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Bacterial phosphorus turnover in agricultural soils and the effect of different fertilizer amendments

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Meiner Familie

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Zusammenfassung

Posphor (P) ist ein unersetzliches Element für alle Lebensformen auf der Erde. Seine Bedeutung für die landwirtschaftliche Produktion spiegelt sich darin wider, dass P einer der wichtigsten pflanzlichen Makronährstoffe ist, so dass eine ausreichende Versorgung notwendig ist, um hohe Ernteerträge zu erzielen. Es gibt jedoch auch die Kehrseite der Medaille. Einerseits stammt der Großteil des in der Landwirtschaft ausgebrachten P aus Rohphosphat, das eine begrenzte Ressource ist. Andererseits hat die übermäßige P-Düngung in den letzten Jahrzehnten zu negativen Folgen für die Umwelt geführt. Mikroorganismen im Boden sind Schlüsselkomponenten für zahlreiche Ökosystemfunktionen, einschließlich des P-Zyklus. Durch Mineralisierungs- und Solubilisierungsprozesse sowie den Einbau und die Speicherung von P in ihrer Biomasse wirken Bakterien sowohl als Senke als auch als Quelle für P im Boden.

Daher ist es notwendig, die Einflussfaktoren des P-Zyklus genauer zu verstehen. Diese Erkenntnisse werden durch nachhaltige Düngestrategien und alternative P-Dünger ergänzt, die dazu beitragen können, hohe Ernteerträge für eine ständig wachsende Bevölkerung zu sichern und gleichzeitig nicht erneuerbare Ressourcen nachhaltig zu nutzen und somit die Umwelt zu schützen. Das Hauptziel dieser Doktorarbeit war es, das Potenzial des bakteriellen P-Umsatzes zur Verringerung des P-Düngereinsatzes in der Landwirtschaft zu erforschen und die Realisierbarkeit alternativer P-Quellen zu untersuchen.

Dazu wurden sowohl der metagenomische als auch der quantitative Polymerase-Kettenreaktion (qPCR) Ansatz angewandt. Die Abundanz von Bakteriengenen, von denen bekannt ist, dass sie an P-Umsetzungsprozessen im Boden beteiligt sind, dient stellvertretend für die Gesamt- und P-Cycling-Gemeinschaftsstruktur sowie für P-Umsetzungsprozesse unter organischen, mineralischen und einer Kombination aus organischen und mineralischen sowie entweder konventionellen (TSP) oder alternativen P-Düngern (BC und BC^{plus}). Die Ergebnisse wurden des Weiteren durch Daten über die potenziellen Aktivitäten der P-akquirierenden Enzyme ergänzt.

Die metagenomische Analyse ergab, dass die Gesamtstruktur der Bakteriengemeinschaft weder von der Düngung noch von der Zeit der Probenahme beeinflusst wurde. Der Aufbau der Familien, die Gene beherbergten, welche am P-Umsatz beteiligt sind, wurde jedoch durch die Zugabe von

organischem Dünger beeinflusst. Die Familien, die in den Datensätzen am häufigsten vorkamen, waren *Acidobacteriaceae*, *Bradyrhizobiaceae*, *Verrucomicrobia* subd. 3, *Planctomycetaceae* und *Chitinophagaceae*. Keine der identifizierten Familien deckte alle bekannten Prozesse ab, die mit dem bakteriellen P-Umsatz im Boden verbunden sind, d. h. Solubilisierung, Mineralisierung und P-Aufnahme. Einige Familien leisteten einen eher universellen Beitrag zum P-Umsatz, während andere lediglich Gene zur P-Aufnahme aufwiesen, ohne nachweisbare Fähigkeit zur P-Solubilisierung oder Mineralisierung. Unabhängig von der angewandten Methode (Metagenomik oder qPCR), der geografischen Lage, Jahreszeit oder der Art von eingesetztem Dünger war das am häufigsten vorkommenden Gen in den Datensätzen die Quinoprotein-Glukose-Dehydrogenase (*gcd*), einem Enzym, das an der Solubilisierung von anorganisch gebundenem P beteiligt ist. Obwohl die potenziellen Phosphatase-Aktivitäten unter organischer Düngung, signifikant erhöht waren, gab es keine entsprechende Änderung der Abundanz von Phosphatase-Genen. Dies deutet darauf hin, dass die Wirkung eher auf die Transkriptionsrate zurückzuführen ist. Darüber hinaus war die Abundanz von Genen, die für Phosphatasen kodieren, in Böden mit sehr niedrigem, niedrigem und optimalem P-Gehalt stabil und zeigte keine signifikante Reaktion auf konventionelle oder alternative P-Düngerzusätze. Im Allgemeinen beeinflussten alternative P-Dünger die P-Mobilisierung, jedoch begünstigte ihr Einsatz unterschiedliche Prozesse. BCplus begünstigte die bakterielle P-Solubilisierung, was sich in einer höheren *gcd*-Abundanz widerspiegelt, während BC das P-Recycling aus der Biomasse förderte. Darüber hinaus waren die Auswirkungen stark mit dem anfänglichen P-Gehalt im Boden und dem Wachstumsstadium der Pflanzen verknüpft. Diese Arbeit bietet grundlegende Einblicke in die mikrobielle Gesamt- und P-Kreislauf-Gemeinschaft und zeigt die Auswirkungen verschiedener Düngemittelzusätze auf das mikrobielle Potenzial für den P-Umsatz im Boden in landwirtschaftlichen Ökosystemen.

Summary

Phosphorus (P) is an irreplaceable element for all life forms on earth. Its significance for agricultural production is reflected by P being one of the most important plant macronutrients, and consequently an adequate supply is necessary for maintaining high crop yields. There is, however, the flipside of the coin. On the one hand, the majority of P applied in agriculture comes from rock phosphate, which is a limited resource. On the other hand, excessive P fertilization over the past few decades has led to negative environmental consequences. Soil microorganisms are key components for numerous ecosystem functions, including P cycling. Through processes of mineralization, solubilization, as well as incorporation and storage of P in their biomass, bacteria act as both sink and source of P in soil.

Therefore, it is necessary to obtain a more detailed understanding of P cycle drivers. Soil microbes have the potential to mobilize soil P reserves and can increase plant-available P. These findings are complemented by sustainable fertilizing strategies and alternative P fertilizers which can assist to maintain high crop yields for an ever-growing human population while sustainably using non-renewable resources and protecting the environment. Thus, the main aim of this PhD thesis was to explore bacterial P turnover potential to reduce P fertilizer input in agriculture and to investigate the viability of alternative P sources. This was accomplished by applying metagenomic and quantitative polymerase chain reaction (qPCR) approaches where abundances of bacterial genes known to be involved in soil P turnover processes were used as a proxy to investigate the total and P-cycling community structure as well as P turnover processes under organic, mineral, and a combination of organic and mineral, as well as either conventional (TSP) or alternative P fertilizers (BC and BC^{plus}).

These results were complimented by data on potential activities of P acquiring enzymes.

Metagenomic analysis revealed that the total bacterial community structure was neither influenced by fertilization nor sampling season. However, community structure of families harboring genes involved in P turnover was affected by organic fertilizer amendment. The most abundant families were *Acidobacteriaceae*, *Bradyrhizobiaceae*, *Verrucomicrobia* subd. 3, *Planctomycetaceae* and *Chitinophagaceae*. None of the identified families covered all known processes associated to bacterial P turnover in soil, i.e., solubilization, mineralization, and P

uptake. Some families showed a more universal contribution to P turnover, whereas others harbored only P uptake genes, without capabilities for P solubilization or mineralization. Irrespective of the method applied (metagenomic or qPCR), geographical location, season, or fertilization treatment, the most abundant gene in the datasets was that of quinoprotein glucose dehydrogenase (*gcd*), an enzyme involved in solubilization of inorganically bound P. The second most abundant genes were those involved in P uptake, followed by genes involved in P mineralization. Although potential phosphatase activities were significantly increased in treatments where organic fertilizer was applied, there was no corresponding change in phosphatase gene abundances, suggesting that the effect more likely occurs on rate of transcription rather than on the DNA level. Moreover, the abundances of genes encoding phosphatases were stable across soils with very low, low, and optimal P content, and showed no significant response to either conventional or alternative P fertilizer amendments. In general, alternative P fertilizers affected P-mobilization, but they differed in the processes they promoted. BC^{plus} favored bacterial P solubilization, as indicated by higher *gcd* abundance, whereas BC favored P recycling from biomass. Additionally, the effects were strongly interlinked with initial soil P content and plant growth stage. This study provides fundamental insights into the total and P cycling microbial community and reveals the effects of different fertilizer amendments on the microbial potential for soil P turnover in agricultural ecosystems.

I Introduction

1. The essential role of P

Phosphorus (P) is an essential nutrient for all forms of life. It is a key element for numerous biological processes, including storage of genetic information, cell development via synthesis of nucleic acids and cell membranes, as well as synthesis of adenosine triphosphate and energy transfer (Caballero et al., 2003). P is therefore a ubiquitous element vital for the survival and proper functioning of all living organisms.

In crop production, phosphorus is the second most limiting macronutrient after nitrogen (N) and its deficiency delays plant maturity and reduces yields (Carstensen et al., 2019; He et al., 2017). Unlike other macronutrients (carbon, sulphur, and nitrogen), P lacks an atmospheric gaseous component, which is why the P cycle has often been described as the “broken cycle” (Slocombe et al., 2020). Most of the phosphorus is locked in sediments and rocks, and as such cannot be directly taken up and used by plants or microorganisms (Rengel & Marschner, 2005). The release of bound P in soil is dependent on the interplay of abiotic factors such as soil type, pH, temperature, and soil biota, especially microorganisms and plant roots.

The modern terrestrial P cycle (Fig. 1) is dominated by agriculture and human activity, with fertilization playing a significant role (Filippelli, 2008).

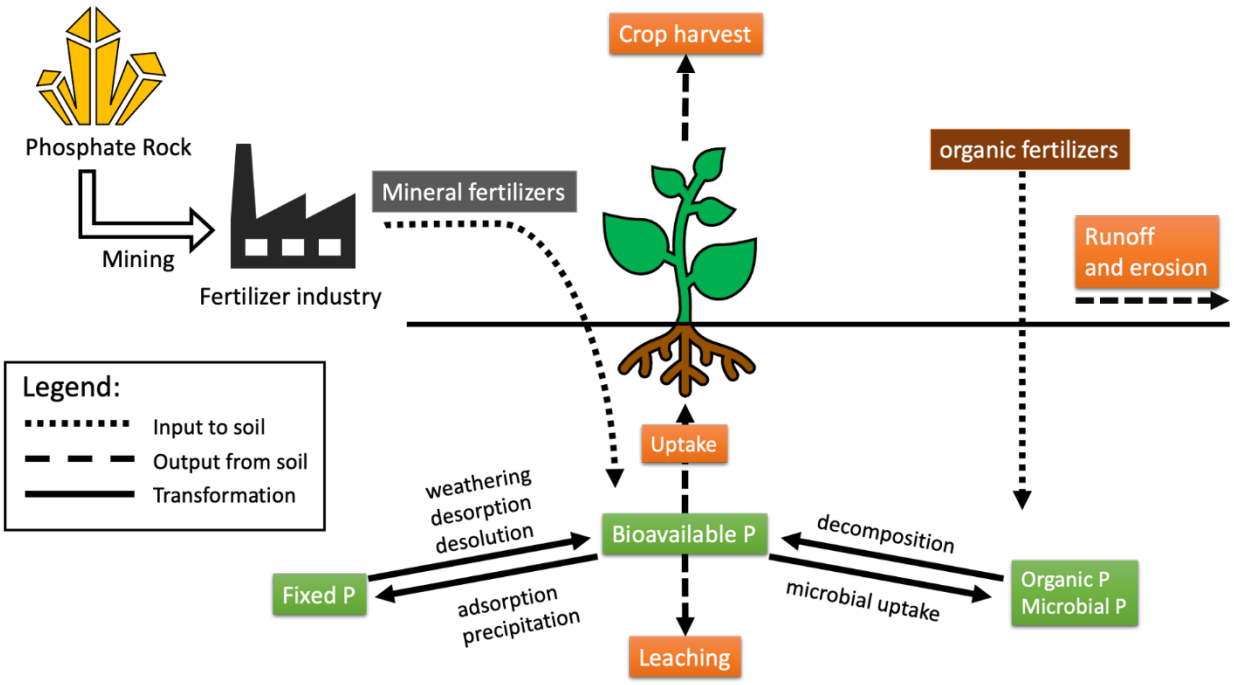

Fig. 1 Terrestrial Phosphorus Cycle. Schematic representation of the terrestrial Phosphorus (P) cycle. Orange labels depict loss of P from soil, green labels depict forms of P present in soil.

To ensure high crop yields necessary to provide food security for an ever-growing human population, agriculture relies on the addition of external phosphorus derived from geological sources (Elser & Bennett, 2011). Unfortunately, excessive amounts of applied P in the last decades disregarded the existing content and dynamics of P in soil, and often exceeded plant P requirements leading to serious ecological problems. Considering that more than 85% of P applied in agriculture comes from non-renewable phosphate rock on one hand, and considerable amounts of bound P in soils with potentially negative ecological implications on the other(Cordell & White, 2011; Nedelciu et al., 2019), sustainable crop production will have to rely on alternative sources for P fertilization and utilizing microorganisms to access soil P reserves.

2. Forms of P in soil

Phosphorus exists in soil in inorganic P (P_i) and organic P (P_o) forms, which differ in their behavior and fate (Fig. 2) (Hansen et al., 2004; Turner, 2008).

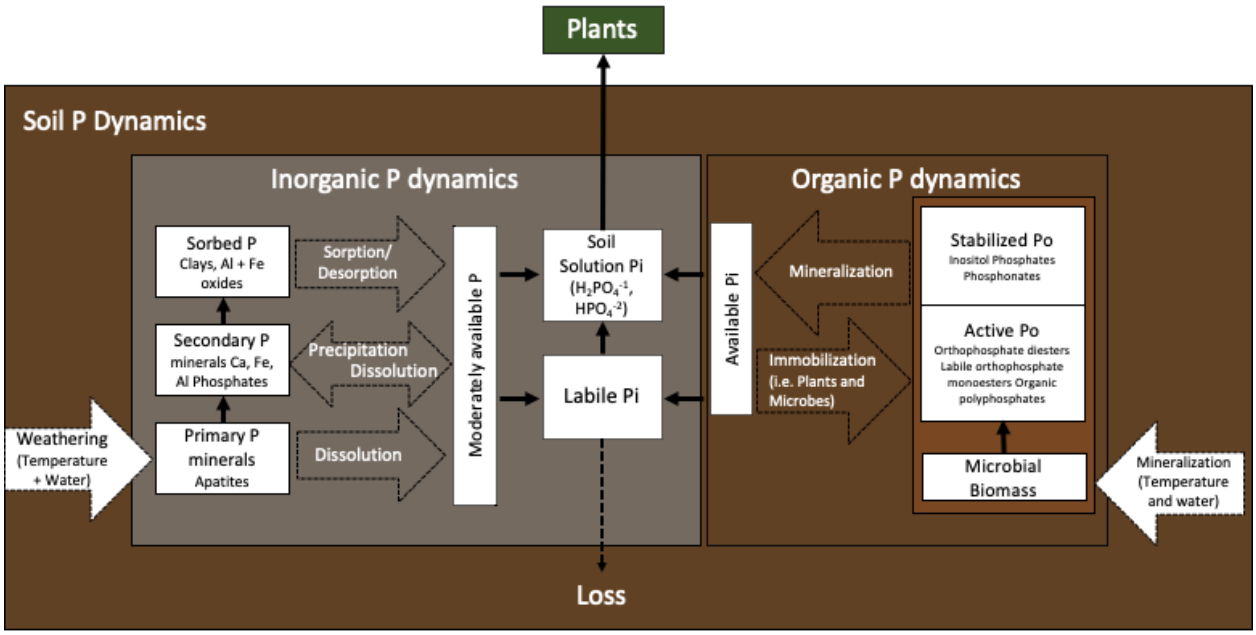

Fig. 2 Soil P Dynamics: Schematic representation of the Phosphorus cycle in soil.

Depending on soil type, parent material, and climate, P_i usually accounts for 35% to 70% of total P in soil (Harrison, 1987). P_i exists in following forms: primary P minerals (apatites, strengite, and variscite), which are very stable and from which available P can be released by weathering; secondary P minerals, such as Al^{3+}, Fe^{3+}, and Ca^{2+} phosphates, whose dissolution rates depend on size of mineral particles and soil pH (Oelkers & Valsami-Jones, 2008; Pierzynski et al., 2015); P adsorbed on clay particles and Al/Fe oxides, which can be released by desorption reactions (Shen et al., 2011). P_o accounts for 30% to 65% of the total P in soil (Sims et al., 2005) and mainly exists in stabilized forms as inositol phosphates and phosphonates, active forms as orthophosphate diesters, labile orthophosphate monoesters, and organic polyphosphates (Sims et al., 2005; Turner et al., 2002). P_o can be released by processes of mineralization through microbial activity and plant root exudates, which are influenced by soil moisture, temperature, surface physicochemical properties, soil pH, and soil redox potential (Shen et al., 2011).

Plants take up P dissolved in the soil solution in form of $H_2PO_4^-$ and HPO_4^{2-}(Bucher & Bucher, 2007; Richardson & Simpson, 2011). Although P reserves in soil might far exceed the plants' needs, only a small fraction is available for plant uptake (Smil, 2000; Sohrt et al., 2017). In comparison to other macronutrients, phosphorus concentration in soil solution is much lower and ranges from 0.001 mg L^{-1} to 1 mg L^{-1} (Brady & Weil, 2008). Crop requirement for P varies between species and cultivars, but generally ranges between 0.3 and 0.5 kg P ha^{-1} each day during phase of rapid growth (Johnston et al., 2014), and adequate supply of available P is crucial for obtaining high yields.

3. Environmental drivers for P turnover

The main environmental factors for P turnover are soil edaphic parameters such as soil type, pH, temperature, moisture, aeration, and salinity as well as plant growth phase and species. P availability from the different bound forms in soil depends largely on weathering of the parent material which is dependent on the soil type (Audette et al., 2016; Börling et al., 2001; Brennan et al., 1994; H. Li et al., 2020; L. Li et al., 2007; Luo et al., 2009; Walker & Syers, 1976; Wang & Tzou, 1995; Zhang et al., 2015). Organic (P_o) and inorganic (P_i) soil P pools are differently affected by abiotic factors. P_o in its stable form is present as inositol phosphates and phosphonates. By climate weathering it is then converted to its active forms, which are either orthophosphate diesters, labile orthophosphate monoesters, or organic polyphosphate. Unlike P_o, P_i is sorbed on clays as well as bound by metals (Al and Fe) and is released through desorption. P can be immobilized by precipitation forming secondary P minerals with Ca, Al, and Fe (i.e., apatites) and can be made available through dissolution. Sorption of P by secondary minerals is pH dependent. The optimum pH for P availability is between 6 and 7.5, whereas under low or high pH conditions P binds with divalent cations such as Al^{2+}, Fe^{2+} or Ca^{2+} (Mora et al., 2004). Temperature also plays a vital role as it influences soil organic matter (SOM) decomposition thereby affecting the rate of P release. This has been demonstrated recently by Geng et al. (2017) and Jiao et al. (2016) who showed that SOM decomposition and P release increased with increased temperature. Soil moisture, aeration, and salinity also affect organic matter decomposition thereby affecting the rate of P mineralization (USDA - NRCS). Plant species and growth phase affect P availability through uptake and release of P solubilizing compounds (Hirzel & Undurraga, 2013). Plants exude

organic acids, such as malate and citrate, which in their deprotonated form chelate Al^{3+}, Fe^{3+}, and Ca^{2+} thereby mobilizing bound P_i (Hinsinger, 2001). However, this metabolic adaptation to low P availability is not present in all species, and even differs among cultivars and genotypes (Wang & Lambers, 2020). Additionally, the composition of exuded organic acids is highly variable and dependents on plant species (Badri & Vivanco, 2009; Curl & Truelove, 1986; Hale et al., 1971). To access P that is bound in organic compounds, plants can secrete phosphohydrolase (Abel et al., 2002; Marschner, 1995; Vance et al., 2003), but the importance of this P acquiring strategy has been shown to vary with species, cropping system, and forms of organic P in the soil (George et al., 2005; Liang & Li, 2003; Yun & Kaeppler, 2001). Plants' uptake of P varies during the season with about 50% of the plants' total requirement being absorbed by the time they have accumulated 25% of their total seasonal dry mass (Black, 1968).

4. Fertilization practices to ensure sufficient P supply

Although cultivation and farming go back to the Neolithic era, and through centuries many attempts to increase yields by adding various mineral and organic substances have been applied, until the mid 19th century the approach to fertilization was highly empirical (Hignett, 1985). The foundations of the modern fertilizer industry were laid by Justus von Liebig in 1840, who recognized the importance of inorganic minerals in plant nutrition, and Lawes, who in 1842 started the first commercial superphosphate production (Hignett, 1985). These discoveries prompted the development of a variety of P fertilizers such as Single super phosphate (SSP), Triple super phosphate (TSP), Monoammonium phosphate (MAP), Diammonium phosphate (DAP) and Nitrophosphate, most of which are based on treatment of phosphate rock with sulfuric acid.

The application of P fertilizers has quadrupled since 1961, from 4.6 million tons to approximately 21 million tons in 2015 (Mogollón et al., 2018). On the one hand, this has enabled considerable increases in yield, contributing to the green revolution and food security (Sharpley et al., 2018), but on the other hand, the lack of scientific and public understanding has led to overuse of P fertilizers (Haygarth et al., 2013). For example, for the period between 1967 and 2007 it has been calculated that the cumulative P application (550 kg ha^{-1}) was more than double of the crop cumulative uptake (225 kg ha^{-1}) globally (MacDonald et al., 2011), and recent research shows

that global surpluses have built up at an estimated 11–16 MT P $year^{-1}$ (Bouwman et al., 2017; Lu & Tian, 2017; Sattari et al., 2012). This excess P application has no added benefit for yield – a comparison of P input for maize, rice, and wheat with simulation where P input was optimized for crop uptake, suggested that currently 34%, 82%, and 70% of P applications for the three crops respectively, could be eliminated without reducing crop yields (J. Liu et al., 2018). Although much of the applied P binds to soil, creating a pool of residual P, a considerable amount is lost via leaching, runoff, and/or erosion and may reach waterbodies, leading to eutrophication (Carpenter, 2008; Childers et al., 2011; Conijn et al., 2018; Grizzetti, 2012; W. Liu et al., 2018; Powers et al., 2016).

However, 32% of the world's cropland and 43% of pastures are still P deficient (Lun et al., 2017), making P fertilization unavoidable. Following a growing demand on P fertilizers, the mining of rock phosphate has also quadrupled over the past half century (Scholz et al., 2013), raising concerns about future shortages and food security (Cordell et al., 2009; Peñuelas et al., 2013; Vuuren et al., 2010). The increasing scarcity of high-quality rock phosphates prompted the search for alternative P sources for fertilizers (Cordell et al., 2009).

One option is the use of organic fertilizers such as animal manures, sewage sludge, plant substances, and compost. In addition to providing macro- and micronutrients to plants and soil microorganisms, thereby positively affecting soil biodiversity and biological activity, organic fertilizers improve soil quality by increasing SOM and moisture retention, improving soil structure, and protecting soil against erosion (Roba, 2018) However, it has to be taken into account that organic fertilizers have relatively low nutrient content and highly variable composition, which makes the accurate application of nutrients to meet plants' needs difficult. In addition, transformations of organic compounds and nutrient release are dependent on microbial activities and therefore, affected by seasonal dynamics (Djukic et al., 2018). The amount of P in organic fertilizers varies between different materials, for example, green waste compost contains on average 0.22% P, liquid biosolids 3.75% P, and 0.3 - 1.4% P in dried manure (Möller et al., 2018).

Another type of fertilizer that has gained popularity in the last few decades is biochar. Biochar is a carbon-rich solid material obtained from the carbonization of biomass which is produced

through application of heat or chemicals on organic materials (Atkinson et al., 2010; Karhu et al., 2011; Lehmann et al., 2007; Novak et al., 2009) by processes such as pyrolysis (Ronsse et al., 2013), gasification, hydrothermal carbonization, flash carbonization (Antal & Grønli, 2003; Chen et al., 2016; Wade et al., 2006), and torrefaction (Benavente & Fullana, 2015). Numerous recent studies have shown that application of biochar has multiple positive effects on soil quality (Barrow, 2012), including high nutrient content and increased soil C through sequestration (Lehmann et al., 2007; Li et al., 2019), increased water-holding capacity and nutrient retention, correction of soil acidity, habitats for soil microbes, control of plant pathogens, and improved mycorrhizal competence (Hammer et al., 2015), as well as positive environmental effects such as reduction in greenhouse gas emissions, and adsorption of heavy metals (Hagemann et al., 2018; Jia et al., 2018; Kong et al., 2018; Thangarajan et al., 2018; Turk Sekulić et al., 2018; Xu et al., 2016). Biochar characteristics differ depending on type of feedstock and the production variables such as highest treatment temperature and holding time at the highest temperature (Enders et al., 2012; Novak et al., 2009).

P content of biochar highly depends on the type of feedstock used to produce biochar. A study comparing different feedstocks showed that bonechar (BC) – a biochar obtained by pyrolyzing defatted animal bones at 600 to 800 °C (Siebers & Leinweber, 2013) – has highest content of both total, as well as bioavailable P (Li et al., 2019).

BC contains roughly 130 to 150 g P kg^{-1}, 280 g Ca kg^{-1}, and 6.5 g Mg kg^{-1} (Siebers et al., 2012; Warren et al., 2009), and in contrast to biochar that is obtained from plant material, contains low amounts of organic C (Zwetsloot et al., 2016). Therefore, BC is a strong candidate as an alternative to mineral P fertilizers (Morshedizad et al., 2016; Morshedizad & Leinweber, 2017; Siebers & Leinweber, 2013; Warren et al., 2009; Zwetsloot et al., 2016). The solubility of BC has been reported to be between the solubility of rock phosphate and that of TSP, and highly depends on abiotic factors such as pH, soil type, temperature, and water content (Warren et al., 2009).

Since many studies have proven that microbial sulfur (S) oxidation enhances solubilization of P from rock phosphate, and thus facilitates P uptake by plants (Besharati & Atashnama, 2007; Pathiratna et al., 1989), a novel approach to increase P solubility of BC is a surface modification by adding S. An incubation experiment with surface-modified BC (BC^{plus}) under equilibrium

conditions revealed larger P dissolution than treatments with BC in all studied soils over a wide range of pH (Morshedizad et al., 2016). The pot experiment with annual rye grass and treatments without P (P0), BC, BC^{plus} and TSP showed that the cumulative dry matter yield of BC was similar to P0, and that of BC^{plus} similar to TSP, concluding that BC^{plus} has a potential as a P and S fertilizer (Zimmer et al., 2019).

5. Microbial P turnover in agricultural soils

Soil microorganisms play a vital role in nutrient cycling, energy flow, and ecosystem productivity (Ohtonen et al., 1997). In agricultural soils they can enhance plant nutrient acquisition by transforming insoluble soil nutrients (Babalola & Glick, 2012). Soil P dynamic is highly dependent on soil microorganisms (Bünemann et al., 2011; Richardson & Simpson, 2011). Soil microbial P pool (P_{mic}) is a critical part of P turnover, as it serves both as P source for plants by releasing bound P, as well as a temporal P sink by immobilizing inorganic P (Bilyera et al., 2018). Amount of P captured in microorganisms is estimated to be 2% to 10% of total soil P (Achat et al., 2010), and is generally equivalent or higher than that held in the plant biomass (Richardson & Simpson, 2011). P_{mic} content of agricultural soils varies in range from 5 to 70 kg P ha^{-1}, with turnover times of a few months, depending on management and C inputs (Oehl et al., 2001). Main factors affecting P_{mic} are soil type, soil texture, and SOM (Turner & Haygarth, 2005). Soils in organic farming systems where no pesticides and only organic fertilizers are applied contain almost twice as much P_{mic} as conventionally cultivated soils fertilized with mineral fertilizers (Oberson et al., 1996; Oehl et al., 2001). The uptake and release of P by microorganisms strongly affects the amount of P that is available for plant uptake (Turner & Haygarth, 2005). P incorporated in microbial biomass represents a temporarily unavailable P pool for plant uptake, but P immobilized in this way is released when cells die, so in the long term, all microbial P is potentially available. Therefore, it has been suggested that the immobilization of P in microbial biomass is an important mechanism for regulating P supply in soil solution (Seeling & Zasoski, 1993) and for preventing it from reactions with soil particles (Olander & Vitousek, 2004).

6. Microorganisms facilitating P transformations in soil

Figure 3 depicts P transformations in soil. A group of microorganisms involved in solubilization and mineralization of bound P is referred to as Phosphorus Solubilizing Microorganisms (PSM), and includes bacteria, fungi, actinomycetes, and algae (Alori et al., 2017). PSM are able to solubilize P_i by secreting organic acids, such as citric, oxalic, and succinic acids, in the surrounding area, and mineralize P_o by secreting enzymes such as phosphatases and phytases that hydrolytically cleave bound P (Tomer et al., 2017).

