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Phosphorus limitation in a Ferralsol: Impact on microbial activity and cell internal P pools

Knut Ehlers [a,*], Lars R. Bakken [b], Åsa Frostegård [c], Emmanuel Frossard [a], Else K. Bünemann [a]

[a] Institute of Plant Sciences, Swiss Federal Institute of Technology (ETH), Zürich, Eschikon 33, 8315 Lindau, Switzerland
[b] Department of Plant and Environmental Sciences, Norwegian University of Life Sciences (UMB), Ås, Norway
[c] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences (UMB), Ås, Norway

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ABSTRACT

Soil microorganisms are key regulators of the biogeochemical phosphorus (P) cycle. Microbial P limitation in highly weathered tropical soils has been reported, but whether it affects the cellular P content of indigenous soil microorganisms and its biochemical composition is unknown. We investigated the effect of microbial P limitation by measuring respiration, microbial growth, community composition and P content of microbial cells in a Ferralsol with low amounts of available P as affected by amendments with C substrates with ample nitrogen (CN) with and without extra phosphate (P). Microbial biomass and community composition were quantified by phospholipid fatty acid (PLFA) analyses. Cellular P content and P pools (PLFA, DNA and RNA per cell) were determined after extraction of microbial cells from soil by density gradient centrifugation. The apparent microbial growth rate during exponential increase in respiration rates in response to CNP addition was 0.072 h^{-1}, compared to 0.017 h^{-1} for the CN amendment (no extra P added). This suggests that the microbial growth after a combined C and N addition was retarded by P limitation in the native soil (without added P). The net increase in microbial biomass, however, reached similar levels for both the CN and CNP treatment (measured at the point in time when respiration rates peaked). This outcome was unexpected since maximum respiration rates were about three times higher in the CNP compared to the CN treatment. Total P in extracted cells ranged from 2.1 to 8.9 fg P $cell^{-1}$ (microscopic counts), with a tendency for lower values for treatments without C amendments. Only 10–25% of the measured total P in extracted cells was accounted for by the measured RNA, DNA and PLFA. This low percentage could partly be due to underestimation of the RNA pool (degradation during extraction). PLFA analyses showed that substrate induced growth, regardless of P addition, led to a change in microbial community composition and was dominated by fungi. The extraction of microbial cells from soil by density gradient centrifugation, however, discriminates against fungi. Accordingly, the extracted cells were not fully representative for the entire soil microbiota regarding the community composition and metabolic state. Nevertheless, for the first time microbial cell P content and P pools are reported for microorganisms that actually grew in soil and not in chemostat or batch cultures.

1. Introduction

Low phosphorus (P) availability due to strong P sorption is often the main constraint for plant growth and biomass production on highly weathered tropical soils (Vitousek, 1984). Soil microorganisms act as a sink and source of available P by mediating key processes in the biogeochemical P cycling. Even though microbial demand for P seems to prevail over geochemical sorption when competing for available P on soils with high sorption capacities (Olander and Vitousek, 2005), Cleveland et al. (2002) showed that in a Ferralsol under tropical rainforest microbial carbon degradation was strongly P limited. Similar results on microbial P limitation (if C and N are given in ample amounts) have been presented for other tropical soils and ecosystems (Duah-Yentumi et al., 1998; Ilstedt and Singh, 2005; Gnankambary et al., 2008).

In the above mentioned studies, microbial P limitation was derived from interpretation of soil respiration responses to additions of C substrates and mineral P. Such respiration kinetics reveal the effect of a given substrate on activity and growth of soil microorganisms (Stotzky and Norman, 1961). When a labile C source is added, the microbial respiration rate increases instantly. This substrate induced respiration (SIR) correlates with active

* Corresponding author. Tel.: +41 52 3549188; fax: +41 52 3549119.
E-mail address: knut.ehlers@ipw.agrl.ethz.ch (K. Ehlers).

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microbial biomass already present in the soil (Anderson and Domsch, 1978). After some time with nearly constant respiration rate, an exponential increase is normally observed. According to Scheu (1993), this additional microbial respiration (AMR) reflects the growth of microorganisms on the added substrate which can also be characterized by the exponential growth rate μ (Nordgren et al., 1988). The ability of soil microorganisms to grow depends not only on the availability of C but also on that of other nutrients. Hence, μ and maximum AMR reflect the microbial nutrient status (Scheu, 1993). Recently, Iovieno and Bååth (2008) were able to verify that respiration in response to substrate incorporation is a reasonable estimator of microbial growth dynamics as measured by leucine and thymidine.

Apart from respiration kinetics, information on the content and forms of limiting elements in the microbial biomass can reveal valuable further information as the chemical composition of microorganisms may change with environmental conditions, in particular with nutrient availability (Herbert, 1961). For example, microbes can accumulate P as polyphosphate when exposed to P sufficient conditions (Hupfer et al., 2007). In a batch culture of P-limited aquatic bacteria, Vadstein (1998) showed that nucleic acids and phospholipids constituted 60% of the cell P. Chemostat studies have revealed that under P limitation, cell P and RNA content per cell increase with growth rate (Elser et al., 2003), and that RNA may contribute 70–90% of total cell P in P-limited cultures (Makino and Cotner, 2004). Vrede et al. (2002) found P contents between 2 and 12 fg P cell^{-1} for a marine bacterial community, depending on the metabolic state. Lovdal et al. (2008) reported 0.8–3.3 fg P cell^{-1} for a chemostat study on *Vibrio splendidus*. Analysis of bacterial cells *in situ* showed P contents of 0.5–1.5 fg P cell^{-1} in aquatic bacteria (Tuomi et al., 1995; Fagerbakke et al., 1996), while Gundersen et al. (2002) reported a greater range (0.01–10 fg P cell^{-1}). However, no data on P content and chemical P composition are available for soil microorganisms that actually grew in soil, although their contents of DNA cell^{-1} (Torsvik and Goksoyr, 1978; Bakken and Olsen, 1989; Sandaa et al., 1998) and of phospholipid fatty acids (PLFA) cell^{-1} (Frostegård and Bååth, 1996) have been determined for microbial cells grown in temperate soils.

