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Distribution of phosphatase activity and various bacterial phyla in the rhizosphere of *Hordeum vulgare* L. depending on P availability

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ABSTRACT

Despite the importance of the rhizosphere for nutrient turnover, little is known about the spatial patterns of organic phosphorus mineralization by plants and by microorganisms in the rhizosphere. Therefore, the distribution of acid and alkaline phosphatase activity and the abundance of bacteria belonging to various bacterial phyla were investigated in the rhizosphere of barley (*Hordeum vulgare* L.) as dependent on the availability of inorganic P. For this purpose, we conducted a greenhouse experiment with barley growing in inclined boxes that can be opened to the bottom side (rhizoboxes), and applied soil zymography and fluorescence-*in situ*-hybridization (FISH). Acid phosphatase activity was strongly associated with the root and was highest at the root tips. Due to P fertilization, acid phosphatase activity decreased in the bulk soil, and less strongly in the rhizosphere. Alkaline phosphatase activity, i.e., microbial phosphatase activity was high throughout the soil in the control treatment and was reduced due to inorganic P fertilization especially in the rhizosphere and less strongly in the bulk soil. P-fertilization slightly increased the total number of bacteria in the rhizosphere. Moreover, P-fertilization decreased the abundance of *Firmicutes* and increased the abundances of *Beta-* and *Gamma-Proteobacteria*. The total number of bacterial cells was significantly higher at the root surface than at the root tip and at a distance of 30 μm from the root surface. Our results show that alkaline phosphatase activity decreased more strongly in the rhizosphere than in the bulk soil due to P fertilization, which might be because of greater C deficiency in the bulk soil compared to the rhizosphere. Furthermore, the results indicate a spatial separation between hotspots of acid phosphatase activity and hotspots of bacteria in the rhizosphere of *H. vulgare*. Taken together, our study shows that bacteria and phosphatase activity were very heterogeneously distributed in soil, and that the effects of P fertilization on phosphatase activity differed strongly between bulk soil and rhizosphere as well as between various zones of the rhizosphere.

1. Introduction

The rhizosphere is one of the hotspots of soil organic P mineralization in soil (Richardson et al., 2009; Marschner et al., 2011; Spohn and Kuzyakov, 2013a). Due to the decrease in rock phosphate that is suitable for fertilizer production (Cordell et al., 2009) on the one hand site, and eutrophication caused by excessive phosphorus (P) fertilizer runoff on the other hand site, a better understanding of the effects of P fertilizer on phosphatase activity and the abundance of microorganisms in the rhizosphere is required. Since the root comprises various zones, such as the root tip, the extension zone, and the root hair zone, which might differently influence the rhizosphere (Watt et al., 2006; Hinsinger et al., 2009), the study of the rhizosphere requires spatially explicit methods (Neumann et al., 2009; Marschner et al., 2011; Burns et al., 2013; Philippot et al., 2013; Ziegler et al., 2013).

Plants and microorganisms have developed several mechanisms to mobilize P, i.e., to mineralize organic P and to solubilize bound inorganic P. They can release (i) protons that solubilize P by modulating the soil pH, (ii) organic ligands such as oxalate and citrate that solubilize inorganic P by chelating iron and aluminum cations P is bound to, and (iii) extracellular phosphatases that mineralize organic P (Illmer et al., 1995; Hinsinger, 2001; Richardson et al., 2009). While microbes are capable of producing

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both acid and alkaline phosphatases, plants only can produce acid phosphatases (Dick et al., 1983; Juma and Tabatabai, 1988; Nannipieri et al., 2011). In the rhizosphere of plants in unfertilized soil, both acid and alkaline phosphatase activity are usually high (Tarafdar and Jungk, 1987; Kandeler et al., 2002; Marschner et al., 2005; Spohn and Kuzyakov, 2013a).

The activity of extracellular phosphatases in soil is generally negatively correlated with the availability of inorganic P (Olander and Vitousek, 2000; Sinsabaugh et al., 2008). However, it is likely that the activities of microbial and plant phosphatases are regulated differently since for plants, phosphatases only fulfill the function of mineralizing organic P, while for microorganisms, phosphatases additionally fulfill the function of rendering phosphorylated compounds available as a carbon (C) source by dephosphorylating them (Hoppe, 2003; Heath, 2005; Steenbergh et al., 2011; Spohn and Kuzyakov, 2013b; Heuck et al., 2015). There are two reasons for the extracellular dephosphorylation of organic compounds by microorganisms. Some phosphomonoesters, such as adenosine-5-monophosphate (AMP), cannot be taken up in their phosphorylate state by bacteria (Yagil and Beacham, 1975), for others, such as glucose–phosphate, exists a transport system in bacteria, but its expression is only induced by high glucose–phosphate concentrations (Dietz and Heppel, 1971) that hardly ever occur in soils. The regulation of phosphatase activity by microbial C demand has been described already some time ago in aquatic ecosystems (Hernandez et al., 1996; Hoppe and Sören, 1999; Hoppe, 2003; Heath, 2005; Steenbergh et al., 2011), but has only recently been discussed with respect to soils (Spohn and Kuzyakov, 2013b; Heuck et al., 2015).

