, the P nutrition of forest ecosystems shifts to a recycling strategy, which relies on tight cycling processes to prevent losses of P from the ecosystem (P recycling system).

4. Soil as a sink for P

The element P is chemically highly reactive and is considered as the most inaccessible and unavailable nutrient in soil (Holford, 1997). Inherently, the form and the solubility of the prevailing P form is influenced by the soil physical and chemical characteristics. This applies to soil pH, the concentration of iron and cations as well as the nature and surface structure of soil particles. Originally, developing soils comprise a significant proportion of mineral-P, while particularly in

unfertilized soils a certain portion of soil P is also adsorbed to clay minerals, in forms of aluminum (Al) or iron (Fe) hydrous oxides. Depending on the soil pH, P is adsorbed to the surface of calcium (Ca) and magnesium (Mg) carbonates accordingly (Holford, 1997). With ongoing pedogenesis, an increased fraction of secondary mineral-P occurs, in forms of Al- and Fe-phosphates, respectively, Ca- and Mg-phosphates. In addition, a significant portion of soil P is bound to organic matter complexes. Depending on the soil age, the fraction of this organic-P pool (P_O) might comprise up to forty percent of the total-P content (Turner et al., 2013). However, merely the smallest portion of P_O is biologically active. This predominantly applies to the fraction of microbial biomass P. Depending on the environmental parameters, substantial amounts of microbial biomass P are rapidly turned over, while the size of the total-P_O pool remains largely stable. After all, the processes of P mineralization and immobilization might occur simultaneously (Stewart and Tiessen, 1987). In this regard, microbial biomass P can be cycled in different ways: The direct uptake of biomass P by microbial grazers like amoeba or nematodes (i.e. micro- and mesofauna) is the most rapid mechanism. Moreover, organic-P compounds are also actively secreted into the soil solution or released after cell lysis, and can be taken up by microorganisms and plants again. Otherwise, these compounds are stabilized in the soil matrix by mineral components and contribute to the labile soil organic-P pool (Stewart and Tiessen, 1987). The composition of P_O is highly diverse and comprises various forms of phosphomono- and phosphodiesters (e.g. phospholipids, glycerol phosphates, phosphatidyl cholines, nucleic acids), phosphonates, polyphosphates as well as orthophosphate (P_i) that is loosely absorbed to organic moieties (Stewart and Tiessen, 1987). Moreover, inositol phosphates (e.g. IP_6) contribute significantly to the organic-P pool. Actually, IP_6 is frequently cited as the major component of P_O in soils (Turner et al., 2002). In the end, however, plants are only able to absorb free and soluble P_i from the soil solution, which is sparingly available in most soils (Holford, 1997). Once the pool of bioavailable-P is entirely depleted due to plant and microbial uptake or sorption, it has to be replenished accordingly to maintain plant growth. The replenishment is based on the soil labile-P pool, while the replenishment capacity is influenced by the size of the labile-P pool and the ease of P release into the soil solution. The latter process again is determined by the sorption isotherm of the individual soil (Holford, 1997). The labile-P pool for its part is replenished by the mineralization of active- and stabilized-P_O compounds as well as by desorption of P from hydrous oxides and dissolution from primary and secondary P minerals (Shen et al., 2011). According to the conceptual model of Walker and Syers (1976), especially ancient soils comprise another substantial fraction of P: the pool of occluded-P. This pool constitutes a sink for P, since occluded-P is generally insoluble and thus inaccessible for organisms (Vitousek et al., 2010). The encapsulation of P by soil minerals induces the physical protection from biological degradation (Smeck, 1985). Finally, the unidirectional formation of occluded-P starts out from primary mineral-P and proceeds via the intermediate forms of soluble-,

labile- and secondary mineral-P in geological timeframes. Together with the pool of stable organic-P, the occluded-P represents the terminal state of soil P transformation (Smeck, 1985).

5. The role of microorganisms for the turnover of soil P: sink and source of P

The microbial world comprises a wealth of unique features, which constitute microbes as the key drivers for ecosystem services. Although merely a fraction of their entire genetic potential might be uncovered yet, the microbial contribution to nutrient cycling processes is of fundamental importance. Thus, the microbial degradation and turnover of C, N and P as well as micronutrients in soil ecosystems provides the basis for soil fertility and sustainable plant growth (Stevenson and Cole, 1999). While the supply with C and N is progressively improving during ecosystem maturation, particularly the availability of P hampers optimal biomass growth ultimately (Vitousek et al., 2010). Therefore, the possible role of plants, fungi and bacteria in mobilization of soil P has been investigated thoroughly since decades (Willsky et al., 1973; Eivazi and Tabatabai, 1977; Nakas et al., 1987). In particular, the application of plant growth promoting bacteria (PGPB) as prospective biofertilizers for agricultural crops has attracted increasing attention (Rodríguez and Fraga, 1999). In fact, the beneficial effects of soil microorganisms on the capacity of plants for P acquisition are of different origin as reviewed by Richardson and Simpson (2011): **i)** The plant root system can be extended by hormonal stimulation, extensive root hair development or branching. Additionally, mycorrhizal associations increase the range of the existing rooting system. **ii)** Microbial activity can enhance the net transfer of P_i into the soil solution as well as the mobility of organic-P forms and not least **iii)** microorganisms are a rich source of distinct metabolic traits that mobilize soil P from sparingly available forms and convert it into bioavailable-P. Regarding the latter mechanism, however, microorganisms might primarily meet their own demand, while plants rather profit from the subsequently increased turnover rate of microbial biomass P (Richardson and Simpson, 2011). After all, microbial biomass P represents a substantial pool of soil P that typically accounts for up to ten percent of the soil total-P content. Depending on the season and the soil horizon, this fraction might even come close to fifty percent (Richardson and Simpson, 2011). Thus, although microorganisms effectively compete with plants and other biota for the bioavailable-P by incorporating it into their biomass where it is temporarily unavailable for plants, they fulfill a crucial role in regulating the plant P supply in the long run. After all, the biomass P is immobilized, respectively, bound and thus protected from soil sorption reactions or leaching. Since microbial biomass P is a highly dynamic pool, significant amounts of P are periodically released to the soil solution in response to environmental factors. Ultimately, plants profit and take up the released P_i as well as the rapidly mineralized forms of organic-P (Richardson and Simpson, 2011). Consequently, the microbial impact on the soil P turnover can be described as a wheel which is rotating in the soil

(National Research Council, 1987): On the one hand microorganisms mobilize the sparingly available forms of organic and inorganic soil P. The solubilized P_i might be taken up directly or subsequently by other biota. On the other side of the fence, microorganisms effectively compete for the bioavailable-P and immobilize a certain fraction into their biomass, before it is released and re-metabolized by plants, microbial grazers or other biota again (Richardson and Simpson, 2011).

In more detail, the microbial mobilization of soil P is attributed to different types of secreted hydrolytic enzymes and organic acids (Figure 3). The basic hydrolysis of ester- and anhydride-bonds of phosphoric acid (i.e. phosphomonoesters) is performed by several classes of nonspecific acid and alkaline phosphatases (Nannipieri et al., 2011). The underlying genes are known to be harbored by a broad diversity of different bacterial and fungal species (Rodríguez and Fraga, 1999; Habib et al., 2013). To date, three distinct classes (A, B, C) of bacterial nonspecific acid phosphatases (NSAPs) are known, which were originally associated to the genes *phoN*, *aphA* and *olpA*, respectively (Rossolini et al., 1998). All currently described NSAPs are secreted enzymes that either act as soluble periplasmic proteins or as membrane-bound lipoproteins (Rossolini et al., 1998). The counterpart comprises three distinct families (PhoA, PhoD, PhoX) of alkaline phosphatases (ALPs), which are encoded by the genes *phoA*, *phoD* and *phoX* (Torriani, 1960; Eder et al., 1996; Monds et al., 2006). While the PhoA and the PhoX family predominantly comprise phosphomonoesterases, the enzymes of the PhoD group also show phosphodiesterase activity. Principally, PhoD is the dominating ALP in soil derived metagenomic datasets (Tan et al., 2013). The prevalence of either acid or alkaline phosphatase activity is strongly correlated to the soil pH, while the activity of acid phosphatases prevails in acid soils and *vice versa* (Nannipieri et al., 2011).

In addition, also phosphodiesters are a main source of continuous organic-P input into soils (e.g. nucleic acids, phospholipids) (Nannipieri et al., 2011). As mentioned previously, their degradation is performed by enzymes of the PhoD family, but also by two further types of phosphodiesterases UgpQ and PhnP (*ugpQ*, *phnP*). The former enzyme specifically hydrolyzes glycerophosphoryl diesters into glycerol-3-phosphate (G3P) and the corresponding alcohols. The reaction is performed during the intracellular transport via a specific G3P transport system. In other respects, the unmodified form of glycerol-3-phosphate is directly transported into the cytoplasm via the G3P transport system. Noteworthy, the genes for UgpQ and the G3P transporter are located within one transcriptional unit (*ugpBAECQ*) (Brzoska and Boos, 1988).

In contrast, the phosphodiesterase PhnP is part of the carbon-phosphorus (C-P) lyase pathway, which enables the degradation of various soil organophosphonates into alkanes and P_i via cleavage of the chemically inert C-P bond (McGrath et al., 2013). The catalytic machinery of this multistep process relies on the *phnCDEFGHIJKLMNOP* gene cluster, which is under the control of one single promoter. While the proteins PhnCDE form a phosphonate-specific ABC transporter, PhnF acts as the *phn*

operon repressor and the *N*-acetyltransferase PhnO is associated with the catabolism of aminoalkylphosphonates. The exact role of PhnK still remains unresolved (McGrath et al., 2013). The actual "core" C-P lyase reaction is initiated by the nucleotide phosphorylase PhnI, which catalyzes the ATP-mediated activation of the phosphonate group through the formation of a triphosphate ester in the presence of PhnGHL. The ester bond is subsequently hydrolyzed by the phosphohydrolase PhnM into pyrophosphate and 5-phosphoribosyl-1-phosphonate, which in turn is the substrate for the C-P bond cleavage by PhnJ (McGrath et al., 2013). The resulting products are the respective alkyl group together with 5-phosphoribosyl 1,2-cyclic phosphate, which is subsequently hydrolyzed by the phosphodiesterase PhnP. Finally, the bisphosphokinase PhnN enables the allocation of P_i to the intermediary metabolism (McGrath et al., 2013).

In addition to the broad-specific degradation of organophosphonates via the C-P lyase pathway, further microbial mechanisms for the specific breakdown of 2-aminoethylphosphonic acid (2-AEP) are known, which is the prevalent form of soil phosphonates (McGrath et al., 2013). On the one hand, this process is attributed to the initial transamination of 2-AEP into phosphonoacetaldehyde by PhnW (*phnW*) and the subsequent cleavage of the C-P bond by the phosphonatase PhnX (*phnX*) (McGrath et al., 2013). Another route for the microbial degradation of 2-AEP is encoded by an operon comprising the genes *phnW*, *phnY* and *phnA*. Here, the phosphonoacetaldehyde dehydrogenase PhnY provides phosphonoacetate, which is finally hydrolyzed into acetate and P_i by PhnA (McGrath et al., 2013).

Beyond that, the microbial potential for the broad-specific catalytic breakdown of organophosphorus phosphotriesters (e.g. as a component of agricultural pesticides) was proven for the plasmid-encoded *opd* gene (McDaniel et al., 1988). The microbial utilization of inositol phosphates (e.g. IP_6) as the P source is accomplished by the stepwise hydrolysis of IP_6 to myo-inositol and P_i. Depending on the initial hydrolysation step, the respective enzymes are classified as 3-, 6- or 5-phytases. However, with respect to the catalytic mechanism, the proteins are characterized as cysteine phytases, histidine acid phosphatases (HAP), β-propeller phytases or purple acid phosphatases (Jorquera et al., 2008). The majority of the hitherto known phytases belong to the class of HAP and perform the cleavage of the IP_6 phosphomonoesters via a two-step mechanism (*appA*) (Mullaney and Ullah, 2003).

Furthermore, microorganisms are also effective in mobilizing P from inorganic-P species. On that note, the utilization of P from polyphosphate (poly-P) sources is enabled via the housekeeping enzyme inorganic pyrophosphatase (*ppA*) (Lahti et al., 1988) and the genes of the poly-P operon (*ppK*, *ppX*). While the polyphosphate kinase PPK either generates poly-P from ATP or converts it back accordingly, the exopolyphosphatase PPX releases P_i processively from the end of long poly-P chains (~500 residues) (Akiyama et al., 1993). Another well-studied mechanism for the microbial utilization

of mineral-P as the sole P source relies on the "mineral-phosphate solubilizing" (mps) phenotype. Initially, this trait was described in gram-negative bacteria, where the direct oxidation of glucose and other aldose sugars in the periplasmic space by the quinoprotein glucose dehydrogenase (*gcd*) enabled the dissolution of poorly soluble mineral-P and the release of P_i via proton substitution (Goldstein, 1994).

The intracellular uptake of P_i from the soil solution is performed by two microbial transport systems: the "phosphate inorganic transporter" Pit (*pitA*) and the "phosphate specific transporter" Pst (*pstSCAB*). While the Pit system predominantly transports divalent metal cations together with P_i, the latter multi-protein complex serves as the major route for P_i uptake, regardless whether extracellular P_i is limiting or in excess (Elvin et al., 1986; Hsieh and Wanner, 2010). Remarkably enough, the majority of the hitherto discussed genes that are involved in the microbial turnover and uptake of soil P are part of the phosphate (Pho) regulon, which is coregulated by the environmental P_i concentration (Hsieh and Wanner, 2010). In *Escherichia coli*, the Pho regulon comprises at least thirty-one genes, which are organized in nine transcriptional units. The entirety of genes is associated to the assimilation of P from a variety of different environmental sources. This includes genes coding for alkaline phosphatases (PhoA, PhoD), the entire C-P lyase pathway, the phosphonate and glycerol-3-phosphate transporter, the Pst transporter, the outer membrane phosphoporin protein E (*phoE*) as well as the PhoR/PhoB two-component system (TCS) (Hsieh and Wanner, 2010). The latter TCS comprises the sensory histidine kinase PhoR (*phoR*), the DNA-binding response regulator PhoB (*phoB*) and the chaperone-like PhoR/PhoB inhibitory protein PhoU (*phoU*) and regulates the gene expression of the Pho regulon depending on the environmental P_i supply (Hsieh and Wanner, 2010).

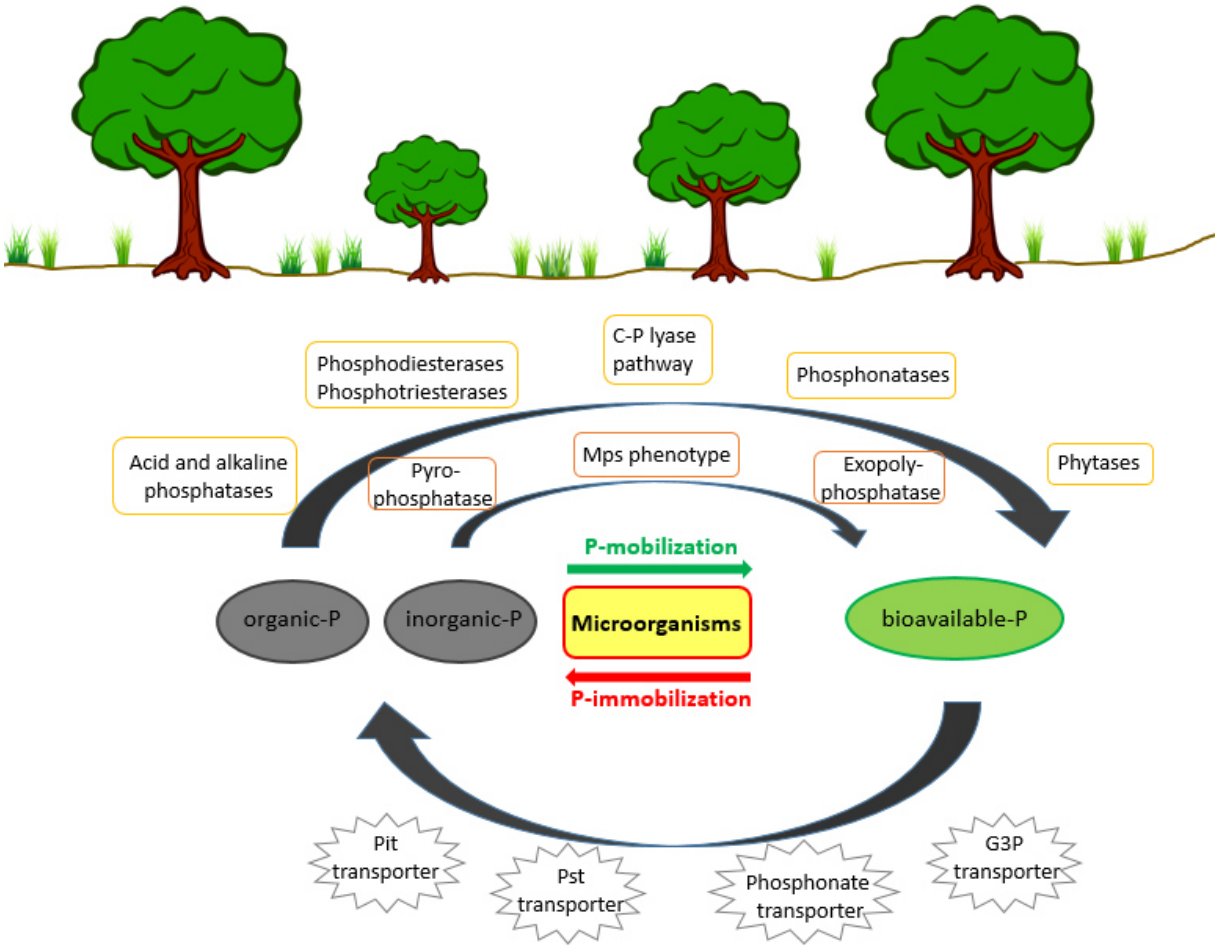

Figure 3 The contribution of microorganisms to the turnover of soil P.

Depicted are important groups of enzymes that perform the mobilization of soil organic- and inorganic-P (yellow or orange boxes), as well as the intracellular P uptake (grey stars).

6. The P geosequence approach in forest ecosystems for the reconstruction of the microbial soil P turnover

Forest ecosystems comprise a variety of unique features that clearly separate them from their agricultural or grassland counterparts. For a start, forest ecosystems represent an important shelter for the preservation of biological diversity. Depending on estimations, half of all known plant and animal species are resident in forest ecosystems. Especially in close to nature forests, the conservation value of biodiversity clearly exceeds that of agricultural systems (Brockerhoff et al., 2008). Since more than forty-two percent of the arable land in Europe are covered by wood or wooden plants at present day, forests constitute an important and representative ecosystem type of

the northern hemisphere (Second Ministerial Conference on the Protection of Forests in Europe, 1993). Inherently, the microbial diversity in soils is strongly affected by the type of ecosystem per se. Besides other factors that influence the microbial life in soil, including the main plant species or the soil type, particularly the management regime is of relevance for the microbial diversity (Garbeva et al., 2004). In this respect, forest ecosystems lack the effects of tillage treatments and irrigation. Compared to agricultural soils, the system is less disturbed and the durable formation of horizons in the mineral topsoil is feasible (Perry and Amacher, 2009). In consequence, the bacterial and fungal communities are significantly vertically stratified in the litter- and humus-layer of close to nature forest ecosystems (Baldrian et al., 2012). Most notably, forest ecosystems are spared from pesticides and fertilizer input, which otherwise induces significant shifts in the total microbial community composition, and moreover affects the contribution of specific taxa to nutrient cycling processes (Garbeva et al., 2004; Tan et al., 2013). As a consequence, the nutrition of forest ecosystems strongly depends on an efficient cycling of the inherent nutrient stocks, whereby the formation of the forest floor is of particular and unique importance (Perry and Amacher, 2009). In this context, Lang and colleagues (2016) characterized forest ecosystems either as “P acquiring” or as “P recycling” systems (Figure 2). Particularly on mineral-P rich sites, P is acquired, respectively, introduced from primary mineral-P into the ecosystem. In contrast, P-depleted forest sites rely on highly efficient recycling processes of the available-P, to maintain the P supply in the long run. The P nutrition strategy of forest ecosystems, regarding the domination of either P acquiring or P recycling processes, might affect the entirety of ecosystem plant and microbial communities (Lang et al., 2016). On that account, forest ecosystems serve as ideal model systems, as they presumably represent the original, undistorted state of the microbial soil P turnover. In addition, the “P geosequence” approach allows to investigate the specific impact of the soil P content and the P species composition on the microbial traits and the microbial key players that are associated to the turnover of soil P. In this context, the P geosequence refers to an array of five beech forest sites that developed from parent materials with distinct P stocks. While all of the sites represent climax ecosystems, the underlying soils are characterized by distinct P fractionations and gradually increasing stocks of mineral- and total-P. Thus, the content of total-P differs by a factor of fifteen between the respective endmembers of the P geosequence (**M6**).

Apart from that, the absence of chemical fertilizer input has further, considerable impact on the nutrient supply and consequently also on the future development of forest ecosystems. Due to anthropogenic activity, the input of carbon and nitrogen into terrestrial ecosystems was strongly increased in the past centuries (Jonard et al., 2015). As a result, the tree productivity in forest ecosystems is generally stimulated. This effect is welcome on the one hand, however, it likewise induces an increased nutrient demand of trees by all accounts. Thus, the forest ecosystems of the

northern hemisphere are increasingly limited by the supply with P and base cations (Jonard et al., 2015). Moreover, the increased ratio of N to P affects tree health and ultimately limits the C sequestration capacity of forest ecosystems (Jonard et al., 2015). Moreover, the general environmental N to P imbalance affects the entirety of ecosystem structure and function. With respect to the microbial world, the elemental stoichiometry of organisms is altered, which impacts metabolic processes like the individual growth rate and ultimately affects both, the species composition and functioning (Peñuelas et al., 2013). Consequently, the reconstruction of the microbial soil P turnover and the actively involved microbial community elucidates how microorganisms cope with this kind of nutrient limitation and the rising imbalance of N and P in close to nature ecosystems. This knowledge might ultimately help to maintain or even improve the P nutrition of forest ecosystems.

7. Aims and hypotheses of the Ph.D. thesis

The microbial performance regarding the mobilization of soil P has been an object of investigation for decades. Thus, different types of microbial enzymes, which enable the specific breakdown of various organic-P compounds have been studied on functional level, in terms of the substrate specificity, the reaction mechanism or the reaction kinetics. Moreover, in recent years, there has been an increasing supply of sequencing data of targeted enzymes from a broad diversity of microorganisms. This in turn helped to uncover the gene expression pattern of major P cycle associated enzymes. The discovery of the bacterial mps phenotype, together with further microbial traits that enhance the P supply of plants, manifested the prominent role of soil microorganisms in plant growth promotion and suggested their prospective role as biofertilizers. Still, one major issue yet remained unacknowledged: The majority of the previously described data was gained from laboratory and microcosm experiments, where merely few, selected and well-characterized isolates were applied. Thus, it remains unclear whether the entire set of beneficial microbial traits is actually present in real, unfactitious ecosystems. Moreover it is questionable, if the hitherto described PGPB indeed represent the key players of the microbial soil P turnover, or if further yet unknown taxa are of major importance. Another unknown variable in this respect is the impact of the soil P stock. Depending on the size and the proportion of organic- and inorganic-P, the individual processes of the P turnover might be differentially pronounced accordingly.

Therefore, the following hypotheses were tested in this study:

i) The microbial potential for the solubilization of inorganic-P is dominating in close to nature forest soils with high amounts of total- and mineral-P in the topsoil. As opposed to this, the microbial P turnover in P-depleted forest soils is more relying on recycling processes of soil organic-P instead.

ii) Moreover, a stronger microbial potential for efficient P transporters is expected in P-depleted forest soils, due to the tighter recycling processes of soil P to prevent nutrient losses from the ecosystem.

iii) The majority of soil microorganisms is simultaneously occurring at distinct forest sites and forms a stable core microbiome, despite significant differences regarding the soil nutrient stocks.

iv) The microbial potential for the mineralization of soil organic-P strongly depends on the soil horizon and is highly variable on temporal and spatial scale.

To address these research questions, several experiments were performed in soils that were derived from a P geosequence of five German close to nature forest ecosystems with comparable management practices and similar main tree species. Apart from that, the forest sites were spatially separated and highly diverse regarding the soil type, the nutrient stocks and the environmental conditions. The main objectives of the underlying study comprised:

- The entire microbial P turnover in forest soils was reconstructed and the respective microbial key processes and players were assigned. Therefore, shotgun sequencing (SGS) of total genomic DNA was performed, which was derived from the mineral topsoil of the two most diverse forest sites of the P geosequence, regarding the contents of total- and mineral-P (**M1**).
- Novel oligonucleotide primer systems were developed *in silico*, which target marker genes that code for the major steps of the microbial P mobilization and uptake in soils (*phoD*, *phoN*, *phnX*, *appA*, *gcd*, *pitA* and *pstS*), considering the results obtained by metagenomics (**M1**). The in-depth diversity analysis of the associated microbial communities was conducted by amplicon sequencing of total genomic DNA derived from the organic layer of the P-richest forest site (**M3**).
- The applicability of the introduced oligonucleotide primers for quantitative analysis of environmental samples was proven for the *phoN* gene. To investigate the rate of seasonal fluctuation and highlight the impact of the distinct soil horizons on the microbial potential for

the mineralization of soil organic-P compounds, soil samples from the organic layer and the mineral topsoil of two different forest soils were analyzed as a function of the season (**M5**).

- The impact of distinct soil nutrient stocks on the diversity and the composition of microbial communities in different forest soils was investigated. As previous data was primarily derived from manipulation experiments, where the P content of agricultural sites was artificially increased by fertilization or manuring, it remained unclear, whether the response pattern of soil microbial communities to P fertilization is transferable to ecosystems with naturally diverging P stocks. Therefore, total genomic DNA from the mineral topsoil of the five forest sites was applied to bacterial fingerprinting of the 16S rRNA gene and subsequently correlated to various soil physicochemical parameters in terms of the total P, N, C contents and pH. The occurrence and the size of a common core microbiome in the soils of the P geosequence was investigated by the calculation of a VENN diagram based on fingerprinting results (**M2**).
- In frame of an interlaboratory test, six variations of the two basic principles for the measurement of microbial biomass P were compared. Microbial biomass P was determined in the mineral topsoil of the five forest sites, using gaseous or liquid fumigation based methods, and subsequently compared regarding the absolute values and the relative gradient along the soils of the P geosequence (**M4**).

II. Materials and Methods

1. Study site description

The experiments of this study were performed in frame of the Priority Program Ecosystem Nutrition: "Forest Strategies for Limited Phosphorus Resources" (SPP1685). The five central study sites of the SPP1685 are uniformly dominated by European beech (*Fagus sylvatica* L.) and are part of the ICP Level II forest monitoring program (International Co-operative Program on Assessment and Monitoring of Air Pollution Effects on Forests). The sites have been intensively monitored for the past two decades and were reliably spared from chemical fertilizer input. In addition to a similar main tree species (*Fagus sylvatica* L.), the five forest ecosystems are consistently based on silicate rock and have a comparable stand age of approximately 120 years in common. While four of the core sites are located in the central German uplands, site "LUE" is located in the north German plain near the city of Unterluess at an altitude of 115 meter (m) above sea level (asl). The mean annual precipitation and temperature are 779 millimeters (mm) and 8 °C, respectively. In contrast, the forest sites Conventwald ("CON") and Vessertal ("VES") are located in the Black Forest and the Thuringian Forest at an elevation of 840 m, respectively, 810 m (asl). The mean annual precipitation and temperature are 1749 mm and 6.8 °C (CON) or 1200 mm and 5.5 °C (VES). The two remaining sites Mitterfels ("MIT") and Bad Brueckenau ("BBR") are situated in the Bavarian Forest and in the Bavarian Rhoen Mountains at an altitude of 1023 m or 809 m (asl), respectively. The two sites are characterized by a mean annual precipitation and temperature of 1299 mm and 4.5 °C (MIT), respectively, 1031 mm and 5.8 °C (BBR) (**M6**).

2. Abiotic soil properties

The soils of the five core sites developed from contrasting parent materials. While the parent material of site BBR is basalt, the soils in MIT and CON developed from paragneiss instead. The parent material of site VES is trachyandesite, while the soil at site LUE is based on sandy till. Inherently, the five sites comprise different types of Cambisol soils. For a detailed description of the individual soil types see Table 1. The dominating humus form ranges from Mull-like Moder at site BBR, to Moder in MIT and VES, and Mor-like Moder at sites CON and LUE. According to the World Reference Base for Soil Resources (WRB, 2015) the texture of the mineral topsoil is classified as silty clay loam at site BBR or loam in MIT, VES and CON, and as loamy sand at site LUE (Table 1) (**M6**).

The basic abiotic soil properties of the five core sites were determined by different research groups of the SPP1685. All measurements were conducted in "quantitative pit" (QP) samples that were also used for measurement of the microbial biomass parameters in frame of an interlaboratory test (**M4**).

The basic characterization of the soils included the determination of the cation exchange capacity, the measurement of the soil total C, N, P stocks and pH in the mineral topsoil, an entire phosphorus fractionation (Hedley and Stewart, 1982), the determination of the P species composition using nuclear magnetic resonance (NMR) spectroscopy, the determination of enzymatic activities and further measurements. The results that were obtained by the different research groups are summarized in **M6**.

Noteworthy, the five forest sites were selected to represent a P geosequence (i.e. soils that developed from parent materials with distinct P stocks), regarding the contents of easily available mineral- and total-P in the topsoil. The order of the P geosequence is as follows: BBR>MIT>VES>CON>LUE. The distinct P stocks enabled the direct linkage to the conceptual model regarding ecosystem P nutrition strategies (Lang et al., 2016): While site BBR ought to fulfill the criteria for a P acquiring system, site LUE can be assumed as a P recycling system instead. The precise classification of the three remaining sites is ambitious, since the processes of P acquisition and recycling might occur simultaneously and in equal measure. According to the P geosequence, the stocks of soil total C and N likewise reach maximum at site BBR and decrease towards site LUE (Table 1).

In contrast, a different sequence exists regarding the contents of resin extractable P in the mineral topsoil (BBR>VES>MIT>CON>LUE) and the extractable fractions of dissolved organic carbon (DOC: CON>BBR>MIT>VES>LUE) and nitrogen (DON: BBR>CON>VES>MIT>LUE) (**M4**).

With respect to the endmembers of the P geosequence, the stocks of labile organic- and inorganic-P are highest at site BBR and decrease by a factor of twenty-one related to site LUE. The ratios of primary and secondary mineral-P related to total-P are likewise highest at site BBR and lowest in LUE. In contrast, the latter soil has an increased ratio of organic-P related to mineral-P in the topsoil. Regarding the composition of the organic-P species in the mineral topsoil, the lowest ratio of diester-P to monoester-P was detected at site BBR. Consequently, an increased relative abundance of diester-P is present at sites MIT, VES, CON and LUE. The latter soil likewise shows the highest percentage of phosphonates in the mineral topsoil (**M6**).

Table 1 Geographic location and description of the basic physical and chemical parameters of the investigated soils.

The texture as well as the nutrient contents and pH refer to the mineral topsoil (Ah-horizon; 0-5 cm). The data was derived from various research groups of the SPP1685 as summarized in **M6**.

Study site	**BBR**	**MIT**	**VES**	**CON**	**LUE**
Geographic location	50°21'7.26" N, 9°55'44.53" E	48°58'34.18" N, 12°52'46.74" E	50°36'23.84" N, 10°46'14.1" E	48°1'21.4" N, 7°57'50.65" E	52°50'21.77" N, 10°16'2.37" E
Soil type (WRB, 2015)	Dystric Skeletic Cambisol	Hyperdystric Chromic Folic Cambisol	Hyperdystric Skeletic Chromic Cambisol	Hyperdystric Skeletic Folic Cambisol	Hyperdystric Folic Cambisol
Humus form	Mull-like Moder	Moder	Moder	Mor-like Moder	Mor-like Moder
Texture (WRB, 2015)	Silty clay loam	Loam	Loam	Loam	Loamy sand
Clay (%)	37	24	24	27	6
Silt (%)	55	32	46	33	19
Sand (%)	8	44	30	40	75
C_{total} (mg g^{-1})	174.83	173.99	125.95	148.90	96.47
N_{total} (mg g^{-1})	11.16	9.62	7.20	6.91	3.76
P_{total} (mg kg^{-1})	2965.78	1375.19	1017.14	929.03	195.77
$pH_{(H2O)}$	3.84	3.57	3.36	4.03	3.52

3. Soil sampling

The soil samples for the respective experiments were taken in frame of individual sampling campaigns between October 2013 and September 2015. Soil samples that were used for whole genome shotgun sequencing and bacterial community fingerprinting (**M1**, **M2**) were taken from the mineral topsoil (Ah-horizon) of the five core sites within one week in October 2013 using a soil auger (8 cm diameter) to a depth of 20 cm. Five contiguous soil cores were taken circularly within a radius of 2 m. Subsequently, the organic layers (Of-, Oh-layer) were removed before the mineral topsoil (Ah-horizon) of five soil cores was pooled for one field-replicate. In total, five field-replicates were

taken in the direct surrounding of the Level II plot at each core site (n=5). The distance between the individual field-replicates was approximately 200 m.

For the measurement of microbial biomass C, N and P (C_{mic}, N_{mic}, P_{mic}) in frame of an interlaboratory test (**M4**), soil samples were derived from quantitative soil pits (QP) as described by Vadebonceur et al. (2012). During October and November 2013 soil samples were taken from the mineral topsoil (Ah-horizon) of the five core sites.

In 2015 a grid sampling campaign was conducted at sites BBR and LUE. Each grid comprised a core area of 50 m x 50 m and was installed in the direct surrounding of the Level II plot. The grids were designed to be representative for the entire forest site regarding biotic and abiotic factors (e.g. stand density, secondary vegetation, hill slope, aboveground rocks and stones). Within the core area, sixteen georeferenced grid points were located in a 4 x 4 pattern with a distance of 10 m to each other. Additionally, each grid point comprised three random satellite points within a distance of 5 m and a maximum azimuth of 360 °. The exact geographic location of the satellite points was specified using the "runif" function in the R environment (R Core Team, 2015). In total, soil samples were taken from forty-eight individual, randomly selected satellite points. While in BBR the grid sampling was performed in April, August and September to cover the entire vegetation period, at site LUE the sampling was performed once in October. At each sampling point, material from the L-, the Of- and the Oh-layer was manually collected. In addition, two different depths of the mineral topsoil (0-7 cm, 7-15 cm) were sampled using a soil auger (8 cm diameter). At site BBR, a thinner soil auger (1 cm diameter) was used for sampling of the mineral topsoil horizon in August and September. During the latter sampling campaign, the two different depths of the mineral topsoil were pooled from five individual, closely adjacent soil cores into one composite sample, to increase the amount of soil material.

In addition, soil samples were derived from a Phosphorus-33 (^{33}P) labeling experiment that was performed at the university of Göttingen and that was analysed in a joint approach with several research groups of the SPP1685 (**M5**). Briefly, one hundred and fifty young beech trees (height: 0.3 - 0.4 m) with intact soil cores (diameter: 0.12 m; height: 0.2 m) were excavated at sites BBR and LUE in October 2013 and transferred to a greenhouse. The labeling experiment was performed for one year and covered five different phenological stages of beech tree development. The labeling time points were: April 2nd (2014), May 12th, July 21st, September 22nd and February 9th (2015). While fifteen plants at a time were irrigated with 40 milliliters (ml) of labeled water (1912 MBq $H_3{}^{33}PO_4$; 0.017 nmol P per plant; Hartmann Analytic GmbH, Braunschweig, Germany), five additional plants of each site were destructively harvested immediately, without labeling (t_0). The unlabeled soil samples (t_0) were derived from both, the organic layer and the mineral topsoil, of sites BBR and LUE. The remaining plants were harvested one day, one week or thirty days after the ^{33}P-labeling, respectively.

In total, 100 unlabeled soil samples (t_0), comprising two different soils (BBR, LUE), two distinct soil horizons (Oh-layer, mineral topsoil), five individual sampling time points and five plants per harvest, were analyzed. In addition, material of the different plant and root compartments was analyzed by the participating research groups.

Generally, for extraction of total genomic DNA soil samples were immediately frozen with dry ice after sampling and subsequently stored at -80 °C. For the measurement of microbial biomass parameters and determination of total organic C, N and P contents fresh soil samples were taken and subsequently stored at 4 °C.

4. Microbial biomass carbon, nitrogen and phosphorus

The measurement of soil microbial biomass C, N and P (C_{mic}, N_{mic}, P_{mic}) was performed in fresh QP soil samples that were stored at 4 °C. Prior to analysis, the samples (Ah-horizon) were homogenized and sieved (<2 mm) to exclude root biomass and stones. The extraction of soil samples was performed as described by Brankatschk et al. (2011) using a 1:4 ratio of soil and 0.01 M $CaCl_2$ during 30 minutes of extraction. The total contents of organic carbon and bound nitrogen in the extraction were determined on a DIMATOC 1000 analyzer (Dimatec, Germany). For the measurement of C_{mic} and N_{mic} the chloroform fumigation-extraction method after Vance et al. (1987) was performed. According to Joergensen (1996) and Joergensen & Müller (1996), a k_{EC} value of 0.45 and a k_{EN} value of 0.54 was applied, respectively. Microbial biomass P was measured from the same soil extract as C_{mic} and N_{mic} according to Brookes et al. (1982) (k_{EP} 0.4). However, 0.01 M $CaCl_2$ was used during extraction instead of 0.5 M $NaHCO_3$. The concentration of orthophosphate was measured as molybdenum-blue using commercial tube test “NANOCOLOR ortho- and total-Phosphate 1” (Macherey-Nagel, Germany).

In frame of an interlaboratory test, six variations of two basic principles for P_{mic} measurement were compared by different research groups of the SPP1685 (**M4**). All measurements were performed in fresh QP soil samples (Ah-horizon). In addition to the abovementioned method using 0.01 M $CaCl_2$ during extraction (“CFE 3”), two further variations of the gaseous chloroform fumigation-extraction method described by Brookes et al. (1982) were performed. Accordingly, soil samples were extracted for 30 minutes in a 1:10 ratio with Bray-1 solution (Oberson et al., 1997; Khan and Joergensen, 2012) (“CFE 1”) or in a 1:20 ratio with Bray-2 solution (Bray and Kurtz, 1945) (“CFE 2”). In contrast, three variations of a liquid fumigation, using anion-exchange resin membranes were performed according to Kouno et al. (1995) (“Resin 1-3”). However, hexanol was used instead of chloroform as proposed by Bünemann et al. (2004). Soil samples were extracted for 16 hours in a 1:15 ratio (“Resin 1-2”) or in a 1:12 ratio (“Resin 3”) with distilled water with or without the addition of liquid hexanol. After extraction, the concentration of orthophosphate was measured according to Murphy and Riley (1962) (“CFE 1-2”, “Resin 2-3”) or Ohno & Zibilske (1991) (“Resin 1”). In total, seven subsamples were

analyzed for the determination of P_{mic} for each soil, irrespective of the applied method. Besides one fumigated and one non-fumigated sample, five different P spikes were applied to take the specific P sorption capacity of each soil into account. Thus, KH_2PO_4 equivalent to 10 µg, 25 µg, 50 µg, 100 µg and 200 µg P g^{-1} soil dry-weight was applied to the soil prior to extraction. According to Kouno et al. (1995), P_{mic} was uniformly calculated as: P_{mic} = (Ep/Kp*100/R). While Ep determines the P concentration after subtracting the non-fumigated from the fumigated sample, Kp defines the percentage of biomass P that is released and extracted after fumigation as orthophosphate (k_{EP} 0.4) (Brookes et al., 1982). In addition, R represents the percentage of recovered P from the soil. The calculation of P_{mic} was based on the mean recovery of the spikes 10 µg, 25 µg and 50 µg g^{-1}, except for method "CFE 3" where the mean recovery of spikes 100 µg and 200 µg g^{-1} was used instead. All analyses were performed for four analytical replicates.

5. Nucleic acid extraction and quantification

For whole genome shotgun sequencing (SGS) and the bacterial community fingerprinting approach (**M1**, **M2**) total genomic DNA was extracted from frozen soil samples (-80 °C) that were taken in October 2013 from the mineral topsoil horizon (Ah-horizon) at the five core sites. For the amplicon sequencing approach using newly designed oligonucleotide primers (**M3**), nucleic acids were extracted from the Oh-layer of site BBR taken in September 2015. According to the phenol-chloroform extraction procedure described by Töwe et al. (2011), 0.5 g of frozen soil were homogenized using Lysing Matrix E tubes (MP Biomedicals, France) and Precellys 24 (Bertin Technologies, France). In contrast, soil samples that were derived from the ^{33}P-labeling experiment were further processed using a commercial DNA extraction kit (NucleoSpin Soil, Macherey-Nagel, Germany) according to the manufacturer's protocol. Generally, the extracted DNA was quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fischer Scientific, USA) and a SpectraMax Gemini EM Fluorescence Plate Reader Spectrometer (Molecular Devices, USA). Additionally, the purity of the extracted DNA was investigated photometrically (Nanodrop ND-1000, Thermo Fischer Scientific, USA).

6. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed on a 7300 Real-Time PCR System (Applied Biosystems, Germany) using SYBR Green as a fluorescent dye. For the quantification of bacterial communities (**M2**) the 16S rRNA gene was amplified as described in de Vries et al. (2015). The template DNA was diluted 128-fold, while serial dilutions of plasmid encoded 16S rRNA genes (*Pseudomonas putida* S16) ranging from 10^7 to 10^1 gene copies $µl^{-1}$ were used for quantification.

For the quantification of the *phoN* gene (**M5**) newly designed primers (*phoN*-FW: GGAAGAACGGCTCCTACCCIWSNGGNCA, *phoN*-RW: CACGTCGGACTGCCAGTGIDMIYYRCA) (**M3**) were used. The qPCR reaction assay comprised: 12.5 µl Power SYBR Green (Life Technologies, USA), 5.3 µl molecular biology water (Lonza, Belgium), 0.5 µl bovine serum albumin (3%), 1 µl of each primer (10 pmol), 2.5 µl of a (10x) FastStart High Fidelity Reaction Buffer (Roche, Germany) and 0.2 µl of a FastStart High Fidelity Enzyme Blend (5 U/µl; Roche, Germany). The target DNA was diluted 16-fold and the amplification was performed as follows: Initial denaturation (95 °C; 10 minutes) followed by 5 cycles of denaturation (95 °C; 15 seconds), annealing (65 °C; -1 °C/cycle; 30 seconds) and elongation (72 °C; 45 seconds). After 5 cycles of touchdown (-1 °C/cycle) the remaining 40 cycles were performed at a constant annealing temperature of 60 °C. For quantification, serial dilutions of plasmid encoded *phoN* genes (10^7 to 10^1 gene copies μl^{-1}) derived from *Salmonella enterica* DSM 10062 were used. Inherently, the amplicon specificity was confirmed by conducting a melting curve after each run and a gel electrophoresis (2%) of selected samples, while the qPCR efficiency was calculated as follows: Efficiency (%) = $[10^{(-1/\text{slope})} - 1]$. To ensure a robust and reproducible amplification, the qPCR efficiency was always in the range between 85% and 110% and the R^2 of the standard curve was always above 0.98.

7. Terminal-Restriction Fragment Length Polymorphism

Terminal-Restriction Fragment Length Polymorphism (t-RFLP) analysis of the 16S rRNA gene (**M2**) was performed using the following components: 29 µl DEPC treated water, 5 µl TopTaq PCR Buffer (10x), 5 µl CoralLoad Concentrate (10x), 5 µl Q-Solution (5x), 0.5 µl TopTaq DNA Polymerase (5 U/µl; all Qiagen, Germany), 2.5 µl dNTPs (2 nM each), 1 µl of each primer (10 pmol), and 1 µl of template DNA (20 ng). Primers were used according to Bruce et al. (1992) (pA) and Wawrik et al. (2005) (1401R), whereas the forward primer (pA) was labeled with 5'-FAM (6-Carboxyfluorescein). The amplification was conducted in the following way: Initial hotstart (95 °C; 5 minutes), 30 cycles of denaturation (94 °C; 45 seconds), annealing (56.5 °C; 45 seconds) and elongation (72 °C; 1 minute) followed by a final elongation step (72 °C; 10 minutes). Subsequently the PCR products were purified (NucleoSpin Gel and PCR Clean-up Kit, Macherey-Nagel, Germany; always according to the manufacturer's protocol) before 400 ng of amplicons were enzymatically digested using MspI (Fermentas, Germany) as proposed by the manufacturer. Following this, 5 ng of restricted and purified sample (NucleoSpin Gel and PCR Clean-up Kit, Macherey-Nagel, Germany) was used for t-RFLP analysis according to Töwe et al. (2011). However, an 800-fold dilution of MapMarker 1000 ladder (Bio-Ventures, USA) was used. Electropherograms were analyzed using the software PeakScanner 2 (Life Technologies, USA) and were further processed using T-REX software (Culman et al., 2009) including a noise filtering step ("Std dev multiplier for fluor B" set to 0.8 using peak height).

Fragments shorter than 50 bp were excluded from the analysis. Finally, operational taxonomic units (OTUs) were defined as peaks within a clustering threshold of 1 bp.

8. Oligonucleotide primer design

Oligonucleotide primers were developed for the amplification of microbial genes that code for enzymes, which perform important steps of the soil microbial P turnover (**M3**). This includes genes that code for enzymes involved in mineralization of soil organic-P (*phoD*, *phoN*, *appA*, *phnX*), in solubilization of inorganic-P (*gcd*) and in cellular P uptake (*pitA*, *pstS*). To enable both, the amplification of genes from a broad diversity of different microorganisms and simultaneously ensure a high degree of primer specificity towards the target gene, the primers were degenerated to a limited extent (64-fold). Most importantly, the primers were designed to generate amplicons in the range between 150 bp and 375 bp to allow an application in both, next generation sequencing approaches ("16S Metagenomic Sequencing Library Preparation" protocol, 2013; Illumina Inc., USA) as well as quantitative real-time PCR approaches (Karsai et al., 2002). For the primer design, database entries (NCBI Protein database; accessed: May 2015) of representative genes (i.e. based on previous metagenomics) (**M1**) were aligned and analyzed for conserved domains using Clustal Omega (Sievers et al., 2011). The ultimate primer design was performed using the CODEHOP program (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) (Rose et al., 2003).

9. Shotgun sequencing and data processing

Shotgun sequencing of total genomic DNA (SGS) extracted from the Ah-horizon at sites BBR and LUE (**M1**), was performed on a Genome Sequencer FLX+ instrument (454 Life Sciences, Roche, USA) as described by de Vries et al. (2015). However, the libraries of three replicates were pooled in a 2:1:1 ratio, respectively, instead of equimolar pooling. Filtering and trimming of raw sequences as well as taxonomic and functional annotation of metagenomic datasets was conducted as described in de Vries et al. (2015) using MEGAN5 (version 5.6.5) (Huson et al., 2011). Prior to analysis, the filtered datasets were subsampled according to the lowest number of sequences obtained in one of the datasets (133,179 sequences) (http://biopieces.org). The statistical analysis on functional level was based on KEGG database results (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa and Goto, 2000) and focused on genes that code for enzymes which are involved in the soil microbial P turnover. In addition, metagenomic datasets were scanned for profile Hidden Markov Models (HMM) of enzymes that are involved in the soil microbial P turnover (de Vries et al., 2015). Finally, sequences of predicted genes that were obtained from the KEGG database were taxonomically annotated as described by de Vries et al. (2015).

10. Amplicon sequencing and data processing

Next generation amplicon sequencing was performed on the Illumina MiSeq platform (Illumina Inc., USA). Library preparation was adapted to the protocol "16S Metagenomic Sequencing Library Preparation" (version 2013; Illumina Inc., USA). In a first round of amplicon PCR (PCR1), target specific primers were used for amplification of genomic DNA extracted from the Oh-horizon of site BBR. The PCR was performed in triplicates and comprised: 2.5 µl of a (10x) FastStart High Fidelity Reaction Buffer (Roche, Germany), 2 µl dNTPs (2 nM each), 1 µl of each primer (10 pmol), 0.5 µl bovine serum albumin (3%), 1.25 U FastStart High Fidelity Enzyme Blend (Roche, Germany), 25 ng of genomic DNA (or 50 ng for primers *appA*-FW/RW and *phoN*-FW/RW) and ad water 25 µl (Lonza, Belgium). The cycling conditions were as listed below: Hotstart (95 °C; 7 minutes), 30 cycles of denaturation (95 °C; 1 minute), annealing (60 °C; 1 minute) and elongation (72 °C; 45 seconds) followed by a final elongation step (72 °C; 7 minutes). In a second round of amplification (PCR2), specific Illumina overhang adapter sequences were added to the amplicons by using 10 pmol of target specific primers with attached adapter overhangs and 2 µl of PCR1 as the template. The ten cycles of amplification were performed as previously stated. The respective triplicates of PCR2 were pooled and purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) according the manufacturer's protocol, except that a 1:4 dilution of washing-buffer (NTI) was used. Since the *phoD*-FW/RW primers generated three distinct bands after PCR2, the respective bands were separated by gel extraction (2% agarose) (NucleoSpin Gel and PCR Clean-up Kit, Macherey-Nagel, Germany) and treated as individual amplicons henceforth (datasets named: phoD-K, phoD-M, phoD-L). In a second approach, the amplicons were directly purified after PCR2 without the separation of individual bands (dataset named: phoD). Thus, a total of ten amplicon samples together with sixteen negative controls of the amplification (NKP) and the DNA extraction procedure (NKE) were further processed. While the correct amplicon size was verified on a Bioanalyzer 2100 instrument (Agilent Technologies, USA) using the DNA 7500 Kit (Agilent Technologies, USA), the concentration of the purified samples was measured applying the Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fischer Scientific, USA). The library preparation was implemented using the Nextera XT v2 Index Kit set A (Illumina Inc., USA). Subsequently, the indexing PCR was performed as follows: 12.5 µl NEBNext High-Fidelity Master Mix (New England Biolabs, USA), 2.5 µl of each Indexing primer, 10 ng of purified amplicons and 6.5 µl of DEPC treated water. The amplification procedure included an initial denaturation step (98 °C; 30 seconds), 8 cycles of denaturation (98 °C; 10 seconds), annealing (55 °C; 30 seconds) and elongation (72 °C; 30 seconds), followed by a final elongation step (72 °C; 5 minutes). The amplicons were checked on a 2% agarose gel, purified, analyzed for the correct size and quantified as mentioned previously. Finally, the amplicons were

pooled equimolar to 4 nM and sequenced using the MiSeq Reagent Kit v3 (600 cycles) (Illumina Inc., USA) for paired end sequencing.

Trimming and merging of FASTQ files was performed using AdapterRemoval v2 (minimum read length = 50; minimum Phred quality = 15) (Schubert et al., 2016). For quality filtering the QIIME suite was applied (version 1.9.1) (Caporaso et al., 2010) using the script "*split_libraries_fastq.py*" (minimum per read length fraction = 0.01; Phred quality threshold = 20; length filtering = 100 – 600 bp). The amplicon datasets were subsampled to 130,377 sequences according to the lowest number of reads obtained within one dataset (http://biopieces.org). FragGeneScan (version 1.19) (train = illumina_5; thread = 15) (Rho et al., 2010) was used for prediction of open reading frames (ORF), before hmmsearch (http://hmmer.org; HMMER 3.0) was performed against the Pfam (version 27.0) (Finn et al., 2014), respectively, TIGRFAMs database (version 15) (Haft et al., 2013). Overlapping HMMs were removed and results were quality filtered (expect value = 10^{-3}). The proportion of defined HMMs, that specifically comprised conserved domains of proteins encoded by the individual target genes, in relation to the total number of detected HMMs in the respective datasets, served as a basis for assessing the primer specificity. All sequences that comprised the favored HMM were taxonomically annotated using the NCBI RefSeq database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/; accessed: February 2016) (Tatusova et al., 2014) and DIAMOND with default settings plus enabled "sensitive" option (Buchfink et al., 2015). The taxonomic analysis was performed in MEGAN (version 5.6.5) (Huson et al., 2011) with the following parameters: Min Score: 50, Max Expected: 10^{-4}, Top Percent: 10, Min Support Percent: 0, Min Support: 1, LCA Percent: 50, Min complexity: 0.0.

11. Statistical analysis

The statistical analysis was performed using the R environment (version 3.2.0) (R Core Team, 2015). The analysis of metagenomic data, both on taxonomic and functional level was performed on filtered, subsampled datasets as described by de Vries et al. (2015) (**M1**). Likewise the rarefaction analysis of amplicon data was based on filtered, subsampled datasets (**M3**). The impact of abiotic soil properties on logarithmic 16S rRNA gene abundance data was investigated by an analysis of variance (ANOVA), while regression coefficients and correlations between two data matrices were calculated using Pearson correlation (**M2**). In addition, the impact of abiotic soil properties on the diversity of the 16S rRNA gene was investigated by permutational multivariate analysis of variance using distance matrices, by regression based canonical correspondence analysis (CCA) and by regularized canonical correlation analysis (RCC) (correlation based). To stabilize the datasets against noise variance, an Anscombe square root transformation was conducted prior to CCA and RCC (Anscombe, 1948; Green, 1979). The calculation of a bacterial core microbiome was performed applying VENN diagrams

(http://bioinformatics.psb.ugent.be/webtools/Venn/). The analysis was based on filtered 16S rRNA gene diversity data, whereby fragments that occurred in less than two replicates were excluded.

III. Manuscript overview

1. List of manuscripts

- Bergkemper, F., Schöler, A., Engel, M., Lang, F., Krüger, J., Schloter, M. & Schulz, S. (2015) Phosphorus depletion in forest soils shapes bacterial communities towards phosphorus recycling systems. *Environ Microbiol*, 18(6), 1988-2000.
  (M1, first author, published)

- Bergkemper, F., Welzl, G., Lang, F., Krüger, J., Schloter, M. & Schulz, S. (2016) The importance of C, N and P as driver for bacterial community structure in German beech dominated forest soils. *Journal of Plant Nutrition and Soil Science*, 179(4), 472-480.
  (M2, first author, published)

- Bergkemper, F., Kublik, S., Lang, F., Krüger, J., Vestergaard, G., Schloter, M. & Schulz, S. (2016) Novel oligonucleotide primers reveal a high diversity of microbes which drive phosphorous turnover in soil. *Journal of Microbiological Methods*, 125, 91-97.
  (M3, first author, published)

- Bergkemper, F., Bünemann, E. K., Hauenstein, S., Heuck, C., Kandeler, E., Krüger, J., Marhan, S., Mészáros, É., Nassal, D., Nassal, P., Oelmann, Y., Pistocchi, C., Schloter, M., Spohn, M., Talkner, U., Zederer, D. P. & Schulz, S. (2016) An inter-laboratory comparison of gaseous and liquid fumigation based methods for measuring microbial phosphorus (P_{mic}) in forest soils with differing P stocks. *Journal of Microbiological Methods*, 128, 66-68.
  (M4, first author, published)

- Spohn, M., Zavišić, A., Nassal, P., Bergkemper, F., Schulz, S., Marhan, S., Schloter, M., Kandeler, E. & Polle, A. (2017) Temporal variations of phosphorus uptake by ectomycorrhizal fungi, soil microbial biomass, and young beech trees in two forest soils with contrasting P stocks. *New Phytologist*.
  (M5, contributing author, in revision)

- Lang, F., Krüger, J., Amelung, W., Willbold, S., Frossard, E., Bünemann, E., Bauhus, J., Nitschke, R., Kandeler, E., Marhan, S., Schulz, S., Bergkemper, F., Schloter, M., Luster, J., Guggisberg, F., Kaiser, K., Mikutta, R., Guggenberger, G., Polle, A., Pena, R., Prietzel, J., Rodionov, A., Talkner, U., Meesenburg, H., von Wilpert, K., Hölscher, A., Dietrich, H. P. & Chmara, I. (2017) Soil phosphorus supply controls P nutrition strategies of beech forest ecosystems in Central Europe. *Biogeochmistry*.
  (M6, contributing author, in revision)

2. Manuscript description and contributions

Manuscript 1

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

Fabian Bergkemper, Anne Schöler, Marion Engel, Friederike Lang, Jaane Krüger, Michael Schloter, Steffanie Schulz

Short description:

Microorganisms are integral to the mobilization and the turnover of soil P. Previous data regarding genetic potentials and enzymatic activities was restricted to culture dependent approaches using selected isolates. For a representative reconstruction of the actual microbial P turnover in close to nature ecosystems, whole genome shotgun pyrosequencing was performed for two forest soils with distinct contents of total- and mineral-P in the topsoil. In both soils, particularly the strong microbial potential for P starvation-inducible gene regulation as well as P_i uptake systems attracted attention. Both traits were highly abundant and exceeded the processes that perform the hydrolytic breakdown of soil organic-P compounds by far. With respect to the soil P stock, a significantly higher microbial potential for the mobilization of inorganic-P was detected in the mineral-P rich soil. In terms of ecosystem nutrition this indicates a P acquiring strategy at the P-rich site. In contrast, the higher microbial potential for efficient P_i transporters in the P-depleted soil implies a P recycling nutrition strategy for the respective ecosystem. The taxonomic affiliation of the investigated processes confirmed previous data to some extent, but moreover revealed an adaption of the P cycle associated microbial community to the specific soil characteristics. While at the P-rich site especially the copiotrophic members of Rhizobiales were of importance, a stronger contribution of oligotrophic taxa like Acidobacteriales and Actinomycetales to the P turnover was detected in the P-depleted soil.

Contributions:

- contributed to experimental planning and design
- performed sample preparation and shotgun sequencing
- performed analysis of sequencing data
- wrote the manuscript

Environ Microbiol, 18(6), 1988-2000

Manuscript 2

The importance of C, N and P as driver for bacterial community structure in German beech dominated forest soils

Fabian Bergkemper, Gerhard Welzl, Friederike Lang, Jaane Krüger, Michael Schloter, Steffanie Schulz

Short description:

Previous data revealed a strong impact of the soil P content on microbial community structure and diversity. Since most of these observations were restricted to P fertilization experiments, the respective impact of naturally diverging P stocks, in comparison to further nutrient contents and pH, was investigated in this study. Bacterial community fingerprinting in the mineral topsoil of five forest sites revealed soil pH as the decisive factor regarding the bacterial community composition. In contrast, the contrasting stocks of total C, N and P predominantly affected the bacterial abundance instead. Although the structure of the five bacterial communities was highly diverse, a homogeneous cluster of microbes was simultaneously occurring at the different sites. After all, this stable core comprised forty-three percent of all detected OTUs. Presumably, the type of ecosystem in combination with the main tree species triggered the existence of the bacterial core microbiome, while the environmental conditions merely affected an adaption of the respective bacterial communities, regarding the abundance of specific OTUs or, to a lesser extent, the occurrence of unique species.

Contributions:

- contributed to experimental planning and design
- performed bacterial community fingerprinting and qPCR measurements
- performed data analysis and interpretation
- wrote the manuscript

Journal of Plant Nutrition and Soil Science, 179(4), 472-480.

Manuscript 3

Novel oligonucleotide primers reveal a high diversity of microbes which drive phosphorous turnover in soil

Fabian Bergkemper, Susanne Kublik, Friederike Lang, Jaane Krüger, Gisle Vestergaard, Michael Schloter, Steffanie Schulz

Short description:

The microbial genes that code for the individual processes of the soil P turnover occasionally are scarce in specific soils and moreover are distributed heterogeneously on temporal and spatial scale. To thoroughly investigate the underlying microbial communities and moreover allow the quantification of the gene abundance in a high-throughput manner, novel oligonucleotide primer systems for the major processes of the microbial soil P turnover were developed. The primers were designed to be suitable for both, amplicon sequencing as well as qPCR approaches. Based on previous metagenomics of forest soils, the most abundant processes of the P turnover were selected, while particularly genes of the respective microbial key players were used for primer design. The seven targeted genes code for enzymes involved in organic-P mineralization, inorganic-P solubilization and cellular P uptake. Although the primers were degenerated to allow the amplification of target genes from a broad diversity of different microorganisms, the primer specificity generally was sufficiently high, which allows the prospective application in qPCR approaches. As revealed by amplicon sequencing, the results obtained by metagenomics were confirmed, while the increased sequencing depth enabled a more precise view into the P cycle associated microbial community and uncovered the contribution of hitherto ignored microorganisms.

Contributions:

- contributed to experimental planning and design
- performed sample preparation and amplicon sequencing
- performed sequencing data analysis
- wrote the manuscript

Journal of Microbiological Methods, 125, 91-97.

Manuscript 4

An inter-laboratory comparison of gaseous and liquid fumigation based methods for measuring microbial phosphorus (P_{mic}) in forest soils with differing P stocks

Fabian Bergkemper, Else K. Bünemann, Simon Hauenstein, Christiane Heuck, Ellen Kandeler, Jaane Krüger, Sven Marhan, Éva Mészáros, Dinah Nassal, Pascal Nassal, Yvonne Oelmann, Chiara Pistocchi, Michael Schloter, Marie Spohn, Ulrike Talkner, Dan P. Zederer, Steffanie Schulz

Short description:

The size and the dynamics of the soil microbial biomass P pool are crucial parameters for soil fertility and plant growth. Depending on the environmental conditions, significant amounts of P are periodically released from the microbial biomass to the soil solution whereby the temporarily fixed P becomes bioavailable again. However, the measurement procedure of P_{mic} is not standardized, while two different basic principles are frequently applied. Thus, it was the aim of this study to compare three different variations of liquid, respectively, gaseous fumigation for the measurement of P_{mic} in the mineral topsoil of five different forest sites with contrasting P stocks. For a start, the six protocols uniformly detected the same gradient of P_{mic} along the investigated sites. With respect to the absolute P_{mic} values, however, significant differences between the two basic principles manifested. Inherently, the liquid fumigation based methods using anion-exchange resin membranes, were characterized by a higher recovery rate of P from the soil and consequently yielded consistently lower P_{mic} values. The obtained results clustered tightly and thus indicated a high rate of reproducibility, regardless of the analyzed soil type. In contrast, the methods that were based on gaseous chloroform fumigation-extraction were severely affected by the soil physicochemical parameters. While the recovery of P from the soil was consistently lower, the obtained P_{mic} values exceeded the former methods by a factor of two. As different solvents and ion strengths were applied during extraction, the P_{mic} of the latter methods was more variable accordingly.

