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COMPOSITION AND DYNAMICS OF THE MICROBIAL PHOSPHORUS POOL IN A FERRALSOL

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Table of contents

List of abbreviations

ANOVA	analysis of variance
AMR	additional microbial respiration
C	carbon
Cmic	microbial carbon
Ctot	total carbon
DGGE	denaturing gradient gel electrophoresis
DRW	drying and rewetting
IEP	isotopically exchangeable phosphorus
N	nitrogen
n	rate of disappearance of radioactivity from the solution for >1 min
$NaHCO_3$	organic and inorganic P solubilized by 0.5 M $NaHCO_3$
NaOH-Pi	inorganic P extractable with 0.1M NaOH
Nmic	microbial nitrogen
Nmin	mineral nitrogen (NH_4^+ and NO_3^-)
ns	not significant
Ntot	total nitrogen
M	constantly moist
OD	optical density
OD_{530}	optical density at 530 nm wavelength
OMloss	organic matter loss
P	phosphorus
p	probability level
PCA	principal component analysis
Pfum	P that is additionally resin extractable in the presence of a fumigant
PLFA	phospholipid fatty acid
Pmic	microbial P
Presin	resin extractable phosphorus
Ptot	total P
PZC	point of zero charge

R	total introduced radioactivity
r_1	radioactivity remaining in the solution after 1 minute of isotopic exchange
SA	specific activity
SD	standard deviation
SE	standard error
SIR	substrate induced respiration
Smod	dispersion in salt solution with pH modification
Snon	dispersion in salt solution without pH modification
WHC	water holding capacity
Wmod	dispersion in water with pH modification
Wnon	dispersion in water without pH modification
μ	apparent microbial growth rate, calculated as the slope of the log-transformed respiration rate plotted against time during exponential increase

Abstract

Soil microorganisms mediate key processes in the soil phosphorus (P) cycle. Particularly in highly weathered tropical soils like Ferralsols where strong P sorption limits P availability to crops, a capital role is assigned to the microbial P pool as sink and source of soil P. However, a thorough understanding of the composition and dynamics of the microbial P pool is lacking. Until today the microbial P pool has only been investigated after cell lysis by the fumigation-extraction method. This method has several known biases and as the extracted P compounds are hydrolyzed during the extraction it reveals no information on the chemical composition of the soil microbial P pool.

As a prerequisite for more detailed analysis of the microbial P pool, the method of microbial cell extraction by density gradient centrifugation was adapted to a Ferralsol. This soil type presented a challenge to this method due to a low cell yield and a substantial contamination of the extracted microbial fraction by soil material. The combination of modifying pH from originally 4.9 to 7.5 by NaOH addition and adding NaCl (8 g l^{-1}) during soil dispersion secured reasonable cell yield (4.6%) and low contamination, and the representativeness was biased within an acceptable range.

Incubating the soil with carbon substrates and ample nitrogen (CN) with and without extra P showed that microbial respiration after CN addition was retarded by P-limitation but microbial growth was not hampered. Substrate induced growth was dominated by fungi, irrespective of P addition. Total P in extracted cells ranged from 2.1 to 8.9 fg P $cell^{-1}$, with a tendency for lower values in less active soil microbial communities. Between 10-25% of the measured total P in extracted cells was accounted for by the sum of measured RNA-P, DNA-P and phospholipid fatty acid P. Presumably, the RNA-P pool was underestimated because of degradation during extraction.

N and P fertilization in the field did not affect soil microbial biomass C, the microbial P pool, plant litter degradation and soil basal respiration. However, the microbial N pool and the glucose induced respiration were sensitive to N fertilization. In contrast to plant

N:P ratios, soil microbial N:P ratios were not affected by soil total N:P ratios and averaged around 11 ± 7 and 9 ± 2 for fumigation-extraction and extraction of cells, respectively. These data reinforce the hypothesis that plant nutrient concentrations depend more on the nutrient concentrations in the surrounding environment than microbial nutrient ratios which are independent from the surrounding environment and thus homeostatic.

Drying and rewetting of the soil led to an increase in available inorganic P and a decrease in microbial P. Labeling the soil with ^{33}P indicated that P is rapidly cycled between the available inorganic P pool and the microbial P pool. Comparison of the isotopic composition in the investigated P pools with and without drying and rewetting indicated that the microbial P pool is the source for an increase in available inorganic P. However, this flush in available P disappeared quickly and presumably, the released microbial P was sorbed to soil particles.

The results show that the soil microbial biomass represents a dynamic and labile P pool. Low P availability in the investigated Ferralsol may slow down soil microbes but does not seem to limit them in absolute terms. Cell extraction confirmed results from fumigation-extraction, indicating that P concentration in the soil microbial biomass does not depend on the P availability. It thus seems that on this soil, microorganisms are able to take up enough P from the soil to satisfy their needs if carbon availability allows microbial growth and that soil drying and rewetting is one possible process that leads to a release of microbial P.

Zusammenfassung

Bodenmikroorganismen sind für wesentliche Prozesse des Phosphorkreislaufs im Boden verantwortlich. Es wird davon ausgegangen, dass sie insbesondere auf stark verwitterten tropischen Böden wie Ferralsolen, auf denen eine starke Sorption von Phosphor (P) die P-Verfügbarkeit für Pflanzen einschränken kann, eine bedeutende Funktion als Senke und Quelle für den Bodenphosphor haben. Jedoch fehlt bislang das Wissen über die Zusammensetzung und Dynamik des mikrobiellen P im Boden. Bisher wurde der mikrobielle P nur über eine Zerstörung mikrobieller Zellen mittels der Fumigation-Extraktionsmethode bestimmt. Diese Methode ist bekannt dafür verzerrte Resultate zu generieren. Da der mikrobielle P bei dieser Methode hydrolisiert wird, kann auch seine Zusammensetzung nicht weiter bestimmt werden.

Als Vorrausetzung für eine genauere Untersuchung der Zusammensetzung des mikrobiellen P wurde die Methode zur Zellextraktion von Bodenmikroorganismen mittels Zentrifugierung über einem Dichtemedium an den Bodentyp Ferralsol angepasst. Bisher war die Extraktion von Mikroorganismen aus diesem Bodentyp wegen geringer Zellausbeute und starker Verunreinigung der extrahierten Zellen durch Bodenpartikel nur eingeschränkt erfolgreich. Eine Kombination von pH Erhöhung (von 4.9 auf 7.5 mittels NaOH Zugabe) und eine Salzzugabe von 8 g NaCl l^{-1} während der Dispergierung ermöglichte eine ausreichende Zellausbeute (4.6%) und geringe Kontamination. Die Repräsentativität der extrahierten Bodenmikroorganismen wurde durch diese Behandlung in vertretbarem Rahmen beeinträchtigt.