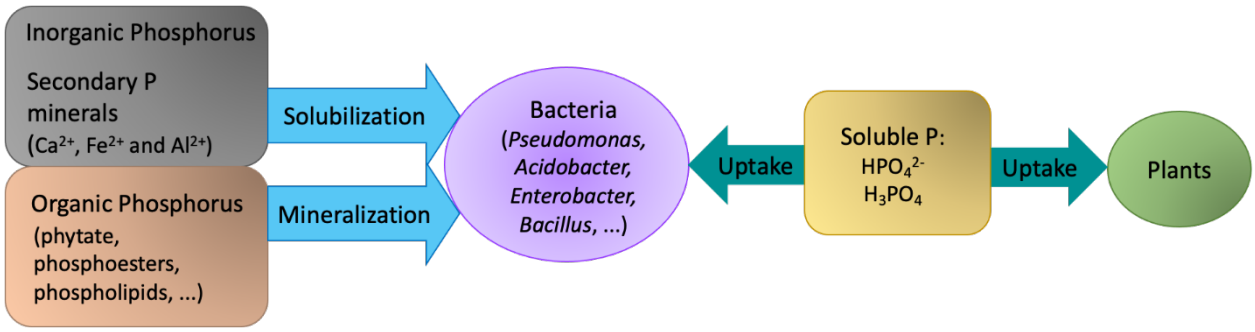

Fig. 3 Schematic representation of the central role of P solubilizing bacteria in soil and their contribution on accessing inorganic and organic sources of P.

P is then released into the soil solution thereby increasing its bioavailability for plant uptake (Bergkemper, et al., 2016a; Khan et al., 2009; Richardson & Simpson, 2011; Zhu et al., 2011). Phosphatases catalyze the hydrolysis of both esters and anhydrides of phosphoric acid (Condron et al., 2015), and can be classified as phosphomonoesterases, phosphodiesterases, triphosphoric diester hydrolases, and enzymes acting on phosphoryl-containing anhydrides, and on P-N bonds (Nannipieri et al., 2011b). Since phosphomonoesters comprise approximately 68% to 98% of organic P (Nannipieri et al., 2011b; Turner & Engelbrecht, 2011; Turner & Haygarth, 2005), it is not surprising that they are the most studied group of P mineralizing enzymes. They include acid and alkaline phosphomonoesterases (ACP and ALP, respectively), phosphoprotein phosphatases, phythases, and nucleotidases. Unlike phosphodiesterases, that hydrolyze ester bonds in phosphodiester compounds and do not produce inorganic phosphates, ACP and ALP can hydrolyze lower-order inositol phosphates thereby releasing free phosphate for biological uptake (Makoi & Ndakidemi, 2008). Whereas ALPs are mainly produced by bacteria, ACP and phytases can be derived from plants, fungi, and bacteria (Nannipieri et al., 2011b). The quantity of

phosphatases in soil varies depending on microbial biomass, amount of SOM, as well as on applied agricultural practices, with fertilization and tillage being the most important ones (Banerjee et al., 2012).

Another important mechanism for sufficient P supply of plants in agricultural soils is symbiotic relationship with the arbuscular mycorrhizal (AM) fungi which are known for their ability to take up and transfer P and other growth limiting nutrients from soil to plants. AM hyphae extend the plant's rooting zone thereby providing access to P that otherwise would not be reachable. Hyphae have higher affinity for phosphate and lower threshold concentration for absorption, which allows for P uptake under low P concentrations (Bolan, 1991). AM fungi can increase P availability also through solubilization by excretion of organic acids (Tawaraya et al., 2006) as well as through mineralization of organic P by production of phosphatases (Koide & Kabir, 2000).

Understanding the microbial P turnover processes in agricultural ecosystems facilitates the development of sustainable strategies for food production. Although it is well known that microorganisms such as fungi contribute immensely to plant phosphorus uptake and overall nutrition, the focus of this PhD thesis was placed on bacteria for the following reasons. Firstly, at the timepoint of investigation, databases showed biases towards sequences from often studied organisms with emphasis on bacteria (Wooley, Godzik and Friedberg, 2010). Secondly, although fungi can significantly contribute to plant P uptake and overall nutrition, this applies mainly for undisturbed soil. Lower amounts of fungi are found in agricultural soil due to constant disruption of the communities through tillage (Miller & Lodge 1997) as well as the application of fertilizers and fungicides (De Vries et al. 2015). Another problem is that fungal genes contain many intronic sequences which require long reads in order to accurately annotate them (De Vries et al. 2015). Lastly, all investigations presented here were performed on bulk soil, excluding roots and therefor a great part of mycorrhiza.

7. Bacterial P mobilizing processes and underlying molecular mechanisms

The main bacterial P turnover processes encompass solubilization of inorganically bound P, mineralization of organically bound P, as well as immobilization of P in microbial biomass and subsequent release upon cell death. Bacterial genera that have been recognized as P solubilizing bacteria include different strains of *Pseudomonas*, *Bacillus*, *Burkholderia*, *Agrobacterium*,

Micrococcus, *Achromobacter*, *Aerobacter*, *Flavobacterium*, *Rhizobium*, *Enterobacter*, *Aeromonas*, *Klebsiella*, *Mycobacterium*, *Acetobacter*, *Corynebacterium*, *Gluconacetobacter*, *Escherichia*, *Ralstonia*, *Serratia*, and *Erwinia* (Kaur and Kaur, 2020). These bacteria can solubilize fixed P_i by production of organic acids, chelation, and formation of soluble complexes with metal ions, as well as mineralize P_o by secretion of enzymes like phosphatases, phytases, and C-P lyases (Billah et al., 2019).

Based on Bergkemper et al. (2016b), bacterial genes coding for P transformation processes can be broadly categorized in six functional categories. These include genes for phosphoesterase, phytase, phosphonate degradation, inorganic phosphate solubilization, P transport, and regulation of phosphate starvation. The majority of genes involved in P turnover are located on the PHO regulon (Baek et al., 2006; Santos-Beneit, 2015), and are inducible by P_i limiting conditions in the environment (Wanner, 1996). P turnover genes that are not regulated by PHO regulon include those encoding for solubilization (*gcd*), nonspecific acid phosphohydrolases (*phoN*), and specific phytases (e.g., *appA*).

7.1. Bacterial P-uptake

The preferential source of P for bacteria is its inorganic form (P_i). Bacteria have two main uptake mechanisms for obtaining P_i which are the phosphorus specific transporter (PstS) and inorganic phosphorus transporters (PitA and PitB). PstS is a high velocity, high affinity, and highly specific ABC-type P_i transport system PstSCAB (Amemura et al., 1982; Magota et al., 1984; Nakata et al., 1984; Rees et al., 2009; Willsky & Malamy, 1980; Yuan et al., 2006) which is active under P limiting conditions (Richardson & Simpson, 2011). It transports P_i and polyphosphates into the cytoplasm and also functions as a sensor of external P_i concentrations (Gardner et al., 2014; Kimura et al., 2009; Peterson et al., 2005; Shi & Hulett, 1999). Another P_i transport mechanism is mediated by the phosphorus inorganic transport systems – PitA and PitB (Harris et al., 2001). *PitA* gene is expressed constitutively and uses proton motive force within the periplasmic space of the cell. In contrast, *pitB* is expressed in low-phosphate environments and, although it shares 81% sequence identity with *pitA*, it is repressed or inactivated by the PHO regulon (Harris et al., 2001; Rao & Torhani, 1990). This was also observed on the field scale by Dai et al. (2020) who reported a

general increase in the relative abundance of *pit* gene copies in agricultural soil under long term P fertilization. Furthermore, Tanuwidjaja et al. (2020) found a higher abundance of *pst* gene in different soil mixtures with low P but did not detect gene copies of *pit*.

Due to the possible limitation of P_i in the soil ecosystem, bacteria possess additional mechanisms to cover their needs. Alternative P sources are present as phosphate esters, phosphonic acids, and other reduced P compounds in soil (Wanner, 1996; White & Metcalf, 2007). Phosphorus esters can be utilized in two ways by bacterial cells. The first form is the release of extracellular phosphatase, mineralizing P and subsequently transporting it into the cell, and the other is transporting the esterized P as a whole and hydrolyzing it in the cytoplasm (Yang et al., 2009).

P esters are broken down and then taken up in form of glycerol-3-phosphate (G3P), glyceryl phosphoryl phosphodiesters by the Ugp (uptake of glycerol phosphate) transport system (Brzoska et al., 1994). Similar to PstSCAB, Ugp is a multicomponent transport system which utilizes ATP as an energy source (Brzoska et al., 1994). It has been reported to specifically transport glycerol-2-phosphate (G2P) and glycerol-3-phosphate (G3P) and is unable to transport P_i (Stasi et al., 2019). Besides P_i and esterized P, bacteria can access phosphonate by the phosphonate transporter (Phn). Stasi et al. (2019) have proven that this system is also able to transport phosphite, P-esters, and even P_i. However, these observations were made in pure culture studies and have not been further investigated in environmental samples.

7.2. Solubilization of P by bacteria

Solubilization of P by microorganisms was first reported back in 1948 by Pikovskaya. Since then, our knowledge on P solubilizing microorganisms has increased significantly (Bergkemper, et al., 2016a; Richardson & Simpson, 2011; Rodríguez & Fraga, 1999). Of microorganisms which can exploit minerally bound P, bacteria make up between 1% and 50% and fungi between 0.1% and 0.5% of the total population (Sharma et al., 2013).

The primary mechanism of orthophosphate release is by compounds which can dissolve it. These include organic acids, siderophores, protons, hydroxyl ions, and even CO_2 (Rodríguez & Fraga, 1999; Sharma et al., 2013). Bacteria produce organic acids such as citric, oxalic, malic, and gluconic acids in the periplasm via the direct oxidation pathway (Zhao et al., 2014), and

subsequently excrete these into the extracellular space (Goldstein, 1994a, 1994b; Liang et al., 2020). The acids then release mineral P by chelating phosphate-bound cations (i.e., Mg^{2+}, Ca^{2+}, Fe^{2+}, Al^{2+}) through their hydroxyl and carboxyl groups or by reducing the surrounding pH (acidification) and releasing P for plants to take up (Castagno et al., 2011; Kim et al., 1997; Seshachala & Tallapragada, 2012). Of the aforementioned organic acids, mineral P release mediated by gluconic acid production is the most extensively studied mechanism (Goldstein, 1995). The glucose dehydrogenase (GDH) enzyme is encoded by the *gcd* gene and is responsible for the conversion of glucose and other aldose sugars to gluconic acid. However, it requires the cofactor pyrroloquinoline quinone (PQQ) for its full function. Besides the cofactor, solubilization of P is influenced by P and C availability in the given environment. Although PQQ is regarded as essential for the activity of the GDH enzyme, evidence suggests that there is no correlation between the PQQ and GDH levels (Schie et al., 1984; van Kleef & Duine, 1989). Nevertheless, An & Moe (2016) observed that PQQ levels are not random and its transcription follows the C availability. Additionally, they found that *gcd* expression is not constitutive and is increased under low P levels. High abundance of *gcd* in sites with low soil P was also observed by Liang et al. (2020) who were searching for novel phosphate solubilizing bacteria in former mining sites undergoing ecological restoration. Interestingly, relative abundance of *gcd* did not decrease when fields received additional P input (Dai et al., 2020). Regarding the taxonomic affiliation of *gcd* harboring bacteria, only a few culture-free studies in environmental samples have been conducted by the time of writing this thesis. Yang et al. (2012) identified 23 strains of *gcd*-harboring bacteria in soil samples of the Dianchi Lake drainage area in China, which were affiliated to three phyla and 12 genera. The most dominant *gcd*-harbouring genera were *Rhizobium*, *Ensifer*, *Shinella*, and *Sinorhizobium*, all belonging to the phylum of Proteobacteria. Bergkemper et al (2016a) obtained six phyla, 17 orders, and 24 families of *gcd*-harboring bacteria from forest soil samples, where most abundant were genes harbored by Rhizobiales, Rhodospirillales, and Burkholderiales.

P solubilization can also be increased through the excretion of H^+ ions which assimilates cations such as NH_4^+ therefore solubilizing P. Alternatively, Rodríguez & Fraga (1999) proposed an H^+ excretion to the outer surface in exchange for cation uptake or translocation of H^+ via an ATPase

as ways to solubilize mineral P. This assimilation of NH_4^+ within cells and the release of H^+ solubilizes mineral P without the production of any organic acids (Sharma et al., 2013).

Beside solubilization via organic acid and H^+ excretion, microorganisms can solubilize P via the production of inorganic acids (i.e., sulphuric, nitric, and carbonic) and accompanying chelating substances (Alori et al., 2017). However, the effectiveness of this P solubilization mechanism has been reported to be lower than that of organic acids (Jordan, 1997).

7.3. Bacterial P mineralization

Up to 85% of total P in agricultural soil can be found in organic form as inositol phosphate esters, phospholipids, nucleic acids, sugar phosphates, and phosphoric acid derivatives (Tarafdar et al., 2001). Microorganisms can mineralize these recalcitrant organic P sources both intra- and extracellularly (Heath, 2005; Winkler, 1973) and thus survive in environments with very low P availability (Godwin & Cotner, 2015; Wei et al., 2017).

Under alkaline conditions bacteria use alkaline phosphatases (ALPs) for P acquisition. These are common in the environment and are principally assigned to bacteria (Tan et al., 2013) although they have also been reported for archaea and fungi (Ragot et al., 2015). ALPs are grouped into three distinct families, namely PhoA, PhoD, and PhoX (Boulanger & Kantrowitz, 2003; Eder et al., 1996; Hsieh & Wanner, 2010; Kageyama et al., 2011; Monds et al., 2006; Torriani, 1960; Wanner & Chang, 1987; Wu et al., 2007). These have in common that they are located in the periplasm (Luo et al., 2009) but differ in their target substrates (Coleman, 1992; Kageyama et al., 2011; Ragot et al., 2015; Wu et al., 2007; Yang et al., 2009). PhoD is the most abundant ALP in soil and is also present in aquatic environments, thus it is the most extensively investigated ALP (Luo et al., 2017; Ragot et al., 2015; Tan et al., 2013). According to Bergkemper et al. (2016b), dominant bacterial orders harbouring *phoD* genes include Planctomycetales, Burkholderiales, Rhizobiales, Pseudomonadales, Rhodospirillales, and Actinomycetales. So far, the studies investigating the effect of fertilization on *phoD* abundance showed contrasting outcomes (Chen et al., 2017; Luo et al., 2017; Tan et al., 2013). Hu et al. (2018) suggested that these inconsistencies might be due to the sensitivity of *phoD* harboring microbes which respond differently depending on the fertilizer and soil type. In a study with long term organic fertilizer application, the quantity and

composition of applied fertilizer affected the composition and structure of the *phoD* harboring bacterial community (Hu et al., 2018). Additionally, type of applied fertilizer, i.e., mineral vs. organic, also affects composition of the *phoD* harboring community (Fraser et al., 2015; Luo et al., 2017; Ragot et al., 2016). Regarding the relationship between soil P content and *phoD* abundance, it has been observed that *phoD* increases with decreasing soil P (Wei et al., 2019). Conversely, the application of P fertilizer has led to decrease of *phoD* abundance, suggesting that the expression of *phoD* is starvation induced (Apel et al., 2007).

Under acidic conditions bacteria can hydrolyze phosphate esters utilizing specific and nonspecific acid phosphohydrolases (SAPs and NSAPs) (Vincent et al., 1992), which have their optimum catalytic activity at acidic to neutral pH (Rossolini et al., 1998). Acid phosphohydrolases (ACPs) can be grouped into three distinct families (A, B, and C) based on amino acid sequence similarities (Thaller et al., 1995). NSAPs target a broad spectrum of phosphoesters and release P_i from nucleotides, sugar phosphates, and phytic acid, to name a few. They are either excreted into the periplasm or located in the membrane as lipoproteins (Rodríguez & Fraga, 1999). Interestingly, little was known about the different NSAPs and their distribution in natural and managed environments until Neal et al. (2018) investigated the distribution of the different classes and discovered that it is highly ecosystem dependent. Specifically, whereas class B NSAPs were associated with pathogenic bacteria, class A and C were dispersed more widely. The most widely spread was class C NSAP, which is located on the outer cell membrane, and enables the hydrolyzation of large organic compounds, thus having an important role in soil P cycling (Neal et al., 2018). Specific acid phosphatases (SAPs) are highly substrate specific and have wide biotechnological applications. However, to my knowledge, these have been discovered in single cell cultures and have not been further investigated in an environmental context.

The predominant form of organic phosphorus are phytates, which constitute up to 60% of soil organic phosphorus (Singh & Satyanarayana, 2011). They are present in form of inositol hexakisphosphate (IP6), inositol polyphosphate, or phytate. Though phytate is highly abundant in the organic P moiety, most plants are incapable of accessing it (Richardson & Simpson, 2011). Soil microorganisms actively secrete phytases into soil, facilitating P availability for plants (Balaban et al., 2017; Greiner et al., 1993; Lim et al., 1973; Touati & Danchin, 1987). Phytases are

grouped into four classes: i) histidine acid phosphatases (HAPS), ii) beta-propeller phytases, iii) purple acid phosphatases, and vi) protein tyrosine phosphatase like phytases (Mullaney & Ullah, 2003; Puhl et al., 2007). These are all reported to be unique, both structurally and catalytically (Castillo Villamizar et al., 2019). Although phytases have been studied in cultured organisms, environmental phytases still remain largely unexplored. Recently, this problem was addressed by Castillo Villamizar et al. (2019) who used a metagenomics approach and discovered novel phosphatases with phytase activity.

Organophosphate compounds represent the last source of P which bacteria can access. These highly stable sources are exploited by the release of enzymes which specifically target C-P bonds (Bujacz et al., 1995; McGrath et al., 2013; McGrath et al., 1995; Ohtake et al., 1996; Quinn et al., 2007). To date, two enzyme systems are known, namely, C-P lyase and C-P hydrolase (Quinn et al., 2007; White & Metcalf, 2007). While C-P lyases are utilized only to obtain P (Dyhrman et al., 2006; Wanner, 1994), C-P hydrolases allow microbes to acquire C, N, P, and energy from phosphonates (Gilbert, 2009; Kulakova et al., 2009; McGrath et al., 1997; McMullan & Quinn, 1994; O'Loughlin et al., 2006). Research on organophosphate degradation by bacteria is of high importance for biodegradation of pesticides (Dar et al., 2020; Kumar et al., 2016).

8. Microbial strategies to maintain their C:N:P stoichiometry

Studies have shown that although the range of C, N, and P in microbial biomass (C_{mic}, N_{mic}, P_{mic}) spans several orders of magnitude, the relationship between these elements is linear and isometric suggesting that soil microorganisms are largely homeostatic in terms of C:N:P stoichiometry (Cleveland & Liptzin, 2007; Hartman & Richardson, 2013; X. Li et al., 2014; Sistla & Schimel, 2012; Xu et al., 2013), i.e., they keep their C:N:P ratios constant under variable resource stoichiometry. Cleveland & Liptzin (2007) analyzed C:N:P ratios of soils and microbial biomass from 187 samples and have found this ratio to be 186:13:1 in soils and 60:7:1 in microbial biomass, whereas Xu et al. (2013) reported averages of 287:17:1 and 42:6:1 in 3422 analyzed samples for soil and microbial biomass, respectively. Nevertheless, there is also evidence for some stoichiometric flexibility of microbial communities (Fanin et al., 2013; Hartman & Richardson, 2013; Li et al., 2012). However, this non-homeostatic behavior appears to be a result of a shift in microbial community structure rather than physiological adaptation of the same

community (Fanin et al., 2013). The resources for soil microorganisms include dead plant biomass, root exudates, and SOM, all of which exhibit much wider and more variable C:N:P ratios than microorganisms (Sistla & Schimel, 2012). This imbalance between resource stoichiometry and microbial nutrient demands leads to microbial regulatory mechanisms for maintaining stoichiometric homeostasis. The regulatory mechanisms include regulation of element use efficiency (defined as "ratio of element invested in growth over total element uptake" by Sterner & Elser, 2002) by releasing elements in excess and optimizing the use of limiting elements, as well as production of extracellular C-, N-, and P acquiring enzymes to mobilize resources to meet their demands (Mooshammer et al., 2014). For example, from C-rich resources, such as leaf and root litter, and woody debris, excess C is released through high respiration, whereas N and P limitation is overcome by high N and P use efficiency and production of N- and P- acquiring enzymes (Zechmeister-Boltenstern et al., 2015). The regulation of extracellular enzymes (EE) production is complex, and it includes constitutive secretion of low levels of EE to detect suitable substrates, induction of EE synthesis in response to increasing availability of complex substrates, and feedback inhibition of EE activity by their products (Burns et al., 2013; Wallenstein & Weintraub, 2008). Aside from being a trigger for regulatory mechanisms of microbes, the quality and nutrient content of the resource shapes microbial community composition on taxonomic and physiological level by promoting microorganisms with certain enzyme capabilities, and metabolic and stoichiometric characteristics. For example, nutrient poor organic matter is dominated by fungi because they have lower P requirements than bacteria (Güsewell & Gessner, 2009). Beside fungi, environments of lower quality are usually favored by Gram-positive bacteria, especially *Acidobacteria* and *Actinobacteria* (Strickland et al., 2009), while soils with high SOM content and N availability tend to be dominated by Gram-negative bacteria (Bray et al., 2012; Fierer et al., 2003; Hossain et al., 2010). Resource quality affects also lifestyle of microorganism, differentiating between copiotrophic microorganisms, which are selectively advantaged by high nutrient conditions, use broadly different pools of organic matter, and show high growth rates as they have a high affinity to soluble substrates (Moorhead & Sinsabaugh, 2006), and oligotrophic microorganisms, which are advantaged under low nutrient conditions, utilize more complex C sources, and have slower growth rates (Hättenschwiler et al., 2011; Moorhead &

Sinsabaugh, 2006). Copiotrophs have been reported to exhibit lower biomass C:N:P ratios than oligotrophs (Elser & Hamilton, 2007; Fierer et al., 2007). Although agricultural practices aim at improving crop yields, addition of C and nutrients by fertilization, rhizodeposition, and incorporation of plant residues into the soil also influence soil microbiota. For example, Chen et al. (2014) showed that long-term P application led to a shift towards a more bacterially dominated system, with greater microbial biomass, different community structure, and altered P metabolism. Recent studies investigating the fertilization effect on microbial C:N:P stoichiometry revealed tighter constraints of microbial C:N ratios in response to N additions as compared to C:P ratios in response to P additions (Soong et al., 2018). This was explained by the ability of microorganisms to store P as polyphosphate when excess P is available (Kornberg, 1995; Mooshammer et al., 2014), suggesting that microbial C:P might reflect P availability in soils (Fujita et al., 2019). High N:P ratio of the substrate leads to microbial P limitation, increase in P use efficiency, and production of phosphatases. Thus, understanding the interplay between microbial and soil C:N:P ratios, and how they respond to fertilization, is of utmost importance as they affect biogeochemical fluxes and have implications for C sequestration and nutrient losses from terrestrial ecosystems (Mooshammer et al., 2014).

9. Objectives and Hypotheses of the PhD thesis

Given the non-renewable nature of rock phosphates as a source of P fertilizers on the one hand, and negative ecological implications of inadequate P fertilization on the other, it is necessary to deepen our understanding of the soil P cycle drivers and complement these findings with sustainable fertilization strategies and alternative P fertilizers.

Gaining deeper understanding of soil P transformations mediated by bacteria is of special interest, since these microbes can mobilize recalcitrant forms of P in soil, thereby narrowing the gap between soil legacy P, fertilizer application, and plant-available P.

To address these issues, this thesis aimed at (i) investigating bacterial P mobilization potential to reduce P fertilizer input in agriculture and (ii) investigating the viability of alternative P fertilizers (BC and BC^{plus}).

Therefore, the following hypotheses were tested:

1. Bacterial community composition will be affected by organic fertilization application, as it has been shown that the changes in microbial biomass C:N:P ratio under changing soil C:N:P are a consequence of bacterial community shifts rather than physiological adaptation of the present community.
2. P release from mineral, organic, organo-mineral fertilizer amendments depends on treatment-specific microbial P-turnover. These fertilizers exhibit different P-pools and thus selectively trigger microbial P transformations.
3. Organic fertilization will increase bacterial demand on P and consequently stimulate bacterial P mobilization which will be reflected in increased abundance of genes coding for extracellular P-acquiring enzymes.
4. Viability of the alternative mineral P fertilizers (BC and BC^{plus}) will be reflected in the abundance of bacteria mediating P turnover processes that is comparable to that of conventional mineral fertilizer (TSP).
5. The effect of fertilization on bacterial P turnover will vary depending on the initial soil P content and plant growth stage leading to changes in potential activities of P-acquiring extracellular enzymes during the growing season in agricultural soils with very low, low, and optimal P content.

These hypotheses were tested in three field experiments carried out on trial sites in three locations in Germany. The first experiment investigated the effect of organic fertilizer in two long-term field trials located in Freising, Bavaria, and Rostock, Mecklenburg Western-Pommerania (Manuscript 1). The second experiment evaluated the impact of inorganic, organic, and combined inorganic/organic fertilizer on the microbial P transformation on a long-term field experiment under maize (*Zea mays* L.) in Rostock (Manuscript 2). The third field experiment, located in Braunschweig, Lower Saxony, investigated the viability of novel alternative P fertilizers (BC and BC^{plus}) using soils from three different initial soil P test classes during the growing season of winter wheat (Manuscript 3).

II Materials and Methods

1. Site descriptions and sampling

All experiments were supported by the German Federal Ministry of Education and research (BMBF), funding the "**Inno**vative Solutions to sustainable **Soil Phos**phorus management" (InnoSoilPhos) project (No.: 031A558A) in the frame of BONARES (www.bonares.de/innosoilphos-de).

1.1. Experiment I (M1)

Two long-term field trial sites were used in this experiment. The first one is located in southern Germany in Freising/Dürnast, Bavaria (48°24' N, 11°41' E), and was established in 1979. The test site is 470 m above sea level with a mean annual precipitation and temperature of 791 mm and 8.4 °C, respectively. The soil type was classified as Cambisol, the soil texture as silty loam and the pH was 6.35. The test site has a split plot design where two treatments were analyzed in the frame of this study: 1) control, without organic fertilization; 2) organic fertilization with straw and catch crops as green manure (ORG). In control, the residue of cereals was removed after harvest, whereas it remained on the ORG plots (straw and green manure). In ORG, green manure was applied by sowing catch crops (turnip rape, rape, mustard, or phacelia) after the harvest of winter or summer barley and prior to the cultivation of potatoes or summer cereals. The plots used in this study had received 150 kg N ha^{-1} and no mineral P fertilizer since 1979. Each plot had a size of 32 m^2. Each treatment was three times replicated. In the long-term crop rotation, potato, winter or summer wheat followed by winter or summer barley were grown. However, winter barley in 2013 was followed by maize in 2014 and 2015. The mineral N fertilizer was applied on May 28, 2015, as calcium ammonium nitrate. The most recent application of green manure occurred in autumn 2010 (phacelia) and that of straw (winter barley) in 2013.

The second experimental site is a long-term field trial site in Rostock, Mecklenburg Western Pomerania, Germany (54°03' N, 12°05' E), which was established in 1998. The test site is 46 m above sea level, characterized by an annual mean precipitation of 600 mm and a temperature of 8.1 °C. The soil texture was classified as loamy sand, and the dominating soil type as Stagnic cambisol with a pH of 6.3. The site has a split plot design with four replicates, where two

treatments were analyzed in the frame of this study: 1) control, without organic fertilization; 2) organic fertilization in form of biowaste compost (30 t ha^{-1}) (ORG). Biowaste was applied every three years (last application in September 2013) and 120 kg ha^{-1} N was applied every year in May (80 kg N ha^{-1}) and June (40 kg N ha^{-1}). Each treatment had three replicates. Plots selected for this study were cultivated with maize from 2013 – 2015, apart from one sample, where a mix of bean and maize was grown. This sample belonged to the biowaste treatment (Sample M11 in Table S1). Each plot has a size of 30 m².

Table 1 Displayed are the initial measurements for soil total carbon (TC), total nitrogen (TN), total phosphorus (TP) and pH of long-term field trial sites in Freising and Rostock. Measurements for the long-term field trial in Munich were performed by Dr. Sabine von Tucher, chair of Plant nutrition at the Technical University of Munich School of Life Sciences (WZW-TUM). The measurements of the Rostock experimental site were performed by Dr. Bettina Eichler-Löbermann, Faculty of agricultural and environmental sciences at the University of Rostock (UoR).

	Freising		**Rostock**	
Treatment	**C**	**ORG**	**C**	**ORG**
TOC (g kg^{-1})	12.30	11.6	7.15	10.4
TN (g kg^{-1})	1.42	1.36	0.82	1.05
TP (mg kg^{-1})	679.4	609.0	544	622
pH	6.55	6.6	5.97	5.95

Samples were taken from both sites in April and September 2015 with a soil auger (3 cm diameter) from the upper topsoil (0 to 10 cm soil depth) in three replicates per plot, which were subsequently pooled and homogenized using 5 mm sieve. A total of 24 samples were taken (6 per site, three replicates, two seasons).

1.2. Experiment II (M2)

Experiment was conducted in 2015 and 2016 in Rostock, Mecklenburg Western Pomerania, Germany (site and experimental design description as in “Experiment I”). Test years differed in precipitation, with 2016 being dryer than 2015. The crop rotation was dominated by maize. The experiment included control (C – no additional P fertilizer), and three treatments: triple superphosphate (MIN), biowaste compost (ORG), biowaste compost + triple superphosphate

(OMI). Triple superphosphate (TSP) was applied annually at rates of 21.8 kg P ha^{-1} until 2013, and at rates of 30 kg P ha^{-1} from 2014. Biowaste compost was applied at rates of 30 t ha^{-1} in autumn every third year since 1998 (latest application before the test period in 2013). Since establishment of the test site in 1998 until the beginning of this study in spring 2015, the treatments with mineral and organic fertilizers received approx. 400 kg P ha^{-1}, combined treatments with TSP and biowaste compost approx. 800 kg P ha^{-1} and the control has not received any P since 1998. The initial concentration of double lactate P (P_{dl}) was 42.2 mg P kg^{-1}. Maize was fertilized annually with 120 kg N ha^{-1} as calcium ammonium nitrate and 125 kg ha^{-1} potassium during the whole duration of the field experiment. Within the period of investigation, the fertilizers were applied after the spring sampling. Soil management included annual tillage up to a depth of 20 cm after the autumn sampling.