The major reason for why microbes might use organic phosphorylated compounds as a C source, is that they are usually C limited in mineral soil (Joergensen et al., 1990; Vance and Chapin, 2001). However, in the rhizosphere a large amount of are roots exudates are released into soils that are a readily available C source for microorganisms, turning the rhizosphere into a hotspot of microbial abundance and activity (Watt et al., 2006; Jones et al., 2009). The release of root exudates is usually highest at the root tip (Watt et al., 2006; Hinsinger et al., 2009; Jones et al., 2009).

With respect to P acquisition, plant–microbe interactions can be mutualistic as well as competitive. Microorganisms in the rhizosphere can strongly mineralize organic P and solubilize bound inorganic P (Richardson et al., 2009; Spohn et al., 2013b). However, microorganisms can also decrease the availability of P to plants by immobilizing P in their biomass, by decomposing P-mobilizing organic compounds released by roots, and by counteracting root-induced pH decreases by proton consumption during ammonification (Richardson et al., 2009; Marschner et al., 2011). According to Marschner et al. (2011) microbial and plant P foraging occur in different regions of the rhizosphere, which might alleviate a potential competition between plants and microbes for P. To our knowledge, this concept has not been fully tested yet. However, recently a spatial separation between plant and microbial organic P mineralization in the rhizosphere of *Lupinus albus* has been shown (Spohn and Kuzyakov, 2013a), which seems to support the concept by Marschner et al. (2011) of a spatial differentiation of organic P mineralization in the rhizosphere.

The objective of this study was to analyze the distribution patterns of acid and alkaline phosphatase activity in relation to the presence of bacteria of different phyla in various zones of the rhizosphere of barley depending on P availability. We hypothesized that alkaline, i.e., microbial phosphatase activity decreases in the rhizosphere but not in the bulk soil due to P fertilization. Microbes in the bulk soil are usually C limited and might release phosphatases not only to mineralize organic P but also to render organic phosphorylated compounds available as a C source by dephosphorylating them. In contrast, rhizosphere microbes might not be C limited since they are provided with exudates by the plant. Consequently, we expected that microorganisms in the rhizosphere stop producing phosphatases when inorganic P is available, while in the bulk soil they keep dephosphorylating organic compounds to meet their C requirement. Furthermore, we hypothesized that the number of bacteria in the rhizosphere is highest at the root tips due to high root exudation in this zone. To test the two hypotheses, we conducted a greenhouse experiment with barley (*Hordeum vulgare* L.), growing in rhizoboxes. The distribution of phosphatase activity in the rhizosphere was analyzed non-destructively by soil zymography (Spohn and Kuzyakov, 2013a), and bacteria colonizing the rhizosphere were identified and localized by fluorescence-*in situ*-hybridization (FISH) using domain-, phylum- and class-specific probes. We choose to study young barley plants because barley is one of the most often cultivated crops and because of the importance of juvenile roots for subsequent crop yields (White et al., 2014). We concentrated on bacteria since they play a crucial role for nutrient cycling in the rhizosphere (Richardson et al., 2009), and we did not consider mycorrhiza, since young barley roots are usually not immediately colonized even in P poor soils (Castillo et al., 2012).

2. Material and methods

2.1. Experimental setup

H. vulgare L. subsp. *vulgare* cultivar Barke (Saatzucht Josef Breun GdbR, Herzogenaurach, Germany) was grown in rhizoboxes in a sandy soil under greenhouse conditions in May 2014. The soil was taken from the Ah horizon of a Cambisol in the vicinity of Bayreuth, Germany (49°58′58″ N and 11°34′50″E) that is used as a pasture and has a low P content. The properties of the soil are as follows: the texture was sandy loam, pH 5.2, 16.0 g kg^{-1} C, 1.4 g kg^{-1} N, and 30.0 mg g^{-1} resin P. The soil was passed through a 2-mm sieve and was filled into the rhizoboxes and compacted to a density of 1.2 g cm^{-3}. The rhizoboxes were plexiglass boxes that were fixed in a rack which keeps them inclined by 50° in order to make the roots grow along the lower wall of the rhizobox, which can be opened. The rhizoboxes had an inner size of 12.3 × 12.5 × 2.3 cm. The same type of rhizobox has been used in prior studies on exoenzyme activity in the rhizosphere (Spohn and Kuzyakov, 2013a, 2014). In half of the boxes, the soil was amended with 0.1 mg P g^{-1} as KH_2PO_4 dissolved in water according to Aldén et al. (2001). The water content in the rhizoboxes was adjusted to 50% field capacity, and was kept stable throughout the experiment. In total, we set up four replicates of P-fertilized rhizoboxes and four rhizoboxes as a control treatment without fertilization. The plants were directly sown in the soil, one seed per rhizobox. After 10 days, soil zymography was performed, and subsequently, the roots were harvested for the FISH analyses.