Eine Inkubation des Bodens mit leicht verfügbaren Quellen von Kohlenstoff (C) und Stickstoff (N) mit oder ohne P-Zugabe zeigte, dass die mikrobielle Atmungsrate und das mikrobielle Wachstum nach CN-Zugabe durch eine P-Limitierung verlangsamt, aber nicht gänzlich eingeschränkt ist. Das durch die Nährstoffzugabe induzierte mikrobielle Wachstum wurde von Pilzen dominiert, unabhängig davon, ob zusätzliches P zugegeben wurde oder nicht. Der Gesamtgehalt an P in den Zellen schwankte zwischen 2.1 bis 8.9 fg P pro Zelle, mit einer Tendenz zu niedrigeren Werten in weniger aktiven mikrobiellen

Gemeinschaften. 10-25% des gemessenen gesamten zellulären P konnte als RNA-P, DNA-P und PLFA-P identifiziert werden. Diese niedrigen Werte führe ich auf eine Unterschätzung des RNA-P zurück. Vermutlich wird RNA bei der Extraktion der Zellen teilweise zersetzt.

N- und P-Düngung im Boden hatte keinen Einfluss auf die mikrobielle Biomasse, die Menge des mikrobiellen P, die Zersetzung von Pflanzenrückständen und die mikrobielle Basalatmung. Die Menge des mikrobiellen N und auch die Atmung nach Glukosezugabe wurde durch N-Düngung erhöht. Im Gegensatz zu N:P-Verhältnissen in Pflanzen war das N:P-Verhältnis in Bodenmikroorganismen nicht durch N:P-Verhältnisse im Boden beeinflusst. Das mikrobielle N:P-Verhältnis schwankte zwischen 11 ± 7 für die Fumigation-Extraktionsmethode und 9 ± 2 für extrahierte Zellen. Diese Ergebnisse unterstützen die These, dass die Nährstoffverhältnisse in Pflanzen in einem gewissen Rahmen von der Umgebung abhängig sind, während mikrobielle Nährstoffverhältnisse unabhängig von den Nährstoffverhältnissen der Umgebung und somit homeostatisch sind.

Das Austrocknen und Wiederbefeuchten von Boden führte zu einem Anstieg des verfügbaren anorganischen P Gehaltes des Bodens und einer Abnahme des mikrobiellen P. Durch das Markieren des Bodens mit ^{33}P wurde deutlich, dass P zwischen dem verfügbaren anorganischen P und dem mikrobiellem P zügig ausgetauscht wird. Der Vergleich der Isotopenzusammensetzung in den P-Pools des Bodens mit und ohne Bodenaustrocknung und Wiederbefeuchtung deutete an, dass das mikrobielle P die Quelle für den Anstieg des verfügbaren anorganischen P ist. Dieser Anstieg an verfügbarem anorganischen P verschwand allerdings recht schnell und wir gehen davon aus, dass er an Bodenpartikel sorbiert wurde.

Die Ergebnisse zeigen, dass die mikrobielle Biomasse im Boden einen labilen und dynamischen P-Pool darstellt. Eine niedrige P-Verfügbarkeit im Boden mag Bodenmikroorganismen beeinträchtigen, scheint auf diesem Standort jedoch keine absolute Limitation darzustellen. Die Zellextraktion bestätigte die Ergebnisse der

Fumigation-Extraktionsmethode, dass die P-Konzentration in der mikrobiellen Biomasse nicht von der P Verfügbarkeit abhängt. Dementsprechend scheint es, dass auf diesem Boden Mikroorganismen ihren Bedarf an P decken können, wenn die C-Verfügbarkeit ein mikrobielles Wachstum zulässt. Bodenaustrocknung und Wiederbefeuchtung ist ein Prozess, der zur Freisetzung von mikrobiellem P führen kann.

General introduction

Phosphorus – a limiting and limited element

Phosphorus (P) is an essential element for any living organism. It is needed as part of the sugar-phosphate backbone in nucleic acids (DNA and RNA), in multifunctional compounds like adenosine triphosphate (ATP) and guanosine triphosphate (GTP), in phospholipids which are major components of all cell membranes and in sugar phosphates like glucose–6-phosphate that serves as an important metabolite. Furthermore, inorganic P (Pi) controls various enzymatic reactions. A lack of P will result in various deficiency symptoms and even death of the suffering organism.

The major deposit of P is the lithosphere, in which, due to its high reactivity, P is bound in minerals. Apatite is among the most relevant phosphate minerals and can be found in high concentrations in rock phosphates. Rock phosphates are the economically most important P source and the bulk of it is used for production of agricultural fertilizers (Gilbert, 2009). As high grade rock phosphate resources are declining worldwide there is an urgent need for improved knowledge on the fate of P fertilizers in the soil and on soil P cycling in general.

Phosphorus in Ferralsols – a major constraint for plant growth

Plants typically take up P from the soil solution as $H_2PO_4^-$ or HPO_4^{2-}, depending on the pH. The fraction of soil P, however, that is actually dissolved in the soil solution represents only around 0.1% of the total soil P. The vast majority of P in soil is usually bound in minerals, immobilized in living or dead organic matter or sorbed to soil particles (Frossard et al., 1995). The relevance of these P binding forms heavily depends on soil age and pH. Young soils are generally rich in primary P containing minerals and secondary Ca-phosphates. With increasing age and weathering, however, relatively more P is bound in organic matter and sorbed to iron (Fe) and aluminum (Al) oxides. Due to leaching, runoff and erosion, total P content in soil also decreases with time (Walker and Syers, 1976; Smeck, 1985).

Ferralsols are very old and highly weathered tropical soils (FAO/ISRIC/ISSS, 1998). Most of the primary minerals in this soil type have already disappeared and only the very recalcitrant ones (e.g. quartz) remain. Thus, the release of P by weathering of P containing primary minerals is insignificant. Similarly, Ca compounds that could form

secondary Ca-phosphates have been leached. On the other side, the intense weathering led to formation of 1:1 layer silicates (e.g. kaolinite) and Fe- and Al-oxides. Those oxides belong to the most reactive components in the soil (Loeppert and Inskeep, 1996) and in combination with the typically low pH in Ferralsols they become strong sorption sites for P (Parfitt, 1980). The P sorption to soil particles leads to low amounts of plant available P in Ferralsols turning P into one of the main nutritional constraints for plant growth (Fairhurst et al., 1999).