The objective of our study was to investigate how P availability affects soil microbial activity (soil respiration), biomass and community composition (PLFA analyses) and the P content and major P pools of soil microorganisms growing in a Ferralsol low in available P. For this novel approach we conducted an incubation experiment with C, N and P additions and extracted microbial cells from soil by density gradient centrifugation (Bakken and Lindahl, 1995) as adapted to highly weathered tropical soils (Ehlers et al., 2008). The extracted cells were analyzed for total P content as well as for P contained in RNA, DNA and PLFA. We hypothesized that (I) microbial activity in our Ferralsol is P limited when given ample amounts of easily available C and N, and that (II) metabolic state rather than soil P status affects cellular P content and P pools.

2. Materials and methods

2.1. Soil

Soil was collected in July 2007 from 0 to 20 cm depth of a field trial site in Nyabeda, Western Kenya (altitude: 1420 m, latitude: 0° 06′ N, longitude 34° 34′ E) under a maize–legume crop rotation without P fertilisation. The soil was stored at 4 °C in moist condition (0.23 ml water g^{-1} soil dry weight, equal to 40% field capacity) for three to five weeks and sieved at 2 mm before use. The soil contains 64% clay, 21% silt and 15% sand, has a pH (H_20) of 4.9 and is classified as a Ferralsol (FAO/ISRIC/ISSS, 1998). The soil contains very low available P of 1.8 ± 0.3 μg g^{-1} (average ± standard deviation) as measured with anion exchange resin membranes (Kouno et al., 1995). The concentration of organic P as measured by ignition and extraction with 0.5 M H_2SO_4 (Saunders and Williams, 1955) is 323 μg g^{-1}. Total P content measured by H_2SO_4/H_2O_2 digestion (Anderson and Ingram, 1993) is 636 μg g^{-1}. Isotopic exchange kinetics with ^{33}P (Frossard et al., 1993) revealed that the soil has a very high P sorption capacity (data not shown). Organic C content measured with a CNS Analyzer (FlashEA 1112, Thermo Electron Corporation) is 22 mg g^{-1}. The mineralogical composition of the soil is given in Ehlers et al. (2008).

2.2. Experimental design and set-up

Four different treatments with a combined C and N addition and a P addition in a factorial design were chosen (Table 1). In each case, the soil was brought to 60% field capacity by addition of 0.13 ml of liquid g^{-1} dry soil. In the H_2O treatment only water was added to the soil, while in the P treatment a P-solution was used to add 0.13 mg P g^{-1} soil. In the CN treatment, 3.25 mg C and 0.65 mg N were added per g of soil, using a nutrient cocktail containing various labile C and N sources (Table 1). In the CNP treatment, the added nutrient cocktail contained 3.25 mg C, 0.65 mg N and 0.13 mg P g^{-1} soil. We decided to apply all nutrients in liquid form to avoid an uneven distribution in the soil. Therefore, the dissolved nutrients were applied together with the added water. Different C and N sources were used in order to obtain a response from a broader fraction of the soil microbial community compared to the addition of only one substrate such as glucose. Because of its tropical origin the soil was incubated at 25 °C. For the different analyses different amounts of soil were incubated. For the cell extraction and PLFA analysis, 31 and 3.08 g of soil, respectively, were incubated. In both cases the soil was incubated in 50 ml tubes loosely covered with aluminum foil. The samples were aerated and soil water content adjusted daily. For respiration measurements 0.77 g soil was weighed into a 2 ml Eppendorf tube which was placed in a crimp sealed serum flask (120 ml). A small amount of water was added into the flasks to sustain high air humidity and prevent drying of the soil sample.

Prior to nutrient addition and incubation, the moist soil (40% WHC) was stored at room temperature for 24 h. Incubation of soil for the various analyses started at different times within a period of 3 weeks. Unless otherwise stated, all analyses were performed with three independent replicates per treatment.

2.3. Respiration measurements

Respiration measurements were done using the robotized incubation system described by Molstad et al. (2007). The first gas measurement started minutes after start of the incubation. CO_2 concentrations were measured every 4 h. The incubation robot also monitors the O_2 concentrations in the headspace which is useful to

Table 1
Description of incubation treatments.

Treatment	Liquid addition (ml g^{-1} soil)	C addition[a] (mg g^{-1} soil)	N addition[a] (mg g^{-1} soil)	P addition[a] (mg g^{-1} soil)
H_20	0.13	–	–	–
P	0.13	–	–	0.13
CN	0.13	3.25	0.65	–
CNP	0.13	3.25	0.65	0.13

[a] C and N were added as a combined nutrient cocktail containing 1.78 mg Na-acetate, 1.23 mg sucrose, 1.3 mg glucose, 1.38 mg fructose, 0.4 mg D-alanine, 0.4 mg arginine, 0.4 mg glutamine, 0.51 mg glycine, 0.4 mg threonine, 0.31 mg proline, 0.31 mg valine, 0.74 mg ammonium nitrate g^{-1} dry soil. P was added as 0.65 mg $NaH_2PO_4 \cdot 2H_2O$ g^{-1} dry soil. All nutrients were added in solution.

avoid O_2-limitation. The O_2 concentrations in headspace never reached below 8.5 vol.% in our incubations.