Contributions:

- contributed to experimental planning and design
- contributed to P_{mic} measurement
- performed data analysis and interpretation
- wrote the manuscript

Journal of Microbiological Methods, 128, 66-68.

Manuscript 5

Temporal variations of phosphorus uptake by ectomycorrhizal fungi, soil microbial biomass, and young beech trees in two forest soils with contrasting P stocks

Marie Spohn, Aljosa Zavišić, Pascal Nassal, Fabian Bergkemper, Stefanie Schulz, Sven Marhan, Michael Schloter, Ellen Kandeler, Andrea Polle

Short description:

P is a major growth limiting factor for plants in terrestrial ecosystems. Particularly in forest soils, which are spared from chemical fertilizer input, the P availability and consequently also the uptake rate of P into plants is of major importance. Therefore, it was the aim of this study to investigate the seasonal variations of P uptake by beech trees, ectomycorrhizal fungi (EMF) and the soil microbial biomass (SMB) and the factors that impact the respective P uptake rate in two forest soils with distinct P stocks. In a mesocosm experiment, juvenile beech trees were labeled with ^{33}P, before samples of the individual plant compartments and the different soil horizons were taken. The P uptake of plants, EMF and soil microorganisms in combination with the potential phosphatase activity and the *phoN* gene abundance in soil was determined for five different time points within one year. The P uptake rate of beech trees at the P-rich site was relatively stable throughout the season, except for an increase in autumn. In contrast, the plant P uptake at the P-poor site exceeded the P-rich site, and was highly dynamic with its maximum during summer. Likewise, the P uptake rate of EMF and SMB (in the organic layer) was consistently higher in the P-poor soil. The potential phosphatase activity and the *phoN* gene abundance was highest in the organic layer of both sites. While a significant influence of the season was not observed, the abundance of *phoN* genes was particularly increased in the organic layer of the P-poor soil in spring. In conclusion, the P uptake rate of beech trees, EMF and SMB varies independently throughout the year with its minimum during winter. The P immobilization of SMB and the EMF community structure was without effect on the rate of beech P acquisition.

Contributions:

- performed qPCR measurements
- conducted qPCR data analysis
- critically revised the manuscript

Manuscript 6

Soil phosphorus supply controls P nutrition strategies of beech forest ecosystems in Central Europe

Friederike Lang, Jaane Krüger, Wulf Amelung, Sabine Willbold, Emmanuel Frossard, Else Bünemann, Jürgen Bauhus, Renate Nitschke, Ellen Kandeler, Sven Marhan, Stefanie Schulz, Fabian Bergkemper, Michael Schloter, Jörg Luster, Fabian Guggisberg, Klaus Kaiser, Robert Mikutta, Georg Guggenberger, Andrea Polle, Rodica Pena, Jörg Prietzel, Andrei Rodionov, Ulrike Talkner, Henning Meesenburg, Klaus von Wilpert, Andrea Hölscher, Hans-Peter Dietrich, Ines Chmara

Short description:

The P content of the soil parent material is a decisive factor for ecosystem development. This particularly refers to the soil compartment. On the one hand, the soil P content affects the nutrition strategies of plants and microorganisms, while the adaptations of individual lifestyles and metabolic processes shape the soil properties and the soil P pools in turn. Thus, it was the aim of this study to investigate the link between the P supply of soils and the P nutrition strategy of European beech forest ecosystems. The analysis of five forest soils that developed from contrasting parent materials and which differed in their total-P stocks included a wide spectrum of soil chemical and biological properties. In the course of the study, indicators for P acquisition and P recycling, regarding the rate of P mobilization from the mineral soil and the soil organic layer, were postulated and subsequently validated using data obtained from the analyzed beech forests. Generally, the turnover rate of the forest floor correlated with the P content, while the proportions of the fine-root biomass in the mineral topsoil and in the forest floor increased with decreasing P supply. Likewise the ratio of P in the fine-root biomass and P in the microbial biomass increased with decreasing P supply. In general, the P depletion of soils and the tighter P cycling processes in forest ecosystems are realized by the joint adaption of microbial and plant communities.

It is stated, that data discussed here and throughout this Ph.D. thesis, which is cited as **M6** was not necessarily derived from this Ph.D. thesis, but from any of the research groups participating in the introduced manuscript **M6**.

Contributions:

- performed measurement of microbial biomass carbon, nitrogen and phosphorus
- critically revised the manuscript

IV. Discussion

The present study was part of the Priority Program “Ecosystem Nutrition: Forest Strategies for Limited Phosphorus Resources” (SPP1685) and was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft; DFG). The central hypothesis of the priority program proposed that P depletion of soils drives forest ecosystems from P acquiring systems, where P is continuously introduced from primary mineral-P into the ecosystem, to P recycling systems which are characterized by efficient and tight recycling processes of P to reduce permanent losses of this crucial compound from the ecosystem. In a joint approach, the participating research groups of the SPP1685 investigated the adaptation mechanisms of entire forest ecosystems to soil P depletion in terms of ecological interplays. On that account, the different processes of phosphorus mobilization and uptake, usage and storage as well as translocation were reconstructed from the micro- up to the macro-scale considering the major components of forest ecosystems: the soil and the bedrock material, the ground and stream water, plants and trees together with anthropogenic factors. As was intended, the five central study sites of the SPP1685 formed a natural gradient regarding the contents of total- and mineral-P in the topsoil. In combination with a permanent forest monitoring at all sites by federal research institutes, an ideal basis for the investigation of the central hypothesis was provided.

The central focus of this Ph.D. thesis was on the microbial contribution to the turnover of soil P in forest ecosystems. In contrast to C and N, external inputs of P into terrestrial ecosystems are scarce. Consequently, the efficient mobilization and uptake of P from the soil solution is a crucial property of soil microorganisms. This study revealed profound insights into the microbial turnover and uptake mechanisms of soil P in close to nature forest ecosystems, and identified the respective microbial key players in soils with contrasting P stocks. In addition, the actual impact of gradually decreasing soil P contents on bacterial community composition in comparison to other soil physicochemical parameters was investigated.

1. Strategies to unravel unknown microbial nutrient cycles

1.1 Reconstruction of microbial nutrient cycles using metagenomics: gains and frontiers

Metagenomics is a precious and powerful tool for assessing the genetic diversity of environmental samples. Shotgun sequencing of total genomic DNA (SGS) provides complex information about microbial community structures and enables the linkage of genomic function and phylogeny (Thomas et al., 2012). The direct sequencing approach without further cultivation steps includes previously unculturable and unknown organisms in the analysis and simultaneously prevents an amplification bias during library preparation (Tringe and Rubin, 2005; Aird et al., 2011). In contrast to phylogenetic surveys, which exclusively focus on the diversity analysis of individual genes (e.g. 16S rRNA), metagenomics provide a broader view into the entire genetic potential of microbial communities (Thomas et al., 2012). This qualifies SGS as an attractive tool for the reconstruction of metabolic pathways or nutrient cycles, like the microbial turnover of soil P. The technique is perfectly suitable to proof the transferability of laboratory-gained data on natural, unfactitious habitats. Consequently, metagenomics can validate and confirm existing data, but most notably also extend the knowledge by providing direct insights into the microbial life in different ecosystems. While the benefits of metagenomics are beyond dispute, the technique is also facing its limitations. The coverage of the entire metagenome of complex environments like soil is hardly feasible with respect to the sequencing effort and costs (Riesenfeld et al., 2004). Since the statistical analysis of datasets requires multiple replicates, the sequencing depth of individual samples is limited. A sufficient sequencing depth, however, is of special importance when microbial pathways are being reconstructed for the first time. While the microbial key players of certain processes are readily uncovered, rare species might be missing in the analysis (Tringe et al., 2005). In addition, the validity of metagenomics strongly depends on the applied DNA extraction method and the choice of the database (Delmont et al., 2011). It is beyond debate, that sequences of certain microbial groups and traits are underrepresented, lacking or wrongly annotated in public databases, which in turn affects the interpretation of metagenomic data (Nilsson et al., 2005; Nilsson et al., 2006; Wooley et al., 2010). Although the sequencing costs per megabase of DNA sharply decreased most recently (Wetterstrand, 2017), the temporal or spatial resolution of processes using metagenomics is still not feasible. Indeed, these factors are of great relevance, since the structure and the genetic potential of soil microbial communities are highly heterogeneous on both the temporal and the spatial scale (Torsvik et al., 2002). Consequently, the in-depth characterization of microbial pathways has to rely on more appropriate tools regarding the high-throughput analysis of multiple samples. Most suitable in this

respect is the quantification of the gene abundance using quantitative real-time PCR (Bustin et al., 2009). After all, the quantification of entire microbial pathways in a high-throughput manner enables the linkage between different nutrient cycles, like the turnover of N and P in soil, and thereby provides information about the nutrient status of an ecosystem. It was one central aim of this study to pave the way from the fundamental reconstruction of the soil microbial P turnover towards the quantification of the major turnover processes in environmental samples. Therefore, a two-phasic approach was applied: **i)** Initially, metagenomics identified the microbial traits as well as the respective key players of the P turnover in two forest soils (**M1**) and provided the basis for **ii)** the development of specific oligonucleotide primer systems for the amplification of selected target genes that code for enzymes of the microbial soil P turnover (**M3**). The downstream application of the primer systems in qPCR approaches constitutes the third, designated stage of the proposed approach, and enables the precise quantification of major P cycle associated processes (regarding the genetic potential) in multiple environmental samples (**M5**).

1.2 Pitfalls of environmental primer design

The development of oligonucleotide primers for the specific amplification of target genes from environmental total genomic DNA is facing a general, crucial obstacle. Due to the high diversity of microorganisms that are present in complex environments like soil (Torsvik et al., 2002), a tremendous number of homologous target genes is present in the extracted DNA (Kloos et al., 2006). For the amplification of a broad diversity of homologous genes, the primer systems have to be degenerated. Accordingly, some positions of the primers have different base combinations (Linhart and Shamir, 2005). In this regard, the degeneracy increases with the number of possible sequence combinations. In theory, the primer system should be suitable for the amplification of each homologous target gene within a sample (Linhart and Shamir, 2005). However, with increasing degeneracy the amplification of unrelated, unwanted sequences occurs. In consequence, the design of degenerated primers is a tradeoff between the coverage of homologous target genes and degeneracy (Linhart and Shamir, 2005). Conventionally, primer design was based on database derived sequences of selected isolates, irrespective of their phylogenetic position or their actual occurrence in specific environments. While the alignment of few rather similar sequences during primer design reduces the degeneracy, the covered microbial diversity is restricted. In consequence, the primers are biased towards certain microbial groups, since their preferential amplification leads to an overrepresentation. In this regard Tan et al. (2013) detected an overabundance of Alphaproteobacterial sequences in soil derived datasets using the alkaline phosphatase specific initial primer system developed by Sakurai and colleagues (2008). In a more promising approach, Ragot et al. (2015) included the entire set of target gene specific database entries (*phoD*) in their primer

design. In consequence, the amplified microbial diversity of the *phoD* gene was significantly increased by a factor of seven, compared to the former primers (Sakurai et al., 2008). However, the approach strongly depends on the existence of highly conserved regions within the corresponding protein. Otherwise, an alignment of hundreds of sequences is not feasible, if the primer degeneracy is going to be kept low. On that account, the conservation of further proteins that are associated to the microbial soil P turnover besides PhoD, is barely sufficient for primer design. This particularly applies to the members of histidine acid phytases or class A acid phosphatases. Although both groups of proteins comprise well defined signature sequence motifs (Rossolini et al., 1998; Mullaney and Ullah, 2003), the simple alignment of multiple database derived sequences was not promising for primer design (**M3**). Aside from the respective signature motifs, the remaining parts of the alignments did not contain any further putative primer binding sites. Consequently, the number of aligned sequences had to be reduced in a sensible way, until at least a second conserved region was obtained for the development of an entire primer system. At the same time, the covered microbial diversity should preferably not be narrowed.

1.3 A two-phasic pipeline for oligonucleotide primer design

The first essential step of the introduced primer design pipeline comprised the identification of the most important processes of the microbial P turnover in two forest soils by metagenomics (**M1**) (Figure 4). Initially, the SGS datasets were aligned against the KEGG database, which provides an interface between genomic and higher order functional information (Kanehisa and Goto, 2000). After all, a set of forty P cycle associated genes was detected in the soils of the two forest sites. Therefrom, seven genes which were highly abundant in the datasets were selected for the subsequent primer design. Noteworthy, all major processes of the soil microbial P turnover were covered: the mineralization of organic-P (*phoD*, *phoN*, *appA*, *phnX*), the solubilization of inorganic-P (*gcd*) and the cellular P uptake (*pitA*, *pstS*) (**M3**). Although a working set of *phoD* primers was recently introduced by Ragot et al. (2015), the gene was still included in the primer design pipeline to benchmark the new approach. In a second step, the microbial key players that were predominantly harboring the selected genes in the two soils were analyzed. Since the SGS datasets rather contained gene fragments instead of entire open reading frames (ORF), curated database entries of the corresponding proteins were used for primer design. On that note, exclusively the database sequences of representative microorganisms that substantially contributed to the individual processes of P turnover in the two soils were applied. In this way, the number of aligned protein sequences was kept down, while the most important microorganisms were reliably covered. This strategy is of special importance for the determination of putative primer binding sites within poorly conserved proteins. Compared to Ragot and colleagues (2015), who implemented hundreds of

individual sequences in their *phoD* primer design, the introduced two-phasic approach principally focused on few, highly representative sequences. Inasmuch as a protein comprised strongly conserved domains (e.g. PhoD), the alignment included one representative sequence from each microbial phylum with a curated database entry, even if members were lacking in the metagenomic datasets. Notwithstanding, the upper limit of each alignment was ten sequences. Having said that, the detection of putative primer binding sites within the alignment came along with the consideration of the basic principles of primer design (Dieffenbach et al., 1993). Since the new primers ought to be suitable for application in qPCR approaches, especially the amplicon size was of relevance (Karsai et al., 2002). Indeed, this factor severely narrowed down the number of putative primer binding sites. If necessary, the quantity and diversity of aligned sequences was reduced accordingly, until the intended primer criteria were fulfilled. However, the sequences of the most important microbial key players were considered by all means, before the primers were finally designed *in silico*. In consequence, the introduced strategy allowed the development of degenerated primers for the amplification of target genes with high specificity from a broad diversity of different microorganisms, while the microbial key players of the investigated environment were reliably covered (**M3**). Primarily, the primers were customized for a specific habitat where they perform best (i.e. beech forest soil). However, since an initial metagenomic analysis is not feasible for each sample, the primers are supposedly suitable for application in comparable sample types as well.

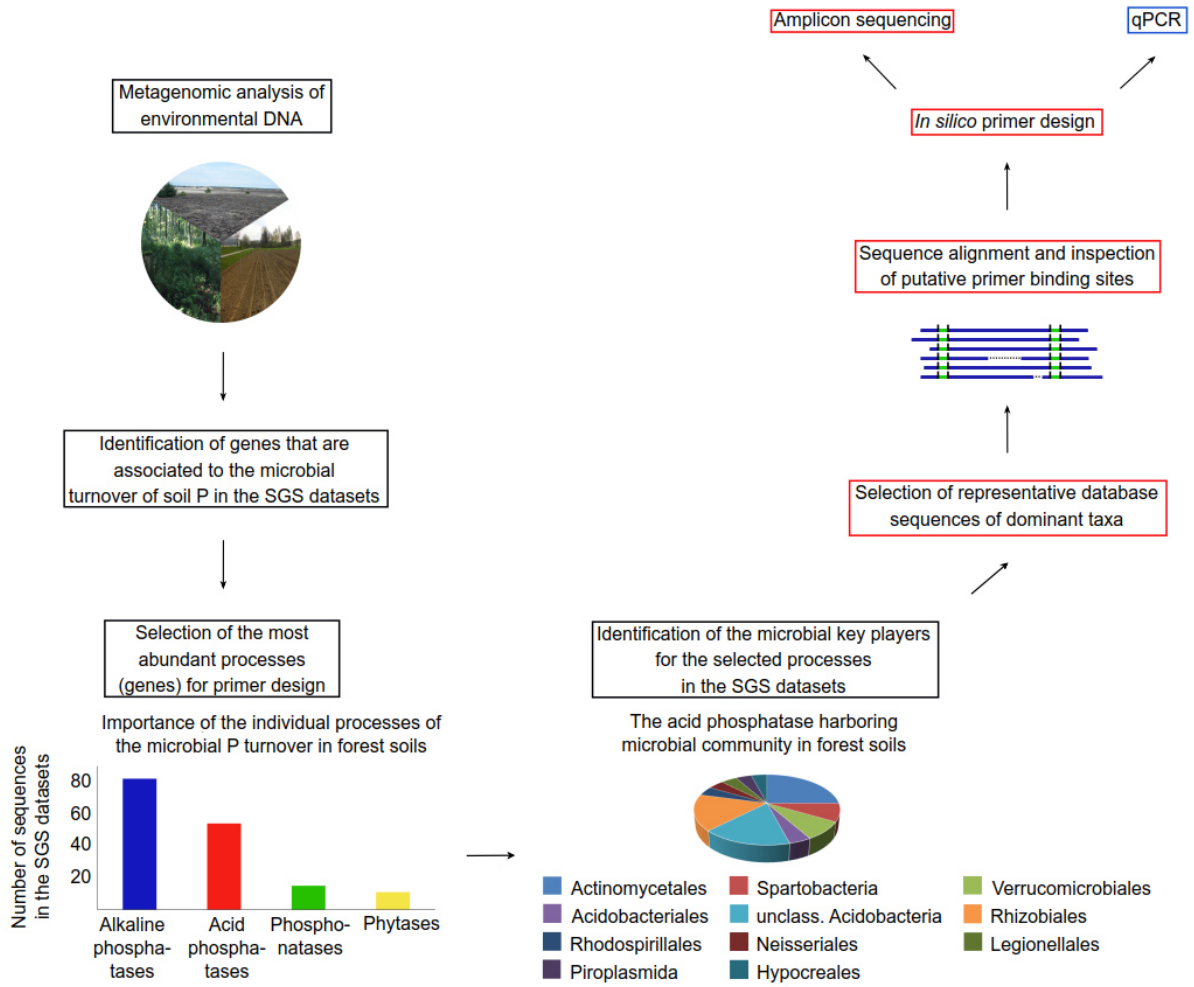

Figure 4 Structure of the introduced two-phasic approach for the design of oligonucleotide primers. The initial identification of processes (i.e. genes) that are associated to the microbial P turnover in two different forest soils was accomplished by shotgun sequencing of total genomic DNA. The microbial key players of the most important (i.e. most abundant) processes were identified by comparison of sequencing data against a curated public database (black boxes; phase one). Database sequences of representative microorganisms that harbored the respective genes in the different forest soils were aligned and used for *in silico* oligonucleotide primer design (red boxes; phase two). Subsequent amplicon sequencing of total genomic DNA confirmed the primer specificity. The designated downstream application of the oligonucleotide primers is the real-time quantification of target genes in environmental samples (blue box).

1.4 Performance of the introduced primer design pipeline

Prior to application of the newly designed oligonucleotide primers in quantitative real-time PCR approaches, the specificity towards the respective target genes was verified (**M3**). In fact, the occurrence of unspecific amplicons would cause an overestimation of the gene abundance due to false-positive signals (Sundquist, 2005). As revealed by Illumina amplicon sequencing of genomic DNA from the organic layer of site BBR, the primers preferably amplified DNA from the respective target genes, since the specificity generally reached ninety-eight percent or more. Sole exception in this regard was the acid phosphatase class A (*phoN*), where a marginal fraction of unspecific amplicons was detected (13%). Unfortunately the metalloenzyme Enolase (EC 4.2.1.11), which is involved in the glycolysis, comprises several conserved amino acids within the signature sequence motif of class A acid phosphatases (G-S-Y-P-S-G-H-T) (Rossolini et al., 1998). Still, the latter conserved region was targeted by the *phoN* primers, since alternative putative primer binding sites were lacking in the alignment.

Regarding the *phoD* primers, three distinct amplicons were detected after PCR that differed by approximately 60 bp, but nonetheless uniformly represented the target gene. The group of Ragot et al. (2015) did not report the occurrence of distinct amplicon sizes, while their primer system targeted a different section of the *phoD* gene. Thus, the variable region is likely to be situated in the upstream region of the PhoD conserved domain (W-D-D-H-E), which is involved in the coordination of metal ions in the PhoD active site of *Bacillus subtilis* (Rodríguez et al., 2014). The length variation in the PhoD polypeptide chain likewise comes into view by visual inspection of the "Clusters of Orthologous Groups" number 3540 within the Conserved Domain Database (Marchler-Bauer et al., 2015). While the corresponding PhoD protein of *Caulobacter crescentus* comprises 564 amino acids, the respective enzyme of *Corynebacterium glutamicum* is significantly shorter (516 amino acids). In this context, the three different amplicon sizes were likewise affiliated to distinct *phoD* harboring microbial communities (**M3**). After all, the introduced *phoD* primers amplified the target gene from fifteen distinct bacterial and fungal phyla, which is comparable to the results described by Ragot et al. (2015). While the authors detected a slightly lower diversity of thirteen bacterial phyla using their primer system on six different grassland soils, the sequencing depth was limited on the other hand. In comparison, the primer system by Sakurai et al. (2008) merely amplified *phoD* genes from six bacterial phyla using the same soil samples as Ragot and colleagues (2015). In accordance with Tan et al. (2013), the latter primer system produced an amplification bias leading to an overrepresentation of Alphaproteobacteria in the datasets (Ragot et al., 2015). Obviously the amplified microbial diversity was strongly affected by the sample type per se. Recently, Ragot et al. (2017) performed *phoD* amplicon sequencing of thirty different soil samples, comprising forest and grassland ecosystems with distinct soil types, vegetation and climatic conditions. This time the authors were

able to amplify the target gene from sixteen microbial phyla, including one archaeal and two fungal phyla. Although archaeal sequences were lacking in the underlying amplicon datasets of this study (**M3**), this does not necessarily constrain the performance of the newly introduced *phoD* primers. Taking into account, that merely one single soil sample was applied for amplicon sequencing, the covered microbial diversity is likely to increase when several samples from distinct ecosystems are compared.

To sum up, the amplicon sequencing approach (**M3**) basically reproduced the results obtained by SGS (**M1**) to the greatest extent. The introduced oligonucleotide primers reliably covered the designated microbial key players of the individual processes, while the relative abundance of specific taxa varied slightly between both approaches. Most probably this referred to the investigated sample type (organic layer or mineral topsoil of beech forest soil), since microorganisms are known to be stratified vertically along the different soil horizons (Baldrian et al., 2012). Beyond that, the diversity analysis using the newly designed oligonucleotide primers (**M3**) revealed a tremendous contribution of various low-abundant taxa to the processes of soil P turnover, which were not detected during SGS (**M1**). Consequently, the constraints of SGS were overruled by targeted, deep amplicon sequencing.

Noteworthy, the covered microbial diversity varied substantially among the investigated genes (**M3**). While *phoD* and *pitA* were amplified from fifteen, respectively, thirteen microbial phyla, the lower limit of two amplified bacterial phyla was observed for the phosphonatase (*phnX*) and the histidine acid phosphatase (*appA*). Regarding the latter one, the low diversity was barely influenced by the primer system itself, but rather by the applied database and the low number of curated sequences. According to Jorquera et al. (2008) database entries of corresponding proteins are restricted to members of Alpha-, Delta-, Gammaproteobacteria and Acidobacteria, which were already covered by the *appA* primers. Although the introduced oligonucleotide primers represent a valuable tool for an in depth diversity analysis the accurate annotation of datasets depends on reliable databases, which still appears as the bottleneck of data evaluation (Hugenholtz, 2002; Nilsson et al., 2006). Thereby the validity of sequencing approaches is constrained to some extent.

In contrast to the histidine acid phosphatase, the reduced diversity of amplified *phnX* genes was actually induced by the primer system itself. The corresponding phosphonatase (PhnX) is a member of the Haloacid Dehalogenase (HAD) superfamily of aspartate-nucleophile hydrolases. Yet, all known phosphonatases comprise a modified version of the HAD conserved catalytic motif, which comprises four amino acids (D-12, A-14, T-16 and D-186) (Morais et al., 2000). Besides this catalytic motif, however, the individual members of the HAD superfamily show very weak sequence similarity (Morais et al., 2000). In consequence, the number and especially the diversity of the aligned sequences had to be reduced during primer design, thereby providing the opportunity to play off the

major strength of the introduced pipeline. As revealed by metagenomics (**M1**), *phnX* was exclusively harbored by members of Proteobacteria and Firmicutes in the investigated soils. Consequently, merely sequences of the latter phyla were included in the alignment to reduce the degeneracy and ensure primer specificity (**M3**).

In conclusion, solely the primer systems of Sakurai et al. (2008) and Ragot and colleagues (2015) may benchmark the performance of the introduced pipeline, since further primers targeting processes of the microbial soil P turnover are lacking in literature. The former primer system was clearly outperformed regarding the amplified microbial diversity in soil samples, while the latter was on a par with the newly introduced primers. Ragot et al. (2017) recently developed another primer system for amplification of the alkaline phosphatase PhoX (*phoX*) from environmental samples. Since *phoX* was not detected during SGS (**M1**), the gene was obviously low abundant if not absent in the two forest soils and consequently was omitted from primer design.

Once the specificity as well as the covered microbial diversity was verified (**M3**), the primers were used for downstream application in qPCR approaches (**M5**). In this respect, the quantification of the *phoN* gene abundance succeeded readily in samples that were derived from the organic layer and the mineral topsoil of the two forest sites BBR and LUE. Following this, the prospective high-throughput implementation of the entire primer set enables the quantification of microbial potentials (based on DNA) and activities (based on mRNA) on the field-scale, thereby addressing questions of spatial and temporal heterogeneity regarding the microbial turnover of soil P.

In summary, the introduced pipeline represents an effective and straightforward strategy for the development of oligonucleotide primer systems that enable the specific amplification of target genes from a broad diversity of distinct microorganisms in environmental samples (**M3**). The pipeline is more than helpful in case of poorly conserved proteins, since it specifies which taxa to focus on whereby the degeneracy is reduced. The two-phasic approach ensures that the target genes of all microbial key players that are actually present in an investigated sample are amplified. In principle, the entire set of primers was designed for application in the two forest soils that were also used for SGS (**M1**). As shown by Ragot et al. (2015; 2017) degenerated primers might generally be suitable for a range of comparable ecosystems. However, it has to be considered, that predominantly target genes of ubiquitous, dominant microorganisms are amplified, while other taxa that are specific to a certain environment might remain undiscovered.

2. Microbial community structure in soils of beech forest ecosystems along a P geosequence

2.1 Adaption of soil microbial communities along the P geosequence

The bacterial communities in the five soils of the P geosequence were distinctly separated into three individual clusters as revealed by diversity analysis of the 16S rRNA gene (**M2**). In a principal component analysis, the bacterial communities were separated alongside principal component 1 according to the order of the P geosequence. The bacterial communities of the endmembers BBR and LUE were significantly separated, while the respective communities of the soils with median P stocks (CON, MIT and VES) were located in-between. Inherently, this separation was attributed to changes in the relative abundance of specific taxa and, to a lesser extent, to the occurrence of unique operational taxonomic units in a certain soil. The latter was especially true for site LUE (12 unique OTUs), while in BBR merely two unique OTUs were detected. In general, site LUE can be considered as a fairly nutrient poor soil regarding the absolute contents of soil C, N and P. Moreover, the extractable fractions of DOC, DON and bioavailable-P are consistently limited (**M4**). Accordingly, canonical correspondence analysis based on bacterial community fingerprinting demonstrated the separation of the LUE community from the remainder due to the restricted nutrient contents (**M2**). In this context, the strongly increased abundance of certain OTUs in this particular soil, underlined the special adaption of the microbial community to the site specific conditions. These findings are in accordance with results obtained by SGS (**M1**), since the total microbial diversity was higher in LUE compared to site BBR. Especially the archaeal and fungal diversity was increased, while the number of detected bacterial orders was slightly reduced at site LUE. Notably, this pattern was consistent during SGS data analysis, irrespective of the applied database (i.e. NCBI Non-redundant protein or SILVA SSU database). That being said, the marked overall domination of Planctomycetales and Acidobacteriales at site LUE was attracting attention. Particularly the members of the latter order are known to effectively cope with nutrient limitation, while their abundance is negatively correlated to the soil C supply (Ward et al., 2009; Fierer et al., 2007).

In addition, the significantly higher abundance of fungal sequences in the SGS datasets of site LUE (**M1**), in combination with increased ratios of C_{mic}:N_{mic} and C_{mic}:P_{mic} indicated a stronger fungal contribution to the microbial biomass at site LUE, compared to site BBR. In general, the fungal abundance is correlated to the ratio of soil C:N (Mouginot et al. 2014; Fierer et al., 2009), which is increased at site LUE. However, the ecosystem nutrition strategy and the adaption of the microbial community to the characteristic nutrient supply in the latter soil, might be of relevance as well. In this regard, especially the group of ectomycorrhizal fungi (EMF) is of central importance for the P

nutrition of beech trees (Zavišić et al., 2016), due to their vital role in mobilization of soil P (Read and Perez-Moreno, 2003; Habib et al., 2013). Accordingly, an increasing impact of EMF with ongoing soil P depletion stands to reason. While Zavišić et al. (2016) detected a tremendous rate of root tip colonization at all five sites of the P geosequence, the anticipated relationship between decreasing soil P contents and the EMF species diversity and the extent of hyphal soil exploration was not observed. After all, the EMF community structure at site LUE was significantly different from those of sites CON, MIT and VES. In contrast, the EMF community at site BBR showed the largest functional diversity regarding the exploration types and therefore overlapped with the remaining sites (Zavišić et al., 2016). The significant separation of the EMF community at site LUE from the remainder was also reflected on bacterial level (**M2**). An in-depth comparison of the EMF species diversity as described by Zavišić et al. (2016) with SGS data (**M1**) was hampered by the limited quantity of obtained fungal sequences. However, on phylum level the majority of detected fungal sequences at sites BBR and LUE corresponded to members of Ascomycota and Basidiomycota. Both groups comprise a variety of EMF species (Tedersoo et al., 2010) and are known to be the dominating groups in forest soils (Buée et al., 2009). In this respect, the fungal species diversity was slightly higher in LUE, where forty-three different orders were detected compared to thirty-eight orders in BBR. This is in contrast to Zavišić et al. (2016), who demonstrated the highest cumulative EMF species richness at site CON, followed by BBR, VES, LUE and MIT. Since both, SGS data (**M1**) and the analysis of Zavišić et al. (2016) were based on identical soil samples, the observed differences might be primarily caused by the methodological background. After all, the ITS amplicon sequencing approach of Zavišić et al. (2016) focused on fungal DNA derived from root tips and included samples from the soil organic layer as well as the mineral topsoil. Moreover, the DNA extraction procedure as well as the applied database differed substantially compared to the SGS approach (**M1**), which focused on the mineral topsoil exclusively while root material was excluded. Notably, the authors detected saturation after not more than eight EMF species at site BBR, respectively, six in LUE. Consequently, the SGS datasets (**M1**) most probably comprised further mutualistic fungal groups (ericoid or arbuscular mycorrhiza) (Read and Perez-Moreno, 2003) as well as saprotrophic and parasitic species (Buée et al., 2009).

In addition to the characteristic and unique microbial community composition at site LUE, the soil was isolated from the remainder by the considerably lowest abundance of 16S rRNA genes (**M2**). This in turn was significantly affected by the reduced contents of soil total C, N and P at site LUE. Interestingly enough, the highest number of 16S rRNA genes was detected at site CON, although total nutrient contents reached maximum in BBR. However, these findings were in accordance with data on microbial biomass C, since highest values were measured at site CON again, followed by BBR and ending up in LUE. The same pattern was observed for microbial biomass N and P values. Regarding the methodological uncertainties during measurement of P_{mic}, several different variations

of gaseous and liquid based fumigation consistently confirmed the P_{mic} gradient along the soils of the P geosequence (P_{mic}: CON>BBR>VES>MIT>LUE) (**M4**). Apparently, this is in contradiction to the P geosequence based on total-P stocks (BBR>MIT>VES>CON>LUE) (**M6**). Remarkably enough, the quantities of DOC were rather comparable between sites BBR (mean of four technical replicates: 56.85 µg g^{-1} ± 14.82 µg g^{-1}) and CON (57.33 µg g^{-1} ± 3.06 µg g^{-1}), while the levels of DON and bioavailable-P at site BBR (DON: 57.62 µg g^{-1} ± 0.20 µg g^{-1}; mean of resin extractable P: 9.62 µg g^{-1} ± 1.52 µg g^{-1}) exceeded site CON (35.13 µg g^{-1} ± 0.21 µg g^{-1}; 1.48 µg g^{-1} ± 0.87 µg g^{-1}) and the remainder by far (**M4**). In fact, the concentration of bioavailable-P at site CON was rather in the range of site LUE (1.10 µg g^{-1} ± 0.73 µg g^{-1}), and significantly lower compared to sites MIT (1.92 µg g^{-1} ± 0.66 µg g^{-1}) and VES (5.83 µg g^{-1} ± 0.40 µg g^{-1}). Presumably, the increased ratio of DOC:bioavailable-P in CON enhanced microbial growth, while the P surplus in BBR was pointless due to the relative shortage of DOC. Therefore, the microbial biomass at site BBR seemed to be growth-limited by the soil C supply or by further environmental factors. In line with this, the increased ratio of microbial biomass C:P at site LUE was presumably likewise attributed to the soil nutrient supply, which affected the microbial community composition and the microbial growth strategy accordingly. The latter includes mechanisms like the intracellular storage of C surplus (Wilson et al., 2010), the extension of the cell size (Thingstad et al., 2005) or the reduction of the cellular P requirements (Van Mooy et al., 2009). Taken together, the results obtained by SGS (**M1**), by diversity analysis of the 16S rRNA gene (**M2**), and measurement of microbial biomass parameters (**M4**) consistently underlined the special adaption of the LUE microbial community to the site specific conditions, concerning the increased abundance of unique and oligotrophic taxa, the enhanced impact of fungal species, and the microbial biomass stoichiometry.

In a similar way, the microbial communities of sites BBR and CON were separated from the remainder. Although both soils represented contrasting stages of the P geosequence, the respective communities showed astonishing similarities, which indicated a common, selective pressure. Hence, the distinct contents of bioavailable- and total-P were of minor importance, while especially the elevated soil pH was a decisive factor (**M2**) (Lauber et al., 2009). The separation of the communities was once again influenced by a strongly increased abundance of specific OTUs, while the total number of detected OTUs was lower compared to the other sites. At least in BBR, where SGS data (**M1**) is available, the microbial community shifted towards more copiotrophic microorganisms. Taking the strong similarities during bacterial fingerprinting (**M2**) into account, the hereafter discussed trends presumably also apply to site CON. In BBR, especially members of Proteobacteria were highly abundant, while the opposite was true for Acidobacteria. After all, both soils (BBR and CON) were characterized by the highest contents of DOC along the five sites (**M4**). Thus, the findings were in accordance with Smit et al. (2001), who proposed a correlation between the ratio of

Proteobacteria to Acidobacteria and the trophic level of soils. In this regard, the highest ratio is to be expected in nutrient rich environments. Even on global scale, the abundance of Protebacteria is positively correlated to the soil C availability (Fierer et al., 2007). In addition, a significantly increased abundance of Verrucomicrobia was detected in the SGS datasets (**M1**) of site BBR. According to Buckley and Schmidt (2001), their abundance is positively correlated to the soil moisture content. In this regard, the soil texture of site LUE and the high content of sand in the mineral topsoil might be of vital importance. In addition, however, also a negative correlation of Verrucomicrobia and Gemmatimonadetes with the contents of total C and N was recently described. Still, this effect is overruled by the positive correlation with soil pH (Zhalnina et al., 2015), which might explain the increased abundance of the latter phyla at site BBR. Interestingly, also the abundance of Chloroflexi was significantly enhanced in BBR, although members of this phylum are known as oligotrophic organisms that prevail in nutrient poor soils (Ding et al., 2013). Especially the N level is of relevance (Zhang et al., 2014), while the abundance is negatively correlated to soil pH. Consequently, a converse pattern would be expected at the sites BBR and LUE (Zhalnina et al., 2015). Apart from this, the significantly reduced abundance of Acidobacteria in BBR was attributed to the elevated soil pH in this soil (Jones et al., 2009). On balance, these observations emphasized an adaption of the microbial communities at site BBR to the site specific soil conditions, which presumably is likewise true for site CON.

Finally, the observed clustering of the bacterial communities at sites MIT and VES (**M2**) came as no big surprise. According to the position of the sites within the P geosequence, both soils are characterized by intermediate stocks of total C, N and P. Likewise the extractable fractions of DOC, DON and bioavailable-P (**M4**) do not suggest nutrient limitation, while the intermediate soil pH is without significant relevance. Consequently, microbial growth is neither limited as in LUE nor enhanced as detected at site CON. This assumption was corroborated by the moderate abundance of 16S rRNA genes (**M2**) and the values of microbial biomass C (**M4**). Both values were situated in between the respective endmembers of the P geosequence. Regarding the bacterial community composition, a specific adaption as described for sites BBR and LUE in terms of relative shifts towards either copiotrophic or oligotrophic taxa is unlikely. Although the highest number of OTUs was detected at site MIT (**M2**), an enrichment of specific or unique taxa was not observed. As revealed by correlation analysis, the most abundant OTUs in the two soils showed no clear correlation to soil nutrient contents or pH. Consequently, the microbial communities at sites MIT and VES might be similarly shaped by another variable instead, like the soil texture. Recently, Lauber et al. (2008) detected the strong impact of the soil texture on microbial community composition, which predominantly refers to the soil moisture regime and the nutrient status. In fact, Girvan et al. (2003) described the soil type as the key factor for microbial community composition in arable soils. The soil

texture at sites MIT and VES might uniformly shape the underlying communities to some extent and moreover explain the slight similarities to the bacterial community at site CON. After all, the texture of the three soils is characterized as loam. Therefore, the sites MIT and VES presumably represent microbial communities typical of European forest soils, without special adaptations to the ecosystem characteristics. Besides the dominating bacterial phyla that were detected during SGS (**M1**) and in previous studies (Baldrian et al., 2012), this might comprise an underrated diversity of saprotrophic, parasitic and mycorrhizal fungi (Buée et al., 2009). In conclusion, the soil microbial communities were impressively separated along the P geosequence, except for site CON (**M2**). Although this separation was barely influenced by the soil P stock per se, particularly the microbial communities of ecosystems with disparate nutrition strategies (BBR and LUE) were distinctly separated.

2.2 Bacterial core microbiome in soils of beech forest ecosystems

Diversity analysis of the 16S rRNA gene revealed the existence of a considerable bacterial core microbiome in the five soils of the P geosequence, in defiance of the site specific adaptations (**M2**). In this regard, the core referred to all OTUs that were common to the analyzed microbial communities, while the relative abundance was not considered (Shade and Handelsman, 2012). Since the investigated soils were derived from ecosystems with contrasting nutrition strategies, parent materials, nutrient stocks, soil types and climatic conditions, and moreover taking the strong and indisputable impact of the soil physical and chemical parameters as well as the soil structure on community composition into account (Rousk et al., 2010; Pett-Ridge and Firestone, 2005; Torsvik and Øvreås, 2002), the occurrence of a stable core of microorganisms was remarkable. As revealed by SGS (**M1**), even the end members of the P geosequence were similarly dominated by the identical microbial groups. Apparently, the five communities (**M2**) were consistently shaped by a ubiquitous, pervasive variable. Presumably, this applied in particular to the type of ecosystem and to the main tree species. The latter affects the soil microbial community by various extents including rhizodeposition, selective interactions with symbionts or alteration of the soil microclimate (Urbanová et al., 2015). In addition, the tree specific composition of the leaf litter is of importance for the composition of soil microbial communities (Prescott and Grayston, 2013). In line with this, Urbanová et al. (2015) demonstrated the strong impact of the tree species on both, the soil bacterial and fungal community composition in a common garden experiment. The observed effect was more pronounced for fungal species, while the influence of further soil properties was overruled. Consequently, the similar main tree species along the sites of the P geosequence was likely to trigger the formation of a common bacterial core microbiome (**M2**). The site specific soil characteristics provoked further adaptions of the bacterial communities regarding the relative abundance of taxa or the occurrence of unique OTUs. Generally, the impact of unique OTUs on community composition

was lower, compared to the common OTUs. Nevertheless, particularly rare species might serve as keystone species with overproportional strong effects on ecosystem function, as they provide a tremendous reservoir of genetic and functional diversity. This predominantly refers to the site specific ecosystem nutrition strategies, in terms of soil nutrient turnover and fixation (Lynch and Neufeld, 2015).

For fungi, the existence of a stable core mycobiome was recently shown in a variety of distinct soils, irrespective of the ecosystem type (Orgiazzi et al., 2013). While this mycobiome was hardly triggered by the vegetation itself, the core size was limited to few, generalist species with oligotrophic and chitinolytic traits in turn (Orgiazzi et al., 2013). Taking the importance of spatial heterogeneity in soils into account, the detected bacterial core microbiome along the P geosequence (**M2**) is primarily restricted to the investigated topsoil horizon and might be downscaled accordingly when considering each soil as a whole. As especially the P nutrition of P-depleted forest ecosystems strongly relies on recycling processes in the forest floor, in contrast to P acquiring systems (Lang et al., 2016), the ecosystem nutrition strategy might affect the size of the core microbiome as well, when comparing the different soil horizons per forest site. In this respect, Uksa and colleagues (2015) reported a high degree of microbial vertical stratification within distinct soil compartments including bulk, rhizosphere and drilosphere. Particularly in the subsoil horizons, the availability and quality of nutrients shaped individual, well-defined microbial clusters. Still, the authors detected an intrinsic bacterial core microbiome of moderate size throughout the different soil compartments, while the archaeal core backbone was less variable instead. In conclusion, despite certain limitations, the occurrence of a bacterial core microbiome in the mineral topsoil of spatially separated European beech forests could be demonstrated.

2.3 The impact of the soil P stock on microbial community composition: Is it overprized?

The essential role of P for all kinds of cellular life on our planet is beyond any question (Elser, 2012). Since the P availability in terrestrial ecosystems is severely restricted (Holford, 1997), P is considered as a major growth limiting factor for plants (Schachtman et al., 1998). In contrast, microbial life primarily tends to be limited by the soil C supply. In a recent comparison of twenty-eight different forest and grassland soils, Demoling et al. (2007) determined C as the major factor limiting microbial growth, while P surplus had little effect and N addition commonly decreased growth instead. This findings are in line with Wallenstein et al. (2006), who reported a consistent decrease in the soil microbial biomass and likewise in the fungal to bacterial ratio due to long-term N application in a cross-site comparison of different forest stands. In addition, Ramirez and colleagues (2010)

illustrated the contrary effect of continuous N fertilization on specific microbial groups in grassland and agricultural soils. While the relative abundance of Gammaproteobacteria and Actinobacteria was positively correlated to N inputs, the opposite was true for Acidobacteria, Cyanobacteria and Nitrospira. At the same time, the richness and the diversity of the bacterial communities was not altered significantly. Regarding the soil P content, the group of Wakelin et al. (2012) revealed merely a weak, inconsistent effect on the total bacterial and fungal community structure in pasture soils under different P fertilization regimes. However, in more detail, significant changes within the Actinobacteria and Pseudomonas group were detected as a function of the soil P content, while other taxa like Alphaproteobacteria were unaffected. In the same way, primarily the community of arbuscular mycorrhizal fungi was severely changed depending on the soil P content. In particular, those taxa which are integral to the biogeochemical turnover of soil P were affected by the selective pressure of the soil P content, while the total bacterial and fungal community remained largely stable (Wakelin et al., 2012). In this context, Wakelin et al. (2012) assumed that Alphaproteobacteria serve as a reference group, which is less dominated by taxa that are associated to the turnover of soil P. This is in contrast to the work of Ragot et al. (2015), who detected a strong genetic potential for ALPs in Alphaproteobacterial genomes derived from public databases. Moreover, as revealed by SGS (**M1**), members of the latter phylum were strongly associated to the different processes of microbial P turnover in forest soils.

Nonetheless, several further studies uniformly reported the significant impact of the soil P content on the frequency and the composition of P mobilizing soil microorganisms (Sakurai et al., 2008; Mander et al., 2012; Wakelin et al., 2012). In this regard, a reduced diversity of the PhoD harboring microbial community was detected in unfertilized control samples compared to P fertilized soils. Especially the members of the Pseudomonas group were selectively enriched (Wakelin et al., 2012; Tan et al., 2013). Moreover, also the way of fertilization (chemical or organic) severely affected the PhoD harboring bacterial community as well as the respective level of phosphatase activity (Sakurai et al., 2008). In addition, Tan and colleagues (2013) detected a general shift in the total bacterial community composition due to P fertilization. The same trend was also detected in unfertilized forest ecosystems by SGS (**M1**). While the relative abundance of oligotrophic Acidobacteria was highest in the P-depleted soil, the copiotrophic members of Alphaproteobacteria were enriched in the soil with higher P stocks. In conclusion, the impact of the soil P content on the total microbial community composition as well as on the group of P mobilizing microorganisms is generally accepted. However, it has to be considered that the majority of data was derived from manipulation experiments where elevated levels of soil P were generated through chemical fertilization.

On that account it came as no big surprise that the soil P content did not significantly affect the soil microbial community structure in close to nature forest ecosystems with native, distinct soil P

contents (**M2**). After all, the stocks of total-P differed by a factor of fifteen within the five soils of the P geosequence. More to the point, the levels of bioavailable-P differed substantially by a factor of sixteen between the respective endmembers BBR and LUE (**M4**). Still, as revealed by fingerprinting of the 16S rRNA gene (**M2**), the impact of the soil P content on the bacterial community structure was not significant (P<0.085), which was likewise true for the contents of total-C and N (P<0.233; P<0.133). Merely soil pH was significantly affecting the bacterial community composition along the P geosequence (P<0.003).

In contrast, the bacterial abundance was strongly correlated to the stocks of soil total P (P<0.004), respectively C (P<0.001) and N (P<0.004).

The bottom line is that previous results on the impact of the soil P content on microbial community structure should be taken with a grain of salt. The general transferability of data that was derived from P fertilization experiments to natural, unmanipulated ecosystems is inadvisable, since the observed effects might be overpowered by other biotic and abiotic factors.

3. Microbial P turnover in forest soils with distinct P stocks

3.1 The significance of gene regulation induced by P starvation

For the reconstruction of the microbial P turnover in forest soils, exclusively the two endmembers of the P geosequence were analyzed. The aim was to cover the entire range of microbial processes that either predominate in a P acquiring or in a P recycling ecosystem. In essence, a stronger relevance for the microbial solubilization of mineral-P was assumed at the P-rich site BBR, while in LUE the effective recycling of organic-P ought to be of major importance. For a start, shotgun sequencing of metagenomic DNA underlined the prominent role of microorganisms for the turnover of soil P in forest ecosystems (**M1**). The entire set of microbial traits that are known from literature to be associated to the processes of organic-P mineralization, inorganic-P solubilization and intracellular P uptake were detected at sites BBR and LUE. This comprised about 0.82% of functionally annotated sequences, which is likewise typically detected for the genes of the microbial nitrogen cycle in soil derived datasets (Fierer et al., 2012). Interestingly, Fierer et al. (2012) detected a twofold increased relative abundance of genes that are related to the microbial phosphorus metabolism in grassland and agricultural soils, compared to the SGS datasets of the underlying study (**M1**). However, this apparent contradiction is mainly caused by the applied data analysis pipeline. The work of Fierer and colleagues (2012) was based on the SEED environment, where functional roles are grouped into subsystems inasmuch as they implement certain pathways or structural complexes (Overbeek et al., 2005). The respective subsystem detected by Fierer et al. (2012) is not restricted to the processes of P mobilization and uptake, but moreover includes a wide range of mechanisms that are involved in the cellular P metabolism. The latter processes were disregarded during SGS data analysis (**M1**), while the focus was on the turnover processes of soil P exclusively.

Apart from that, metagenomics highlighted the major importance of effective P_i uptake systems for soil microorganisms, irrespective of the soil type. The genes encoding the highly efficient Pst transporter, which is likewise involved in extracellular P signaling, were highly abundant compared to the processes of P mobilization. This was especially true for site LUE, where likewise an increased genetic potential for a second type of P_i transporter (PitA) was detected. These findings presumably are attributed to the soil P supply itself. After all, the quantity of bioavailable-P is severely restricted in LUE (**M4**). Thus, the Pst system is vital to ensure a sufficient P supply for microorganisms in the competition with plants (Yuan et al., 2006; Schachtman et al., 1998). This finding gives direct evidence to the conceptual model of Lang et al. (2016), since particularly in P-depleted soils the tight recycling of P is highly important to prevent nutrient losses from the ecosystem. Moreover, the strong genetic potential for P_i uptake systems might also refer to the high energy consumption,

which is required during protein biosynthesis (Russell and Cook, 1995). The microbial focus might rather be on the direct uptake of bioavailable-P from the soil solution, while the production of secreted, hydrolytic P mobilizing enzymes takes a back seat instead. In contrast to bioavailable-P, the uptake of soil organic-P in forms of glycerol-3-phosphates or phosphonates was of minor relevance in both soils, although especially the latter form constituted the majority of organic-P at site LUE (22%) (**M6**). The low genetic potential (**M1**) is presumably affected by the additional effort that is required during intracellular breakdown of these compounds prior to P_i release. As opposed to this, the relevance of the Pst transporter obviously correlated with the microbial TCS PhoR/PhoB, which is involved in Pho regulon control and consequently is integral to the cellular P supply. Under conditions of extracellular P_i limitation (<4 µM), the membrane-bound sensory histidine kinase PhoR promotes phosphorylation of the DNA-binding response regulator PhoB, which in turn acts as a transcription factor for the promotors of the Pho regulon (Hsieh and Wanner, 2010). Alternatively, if environmental P_i is in excess, the periplasmic-binding protein (PstS) of the Pst transporter is fully saturated, whereby the conformation of PhoR is changed and the phosphorylation of PhoB is inhibited. Thus, under P_i saturation the Pst transporter serves as the primary route for cellular uptake, while during P_i limitation the expression of the Pho regulon genes is induced, which enables P assimilation from a variety of different sources (Hsieh and Wanner, 2010). The particular importance of efficient P uptake systems and sufficient Pho regulon control for the microbial P nutrition is pronounced in the SGS datasets of both forest sites, irrespective of the soil P stock (**M1**).

3.2 The microbial mobilization of soil inorganic-P

With respect to the microbial solubilization of inorganic-P, a unidirectional impact of the soil P stock on the microbial P nutrition was proven by the significantly increased genetic potential for the quinoprotein glucose dehydrogenase (PQQGDH) at site BBR (**M1**). More precisely, this finding was attributed to the P species composition in the investigated soils, since the relative fraction of mineral-P is distinctly increased in the mineral topsoil of site BBR compared to LUE (**M6**)**.** The PQQGDH enables microorganisms to dissolve and assimilate P_i from mineral-P via the direct, nonphosphorylating oxidation of glucose in the periplasmic space (Goldstein, 1995). It should be noted, that the latter process represents merely one of several options for the mps phenotype in bacteria (Rodríguez et al., 2006). Principally this trait is attributed to the secretion of low molecular weight organic acids by bacterial or fungal species (Khan et al., 2014). However, these findings indicate that the microbial P nutrition strategy was affected by the soil P stock itself. This is in line with the feedback loop that is assumed between the soil P characteristics and the ecosystem P nutrition strategy (Lang et al., 2016): On the one hand the parent material, in terms of P speciation and allocation, initially controls the evolution of plant and microbial nutrition strategies. On the other

hand, the arising microbial traits and their demand for P shape soil properties and P pools again. Alien from Odum's hypothesis (1969), which describes the tightening of nutrient cycles in ecosystems as a result of maturity, the authors hypothesize that rather the soil P status than maturity itself is affecting the tightness of P cycling. Consequently, even mature ecosystems on P rich soils (e.g. site BBR) can be characterized by an acquiring strategy, whereas P cycling tightens with ongoing soil P depletion (e.g. site LUE) (Lang et al., 2016). From the microbial point of view, a significantly higher potential for P acquisition was detected in the mineral-P rich soil at site BBR. Certainly, the general significance of this finding still is restricted, due the limited amount of analyzed samples and obtained gene sequences (**M1**). However, the quantification of the microbial mps phenotype using the introduced *gcd* primers (**M3**) in qPCR approaches for ecosystems with contrasting stocks of mineral-P would further address the posed assumption.

3.3 The microbial mobilization of soil organic-P

With regard to the hydrolytic enzymes that are involved in mineralization of soil organic-P, the strongest genetic potential was detected for the different types of alkaline and acid phosphatases (**M1**). Surprisingly, the respective gene abundance was highest at site BBR in either case, although effective P recycling was rather expected at site LUE. Regarding the abundance of NSAPs, the higher potential in BBR was not necessarily linked to the microbial P nutrition strategy or the soil P content per se. On the one hand, acid phosphatases commonly are regulated in a P_i irrepressible manner, which decouples gene expression from the soil P supply (Rodríguez and Fraga, 1999). On the other hand, this type of enzyme performs an unspecific degradation of various organic phosphomonoesters, whereby P_i and further essential nutrients are provided to cells (Rossolini et al., 1998). Consequently, acid phosphatases are rather integral to the microbial nutrition per se, and not exclusively in terms of the P supply. At first glance, the relatively low abundance of acid phosphatase encoding genes in LUE is in line with the reduced level of actual phosphomonoesterase activity that was detected in this soil (**M6**). However, taking the role of plants and other soil biota in production of phosphomonoesterases, as well as the stability of enzymes that are immobilized to the soil matrix into account (Burns and Dick, 2002; Martin and Byers, 1976), the direct correlation between potential enzyme activity and SGS data (**M1**) might not be valid.

In contrast to acid phosphatases, the different families of ALPs usually are part of the Pho regulon (Wanner, 1993; Eder et al., 1996). Their expression is actively upregulated during P starvation to meet the microbial needs for P. Consequently, the respective gene abundance is likely to be affected by the soil P supply to some extent. Again, however, the highest gene abundance was detected at site BBR (**M1**). In general, the higher microbial potential for acid and alkaline phosphomonoesterases in the latter soil is presumably affected by the soil P characteristics. After all, an increased portion of

P monoesters, which exceeded the values of site LUE by a factor of two, was detected in the mineral topsoil of site BBR (**M6**). Accordingly, the microbial community adapted to this enrichment in terms of a higher genetic potential for phosphomonoesterases. Certainly, taking the importance of the forest floor for the P nutrition of recycling ecosystems into account (Lang et al., 2016), the situation might change completely in the litter- or humus-layer. On that note, the potential phosphomonoesterase activity generally reaches maximum in the uppermost forest floor and strongly decreases with ongoing soil depth (Pang and Kolenko, 1986). Taking into account, that the relative fraction of organic-P (related to total-P) is distinctly increased in the Oi- and Oe-layer of site LUE compared to site BBR (**M6**), the property of tight P cycling should be accompanied by an enhanced microbial potential, and most notably by an increased level of gene expression regarding the turnover processes of soil organic-P compounds, especially in the forest floor of site LUE.

Indeed, this assumption was confirmed for the acid phosphatase class A using qPCR (**M5**) and target gene (*phoN*) specific oligonucleotide primers (**M3**) for the amplification of total genomic DNA. Regarding the quantity of *phoN* genes in the humus layer and in the mineral topsoil of sites BBR and LUE, two basic trends were detected (**M5**). For a start, the strong effects of seasonality on the *phoN* gene abundance became visible, especially in the humus layer of site LUE. In addition, the assumed impact of the soil horizon on the gene abundance was validated. In this regard, the highest microbial potential for class A acid phosphatases was detected in the humus layer of site LUE in spring, during the stage of bud swelling in *Fagus sylvatica* L., while the humus layer of site BBR was clearly outreached. As expected, the *phoN* gene abundance varied markedly between the humus layer and the bulk soil of site LUE. After all, the gene copy number in the mineral topsoil of site LUE decreased by a factor of eight, while in BBR the effect was less pronounced (factor of three). These findings nicely demonstrate the dependency of P-poor ecosystems, especially on the forest floor. Moreover, these results underline the importance and likewise the strong microbial potential for tight cycling of organic-P in the humus layer of P-depleted soils (Lang et al., 2016). In the same way, the quantification of further microbial turnover processes of soil organic-P using qPCR and target gene specific oligonucleotide primer systems (e.g. *phoD*, *appA*, *phnX*), especially on activity level, might provide evidence for tighter P cycling in P-depleted soils. Throughout the season, the *phoN* gene abundance generally decreased in all soil horizons (**M5**). While the gene copy number still was highest in the respective humus layers of both sites, particularly at site BBR the gap between the humus layer and the mineral topsoil was almost filled. In contrast, the *phoN* gene abundance in the mineral topsoil of site LUE was consistently lagging behind. Soon after a slight increase of the *phoN* gene abundance in autumn, the gene copy numbers similarly collapsed during winter. The significantly lower *phoN* gene abundance in the mineral topsoil of site LUE compared to BBR in autumn, was in agreement with SGS data (**M1**), provided that results of the two NSAPs specific KEGG

KO numbers (K09474 and K01078) were pooled. As a consequence, it can be assumed that also the remaining processes of the microbial P turnover having a low abundance in the SGS datasets of the mineral topsoil (e.g. phosphonatases, phytases, phosphotriesterases), are likewise more abundant in the uppermost forest floor. Once again, this effect should be more pronounced at site LUE. However, it was necessary to focus on the mineral topsoil during SGS, to also capture the microbial potential for the solubilization of inorganic-P, which is considered to predominate in the deeper soil horizons.

Regarding the vertical separation of P mobilization processes, also the microbial potential for the degradation of diester-P is of interest. After all, the highest actual phosphodiesterase activity was reached in the forest floor of both sites again (**M6**), while a decrease was detected with ongoing soil depth. As revealed by SGS (**M1**), the respective gene abundance (*uqpQ*, *phnP*) was almost comparable within the mineral topsoil of sites BBR and LUE. In line with this, the level of the actual phosphodiesterase activity was not increased at site LUE (**M6**). The latter resulted in higher ratios of phosphomonoesterase to phosphodiesterase activity especially in the mineral topsoil, compared to site BBR. This finding might give an explanation for the enrichment of diester-P in the mineral topsoil of site LUE (**M6**). In this particular case, a direct effect of the soil P species composition on the respective microbial potential was not detected.

The opposite is true regarding the degradation of organophosphonates. The genes that code for both, the phosphonatase PhnX and the C-P lyase pathway were basically more abundant at site LUE (**M1**). Presumably, this is an adaption to the sevenfold higher portion of organophosphonates in the mineral topsoil of site LUE (**M6**).

In sum, the soil P content and the P species composition had a severe impact on the structure and the ability of the microbial soil P turnover. In accordance with the site specific adaptions of the microbial communities that were observed on taxonomic level (**M2**), also on functional level (**M1**) an adaption to the specific soil characteristics occurred, which resulted in an intrinsic, unique emphasis of the individual processes of the soil microbial P turnover at different forest sites.

3.4 Microorganisms driving soil P turnover: exclusively site specific adaptions or core community?

The microbial potential for the processes of soil P turnover at sites BBR and LUE was harbored by not less than fifty-five distinct microbial orders (**M1**). In particular, the members of Rhizobiales, Actinomycetales, Acidobacteriales as well as Solibacterales and Burkholderiales were of importance. Once the focus was on microbial P mobilizing enzymes exclusively, inasmuch as P transport systems were omitted, the relevance of Solibacterales and Planctomycetales was particularly enlarged. All in all, the latter six orders comprised seventy-three percent of the detected genes, which is a

consequence of their overall domination at the two sites. On that note, the previously described capacity of rhizobial strains for the mineralization of soil organic-P was impressively underlined also in close to nature ecosystems. However, further species that are known from literature for their acid phosphatase activity including the genera of Pseudomonas, Bacillus, Serratia or Enterobacter (Rodríguez and Fraga, 1999), did barely contribute to the P turnover in the two soils. Likewise the solubilization of inorganic-P, which is known to be performed by members of Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium or Micrococcus (Rodríguez and Fraga, 1999), was predominantly associated to the members of Solibacterales in the two soils BBR and LUE. Consequently, the implemented SGS approach (**M1**) partially verified the existing data, but more importantly unraveled the actual microbial key players in close to nature ecosystems.

Apart from the above-mentioned phyla, the impact of the remaining detected taxa to the processes of P turnover was of minor importance. From this point of view, the existence of a stable core of microbes that performs the turnover of soil P in distinct forest ecosystems sounds reasonable. However, the relative contribution of specific taxa to the individual processes varied substantially with respect to the different soils. In accordance with diversity analysis (**M1**), at site BBR particularly the members of copiotrophic Alphaproteobacteria harbored the majority of investigated genes (40%). However, the soil nutrient content was not the sole factor that influenced the composition of the P cycling microbial community. After all, the soil P species composition and hence the significantly higher potential for the solubilization of mineral-P at site BBR induced a stronger contribution of Solibacterales in the latter soil, since members of Solibacterales were discovered as the major source for PQQGDH encoding genes. In addition, several taxa exclusively contributed to the soil P turnover at site BBR, while their impact was lacking in LUE. This includes members of Gemmatimonadetes, Poribacteria, Ktedonobacterales and Firmicutes.