Samples were taken in spring before sowing and in autumn before harvesting in 2015 and 2016, respectively, with a soil auger (3 cm diameter) from the upper topsoil (0 to 10 cm soil depth) in three of the four replicate plots for each fertilization treatment. Samples were pooled and subsamples were stored at 4 °C for microbial analyses and dried at 60°C for chemical analyses.

1.3. Experiment III (M3)

The study was performed at a field trial in the north-west of Braunschweig in Lower Saxony, Germany (52°18'N 10°27'E). The test site is situated 81 m above sea level with a mean annual precipitation of 620 mm and a mean temperature of 9 °C. The dominating soil types are Dystric Cambisol and Orthic Luvisol (according to IUSS Working Group WRB, 2015), which developed in silty-loamy sand with an average pH of 5.2. On this site, a long-term P field experiment was carried out between 1985 and 2008 (Vogeler et al., 2009). Briefly, five different mineral P fertilization regimes were applied including T1 – no P fertilization, T2 – 21.8 kg P ha^{-1} once a year (spring), T3 – 21.8 kg P ha^{-1} twice a year (spring and autumn), T4 – addition of P uptake of previous crop, and T5 – 1.5-times addition of P taken-up by the previous crop. In 1998, the experiment was split in two blocks to compare conventional and conservational tillage regimes for all treatments. The fertilization treatments included (1) no P fertilization, (2) organic P fertilization (T2, farmyard manure) and (3) a mixture of mineral fertilizer (T3, 45 kg P ha^{-1}) and

farmyard manure. Because of this previous experiment, significant differences in concentrations of plant available P were present in the plots. According to the guidelines of the Association of German Agricultural Analytic and Research Institutes (VDLUFA), the plots were assigned to different classes of available P in soil, namely initial soil test class A (very low, < 15 mg P_{CAL} kg^{-1}), B (low, 15 – 30 mg P_{CAL} kg^{-1}), and C (optimal, 31 – 60 mg P_{CAL} kg^{-1}).

The experiment was set up as a randomized complete block design with three replicates and a plot size of 5.75 * 17.5 m. The crop rotation included winter barley, winter oilseed rape, winter wheat, lupine, and winter rye. In addition to control without P fertilization, three distinct types of P fertilizer have been applied to an equivalent of 45 kg ha^{-1} P once a year shortly before sowing since fall 2013, namely, bone char (BC), surface-modified bone char (BC^{plus}) with sulfur compounds from biogas streams (patent DE102011010525), and triple super phosphate (TSP). The proportion of P of the different fertilizer was 14.81% for BC, 10.72% for BC^{plus} and 20.04% for TSP. Next to P, BC and BC^{plus} contained considerable amounts of Zn and Ca, but were depleted in As, Cd, Cr, Cu, Ni, and U compared to TSP. The detailed elemental composition of the fertilizers was described previously (Panten & Leinweber et al. 2020, Zimmer et al. 2019). A detailed description of the experiment including all agronomic measures, and the P uptake by crops, crop yield, and the fertilizer efficiencies are published by Panten & Leinweber (2020).

This study investigated the third year of the newly established trial during the vegetation period in 2015/2016. In 2015 winter wheat (*Triticum aestivum* L., variety ASANO) was sown after ploughing to a depth of 25 cm. Fertilization with N (130 kg ha^{-1}), K (100 kg ha^{-1}), Mg (11 kg ha^{-1}), and S (12 kg ha^{-1}), as well as plant protection were uniform in all treatments. The trial was irrigated once with 30 l m^{-2} on May 10th, 2016 and harvested on July 28th, 2016. Crop yields ranged from 5.7 (control) to 6.0 t dry matter (DM) ha^{-1} (TSP) in the plots with optimal P concentrations, from 5.38 (BC) to 6.02 DM ha^{-1} (BC^{plus}) in plots with low P concentrations and from 5.41 (control) to 5.87 DM ha^{-1} (TSP) in the plots with very low P concentrations (Panten & Leinweber et al. 2020).

Bulk soil samples were collected three times during the vegetation period in 2016 (April/BBCH 33/34 – stem elongation, May/BBCH 53 – heading, June/BBCH 73-75 – ripening). In the following, the terms stem elongation, heading, and ripening were used to improve readability of the

manuscript. Per plot, three cores (up to 10 cm soil depth) were collected, pooled, and subsequently homogenized using a 5 mm sieve. In total, 108 samples were taken (three samplings, three soil P classes (A, B, C), four treatments, and three replicates). The samples for the determination of potential acidic and alkaline phosphatase activities were stored at 4 °C and analyzed within one week. The samples for nucleic acid extraction were collected and immediately frozen on the field using dry ice. Samples for pH, water soluble P (P_{water}) and plant available P (P_{CAL}) analysis were air-dried and sieved < 2 mm.

Table 2 Displayed are the means of soil parameters in the top 30 cm of three different initial soil P test classes after harvest in 2013. Measurements were performed by Dr. Kerstin Panten from the Institute for Crop production and Soil Science, Julius Kühn-Institut (JKI) in Braunschweig, Germany. These measurements were conducted before the set-up of the present experiment.

Soil parameter	**Soil A**	**Soil B**	**Soil C**
P_{water} [mg kg^{-1}]	2.8 (2.0-3.9)	4.4 (3.5-5.5)	9.4 (8.0-10.5)
P_{CAL} [mg kg^{-1}]	11.2 (6.9-17.8)	20.7 (16.5-25.1)	47.2 (41.4-53.0)
Total P [mg kg^{-1}]	217 (204-241)	273 (230-324)	399 (378-424)
Total C [%]	1.3 (1.2-1.4)	1.4 (1.3-1.5)	1.4 (1.4-1.4)
Total N [%]	0.10 (0.09-0.10)	0.10 (0.10-0.11)	0.10 (0.10-0.11)
pH	5.1 ± 0.1	5.2 (5.0-5.2)	5.2 (5.1-5.3)

2. Soil abiotic properties

Measurements of dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) concentrations were determined after extraction in 0.01 M $CaCl_2$ solution (4 g soil in 20 ml $CaCl_2$) using a photometric autoanalyzer (CFA-SAN Plus; Skalar Analytik, Germany). DOC and DON concentrations were measured with a DIMA-TOC 100 (Dima Tec, Langenhagen, Germany). Further soil abiotic properties (pH, TC, TN, C_{org}, TP, and P_{dl}) of Manuscripts I and II were measured by the research group of Dr. Baum from the Faculty of agricultural and environmental sciences at the University of Rostock. Measurements of soil abiotic properties (pH, P_{cal}, P_{Water}) for Manuscript III were performed by the research group of Dr. Panten of the Institute for Crop production and Soil Science, Julius Kühn-Institut (JKI) in Braunschweig, Germany.

3. Microbial biomass C, N, and P and soil enzyme activities

For the assessment of C_{mic} and N_{mic}, the chloroform-fumigation-extraction method (Vance et al., 1987) with 0.01 M $CaCl_2$ for extraction was used. Correction factors were K_{EC}=0.45 for C_{mic} and K_{EN}=0.54 for N_{mic} according to Joergensen (1996) and Joergensen & Mueller (1996), respectively. Measurements of microbial P (P_{mic}), potential enzymatic activities for acid and alkaline phosphomonoesterases (ACP and ALP), as well as phosphodiesterases (PDE) and ß-glucosidase (GLA) were performed by the research group of Dr. Baum for M1 and M2. ACP and ALP activities in Experiment III were measured by the research group of Dr. Panten Institute for Crop production and Soil Science, Julius Kühn- Institut (JKI) in Braunschweig, using the same protocol.

4. Nucleic acid extraction

DNA was directly extracted from 0.5 g of frozen soil (−80°C) using Precellys 24 (Bertin Technologies, France) and phenol-chloroform based protocol modified according to Lueders et al. (2004) and Töwe et al. (2011). Total genomic DNA quality was determined photometrically (Nanodrop ND-1000; Thermo Fischer Scientific, USA) and the quantity of the DNA was assessed using the QuantiT PicoGreen kit (Life Technologies, USA). The obtained DNA concentrations were used to calculate microbial biomass in the extracted samples. Extracted samples were stored at −20°C before further processing for whole shotgun sequencing and qPCR analysis.

5. Quantitative polymerase chain reaction measurements (qPCR)

Real-time quantitative PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems, Germany) using SyBr Green as fluorescent dye to quantify maker genes for different processes of P turnover including *gcd, phoD*, *phoN*, *phnX*, *appA, pstS,* and *pitA*. The reaction mix contained 12.5 µl of SYBR Green® (Thermo Fisher Scientific, USA), forward (F) and reverse (R) primers (Metabion, Germany), 0.5 µl BSA (3%, Sigma, Germany) and DEPC-treated water and was set to 25 µl. The source of the standard, primer sequences and reaction mixture components are summarized in Table 2 (Bergkemper, et al., 2016b). The thermal profile consisted of a touchdown of 5 cycles starting with a denaturation at 95 °C for 15 sec followed by primer annealing at 65 °C for 30 sec and finished by elongation at 72 °C for 45 sec. After the touchdown with a reduction of the annealing temperature of 1 °C per qPCR cycle, 40 qPCR cycles followed with an annealing temperature of 60 °C. Serial plasmid dilutions (10^1–10^7 gene copies per µl) were used for standard curve calculations. In a pre-experiment, performed to avoid reaction inhibition effects, the optimal sample dilution was determined as 1:16 (data not shown). Additionally, a melting curve analysis was performed by adding a dissociation stage after each run, to prove the specificity of the amplified qPCR products. To confirm the correct size of the amplified fragments further, gel electrophoresis for randomly selected samples was conducted on a 1% agarose gel. Efficiencies ($E = 10^{(-1/slope)}-1$) obtained were as follows: 81,9% for *phoD*, 73,5% for *pstS*, 89,3% for *pitA,* 90,3% *phnX,* and 85,0% for *gcd*, respectively. R^2 was determined to be above 0.99 for each qPCR assay. The abundance of *appA* and *phoN* was below detection limit in all samples (less than 10 copies per µl).

6. Library preparation for whole shotgun sequencing

Prior to library preparation 1 µg of DNA was shared on a COVARIS® E220 (Covaris®) according to the protocol provided by the manufacturer (Conditions: Peak incident Factor: 175 W; Duty Factor: 5%; Cycles per burst: 200; Treatment time: 35 sec; Temperature: 7 °C; Water level: 6; Intensifier: Yes). 100-200 ng of the shared DNA were used for the library preparation with NEBNext® Ultra™ DNA Library Prep Kit (New England Biolabs® Inc.). All steps were carried out according to the NEBNext® Ultra™ manual (New England Biolabs® Inc.). As described in the

manual, the adapters for the ligation were diluted 1:10 to prevent the occurrence of primer dimers. Size selection of fragments was performed with Agencourt® AMPure® XP (Beckman Coulter, USA) to obtain a total library size of 500-600 bp. After the PCR, an additional cleaning up step with a bead to DNA ratio of 0.6:1 was necessary to eliminate residual primer dimers. Libraries were pooled equimolar (4nM). 10 pM of the mixture was spiked with 30 % PhiX, used as a quality and calibration control (Mukherjee et al., 2015). Sequencing was performed on an Illumina® Miseq® (Illumina®) sequencing machine using the MiSeq® Reagent Kit v3 (600 cycles) (Illumina®) for paired end sequencing. The raw sequencing data is available at the sequencing read archive (SRA) under the BioProject ID PRJNA385596 (SAMN06894543- SAMN06894566).

7. Sequence processing and taxonomic and functional annotation

MiSeq fastq files were processed by removing the remnant adaptor sequences and subsequently trimming the reads via AdapterRemoval (Schubert et al., 2016) by applying the following settings: 5'/3'- terminal minimum Phred quality = 15, minimum read length = 50. Furthermore, PhiX contamination was used as a quality and calibration control during the sequencing run (Mukherjee et al., 2015). PhiX was removed using DeconSeq (Schmieder & Edwards, 2011).

For taxonomic and functional annotation, the sequences were blasted against the NCBI-nr database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (June 2011) (Kanehisa et al., 2016) using DIAMOND (0.5.2.32) on sensitive settings (Buchfink et al., 2014). Taxonomic and functional assignment was performed using MEGAN5 v 5.10.6 (Huson et al., 2011). This was based on the top 25 blast results (i.e., hits with the lowest e- value). Following parameters were applied during MEGAN analysis: MinScore=50.0, MaxExpected=0.01, TopPercent=10.0, MinSupport=1, MinComplexity=0. Hits with organism names containing words "environmental samples", "unclassified", and "unidentified" were excluded from further analysis. This was done to avoid confusion during visualization (Mizuno et al., 2001; Q. Zhu et al., 2014).

8. Statistical analysis

Statistical analyses and visualization of all experiments were performed using Rstudio (https://www.r-project.org; versions 3.6.1 and 4.0-2).

For Experiment I, statistical analysis and visualization were based upon the relative abundance of reads. Relative abundances were obtained by dividing the number of reads by the total number of filtered reads per sample and multiplication by 100. Using two-way analysis of variance (ANOVA), the effects of season, fertilizer, and interaction of both, on taxonomic as well as the functional profiles of the samples were detected. Pairwise differences were identified using the Fisher's least significant difference (LSD) method. Prior to testing for differences, normality of the data was ascertained with Q-Q plots and Shapiro-Wilk tests. Differences were considered significant when the p-value was below 5% ($p < 0.05$). Only taxa with a relative abundance of 0.01% and above in one of the samples were included in the statistical analysis of taxonomic profiles. This cut-off was not applied to functional data due to the low number of attained reads. To assess the level of dissimilarity between the samples, non-metric multidimensional scaling (NMDS) ordination plots were created. These are based on the Bray-Curtis distance metrics, which uses the metaMDS function in the R vegan package (Oksanen et al., 2015). All R packages used for this study can be found in Table S6. Visualization of VENN diagrams was performed using InteractiVENN (Heberle et al., 2015). To create these diagrams a family was regarded as present if it occurred in at least two out of three samples.

For Experiment II, R packages hmisc (https://www.r-project.org; version 4.0-2) and agricolae (https://cran.r-project.org/web/packages/agricolae/ index.html; version 1.2-4) were used. Soil properties, parameters of microbial activity, and mycorrhizal colonization were analyzed using the Kruskal test. Significance was considered at $p < 0.05$ and significant differences between treatments were indicated by using different letters. The treatment specific changes in the activities of hydrolytic enzymes were tested with principal component analysis (PCA) using the software package PAST (Hammer et al., 2001).

For Experiment III, the crossbar plots were created using ggplot2 package (Wickham, 2016). Linear models on log-transformed data and ANOVA were applied to evaluate variances caused by sampling time point, initial soil P class, and treatment on the total sample set. Further, samples were separated by sampling time point to detect different reactions caused by initial soil P concentration or treatment. Residual vs. fitted plots and sample quantiles vs. theoretical quantiles plots based on the model were tested for normal distribution and homogenous

variance to verify the models. As conditions were not met, data was log transformed. To test for significant differences ($p < 0.05$) between the investigated factors, pairwise comparisons were conducted by a Tukey Post hoc test (R package lsmeans) (Lenth, 2017). To visualize how gene abundances differ between control and fertilized plots, average gene abundances were calculated for each treatment (control, TSP, BC and BC^{plus}) and subsequently normalized to the control. In case the values of treatments were higher than control values, this was calculated as r = treatment/control, else as r = - control/treatments. $r > 1$ indicate higher abundances in the fertilization treatment, while $r < -1$ indicate higher values in the control treatment.

III Manuscript overview

List of manuscripts

- Grafe M, Goers M, von Tucher S, Baum C, Zimmer D, Leinweber P, Vestergaard G, Kublik S, Schloter M, Schulz S (2018) Bacterial potentials for uptake, solubilization and mineralization of extracellular phosphorus in agricultural soils are highly stable under different fertilization regimes. Environ Microbiol Rep 10:320–327. DOI: https://doi.org/10.1111/1758-2229.12651
  (M1, first author, published)

- Peine M, Vitow N, Grafe M, Baum C, Zicker T, Eichler-Löbermann B, Schulz S, Schloter M, Leinweber P (2019) Effect of triple superphosphate and biowaste compost on mycorrhizal colonization and enzymatic P mobilization under maize in a long-term field experiment, J Plant Nutr Soil Sci, 182 167. DOI: 10.1002/jpln.201800499
  (M2, coauthor, published)

- Grafe M, Kurth JK, Panten K, Durai Raj A, Baum C, Zimmer D, Leinweber P, Schloter M, Schulz S (2021) Effects of different innovative bone char based P fertilizers on microbiota catalyzing P turnover in agricultural soils. Agriculture, Ecosystems & Environment, 314, 107419. DOI: https://doi.org/10.1016/j.agee.2021.107419
  (M3, first author, published)

Manuscript description and contributions

Manuscript I

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

Martin Grafe, Manuela Goers, Sabine v. Tucher, Christel Baum, Dana Zimmer, Peter Leinweber, Gisle Vestergaard, Susanne Kublik, Michael Schloter, Stefanie Schulz

Brief description:

Bacteria play a crucial role in soil P turnover, affecting agricultural productivity. Since the application of fertilizers changes C:N:P ratios of soil, it is important to understand its effects on microbial C:N:P stoichiometry, P turnover processes, and community structure. These questions were addressed in two long-term field trials located in Freising, Bavaria, and Rostock, Mecklenburg Western-Pommerania using a metagenomic approach. The results showed highly stable microbial C:N:P ratios, that were not affected by fertilization, season, or location. Neither season nor the fertilization treatment had a significant effect on the relative abundance of the analyzed P turnover genes, with genes encoding for a glucose dehydrogenase (*gcd*) being the most abundant, followed by genes encoding for the uptake of inorganic P (*pst* and *pit*) and genes encoding for phosphatases (*phoA*, *phoD*, and olpA). However, a fertilization treatment had a significant effect on community structure of bacteria harboring P turnover genes, suggesting that fertilization could change P turnover efficiency by favoring different families.

My contributions:

- performed sample preparation and metagenome sequencing
- conducted subsequent analyses and interpretation of metagenomic data
- contributed to bacterial biomass C, N, and P measurements
- wrote the manuscript

Environ Microbiol Rep 10:320–327

Manuscript II

Effect of triple superphosphate and biowaste compost on mycorrhizal colonization and enzymatic P mobilization under maize in a long-term field experiment

Manuela Peine, Nora Vitow, Martin Grafe, Christel Baum, Theresa Zicker, Bettina Eichler-Löbermann, Stefanie Schulz, Michael Schloter, Peter Leinweber

Brief description:

This experiment aimed at finding the favorable fertilization strategies by evaluating the impact of inorganic, organic and combined inorganic / organic fertilization on the microbial P transformation, mycorrhizal colonization, and the supply of crops on a long-term field experiment under maize (*Zea mays* L.) in Rostock.

The objective was to test whether the different P-pools and C:N:P ratios of applied fertilizers selectively trigger microbial P transformation, deciding whether microbial biomass acts as a P sink or source. The results showed no effect of fertilization treatments on crop yield. However, the soil enzyme activities involved in P turnover showed clear splitting between treatments without (C, MIN) and with organic amendments, where organic fertilizer resulted in significantly increased potential activities.

My contributions:

- contributed to measurement of microbial biomass C and N
- contributed to data analysis and interpretation
- edited first manuscript draft provided by the first author

Journal of Plant Nutrition and Soil Science, 182 (2019) 167.

Manuscript III

Effects of different innovative bone char based P fertilizers on microbiota catalyzing P turnover in agricultural soils

Martin Grafe, Julia Katharina Kurth, Kerstin Panten, Abilash Durai Raj, Christel Baum, Dana Zimmer, Peter Leinweber, Michael Schloter, Stefanie Schulz

Brief description:

The finite nature of natural P sources stresses the importance of improved P recycling. This study evaluated the effect of bone char (BC) and bone char plus (BC^{plus}) as recycling products and alternative to conventional P fertilizers, such as triple super phosphate (TSP), on the abundance of microorganisms catalyzing the major steps in P turnover in a field experiment in Central Germany. Samples were analyzed from soils with three different initial soil P concentrations (very low, low, optimal) and three times during winter wheat cultivation (stem elongation, heading, ripening). The gene abundances, assessed by qPCR, were complemented by acidic- and alkaline phosphatase activity, water extractable P, and plant available P measurements. Results showed that fertilization effects were strongly interrelated with plant growth stage and initial soil P concentrations and effects were most pronounced at heading of wheat and in soils with optimal initial P concentration. The addition of BC^{plus} increased the solubilization potential, while the slow release of P from BC favored P recycling from biomass and P inducible uptake systems.

My Contributions:

- Performed sampling and DNA extraction
- performed qPCR measurements
- performed data analysis and interpretation
- wrote the manuscript

Agriculture, Ecosystems & Environment, Volume 314, 2021

IV Discussion

The present study was part of the collaborative InnoSoilPhos project (Innovative solutions to sustainable Soil Phosphorus management) and was funded by BonaRes ("Soil as a sustainable resource for the bioeconomy") funding initiative of the German Federal Ministry for Education and Research (BMBF) with focus on the sustainable use of soils as a limited resource. The main aim of the InnoSoilPhos project is the optimization of the soil-P-fertility to lower the dependency on phosphate rock-derived P-fertilizers.

The central focus of this PhD thesis was the microbial contribution to the soil P turnover and its response to different fertilization treatments as well as the response to the application of alternative P fertilizers.

1. Bacterial community composition and P turnover potential is affected by organic amendment

The first hypothesis of this thesis postulated that organic fertilization will lead to changes in microbial community composition, as it has been shown that the changes in microbial biomass C:N:P ratio under changing soil C:N:P are a consequence of bacterial community shifts rather than physiological adaptation of the present community. To test this hypothesis, first the effect of organic amendment on C:N:P ratio of soil microorganisms was obtained, and then the structure of bacterial P turnover community in agricultural soil was addressed using a metagenomic approach in two long-term field trials located in Freising, Bavaria, and Rostock, Mecklenburg Western-Pommerania (M1).

1.1. Microbial C:N:P ratios reflected the soil nutrient stochiometry

As microorganisms take up required nutrients from their environment, carbon-to-nutrient ratios (C:N and C:P) of the resource determines whether nutrients are immobilized in the microbial biomass or mineralized to become available for uptake (Griffiths et al., 2012). The microbial C:N:P ratio has been subject to numerous studies with results suggesting a highly constrained relationship between these elemental ratios (Cleveland & Liptzin, 2007; Hartman & Richardson,

2013). However, there is also evidence of stoichiometric flexibility of microbial communities (Fanin et al., 2013; Hartman & Richardson, 2013; Li et al., 2012).

The results obtained in M1 showed highly stable ratios that were not affected by fertilization treatment. Interestingly, the observed C_{mic}:N_{mic}:P_{mic} ratios of 25:1.2: 1 in Freising and 36:1.2:1 in Rostock deviated from the 60:7:1 reported by Cleveland & Liptzin (2007) and 42:6:1 reported by Xu et al (2013). However, Xu et al (2013) also stated that microbial C:N:P ratios differed at ecosystem and biome level, following a linear correlation with soil C:N:P ratios. They observed that cropland has a narrow microbial C:N:P, ascribing it to low C density and N and P input. This is in line with the observation made in M1, where ratios of C_{mic}:N_{mic}:P_{mic} reflected the total C:N:P ratios of soils which was 20:2:1.

1.2 Distribution of genes coding for enzymes catalyzing different steps in P turnover among bacterial families

Metagenomic analysis identified 295 bacterial families in total. The most abundant families were *Acidobacteriaceae, Bradyrhizobiaceae, Verrucomicrobia* subd. 3, *Planctomycetaceae,* and *Chitinophagaceae*, all of which are known to be widely spread throughout terrestrial ecosystems (Rosenberg et al., 2014). None of the families covered all P turnover processes and therefore did not invest the same amount of energy in P turnover. This analysis was done by pooling the genes of all samples based on their function into regulation (3 genes), mineralization (23 genes), solubilization (1 gene) as well as organic and inorganic P uptake (7 genes) (M1).

Some of the identified families appeared to be more universal in the variety of genes which could be used in P turnover. In contrast, few families were detected which appeared to only have the ability for P uptake, without P solubilization and mineralization potential. Amongst those with a wider range were *Verrucomicrobiaceae* subdivision 3, *Sphingomonadaceae*, *Anaerolinaceae*, *Planctomycetaceae*, *Chitinophagaceae*, *Acidobacteriaceae,* and *Bradyrhizobiaceae*. This finding is especially surprising when considering that most of these families are known to be oligotrophic. Because the investigated soil is used for agriculture, and consequently is nutrient rich, one would expect to find mainly copiotrophic bacteria (Fierer et al., 2007). This finding highlights the hierarchical structure of soils, which is linked to the formation of niches with varying conditions (Kaminsky et al., 2017; Oades et al., 1991). What made this discovery even more interesting is

the association of some families, e.g., *Bradyrhizobiaceae* and *Nitrospiraceae*, with N turnover. The capability of fixing N and the effective use of both organic and inorganic P compounds has also been documented for other organisms like Azotobacter (Nosrati et al., 2014; Zaidi et al., 2009).

The more specialized families can be split in those which are only involved in organic and inorganic P uptake and those that seem to act as suppliers. The prior pursued a rather copiotrophic lifestyle. This observation fits nicely especially because the fast-growing copiotrophs prefer to use easily available nutrients instead of investing energy into P solubilization or mineralization. These include *Rhodocyclaceae*, *Chlorobiaceae*, *Geobacteraceae*, *Flavobacteriaceae* and *Opitutaceae* (Uksa et al., 2015). Belonging to the supplier group were families such as *Verrucomicrobiaceae* and *Solibacteraceae*, which exhibited preferences either for mineralization or solubilization of P but in which, intriguingly, the P uptake genes were not detected (M1). This could suggest that P is being released as a by-product of the bacterial C metabolism (Heuck et al., 2015). However, it is also possible that P uptake genes were present only in very few taxa and the relative abundance of these was too low to be detected in the analyzed metagenomes. Alternatively, a possible explanation is the existence of other uncharacterized uptake mechanisms. This would make a detection difficult by the applied approach. This is especially the case when considering that a slightly divergent organic phosphate transporter can appear as a sulphate transporter, or even as a carboxylate transporter, depending on the organic moiety involved.

1.3. Effect of organic fertilization on bacterial community structure

Of the 295 total identified families, 258 harbored genes encoding for proteins involved in extracellular P turnover. Of these, 85 families comprised a core microbiome (as defined by Shade & Handelsman (2012)), which was present at both sites, independent of season (spring, before sowing, and autumn, before harvest of maize) and treatments (organic fertilizer vs. control). The observed similarity of the communities between both sites corroborates previous reports that total bacterial community structure is primarily shaped by ecosystem type rather than location (Cao et al., 2016; Fierer & Jackson, 2006) and is further backed by Allison & Martiny, (2008) who argue that important ecosystem functions are preserved even if changes in microbial community structure occur. Fierer (2017) also postulated that the major determining factor for the community structure is pH, which was similar at both sites.

Total community structure was not significantly affected by organic fertilizer amendment (M1). The insensitivity of microbial community composition to fertilization has been reported by Beauregard et al. (2010) who found limited alterations of bacterial communities in response to 8 years of P fertilization in perennial forage stands. The group of Li et al. (2020) also reported that P fertilization had an insignificant influence on the overall structure of soil microbial communities and concluded that N fertilization is a main driver of soil microbial diversity, community structure, and function in a wheat producing agroecosystem. This could also be an explanation for the similarity of the total community structure in the tested soils, since all plots were amended with comparable amounts of mineral N.

However, the hypothesis that organic fertilization influences microbial community composition by changing soil C:N:P stoichiometry was corroborated by the analysis of the bacterial community involved in P turnover, which showed that its composition was influenced by fertilization, and the effect of season was strongly connected to fertilization effect. It has been shown that in agricultural soil, the addition of C-rich organic fertilizers results in strong coupling of C and P cycles (Bünemann et al., 2006), where increased availability of P in low-P soils leads to increased rates of C-mineralization (Wakelin et al., 2014). Increased bioavailability of C drives ecosystem processes and affects sequestration and supply of soil P. Consequentially, this leads to change in soil P species (Bradford et al., 2008; Gressel et al., 1996; Kirkby et al., 2013; Spohn & Kuzyakov,

2013), and thus, influences microbial P turnover, which could explain the change in the P-cycling community structure under long-term organic amendment. Moreover, Mander et al. (2012) showed that C:P ratio is a strong driver of microbial community composition, as it directly affects the abundance and taxonomic composition of P solubilizing bacteria.

Yet, metagenomics only provides information on microbial potential, and not their actual activity (Prosser, 2015). The fact that genes necessary for P turnover were harbored by different families gives rise to the possibility that these could perform the same function with different efficiencies. Indeed, it has been shown by Ragot et al. (2016) that for some taxa there are significant differences between the presence and expression of genes involved in P turnover. Therefore, the observed influence of fertilizer on the gene distribution pattern in different bacterial families could cause substantial changes in activity pattern. This hypothesis could be strengthened by the high abundance of regulatory genes that could influence the efficiency of P turnover.

2. The effect of different fertilizers on the abundance of bacterial P turnover genes

The second hypothesis of this thesis postulated that fertilization treatments – i.e., mineral, organic, organo-mineral – constitute different P sources, thus, their application will selectively trigger microbial P transformation processes.

Although the amount of total P in soil is often large and usually ranges from 100 to 2000 mg P kg^{-1} representing approximately 350 to 7000 kg P ha^{-1} in the surface 25 cm of the soil, due to the high reactivity of P, only a small fraction is available for plant uptake (Syers et al., 2008). Additionally, P is the most immobile macronutrient in soil. Altogether this led to P fertilization being a common agricultural practice to sustain optimal plant growth and high yields. Nevertheless, accumulated soil P, both in organic and inorganic forms, can be accessed by soil microorganisms.