Apart from these abiotic processes 20-80% of the soil total P is immobilized in soil organic matter (Dalal, 1977). This organic P (Po) is always of biological origin and can be bound either in dead organic matter or in living organisms. 99% of the living soil Po pool (excluding roots) is estimated to be present in soil microorganisms (Magid et al., 1996).

The role of the microbial biomass in P cycling

The microbial biomass is a very dynamic P pool, maintaining P in labile and thus potentially plant available forms and protecting it from P sorption (Olander and Vitousek, 2005). In Ferralsols the microbial P pool represents 1-2% of total soil P and is thus rather small (Oberson et al., 2001; Bünemann et al., 2004). Nevertheless a high importance is assigned to it as the P fluxes passing through this pool are high (Oberson et al., 2001; Oehl et al., 2001; Kouno et al., 2002). Microorganisms mediate several key processes in the P cycle (Figure 1.1) that can affect P availability to plants (Oberson and Joner 2005). They access the inorganic P pool via P uptake from the soil solution (Oehl et al., 2001) and have the ability to solubilize poorly available inorganic P by releasing organic acids and modifying the pH (Kucey et al., 1989; Richardson, 2001). By decomposing soil organic matter soil microbes incorporate organically bound P and they can exude phosphatases in order to hydrolyse organic P extracellularly (Quiquampoix and Mousain, 2005). Hence, soil microorganisms are a major factor controlling organic and inorganic P concentration in the soil solution (Seeling and Zasoski, 1993).

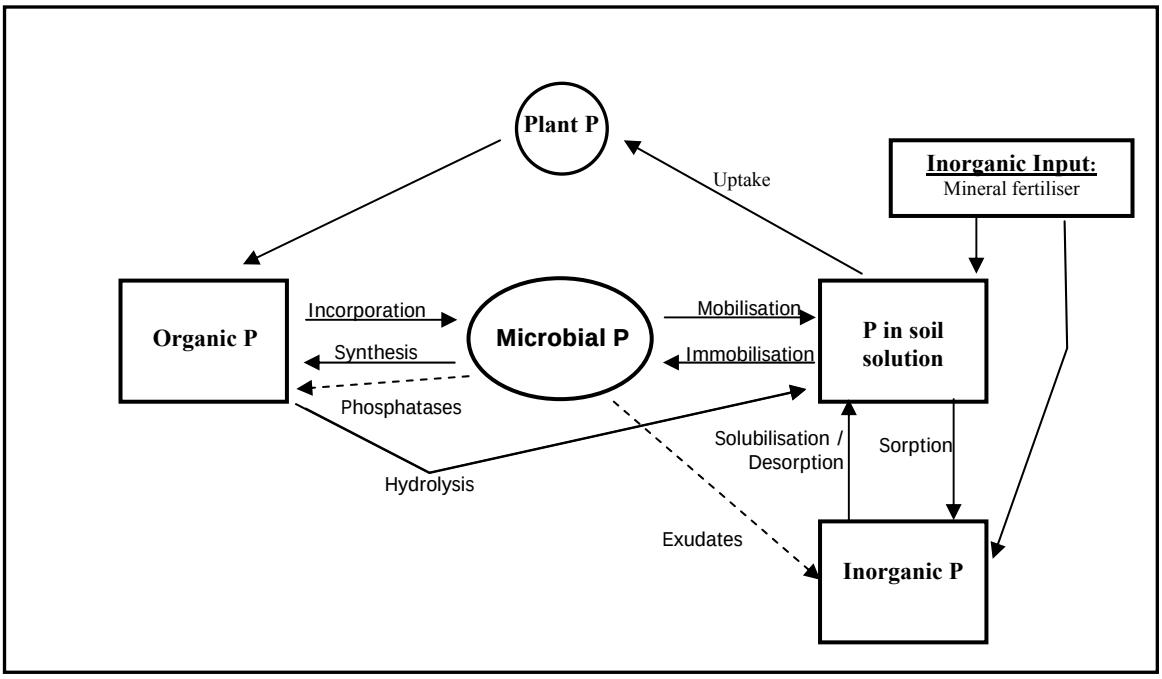

Figure 1.1: Conceptual diagram of soil P dynamics with a focus on role of the microbial biomass.

Determinating microbial P

The size of the microbial P pool is commonly determined by fumigation-extraction (Brookes et al., 1982; Hedley and Stewart, 1982). In this approach, the amount of P that is additionally released by the presence of a fumigant compared to the amount of P that is released without the fumigant is considered to be of microbial origin. The underlying mechanism is that microbial cells lyse in the presence of a fumigant and the P bound in them is released. Chloroform and hexanol are among the commonly used fumigants and both give similar results (McLaughlin et al., 1986). The method is well established and used frequently on tropical soils following the procedures of Oberson et al. (1997) or Kouno et al. (1995). It has, however, several known biases like incomplete cell lysis, incomplete hydrolysis of the released organic P, release of P that is not derived from soil microbes but plant roots, and sorption of the released and hydrolyzed microbial P to soil particles during the extraction. The sorption during the extraction can be taken into account by measuring the recovery of a known inorganic P spike that is added before the extraction starts (Morel et al., 1996). Conversion factors correcting for incomplete cell

lysis have been proposed (Brookes et al., 1982; Hedley and Stewart, 1982; McLaughlin et al., 1986; Wu et al., 2000) but their use is seldom suitable for two major reasons: First, they are derived from the P-release of added cultured microorganisms during the extraction. Freshly added cultured microorganisms might, however, react differently upon fumigation than indigenous soil microorganisms. Second, those conversion factors are depending on soil properties and thus should be determined for each soil individually (Oberson and Joner, 2005).

Composition of the microbial P pool

Fumigation-extraction hydrolyzes microbial P compounds and thus does not deliver information on the composition (i.e. P forms) of the microbial P pool. In general, the majority of P in microorganisms is present as P bound to DNA, RNA and phospholipids as shown by Vadstein (1998) for aquatic bacteria. Yet, cell internal P-forms and nutrient ratios are known to change with environmental conditions (Herbert, 1961). For example, some microorganisms can accumulate P as polyphosphate when exposed to P sufficient conditions (Hupfer et al., 2007). 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).

Thus, improved information on the cell internal P pools and nutrient ratios of soil microorganisms could give new insights and improve our understanding on how they react to P surplus or limitation. Nevertheless, this kind of investigation has so far been limited to microorganisms that grew in water or culture media. Similar analyses of soil microorganisms that actually grew in soil were hampered by the presence of soil particles, since most soil microorganisms are attached to soil surfaces and/or trapped within soil aggregates (Mills, 2003). The extraction of microbial cells from soil by density gradient centrifugation as described by Bakken and Lindahl (1995) is a way to overcome this obstacle and thus may facilitate chemical analysis of microbial cells (Oberson and Joner, 2005).