In contrast, the P cycling microbial community at site LUE was shaped more homogeneously (**M1**). Several different taxa were contributing to the individual processes of the soil P turnover in equal measure. While the prominent impact of copiotrophic taxa was dramatically reduced, the members of oligotrophic Acidobacteriales and Actinomycetales were of major importance. If the focus was exclusively on the alkaline phosphatase (PhoA, PhoD) harboring microbial community, a considerable decrease in diversity was detected from ten microbial orders in BBR to six orders with ongoing soil P depletion (**M1**), which is in accordance with the work of Tan et al. (2013). However, the authors reported an enrichment of members of the Pseudomonas group in P-depleted soils, while at site LUE particularly Burkholderial genes were dominating (**M1**). In this regard, Ragot et al. (2015) detected a deeply contrasting Burkholderial *phoD* gene abundance in distinct soil samples, whereby the singularity of the underlying microbial communities was determined. Taken together, these findings indicate an adaptation of the P cycling microbial community to the site specific conditions, regarding

resource limitation and soil pH (Jones et al., 2009; Ward et al., 2009). Likewise for site LUE, several taxa including Cytophagales, Rhodobacterales and Desulfobacterales were associated to the P turnover exclusively in this soil. Thus, the occurrence of unique OTUs at the respective sites, which was already detected during diversity analysis (**M2**), was similarly reflected on functional level in terms of an exclusive contribution of specific taxa to the P turnover in a certain soil (**M1**). However, taking the restricted sequencing depth during metagenomics into account, the description of unique taxa might be misleading. On the one hand, deep sequencing of soil metagenomic DNA might reveal an even higher degree of site specific adaption, regarding the microbial community structure and traits. After all, SGS (**M1**) merely scratched the surface of the entire soil metagenome (Tringe and Rubin, 2005). Consequently, the impact of unique taxa on the soil P turnover could still be underestimated. On the other hand, the theory of Baas Becking (1934) assuming that "*Everything is everywhere*, but, *the environment selects*", might apply on functional level as well (De Wit and Bouvier, 2006). Thus, exactly the same microbial community with identical traits could perform the turnover of soil P in distinct ecosystems. While the relative contribution of individual taxa could vary, unique taxa were rare if not lacking entirely.

In this regard, amplicon sequencing of P cycle associated genes brought new facts to light (**M3**). The quantity of obtained sequences exceeded the respective SGS results (**M1**) to a considerable degree (e.g. 117,161 vs. 38 *phoD* sequences) (**M3**, **M1**). Basically, the amplicon sequencing approach (**M3**) revealed a significantly higher diversity within the P cycling microbial community, since investigated genes from seventy-four different orders were detected compared to thirty-one orders in the SGS datasets (**M1**) of site BBR. This gap might to some extent be caused by the analyzed soil horizon (mineral topsoil or organic layer) since microorganisms are vertically highly stratified (Baldrian et al., 2012) and the forest floor is of peculiar importance for ecosystem P nutrition. However, the severely enhanced sequencing depth (**M3**) might still be the decisive factor in this respect. One the one hand, the microbial key players of the P turnover that were detected during metagenomics (**M1**) were also verified during amplicon sequencing, while moreover a tremendous number of genes from rare species were discovered with a low frequency (**M3**). In this regard, the majority of microbial orders (70%) that contributed to the P turnover at site BBR, had a low abundance (<1%) related to the total P cycle associated community. This weak, however important contribution of diverse taxa remained undetected during metagenomics (**M1**).

Still, it has to be taken into account that the covered microbial diversity strongly depends on the database background. As already discussed for the microbial community composition at the respective sites, also on functional level the fungal contribution to the turnover of soil P is certainly underestimated in the SGS datasets of sites BBR and LUE (**M1**). After all, amplicon sequencing

detected the contribution of eight Ascomycotal and Basidiomycotal orders to the mobilization of phosphomonoesters at site BBR (**M3**).

In conclusion, since amplicon sequencing was merely performed for one soil type (**M3**), the question regarding the existence of a stable core of P cycling microorganisms in distinct ecosystems cannot be answered ultimately. However, the latter approach demonstrated that the composition of the P cycle associated microbial community is more complex than it appeared during metagenomics. Certainly, the initially detected microbial key players are of major importance in terms of ecosystem P nutrition, since they perform the bulk of soil P mobilization. In contrast, the majority of low-abundant taxa presumably is part of the rare-biosphere. While their growth strategy is preferably *K*-selected, these taxa are substantial contributors to ecological resilience instead (Lynch and Neufeld, 2015).

4. Outlook and perspectives

In terrestrial ecosystems the progression of nutrient contents is contrary. While the fixed P content of initial ecosystems successively decreases, the N content depends on biological N_2 fixation or atmospheric deposition and consequently increases over time (Vitousek et al., 2010). In the same way, the C stocks prosper during ecosystem maturation. This succession eventuates in a state of N and P co-limitation in terrestrial ecosystems (Elser et al., 2007). In consequence, additional nutrient input presumably affects the entirety of ecosystem processes. However, the response pattern differs with respect to the habitat type. In this regard, tropical ecosystems which have not undergone glacial periods are more affected by P inputs. In contrast, tundra sites show a pronounced response to N addition instead, since they represent an earlier stage of ecosystem development (Elser et al., 2007). On that account, the response pattern of typical European beech forest ecosystems with distinct soil ages and nutrient stocks to P and N addition is of peculiar interest. In this context, a special focus is to be on the microbial feedback on temporal and spatial scale, due to their vital role in ecosystem N and P supply. In soils which are P-limited, the additional input of N presumably increases the rate of biological P mobilization, especially in compartments where C is not limited. This might affect both, the composition and the activity of the P cycle associated microbial community. Ultimately, the pool of biomass P would be increased, while the P turnover rate of the entire ecosystem is increased. On the contrary, in P-rich soils the additional input of N is without effect regarding the P cycle associated microbial community. However, the beneficial N supply might affect the structure of the entire soil microbiome in turn. If forest soils are fertilized with P instead, the anticipated effects on the microbial community are less pronounced. After all, the majority of forest ecosystems is limited by the N supply. In addition, the role of plants as drivers for the microbial P mobilization is of interest. By disruption of the plant C fluxes into the rhizosphere, the beneficial nutrient situation, which microorganisms have adapted to, is severely altered. Particularly in P recycling forest ecosystems, the shortage of available-C might strongly affect the rate of microbial P mobilization, due to the high energy consumption during breakdown of organic-P compounds. Moreover, the resulting competition of bacteria, fungi and plants for C but ultimately also for N and P, is likely to affect the composition and the activity of the entire soil microbiome. In this respect, the microbiome might adapt to the altered nutrient situation or new network structures could be established. Investigating the differential effect of N input into terrestrial ecosystems with or without simultaneous P addition on nutrient turnover processes in soils, helps to understand and maintain ecosystem service in future times of increasing atmospheric N deposition (Reay et al., 2008).

5. Conclusions

Microorganisms are integral to the turnover of soil P and severely control plant performance. While plant P uptake can be increased by mycorrhizal associations or hormonal stimulation, the P mobilizing activity of free-living microorganisms relies on the production and secretion of hydrolytic enzymes and organic acids (Richardson and Simpson, 2011). Due to these features, microorganisms play a prominent role in the entire process of ecosystem P nutrition. In this regard, the P nutrition strategy of forest ecosystems predominantly depends on the P status of the soil (Lang et al., 2016). Consequently, also the microbial P nutrition, in terms of the individual processes of P mobilization and uptake, should be affected by the soil P status as well. At the P-rich site BBR, the microbial nutrition strategy has adapted to the increased content of mineral-P, since the genetic potential for inorganic-P solubilization was significantly enhanced in this soil (**M1**). In contrast, the microbial potential for the mineralization of organic-P in the mineral topsoil of both forest sites does not permit definite inferences regarding the P nutrition. However, the quantification of the *phoN* gene abundance revealed a significantly increased potential for acid phosphomonoesterases in the humus layers of both sites, compared to the mineral topsoil especially during the growth season of *Fagus sylvatica* L. (**M5**). This trend was most pronounced at site LUE, whereby the strong dependency of this ecosystem on the forest floor is underlined. On condition that the remaining processes of organic-P mineralization are likewise more abundant in the forest floor of site LUE, this characterizes the latter site as a P recycling ecosystem, compared to the P acquiring site BBR (Lang et al., 2016). The microbial potential for soil P turnover was predominantly harbored by few, identical taxa in both soils (**M1**). Only deep amplicon sequencing revealed the complex structure of the P cycle associated microbial community, since a tremendous diversity and a large number of low-abundant taxa was detected alongside the microbial key players (**M3**). In contrast to the microbial P nutrition strategy, the total bacterial community composition was not affected by the soil P status (**M2**). Along the soils of the P geosequence, merely pH had a significant impact on the bacterial community structure. The content of soil total-P was of minor importance, while it merely affected the bacterial abundance instead (**M2**, **M4**). Thus, despite the distinct soil characteristics in terms of total C, N and P along the five forest sites, a considerable, stable bacterial core microbiome was detected in the respective soils (**M2**). This core microbiome was primarily shaped by the ecosystem type and the main tree species, and remained stable irrespective of the ecosystem P nutrition strategy.

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List of abbreviations

16S rRNA	Component of the small subunit of prokaryotic ribosomes
2-AEP	2-aminoethylphosphonic acid
^{33}P	Phosphorus-33
Al	Aluminium
ALP	Alkaline phosphatase
asl	Above sea level
ATP	Adenosine triphosphate
bp	Base pair
C	Carbon
Ca	Calcium
CCA	Canonical correspondence analysis
cm	Centimeter
C_{mic}	Microbial biomass carbon
C-P	Carbon-phosphorus
DEPC	Diethyl dicarbonate
DFG	Deutsche Forschungsgemeinschaft
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DON	Dissolved organic nitrogen
e.g.	Exempli gratia
EMF	Ectomycorrhizal fungi
Fe	Iron
g	Gram
G3P	Glycerol-3-phosphate
GRH	Growth Rate Hypothesis
HAD	Haloacid dehalogenase
HAP	Histidine acid phosphatase
HMM	Profile Hidden Markov Model
ICP	International Co-operative Program on Assessment and Monitoring of Air Pollution Effects on Forests
i.e.	Id est
IP_6	Inositol hexaphosphate
ITS	Internal transcribed spacer

K	Potassium
L	Litter layer
m	Meter
M	Molar
MBq	Millibecquerel
Mg	Magnesium
ml	Milliliter
mm	Millimeter
mps	Mineral phosphate solubilizing phenotype
mRNA	Messenger RNA
N	Nitrogen
ng	Nanogram
NKE	Negative control during DNA extraction
NKP	Negative control during PCR amplification
nM	Nanomolar
N_{mic}	Microbial biomass nitrogen
nmol	Nanomol
NMR	Nuclear magnetic resonance
NSAP	Nonspecific acid phosphatase
Oe	Soil organic layer (hemic)
Of	Soil organic layer (mouldered)
Oh	Soil organic layer (humous)
Oi	Soil organic layer (fibric)
ORF	Open reading frame
OTU	Operational taxonomic unit
P	Phosphorus
PCR	Polymerase chain reaction
PGPB	Plant growth promoting bacteria
Pho	Phosphate regulon
P_i	Orthophosphate
Pit	Phosphate inorganic transporter
P_{mic}	Microbial biomass phosphorus
pmol	Picomol
P_o	Organic phosphorus
poly-P	Polyphosphate

ppm	Part per million
PQQGDH	Quinoprotein glucose dehydrogenase
Pst	Phosphate specific transporter
QP	Quantitative pit
qPCR	Quantitative real-time PCR
RCC	Regularized canonical correlation analysis
rRNA	Ribosomal ribonucleic acid
SGS	Shotgun sequencing
t_0	Timepoint zero
TCS	Two-component system
t-RFLP	Terminal-Restriction Fragment Length Polymorphism
U	Enzymatic unit
µg	Microgram
µM	Micromolar

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Appendix

A Manuscript 1

environmental microbiology

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

Fabian Bergkemper,[1]* Anne Schöler,[1] Marion Engel,[2] Friederike Lang,[3] Jaane Krüger,[3] Michael Schloter[1] and Stefanie Schulz[1]

[1]*Research Unit Environmental Genomics and*
[2]*Scientific Computing Research Unit, Helmholtz Zentrum München, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany.*
[3]*Professur für Bodenökologie, Albert-Ludwigs-Universität Freiburg, Bertoldstr. 17, 79085 Freiburg i. Br, Germany.*

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 analysed the role of microbes in two beech forest soils with high and low P content by direct sequencing of metagenomic deoxyribonucleic acid. 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 data sets. 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 harboured 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 and 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-P, plant-P, occluded-P 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 and 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 and 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 and Simpson, 2011). Depending on the substrate, microbial enzymes releasing P from organic compounds can be classified into three distinct groups: (i) nonspecific phosphatases (phosphohydrolases), (ii) phytases and (iii) phosphonatases and C-P lyases (Rodríguez *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 and 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

Received 14 August, 2015; revised 13 November, 2015; accepted 11 December, 2015. *For correspondence. E-mail fabian.bergkemper@helmholtz-muenchen.de; Tel. +49 (0) 89 3187 3048; Fax +49 (0) 89 3187 2136.

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 were analysed 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 [Bad Brueckenau (BBR)] revealed more than 10 times higher Pmic values (105 μg P g^{-1}) compared with the P-depleted soil [Luess (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 with 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.

Phylogenetic annotation of metagenomic data sets

Six soil samples from two German forest soils were used for metagenomic sequencing. Three hundred eighty-eight megabases of data were generated in total using 454 technology. This corresponded to 1 122 938 filtered sequences with an average read length of 344 bp. All details of the sequencing run are summarized in Table S1. Subsampled data sets were phylogenetically analysed using BLASTN (Camacho *et al.*, 2009) against the SILVA SSU database (Pruesse *et al.*, 2007) and MEGAN (Huson *et al.*, 2011). The majority of assigned sequences referred to Bacteria (91.08%), followed by Eukaryotes (8.22%) and Archaea (0.70%). As only a small proportion of all reads (0.05%) could be aligned to the ribosomal database, analysis focused on phylum level exclusively. Both forest soils were dominated by Proteobacteria, Acidobacteria and Actinobacteria (Fig. S1).

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

For a broader characterization of the microbial communities of the two soils, subsampled data sets were aligned against the National Center for Biotechnology Information (NCBI) Non-redundant protein sequences (nr) database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz; October 2014). In total 464 438 sequences could be assigned. To detect global differences within the community structures of the samples, a principal component analysis was performed (Fig. S2). Depending on the soil type, a clear separation of the two forest sites was detected on order level. Principal Component 1 explained about 85% of the total variance within the metagenomic data sets. To estimate the coverage of microbial diversity, rarefaction analysis was performed based on subsampled, phylogenetically annotated reads. In Fig. S3, the number of annotated reads on order level is plotted against the amount of sequenced reads. The rarefaction curves showed a sufficient coverage of the microbial diversity for all six samples. Curves depicting biological replicates were comparable; overall a slightly higher microbial richness was detected in LUE. Phylogenetic annotation of sequencing reads highlighted 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 data sets. Most abundant fungal orders referring to Eurotiales, Agaricales and Hypocreales were dominating in LUE. See Table S2 for absolute number of sequences annotated to the most abundant microbial phyla, respectively orders, in the data sets. To detect significant differences within the microbial communities of BBR and LUE, the abundance of all annotated taxa was statistically compared. 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

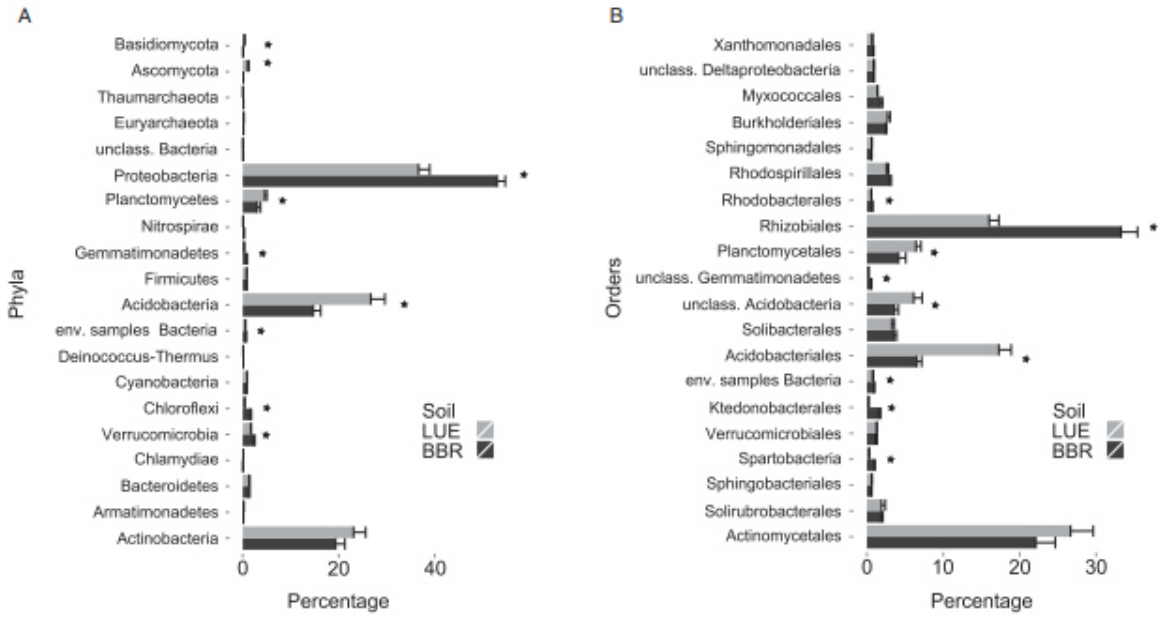

Fig. 1. Relative abundance of microbial phyla (A) and orders (B) in metagenomic data sets of two forest soils. Sequences were assigned using DIAMOND against the NCBI Non-redundant protein sequences (nr) database and MEGAN. Shown are the 20 most abundant taxa. Significant differences in the number of annotated reads among both sites are shown ($n = 3$). *$P < 0.05$.

the total number of assigned reads) were included. On phylum level, nine taxa were found to fulfil 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 two classes of fungi were detected. Both Eurotiomycetes and Agaricomycetes showed a significantly higher abundance in the P-depleted soil.

Functional annotation of metagenomic data sets

Functional annotation of metagenomic data sets was performed against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000). Based on subsampled data, 266 415 sequences were assigned and further analysed using MEGAN (Huson *et al.*, 2011). Genes encoding pathways for two-component systems, ABC transporters and purine metabolism were most abundant in both soils (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 hydrolysing phosphoester bonds) which are involved in metabolic processes were disregarded, since they are not directly contributing to the turnover of soil P. Table S4 comprises all enzymes, corresponding genes and KEGG orthology (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 KO numbers that were detected in the subsampled data sets are shown in 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 data sets 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 with the soil rich in P (BBR). In addition, genes coding for the low-affinity phosphate inorganic transporter (*pit*) were more abundant in LUE. Compared with the Pst system, sequences referring to glycerol-3-phosphate transporter (*ugpBAEC*) and genes coding for phosphonate transporter (*phnCDE*) were less abundant by seven or 10 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

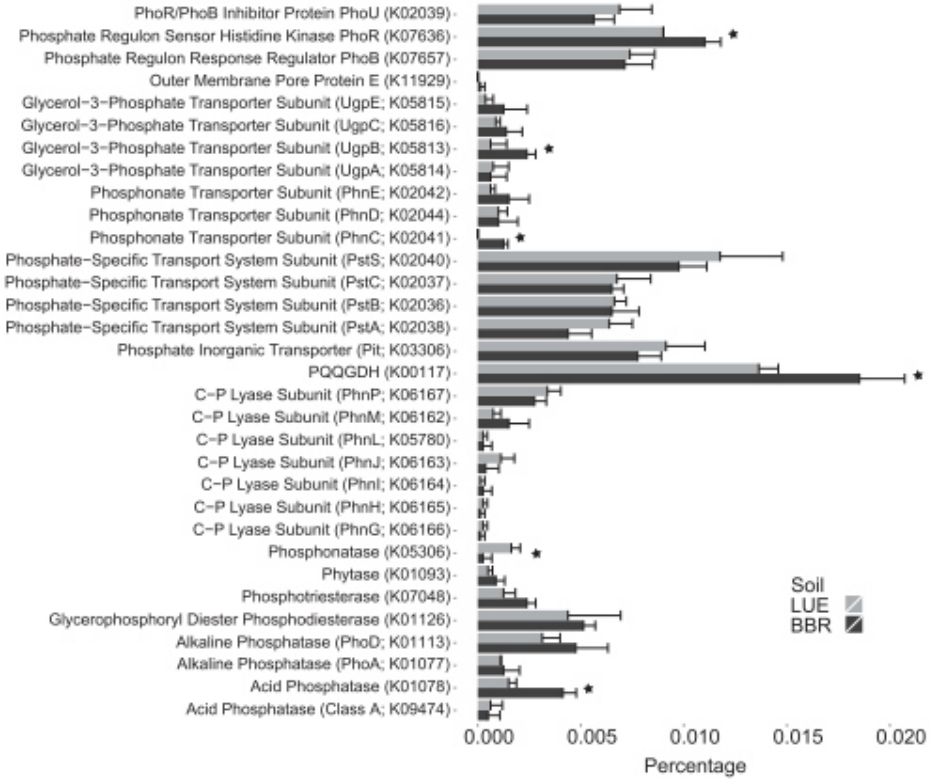

Fig. 2. Relative abundance of microbial genes coding for enzymes involved in soil phosphorus mineralization and solubilization as well as P uptake and P starvation response regulation. Metagenomic data sets of two forest soils were aligned against the KEGG database using DIAMOND. Significant differences in the number of annotated reads among both soils are shown ($n = 3$). *$P < 0.05$.

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 data sets. 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 with 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 with PhoA independent from the soil investigated. Fifty-four 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 data sets 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 four 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

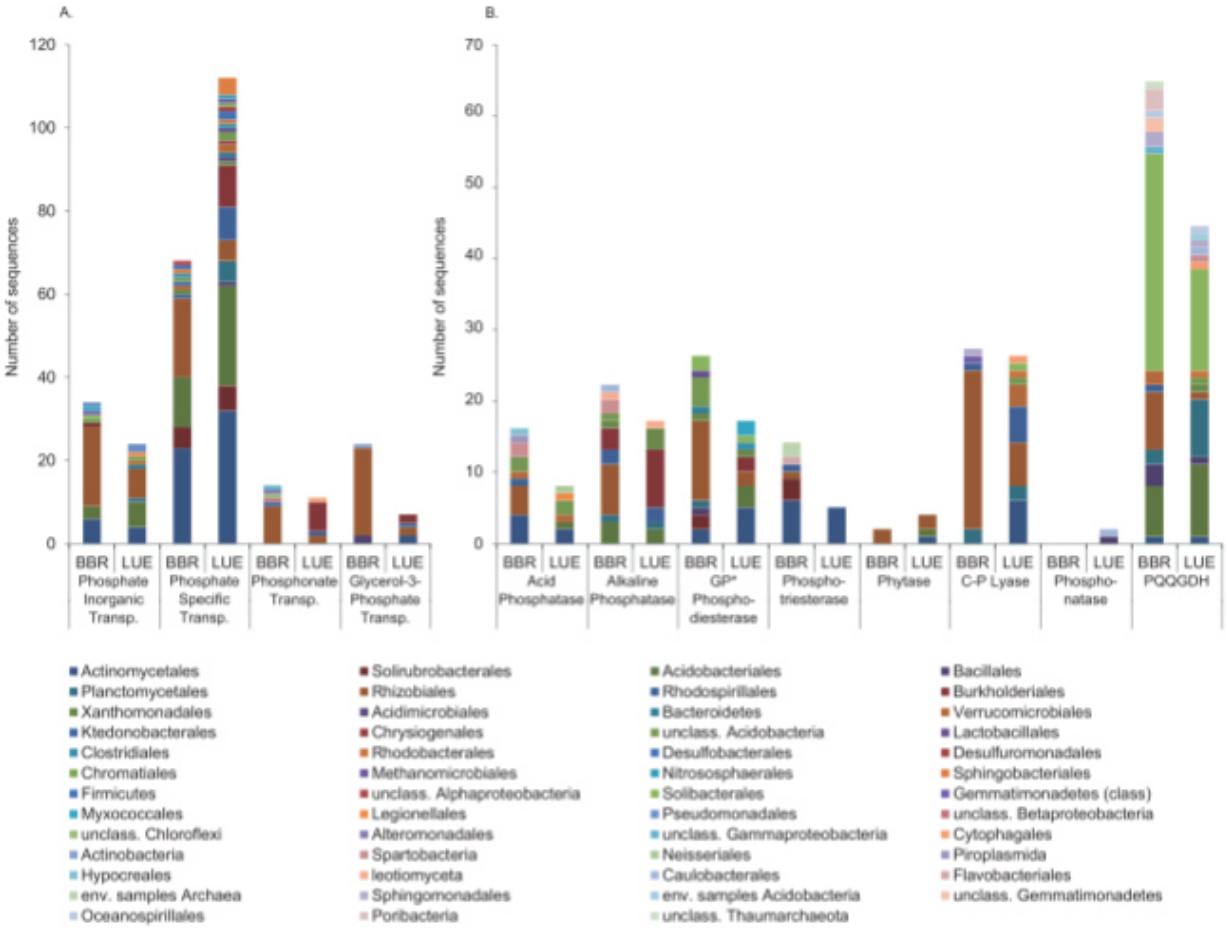

Fig. 3. Taxonomic assignment of microbial genes coding for enzymes involved in the turnover of soil P. Metagenomic data sets of two forest soils were assigned on functional level using DIAMOND against the KEGG database. Sequences coding for microbial phosphate uptake systems (pooled subunits) (A) and enzymes performing mineralization and solubilization of soil P (B) were taxonomically assigned (DIAMOND against NCBI Non-redundant protein sequences (nr) database). Shown are absolute numbers of assigned sequences; (*Glycerophosphoryl Phosphodiesterase).

data sets, open-reading frames were predicted and subsequently scanned for a set of Hidden Markov Models (HMM), comprising conserved domains of investigated proteins (Table S4). Basically, both approaches led to similar results concerning the relative abundance of genes, with respect to the different soil types (Fig. S6). However, the absolute numbers of predicted genes varied slightly. Sole exception was *phoR*, where a relative decrease in abundance related to *phoB* was detected at both sites.

Taxonomic assignment of investigated genes

The taxonomic assignment of investigated genes was based on KEGG database results. Sequencing reads were aligned against the NCBI Non-redundant protein sequences (nr) database using DIAMOND (Buchfink *et al.*, 2015) and further analysed employing MEGAN (Huson *et al.*, 2011). Subunits of P transporters and the C-P lyase multienzyme complex as well as different classes of acid and alkaline phosphatases were pooled respectively. Results are shown on phylum level (Fig. S7A, and B) and reflected the overall abundance of taxa in the metagenomic data sets. Most of the predicted genes were harboured by Proteobacteria (50%), Acidobacteria (24.8%), Actinobacteria (14.4%), Planctomycetes (2.6%), Firmicutes (1.9%) and Verrucomicrobia (1.9%). While the phylum Proteobacteria covered all groups of predicted genes, Acidobacteria especially harboured genes coding for the PQQGDH and the Pst transporter. On order level (Fig. 3A and B), Rhizobiales (25.5%), Actinomycetales (17%), Acidobacteriales (12.2%), Burkholderiales (5.6%)

and Rhodospirillales (4.3%) were among the most abundant taxa. Especially in the P-rich soil (BBR), a substantial amount of genes referring to acid and alkaline phosphatases, phosphodiesterases, C-P lyases, PQQGDH, Pst-, Pit-, phosphonate- and glycerol-3-phosphate transporters were harboured by Rhizobiales. By contrast in LUE different orders, including Actinomycetales, Acidobacteriales, Burkholderiales and Rhodospirillales, contributed to the soil 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 data sets (3.4%).

Discussion

Microbial phosphate uptake systems and Pho regulon control

Functional annotation of metagenomic data sets 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 data sets. 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 signalling and gene regulation (Wanner, 1993; 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 and Wanner, 2010). The high abundance of P signalling and Pho regulation genes in the data sets 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 PQQGDH 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 data sets 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 and Sode, 2003). Goldstein and colleagues (2003) reported induction of PQQGDH activity through novel deoxyribonucleic acid (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 and Simpson, 2011).

Microbial organic phosphorus mineralization

Regarding organic P mineralization a significantly higher abundance of genes coding for nonspecific acid phosphatases was detected in BBR compared with LUE. This group of enzymes hydrolyses 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 and 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 with acid phosphatases) was detected in the data sets, 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 and 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 and Frey, 2002). Especially forest litter and organic layers are hotspots of microbial phosphatase activity, whereas a decline was observed in mineral soils (Pang and 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 and Cooper, 2002). In contrast to acid phosphatases microbial genes coding for ALP are upregulated during phosphate starvation, thereby enabling usage of alternative P sources. The high abundance of Pho-regulated ALP-encoding genes underlines their importance for microbes in the struggle for P. Alkaline phosphatase PhoD was found to be three times more abundant in the data sets compared with PhoA. This is in accordance with previous studies, since PhoD is the most frequently found ALP in metagenomic data sets derived from soil and water samples (Luo *et al.*, 2009; Tan *et al.*, 2013). While enzymes of the PhoA family predominantly dephosphorylate monoester bonds, PhoD also shows phosphodiesterase activity against cell wall teichoic acids and phospholipids (Rodriguez *et al.*, 2014). The broader substrate specificity allows usage of various P sources and might be one reason for the higher gene abundance in the data sets. However, taking into account that the investigated soils are classified as extremely acid according to the Soil Survey Manual (Soil Survey Division Staff, 1993), the expression of the related genes, that we have identified in our metagenomics library needs to be confirmed in future studies focusing on gene expression.

Phytate (myo-Inositol-1,2,3,4,5,6-hexakisphosphate, IP_6) degrading enzymes were rarely detected, although the substrate makes up a major fraction of organic P in many soils (Turner, 2007). In terrestrial ecosystems IP_6 is mainly derived from storage compounds of plants, especially seeds (Turner *et al.*, 2002). Inherently phytate tends to accumulate in top horizons due to the formation of insoluble complexes with metallic cations or adsorption to clay minerals (Bowman *et al.*, 1967; Turner *et al.*, 2002). Especially in soils classified as extremely acid (Soil Survey Division Staff, 1993), phytate is stabilized effectively, leading to increased absolute and relative phytate levels (as a fraction of soil organic P) (Turner and Blackwell, 2013). This might explain the low abundance of phytase genes in the present study, since soil samples were derived from the Ah-horizon exclusively. A higher potential for phytase mineralization can be expected in the organic or litter layer.

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 data sets. 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 and 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 favouring oligotrophic microorganisms. Ratios of Cmic:Pmic indicated a higher P content in the BBR biomass compared with 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 data sets, the majority of rhizobial sequences (40%; data not shown) was assigned to the N-fixing genus of *Bradyrhizobium*. However, symbiotic 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 symbiotic 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 favour growth under resource limitation (Ward *et al.*, 2009). Fierer and colleagues (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 with 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 and colleagues (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 data sets compared with 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 and colleagues (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 and colleagues (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 data sets, 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 harboured 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 data sets, 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 unfavourable environmental conditions (Challacombe *et al.*, 2011). Ward and colleagues (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 data sets solely Ascomycota harboured 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 and colleagues (2005) detected highest fungal richness in forest organic horizons with a consistently decrease in deeper soil layers. Baldrian and colleagues (2012) reported a decline of the fungal to bacterial ribosomal DNA 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 data sets 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 data sets.

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

Soil samples were taken from two beech (*Fagus sylvatica*) dominated German forest soils. Both sites are ICP Level II forests (International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests) namely Bad Brueckenau (BBR) and Luess (LUE). The stands possess an average age of 120 years and have been intensively monitored since 1995 and 1990 respectively. Both soils have been spared from chemical fertilizer input. The forest site near Bad Brueckenau (BBR) is located in the Bavarian Rhoen Mountains (50°21′7.26″ N, 9°55′44.53″ E) and reaches up to 850 m above sea level. The mean annual temperature and precipitation are 5.8 °C and 1031 mm respectively. According to the World Reference Base for Soil Resources (WRB) the soil is classified as Dystric Skeletic Cambisol with Mull-like Moder and alkaline igneous rock/metamorphite as the substrate. The soil (Ah-horizon) has a pH_{H2O} of 3.84 and consists of sand (8%), silt (55%) and clay (37%). It is characterized by a total carbon 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 kg^{-1}, an N:P ratio of 3.76 and a C:P ratio of 58.9.

In contrast, the forest stand near Unterluess (LUE) has a soil (Ah-horizon) N:P ratio of 19.2, a C:P ratio of 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 phosphorus content of 195.8 mg kg^{-1}. The soil has a pH_{H2O} of 3.52 and consists of sand (75%), silt (19%) and clay (6%). According to the WRB, it is classified as Hyperdystric Folic Cambisol with Moder and poor pleistocene sands as substrate. The mean annual temperature and precipitation, respectively, are 8 °C and 730 mm. The forest stand has an elevation of 150 m above sea level and is situated in the Lower Saxon Plain (52°50′21.77″ N, 10°16′2.37″ E).

Soil samples from the Ah-horizon were taken in October 2013 using a soil auger with a diameter of 8 cm up to a depth of 20 cm. At both forest sites, three biological replicates, each pooled from five contiguous soil cores, were sampled. Samples were taken in the direct surroundings of the Level II plots. After pooling, aliquots of the three replicates were immediately deep frozen on dry ice for nucleic acid extraction. The remaining soil was stored at 4 °C for further analysis.

Microbial biomass C, N and P

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

Nucleic acid isolation

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

Pyrosequencing

Total genomic DNA of six soil samples was sequenced. Pyrosequencing was performed on a Genome Sequencer FLX+ instrument (454 Life Sciences, Roche, USA). Library preparation was accomplished according to the Roche protocol 'Rapid Library Preparation Method Manual' using Roche MID Adaptors. As different sequencing depths were applied, libraries of replicates were pooled in a 2:1:1 ratio. Subsequent emulsion polymerase chain reaction (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 the Sequence Read Archive (SRA) under the accession number: PRJNA288276.

Analysis of sequencing data

Roche SFF files (Standard flowgram format) 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 l = 50. Remaining adaptor sequences and duplicated sequences were removed using BIOPIECES (http://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 (June 2011) (Kanehisa and 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 Table S4 for all enzymes associated with the soil microbial P turnover that were investigated in this study. Corresponding KO numbers were searched within the functionally annotated data sets. 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 (Table S4) using HMMSCAN (HMMER 3.0) (http://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 were 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 data sets. Subsampling using BIOPIECES (http://www.biopieces.org) corresponded to the lowest quantity of filtered sequences achieved in one of the data sets (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 data sets, were included in the analysis.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

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

Fig. S1. Relative distribution of microbial phyla in metagenomic data sets of two forest soils. Sequences were aligned against the SILVA database using BLASTN (e-value 0.001). Significant differences in the amount of annotated reads among both sites are shown ($n = 3$). $^*P < 0.05$.

Fig. S2. Principal component analysis of metagenomic data sets derived from two forest soils. Shown are the first two components based on relative abundance of microbial orders. Sequences were assigned using DIAMOND against the NBCI Non-redundant protein sequences (nr) database and MEGAN ($n = 3$).

Fig. S3. Rarefaction analysis of metagenomic data sets derived from two forest soils. Depicted is the number of assigned microbial orders (DIAMOND against the NBCI Non-redundant protein sequences (nr) database and MEGAN) as a function of sequencing depth ($n = 3$).

Fig. S4. Relative abundance of KEGG pathways (DIAMOND against KEGG database and MEGAN) in metagenomic data sets of two forest soils. Shown are the 30 most abundant pathways. Significant differences in the number of assigned sequences among both soils are shown ($n = 3$). $^*P < 0.05$.

Fig. S5. Relative abundance of all investigated genes coding for enzymes involved in soil microbial P cycling and

uptake. Metagenomic data sets of two forest soils were aligned against the KEGG database using DIAMOND. Significant differences in the amount of annotated reads among both soils are shown ($n = 3$). $^*P < 0.05$.

Fig. S6. Relative abundance of microbial genes coding for enzymes involved in soil microbial P cycling and uptake. Open reading frames were predicted in metagenomic data sets of two different forest soils and subsequently scanned for profile Hidden Markov Models of investigated proteins. Significant differences in the amount of annotated reads among both sites are shown ($n = 3$). $^*P < 0.05$

Fig. S7. Taxonomic assignment of microbial genes coding for enzymes involved in the microbial turnover of soil P (phylum level). Metagenomic data sets of two forest soils were assigned on functional level using DIAMOND against the KEGG database. Sequences coding for microbial phosphate uptake systems (pooled subunits) (A) and enzymes performing the mineralization and solubilization of soil P (B) were taxonomically assigned (DIAMOND against NCBI Non-redundant protein sequences (nr) database). Shown are absolute numbers of assigned sequences; (*Glycerophosphoryl Phosphodiesterase).

Table S1. Details of the sequencing run: Shown are number of obtained reads and average read length per replicate before and after quality filtering.

Table S2. Absolute number of sequences annotated to the 20 most abundant microbial phyla and orders in metagenomic data sets of two forest soils. Sequences were assigned using the NCBI Non-redundant protein sequences (nr) database and MEGAN.

Table S3. Absolute number of sequences annotated to microbial taxa (DIAMOND against NCBI Non-redundant protein sequences (nr) database) that differ significantly ($P < 0.05$) in abundance among both soils. Only taxa with an abundance of at least 0.05% in one data set (related to the total number of assigned reads) are included.

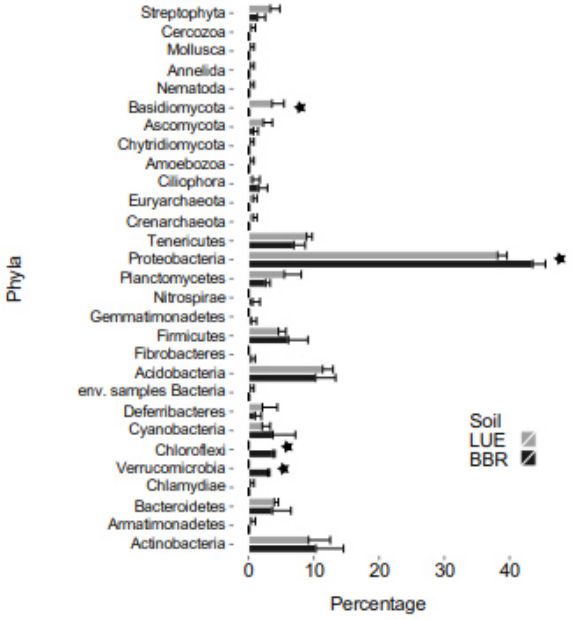
Table S4. Investigated enzymes related to the microbial turnover of soil P, corresponding genes and origin of Hidden Markov Models.

Table S5. Keywords used for quality filtering of predicted enzymes. Open reading frames were predicted in metagenomic data sets of two forest soils and scanned (HMMSCAN) for HMM of investigated proteins. Positive hits were aligned against the NCBI REFSEQ database (BLASTP) and checked for keywords. Keywords correspond to NCBI REFSEQ database annotations of curated proteins.

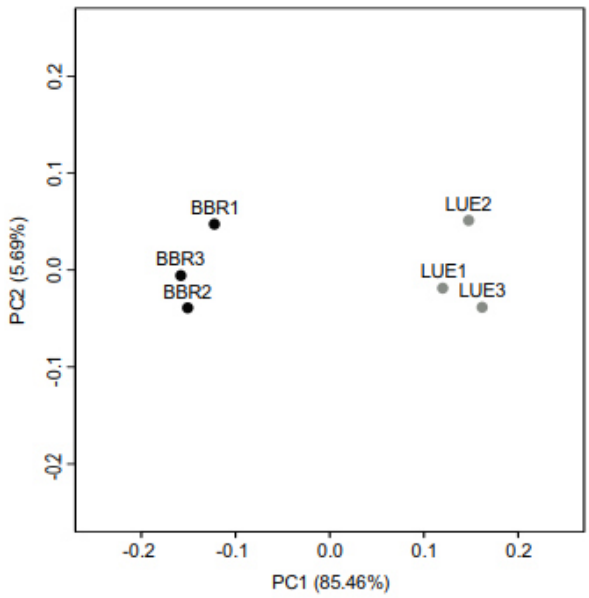
Supplemental Information Manuscript 1

Supporting Information Experimental Procedures

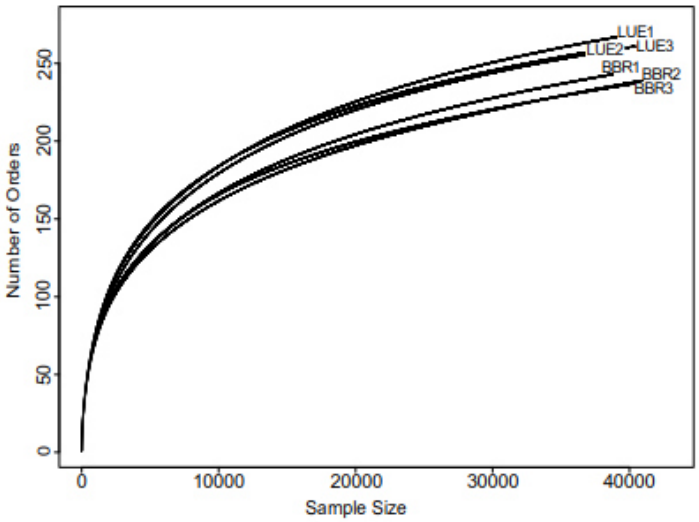
Functional annotation of metagenomic datasets was performed using DIAMOND (Buchfink *et al.*, 2015) against the KEGG database (Kanehisa & Goto, 2000). To confirm the obtained results with a second approach, open-reading frames were predicted based on filtered sequencing reads using FragGeneScan (version 1.18) (Rho *et al.*, 2010) and subsequently scanned for Profile Hidden Markov Models (HMM) using hmmscan (HMMER 3.0) (www.hmmer.org). Protein sequences of all investigated enzymes were obtained from the NCBI Protein database (preferably functionally proven entries) (October 2014) and searched for conserved domains using CD-Search (Marchler-Bauer *et al.*, 2015) and TIGR FAMs HMM Search (http://blast.jcvi.org/web-hmm) with default settings. Profile Hidden Markov Models of conserved domains (Supporting Information Table S4) were derived from the Pfam database (version 27.0) (Finn *et al.*, 2014) and the TIGRFAMs database (version 15) (Haft *et al.*, 2013). Subsequently predicted protein sequences of metagenomic datasets were scanned for the obtained HMM using hmmscan with an expect-value of 10^{-5}. Since some Hidden Markov Models are not specific for a certain enzyme function, sequences of predicted proteins were additionally verified using blastp (BLAST+ suite version 2.2.27+) (Camacho *et al.*, 2009) against the NCBI RefSeq database (Tatusova *et al.*, 2014) (October 2014). The top 20 BLAST hits (expect value) per predicted enzyme were afterwards scanned for a list of keywords characterizing the distinct enzyme functions. Headwords corresponded to NCBI RefSeq database annotations of, if available, functionally proven enzymes and are listed in Supporting Information Table S5. If at least 50% of the top BLAST hits harbored one or more of the given keywords than the underlying sequence was counted as a positive hit. Thereby all sequences potentially coding for enzymes related to the soil microbial phosphorus turnover could be identified.



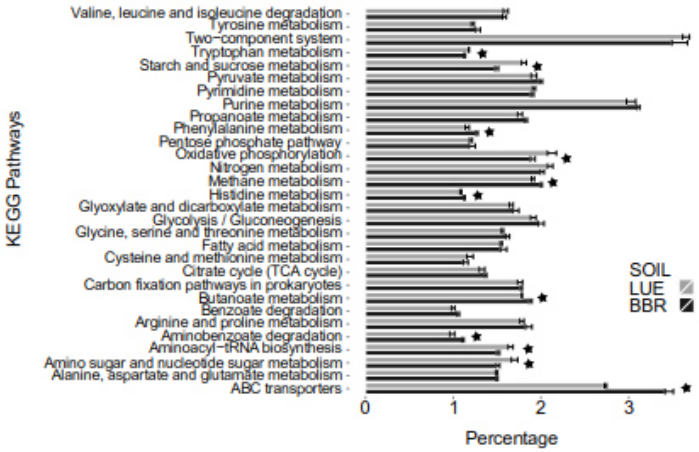
Supporting Information Figure S1 Relative distribution of microbial phyla in metagenomic datasets of two forest soils. Sequences were aligned against the SILVA database using blastn (e-value 0.001). Significant differences in the amount of annotated reads among both sites are shown (n=3).
*P<0.05



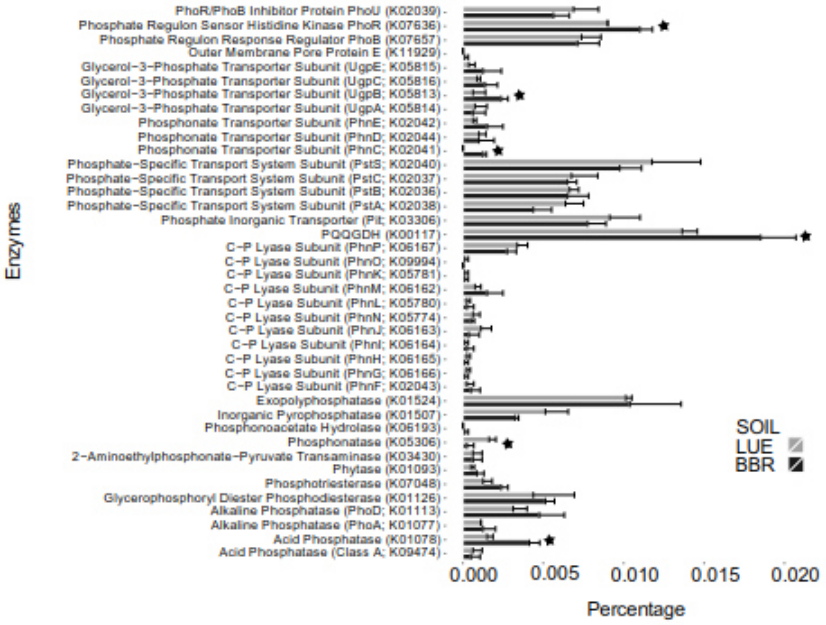
Supporting Information Figure S2 Principal Component Analysis of metagenomic datasets derived from two forest soils. Shown are the first two components based on relative abundance of microbial orders. Sequences were assigned using DIAMOND against the NBCI Non-redundant protein sequences (nr) database and MEGAN (n=3).



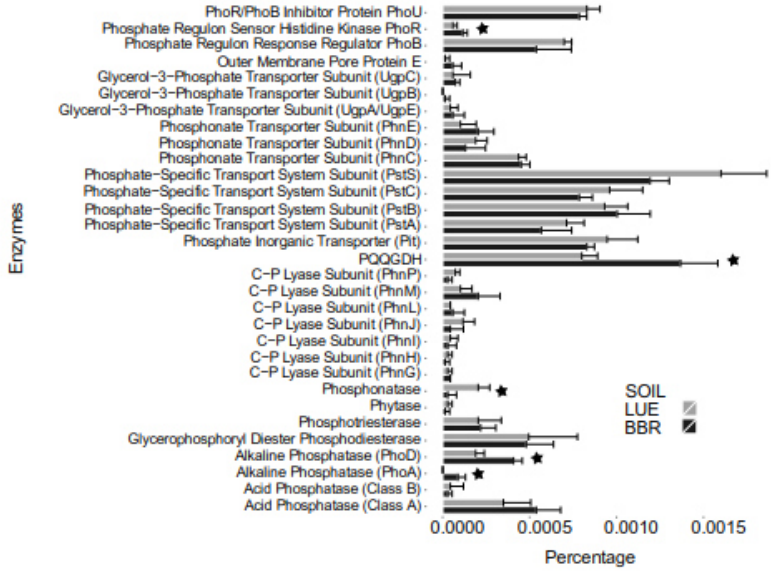
Supporting Information Figure S3 Rarefaction analysis of metagenomic datasets derived from two forest soils. Depicted is the number of assigned microbial orders (DIAMOND against the NBCI Non-redundant protein sequences (nr) database and MEGAN) as a function of sequencing depth (n=3).



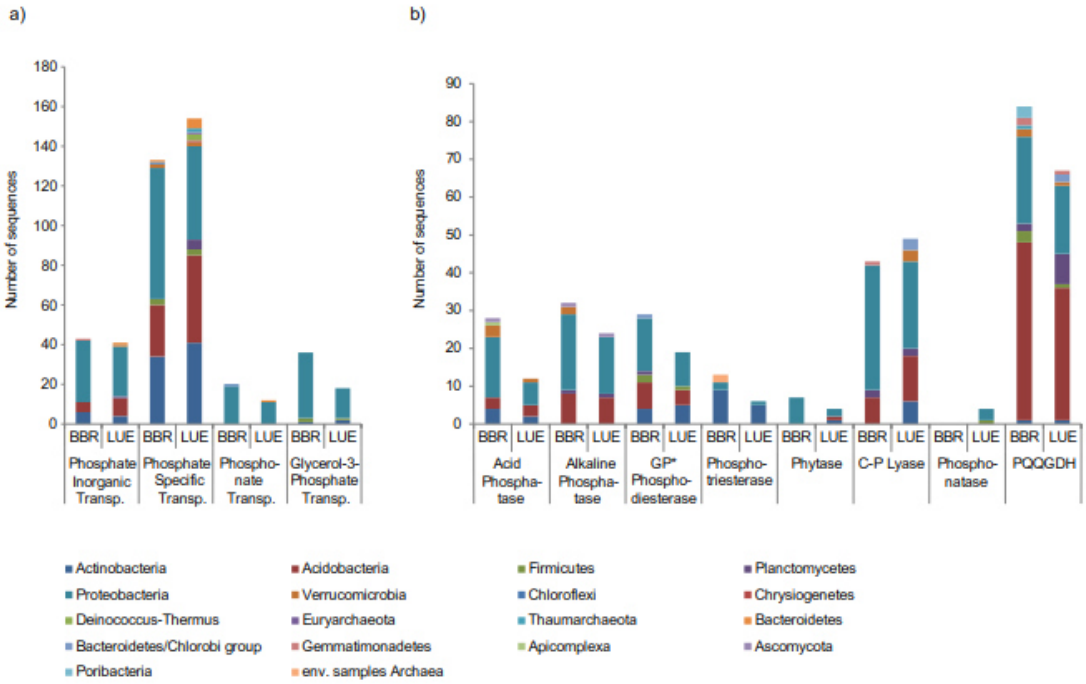
Supporting Information Figure S4 Relative abundance of KEGG pathways (DIAMOND against KEGG database and MEGAN) in metagenomic datasets of two forest soils. Shown are the 30 most abundant pathways. Significant differences in the number of assigned sequences among both soils are shown (n=3).
*P<0.05



Supporting Information Figure S5 Relative abundance of all investigated genes coding for enzymes involved in soil microbial P cycling and uptake. Metagenomic datasets of two forest soils were aligned against the KEGG database using DIAMOND. Significant differences in the amount of annotated reads among both soils are shown (n=3).
*P<0.05



Supporting Information Figure S6 Relative abundance of microbial genes coding for enzymes involved in soil microbial P cycling and uptake. Open reading frames were predicted in metagenomic datasets of two different forest soils and subsequently scanned for profile Hidden Markov Models of investigated proteins. Significant differences in the amount of annotated reads among both sites are shown (n=3). **P*<0.05



Supporting Information Figure S7 Taxonomic assignment of microbial genes coding for enzymes involved in the microbial turnover of soil P (phylum level). Metagenomic datasets of two forest soils were assigned on functional level using DIAMOND against the KEGG database. Sequences coding for microbial phosphate uptake systems (pooled subunits) (a) and enzymes performing the mineralization and solubilization of soil P (b) were taxonomically assigned (DIAMOND against NCBI Non-redundant protein sequences (nr) database). Shown are absolute numbers of assigned sequences; (*Glycerophosphoryl Phosphodiesterase).

Supporting Information Table S1 Details of the sequencing run: Shown are number of obtained reads and average read length per replicate before and after quality filtering.

Raw data	BBR1	BBR2	BBR3	LUE1	LUE2	LUE3
Number of reads	195,392	211,115	163,110	332,683	174,432	142,422
Average read length (bp)	584	596	582	577	570	579
Filtered data						
Number of reads	176,175	193,130	148,110	310,866	161,478	133,179
Average read length (bp)	338	347	335	357	330	359

Supporting Information Table S2 Absolute number of sequences annotated to the 20 most abundant microbial phyla and orders in metagenomic datasets of two forest soils. Sequences were assigned using the NCBI Non-redundant protein sequences (nr) database and MEGAN.

Taxa	Name	BBR1	BBR2	BBR3	LUE1	LUE2	LUE3
Phylum	Actinobacteria	9,519	12,204	11,027	12,916	10,759	14,445
Phylum	Armatimonadetes	86	73	67	73	70	130
Phylum	Bacteroidetes	824	583	615	646	688	825
Phylum	Chlamydiae	22	30	36	72	53	55
Phylum	Verrucomicrobia	1,277	1,437	1,119	864	904	983
Phylum	Chloroflexi	856	1,032	813	229	207	193
Phylum	Cyanobacteria	479	440	407	507	486	480
Phylum	Deinococcus-Thermus	70	74	77	50	61	53
Phylum	env. samples Bacteria	383	382	369	320	293	298
Phylum	Acidobacteria	9,012	7,971	7,805	13,162	15,451	15,207
Phylum	Firmicutes	491	443	435	477	441	458
Phylum	Gemmatimonadetes	532	429	370	155	170	112
Phylum	Nitrospirae	110	153	132	57	38	36
Phylum	Planctomycetes	2,032	1,338	1,723	2,835	2,312	2,447
Phylum	Proteobacteria	28,312	29,998	31,293	21,722	19,131	19,433
Phylum	unclass. Bacteria	42	46	35	33	34	34
Phylum	Euryarchaeota	80	87	88	106	95	108
Phylum	Thaumarchaeota	61	94	63	29	34	29
Phylum	Ascomycota	71	70	82	715	456	584
Phylum	Basidiomycota	47	31	40	148	221	168
Order	Actinomycetales	7,676	10,167	8,749	10,325	8,764	12,018
Order	Solirubrobacterales	685	644	861	972	598	670
Order	Sphingobacteriales	262	195	186	232	234	274
Order	Spartobacteria	321	449	309	105	117	106
Order	Verrucomicrobiales	525	451	356	446	498	510
Order	Ktedonobacterales	588	745	557	87	81	63
Order	env. samples Bacteria	383	382	369	320	293	298
Order	Acidobacteriales	2,810	2,501	2,559	6,057	6,855	7,145
Order	Solibacterales	1,533	1,374	1,304	1,219	1,348	1,225
Order	unclass. Acidobacteria	1,629	1,397	1,303	1,963	2,651	2,492
Order	unclass. Gemmatimonadetes	253	193	177	81	82	67
Order	Planctomycetales	1,968	1,295	1,686	2,799	2,274	2,402
Order	Rhizobiales	12,273	13,289	14,405	6,703	5,960	5,921
Order	Rhodobacterales	285	268	295	223	221	234
Order	Rhodospirillales	1,090	1,306	1,195	1,128	867	970
Order	Sphingomonadales	256	227	222	262	203	253
Order	Burkholderiales	1,017	948	935	1,085	1,105	955
Order	Myxococcales	810	644	670	552	500	541
Order	unclass. Deltaproteobacteria	345	383	352	377	324	297
Order	Xanthomonadales	340	356	299	298	246	266

Supporting Information Table S3 Absolute number of sequences annotated to microbial taxa (DIAMOND against NCBI Non-redundant protein sequences (nr) database) that differ significantly ($P<0.05$) in abundance among both soils. Only taxa with an abundance of at least 0.05% in one dataset (related to the total number of assigned reads) are included.

Taxa	Name	BBR1	BBR2	BBR3	LUE1	LUE2	LUE3
Phylum	Verrucomicrobia	1,277	1,437	1,119	864	904	983
Phylum	Chloroflexi	856	1,032	813	229	207	193
Phylum	environmental samples Bacteria	383	382	369	320	293	298
Phylum	Acidobacteria	9,012	7,971	7,805	13,162	15,451	15,207
Phylum	Gemmatimonadetes	532	429	370	155	170	112
Phylum	Planctomycetes	2,032	1,338	1,723	2,835	2,312	2,447
Phylum	Proteobacteria	28,312	29,998	31,293	21,722	19,131	19,433
Phylum	Ascomycota	71	70	82	715	456	584
Phylum	Basidiomycota	47	31	40	148	221	168
Class	Spartobacteria	321	449	309	105	117	106
Class	Ktedonobacteria	588	745	557	87	81	63
Class	environmental samples Bacteria	383	382	369	320	293	298
Class	Acidobacteriia	2,810	2,501	2,559	6,057	6,855	7,145
Class	unclassified Acidobacteria	1,629	1,397	1,303	1,963	2,651	2,492
Class	Gemmatimonadetes	532	429	370	155	170	112
Class	Nitrospira	110	153	131	57	38	36
Class	Planctomycetia	1,999	1,321	1,703	2,816	2,287	2,421
Class	Alphaproteobacteria	18,537	20,066	21,434	12,445	10,851	11,063
Class	Eurotiomycetes	8	7	8	205	123	195
Class	Agaricomycetes	37	20	31	140	200	143
Order	Acidimicrobiales	60	87	61	118	108	124
Order	Spartobacteria	321	449	309	105	117	106
Order	Ktedonobacterales	588	745	557	87	81	63
Order	environmental samples Bacteria	383	382	369	320	293	298
Order	Acidobacteriales	2,810	2,501	2,559	6,057	6,855	7,145
Order	unclassified Acidobacteria	1,629	1,397	1,303	1,963	2,651	2,492
Order	unclassified Gemmatimonadetes	253	193	177	81	82	67
Order	Nitrospirales	110	153	131	57	38	36
Order	Planctomycetales	1,968	1,295	1,686	2,799	2,274	2,402
Order	Caulobacterales	202	174	169	133	100	111
Order	Rhizobiales	12,273	13,289	14,405	6,703	5,960	5,921
Order	Rhodobacterales	285	268	295	223	221	234
Family	Acidimicrobiaceae	60	87	61	118	108	124
Family	Spartobacteria	321	449	309	105	117	106
Family	Ktedonobacteraceae	588	745	557	87	81	63
Family	environmental samples Bacteria	383	382	369	320	293	298
Family	Acidobacteriaceae	2,808	2,501	2,559	6,053	6,855	7,145
Family	unclassified Acidobacteria	1,629	1,397	1,303	1,963	2,651	2,492
Family	Gemmatimonadaceae	94	70	71	29	42	26
Family	unclassified Gemmatimonadetes	253	193	177	81	82	67
Family	Nitrospiraceae	110	153	131	57	38	36
Family	Planctomycetaceae	1,920	1,248	1,651	2,746	2,220	2,343
Family	Caulobacteraceae	202	174	169	133	100	111
Family	Bradyrhizobiaceae	8,861	9,671	10,356	3,417	3,071	2,868
Family	Hyphomicrobiaceae	146	133	171	80	63	60
Family	Methylobacteriaceae	169	186	182	135	121	141
Family	Methylocystaceae	96	101	113	122	121	117
Family	Rhodobacteraceae	262	249	278	206	198	206
Family	Rhodospirillaceae	274	317	282	242	218	209
Family	unclassified Rhodospirillales	263	347	294	220	169	181
Family	Burkholderiaceae	521	467	474	720	772	625
Family	Comamonadaceae	139	119	139	82	89	72
Family	Polyangiaceae	324	254	305	205	185	208
Genus	*Acidimicrobium*	36	49	33	82	78	79
Genus	*Catenulispora*	31	35	38	53	43	72
Genus	*Chthoniobacter*	321	449	309	105	117	106
Genus	*Ktedonobacter*	588	745	557	87	81	63
Genus	*environmental samples Bacteria*	383	382	369	320	293	298
Genus	*Acidobacterium*	158	153	145	512	566	614

Genus	*Granulicella*	196	185	225	495	525	579
Genus	*Terriglobus*	97	97	74	197	198	190
Genus	*unclassified Acidobacteriaceae*	1,510	1,290	1,359	2,621	3,109	3,221
Genus	*Candidatus* Koribacter	1,334	1,160	1,073	1,734	2,361	2,233
Genus	*Gemmatimonas*	94	70	71	29	42	26
Genus	*unclassified Gemmatimonadetes*	253	193	177	81	82	67
Genus	*Nitrospira*	97	144	122	39	25	25
Genus	*Gemmata*	120	77	127	230	182	247
Genus	*Planctomyces*	113	83	87	137	120	125
Genus	*Singulisphaera*	382	256	370	885	646	599
Genus	*Zavarzinella*	183	124	125	231	228	209
Genus	*Afipia*	96	124	100	65	57	52
Genus	*Bradyrhizobium*	5,523	6,215	6,450	1,977	1,780	1,628
Genus	*Nitrobacter*	61	64	83	40	29	33
Genus	*Rhodopseudomonas*	87	94	127	53	52	64
Genus	*Hyphomicrobium*	98	88	121	54	41	34
Genus	*unclass. Rhodospirillales (miscellaneous)*	234	333	277	211	164	168
Genus	*Burkholderia*	356	319	301	545	624	476
Species	*Acidimicrobium ferrooxidans*	36	49	33	82	78	79
Species	*Mycobacterium smegmatis*	45	62	32	24	23	10
Species	*Chthoniobacter flavus*	321	449	309	105	117	106
Species	*Ktedonobacter racemifer*	588	745	557	87	81	63
Species	*Chloroflexi bacterium JGI 0002000-F10*	43	57	48	8	13	13
Species	*uncultured bacterium*	196	207	213	170	163	147
Species	*Acidobacterium capsulatum*	65	74	66	186	208	217
Species	*Acidobacterium sp. PMMR2*	73	66	66	233	270	288
Species	*Granulicella mallensis*	115	110	138	313	338	350
Species	*Granulicella tundricola*	61	53	67	141	137	158
Species	*Terriglobus roseus*	36	27	22	58	63	64
Species	*Terriglobus saanensis*	49	61	43	124	109	101
Species	*Acidobacteria bacterium KBS 146*	53	38	54	94	99	121
Species	*Acidobacteriaceae bacterium KBS 83*	247	212	233	466	528	484
Species	*Acidobacteriaceae bacterium KBS 89*	220	213	211	371	500	629
Species	*Acidobacteriaceae bacterium TAA166*	74	42	58	109	129	128
Species	*Acidobacteriaceae bacterium URHE0068*	270	226	235	683	773	813
Species	*Candidatus* Solibacter usitatus	1,533	1,374	1,304	1,219	1,346	1,225
Species	*Candidatus* Koribacter versatilis	1,334	1,160	1,073	1,734	2,361	2,233
Species	*Gemmatimonadetes bacterium KBS708*	249	191	174	79	80	65
Species	*Candidatus* Nitrospira defluvii	97	144	122	39	25	25
Species	*Gemmata obscuriglobus*	115	72	121	215	174	237
Species	*Singulisphaera acidiphila*	382	256	370	885	646	599
Species	*Zavarzinella formosa*	183	124	125	231	228	209
Species	*Bradyrhizobium elkanii*	164	163	166	75	74	58
Species	*Bradyrhizobium japonicum*	105	132	138	54	57	45
Species	*Bradyrhizobium sp. ARR65*	91	115	115	60	44	44
Species	*Bradyrhizobium sp. Ec3.3*	55	74	82	25	31	31
Species	*Bradyrhizobium sp. STM 3843*	61	53	63	33	24	29
Species	*Bradyrhizobium sp. Tv2a-2*	173	186	208	70	55	72
Species	*Bradyrhizobium sp. URHA0002*	94	85	90	21	24	19
Species	*Bradyrhizobium sp. URHD0069*	212	224	237	71	63	54
Species	*Rhodopseudomonas palustris*	70	81	96	35	37	33
Species	*Rhodospirillales bacterium URHD0088*	234	333	277	211	164	168
Species	*Haliangium ochraceum*	71	35	48	18	17	27
Species	*Candidatus* Entotheonella sp. TSY2	87	90	100	75	74	54

Supporting Information Table S4 Investigated enzymes related to the microbial turnover of soil P, corresponding genes and origin of Hidden Markov Models.