2.2. Soil zymography

The *in situ* distribution of extracellular acid and alkaline phosphatases was analyzed by soil zymography according to Spohn et al. (2013a). The approach is based on the incubation of the soil attached to an agarose gel that is covered by a nylon membrane coated with a substrate that becomes fluorescent once it gets hydrolyzed (Spohn and Kuzyakov, 2013a, 2014). For the preparation of the gels, 1% agarose was dissolved at 80 °C in universal buffer with pH 6.5 for acid phosphatase and pH 11.0 for alkaline phosphatase. Gels were cast in systems usually used for vertical gel-electrophoresis (Biometra) with an inner size of 12 × 11 × 0.1 cm. As substrate phosphate-methylumberlliferyl

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(Sigma–Aldrich, Germany) was used and dissolved in modified universal buffer (Skujins et al., 1962) to a concentration of 12 mM. The buffer had either been adjusted to pH 6.5 for acid phosphatase activity or to pH 11.0 for determining alkaline phosphatase activity. The different pH values of the buffer allow to largely separate acid and alkaline phosphatase (Nannipieri et al., 2011) although a complete separation may not be achieved. Membrane filters of polyamide (Satorius) with a diameter of 14.2 cm and a pore size of 0.45 μm were coated with the substrate. For the incubation, the lower side of the rhizoboxes was opened exposing the roots. The agarose gel was attached to the soil, and on top of the gel the membrane was placed. After incubation at 20 °C, the membrane was placed on an epi-UV-desk (Desaga) in the dark, and lightened at 360 nm wavelength. A picture of the membrane was taken with a digital camera (D60, Nikon). The distribution of alkaline phosphatase activity was analyzed in the same sample and by the same technique directly after the determination of acid phosphatase activity. The incubation time was 20 min for the acid phosphatase and 25 min for the alkaline phosphatase activity. Different incubation times were required to obtain a strong contrast in the zymograms of both enzymes that slightly differed in activity. A calibration line was prepared from membranes with 4-methylumbelliferone (MUF) of different concentrations (0, 35, 70, 130, 200 μM), and a picture of the calibration line was taken in the same was as of the zymograms. The enzyme activity was calculated based on a linear correlation between the gray values and the MUF concentration as described in Spohn and Kuzyakov (2013a). Image processing and analysis of the zymograms was done using the open source software ImageJ. The digital images were transformed to 8-bit, i.e., grayscale images. To illustrate the results, we depicted the values of the grayscale image in color. Both acid and alkaline phosphatase activity were measured using imageJ in five randomly chosen areas both in the bulk soil and in the rhizosphere in each rhizobox, and one mean per rhizobox was calculated. The areas had a size of 150 × 150 pixels (bulk soil) and 10 × 10 pixels (rhizosphere). Homogeneity of variance of the phosphatase activities was tested by the Levene-test. Significance of differences between bulk soil and rhizosphere and between control and P-fertilized treatments were tested by ANOVA followed by the Duncan-test, where $\alpha < 0.05$ was considered as the threshold value for significance. Statistical analyses were performed using R environment for statistical computing (R Core Team, 2013).

2.3. Fluorescence-in situ-hybridization (FISH) and confocal laser scanning microscopy (CLSM)

After soil zymography was performed, fluorescence-*in situ*-Hybridization (FISH, DeLong et al., 1989; Amann et al., 1990) was done in three parts of the rhizosphere – the root tip, the root surface, and at a distance of 30 μm from the root surface. For this purpose, approximately 7 mm long root pieces were cut with sterilized scissors and incubated for seven days in fixation buffer (for Gram-negative bacteria in paraformaldehyde 4% in PBS respectively for Gram-positive bacteria in EtOH:PBS (55:45 v/v)) at 4 °C, and washed carefully with EtOH:PBS (55:45 v/v) twice. The root pieces were stored in EtOH:PBS (55:45 v/v) at 4 °C until analysis. The root pieces were taken at a distance of 2 cm or more from the seed and at least 5 mm from the root tip. The analysis of the root tips was conducted in an area of less than 1 mm around the root apical meristem.