Basal soil respiration was calculated as the average of all measurements from the H_2O treatment. Fig. 1 depicts in a schematic manner how values for SIR and maximum AMR were derived. In detail, SIR was calculated by subtracting the respiration rates of the H_2O treatment from the respiration rates of the CNP or CN treatment. The first 17 h after the start of the incubation start were used to calculate SIR, as this was the time span before exponential increase occurred in our experiment. The time to reach maximum AMR for the CN and CNP treatment was derived from the time span between the start of the incubation and the peak respiration rate. Maximum AMR for the CN and CNP treatments was calculated as the difference between peak respiration rate and average respiration rate before exponential increase. The apparent microbial growth was taken as the exponential growth rate μ, which was calculated as the slope of the log-transformed respiration rate plotted against time during exponential increase (Nordgren et al., 1988).

For all subsequent analyses (extraction and analysis of cells, bacterial cell counts and PLFA analysis) we analyzed the microbial communities in their most active state, as given by the peak of the respiration rates. Accordingly, the microbial community of the CNP treatment was analyzed after 50 h, whereas that of the CN treatment was analyzed after 91 h. Since the H_2O and P treatment did not show a peak in the respiration curves, the microbial community was analyzed at the same time as that of the CN treatment (Fig. 2).

2.4. Extraction of cells from soil

Cell extraction was done by dispersion of soil and centrifugation at high speed over cushions of Nycodenz (Nycomed Pharma, Norway), a nonionic density gradient medium. The procedure was that described by Bakken and Lindahl (1995), with modifications for optimal results with Ferralsols as described in Ehlers et al. (2008). Briefly, 31 g of soil were dispersed in 800 ml 0.8% NaCl solution and brought to a pH of 7.5. After soil dispersion in a Waring Commercial Blender for 3 × 1 min with intermittent cooling on ice, 200 ml aliquots of the soil suspension were transferred to centrifugation tubes and 40 ml of Nycodenz solution (0.8 g ml^{-1} H_2O) were placed underneath using a syringe with a 15 cm steel needle.

The samples were centrifuged for 2 h at 4 °C and 10 000 g (Avanti J-25 Centrifuge, JS-7.5 swinging bucket rotor, Beckmann Coulter International). After centrifugation, we siphoned off and discarded

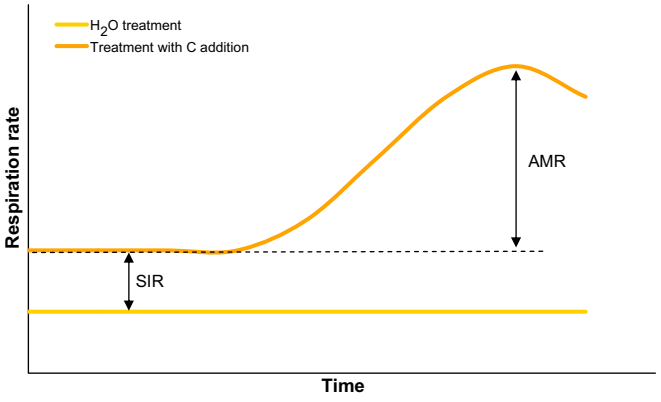

Fig. 1. Schematic diagram explaining the calculation of substrate induced respiration (SIR) and additional microbial respiration (AMR).

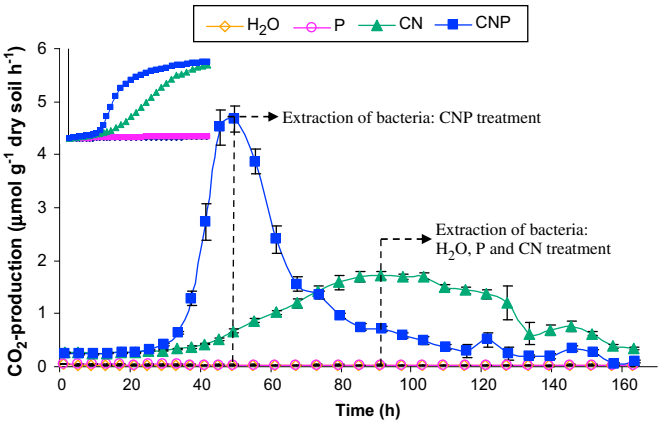

Fig. 2. Respiration rates for the four treatments during the first 163 h of incubation. Error bars show the SD derived from three independent replicates. Arrows indicate the time of extraction of bacteria from soil for different treatments. The small figure in the upper left corner shows the cumulative CO_2 production.

the supernatant down to approximately 2 cm above the Nycodenz cushion. The remaining supernatant with cells floating on top of the Nycodenz phase was harvested by siphoning off and the extracted cells were collected by filtering the cell suspension through polycarbonate filters (pore size 0.2 µm). Filters with cells were stored at −20 °C before further analysis.

2.5. Cell counts

Cell counts in soil were done in order to estimate differences in bacterial cell numbers between treatments while cell counts of extracted cells were performed in order to allow calculation of total P, DNA-P, RNA-P, and PLFA-P on a per cell basis. Cells numbers were quantified at the time of highest microbial activity as indicated by respiration rates (H_2O, P and CN after 91 h; CNP after 50 h). Cells counts were done by acridine orange direct counts as described by Hobbie et al. (1977). Cells in the soil were quantified by taking an aliquot of the soil suspension after dispersion in the Waring blender and diluting it 1/100 in 0.8% NaCl solution. 1 ml of this suspension was stained with 1 ml of acridine orange for 5 min before filtering on black polycarbonate filters (pore size 0.2 µm). Similarly, extracted cells were quantified by staining 0.2 ml of the cell suspension with 0.2 ml of acridine orange.