Dynamics of the microbial P pool

As mentioned above, the composition of the microbial P pool may be dynamic if it changes with the metabolic state of soil microorganisms. According to Sterner and Elser (2002) and Makino et al. (2003), not only P forms but also microbial P concentrations may change with microbial activity. According to the so-called growth rate hypothesis, the P content in microbial cells increases with increasing microbial activity (Elser et al., 2000). For example, 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. This has however not been checked for microorganisms that actually grew in soil. According to Sterner and Elser (2002), microorganisms are generally homeostatic. Koojiman (1995) defines the term homeostasis in a stoichiometric context as follows: ”The term homeostasis is used to indicate the ability of most organisms to keep the chemical composition of their body constant, despite changes in the chemical composition of the environment, including their food”. Accordingly, the nutrient ratios and P concentrations in soil microbial cells should not reflect the nutrient ratios and P concentration in the soil but should be rather fixed. In a metaanalysis of soil microorganisms analyzed by fumigation-extraction Cleveland and Liptzin (2007) approve this assumption. However, due to potential errors associated with the fumigation-extraction technique these results need further confirmation.

Soil microorganisms take up and immobilize P as they grow (Oehl et al., 2001; Bünemann et al., 2004). Whether microorganisms in soil grow or not can be explained by Liebigs’s “law of the minimum” (1840), which states that growth is controlled by the scarcest resource and not by the total amounts of nutrients that are available. As soil microorganisms are mainly heterotrophic, carbon (C) availability and degradability is generally the most important factor limiting microbial growth (Anderson and Domsch, 1985; Wardle, 1992; Tate, 2009).

Several studies have described that under sufficient C supply nitrogen (N) and/or P may become limiting for microorganisms (Nordgren, 1992; Duah-Yentumi et al., 1998; Joergensen and Scheu, 1999; Teklay et al., 2006). Whether it is N or P that is the second limiting factor (after C) depends on the microbial demand and the availability of each

nutrient in the respective soil. Thus, different soils show different limitation pattern. On highly weathered tropical soils with low amounts of available P and strong P sorption capacities P has been shown to be a major constraint for microbial growth (Cleveland et al., 2002; Ilstedt and Singh, 2005; Esberg et al., 2010). However, Bünemann et al. (2004) showed that in a Ferralsol with low P availability the microbial biomass can grow rapidly and increase its size substantially when readily degradable C is available.
The P immobilized in soil microorganisms can be mobilized (i.e. released into the soil solution) when soil microbes are dying. For instance, protozoa feeding on bacteria might cause a net release of P from the consumed microorganisms (Bonkowski, 2004). Furthermore a significant decrease in the microbial biomass may result from sudden changes in environmental conditions. For example, P bound in the microbial biomass is released when microbial cells are disrupted in response to freeze thawing (Chapin et al., 1978). Similarly, sudden changes in soil water content may lead to microbial cell lysis and a release of microbial P (Chepkwony et al., 2001; Turner et al., 2003).

Objectives and structure of this thesis

In this thesis the composition and dynamics of the microbial P pool in a Ferralsol from western Kenya with low amounts of available P were investigated. The overall aim was to improve the understanding of factors that influence the composition and the size of the microbial P pool. This knowledge is important as 80% of the land held by small-scale owners in western Kenya is extremely deficient in P (Sanchez, 2002) and new insights might lead to improved cultivation strategies adapted to this area and soil type. In order to enable analyses that might give a more precise picture of the microbial P pool than that derived by fumigation-extraction the method for extraction of microbial cells from soil by density gradient centrifugation was adapted to a Ferralsol in Chapter 1. In chapters 2 and 3, I turned towards the microbial P pool, microbial biomass and microbial activity as affected by different nutrient additions, and finally investigated the release of microbial P due to soil drying and rewetting in chapter 4.

In the first chapter the method for extraction of microorganisms from soil by density gradient centrifugation (Bakken and Lindahl, 1995) was adapted to the Ferralsol used in

this thesis. An adaptation to this soil type was necessary as highly weathered, clayey and acidic tropical soils like Ferralsols represent a challenge to the method due to very low cell yields (Maron et al., 2006). The main objective for the method adaptation was to develop a procedure that enables a reasonable cell yield combined with low contamination of the extracted microbial cells with soil particles. A further objective was to assess the representativeness of the extracted microbial community with respect to the soil microbial community.

The objective of the second chapter was to investigate the effect of microbial P limitation on respiration, microbial growth, community composition and P content of microbial cells in a Ferralsol as affected by amendments with C-substrates with ample N with and without extra P. In this context the method from chapter one was applied and delivered information on cell internal P pools.

The objective of the third chapter was to investigate if N and/or P fertilization in the field affects microbial nutrient pools and microbial N:P ratios. Furthermore the effect of N and/or P fertilization on microbial litter degradation, basal soil respiration and soil respiration after glucose addition was investigated in order to assess potential differences in microbial activity. Again, the method for extraction of soil microorganisms as adapted to the Ferralsol came to use, and N:P ratios of the extracted cells were assessed.

Finally, in the fourth chapter I turned towards studying the potential release of microbial P. The objective was to assess the size of flushes in available P in this soil type due to drying and rewetting. Furthermore, I investigated if the size of the P flush is affected by the soil inorganic P status or the size of the microbial P pool. The soil inorganic P status was raised by addition of inorganic P and the microbial P pool was increased by inducing microbial growth by a C and N addition. Labelling with ^{33}P was used in order to identify the source of drying and rewetting P flushes in this soil type.

In the general discussion the results of the four chapters are synthesized and conclusions are drawn.