The labeling was conducted in a 2 ml tube according to Hofmann et al. (2014) with modifications (probes and hybridization conditions are listed in Table 1). To avoid loss of rhizosphere soil adhering to the root, the samples were treated very carefully and all steps were subsequently performed in the same tube without touching the sample with the tip of the pipette. For the hybridization, the root pieces were dehydrated by a rising ethanol gradient (50%, 80%, and 100%) for 3 min each before they were air dried. One root piece was incubated with 100 μl hybridization buffer [0.9 M NaCl, 20 mM Tris–HCl (pH 8), 0.01% (v/v) SDS, 35% (v/v) formamide], 10 μL oligonucleotide probes solution (50 ng/μL; Metabion GmbH) and, if required, a competitor probe for 1.5 h at 46 °C. After carefully removing the hybridization solution, the roots pieces were washed twice in stringent washing procedures with 1 ml pre-warmed washing buffer [0.09 M NaCl, 20 mM Tris–HCl (pH 8), 0.01% (v/v) SDS, 5 mM EDTA] and then incubated in 2 ml washing buffer for 15 min at 48 °C. The washing buffer was removed and the root pieces were washed with 1 ml of sterile millipore water. The root section was transferred to a glass slide, embedded in Citifluor AF1™ (Citifluor Ltd.) and covered with a slide.

Confocal laser scanning microscopy was performed using a Zeiss LSM 510.meta microscope (Zeiss, Oberkochen, Germany) with 40 × (C-Apochromat® 40×/1.2 W Korr) and 63 × (C-Apochromat® 63×/1.2 W Korr) water immersion objectives. Representable areas of the sample were chosen for acquiring image stacks (z = 1 μm).

The Zeiss LSM Image Browser version 4.2 software was used for image analysis of FISH. For evaluation of the fluorescence images, the number of bacteria labeled by each probe was counted in a square of 100 × 100 μm. The number of each selected bacteria type was compared to the total amount of bacteria in the counted square (EUB 338 Mix). A mean of the bacterial numbers per rhizobox was calculated for each of the three zones of the rhizosphere (root tip, root surface, distance of 30 μm from the root surface). Statistical significance was tested by an unpaired t-test with the SigmaPlot 12.0 software, which contained a normality test after Shapiro and Wilk (1965) and an equal variance test. If the equal variance test failed, a Mann–Whitney rank sum test (Mann and Whitney, 1947) was conducted.

3. Results

Ten days after sowing, root length varied strongly and did not show a significant difference between the control treatment and the P-fertilized treatment (6.2 ± 3.3 cm and 6.6 ± 3.5 cm, respectively). We found that acid phosphatase activity was significantly higher in the rhizosphere than in the bulk soil both in the control treatment and in the P-fertilized treatment (Figs. 1 and 2A). Due to P fertilization, acid phosphatase activity decreased in the bulk soil, and less strongly in the rhizosphere (Figs. 1 and 2A). Acid phosphatase activity was highest at the root tips both in the control and in the P-fertilized treatment (especially in rhizobox b, e and f; Fig. 1).

Alkaline phosphatase activity was not associated with the roots in the control treatment, and it was high both in the bulk soil and in the rhizosphere (Figs. 1 and 2B). Due to P fertilization, alkaline phosphatase activity decreased in the rhizosphere and less strongly in the bulk soil (Figs. 1 and 2B). The low alkaline phosphatase activity in the rhizosphere compared to the bulk soil can be seen especially in rhizobox f and h (Fig. 1).

Fluorescence-*in situ*-hybridization of bacteria in the rhizosphere revealed a dense colonization both in P-fertilized and in the control treatments (Fig. 3). The total number of bacteria was almost twice as high at the root surface as at the root tip, and the number of bacteria decreased in the order root surface > root tip > distance of 30 μm from the root surface (Fig. 4). The total numbers of bacteria in all three zones of the rhizosphere were slightly higher in the P fertilization treatment than in the control (Fig. 4), but these differences were not statistically significant.

The quantitative analysis of the bacteria at the root surface showed the same bacterial clades in the P-fertilized and control

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Table 1
The 16S rRNA-targeted oligonucleotide probes used in this study for the detection of different bacterial clades.