2.6. PLFA analysis

PLFA composition of the soil and of extracted cells was analyzed to investigate treatment effects on microbial community composition, total microbial biomass (as indicated by total amount of PLFAs), and to assess the representativeness of the extracted cells for the microbial community in the soil. For soil PLFA analysis, 3.08 g soil were extracted. For PLFA analysis of extracted cells we used the cells collected on polycarbonate filters. Lipid extraction and preparation of fatty acid methyl esters followed the method described by Frostegård et al. (1993). Conditions for gas chromatography were as described by Jia et al. (2006).

Microbial community composition was described using signature PLFAs that are considered to be indicators for specific groups of soil microorganisms. The PLFA 18:2ω6,9 is considered a fungal biomarker, whereas the detected PLFAs i15:0, a15:0, 15:0, i16:0, i17:0, a17:0, 17:0, cy17:0, 18:1ω7 and cy19:0 are considered to be of bacterial origin (Frostegård et al., 1993; Frostegård and Bååth, 1996). Accordingly the fungal:bacterial ratio was calculated by dividing

the amount of 18:2ω6,9 by the sum of the bacterial fatty acids. The PLFAs cy17:0, cy19:0 and 18:1ω7 were used as indicators for Gram-negative bacteria (Wilkinson, 1988; Joner et al., 2005) and the anteiso- and iso-branched PLFAs a15:0, i15:0, i16.0, a17:0, i17:0, br17:0 as well as br18:0 were considered as indicators for Gram-positive bacteria (ÓLeary and Wilkinson, 1988). In addition, 10Me17:0 and 10Me18:0 were used as indicators for actinomycetes (Kroppenstedt, 1985, 1992).

2.7. Total P measurements

For total P measurements, the cells extracted from soil were digested by peroxidisulfate oxidation while autoclaving at 120 °C in an oxidizing solution (0.074 M $K_2S_2O_8$; 0.075 M NaOH) (Ebina et al., 1983). We tested this method previously with cultured *Escherichia coli* and soil samples and found that it combines a full recovery of bacterial P with a low recovery (3%) of total soil P (data not shown). Nevertheless, a slight overestimation of cell bound P is possible, since a limited contamination of the extracted cells with remnant soil particles is inevitable (Ehlers et al., 2008). The P concentration in the digest was measured colorimetrically using malachite green at a wavelength of 610 nm (Ohno and Zibilske, 1991). Total P measurements were performed in duplicate for each of the three independent replicates per treatment.

As microbial cell volume might vary between the treatments P content per cell does not necessarily reflect P concentration in the cells. Therefore, we used the ratio between PLFA and total P per cell as a proxy for the P concentration in the extracted cells, since PLFA content is proportional to cell size (Lindahl et al., 1997).

2.8. Quantification of RNA-P and DNA-P

Quantification of RNA and DNA from previously extracted cells was done by sonication followed by staining of nucleic acids with the fluorescent stain RiboGreen (Quant-iT^{tm} RiboGreen, Invitrogen) as described by Cotner et al. (2006). In detail, polycarbonate filters with extracted cells were placed in test tubes with 5 ml TE buffer (Fluka Biochemika) and 0.2% (w/v) N-laurosarcosyl (Fluka Biochemika) and sonicated for 3 min. Samples were then incubated at room temperature on a shaker (150 rpm) for 20 min. Preliminary tests had shown no difference to incubation for 2 h as performed by Cotner et al. in 2006. Subsequently, 2 ml of sample were stained with 2 ml freshly prepared RiboGreen working solution (Quant-iT^{tm} RiboGreen 200fold diluted in TE buffer). Samples were incubated for 5 min in the dark and fluorescence was measured in a luminescence spectrometer (LS 500, Perkin Elmer) with an excitation of 480 nm and emission at 520 nm. Afterwards, 75 units of RNase (RNase ONE™ Ribonuclease, Promega) were added and the samples were rescanned after incubating for 30 min in the dark. RNA and DNA contents were calculated by difference in fluorescence before and after RNase addition. Nucleic acid P content was calculated assuming that DNA and RNA contain 9% P (Sterner, 1995; Elser et al., 1996).

2.9. Quantification of PLFA-P

The amount of PLFA-P was determined in two different, yet not fully independent ways. For one, PLFA-P was calculated from the total amount of PLFAs based on the assumption that for 2 mol of PLFA there is 1 mol of PLFA-P. For the other, the amount of PLFA-P was measured by isolating the P-containing polar head of the phospholipids from the apolar fatty acids during the trans-esterification of PLFAs, followed by digestion and measurement of P content as described in Chapter 2.7.

2.10. Statistical analysis

To analyze treatment differences in microbial community composition, principal component analyses (PCA) were performed on the relative abundance of individual PLFAs (mol% values; standardized data) using Sirius 6.5 (Pattern Recognition Systems, http://www.prs.no/). The PLFAs 16:0, 18:1ω9 and 18:2ω6,9 were excluded from the PCA analysis in order to focus on bacterial PLFAs. All other data were analyzed by a one way ANOVA using SYSTAT 11 (SYSTAT Software Inc. 2004), followed by Tukey's pairwise comparison if significant differences were indicated. All data are presented as average ± standard deviation unless otherwise stated.