Chapter 1: Extraction of soil bacteria from a Ferralsol

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Abstract

Extraction of intact bacteria from soil by dispersion and density gradient centrifugation may facilitate analyses of soil bacterial communities which are otherwise hampered by soil particles. Reasonable cell yield and representative fractions with sufficient purity can be extracted from most soils, but highly weathered, clayey and acidic tropical soils like Ferralsols represent a challenge to the method due to low cell yields. At an early stage of our studies with Ferralsols we also found substantial contamination of the extracted bacterial fractions by soil material, measured as total Al and Fe. We have adapted the method to a Ferralsol (pH 4.9, 64% clay, 21% silt, 15% sand) by factorial combinations of pH modification and salt (NaCl) concentration during soil dispersion, and evaluated the yield (microscopic cell counts), purity (optical density and Al + Fe content), and compared the composition of the extracted bacteria (phospholipid fatty acid analysis and 16S rDNA-denaturing gradient gel electrophoresis) versus that of the intact soil community. The cell yield was increased with a factor 2-3 by increasing the pH to 7.5, while it was decreased to a similar extent by adding NaCl (8 g l^{-1}). However, NaCl removed more than 99% of the Al + Fe contamination of the bacterial extracts, and the combination of modified pH and NaCl addition secured reasonable cell yield (4.6% of total number) and low contamination. The observed effects of pH and NaCl are probably due to changes in variable charge (by pH) and ion distribution (NaCl) around interacting particles (soil and bacteria), thus affecting their flocculation. Phospholipid fatty acid and denaturing gradient gel electrophoresis analysis indicate that the bias of the bacterial extracts compared to direct soil extracts increases with the addition of NaCl as well as by pH manipulation. Nevertheless, representativeness was acceptable as indicated by a Bray-Curtis similarity index (bacterial extracts versus soil) of 70% and 87% for phospholipid fatty acid profiles and denaturing gradient gel electrophoresis, respectively. Overall, the results reveal a trade-off between yield, purity and representativeness. Thus, depending on application and analyses, future users can choose the right treatment according to their specific purpose.

1. Introduction

Analyses of soil bacteria are often hampered by soil particles, since most soil bacteria are attached to soil surfaces and/or trapped within soil aggregates (Mills, 2003). The extraction of bacteria from soil by density gradient centrifugation as described by Bakken and Lindahl (1995) is a way to overcome this obstacle. The method has already been used for a wide range of purposes, e.g. to study heavy metals in and adsorbed onto soil bacteria (Sitaula et al., 1999), Zn and Cd tolerance of soil bacteria (Almas et al., 2004; Almas et al., 2005) as well as for determination of cell size by flow cytometric analysis (Christensen et al., 1994) and for detection of microorganisms by fluorescence in situ hybridization (Caracciolo et al., 2005; Bertaux et al., 2007). In addition, it can be used to obtain nucleic acids of soil bacteria for molecular genetic analysis (Bakken and Lindahl, 1995; Courtois et al., 2001; Bakken and Frostegård, 2006).

The extraction of bacteria by density gradient centrifugation comprises two steps. The first step is to detach bacteria from soil particles by physical or chemical dispersion or by a combination of both. The second step is the separation of detached bacteria from soil by high speed centrifugation over Nycodenz, a nonionic density gradient medium. Due to differences in buoyant densities, soil particles will sink through the Nycodenz to the bottom of the centrifugation tube, whereas bacteria will float on top of the Nycodenz and can be harvested by siphoning off the supernatant. Bacteria attached to soil particles will sink together with soil, hence an imperfect detachment of bacterial cells from soil results in low cell yield and representativeness. The extent of dispersion and detachment of bacterial cells depends on soil type and texture, with lower yields generally observed on soils with higher clay content (Bakken, 1985; Bakken and Lindahl, 1995). Yields in most studies vary between 10 to 20 % of the total number of bacteria in the soil (Bakken and Frostegård, 2006). For example, Maron et al. (2006) were able to extract 9% of all soil bacteria on a sandy soil from France (48% sand, 35% silt, 17% clay), but only 0.5% of the cells from a clayey, acidic, tropical forest soil (24% sand, 6% silt, 70% clay). The difficulties in extracting cells from such highly weathered, clayey, tropical soils are probably due to a combination of low pH and high contents of metal oxides and clay. According to Stotzky (1985) and Mills (2003), electrostatic interaction (caused by surface

charges) and van der Waals forces play a key role in the attachment of bacteria to soil surfaces. Furthermore, at low pH, surfaces with variable charge carry a net positive charge while with increasing pH the net charge turns negative. The pH at which the total number of positive charges equals the number of negative charges is referred to as the point of zero charge (PZC). Bacterial cells have a PZC in the region of pH 1.75 to 4.15 (Harden and Harris, 1953; Wicken, 1985). Therefore, the positive charge of some clays and metal oxides causes bacterial sorption (Mills, 2003).

A wide range of physical and chemical dispersion techniques have been used to promote bacterial detachment and thus to increase the yield of extracted bacteria. As for the physical dispersion, treatment in a Waring blender for 3 x 1 minute appears to provide reasonable cell yields, while minimizing mechanical cell damage (Lindahl and Bakken, 1995; (Bakken and Lindahl, 1995). Chemical agents to promote dispersion include ligand exchangers and chelating agents such as oxalate, citrate and EDTA (Katayama et al. 1997), Tris buffer (Niepold et al., 1979; Hopkins and O'Donnell, 1992), pyrophosphate (Lindahl, 1996) and the use of cation exchange resins (Hopkins and O'Donnell, 1992). The intended function of these chemical agents is to mask positive charges of clay minerals and to chelate multivalent cations in order to increase net repulsion, thus enhancing detachment of cells from soil particles (Niepold et al., 1979; Hopkins and O'Donnell, 1992) and hindering reattachment of cells already released (Bakken and Lindahl, 1995,).

The fact that Ferralsols, like other highly weathered, tropical soils, have a large proportion of variable charge on the mineral particles (Parfitt, 1980) could open for an alternative approach. Instead of masking positive surface charges with ligand exchangers, it is possible to change variable charges from positive to negative by increasing the surrounding pH (Parfitt, 1980). Thus, electrostatic repulsion between bacteria and soil particles should be increased by increasing the pH during soil dispersion in the blender. As a second factor, the ion density during soil dispersion could be modified. The ion density determines the thickness of the diffuse layer of ions that surrounds a charged surface. A low ion density increases the thickness of the diffusive double layer, making an attachment due to van der Waals forces less likely to occur (Loeb, 1985).

The aim of our study was to find an improved protocol for extraction of bacteria from highly weathered, clayey and acidic tropical soils. Our tentative criteria for an acceptable protocol were high cell yields, combined with acceptable purity and representativeness to allow a broad range of applications. In preliminary experiments, we tried some of the mentioned chemical dispersion agents, i.e. oxalate, citrate and EDTA (at 10 mM each) during dispersion of our Ferralsol in a Waring blender. None of the agents improved the cell yield significantly compared to the use of distilled water. The experiments further demonstrated that the suspensions of cells extracted from the Ferralsol were heavily contaminated with iron oxides. We hypothesized a differential effect of pH and ion concentrations on the repulsion between soil colloids versus that between bacterial cells and soil colloids, which would open for a selective enhancement of bacterial dispersion versus that of soil colloids. In total, four different dispersion treatments with a factorial combination of pH modification and ion density were tested. The estimated yields of extracted bacteria were based on epifluorescence direct counts. The purity was estimated by measuring the optical density and total Al and Fe content in the extracted bacterial suspension. Representativeness was assessed by analyses of phospholipid fatty acid (PLFA) and 16S rDNA-denaturing gradient gel electrophoresis (DGGE) patterns of the various cell extracts versus those of intact soil.

