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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 $E = (10^{(-1/slope)}\text{-}1) \times 100$ 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<br>phoD-RW-CGTTCGCGACCTCGTGRTCRTCCCA | 1.0 | – |
| *phoN* | *Salmonella enterica* DSM 10062 | 159 | phoN-FW-GGAAGAACGGCTCCTACCCIWSNGGNCA<br>phoN-RW-CACGTCGGACTGCCAGTGIDMIYYRCA | 1.0 | 0.2 |
| *appA* | *Escherichia coli* DSM 30083 | 375 | appA-FW-AGAGGGTGGTGATCGTGATGMGICAYGGNRT<br>appA-RW-GCCTCGATGGGGTTGAAIADNGGRTC | 0.75 | 0.1 |
| *phnX* | *Salmonella enterica* DSM 17058 | 147 | phnX-FW-CGTGATCTTCGACtGGGCNGGNAC<br>phnX-RW-GTGGTCCCACTTCCCCADICCCATNGG | 0.2 | – |
| *gcd* | *Salmonella enterica* DSM 17058 | 330 | gcd-FW-CGGCGTCATCCGGGSITIYRAYRT<br>gcd_RW-GGGCATGTCCATGTCCCAIADRTCRTG | 0.75 | 0.1 |
| *pitA* | *Pseudomonas fluorescens* | 270 | pitA-FW-GGTCTTCGAGTTCATGAACGGNTTYCAYGA<br>pitA-RW-CCAGGTGACCAGGTTCCAIRNDAT | 0.5 | 0.2 |
| *pstS* | *Bradyrhizobium japonicum* | 221 | pstS-FW-TCTACCTGGGGAAGATCACAAARTGGRAYGA<br>pstS-RW-TGCCGACGGGCCAITYNWC | 1.0 | 0.1 |