Target	Probe name	Probe sequence (5′–3′)	References
Most Bacteria	EUB 338 I	GCT GCC TCC CGT AGG AGT	Amann et al. (1990)
Planctomycetales	EUB 338 II	GCA GCC ACC CGT AGG TGT	Daims et al. (1999)
Verrucomicrobiales	EUB 338 III	GCT GCC ACC CGT AGG TGT	Daims et al. (1999)
None (control probe)	NON EUB 338	ACT CCT ACG GGA GGC AGC	Wallner et al. (1993)
Alpha-Proteobacteria, except of *Rickettsiales*	ALF 968	GGT AAG GTT CTG CGC GTT	Neef (1997)
Beta-Proteobacteria[a]	BET 42a	GCC TTC CCA CTT CGT TT	Manz et al. (1992)
Gamma-Proteobacteria[a]	GAM 42a	GCC TTC CCA CAT CGT TT	Manz et al. (1992)
Most *Delta-Proteobacteria*, most *Gemmatimonadetes*[a]	DELTA 495a	AGT TAG CCG GTG CTT CCT	Loy et al. (2002)
Actinobacteria (high G + C Gram-positive bacteria)[a]	HGC 69a	TAT AGT TAC CAC CGC CGT	Roller et al. (1994)
Firmicutes (low G + C Gram-positive bacteria)	LGC 354A	TGG AAG ATT CCC TAC TGC	Meier et al. (1999)
Firmicutes (low G + C Gram-positive bacteria)	LGC 354B	CGG AAG ATT CCC TAC TGC	Meier et al. (1999)
Firmicutes (low G + C Gram-positive bacteria)	LGC 354C	CCG AAG ATT CCC TAC TGC	Meier et al. (1999)

[a] Competitor probe was used.

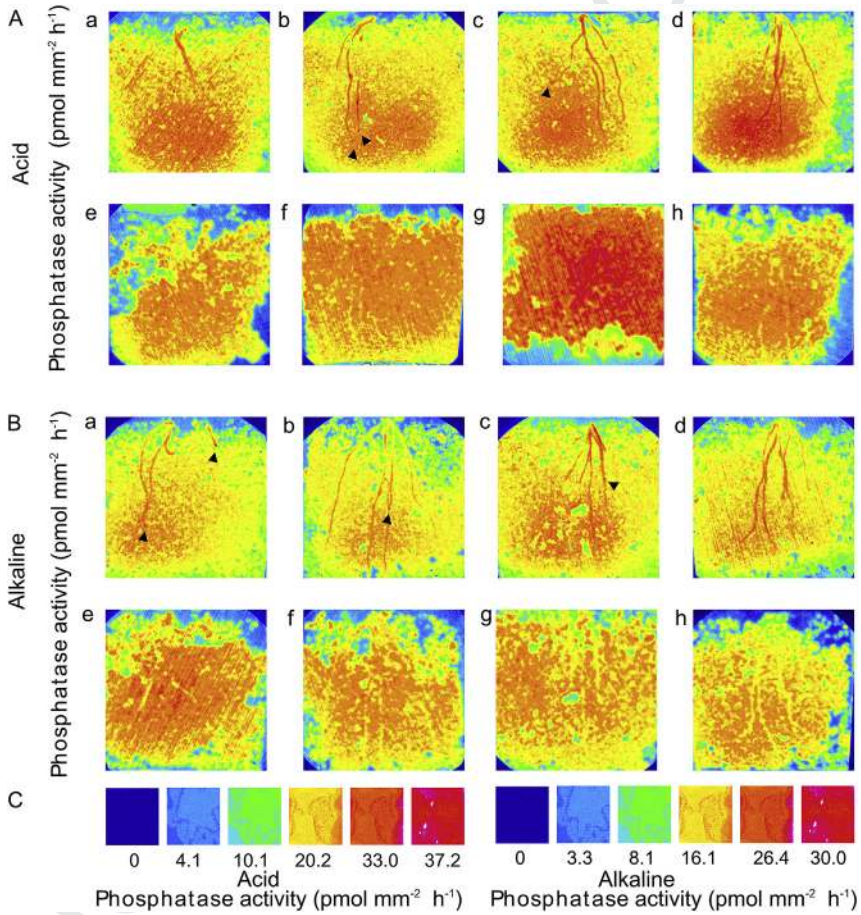

Fig. 1. Zymograms showing the distribution of acid (A) and alkaline (B) phosphatase activity together with the calibration lines (C). Images a–d show P-fertilized rhizoboxes and images e–h show the controls. The width of each single image corresponds to 11.5 cm. Black arrows point to the root tips with elevated acid phosphatase activity.

treatment but with different abundances (Fig. 5). Relative to the total number of bacteria, more *Firmicutes* could be detected in the control treatment than in the P-fertilized treatment (34% vs. 10% of the total number of bacteria). The relative abundance of *Actinobacteria* was higher in the control treatment (7%) than in the fertilized treatment (5%). More *Beta-Proteobacteria* and *Gamma-Proteobacteria* were localized in the rhizosphere of the P-fertilized treatment (18 and 11% of the total number of bacteria, respectively) compared to the control treatment (13 and 7%, respectively). Other bacteria (which could not be identified with the previously described bacterial clade specific probes) constituted 49% of the total number of bacterial cells in the fertilized treatment and 31% in the control treatment.