Enzyme Name	KEGG KO Number	HMM Name	HMM Database	Corresponding Gene	Reference
Acid Phosphatase (Class A)	K09474	Pf01569	Pfam	*phoN*	Rossolini *et al*., 1998
Acid Phosphatase (Class B)	K03788 (no KEGG Hits in datasets detected)	Pf03767	Pfam	*aphA*	Rossolini *et al*., 1998
Acid Phosphatase (Class C)	K01078 (not specific for class C)	TIGR01533 (no Hits in datasets detected)	TIGR	*olpA*	Rossolini *et al*., 1998
Alkaline Phosphatase (PhoA)	K01077	Pf00245	Pfam	*phoA*	Torriani, 1960
Alkaline Phosphatase (PhoD)	K01113	Pf09423	Pfam	*phoD*	Eder *et al*., 1996
Alkaline Phosphatase (PhoX)	(no KEGG KO number available)	Pf05787 (no Hits in datasets detected)	Pfam	*phoX*	Monds *et al*., 2006
Glycerophosphoryl Diester Phosphodiesterase	K01126	Pf03009	Pfam	*ugpQ*	Brzoska & Boos, 1988
Phosphotriesterase	K07048	Pf02126	Pfam	*opd*	McDaniel *et al*., 1988
Phytase	K01093 (K01083: no KEGG Hits in datasets detected)	Pf00328	Pfam	*appA*	Golovan *et al*., 2000
2-Aminoethylphosphonate (AEP) - Pyruvate Transaminase	K03430	TIGR02326	TIGR	*phnW*	McGrath *et al*., 2013
Phosphonatase	K05306	TIGR01422	TIGR	*phnX*	McGrath *et al*., 2013
Phosphonoacetate Hydrolase	K06193	TIGR02335	TIGR	*phnA*	McGrath *et al*., 2013
Inorganic Pyrophosphatase	K01507	Pf00719	Pfam	*ppa*	Lahti *et al*., 1988
Exopolyphosphatase	K01524	Pf02541	Pfam	*ppx*	Akiyama *et al*., 1993
C–P Lyase Subunit (PhnF)	K02043	TIGR02325	TIGR	*phnF*	McGrath *et al*., 2013
C–P Lyase Subunit (PhnG)	K06166	Pf06754	Pfam	*phnG*	McGrath *et al*., 2013
C–P Lyase Subunit (PhnH)	K06165	Pf05845	Pfam	*phnH*	McGrath *et al*., 2013
C–P Lyase Subunit (PhnI)	K06164	Pf05861	Pfam	*phnI*	McGrath *et al*., 2013
C–P Lyase Subunit (PhnJ)	K06163	Pf06007	Pfam	*phnJ*	McGrath *et al*., 2013
C–P Lyase Subunit (PhnK)	K05781	TIGR02323	TIGR	*phnK*	McGrath *et al*., 2013
C–P Lyase Subunit (PhnL)	K05780	TIGR02324	TIGR	*phnL*	McGrath *et al*., 2013
C–P Lyase Subunit (PhnM)	K06162	TIGR02318	TIGR	*phnM*	McGrath *et al*., 2013
C–P Lyase Subunit (PhnN)	K05774	TIGR02322	TIGR	*phnN*	McGrath *et al*., 2013
C–P Lyase Subunit (PhnO)	K09994	Pf00583	Pfam	*phnO*	McGrath *et al*., 2013
C–P Lyase Subunit (PhnP)	K06167	TIGR03307	TIGR	*phnP*	McGrath *et al*., 2013
PQQGDH (Quinoprotein Glucose Dehydrogenase)	K00117	TIGR03074	TIGR	*gcd*	Cleton-Jansen *et al*., 1990
Phosphate Inorganic Transporter (Pit)	K03306	Pf01384	Pfam	*pit*	Elvin *et al*., 1986
Phosphate–Specific Transport System Subunit (PstA)	K02038	TIGR00974	TIGR	*pstA*	Hsieh & Wanner, 2010
Phosphate–Specific Transport System Subunit (PstB)	K02036	TIGR00972	TIGR	*pstB*	Hsieh & Wanner, 2010
Phosphate–Specific Transport System Subunit (PstC)	K02037	TIGR02138	TIGR	*pstC*	Hsieh & Wanner, 2010
Phosphate–Specific Transport System Subunit (PstS)	K02040	TIGR00975	TIGR	*pstS*	Hsieh & Wanner, 2010
Phosphonate Transporter Subunit (PhnC)	K02041	TIGR02315	TIGR	*phnC*	Hsieh & Wanner, 2010
Phosphonate Transporter Subunit (PhnD)	K02044	TIGR03431	TIGR	*phnD*	Hsieh & Wanner, 2010
Phosphonate Transporter Subunit (PhnE)	K02042	TIGR01097	TIGR	*phnE*	Hsieh & Wanner, 2010
Glycerol–3–Phosphate Transporter Subunit (UgpA)	K05814	Pf00528	Pfam	*ugpA*	Hsieh & Wanner, 2010
Glycerol–3–Phosphate Transporter Subunit (UgpB)	K05813	Pf01547	Pfam	*ugpB*	Hsieh & Wanner, 2010
Glycerol–3–Phosphate Transporter Subunit (UgpC)	K05816	Pf00005	Pfam	*ugpC*	Hsieh & Wanner, 2010
Glycerol–3–Phosphate Transporter Subunit (UgpE)	K05815	Pf00528	Pfam	*ugpE*	Hsieh & Wanner, 2010
Outer Membrane Pore Protein E	K11929	Pf00267	Pfam	*phoE*	Hsieh & Wanner, 2010
Phosphate Regulon Response Regulator (PhoB)	K07657	TIGR02154	TIGR	*phoB*	Hsieh & Wanner, 2010
Phosphate Regulon Sensor Histidine Kinase (PhoR)	K07636	TIGR02966	TIGR	*phoR*	Hsieh & Wanner, 2010
PhoR/PhoB Inhibitor Protein (PhoU)	K02039	TIGR02135	TIGR	*phoU*	Hsieh & Wanner, 2010

Supporting Information Table S5 Keywords used for quality filtering of predicted enzymes. Open reading frames were predicted in metagenomic datasets of two forest soils and scanned (hmmscan) for HMM of investigated proteins. Positive hits were aligned against the NCBI RefSeq database (blastp) and checked for keywords. Keywords correspond to NCBI RefSeq database annotations of curated proteins.

Keywords
glycerol-3-phosphate transporter membrane protein
glycerol-3-phosphate transporter permease
sn-glycerol-3-phosphate ABC transporter substrate-binding protein
glycerol 3-phosphate ABC transporter
sn-glycerol-3-phosphate import ATP-binding protein UgpC
glycerol-3-phosphate ABC transporter ATPase
phosphonates metabolism transcriptional regulator PhnF
putative transcriptional regulator phnF
phosphonate metabolism transcriptional regulator PhnF
putative DNA-binding transcriptional regulator of phosphonate uptake and biodegradation
ribose 1,5-bisphosphokinase
MULTISPECIES: ribose 1,5-bisphosphate phosphokinase
phosphonate metabolism protein/1,5-bisphosphokinase PhnN
aminoalkylphosphonate N-acetyltransferase
MULTISPECIES: aminoalkylphosphonate N-acetyltransferase
aminoalkylphosphonic acid N-acetyltransferase
MULTISPECIES: aminoalkylphosphonic acid N-acetyltransferase
Protein phnO
DNA-binding transcriptional regulator of phosphonate uptake and biodegradation PhnF
transcriptional regulator of phosphonate uptake and biodegradation
phosphonate metabolism protein
phosphonate C-P lyase system protein PhnG
phosphonate C-P lyase
MULTISPECIES: phosphonate C-P lyase system protein PhnG
protein phnG
phosphonate metabolism protein PhnG
MULTISPECIES: protein phnG
PhnG protein
protein phnH
carbon-phosphorus lyase
phosphonate C-P lyase system protein PhnK
Phosphonates transport ATP-binding protein phnK
phosphonate C-P lyase system protein PhnL
carbon-phosphorus lyase complex subunit
membrane-bound PQQ-dependent dehydrogenase, glucose/quinate/shikimate family protein
MULTISPECIES: exopolyphosphatase
MULTISPECIES: PhoB family transcriptional regulator
putative alkaline phosphatase D
MULTISPECIES: sensory histidine kinase in two-component regulatory system with PhoB
PhnI protein
MULTISPECIES: quinoprotein glucose dehydrogenase
carbon-phosphorus lyase complex subunit PhnI

putative PhnI protein, phosphonate metabolism
MULTISPECIES: carbon-phosphorus lyase complex subunit PhnI
PhnJ protein
carbon-phosphorus lyase complex subunit PhnJ
putative phosphonate metabolism protein PhnJ
Phosphonate metabolism protein PhnJ
MULTISPECIES: carbon-phosphorus lyase complex subunit PhnJ
MULTISPECIES: carbon-phosphorus lyase
PhnJ
phosphonate metabolism protein PhnM
MULTISPECIES: phosphonate metabolism protein PhnM
quinoprotein glucose dehydrogenase
putative quinoprotein glucose dehydrogenase
membrane-bound PQQ-dependent dehydrogenase glucose/quinate/shikimate family
Glucose dehydrogenase, PQQ-dependent
glucose dehydrogenase
MULTISPECIES: glucose dehydrogenase
glucose/quinate/shikimate family membrane-bound PQQ-dependent dehydrogenase
MULTISPECIES: phosphonate ABC transporter ATP-binding protein
phosphonate ABC transporter ATPase
phosphonate import ATP-binding protein PhnC
phosphonate ABC transporter, ATP-binding protein
phosphonate ABC transporter ATP-binding protein
phosphonate/organophosphate ester transporter subunit
phosphonate/organophosphate ester transporter subunit PhnC
phosphonate ABC transporter ATP-binding protein
phosphonate ABC transporter substrate-binding protein
MULTISPECIES: phosphonate ABC transporter substrate-binding protein
phosphate/phosphite/phosphonate ABC transporter, periplasmic binding protein
ABC-type phosphate/phosphonate transport system periplasmic component-like protein
ABC-type phosphate/phosphonate transport system periplasmic component
phosphate/phosphonate ABC transporter substrate-binding protein
phosphonate ABC transporter periplasmic phosphonate-binding protein
ABC-type phosphate/phosphonate transport system periplasmic component
ABC-type phosphate/phosphonate transport system, periplasmic component
phosphate ABC transporter substrate-binding protein
phosphate/phosphonate ABC transporter periplasmic protein
phosphate/phosphonate ABC transporter substrate-binding protein
phosphonate ABC transporter permease
MULTISPECIES: phosphonate ABC transporter permease
membrane channel protein component of Pn transporter
phosphonate/organophosphate ester ABC transporter permease
phosphonates transport system, permease protein
inorganic phosphate transporter
MULTISPECIES: inorganic phosphate transporter
phosphate transporter
low-affinity phosphate transport protein
MULTISPECIES: phosphate transporter permease subunit PtsA
phosphate transporter permease subunit PtsA
phosphate ABC transporter, permease protein PstA

phosphate ABC transporter permease
phosphate transport system permease PstA
phosphate ABC transporter ATP-binding protein
phosphate ABC transporter, ATP-binding protein
MULTISPECIES: phosphate ABC transporter ATP-binding protein
phosphate import ATP-binding protein PstB
phosphonate ABC transporter ATP-binding protein
MULTISPECIES: Phosphate import ATP-binding protein PstB
phosphate ABC transporter permease
phosphate ABC transporter, permease protein PstC
phosphate transporter permease subunit PstC
MULTISPECIES: phosphate ABC transporter permease
phosphate ABC transporter substrate-binding protein
Periplasmic phosphate binding protein
phosphate ABC transporter, phosphate-binding protein
ABC-type phosphate transport system, periplasmic component
MULTISPECIES: phosphate ABC transporter substrate-binding protein
phosphate ABC transporter periplasmic substrate-binding protein PstS
phosphate ABC transporter, periplasmic phosphate-binding protein PstS
nuclease PIN
phosphate-binding protein
MULTISPECIES: phosphate-binding protein
MULTISPECIES: phosphate ABC transporter, phosphate-binding protein PstS
phosphoesterase
PAP2 family phosphoesterase
MULTISPECIES: phosphoesterase
acid phosphatase
putative acid phosphatase
major phosphate-irrepressible acid phosphatase PhoC
MULTISPECIES: acid phosphatase
MULTISPECIES: Acid phosphatase
non-specific acid phosphatase
nonspecific acid phosphatase
Non-specific acid phosphatase
Non-specific acid phosphatase PhoN
acid phosphatase (class B)
putative secreted acid phosphatase
alkaline phosphatase
alkaline phosphatase A
Alkaline phosphatase
phosphodiesterase
phosphodiesterase/alkaline phosphatase D
alkaline phosphatase D
secreted alkaline phosphatase
alkaline phosphatase protein
alkaline phosphatase family protein
putative alkaline phosphatase
Alkaline phosphatase D-related protein
putative phosphodiesterase/alkaline phosphatase
MULTISPECIES: alkaline phosphatase

Phosphodiesterase/alkaline phosphatase D
putative phosphodiesterase/alkaline phosphatase D
phosphodiesterase/alkaline phosphatase D-like protein
phosphodiesterase/alkaline phosphatase d
exopolyphosphatase
putative exopolyphosphatase
MULTISPECIES: exopolyphosphatase
Ppx/GppA phosphatase
ppx/GppA phosphatase family protein
Exopolyphosphatase
glycerophosphoryl diester phosphodiesterase
glycerophosphodiester phosphodiesterase
MULTISPECIES: glycerophosphodiester phosphodiesterase
MULTISPECIES: glycerophosphoryl diester phosphodiesterase
Glycerophosphoryl diester phosphodiesterase
PhnP
phnP protein
carbon-phosphorus lyase complex accessory protein
phosphonoacetaldehyde hydrolase
putative phosphonoacetaldehyde hydrolase
MULTISPECIES: phosphonoacetaldehyde hydrolase
MULTISPECIES: Phosphonoacetaldehyde hydrolase
phosphonoacetate hydrolase
putative phosphonoacetate hydrolase
putative phosphonopyruvate hydrolase PalA
MULTISPECIES: phosphonopyruvate hydrolase
phosphonopyruvate hydrolase
Phosphonopyruvate hydrolase
aryldialkylphosphatase
MULTISPECIES: aryldialkylphosphatase
phosphotriesterase
putative phosphotriesterase
MULTISPECIES: phosphotriesterase
Putative phosphotriesterase Php
parathion hydrolase
Parathion hydrolase
putative aryldialkylphosphatase
putative Aryldialkylphosphatase
MULTISPECIES: parathion hydrolase
phosphotriesterase protein Php
phosphotriesterase PHP
Phosphotriesterase family protein
phosphotriesterase family protein
4-phytase
phosphoanhydride phosphorylase
histidine acid phosphatase
Phosphoanhydride phosphohydrolase (pH 2.5 acid phosphatase) (AP) / 4-phytase
6-phytase
MULTISPECIES: 6-phytase
4-phytase / acid phosphatase

inorganic pyrophosphatase
MULTISPECIES: inorganic pyrophosphatase
putative aminotransferase PalB
2-aminoethylphosphonate--pyruvate aminotransferase
2-aminoethylphosphonate:pyruvate aminotransferase
MULTISPECIES: 2-aminoethylphosphonate:pyruvate aminotransferase
2-aminoethylphosphonate--pyruvate transaminase
putative phosphonoacetaldehyde dehydrogenase
MULTISPECIES: PhoB family transcriptional regulator
transcriptional regulator PhoB
PhoB family transcriptional regulator
phosphate regulon transcriptional regulatory protein PhoB
porin
MULTISPECIES: porin
putative phosphate regulon sensor histidine kinase PhoR
phosphate regulon sensor protein
sensory histidine kinase in two-component regulatory system with PhoB
MULTISPECIES: phosphate regulon sensor protein
phosphate regulon sensor protein phoR
PAS/PAC sensor signal transduction histidine kinase
signal transduction histidine kinase, with phosphoacceptor and ATP binding domain
PhoR
phosphate regulon sensor kinase PhoR, phoR
Phosphate regulon sensor protein phoR
phosphate regulon sensor kinase PhoR
Phosphate regulon sensor protein PhoR (SphS)
PhoU family transcriptional regulator
PhoU-like phosphate transport system protein
phosphate uptake regulator PhoU
MULTISPECIES: phosphate transport system protein PhoU
phosphate uptake regulator, PhoU
MULTISPECIES: PhoU family transcriptional regulator
transcriptional regulator PhoU

B Manuscript 2

The importance of C, N and P as driver for bacterial community structure in German beech dominated forest soils

Fabian Bergkemper[1]*, Gerhard Welzl[1], Friederike Lang[2], Jaane Krüger[2], Michael Schloter[1], and Stefanie Schulz[1]

[1] Research Unit Environmental Genomics, Helmholtz Zentrum München, Ingolstädter Landstr. 1, 85764 Neuherberg
[2] Professur für Bodenökologie, Albert-Ludwigs-Universität Freiburg, Bertoldstr. 17, 79085 Freiburg i. Br.

Abstract

Among several environmental factors shaping soil microbial communities the impact of soil nutrients is of special interest. While continuous application mainly of N and P dramatically shifts community composition during fertilization, it remains unclear whether this effect is consistent in generic, unfertilized beech forest ecosystems of Germany, where differences in nutrient contents are mostly a result of the parental material and climatic conditions. We postulate that in such ecosystems nutrient effects are less pronounced due to the possibility of the soil microbiome to adapt to the corresponding conditions over decades and the vegetation acts as the major driver. To test this hypothesis, we investigated the bacterial community composition in five different German beech dominated forest soils, representing a natural gradient of total- and easily available mineral-P. A community fingerprinting approach was performed using terminal-Restriction Fragment Length Polymorphism analysis of the 16S rRNA gene, while abundance of bacteria was measured applying quantitative real-time PCR. Bacterial communities at the five forest sites were distinctly separated, with strongest differences between the end-members of the P-gradient. However the majority of identified microbial groups (43%) were present at all sites, forming a core microbiome independent from the differences in soil chemical properties. Especially in the P-deficient soil the abundance of unique bacterial groups was highly increased, indicating a special adaption of the community to P limitation at this site. In this regard Correspondence Analysis elucidated that exclusively soil pH significantly affected community composition at the investigated sites. In contrast soil C, N and P contents did mainly affect the overall abundance of bacteria.

Key words: core microbiome / forest soil / nutrient content / diversity

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1 Introduction

Soils are considered as one of the hotspots for biodiversity on Earth, which can be explained by the highly heterogeneous habitat structure both on the micro- and macroscale (*Torsvik* and *Øvreås*, 2002). Besides a wide range of physical factors, including soil pH (*Rousk* et al., 2010), redox conditions (*Pett-Ridge* and *Firestone*, 2005) or water availability, chemical characteristics of soils drive the composition of soil (micro) biota (*Torsvik* and *Øvreås*, 2002). In addition to the amount and quality of bioavailable carbon (C), macronutrients like nitrogen (N) and phosphorous (P) strongly influence the community structure and function of bacteria, fungi, and archaea living in soils. In turn the soil microbiome acts as the major catalyst for the transformation of organically bound C, N, and P and determines consequently the performance of other biota like plants or soil animals (*Stevenson* and *Cole*, 1999). As nitrogen can act either as an important nutrient for higher organisms like plants or induce pollution due to the leaching of nitrate to the groundwater and the formation of N_2O during denitrification (*Ollivier* et al., 2011), in the past many studies focused on the dynamics of N turnover and the identification of microorganisms driving the major processes. Most results suggest a strong influence of N on microbial community structure. For example, a continuous application of nitrogen to soil changed microbial communities in soil leading to an increased abundance of specific phyla (*e.g.*, Gammaproteobacteria, Actinobacteria), whereas others were negatively correlated to the treatment (*e.g.*, Acidobacteria, Cyanobacteria) (*Ramirez* et al., 2010). Also the influence of C on the soil microbiome has been well proven. *Fierer* et al. (2007) detected a positive correlation between certain phyla (*e.g.*, Betaproteobacteria) and C availability in a broad range of different soils, whereas a contrary trend was observed for other taxa (*e.g.*, Acidobacteria).

In comparison to C and N, the impact of naturally diverging soil P stocks on microbial community structure and function has been less intensively studied in the past. Taking the high affinity of P to the solid phase of soils into account, microbial growth is often limited. Even if soils contain sufficient P stocks, the amount of bioavailable P is strongly reduced. De-

* Correspondence: F. Bergkemper;
e-Mail: fabian.bergkemper@helmholtz-muenchen.de

pending on the soil type, P can be immobilized in different forms of phosphate-minerals, attach to clay particles or bind to organic matter complexes, respectively. Therefore it has been concluded that P represents the most inaccessible macronutrient in soils (*Holford*, 1997). Thus, not surprisingly first studies of long-term field trials have indicated that a continuous P amendment induces changes in bacterial community composition and increased species richness (*Tan* et al., 2013; *Liu* et al., 2012). Overall, most studies on the importance of C, N, and P as driver for microbial community structure were performed on sites under agricultural use, where one factor was manipulated (*e.g.*, by fertilization) and the other factors were not changed. Thus, the observed response pattern may not necessarily apply for natural ecosystems without external nutrient input, as on the one hand microbes had the chance to adapt to the site specific conditions for decades and on the other hand several other factors, differing between the studied sites, might mask the consequences of differences in amount and quality of a single nutrient on microbial community structures. However, the impact of macronutrients on community structure merely affects the relative abundance of taxa while the composition of the core community may remain largely stable. In fact, *Orgiazzi* et al. (2013) detected a small set of ubiquitous fungal taxa in soils from distinct ecosystems. It comes as no surprise that this "core mycobiome" comprises generalist fungi which are adapted to nutrient-poor soils and are capable to degrade complex organic compounds.

In this study we compared soil samples from 5 different beech dominated forests (climax ecosystems), which are spatially separated but comprise comparable management practices, geographic location, and type of ecosystem. They represented a cross section of sites with differing amounts of easily available mineral- and total-P contents typical for Middle Europe; furthermore, the investigated sites also differed in total C and N stocks. We asked the question (1) if a bacterial core microbiome could be identified that is typically occurring at all sites despite the significant differences in soil C, N and P. We further aimed (2) to assess the size of this core microbiome and addressed the question (3) about differences in total bacterial community structure in response to the different environmental conditions. We postulated that major parts of the microbiome at the five sites are part of a core microbiome, as all sites are characterized as beech dominated forests and plants are known as strong drivers for the soil microbiome (*Berg* et al., 2014) and only a small proportion of the microbes differs between the sites as a result of the varying conditions in C, N, and P. In the present study we focused on bacterial community structure as bacteria are known also in fungal dominated forest soils to drive turnover of nutrients like P and N and act as storage pool for labile P and N (*Rodríguez* and *Fraga*, 1999; *Gyaneshwar* et al., 2002; *Rösch* et al., 2002; *Boyle* et al., 2008). We quantified the abundance of bacteria by quantitative real-time PCR and analyzed their diversity by terminal-Restriction Fragment Length Polymorphism targeting the 16S rRNA gene. Both analyses were correlated to various soil parameters including total C, N, P, as well as pH.

2 Material and Methods

2.1 Study sites

In the frame of this project, soil samples from five beech dominated forests (*Fagus sylvatica*) across Germany were investigated. All sites are part of the ICP Level II forest monitoring program (International Co-operative Program on Assessment and Monitoring of Air Pollution Effects on Forests) and have been intensively monitored for the past two decades. Besides identical main tree species, the investigated forests have a similar stand age in common, ranging from 80 to 120 years; at none of the sites chemical fertilizers have been applied. However, considerable differences regarding the soil type and the parent material, climate, and topography exist between the five sites. Four of the investigated beech forests are located in the Central German Uplands (Table 1): Site "Conventwald" (CON) is situated in the Black Forest and the soil (Ah-horizon) contains 8.7% primary and secondary min-

Table 1: Description of forest sites that were investigated in this study. The exact geographic location of the Level II plots, mean annual temperature and precipitation rates as well as the classification according to the World Reference Base for Soil Resources are listed.

Forest Site	Geographic Location	Altitude (asl)	Mean annual precipitation	Mean annual temperature	Humus	Bulk	Substrate
BBR	50°21′7.26″ N, 9°55′44.53″ E	850 m	1031 mm	5.8°C	Mull-like Moder	Dystric Skeletic Cambisol	Basalt
CON	48°1′21.4″ N, 7°57′50.65″ E	850 m	1749 mm	6.8°C	More-like Moder	Hyperdystric Skeletic Folic Cambisol	Paragneiss
MIT	48°58′34.18″ N, 12°52′46.74″ E	1050 m	1299 mm	4.99°C	Moder	Hyperdystric Chromic Folic Cambisol	Paragneiss
VES	50°36′23.84″ N, 10°46′14.1″ E	850 m	1446 mm	5.3°C	Moder	Hyperdystric Skeletic Chromic Cambisol	Trachyandisite
LUE	52°50′21.77″ N, 10°16′2.37″ E	150 m	730 mm	8°C	More-like Moder	Hyperdystric Folic Cambisol	Sandy Till

eral-P and 26.5% organic-P (related to total-P; *Hedley* and *Stewart*, 1982); site "Mitterfels" (MIT) is located in the Bavarian Forest (mineral-P: 6.3%, organic-P: 28.1%), site "Vessertal" (VES) in the Thuringian Forest (mineral-P: 4.7%, organic-P: 26.8%), and site "Bad Brueckenau" (BBR) in the Bavarian Rhoen Mountains (mineral-P: 21%, organic-P: 33.2%). The fifth site is situated in the North German Plain near Unterlueß (LUE) in Lower Saxony (mineral-P: 1.8%, organic-P: 25.7%).

2.2 Soil sampling and nucleic acid extraction

Soil samples were taken from the five investigated forest sites within one week in October 2013 to avoid influences of differing climatic conditions. At each site 5 circular subplots (diameter 4 m) were selected in the surrounding of the ICP Level II plots. The distance between the individual subplots was 200 m. Thus, samples from the individual subplots were treated as true replicates in this study (n = 5). For each subplot five samples were taken using a soil auger to a depth of 20 cm (diameter 8 cm), including the Of-, Oh-, and the Ah-horizon. For this study, the Ah-horizon was separated from the rest and used for further analyses. To reduce heterogeneity, the separated Ah-fractions from the five cores per subplot were mixed at the field site and stored on dry ice for nucleic acid extraction. For measurements of chemical and physical soil properties (total carbon, nitrogen, phosphorus and pH) a part of the samples was additionally stored at 4°C. Total nucleic acids were co-extracted from frozen soil samples (–80°C) using the phenol–chloroform extraction method described by *Töwe* et al. (2011). The extracted DNA was quantified by fluorescent nucleic acid staining using the Quant-iT PicoGreen Kit (Life Technologies, USA) and stored at –20°C.

2.3 Soil analysis

Total contents of soil total C and N were measured in milled samples dried at 105°C using an elemental analyzer (Vario EL cube, Elementar, Germany). Contents of soil total P were also determined in milled samples dried at 105°C after microwave-digestion with 65% HNO_3 and H_2O_2 (both Suprapur, Merck Millipore, Germany) using ICP-OES (CIROS CCD, Side-On plasma, Spectro, Germany). Soil pH of dried samples (60°C) was measured in deionized water (EC < 0.06 µS cm^{-1}) with a soil: solution ratio of 1:10 after equilibration for 2 hours.

2.4 Quantitative real-time PCR (qPCR) assay

Bacterial communities were quantified using quantitative real-time PCR targeting the 16S rRNA gene. For the qPCR primers FP*16S* and RP*16S* were used corresponding to *Bach* et al. (2002). Amplification was performed on a 7300 Real-Time PCR System (Applied Biosystems, Germany) with SybrGreen (Life Technologies, USA) as fluorescent dye. The cycling conditions were as follows: initial denaturation (95°C; 10 minutes) followed by 40 cycles of denaturation (95°C; 45 seconds), annealing (58°C; 45 seconds), and elongation (72°C; 45 seconds). The specificity of the amplicons was confirmed by conducting a melting curve after each PCR run. For quantification, serial dilutions of plasmid encoded 16S rRNA genes (originating from *Pseudomonas putida* S16) were used (10^7 to 10^1 gene copies μL^{-1}). To exclude inhibition of co-extracted humic substances, a dilution test was performed in advance. Based on that results all samples were diluted in a ratio of 1:128. The reaction components per 25 µL assay were: Power SYBR Green (12.5 µL) (Life Technologies, USA), DEPC treated water (9 µL), 2 µL of template DNA, 0.5 µL BSA (3%) and 0.5 µL of each primer (5 pmol). qPCR efficiency was calculated with the folllowing equation and resulted in 99.83%:

$$\text{efficiency (\%)} = [10^{(-1/\text{slope})} - 1]. \quad (1)$$

The R^2 of the standard curve was always above 0.98.

2.5 Terminal-Restriction Fragment Length Polymorphism (t-RFLP)

Bacterial community fingerprinting based on 16S rRNA gene t-RFLP analysis was performed for all samples. PCR amplification of the target gene comprised the following components: 2.5 units TopTaq DNA Polymerase (Qiagen, Germany), 5 µL TopTaq PCR buffer (10×), 5 µL CoralLoad concentrate (10×), 5 µL Q-solution (5×), 2.5 µL dNTPs (2 nM), 1 µL of each primer (10 pmol), 20 ng of template DNA and ad water to 50 µL. Target specific primers were used according to *Bruce* et al. (1992) (pA) and *Wawrik* et al. (2005) (1401R). Forward primer (pA) was additionally labeled with 5′-FAM (6-carboxyfluorescein). PCR cycling conditions are listed below: hotstart (95°C; 5 minutes), 30 cycles of denaturation (94°C; 45 seconds), annealing (56.5°C; 45 seconds), and elongation (72°C; 1 minute), followed by a final elongation step (72°C; 10 minutes). PCR products were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). 400 ng of amplicons were enzymatically digested using MspI (Fermentas, Germany) according to the manufacturer's protocol. Restricted fragments were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). Subsequent t-RFLP was conducted using 5 ng of sample according to *Töwe* et al. (2011). The t-RFLP assay contained an 800-fold dilution of MapMarker 1000 ladder (Bio-Ventures, USA). Analysis of electropherograms was performed using the software PeakScanner 2 (Life Technologies, USA). Fragments shorter than 50 bp were omitted before datasets were further processed using T-REX software (*Culman* et al., 2009). For noise filtering the "Std dev multiplier for fluor B" was set to 0.8 using peak height. Operational taxonomic units (OTUs) were defined as peaks within a clustering threshold of 1 bp.

2.6 Statistical Analysis

Statistical analysis was implemented using the R environment (version 3.2.0) (*R Core Team*, 2015). The impact of abiotic soil properties on abundance of 16S rRNA genes (logarithmic) was tested by analysis of variance (ANOVA). Here, normal distribution was verified using the "Shapiro–Wilk normality test" in R. The effect of abiotic soil properties on diversity of 16S rRNA genes was investigated by permutational multivariate analysis of variance using distance matrices.

For determination of a core microbiome VENN diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/) were calculated based on filtered t-RFLP data (fragments occurring in less than 2 replicates were omitted). To analyse regression-coefficients and correlations between two data matrices, Pearson correlation was applied for soil parameter and 16S rRNA gene abundance data (qPCR; logarithmic). Canonical correspondence analysis (CCA) (regression based) and regularized canonical correlation analysis (RCC) (correlation based) were applied for soil parameter compared with 16S rRNA gene diversity data (t-RFLP). For CCA and RCC an Anscombe square root transformation was applied to the t-RFLP data to stabilize the noise variance (*Anscombe*, 1948; *Green*, 1979).

3 Results

3.1 Soil properties

Contents of soil total C, N, and P as well as pH were determined in the mineral topsoil (Ah-horizon) of five different beech forest soils. As shown in Table 2, the five sites formed a natural "gradient" regarding the total-P contents but also differed in their C and N stocks. Highest amounts of soil C and P were detected at site BBR, exceeding the measured amounts at site LUE, where lowest values were detected threefold respective twentyfold. Regarding soil total-P contents, the remaining sites CON, MIT and VES are located in-between these two "extreme" sites. Soil pH was highest at sites BBR and CON (pH > 4) and lowest at MIT (pH < 3.8). Thereby all investigated soils were classified as extremely acid (*Soil Survey Division Staff*, 1993).

3.2 Bacterial 16S rRNA gene abundance (qPCR)

An analysis of variance (ANOVA) revealed a significant influence of the sampling site on the abundance of the 16S rRNA genes ($P < 0.001$). In detail, 16S rRNA gene abundances ranged from 3.14×10^{10} copies g^{-1} soil dry weight (LUE) to 2.05×10^{11} gene copies g^{-1} soil (CON). While the LUE samples revealed significantly lowest gene copy numbers, samples from VES, BBR and MIT ranged between 1.12×10^{11} and 1.76×10^{11} 16S rRNA gene copies per g^{-1} soil (Fig. 1).

When the quantitative real-time PCR data was correlated to the analyzed soil parameters (Table 2), a significant positive correlation ($P < 0.001$) between soil total C content and the abundance of 16S rRNA genes was detected (Table 3). Furthermore, total N and P contents ($P < 0.001$; $P < 0.004$) were positively correlated to the abundance of 16S rRNA genes. Likewise soil pH was positively correlated to 16S rRNA gene abundance, but the level did not reach significance ($P = 0.166$).

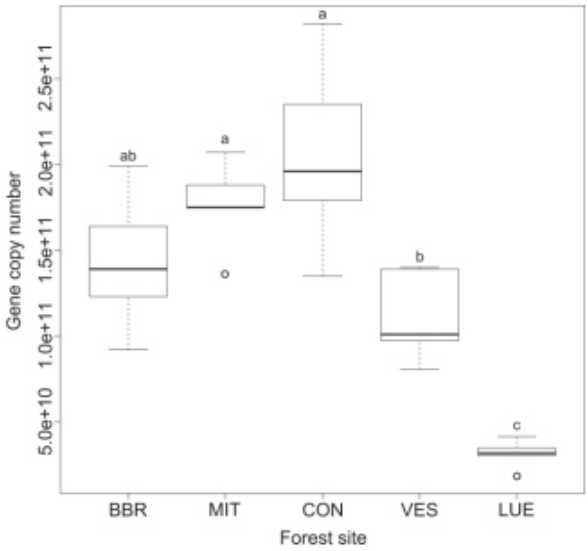

Figure 1: Abundance of 16S rRNA genes in five different forest soils. Shown are gene copy numbers (16S rRNA gene) per gram soil dry weight for five different forest sites. Significant differences between the soils are indicated ($n = 5$).

Table 3: Pearson correlation analysis of logarithmic qPCR data and contents of soil C, N, P and pH in five different forest soils. Listed are Pearson correlation coefficients and levels of significance for the investigated soil parameters ($n = 5$).

Soil parameter	Pearson correlation coefficient	*p*-value
C	0.779	< 0.001
N	0.792	< 0.001
P	0.594	0.004
pH	0.286	0.166

Table 2: Contents of total carbon, nitrogen and phosphorus together with pH in investigated soil samples from five different forest sites. Given are means and standard deviations of five replicates ($n = 5$).

Sample	C / mg g^{-1}	SD	N / mg g^{-1}	SD	P / mg g^{-1}	SD	pH_{H_2O}	SD
BBR	129.96	33.19	9.02	2.37	2.27	0.89	4.35	0.18
CON	110.03	28.61	6.89	0.82	1.35	0.81	4.22	0.29
MIT	117.21	27.53	6.85	1.41	1.29	0.37	3.73	0.11
VES	95.63	24.49	5.92	1.12	1.11	0.54	3.77	0.14
LUE	35.67	9.15	1.94	0.46	0.11	0.01	3.82	0.09

3.3 Bacterial community fingerprinting (t-RFLP)

After filtering, a total of 136 different terminal-restriction fragments (t-RFs) were detected in the datasets. Highest bacterial richness was observed in MIT (105 different t-RFs), followed by LUE (97), VES (95), and CON (93). In contrast, BBR merely yielded 83 distinct t-RFs. As revealed by the VENN-diagram (Fig. 2), a considerable amount of t-RFs (59) (43% referred to the total number of OTUs) was shared by the five forest soils in common. The majority of the shared t-RFs (64%) accounted for 2% or less of the total community at each investigated site. The amount of unique t-RFs varied between the 5 investigated soils. While 12 t-RFs were exclusively found at LUE, soils MIT (7), Con (6), VES (3), and BBR (2) showed decreasing numbers (Fig. 2). Against our initial postulation, the impact of the unique t-RFs on community composition was lower compared to the common t-RFs and ranged from 0.3% to 1% of the respective community. Furthermore specific overlaps between the microbial communities of two or more different soils were observed (Fig. 2). While BBR and LUE did not share any t-RFs except for the 59 common ones, BBR and CON harbored five t-RFs exclusively occurring in these two soils. In contrast seven t-RFs were detected likewise in MIT, VES, CON and LUE, but were absent in BBR.

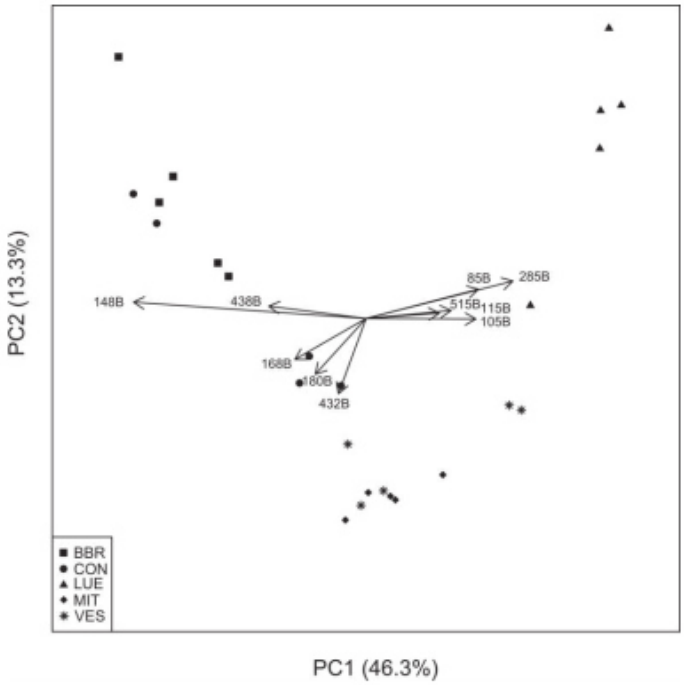

Figure 3: Principal Component Analysis. The Principal Component Analysis was based on t-RFLP data (16S rRNA gene) from five different forest soils. The influence of certain OTUs on bacterial community separation is shown ($n = 5$).

Based on t-RFLP data a Principal Component Analysis (PCA) was performed (Fig. 3). While PC1 explained 46.3% of the total variance in the datasets, PC2 accounted for 13.3%. An analysis of variance revealed a significant influence of the measured abiotic soil properties present at each site on the bacterial community composition ($P < 0.001$). Depending on the forest site three distinct clusters were detected. The LUE and BBR samples comprised their own cluster, respectively. Samples from MIT and VES formed a third cluster, while samples from CON overlapped with the BBR and MIT/VES cluster. The prominent separation of the LUE samples was mainly caused by the high abundance of five t-RFs (85B, 105B, 115B, 285B, and 515B), which comprised 21.12% of the total LUE community. In contrast, the BBR community was mainly affected by the presence of two t-RFs (148B and 438B), whereas the CON soil was predominantly influenced by three t-RFs (168B, 180B, and 432B).

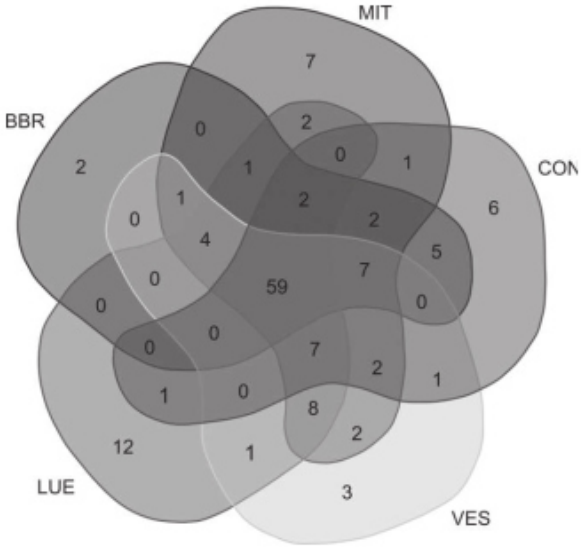

Figure 2: Definition of a core microbiome in five different forest soils. Based on t-RFLP data (16S rRNA gene) of five different forest soils a VENN diagram was calculated. Shared OTUs representing the core microbiome (59 t-RFs) as well as uniquely found OTUs are shown ($n = 5$).

For the relation of t-RFLP data to soil parameters a Canonical Correspondence Analysis (CCA) (Fig. 4) and a Canonical Correlation analysis (Fig. 5) were performed. The CCA captured 36.8% of the total variability within the datasets and revealed a clustering of t-RFLP data into distinct groups. CCA1 accounted for 62.8% of the constrained variability followed by CCA2 with 21.7%, CCA3 with 9.4% and CCA4 with 6%. While the bacterial communities in LUE and MIT/VES formed individual cluster, the communities in BBR and CON were strongly overlapping and therefore represented a common, third cluster (Fig. 4). Apparently the latter communities were strongly linked to higher soil pH. In contrast, soil C, N, and P contents did not correlate with one single cluster. In fact, the soil nutrient contents were

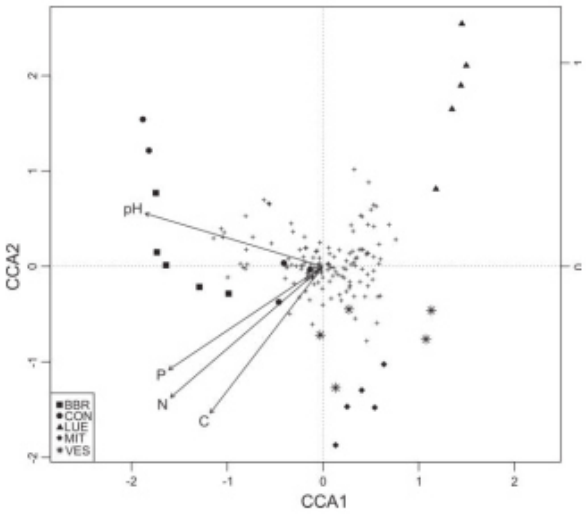

Figure 4: Canonical Correspondence Analysis. T-RFLP data (16S rRNA gene) from five different forest soils was related to contents of soil total C, N, P and pH. Shown is the impact of the investigated macronutrients and pH on community separation as well as the distribution of OTUs within the five soils ($n = 5$).

polar to the LUE cluster and therefore responsible for the overall separation of the LUE samples from all others. As revealed by analysis of variance the stocks of soil C, N, and P were not significantly influencing community composition at the respective sites ($P < 0.233$; $P < 0.133$; $P < 0.085$). In contrast, soil pH had a significant impact on the bacterial community structure ($P < 0.003$). The Canonical Correlation analysis revealed certain t-RFs clustering particularly with soil pH (60B, 70B, 483B, 595B, and 802B; Fig. 5). Interestingly, these t-RFs were predominantly found in BBR and CON or were actually absent in the remaining soils. Two fragments (148B and 438B), which were specifically inducing separation of the BBR/CON cluster (Fig. 3), were likewise correlated with pH. In contrast, fragments that were specifically correlated to soil C, N, and P (138B, 168B, 189B, 430B, and 479B) were consistently detected within samples from the BBR/CON and MIT/VES cluster but were scarce respectively lacking in the LUE soil. However, t-RFs that were highly abundant in LUE (85B, 105B, 115B, 263B, 285B, and 515B) were all located in the periphery of the upper-left quartile of the correlation circle (Fig. 5) and thereby negatively correlated to all investigated soil parameters. Most abundant t-RFs in MIT and VES (90B, 136B, 146B, 148B, 304B, 432B, and 492B) basically scattered across the entire circle, thereby showing no consistent correlation (either positive or negative) to soil nutrient contents or pH.

4 Discussion

Soil microorganisms are influenced by a number of environmental factors including soil physical parameters like pH as well as chemical parameters like the availability of C, N, and P (*Fierer* and *Jackson*, 2006; *Fierer* et al., 2007; *Ramirez* et al., 2010; *Tan* et al., 2013). Our data indicate that although nutrient contents and pH differed substantially across the investigated sites, the five forest soils share a common core microbiome, which confirms our initial hypothesis. It comprises 43% of all bacterial OTUs that were detected in our datasets. This indicates that environmental factors like the type of ecosystem (forest) and the main tree species (*Fagus sylvatica*) might trigger microbial communities to a large extent and thereby induce the formation of a core microbiome which is present at all investigated sites. Accordingly, a study of *Urbanová* et al. (2015) detected a strong impact of the tree species on microbial community structures. Especially litter composition and pH are decisive factors in this respect. Furthermore, our data imply that this community shaping effect induced by the tree species overpowers the impact of distinct soil nutrient contents and pH on a broader scale. In fact, we investigated samples that represent deeply contrasting forest soils in terms of total C, N, P contents, pH, types of humus and bulk soil as well as mean temperature and precipitation rates. Although the t-RFLP fingerprinting approach we applied in this study does not allow a clear identification of particular microbial groups in terms of their phylogenetic position, it is considered as a suitable tool for the comparison of the relative diversity of samples comparable to sequencing the amplified 16S rRNA gene (*Orcutt* et al., 2009; *Van Dorst* et al.,

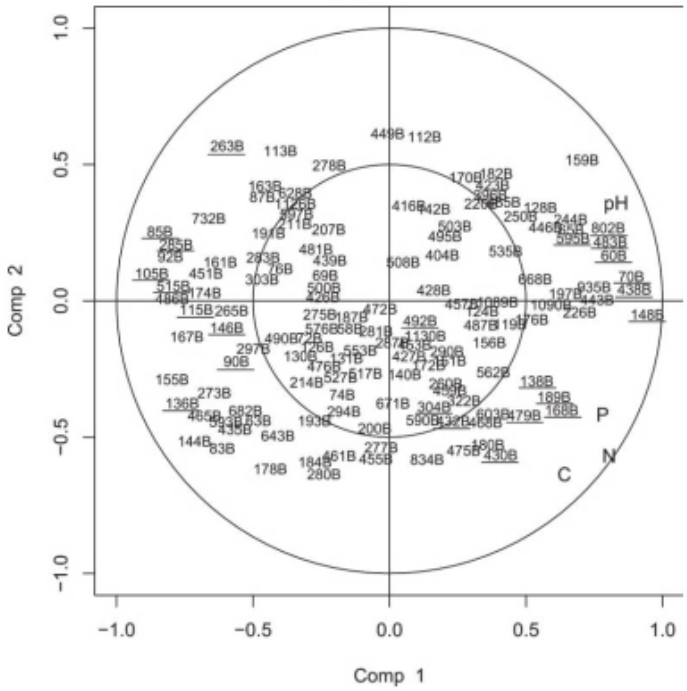

Figure 5: Canonical Correlation Analysis. T-RFLP data (16S rRNA gene) from five forest sites was correlated to contents of soil C, N, P, and pH. Most abundant t-RFs in the respective soils are underlined ($n = 5$).

2014). However, t-RFLP analysis allows the high throughput analysis of a larger number of samples compared to sequencing, which makes the analysis of replicates possible. This in turn is of high importance to take spatial heterogeneity of the soils into account. Based on *in silico* analysis of the t-RFs, the core microbiome we detected might include Acidobacteria, Proteobacteria, Verrucomicrobia, Actinobacteria, and Firmicutes (data not shown), phyla that have been also found in forest soils by other groups (*Rasche* et al., 2011; *Baldrian* et al., 2012; *Bergkemper* et al., 2015). Obviously, nutrient contents and pH cause an adaption of the core microbiome to the respective soil conditions in terms of the relative contribution of the mentioned phyla to the core microbiome. Furthermore, especially in LUE the occurrence of several unique t-RFs indicates an adaption to nutrient limitation (Fig. 2). The relatively low abundance of unique OTUs observed in all soils (0.3% to 1% of the respective community) is in line with the concept of the "rare biosphere" (*Lynch* and *Neufeld*, 2015). Rare taxa could serve as keystone species, having exceptionally strong effects on ecosystem service including nutrient cycling processes despite their low abundance. Keystone species tremendously increase the genetic and functional diversity of microbial communities thereby maintaining ecosystems stability (*Lynch* and *Neufeld*, 2015).

Despite the presence of a core microbiome, the PCA based analysis of the t-RFLP fingerprints (Fig. 3) indicated three discrete groups, which was mostly linked to differences in relative abundance of certain t-RFs and only in some cases to the presence/absence of t-RFs when different samples were compared. Unique t-RFs were mainly observed at the LUE site. This indicates that the abundance of certain taxa is driven by the soil conditions present at a site. For example the low contents of C, N, and P in LUE (Table 2) might favor the growth of oligotrophic taxa. This applies in particular for members of the Acidobacteria. Their abundance is negatively correlated to C availability (*Fierer* et al., 2007), while they are characterized by slow growth rates in nutrient-limited soils (*Ward* et al., 2009). The effect of nutrient limitation in LUE on microbial life also becomes obvious in a significantly lower abundance of bacteria as indicated by the quantification of 16S rRNA gene copy numbers (Fig. 1). In contrast, the sites BBR and CON are rather rich in macronutrients. Several t-RFs, which are predominantly influencing the BBR and CON samples (148B, 168B, 180B, 432B, 438B) (Fig. 3), indicate an increased abundance of more copiotrophic taxa in these soils. This might especially comprise members of Proteobacteria (*Smit* et al., 2001) and Bacteroidetes (*Fierer* et al., 2007). For site BBR an exceptionally high abundance of especially Alphaproteobacteria has recently been shown (*Bergkemper* et al., 2015). The distinct separation of the bacterial communities in BBR/CON and LUE can probably be explained by the soil nutrient status, leading to characteristic ratios of oligotrophic and copiotrophic taxa as proposed by *Smit* et al. (2001). The bacterial communities in MIT and VES are virtually located in-between the abovementioned "extreme" sites since both soils are lacking the impact of specialized taxa (either oligotrophic or copiotrophic).

To further investigate the individual influence of crucial soil parameters on community structure a CCA was performed (Fig. 4). Surprisingly, despite the tremendous differences in soil total C, N and P contents between the five soils, merely pH significantly influenced community structure. While the bacterial abundance was significantly positive correlated to soil C, N, and P stocks, the soil pH had a significant impact on community composition instead (Table 3). Regarding soil nutrients the contents of P had the strongest impact on community structure, followed by N and C. Indeed, these trends did not reach the level of significance which is surprising since P stocks in BBR exceeded LUE twentyfold. At the same time the strong effect of soil pH on bacterial community composition we detected in our datasets is in accordance with previous studies (*Lauber* et al., 2009). Evidently, the BBR and CON bacterial communities are primarily shaped and thereby separated from the remaining soils by the higher pH (> 4). In contrast, the rising contents of soil C, N, and P similarly influenced the community compositions at BBR, CON, MIT, and VES. Therefore, one particular soil parameter that would significantly induce community separation at one site (*e.g.*, the enormous P stocks in BBR) was not detected. The general limitation of soil nutrients in LUE leads to an adaption of the respective bacterial community and induces the separation of the LUE cluster compared to the remaining samples. This adaption is also reflected by the highest number of unique OTUs detected in LUE (12% of the total bacterial community). However, the distinct separation of the BBR/CON and the LUE cluster is probably not exclusively caused by the differences regarding pH or contents of C, N, and P. Also the prevalent form of soil P might influence the community structure to some extent. In this regard BBR and CON contain the highest amounts of mineral-P, while site LUE has an increased proportion of soil organic-P (compared to mineral-P). According to *Goldstein* (1994; 1995), the bacterial solubilization of calcium and mineral phosphates is attributed to the acidification of the periplasmic space *via* the quinoprotein glucose dehydrogenase or oxidation of other aldose sugars. The higher contents of mineral-P in BBR and CON could selectively enrich taxa performing the solubilization of inorganic phosphorus. In contrast, in LUE mineral-P is hardly present, while the relative percentage of organic-P is strongly increased. Since soil bacteria are famous for their ability to release orthophosphate from phosphomono- and diesters (*Rodríguez* and *Fraga*, 1999), phytate (myo-inositol hexakisphosphate) (*Rodríguez* et al., 2006) or phosphonates (*McGrath* et al., 2013), the LUE site might favor growth of microorganisms that are able to mineralize different forms of organic-P instead. Consequently, the bacterial communities in BBR/CON and LUE, which are the respective end-members of the detected P-gradient, are likely to be separated from each other by the form and content of soil P. These findings are in accordance with previous studies likewise demonstrating the strong impact of soil P on microbial community structures in fertilization experiments (*Sakurai* et al., 2008; *Liu* et al., 2012; *Tan* et al., 2013). However, our study included a broader range of different soil parameters and further differentiated their respective impact on community composition. Regarding the investigated beech forest sites the effect of soil P content on community separation was comparable to that of C and N. Yet it was overpowered by the impact of soil pH.

5 Conclusions

In conclusion, our data revealed the existence of a considerable, stable core microbiome within five different German forest soils, despite distinct differences regarding soil physicochemical parameters. We propose that this core microbiome is mainly triggered by the type of ecosystem and the main tree species. In the compared ecosystems, which are highly diverse in terms of their C, N and P content, the effect of phosphorus on community structures was slightly stronger compared to that of carbon and nitrogen. However, soil pH had the strongest impact on bacterial community composition by far. Therefore, soil physicochemical parameters lead to an adaption of the core microbiome which mostly changes the relative abundance of certain bacterial groups.

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Novel oligonucleotide primers reveal a high diversity of microbes which drive phosphorous turnover in soil

Fabian Bergkemper [a], Susanne Kublik [a], Friederike Lang [b], Jaane Krüger [b], Gisle Vestergaard [a], Michael Schloter [a,*], Stefanie Schulz [a]

[a] Research Unit Environmental Genomics, Helmholtz Zentrum München, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany
[b] Professur für Bodenökologie, Albert-Ludwigs-Universität Freiburg, Bertoldstr. 17, 79085 Freiburg i. Br., Germany

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ABSTRACT

Phosphorus (P) is of central importance for cellular life but likewise a limiting macronutrient in numerous environments. Certainly microorganisms have proven their ability to increase the phosphorus bioavailability by mineralization of organic-P and solubilization of inorganic-P. On the other hand they efficiently take up P and compete with other biota for phosphorus. However the actual microbial community that is associated to the turnover of this crucial macronutrient in different ecosystems remains largely anonymous especially taking effects of seasonality and spatial heterogeneity into account. In this study seven oligonucleotide primers are presented which target genes coding for microbial acid and alkaline phosphatases (*phoN*, *phoD*), phytases (*appA*), phosphonatases (*phnX*) as well as the quinoprotein glucose dehydrogenase (*gcd*) and different P transporters (*pitA*, *pstS*). Illumina amplicon sequencing of soil genomic DNA underlined the high rate of primer specificity towards the respective target gene which usually ranged between 98% and 100% (*phoN*: 87%). As expected the primers amplified genes from a broad diversity of distinct microorganisms. Using DNA from a beech dominated forest soil, the highest microbial diversity was detected for the alkaline phosphatase (*phoD*) gene which was amplified from 15 distinct phyla respectively 81 families. Noteworthy the primers also allowed amplification of *phoD* from 6 fungal orders. The genes coding for acid phosphatase (*phoN*) and the quinoprotein glucose dehydrogenase (*gcd*) were amplified from 20 respectively 17 different microbial orders. In comparison the phytase and phosphonatase (*appA*, *phnX*) primers covered 13 bacterial orders from 2 different phyla respectively. Although the amplified microbial diversity was apparently limited both primers reliably detected all orders that contributed to the P turnover in the investigated soil as revealed by a previous metagenomic approach. Genes that code for microbial P transporter (*pitA*, *pstS*) were amplified from 13 respectively 9 distinct microbial orders. Accordingly the introduced primers represent a valuable tool for further analysis of the microbial community involved in the turnover of phosphorus in soils but most likely also in other environments.

1. Introduction

Phosphorus (P) is an essential macronutrient for all biota on earth as it is integral for processes of cellular bioenergetics, the formation of lipid bilayers and the genetic backup (Elser, 2012). In most natural ecosystems in addition to nitrogen (N), P is a major growth limiting factor of primary production (Vitousek et al., 2010). Although many soils contain extensive stocks of total P, the bioavailability of soluble orthophosphate, which can be used by most biota is low (Rodriguez and Fraga, 1999). Consequently P is considered as the most inaccessible and unavailable of all soil nutrients (Holford, 1997). Therefore the prominent role of microorganisms for the turnover of soil phosphorus is generally accepted, since they can increase the P availability by different means, which lead to improved P nutrition of plants and other biota (Richardson and Simpson, 2011). Especially the metabolic traits which perform the mineralization of organic-P and the solubilization of inorganic-P are of peculiar interest. Most previous studies mainly focused on the characterization of the respective enzymes using cultivated bacterial and fungal strains (Rodriguez and Fraga, 1999). However only few attempts have been made to directly target genes that drive the turnover of soil P in microbial communities derived from environmental samples without introducing the bias caused by isolation. This might be of great relevance since strains that perform well under controlled conditions might easily be outcompeted in natural environments (Rodriguez and Fraga, 1999). It was not until 2008 that Sakurai et al. (2008) developed PCR primers specifically targeting the alkaline phosphatase *phoD* gene. Since an amplification bias towards Alphaproteobacteria was recently observed (Tan et al., 2013), Ragot et al. (2015) introduced a new set of primers which increased the covered diversity of *phoD* genes in soils by the factor of 7. For genes encoding other key processes of the

* Corresponding author at: Research Unit Environmental Genomics, Helmholtz Zentrum München, Ingolstädter Landstr. 1, 85764, Germany.
E-mail address: schloter@helmholtz-muenchen.de (M. Schloter).

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microbial P turnover like the mineralization of organic-P, the solubilization of inorganic-P or phosphorus uptake, primer systems are still missing.

Recently, we performed a metagenomic analysis based on whole genome shotgun (WGS) sequencing in two undistorted beech forest soils to gain insights into the microbial P turnover in soil (Bergkemper et al., 2015). The results highlighted Rhizobiales, Actinomycetales and Acidobacteriales as drivers for P turnover but also rare orders like Solibacterales contributed to the turnover of soil P. Dominating processes were the uptake of P by the phosphate inorganic transporter (Pit) and the phosphate specific transport (Pst) system, the solubilization of inorganic P and the mineralization of organic P by alkaline and acid phosphatases, phosphonatases and phytases. In contrast, Glycerol-3-phosphate transporter or C–P lyases were of minor importance. As the obtained sequencing depth of metagenomic approaches is still far away from allowing quantitative conclusions (Delmont et al., 2011), metagenomic data can serve as a starting point for a targeted primer development to answer questions about spatial and temporal distribution of microbial key players (Schöler et al., 2016). Moreover due to the important role of microorganisms for P turnover we propose that there is a need to investigate the entire set of enzymes (genes) involved in processes of microbial P mineralization, solubilization and uptake to better understand the turnover of this crucial nutrient in soil. Thus it was the aim of this study to develop primer systems suitable for high-throughput amplicon sequencing as well as quantitative real-time PCR approaches. Based on the obtained metagenomic data we chose 7 marker genes, which were highly dominant in the metagenomes of the two forest soils. Overall we covered important steps of the solubilization of inorganic P, the mineralization of organic P and the cellular P uptake. For targeting P solubilization processes, which are mainly attributed to the efflux of protons and organic anions during the oxidation of glucose and other aldose sugars (Goldstein, 1994), we targeted the quinoprotein glucose dehydrogenase (*gcd*) gene (Cleton-Jansen et al., 1990). With respect to the mineralization of organic-P three different classes of enzymes were investigated: (i) Nonspecific acid phosphohydrolases (NSAPs) and alkaline phosphatases (ALPs) perform the dephosphorylation of phosphoester and — anhydride bonds. Here we focused on the NSAP class A (*phoN*) (Rossolini et al., 1998) and the ALP PhoD (*phoD*) (Eder et al., 1996). (ii) The mineralization of more complex myo-Inositol-1,2,3,4,5,6-hexakisphosphates (IP_6) is catalyzed by microbial phytases. Especially enzymes which are classified as 6-phytases (*appA*) (Golovan et al., 2000) were targeted here. (iii) The enzymatic cleavage of relatively stable carbon–phosphorus bonds, which occur in natural and synthetic organophosphonates, is performed by C–P lyases and phosphonoacetaldehyde hydrolases. The latter one (phosphonatase; *phnX*) (Hsieh and Wanner, 2010) was further investigated in this study. Moreover, microbes also compete for the available P with other biota as they have efficient phosphate uptake systems (Pst, Pit transporter). Therefore, we also targeted the key genes *pitA* and *pstS* (Hsieh and Wanner, 2010).