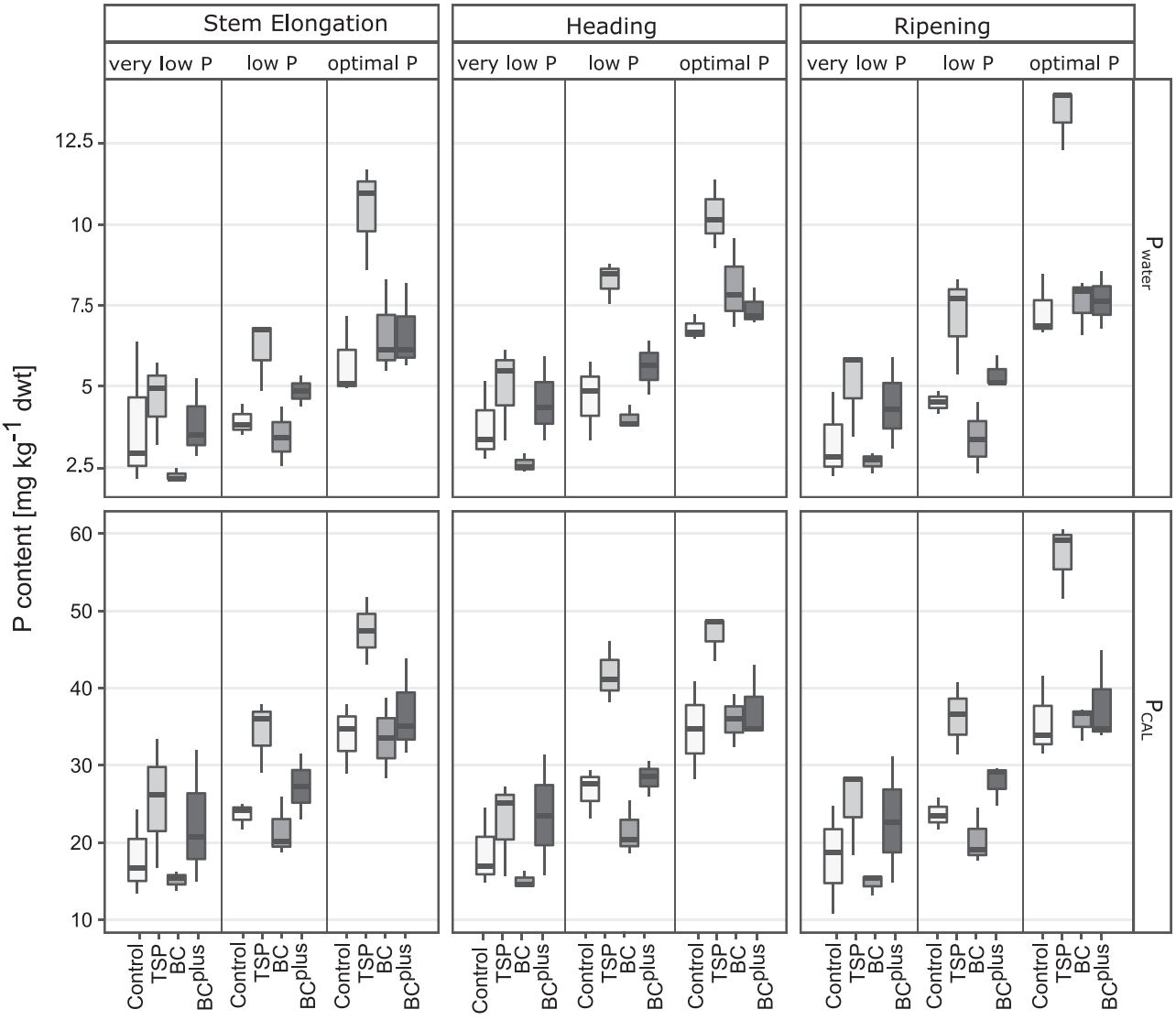


**Fig. 1.** Concentrations of $P_{CAL}$ and $P_{water}$ (n = 3) as box plots. Samples were taken from experimental fields that were classified into initial soil P test classes with very low, low and optimal $P_{CAL}$ concentrations and sampled at three different growth stages of winter wheat (stem elongation, 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 $6.9 \times 10^5$ and $5.9 \times 10^5$ gene copies $g^{-1}$ dwt for *pstS* and $3.7 \times 10^7$ and $2.9 \times 10^7$ 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 ($8.4 \times 10^5$ gene copies $g^{-1}$ dwt) compared to the TSP treatment ($3.7 \times 10^5$ 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 ($2.2 \times 10^6$ gene copies $g^{-1}$ dwt). For all other samples, copy numbers between $2.6 \times 10^5$ and $1 \times 10^6$ 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 $1 \times 10^6$ gene copies $g^{-1}$ dwt, while in soils with optimal initial P highest gene abundances of $1 \times 10^6$ 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 $1.2 \times 10^7$ and $1.9 \times 10^7$ gene copies $g^{-1}$ dwt, respectively. Interestingly, the BC treatment revealed lowest *gcd* gene copy numbers during heading accounting for $3.2 \times 10^6$ in soil with low initial P and $4.1 \times 10^6$ 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 $7.2 \times 10^6$ 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