2. Materials and Methods

2.1. Soil

Soil was collected in August 2006 from 0-20 cm depth of a field trial site in Nyabeda, Western Kenya (altitude: 1420m, latitude: 0° 06’ N, longitude 34° 34’ E) under a maize-soybean crop rotation. The soil was stored at 4°C in moist condition (0.25 ml water g^{-1} soil dry weight) for two months before use. It 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). Organic C content measured with a CNS Analyzer (FlashEA 1112, Thermo Electron Corporation) is 22 g kg^{-1}.

According to X-ray diffraction (XRD) and Rietveld analyses (Jenkins, 2000), the soil contained 40% kaolinite, 18% quartz, 17% goethite, 3% potash feldspar, 2% hematite, 2% anatase, and 1% crandallite. Amorphous compounds accounted for 18%.
Oxalate extractable Fe and Al (McKeague and Day(1966) are 2.6 g kg^{-1} (± 0.04) and 1.3 g kg^{-1} (± 0.01), respectively. Dithionite-citrate-bicarbonate extractable Fe and Al (Mehra and Jackson, (1960) are 51.7 g kg^{-1} (± 0.51) and 3.8 g kg^{-1} (± 0.12), respectively.
Regarding the soil properties, the Ferralsol being used in our study is typical for a highly weathered, clayey and acidic tropical soil.

2.2. Extraction of bacteria from soil

Bacteria were extracted from soil by high speed centrifugation over Nycodenz (Nycomed Pharma, Norway), a nonionic density gradient medium, as described by Bakken and Lindahl (1995). Ten g of soil (fresh weight) and 200 ml of liquid were dispersed using a Waring Commercial Blender with 1 l glass containers, run at full speed (18 000 rev min^{-1}) for 3x1 min, with intermittent cooling on ice for 1-2 min to sustain low temperature. Aliquots of the soil suspension were then transferred to centrifugation tubes and the Nycodenz solution was placed underneath using a syringe with a 15 cm steel needle.
For centrifugation two different volumes were used. For the determination of yield as well as for the DGGE and PLFA analysis, 250 ml centrifugation tubes with 200 ml of soil suspension and 40 ml Nycodenz solution (0.8 g ml^{-1} H_2O) were used. The samples were centrifuged for 2 h (4°C) at 10000 *g* (Avanti J-25 Centrifuge, JS-7.5 swinging bucket rotor, both Beckmann Coulter International). For purity measurements, 50 ml centrifugation tubes with 30 ml of soil suspension and 8 ml of Nycodenz solution were used. This time centrifugation for 2 h at 4°C was performed at 15000 *g* with a JS-13.1 swinging bucket rotor (Beckmann Coulter International). The different *g*-forces (10000 *g* with 250 ml tubes, 15000 *g* with 50 ml tubes) did not affect the yield (data not shown), yet using the 50 ml centrifugation tubes proved to be more practical since the separation between phases was more distinct and reliable.
After centrifugation, we siphoned off and discarded the supernatant down to approximately 1 cm above the Nycodenz cushion. The remaining supernatant with bacteria floating on top of the Nycodenz phase was then harvested by siphoning off and

the extracted bacterial suspension was stored at 4°C. For PLFA and DGGE analysis the extracted bacteria were collected by filtering through polycarbonate filters (pore size 0.2 μm). Filters with bacteria were then stored at -20°C.

Four different soil dispersion treatments, a factorial combination of pH modification and salt concentration in the soil suspension (Table 2.1), were tested. In the water (W)-treatments (Wnon and Wmod), filter-sterilized ultrapure water was used as dispersion liquid, whereas in the salt (S)-treatments (Snon and Smod) a 0.8 % NaCl solution was used. The pH in soil suspension remained nonmodified in treatments Wnon and Snon while it was modified to pH 7.5 by adding 1 M NaOH in treatments Wmod and Smod. The amount of 1 M NaOH needed was determined in a separate titration experiment where the pH response after 10 minutes was measured in the soil suspension. This time span was chosen since it is approximately the time the dispersion of the soil in the Waring Blender takes. The extraction was performed in triplicate for each treatment.

Table 2.1: Modifications of the dispersion step in extraction of soil bacteria from a Ferralsol: Treatment abbreviations.

Treatment	Dispersion medium	pH modification
Wnon	H_2O	none
Snon	0.8% NaCl	none
Wmod	H_2O	raised to 7.5
Smod	0.8% NaCl	raised to 7.5

2.3. Bacterial counts

The numbers of bacterial cells in the soil and in the extracted bacterial suspension were quantified by acridine orange direct counts as described by Hobbie et al. (1977). The cell yield was calculated as number of extracted bacteria as per cent of the total number of bacteria in the soil.

2.4. Determination of purity

Purity was assessed by determining the optical density (OD), which provides an estimate of soil particle concentration in a suspension (Hesterberg and Page, 1990), and by measuring the Fe and Al content of the extracted bacterial suspension. The OD was determined as % absorbance at 530 nm wavelength compared to pure water using a spectrophotometer (UV-1601, Shimadzu). Fe and Al were measured by ICP-MS analyses after sodium hydroxide-persulfate digestion according to Ebina et al. (1983).

2.5. PLFA analysis

PLFA composition of the soil and extracted bacteria was analyzed to asses the representativeness of the extracted soil bacteria for the bacterial community in the soil. The PLFAs were extracted from soil and from extracted bacteria in three independent replicates for each treatment. 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).

The PLFA 18:2ω6 is considered a fungal biomarker (Vestal and White, 1989; Frostegård and Bååth, 1996; Klamer and Baath, 2004), whereas a15:0, i16:0, 16:1ω7t, 16:1ω5, i17:0, a17:0, 18:1ω7 and cy19:0 are all considered to be of bacterial origin (Tunlid and White, 1992; Frostegård et al., 1993; Frostegård and Bååth, 1996). Accordingly, the fungal:bacterial ratio was calculated by dividing the amount of 18:2ω6 by the sum of the bacterial fatty acids. Furthermore the PLFAs cy17:0 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, a17:0, i16.0, i17:0 and br18:0 as well as br17:0 were considered as indicators for Gram-positive bacteria (O´Leary and Wilkinson, 1988). In addition, 10Me17:0 and 10Me18:0 were used as indicators for actinomycetes (Kroppenstedt, 1985; Kroppenstedt, 1992).