We aimed to cover a broad diversity of distinct microorganisms for the mentioned processes. The primer specificity towards the individual target genes and the diversity of the amplified microbial communities were investigated by Illumina amplicon sequencing of genomic DNA extracted from beech forest soil, which has also been included in the metagenomic analysis described above.

2. Material and methods

2.1. Site description and soil sampling

For this study soil samples were taken from a beech (*Fagus sylvatica*) dominated German forest site located in the Bavarian Rhoen Mountains near Bad Brueckenau (BBR) (50.352009°N, 9.929028°E). The stand has an average age of 120 years and is part of the International Cooperative Program for the Assessment and Monitoring of Air Pollution Effects on Forests (ICP Level II). The forest site reaches up to 850 m above sea level and the mean annual precipitation and temperature are 1031 mm respectively 5.8 °C. According to the World Reference Base (WRB) for Soil Resources the soil is classified as Dystric Skeletic Cambisol with Mull-like Moder and basalt as the substrate. The soil organic layer (Of-horizon) is characterized by a total carbon (C) content of 481.81 mg/g, a total nitrogen (N) content of 20.66 mg/g, a total phosphorus (P) content of 1.57 mg/g and a pH (H_2O) of 5.30 (pers. comm. F. Lang, Freiburg). Soil samples were taken from the organic layer in September 2015 after the removal of forest litter and immediately stored on dry ice.

2.2. Nucleic acid extraction

DNA was directly extracted from 0.5 g of frozen soil (−80 °C) using Lysing Matrix E tubes (MP Biomedicals, France) and Precellys 24 (Bertin Technologies, France) according to Töwe et al. (2011). Additionally a negative control of the extraction procedure was performed without soil input (NKE). Total genomic DNA was quantified using the Quant-iT PicoGreen kit (Life Technologies, USA) and stored at −20 °C for further analysis.

2.3. Primer development

Oligonucleotide primers were designed for microbial genes encoding enzymes that catalyze the mineralization of soil organic phosphorus (P) (*phoD*, *phoN*, *appA*, *phnX*), the solubilization of inorganic-P (*gcd*) as well as genes encoding microbial phosphate transport systems (*pitA*, *pstS*). The investigated genes together with the EC (TC) numbers of corresponding proteins are listed in Table 1. Oligonucleotide primers were designed in a way to amplify target genes from a broad diversity of different soil microorganisms. However to ensure a high level of specificity during PCR the grade of primer degeneracy was limited to a maximum of 64-fold. Since the amplicon size ranged from 147 bp to 375 bp the primers were suitable for both next generation sequencing (Illumina manual "16S Metagenomic Sequencing Library Preparation") as well as quantitative real-time PCR approaches (Karsai et al., 2002). Moreover the calculated annealing temperature of all primers was set to 60 °C. For primer development sequences of the investigated genes were derived from the NCBI Protein database (May 2015). The obtained protein sequences were aligned using Clustal Omega (Sievers et al., 2011) and analyzed for conserved domains. The conserved regions that were targeted by the individual primers as well as corresponding reference entries from the NCBI Conserved Domain Database (Marchler-Bauer et al., 2015) are listed in Supplementary Table S1. Potential oligonucleotide primers were designed using the CODEHOP program (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) (Rose et al., 2003). Sequences of primers developed in this study are listed in Table 1.

2.4. Amplicon sequencing

Next generation amplicon sequencing was performed for seven primer pairs using the Illumina MiSeq platform (Illumina Inc., USA). Library preparation basically followed the protocol "16S Metagenomic Sequencing Library Preparation" (Illumina Inc., USA) but included further adaption steps to the specific characteristics of the primers. Briefly, a first round of amplicon PCR (PCR1) was performed using target specific primers (Table 1) and genomic DNA extracted from forest soil. The reaction was performed in triplicates and comprised: 2.5 μl 10× FastStart High Fidelity Reaction Buffer (Roche Diagnostics, Germany), 2 μl dNTPs (2 nmol each), 1 μl of each primer (10 pmol), 0.5 μl of BSA (3%), 1.25 U FastStart High Fidelity Enzyme Blend (Roche Diagnostics, Germany), 25 ng of genomic DNA (50 ng for *appA* and *phoN*) and ad water 25 μl. The PCR cycling conditions are listed below: Hotstart

Table 1
Listed are investigated genes, classification of corresponding proteins, the mean amplicon length and nucleotide sequences of developed primers.

Protein	Classification	Target gene	Mean expected amplicon length (bp)	Reference	Primer name	Primer sequence 5′–3′
Alkaline phosphatase (phoD)	EC 3.1.3.1	*phoD*	208	Eder et al. (1996)	*phoD*-FW	TGTTCCACCTGGGCGA YWMIATHTAYG
					phoD-RW	CGTTCGCGACCTCGTG RTCRTCCCA
Acid phosphatase (class A)	EC 3.1.3.2	*phoN*	159	Rossolini et al. (1998)	*phoN*-FW	GGAAGAACGGCTCCTA CCCIWSNGGNCA
					phoN-RW	CACGTCGGACTGCCAG TGIDMIYYRCA
6-Phytase/acid phosphatase	EC 3.1.3.26/EC 3.1.3.2	*appA*	375	Golovan et al. (2000)	*appA*-FW	AGAGGGTGGTGATCGTGATG MGICAYGGNRT
					appA-RW	GCCTCGATGGGGTTGA AIADNGGRTC
Phosphonoacetaldehyde hydrolase	EC 3.11.3.1	*phnX*	147	McGrath et al. (2013)	*phnX*-FW	CGTGATCTTCGACtGGGCNGGNAC
					phnX-RW	GTGGTCCCACTTCCCC ADICCCATNGG
Quinoprotein glucose dehydrogenase	EC 1.1.5.2	*gcd*	330	Cleton-Jansen et al. (1990)	*gcd*-FW	CGGCGTCATCCGGGSITIYRAYRT
					gcd-RW	GGGCATGTCCATGTCC CAIADRTCRTG
Phosphate inorganic transporter	TC 2.A.20	*pitA*	270	Elvin et al. (1986)	*pitA*-FW	GGTCTTCGAGTTCATGAACG GNTTYCAYGA
					pitA-RW	CCAGGTGACCAGGTTCCAIRNDAT
Phosphate-specific transporter (periplasmic phosphate-binding protein)	TC 3.A.1.7.1/EC 3.6.3.27	*pstS*	221	Hsieh and Wanner (2010)	*pstS*-FW	TCTACCTGGGGAAGATCACA AARTGGRAYGA
					pstS-RW	TGCCGACGGGCCAITYNWC

(95 °C; 7 min), 30 cycles of denaturation (95 °C; 1 min), annealing (60 °C; 1 min) and elongation (72 °C; 45 s) followed by a final elongation step (72 °C; 7 min). The optimum annealing temperature of the individual primers was investigated in a preliminary test performing a gradient PCR (annealing temperature: 55 °C to 65 °C). Further on a second amplicon PCR (PCR2) was performed using target specific primers comprising Illumina overhang adapter sequences. The amplification was conducted as previously stated. However 2 μl of PCR1 were used as template, 10 pmol of each primer containing adapter overhangs were applied and the cycle number was limited to ten cycles. The amplified triplicates (PCR2) were analyzed on a 2% agarose gel, pooled and subsequently purified using the PCR clean-up /Gel extraction kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. However the washing-buffer (NT1) was diluted in a 1:4 ratio with DEPC treated water prior to elution. Regarding the *phoD* primers three distinct bands were detected after amplification (Supplementary Fig. S1). To check whether all bands represented the target gene the three bands were extracted separately from the agarose gel (PCR clean-up /Gel extraction kit; Macherey-Nagel, Germany) and subsequently treated as individual amplicons (datasets named: *phoD*-K, *phoD*-M, *phoD*-L). Additionally *phoD* amplicons were purified directly from the PCR2 reaction in a second approach (without separating individual bands) (dataset named: *phoD*). Moreover negative controls of the PCR (NKP) as well as negative controls of the DNA-extraction procedure (NKE) were performed for all primers. Finally a total of ten amplicon samples (Supplementary Table S2) plus sixteen negative controls were further processed after PCR2. The correct amplicon sizes were checked on a Bioanalyzer 2100 instrument (Agilent Technologies, USA) using the DNA 7500 kit (Agilent Technologies, USA). The concentration of the purified samples was measured by the Quant-iT PicoGreen kit (Life Technologies, USA). For library preparation the Nextera XT v2 Index kit set A was used (Illumina Inc., USA). The Indexing PCR was performed in 25 μl reactions containing: 12.5 μl NEBNext High-Fidelity Master Mix (New England Biolabs, USA), 2.5 μl of each Indexing primer, 10 ng of purified amplicons and 6.5 μl DECP treated water. The amplification procedure included an initial denaturation step (98 °C; 30 s), 8 cycles of denaturation (98 °C; 10 s), annealing (55 °C; 30 s) and elongation (72 °C; 30 s) followed by a final extension step (72 °C; 5 min). The amplicons were checked on a 2% agarose gel, purified as mentioned previously, analyzed on a Bioanalyzer 2100 instrument (Agilent Technologies, USA) using a DNA 7500 chip (Agilent Technologies, USA) and finally quantified using the Quant-iT PicoGreen kit (Life Technologies, USA). Amplicons were pooled equimolar to 4 nM and sequenced using the MiSeq Reagent kit v3 (600 cycles) (Illumina Inc., USA) for paired end sequencing.

2.5. Amplicon sequencing analysis

FASTQ files of sequenced amplicons were trimmed and merged using AdapterRemoval v2 (minimum read length = 50; minimum Phred quality = 15) (Schubert et al., 2016). Datasets were quality filtered applying the QIIME suite (version 1.9.1) (Caporaso et al., 2010) calling the script "*split_libraries_fastq.py*" (minimum per read length fraction = 0.01; Phred quality threshold = 20; length filtering = 100–600 bp). Details of the sequencing run including number of merged reads and percentage of high quality sequences per dataset are summarized in Supplementary Table S2. All samples were subsampled to 130,377 sequences according to the lowest number of reads obtained within the datasets after quality filtering (Supplementary Table S2) (http://biopieces.org). Subsequently open reading frames (ORF) were predicted utilizing FragGeneScan (version 1.19) (train = illumina_5; thread = 15) (Rho et al., 2010). For functional annotation of datasets hmmsearch (http://hmmer.org; HMMER 3.0) was performed on predicted ORF using the Pfam (version 27.0) (Finn et al., 2014) respectively TIGRFAMs database (version 15) (Haft et al., 2013). Subsequently overlapping profile Hidden Markov Models (HMMs) were removed and results were quality filtered (expect value = 10^{-3}). The quantity of predicted ORF per dataset and the total number of HMMs detected per dataset before and after quality filtering are listed in Table 2 (based on the entire Pfam respectively TIGRFAMs database). To assess the specificity of the primers with respect to the individual targeted genes, the relative percentage of particular HMMs is stated that specifically comprise conserved domains of the proteins encoded by the investigated genes (percentage related to the total number of HMMs detected; Table 2). For taxonomic annotation of the datasets all "positive" sequences (i.e. sequences comprising the conserved domain of the respective HMM)

Table 2
Listed are number of predicted open-reading frames (ORF) per dataset, total number of detected HMMs before and after quality filtering as well as number and percentage of specific HMMs with conserved domains of targeted genes.

Amplicon name	Database	Number of predicted ORF	Total number of HMMs (complete database)	Total number of filtered HMMs (complete database)	Specific HMM	Number of specific HMMs	Percentage of specific HMMs
phoD	Pfam	130,500	125,702	117,210	PF09423	117,161	99.96
phoD-K	Pfam	130,431	121,727	113,543	PF09423	113,504	99.97
phoD-M	Pfam	130,428	122,100	114,914	PF09423	114,888	99.98
phoD-L	Pfam	130,529	133,071	121,738	PF09423	121,463	99.77
phoN	Pfam	129,784	119,958	87,388	PF01569	75,736	86.67
appA	Pfam	131,002	239,090	123,070	PF00328	120,980	98.30
phnX	TIGRFAMs	130,333	256,412	128,376	TIGR01422	128,189	99.85
gcd	TIGRFAMs	131,090	466,310	130,909	TIGR03074	130,536	99.72
pitA	Pfam	130,560	157,192	130,356	PF01384	130,351	100.00
pstS	TIGRFAMs	130,415	248,069	130,264	TIGR00975	130,252	99.99

were aligned against the NCBI RefSeq database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/; February 2016) (Tatusova et al., 2014) using DIAMOND with default settings (Buchfink et al., 2015). Since the *appA* amplicons reached an average length of 375 bp the "sensitive" option in DIAMOND was applied for all datasets. DIAMOND results were imported into MEGAN (version 5.6.5) (Huson et al., 2011) and analyzed on taxonomic level applying the parameters given below: Min Score: 50, Max Expected: 10^{-4}, Top Percent: 10, Min Support Percent: 0, Min Support: 1, LCA Percent: 50, Min complexity: 0.0. Rarefaction analysis of taxonomically annotated datasets was implemented using the R environment (R Core Team, 2015) and package "vegan" (Oksanen et al., 2015). Sequences are stored in the European Nucleotide Archive (Accession number: PRJEB13290).

3. Results and discussion

3.1. Primer specificity

In total seven oligonucleotide primers were developed for genes that code for enzymes which perform major processes of the soil microbial phosphorus (P) turnover. For the entire set of primers a high degree of specificity was determined during amplification of genomic DNA extracted from forest soil. The PCR consistently generated distinct bands of the expected size while unspecific amplification was not observed (Supplementary Fig. S1). Marginal levels of smear around the central band were caused by the amplification procedure since two different types of the individual primers were used in preparation for Illumina sequencing. While the first round of amplicon PCR (PCR1) was performed with the target specific primers (Table 1), the subsequent PCR (PCR2) required Illumina specific adapter overhangs which increased the mean amplicon length by approximately 70 bp. Minor levels of "shorter" fragments from PCR1 were still detectable after PCR2. Regarding the *phoD* gene three distinct amplicon sizes (bands) were detected after amplification (Supplementary Fig. S1). To investigate whether all of them corresponded to the *phoD* gene the bands were separated and subsequently treated as individual amplicons.

The specific amplification of the respective target genes was demonstrated by amplicon sequencing on an Illumina MiSeq instrument. After quality filtering 207,661 sequences were obtained per dataset on average (±37,263) (Supplementary Table S2). Open-reading frames (ORF) were predicted in subsampled datasets (130,377 sequences; according to the lowest number obtained in one dataset) and subsequently scanned against the Pfam (Finn et al., 2014) respectively TIGRFAMs database (Haft et al., 2013) (Table 2). After quality filtering the smallest number of profile Hidden Markov Models (HMMs) was detected in the *phoN* dataset (87,388) while for the other genes 123,375 HMMs on average were obtained (Table 2). Subsequently the relative percentage of specific HMMs, which comprised conserved domains of the proteins encoded by the investigated genes, was determined (Table 2). The sequences that gave a match to the respective (specific) HMM were considered as correctly amplified target genes. After all a satisfactorily high rate of primer specificity was proven. Regarding the primers for *phoD*, *phnX*, *gcd*, *pitA* and *pstS* more than 99% of all sequences that contained an ORF corresponded to the respective target gene (Table 2); for the *appA* primers a specificity of 98% was reached; only for *phoN* specificity levels were below 90% (87%). Regarding the unspecific sequences (*phoN*) the majority of reads represented genes coding for phosphopyruvate hydratases (Pfam family: PF00113). The corresponding proteins comprise certain conserved amino acids within the signature sequence motif of class A acid phosphatases (G-S-Y-P-S-G-H-T) which is targeted by the *phoN*-FW primer (Supplementary Table S1). Since the degree of primer degeneracy is relatively high (*phoN*-FW: 64×) to cover a broad diversity of different microbes, a minor fraction of unspecific sequences was amplified. However the vast majority of amplicons corresponded to the target gene. Therefore the latter primer can also be considered as a suitable tool for the detection of microbes harboring the *phoN* gene. Remarkably the three distinct bands that were amplified by the *phoD* primers (Supplementary Fig. S1) uniformly represented the *phoD* gene. The primer specificity towards the *phoD* gene consistently reached 99% for the individual datasets *phoD*-K, *phoD*-M and *phoD*-L (Table 2). This underlines the broad diversity of microbial *phoD* genes existing in environmental samples which has been previously described (Bergkemper et al., 2015). The amplicon size of *phoD*-K and *phoD*-L differed by sixty base pairs on average. The variable region is likely to be situated in the front section of the *phoD* gene since the primers amplified the upstream region of the *phoD* conserved domain W-D-D-H-E (Supplementary Table S1) (position 621 to 831 of *phoD* from *Bacillus subtilis* subsp. subtilis str. 168; Accession number: gi|255767073). In contrast the PHOD primers introduced by Ragot et al. (2015) target the downstream region of the *phoD* conserved domain; here the occurrence of distinct amplicon sizes was not reported.

Due to the high degree of specificity towards the individual target genes together with the medium amplicon size ranging from 147 bp to 375 bp (Table 1) the introduced primers are not only suitable for diversity analysis but also for quantitative real-time PCR approaches (Karsai et al., 2002).

3.2. Microbial diversity of amplified genes

Insights into the microbial community harboring the investigated genes were gained by taxonomic annotation of amplicon datasets using DIAMOND (Buchfink et al., 2015) against the NCBI RefSeq database (Tatusova et al., 2014) and MEGAN (Huson et al., 2011). Rarefaction analysis based on the number of assigned orders revealed a sufficient coverage of the microbial diversity for all primers (Fig. 1). Indeed the rarefaction curves for *phnX* and *appA* reached a plateau already after 13 detected microbial orders (corresponding to 80,000 reads analyzed), while for *phoD*-L saturation was reached not before 42 detected orders respectively 100,000 analyzed sequences. Thus additional sequencing effort is unlikely to increase the detected microbial diversity in the investigated soil.

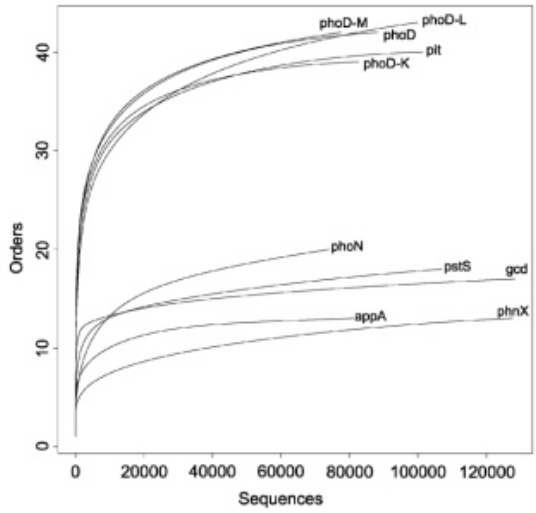

Fig. 1. Rarefaction analysis. Depicted is the number of microbial orders as a function of sequencing depth that were detected in amplicon datasets using seven newly designed oligonucleotide primers and forest soil genomic DNA. Sequences were aligned against the NCBI RefSeq database applying DIAMOND and MEGAN5.

In Fig. 2 the relative abundance of microbial orders that were amplified with the individual primers is shown. The highest microbial diversity was detected for the *phoD* and *pitA* genes. After all genes from 15 respectively 13 different microbial phyla were amplified which corresponded to 43 (40) orders or 81 (68) distinct families (Table 3). Dominating orders for *phoD* were Planctomycetales (33%), Burkholderiales (16%), Rhizobiales (14%), Pseudomonadales (12%), Rhodospirillales (9%) and Actinomycetales (6%); 25 orders had a relatively low abundance of 1% or less. In comparison WGS of the investigated soil and filtering of reads related to *phoD* genes revealed a dominance of Acidobacteriales, Rhizobiales, Burkholderiales, Rhodospirillales, Xanthomonadales and Spartobacteria (Bergkemper et al., 2015). Hence the new *phoD* primers covered all taxa that were revealed by WGS (although not necessarily with the same abundance) and further indicated the impact of 38 additional microbial orders on organic-P mineralization by alkaline phosphatase (PhoD) in soil. While the primers were designed based on bacterial sequences exclusively also six fungal orders were detected in the *phoD* dataset:

Table 3
Listed are number of detected microbial phyla, orders and families that were amplified by seven oligonucleotide primers from forest soil genomic DNA. Amplified sequences were aligned against the NCBI RefSeq database (DIAMOND) and analyzed using MEGAN5.

Amplicon name	Gene	Number of detected		
		Phyla	Orders	Families
phoD	*phoD*	15	42	77
phoD-K	*phoD*	14	39	69
phoD-M	*phoD*	14	42	78
phoD-L	*phoD*	15	43	81
phoN	*phoN*	7	20	36
appA	*appA*	2	13	13
phnX	*phnX*	2	13	19
gcd	*gcd*	6	17	24
pitA	*pitA*	13	40	68
pstS	*pstS*	9	18	28

Pleosporales, Chaetothyriales, Onygenales, Hypocreales, Sordariales and Dacrymycetales. In comparison Ragot et al. (2015) amplified the *phoD* gene from 13 different (exclusively) bacterial phyla respectively 38 orders using their primer system. While dominating orders were similar to the presented study, the authors detected also members of Bacillales and Gloeobacterales harboring *phoD* genes in soils. However taking into account that Ragot et al. (2015) sequenced DNA from six different grassland soils with a consistently lower sequencing depth (<5000 sequences per library), the results are not absolutely transferable but may benchmark the efficiency of our newly developed *phoD* primers and validate their efficiency. Regarding the three distinct fragment sizes amplified by the *phoD* primers the underlying microbial community differed for *phoD*-K respectively *phoD*-L (Fig. 2). While longer amplicon sizes (*phoD*-L) predominantly corresponded to a higher abundance of Burkholderiales, Rhizobiales and Pseudomonadales shorter fragments (*phoD*-K) primarily represented genes harbored by Planctomycetales and Rhodospirillales.

Using the newly developed primer system for *phoN* genes 20 different microbial orders were amplified including Rhizobiales (57%), Xanthomonadales (29%), Enterobacteriales (8%) and Burkholderiales (6%), which corresponds again nicely with the WGS dataset from Bergkemper et al. (2015) where the dominance of Rhizobiales and Xanthomonadales in the group of *phoN* harboring bacteria was shown for the same soil. Since sequencing depth has been limited for WGS again additional orders were detected by the amplicon based approach, which were not described in the metagenomes.

Microbial enzymes that degrade soil organic myo-Inositol-1,2,3,4,5,6-hexakisphosphates (IP_6) have been classified as 3-phytases

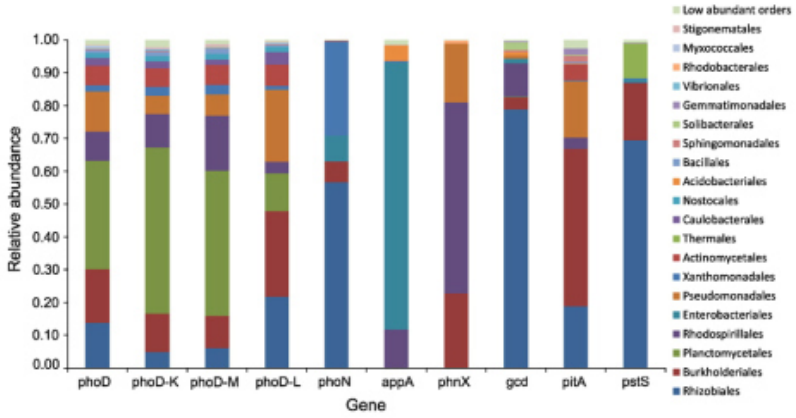

Fig. 2. Taxonomic diversity of amplified genes. Seven newly designed oligonucleotide primers were applied for amplicon sequencing of forest soil genomic DNA. Sequences of amplified target genes were aligned against the NCBI RefSeq database using DIAMOND and analyzed on taxonomic level using MEGAN.

and 6-phytases depending on the initial dephosphorylation (Jorquera et al., 2008). The primers introduced in this study specifically targeted the *appA* gene, which codes for a bifunctional enzyme with phytase and acid phosphatase activity that is characterized as histidine-acid phosphatase (HAP; 6-phytase) (Golovan et al., 2000). In total the *appA* primers amplified genes from 2 different bacterial phyla and 13 distinct orders (Table 3). Compared to the microbial diversity that was covered by the *phoD* primers this number appeared to be insufficient given the fact that IP_6 is considered as the major storage form of soil organic P (Turner et al., 2002). However HAP represents only one single class of phytases beyond beta propeller phytases (BPP) and purple acid phosphatases (PAP). Unfortunately conserved domains suitable for primer design were only present in HAP (Mullaney and Ullah, 2003). Moreover database entries of sequenced HAP are rare and generally restricted to members of Alpha-, Delta-, Gammaproteobacteria and Acidobacteria (Jorquera et al., 2008). Therefrom the *appA* primers amplified genes from all classes of microorganisms that are known for harboring HAP genes (Fig. 2).

Similarly a relatively low microbial diversity was amplified by the *phnX* primers, which target the gene coding for a phosphonatase (McGrath et al., 2013). Two different bacterial phyla (Proteobacteria, Firmicutes) and 13 orders were detected. Database entries of *phnX* genes are limited (<10% compared to *phoD*; NCBI RefSeq; March 2016) and comprise predominantly Actinobacteria, CFB group bacteria (Cytophaga–Flavobacter–Bacteroides), Proteobacteria and Firmicutes. The latter two phyla were covered by the *phnX* primers while Actinobacterial sequences were not amplified (Fig. 2). This is caused by the fact that phosphonatases are not well conserved on amino acid level when comparing microorganisms from distinct phyla. Therefore the *phnX* primers were exclusively designed based on sequences derived from different classes of Proteobacteria and Firmicutes while Actinobacteria and CFB group bacteria were omitted. In a preliminary experiment, an additional set of highly degenerated primers was investigated that also targeted the *phnX* gene of Actinobacteria. As degeneracy increased manifold the PCR failed to produce target specific amplicons (data not shown). Therefore *phnX* primers were designed to reliably amplify target genes while the primer degeneracy was minimized.

In contrast to the enzymes previously discussed which are involved in soil organic-P mineralization the quinoprotein glucose dehydrogenase (PQQGDH; *gcd*) facilitates the solubilization of mineral-P (Goldstein, 1994) and serves as an indicator for the microbial potential of inorganic-P solubilization. The *gcd* primers amplified target genes from 6 distinct bacterial phyla and 17 orders. Most abundant were genes harbored by Rhizobiales (79%), Rhodospirillales (10%) and Burkholderiales (4%) which are known for their ability to solubilize inorganic-P compounds (Rodriguez and Fraga, 1999) (Fig. 2). The strikingly high abundance of Rhizobial *gcd* genes certainly is influenced by the strong domination of this order in the investigated soil (Bergkemper et al., 2015). WGS revealed also members of Solibacterales and Acidobacteriales as important sources for *gcd* in BBR. Both orders were likewise covered by the *gcd* primers although their relative abundance (2% respectively 1% of amplified sequences) was low compared to the WGS approach (Bergkemper et al., 2015). On the one hand this could be related to an overrepresentation of Proteobacterial *gcd* sequences in common databases (>80% of *gcd* sequences in NCBI RefSeq; March 2016). On the other hand microorganisms are highly stratified in the different forest soil horizons (Baldrian et al., 2012). Hence the *gcd* harboring microbial community might shift from the soil organic horizon used in the present study to the mineral topsoil applied for WGS (Bergkemper et al., 2015) especially since inorganic-P solubilization might play a major role once the content of soil organic-P is decreased (e.g. in mineral soil horizons) (Talkner et al., 2009).

Genes coding for the phosphate inorganic transporter (Pit) were amplified from a broad diversity of microorganisms including 13 different bacterial phyla and 40 orders (Table 3). Dominating taxa were Burkholderiales (48%), Rhizobiales (19%), Pseudomonadales (17%), Actinomycetales (5%) and Rhodospirillales (3%) (Fig. 2). In addition all remaining orders that were known for harboring the *pitA* gene in the investigated soil were covered by the *pitA* primers (Acidobacteriales, Solibacterales, Myxococcales, Gemmatimonadales) (Bergkemper et al., 2015). The gene *pstS* that codes for the periplasmic binding protein of the phosphate-specific transporter (Pst) was amplified from 9 different bacterial phyla respectively 18 orders (Table 3). Most abundant were members of Rhizobiales (70%), Burkholderiales (17%), Thermales (11%) and Xanthomonadales (0.4%) (Fig. 2). Therefrom the primers covered 7 (of 11) *pstS* harboring orders that were detected by WGS while the target gene was amplified from 11 additional orders using the *pstS* primers.

4. Conclusion

In conclusion a new set of oligonucleotide primers was introduced in this study that covers the major processes of the soil microbial phosphorus turnover. The seven primers target genes which code for proteins involved in mineralization of various forms of soil organic-P, solubilization of inorganic-P as well as cellular P uptake. A novel strategy for primer design was applied to allow both the amplification of target genes from a broad diversity of distinct microorganisms and simultaneously minimize the grade of primer degeneracy. Microbial key players that strongly contributed to the turnover of soil P in beech forests were identified by whole genome shotgun sequencing while corresponding database sequences of the individual enzymes were used for primer design. The new primers showed a high degree of specificity during amplification while simultaneously a broad diversity of distinct microbial phyla was covered and the results obtained by metagenomics were nearly reproduced. On this account the introduced primers represent a valuable tool to deepen our knowledge on key players of this crucial nutrient turnover in soils and other environments. Moreover the primers are suitable for application in quantitative real-time PCR approaches which allows the quantification of important transformation steps in P turnover both on temporal and spatial scale using DNA (as a proxy for potentials) and mRNA (as a proxy for activity).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2016.04.011.

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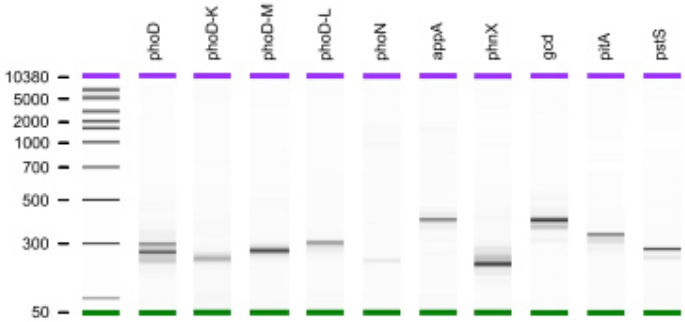
Supplemental Information Manuscript 3



Supplementary Figure S1 Gel electrophoresis of ten amplicons using seven newly designed oligonucleotide primers and genomic DNA from forest soil. The initial PCR included target specific primers before Illumina adapter overhangs were added to the amplicons during a second round of amplification. Multiple bands (phoD) were treated as individual amplicons.

Supplementary Table S1 Listed are conserved domains of investigated genes (together with database entries) that were targeted by seven newly designed oligonucleotide primers

Target Gene	Corresponding Enzyme	Conserved Domain	Targeted By Primer	Database Entry
phoD	Alkaline Phosphatase PhoD	W-D-D-H-E	Reverse Primer	"MPP_PhoD" (cd07389)
phoN	Acid Phosphatase Class A	G-S-Y-P-S-G-H-T	Forward Primer	"PAP2_acid_phosphatase" (cd03397)
appA	4-Phytase/Acid Phosphatase	S-R-H-G-D	Forward Primer	"HP_HAP_like" (cd07061)
phnX	Phosphonoacetaldehyde Hydrolase	D-W-A-G-T	Forward Primer	"phosphonatase" (TIGR01422)
gcd	Quinoprotein Glucose Dehydrogenase	V-H-H-D-L	Reverse Primer	"PQQ_mGDH" (cd10280)
pitA	Phosphate Inorganic Transporter	G-F-H-D	Forward Primer	"PHO4" (pfam01384)
pstS	Phosphate-specific Transporter (Periplasmic Phosphate-binding Protein)	W-N-D-P-A	Forward Primer	"3a0107s03" (TIGR00975)

Supplementary Table S2 Listed are number of merged reads for ten amplicon datasets before and after quality filtering together with percentage of high quality reads.

Amplicon Name	Target Gene	Number Of Merged Reads	Number Of Reads After Quality Filtering	Percentage Of High Quality Reads
phoD	*phoD*	218,451	217,758	99.7
phoD-K	*phoD*	207,874	207,553	99.8
phoD-M	*phoD*	214,714	214,396	99.9
phoD-L	*phoD*	251,812	251,275	99.8
phoN	*phoN*	144,044	130,377	90.5
appA	*appA*	193,331	190,454	98.5
phnX	*phnX*	251,711	248,383	98.7
gcd	*gcd*	190,965	189,681	99.3
pitA	*pitA*	180,973	180,721	99.9
pstS	*pstS*	246,266	246,015	99.9

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Note

An inter-laboratory comparison of gaseous and liquid fumigation based methods for measuring microbial phosphorus (P_{mic}) in forest soils with differing P stocks

Fabian Bergkemper [a], Else K. Bünemann [b,c], Simon Hauenstein [d], Christine Heuck [e], Ellen Kandeler [f], Jaane Krüger [g], Sven Marhan [f], Éva Mészáros [b], Dinah Nassal [f], Pascal Nassal [f], Yvonne Oelmann [d], Chiara Pistocchi [b], Michael Schloter [a,*], Marie Spohn [e], Ulrike Talkner [h], Dan P. Zederer [h], Stefanie Schulz [a]

[a] Research Unit Environmental Genomics, Helmholtz Zentrum München, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany
[b] Institute of Agricultural Sciences, ETH Zurich, Eschikon 33, 8315 Lindau, Switzerland
[c] Research Institute of Organic Agriculture (FiBL), Ackerstrasse 113, 5070 Frick, Switzerland
[d] Geoecology, University of Tübingen, Rümelinstrasse 19-23, 72070 Tübingen, Germany
[e] Department of Soil Ecology, Bayreuth Center of Ecology and Environmental Research (BayCEER), University Bayreuth, Dr.-Hans-Frisch-Straße 1-3, 95448 Bayreuth, Germany
[f] Institute of Soil Science and Land Evaluation, Soil Biology Department, University of Hohenheim, Emil-Wolff-Str. 27, 70599 Stuttgart, Germany
[g] Chair of Soil Ecology, Albert-Ludwigs-University Freiburg, Bertoldstr. 17, 79085 Freiburg i. Br., Germany
[h] Northwest German Forest Research Institute, Grätzelstraße 2, 37079 Göttingen, Germany

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Forest soils

ABSTRACT

In an inter-laboratory trial, gaseous ("CFE") and liquid fumigation ("Resin") based methods for measuring microbial phosphorus (P_{mic}) were compared, based on the analysis of soil samples from five forests, which differ in their P stocks. Both methods reliably detected the same P_{mic} gradient in the different soils. However, when the individual recovery rates of spiked P were taken into account, the "CFE" based methods consistently generated higher P_{mic} values (factor 2) compared to the "Resin" based approaches.

1. Text

Phosphorus (P) is of central importance for all forms of cellular life but likewise considered as the most inaccessible macronutrient in soils (Elser, 2012; Holford, 1997). Microorganisms are integral to the turnover of soil phosphorus since they mobilize different forms of soil P, but also compete with other biota for available P (Richardson and Simpson, 2011). The microbial P pool (P_{mic}) is highly variable and represents a substantial fraction of total-P in mineral topsoils (up to 11%; Heuck et al., 2015; Oberson and Joner, 2005). Significant amounts of P are released to the soil solution as a result of a continuous recycling of the microbial biomass (Richardson and Simpson, 2011). Thus, reliable measurements regarding the size and dynamics of this P pool (P_{mic}) are crucial for assessing soil fertility and plant P nutrition.

Whereas for the assessment of microbial C and N more or less standardized methods are used (ISO 14240-2, 1997; Beck et al., 1997), the analysis of P_{mic} relies on different protocols which hampers the direct comparison of data. While Brookes et al. (1982) proposed the determination of P_{mic} after gaseous fumigation using chloroform ("CFE"), the method by Kouno et al. (1995) is based on liquid fumigation in the presence of anion-exchange resin membranes ("Resin") instead. It remains unclear whether both methods provide similar P_{mic} values when comparing soils with distinct properties. In this regard, the contents of soil total carbon (C), nitrogen (N) and P as well as the texture are of relevance. Thus it was the aim of this inter-laboratory study to compare P_{mic} values and gradients along a sequence of unfertilized forest soils with different P stocks in the mineral topsoil, derived by different methods based on gaseous or liquid fumigation.

Soil samples were taken from five beech-dominated (*Fagus sylvatica* L.) German forests in autumn 2013 and included a wide range of distinct properties regarding the contents of total-C, N, P, texture and, though less pronounced, soil pH (Supplementary Table S1). Regarding the content of total-P, the five soils formed a natural gradient that ranged from 3.16 mg g^{-1} to 0.14 mg g^{-1} (Bad Brueckenau, BBR > Mitterfels, MIT > Vessertal, VES > Conventwald, CON > Luess, LUE; Supplementary

* Corresponding author.
E-mail address: schloter@helmholtz-muenchen.de (M. Schloter).

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Table S1). One composite sample of mineral topsoil (0–23 cm depth) was created from the individual topsoil layers (Supplementary Table S1). Subsequently composite samples were homogenized by sieving (<2 mm) and distributed to the six laboratories.

In total six variations of gaseous ("CFE") and liquid ("Resin") fumigation based methods were compared by six laboratories in Germany and Switzerland. For each soil sample, seven subsamples were used for measurement of P_{mic}: i) one non-fumigated sample, ii) five non-fumigated samples which received an additional P-spike to take the specific sorption capacities of the distinct soils into account (KH_2PO_4 equivalent to 10 μg, 25 μg, 50 μg, 100 μg, 200 μg P g^{-1} soil dry-weight) and iii) one fumigated subsample. All analyses were carried out using four analytical replicates.

The "CFE" methods were performed as described by Brookes et al. (1982) with slight modifications. Briefly, field-moist soil was shaken for 30 min with Bray-1 solution (Khan and Joergensen, 2012; Oberson et al., 1997; "CFE 1"), with Bray-2 solution (Bray and Kurtz, 1945; "CFE 2") or 0.01 M $CaCl_2$ ("CFE 3"). The latter extraction method is frequently used for the determination of C_{mic} and N_{mic} (Brankatschk et al., 2011), which allows the measurement of C_{mic}, N_{mic} and P_{mic} from the same extract. The "Resin" method to determine P_{mic} was based on the protocol published by Kouno et al. (1995; "Resin 1–3"). However, hexanol was used instead of liquid chloroform (Bünemann et al., 2004), because chloroform damages the anion-exchange membranes and hexanol is as efficient as a fumigant as chloroform (Mclaughlin, 1986). Field-moist soil was shaken with anion-exchange resins and distilled water with or without liquid hexanol. The concentration of ortho-P in the eluate was determined according to Murphy and Riley (1962; "CFE 1–3", "Resin 2–3") respectively Ohno and Zibilske (1991; "Resin 1"). See Table 1 for a methodological comparison of the two original methods (Brookes et al., 1982; Kouno et al., 1995) and the interlaboratory variations that were applied in this study as well as Supplementary Table S3 for further details on the individual measurement procedures.

Since the majority of laboratories (except "CFE 3") detected an approximately linear recovery of the applied spike concentrations (Supplementary Table S2), the mean recovery of the spikes 10 μg, 25 μg and 50 μg g^{-1} was used for calculation of P_{mic}. This range also covered the amount of added P proposed by Brookes et al. (1982; 25 μg g^{-1}). For "CFE 3", where the lowest spike concentrations were not reliably recovered from certain soils, the recovery of spikes 100 μg and 200 μg g^{-1} was used. Finally, P_{mic} was uniformly calculated as proposed by Kouno et al. (1995): $P_{mic} = (Ep / Kp * 100/R)$, where Ep = (P released and extracted after fumigation) minus (P released from non-fumigated soil), Kp = biomass P released and extracted after fumigation as P (k_{EP} = 0.4) and R = percentage recovery of added P. In all methods, only the concentration of inorganic-P is determined. Thus all methods rely on enzymatic hydrolysis of DNA, RNA or phospholipids. Therefore an additional correction factor (k_{EP}) has been introduced, which takes into account that only 40% of the microbial P is extracted as inorganic P (Brookes et al., 1982).

However, since a certain proportion of orthophosphate attaches to soil particles during extraction, the individual soils were spiked with known amounts of P to estimate the fraction of "fixed" P (Brookes et al., 1982). The recovery of P added to the different soil samples was consistently higher using the methods "Resin 1–3" compared to the "CFE" approaches (Supplementary Table S2). Depending on the soil type, the mean recovery (spikes 10 μg–50 μg g^{-1}) of "Resin 1–3" ranged from 55% (±9.5%) at the site CON to 89% (±7.6%) at the site LUE. In contrast, the recovery rates of the "CFE" methods were generally lower and severely affected by the soil type and the applied extracting solution. While "CFE 1–2" recovered 80% of P added to the soil at the site LUE (±23.2%), this rate decreased to 12% at the site CON (±7.2%). For "CFE 3", the lower P spikes (10 μg–50 μg g^{-1}) were not reliably recovered from the different soils. The mean recovery of "CFE 3" (spikes 100 μg–200 μg g^{-1}) was always below 1% except for site the LUE (31%, ±8.5%). Thus the impact of soil physicochemical parameters (e.g. texture, clay content) on the P extraction efficiency is more pronounced for "CFE" than for the "Resin" methods. This is in accordance with Kouno et al. (1995) who reported a distinctly increased recovery of P from Andosols when using anion-exchange resins rather than applying chemical extractants.

Nonetheless, all laboratories detected the same P_{mic} gradient within the investigated samples (i.e. the same sequence of the forest sites regarding the magnitude of P_{mic}; Fig. 1), as the marked differences for P extraction efficiency were taken into account for the calculation of P_{mic}. Values ranged from 5 μg g^{-1} (±2 μg g^{-1}; LUE) to 147 μg g^{-1} (±31 μg g^{-1}; CON), which is in agreement with previous data obtained from mineral topsoils of beech forests (Joergensen et al., 1995). Overall, there was merely a slight positive correlation between P_{mic} and the soil total-P contents (mean of "CFE 2–3" and "Resin 1–3": $r = 0.3151 \pm 0.05$; "CFE1": $r = 0.6563$). This finding comes as no surprise, since P_{mic} in forest soils shows strongest correlation to the total-N and organic-C contents (Joergensen et al., 1995). Consistently, the highest P_{mic} values were measured at the sites CON and BBR. While the differences (P_{mic}) were marginal between VES and MIT, distinctly lowest values were detected at the site LUE (Fig. 1; Supplementary Table S3). Accordingly, an analysis of variance based on cube root transformed data (R Core Team, 2015) uniformly revealed a significant influence of the forest site on P_{mic} for all laboratories ($P < 0.001$). Overall, absolute P_{mic} values obtained by "CFE" based methods were higher by a factor of 1.5 (CON), 2 (BBR), 2.4 (VES), 2.7 (MIT) and 1.3 (LUE; except "CFE 3") respectively, compared to the "Resin" based approaches. Taking the sorption of P to the soil solid phase during 24 h of fumigation into account the P_{mic} values obtained by the CFE based methods might still be underestimated (Morel et al., 1996). In contrast for the "Resin" methods, the fumigation and extraction procedure were performed simultaneously.

Table 1
Comparison of measurement procedures for determination of microbial biomass phosphorus in soil. Shown are details of the method proposed by Brookes et al. (1982) for gaseous fumigation and the variations that were compared in this study ("CFE 1–3"), respectively, the method by Kouno et al. (1995) for liquid fumigation and the variations thereof ("Resin 1–3").

Method	Extraction procedure	Duration of extraction	Fumigation medium	Duration of fumigation	Ratio extracting solution: soil
Brookes et al., 1982	0.5 M $NaHCO_3$ (pH 8.5); filtration without centrifugation	0.5 h	Chloroform (vapor)	24 h	20
CFE 1	Bray-1 solution (0.03 M NH_4F, 0.025 M HCl); filtration without centrifugation	0.5 h	Chloroform (vapor)	24 h	10
CFE 2	Bray-2 solution (0.03 M NH_4F, 0.1 M HCl); filtration without centrifugation	0.5 h	Chloroform (vapor)	24 h	20
CFE 3	0.01 M $CaCl_2$; filtration without centrifugation	0.5 h	Chloroform (vapor)	24 h	4
Kouno et al., 1995	Anion-exchange resin membranes in bicarbonate form, distilled water; elution of resins in 0.5 M HCL	24 h	Chloroform (liquid)	24 h	15
Resin 1	Anion-exchange resin membranes in bicarbonate form, nanopure water; elution of resins in 0.1 M NaCl + 0.1 M HCL	16 h	Hexanol (liquid)	16 h	15
Resin 2	Anion-exchange resin membranes in bicarbonate form, deionized water; elution of resins in 0.5 M HCL	16 h	Hexanol (liquid)	16 h	15
Resin 3	Anion-exchange resin membranes in bicarbonate form, nanopure water; elution of resins in 0.2 M HNO_3	16 h	Hexanol (liquid)	16 h	12

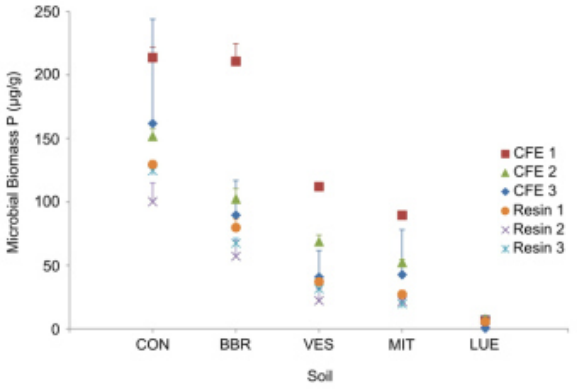

Fig. 1. Microbial biomass P (P_{mic}) values per laboratory and soil type. Shown are means of P_{mic} in the mineral topsoil of five beech-dominated forest soils derived from six different laboratories using gaseous and liquid fumigation respectively (n = 4). Three different variations based on gaseous fumigation using chloroform ("CFE 1–3") were compared to liquid fumigation with hexanol ("Resin 1–3"). The investigated forest soils form a natural gradient of total phosphorus in the mineral topsoil (BBR > MIT > VES > CON > LUE).

The P_{mic} of the "Resin" based methods ("Resin 1–3") clustered closely for all five soils (mean coefficient of variation, cv = 18%; Supplementary Table S3), indicating a good reproducibility of the method in different laboratories. For the "CFE" based methods as expected, variabilities were significantly higher due to different solvents, ionic strength and soil:solution ratios used in the different labs. The P_{mic} values of "CFE 2" and "CFE 3" were in a comparable range (cv = 16%; except LUE), while consistently higher values were detected by "CFE 1" (factor 1.4–2.2). Thereby the mean coefficient of variation was increased to 39% for "CFE 1–3" (Supplementary Table S3).

Although 0.01 M $CaCl_2$ ("CFE 3") is known as a weak extracting solution (Neyroud and Lischer, 2003) the obtained P_{mic} values were fairly comparable to those of "CFE 2" (Bray-2 solution). In fact, P_{mic} of "CFE 2" clustered more strongly with "CFE 3" than with "CFE 1" (Bray-1 solution). The only exception in this context was the P_{mic} value obtained by method "CFE 3" for site LUE. Here, the differences compared to other laboratories were rather high ("CFE 2": 7.78 µg g^{-1}, "CFE 3": 0.24 µg g^{-1}), indicating that the "CFE 3" method might not be useful for soils with very low contents of P_{mic} (i.e. reduced Ep value). Nevertheless, the co-extraction of C_{mic}, N_{mic} and P_{mic} by one extraction makes the latter method attractive for soils with medium or high P stocks.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2016.07.006.

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Supplemental Information Manuscript 4

Supplementary Table S1 Listed are geographic location, soil classification according to the World Reference Base for soil resources (WRB, 2015), sampled horizons as well as physical and chemical parameters and microbial biomass carbon (C_{mic}) of five investigated beech forest soils.

Soil	Geographic Location	Soil Type (WRB, 2015)	Horizon	Depth (cm)	Total C (mg/g)	Total N (mg/g)	Total P (µg/g)	pH (H_2O)	Sand (%)	Silt (%)	Clay (%)	Texture (WRB, 2015)	C_{mic} (µg/g)
Conventwald (CON)	48°1'21.4" N, 7°57'50.65" E	Hyperdystric Skeletic Folic Cambisol	Ah1 Ah-Bv	0-23	114.54	5.20	796.27	4.18	39.50	35.50	25.00	Loam	1305.54
Bad Brueckenau (BBR)	50°21'7.26" N, 9°55'44.53" E	Dystric Skeletic Cambisol	Ah1 Ah2 Ah-Bv	0-23	116.36	7.75	3164.78	4.17	8.67	55.00	36.33	Silt clay loam	948.11
Vessertal (VES)	50°36'23.84" N, 10°46'14.1" E	Hyperdystric Skeletic Chromic Cambisol	Ah1 Ah2 Ah-Bv Bv	0-21	71.52	4.32	985.94	3.86	33.00	46.25	20.50	Loam	764.14
Mitterfels (MIT)	48°58'34.18" N, 12°52'46.74" E	Hyperdystric Chromic Folic Cambisol	Ah1 Ah2 Ah-Bv	0-17	99.10	5.59	1161.98	3.88	49.67	31.33	19.67	Loam/ Sandy loam	740.63
Luess (LUE)	52°50'21.77" N, 10°16'2.37" E	Hyperdystric Folic Cambisol	Ahe Ae Bsh	0-20	42.99	1.70	144.34	3.72	77.67	17.33	5.33	Loamy sand	287.53

Supplementary Table S2 Recovery of added P from soil per method (laboratory) and soil type (n=4).

		CFE 1		CFE 2		CFE 3			Resin 1			Resin 2		Resin 3	
Soil	P Spike (µg/g)	P recovered (µg/g)	SD	P recovered (µg/g)	SD	P recovered (µg/g)	SD	P Spike (µg/g)	P recovered (µg/g)	SD	P Spike (µg/g)	P recovered (µg/g)	SD	P recovered (µg/g)	SD
CON	0	1.47	0.08	3.79	0.25	0.19	0.02	0	1.28	0.29	0	2.43	2.29	0.72	0.50
	10	2.36	0.01	5.88	1.14	0.17	0.01	10	7.38	0.77	10	7.10	1.87	3.84	2.01
	25	3.08	0.02	7.30	0.57	0.18	0.02	23	17.48	1.10	25	14.46	1.37	13.56	1.58
	50	4.38	0.06	12.18	0.58	0.20	0.01	45	29.98	0.93	50	30.94	0.85	28.63	2.37
	100	8.39	0.08	24.00	1.72	0.26	0.01	83	62.70	1.19	100	76.93	10.27	64.59	5.61
	200	21.46	0.63	42.14	3.58	0.58	0.02	145	115.05	2.12	200	154.82	9.61	138.90	10.03
BBR	0	6.97	0.07	37.16	0.56	0.43	0.03	0	11.38	0.69	0	8.84	2.19	8.65	0.32
	10	8.20	0.25	40.87	1.36	0.35	0.01	10	18.75	0.69	10	14.38	2.51	15.05	0.43
	25	10.00	0.76	43.30	1.15	0.39	0.02	23	29.25	1.10	25	23.03	2.61	24.87	0.16
	50	12.23	0.35	50.48	0.74	0.38	0.03	45	43.78	1.18	50	39.39	1.52	41.86	0.89
	100	19.68	0.46	62.03	2.37	0.58	0.01	83	77.65	3.12	100	82.90	13.98	81.63	1.18
	200	40.61	0.62	88.71	0.02	0.96	0.03	145	132.65	1.05	200	83.60	10.10	164.71	5.04
VES	0	20.23	0.33	54.13	0.51	0.24	0.02	0	6.15	0.39	0	5.95	2.17	5.38	0.29
	10	24.18	0.32	58.84	0.78	0.26	0.04	10	13.38	2.31	10	13.01	1.80	13.66	0.20
	25	29.26	0.42	69.33	0.68	0.31	0.03	25	27.80	1.51	25	24.23	1.63	26.40	0.67
	50	41.18	0.97	91.25	1.77	0.36	0.05	50	47.03	0.80	50	47.15	5.96	49.20	0.89
	100	65.31	0.77	123.88	3.14	0.67	0.02	100	89.80	4.80	100	96.20	9.81	95.49	0.76
	200	115.05	2.17	192.95	5.36	1.91	0.14	200	164.48	22.68	200	204.43	12.46	188.15	3.93
MIT	0	5.54	0.17	12.40	0.25	0.28	0.00	0	1.55	0.21	0	2.68	2.08	1.52	0.09
	10	6.91	0.07	15.95	2.71	0.25	0.02	10	6.80	0.55	10	7.47	2.26	7.14	0.24
	25	8.64	0.18	17.80	0.94	0.29	0.05	25	18.40	1.13	25	15.79	1.51	16.97	0.46
	50	12.22	0.58	24.50	0.62	0.28	0.03	50	32.55	5.61	50	32.44	2.74	33.55	1.17
	100	20.48	0.68	37.15	2.36	0.41	0.05	100	78.25	2.09	100	75.75	14.82	75.83	1.97
	200	45.83	1.47	64.02	0.97	0.76	0.02	200	160.83	1.70	200	170.43	10.11	157.66	4.09
LUE	0	2.93	0.20	3.34	0.40	0.28	0.00	0	0.70	0.34	0	1.94	1.96	0.66	0.16
	10	8.00	0.20	14.31	3.86	0.74	0.04	10	7.40	0.20	10	10.99	1.36	10.57	0.26
	25	19.31	0.14	25.64	0.40	2.35	0.09	25	23.33	0.33	25	22.79	1.06	24.78	0.15
	50	40.52	0.17	48.77	0.86	7.59	0.29	50	45.30	0.81	50	46.18	3.01	48.83	0.66
	100	82.03	0.68	98.41	1.86	25.33	0.90	100	94.73	0.96	100	96.44	15.16	96.77	1.24
	200	170.91	7.26	194.21	2.48	74.48	1.51	200	194.40	3.60	200	214.37	19.04	194.78	2.66

Supplementary Table S3 Details of the P_{mic} measurement procedures and P_{mic} values per laboratory and soil type (n=4).

Method	Extraction procedure	Duration of extraction	Shaking intensity (extraction)	Fumigation medium	Duration of fumigation	Ratio extracting solution:soil	Soil	Pmic (µg/g)	StabW
CFE1	Bray-1 solution (0.03M NH_4F, 0.025M HCl); Filtration without centrifugation (Rotilabo 113P, Roth, Germany)	0.5h	150 rotations per minute (horizontal)	Chloroform	24h	10	CON	213.64	8.53
							BBR	210.62	14.07
							VES	111.84	2.62
							MIT	89.51	3.46
							LUE	6.78	0.65
CFE2	Bray-2 solution (0.03M NH_4F, 0.1M HCl); Filtration without centrifugation (Whatman 595 1/2, GE Healthcare, Germany)	0.5h	150 rotations per minute (horizontal)	Chloroform	24h	20	CON	151.43	5.80
							BBR	102.26	7.96
							VES	68.59	5.40
							MIT	52.28	2.35
							LUE	7.78	0.47
CFE3	0.01M $CaCl_2$; Filtration without centrifugation (Whatman 595 1/2, GE Healthcare, Germany)	0.5h	60 rotations per minute (overhead)	Chloroform	24h	4	CON	161.55	82.48
							BBR	89.60	27.12
							VES	40.67	21.03
							MIT	42.57	35.64
							LUE	0.24	0.39
Resin1	Anion-exchange resin membranes in bicarbonate form (BDH #55164), nanopure water; elution of resins in 0.1M NaCl + 0.1M HCL (2h; 150rpm)	16h	150 rotations per minute (horizontal)	Hexanol	16h	15	CON	129.28	3.22
							BBR	79.78	6.71
							VES	37.09	2.95
							MIT	27.11	3.22
							LUE	5.55	0.46
Resin2	Anion-exchange resin membranes in bicarbonate form (VWR, Radnor, USA), deionized water; elution of resins in 0.5M HCL (1h; 170rpm)	16h	170 rotations per minute (horizontal)	Hexanol	16h	15	CON	100.13	14.63
							BBR	57.21	12.79
							VES	22.13	9.41
							MIT	21.44	7.44
							LUE	4.27	5.36
Resin3	Anion-exchange resin membranes in bicarbonate form (VWR,BDH Prolabo #551642S), nanopure water; elution of resins in 0.2M HNO3 (2h; 150rpm)	16h	150 rotations per minute (horizontal)	Hexanol	16h	12	CON	124.25	8.18
							BBR	67.42	4.09
							VES	31.38	1.05
							MIT	19.55	1.17
							LUE	7.02	0.39

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Temporal variations of phosphorus uptake by ectomycorrhizal fungi, soil microbial biomass, and young beech trees in two forest soils with contrasting P stocks

Marie Spohn[1*§], Aljoša Zavišić[2,3*], Pascal Nassal[4], Fabian Bergkemper[5], Stefanie Schulz[5], Sven Marhan[4], Michael Schloter[5], Ellen Kandeler[4], Andrea Polle[2,3]

[1] *Soil Biogeochemistry, Bayreuth Center of Ecology and Environmental Research (BayCEER), University of Bayreuth, Germany*

[2] *Forest Botany and Tree Physiology, Georg-August University of Göttingen, Germany*

[3] *Laboratory for Radio-Isotopes, Georg-August University of Göttingen, Germany*

[4] *Institute of Soil Science and Land Evaluation, Soil Biology Department, University of Hohenheim, Stuttgart, Germany*

[5] *Research Unit Comparative Microbiome Analysis, Helmholtz Zentrum München, Germany*

* shared first authorship

§ correspondence: phone: +49 +921/555762

Email: marie.spohn@uni-bayreuth.de

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Summary

- The objective of this study was to determine temporal variations of phosphorus (P) uptake by young beech trees (*Fagus sylvatica* L.) and soil microbial biomass (SMB), as well as ectomycorrhizal fungal (EMF) community assemblage with the aim to better understand the factors affecting P uptake by forest trees.
- We conducted a mesocosm-labeling experiment with juvenile beech trees from two forests differing in total soil P stocks. At five times of the year, we determined P uptake by SMB, EMF and *F. sylvatica* by ^{33}P labeling, and measured EMF community composition and potential acid phosphatase activity (APA).
- In mesocosms from the P-poor site, highest plant P uptake took place in summer and coincided with the highest P uptake by SMB. In contrast, in mesocosms from the P-rich site, plant P uptake was highest in autumn, while P uptake by the SMB was highest in spring, coinciding with highest APA. EMF community composition was very variable over the year, suggesting functional redundancy of P acquisition.
- We conclude that P uptake rates of beech, EMF and SMB varied independently over the year. We did not find indications that P immobilization by the SMB, EMF community composition or APA affected plant P acquisition.

Key words: Forest phosphorus nutrition; Phosphorus uptake kinetics; Plant-microbe interactions; Ectomycorrhizal community; Seasonal dynamics; Phosphatase activity

Introduction

Despite the importance of phosphorus (P) for plant nutrition, little is known about the controls of plant P uptake in forest ecosystems (Vance *et al*., 2003; Plassard & Dell, 2010). During the last decades, foliar P concentrations of several tree species in temperate forests have decreased, and the reasons for this decrease are not known yet (Flückiger & Braun 1998, Duquesnay *et al*., 2000; Ilg *et al.*, 2009; Braun *et al*., 2010; Crowley *et al*., 2012; Jonard *et al*., 2015; Talkner *et al*., 2015; Lang *et al.* 2016). This calls for a better understanding of P dynamics in the interplay of soil, ectomycorrhizal fungi (EMF), soil microbial biomass (SMB), and plant activities in temperate forests.

P availability in soil is largely affected by sorption. Since phosphate is rapidly sorped to the soil matrix, only a small proportion of the total soil P is plant available. Thus, plants, bacteria and especially EMF have developed several mechanisms to solubilize bound inorganic P by releasing organic acid anions, protons and siderophores (Jones & Oburger, 2011; Jansa *et al*., 2011; Smits *et al*., 2012). Furthermore, they can mineralize organic P by releasing extracellular phosphatases, which renders P plant available (Plassard & Dell, 2010; Nannipieri *et al*., 2011). Besides mobilizing P, EMF can be very efficient in P uptake from soil because their hyphae reach micropores that are not accessible to roots and have a very high surface-to-volume ratio (Jansa *et al*., 2011).