2.1. Bacterial P solubilization

Microorganisms can solubilize inorganically bound P by producing organic acids, through release of protons which release P by decreasing soil pH, chelating cations (Al^{3+}, Fe^{3+}, Ca^{2+}), and competition for adsorption sites (Khan et al., 2014; Uroz et al., 2009). The most important and main organic acids in microbial P solubilization is gluconic acid (GA). GA is produced by oxidation

of glucose in a reaction catalyzed by the enzyme glucose dehydrogenase with pyrroloquinoline quinon (PQQ) as cofactor. The gene coding for this enzyme is quinoprotein glucose dehydrogenase (*gcd*) (Cleton-Jansen et al., 1988). g*cd* was the most abundant gene both in dataset from Freising and Rostock (M1), as well as in dataset obtained by qPCR from Braunschweig (M3). This is in accordance with Bergkemper et al. (2016a) who proposed that solubilization is the main driver of microbial P turnover in soils rich in mineral P, which is true for most agricultural soils (Stutter et al., 2015). The studies have yielded contradicting results regarding the influence of fertilization on the abundance of *gcd*. Zeng et al. (2016) reported that *gcd* was suppressed by elevated levels of soluble phosphate. However, this study investigated the effect of soluble phosphate concentration on *gcd* expression in laboratory settings and only included one PSB strain (*Pseudomonas frederiksbergensis* JW-SD2). In a controlled pot experiment containing soil amended with three different levels of P fertilizer, Li et al. (2020) observed increased abundance of *gcd* with increasing P treatment. They suggested that rare taxa have the potential to release inorganically bound P when the supply is sufficient, which might allow them to be more competitive against dominant taxa. The experiments conducted in the scope of this PhD thesis showed that neither organic fertilization with compost in Rostock and Freising (M1) nor application of mineral P fertilizers (TSP, alternative fertilizers in form of bonechar and sulfur-enriched bonechar) in Braunschweig (M3) influenced *gcd* abundance. This is in line with Dai et al. (2020) who found no effect of long-term P fertilization on total relative abundance of genes involved in P_i solubilization. Thus, it can be assumed that stable *gcd* abundances reflect changes in the community structure of PSB, which was corroborated by the results of metagenomic analysis in M1, where addition of organic fertilizer had an effect on community structure of microorganisms harbouring P turnover genes.

2.2. Bacterial P mineralization

Microorganisms mineralize P by excreting extracellular enzymes which release P bound in organic compounds. The main enzymes involved in P_o transformations can be grouped into three categories depending on their substrate: (i) phosphatases, which hydrolyze ester-phosphate bonds form inositol phosphates, nucleotides, phosphoproteins, and sugar phosphates

(Nannipieri et al., 2011a; Turner et al., 2004); ii) Phytases, which degrade soil organic myo-Inositol1,2,3,4,5,6-hexakisphosphates (IP6) (Jorquera et al., 2008), and (iii) Phosphonatases and C-P lyases mineralize organophosphonates – organophosphorus compounds that contain at least one stable carbon to phosphorus bond and are mainly found in prokaryotes, mostly in peptide, glycan, or lipid conjugates (Hilderbrand, 2018).

The genes coding for mineralization enzymes were less abundant than solubilization genes in metagenomic dataset (M1) and their abundance was not affected by organic fertilization. The most abundant ones were the alkaline phosphatase genes (ALP) *phoA*, *phoD,* and a class C acid phosphatase *olpA*, while the abundances of genes coding for enzymes that specifically target substrates, such as phosphonate or phytate, were low. Lower abundance of mineralization genes compared to solubilization could be explained by the fact that, even with organic fertilization, the prevailing form of P in agricultural soils is P_i (Stutter et al., 2015).

In M3, the bacterial mineralization potential was investigated using qPCR to measure gene abundances: NSAP class A (*phoN*) (Rossolini et al., 1998) and the ALP (*phoD*) (Eder et al., 1996) for phosphatases; *appA* for phytase (Lim et al., 2000), and *phnX* for phosphonatase (Hsieh & Wanner, 2010). The primers were developed by Bergkemper et al. (2016b) who proposed that there is a need to investigate the complete set of enzymes (genes) involved in processes of microbial P mineralization, solubilization, and uptake to better understand the P turnover in soil. They used metagenomic datasets as a starting point for targeted primer development.

In all samples, *phoN* and *appA* were below detection limit. The low abundance of *appA* does not necessarily imply a low abundance of phytases, since this gene codes for histidine-acid phosphatase (HAP; 6-phytase) (Lim et al., 2000), which represents only one class of phytases, next to beta propeller phytases (BPP) and purple acid phosphatases (PAP) (Bergkemper et al., 2016b). Since *phoN* is mostly present in Rhizobiales (Bergkemper et al., 2016b), its low abundance in soil under wheat growth is not surprising. This was confirmed by the analysis of samples from the same plots one year later, when there was lupine growing, which revealed significantly increased abundance of *phoN* (data not shown). Lupins fix atmospheric N by forming a symbiosis with soil bacteria of the genus Bradyrhizobium, which belongs to the order Rhizobiales.

It has been reported that phosphomonoesterases are induced by P limitation (Chen et al., 2019; Saha et al., 2008; Zhang et al., 2014) and that *phoD* abundance is negatively related to P availability (Luo et al., 2017; Tan et al., 2013). However, contradicting observations have also been made. Randall et al. (2019) found an increase in *phoD* gene abundance under P_i fertilization and Wei et al. (2021) observed a significantly increased abundance of *phoD*-harbouring bacteria and organic-P functional bacteria following both organic and mineral fertilization.

Contrary to those findings, in M3 the *phoD* and *phnX* gene abundances did not differ between soils with different initial P levels (very low, low, optimal) and were not influenced by mineral P amendments (TSP, BC, BC^{plus}). A similar observation was made by Long et al. (2018), who found no difference in the abundance of genes involved in P_o mineralization with increasing P treatment. It is possible that fertilization effects strongly interacted with initial soil P content and sampling date, which prevented the clear differentiation. Another explanation for the lacking effect of applied fertilizers on *phoD* gene abundances could be the increase of microbial P uptake when P is available in excess. It has been shown that *phoD* harboring microorganisms can modulate their P use strategies in response to P availability by stimulating intracellular P accumulation under high P conditions, and mineralization of organically bound P under P limitation (Raiesi & Hosseinpur, 2017; Zhang et al., 2015), which could explain the stable abundance of *phoD* genes regarding fertilization.

2.3. Bacterial P uptake

Bacteria have two main systems for uptake of inorganic phosphate. Low affinity transporter Pit (phosphate inorganic transporter) is active under high P_i availability, when P_i concentration is above 4 µM, whereas extracellular P_i concentration below this value induces starvation response which activates expression of high affinity phosphate specific transporter (Pst) (Hsieh & Wanner, 2010). Interestingly, in metagenomic dataset (M1), genes for both P_i transport systems were equally abundant and not affected by organic fertilization. In the experiment with mineral P fertilizers applied to soils with three different initial P levels (M3), the abundance of *pitA* was significantly higher than the abundance of *pstS*, indicating sufficient P availability in all of the tested soils.

3. The effect of organic amendments on bacterial P turnover

The next hypothesis postulated that the addition of organic amendments will stimulate bacterial growth by providing C and N. This will consequentially increase the bacterial demand on P leading to increased production of P-acquiring extracellular enzymes, which will be reflected in the abundance of bacterial genes involved in P solubilization, mineralization and uptake, as well as potential phosphatase activity.

3.1. Metagenomic analysis of bacterial P turnover genes as affected by organic fertilization

To assess the dominant bacterial P turnover processes and link them to the potential effect of organic fertilization, a metagenomic approach was applied and the genes encoding proteins linked to regulation, mineralization, solubilization, and uptake of extracellular P sources were detected (M1). Of the total reads, those which could be assigned to the P cycle accounted for 0.34% in Freising and 0.33% in Rostock. From a total of 40 P-turnover genes, 27 were included into further analysis, whilst the rest were excluded due to their low abundance. The relative abundance of the analyzed genes was similar between the investigated sites.

Contrary to the hypothesis, the application of organic fertilizer did not have a significant influence on the abundance of analyzed bacterial P turnover genes.

The most abundant gene in the metagenomic dataset was that encoding for glucose dehydrogenase enzyme (*gcd*) which oxidizes glucose and other aldose sugars resulting in the excretion of gluconic acid which acidifies the surrounding area thereby facilitating the solubilization of P bound in Ca- and Mg- secondary minerals. Normally, Gcd requires the co-factor pyrroloquinone (PQQ) to form the active PQQGDH holoenzyme (Goldstein, 1995). However, in M1 genes linked to this cofactor were very low in abundance. This could be due to partly PQQ independent activation of GDH, as described by (Goldstein et al., 2003) and although PQQ is regarded as essential for the activity of the GDH enzyme, evidence suggests that there is no correlation between the PQQ and GDH levels (Schie et al., 1984; van Kleef & Duine, 1989). It has been found that *gcd* expression is increased under low soil P conditions (An & Moe, 2016; Liang et al., 2020), which is in line with findings of M1 and M2, since the test sites have not received any P fertilizer in M1, and in M2 two out of three investigated soils contained suboptimal P levels.

The second most abundant genes in the dataset were those encoding for proteins involved in P_i uptake, namely *pst* and *pit*, followed by alkaline phosphatases (ALP) *phoA*, *phoD*, and a class C acid phosphatases (ACP) *olpA*, all of which are genes involved in P mineralization. Genes coding for substrate specific enzymes (phosphonatases, C-P lyases, and phytases) capable of accessing sources of P that are lower in concentration or more difficult to obtain (e.g., phytate) were very low in abundance. It has been suggested that when P utilization preference is examined, the most readily metabolized form of P determines which enzymes are produced, whereas the enzymes for accessing alternative P sources are usually not expressed (Tapia-Torres et al., 2016). This suggests that the prevalent form of P_o in the investigated soils were phosphoesters, which are mineralized more readily than phosphonates since C-P bonds are highly stable, as well as that P pools in the investigated agricultural soils are rich in inorganic orthophosphate (Stutter et al., 2015).

3.2. The effect of organic amendments on P_{mic} and potential phosphatase activity

In the experiment testing mineral P and organic amendments (M2), P_{mic} content ranged from 2.1 to 46.4 mg kg^{-1} and was generally lowest in control plots (average of 7.2 mg kg^{-1}). The content of P_{mic} in agricultural soils is generally lower than in forest and grassland soils with determined ranges between 2.4 and 21 mg kg^{-1} (Turner & Haygarth, 2005). This is attributed to lower soil organic C in agricultural soils, and further supported by an observed increase in P_{mic} content upon repeated addition of organic materials to cropped soils (Turner et al., 2004). Opposing to the previous findings where soils fertilized with organic amendments contained almost twice as much P_{mic} as soils receiving only mineral fertilizers (Oberson et al., 1996; Oehl et al., 2001), in the aforementioned experiment, P_{mic} content was highest in MIN (average of 26.6 mg kg^{-1}), and lowest in ORG (16.6 mg kg^{-1}), suggesting that microbes responded to readily available inorganic phosphate.

Amendments containing organic fertilizer (ORG and OMI) resulted in increased potential phosphatase activities compared to MIN and control. This is in line with Colvan et al. (2001), who found that meadow soils fertilized with farmyard manure for about 100 years had higher phosphatase activities than those receiving mineral P. The increase in phosphatase activities

following the organic fertilization was addressed in multiple studies (Criquet et al., 2007; Nannipieri et al., 2011b; Piotrowska-Długosz & Wilczewski, 2014) and can be explained by stimulation of microbial activity and improvement of soil properties through enrichment of SOM (Adetunji et al., 2017; Jones et al., 2011). In M2, soils with ORG and OMI amendments had significantly higher TN and C_{org} compared to MIN and control. Marklein & Houlton (2012) found that TN is strongly positively correlated with phosphatase activity. This can be explained by high N availability relative to P being required for the microorganisms to start investing their energy into production of phosphatases since N is a necessary element for the synthesis of enzymes (Houlton et al., 2008; Olander & Vitousek, 2000; Treseder & Vitousek, 2001).

Therefore, although the hypothesis that organic amendments will result in higher abundance of bacterial genes involved in soil P transformations was not confirmed using metagenomic approach in M1, the results obtained in M2 regarding potential phosphatase activities suggest that organic amendments do stimulate bacterial P acquisition.

4. Alternative P fertilizers

In M3, the response of the soil P turnover bacteria to the application of the alternative P fertilizers obtained by carbonizing animal bones (bonechar – BC, and sulphur enriched bonechar – BC^{plus}) was compared to TSP fertilization and control (no P). The experiment was conducted during one growing season of winter wheat on soils with three different initial P levels which were classified based on available P content as very low (11.2 mg kg^{-1}), low (20.7 mg kg^{-1}), and optimal (47.2 mg kg^{-1}) (according to VDLUFA guidelines), and contained different levels of TP (217, 273, 399 mg kg^{-1}, respectively), while TC (1.3 – 1.4%) and TN (0.1%) were constant.

The fourth hypothesis of this thesis postulated that viability of the alternative mineral P fertilizers will be reflected in the abundance of bacteria mediating P turnover processes that is comparable to that of conventional mineral fertilizer (TSP). It has also been postulated that the effect of fertilization on bacterial P turnover will vary depending on the initial soil P content and plant growth stage. This will lead to changes in potential activities of P-acquiring extracellular enzymes during the growing season of winter wheat in agricultural soils with very low, low, and optimal P content (hypothesis five).

The effect of the different P fertilization regimes was most obvious in the P_{CAL} and P_{water} values that were, compared to the control, the highest in TSP amended plots, irrespective of soil class or plant growth stage. This might be due to TSP being the best water-soluble fertilizer and thus most of it could be reextracted, or most of the TSP remained unused and at risk of being lost by leaching to the groundwater (Djodjic et al., 2004). In the BC and BC^{plus} treatments, the same amount of P was applied as in TSP plots, but no accumulation of P_{water} or P_{CAL} was observed for the alternative fertilizers. It is unlikely that no P was released from BC and BC^{plus}, since this has already been proven, and it has also been shown that P release from BC^{plus} is higher than that from BC due to activation by S, the oxidation of which results in pH reduction which promotes P dissolution (Zimmer et al., 2018, 2019). The other possibility is the binding of released P to the bone char particles, which is also unlikely because the bone char surface at soil pH between 5 and 5.3 is negatively charged and repels anions such as phosphate (Leinweber, unpublished data). Thus, it seems that all P released from BC and BC^{plus} has been taken up, which was corroborated by higher abundance of P solubilization genes (*gcd*) as well as P uptake genes (*pitA*

and *pstS*). This has also been observed for rock phosphate as alternative P fertilizer, which resulted in increased organic P mineralization and the immediate immobilization of P in the microbial biomass, while the fertilization with TSP caused an increase of P_i (Margenot, 2016).

The higher abundance of *gcd* genes in BC^{plus} treatments suggests that this form of P fertilizer increases the microbial potential to solubilize inorganic P. This is in line with Postma et al. (2010), who demonstrated that the addition of P solubilizing microbes to animal bone charcoal particles improved the dissolution of P. Although the addition of P solubilizing bacteria to the soil had been recommended for improving the sustainability of P fertilization in agriculture (Alori et al., 2017; Ditta et al., 2018), the results imply that the application of alternative P fertilizers stimulates the inherent P solubilizing microbes, especially under specific circumstances such as particular plant growth stages (heading) or different initial soil P concentrations (low, optimal). Additionally, application of BC^{plus} to the soil with low initial P level resulted in slightly higher crop yield (Panten & Leinweber, 2020).

The increased abundance of *phoD* genes in plots receiving BC fertilizer suggests that the fertilization with BC favored P_o mineralization. In the plots with optimal initial soil P content, higher abundance of *phoD* was accompanied by higher abundance of *pitA* genes, which code for low-affinity P_i transporter responsible for bacterial Pi uptake under sufficient P availability (Wanner, 1993; Willsky et al., 1973). Therefore, it seems that BC application provides favorable conditions for a closed P cycle. However, potential ALP activities were not increased, which might be due to low soil pH not being optimal for their activity.

Regarding the P uptake genes, the abundance of *pitA* was always 10 to 100-times higher than the abundance of *pstS*, indicating sufficient P availability in all tested soils.

4.1. Fertilizer effect is highly interlinked with plant growth stage and initial soil P concentration

In addition to the initial soil P content, the effect of alternative fertilizers was also strongly interlinked with plant growth stage (M3). During the heading of winter wheat, the abundances of P-turnover genes were higher in control plots compared to P fertilized plots. This points toward P_i limiting conditions for the microorganisms when no P fertilizer was applied and after wheat had met most of its own P demands during highest biomass growth (Römer & Schilling, 1986).

Especially the abundance of *pstS* gene, which codes for a highly specific P_i transporter and is starvation induced, was significantly higher compared to the other sampling dates ($p = 0.03$). Interestingly, both P uptake genes – low-affinity, constitutively expressed *pitA* and highly specific, starvation-induced *pstS*, were influenced by initial soil P content, with highest values detected in soils with optimal initial P content. This indicates that microbial community of those soils might have adapted to higher levels of available P by switching to "luxury phosphorus uptake" (Ohtake et al., 1985) as it has been shown that many microorganisms accumulate P_i in the form of polyphosphate in P rich environments (Akbari et al., 2021; Kulaev & Vagabov, 1983; S. Li et al., 2014).

Even more interestingly, the potential phosphatase activities did not significantly differ between three soil classes used in this experiment. Addition of mineral P fertilizers has been shown to increase the phosphatase activity in soils with low organic matter but had no effect when the soil contained high organic matter (Piotrowska-Długosz & Wilczewski, 2014). Margalef et al. (2017) used a total of 378 sites around the world to analyze patterns of phosphatase activity and found no correlation between available P and phosphatase activity, suggesting that available P does not relate to potential capacity of the system to release P with the help of phosphatases. They proposed that phosphatases relate only to the source (organic matter) whereas available P is controlled both by the sink (plant and microbial P uptake) and the source. They also found TP to be a poor predictor of phosphatase activity because it comprises the phosphorus part of primary mineral and occluded recalcitrant forms, both being reservoirs that cannot be a substrate for phosphatase enzymatic activity.

Surprisingly, the increased abundance of *phoD* genes in plots receiving BC fertilizer was most pronounced in soils with optimal initial P content. This is unexpected because *phoD* is part of the PHO regulon which detects P starvation and controls several phosphate starvation inducible genes (Hsieh & Wanner, 2010). In a long-term fertilization study, Fraser et al. (2015) found highest mineralization potential, as shown by increased *phoD* abundance and high potential ALP activity, in soils with lowest available P concentration. Thus, one would expect to find highest *phoD* abundance in soils with lowest initial P content. However, it seems that the bacterial

community in a very low P environment is adapted to low P availability, whereas slow rate of P release from BC (Leinweber et al., 2018) could promote bacterial mineralization potential.

Control plots showed similar gene abundance pattern irrespective of the soil class, suggesting an equilibrium between P uptake, P turnover of the bacterial biomass and P solubilization from the soil. Under constant depletion of P pools by microbial and plant uptake, this equilibrium is essential for meeting their P-demands. Similar observations were made in forest sites with low P concentrations, which showed an efficient recycling of P (Bergkemper et al. 2016a, Lang et al. 2017).

In general, alternative P fertilizers affected P-mobilization, but they differed in the processes which they promote. BC^{plus} favored bacterial P solubilization, as indicated by higher *gcd* abundance, whereas BC favored P recycling from biomass, which was displayed by high abundance of *phoD* carrying bacteria. Additionally, the effects were strongly interlinked with initial soil P content and plant growth stage.

V Conclusions and outlook

Soil is a heterogeneous environment with complex interactions between plants and their surroundings. Microorganisms no doubt are at the heart of the P cycle in soil and contribute a large part to the P nutrition of plants. Throughout this study the predominant processes and families involved in P turnover in the scope of agricultural soil were uncovered. More specifically, it has been found that in agricultural soils the primary acquisition of P by bacteria was through solubilization of inorganic P indicative of sufficient reserves still being present. Moreover, it was confirmed that bacterial family composition and P transformation are not affected by geographical location, climate, soil genesis, and parent material but rather by management and ecosystem type.

Type of applied fertilizer has a profound effect on P-turnover, which was demonstrated by a clear splitting in phosphatase activities between treatments without and with organic amendments.

Furthermore, the experiment with conventional and alternative mineral P fertilizers during one growth season of winter wheat on soils with different initial P content further revealed the complexity of the different interactions. Alternative P fertilizers affected P-mobilization, but they differed in the processes which they promote. Additionally, the effects were strongly interlinked with initial soil P content and plant growth stage. However, the results did show that even here, P solubilization and uptake are the predominant processes.

Future work aimed at obtaining a more complete picture of microbial P turnover in soil undoubtedly involves the analysis of transcription rates of P turnover genes as well as the expansion of databases. Furthermore, the diversity of the soil microbial community should be complemented by deeper understanding of complex interactions between key players. Additionally, rhizosphere soil should be investigated and compared to bulk soil, and ideally sampling should take place multiple times during different growth stages of a crop as well as between growth seasons. Together these findings would allow for more sustainable P application in agriculture, improved crop production, as well as protection of environmental health.

VI Methodological caveats

1. Sampling and DNA extraction

It has been shown that one gram of soil contains around 100 million microbial cells (Prosser, 2015). This vast number is accompanied by the complex soil ecosystems compiling a plethora of diverse microhabitats, each defined by unique physical, chemical, and biological properties (Totsche et al., 2010). This level of complexity is complemented by aspects of temporal heterogeneity signified by the aboveground plant diversity and growth stage as well as season (Petersen & Esbensen, 2005). Diversity and distribution of soil microorganisms are strongly interlinked with these factors (Prosser, 2015; Richardson & Simpson, 2011; Vestergaard et al., 2017), resulting in numerous micro-niches and hotspots. Although this is well known, most studies on microbial distribution focus on large-scale differences, comparing land use types, position in landscape, or different treatments (Du et al., 2015; Francioli et al., 2014; Garciá-Orenes et al., 2013). Capturing this vast microbial diversity requires a carefully designed and executed sampling plan, appropriate downstream processing of samples, and efficient DNA extraction. Each of these steps poses a potential source of biases, from choosing inappropriate sampling scheme, to getting distorted picture of microbial community composition because of the biased DNA extraction. The extracellular and the DNA deriving from dormant and/or dead cells further complicates obtaining a meaningful picture of soil functional diversity. The extracted functional gene may also be inactive because it relies on other metabolic pathways for its activity (Prosser, 2015) A method which separates intracellular from extracellular DNA (Pietramellara et al., 2008) could eliminate this bias.

2. Limitations of metagenomic analysis

Next generation Sequencing (NGS) has revolutionized the way we look at complex environments and has allowed for culture independent, simultaneous detection, and characterization of microorganisms in different environments (Goodwin et al., 2016; Prosser, 2015). For the past 15 years, technological advances in NGS have produced innumerable studies – with ascending tendency. One of the main advantages of this culture independent approach is the identification

of untargeted microbiota and their characterization. Shotgun sequencing alone can provide a holistic image of communities based on relative abundances. This means that the obtained information reflects microbial potential and not actual activity (Knight et al., 2012).

Processing of the sequencing data is susceptible to limitations and biases (Lombard et al., 2011; Prosser, 2017), especially considering that most of the databases, such as the NCBI non-redundant protein database, show biases towards medically relevant, eukaryotic, or model organisms (Darzi et al., 2016; Vestergaard et al., 2017). The use of these general databases also impacts coverage estimations after sequencing runs of environmental samples. This is likely to change with databases focusing on environmental genomic data such as the earth microbiome project (Gilbert et al., 2014). But until then database independent coverage estimation through approaches such as nonpareil will still be important (Rodriguez & Konstantinidis, 2014).

As it is the case for microbial diversity, functional annotation also relies heavily on publicly accessible databases such as KEGG (Kanehisa et al., 2016), SEED (Overbeek et al., 2005), and COG (Tatusov et al., 2000) which are useful to get a first overview. However, further development and utilization of databases such as FOAM (Prestat et al., 2014) that are specifically designed for soil related studies will give a more specific picture.

To obtain a better understanding on actual P-turnover activity of microorganisms, future research should include data on transcriptional level using metatranscriptomic and/or metaproteomic approaches. Lastly, spatio-temporal data on physicochemical measurements, water availability, temperature, as well as plant species and growth stage should be well documented and included in analyses, as they are particularly important for obtaining a full picture of microbial activities, especially when investigating the effects of fertilization on microbial potentials to metabolize an extremely immobile element such as P.

3. qPCR

Quantitative polymerase chain reaction (qPCR) allows for cultivation independent detection and analysis of a gene of interest in real time and is widely used in microbial ecology. It is known to be a sensitive, reproducible, and robust method with which the changes in abundance of phylogenetic and functional genes can be tracked across temporal and spatial scales under changing environmental conditions.

However, qPCR is susceptible to limitations that could affect interpretation. As mentioned, the most crucial step which can affect the outcome and quality is the nucleic acid extraction protocol. In environmental sample analyses these vary greatly, especially because the extraction protocol is adjusted to the studied sample (Martin-Laurent et al., 2001). Agricultural soil can be particularly challenging depending on clay content and a number of various inhibitors (i.e., humic acids) which must be removed to obtain high grade DNA for downstream processing (Frostegård et al., 1999; Stults et al., 2001; Vestergaard et al., 2017). Moreover, the specificity of any qPCR assay is highly dependent on the primer design. This is particularly problematic when considering that some functional genes are not well conserved on the amino acid level when comparing microorganisms from distinct phyla (Bergkemper et al., 2016b). Furthermore, primer design could be influenced by biases in the employed database (Bergkemper et al., 2016b). These will likely be alleviated with further expansion of publicly accessible databases which focus on specific environments.

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List of abbreviations

µg	**Microgram**
µl	Microliter
ACP	Acid phosphatase
Al	Aluminium
ALP	Alkaline phosphatase
AMF	Arbuscular mycorrhiza
ATP	Adenosine triphosphate
BC	Bonechar
BC^{plus}	Sulfur enriched bonechar
BMBF	Bundesministerium für Bildung und Forschung
bp	Base pair
C	Carbon
Ca	Calcium
CaCl2	Calcium chloride
cm	Centimeter
Cmic	Microbial biomass carbon
CO2	Carbon dioxide
DAP	Diammonium phosphate
DM	Dry matter
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DON	Dissolved organic nitrogen
dwt	Dry weight
EE	Extracellular enzymes
Fe	Iron
g	Gram

G2P	Glycerol-2-phosphate
G3P	Glycerol-3-phosphate
GDH	Glucose dehydrogenase
H+	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen peroxide
ha	Hectare
HAPS	Histidine acid phosphatase
HCl	Hydrochloric acid
HNO_3	Nitric acid
IP6	Inositol hexakisphosphate
iSPTC	Initial soil phosphorus test class
K	Potassium
kg	Kilogram
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOH	Potassium hydroxide
m	Meter
M	Molar
m2	Square meter
MAP	Monoammonium phosphate
Mg	Magnesium
mg	Milligram
ml	Milliliter
mmol	Millimol
N	Nitrogen
NH4+	Ammonium
ng	Nanogram
nM	Nanomol

pM	Picomol
NMDS	Non-metric multidimensional scaling
Nmic	Microbial biomass nitrogen
NSAP	Nonspecific acid phosphatase
OTU	Operational taxonomic unit
P	Phosphorus
PCA	Principal Component Analysis
Pcal	Polymerase chain reaction
Pcal	Calcium acetat lactate-extractable phosphorus
PDE	Phosphodiesterase
Pdl	Double lactate-extractable phosphorus
Pi	Orthophosphate
Pit	Phosphate inorganic transporter
Pmic	Microbial biomass phosphorus
p-NP	para-nitrophenol
Po	Organic phosphorus
PQQ	Pyrroloquinoline quinone
PQQGDH	Quinoprotein glucose dehydrogenase
PSM	Phosphorus solubilizing microorganisms
Pst	Phosphate specific transporter
Pwater	Water-extractable phosphorus
qPCR	Quantitative real-time PCR
S	Sulfur
SOM	Soil organic matter
TSP	Triple Superphosphate
VDLUFA	Der Verband deutscher landwirtschaftlicher Untersuchungs und Forschungsanstalten e.V.
SAP	Specific acid phosphatases

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Appendix

Manuscript I

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

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Summary

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

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Introduction

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

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

the bacterial demands for P and thus stimulate P mineralization from soil if the amount of easily degradable organic carbon is not limiting microbial activities.

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

Results and discussion

Microbial C, N and P pools

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

Functional profiles of microbes triggering P transformation

P-turnover was analyzed by comparing the relative abundances of 40 bacterial genes encoding proteins which are linked to regulation, mineralization, solubilization and uptake of extracellular P sources; genes that are involved in intracellular P turnover were excluded from further analysis (Supporting Information Table S2; Bergkemper *et al.*, 2016). Therefore, the filtered reads obtained by metagenomics sequencing were mapped against sequences of the KEGG database [using BLAST (Altschul *et al.*, 1990)] for gene annotation (for details see Supporting Information materials and methods) (Table S1). After subsequent filtering, we obtained 52,654,918 reads with an average read length of 296 bp

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

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

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

after trimming (Table S1). Of these, genes linked to P turnover made up approximately 0.34% at both sites. Rarefaction analysis (Supporting Information Fig. S2) revealed sufficient coverage in all samples. For further analysis, 27 genes were processed. The analysis of the relative abundances of the targeted genes indicated a strong similarity of the two sites in Rostock and Freising. Further the data indicated, that neither season nor the fertilization treatment had a significant effect on the relative abundance of the analyzed genes.