2.6. DNA extraction, amplification and DGGE fingerprinting

DNA was extracted in three independent replicates from 0.5 g of soil and from extracted cells collected on polycarbonate filters using a soil DNA extraction and purification kit (FastDNA SPIN for Soil Kit from Q-BIOgene). DNA concentrations were measured with

a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies Inc.). Partial 16S rRNA gene was amplified by PCR using the eubacterial universal forward primers 8F-GC clamp (5'-cgcccgccgcgcgcggcgggcggggcgggggcacgggggg AGA GTT TGA TCC TGG CTC AG-3') and 518R (5'- ATT ACC GCG GCT GCT GG-3') (Hicks et al., 1992; Muyzer et al., 1993). A 25 µl reaction mixture contained 1 U of Promega Taq DNA polymerase (Promega Co.), 1× PCR buffer (Mg2+ free), 2 mM MgCl2, 10 pmol of each primer, 200 µM of each deoxynucleoside triphosphate (dNTP), and 20 ng of DNA. The samples were amplified in a thermocycler PCR system (GeneAmp PCR System, Applied Biosystems). PCR products were checked by agarose gel electrophoresis (0.8% wt/vol) stained by ethidium bromide and photographed by Bio-Rad Gel Doc 2000 (Bio-Rad Laboratories, Hercules).

PCR amplicons were separated on 8% (wt/vol) polyacrylamide gels with a denaturing gradient from 40% to 58% (100% denaturant corresponds to 7 M urea and 40% deionized formamide) using a Dcode System apparatus (Bio-Rad, Hercules). DGGE was run in 1 × TAE buffer at constant voltage (160 V) and temperature (60 °C) for 240min. Gels were stained by SYBR green (Amresco, Solon) for 1h and photographed by Bio-Rad Gel Doc 2000 (Bio-Rad Laboratories, Hercules). The DGGE gels were analyzed by Quantity One (version 4.4.0, Bio-Rad Laboratories, Hercules) using all the bands.

2.7. Statistical analysis

To analyze differences in microbial community composition between the treatments, principal component analyses (PCA) were performed on the PLFA and the DGGE data sets.

The PLFA composition was analyzed based on the relative abundance of individual PLFAs (mol% values). The PLFA 18:2ω6 was disregarded for PCA since it is considered to be a fungal biomarker (Vestal and White, 1989; Frostegård and Bååth, 1996). For the DGGE data, the relative intensity of individual bands (% of the summed intensity of all selected bands in a treatment) was used. The % values both for the PLFA and DGGE data sets were standardized before PCA analysis.

Similarity of the PLFA and DGGE profiles was analyzed using the Bray-Curtis coefficient. The Bray Curtis is a proportion coefficient for abundance data and was calculated as follows:

$$BC_{jk} = 1 - \frac{\sum_{i=1}^{n} \left| y_{ij} - y_{ik} \right|}{\sum_{i=1}^{n} \left(y_{ij} + y_{ik} \right)}$$

Where y is the relative band density (DGGE) or lipid concentration (PLFA), while i denotes the band/lipid identity across j and k samples (Bray and Curtis, 1957; (McCune and Grace, 2002)). The remaining statistical analyses were done using SYSTAT 11 (SYSTAT Software Inc. 2004). The impact of pH, NaCl and their interaction were analysed by a two way ANOVA followed by Tukey's multiple comparison test whenever significant differences (P<0.05) were indicated.

3. Results

3.1 Yield of soil bacteria

Extraction efficiencies (Table 2.2) are expressed as percent of the bacterial numbers in the dispersed soil suspension, which was $3.7*10^9$ g^{-1} fresh soil with a standard deviation (SD) of $1.3*10^8$. The highest yield (10.7 %) was obtained with high pH and no NaCl added (Wmod). The yields of the Wnon and Smod treatments did not differ significantly and were just below 5%. The Snon treatment had the lowest yield with extraction of 1.6% of total bacteria in soil. Thus, raising the pH to 7.5 increased the yield, whereas the addition of NaCl to the dispersion liquid had a lowering effect.

Table 2.2: Treatment effects on yield of extracted soil bacteria and on optical density (OD, 530 nm wavelength) and sum of Fe and Al in the extracted bacterial suspension.[a]

Treatment	Yield			OD_{530}			Sum of Fe and Al		
	% of total soil bacteria			%			mg l^{-1}		
Wnon	4.94	(0.07)	b	9.41	(4.23)	a	34.06	(12.3)	b
Snon	1.56	(0.14)	c	0.12	(0.06)	b	0.04	(0.07)	c
Wmod	10.65	(0.19)	a	9.33	(1.53)	a	65.13	(6.12)	a
Smod	4.61	(0.30)	b	0.33	(0.22)	b	0.05	(0.05)	c
Anova:									
pH	***			n.s.			**		
NaCl	***			***			***		
pHxNaCl	***			n.s.			**		

[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.

*** Indicates significance at $P<0.001$

** Indicates significance at $P<0.01$

n.s. No significance ($P\geq0.05$)

3.2 Estimation of impurities

We observed that extracted bacterial suspensions of the W-treatments had a brown-reddish color while the extracted bacterial suspensions of both S-treatments were practically colorless. Around 9 % of the light was absorbed in the W-treatments compared to less than 0.5% in the S-treatments (Table 2.2). The bacterial suspensions from the S-treatments contained low amounts of impurities (sum of Fe and Al below 0.1 mg l^{-1}), while the suspensions from the Wnon and Wmod treatments contained 34 and 65 mg l^{-1}, respectively. Thus, the amount of Fe and Al in the extracted bacterial suspension was grossly reduced by NaCl, while it was increased by raising the pH.

3.3 Representativeness of extracted bacterial communities

A PCA was performed using the relative abundance of the selected PLFAs to compare the PLFA composition of the four dispersion treatments and the soil (Figure 2.1). The fungal PLFA 18:2ω6 was excluded from this analysis. The first principal component (PC1) explained 40.1% and the second principal component (PC2) explained 27.6% of the total variation. The PLFA profile of the Wmod treatment was most similar to that of the soil while the salt treatments, Smod and Snon, were the least similar to the soil samples, as seen by the separation along PC2. The PLFA patterns did not reveal consistent treatment effect on broad bacterial groups such as Gram negatives and actinomycetes, as judged by their signature lipids. For example, the PLFA 18:1w7, which is representative of Gram negative bacteria, showed the highest relative abundance in the Smod treatment while the opposite was true for cy17:0, which is another PLFA representative of Gram negative bacteria (Figure 2.1B). Similarly, 10Me18:0, which is specific to actinomycetes, had the highest relative abundance in the Smod treatment, while 10Me17:0, also specific to actinomycetes, was most abundant in the samples from whole soil, Wnon and Wmod. PLFAs representative of Gram positive bacteria were also scattered in the loading plot, e.g. i16:0, i17:0 and br18:0 showed the highest relative abundance in the whole soil and in the Wmod treatments, while a15:0, a17:0 and br17:0 were most abundant in the Wnon and Snon.