3. Results

3.1. Respiration

Respiration rates (in μmol CO_2 g^{-1} soil h^{-1}) during the first 163 h of incubation are shown in Fig. 2. Both the P and the H_2O treatment showed stable and low respiration rates. Basal soil respiration as indicated by the H_2O treatment was around 0.024 ± 0.001 μmol CO_2 g^{-1} h^{-1}. Respiration in the P treatment was with 0.032 ± 0.008 μmol CO_2 g^{-1} h^{-1} significantly ($P < 0.001$) higher than the basal respiration in the H_2O treatment. In the CN and CNP treatments, respiration remained constant within the first 17 h and SIR was similar for both treatments (Table 2). After that, however, differences between the CN and CNP treatment became clear. In the CNP treatment, respiration peaked after 50 h and thus much earlier than in the CN treatment (91 h). Maximum AMR in the CNP treatment was about three times higher than in the CN treatment. Accordingly, the exponential growth rate μ in the CNP treatment was about four times higher than in the CN treatment. Until the moment of maximum AMR, microorganisms cumulatively respired 27.51 % of the added C in the CN treatment compared to 23.51% in the CNP treatment.

3.2. Soil cell counts

No significant differences of cell counts in the soil suspension between treatments were detected (data not shown). On average, the soil contained 6.4 × 10^9 (±1.8 × 10^9) bacteria g^{-1} soil for all treatments.

Table 2
SIR, max. AMR, cumulative respiration of added C and the exponential growth rate μ of CN and CNP treatment.[a]

Treatment	SIR (μmol CO_2 g^{-1} dry soil h^{-1})	Max. AMR (μmol CO_2 g^{-1} dry soil h^{-1})	Exp. growth rate μ (h^{-1})	Cum. respiration of added C until moment of max. AMR (%)
CN	0.227 (0.004)	1.467 (0.077)	0.017 (0.001)	27.51 (1.40)
CNP	0.214 (0.011)	4.420 (0.255)	0.072 (0.002)	23.51 (0.97)
ANOVA				
P	=0.116	<0.001	=0.015	<0.001

[a] All values are means of three replicates, SD in brackets, $n = 6$.

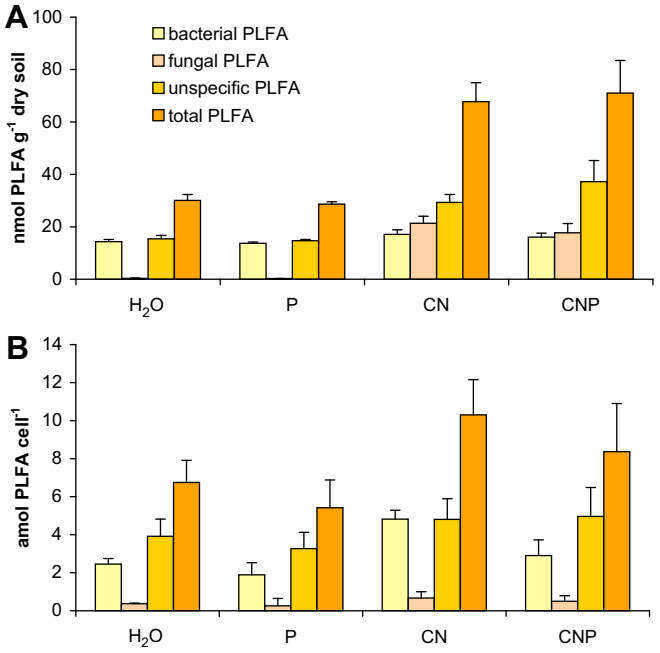

Fig. 3. PLFA content in soil (A) and extracted cells (B). Error bars show the standard deviation from total amount of PLFA.

3.3. PLFA analysis

The amounts of total, bacterial, fungal and unspecific PLFA in the soil and in the extracted cells are given in Fig. 3. For the soil, these results suggest that P addition alone did not lead to significant increase in microbial biomass compared to the H_2O treatment (both 29.4 ± 1.7 nmol PLFA g^{-1} soil). Substrate addition more than doubled microbial biomass and similar amounts were present in the CN and CNP treatment at maximum AMR (69.4 ± 9.3 nmol PLFA g^{-1} soil). The fungal signature fatty acid 18:2ω6,9 increased more than 50 fold from 0.3 ± 0.1 nmol PLFA g^{-1} soil in the H_2O and P treatments to 19.5 ± 3.5 nmol g^{-1} soil in the CN and CNP treatments. In contrast, bacterial PLFAs increased by less than 20% from 14 ± 0.7 nmol PLFA g^{-1} soil to 16.6 ± 1.5 nmol PLFA g^{-1} soil. Accordingly, the fungal:bacterial ratio in the CN and CNP treatments of 1.17 ± 0.162 differed strongly from that of the H_2O and P treatments (0.02 ± 0.01).

Fig. 3B shows that the extracted cells, however, did not reflect the differences in fungal biomass. Thus, fungal:bacterial ratios in extracted cells were similar for all treatments (0.15 ± 0.11). There was a tendency towards higher total PLFA content per cell in the CN and CNP treatments compared to the H_2O and P treatments.

In the PCA of bacterial PLFAs (Fig. 4), the first principal component (PC1) explained 50.4% and the second (PC2) 17.1% of the total variation. As for the soil PLFA profiles, P addition itself had no effect when comparing it to the H_2O treatment (Fig. 4A). The combined addition of C and N, however, changed soil PLFA composition so that CN and CNP were separated from the P and H_2O treatment along PC2.

The PLFA profiles in the extracted cells were clearly separated from the soil PLFA profiles along PC1. Within the extracted cells the CNP and CN treatments formed separate clusters, whereas the cells from the H_2O and P treatment were grouped together.

PLFA patterns did not reveal a consistent effect on broad bacterial groups (Fig. 4B). For example, 10Me18:0 which is specific for actinomycetes had the highest relative abundance in the soil treatments, while 10Me17:0, also specific to actinomycetes, was most abundant in the extracted cells. PLFAs representative of Gram-positive bacteria like i15:0 and i17:0 showed highest relative abundance in the soil, while br17:0 as another Gram-positive indicator was most abundant in the extracted cells in general and br18:0 and a15:0 were most abundant in the extracted cells from the CN treatment. Similarly, the PLFAs 18:1ω7, cy17:0 and cy19:0, all being representative of Gram-negative bacteria, did not show a uniform picture.