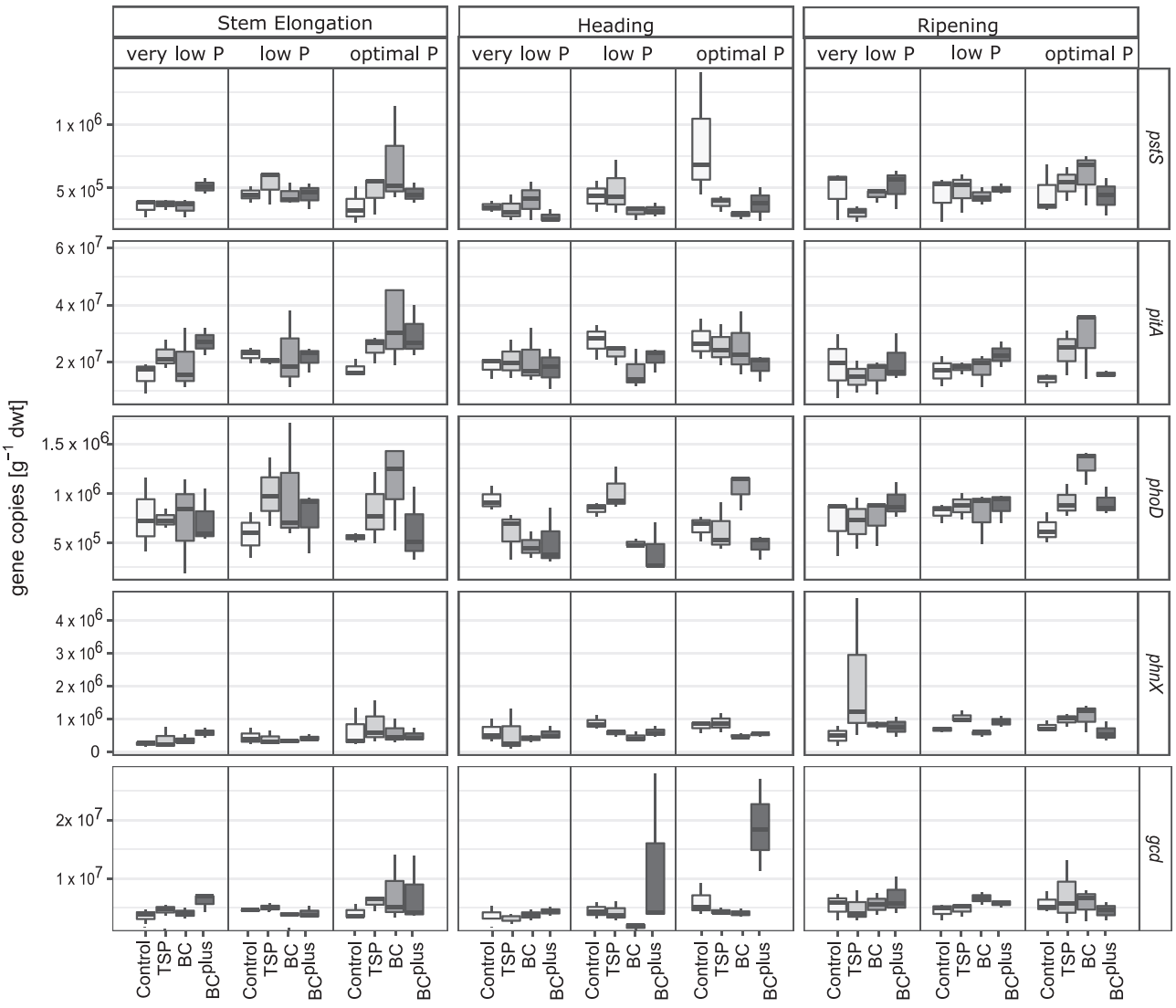


**Fig. 2.** Gene copy numbers of five genes involved in P turnover (*pstS, pitA, phoD, phnX, gcd*). Gene copy numbers were calculated per gram dry weight and are plotted on a linear scale as box plots (n = 3). Samples were taken at three plant development stages (stem elongation, heading, ripening), 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

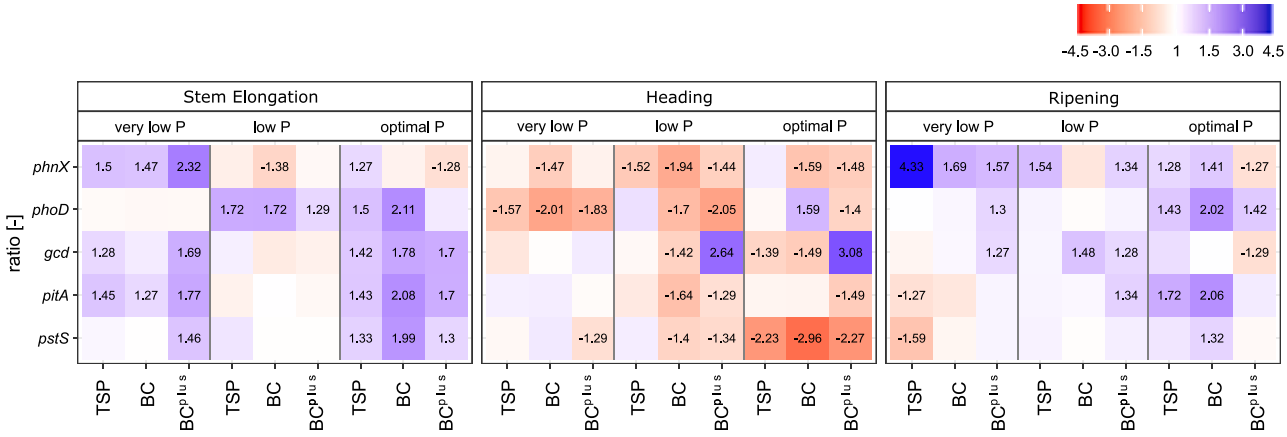


**Fig. 3.** Ratio between gene copy numbers in the control and treatment in form of a heatmap. Average gene abundances were calculated for each treatment (control, TSP, BC and BC$^{plus}$) and subsequently normalized to the control. In case the values of treatments were higher than control values, this 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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