Only few studies explored P uptake kinetics of ectomycorrhizal-forming tree species (Van Tichelen & Colpaert, 2000; Desai *et al*., 2014; Kavka & Polle, 2016). Tracer studies with ^{33}P showed that P uptake systems of non-mycorrhizal roots are limiting because their K_m values are higher than typical concentrations of free P_i in the soil solution (Van Tichelen & Colpaert, 2000; Desai *et al*., 2014; Kavka & Polle, 2016). In mycorrhizal trees, the K_m is strongly decreased and the uptake rate drastically enhanced (Van Tichelen & Colpaert, 2000; Desai *et al*., 2014), which underpins the relevance of EMF for plant P uptake. However, since previous experiments were conducted in hydroponic solutions in the absence of soil bacteria and saprotrophic fungi, which may strongly affect tree nutrient uptake, the environmental factors that influence P acquisition of EMF and their host trees are still unknown.

The relationship between plants and saprotrophic microorganisms can be either mutualistic or competitive. Microorganisms can mineralize more organic P than they need for themselves, which is beneficial for plants because it provides them with inorganic P (Richardson *et al*., 2009; Marschner *et al*., 2011). However, saprotrophic microbes might also compete with plants for P, and both saprotrophic microorganisms as well as EMF can immobilize a large amount of P in their biomass, which might decrease plant P uptake (Richardson *et al*., 2009; Marschner *et al*., 2011). Microbial biomass P can represent a substantial fraction of the total soil P. In temperate coniferous and broadleaf forests it amounts on average to 4.3 and 8.6% of the total soil P, respectively (Xu *et al*., 2013).

While there are only few studies that explored P uptake kinetics of trees and microorganisms using ^{33}P, uptake kinetics have been studied more intensively for nitrogen (N) using ^{15}N. In many studies, in which N uptake by trees and SMB was compared, it was found that initially the SMB took up a significantly larger percentage of the added ^{15}N than the tree. This was documented for *Acer saccharum* in northern hardwood forests (Zogg *et al*., 2000), for *Quercus douglasii* in California (Cheng & Bledsoe, 2004), for birch forests in subarctic Sweden (Grogan & Jonasson, 2003), for *Fraxinus excelsior* in France (Bloor *et al*., 2009), and for *Fagus sylvatica* in Germany (Pena *et al*., 2013; Leberecht *et al*., 2015; Dannenmann *et al*., 2016). ^{15}N immobilized in the SMB was only very slowly released during the following months (Zogg *et al*., 2000; Grogan & Jonasson, 2003).

In ecosystems with pronounced seasonality, such as temperate and alpine ecosystems, plant N uptake and microbial N uptake are often anticyclical in the way that plants take up N mostly during the growing season, while microbial N uptake is highest in autumn, stimulated by high inputs of plant detritus during this time of the year (Jaeger *et al*., 1999; Lipson *et al.*, 1999; Kaiser *et al*., 2011). At the end of winter, microbial N decreases again due to thawing-and-freezing events that induce microbial cell lysis (Jaeger *et al*., 1999; Lipson *et al*., 1999; Kaiser *et al*., 2011). It is not known yet, whether such dynamics also occur in temperate forest soils with respect to P uptake.

The objective of this study was to determine temporal variations of P uptake by SMB and by young beech trees (*Fagus sylvatica*) along with their root-associated EMF assemblages, P sorption, potential acid phosphatase activity and the abundance of the bacterial acid phosphatase (*phoN)* genes to clarify whether P acquisition of plants and microorganisms is synchronized. For this purpose, young beech trees were extracted with intact soil cores from two forest sites that differ in total soil P stocks and P availability (Zavišić *et al*., 2016), and were exposed to ambient conditions in a common garden study. We hypothesized, first, that uptake of P by beech trees and EMF is higher in summer, when the trees are photosynthetically more active than in autumn (Yang *et al*., 2016), while P uptake by the SMB is highest in autumn due to inputs of plant detritus. Second, we hypothesized that P uptake by EMF is equally high in P-poor and in the P-rich soil, because EMF are able to mobilize P, which might compensate low P availability and supports plant P acquisition in the P-poor soil. Third, we hypothesized that the potential acid phosphatase activity (APA) as well as abundance of bacterial *phoN* genes are high throughout the year, and that a substantial proportion of P is immobilized in the SMB.

Materials and Methods

Study site

Soils and juvenile trees were collected at two sites with contrasting soil P availability (Table 1). The site Bad Brueckenau (BBR) that has a high soil P availability is located in the Rhoen Mountains, close

to the city of Fulda, Germany (N 50° 21.38', E 9° 55.71') at 825 m above sea-level. The mean annual rainfall is 1031 mm and the mean annual temperature is 5.8°C. The soil is a Dystric Skeletic Cambisol derived from basalt, and the prevailing tree species is European beech (*Fagus sylvatica* L.). The site Luess (LUE) that has a low soil P availability is located in the Lueneburg Heath, close to the city of Hamburg, Germany (N 52° 50.32', E 10° 16.06') at 115 m above sea-level. The mean annual rainfall amounts to 730 mm and the mean annual temperature is 8°C. The soil is a Hyperdystric Folic Cambisol developed from sandy Pleistocene sediments, and the dominant tree species is also *Fagus sylvatica* L. More details have been reported by Zavišić *et al*. (2016).

Sampling and experimental setup

Young beech trees of 0.3 to 0.4 m height were excavated on the sites BBR and LUE at the end of October 2013. For this purpose, polymer tubes with a diameter of 12 cm and a height of 20 cm were hammered into the soil around the young tree and subsequently extracted with the plant and the intact soil core. 150 tubes with young beech trees and soil from each forest site (Table 1) were transported to the Forest Botanical Garden of the University of Goettingen, where they were kept outdoors, under natural climatic conditions throughout the experiment (for air temperature and soil water content during the experiments see Additional online material Fig. 1). Because root damage could not be entirely excluded, when harvesting field-grown young trees, the beech trees in the tubes were left undisturbed for six months before using them for experiments.

Labeling and harvest

At five dates during the first year, which corresponded to five phenological stages as described by Yang *et al*. (2016), 15 mesocosms from each of the two sites were labeled with ^{33}P. Additionally, another 15 mesocosms from each site were labeled in the following year in summer, leading to a total of six labeling dates; spring (April 2nd, 2014): bud swelling; early summer (May 12th, 2014): young leaves, late summer (July 21st, 2014): mature leaves; fall (September 22nd, 2014): leaf senescence; and winter (February 9th, 2015): dormancy (no leaves), and additionally, a second late summer (July 16, 2015). For this purpose, 1912 MBq carrier free $H_3{}^{33}PO_4$ (Hartmann Analytic GmbH, Braunschweig, Germany) was added in 40 ml of tap water, amounting to a total of 0.017 nmol P per plant. It should be noted that the amount of P added is negligible, and ^{33}P served only as a tracer without affecting the P availability. Subsequently, each plant was watered with 40 ml tap water in order to distribute the tracer throughout the soil core. To avoid loss of label via leaching, a plastic saucer was placed underneath each soil core and if leachate appeared it was immediately re-applied on the soil. Five non-labeled mesocosms of each site were destructively sampled at the day of tracer application. Labeled mesocosms of each forest site were destructively sampled 1, 7 and thirty days

after labeling (n = 5 mesocosms per time point and site). For this purpose, the plant with the soil core was completely removed from the tube. The organic layer and the mineral soil of each mesocosm was separated and weighed. Subsequently, the stem was cut at the shoot-root junction and the roots were carefully removed from the soil to avoid damaging EMF. The mineral soil and the organic layer were kept separately and each fraction was thoroughly mixed. Leaves, buds, coarse roots and fine roots (<2 mm diameter) were separated using scissors, and an aliquot of the plant material was shock frozen and stored at -80°C. The remaining plant material was oven-dried at 40°C for one week. Fine roots were dried after morphotyping. Fine root distribution was determined for selected samples that were not included in other analyses. For this purpose soil cores were cut at the border of the mineral soil and organic layer, all roots per soil layer were carefully removed, separated according to coarse and fine roots and dried at 40°C for one week. Total dry weight of all plant components (leaves, buds, stem, coarse roots, and fine roots) and mineral and organic soil was measured. An aliquot of each fresh soil fraction was stored frozen at -80°C for measurements of phosphatase activity and *phoN* gene abundance measurements. Further fresh soil aliquots were used for determination of microbial biomass C and P, labile soil P, soil moisture and dry mass.

Ectomycorrhizal species composition

Vital and dead roots tips of each plant were identified by their turgescent or dry and shrunken appearance under a dissecting microscope (Leica M205 FA; Leica, Wetzlar, Germany). Labeling studies with ^{15}N showed that dry and shrunken root tips were physiologically inactive (Pena & Polle, 2014). Therefore these root tips were considered as dead root tips. Vital and dead root tips were counted. Ectomycorrhizal morphotypes were identified based on color, shape, branching pattern, mantle surface texture, hyphal morphology and rhizomorph connection/shape according to Agerer *et al*. (2006). Each morphotype was collected (n = 5-20 root tips) and stored at -20°C. Total genomic DNA was isolated from the morphotypes using the innuPREP Plant DNA Kit (Analytik Jena AG, Jena, Germany) as described by the manufacturer. The rRNA ITS-region was amplified by the polymerase chain reaction (PCR) with the primer pair ITS1F and ITS4 (Eurofins MWG Operon, Ebersberg, Germany) (White *et al*., 1990) using either the original genomic DNA sample or a dilution (1:10 or 1:100) to remove inhibitors of the PCR as described previously (Lang & Polle, 2011). Purification of all PCR products was done by addition of 35 mL 2-propanol (Roth, Karlsruhe, Germany). DNA was subsequently precipitated for 1 h at room temperature, and centrifuged for 30 min at 20,000 x g (Centrifuge 5417 R, Eppendorf, Hamburg, Germany). The obtained pellet was fully dehydrated for 10 min at 50°C (Concentrator 5301, Eppendorf, Hamburg, Germany) and dissolved in 30 mL ultrapure H_2O (Applichem GmbH, Darmstadt, Germany). The purified DNA was used for Sanger sequencing (Applied Biosystems 3730XL DNA Analyzer, Seqlab GmbH, Göttingen, Germany). Sequence alignment

was done using the Staden Package software (4.10, http://staden.sourceforge.net) and blasted in the NCBI GenBank. With a sequence homology of >97% and a score >900 bits, the name suggested by the database was accepted. Sequences were deposited in the NCBI Genbank with the accession numbers KX168637-KX168665 (see also Additional online material Table 1)

Soil microbial biomass, labile P and total C and N

Soil microbial biomass C and P (SMB_C and SMB_P), comprising C and P of archaea, protozoa, bacteria well as saprotrophic and mycorrhizal fungi, were determined in fresh soil using the chloroform fumigation extraction method (Brookes *et al*., 1982; Vance et *al*., 1987). 10 g of fresh mineral topsoil and 5 g of organic layer were fumigated with chloroform for 24 h. P was extracted from the fumigated and the non-fumigated soil in Bray-1 solution (0.03 M NH_4F - 0.025 M HCl) and quantified by the molybdate blue assay using an injection flow photometer (FIA-LAB, MLE Dresden, Germany). The conversion factor of 2.5 was used to calculate SMB_P (Brookes *et al*., 1982). The inorganic P concentration in the Bray-1 extracts of the non-fumigated soils (see above) were considered as the labile P fraction. C was extracted from the fumigated and the non-fumigated soil in 40 ml 0.5 M K_2SO_4 and were measured using a TOC/TN analyzer (Multi N/C 2100S, Analytik Jena AG, Jena, Germany). A conversion factor of 2.2 was used to calculate SMB_C (Joergensen, 1996). Total C and N were determined using a CNS elemental analyzer (EA1108, Carbo Erba Strumentazione, Rodano, Italy).

^{33}P activity

Plant components (leaves, buds, stem, coarse roots, and fine roots) and EMF samples were milled (Retsch MN 400, Haan, Germany) and extracted in 65% HNO_3 at 160 °C for 12 h according to (Heinrichs *et al.*, 1986). Total P in the extracts was measured by ICP-OES (iCAP 6000 Series ICP–OES, Thermo Fisher Scientific, Dreieich, Germany). ^{33}P in all HNO_3 and in Bray-1 extracts was determined by a Perkin-Elmer scintillation counter (Tri-Carb TR/SL 3180, Waltham, MA, USA), using scintillation cocktail (Rotiszint eco plus, Roth, Karlsruhe, Germany). The signal was corrected for radioactive decay, and a background correction value was subtracted that was measured using only scintillation cocktail and extraction solution.

Potential acid phosphatase activity (APA)

Potential APA was measured using a modified disodium phenylphosphate method. 5 g of soil was incubated in 10 ml of acetate buffer (pH 5) and 5 ml of 20 mM buffered disodium phenylphosphate solution at 37°C for 3 h; the release of phenol was determined colorimetrically at 614 nm, using 2,6-dibromchinone-chlorimide as coloring reagent (Hoffmann, 1968; modified by Öhlinger, 1996).

Abundance of phoN genes

Total genomic DNA was extracted from the soil of day 0 of each labeling experiment using the "NucleoSpin Soil Kit" (Macherey-Nagel, Germany) according to the manufacturer's protocol. Quantification of the bacterial *phoN* gene was performed using quantitative real-time PCR and target specific primers *phoN*-FW and *phoN*-RW (Bergkemper *et al*., 2016) on a 7300 Real-Time PCR System (Applied Biosystems, Germany) together with SybrGreen (Life Technologies, USA) as fluorescent dye. After each qPCR run a melting curve analysis was performed to verify the amplicon specificity. The quantification of the target gene was conducted by using serial dilutions of plasmid encoded *phoN* genes (10^7 to 10^1 gene copies μl^{-1}) derived from *Salmonella enterica* DSM 10062. Based on a previous dilution test all samples were diluted in a 1:16 ratio to avoid inhibition during amplification caused by co-extracted humic substances. The efficiency of the qPCR was calculated as: Efficiency (%) = $[10^{(-1/\text{slope})} - 1]$ and was always above 86% while the R^2 of the standard curve was above 0.99.

Calculations and Statistical Analyses

We calculated the total ^{33}P taken up by the plant for distinct points in time as follows.

$$Total\ 33P_{Plant}\ (Bq) = Total\ biomass_{Leaves}(g) \times 33P_{Leaves}(Bq\ g^{-1}) + Total\ biomass_{Stem}(g) \times 33P_{Stem}(Bq\ g^{-1}) + Total\ biomass_{Coase\ roots}(g) \times 33P_{Coarse\ roots}(Bq\ g^{-1}) + Total\ biomass_{Fine\ roots}(g) \times 33P_{Fine\ roots}(Bq\ g^{-1}) + Total\ biomass_{Buds}(g) \times 33P_{Buds}(Bq\ g^{-1})$$

The ^{33}P enrichment of the plant biomass was calculated as follows.

$$33P\ enrichment\ (Bq\ (g\ biomass)^{-1}) = \frac{Total\ 33P_{Plant}(Bq)}{Plant\ biomass\ (g)}$$

The total ^{33}P of EMF in one mesocosm was calculated based on the ^{33}P enrichment of the EMF biomass as follows.

$$Total\ 33P_{EMF}\ (Bq) = 33P_{EMF}\ (Bq(g\ biomass)^{-1}) \times Biomass\ of\ EMF\ on\ one\ root\ tip\ (\mu g) \times Number\ mycorrhizal\ root\ tips$$

The ^{33}P enrichment of the SMB per gram dry soil was calculated separately for the mineral soil and the organic layer.

$$33P_{SMB}(Bq\ g^{-1}\ soil) = 33P_{Fumigated\ soil}(Bq\ g^{-1}\ soil) - 33P_{Non-fumigated\ soil}(Bq\ g^{-1}\ soil)$$

The total amount of ^{33}P taken in the SMB in one mesocosm was calculated separately for the mineral soil and the organic layer as follows.

$$Total\ 33P_{SMB}\ (Bq) = 33P_{SMB}(Bq\ g^{-1}\ soil) \times Dry\ mass_{Soil\ mesocom}(g)$$

Total ^{33}P in the labile P fraction was calculated separately for the mineral soil and the organic layer for the harvest thirty days after ^{33}P application as follows.

$$Total\ 33P_{Labile\ P\ fraction}(Bq) = 33P_{Labile\ P\ fraction}(Bq\ g^{-1}\ soil) \times Dry\ mass_{Soil\ mesocom}\ (g)$$

Total P taken up by the microbial biomass during thirty days was calculated based on the assumption that microbes take up P from the labile soil P fraction separately for the organic layer and the mineral soil as follows.

$$P\ taken\ up\ by\ SMB(\mu g) = Total\ 33P\ in\ SMB\ (Bq) / Specific\ 33P\ activity\ (Bq\ \mu g\ P^{-1})\ of\ labile\ P\ fraction$$

Specific ^{33}P activity of a soil P pool is defined as follows

$$Specific\ 33P\ activity\ (Bq\ \mu g\ P^{-1}) = \frac{33P\ (Bq\ g^{-1}\ soil)}{P(\mu g\ g^{-1} soil)}$$

Total P taken up by *F. sylvatica* during thirty days was calculated based on the relative P availability and the relative root abundance in the mineral soil and the organic layer as follows.

$$Total\ P\ taken\ up\ by\ plant\ (\mu g\ P) = Total\ 33P_{Plant}\ (Bq) \times ((Specific\ activity\ in\ labile\ P\ fraction\ in\ organic\ layer\ (Bq\ \mu g\ P^{-1}) \times (relative\ P\ availability\ in\ organic\ layer + relative\ root\ abundance\ in\ organic\ layer)/2) + (Specific\ activity\ in\ labile\ P\ fraction\ in\ mineral\ soil\ (Bq\ \mu g\ P^{-1}) \times (relative\ P\ availability\ in\ mineral\ soil + relative\ root\ abundance\ in\ the\ mineral\ soil\ )/2))$$

where

$$Relative\ P\ availability_{Organic\ layer} = \frac{Labile\ P_{organic\ layer}}{(Labile\ P_{organic\ layer} + Labile\ P_{Mineral\ soil})}$$

and

$$Relative\ root\ abundace_{Organic\ layer} = \frac{DW\ of\ fine\ roots_{Organic\ layer}}{(DW\ of\ fine\ roots_{Organic\ layer} + DW\ of\ fine\ roots_{Mineral\ soil})}$$

The relative P availability in the mineral soil and the relative root distribution in the mineral soil were calculated accordingly. The total P taken up by EMF was calculated in the same way as the total P uptake by the plant.

Total P taken up by the SMB in each mesocosm was calculated as follows

$$Total\ P\ taken\ up\ by\ SMB\ (\mu g) = Total\ 33P\ in\ SMB\ (Bq)/Specific\ activity\ in\ labile\ P\ fraction\ (Bq\ \mu g\ P^{-1})$$

However, based on ^{33}P per unit EMF dry weight, we calculated the amount of P taken up per unit EMF dry weight as follows.

$$\text{P taken up by EMF } (\mu g\ P\ mg^{-1}\ dw) = 33P\ in\ EMF\ (Bq\ mg^{-1}\ dw) / Specific\ activity\ in\ labile\ P\ fraction\ (Bq\ \mu g\ P^{-1})$$

Homogeneity of variance was tested by the Levene-test. Differences between sites and dates or seasons were tested by ANOVA followed by Scheffé post-hoc test using R (R Core Team, 2013). We

used the software package PAST 3.08 (http://folk.uio.no/ohammer/past/, Hammer *et al*., 2001) to calculate Shannon index (H), Simpson index, species richness and evenness of the EMF. To achieve species saturation, the data of the root systems of five plants sampled per time point, soil type and season were pooled. To compare the diversity of EMF in different soil types, all values obtained across the whole year were compared by a paired rank test. The similarity of the EMF community structures was analyzed by non-metric multidimensional scaling (NMDS) using Bray Curtis as the similarity measure.

Results

EMF, SMB and labile P

The number of root tips colonized by EMF was lower in the mesocosms from the P-poor site LUE than in the mesocosms from the P-rich site BBR throughout the year ($p<0.001$; Fig. 1a). The community composition of EM taxa showed seasonal fluctuations indicated by the separation along coordinate 1 of an NMDS (Fig. 2). The communities in spring and early summer clustered, and the communities of late summer, autumn, and winter also showed some overlap (Fig. 2, Additional online material Table 2). The communities from LUE and BBR differed significantly in their structure shown by the separation along coordinate 2 (Fig. 2, Additional online material Table 2). This separation was due to higher EMF species richness on the roots from BBR than from LUE, and divergent composition of the assemblages (Additional online material Table 3). In the EMF assemblages of BBR, *Xerocomellus pruinatus*, *Tuber* sp, *Tomentellopsis* sp. and *Clavulina coralloides* appeared seasonally with abundances of >10%, whereas in LUE *Russula ochroleuca*, *Lactarius blennius*, *Scleroderma citrinum*, and *Genea* cf. *anthracina* were predominant (Additional online material Table 1).

Concentrations of labile P were higher in the organic layer than in the mineral soil in the mesocosms from both forest sites ($p<0.001$; Fig. 1b). While there was no significant difference in the labile P concentration in the mineral soil between the sites ($p>0.05$), the labile P concentration in the organic layer was on average 2.4-times higher ($p<0.01$) in the mesocosms from BBR than in the mesocosms from LUE throughout the year (Fig. 1b). Concentrations of labile P showed large variations in the organic layer between the mesocosms from one site, and no clear seasonal trend was observable neither in the organic layers nor in the mineral soils.

The SMB_C concentration was higher in the organic layer than in the mineral soil of both sites ($p<0.01$). In the mineral soil, the SMB_C concentration at BBR was higher than at LUE throughout the year ($p<0.01$; Fig 1c). No significant seasonal variation in SMB_C in the mineral soil and in the organic layer of both soils was found ($p>0.05$; Fig. 1c). In the organic layer, but not in the mineral soil, the

SMB_C was significantly correlated with the gravimetric water content across seasons (R^2=0.30 and 0.25 in LUE and BBR, respectively).

^{33}P enrichment and recovery

The young beech trees from the P-rich site BBR showed similar ^{33}P enrichment during all seasons, except for autumn when higher ^{33}P enrichment was found ($p<0.01$, Fig. 3a). In contrast, ^{33}P uptake by the young beech trees from the P-poor site LUE showed a more pronounced seasonal dynamic (Fig. 3a). The plants from LUE exhibited increased ^{33}P enrichment in early summer compared to spring ($p<0.01$), and they further increased the ^{33}P uptake during late summer ($p<0.01$). ^{33}P enrichment in EMF was fast during the first seven days after tracer addition and slowed strongly afterwards (Fig. 3b). The same trend was observed for ^{33}P uptake by the SMB in the mineral soil and especially in the organic layer (Fig. 3c and d). The dynamics observed in late summer, were confirmed in the second year (Additional online material, Fig. 2).

Taking into account the soil dry mass in the mesocosms, more ^{33}P was recovered from the organic layer than from the mineral soil in LUE than in BBR, whereas the mineral soil sequestered more ^{33}P in BBR than in LUE (Fig. 4). The total amount of ^{33}P recovered from the plant, the SMB and the labile P pool in the organic layer and in the mineral soil together, was quite stable across all five experiments (Fig. 4), and amounted on average to 44.4 and 23.4% of the initially added tracer in LUE and BBR, respectively. The largest proportion of added ^{33}P was sorped in abiotic soil P fractions, and was not recovered in the labile P fraction or in the biotic pools (Fig. 4).

The specific ^{33}P activity of the labile P pool was higher in LUE than in BBR both in the organic layer and in the mineral soil throughout the year (Additional online material Fig. 3). It was also larger in spring and in winter than in summer (Additional online material Fig. 3), which is important for the calculation of the total P uptake.

Total P uptake

Total P taken up by the beech trees over thirty days in late summer amounted to 15.10 mg in the mesocosms from LUE and was significantly higher than P uptake by the young beech trees from BBR during the same time (1.75 mg in thirty days, Fig. 5). However, in autumn, total plant P uptake over thirty days was similar in LUE and BBR and amount to 10.04 and 13.13 mg P per plant, respectively (Fig. 5). P uptake by EMF collected from the fine roots did not differ significantly between sites and seasons ($p>0.05$), although the uptake was increased in autumn (Fig. 5). The net amount of new P taken up during thirty days by EMF collected at the fine roots did not exceed 0.02 mg P per mesocosm during most of the year, and only increased to 0.20 and 0.25 mg P per mesocosm, in LUE and BBR respectively, in autumn (Fig. 5). However, it has to be taken into account that here root-

associated EMF were measured, whereas mycorrhizal hyphal P uptake was part of the microbial biomass, and thus, the total amount of P taken up by the EMF is very likely larger.

The amount of P taken up by the SMB in the mineral soil both in mesocosm from LUE and BBR did not differ significantly between the seasons ($p<0.05$), whereas we found seasonal differences for the organic layer ($p<0.01$). In LUE, P uptake by SMB in the organic layer was increased in spring, summer and autumn compared to winter, and it was significantly higher in summer than in winter ($p<0.01$). In BBR, P uptake by SMB in the organic layer was highest in spring, and was significantly higher in spring than in winter ($p<0.01$). The total amount of P taken up over thirty days by the SMB in the organic layer in LUE was higher than in BBR throughout the year (although the differences were only statistically significant ($p<0.05$) in three seasons; Fig. 5). The reason for this is mostly the larger mass of the organic layer in LUE than in BBR. The amount of P taken up by the SMB in the organic layer and in the mineral soil together was higher than the amount of P taken up by the plant throughout the year (Fig 5).

Acid Phosphatases

Potential acid phosphatase activity (APA) was highest in spring in both the organic layer and the mineral soil in the mesocosm from both sites, while lowest APA was observed in summer (Table 2). APA in the organic layer was correlated with the gravimetric water content (Supplementary material Fig. 1B) across seasons (R^2=0.30 and 0.51 in BBR and LUE, respectively), while no significant correlation between water content and phosphatase activity was found for the mineral soil. APA was correlated with SMB_C, especially in the mineral soil, and with the abundance of the bacterial *phoN* genes (Table 3).

Discussion

P uptake by EMF and EMF community composition

This is the first study to show that P uptake rates by a young tree, by EMF and SMB differ seasonally, and between contrasting forest sites. In mesocosms from the P-poor site LUE, highest plant P uptake took place in summer and coincided with the highest P uptake by SMB in the organic layer. In contrast, in mesocosms from the P-rich site BBR, plant P uptake was highest in autumn, while P uptake by the SMB in the organic layer was highest in spring, and the latter coincided with the highest potential APA. Since our study was conducted in a common garden, effects of low water availability were avoided, which are known to impact nutrient uptake under field conditions (Bimüller *et al.* 2014; Dannenmann *et al*., 2016; Leberecht *et al*. 2016).

The highest plant P uptake did not go along with significant increases in P uptake by EMF (Fig. 5). This result could imply that plant P uptake was not controlled by EMF, but by plant demand.

However, it has to be taken into account, first, that the EMF pool measured here represents only a small proportion of the total EMF biomass, and second, that P taken up by EMF is continuously delivered to the plant (Van Tichelen & Colpaert, 2000; Desai *et al*., 2014; Kavka & Polle, 2016). Our results show that EMF in LUE and BBR took up very similar amounts of P throughout the year (Fig. 5), indicating that EMF in LUE were able to compensate for the lower soil P availability at this site (Fig. 1b).

The EMF community was affected by season as well as by site and showed a large spatial and temporal variability (Fig. 2) similar as previously described for other forests (van der Heijden *et al*., 2000; Koide *et al*., 2007; Walker *et al*., 2008; Pena *et al*., 2010). The EMF communities from LUE and BBR were different from each other during all seasons of the year. During the seasons, in which plant P uptake was increased in the mesocosms from both sites (autumn), the structures of the EMF assemblages of BBR and LUE plants were significantly different suggesting functional redundancy for P uptake by EMF.

Scleroderma citrinum, which dominated the EMF community on the plants from LUE, was found to often coexist with biotite-weathering bacteria (Uroz *et al*., 2009). This is in agreement with a study on the microbiome of the soils from LUE and BBR, reporting a high number of Proteobacteria and Acidobacteria in LUE known to be able to mineralize biotite (Bergkemper *et al*., 2015). Russulaceae such as *Lactarius blennius* and *Russula ochroleuca* were also abundant in P poor soils (Zavišić et al., 2016; this study); these species are known to produce organic acids to mobilize inorganic P (Courty et al. 2010). In contrast, roots grown in soil from BBR were dominated by the EMF *Hydnum repandum* during late spring, which can produce high phytase activity (Goud & Suryam, 2009). During summer and autumn the EMF species *Tuber sp.* was found to be dominant on the root tips of plants from BBR, which produces a high amount of phosphatases, and has a high ability to mineralize N from N-containing biopolymers (Pena & Polle 2014), along with high storage of nutrients in its mantle (Walker *et al*., 2014). The different P availability of the sites was likely only one factor among others that shaped the EMF community as indicated also by a recent study on the sites BBR and LUE (Zavišić *et al*., 2016).

P uptake by SMB and P sorption

We found relatively stable SMB_C concentrations over the year (Fig. 1c), which is in accordance with previous studies on beech and spruce forests in Germany (von Lützow *et al*., 1992; Bauhus & Barthel, 1995). P uptake by the SMB in the organic layer in LUE was highest in summer, coinciding with highest plant P uptake in the mesocosms from this site. Thus, P uptake by SMB in LUE was not anticyclical to plant P uptake as hypothesized. The reason for this might be that there was no pronounced input of easily available C in autumn, leading to growth and P immobilization of

saprotrophic microorganisms in autumn. However, it seems rather likely that the SMB in the organic layer in LUE was dominated not by saprotrophic microorganisms but by EMF as indicated by the similar ^{33}P uptake kinetics of EMF and SMB in the organic layer (Fig. 2b and c), and as also suggested by a study on the microbial community of LUE and BBR (Zavišić et al., 2016). However, in the mesocosms from BBR, plant P uptake was elevated in autumn, whereas P uptake by the SMB in the organic layer was increased in spring. The reason for the different seasonal dynamics of P uptake by the SMB in the organic layer between LUE and BBR is most likely a higher dominance of EMF over saprotrophic microorganisms in the organic layer in LUE compared to BBR.

In accordance with studies on N uptake by microorganisms and trees (Zak *et al*., 1990; Zogg *et al*., 2000; Cheng & Bledsoe, 2004; Grogan & Jonasson, 2003; Bloor *et al*., 2009; Dannenmann *et al*., 2016), a larger proportion of the tracer was immobilized in the SMB than in the plant during most seasons (Fig. 5). Similar as for N uptake, this was mostly due to the ubiquitous presence of microorganisms in soil and due to the relatively large mass of soil with respect to the plant biomass. In contrast to studies on N dynamics (Zak *et al*., 1990; Zogg *et al*., 2000; Cheng & Bledsoe, 2004; Grogan & Jonasson, 2003; Bloor *et al*., 2009; Dannenmann *et al*., 2016), we found that most of the added P tracer was sequestered in abiotic P pools, and was not recovered in the labile P pool (Fig. 4), showing that sorption plays a very important role for P availability in these soils, and has a larger effect on the concentration of labile P than microbial P immobilization. Moreover, we found that despite a larger plant P uptake in summer and autumn, total ^{33}P recovery did not increase in theses seasons (Fig.4), indicating that the plants acquired P from labile sources, but not P from more stable soil P pools.

More ^{33}P was sequestered in the organic layer in LUE than in BBR (Fig. 4). This can be attributed to the higher mass of the organic layer in LUE (Table 1) since the uptake of ^{33}P by the SMB per g soil (data not shown) was in the same range in the organic layer in LUE and in BBR. The relatively large proportion of ^{33}P sequestered in the organic layer of both soils is most likely due to the fact that the tracer was added with the irrigation water from the top of the mesocosms, and only a small proportion of the added tracer (larger in BBR than in LUE) reached the mineral soil. However, this did not influence our calculations of the total amounts of P taken up by SMB, EMF and *F. sylvatica* since they were based on the specific ^{33}P activity of the labile P pool in the organic layer and the mineral soil. The specific ^{33}P activity of the labile P pool was higher in LUE than in BBR both in the organic layer and in the mineral soil throughout the year (Additional online material Fig. 3). The reason for this is likely twofold, first, the sorption capacity of the mineral soil from LUE is lower than the sorption capacities of the mineral soil from BBR (Bünemann *et al*., 2016). Second, the gross organic P mineralization in LUE might have been lower due to the lower SMB in LUE (Fig. 1c), leading to a

slower dilution of the added ^{33}P-orthophosphate tracer with ^{31}P-orthophosphate (Bünemann *et al*., 2016).

Phosphatases and P mineralization

The correlation between potential APA and copy number of bacterial *phoN* genes was highest for the mineral soil (R^2=0.40, p<0.001), suggesting that the phosphatase activity in the mineral soil largely stemmed from bacterial acid phosphatases, while in the organic layer, plant and fungal phosphatases seem to have contributed more strongly to phosphatase activity. This is supported by the correlation of phosphatase activity and SMB_C, which was highest in the mineral soil (Table 3). This is also in agreement with Zavišić et al., (2016) reporting that the vast majority of mycorrhizal root tips in LUE and BBR were found in the organic layer.

Our finding that soil phosphatase activity underwent seasonal changes is in disagreement with a recent study that did not find seasonal differences in phosphatase activity in the rhizosphere of *F. sylvatica* and in the bulk soil in BBR and LUE during all four seasons of the year (Hofmann *et al*., 2016). The reason for the disagreement is likely, that the soil water content was maintained constant in the previous experiment, while it was variable in the present study, and positively correlated with the phosphatase activity in the organic layer (R^2=0.30 and 0.51 in BBR and LUE, respectively). Despite the elevated phosphatase activity in spring, labile P concentrations (Fig. 1b) were not increased during this time of the year, suggesting that the high potential enzyme activities did not translate into increased net P mineralization rates.

Conclusions

Revisiting the hypotheses, we conclude, first, that in contrast to our expectations, we only found an anticyclical uptake of P by *F. sylvatica* and the SMB in BBR but not in LUE. The reason for the different seasonal dynamics of P uptake by the SMB between LUE and BBR is most likely a higher dominance of EMF over saprotrophic microorganisms in the organic layer in LUE compared to BBR. Second, P uptake rates by EMF in the mesocosms from the two forest sites were in the same range throughout the year, indicating that EMF in the P-poor soil could compensate for the lower P availability, as hypothesized. In accordance with the third hypothesis, we found that the SMB took up more P than the plant throughout the year. However, the vast majority of the P tracer was sequestered by abiotic soil P fractions, suggesting that sorption rather than microbial immobilization of P mainly affected the concentration of labile P. Moreover, the EMF community composition was very variable over the year, suggesting functional redundancy of P acquisition. Our results indicate that phosphatase activity, EMF community composition or competition between plants and microbes for P are not the reason for the observed decrease in foliar P contents in *F. sylvatica* across Europe.

Future research should test whether instead increased atmospheric N deposition and drought impede P cycling in temperate forests.

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Author contributions

AP designed the experiments, analyzed data, wrote and commented on the manuscript. AZ conducted field work, analyzed samples, analyzed data, wrote and commented on the manuscript. MS analyzed samples, conducted data analysis and wrote the manuscript. PN, SM and EK analyzed samples and commented on the manuscript. SS, MSch and FB analyzed qPCR data and commented on the manuscript. All authors approved the final version of the manuscript.

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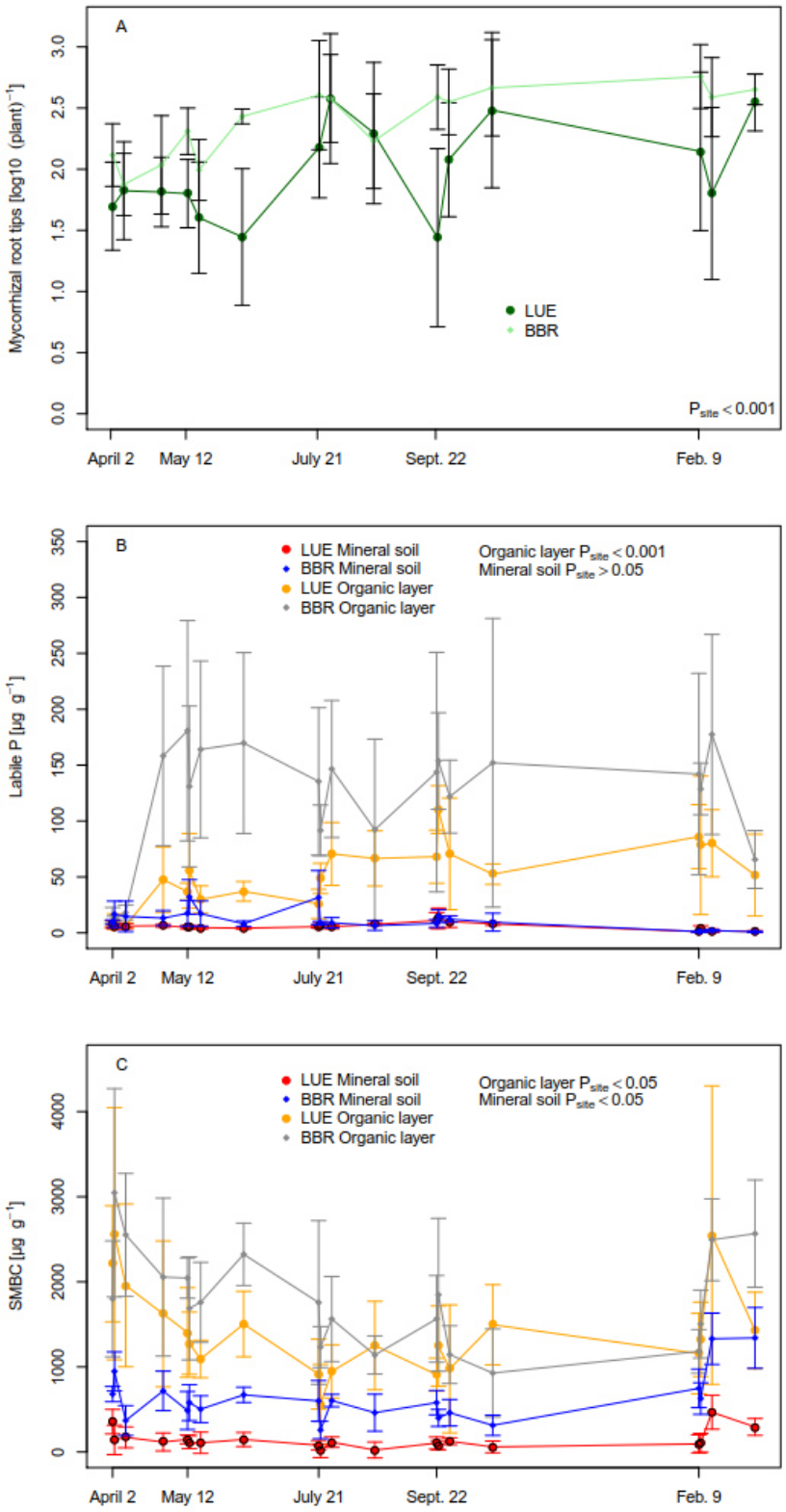

Figure 1 Mycorrhizal root tips (A), labile inorganic P (B) and the soil microbial biomass C (C) in mesocosms from the P-poor site LUE and the P-rich site BBR during all five labeling experiments. The number of mycorrhizal root tips was log10 transformed to meet the requirements of normal distribution. P_{sites} indicates significant differences between sites. Each data point was calculated based on five mesocosms (n=5). Error bars show standard deviations.

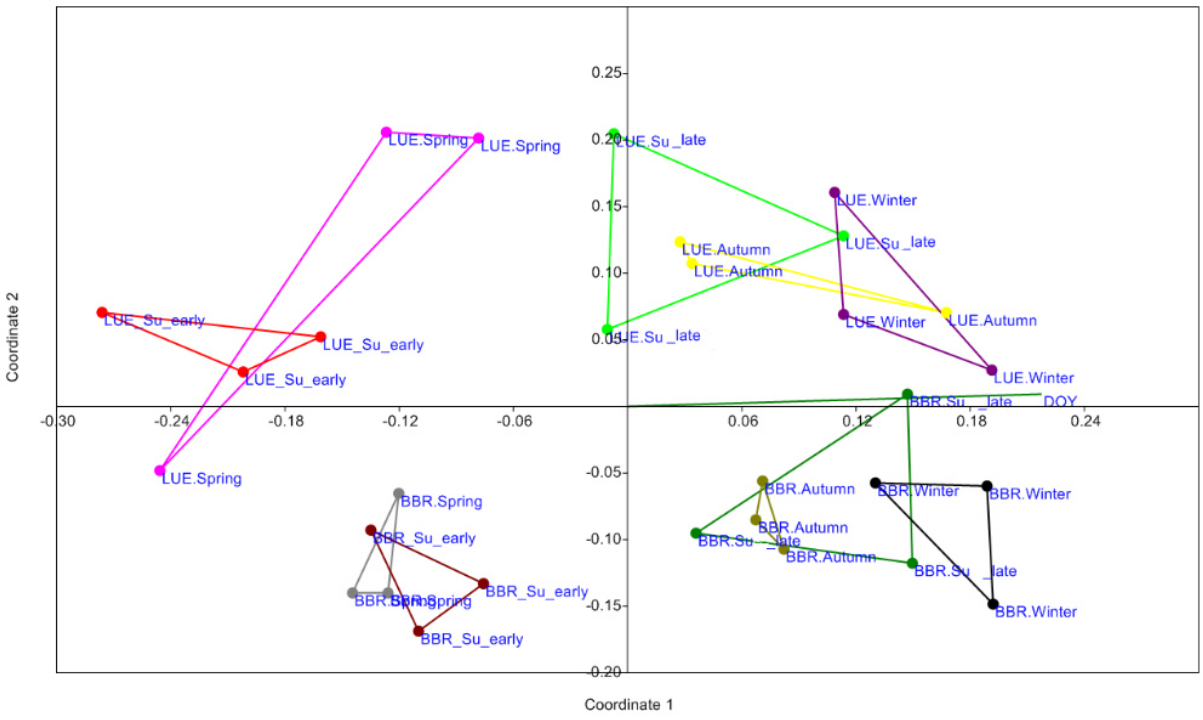

Figure 2 Non-metric multidimensional scaling of the ectomycorrhizal fungal communities (with Bray Curtis as similarity index). Su stands for summer.

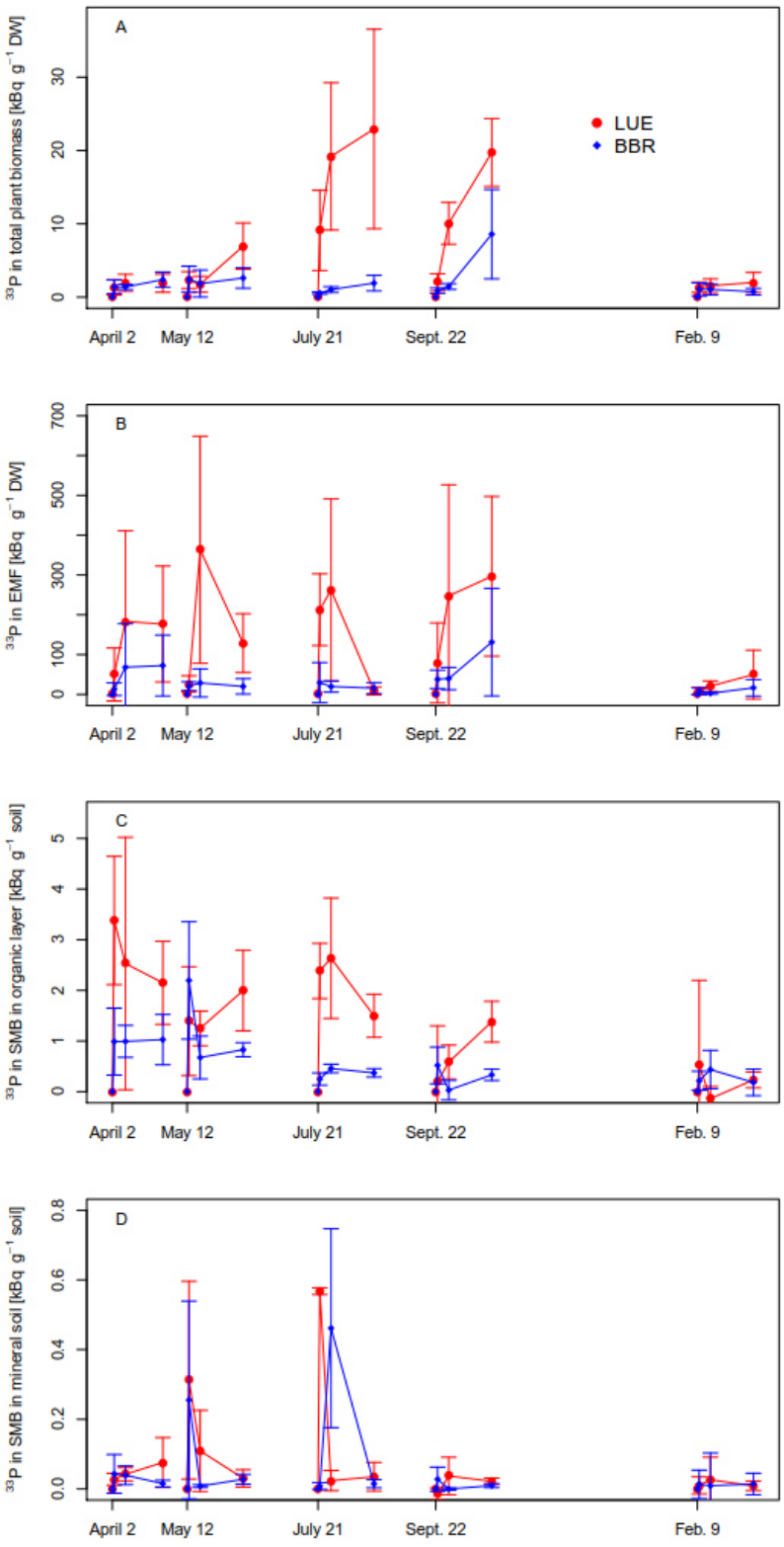

Figure 3 ^{33}P enrichment of young beech trees (plant, A), ectomycorrhizal fungi (EMF, B), and soil microbial biomass (SMB) in the organic layer (C) and SMB in the mineral soil (D) in mesocosms from the P-poor site LUE and the P-rich site BBR during five consecutive labeling experiments. Each data point was calculated based on five mesocosms (n=5). Error bars show standard deviations.

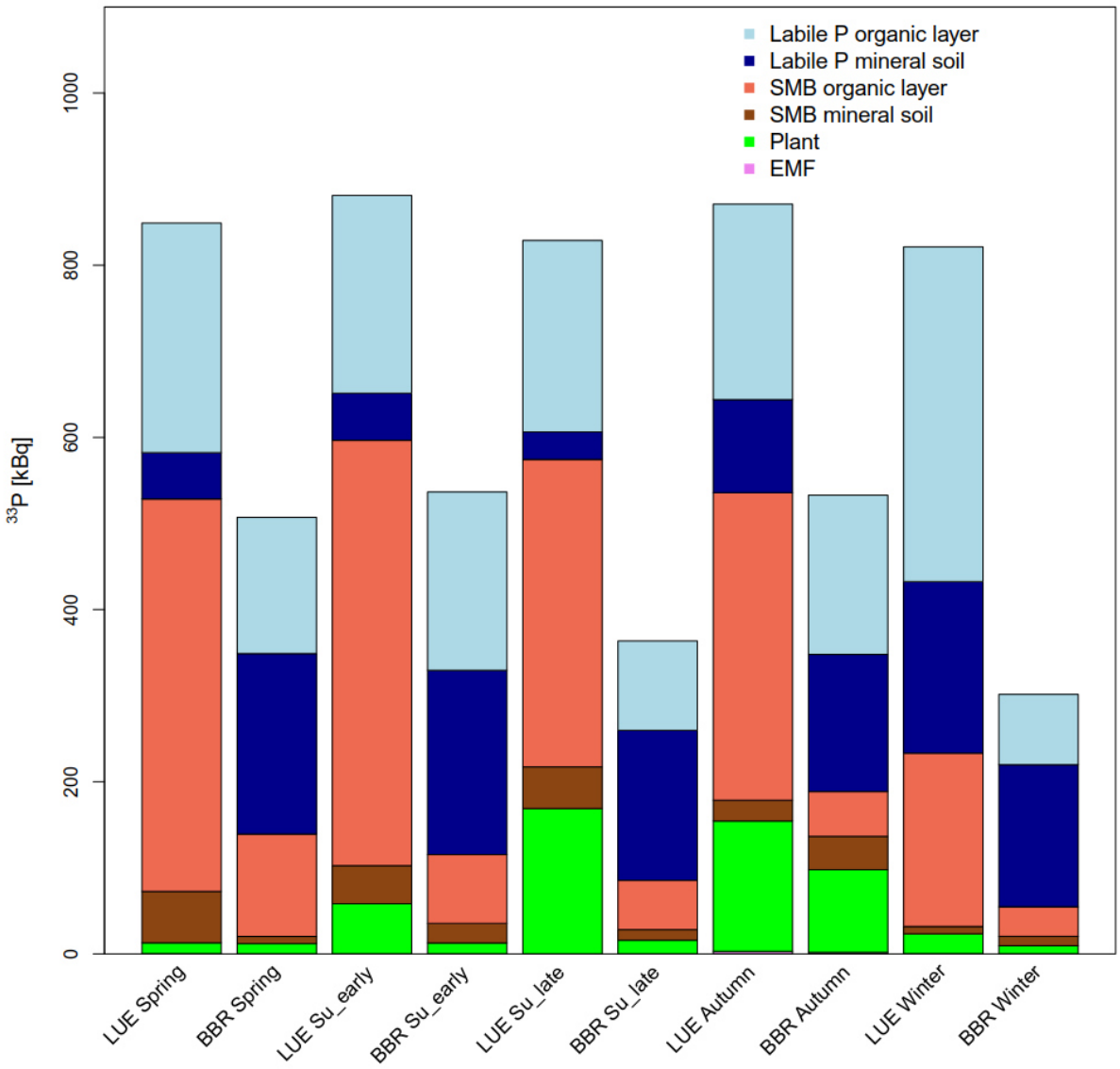

Figure 4 Total amount of ^{33}P in young beech trees (plant), in ectomycrorrhizal fungi (EMF), in the soil microbial biomass (SMB), and in the labile P pool in the mineral soil and in the organic layer in mesocosms from the P-poor site LUE and the P-rich site BBR thirty days after tracer application at five different times of the year. Each data point was calculated based on five mesocosms (n=5), taking into account the mass of the organic and the mineral soil in each mesocosm.

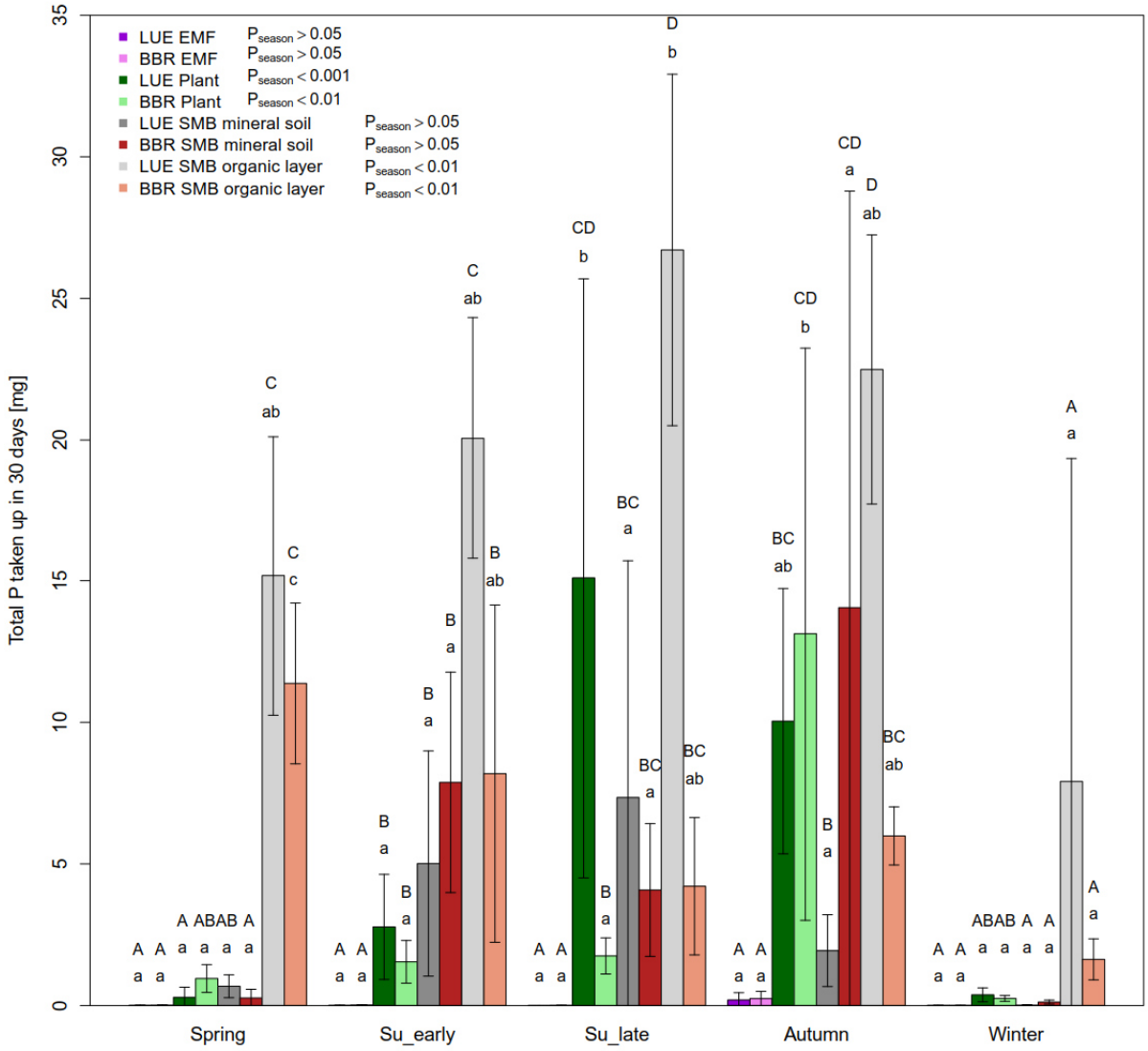

Figure 5 Total P taken up during thirty days by ectomycorrhizal fungi (EMF), *F. sylvatica* (plant) and the soil microbial biomass (SMB) in mesocosms from the P-poor site LUE and the P-rich site BBR at five different times of the year. Each data point was calculated based on five mesocosms (n=5), taking into account the mass of the organic and the mineral soil in each mesocosm. Capital letters indicate significant differences ($p<0.05$) between different parameters in one season, while lowercase letters indicate significant differences ($p<0.05$) in one parameter across seasons.

Table 1 Properties of the soils of the P-poor site LUE and the P-rich site BBR together with the soil chemical features of the undisturbed soil pedons in the mesocosms from the two sites, and the average soil mass, fine root abundance, and water content during the experiment. Letters indicate significant differences ($p<0.05$) in soil chemical parameters between all four soil layers.

	LUE		**BBR**	
Soil order (WRB)	Cambisol		Dystric Skeletic Cambisol	
Parent material	Glacial sands		Basalt	
Humus form	Mor-like Moder		Mull-like Moder	
Depth of the organic layer [cm]	12		7	
	Organic layer	Mineral soil	Organic layer	Mineral soil
Dry weight per mesocosm [g]*	276 (± 133)	1226 (± 391)	180 (± 76)	788 (± 184)
Relative fine root abundance×	0.64 (± 0.05)	0.36 (± 0.05)	0.32 (± 0.02)	0.68 (± 0.03)
Average water content [%]*	18.8 (± 4.6)	50.3 (± 4.6)	33.9 (± 6.7)	42.4 (± 10.8)
Total C [g kg^{-1}]×	223.6 (± 19.1)^{c}	48.4 (± 4.9)^{a}	141.5 (± 7.6)^{b}	62.7 (± 2.7)^{a}
Total N [g kg^{-1}]×	8.96 (± 0.76)^{c}	1.75 (± 0.19)^{a}	7.88 (± 0.36)^{c}	3.79 (± 0.12)^{b}
Total P [g kg^{-1}]*	0.5 (± 0.2)^{b}	0.1 (± 0.0)^{a}	2.8 (± 0.3)^{c}	2.8 (± 0.4)^{c}
Labile P [mg kg^{-1}]*	52.7 (± 37.8)^{b}	5.9 (± 4.2)^{a}	120.0 (± 81.9)^{c}	11.9 (± 37.8)^{a}
Soil pH_{H2O}×	4.3^{b}	3.8^{a}	5.1^{c}	4.3^{b}

*n=100, ×n=5

Table 2 Phosphatase activity and copy numbers of the bacterial acid phosphatase gene *phoN* in soil of mesocosms from the P-poor site LUE and the P-rich site BBR at five different times of the year. Each data point was calculated based on five mesocosms (n=5) at the first day of each labeling experiment. Significant seasonal differences ($p<0.05$) in one parameter tested separately for the organic layer and the mineral soil by Scheffé test are indicated by letters. P_{season} indicates the P value of the ANOVA testing difference between seasons. Su stands for summer.

		LUE						BBR					
		Spring	Su_early	Su_late	Autumn	Winter	P_{season}	Spring	Su_early	Su_late	Autumn	Winter	P_{season}
Organic layer	Phosphatase activity [mg Phenol (3 h)$^{-1}$ g^{-1}]	30.14^{b} (±15.96)	15.48^{ab} (±5.54)	8.07^{a} (±1.61)	19.07^{ab} (±5.35)	9.37^{a} (±3.05)	<0.01	16.19^{b} (±2.83)	12.40^{ab} (±4.26)	7.71^{a} (±3.77)	8.40^{a} (±3.65)	7.28^{a} (±2.70)	<0.01
	Number of *phoN* genes [×100000 g^{-1}]	2.22 (±0.89)	0.58 (±0.49)	0.68 (±0.44)	0.76 (±0.29)	0.21 (±0.06)	>0.05	1.46 (±0.72)	0.52 (±0.21)	0.57 (±0.31)	0.88 (±0.30)	0.25 (±0.11)	>0.05
Mineral soil	Phosphatase activity [mg Phenol (3 h)$^{-1}$ g^{-1}]	1.49^{b} (±0.63)	0.59^{a} (±0.23)	1.01^{ab} (±0.13)	0.93^{ab} (±0.30)	0.73^{ab} (±0.04)	<0.01	2.23^{b} (±0.81)	2.28^{b} (±0.52)	1.19^{a} (±0.22)	1.79^{ab} (±0.64)	1.73^{ab} (±0.38)	<0.05
	Number of *phoN* genes [×100000 g^{-1}]	0.27 (±0.11)	0.14 (±0.05)	0.08 (±0.09)	0.15 (±0.05)	0.05 (±0.04)	>0.05	0.82 (±0.24)	0.64 (±0.23)	0.36 (±0.19)	0.64 (±0.23)	0.05 (±0.04)	>0.05

Table 3 Correlation coefficients (R^2) for correlations of potential acid phosphatase activity, copy numbers of the bacterial acid phosphatase *phoN* genes, and soil microbial biomass carbon (SMB_C) in the mineral soil and the organic layer of the mesocosms across seasons. *** p<0.001; * p<0.05.