3.4. P content and P pools of extracted cells

The amounts of total P, DNA-P, RNA-P and PLFA-P per extracted cell and the PLFA:total P ratio in cells are given in Table 3. Overall, total P measurements ranged from 2.1 to 8.9 fg $cell^{-1}$, with a tendency for higher total P content in the CN and CNP treatments. DNA-P content per cell was similar in all treatments, with a trend to higher amounts in the CN treatment. Similarly, RNA-P did not differ between H_2O, P and CNP treatments, but the CN treatment showed significantly higher amounts. There was a reasonable agreement between measured and calculated PLFA-P. Due to high variation between replicates, the differences in PLFA-P between treatments were not significant. The proportion of DNA-P to total P ranged from 9% for the CNP treatment over 16% for the H_2O and CN treatments to 21% in the P treatment. RNA-P proportion of total P was rather low with 1% (CNP), 2% (H_2O), 3% (P) and 6% (CN). The measured and calculated PLFA-P proportions averaged around 3%. Altogether, the investigated P pools explained 10% (CNP), 18% (H_2O), 21% (CN) and 25% (P) of the total P content per cell.

There was no significant treatment effect in the PLFA:total P ratios due to the high variation within replicates, yet there is a tendency for a lower ratio (i.e. higher relative P concentration) in the cells of the CNP treatment than in the other treatments. Maximum AMR and total P content per cell were positively correlated ($r = 0.59$, $p = 0.003$), whereas PLFA:total P ratios and maximum AMR were negatively correlated ($r = 0.47$, $p = 0.028$).

4. Discussion

4.1. Microbial activity, biomass and community composition in the soil

The CN and CNP treatments showed the same initial substrate induced increase in respiration (SIR) and thus microbial biomass that was activated by the carbon substrate addition was unaffected by the addition of P. The lower maximum AMR and the lower exponential growth rate μ of the microbial community in the CN treatment compared to the CNP treatment, however, suggest a retardation of microbial growth due to a slow release of soil-derived P in the CN treatment. This finding is in accordance with other studies on tropical (Duah-Yentumi et al., 1998; Cleveland et al., 2002; Ilstedt and Singh, 2005; Gnankambary et al., 2008) and temperate soils (Demetz and Insam, 1999; Giesler et al., 2004).

Assuming that the maximum AMR occur simultaneously with a maximum of microbial biomass, respiration data indicate that low amounts of easily available P in the CN treatment retarded microbial growth rate but not the overall size of microbial biomass produced (as indicated by the PLFA data). The microbial biomass in the CN treatment simply took longer to grow to the same size as in the CNP treatment (91 h vs. 50 h). The fact that the increase in microbial biomass was similar, while the AMR in the CNP treatment was three times higher than in the CN treatment indicates that the AMR might not be useful in describing microbial growth quantitatively. This outcome is in agreement with Marstorp and Witter (1999) who did not find a correlation between chloroform labile C

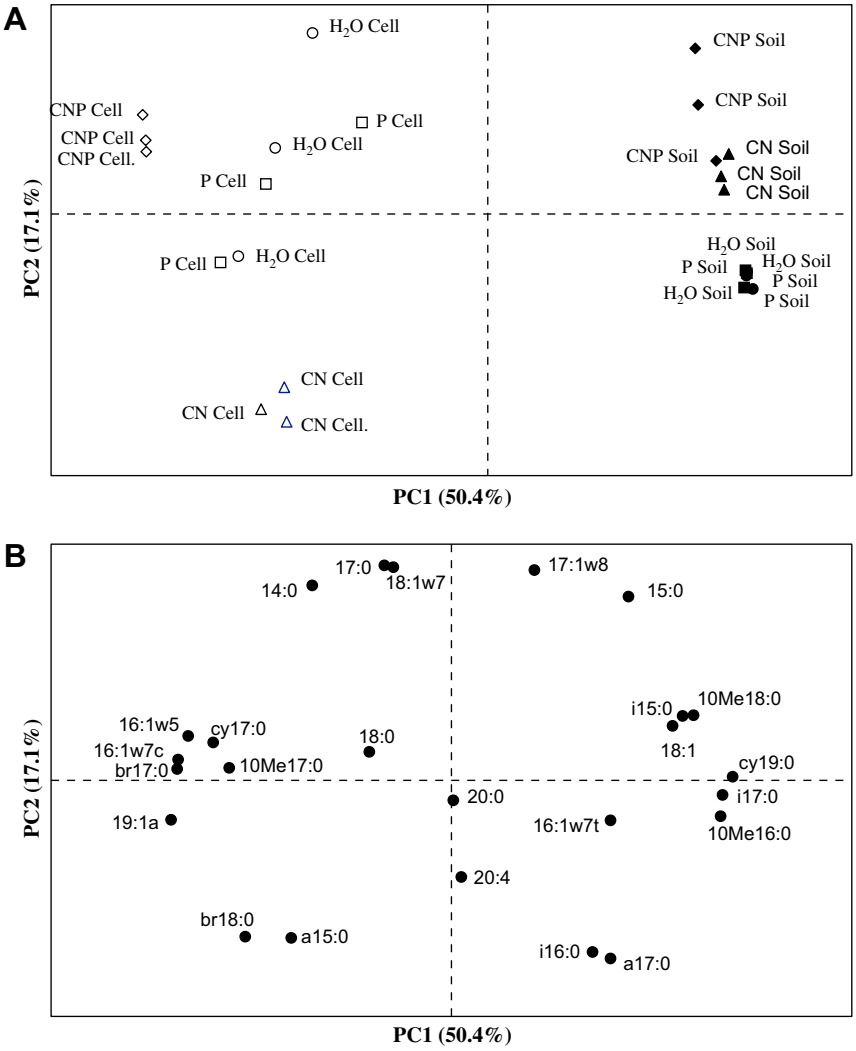

Fig. 4. PCA for relative abundance of PLFAs in soil and in extracted cells. PLFAs considered to represent mainly fungal biomass (16:0, 18:1ω9 and 18:2ω6,9) were excluded from the analysis.

and respiration rate. The amount of extractable soil DNA, however, reflected microbial respiration rate in their study.