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

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

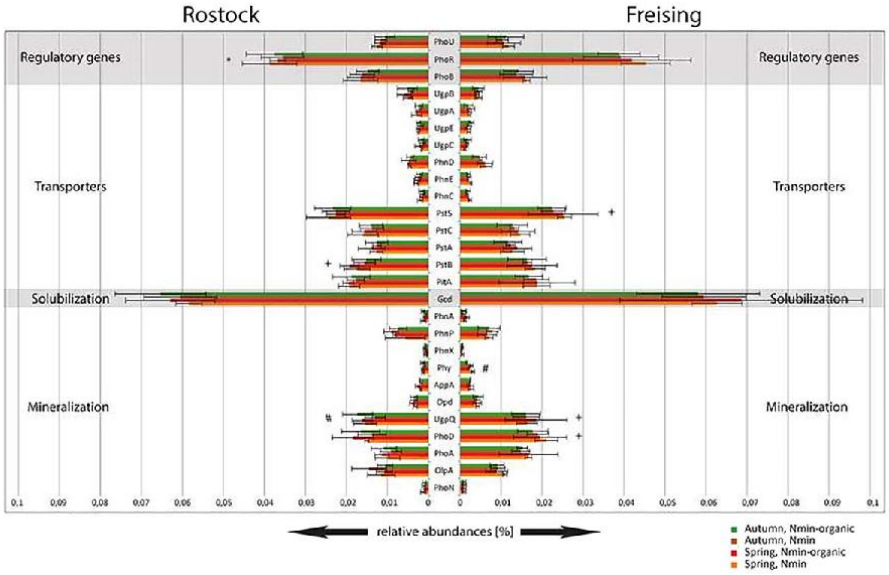

Fig. 1. Barplot showing relative abundances of genes for proteins, which are involved in P turnover in Freising and Rostock. Metagenomic data presented is of both test sites, over the change of the season, as well as two treatments. The quality filtered reads were blasted against the KEGG database and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

also found in our datasets; however, they were far less abundant. Their low abundance could be surprising, considering that organic fertilizers contain large amounts of organically bound P.

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

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

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

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

Structure of bacterial communities triggering P transformation

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

Overall, 295 bacterial families were identified, of which 292 families were present at both sites. The most abundant families were Acidobacteriaceae, Bradyrhizobiaceae, Verrucomicrobia subd. 3, Planctomycetaceae and Chitinophagaceae. This is not surprising because they are known to be widely spread throughout terrestrial ecosystems (Rosenberg, 2014). The effect of season was much stronger than that of the fertilizer addition as indicated by means of a two-way blocked ANOVA. Of the 295 families, 36 were influenced by season, 14 by fertilizer and 18 by the interaction of both (Supporting Information Table S3).

Of the 295 total families, 258 harboured genes encoding for proteins involved in extracellular P turnover. Of these, 85 families comprised a core microbiome (as defined by Shade and Handelsman, 2012), which was present at both sites, independent from seasons and treatments. 91% of all filtered reads could be assigned to these families (Supporting Information Fig. S3).

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

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

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

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

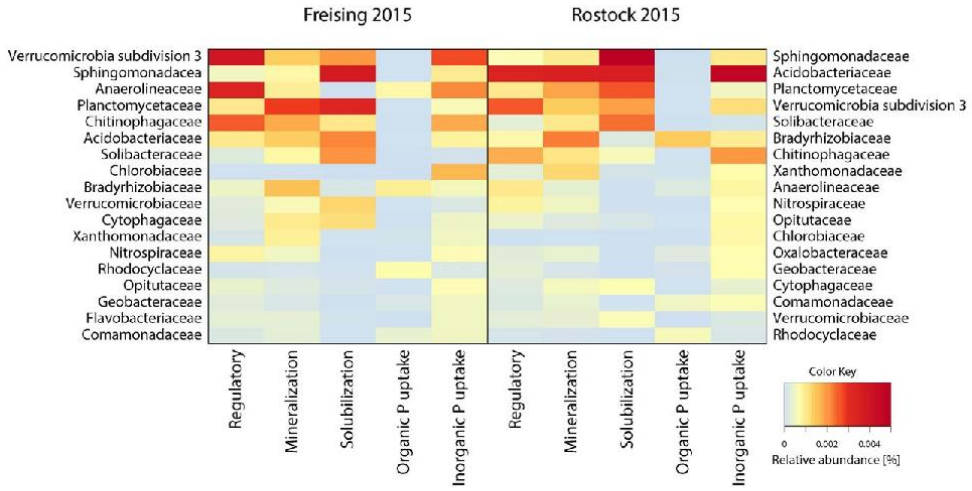

Fig. 2. Visualization of mean relative abundances of genes harboured by different families in Freising and Rostock independent of season and fertilizer, by means of a heatmap. Genes involved in P turnover were selected by their K0 numbers and grouped depending on their function. These were then assigned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

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

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

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

Experimental procedures

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

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

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

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

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Supporting Information

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

Text S1. Materials and Methods

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

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

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

Table S4. R packages used for data visualization.

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

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

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

Supplemental Information Manuscript I

Bacterial potentials for uptake, solubilization and mineralization of extracellular phosphorus in agricultural soils is highly stable under carbon and nitrogen amendment

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Text S1: Materials and Methods

Site description and sampling

The long-term field trial (established in 1979) in southern Germany is situated north of Munich in Freising/Dürnast, Bavaria (FS) (48°24'13.38" N, 11°41'32.93" E). The test site is 470m above sea level with a mean annual precipitation and temperature of 791mm and 8.4°C, respectively. The soil type was classified as Cambisol, the soil texture silty loam and the pH was 6.35. The test site has a split plot design where two treatments were analyzed in the frame of this study: 1) mineral N fertilization (2015) 150 kg ha^{-1}, no additional organic fertilization (Nmin); 2) mineral N fertilization (2015) 150 kg ha^{-1}, and organic fertilization with straw and catch crops as green manure (Nmin-organic). In treatment 1 (Nmin) the straw as harvest residue of cereals was removed whereas it remained at the plots in treatment 2 (Nmin-organic). In treatment 2, green manure was applied by the sowing of catch crops (turnip rape, rape, mustard, or phacelia) after the harvest of winter or summer barley prior to the cropping of potatoes or summer cereals. The plots used in this study had received no mineral P fertilizer since 1979. Each plot had a size of 32m^2. Each treatment was three times replicated. In the long-term crop rotation potato, winter or summer wheat followed by winter or summer barley were grown. However, winter barley in 2013 was followed by maize in 2014 and 2015. The mineral N fertilizer was applied at May 28, 2015 as calcium ammonium nitrate. The most recent application green manure occurred in autumn 2010 (phacelia) and that of straw (winter barley) in 2013. As tillage treatment ploughing up to 25 cm was performed. The obtained yield of the N fertilized plots in 2015 was 206 dt dry matter ha^{-1} (standard deviation 28 dt ha^{-1}) and of the N and organic fertilized plots was 197 dt dry matter ha^{-1} (standard deviation 32 dt ha^{-1}).

The long-term field trial (established in 1998) in northern Germany is located in Rostock (HRO), Mecklenburg Western Pommerania, (54°03'42.44" N, 12°05'07.32" E). The test site is 46 m above sea level, characterized by annual means of 600 mm precipitation and 8.1°C temperature. The soil texture is loamy sand, and the dominating soil type is a Stagnic Cambisol according to the World Reference Base of Soil Resources. The soil has a pH_{CaCl2} of 6.3. The site was arranged as a split plot design with four replicates, where two treatments were analysed in the frame of this study: 1) N fertilization 120 kg ha^{-1}, no additional organic fertilization (Nmin); 2) N fertilization

120 kg ha^{-1}, organic fertilization with organic fertilizer (30 t ha^{-1}) (Nmin-organic). Nmin plots, had 10.8 g TOC kg^{-1}, 1.0 g TN kg^{-1}, 547 TP mg kg^{-1} on average. Nmin-organic plots had 12.1 g TOC kg^{-1}, 1.2 g TN kg^{-1} and 603 mg TP kg^{-1} on average. The organic fertilizer was applied every three years (last application in September 2013) and mineral N fertilizers are applied every year in May (80 kg N ha^{-1}) and June (40 kg N ha^{-1}). Each plot has a size of 30 m². Each treatment has three replicates. Plots selected for this study were cultivated with maize from 2013 – 2015, except for one sample, where a mix of bean and maize was grown. This sample belonged to the biowaste treatment (Sample Rostock S+2 in Table S1). For tillage soil was ploughed 25 cm deep in autumn after the harvest and soil sampling. The obtained yield in 2015 was 12.1 t DM ha^{-1} in the N fertilized plots and 12.0 t DM ha^{-1} in the N and organic fertilized plots without significant differences between the treatments.

Samples were taken in April and September of 2015 using a soil auger of 3 cm diameter to a depth of 10 cm. As suggested by Vestergaard et al. (2017) three cores were collected for each plot, pooled, and subsequently homogenized on a 5mm sieve. A total of twenty-four (24) samples were taken (two sites, two sampling time points, three replicates, two treatments). Spring samples were taken before seeding and the initial N - fertilization; the autumn samples were taken with the maize still being present. The samples for the determination of soil microbial biomass were stored at 4°C and analyzed within one week. The samples for nucleic acid extraction were collected and immediately frozen on the field using dry ice.

Determination of soil microbial biomass

For the assessment of Cmic and Nmic the chloroform- fumigation-extraction method as described by (Vance et al., 1987) was used, Correction factors were K_{EC}=0.45 for Cmic and K_{EN}=0.54 for Nmic according to Joergensen (1996) and Joergensen and Mueller (1996) respectively. Microbial P (Pmic) was also analyzed using the chloroform-fumigation-extraction method as described by Brookes et al. (1982) and calculated using K_{EP}=0.4.

Nucleic acid Extraction and Sequencing

DNA was directly extracted from 0.5g of frozen soil (−80°C) using Precellys 24 (Bertin Technologies, France) using a phenol-chloroform based protocol modified according to Lueders

et al. (2004) and Töwe et al. (2011). Total genomic DNA quality was quantified photometrically (Nanodrop ND-1000; Thermo Fischer Scientific, USA); the quality of the DNA was assessed using the QuantiT PicoGreen kit (Life Technologies, USA). DNA content of all samples was in the range of 14-22 ng/µl. Extracted samples were then stored at −20°C before further processing.

Prior to library preparation 1µg of DNA was shared on a COVARIS® E220 (Covaris®) according to the protocol provided by the manufacturer. (Conditions: Peak incident Factor: 175W; Duty Factor: 5%; Cycles per burst: 200; Treatment time: 35 seconds; Temperature: 7°C; Water level: 6; Intensifier: Yes). Of the 100-200 ng of the shared DNA were used for the Library preparation with NEBNext® Ultra™ DNA Library Prep Kit (New England Biolabs® Inc.). All steps were carried out according to the NEBNext® Ultra™ manual (New England Biolabs® Inc.). As described in the manual, the adapters for the ligation were diluted 1:10 to prevent the occurrence of primer dimers. Size selection of fragments was performed with Agencourt® AMPure® XP (Beckman Coulter, USA) to obtain a total library size of 500-600bp. Furthermore, an additional cleaning up step (after the PCR) was necessary to eliminate residual primer dimers. Therefore, a bead to DNA ratio of 0.6:1 was used. Libraries were pooled equimolar (4nM). 10 pM of the mixture was spiked with 30 % PhiX, used as a quality and calibration control (Mukherjee et al., 2015). Sequencing was performed on an Illumina® Miseq® (Illumina®) sequencing machine and using the MiSeq® Reagent Kit v3 (600 cycles) (Illumina®) for paired end sequencing. The raw sequencing data are available at the sequencing read archive (SRA) under the BioProject ID PRJNA385596 (SAMN06894543- SAMN06894566).

Analysis of sequencing data

MiSeq fastq files were processed by first removing the remnant adaptor sequences and subsequently trimming the reads via AdapterRemoval (Schubert et al., 2016) by applying the following settings: 5'/3'- terminal minimum Phred quality = 15, minimum read length = 50. Furthermore, PhiX contamination was used as a quality and calibration control during the sequencing run (Mukherjee et al., 2015). PhiX was removed using DeconSeq (Schmieder and Edwards, 2011).

For taxonomic and functional annotation, the sequences were blasted against the NCBI-nr database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz) and the Kyoto Encyclopedia of Genes

and Genomes (KEGG) database (June 2011) (Kanehisa & Goto, 2000) using DIAMOND (0.5.2.32) on sensitive settings (Buchfink et al., 2014). Taxonomic and functional assignment was performed by using MEGAN5 v 5.10.6 (Huson et al., 2011). This was performed based on the top 25 blast results (i.e., hits with the lowest e- value). Following parameters were applied during MEGAN analysis: MinScore = 50.0, MaxExpected = 0.01, TopPercent = 10.0, MinSupport = 1, MinComplexity = 0. Hits with organism names containing words "environmental samples", "unclassified" and "unidentified" were excluded from further analysis. This was done to avoid confusion during visualization (Mizuno et al., 2001; Zhu et al., 2014).

Statistical analyses and visualization were performed using Rstudio (Team, 2015), based upon the relative abundance of reads. Relative abundances were obtained by dividing the number of reads by the total number of filtered reads per sample and multiplication by 100. Using two-way analysis of variance (ANOVA), the effects of season, fertilizer, and interaction of both, on taxonomic as well as the functional profiles of the samples were detected. Pairwise differences were identified using the Fisher's least significant difference (LSD) method. Prior to testing for differences, normality of the data was ascertained with Q-Q plots and Shapiro-Wilk tests. Differences were considered significant when the p-value was below 5 % ($P < 0.05$). Only taxa with a relative abundance of 0.01% and above in one of the samples were included in the statistical analysis of taxonomic profiles. This cut-off was not applied to functional data due to the low number of attained reads. To assess the level of dissimilarity between the samples, non-metric multidimensional scaling (NMDS) ordination plots were created. These are based on the Bray-Curtis distance metrics, which uses the metaMDS function in the R vegan package (Oksanen et al., 2015). All R packages used for this study can be found in Table S6. Visualization of VENN diagrams was performed using InteractiVENN (Heberle et al., 2015). To create these diagrams a family was regarded as present if it occurred in at least two out of three samples.

		Freising						Rostock					
		Nmin			Nmin-organic			Nmin			Nmin-organic		
Spring	Samples	S-1	S-2	S-3	S+1	S+2	S+3	S-1	S-2	S-3	S+1	S+2	S+3
	Number of reads	1519414	1793634	2014728	3482732	1618966	2477132	2173652	1684256	2227964	2432242	2538612	2368702
	Average length of reads	301	301	301	301	301	301	301	301	301	301	301	301
	Quality filtered data												
	Number of reads	1519109	1793260	2014479	3481603	1618357	2476260	2172948	1683857	2227777	2431901	2538107	2368511
	Average length of reads	296.90	296.00	297.24	296.74	296.58	295.83	295.99	295.92	296.88	296.16	296.67	295.69
	Pfiltered reads	5324	6319	6275	11554	5629	8008	7366	5528	7054	8423	6468	7568
Autumn	Raw data	A-1	A-2	A-3	A+1	A+2	A+3	A-1	A-2	A-3	A+1	A+2	A+3
	Number of reads	1962416	2064692	2444034	2417380	1793994	1740534	1809582	2389842	1914436	2680464	2418498	2700418
	Average length of reads	301	301	301	301	301	301	301	301	301	301	301	301
	Number of reads	2697782	2064385	2443893	2416960	1793125	1739821	1809142	2389425	1913974	2679936	2418081	2697782
	Total length of reads	792376348	613099923	726876595	712436432	531094135	515919695	536941092	708253978	567169242	794836574	715819266	792376348
	Average length of reads	293.71	296.99	297.43	294.77	296.18	296.54	296.79	296.41	296.33	296.59	296.03	293.71
	Pfiltered reads	6566	5747	7694	7466	5526	4953	7221	7150	5525	8737	5672	7747

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

Enzyme Name	KO Number	Gene	Reference
Phosphate Regulon Response Regulator	K07657	*phoB*	Hsieh & Wanner, 2010
Phosphate Regulon Sensor Histidine Kinase	K07636	*phoR*	Hsieh & Wanner, 2010
PhoR/PhoB Inhibitor Protein	K02039	*phoU*	Hsieh & Wanner, 2010
Glycerol−3−Phosphate Transporter Subunit	K05813	*ugpB*	Hsieh & Wanner, 2010
Glycerol−3−Phosphate Transporter Subunit	K05814	*ugpA*	Hsieh & Wanner, 2010
Glycerol−3−Phosphate Transporter Subunit	K05815	*ugpE*	Hsieh & Wanner, 2010
Glycerol−3−Phosphate Transporter Subunit	K05816	*ugpC*	Hsieh & Wanner, 2010
Phosphonate Transporter Subunit	K02044	*phnD*	Hsieh & Wanner, 2010
Phosphonate Transporter Subunit	K02042	*phnE*	Hsieh & Wanner, 2010
Phosphonate Transporter Subunit	K02041	*phnC*	Hsieh & Wanner, 2010
Phosphate−Specific Transport System	K02040	*pstS*	Hsieh & Wanner, 2010
Phosphate−Specific Transport System	K02037	*pstC*	Hsieh & Wanner, 2010
Phosphate−Specific Transport System	K02038	*pstA*	Hsieh & Wanner, 2010
Phosphate−Specific Transport System	K02036	*pstB*	Hsieh & Wanner, 2010
Phosphate Inorganic Transporter	K03306	*pit*	Elvin et al., 1986
Outer Membrane Pore Protein E	K11929	*phoE*	Hsieh & Wanner, 2010
PQQGDH (Quinoprotein Glucose Dehydrogenase)	K00117	*gcd*	Cleton-Jansen et al., 1990
C−P Lyase Subunit	K02043	*phnF*	McGrath et al., 2013
C−P Lyase Subunit	K05774	*phnN*	McGrath et al., 2013
C−P Lyase Subunit	K05780	*phnL*	McGrath et al., 2013
C−P Lyase Subunit	K05781	*phnK*	McGrath et al., 2013
C−P Lyase Subunit	K06162	*phnM*	McGrath et al., 2013
C−P Lyase Subunit	K06163	*phnJ*	McGrath et al., 2013
C−P Lyase Subunit	K06164	*phnI*	McGrath et al., 2013
C−P Lyase Subunit	K06165	*phnH*	McGrath et al., 2013
C−P Lyase Subunit	K06166	*phnG*	McGrath et al., 2013
C−P Lyase Subunit	K06167	*phnP*	McGrath et al., 2013
C−P Lyase Subunit	K09994	*phnO*	McGrath et al., 2013
Phosphonatase	K05306	*phnX*	McGrath et al., 2013
3- Phytase	K01083	E3.1.3.8	Golovan et al., 2000
4- Phytase	K01093	*appA*	Golovan et al., 2000
Phosphotriesterase	K07048	*opd*	McDaniel et al., 1988
Glycerophosphoryl Diester Phosphodiesterase	K01126	*ugpQ*	Brzoska & Boos, 1988
Alkaline Phosphatase	K01113	*phoD*	Eder et al., 1996
Alkaline Phosphatase	K01077	*phoA*	Torriani, 1960
Acid Phosphatase (Class A)	K09474	*phoN*	Rossolini et al., 1998
Acid Phosphatase (Class B)	K03788	*aphA*	Rossolini et al., 1998
Acid Phosphatase (Class C)	K01078	*olpA*	Rossolini et al., 1998
Phosphonoacetate Hydrolase	K06193	*phnA*	McGrath et al., 2013
2-Aminoethylphosphonate (AEP) - Pyruvate Transaminase	K03430	*phnW*	McGrath et al., 2013

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

Factor	Families general	Families P filtered
Season	Cryomorphaceae,Lentisphaeraceae, Fibrobacteraceae, Heliobacteriaceae, Methylophilaceae, Neisseriaceae, Desulfuromonadaceae, Polyangiaceae, Sandaracinaceae, Syntrophorhabdaceae, Campylobacteraceae,Aeromonadaceae, Ferrimonadaceae, Moritellaceae, Shewanellaceae, Methylococcaceae, Alcanivoracaceae, Hahellaceae, Pseudomonadaceae, Acidothermaceae, Chlamydiaceae, Chloroflexaceae, Oscillochloridaceae, Roseiflexaceae, Deinococcaceae, Chitinivibrionaceae, Sporolactobacillaceae, Kiloniellaceae, Rhodobacteraceae, Sulfuricellaceae, Enterobacteriaceae, Competibacteraceae, Methanospirillaceae, Tuberaceae, Tricholomataceae, Siphoviridae	Dictyoglomaceae, Bacillaceae, Streptococcaceae, Flammeovirgaceae, Chrysiogenaceae, Elusimicrobiaceae, Desulfovibrionaceae

Fertilizer	Bacteriovoracaceae, Alcanivoracaceae, Cryomorphaceae, Saprospiraceae, Bacillaceae, Planococcaceae, Thermoactinomycetaceae, Desulfurococcaceae, Chrysochromulinaceae, Tuberaceae, Tricholomataceae, Tremellaceae, Charadriidae, Daphniidae	Acidimicrobiaceae, Acidothermaceae, Geodermatophilaceae, Cyclobacteriaceae, Chlorobiaceae, Opitutaceae, Anaerolineaceae, Nostocaceae, Prochlorococcaceae, Deinococcaceae, Thermaceae, Dictyoglomaceae, Acidobacteriaceae, Bacillaceae, Enterococcaceae, Streptococcaceae, Clostridiaceae, Gemmatimonadaceae, Nitrospiraceae, Rhizobiaceae, Sphingomonadaceae, Burkholderiaceae, Methylophilaceae, Pelobacteraceae, Anaeromyxobacteraceae, Pseudomonadaceae, Mariprofundaceae, Leptospiraceae, Spirochaetaceae, Rhodothermaceae, Rikenellaceae, Ardenticatenaceae, Chrysiogenaceae, Elusimicrobiaceae, Paenibacillaceae, Hyphomonadaceae, Nannocystaceae, Enterobacteriaceae

Interaction	Actinopolysporaceae, Dehalococcoidaceae, Thermoanaerobacterales Family IV. Incertae Sedis, Nitrospiraceae, Kordiimonadaceae, Francisellaceae, Coriobacteriaceae, Lactobacillaceae, Peptoniphilaceae, Acetobacteraceae, Ferrovaceae, Desulfurellaceae, Moritellaceae, Halobacteriaceae, Methanomicrobiaceae, Thermococcaceae, Clavicipitaceae, Pyronemataceae	Nakamurellaceae, Micrococcaceae, Propionibacteriaceae, Solirubrobacteraceae, Rhodothermaceae, Porphyromonadaceae, Cytophagaceae, Flavobacteriaceae, Chitinophagaceae, Sphingobacteriaceae, Verrucomicrobiaceae, Solibacteraceae, Planctomycetaceae, Caulobacteraceae, Bradyrhizobiaceae, Hyphomicrobiaceae, Rhodospirillaceae, Comamonadaceae, Chromobacteriaceae, Neisseriaceae, Nitrosomonadaceae, Rhodocyclaceae, Bdellovibrionaceae, Cystobacteraceae, Myxococcaceae, Polyangiaceae, Methylococcaceae, Salinisphaeraceae, Xanthomonadaceae, Catenulisporaceae, Cryomorphaceae, Clostridiales Family XVII. Incertae Sedis, Thermoanaerobacterales Family III. Incertae Sedis, Acetobacteraceae, Desulfomicrobiaceae, Alteromonadaceae, Moraxellaceae

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

Package	Reference
agricolae	De Mendiburu (2015)
asbio	Aho (2015)
binhf	Nunes (2014)
ggplot2	Wickham (2016)
gmodels	Warnes et al. (2015a)
gplots	Warnes et al. (2015b)
gridExtra	Auguie (2015)
GUniFrac	Chen and Orphaned (2012)
knitr	Yihui (2015)
lattice	Sarkar (2008)
lmPerm	Wheeler (2010)
made4	Culhane et al. (2005)
MASS	Venables and Ripley (2002)
nlme	Pinheiro et al. (2016)
pamr	Hastie et al. (2014)
plyr	Wickham (2011)
RColorBrewer	Neuwirth (2014)
reshape2	Wickham (2007)
sciplot	Morales (2012)
sfsmisc	Maechler (2015)
shape	Soetaert (2014)
vegan	Oksanen et al. (2015)
xlsx	Dragulescu (2014)

Table S4: R packages used for data visualization.

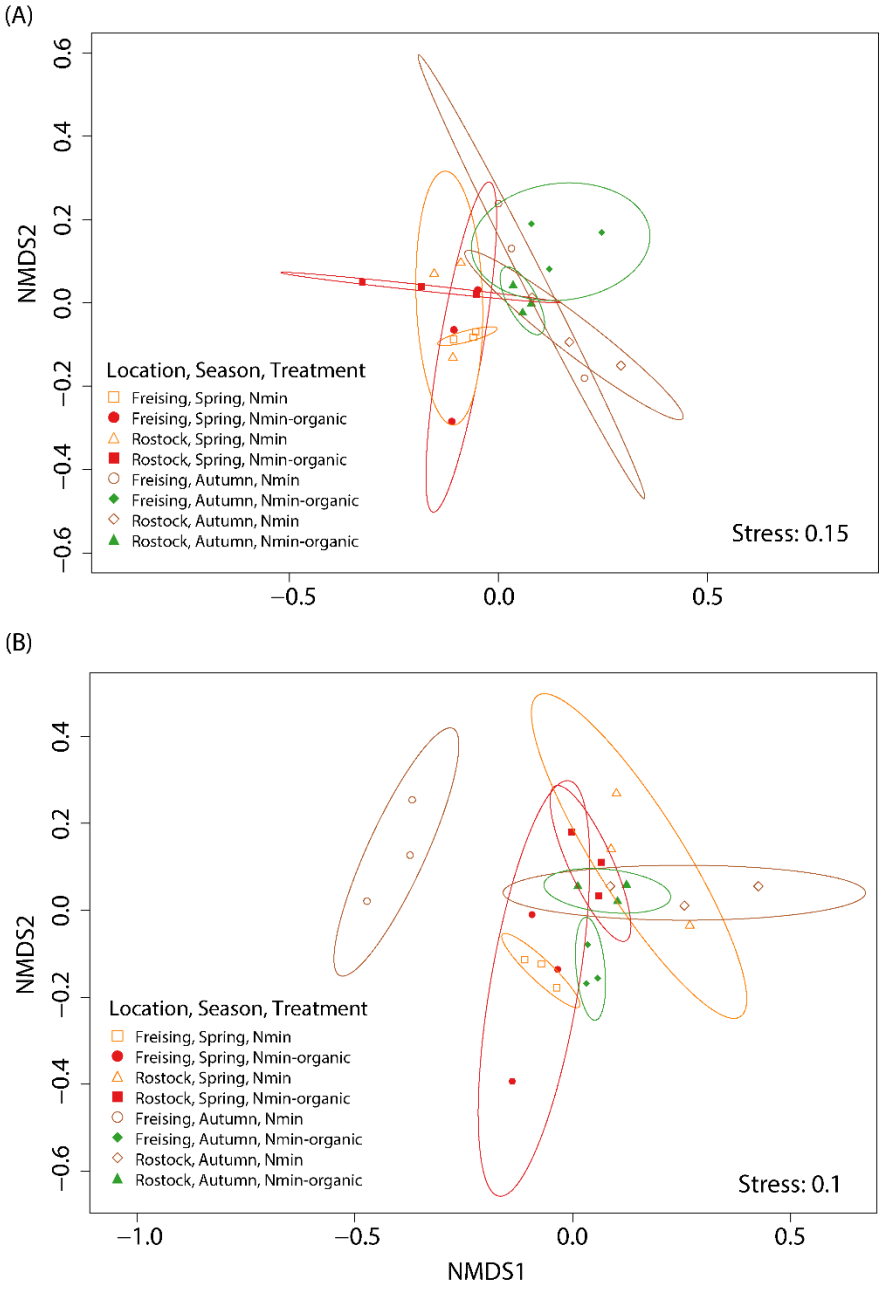

Figure S1: NMDS ordination plots depicting taxonomic profiles on the family level for bacteria found at both sites. Shown in A) is the overall community, and depicted in B) distribution of genes involved in P turnover among bacterial families (n=24). 95% confidence level is depicted as ellipses for each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

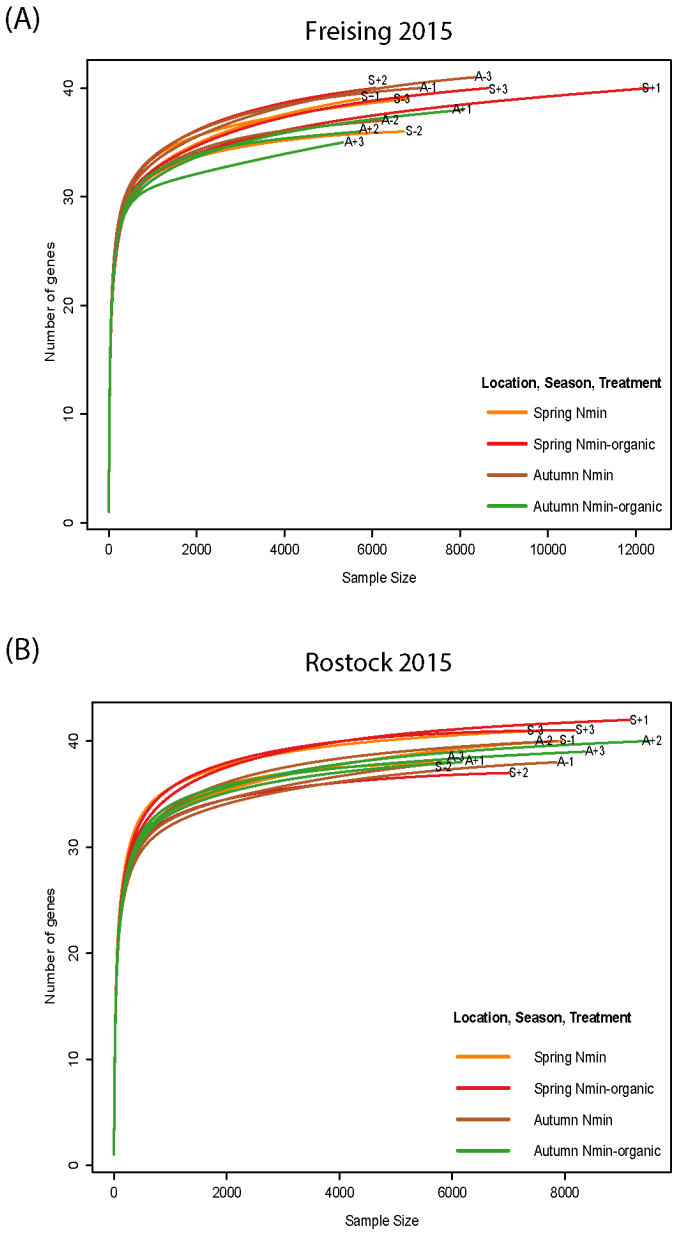

Figure S2: Rarefaction curves of metagenomic datasets from samples taken in spring and autumn from Freising and Rostock. Depicted are the numbers of assigned genes involved in P turnover as a function of sequencing depth. Genes were assigned using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology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