		Phosphatase activity [mg Phenol (3 h)$^{-1}$ g^{-1}]	SMB_C [µg g^{-1}]
Organic layer	Number of *phoN* genes [g^{-1}]	0.21***	0.10*
(LUE and BBR)	Phosphatase activity [mg Phenol (3 h)$^{-1}$ g^{-1}]	-	0.11***
Mineral soil	Number of *phoN* genes [g^{-1}]	0.40***	0.41***
(LUE and BBR)	Phosphatase activity [mg Phenol (3 h)$^{-1}$ g^{-1}]	-	0.35***
Organic layer and mineral soil	Number of *phoN* genes [g^{-1}]	0.33***	0.26***
(LUE and BBR)	Phosphatase activity [mg Phenol (3 h)$^{-1}$ g^{-1}]	-	0.41***

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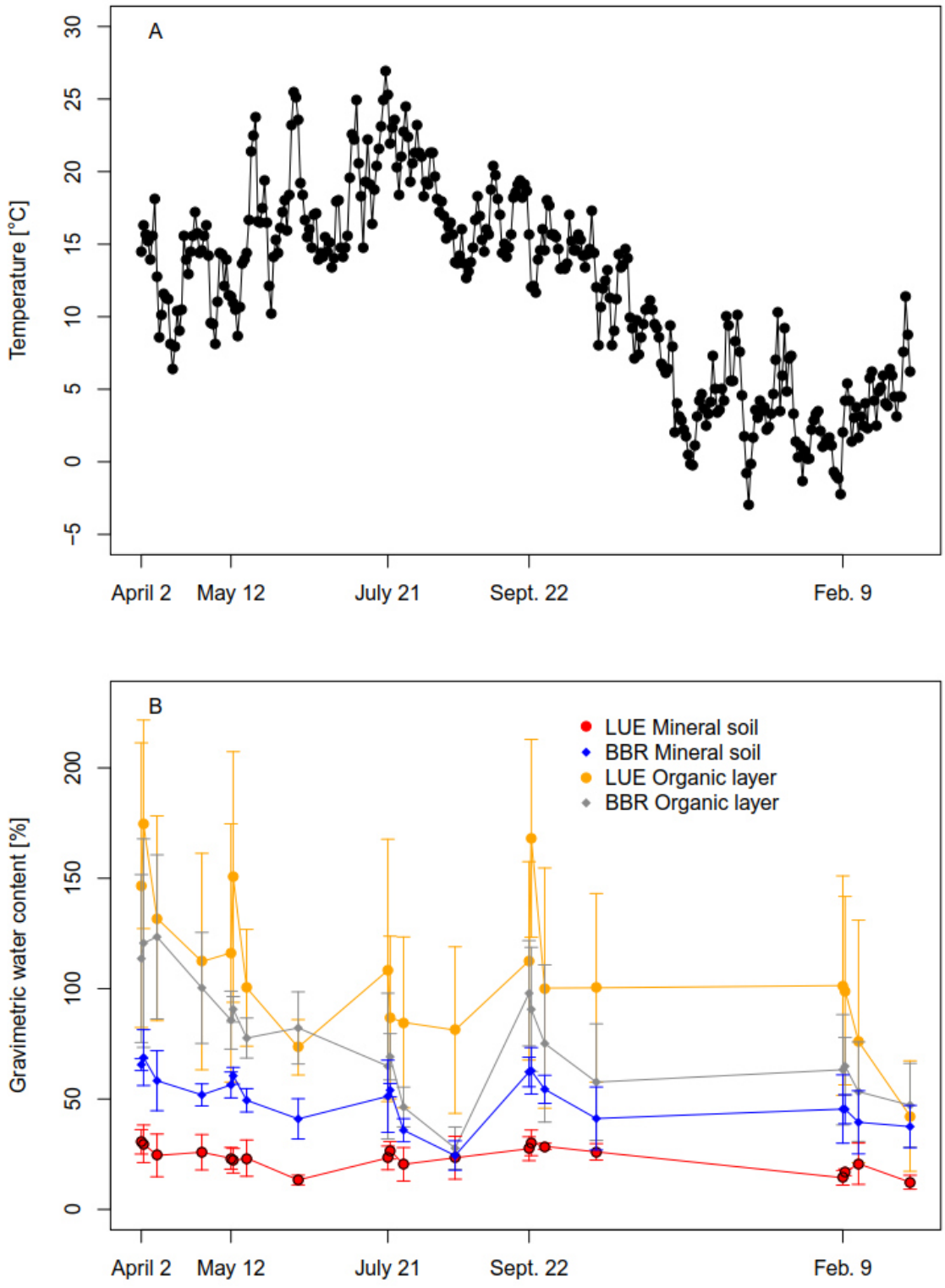
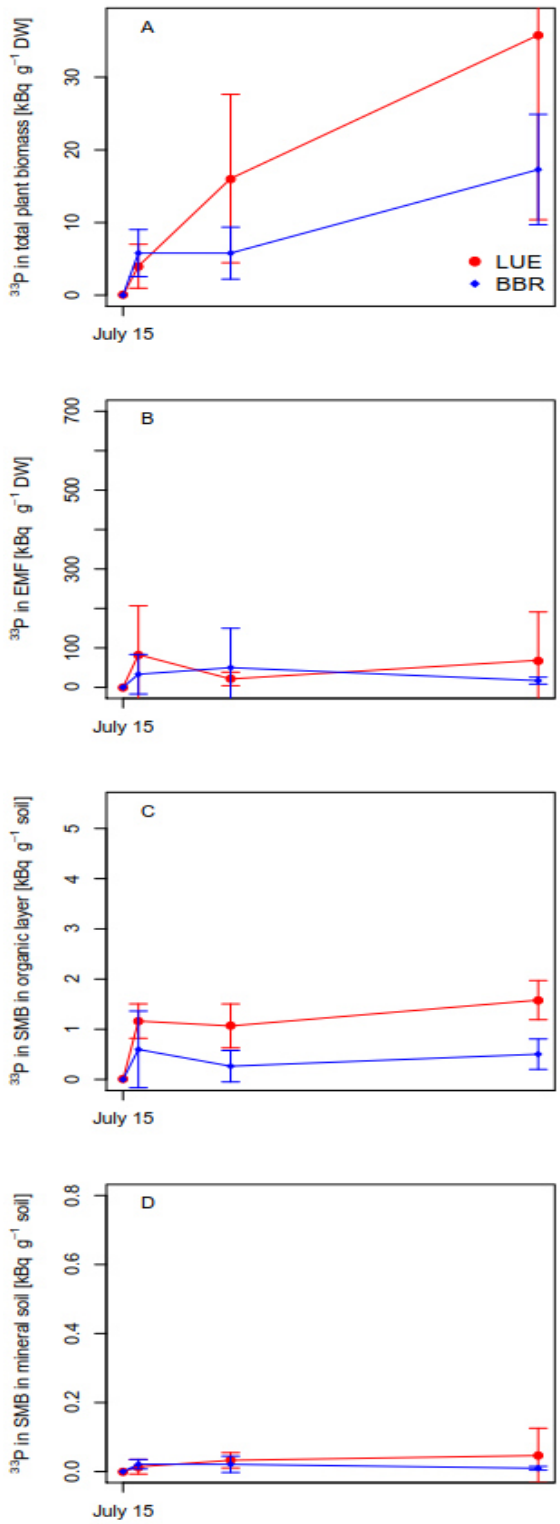
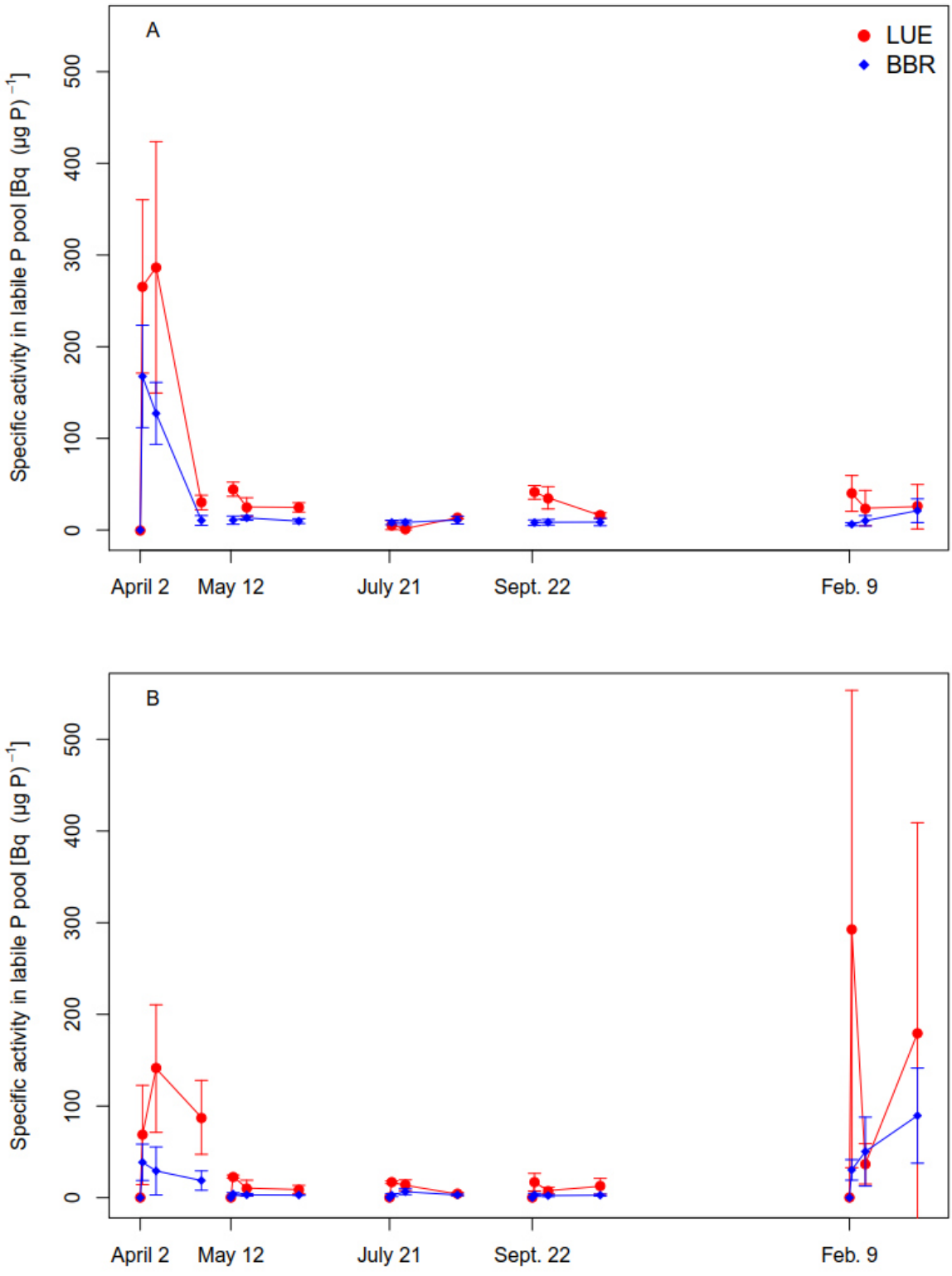


Additional online material Figure 1 Air temperature (A) and gravimetric water content of the organic layer and the mineral soil in the mesocosms from both sites (B) during five consecutive labeling experiments in 2014/2015. Each data point was calculated based on five mesocosms (n=5). Error bars show standard deviations.



Additional online material Figure 2 ^{33}P enrichment of young beech trees (plant, A), ectomycorrhizal fungi (EMF, B), and soil microbial biomass (SMB) in the organic layer (C) and SMB in the mineral soil (D) in mesocosms from the P-poor site LUE and the P-rich site BBR in the summer following the main experiment (summer 2015) over 30 days. Each data point was calculated based on five mesocosms (n=5). Error bars show standard deviations.



Additional online material Figure 3 Specific ^{33}P activity in the labile P fraction in the organic layer (A) and in the mineral soil (B) in mesocosms from the P-poor site LUE and the P-rich site BBR during all five labeling experiments. Each data point was calculated based on five mesocosms (n=5). Error bars show standard deviations.

Additional online material Table 1 Relative abundance of EMF (%). Numbers are means calculated from 15 mesocosms per season (n=15).

	BBR					LUE				
EMF species	Spring	Su_early	Su_late	Autumn	Winter	Spring	Su_early	Su_late	Autumn	Winter
*Helotiale*s sp.	3.7	10.6	0.7	5.6	3.7	0.0	2.6	5.0	0.0	0.8
Hydnum repandum	8.6	57.7	7.8	2.7	1.2	6.6	19.2	0.0	0.0	0.0
MT3	5.8	19.4	1.3	10.5	5.0	10.1	0.0	0.0	0.0	0.0
Cortinarius sp.	3.8	0.4	8.3	3.7	2.4	0.0	8.7	0.0	0.0	0.5
Cortinarius casimiri	0.8	0.5	0.0	2.9	1.2	0.3	0.0	0.0	0.0	0.0
MT8	0.6	0.0	0.6	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Cenococcum geophilum	21.5	4.8	19.2	30.5	25.7	16.7	9.3	14.0	27.3	31.4
Laccaria amethystina	0.4	0.0	1.6	0.0	0.0	0.0	0.0	5.9	3.1	0.0
Russula ochroleuca	2.2	0.0	0.0	0.0	0.0	40.3	45.2	3.4	1.5	0.4
Lactarius blennius	0.4	0.0	0.4	0.0	0.0	12.7	0.0	15.0	33.4	25.7
Cortinaius bolaris	0.2	0.0	0.0	0.1	0.1	5.9	1.8	0.1	0.0	0.1
Melanogaster sp.	0.2	0.0	0.0	0.0	0.0	7.4	0.0	0.7	0.0	0.0
Xerocomellus pruinatus	10.4	6.6	20.1	3.6	7.9	0.0	0.4	0.6	0.0	0.0
Cortinaius flexipes	0.1	0.0	0.0	0.0	0.0	0.0	2.5	0.0	0.0	1.0
MT20	0.3	0.0	0.0	0.0	0.0	0.0	10.4	1.2	0.0	1.7
Scleroderma citrinum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	46.4	2.9	2.3
Tuber sp.	10.5	0.0	25.5	11.8	6.7	0.0	0.0	0.0	0.0	0.0
Genea hispidula	3.7	0.0	6.8	3.0	3.4	0.0	0.0	0.0	1.2	1.4
MT31	5.4	0.0	7.5	0.4	6.0	0.0	0.0	0.0	0.0	0.0
MT32	1.0	0.0	0.4	3.8	1.6	0.0	0.0	0.0	3.7	0.0
Tometellopsis submollis	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.1	2.3
Hydnum ellipsosporum	0.4	0.0	0.0	1.8	0.7	0.0	0.0	0.0	0.0	3.9
Piloderma sp.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0

Tometellopsis sp.	4.5	0.0	0.0	16.9	7.5	0.0	0.0	5.9	0.0	0.0
Russula velenovskyi	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	4.6
Hydnotrya tulasnei	0.5	0.0	0.0	2.1	0.9	0.0	0.0	0.0	0.0	0.0
Pezizales sp.	0.3	0.0	0.0	0.8	0.5	0.0	0.0	0.0	0.0	0.0
MT45	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.8	4.4
Genea cf. *Anthracina*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.4	14.6
Clavulina coralloides	10.0	0.0	0.0	0.0	16.8	0.0	0.0	0.0	7.5	0.0
Melanogaster intermedius	0.4	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0
Russula grisea	2.4	0.0	0.0	0.0	4.0	0.0	0.0	0.0	0.0	0.0
Leptodontidium orchidicola	0.9	0.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0	0.0
Pachyphlodes nemoralis	1.1	0.0	0.0	0.0	1.8	0.0	0.0	0.0	0.0	0.0
Russula nigricans	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4
MT55	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.6

Additional online material Table 2 P values of the similarity analyses of the EMF assemblages. Su stands for summer.

		BBR					LUE				
		Spring	Su_early	Su_late	Autumn	Winter	Spring	Su_early	Su_late	Autumn	Winter
BBR	Spring		0.7155	0.0045	0.005	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045
	Su_early			0.0045	0.005	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045
	Su_late				1.000	0.0045	0.0045	0.0045	0.0045	0.0045	0.009
	Autumn					0.0045	0.0045	0.0045	0.0045	0.0045	0.0045
	Winter						0.0045	0.0045	0.0045	0.0045	0.0045
LUE	Spring							0.09	0.378	0.117	0.0585
	Su_early								0.0045	0.0045	0.0045
	Su_late									0.279	0.261
	Autumn										1.000
	Winter										

Additional online material Table 3 Diversity indices of ectomycorrhizal communities on young beech trees in P-poor (LUE) and P-rich (BBR) soil. For diversity analyses, the total root systems of five plants were analyzed and the data were pooled to achieve species saturation. P_{site} indicates the P value of the paired rank test (signed rank test), testing differences between sites. Su stands for summer.

		Species richness		Simpson		Shannon H		Evenness	
Season	Days after labeling	**LUE**	**BBR**	**LUE**	**BBR**	**LUE**	**BBR**	**LUE**	**BBR**
Spring	0	4	8	0.637	0.788	1.096	1.742	0.748	0.714
Spring	7	3	6	0.520	0.705	0.780	1.370	0.727	0.656
Spring	30	4	7	0.476	0.507	0.881	1.154	0.603	0.453
Su_early	0	6	4	0.672	0.444	1.338	0.762	0.635	0.536
Su_early	7	5	6	0.723	0.412	1.382	0.890	0.797	0.406
Su_early	30	6	5	0.656	0.741	1.263	1.438	0.589	0.843
Su_late	0	7	11	0.717	0.738	1.485	1.646	0.631	0.472
Su_late	7	10	10	0.587	0.752	1.355	1.701	0.388	0.548
Su_late	30	7	6	0.740	0.692	1.502	1.382	0.641	0.664
Autumn	0	3	7	0.529	0.691	0.834	1.434	0.767	0.599
Autumn	7	5	12	0.667	0.814	1.276	2.042	0.717	0.642
Autumn	30	9	11	0.788	0.814	1.765	2.004	0.649	0.674
Winter	0	13	11	0.623	0.604	1.561	1.241	0.366	0.315
Winter	7	7	9	0.598	0.702	1.236	1.494	0.492	0.495
Winter	30	10	12	0.709	0.847	1.506	2.109	0.451	0.687
P_{site}		0.023		0.164		0.065		0.379	

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Soil phosphorus supply controls P nutrition strategies of beech forest ecosystems in Central Europe

Lang, F.[1], Krüger, J.[1], Amelung, W.[2], Willbold, S.[3] , Frossard, E.[4], Bünemann, E.K.[4], Bauhus, J.[6], Nitschke, R.[6], Kandeler, E.[7], Marhan, S.[7], Schulz, S.[8], Bergkemper, F.[8], Schloter, M.[8], Luster, J.[9], Guggisberg, F.[9], Kaiser, K.[10], Mikutta, R.[10], Guggenberger, G.[11], Polle, A.[12], Pena, R.[12], Prietzel, J.[13], Rodionov, A.[2], Talkner, U.[14], Meesenburg, H.[14], von Wilpert, K.[15], Hölscher, A.[15], Dietrich, H.P.[16], Chmara, I.[17]

[1] Albert-Ludwigs-Universität Freiburg, Professur für Bodenökologie, Bertholdstraße 17, 79085 Freiburg (i Br.), Germany; [2] Universität Bonn, Institut für Nutzpflanzenwissenschaften und Ressourcenschutz, Nußallee 13, 53115 Bonn, Germany; [3] Forschungszentrum Jülich, Zentralinstitut für Engineering, Elektronik und Analytik (ZEA), Wilhelm-Johnen-Str., 52428 Jülich, Germany; [4] ETH Zurich, Institute of Agricultural Sciences, Eschikon 33, 8315 Lindau, Switzerland; [6] Albert-Ludwigs Universität Freiburg, Professur für Waldbau, Tennenbacherstr. 4, 79085 Freiburg (i. Br.), Germany; [7] Universität Hohenheim, Soil Biology, Emil-Wolff-Str. 27, 70593 Stuttgart, Germany; [8] Helmholtz Zentrum München, Research Unit for Comparative Microbiome Analyses, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany; [9] Eidg. Forschungsanstalt WSL, Waldböden und Biogeochemie, Zürcherstrasse 111, 8903 Birmensdorf, Switzerland; [10] Martin-Luther-Universität Halle-Wittenberg, Professur für Bodenkunde und Bodenschutz, Von-Seckendorff-Platz 3, 06120 Halle (Saale), Germany; [11] Leibnitz Universität Hannover, Institut für Bodenkunde, Herrenhäuser Str. 2, 30419 Hannover, Germany; [12] Georg-August-Universität Göttingen, Department of Forest Botany and Tree Physiology, Büsgenweg 2, 37077 Göttingen, Germany; [13] Technische Universität München, Lehrstuhl für Bodenkunde, Emil-Ramann-Straße 2, 85354 Freising, Germany; [14] Nordwestdeutsche Forstliche Versuchsanstalt, Grätzelstr. 2, 37079 Göttingen, Germany; [15] Forstliche Versuchs- und Forschungsanstalt Baden-Württemberg, Abteilung Boden und Umwelt, Wonnhaldestraße 4, 79100 Freiburg, Germany; [16] Bayerische Landesanstalt für Wald und Forstwirtschaft, Abteilung Boden und Klima, Hans-Carl-von-Carlowitz-Platz 1, 85354 Freising, Germany; [17] Thüringen Forst, Forstlichen Forschungs- und Kompetenzzentrum Gotha, Jägerstraße 1, 99867 Gotha, Germany

Correspondig author: Prof. Dr. Friederike Lang
phone: +49-(0)761-203-3625
fax: +49-(0)761-2033618
email: fritzi.lang@bodenkunde.uni-freiburg.de

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Abstract

Phosphorus availability is assumed to shape plant–microorganism–soil interactions in forest ecosystems. The aim of our study was to provide quantitative information regarding the link between soil P availability and P nutrition strategies of European beech (*Fagus sylvatica*) forests. The study was motivated by the conceptual model that plant and microbial communities of P-rich forest ecosystems carry over mineral-bound P into the biogeochemical P cycle (acquiring strategy). In contrast, P-poor ecosystems establish tight P cycles to sustain their P demand (recycling strategy). We tested if this conceptual model on supply-controlled P-nutrition strategies is consistent with data obtained from five European beech forest ecosystems with soils derived from different parent materials (geosequence), and thus, different total P stocks (160–900 g P m^{-2}; down to 1 m soil depth). The analyses addressed a wide range of soil chemical (wet chemistry, ^{31}P-NMR, isotopic exchange kinetics) and biological properties (stand characteristics, tissue P concentrations in leaves, litterfall, fine-roots and microbial biomass, mycorrhization, phosphatase activities). Based on evidence from the literature, we developed indicators for P acquisition and recycling, which were related to the intensity of P mobilization from the mineral and the organic soil pool, respectively. In general, our data are in agreement with the assumption of supply-controlled P nutrition strategies of beech forest ecosystems: We found that especially P-rich beech ecosystems accumulated P in topsoil horizons in moderately labile forms. The turnover rate of the forest floor decreased with decreasing P stocks (1/5 per year down to 1/40 per year) while C:P_{org} ratios increased from 110 to 984 (A horizons). High proportions of fine-root biomass in forest floors seemed to favor tight P recycling. Phosphorus in fine-root biomass increased relative to P in microbial biomass with decreasing P stocks. In line with this, the ratio of phosphomonoesterase to phosphodiesterase activity increased, which might explain the increasing proportion of diester-P remaining. The indicator values obtained for P acquisition and recycling changed continuously along the P gradient, implying continuous adjustment of plant–microorganism–soil feedbacks to the P status of soils in beech forest ecosystems.

Key words: forest ecosystem nutrition, P geosequence, P chronosequence, P acquiring, P recycling

1 Introduction

Results obtained from studies along chronosequences showed that aboveground and belowground P pools of ecosystems reflect the current state of interactions between P availability of soils and the quality and quantity of P input from plants and microbes occurring at these sites (Pearson and Vitousek 2002, Selmants and Hart 2010, Turner and Condron 2013, Galván-Tejada et al. 2014, Wu et al. 2014). These studies suggest that the P contents of the parent material influence the development of ecosystems, especially the soil compartment, based on different P-related plant–microorganism–soil interactions. According to Odum's hypothesis on the nutrition strategies of

vegetation, P cycles tend to "tighten" during succession (Odum 1969): Young ecosystems are characterized by open P cycles, whereas mature ecosystems establish closed P cycles. The rate of changes in P pools with succession may depend on the parent material of the soils (Laliberté et al. 2013). Recently, the role of the parent material in the P nutrition of terrestrial ecosystems has gained more and more attention (Augusto et al. 2017). Based on their analyses of the P contents of different types of parent material, Porder et al. suggested, quantifying links between the P content of the parent material and ecosystem P dynamics. Owing to our limited understanding of the underlying processes, it is currently not possible to predict, if P nutrition strategies of late successional forest ecosystems are controlled by the P supply from parent material. .

The recent decline in P nutrition of European beech (*Fagus sylvatica*) documented in several publications (e.g. Talkner et al. 2015) has intensified the scientific debate about possible underlying mechanisms (Jonard et al. 2015). So far no consensus has been reached and many open questions remain regarding the P nutrition of beech forests. Natural *F. sylvatica* forests cover a wide range of soils, which mainly formed since the last ice age and developed from various parent materials (Peters 1997). The prevalence of *F. sylvatica* ecosystems in Central Europe enables exploring the P nutrition strategies of plant and microbial communities with different P supply, yet similar state of forest development. This provides an opportunity to test the conceptual model, that P stocks in parent material control P nutrition of mature forest ecosystems (Lang et al. 2016). According to this model, plant and microbial communities follow a P acquiring strategy at P-rich sites, transferring P from soil minerals into the biogeochemical P cycle. In contrast, tight P cycling is expected at sites poor in P. That means plants and microbes use P from organic sources and minimize P losses from the biogeochemical cycle. The aim of our study was to test if this conceptual model on supply-controlled P-nutrition is consistent with analytical data obtained from beech forest ecosystems. Additionally, we addressed the question *how* P acquisition and recycling changes with changing P supply. First we analysed a variety of P-related ecosystem properties of beech forest ecosystems at sites with different parent material, and thus covering a wide range of different P stocks. Second, we quantified indicator values for the proposed P nutrition strategies and related them to the P soil stocks.

To the best of our knowledge, the study design as well as the diversity of analyses applied is unprecedented: We studied five forest ecosystems dominated by the same tree species but differing in P stocks of soils by a factor of six. In contrast to the concept of chronosequences, the sites represent a geosequence with different P contents in the parent material and are spatially independent. The chemical analyses covered a wide range of P forms, included soil stocks at high depth resolution down to 1 m, as well as P mobilization kinetics. These analyses were combined with

analyses of microbiological and root characteristics as well as P concentrations beech leaves and permitted the solid analysis of soil–plant–microorganism interactions along the P geosequence.

2 Material and Methods

Study sites

We selected five study sites differing in their parent material and supporting 120–140 year old beech forests (Table 1). Methods used for analyzing stand characteristics are summarized in supplementary S1. The parent material ranged from basaltic rock rich in P (Porder and Ramachandran 2013) to P-poor sandy till (Table 1). All sites were in periglacial zones during the last glaciation in central Europe (Fiebig et al. 2011, Geyer and Gwinner 1986, Ergenzinger 1967). Thus, the development of the present soil profiles started at the end of the last ice-age, 10–12,000 years ago (Eberle and Allgaier 2010). The study sites belong as Level II Intensive Monitoring plots to the Pan-European International Co-operative Program on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests) under UNECE (Lorenz 1995, Vries et al. 2003) and soil and tree properties have been monitored for the last 15–25 years. Four sites (Bad Brückenau, BBR; Mitterfels, MIT; Vessertal, VES; Conventwald, CON) are located at intermediate elevation in the German central and southern uplands; the P-poorest site Lüss (LUE) is located in the North German lowlands (Table 1).

Sampling design

The characterization of forest stands, vegetation, and litterfall was performed according to the ICP Forest manual (ICP Forests 2010, http://icp-forests.net/page/icp-forests-manual) at a monitoring plot with an area of 0.25 ha representing the forest stand. The analyses of microbial biomass were performed with samples of soil cores derived from five sampling points distributed within the buffer zone around the monitoring plot (see below). For the other soil analyses, we used soil samples derived from volume based sampling of a complete soil profile performed at an area of 0.25 – 0.56 m^2, down to 90 – 100 cm below the mineral soil surface. Due to the stony forest soils characterizing the study sites, we decided to use the “quantitative soil pit” (QP) approach developed by Hamburg (1984) and adjusted recently by Vadeboncoeur et al. (2012) to quantify C stocks in stony forest soils. The method provides volume-based samples of fine earth material and other constituents, such as roots and stones, of a soil pit. By analyzing a soil volume with a large cross section representing a large portion of the rooting space of an adult tree, QP sampling allows to obtain a more coherent picture of the system than would otherwise be possible by analyzing several small soil volumes.

The QPs were established in the buffer zone of the monitoring plots within the Level II sites. Only one QP per study site could be established. The exact position was determined randomly but had to meet

the following criteria: (1) thickness of forest floor and distribution of mineral soil horizons representative for the forest stand (identified based on auger screening of the monitoring site), (2) a minimum distance of at least 3 m to the next trees (diameter at breast height (DBH) > 10 cm), and (3) not covered by understory or downed deadwood.

Soil sampling and fractionation

For quantitative pit establishment, a square wooden frame with an interior lateral length of 50 cm (75 cm at site BBR) was prepared and registered optical targets for photogrammetric analysis were fixed to the upper side of the frame. The frame was fixed as reference plane to the soil surface using steel pins. Afterwards the whole organic layer was cut off alongside the inner edge of the frame with a knife. The individual humus layers (Oi, Oe, Oa) were sampled separately by hand. Roots crossing different layers were cut off at the layer boundary and removed to prevent mixing of material from different layers. The mineral soil sampling followed layers representing single diagnostic horizons. If the thickness of a diagnostic horizon exceeded 5 cm for A or E horizons and 10 cm for B horizons, a new sample layer was started. The procedure was repeated until a maximum sampling depth of 1 m was reached. Following this procedure, we obtained 10 (BBR) to 15 (MIT and VES) different depth layers per site. Detailed information on soil sampling depths at the different sites is given in the supplementary material (S2). All soil material (including rocks and roots) was placed in containers, brought to the laboratory, and air-dried (40°C). Then, the soil samples were manually separated into the following fractions: fine earth (<2 mm), gravel (2–20 mm), stones (>20 mm), coarse roots (>2 mm), fine roots (<2 mm), and other soil constituents (e.g., wood or seedlings). All fractions were weighed and stored dry, cold, and in the dark for further analysis. Unless otherwise noted, dried fine earth material was used for soil analyses. The volume quantification of the different soil layers was carried out based on photogrammetry (Haas et al. 2016).

For microbiological analyses, five contiguous soil cores (circular distance 2–3m) were taken and the Oe and Oa horizons removed before samples were pooled together and sieved to 2 mm. This procedure was conducted five times to receive five pooled soil samples per site. Soil samples were stored at 4°C prior to analysis.

The analyses of the bulk samples from the quantitative pits were conducted in duplicate (soil chemical analyses) or triplicate (analyses of enzyme activity) to account for analytical variability. The mean coefficients of variation of the analyzed soil samples were below 10% and indicate good reproducibility of applied methods. We do not have exact information regarding the spatial heterogeneity of all the analyzed properties across study sites. However, there are clear indications

that the results obtained from QP sampling represent the study site properties: (1) QP location was determined based on a soil survey of the study area (see description above). (2) In frame of a geostatistical analysis, we determined citrate extractable P concentrations, C/N values and pH values of the forest floor and three soil depth increments at 48 sampling points within 50 × 50 m areas at four of the study sites (LUE, CON, MIT, BBR). The results for the pit samples were within the 68% confidence range of the grid data for the analyzed soil properties and soil horizons. (3) We analyzed 10 – 15 depth intervals at the different sites and outliers would have been identified based on extraordinary discontinuities of analyzed soil properties along the depth gradient. (4) Total P concentrations are assumed to be controlled by the P content of the parent soil material (Turner and Engelbrecht 2011), which is homogeneous within the study sites. Furthermore, published information on the heterogeneity of P concentrations in soil within an area of uniform morphology and geology pointed to only small variation with coefficients of variation smaller than 10% (e.g., Turner et al. 2012, Chen et al. 2015).

Basic soil chemical characterization

Total contents of soil C and N were measured in ground samples dried at 105°C using an elemental analyzer (Vario EL cube, Elementar, Germany). Soil pH of air-dried samples (40°C) was determined in deionized water and in 1 M KCl at a soil-to-solution ratio of 1:2.5 (w:v).

Determination of the cation exchange capacity (CEC) and exchangeable cations was carried out using ammonium acetate at pH 7 and KCl (Hendershot et al. 2008). Concentrations of extracted Ca, Mg, K, and Na were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Ultima 2, Horiba Jobin-Yvon S.A.S., Longjumeau, France); ammonium in KCl extracts was determined using an automated photometer (SANplus, Skalar Analytical B.V., Breda, The Netherlands). The difference between the CEC and the sum of Ca, Mg, K, and Na is an estimate of H^+ and Al^{3+} occupation of the CEC.

The hot dithionite–citrate–bicarbonate extraction of Fe (Fe_{DCB}), as outlined by Mehra and Jackson (1960), was used to estimate total pedogenic Fe oxide phases. Extraction with NH_4 oxalate at pH 3.0 and 2 h shaking in the dark (Schwertmann 1964) was carried out to estimate Al and Fe in short range-ordered forms and organic complexes (Al_{ox} and Fe_{ox}). The concentrations of extracted Al and Fe were determined by ICP-OES.

Soil phosphorus analyses

Total P, citrate extractable P and organic P

Contents of total soil phosphorus were determined on ground samples dried at 105°C after microwave-digestion with 42% HF and H_2O_2 (both: Suprapur©, Merck Millipore, Germany) using ICP-OES (CIROS CDD, Side-On plasma, Spectro, Germany). Organic acid extraction for the quantification of plant-available P in soils has been recommended to simulate organic acid secretion by plant roots (Gerke and Hermann 1992). We analyzed plant-available P by extraction of sieved subsamples with 1% citric acid (50 mM citrate), similar as described by Hayes et al. (2000), at a soil-to-solution ratio of 1:10 (w:v), and with subsequent determination of orthophosphate-P using the ascorbic acid method of Murphy and Riley (1962) as modified by John (1970), and of total extractable P by ICP-OES. Organic P in soil samples was analyzed using the ignition method of Saunders and Williams (1955). Each sample was extracted with or without preceding ignition at 550°C by 0.5 M H_2SO_4 and the fraction of organic P was quantified as difference of extracted orthophosphate quantified using the malachite green colorimetric method (Ohno and Zibilske 1991). We used P*org* obtained by the method of Saunders and Williams for calculating the C:P*org* ratios of soil organic matter. Uncertainties related to this approach are outlined in supplementary material S3.

Hedley fractionation

Soil samples were analyzed by sequential extraction according to Hedley and Stewart (1982) as modified by Tiessen and Moir (2008). We used 24 samples in one batch consisting of 23 individual samples including six replicate samples (as random quality check) and one in-house soil standard for quality testing. Each 0.5 g soil was extracted with solutions of increasing extraction strength, starting with distilled water containing an anion exchange resin (Dowex 18, 20–50 mesh, Sigma-Aldrich, Taufkirchen, Germany), followed by 0.5 M $NaHCO_3$; 0.1 M NaOH; 1 M HCl; HCl (conc.) and a final acid digestion with 65% HNO_3 and 30% H_2O_2. Orthophosphate-P concentrations of the different extractants were determined photometrically (Murphy and Riley 1962). We combined the different fractions according their mobility and speciation (organic vs. inorganic P) to describe the following P pools:

Sorbed inorganic P: inorganic P extractable by resin, $NaHCO_3$, and NaOH and *sorbed organic P*: organic P extractable by $NaHCO_3$ and NaOH, *Ca-phosphates*: the P fraction mobilized by 1 M HCl; *stable P*: sum of the P fractions dissolved by HCl (conc) or acid digestion.

Solution-state P-31 nuclear magnetic resonance (NMR) spectroscopy

For solution-state ^{31}P-NMR analyses, three soil horizons (Ah horizons, as well as two B horizons from about 30 and 90 cm soil depth) were extracted using 0.25 M NaOH plus 0.05 M Na_2EDTA (1:1/v/v) as described by Cade-Menun (2005). Samples were thereafter centrifuged (1500 g, 20 min), and the remaining supernatant was then split into two halves. One half was lyophilized directly (Thermo Freeze Dryer, Heto PowerDry PL6000). The second half of the supernatant was dialyzed (molecular weight cut off, MWCO, was 14,000; thickness 0.041 mm; Visking, Cellulose, Roth, (Sumann et al. 1998, Amelung et al. 2001). To prepare the samples for NMR spectroscopy, the freeze-dried extracts were resolved by 1 ml *aqua* dest., 0.5 ml of D_2O, and 10 M NaOH to increase and to standardize the pH for optimal peak separation (Crouse et al. 2000). Samples were centrifuged (1500 g, 20 min) und decanted into NMR tubes.

Spectra were recorded on a NMR spectrometer (Inova 400, Varian, USA) with power-gated proton decoupling at a temperature of 295 K. An acquisition time of 0.7 s, a 30° pulse, and 0.5 s of relaxation delay were used. Chemical shifts of signals were measured in parts per million (ppm) relative to 85% orthophosphoric acid. Approximately 24,576 scans were acquired for each sample. Spectra for the soil samples were recorded with a line broadening of 3.0 Hz. Terminology and interpretations of the spectra followed Cade-Menun (2005, 2015), Bol et al. (2006), Vincent et al. (2013). The chemical shift of the spectra was analyzed as described by Turner (2004). Further details and a discussion of uncertainties are provided in the supplementary material (S4).

Isotopic exchange kinetic

Briefly, a given amount of $H_3{}^{33}PO_4$ was added to a soil water suspension pre-equilibrated (steady-state conditions for P) and the decrease of radioactivity remaining in the solution was measured over time. At the end of the experiment (after 80 minutes for all samples except for LUE, AE, in which the experiments lasted 420 minutes because of the very low rate of isotopic exchange), the concentration of orthophosphate in the solution (C_P) was measured after centrifugation for 10 min at 10 000 *g* and filtration at 0.2 µm using the malachite green colorimetric method (Ohno and Zibilske 1991). Further details on the isotopic exchange kinetic (IEK) experiment are given elsewhere (Fardeau 1993, 1996, Randriamanantsoa et al. 2013). The decrease with time of the ^{33}P added in solution can be described by the following equation (Fardeau 1993):

$$\frac{r_t}{R} = m \times \left(t + m^{\frac{1}{n}}\right)^{-n} + \frac{r_\infty}{R} \qquad \text{(eq. 1)}$$

where r_t and r_∞ (MBq) are the radioactivity remaining in solution after *t* minutes of exchange and after an infinite time of exchange, respectively; *R* (MBq) is the initially added radioactivity; *t* is the time (in minutes) elapsed after the radioactivity addition and *m* and *n* are soil specific parameters

calculated from a non-linear regression between r_t/R and t. The r_∞/R value is estimated as the ratio of the water soluble P expressed in mg P kg^{-1} to the total inorganic P, which itself was estimated as the difference between the total P content of the soil samples minus the amount of organic P determined according to Saunders and Williams (1955). The amount of water soluble P is calculated as 10 x C_P with C_P expressed in mg P L^{-1} and 10 being the solution to soil ratio (100 ml:10 g).

As described by Fardeau (1993) the amount of soil isotopically exchangeable P (E_t, expressed in mg P kg^{-1} soil) is calculated based on equation 2:

$$E_t = 10 \times C_p \times \left(\frac{R}{r_t}\right) \qquad \text{(eq. 2)}$$

The following variables were calculated for selected samples (Fardeau 1993): *m, n,* C_P, and the amounts of P isotopically exchangeable within 1 minute (E_{1min}, mg P kg^{-1} soil), between 1 minute and 1 day ($E_{1min\text{-}1day}$), between 1 day and 3 months ($E_{1day\text{-}3months}$) and the amount of P that cannot be exchanged within 3 months ($E_{>3months}$) by taking the difference between total inorganic P and $E_{3months}$. The samples were analyzed in duplicate and the average values are shown here.

Fine roots

Fine-root biomass was quantified as described above. Fine roots (<2mm diameter) were weighed and used for mycorrhizal analyses. All root tips of each weighed sample were inspected under a dissecting microscope (M205 FA; Leica, Wetzlar, Germany) and classified as vital non-mycorrhizal, vital mycorrhizal or dead. The percentage of mycorrhizal colonization was calculated as (n vital mycorrhizal root tips/n vital root tips) * 100. Vital root tips were distinguished from dead and dry root tips according to the method used by Winkler et al. (2010). Root tip vitality (%) was calculated (n vital root tips/n total root tips) * 100. For the determination of P concentration in fine roots, fine root material was washed, ground and digested by HNO_3 (conc.). Phosphorus concentrations were analyzed by ICP-OES (CIROS CDD, Side-On plasma, Spectro, Germany).

Microorganisms

Quantification of microbial biomass

Soil samples (A horizon) were extracted according to Brankatschk et al. (2011) for estimation of microbial biomass C (C_{mic}), N (N_{mic}), and P (P_{mic}). Measurements of C_{mic} and N_{mic} were performed using the chloroform fumigation-extraction (CFE) method according to Vance et al. (1987), Joergensen (1996) (k_{EC} 0.45) and Joergensen and Mueller (1996) (k_{EN} 0.54). For determination of microbial biomass P the CFE-method modified from Brookes et al. (1982) (k_{EP} 0.4) was applied. Since C_{mic}, N_{mic} and P_{mic} were intended to be compared from the same extract, 0.01 M calcium chloride was used instead of 0.5 M sodium hydrogen carbonate for inorganic-P extraction. Orthophosphate was

quantified as molybdenum blue using commercial tube tests “NANOCOLOR ortho- and total-Phosphate 1” (Macherey-Nagel, Germany).

Determination of acid phosphomonoesterase and phosphodiesterase activities

Acid phosphomonoesterase (EC 3.1.3.2) activity was determined using a modified disodium phyenylphosphate method. Briefly, each soil sample (field-moist samples, stored at –20°C and sieved) was split into three subsamples and two controls of 1 g each. Soil suspensions were prepared with 10 ml acetate buffer (pH 5) and 5 ml of 20 mM disodium phenylphosphate (EC 3279-54-7) as substrate solution; in controls, substrate solution was replaced by deionized water. All soil suspensions were incubated at 37°C and continuous shaking (100 rpm) for 3 hours. The release of phenol was determined colorimetrically at 614 nm (ELx808, Absorbance Microplate Reader, BioTek Instruments Inc., Winooski, VT, USA), using 2,6-dibromchinone-chlorimide (EC 202-937-2) as coloring reagent (Hoffmann 1968, modified by Öhlinger 1996).

The activity of phosphodiesterase (EC 3.1.4.1) was measured using bis(p-nitrophenyl) phosphate (EC 223-739-2) as substrate and ris(hydroximethyl)aminomethane as the p-nitrophenol color reagent, according to a modified procedure of Margesin (1996). Each fresh soil sample was split into three subsamples and two controls of each 1 g. Soil suspensions were prepared with 4 ml of 0.05 M Tris(THAM) buffer (pH 8.0) and 1 ml of 5 mM substrate solution; in controls, substrate solution is replaced by deionized water. Soil suspensions were incubated at 37°C for 1 hour at continuous shaking (100 rpm). After incubation, 1 ml 0.5 M NaCl solution and 4 ml 0.1 M Tris(THAM) buffer (pH 12.0) were added to each subsample, whereas the controls received additionally 1 ml of the substrate solution. Soil suspensions were filtered and pipetted into 96-well microplate (PS F transparent 96 well; Greiner Bio-one GmbH, Frickenhausen, Germany). The enzyme activity was measured photometrically at 405 nm on a microplate reader (ELx808, Absorbance Microplate Reader, BioTek Instruments Inc., Winooski, VT, USA).

Leaves and litterfall

P concentrations of beech leaves

Current year beech leaves were sampled in July/August from 5 trees (9 at LUE). South-facing branches from the upper third of the crown were selected for leaf sampling (Rautio et al. 2010). Leaf material was washed, ground and digested by HNO_3 (conc.). Phosphorus concentrations were analyzed by ICP-OES. Mean values for leaves from 3–5 years of sampling (between 1995 and 2013, integrating mast years and non-mast years) are presented.

Litterfall

Litterfall at the study sites except VES, where no litterfall sampling took place, was determined according to Pitman et al. (2010) based on 10 (12 at LUE) litter collectors distributed among the monitoring plot. Each litter collector had an area of 0.25 m², litterfall was collected monthly and in periods of strong litterfall even bi-weekly. Values presented here describe the sampling period 2013. For litter weight documentation the total mass of litter was determined. P concentrations were only determined for the leaf component of litter fall.

Indicators for P acquisition and P recycling

The most direct approach for testing the hypothesis that soil P stocks control the P nutrition strategy of beech forest ecosystems would be the analysis of P fluxes within the soil and from soil to plants and microbes. Some of the relevant P fluxes are hard to quantify (Bol et al. 2016) and not yet available at the study sites. However, the different P nutrition strategies should have changed the studied P characteristics of the ecosystems as well as other ecosystem properties. Based on current knowledge and theoretical assumptions we used indicators to assess P nutrition strategies. We defined three indicators for P acquisition and four indicators for P recycling and analyzed how indicator values were related to the soil P stocks at the study sites. The indicators used are listed and their justifications as well as the way the indicator values were calculated are provided in Table 2. In general, indicators representative of acquiring systems address P mobilization from the mineral soil, whereas indicators representative of recycling systems refer to P associated with soil organic matter. To enable the comparison of the different indicators along the geosequence, we normalized all the different indicator values according to equation 3:

$$N_{ai} = \frac{I_{ai}}{I_{am}} \qquad \text{(eq. 3)}$$

Where N represents the normalized indicator value, the index a represents the indicator addressed, the index i represents the study site, I represents the indicator value, and the index m represents the P-richest site (= BBR) for acquisition indicators and the P-poorest site (=LUE) for recycling indicators.

3 Results

3.1 Analyses of the soils' solid phase

Basic soil characteristics along the geosequence

The soils of all study sites were acidic (Table 3, supplementary S2) and showed an intermediate to high OC content. Soil textures were mostly loam, apart from the P-poorest site LUE with a loamy sand texture. The plant availability of the major nutrients as indicated by C/N ratio or exchangeable cations at the different sites differed strongly. Total N contents of the sites were significantly related

to P contents. Yet, the difference in P-related soil properties was larger than that of all the other properties (Table 3).

The P range covered by the geosequence

Total P stocks down to 1m depth of the mineral soil of the geosequence differed by a factor of 5.6 between the poorest site LUE and the richest site BBR (supplementary material S5). The P concentrations in topsoil horizons even differed by a factor of 27 among sites (supplementary material, S5). Nearly all extraction methods confirmed the P gradient for the first meter of the soil profile: BBR > MIT ≥ VES > CON > LUE. The P status of the fine earth of VES and MIT was similar, whereas coarse stony fragments of subsoil material from the site VES showed much higher values than those of the site MIT (supplementary material S5). Depending on the extraction method, soil depth, and whether soil concentrations or stocks were considered, the relative order varied for VES and MIT.

The depth distribution of total P along the geosequence

We observed decreasing concentrations of total P with increasing soil depth at all sites and the decrease was more pronounced at P-rich than at P-poor sites (Fig. 1). While the soil profiles at MIT and LUE indicate vertical translocation of Al and Fe, no such re-location of P during podsolization could be observed (S6). Soil P concentrations in the forest floor horizons covered a much smaller range than the mineral soil horizons (e.g., Oe horizons: 847–1570 mg kg^{-1}; A horizons: 196–3265 mg kg^{-1}).

Hedley P fractionation

Phosphorus concentrations of all the Hedley fractions analyzed decreased along the P geosequence with decreasing total soil P stocks (supplementary material S7). Most pronounced was the increase in P in the $HCl_{(conc)}$ and residual P fractions with decreasing soil P stocks (supplementary material S7). These two fractions contributed up to 20% of the total P at the P-richer sites BBR, MIT and VES. In contrast, at the P-poor sites they accounted for more than 35% in topsoil horizons and up to 60% of total P in subsoil horizons. The stock-based P fractionation at the different sites also highlighted the increasing relevance of these stable P fractions with decreasing total P stock (Fig. 2). In the subsoil horizons, organic Hedley fractions seemed to be of more relevance at sites BBR and MIT compared to the P-poor sites CON and LUE (supplementary material S7). The sum of both organic Hedley fractions corresponded well with the results obtained by the method of Saunders and Williams (1955, see below).

Soil organic P and C:P_{org} *ratios*

With decreasing P supply by the mineral soil, the portion of P located in the forest floor increased, yet it was generally rather small (0.4 to 9.2% of total P stock; supplementary material S5). In addition, the mass of forest floor material tended to increase along the P geosequence and its turnover rate decreased continuously (Table 7). A rather large portion of forest floor P was present in inorganic form (15–52%; Table 4). Amount and speciation of P in the Oi layer varied strongly between the different sites, yet no relation with the P concentration of the soils or the living beech leaves was observed. In general, the percentage of organic P of the mineral soil samples decreased with increasing soil depth and decreasing C_{org} concentration. While there was no clear trend in the percentage of P_{org} along the P geosequence, the C:P_{org} ratio of the mineral soil strongly increased with decreasing P status, which was most clearly pronounced for the A horizons. For the sites BBR, MIT, VES and CON, the C:P_{org} ratio declined with increasing soil depth, while at the site LUE the C:P_{org} ratio increased from the Oe to the AE horizon and decreased only thereafter.

NMR data

The most prominent P species were diester- and monoester-P forms (Table 5). The NMR spectra, however, also revealed that the samples contained up to 15% orthophosphate, which was not expected because sample dialyses preceded NMR analyses of the samples. In addition, the samples contained traces of polyphosphates (1%, detected in surface soils only) and pyrophosphates (6%). Unusually large portions of phosphonates were detected, which reached 22% of total signal intensity at LUE (Table 5). With increasing soil depth, the portions of monoester-P increased while those of phosphonates and orthophosphate decreased. All other P forms did not show consistent changes with soil depth but P recovery decreased with increasing soil depth (Table 5).

The spectra of topsoil samples showed increasing proportions of diester-P with decreasing P supply. The increase in diester-P was accompanied by an increase in phosphonates, while the proportions of monoester-P declined correspondingly. The ratio of diester-P/monoester-P increased in the same direction: BBR < MIT = VES < CON < LUE (Table 5).

Phosphorus isotopic exchange kinetics

The concentrations of water-extractable inorganic P (C_P) ranged between 1.3 and 0.01 mg P L^{-1} in AE LUE and BA CON, respectively (Table 6). In all soils, C_P values decreased with increasing depth. The parameter m followed the same trends as C_P. The parameter n was between 0.4 and 0.8 in all samples except in LUE where values were much lower. The amount of P isotopically exchangeable between 1 minute and 3 months ($E_{1min\text{-}3months}$) in the topsoil horizon followed the order BBR > MIT >

CON > VES > LUE, with very little P found in this pool in LUE. In contrast, the amount of P isotopically exchangeable within 1 minute of the AE horizon of LUE was nearly as high as that determined in the Ah1 horizons of VES and MIT.

3.2 Trees and Soil Microbes

Leaves and litter fall

Despite the strong differences in P stocks and availability, above-ground woody biomass was similar among the sites The P concentrations of beech leaves decreased only slightly with decreasing P stocks in soils (Table 7). Leaf litter P was slightly higher at BBR and MIT compared to CON and LUE (Table 7). The decrease in leaf litter P was much steeper than the decrease in the P concentration of living leaves (Table 7) indicating an increasing P re-sorption efficiency with decreasing P stocks

Fine roots

The total fine-root biomass per area slightly increased along the geosequence (BBR 815 mg m^{-2}, MIT 1160 mg m^{-2}, VES 1098 mg m^{-2}, CON 923 mg m^{-2}, LUE 1668 mg m^{-2}). P concentrations in fine roots were lower than in leaves (Table 7). Especially at the P-poor sites CON and LUE, root P-concentrations in mineral soil horizons were much lower than those of fine roots in the forest floor (Table 7). In addition, root biomass was increasingly concentrated in the forest floor and topsoil horizons with decreasing soil P stocks. Only 20% of the total fine-root biomass within the first meter of the soil profile was located in the forest floor and A horizon at the P-rich site BBR; the share increased to nearly 70% at the P-poor site LUE (Fig. 4). The degree of mycorrhization of the vital root tips was 100% across all sites and soil horizons analyzed. Root vitality was similar along the study sites of the geosequence, but showed decreasing vitality with increasing soil depth, starting at 60–70% in the forest floor and topsoil horizons and decreasing to about 30–40% in the subsoil horizons, respectively.

Microbial biomass

Highest microbial biomass P (P_{mic}) in the topsoil was detected at site MIT (111 µg g^{-1}), followed by CON (104 µg g^{-1}), BBR (100 µg g^{-1}) and VES (79 µg g^{-1}) while LUE showed the lowest values (10 µg g^{-1}). Regarding C_{mic} and N_{mic} a different order was observed. While LUE was again characterized by consistently low values, C_{mic} and N_{mic} reached maxima at the sites CON and BBR (Table 8). The mass ratios of microbial C and P (C_{mic}:P_{mic}) decreased from twenty at LUE to seven at MIT (Table 8). The latter soil likewise showed the lowest ratios of N_{mic}:P_{mic} (0.7) and C_{mic}:N_{mic} (10) while highest values were detected at CON (1.3) and LUE (16). In summary N_{mic}:P_{mic} and C_{mic}:P_{mic} ratios did not strictly follow the P supply of the soils, yet the P-richest site of the geosequence (BBR) had larger N_{mic}:P_{mic}

and C_{mic}:P_{mic} ratios in the Ah horizon compared to the P-poorer sites MIT and VES. At all sites, the C:P ratios of microorganisms were much narrower than the C:P ratio of soil organic matter. These imbalances increased with decreasing P supply by the soil (Table 8).

Enzyme activities involved in P mineralization

Phosphomonoesterase activity (supplementary S8) was much higher in the forest floor material than in the mineral soil samples of all horizons. This was also true for phosphodiesterase activity (S8). Yet, phosphomonoesterase activities decreased more strongly from forest floor to the mineral soil than phosphodiesterase activities resulting in smaller monoesterase:diesterase ratios in the mineral soil compared to the forest floor, except for LUE (Fig. 3). Phosphomonoesterase activities of soil samples from different soil horizons were closely related to fine-root biomass, yet this relationship was characterized by site-specific slopes (data not shown). In contrast phosphodiesterase activities were related to microbial biomass (r^2=0.81; $p < 0.05$).

4 Discussion

4.1 Solid phase properties

The range of soil properties covered by the P geosequence

The study sites differed clearly regarding the total P stocks of the mineral soils. The range in P stocks and concentrations in the soils (fine-earth fractions) represents the upper end of P ranges covered by published chronosequence studies (Table 3, supplementary material S5). The P supply by the soil fine-earth material roughly mirrored the P level of the parent material as described recently (Porder and Ramachandran 2013). The P contents of the parent materials as well as pedogenic processes seem to control the total P stocks of the soils at the different study sites. Based on the total P stocks of the fine-earth material down to 1 m, the study site along the geosequence can be grouped into sites with decreasing P supply following the order BBR > MIT ≥ VES > CON > LUE.

Phosphorus associated to Al and Fe oxy(hydr)oxides

Iron and Al oxy(hydr)oxides are important sorbents for inorganic and organic P components. This function was confirmed for the sites BBR, MIT, and CON based on P K-edge XANES analyses (Prietzel et al. 2016). At all sites, the amount of oxalate-extractable Al and/or Fe increased – at least slightly – with increasing soil depth indicating beginning podsolization (Lundström et al. 2000). Podsolization was most obvious at the site LUE. Yet, P was obviously not leached together with Fe and Al, but it was incorporated into organic P pools (supplementary material S7). In consequence, the degree of P saturation of the soil sorbents decreased with increasing soil depth (S6). In contrast, several studies showed translocation of P by podsolization (Turner et al. 2012, Wu et al. 2014, Celi et al. 2013, Wood

et al. 1985), especially in cold temperate climate (Väänänen et al. 2008). In part, the different findings might be explained by differing biomass growth.

Organic P

The percentage of P_{org} in soils has been assumed to be an emergent ecosystem property (Turner and Engelbrecht 2011). We observed no clear trend of the percentage of P_{org} along our geosequence. Yet – as expected - a clear decrease in the relevance of P_{org} with increasing soil depth was found. The values we observed for the proportion of P_{org} in the forest floor and topsoil horizons cover a range from 35–83% (mean value 63%; Table 4) and extend the range of values found for temperate European beech forest ecosystems established on Loess (44–55%; Talkner et al. 2009). The mean value for the proportion of P_{org} found in our study is very close to the value observed for birch forests and tundra soils (mean value 62%; Turner et al. 2004) and for temperate rain forests in New Zealand (mean value 52%; Turner et al. 2007). In transition and subsoil horizons on average only 44% of P was bound organically at our study sites (range 1–66%; Table 4). Organic P extractable by $NaHCO_3$ and NaOH (Hedley fractionation) was less than P_{org} obtained according to Saunders and Williams (1955). Resulting proportions of organic P stocks extractable by $NaHCO_3$ and NaOH in total P down to 1 m soil depth were 38 – 44 – 38 – 26 and 26% along the P geosequence from highest to lowest P stocks.

Considering the classification of $C:P_{org}$ ratios in Oa and A horizons used in Germany for forest site assessment (Arbeitsgemeinschaft Forsteinrichtung 1996), the site LUE is clearly more P limited than all other sites, which had ratios in the medium (Oa) and moderately close (uppermost A horizon) range. Remarkably, $C:P_{org}$ ratios of soil organic matter of the different study sites converged in subsoil horizons (Table 4). This might be due to specific C:P ratios of dissolved organic matter, which is the most important source of P_{org} in subsoil horizons. This is supported by a recent meta-analysis of soil organic matter stoichiometry in a large number of soils worldwide by Tipping et al. (2016). Their data analysis suggests that relatively P and N-rich organic matter is selectively sorbed by mineral surfaces.

We observed distinct changes in the quality of organic P by NMR analyses. At the P-poor sites, the proportions of phosphonates exceeded by far the levels reported for more fertile soils (e.g., Sumann et al. 1998). Accumulation of phosphonates has been observed for very acidic or water-logged conditions (Condron et al. 2005). In contrast to our observation, phosphonate concentrations showed extremely low concentrations at the late and P-poor states of the Franz-Josef chronosequence (Turner et al. 2007). A relative increase in phosphonates along the geosequence (towards LUE) could be favoured by an elevated chemical stability of these compounds. Biogenic phosphonates are produced by several primitive life forms where they are incorporated into

exopolysaccharides and glycoproteins or polar groups of membrane phosphonolipids (McGrath et al. 2013). The C-P bond is thermally and chemically more stable compared to O-P bonds and therefore organophosphonates often resists thermal and chemical degradation as well as photolysis (Ternan et al. 1998). However, microorganisms are well capable of enzymatic degradation of various forms of organophosphonates. The expression of these enzymes can be both, Pi regulated (as part of the Pho regulon) or substrate-induced. While Pho-regulated phosphonatases are primarily expressed to meet the organisms´ demand for P during phosphate starvation, the latter ones also provide C and N to cells (McGrath et al. 2013). Supposedly (easily degradable) phosphoester-bonds are hydrolyzed first at all sites, while (more stable) organophosphonates remain (accumulate) in the soil. At LUE a higher percentage of phosphoester containing compounds is recycled (compared to BBR), therefore the relative content of phosphonates was increased. In our metagenomic datasets a significantly higher microbial potential for one type of phosphonate degrading enzyme (phosphonoacetaldehyde hydrolase) was detected in LUE (Bergkemper et al. 2016). This finding might indicate that the LUE microbial community has adapted to the elevated levels of organophosphonates, thus, showing a higher abundance of the respective genes.

It has been assumed that diester-P is more readily available to microbial breakdown than monoester-P (Hinedi et al. 1988, Guggenberger et al. 1996). Yet, increased proportions of diester-P have also been observed in acid soils of other studies, which has been explained by inclusion of diester-P into large organic molecules (Turner et al. 2007). Generally, the origin of mono- and diester-P is hard to decipher, as both compounds occur in plants and microorganisms (Makarov et al. 2005). Studies that investigated microbial P transformations with litter decomposition or from coarse to fine soil size separates usually also found an increase in the portions of diester-P at the expense of monoester-P signals (e.g., Gressel et al. 1996, Miltner et al. 1998, Amelung et al. 2001).

Phosphorus mobilization

Whereas the results mentioned above mainly address the amount of P in the solid phase, the analysis of the isotopic exchange kinetics provides information on the exchangeability of P between the soil solid phase and the soil solution (Fardeau 1996) and therefore on the amount of inorganic P that may diffuse to the absorbing surface of roots or hyphae. In addition, the parameter m informs on the ability of the soil to sorb inorganic P (Randriamanantsoa et al. 2013). Small m values indicate a high sorption potential. For the set of soil samples studied here, m was highly significantly correlated to Fe_{CBD} (which is related to the total number of sorption sites) and C_P ($m = 0.47 + 0.39*C_P - 0.016*Fe_{CBD}$; $r^2=0.67$, $p < 0.01$). The values obtained for the parameter m confirm the low sorption capacity for inorganic P of LUE, and the high P sorption capacity of the soil samples at the other sites.

Interestingly, water soluble P was much higher for samples from the LUE AE horizon than for the other samples. We suggest that this high value can be explained by the release of microbial P following drying before analysis and subsequent rewetting during analysis (Bünemann et al. 2013).

E_{1min} values were in most cases equal or higher than 5 mg P kg^{-1}, the level above which the yield of crop plants does not increase following P application (Gallet et al. 2003). This threshold was, however, found to be valid in soils that had sufficient P exchangeable on the medium term (i.e. between 1 minute and 3 months) and were able to sustain a significant P desorption flux into the soil solution. This is not the case at LUE. Samples from LUE AE horizon were characterized by very little P exchangeable between 1 minute and 3 months. In agreement with the results from a subsequent soil sampling at sites BBR and LUE (Bünemann et al. 2016), the results obtained here suggest that diffusion of inorganic P into the solution can sustain P availability at most sites but that this process is probably not sufficient to cover the plant demands at LUE. Thus, beech forests at LUE strongly depend on P recycling from soil organic matter.

4.2 Response of soil organisms and trees to P stocks of soils

Microbial biomass

The C_{mic}:N_{mic}:P_{mic} ratios detected at the five sites (mean molar ratio: 32:2:1) were comparable to results obtained for tropical mountain forest soils (32:3:1; Tischer et al. 2014) but were lower than documented by a global forest dataset (74:9:1; Cleveland and Liptzin 2007). The C_{mic}:P_{mic} ratios were similar to those found in other and also the same beech forests (Joergensen et al. 1995, Heuck et al. 2015). In addition, Turner and Condron (2013) observed similar C_{mic}:P_{mic} values based on the same method for P_{mic} analysis. The C_{mic}:P_{mic} values increased with decreasing P status of the soils.

Pools of microbial C, N and P were lowest at site LUE indicating the general limitation of soil nutrients and the acidic conditions at this site (Table 8). In a previous study the adaptation of the microbial community to nutrient limitation at the site LUE was shown by a significant increase in abundance of oligotrophic taxa like Acidobacteriales compared to BBR (Bergkemper et al. 2016). Surprisingly P_{mic} reached maximum values at sites MIT and CON although concentrations of bioavailable P (P_{Resin}) were highest at site BBR (Table 3). In contrast to the C_{mic}:P_{mic} ratio, P_{mic} in the mineral topsoil did not follow the P gradient described for soil total-P stocks (BBR>MIT≥VES>CON>LUE). Presumably microbial growth in BBR is restricted by other soil nutrients or environmental conditions.

While P_{mic} was lowest at LUE, ratios of C_{mic}:P_{mic}, N_{mic}:P_{mic} and C_{mic}:N_{mic} were higher than at the other study sites (Table 8). The increased ratio of C_{mic}:N_{mic} indicates a higher fungal contribution to the

microbial biomass at site LUE (Mouginot et al. 2014, Fierer et al. 2009). Accordingly, previous data from a metagenomics approach showed a tenfold increase in fungal sequences at LUE compared to BBR, where also the C_{mic}:N_{mic} ratio was much lower (Bergkemper et al. 2016). The high ratio of C_{mic}:P_{mic} in LUE also indicated a strong fungal contribution to the microbial biomass (Heuck et al. 2015). The imbalance of the microbial biomass over the C:P ratio of the soil organic matter ranged from 9 to 45, increased along the geosequence, and was larger at all sites than the average global value for terrestrial ecosystems of 7 (Xu et al., 2013). These results stress the need for P-specific decomposition strategies and for high P uptake efficiency by microorganisms.