4. Discussion

Our results show that P fertilization decreased microbial phosphatase activity in the rhizosphere and to a lesser extent also in the bulk soil (Figs. 1 and 2B). One likely explanation for the difference between the rhizosphere and the bulk soil is that microbial phosphatase activity was not only regulated by microbial P demand but also by microbial need for C, and that microbial phosphatase

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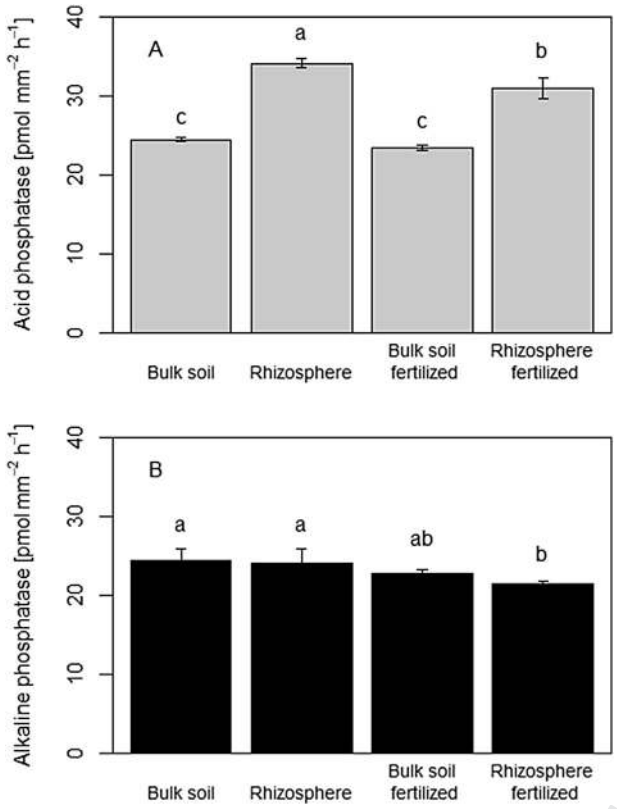

Fig. 2. Acid (A) and alkaline (B) phosphatase activity in the bulk soil and in the rhizosphere of the control and the P-fertilized treatment. Letters indicate significant differences between bulk soil, rhizosphere and P-fertilized bulk soil and rhizosphere. Error bars depict standard deviation calculated from the means of four independent replicates.