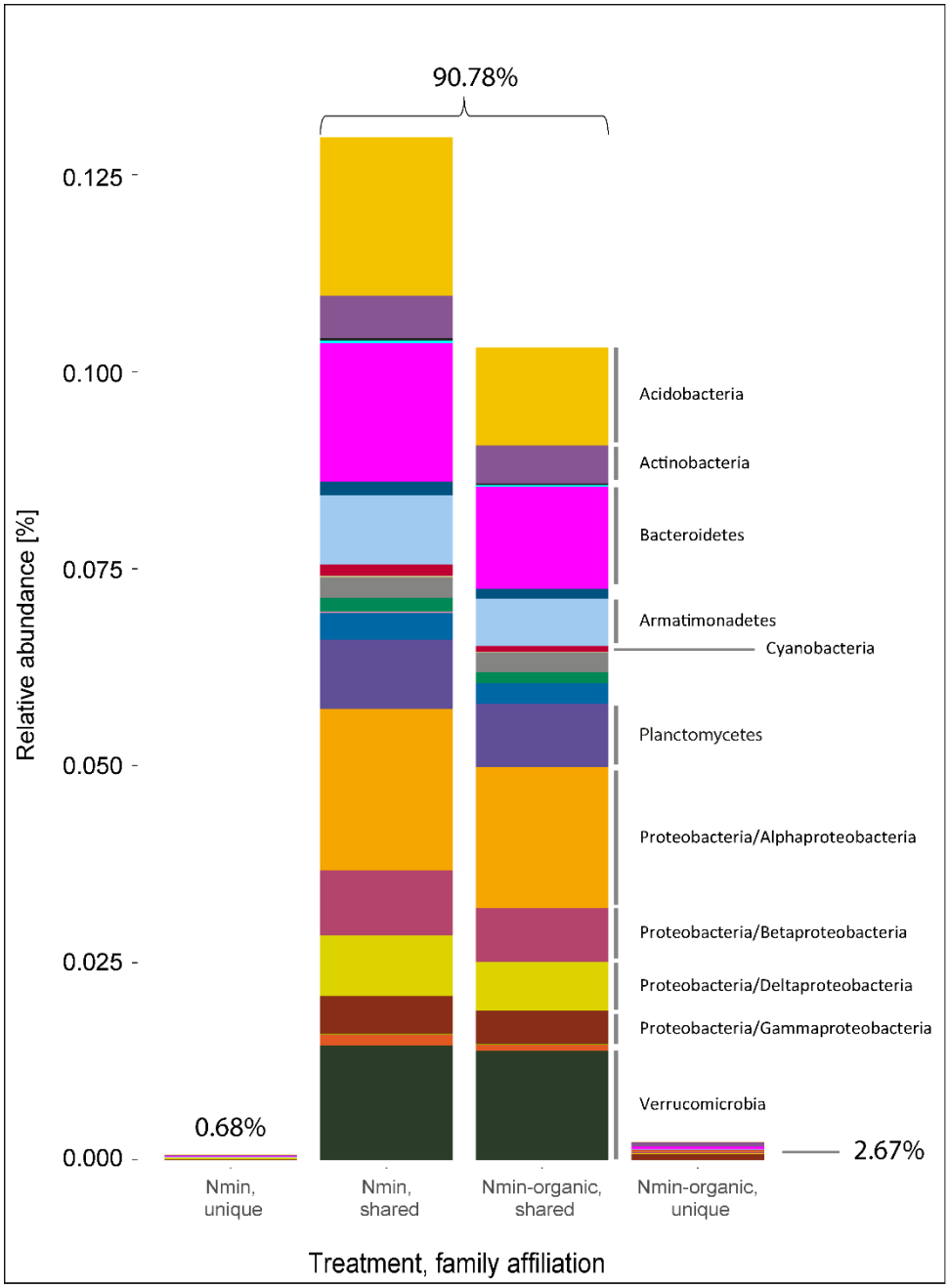

Figure S3: Core microbiome for the comparison of the two fertilization treatments Nmin and Nmin-organic by means of stacked barplots. Depicted are all genes involved in P turnover. Percentages describe the total relative abundance of reads assigned to P turnover.

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Manuscript II

Effect of triple superphosphate and biowaste compost on mycorrhizal colonization and enzymatic P mobilization under maize in a long-term field experiment

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Abstract

Phosphorus (P) fertilizers and mycorrhiza formation can both significantly improve the P supply of plants, but P fertilizers might inhibit mycorrhiza formation and change the microbial P cycling. To test the dimension and consequences of P fertilizer impacts under maize (*Zea mays* L.), three fertilizer treatments (1) triple superphosphate (TSP, 21–30 kg P ha^{-1} annually), biowaste compost (ORG, 30 Mg ha^{-1} wet weight every third year) and a combination of both (OMI) were compared to a non-P-fertilized control (C) in 2015 and 2016. The test site was a long-term field experiment on a Stagnic Cambisol in Rostock (NE Germany). Soil microbial biomass P (P_{mic}) and soil enzyme activities involved in P mobilization (phosphatases and ß-glucosidase), plant-available P content (double lactate-extract; P_{DL}), mycorrhizal colonization, shoot biomass, and shoot P concentrations were determined. P deficiency led to decreased P immobilization in microbial biomass, but the maize growth was not affected. TSP application alone promoted the P uptake by the microbial biomass but reduced the mycorrhizal colonization of maize compared to the control by more than one third. Biowaste compost increased soil enzyme activities in the P cycling, increased P_{mic} and slightly decreased the mycorrhizal colonization of maize. Addition of TSP to biowaste compost increased the content of P_{DL} in soil to the level of optimal plant supply. Single TSP supply decreased the ratio of P_{DL}:P_{mic} to 1:1 from about 4:1 in the control. Decreased plant-benefits from mycorrhizal symbiosis were assumed from decreased mycorrhizal colonization of maize with TSP supply. The undesirable side effects of TSP supply on the microbial P cycling can be alleviated by the use of compost. Thus, it can be concluded that the plant-availability of P from soil amendments is controlled by the amendment-specific microbial P cycling and, likely, P transfer to plants.

Key words: compost / microbial biomass / mycorrhiza / P fertilization / phosphatases

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1 Introduction

Phosphorus (P) is beside nitrogen (N) one of the most important nutrient elements limiting plant growth. However, economically mineable P deposits are finite and often contaminated with cadmium and uranium (*Mar* and *Okazaki*, 2012; *Kratz* et al., 2016). Thus, an adequate P supply to crops combined with minimal nutrient losses into ground and surface water are important for a sustainable agriculture. In general, supply of organic amendments, such as compost, straw or manure, have become increasingly popular strategies in agriculture (*Eichler-Löbermann* et al., 2007; *Nelson* and *Janke*, 2007; *Ohm* et al., 2017), as they combine an improved nutrient availability for plants with other soil ecological benefits, like carbon sequestration and biocontrol of plant pathogens (*Baum* et al., 2015). However, the needed amount of organic fertilizer to ensure an optimal supply of plants with P is often difficult to calculate compared to mineral P fertilizers. This originates from large variations in the composition of organic fertilizers affecting the amounts of organic and inorganic P (*Traoré* et al., 1999) and the manifold transformations of organic compounds and nutrient releases depending on many abiotic and biotic factors, including microbial activities. Microbes catalyze key processes in soil P cycle, such as P mineralization or immobilization, *e.g.*, by the release of hydrolytic enzymes into soil (*Bergkemper* et al., 2016). Consequently, the soil microbial biomass has been considered as temporal sink and source of P (*Oberson* and *Joner*, 2005). Lack of P leads to a higher P use efficiency of microbes (*Bünemann* et al., 2004; *Olander* and *Vitousek*, 2004). There-

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fore, favorable fertilization strategies have to consider the soil microbial resources, which are insufficiently understood and applied in agronomic practice so far (*Bargaz* et al., 2018). Recently, high stability of bacterial potentials for uptake, solubilization and mineralization of extracellular P in agricultural soils under different fertilization regimes was demonstrated (*Grafe* et al., 2018). Beside bacteria, fungi control the microbial P cycling and transfer to the plants (*Bargaz* et al., 2018). Fungi were revealed to receive maize-derived C to a greater extent than the total soil microbial biomass (*Kramer* et al., 2012), and any P supply, *e.g.*, by fertilizers might affect P plant-availability through the stoichiometric regulation of C cycling by the P availability (*Hartman* et al., 2017). For this reason, we investigated the microbial response on defined mineral and organic fertilization treatments to propose favorable fertilization strategies. We evaluated the impact of inorganic, organic and combined inorganic / organic fertilization on the microbial P transformation and the supply of crops with supply based on a long-term field experiment with maize (*Zea mays* L.). This crop was selected because of its high P demand and high economic significance (*Muhammad* et al., 2007) and because it can have a strong mycorrhizal dependency (*Ortas* and *Akpinar*, 2011).The present study tested the following hypotheses: (1) plant-availability of P from soil amendments is controlled by the amendment-specific microbial P turnover. (2) Different forms of P fertilizer will selectively trigger changes in microbial P transformations and regulate the retention of P in soil or its transfer to the plants.

2 Material and methods

2.1 Study area and sampling

The study was conducted in 2015 and 2016 at a long-term field experimental test site in Northern Germany (54°3′41.47′′N; 12°5′5.59′′E) with consistent fertilization management since 1998. The mean of temperature is 8.1°C and that of annual precipitation 600 mm. The test years differed mainly in their water supply by precipitation with considerably lower precipitation in the sampling periods in 2016 than in 2015 (Fig. 1). The predominant soil unit is a Stagnic Cambisol according to the World References Base for Soil Resources. The soil texture of the topsoil was characterized as loamy sand.

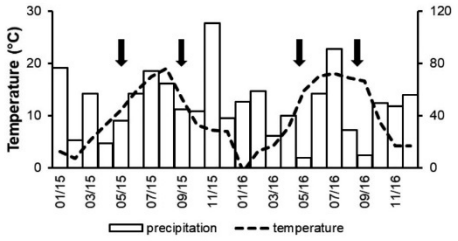

Figure 1: Temperature (line, monthly mean) and precipitation (bars, monthly sum) at the experimental test site in Rostock; arrows indicate spring and autumn samplings in 2015 and 2016.

The crop rotation was dominated by maize which had been grown also in the test years 2015 and 2016. The experiment was designed as randomized split plot in four replicates. The plots of interest for this study comprised three fertilizer treatments: triple superphosphate (MIN), biowaste compost (ORG), biowaste compost + triple superphosphate (OMI), and a control (C) without additional P fertilization. Triple superphosphate (TSP) was applied annually at rates of about 21.8 kg P ha^{-1} until 2013, and at rates of 30 kg P ha^{-1} from 2014. Biowaste compost was applied at rates of 30 t ha^{-1} in autumn every third year since 1998 (latest application before the test period in 2013). Since establishment of the test site in 1998 until the beginning of this study in spring 2015, the treatments with mineral and organic fertilizers received approx. 400 kg P ha^{-1}, combined treatments with TSP and biowaste compost approx. 800 kg P ha^{-1}, and the control has not received any P since 1998. The initial concentration of double lacatate P (P_{dl}) was 42.2 mg P kg^{-1}, indicating a suboptimal P supply according to the German soil phosphorus classification (*Eichler-Löbermann* et al., 2007). For further details on organic and inorganic P forms in the present experiment see *Requejo* and *Eichler-Löbermann* (2014). Maize was fertilized annually with 120 kg N ha^{-1} as calcium ammonium nitrate and 125 kg ha^{-1} potassium during the whole duration of the field experiment. Within the period of investigation, it was applied after the spring samplings. Soil management included annual tillage up to a depth of 20 cm after the autumn sampling.

Soil samples were taken in spring before sowing and in autumn before harvesting in 2015 and 2016, respectively.

Table 1: Soil properties of the different fertilization treatments at the beginning of the experiment at the test site Rostock in 2015. C_{org}: organic C content; N_t: total N content; P_t: total P content. $n = 6$; mean values ± standard deviation; post hoc test Tukey HSD $p < 0.05$; abc = differences between treatments.

Fertilization treatment	pH_{CaCl_2}	C_{org} (g kg^{-1})	N_t (g kg^{-1})	P_t (mg kg^{-1})
Control (C)	5.98 ± 0.32a	7.15 ± 0.32a	0.82 ± 0.03a	544 ± 60a
MIN (+TSP[a])	6.09 ± 0.21a	8.00 ± 1.24a	0.82 ± 0.09a	550 ± 43a
Biowaste compost (ORG)	5.95 ± 0.28a	10.40 ± 0.31b	1.05 ± 0.04b	622 ± 107ab
Biowaste compost +TSP (OMI)	6.13 ± 0.17a	9.78 ± 0.53b	1.06 ± 0.08b	609 ± 31b

[a]TSP: triplesuperphosphate.

Sampling was conducted in three of the four replicate plots for each fertilization treatment. Five soil cores per plot were taken with a corer (3 cm diameter) from the upper topsoil (0 to 10 cm soil depth), bulked and subsamples were stored at 4°C for microbial and dried at 60°C for chemical analyses, respectively.

The total shoot biomass was harvested in both years in autumn when fully ripe (BBCH 89) by cutting the shoots 5 cm above the ground. Biomass samples were dried at 60°C and milled (Retsch GmbH, Haan, Germany) for elemental analyses.

2.2 Analyses of soil properties and plant traits

The soil pH was measured in a 0.01 M $CaCl_2$ solution (w/v 1/2.5). Total concentrations of carbon, nitrogen, and sulfur in soil were determined by a CNS Analyzer (Vario EL Fa. Foss Heraeus, Hanau, Germany) using air dried soil.

Double lactate-extractable P (P_{dl}), considered to be the plant-available P fraction (*e.g.*, *Kerschberger* et al., 1997), was determined by extracting P from 12 g soil with 150 mL lactate solution ($C_6H_{10}CaO_6 \cdot H_2O$ + 10 N HCl) according to Hoffmann (*Hoffmann*, 1991; *VDLUFA*, 1991). Phosphate concentrations in the soil extract were determined by using the vanadate molybdate method with a spectral photometer (Specord 40, Analytik Jena, Germany). P_{dl} represent the plant-available P in soils (*Beyme*, 1980).

For the analysis of the P content, 0.1 g of the ground plant material were digested with 5 mL HNO_3 and 3 mL H_2O_2 in a microwave (Mars Xpress, CEM, Kamp-Lintfort, Germany) and diluted with $H_2O_{dest.}$ to a volume of 25 mL. Concentrations of P were determined with inductively coupled plasma-optical emission spectroscopy (ICP-OES, Optima 8300, Perkin Elmer, Waltham, Massachusetts, USA) at wave lengths of 214.914 nm.

Mycorrhizal colonization density of fine roots in the soil was determined in autumn 2015 and autumn 2016. Fine roots were rinsed and cut into 10-mm segments. The segments were cleared with 10% (w/v) KOH (*Phillips* and *Hayman*, 1970) at room temperature for 24 h and stained with 0.05% (w/v) chlorazol black E (*Brundrett* et al., 1983). The arbuscular mycorrhizal colonization was quantified microscopically using the intersection method of *McGonigle* et al. (1990).

2.3 Analyses of soil microbial biomass and soil enzyme activities

Soil microbial biomass phosphorus (P_{mic}) was assessed by chloroform-fumigation extraction using a k_{EP} factor of 0.4 to convert extracted P into P_{mic}, assuming that 40% of the biomass P are extractable as inorganic P (P_i) (*Brookes* et al., 1982).

P concentrations in the extracts were measured by ICP-OES (Optima 8300, Perkin Elmer, Waltham, Massachusetts, USA) at a wave length of 214.94 nm.

Measurements of potential enzyme activity were conducted for acid and alkaline phosphomonoesterases (ACP and ALP; *Tabatabai* and *Bremner*, 1969), phosphodiesterases (PDE; *Browman* and *Tabatabai*, 1978), and ß-glucosidase (GLA; *Eivazi* and *Tabatabai*, 1977; *Tabatabai*, 1982). The enzyme activities were measured in μmol *p*-nitrophenol as phenol released from a pre-given substrate solution in 1 g field moisture soil within 1 h (μmol *p*-nitrophenol g^{-1} h^{-1}). A slight modification was the refrain from adding toluene as suggested by *Nannipieri* et al. (1978) for this short incubation period.

2.4 Statistical analyses

Analyses were performed with R studio and R packages hmisc (https://www.r-project.org; version 4.0-2) as well as agricolae (https://cran.r-project.org/web/packages/agricolae/index.html; version 1.2-4). Soil properties, parameters of microbial activity and mycorrhizal colonization were analysed using the Kruskal test. Significance was considered at $p < 0.05$ and significant differences between treatments were indicated by using different letters. The treatment specific changes in the activities of hydrolytic enzymes were tested with principal component analysis (PCA) using the software package PAST (*Hammer* et al., 2001).

3 Results

3.1 Plant-available P, mycorrhizal colonization and P in microbial and plant biomass

The application of inorganic and organic fertilizers increased the content of plant-available P in soil and of microbial P but had no significant impact on the biomass production of maize (Tab. 2). The combined organic and inorganic fertilizer (OMI) resulted in the highest concentrations of plant-available P (P_{dl}) in soil that were larger by factor 1.3 to 1.8 than in the control (spring 2016).

The ratio of microbial biomass P on the total P in the soil was significantly increased after fertilization [in the control (C): 1.9% *vs.* fertilized treatments with 8.4% in MIN, 3.7% in ORG and 6.0% in OMI; calculated from Tabs. 1 and 2].

The mycorrhizal colonization in 2015 was constantly higher than in 2016 (Fig. 2). The MIN treatment had the lowest mycorrhizal colonization, 31% of the root length in 2015, and 17% in 2016. Also the combined treatment OMI showed a significant reduction of mycorrhizal colonization from 2015 to 2016. Within 2016, the mineral and the combined amendments differed significantly to the control.

The nutritional status of the maize was in the optimal range in all treatments (according to reference data of *Kerschberger* et al., 1997). The P uptake by maize ranged from 17–21 kg P ha^{-1} in autumn 2015 and 20–28 kg P ha^{-1} in autumn 2016, without significant differences between the treatments.

Table 2: Double lactate-extractable P (P_{dl}), microbial P, shoot biomass and shoot P ($P_{phytomass}$) of maize as affected by different P fertilizer treatments at the test site Rostock (C: control; MIN: triple superphosphate; ORG: compost; OMI: compost + triple superphosphate); n = 9; mean values ± standard deviation; post hoc test Tukey HSD p < 0,05; abc = differences between treatments within the sampling date.

Year	Spring				Autumn			
2015	**C**	**MIN**	**ORG**	**OMI**	**C**	**MIN**	**ORG**	**OMI**
P_{dl} (mg P kg^{-1})	43.3 ± 3.7a	47.1 ± 18.2ab	55.8 ± 0.6b	58.9 ± 1.6b	30.2 ± 1.7a	36.7 ± 3.2b	42.1 ± 5.3bc	48.6 ± 1.9c
Microbial P (mg kg^{-1}) DM)	10.7 ± 4.1a	46.4 ± 7.5c	22.9 ± 3.1b	36.3 ± 5.1c	7.4 ± 3.9a	17.0 ± 5.9b	20.1 ± 6.7b	4.8 ± 1.5a
Shoot biomass (t DM ha^{-1})					12.1 ± 0.3a	10.5 ± 1.1a	10.9 ± 0.8a	12.0 ± 0.7a
$P_{phytomass}$ (g kg^{-1} DM)					1.6 ± 0.2a	1.7 ± 0.1a	1.7 ± 0.2a	1.9 ± 0.4a
2016	**C**	**MIN**	**ORG**	**OMI**	**C**	**MIN**	**ORG**	**OMI**
P_{dl} (mg P kg^{-1})	26.5 ± 2.5a	34.0 ± 5.8bc	35.5 ± 1.3b	48.6 ± 2.9c	33.1 ± 3.9a	36.0 ± 9.3a	44.5 ± 0.8a	46.1 ± 13.7a
Microbial P (mg kg^{-1})	8.8 ± 1.6a	31.4 ± 2.8c	16.2 ± 6.7b	19.0 ± 4.7b	2.1 ± 1.5a	11.7 ± 9.2b	7.2 ± 1.6b	14.0 ± 7.2b
Shoot biomass (t DM ha^{-1})					18.6 ± 3.5a	17.8 ± 3.3a	19.8 ± 0.9a	18.8 ± 2.2a
$P_{phytomass}$ (g kg^{-1} DM)					1.2 ± 0.9a	1.1 ± 0.4a	1.3 ± 0.3a	1.3 ± 0.1a

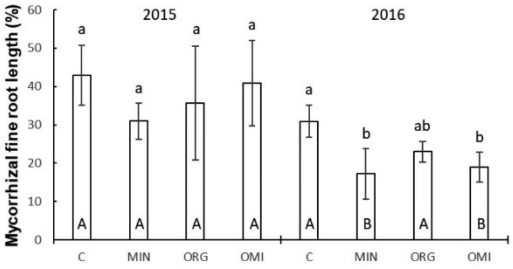

Figure 2: Mycorrhizal colonization of fine roots of maize (Zea mays) as affected by fertilization treatments (C: control; MIN: triple superphosphate; ORG: compost; OMI: compost + triple superphosphate) at the field experiment in Rostock in 2015 and 2016; mean ± SD (n = 9), post hoc test Kruskal–Wallis p < 0.05; abc = differences between treatments within a sampling date; ABC = differences between samplings within a treatment.

3.2 Soil enzyme activities

Supply of organic fertilizer (ORG, OMI) resulted in significantly increased potential activities of ACP, ALP, PDE, and GLA compared to treatments with inorganic (MIN) or without P fertilization (C) (Fig. 3). There were no significant differences between the C and MIN treatments or ORG and OMI, respectively. Increases of the activities of ALP and PDE in response to the fertilizer application were larger by factor of 1.5 for ORG / OMI compared to MIN. In general, fertilization treatment-specific differences were mainly stronger in spring without the influence of the vegetation, with exception of PDE in autumn 2016. ALP and PDE were not affected by fertilization in autumn 2016. Further, enzyme activities varied within a year. Activities for ACP (except 2015), ALP, PDE, and GLA were higher in spring than in autumn at the two samplings.

There was a clear splitting of the joint soil enzyme activities involved in the P cycling between treatments without (C, MIN) and with organic amendments (ORG, OMI; see Fig. 4).

4 Discussion

In response to long-term P-deficiency, low microbial biomass P (2.1 to 10.7 mg kg^{-1}) was revealed in the control (C) of the present experiment. This impact of P-deficiency was also observed in long-term field experiments at Alfisols in China (*Luo* et al., 2015). The range of biomass P in the control of the present study was at a same level as determined in a sandy Haplic Cambisol by *Heinze* et al. (2010). Fertilizer supply significantly increased the microbial P contents in the topsoil of the present experiment, especially in the treatments with inorganic P application in form of TSP (Tab. 2). This indicates a fast use of P from TSP by the soil microorganisms. Significantly lower microbial biomass P in 2015 than in 2016 can be explained by the dryness in the sampling months in 2016 (see Fig. 1). This also decreased the hydrolytic soil enzyme activities (see Fig. 3) due to their general strong correlation with the soil moisture (*Chen* et al., 2003; *Nannipieri* et al., 2011).

Although the portion of microbial biomass P on total P of cropped soil is usually low (mainly < 3%; *Oberson* and *Joner*, 2005) as confirmed in the present experiment, it was significantly increased after TSP supply to up to 8% on the total P

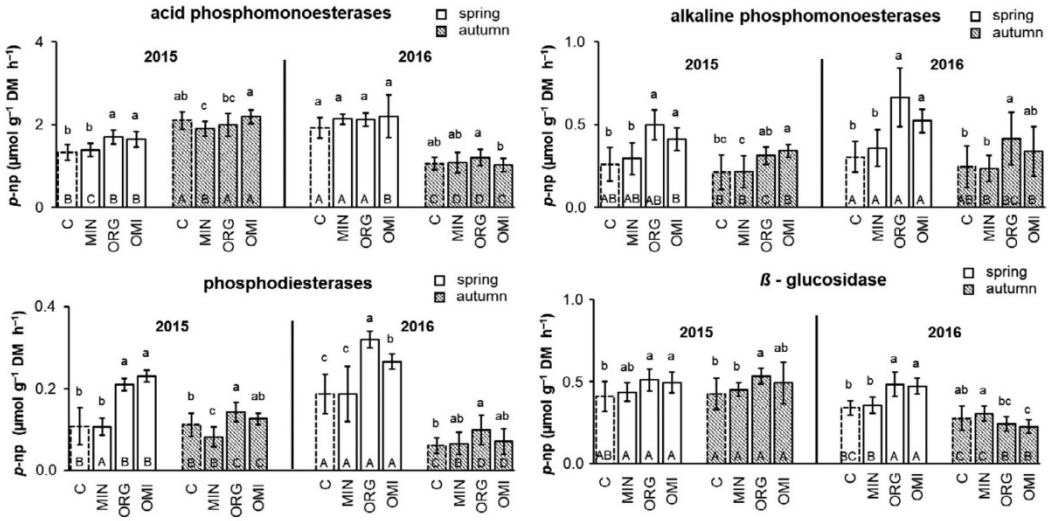

Figure 3: Potential soil enzyme activities in spring and autumn 2015 and 2016 as affected by fertilization treatments (C: control; MIN: triple superphosphate; ORG: compost; OMI: compost + triple superphosphate) at the field experiment in Rostock; mean ± SD (n = 9), post hoc test Kruskal–Wallis $p < 0.05$; abc = differences between treatments within a sampling date; ABC = differences between samplings within a treatment.

(calculated from Tabs. 1 and 2). Therefore, a rising significance of the microbial biomass P pool for the plant supply in this treatment can be derived from this result.

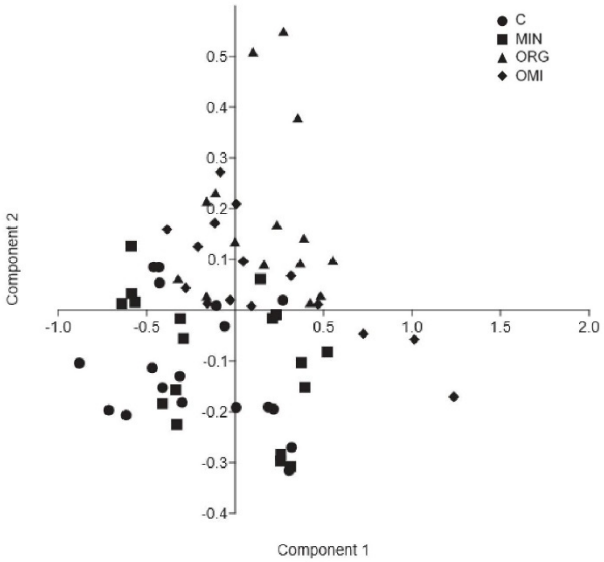

Figure 4: Principal component analysis (PCA) of enzyme activities in soils in spring 2015 and 2016 as affected by fertilization treatments (C: control; MIN: triple superphosphate; ORG: compost; OMI: compost + triple superphosphate) at the field experiment in Rostock.

Oppositely to the microbial biomass P, soil enzyme activities were stronger affected by organic amendments than by TSP supply (see Tab. 2, Fig. 3). This can be explained by the substrate supply and improved soil chemical and physical properties (*Jones* and *Oburger*, 2011). Increased microbial activity and P uptake in response to organic amendments were described previously also by *Oberson* et al. (2001) from a legume rotation. However, an initial promotion of the P mobilization after organic amendments even can be followed by a decreased mobilization in the long-term (*Martens* et al., 1992). In the present experiment, no decreased mobilization by hydrolytic enzymes was indicated so far.

Microbial immobilization of P is affected by the C:P ratio in the soil mass (*Bünemann* et al., 2004; *Achat* et al., 2009). This was confirmed by a smaller ratio of P_{DL}:P_{mic} in MIN with TSP, but no organic C addition (1:1) than in the control (4:1). This might indicate a stronger plant-microbial competition for P in this treatment, which is supported by a decreased P uptake of maize (biomass × P concentration in the shoots; calculated from Tab. 2). The P uptake of maize was even lower with TSP than in the control (2015: control 19.6 *vs*. +TSP 17.6 kg P per hectare and 2016: control 22.3 *vs*. 19.6 kg P per hectare). In this context, *Zhang* et al. (2014) revealed in a pot experiment that reducing the C:P ratio by addition of inorganic P to the soil can lessen the competition for P of the soil microorganisms with maize as test plant. This was supported by jointly higher plant-available and micro-

bial biomass P contents after TSP addition in combination with biowaste compost in comparison to single biowaste compost addition (see Tab. 2).

The impact of the substrate supply by organic amendments caused a clear differentiation of the treatments with (ORG and OMI) from those without organic amendments (C and MIN) in the activities of soil enzymes involved in the hydrolytic P mobilization (see Fig. 4). Increased soil enzyme activities in response to organic amendments with compost and municipal wastewater sludge were described previously also by *Leifeld* et al. (2002) and *Dindar* et al. (2015).