The higher respiration after P addition alone in comparison with basal respiration in the H_2O treatment shows a positive priming effect of P, in accordance with Cleveland et al. (2002). However, we believe that in our case it is rather unlikely that this priming effect is actually due to a previous metabolic P limitation of microorganisms. Instead, it seems possible that the added orthophosphate, being a strong ligand, displaced sorbed organic matter from minerals and therefore rendered more carbon sources available for microorganisms (Jones and Edwards, 1998; Kögel-Knabner et al., 2008). In fact, after 91 h, extractable C (0.5 mol K_2SO_4) was higher in the P treatment (216 ± 3 mg C g^{-1}) than in the H_2O treatment (202 ± 9 mg C g^{-1}) (data not shown). Nevertheless, the release of organic matter was not high enough to cause detectable growth of microbial biomass as seen from the total PLFA data and bacterial cell counts. Accordingly, we believe that P limitation of soil microbes occurred only when ample amounts of easily available C and N were added.

In the CNP and CN treatments, PLFA data show that the substrate induced growth was mainly derived from fungi and only to a minor extent from bacteria. This finding is in accordance with the cell counts, which gave similar amounts of bacterial cells in the soil for all treatments. When using the conversion factor of 11.8 μmol of the fatty acid 18:2ω6,9 g^{-1} fungal biomass C as proposed by Klamer and Bååth (2004), the increase in fungal biomass is around 1.63 mg

Table 3
P content, P pools and PLFA:total P ratio in extracted soil microorganisms.[a]

Treatment	Total P (fg $cell^{-1}$)	DNA-P (fg $cell^{-1}$)	RNA-P (fg $cell^{-1}$)	PLFA-P (meas.) (fg $cell^{-1}$)	PLFA-P (calc.) (fg $cell^{-1}$)	PLFA:total P[b] (mol:mol)
H_2O	3.22 (0.83)ab	0.52 (0.08)a	0.06 (0.01)b	0.15 (0.07)a	0.10 (0.02)a	0.06 (0.01)a
P	2.70 (0.60)b	0.56 (0.08)a	0.09 (0.03)b	0.05 (0.04)a	0.08 (0.02)a	0.06 (0.01)a
CN	4.60 (1.13)ab	0.74 (0.17)a	0.28 (0.01)a	0.15 (0.11)a	0.16 (0.03)a	0.07 (0.01)a
CNP	6.53 (2.46)a	0.57 (0.16)a	0.09 (0.03)b	0.13 (0.08)a	0.13 (0.04)a	0.04 (0.02)a

[a] All values are means of three replicates, SD in brackets, values within a column followed by the same letter do not differ significantly ($P < 0.05$) according to Tukey's test.
[b] Total PLFA $cell^{-1}$ divided by total P $cell^{-1}$ (molar ratio).

fungal biomass C g^{-1} soil in the CN and CNP treatment compared to the H_2O and P treatment. In contrast, bacterial biomass increased only by 0.02 mg C g^{-1} soil (based on an average content of 3.02 amol bacterial PLFA per cell in our study and a C content in bacterial cells of 20 fg $cell^{-1}$ (Bååth, 1994)). Accordingly, the estimated increase in fungal biomass C amounts to 51% of the added substrate C, while the increase in bacterial C was less than 1%. Obviously fungi outcompeted bacteria in rivalry for the added C and N sources, no matter whether available P was scarce or not. Similarly, Griffiths et al. (1998) reported that fungi dominated over bacteria after the addition of high amounts of easily degradable substrates. Furthermore the rather low soil pH might have favored fungal growth (Rousk et al., 2009). The dominance of fungi over bacteria, however, does not necessarily mean that bacteria did not participate in C degradation. Probably bacteria were responding to the C source, but fungi may have prevented bacterial growth.

For estimation of the microbial growth efficiency (Giorgio and Cole, 1998) until maximum AMR, we divided the increase in microbial biomass C by the assimilated C, which is the sum of biomass C increase and respired C. This revealed that growth efficiencies were not significantly different between CN and CNP (0.67 ± 0.03).

4.2. Community composition, P content and P pools in extracted microbial cells

The extracted microbial communities do not reflect the changes that occurred in the soil microbial communities after substrate amendments. This can be at least partially explained by the abundant growth of fungi in CN and CNP treatments, whereas the proportion of fungi extracted together with soil bacteria stayed the same. A low recovery of fungal biomass after extraction by density centrifugation has frequently been observed, and can in part be due to physical disintegration by the dispersion and in part be due to strong attachment to soil particles (Bakken and ÅFrostegård, 2006).

For dominating broad bacterial groups, several studies so far did not reveal a biased cell extraction by the Nycodenz methodology (Mayr et al., 1999; Courtois et al., 2001; Ehlers et al., 2008). This is no proof, however, of the absence of bias for specific groups, as demonstrated for methane oxidizing (Prieme et al., 1996) and ammonia oxidizing bacteria (Aakra et al., 2000). Therefore, our data on P content and major P pools of indigenous soil microorganisms might not be representative for the overall microbial community in the soil.