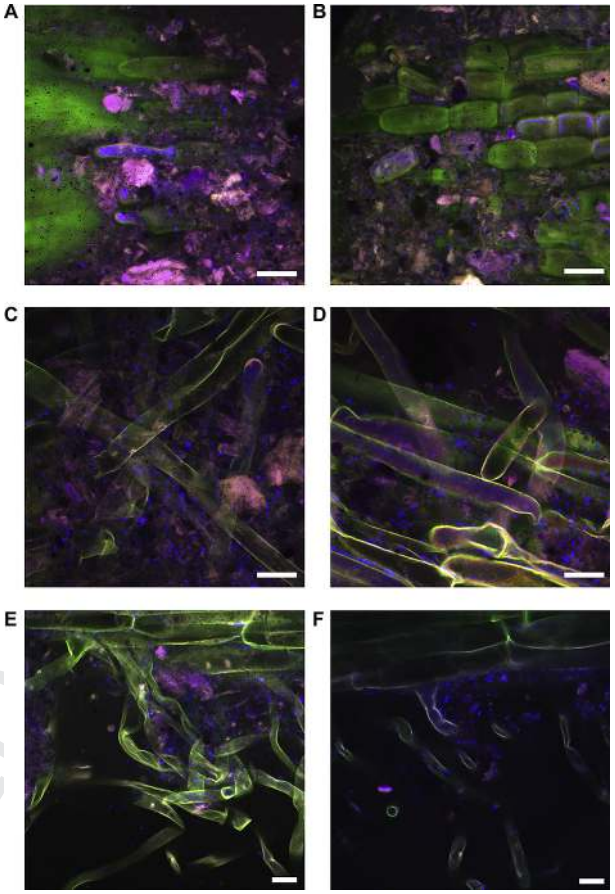

Fig. 3. Fluorescence images of barley root sections labeled by FISH at the root tip (A, B), the root surface (C, D) and the area at a distance of 30 μm from the root surface (E, F). Images A, C, E show the P-fertilized samples, whereas images B, D, F show the controls. *Bacteria* are labeled with the EUB 338 Mix probe (Cy5-labeled, blue) and with either ALF 968 probe for *Alpha-Proteobacteria* (A–E) or GAM 42a probe for *Gamma-Proteobacteria* (F) (Cy3-labeled, red). The images have been recorded at a confocal laser scanning microscope with a 63× water immersion objective. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activity was high in the bulk soil due to the low C availability there. In contrast, in the rhizosphere, microorganisms were provided with easily available C in the form of root exudates, and therefore were less C deficient than in the bulk soil. Evidence that mineralization of organic P might be driven by the microorganisms' need for C rather than for P comes from recent studies that used ^{14}C and ^{33}P labeled phosphomonoesters to investigate the uptake of C and P from organic phosphorylated compounds. In these labeling experiments, it was found that the organic C of glucose-6-phosphate was taken up much faster than the phosphoryl moiety of the molecule, indicating that the mineralization of glucose-6-phosphate was driven by microbial C demand (Spohn and Kuzyakov, 2013; Heuck et al., 2015). The reason for the extracellular dephosphorylation of organic compounds by microorganisms seems so be twofold. Some phosphomonoesters, such as adenosine-5-monophosphate (AMP), cannot be taken up in their phosphorylate state by bacteria (Yagil and Beacham, 1975), for others, such as for glucose–phosphate, exists a transport system in bacteria, but its expression is only induced by high glucose–phosphate concentrations (Dietz and Heppel, 1971) that hardly ever occur in soils.

In agreement with our results, it has been found that phosphatase activity in aquatic ecosystems is not only regulated by P but also by C availability (Hernandez et al., 1996; Hoppe and Sören, 1999; Hoppe, 2003; Heath, 2005; Steenbergh et al., 2011). Organic P mineralization driven by microbial need for C has recently also been reported to likely be the reason for patterns of phosphatase activity observed in a long term fertilization experiment (Turner and Wright, 2014) and in the rhizosphere of maize (Zhang et al., 2014).

Acid phosphatase activity was strongly associated with the roots (Fig. 2), which agrees with previous studies (Tarafdar and Jungk, 1987; Juma and Tabatabai, 1988; Kandeler et al., 2002; Spohn and Kuzyakov, 2013a). Our observation that especially the root tips were hotspots of acid phosphatase is in accordance with the more general finding that the release of organic compounds by plants is highest in this zone of the root (Watt et al., 2006; Hinsinger et al., 2009). The observation that acid phosphatase activity strongly decreased due to P fertilization also goes in line with previous observations (Olander and Vitousek, 2000; Sinsabaugh et al., 2008). Compared with two recent studies in which phosphatase activity was determined by soil zymography (Spohn and Kuzyakov, 2013a, 2014), a less strong contrast between the activity in the rhizosphere and in the bulk soil was found. This is most likely because in the two previous experiments subsoil from the B horizon with lower microbial activity and available nutrients was used, while in the present experiment the plants grew in soil from the A horizon.

The high number of bacteria at the root surface compared to the root tip can be explained by the high growth rate of the roots (about 1 cm per day). Bacteria that started growing at the root tip, where exudation is highest (Jones et al., 2009), likely could not attach quick enough to the root tip, and were passed by the fast moving root tip. Watt et al. (2006) quantified and localized native

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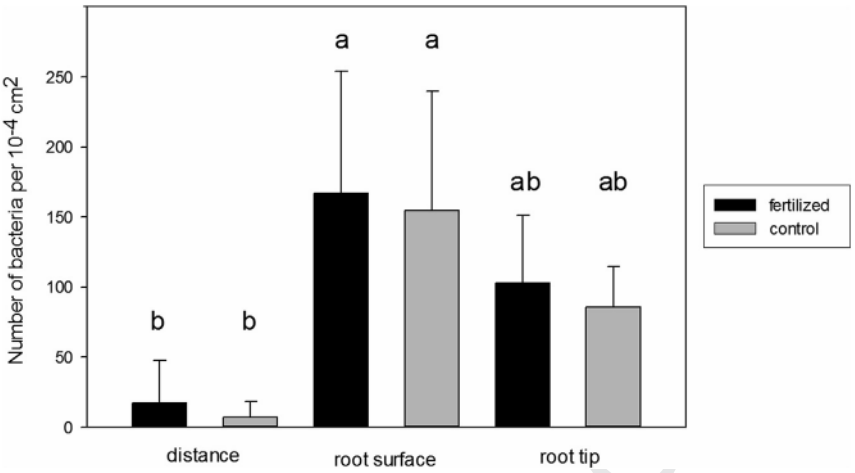

Fig. 4. Total number of bacterial cells in the rhizosphere of the P-fertilized and the control treatment at the root tip (A), at the root surface (B), and at a distance of 30 μm from the root surface (C). Error bars depict standard deviation calculated from the means of four independent replicates. Q3