In the present experiment, contents of P_{dl} in the control (C) were always suboptimal (26.5–43.3 mg P kg^{-1}) and only in the treatment with biowaste compost + TSP (46.1–48.6 mg P kg^{-1}) always in the optimal range (45–90 mg P kg^{-1}) according to German fertilization recommendations (*Kerschberger* et al., 1997). According to these a slight growth promotion after P fertilization was expected, however, no significant impact on the shoot biomass was observed in both test years (see Tab. 2), but only slightly increased total P uptake in the shoot biomass in the treatments with organic amendments (calculated from shoot weight × P concentration; Tab. 2).

As the early growth of maize is the most important period for the yield-relevant P uptake of the crop (*Oberson* et al., 1993), the microbial P cycling in spring should be especially important, whereas increased activities in the autumn might increase the risk of P losses. Thereby, higher phosphatase activities in the soil must not necessarily result in an increased plant P uptake (*De Freitas* et al., 1997). In the present study, increased phosphatase activities were not related with increased shoot biomass of maize. Similar observations were reported from experiments of *Gransee* and *Merbach* (2000) and *Bünemann* et al. (2004).

Since maize usually receives up to 60% of its total P requirements through mycorrhizal symbiosis (*Nurlaeny* et al., 1996), it can be supposed that an inhibited or disturbed mycorrhizal association can be one of the main reasons for P deficiency. In the present experiment, the mycorrhizal colonization of maize in 2015 was with up to 50% of the root length (Fig. 2) within and in 2016 below the level of typical mycorrhizal colonization of maize (*Rakshit* and *Bhadoria*, 2010). Significantly higher mycorrhizal colonization in the control than after TSP application in 2016 (Fig. 2) might have enhanced the drought tolerance of maize in the control, since this impact of mycorrhizal symbiosis in maize was described in advance by *Quiroga* et al. (2017) for drought-sensitive cultivars. Decreased mycorrhizal colonization was particularly observed after TSP application without organic amendments (see Fig. 2) and was linked with the lowest average yield and P uptake of maize (see Tab. 2). This confirms the significance of mycorrhizal formation for maize growth in drought stress. Similarly, a decreased mycorrhizal colonization after application of fertilizer P was already reported for soybean and wheat (*Manna* et al., 2007). It can be suggested, that the impact of TSP on mycorrhizal fungi varies significantly and might partly even include their promotion, *e.g.*, in relation to the presence of organic matter. This was observed in a pot experiment by *Prasad* et al. (2012). However, usually mycorrhizal significance for the P supply of host plants increase under P deficiency (*Smith* et al., 2011), as assumed for control treatment in the present study. Thus, in summary, the present study indicated a general fertilization impact on enzymatic P mobilization, microbial immobilization, and potential P transfer to host plants by mycorrhizal fungi.

5 Conclusions

P deficiency leads to a decreased P immobilization in the microbial biomass and to an increased potential for mycorrhizal P transfer of from soil to the host plant.

Inorganic P supply in P deficiency might favor microbial use instead of plant uptake.

Plant growth promotion by inorganic P might be enhanced by joint C supply by organic amendments, caused by the coupling of C and P cycling and through increased mycorrhizal colonization.

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Effects of different innovative bone char based P fertilizers on bacteria catalyzing P turnover in agricultural soils

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ABSTRACT

Phosphorous (P) is one of the most critical macronutrient elements for plant growth, yield and quality. However, natural P sources are finite and an improved P recycling is necessary. Therefore, we investigated the effect of bone char (BC) and bone char plus (BC^{plus}) as recycling products and alternative P fertilizers, on the abundance of microorganisms, which catalyze major steps in P turnover in a field experiment in Central Germany. The effects were compared to conventional triple super phosphate (TSP) and no P fertilization. Samples were analyzed from soils with three different initial soil P concentrations (very low, low, optimal) and three times during winter wheat cultivation (stem elongation, heading, ripening) to reveal interactions of fertilizers and soil properties. Abundances of microorganisms involved in P uptake, solubilization and mineralization were assessed by quantitative real time PCR (qPCR). Additionally, potential acidic- and alkaline phosphatase activity, water extractable P and plant available P were measured. Bacterial strategies to maintain P pools differed among the treatments. While the addition of BC^{plus} increased the solubilization potential, the low P concentration in control plots and slow release of P from BC favor P recycling from biomass and P inducible uptake systems, which is displayed by either high abundance bacteria harboring the *phoD* or *pstS* gene, respectively. All effects were most pronounced at the time of heading and in soils with optimal initial P concentration. It can be assumed that sulfurization of bone char (BC^{plus}) influences bacterial P turnover by promoting solubilization of the fertilizer thereby increasing P availability for plants. Additionally, plant development stage and initial soil P concentrations hamper the effect of BC and BC^{plus} on bacterial P turnover.

1. Introduction

The availability of phosphorus (P) in soils strongly influences crop yield and quality (Schachtman et al., 1998; Elser and Bennett, 2011). Due to the decreasing sources of high-grade phosphorous rock materials (Chadwick et al., 1999; Cordell et al., 2009; Gilbert, 2009; Kauwenbergh et al., 2013), alternative ways for P fertilization are important to ensure sustainable agriculture in the long-term. This includes the mobilization of labile legacy P from soil (Rowe et al., 2015) or the application of P fertilizers obtained from recycling products.

One example of these recycling products, which has been successfully used as an alternative P fertilizer, is bone char (BC). BC is produced by defatting, degelatinizing and subsequently pyrolyzing animal bone chips at 600–800 °C (LeGeros, 2017). The typical elemental concentrations of BC are 152 g P kg^{-1}, 280 g Ca kg^{-1}, and 6.5 g Mg kg^{-1} (Siebers and Leinweber, 2013). Carbon concentration is often lower than 100 g kg^{-1} (Zimmer et al., 2018; Zimmer et al., 2019). The solubility of BC in soil has been reported to be intermediate between rock phosphates and

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triple super phosphate (TSP) and depends on soil characteristics such as pH and the P sorption capacity of the soil (Warren et al., 2009). To increase the P solubility of BC, a surface modified version enriched with sulfur (S) has been developed (BC^{plus}). First incubation studies and pot experiments confirmed the improved solubility (Morshedizad et al., 2016; Zimmer et al., 2018; Zimmer et al., 2019) and a recent field study proved the potential of BC^{plus} to increase available P concentrations in soils with low initial P concentration (Panten and Leinweber, 2020). However, if this was caused by a response of the soil microbiome towards the application of BC and BC^{plus} is still unclear and is thus a major aim of this study.

Generally, the soil microbiome plays an important role for the mobilization of P in soil by catalyzing P solubilization and mineralization (van Loon, 2007; Richardson and Simpson, 2011). However, microorganisms also compete for P resources with plants, as they possess effective and inducible P uptake systems. It was reported previously that both the initial soil P content and the P fertilizer regime including the type of P fertilizer influence the bacterial P turnover. Regarding the initial soil P content it was demonstrated that in P-rich soils, first the inorganic P (P_i) pool is depleted, and the pool of organic P (P_o) becomes more important under P_i limitation (Blake et al., 2003; Gallet et al., 2003; Bergkemper et al., 2016b). This is also in line with a long-term fertilization study from Fraser et al. (2015), who demonstrated that the abundance of P mineralizing bacteria carrying the *phoD* gene, which codes for the alkaline phosphatase and the potential alkaline phosphatase activity were highest in plots with lowest bioavailable P concentrations. With respect to P fertilizer type it was demonstrated, that the application of slow release P-fertilizers like rock phosphate (Margenot et al. 2017) or biochar (Jin et al. 2016) increases potential phosphatase activities and promotes P solubilizing bacteria compared to non-fertilized soils (Anderson et al., 2011). In contrast, long-term fertilization of highly soluble mineral P reduced the abundance and diversity of P solubilizing bacteria in managed grassland soils (Mander et al. 2012). Thus, it can be assumed that the bacterial potential to utilize the applied P in agricultural soils is determined by both, the nature of the fertilizer and the initial soil P concentration, but so far comprehensive studies investigating this link and its consequences for the bacterial potential for uptake, solubilization and mineralization of P are missing.

To address these open questions, we analyzed bulk soil samples from a field experiment in the third experimental year during one growing season of winter wheat. The field experiment included different P fertilization treatments (control, TSP, BC, BC^{plus}) and soils from three different initial soil P test classes, which reflect very low, low and optimal plant available P concentrations (P_{CAL}). In those samples, we compared population sizes of bacteria, which take up (based on the marker genes *pitA, pstS),* solubilize (*gcd*) and mineralize P (*phoD, phnX, phoN, appA*) using quantitative real time PCR, with potential activities of phosphatases and P concentrations.

We hypothesized that, (i) long-term P depletion increases the abundance of P mineralizing and solubilizing bacteria as well as bacteria able to induce P uptake on demand. Therefore, the addition of slow release fertilizers like BC and BC^{plus} will promote P mobilizing bacteria, while the addition of easily available P like from TSP will promote bacteria able to take up P directly and suppresses the abundance of P mobilizing bacteria. (ii) Fertilizer induced differences of bacterial P turnover will become less obvious with increasing initial soil P concentrations. (iii) Besides the bacterial need for P, wheat has to cover its demand for P as well, which is highest during the period of major biomass increase (Römer and Schilling, 1986). In contrast, bacterial biomass in soil increases with higher root exudation and reaches its maximum during the flowering period, where consequently bacterial demand for P will be highest. Thus, we postulated that the bacterial mobilization of P increases during the vegetation period.

2. Materials and methods

2.1. Experimental set up and sampling

The study was performed at a field trial close to Braunschweig in Lower Saxony, Germany (52°18'N 10°27'E). This site is located 81 m above sea level with a mean annual precipitation of 620 mm and a mean temperature of 9 °C. The dominating soil units are Dystric Cambisol and Orthic Luvisol (according to IUSS Working Group WRB, 2015), which developed in silty-loamy sand with an average pH of 5.2 in topsoil. Two consecutive long-term P field experiments were carried out between 1985 and 2008 on this site (Vogeler et al., 2009). Briefly, five different mineral P fertilization regimes were applied including (T1) no P fertilization, (T2) 21.8 kg P ha^{-1} once a year (spring), (T3) 21.8 kg P ha^{-1} twice a year (spring and autumn), (T4) addition of P uptake of previous crop and (T5) 1.5-times addition of the P uptake of the previous crop. In 1998, the experiment was split in two blocks to compare conventional and conservational tillage regimes. The fertilization treatments included (T1) no P fertilization, (T2) organic P fertilization (farmyard manure, ~ every third year, last applied 2004; 20–40 kg P ha^{-1}) and (T3) farmyard manure like T2 and annual mineral fertilizer application (30–45 kg P ha^{-1}). Because of this previous experiments, significant differences in concentrations of plant available P were present in the plots. According to the guidelines of the Association of German Agricultural Analytic and Research Institutes (VDLUFA) (Wiesler et al., 2018), the plots were assigned to different classes of P_{CAL} in soil, namely initial soil P test class A (very low, < 15 mg P_{CAL} kg^{-1}), B (low, 15–30 mg P_{CAL} kg^{-1}), and C (optimal, 31–60 mg P_{CAL} kg^{-1}). Thus, throughout the manuscript we will use the terms very low P, low P and optimal P to describe the different initial soil P test classes determined in 2013. Table S1 provides an overview about initial P, N and C concentrations as well as pH before the start of the experiment as described by Panten and Leinweber (2020). The differences in P availability were maintained until 2013 by cultivation of an extensively managed grassland. In 2013, the experiment was ploughed to a depth of 25 cm and oat was sown.

After the harvest of oat in 2013, a new experiment was established using the old plots to test the potential of BC and BC^{plus} as fertilizer in soils with different initial levels of plant available P (Panten and Leinweber, 2020). The plots with very low initial P concentrations correspond to the P0 treatment of the previous experiment, the plots with low P concentrations to the organically fertilized plots and the plots with optimal P concentrations to the mineral and organically fertilized plots, respectively. The experiment was set up as a completely randomized block design with three replicates, plot sizes of 5.75 m * 17.50 m, and a 5-year crop rotation of winter barley, winter oilseed rape, winter wheat, lupin and winter rye. A combination of chisel ploughing and conventional ploughing to a depth of 25 cm was used to incorporate remaining straw and stubble before sowing. In addition to a control without P fertilization, three different types of P fertilizer were applied to an equivalent of 45 kg ha^{-1} P once a year shortly before seeding since autumn 2013, namely, bone char (BC), surface-modified bone char (BC^{plus}) with sulfur compounds from biogas streams (patent DE102011010525), and highly water soluble triple super phosphate (TSP). The proportion of P of the different fertilizer was 14.81% for BC, 10.72% for BC^{plus} and 20.04% for TSP. Besides P, BC and BC^{plus} contained high amounts of Zn and Ca, but were depleted in As, Cd, Cr, Cu, Ni, and U compared to TSP. The detailed elemental composition of the fertilizers was described previously (Panten and Leinweber, 2020, Zimmer et al. 2019). A detailed description of the experiment including all agronomic measures, and the P uptake by crops, crop yield and the fertilizer efficiencies are published by Panten and Leinweber (2020).

In frame of this study, we investigated the third year of the newly established field trial during the vegetation period in 2015/2016. In 2015 winter wheat (*Triticum aestivum* L. cv. JB Asano) was sown after ploughing to a depth of 25 cm. Fertilization with nitrogen (130 kg ha^{-1}), potassium (100 kg ha^{-1}), magnesium (11 kg ha^{-1}), and sulfur (12 kg ha

^{1}), as well as plant protection was uniform in all treatments. The trial was irrigated once with 30 l m^2 on the 10th of May 2016 and harvested on the 28th of July 2016. Crop yields ranged from 5.7 (control) to 6.0 t dry matter (DM) ha^{-1} (TSP) in the plots with optimal P concentrations, from 5.4 (BC) to 6.0 DM ha^{-1} (BC^{plus}) in plots with low P concentrations and from 5.4 (control) to 5.9 DM ha^{-1} (TSP) in the plots with very low P concentrations (Panten und Leinweber, 2020). Bulk soil samples were collected three times during the vegetation period (April/BBCH 33/34 - stem elongation, May/BBCH 53 - heading, June/BBCH 73–75 - ripening). From this point forward, the terms stem elongation, heading and ripening are used to improve the readability of the manuscript. Per plot, three soil cores (up to 10 cm soil depth) were collected, pooled and subsequently homogenized using a 5 mm sieve. In total, 108 samples were taken (three samplings, three soil P classes, four fertilization treatments, and three replicates). The samples for the determination of potential acidic and alkaline phosphatase activities were stored at 4 °C and analyzed within one week. The samples for nucleic acid extraction were collected and immediately frozen on the field using dry ice. Samples for pH, water soluble P (P_{water}) and plant available P (P_{CAL}) analyses were air-dried and sieved < 2 mm.

2.2. Physico-chemical analyses of soil samples

Soil pH values were measured in 0.01 M $CaCl_2$ (10 g soil in 25 ml $CaCl_2$); P_{water} was extracted according to van der Paauw (1971) and analyzed colorimetrically at a wavelength of 882 nm (Specord 50, Analytik Jena, Germany). P_{CAL} was extracted with calcium acetate lactate (soil P_{CAL}) (Schüller, 1969) and measured with an ICP-OES (icap 6000, Thermo Fisher, United Kingdom) at a wavelength of 213.6 nm. Dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) were extracted with 0.01 M $CaCl_2$ (4 g soil in 20 ml $CaCl_2$). Microbial biomass C (C_{mic}) was measured with the chloroform-fumigation-extraction method (Vance et al., 1987) using 0.01 M $CaCl_2$ for extraction and calculated using the correction factor k_{EC}= 0.45 (Joergensen, 1996). DOC and DON concentrations were measured with a DIMA-TOC 100 (Dima Tec, Langenhagen, Germany).

2.3. Acidic and alkaline phosphatases

Potential acidic and alkaline phosphatase activity was determined according to Schinner et al. (1991), based on the method developed by Tabatabai and Bremner (1969) and Eivazi and Tabatabai (1977). Colorimetric analysis was carried out at 400 nm (Specord 50, Analytik Jena, Germany).

2.4. DNA extraction

DNA was directly extracted from 0.5 g of frozen soil (−80 °C) using Precellys 24 (Bertin Technologies, France) based on a phenol-chloroform based protocol modified according to Lueders et al. (2004) and Töwe et al. (2011). Total genomic DNA quality was determined photometrically (Nanodrop ND-1000; Thermo Fischer Scientific, MA, USA). The quantity of the DNA was assessed using the QuantiT Pico-Green kit (Thermo Fischer Scientific, MA, USA). Extracted samples were stored at −20 °C before further processing by qPCR.

2.5. Quantitative Polymerase Chain Reaction measurements (qPCR)

Real-time quantitative PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems, Germany) using Sybr Green as fluorescent dye to quantify marker genes for different processes of P turnover including *gcd, phoD*, *phoN*, *phnX*, *appA, pstS,* and *pitA*. The reaction mix contained 12.5 µl of SYBR Green® (Thermo Fisher Scientific, USA), forward (F) and reverse (R) primers (Metabion, Germany), 0.5 µl BSA (3%, Sigma, Germany) and DEPC-treated water and was set to 25 µl. The source of the standard, primer sequences and reaction mixture components are summarized in Table 1 (Bergkemper et al., 2016a). The thermal profile consisted of a touchdown of 5 cycles starting with a denaturation at 95 °C for 15'' followed by primer annealing at 65 °C for 30'' and finished by elongation at 72 °C for 45''. After the touchdown with a reduction of the annealing temperature of 1 °C per qPCR cycle, 40 qPCR cycles followed with an annealing temperature of 60 °C. Serial plasmid dilutions (10^1 to 10^7 gene copies μl^{-1}) were used for standard curve calculations. In a pre-experiment, performed to avoid reaction inhibition effects, the optimal sample dilution was determined as 1:16 (data not shown). Additionally, a melting curve analysis was performed by adding a dissociation stage after each run in order to prove the specificity of the amplified qPCR products. To confirm the correct size of the amplified fragments further, gel electrophoresis for randomly selected samples was conducted on a 1% agarose gel. Efficiencies of qPCRs were calculated as $E = (10^{(-1/slope)}-1) \times 100$ and were as follows: 81.9% for *phoD*, 73.5% for *pstS*, 89.3% for *pitA,* 90.3% *phnX* and 85.0% for *gcd*. R^2 was determined to be above 0.99 for each qPCR assay. The abundance of *appA* and *phoN* was below detection limit in all samples (less than 10 copies μl^{-1}).

2.6. Statistical analysis

Data analysis was performed with R version 3.6.1 (R Core Team, 2019). The crossbar plots were created using ggplot2 package (Wickham, 2009). Linear models on log-transformed data were applied and an ANOVA was performed to evaluate variances caused by sampling time point, initial soil P class, and fertilizer treatment on the total sample set. Further, samples were analyzed separately by sampling date to detect different reactions caused by initial soil P concentration or fertilizer treatment. The Tukey Post hoc test was performed to test for significant differences ($p < 0.05$) between the investigated factors (R package lsmeans) (Lenth, 2016).

To visualize how gene abundances differ between control and fertilized plots, average gene abundances were calculated for each treatment (control, TSP, BC and BC^{plus}) and subsequently normalized to the control. In case the values of treatments were higher than control values, this was calculated as r = treatment/control, else as r = - control/treatments. $r > 1$ indicate higher abundances in the fertilization treatment, while $r < -1$ indicate higher values in the control treatment.

3. Results

3.1. Soil chemical properties

The concentrations of P_{CAL} and P_{water} are summarized in Fig. 1. Both were significantly influenced by the initial soil P class, fertilizer treatment, and P_{water} additionally by sampling date (Table 2, Table S3). P_{CAL} concentrations ranged between 13.4 (stem elongation: control treatment on, very low and low initial P) and 57.1 mg kg^{-1} dry weight (dwt) (ripening: TSP treatment on optimal initial P). P_{CAL} concentrations of the controls, the TSP and the BC fertilized plots were significantly higher in plots with optimal initial P concentrations throughout the growing season of wheat. Significant differences between fertilizer treatments were only observed in soils with low initial P during wheat heading. There, the fertilization with TSP resulted in significantly higher values compared to the BC treatment and peaked in 41.8 mg kg^{-1} P_{CAL} dwt.

The P_{water} concentrations were four to five times lower than P_{CAL} concentrations and ranged between 2.3 mg kg^{-1} dwt (stem elongation: BC treatment on very low initial P) and 13.4 mg kg^{-1} dwt (ripening: TSP on optimal initial P). Similar to P_{CAL}, a significant increase from soil with very low initial P to optimal initial P was observed, but only in the TSP and BC fertilized plots during stem elongation and heading of wheat. Differences of the fertilization regimes were most pronounced during wheat heading. This includes significantly higher values in the TSP treatment and lowest values in the BC treatment in plots with very low and low initial P, resulting in P_{water} concentrations of 5.0 mg kg^{-1} dwt

Table 1
qPCR reaction conditions, standard sources and primer sequences used for qPCR of target genes.

Target gene	Source of standard	Mean expected amplicon length	Primer sequence	Primer (10 μM)	Polymerase
phoD	*Bradyrhizobium japonicum*	208	phoD-FW-TGTTCCACCTGGGCGAYWMIATHTAYG phoD-RW-CGTTCGCGACCTCGTGRTCRTCCCA	1.0	–
phoN	*Salmonella enterica* DSM 10062	159	phoN-FW-GGAAGAACGGCTCCTACCCIWSNGGNCA phoN-RW-CACGTCGGACTGCCAGTGIDMIYYRCA	1.0	0.2
appA	*Escherichia coli* DSM 30083	375	appA-FW-AGAGGGTGGTGATCGTGATGMGICAYGGNRT appA-RW-GCCTCGATGGGGTTGAAIADNGGRTC	0.75	0.1
phnX	*Salmonella enterica* DSM 17058	147	phnX-FW-CGTGATCTTCGACtGGGCNGGNAC phnX-RW-GTGGTCCCACTTCCCCADICCCATNGG	0.2	–
gcd	*Salmonella enterica* DSM 17058	330	gcd-FW-CGGCGTCATCCGGGSITIYRAYRT gcd_RW-GGGCATGTCCATGTCCCAIADRTCRTG	0.75	0.1
pitA	*Pseudomonas fluorescens*	270	pitA-FW-GGTCTTCGAGTTCATGAACGGNTTYCAYGA pitA-RW-CCAGGTGACCAGGTTCCAIRNDAT	0.5	0.2
pstS	*Bradyrhizobium japonicum*	221	pstS-FW-TCTACCTGGGGAAGATCACAAARTGGRAYGA pstS-RW-TGCCGACGGGCCAITYNWC	1.0	0.1

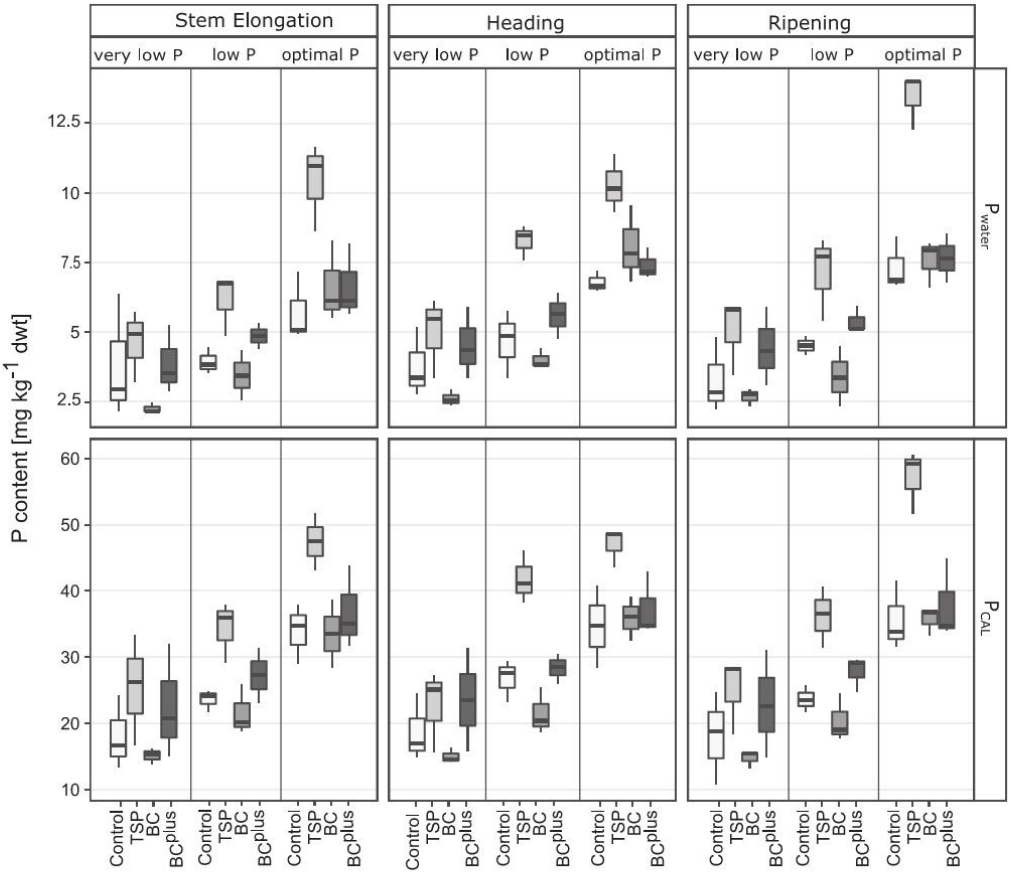

Fig. 1. Concentrations of P_{CAL} and P_{water} (n = 3) as box plots. Samples were taken from experimental fields that were classified into initial soil P test classes with very low, low and optimal P_{CAL} concentrations and sampled at three different growth stages of winter wheat (stem elongation, heading, ripening) and four fertilizer treatments (control, TSP, BC, BC^{plus}).

and 8.3 mg kg^{-1} dwt for TSP and 2.6 mg kg^{-1} dwt and 4.0 mg kg^{-1} dwt for BC, respectively. Only in soils with low initial P, this significant difference was also detectable at wheat ripening.

In addition to the P concentrations in soil, we included pH, dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) as well as C_{mic} measurements in the analysis. These data are summarized in Table S2. The C_{mic} concentrations and pH values did not differ significantly among the samples and revealed mean values of 246 μg g^{-1} dwt

Table 2
p values of ANOVA analysis on Linear Models of the factors sampling date, initial soil P test class and treatment for potential enzyme activities and plant-available P concentrations in soil. Significant values ($p < 0.05$) are shown in italics.

	Available P		Potential enzyme activity	
Total	P_{water}	P_{CAL}	ACP	ALP
Sampling	*0,02*	0,71	*<0,01*	*0,02*
Soil class	*<0,01*	*<0,01*	0,43	0,7
Treatment	*<0,01*	*<0,01*	0,14	0,69
Sampling x Soil class	0,67	0,74	0,97	0,46
Sampling x Treatment	0,99	0,99	0,99	0,95
Soil class x Treatment	*0,02*	0,22	0,69	0,37
Sampling x Soil class x Treatment	0,98	0,99	0,89	0,76
Stem elongation				
Soil class	*<0,01*	*<0,01*	0,97	0,68
Treatment	*<0,01*	*<0,01*	0,32	0,57
Soil class x Treatment	0,57	0,57	0,39	0,56
Heading				
Soil class	*<0,01*	*<0,01*	0,66	0,42
Treatment	*<0,01*	*<0,01*	0,76	0,9
Soil class x Treatment	0,14	0,31	0,95	0,91
Ripening				
Soil class	*<0,01*	*<0,01*	0,57	0,37
Treatment	*<0,01*	*<0,01*	0,6	1
Soil class x Treatment	0,47	0,83	0,83	0,1

and 5.2, respectively. DOC concentrations were highest during stem elongation in the BC^{plus} treatment revealing 91.6, 237.8 and 92.5 μg g^{-1} dwt in soil with very low, low and optimal initial P, respectively. In most other samples, values were up to 4-times lower. DON concentration was highest in soils with very low initial P during stem elongation and ranged from 10.5 μg g^{-1} dwt in the TSP treatment to 24.5 μg g^{-1} dwt in the control treatment. Afterwards, the DON concentration dropped below 10 μg g^{-1} dwt in all other samples, except the control treatment in soils with very low initial P during heading where still a DON concentration of 12.6 μg g^{-1} dwt was measured.

3.2. Potential enzyme activities

Potential enzyme activities are summarized in Table S2. Initial soil P class and fertilizer treatment had no significant influence on potential enzyme activities, but potential alkaline (ALP) and acid phosphatase activity (ACP) weresignificantly influenced by the sampling date (Table 2). In general, potential enzyme activities were lowest during stem elongation. Regarding ACP, the values ranged from 248 to 301 μg p-NP g^{-1} dwt h^{-1} during stem elongation, while ALP was much lower and did not exceed 73 μg p-NP g^{-1} dwt h^{-1}. At later sampling dates, no significant changes were observed and the mean potential ALP activity across all initial soil P classes and fertilization treatments was 74.3 μg p-NP g^{-1} dwt h^{-1} (± 6.6) and for ACP 313 μg p-NP g^{-1} dwt h^{-1} (± 14.5).

3.3. Abundance of bacteria catalyzing different steps in P turnover

As the microbial biomass C did not differ significantly among the samples the gene copy numbers were related to gram dry weight. The results are depicted in Fig. 2.

For P uptake, the abundance of *pitA* was always 10–100-times higher than the abundance of *pstS* in our samples. Gene copy numbers of both genes were significantly influenced by initial soil P class and sampling date. Highest values were detected in soils with optimal initial P at all sampling dates. During stem elongation and ripening, the BC treatment revealed the highest gene abundances of 6.9×10^5 and 5.9×10^5 gene copies g^{-1} dwt for *pstS* and 3.7×10^7 and 2.9×10^7 gene copies g^{-1} dwt for *pitA*. Fertilizer effects and sampling date significantly interacted. Thus fertilizer effects were only obvious during heading (Table 3, Table S3) and resulted in significantly higher *pstS* gene copy numbers in the control (8.4×10^5 gene copies g^{-1} dwt) compared to the TSP treatment (3.7×10^5 gene copies g^{-1} dwt) in soils with optimal initial P.