Activities of phosphomonoesterase and phosphodiesterase

Phosphatases hydrolyse different phosphate ester bonds to release inorganic P. Therefore, phosphomonoesterases cleave P from monoester forms, such as phospholipids or nucleotides and phosphodiesterases release P from compounds like nucleic acids (Keller et al. 2012, Paul 2015). Since plants secrete phosphodiesterase only under severe P-deficiency, the production of this enzyme is mainly of microbial origin (Turner and Haygarth 2005). Phosphodiesterase activity showed similar activity pattern within the five studied soil profiles than phosphomonoetserase activity (data not shown). However, phosphodiesterase activity was much lower than phosphomonoesterase activity as already described by Turner and Haygarth (2005) for different pasture soils. The absolute activities of the two different enzymes corresponded to the species composition of organic phosphorus forms in the NMR spectra with a range of 7–34% for diesters and a range of 36–86% for monoesters (Table 5). The most striking result was that the LUE site was characterized by high concentrations of diesters (Table 5), but also by low phosphodiesterase activities resulting in high ratio of phosphomonoesterase to phosphodiesterase activity (Fig. 3). This result can be explained by low enzyme production of soil microorganisms at this site and/or low enzyme stability at low pH values at the LUE site (Table 3; Turner and Haygarth 2005). Limited enzyme concentrations were found as a main reason for recalcitrance of phosphodiesters in different soils (Jarosch 2016). The retarded hydrolysis of diesters might cause the relative high diester concentrations at the LUE site. The high C:P_{org} ratio at the site LUE is in agreement with relative enrichment of diester compounds due to limited enzyme concentrations. Finally retarded hydrolysis of diesters might also be the reason for the thick forest floor forming at the P-poor sites and the low turnover of organic matter (Table 7).

Leaves

According to Mellert and Göttlein (2012), the range of P leaf concentrations observed in our study (1.2–1.7 mg g^{-1}) corresponds with an average P supply. Only beech leaves at sites CON and LUE were in the concentration range of latent P deficiency, with P leaf concentrations of 1.1–1.2 mg g^{-1}. With

N:P ratios of 21 and 19 of leaves, respectively, these sites were also close to or even above the critical N:P ratio of 18.9 given for beech leaves (Talkner et al. 2015, Mellert and Göttlein 2012). The increasing difference between the P concentrations of living leaves and the P concentration of leaf litter showed an increasing P re-sorption by trees from senescing leaves with decreasing P supply by the soils. Likely, high P resorption seems to contribute to the adaptation of European beech to low P supply (Hofmann et al. 2016, Netzer et al. 2017). In summary, neither standing biomass (Table 1) nor the amount of litter produced (Table 7) seem to be related to the P gradient of the soils. However, based on leaf nutrient concentrations, the sites CON and LUE seem to approach P limitation.

Roots

We observed a strong concentration of root biomass in topsoil horizons at the P-poor sites (Fig. 4), while roots penetrated to subsoils especially at P-rich sites (fine root biomass at 80 cm soil depth: BBR 38 mg m^{-2}; LUE 2.7 mg m^{-2}). Despite large differences in soil P availability, the P concentrations in fine roots showed only a moderate decline with decreasing soil P (Zavišić et al. 2016). Yet relative differences in fine-root P concentrations observed at the different sites were still bigger than differences of leaf concentrations (Table 7). Obviously the P-supply to roots was facilitated by mycorrhizal fungi, since all vital root tips were completely enwrapped by the fungal mantles. Only in deep soil horizons, the growth of roots seemed to be P limited due to very low P concentrations of roots (0.3 mg g^{-1}). Very low P concentrations of coarse roots have also been observed at the P-poorest site LUE (Yang et al. 2016). This finding is well in line with those from isotope exchange kinetics, showing that particularly at site LUE there was no release of mineral bound inorganic P into soil solution. Thus, roots are limited to the surface layers with elevated microbial activity and P mobilisation from organic matter. Of the analyzed biological traits, root distribution between topsoil and subsoil compartments seemed the characteristic most sensitive to the P status of soils. The ratio of P in fine-roots to P_{mic} of the topsoil horizons (0-20 cm) tended to increase with decreasing P status of the soils (BBR: 0.05 – LUE: 0.16). This suggests that with decreasing P availability, beech fine roots and/or associated hyphae are more effective at maintaining P concentrations than microorganisms. However, it is not clear whether this is simply a result of more effective uptake or other mechanisms.

4.3 Indicators for P nutrition strategies

We defined ecosystem traits, which indicate the intensity of P acquisition (acquiring indicators) and the intensity of P recycling (recycling indicators) (Table 2). We are aware that the indicator values obtained bear uncertainties, e.g., regarding causes and effects or inter-correlation effects. Thus, our indicator approach provides no concluding proof for underlying mechanisms. Yet, it enables a consistency test for the supply-controlled nutrition strategies of beech forest ecosystems. The

indicator values allow us to relate nutrition strategies in a quantitative way to the P stocks of soils thereby providing novel information on how the adjustment of nutrition strategies to changes in P stocks of forest soils occurs.

We assume that ecosystems with P ***acquiring strategies*** are characterised by spatial (Jobbágy and Jackson 2004) and chemical (Prietzel et al. 2016) redistribution of P in soils, for example through P uptake in deeper horizons and fixation of the P mobilised during litter decay by topsoil minerals. Based on this assumption, we identified the following acquiring indicators: (1) P enrichment in topsoil horizons, (2) share of non-stable P in the profile, and (3) kinetics of phosphate mobilization (Table 2).

All the three acquiring indicators highlight more intense spatial and chemical redistribution of P in P-rich soils than in P-poor soils. We generally observed higher P concentrations in topsoils than in subsoils (Fig. 1). Published data on P in temperate forest soils confirm this observation (e.g. Talkner et al. 2009, Jobbágy and Jackson 2004). At our study sites, the differences between topsoil and subsoil concentrations were more pronounced at P-rich sites than at P-poor sites. At sites with high mineral P concentrations, the C investment for establishing and maintaining a deep root system might pay off through gains in nutrient uptake, whereas at P-poor sites these costs are too high given the low amount of P in the subsoil horizons. The actual depth distribution of fine-root biomass is in line with this explanation, although this does not exclude the possibility that at least some of the roots also acquire P from deeper depths at P-poor sites. The depth distribution of organic P obtained from Hedley fractionation additionally confirms increased P acquisition from subsoil material at P-rich sites. The proportion of organic P in the subsoil horizons is higher at P-rich sites than at P-poor sites (supplementary material S7), which might reflect intense root foraging for P and fine-root turnover in the subsoil horizons of P-rich sites (Cross and Schlesinger, 1995). Hedley fractionation results provide further indications for the assumption that intense P acquisition occurs preferentially at P-rich sites: In general, the proportion of residual P seems to be higher at sites low in P than at those having large P stocks (supplementary material S7, see above). The increasing proportion of stable P with decreasing P stocks might also be explained by increasing fixation of organic and inorganic P by mineral sorbents (Velásquez et al. 2016). Yet there is no reason to assume that the proportion of such fixed P in the stable Hedley P pool increases along the P gradient. If any differences would be expected based on the state of the art, less P fixation should occur at site LUE due to low contents of Fe and Al oxyhydroxides. Mobilization kinetics has often been assumed to indicate plant availability, and P mobilisation determined by isotopic exchange has been shown to be closely related to plant uptake (Achat et al. 2016). In line with this, P exchangeable within one day

from topsoil material was closely related to the P status of the study sites. The rate of P mobilisation might be both, the reason for, but also the consequence of P acquisition from mineral material.

The four ***recycling indicators*** identified were remarkably closely related to the P stocks of the different geosequence sites: (1) accumulation of P in the forest floor (r^2 0.94) (2) fine-root biomass accumulation in the forest floor (r^2 0.95), (3) residence time of the forest floor (r^2 0.99), and (4) contribution of diester-P to total P in A horizons (r^2 0.89; Fig. 4). Furthermore all the four recycling indicators averaged in the normalized form for aggregated illustration (Fig. 5) confirm decreasing intensity of P recycling with increasing P supply (r^2 0.96, $p < 0.01$, for the linear regression of recycling indicator values vs. P soil stock). These results indicate that a majority of P is contained in the surface organic-rich layers at P-poor parent materials. Slow turnover and high rooting intensity seem to foster tight P recycling (Fig. 4). Beyond that, we observed an unexpected close, linear and positive correlation between the diester-P/monoester-P ratio of mineral topsoil horizons and the P stock at a given site (Fig. 4). Despite the uncertainties of NMR interpretation mentioned above, some authors suggested the diester-P/monoester-P ratio as indicator of microbial P transformation (e.g., Turrión et al. 2000). The increase in the diester-P/monoester-P ratio can be seen as a biomarker indicating increased microbial recycling of organic P with increasing deficiency in overall P supply. This supports our hypothesis that there is a shift to P recycling systems in forest surface soils under extreme P limitations. High phosphomonoesterase-to-diesterase ratios at site LUE likely maintain those chemical signatures.

In summary, the indicator values obtained confirm that European beech forests at silicate sites are characterized by different P nutrition strategies depending on the P stocks of the soils. Our results imply that with decreasing P supply by the mineral soil, the intensity of recycling processes increases continuously whereas the intensity of acquisition processes decreases. Future work needs to analyse how these different strategies might be impacted by perturbations or disturbances such as chronic N deposition or climatic extremes. Our results support speculations, which help exemplify the use of such indicators: Interestingly, the peak of biological activity along the P gradient does not seem to be at site BBR (highest P stock) but at sites MIT and VES (intermediate P stocks). These sites showed the highest P leaf concentrations (Table 7), the lowest C_{mic}/P_{mic} values (Table 8), the highest ratio of organic P to total P and the highest C stocks in soils (Table 3) among our study sites. Under intermediate conditions, P nutrition of vegetation and microbes may be best owing to a mixture of recycling and acquisition strategies.

4.5 Geosequence vs. Chronosequence

Chronosequences present a useful design for analyzing the relationships between soil development and biogeochemical P cycles as well as associated succession of plant and microbial communities and ecosystem properties (Laliberté et al. 2013). Complementing this approach with geosequences provides additional information on the role of P in parent material on forest ecosystem development.. Our assumption was that the P speciation along chronosequences as well as along the studied geosequence is controlled by the response of plant and microbial communities to P contents. Accordingly, we expected similar changes of P species along the geosequence as has been found for chronosequences. Although, our sites cover a similar range of total soil P contents as in chronosequence studies (supplementary material S5), the changes in P species along the P gradient (Fig. 2) clearly differed from the pattern described by Syers and Walker (1969). First, chronosequence studies indicate that the proportion of organically bound P increases with increasing age of the soils (e.g., Chen et al. 2015, Turner et al. 2012, Cross and Schlesinger 1995, Syers and Walker 1969). We found no such relative increase in organic P along the geosequence. This might indicate that P nutrition strategies in response to low soil P concentrations are not the reason for the increase in P*org* observed during soil development. Preferential losses of other P fractions or changes in the forms of P*org* produced through successional changes in vegetation might rather be the reason for this observation. In contrast to chronosequence studies, we observed the accumulation of forest floor under low-P conditions. This might also be attributable to the specific conditions of the cool temperate climate at our study sites, which is cooler than at most of the chronosequence studies analyzed until now. Second, we observed an increasing percentage of stable P with decreasing P stocks of the soils; together with the lower percentage of organic P a the P-poor sites. This might indicate less biotransformation of mineral-bound P at P-poor sites. Third, we did not observe relative enrichment of the P associated with sesquioxides as described for chronosequences, neither along the geosequence nor with soil depth.

5 Conclusion

We selected a novel P-geosequence approach on silicate material to unravel the response of European beech forests to different soil P stocks. Our results are in line with the hypothesis of a tightening of P cycles with decreasing soil P and provide evidence for the existence of adaptation processes at the ecosystem level. In P-rich and acid soils plants and soil organisms mobilize P from primary and secondary minerals, in P-poor soils, roots and fungi seem to sustain their P demand more successfully than bacteria and mainly from the forest floor and soil horizons rich in organic matter. In turn, this influences the quality of organic P compounds in soils which underwent pronounced changes along the P geosequence. The indicators for P-recycling and P-acquisition

pointed to a continuous change in nutrition strategies with changing P supply. However, highest proportions of organic P, closest C:P ratios in microbial biomass and highest P concentrations in beech leaves were observed at sites of intermediate P stocks, where the combination of different nutrition strategies might favor P uptake. Finally, our results emphasize the enormous adaptability of beech forest ecosystems to changing P supply. We conclude that within the range of P stocks analyzed, P deficiency in beech forest ecosystems is unlikely the result of a low P supply per se, yet sufficient P nutrition depends on supply-specific plant–microorganism–soil interactions.

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Figure 1 Total P concentrations and depth distributions of P along the analyzed geosequence.

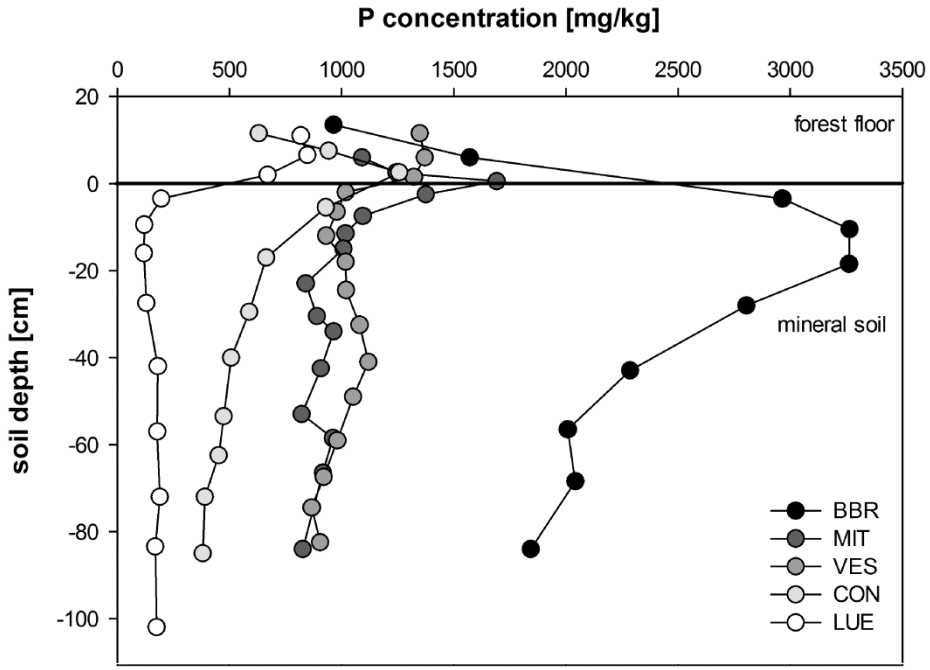

Figure 2 Stocks of stable P (P HCl_{con}, P residual (acid digestion)), P Ca (P 1M HCl), sorbed Pi (P resin, P_i $NaHCO_3$, P_i NaOH), and organically bound P (P_o $NaHCO_3$, P_o NaOH, P_{tot} (HF) from organic layer) along the analyzed geosequence.

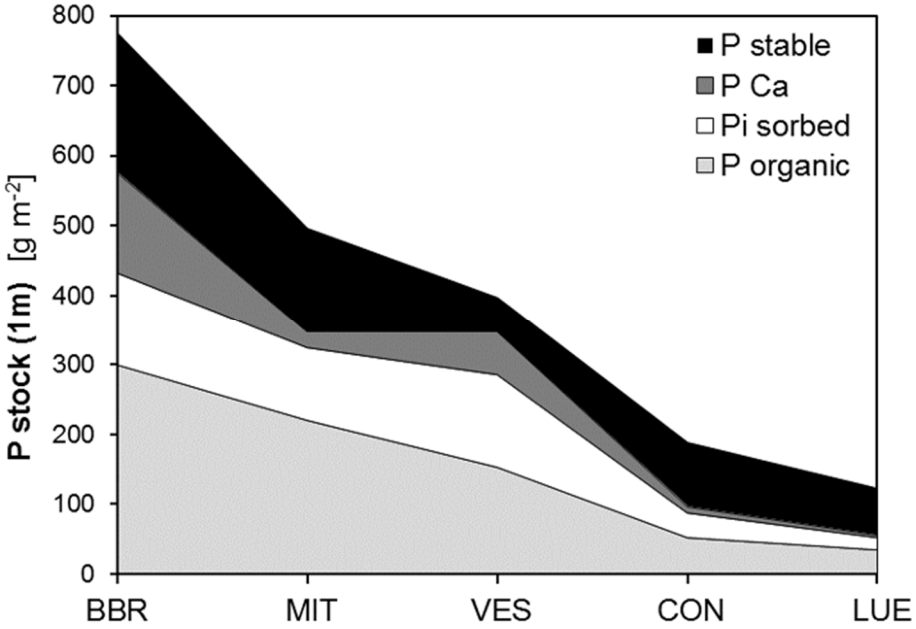

Figure 3 Ratio of monoesterase activity:diesterase activity at different soil depth and study sites.

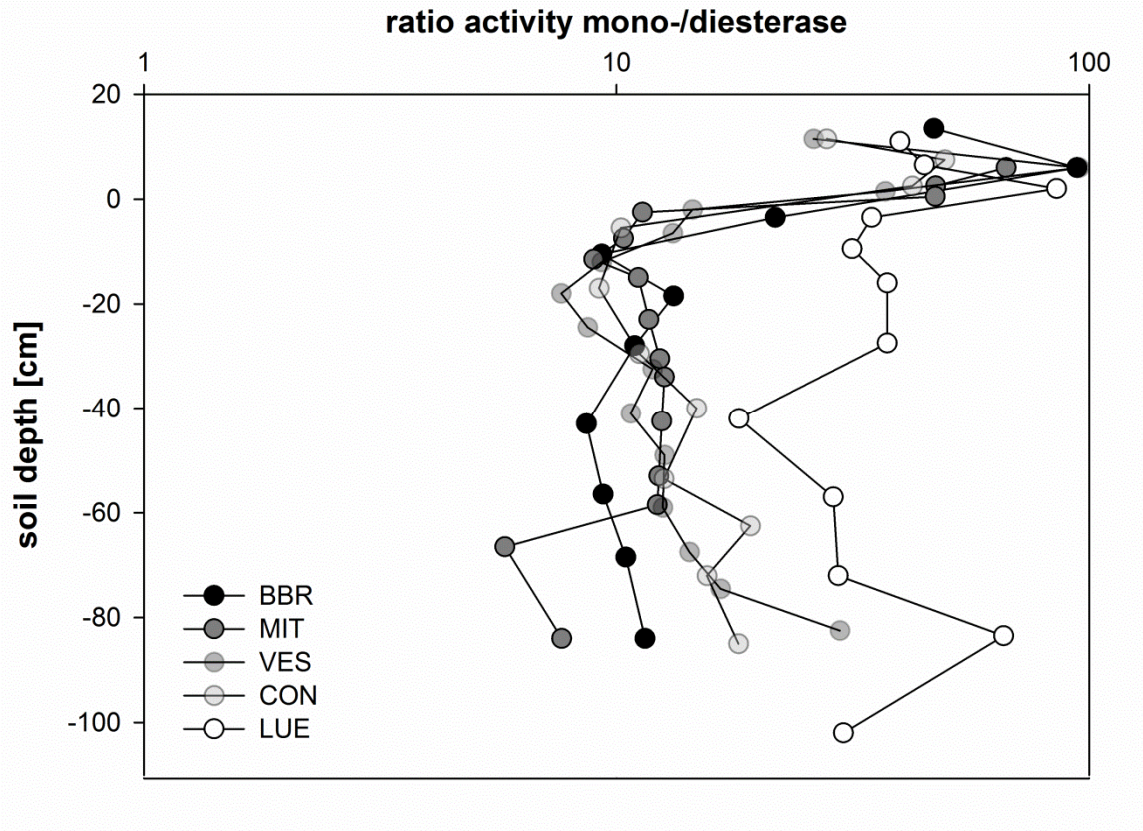

Figure 4 Three of the soil properties applied as indicators for the recycling strategy against the total P stock of the soil at the five study sites.

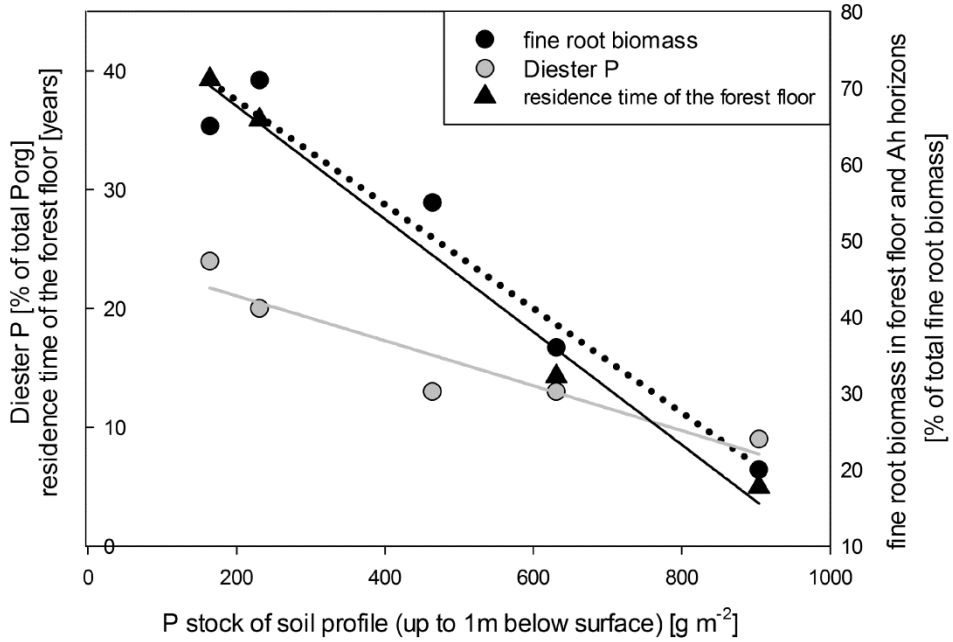

Figure 5 Means of normalized indicator values as calculated according to equation 3 for the different sites. Bars represent the standard error of normalized values.

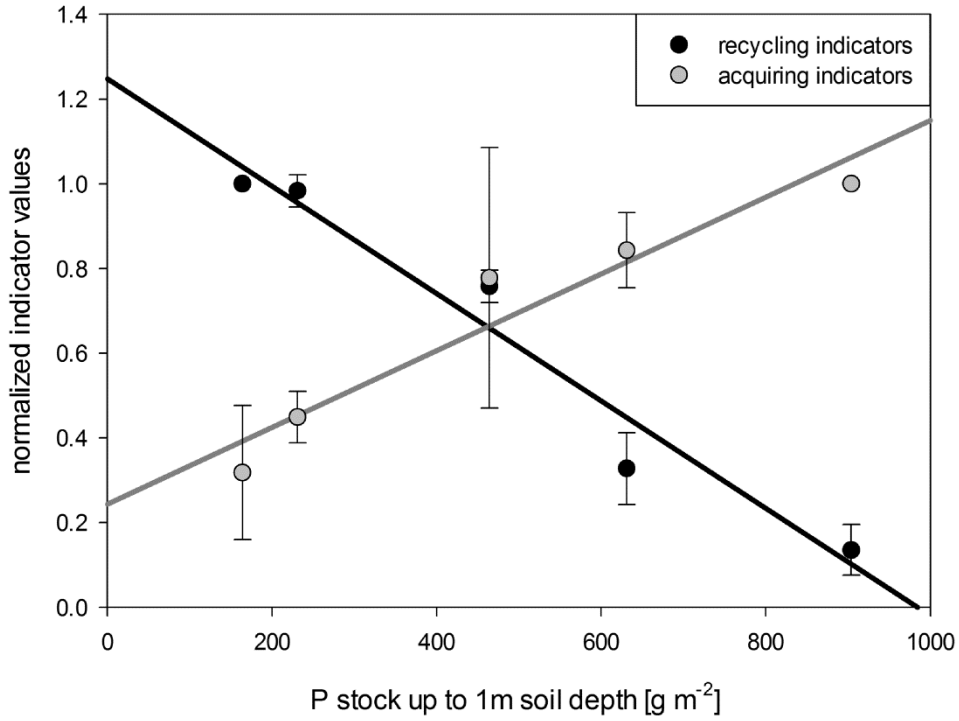

Table 1 Site and stand characteristics of the study sites Bad Brückenau (BBR), Mitterfels (MIT), Vessertal (VES), Conventwald (CON), Lüss (LUE).

Study site	***BBR***	***MIT***	***VES***	***CON***	***LUE***
Soil type (WRB 2015)	Dystric Skeletic Cambisol (Hyperhumic. Loamic)	Hyperdystric Chromic Folic Cambisol (Humic. Loamic. Nechic)	Hyperdystric Skeletic Chromic Cambisol (Hyperhumic. Loamic)	Hyperdystric Skeletic Folic Cambisol (Hyperhumic. Loamic)	Hyperdystric Folic Cambisol (Arenic. Loamic. Nechic. Protospodic)
Parent material	Basalt	Paragneiss	Trachyandesite	Paragneiss	Sandy till (Geschiebedecksand)
Humus form (AK Humusformen 2004)	Mull-like Moder	Moder	Moder	Mor-like Moder	Mor-like Moder
Texture (topsoil) (WRB 2015)	Silty clay loam	Loam	Loam	Loam	Loamy sand
Texture (subsoil) (WRB 2015)	Loam	Sandy loam	Sandy loam	Sandy loam	Sand
Altitude (m a.s.l.)	809	1023	810	840	115
Mean annual precipitation (mm)	1031	1299	1200	1749	779
Tree species composition (%)	*Fagus sylvatica* (99) Acer pseudoplatanus (1)	*Fagus sylvatica* (96) *Picea abies* (2) *Abies alba* (2)	*Fagus sylvatica* (100)	*Fagus sylvatica* (69) *Abies alba* (31)	*Fagus sylvatica* (91) *Quercus petraea* (9)
Age *beeches* (a)	137	131	123	132	132
Height *beech* (mean basal area tree) (m)	26.8	20.8	29.3	27.6	27.3
Diameter at breast height *beeches* (cm)	36.8	37.6	40.1	39.9	27.5
Number of trees (ha^{-1})	335	252	506	312	480
Basal area ($m^2\ ha^{-1}$)	35.6	28.1	31.4	44.0	36.7

Standing volume ($m^3\ ha^{-1}$)	495	274	550	685	529
Vegetation (pot. nat.)	Hordelymo-Fagetum	Dryopteris-Fagetum	Luzulo-Fagetum	Galio-Fagetum	Luzulo-Fagetum

Table 2 Indicators for P-acquisition and P-recycling applied in this study, method of calculation of indicator values and justification of indicator.

Acquiring indicators		
Variable	Calculation of indicator values	Assumed underlying process
P-enrichment in topsoil	P stock in the upper 50% of the soil fine earth mass divided by the total soil P stock (up to 1m).	Spatial redistribution induced by the P pumping of trees: root uptake of P in the subsoil, P deposition with litter at the topsoil and adsorption after mineralization. This mechanism was described by Guckland et al. (2009) for Ca. A general discussion on nutrient pumping was provided by Jobbágy and Jackson (2004).
Proportion of non-stable P in the profile	Stock (up to 1m) of non-stable P. (i.e. sum of Hedley P minus $P_{HCl\ conc}$ and $P_{residual}$) relative to total Hedley P.	Chemical redistribution due to biological mobilization of P from primary minerals (Prietzel et al. 2016). Nutrient demand had been discussed as the reason for root induced weathering (Kelly et al. 1998).
Phosphate mobilization within 1 min and 1 day	Concentration of isotopically exchangeable P within 1 min and 1day of topsoil horizons as described in the methods chapter.	P mobilization based on physicochemical processes; indicator for P availability (Achat et al. 2016, Bünemann et al. 2016, Oehl et al. 2001).
Recycling indicators		
Variable	Calculation of indicator values	Assumed underlying process
Accumulation of P in the forest floor	P stock in the forest floor related to total P stock (up to 1m mineral soil depth).	Forest floor pathways as short cut for plant P uptake without passing of P through the fixing mineral soil. P turnover in the forest floor as an emergent property of beech forest ecosystems had been discussed by Talkner et al. (2009).
Concentration of fine-root biomass in the forest floor	Total fine root biomass in the forest floor and in the upper 0-5 cm mineral soil in relation to total fine root biomass (up to 1m mineral soil depth).	Peak concentrations of fine roots in the forest floor have been assumed to favor tight P cycling in acid temperate forest ecosystems (Wood et al. 1984). Results obtained were much clearer when we added the 0-5 cm increment of the mineral soil. This is in agreement with a smooth transition between Oa and Ah horizons observed at most of the sites.
Enrichment of diester-P	Diester-P / monoester-P ratio in the topsoil horizon as calculated from NMR spectra.	Increased proportions of diester-P have been observed in acid soils and were explained by changes in enzyme activity (Turner and Haygarth 2005), and decreased accessibility of diester-P for microbial decay due to accumulation

		within large organic molecules (Turner et al. 2007).
Mean residence time of the forest floor	Mass of the forest floor related to the mass of annual litter fall.	Limited decay of soil organic matter enhances tight P recycling by providing forest floor P-pathways for tree nutrition.

Table 3 Basic characteristics of the soils at the study sites and their variation as indicated by the coefficient of variation among the different sites.

Study sites	***BBR***	***MIT***	***VES***	***CON***	***LUE***	Coefficient of variation [%]
Element stocks up to 1 m soil depth and forest floor						
P_{tot} [g m^{-2}]	904	678	464	231	164	63
N_{tot} [kg m^{-2}]	1.3	1.4	1.1	0.8	0.7	27
C_{tot} [kg m^{-2}]	18	26	19	18	16	20
Chemical characteristics of A horizons (0-5 cm)						
P_{tot} mg kg^{-1}	2966	1375	1017	929	195	79
P_{resin} mg kg^{-1}	116	70	40	24	11	80
C mg g^{-1}	175	174	126	149	95	24
C/N	16	18	18	22	26	20
C/P	59	127	124	160	493	89
$pH_{(H2O)}$	3.8	3.6	3.4	4.0	3.5	7
$pH_{(KCl)}$	3.3	3.0	2.8	3.2	2.5	11
CEC meq kg^{-1}	371	408	197	293	108	45
$(Al+H)_{ex}$ meq kg^{-1}	297	388	184	286	100	44
Texture of the A horizon (0-5 cm)						
Clay %	37	24	24	27	6	47
Silt %	55	32	46	33	19	37
Sand %	8	44	30	40	75	62
Stone content						
Content of stony fragments (profile average) % w/w	78	25	63	69	43	38

Table 4 C and P_{org} concentration and C:P_{org} mass ratio of the forest floor and the mineral soil horizons. Please consider that sampling depths vary depending on study site. Sampling depths are documented in the supplementary material (S2).

	C_{org}					Proportion P_{org}					C/P_{org}				
	mg g^{-1}					% of total P (HF digestion)					g g^{-1}				
Horizon	**BBR**	**MIT**	**VES**	**CON**	**LUE**	**BBR**	**MIT**	**VES**	**CON**	**LUE**	**BBR**	**MIT**	**VES**	**CON**	**LUE**
Oi	506	517	510	521	502	56	n.d.	55	n.d.	78	931	n.d.	685	n.d.	792
Oe	482	503	484	500	387	48	72	67	45	85	634	565	530	1180	536
Oa	n.p.	349	313	319	463	n.p.	67	83	77	71	n.p.	308	285	330	972
A	175	174	126	149	96	54	69	66	71	50	110	183	188	226	984
	97	73	68		18	49	75	66		35	60	89	105		447
BA	78	50	46	80	14	50	66	59	60	37	47	75	85	200	323
transition	50				10	51				35	36				225
B		45	46	56	13		64	53	61	54	29	70	85	156	132
	34	33	40	35		50	64	51	43			61	78	161	
		34	35	22	10		65	40	40	58		59	81	119	97
		31	29				45	35				73	74		
	22	30	23	18	4	44	46	31	34	54	24	73	69	119	39
		25	17				53	24				58	70		
		33	12				47	20				73	66		
	26	27	8	8	2	41	46	1	34	38	31	64	74	57	27
	11	16		3	2	16	41		28	35	37	47		28	26
CB transition	b.	b.	6	b.	b.	b.	b.	14	b.	b.	b.	b.	45	b.	b.

n.p. *horizon not present*
n.d. *not determined*
b. *below max. sampling depth*

Table 5 Species composition of soil phosphorus forms in EDTA/NaOH extracts after dialysis. For recovery calculations P_{org} as determined according to Saunders and Williams (1955) was used, P_{inorg} was calculated as the difference between the concentrations of HF P and P_{org}.

Site/ horizon	Organic P species according to chemical shift range (calculated as percent of total signal intensity)						Diester-P/ Monoester-P ratio	NMR Recovery in dialyzed samples	
	Ortho-P %	Monoesters %	Diesters %	Pyrophosphate %	Phosphonate %	Polyphosphate %		Organic species relative to P_{org} %	Inorganic species relative to P_{inorg} %
BBR									
Ah1	16.7	68.8	8.8	1.7	3.3	0.7	0.13	37	10
BA2	9.9	81.4	6.4	0.5	1.8	n.d.	0.08	29	4
BC	4.9	85.2	9.0	0.1	0.8	n.d.	0.11	23	0
MIT									
Ah1	12.4	57.3	12.6	4.9	10.1	2.7	0.22	40	14
Bw3	4.1	85.2	8.6	0.0	2.0	n.d.	0.10	68	3
BC	5.7	85.6	6.8	n.d.	1.9	n.d.	0.08	30	0
VES									
Ah1	8.2	57.0	12.5	3.0	15.2	4.2	0.22	28	19
Bw3	5.9	81.5	10.8	n.d.	1.7	n.d.	0.13	26	3
BC	4.4	83.2	11.6	n.d.	0.8	n.d.	0.14	8	0
CON									
Ah1	13.7	48.5	20.3	4.7	9.7	3.2	0.42	39	22
Bw1	11.9	50.7	33.6	2.0	1.8	n.d.	0.66	35	3
BC	9.0	59.5	30.8	0.7	n.d.	n.d.	0.52	50	2
LUE									
AE	11.3	35.6	23.6	3.8	22.1	3.6	0.66	52	12
Bsw	6.0	69.7	21.3	0.6	2.4	n.d.	0.31	8	1
BC	10.7	69.8	13.4	6.1	n.d.	n.d.	0.19	3	0

n.d. *not determined*

Table 6 Results of isotopic exchange analyses for the Ah horizons: Water extractable P (CP), and total inorganic P (P_{inorg}). Fitting parameters describing the decrease of radioactivity in the solution with time (m and n), amount of P isotopically exchangeable within 1 minute (E1min), between 1 minute and 1 day (E1min-1day), between 1 day and 3 months (E1day-3months), and amount of P that cannot be exchanged within 3 months (E>3months).

Site	Horizon	C_P	P_{inorg}	m	n	E_{1min}	$E_{1min-1day}$	$E_{1day-3months}$	$E_{>3months}$
		mg P L^{-1}	mg P kg^{-1}				mg P kg^{-1} soil		
BBR	Ah1	0.21	1375.0	0.18	0.59	13.3	533.8	637.9	190.0
	Ah2	0.07	1660.0	0.06	0.57	11.9	504.9	901.6	241.6
	BA1	0.04	1626.0	0.03	0.55	13.6	494.7	862.1	255.6
	BA2	0.07	1383.0	0.03	0.53	21.6	568.7	640.2	152.5
MIT	Ah1	0.27	422.0	0.34	0.76	9.2	339.0	70.9	3.0
	Ah2	0.05	271.0	0.11	0.56	4.7	130.8	114.1	21.5
	BA	0.02	347.0	0.06	0.60	3.0	138.3	158.3	47.4
VES	Ah1	0.70	348.0	0.68	0.40	11.4	108.8	142.9	84.9
	Ah2	0.10	329.0	0.21	0.60	5.2	172.6	132.7	18.6
	BA	0.04	384.0	0.06	0.51	6.8	157.6	174.4	45.1
CON	Ah	0.04	269.0	0.09	0.57	4.0	126.3	118.3	20.4
	BA	0.01	263.0	0.03	0.50	5.0	104.1	119.3	34.7
LUE	AE	1.31	98.0	0.86	0.03	13.2	2.8	2.0	80.0
	E	0.10	78.0	0.97	0.22	1.0	3.9	7.0	66.1

Table 7 Litterfall and P concentrations in leaves, leaf litter and fine roots as well as the turnover rate of the forest floor calculated based on the mass of forest floor and annual litterfall. Values in parentheses represent standard deviation.

Study site	***BBR***	***MIT***	***VES***	***CON***	***LUE***
P in leaf litter ($g\ m^{-2}\ a^{-1}$)	0.229	0.213	n.d.	n.d.	0.156
P leaves ($mg\ g^{-1}$ d.w.)	1.41 (0.21)	1.66 (0.16)	1.64 (0.14)	1.22 (0.14)	1.21 (0.08)
N leaves ($mg\ g^{-1}$ d.w.)	22.9 (2.27)	26.5 (2.27)	24.0 (1.63)	25.2 (0.49)	22.7 (1.39)
P fine roots forest floor ($mg\ g^{-1}$)	0.96	0.96	0.93	0.80	0.76
P fine roots mineral soil (mean) ($mg\ g^{-1}$)	0.88 (0.27)	0.82 (0.14)	0.77 (0.13)	0.49 (0.17)	0.54 (0.27)
Mass of forest floor ($kg\ m^{-2}$)	1.83	5.05	13.98	11.74	12.84
Turnover rate of the forest floor ($years^{-1}$)	1/5	1/14	n.d.	1/36	1/39

n.d. not determined

Table 8 Microbial biomass and elemental ratios of microbial biomass as well as P of fine root biomass (P_{fr}) of mineral topsoil horizons (0-20 cm) and stoichiometric imbalances between resources (i.e. soil organic matter) and microbes calculated as the mass ratio of $C_{resource}$:$P_{resource}$ over C_{mic}:P_{mic} according to Mooshammer et al. (2014).

Site	C_{mic}	N_{mic}	P_{mic}	P_{fr}	C_{mic}/P_{mic}	N_{mic}/P_{mic}	C_{mic}/N_{mic}	C_{Org}/P_{Org}	N_{Org}/P_{Org}	C_{Org}/N_{Org}	C/P imbalance
	µg g^{-1}				g g^{-1}						
BBR	1223	87	100	5.5	12.2	0.9	14	103	6	15.3	9
MIT	795	82	111	3.9	7.2	0.7	10	198	11	17.5	28
VES	810	64	79	3.3	10.3	0.8	13	226	13	16.8	23
CON	1392	130	104	6.0	13.4	1.3	11	393	18	22.5	30
LUE	192	12	10	1.6	19.8	1.2	16	905	34	25.4	45

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S1 Methods for the assessment of stand characteristics provided in Table 1.

Methods: Stand characteristics and ground vegetation survey

Stand growth characteristics were determined according to (Dobbertin and Neumann, 2010). For the determination of the number of trees all trees with a diameter at breast height bigger than 5 cm at the intensive research area of 0.25 ha were counted. The basal area of these trees was determined based on dendrometer measurements. Furthermore, the mean diameter at breast height of the trees counted was determined. Tree height was assessed at a selection of sample trees at the monitoring plot in order to construct diameter-height curves. The height of the beech stands determined gives the mean stand height of the intense study are, which is determined as the height of the mean basal area diameter trees as derived from the diameter-height curve. Resulting standing volume of the research forests was calculated according to Bergel (1973). Ground vegetation survey also covered the complete intensive research area (Canullo et al., 2010). Here only the determined plant community is presented.

Bergel D (1973) Formzahluntersuchungen an Buche, Fichte, europäischer Lärche und japanischer Lärche zur Aufstellung neuer Massentafeln. Allgemeine Forst- und Jagdzeitung 144: 117-124

Canullo R, Starlinger F, Granke O, Fischer R, Aamlid D, Neville, P. (2010) Assessment of Ground Vegetation. In: ICP Forest (ed) Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests, Part VII.1, 1–19

Dobbertin M, Neumann M (2010) Tree Growth. In: ICP Forest (ed) Manual on methods and criteria for harmonized sampling, assessment, monitoring and analysis of the effects of air pollution on forests, 1–29

S2 Sampling depths and basic soil properties at the studied sites.
Depth distribution of soil horizons, soil sampling depths and basic soil characteristics of study sites Bad Brückenau (BBR), Mitterfels (MIT), Vessertal (VES), Conventwald (CON) and Lüss (LUE).

Horizon / Layer	Upper sampling limit cm	Thickness cm	Bulk density of fine earth material g cm^{-3}	Sand %	Silt %	Clay %	pH (H_2O)	C mg g^{-1}	N mg g^{-1}	P_{tot} (HF-dig.) mg kg^{-1}	P_{citr} (ICP) mg kg^{-1}	P_{citr} (PO_4-P) mg kg^{-1}	CEC mm ol_c kg^{-1}
BBR													
Oi	15	3	0.01	n.d.	n.d	n.d.	5.6	506.4	13.09	964	368	315	417
Oe	12	12	0.01	n.d.	n.d	n.d.	5.3	481.8	20.66	1571	390	326	543
Ah	0	7	0.21	8	56	37	3.8	174.8	11.16	2966	162	58	371
	-7	7	0.73	9	54	37	4.2	96.6	6.48	3266	121	39	260
BA	-14	9	n.d.	9	55	35	4.5	77.6	5.62	3263	101	34	243
	-23	10	0.93	14	53	33	4.8	50.5	3.91	2806	117	35	206
Bw	-33	18	1.24	24	50	26	4.9	33.6	2.51	2286	113	64	149
	-51	11	0.53	26	50	24	5.1	21.5	1.62	2009	103	62	143
	-62	13	n.d.	29	51	20	5.4	26.2	1.94	2042	96	66	130
	-75	18	2.18	37	48	15	5.7	11.2	0.88	1844	174	141	148
MIT													
Oi	8	4	0.02	n.d.	n.d	n.d.	4.95	517.0	16.945	1091	344	307	689
Oe	4	3	0.08	n.d.	n.d	n.d.	3.89	503.2	24.245	1243	223	182	750
Oe+Oa	1	1	0.18	n.d.	n.d	n.d.	3.53	348.5	19.47	1693	231	109	121
Ah	0	5	0.65	44	32	24	3.57	174.0	9.62	1375	132	36	408
	-5	5	0.54	48	33	20	3.86	73.1	4.23	1094	99	17	242
BA	-10	3	0.72	57	29	15	4.20	50.2	2.905	1017	84	17	188
Bw	-13	4	0.59	51	31	18	4.46	44.9	2.485	1008	62	15	181
	-17	12	0.85	52	31	16	4.46	32.8	1.725	840	69	21	165
	-29	3	0.68	58	30	11	4.45	33.9	1.775	890	67	22	158
	-32	4	0.36	58	34	8	4.49	31.4	1.73	964	69	29	156
	-36	13	0.87	57	33	9	4.58	30.0	1.63	906	68	28	152
	-49	8	0.77	56	36	9	4.52	25.3	1.31	822	72.4	31	150
	-57	3	n.d.	56	34	10	4.52	33.2	1.72	961	73	28	146
	-60	13	1.03	56	35	8	4.61	26.8	1.42	916	64	31	133
	-73	22	1.05	57	37	7	4.64	16.1	0.94	827	74	44	103
VES													
Oi	14	5	0.02	n.d.	n.d	n.d.	5.55	510.0	14.4	1348	460	384	444
Oe	9	6	0.05	n.d.	n.d	n.d.	3.96	484.0	23.8	1370	313	242	624
Oa	3	3	0.35	n.d.	n.d	n.d.	3.45	313.0	17.5	1322	174	85	403
Ah	0	4	0.54	30	46	24	3.36	126.0	7.2	1017	103	29	197
	-4	5	0.74	30	47	23	3.66	68.1	4.3	978	122	31	138
BA	-9	6	0.72	33	47	19	4.06	46.5	3.0	931	117	25	136
Bw	-15	6	0.59	39	45	16	4.34	45.5	2.8	1018	136	40	136
	-21	7	0.70	41	46	13	4.49	40.4	2.5	1019	145	62	133

Horizon / Layer	Upper sampling limit cm	Thickness cm	Bulk density of fine earth material g cm^{-3}	Sand %	Silt %	Clay %	pH (H_2O)	C mg g^{-1}	N mg g^{-1}	P_{tot} (HF-dig.) mg kg^{-1}	P_{citr} (ICP) mg kg^{-1}	P_{citr} (PO_4-P) mg kg^{-1}	CEC mm ol_c kg^{-1}
	-28	9	0.78	45	44	11	4.58	35.1	2.1	1080	173	101	131
	-37	8	0.76	50	38	11	4.64	29.2	1.7	1119	193	114	115
	-45	8	1.12	48	43	10	4.70	22.6	1.4	1051	214	136	105
	-53	12	0.91	58	35	8	4.80	16.6	1.0	980	193	134	91
	-65	5	n.d.	63	32	6	4.79	12.0	0.8	920	174	131	68
	-70	9	0.98	63	31	5	4.79	8.1	0.6	867	206	160	59
CB	-79	7	1.33	72	25	4	4.76	5.9	0.4	904	253	205	53
CON													
Oi	13	3	0.03	n.d.	n.d	n.d.	4.91	520.5	12.0	630	178	153	745
Oe	10	5	0.06	n.d.	n.d	n.d.	3.55	500.4	20.7	942	187	163	791
Oa	5	5	0.20	n.d.	n.d	n.d.	3.46	319.3	16.4	1256	131	51	447
Ah	0	11	0.28	40	33	27	4.03	149.0	6.9	929	75	14	294
BA	-11	12	0.47	39	38	23	4.32	80.2	3.5	664	47	8	239
Bw	-23	13	0.55	41	42	17	4.42	56.2	2.4	588	34	8	181
	-36	12	0.74	44	41	16	4.53	35.3	1.6	507	19	8	141
	-48	11	2.59	48	39	12	4.63	22.5	1.1	474	16	10	113
	-59	7	1.40	49	40	11	4.56	18.3	1.0	452	12	8	108
	-66	12	1.19	48	43	10	4.61	7.7	0.6	390	12	8	79
	-78	14	0.71	54	37	8	4.61	3.0	0.4	380	15	12	61
LUE													
Oi	13	4	0.01	n.d.	n.d	n.d.	5.72	502.4	11.0	817	275	226	583
Oe	9	5	0.06	n.d.	n.d	n.d.	4.49	387.2	15.2	847	259	225	695
Oa	4	4	0.25	n.d.	n.d	n.d.	3.29	462.5	17.5	671	62	54	557
AE	0	7	0.53	75	19	6	3.52	96.5	3.8	196	15	10	108
E	-7	5	1.38	77	19	5	3.80	18.3	0.7	119	7	1	24
Bsh	-12	8	1.11	81	14	5	3.84	14.2	0.6	118	9	1	31
Bsw	-20	15	1.87	75	19	6	4.16	10.1	0.5	129	8	2	29
Bw	-35	14	1.12	71	21	8	4.46	12.8	0.6	180	11	2	46
	-49	16	1.22	76	19	6	4.41	10.0	0.5	177	10	3	56
	-65	14	1.69	80	12	7	4.59	4.0	0.3	190	6	3	37
	-79	9	1.85	81	13	6	4.63	1.8	0.2	169	4	3	27
	-88	18	2.06	84	10	6	4.63	1.6	0.2	175	3	3	23

n.d. *not determined*

S3 Details and comparisons of the methods for the determination of organic P.

Method Quality Assessment: Porg

The ratio between organic P determined by the ignition method (Saunders and Williams, 1955) and the sum of organic P in the hydrogencarbonate and hydroxid fractions of the Hedley fractionation (Hedley and Stewart, 1982), increased from between 1 and 1.5 in the topsoil horizons to between 2 and 7 in the subsoil. Furthermore, the subsoil values of this ratio increased along the P geosequence with decreasing total P (data not shown). This difference can very likely be assigned to P associated with particulate organic matter or stable residual organic matter, which is determined by the ignition method, but not extracted by hydrogencarbonate and hydroxid extraction as performed in frame of Hedley fractionation. In consequence some part of the residual P of the Hedley might consist of organic P (Velásquez et al., 2016; Tiessen and Moir, 2008). This is why we decided to use the organic P determined by the ignition method as a proxy for total organic P as has been done before in other studies (Condron et al., 2005). However, this method might overestimate organic P especially in highly weathered soils (Condron et al., 1990). Thus it should be considered that the C:P_{org} values and the values for the portion of organic P (Table 6) represent minimum and maximum values, respectively.

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Velásquez G, Ngo P, Rumpel C, Calabi-Floody M, Redel Y, Turner BL, Condron LM, Mora, María de la Luz (2016) Chemical nature of residual phosphorus in Andisols. Geoderma 271:27–31. doi:10.1016/j.geoderma.2016.01.027

S4 Detailed description and evaluation of 31P NMR analyses and interpretation.

Methods: Detailed description of P NMR methodology applied and discussion of challenges

We faced the problem that no reliable P spectra could be obtained at all from the untreated extracts due to high paramagnetic Fe loads (up to 1,200 mg L^{-1}) interfering with NMR analyses. Also applying high-speed ultracentrifugation and Fe precipitation reactions using Na_2S (Vestergren et al., 2012) did not solve this problem. Hence, we had to use the dialyzed extracts for NMR analyses (Sumann et al., 1998; Amelung et al., 2001), being well aware that this included additional risks of P losses. As we could use the P-NMR data as qualitative indicators for changes in P transformation only, we found this risk acceptable. After dialyses, Fe contents were reduced to 0.2 to 70 mg L^{-1}.

In dialyzed extracts, salt contents are presumably much lower than in samples formerly used for generating spectral libraries. However, as salt content affects chemical shifts (Cade-Menun, 2015). Since we also did not perform spiking experiments, we herein refrain from identifying and interpreting specific NMR signals such as for *chiro-, neo-, myo-*, or *scyllo*-inositol hexaphosphates (Cade-Menun, 2015), but stick to broader classes of P compounds, as summarized with respective chemical shift in Table 2. The chemical shift of the spectra spans signals from phosphonates (17.5 to 20 ppm) to polyphosphates (-18 to -20 ppm). Orthophosphate (5 to 7 ppm) and pyrophosphate (-4 to -5 ppm) are the dominating inorganic forms associated with humic compounds. Diester-P (2.5 to -1 ppm) contains phospholipids and nucleic acids. Monoester-P (3 to 6 ppm) comprises both, labile compounds such as adenosine monophosphate as well as inositol phosphates, which are more stable against microbial and enzymatic attacks in mineral soils, due to strong sorption to Fe and Al oxides in soils (Turner, 2008). In doing so, we do not correct for potential degradation products of RNA or phospholipids, that also end in the monester region, while their precursors actually belong to diester-P (Cade-Menun, 2015).

Amelung W, Rodionov A, Urusevskaja I, Haumaier L, Zech W (2001) Forms of organic phosphorus in zonal steppe soils of Russia assessed by 31P NMR. Geoderma 103(3-4):335–350. doi:10.1016/S0016-7061(01)00047-7

Cade-Menun BJ (2015) Improved peak identification in 31P-NMR spectra of environmental samples with a standardized method and peak library. Geoderma 257-258:102–114. doi:10.1016/j.geoderma.2014.12.016

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S5 The range of P values covered by our study (geosequence approach) and the range of P values covered by chronosequence analyses (literature review).

Soil P characteristics of soils along the P-geosequence in comparison with published results on soils along chronosequences

Study site		Geosequence (this study)					Chronosequence studies (cited literature)
		BBR	***MIT***	***VES***	***CON***	***LUE***	
P_{tot} stony fragments (5 – 2 mm size at 1 m soil depth) mg kg^{-1}		2776	642	2287	825	401	*not determined*
P_{tot} g m^{-2}		904 780	678 400	464 384	231 200	164 100	Hawai: 400 - 5500 (to 0.50 m depth*)* (Vitousek et al., 1995) Borneo: 30-60 (to 0.30 m depth) (Kitayama et al., 2004) Franz-Josef: 1292-76 (0.75 m*)* (Turner et al., 2013) Mexiko: 40 – 200 (Galván-Tejada et al., 2014)
		First line: to 1m soil depth Second line: to 0.75 m soil depth					
P_{tot} forest floor g m^{-2}		2.7	6.9	18.6	13.1	9.2	Franz-Josef. NZ: 0-9.2 (Turner and Condron, 2013)
P_{tot} forest floor % of profile		0.3 0.4	1.1 1.7	4.0 4.8	5.7 6.6	5.6 9.2	0 – 0.6 – 4 – 1.9 (Turner et al., 2013) (75 cm soil depth)
		First line: 1m soil depth Second line: 75 cm soil depth					
P_{tot} mg kg^{-1}	Oi	964	1091	1348	630	817	Mineral soil samples: Hawai: 30 – 2000 (Pearson and Vitousek, 2002) Franz-Josef. NZ: 100-800 (Richardson et al., 2004) Manawatu. NZ: 179-1013 (McDowell et al., 2007) Waitutu: 228 – 866 (Parfitt et al., 2005)
	Oe	1571	1243	1370	942	847	
	Oa	n.p.	1693	1322	1256	671	
	0-5	2966	1375	1017	929	195	
	5-10	3265	1094	978	663	119	

P_{citr} mg kg^{-1}	Oi	368	344	460	178	275	
	Oe	390	222	313	187	258	
	Oa	n.p	160	174	130	62	Arizona: 10-80 (Selmants and Hart, 2010)
	0-5	162	131	103	75	15	
	5-10	121	99	121	47	7	

n.p. horizon not present

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S6 Amount of sesquioxides, amount of labile and moderate labile P (including P extracted during resin, NaHCO3, and NaOH steps of the Hedley fractionation), and amount of total P (Ptot). Z represents the saturation index calculated as suggested by van der Zee and van Riemsdijk (1988): Z = P (labile+moderate labile)/0.5 (Alo + Feo) with all concentrations given in mmol kg^{-1}.

		Soil depth	Labile and moderate labile $P_{(i+o)}$	P_{tot} (HF-dig.)	Fe_O	Fe_{DCB}	Al_O	Z
		cm	mg kg^{-1}	mg kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}	
BBR	Ah1	0-7	1871	2966	29.3	30.4	8.4	0.14
	Ah2	7-14	1953	3266	35.2	36.5	11.8	0.12
	BA	14-23	1812	3263	34.0	35.9	13.7	0.10
	Bw1	33-51	1490	2286	23.6	27.5	12.9	0.11
MIT	Ah1	0-5	817	1375	13.1	13.1	7.8	0.10
	Ah2	5-10	671	1094	13.6	13.8	9.3	0.07
	BA	10-13	601	1017	15.5	15.8	12.9	0.05
	Bw1	13-29	465	1008	15.2	15.8	12.1	0.04
VES	Ah1	0-4	515	1017	3.4	11.4	2.6	0.21
	Ah2	4-9	564	978	9.9	14.9	4.3	0.11
	BA	9-15	594	931	11.1	15.3	7.1	0.08
	Bw1	15-21	633	1018	8.8	13.1	9.9	0.08
CON	Ah1	0-11	425	929	18.5	19.0	10.3	0.04
	BA	11-23	314	664	18.1	18.7	13.6	0.02
	Bw1	23-36	243	588	16.4	17.3	14.5	0.02
LUE	AE	0-7	89	196	0.9	1.7	0.3	0.21
	E	7-12	51	119	1.4	2.8	0.4	0.08
	Bsh	12-20	39	118	3.4	4.1	0.7	0.03
	Bws	20-35	33	129	3.5	4.1	1.2	0.02

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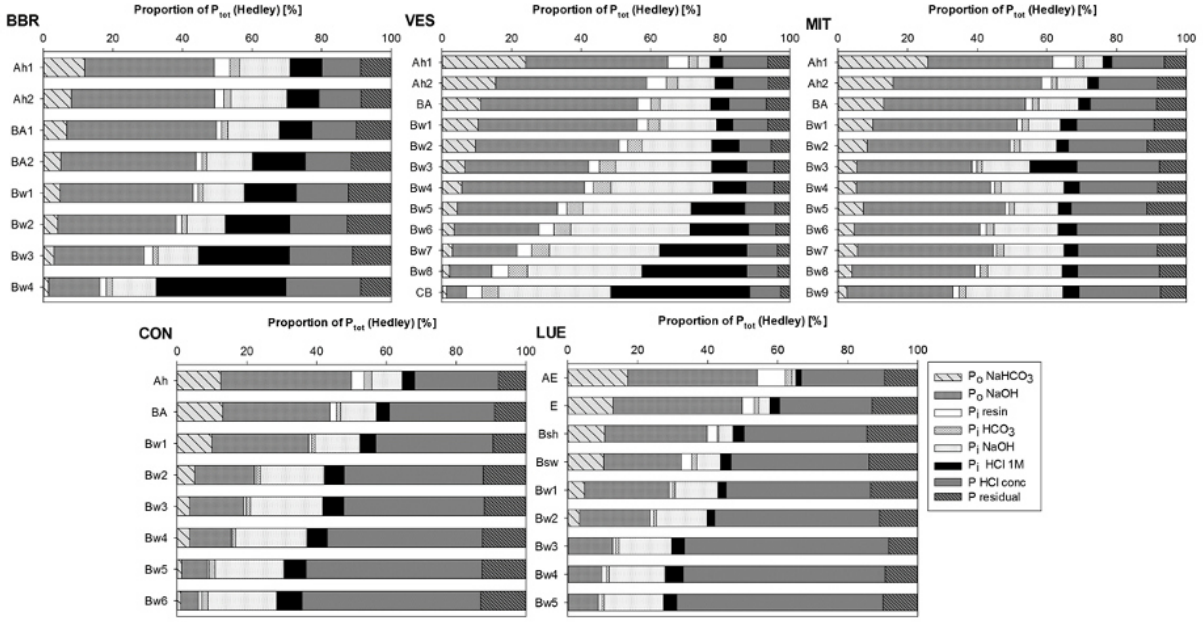
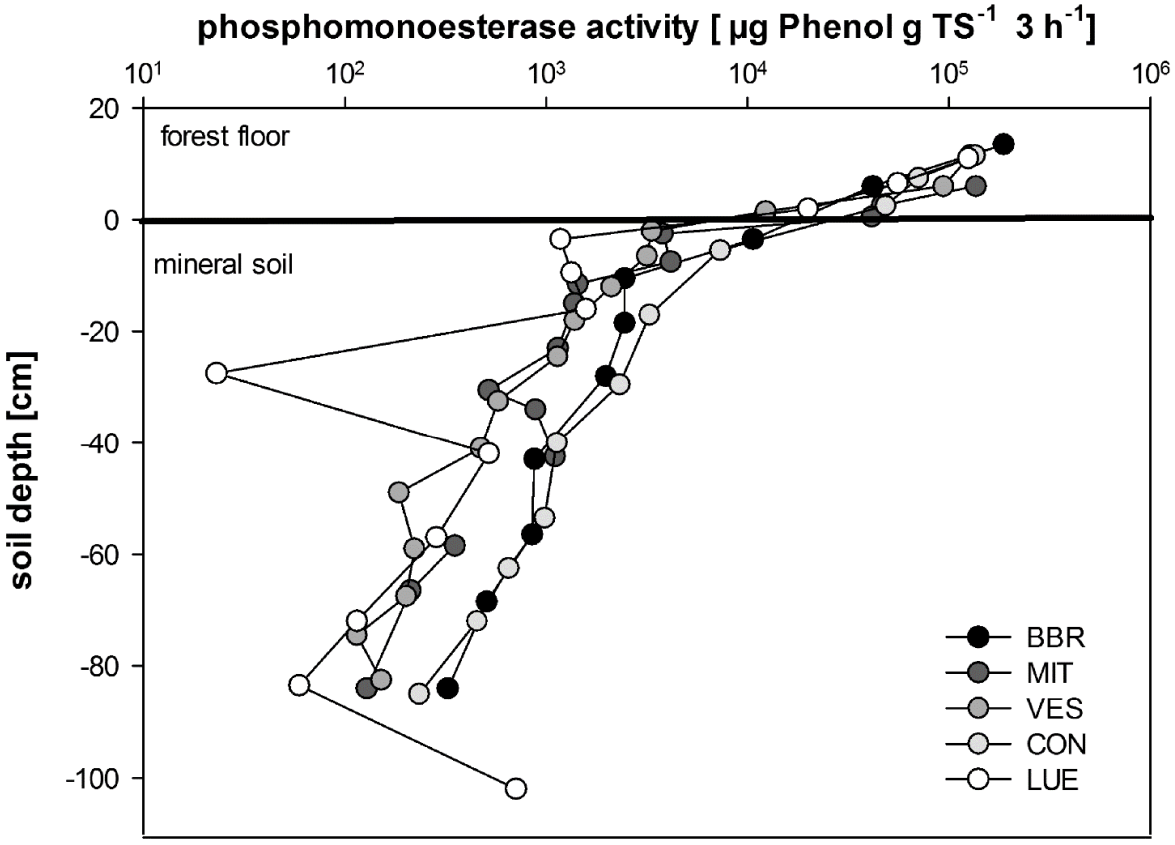
S7 Distribution of P over Hedley fractions in different soil horizons down to 1 m soil depth. Arabic numerals represent different sampling layers of one horizon and not different horizons. Each segment of the bars represents one of the Hedley fractions following the order: P_o NaHCO3, P_o NaOH, P resin, P_i NaHCO3, P_i NaOH, P 1M HCl, P HCl_{con}, P residual (acid digestion).



S8 Phosphomonoesterase activity at different soil depth and study sites.



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H List of publications and manuscripts

(Publications and manuscripts that are included in this study or collaboration work that is related to the topic)

- Bergkemper, F., Schöler, A., Engel, M., Lang, F., Krüger, J., Schloter, M. & Schulz, S. (2015) Phosphorus depletion in forest soils shapes bacterial communities towards phosphorus recycling systems. *Environ Microbiol*, 18(6), 1988-2000.

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