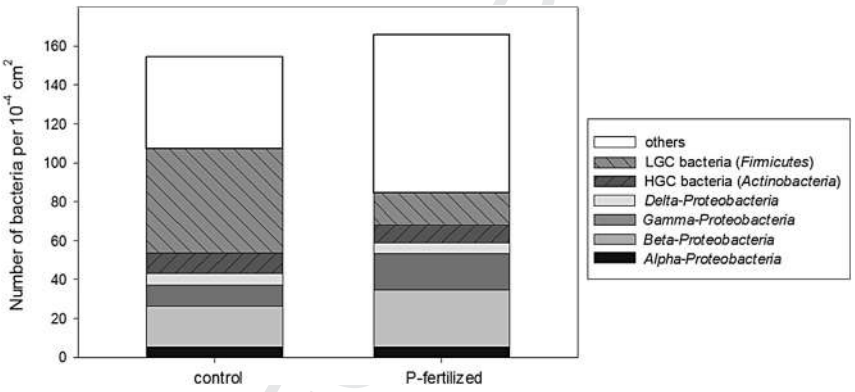

Fig. 5. Number of bacteria per clade, standardized to the total amount of bacteria determined by the labeling with the EUB 338 Mix probes. The numbers were evaluated from confocal laser scanning microscopy (CLSM) pictures of the root surface. The numbers are means calculated from four independent replicates.

Pseudomonas and filamentous bacteria on wheat roots by FISH, and observed a stable biofilm within 11 μm of the root surface. In contrast to our findings, Watt et al. (2006) found that the root cap and the mucilage were the most heavily colonized zone. However, the colonization density is also influenced by the plant vegetation stage. Schmidt and Eickhorst (2013) observed that the highest colonization densities shifted from the root tip towards more mature regions with increasing plant age. The slightly higher number of bacteria in the rhizosphere in the P-fertilized treatment compared to the control treatment indicate that the P amendment led to bacterial growth in the rhizosphere. This might be because bacteria that received P from fertilizer can save C, nitrogen (N), and energy they otherwise would have to invest into extracellular phosphatases and can invest it in somatic growth. P is especially required by r-strategists (copiotroph bacteria), i.e., microorganisms that grow fast in response to available C (Fierer et al., 2007), because they need to synthesize large amounts of RNA (Elser et al., 2003). Makino et al. (2003) showed that bacterial growth rate has a strong effect on the microbial biomass P concentration and on bacterial biomass C:P ratios. Hence, it seems likely that r-strategists react most strongly to changes in P availability. Yet, this concept is not easy to prove since r-strategists are likely found in many bacteria phyla (Fierer et al., 2007).

While the bulk soil represents a niche mainly for oligotrophic bacteria, which are well adapted to low C availability, the rhizosphere represents a hotspot of copiotrophic bacteria because of the high concentrations of easily degradable C sources (Hu et al., 1999; Fierer et al., 2007; Dennis et al., 2010). Due to the ecophysiological diversity within bacterial phyla, it is unlikely that all members of a phylum like *Proteobacteria* share similar ecological characteristics, and react to P fertilization in the same way. Nevertheless, differences in the abundances of bacterial clades due to P-fertilization were observed in this study. *Beta-* and *Gamma-Proteobacteria* were more abundant in the rhizosphere of the P-fertilized plants. Both classes are known to play a key role in rhizosphere colonization (Inceoglu et al., 2010; Hol et al., 2014) and are relatively fast-growing copiotrophic microbes (Philippot et al., 2013). The control treatment showed a higher abundance of *Actinobacteria* and especially *Firmicutes* than the P-fertilization treatment, which are also known to colonize the rhizosphere (DeAngelis et al., 2009; Eastman et al., 2014; Haesler et al., 2014; Hol et al., 2014). Further research is needed to better understand the observed shifts in the abundance of the bacterial phyla.

4.1. Conclusion

This study shows that P fertilization decreases acid and alkaline phosphatase activity as well as the abundance of bacteria in the rhizosphere of *H. vulgare*. Thus, the application of fertilizer likely decreases organic P mineralization and the use of organic P as a P source by the plant. We found some support for our hypothesis that microbial phosphatase activity decreases in the rhizosphere but not

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in the bulk soil due to different C availabilities although the difference was smaller than expected. We observed that the root tips were hotspots of acid phosphatase activity. However, against our hypothesis that the number of bacteria in the rhizosphere is highest at the root tips due to the high root exudation in this zone, we found the highest microbial abundance not at the root tips but at the root surface. These findings indicate a spatial separation between hotspots of acid phosphatase activity and hotspots of bacteria in the rhizosphere of *H. vulgare*. Taken together, our study shows that bacteria and phosphatase activity were very heterogeneously distributed in soil, and that the effects of P fertilization on phosphatase activity differed strongly between bulk soil and rhizosphere as well as between various zones of the rhizosphere.

Uncited reference

De Nobili et al., 2001, Somers et al., 2004, Spiers and McGill, 1979, van Overbeek and van Elsas, 2010.

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