Regarding the mineralization of P, the abundance of the genes *phoD* and *phnX* was significantly influenced by the sampling date. This effect was visible for *phnX* during ripening in the TSP treatment in soils with very low initial P where the highest gene copy numbers were observed (2.2×10^6 gene copies g^{-1} dwt). For all other samples, copy numbers between 2.6×10^5 and 1×10^6 gene copies g^{-1} dwt were measured. Regarding the abundance of the *phoD* gene the fertilizer effects strongly interacted with initial soil P class and sampling date. Thus, effects of fertilizer treatments and initial soil P class were most pronounced during heading. In soils with low initial P significantly higher values were detected in the TSP treatment compared to BC^{plus} reaching up to 1×10^6 gene copies g^{-1} dwt, while in soils with optimal initial P highest gene abundances of 1×10^6 gene copies g^{-1} dwt were detected in the BC treatment.

For the *gcd* gene, which drives the solubilization of P, initial soil P class and fertilizer treatment had a significant effect on its abundance. This was especially apparent during heading where the use of BC^{plus} as fertilizer caused a significant increase of the *gcd* gene abundance in soils with low and optimal initial P, which peaked in 1.2×10^7 and 1.9×10^7 gene copies g^{-1} dwt, respectively. Interestingly, the BC treatment revealed lowest *gcd* gene copy numbers during heading accounting for 3.2×10^6 in soil with low initial P and 4.1×10^6 in soils with optimal initial P, respectively. This is in contrast to the stem elongation period where the *gcd* gene abundance in soils with optimal initial P under BC and BC^{plus} fertilization revealed similar mean abundances of 7.5 and 7.2×10^6 gene copies g^{-1} dwt.

To better visualize the fertilization effects in the soils with different initial P, we calculated ratios between the gene copy numbers in the different fertilization treatments and the control treatments. Ratios are depicted in Fig. 3. This analysis demonstrated that during heading in 35 out of 45 cases (3 soil P classes, 3 fertilization treatments, 5 genes) the abundance of bacteria harboring P turnover genes was higher in the control compared to the fertilization treatments, as indicated by ratios $r < -1$. Interestingly, *gcd* was an exception. Here the gene abundance was 2.6 and 3 times higher in the BC^{plus} treatment compared to the control in soils with low and optimal initial P, respectively. During stem elongation, an opposite pattern for soils with very low and optimal initial P was observed, as gene abundances were higher in the fertilization treatment as indicated by ratios $r > 1$. This was especially pronounced for the BC treatment in soils with optimal initial P for all genes except *phnX*. In soils with low initial P, only *phoD* ratios remained consistently higher in all fertilization treatments. At the ripening stage, the ratio $r > 1$ indicated higher abundances in the fertilization treatments for the majority of genes.

4. Discussion

In this study, we compared the response of the soil microbiome towards the application of the recycling P fertilizers BC and BC^{plus} with no P or TSP fertilization in soils with different initial P concentrations during one growing season of winter wheat.

Our data indicate that the bacterial potential to solubilize P is increased in the BC^{plus} treatment as revealed by higher *gcd* gene abundances. The *gcd* gene codes for the quinoprotein glucose dehydrogenase, which catalyzes the solubilization of inorganically bound P fractions by oxidizing glucose and other aldose sugars to gluconic acid (Goldstein, 1995), which results in a small-scale reduction of the pH. This is in line with Postma et al. (2010), who demonstrated that the addition of P solubilizing bacteria to animal bone charcoal particles improved the dissolution of P. In general, P solubilizing bacteria are discussed as an option to improve the sustainability of P fertilization in agriculture (Alori et al., 2017, Ditta et al., 2018). Our data imply that under specific circumstances like specific plant growth stages (heading) or different initial soil P concentrations (low, optimal) the soil inherent P solubilizing bacteria are stimulated (Table 3, Fig. 2+3). In BC^{plus} fertilized

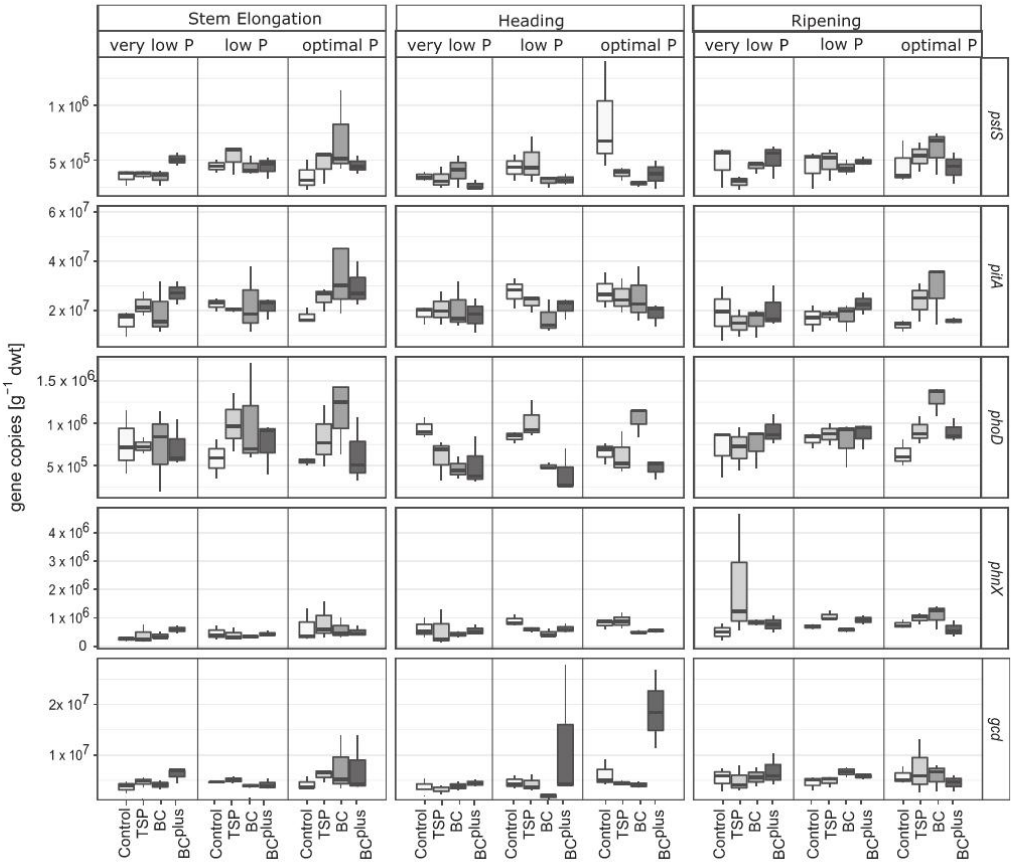

Fig. 2. Gene copy numbers of five genes involved in P turnover (*pstS, pitA, phoD, phnX, gcd*). Gene copy numbers were calculated per gram dry weight and are plotted on a linear scale as box plots (n = 3). Samples were taken at three plant development stages (stem elongation, heading, ripening), from three initial soil P test classes with very low, low and optimal P_{CAL} concentrations and four fertilizer treatments (control, TSP, BC, BC^{plus}).

soils with low initial P, this was accompanied by slightly higher crop yields (Panten and Leinweber, 2020).

The fertilization with BC favored the mineralization of P_o as demonstrated by significantly more bacteria carrying *phoD* throughout the whole season. Interestingly, this pattern was mostly observed in soils with optimal initial P. This was unexpected because *phoD* is under the control of the *phoRB* two component system, which specifically detects P starvation and controls several phosphate starvation inducible genes (Hsieh and Wanner, 2010). Moreover, it was reported previously that the abundance of *phoD*-carrying bacteria and the potential alkaline phosphatase activity was highest in plots with lowest bioavailable P concentrations in a long-term fertilization study (Fraser et al., 2015). Therefore, it would have been expected to detect highest *phoD* abundances in soils with low initial P, where also crop yields were lower compared to soils with optimal initial P (Panten and Leinweber, 2020). However, it can be assumed that the bacterial community in soils with very low initial P is well adapted to low amounts of available P, and that a slow rate of P release from BC (Leinweber et al., 2019) might promote the inherent bacterial community. In contrast, the long-term application of high amounts of P in soils of optimal initial P might have suppressed the bacterial potential to solubilize P as demonstrated by Mander et al. (2012). Moreover, the long-term input of P caused a shift in nutrient stoichiometry, which is confirmed by lower C:P and N:P ratios in soils with optimal initial P (Table S1). Thus, it is likely that also nutrients other than P need to be released from organic pools as it was demonstrated previously (Spohn and Kuzyakov, 2013). Interestingly, the higher abundance of *phoD* carrying bacteria in the BC fertilization treatment in soils with optimal initial P was accompanied by an increase of *pitA* carrying bacteria. The Pit transporter is a low-affinity P_i transporter, which takes up cation-phosphate complexes at sufficient P concentrations (Willsky et al., 1973; Wanner, 1993). Thus, the higher concentrations of P_{CAL} and P_{water} (Fig. 1) and the additional input of cations like Ca and Mg from the BC (Siebers and Leinweber, 2013; Zimmer et al., 2019) might display favorable conditions and promotes a closed P cycle. Surprisingly, the observed higher gene abundance of *phoD* in the BC fertilized plots with optimal initial P was not confirmed by the potential enzyme activity measurements, which did not increase (Table S2). This might be attributed to (i) the acidic pH, which is not optimal for the alkaline phosphatase. The pH was optimal for the acidic phosphatase, which was generally much higher, but rather originated from plants because of the absence of *phoN* carrying bacteria. (ii) Or the survival of extracellular enzymes attached to soil particles, which

Table 3
p values of ANOVA analysis on Linear Models of the factors sampling date, initial soil P test class and treatment for gene abundances in soil. Significant values ($p < 0.05$) are shown in italics.

	Gene abundance				
Total	*pstS*	*pitA*	*phoD*	*gcd*	*phnX*
Sampling	*0.03*	*0.03*	*0.01*	0.52	*< 0.01*
Soil class	*0.04*	*0.04*	0.3	*0.03*	*0.07*
Treatment	0.97	0.64	0.16	*0.01*	0.36
Sampling x Soil class	0.97	0.97	0.95	0.18	0.5
Sampling x Treatment	*0.02*	0.25	*0.02*	*0.02*	*0.09*
Soil class x Treatment	0.44	0.18	*0.01*	0.99	0.2
Sampling x Soil class x Treatment	*0.09*	0.78	0.92	0.25	0.84
Stem elongation					
Soil class	0.26	0.22	0.83	0.25	0.13
Treatment	0.32	0.22	0.43	0.42	0.69
Soil class x Treatment	0.21	0.44	0.73	0.66	0.69
Heading					
Soil class	0.2	0.25	0.64	*0.01*	0.14
Treatment	*0.03*	0.34	*< 0.01*	*< 0.01*	0.19
Soil class x Treatment	0.11	0.68	*0.02*	0.22	0.68
Ripening					
Soil class	0.5	0.52	0.2	1	0.99
Treatment	0.78	0.64	0.21	0.87	*0.04*
Soil class x Treatment	0.5	0.39	0.27	0.82	0.26

accumulate and overlay treatment effects (Nannipieri et al., 2003).

Although the same amount of P was applied in the BC and BC^{plus} fertilized plots as for the TSP plots, no increase of P_{water} or P_{CAL} concentrations was observed for the alternative fertilizers compared to the control. This might have three reasons, (i) no additional P was released from the alternative fertilizers, (ii) released P was bound at the bone char surfaces, or (iii) it was directly taken up by the plants or microbes. The first case is very unlikely as it has been demonstrated previously that the P-release from BC^{plus} is higher than from BC (Zimmer et al., 2018; Zimmer et al., 2019). This was mostly attributed to the internal activation by S, which is oxidized by microorganisms and caused a reduction of the pH, which in turn favors the dissolution of P from hydroxyapatite, the major component of BC^{plus} (Zimmer et al., 2019). Based on recent literature, it is even possible that the oxidation of S and dissolution of P is performed by the same bacteria, as for example members of the *Verrucomicrobiaceae* and *Solibacteraceae* were described as abundant groups for both processes in soil (Grafe et al., 2018; Hausmann et al., 2018). However, a verification of this hypothesis is only possible by additional sequencing approaches. Binding of released phosphate ions by the bone chars is unlikely as well, because BC had a point of zero charge of 4.5 and BC^{plus} of 2.7 (Leinweber, unpublished data). That means that at a soil pH of 5.0–5.3 (Table S2) the BC surfaces are net-charged negatively, indicating repellence of anions rather than adsorption. Thus, a tight coupling of P solubilization and uptake is more likely, which for example is corroborated by higher abundances of bacteria carrying *gcd, pitA* and *pstS* gene abundances in the BC^{plus} treatment of soils with very low initial P. The efficient recycling of P from rock phosphate resulted in increased organic P mineralization and the immediate immobilization of P in the microbial biomass, while the fertilization with easily available TSP caused an increase of mineral bound P (Margenot et al. 2017). Similarly, it could be assumed for the control plots that the available P pools are constantly depleted by the uptake of P by plants and bacteria. Therefore, an equilibrium between P uptake, P turnover of the bacterial biomass and P solubilization from the soil is needed to meet the P-demands of the crop and the bacteria by avoiding a loss of P for example by leaching. Thus, it is not surprising, that in the control treatment the gene abundance pattern did not differ significantly no matter which initial soil class was analyzed (Fig. 2, Table S3). Similar observations were made in forest sites with low P concentrations, which promoted an efficient recycling of P (Bergkemper et al. 2016, Lang et al. 2017). In contrast to the P depleted control plots, the addition of TSP to soils with optimal initial P maintained the P_{water} and P_{CAL} concentrations measured at the beginning of the experiment of 9.4 and 47.2 mg kg^{-1}, respectively (Table S1 and Fig. 1). As this did not result in higher yields, it seems that these P sources remain unused and are at risk for being lost by leaching to the groundwater, especially via preferential flow (Djodjic et al., 2004).

Beside many differences among the treatments, all samples taken during heading had in common that the abundance of P transforming bacteria was higher in the control treatment compared to the P fertilized treatments (Fig. 3). Winter wheat met most of its own P demands during highest biomass growth (Römer and Schilling, 1986). The high P demand during plant growth might be compensated by the presence of arbuscular mycorrhizal fungi in the roots of wheat (Römer and Schilling, 1986; Pellegrino et al., 2015), while bacteria replenish the depleted P pools afterwards. In the control treatment, this was achieved by generally higher abundances of many P transforming bacteria. Especially the abundance of bacteria harboring the *pstS* gene was significantly higher compared to the other sampling dates ($p = 0.03$). This gene codes for a subunit of the highly specific P transporter, which can take up P against a steep concentration gradient under the turnover of ATP (Jansson, 1988). Thus, highest expression levels can be expected under P limitation, when a sufficient amount of bioavailable C and N is available. This

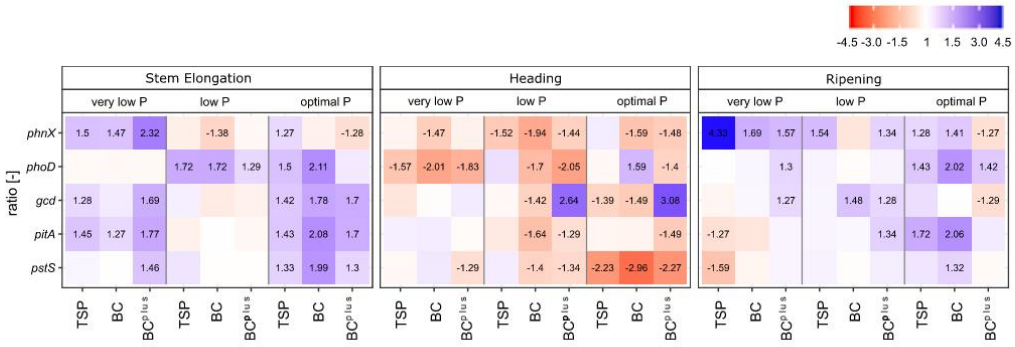

Fig. 3. Ratio between gene copy numbers in the control and treatment in form of a heatmap. Average gene abundances were calculated for each treatment (control, TSP, BC and BC^{plus}) and subsequently normalized to the control. In case the values of treatments were higher than control values, this was calculated as r = treatment/control, else as r = - control/treatment. $r > 1$ indicate higher abundances in the fertilization treatment and are displayed in blue, while $r < -1$ indicate higher values in the control treatment and are color coded in red scale. Only values above 1.25 or below −1.25 are additionally depicted as numbers. The initial soil P test classes are characterized by very low, low and optimal P_{CAL} concentrations.

assumption is corroborated by lower P_{water} concentrations in the control plots during heading compared to the other treatments. At the same time, DOC and DON did not change or even slightly increased (Table S2). This change in nutrient stoichiometry might cause an imbalance of the bacterial C, N and P supply and further favors sufficient P uptake mechanisms (Spohn and Kuzyakov, 2013; Heuck et al., 2015), as it was presumed that bacteria aim for a stable C:N:P ratio (Cleveland and Liptzin, 2007). In soils with optimal initial P and BC fertilization, the abundance of *phoD* increased significantly during heading in comparison to stem elongation, which was underlined by significantly higher potential phosphatase activities (Table 2, Table S2). This corroborates our hypothesis that the importance of the mineralization of P_o increased during the season. Regarding the BC^{plus} treatment, the observed impact on P solubilizing bacteria was even higher during heading. In addition, the BC^{plus} fertilizer might also improve the release of P from legacy P pools as postulated by Rowe et al. (2015), espsecially as the highest abundances were observed in soils with optimal initial P.

5. Conclusions

Our data demonstrate that the effects of alternative P fertilizer on bacterial P turnover are strongly interlinked with the initial soil P concentration as well as the plant growth stage. In general, as hypothesized the addition of BC^{plus} and BC increased the abundance of specific P mobilizing bacteria. However, the difference in P dissolution from BC^{plus} and BC favored different bacterial groups. While BC^{plus} promotes P solubilizing bacteria, the low P concentration in control plots and slow release of P from BC favors P recycling from biomass and inducible uptake systems, which is displayed by either high abundance of *phoD* carrying bacteria in BC fertilized plots or of *pstS* carrying bacteria in control plots, respectively. Surprisingly and contrary to our assumption, many fertilizer effects were more pronounced in soils with optimal initial P concentration. This, might point to the fact that bacterial nutrient turnover is not restricted to the availability of a single nutrient, but relies on a balanced delivery of all macronutrients, leading to unfavorable C:P and N:P ratios under long-term P fertilization causes. Additionally, it became obvious that plant growth stage interferes with fertilizer effects as well. We hypothesized, that bacterial P turnover is increased in P depleted soils. This was only obvious during heading, after wheat had met most of its demand. As assumed, bacteria of the control plots replenished the missing P by mineralizing organic P, but in BC^{plus} fertilized plots this was accomplished by an increased potential for solubilization, which might have been related to a continuous release of P from BC^{plus}. Our study underlines the importance of studying complex experiments to become aware of those interactions and to study them further in follow up experiments. However, based on our results we cannot predict long-term responses of bacteria towards the fertilization regime, as the sampling took place during one year of the first crop rotation. Thus, different plant properties for example caused by symbiosis with nitrogen fixing bacteria or different climatic conditions during the growing season might significantly alter bacterial responses to fertilizer applications by modulating nutrient stoichiometry, water availability and soil temperature. Moreover, our study was restricted to the bacterial potential, but we do not know to which extent this was recalled during the sampled growing season. Thus, future studies need to investigate transcription rates of the different genes involved in P turnover processes and how they differ during a crop rotation. However, as transcripts have a short half-life time many and fast samplings several times a day might be needed to reach statistical significance especially in field studies.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2021.107419.

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Supplemental Information Manuscript III

Table S1. Mean of soil parameters in the top 30 cm of three soils with different initial P concentrations namely very low, low, and optimal P_{CAL} concentrations after harvest in 2013. Measurements were conducted before the set-up of the present experiment and previously published by Panten and Leinweber (2020).

Soil parameter	**Very low P**	**Low P**	**Optimal P**
P_{CAL} [mg kg^{-1}]	11.2 (6.9-17.8)	20.7 (16.5-25.1)	47.2 (41.4-53.0)
P_{water} [mg kg^{-1}]	2.8 (2.0-3.9)	4.4 (3.5-5.5)	9.4 (8.0-10.5)
Total P [mg kg^{-1}]	217 (204-241)	273 (230-324)	399 (378-424)
Total C [%]	1.3 (1.2-1.4)	1.4 (1.3-1.5)	1.4 (1.4-1.4)
Total N [%]	0.10 (0.09-0.10)	0.10 (0.10-0.11)	0.10 (0.10-0.11)
pH	5.1 (5.0-5.2)	5.2 (5.0-5.2)	5.2 (5.1-5.3)

Table S2. Mean values and standard deviation of the potential alkaline (ALP) and acid (ACP) phosphatase activity, dissolved organic carbon (DOC) and nitrogen (DON), microbial biomass carbon (C_{mic}) and the pH. Samples were taken and analyzed from three field replicates.

Sampling Date	Initial P	Treatment	ALP [µg p-NP g^{-1} dwt h^{-1}]	ACP [µg p-NP g^{-1} dwt h^{-1}]	DOC [µg g^{-1} dwt]	DON [µg g^{-1} dwt]	C_{mic} [µg g^{-1} dwt]	pH
Stem elongation	very low	Control	69.4 ± 13	285.1 ± 24.1	21.4 ± 13.5	24.5 ± 20.6	242.5 ± 125.5	5.0 ± 0.2
		TSP	69.2 ± 14.3	248.0 ± 27.8	22.7 ± 11	10.5 ± 4.4	201.7 ± 87.1	5.1 ± 0.2
		BC	73.7 ± 10	301.3 ± 28.1	24.1 ± 9.7	20.0 ± 6.2	243.9 ± 37.8	5.0 ± 0.1
		BC^{plus}	56.9 ± 12.4	254.4 ± 39.3	91.6 ± 115*	16.9 ± 21.0	244.6 ± 130.9	5.2 ± 0.1
	low	Control	75.6 ± 19	266.0 ± 32.1	16.1 ± 2.1	7.7 ± 5.6	225.8 ± 64.2	5.1 ± 0.0
		TSP	54.8 ± 2.5	267.5 ± 13.7	14.3 ± 3.3	2.2 ± 1.1	271.8 ± 155.3	5.1 ± 0.1
		BC	64.3 ± 11.8	283.6 ± 21.4	15.7 ± 0.7	10.3 ± 5.8	210.4 ± 48.2	5.1 ± 0.2
		BC^{plus}	68.3 ± 13.4	265.0 ± 1.6	237.8 ± 382#	6.3 ± 4.8	163.5 ± 75.3	5.1 ± 0.1
	optimal	Control	70.4 ± 14.3	268.9 ± 21.2	20.1 ± 8.8	4.8 ± 4.9	210.6 ± 25.7	5.1 ± 0.1
		TSP	67.9 ± 5.1	260.0 ± 16.6	23.6 ± 11.3	7.0 ± 1.9	251.3 ± 48.4	5.2 ± 0.0
		BC	69.9 ± 7.3	262.6 ± 29.2	16.9 ± 5.6	3.1 ± 1.3	167.4 ± 51.8	5.2 ± 0.0

		BC^{plus}	71.9 ± 23.1	283.5 ± 38.3	92.5 ± 47.2	4.0 ± 4.0	260.3 ± 94.1	5.1 ± 0.1
Heading	very low	Control	81.3 ± 33.5	328.6 ± 43	25.8 ± 8.9	12.6 ± 6.3	225.6 ± 31.2	5.1 ± 0.2
		TSP	74.8 ± 13.5	320.5 ± 42	23.6 ± 4.25	8.5 ± 3.1	258.9 ± 31.7	5.2 ± 0.1
		BC	69.4 ± 17.8	324.4 ± 33.7	23.9 ± 4.95	9.2 ± 1.7	259.2 ± 56.9	5.2 ± 0.2
		BC^{plus}	75.0 ± 5.4	314.3 ± 28.2	20.2 ± 0.58	7.2 ± 1.4	242.5 ± 72.5	5.2 ± 0.1
	low	Control	86.2 ± 9.9	318.0 ± 6.4	23.8 ± 4.85	8.2 ± 1.1	251.8 ± 36.1	5.2 ± 0.1
		TSP	80.1 ± 17.8	313.2 ± 28.9	24.4 ± 1.68	7.2 ± 1.1	231.1 ± 33.9	5.1 ± 0.1
		BC	77.4 ± 10.9	332.9 ± 60.9	21.5 ± 5.82	7.0 ± 0.4	211.8 ± 53.2	5.2 ± 0.1
		BC^{plus}	83.7 ± 18.3	333.6 ± 41.7	18.4 ± 2.79	8.5 ± 0.7	273.9 ± 78.9	5.2 ± 0.1
	optimal	Control	74.9 ± 14	328.0 ± 29.3	22.2 ± 6.8	7.5 ± 1.7	247.7 ± 59.6	5.1 ± 0.1
		TSP	77.2 ± 1.7	301.8 ± 43.6	21.2 ± 1.71	8.0 ± 1.3	270.2 ± 41.3	5.2 ± 0.0
		BC	78.2 ± 9.6	322.6 ± 32.7	18.5 ± 4.95	6.9 ± 1.2	229.2 ± 11.2	5.2 ± 0.0
		BC^{plus}	65.3 ± 15.3	293.1 ± 25.5	20.1 ± 1.62	6.1 ± 1.1	255.4 ± 35.0	5.2 ± 0.1
Ripening	very low	Control	66.9 ± 4.4	321.7 ± 67.6	31.2 ± 13.9	5.8 ± 0.5	249.6 ± 67.7	5.2 ± 0.4
		TSP	73.8 ± 4.2	285.9 ± 1.3	23.9 ± 8.07	5.7 ± 1.4	276.6 ± 18.1	5.4 ± 0.3

	BC	67.9 ± 2.2	329.0 ± 52.2	25.1 ± 1.59	6.6 ± 0.8	270.0 ± 41.0	5.6 ± 0.6
	BC^{plus}	81.7 ± 8	316.6 ± 27.5	23.7 ± 1.35	6.3 ± 0.7	283.4 ± 44.1	5.2 ± 0.2
low	Control	83.9 ± 8.1	294.0 ± 42.5	46.4 ± 34	8.7 ± 2.9	255.7 ± 57.3	5.2 ± 0.0
	TSP	67.7 ± 13.7	308.6 ± 13.6	21.1 ± 3.1	6.6 ± 1.3	282.3 ± 81.8	5.1 ± 0.2
	BC	73.7 ± 14.9	328.0 ± 15.1	30.6 ± 13.4	6.3 ± 1.5	268.1 ± 65.2	5.1 ± 0.2
	BC^{plus}	69.3 ± 7.2	308.0 ± 39.6	29.6 ± 12.6	7.1 ± 1.9	244.5 ± 52.4	5.2 ± 0.1
optimal	Control	64.7 ± 11.1	303.9 ± 21.2	23.7 ± 4.99	5.1 ± 0.7	266.9 ± 35.6	5.1 ± 0.3
	TSP	73.4 ± 3.7	297.4 ± 30.5	29.4 ± 5.82	7.0 ± 0.9	280.1 ± 68.8	5.2 ± 0.1
	BC	72.5 ± 8.1	300.4 ± 11.6	26.3 ± 4.18	6.4 ± 0.9	278.9 ± 72.4	5.3 ± 0.1
	BC^{plus}	62.9 ± 14.7	290.0 ± 21	30.7 ± 9.13	6.6 ± 2.3	260.0 ± 50.8	5.1 ± 0.2

DOC values excluding outlier replicates (n=2): *25.6 µg g^{-1} dwt, # 17.5 µg g^{-1} dwt

Table S3. p values of significant ($p < 0.05$) pairwise comparisons based on a Tukey Post hoc test.

Sampling Date	Contrast (initial P, Treatment)	p Value	Test Variable
Stem elongation	very low, BC - optimal, BC	0.002	P_{water}
	very low, TSP - optimal, TSP	0.023	P_{water}
	very low, BC - optimal, BC	0.005	P_{CAL}
	very low, Control - optimal, Control	0.031	P_{CAL}
	very low, TSP - optimal, TSP	0.028	P_{CAL}
Heading	optimal, BC - optimal, Control	0.027	*pstS*
	low, BCplus - low, TSP	0.033	*phoD*
	optimal, BC - optimal, BC^{plus}	0.012	*gcd*
	very low, BC^{plus} - optimal, BC^{plus}	0.019	*gcd*
	optimal, BC^{plus} - optimal, TSP	0.019	*gcd*
	very low, BC - optimal, BC	0.000	P_{water}
	very low, BC - A, TSP	0.028	P_{water}
	low, BC - optimal, BC	0.010	P_{water}
	low, BC - low, TSP	0.006	P_{water}
	very low, Control - optimal, Control	0.027	P_{water}

	low, Control - low, TSP	0.037	P_{water}
	very low, TSP - optimal, TSP	0.004	P_{water}
	very low, BC - optimal, BC	0.000	P_{CAL}
	low, BC - low, TSP	0.007	P_{CAL}
	very low, Control - optimal, Control	0.013	P_{CAL}
	very low, TSP - low, TSP	0.011	P_{CAL}
	very low, TSP - optimal, TSP	0.002	P_{CAL}
Ripening	very low, BC - optimal, BC	0.000	P_{water}
	low, BC - optimal, BC	0.005	P_{water}
	low, BC - low, TSP	0.014	P_{water}
	very low, Control - optimal, Control	0.004	P_{water}
	very low, TSP - optimal, TSP	0.001	P_{water}
	very low, BC - optimal, BC	0.001	P_{CAL}
	very low, Control - optimal, Control	0.009	P_{CAL}
	very low, TSP - optimal, TSP	0.002	P_{CAL}

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