With the exception of DNA and PLFA quantifications, these data are the first P content values to be reported for soil microorganisms that actually grew in soil. Therefore, our values need to be compared to values of bacteria from other ecosystems (e.g. aquatic bacteria) or to those of cultured bacteria. As mentioned earlier, we cannot exclude a slight overestimation of total P content caused by contamination with soil-derived P. Nevertheless, the observed total P contents of 2.1–8.9 fg $cell^{-1}$ seem to be within a reasonable range compared to 3.4–31 fg P $cell^{-1}$ for cultured aquatic bacteria (Fagerbakke et al., 1996) or values between 0.01 and 10 fg P $cell^{-1}$ for *in situ* analysis of marine bacteria (Gundersen et al., 2002).

The positive correlation between maximum AMR and total P content per cell is in accordance with the growth rate hypothesis, which states that biomass P content increases with growth rate due to increased P allocation to P-rich ribosomal RNA (Elser et al., 2000, 2003). Similarly, Vrede et al. (2002) reported average P contents of 2 and 12 fg P $cell^{-1}$ for marine bacteria in batch culture in stationary and exponential growth phase, respectively. The negative correlation between PLFA:total P ratios and maximum AMR indicates that the increased P content per cell is not only due to an increased cell size in the treatments with C and N addition (as indicated by a tendency of higher PLFA content per cell), but is actually due to an increased P concentration in the cells.

However, those correlations were rather weak and P concentration in the cells (PLFA:total P ratios) did not differ significantly between treatments. This is probably due to the fact that both active and dormant cells were extracted from soil. Therefore, the higher P content in activated cells was presumably diluted by low P content in dormant cells in the analyzed extracts.

According to the growth rate hypothesis, the RNA-P content should be a major P pool in growing microorganisms. For example, Makino and Cotner (2004) reported an RNA-P proportion of 70–90% and 25–43 % in P limited and P sufficient cultures, respectively. Similarly, RNA-P proportions of 40–50% at low growth rate and 70–80% at high growth rate were found for *E. coli* in a chemostat culture (Makino et al., 2003). Compared to these data, our proportions of RNA-P of 1–6% seem unreasonably low. Again, this might partly be due to the fact that even in the treatments with high respiration rates, we extracted both active and dormant cells. Furthermore, it is possible that RNA was partially degraded during the 3–4 h needed for the entire procedure of extracting and filtering cells. A failure in the RNA-quantification itself, however, seems unlikely since tests with cultured *E. coli* gave reasonable proportions of RNA-P (53 (±8) %, data not shown).

DNA-P quantification showed expected amounts. The overall average of 0.6 fg DNA-P $cell^{-1}$ was calculated based on a DNA content of 6.6 fg $cell^{-1}$ which is somewhat higher than the 1.6–2.4 fg $cell^{-1}$ reported by Bakken and Olsen (1989) for indigenous soil bacteria, but well within the range of 2–9 fg DNA $cell^{-1}$ which the same authors found for cultured soil bacteria. Similarly, Torsvik and Goksoyr (1978) and Sandaa et al. (1998) found values of 8.4 fg DNA $cell^{-1}$ and 8.8–11.5 fg DNA $cell^{-1}$, respectively.

As for the PLFA-P values, Frostegård and Bååth (1996) found PLFA values of 0.62×10^{-17}–2.35×10^{-17} mol $cell^{-1}$ for 15 Swedish soils covering a wide range of pH and organic matter contents. Therefore our PLFA-P values based on an overall average of 0.77×10^{-17} mol PLFA $cell^{-1}$ for all treatments seem to be realistic.

Altogether, the investigated P pools explained 10–25% of the measured total P $cell^{-1}$. Compared to Vadstein (1998) who found about 60% of the total P to be bound in DNA, RNA and phospholipids for cultured bacteria, our numbers are rather low. We attribute this to our mentioned difficulties with RNA-P recovery.

Estimating the size of the microbial P pool in the soil on basis of the PLFA:total P ratio and the amount of PLFA g^{-1} soil gives results of around 15 mg P kg^{-1} soil in the H_2O and P treatments compared to 36 mg P kg^{-1} soil in the CN and CNP treatments. This would mean that despite the low P status and the strong P sorption capacity in the soil, microbes have been able to extract around 21 mg P kg^{-1} from the soil in the CN treatment. Compared to 1.8 mg of available P per kg of soil, this number reflects the ability of microbes to extract P from the sorbed P pool after stimulation with C as stated by Olander and Vitousek (2004). Since the PLFA:total P ratio in the soil might differ from that in the extracted cells, the amount of microbial P per kg of soil might be overestimated. Nevertheless, the values seem to be within a reasonable range when applying the conversion factor of 0.18 given by McLaughlin et al. (1986) to the values of P extracted by fumigation extraction with hexanol on a similar Kenyan Ferralsol as given by Bünemann et al. (2004).

4.3. Conclusions

We were able to verify our hypothesis that low P availability in our Ferralsol retards microbial growth in response to added substrates but were surprised to see that similar biomass values were reached with and without added P. The P content per cell was related to microbial respiration rates, while P addition alone did not

lead to an increase in cell P content. This is in agreement with our second hypothesis that metabolic state rather than soil P status affects cellular P content.

The extraction of cells from soil by density gradient centrifugation, followed by further analysis of the extracted cells can give an insight into the P pools of soil microorganisms. However, in this study, the extracted cells were not fully representative for the soil microbiota regarding the community composition and metabolic state. The P content and P pool data are the first to be reported for cells that actually grew in the soil and not in chemostat or batch cultures. Except for RNA-P, cell P contents seem to be within a reasonable range when compared to other studies on aquatic or cultured bacteria.

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