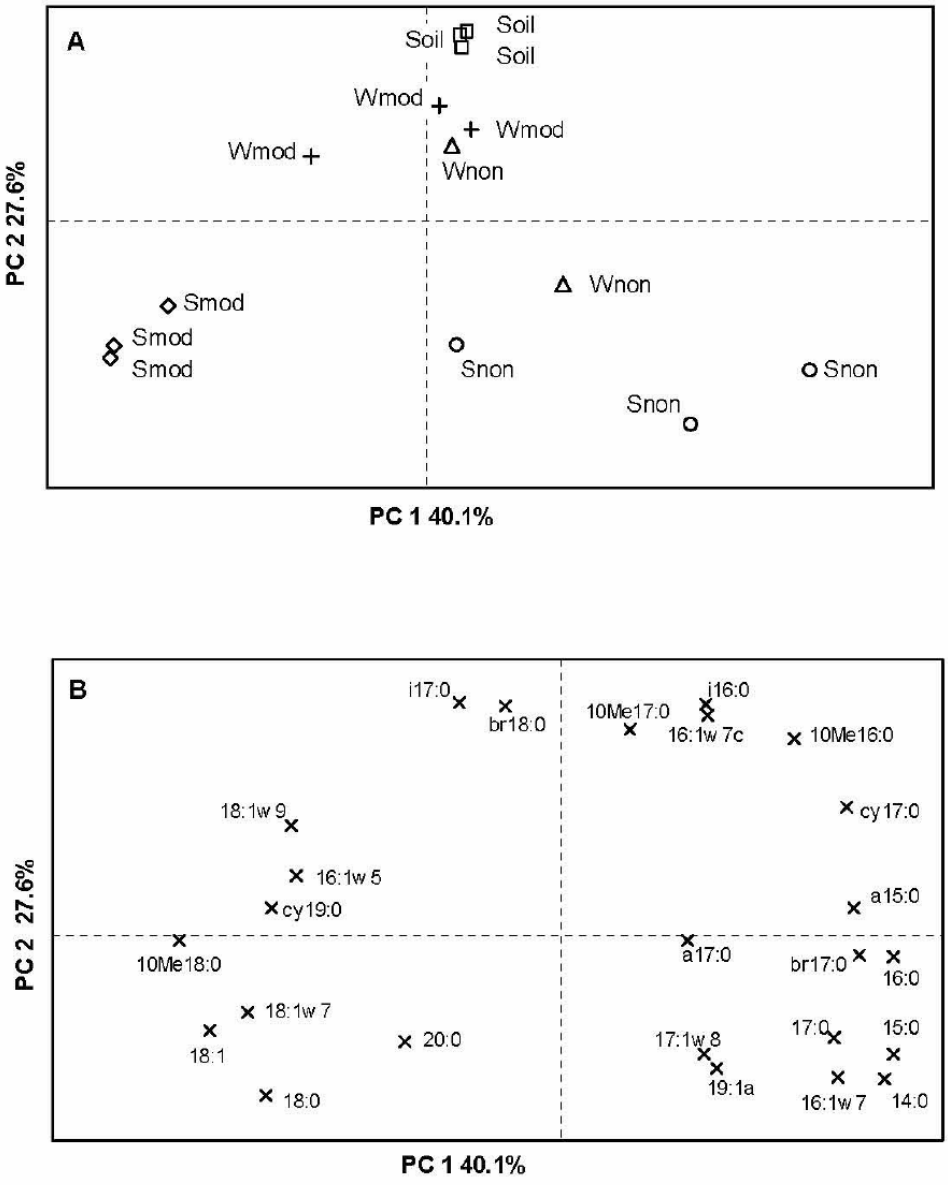

Figure 2.1: PCA generated from PLFA profiles from soil and from extracted bacteria. Profiles were obtained from three replicates except for the Wnon treatment (duplicates).

The Bray-Curtis coefficient (Table 2.3) showed that the Wmod treatment was closest to the soil (83% similarity) while the Smod treatment was the least similar (70%), thus corroborating the results from the PCA.

Table 2.3: Bray-Curtis similarity coefficients calculated for the PLFA profiles of the soil and of extracted bacteria (using 4 different extraction treatments).[a]

	Soil	Wnon	Snon	Wmod	Smod
Soil	0.99	0.77	0.73	0.83	0.70
Wnon		0.83	0.79	0.77	0.66
Snon			0.90	0.73	0.65
Wmod				0.93	0.72
Smod					0.90

[a] Coefficients were calculated on the basis of three replicates except for the Wnon treatment (duplicates).

The relative abundance of the fungal fatty acid 18:2ω6 was highest in both S-treatments with more than 4% (Table 2.4). However, the relative abundance of 18:2ω6 did not differ significantly between the different PLFA profiles due to the high variation. Likewise, the calculated fungal:bacterial ratio did not differ significantly between treatments, although there was a tendency for the ratios to be higher in the S-treatments.

Table 2.4: Relative abundance of the fungal signature fatty acid 18:2ω6 and fungal:bacterial ratio in the extracted bacterial suspensions and in soil.[a]

Treatment	18:2ω6 %			fungal:bacterial ratio		
Soil	1.48	(0.57)	a	0.03	(0.01)	a
Wnon	1.43	(2.02)[b]	a	0.06	[b]	a
Snon	4.50	(0.94)	a	0.11	(0.04)	a
Wmod	1.15	(0.08)	a	0.02	(0.00)	a
Smod	4.03	(4.15)	a	0.09	(0.10)	a
Anova: Treatment	n.s.			n.s.		

[a] All values are means of three replicates except for the Wnon treatment (duplicates), SD in brackets, values within a row followed by the same letter do not differ significantly ($P<0.05$) according to Tukey's test.
[b] 18:2ω6 was detected only in one of the two replicates of the Wnon treatment
n.s. No significance ($P\geq0.05$)

A photograph of the DGGE gel is shown in Figure 2.2. The number of detectable bands were 22 in both S-treatments, 24 in the Wmod and 27 in the Wnon treatments (all with a SD = 0). The results indicate that modifying the pH as well as using a 0.8% NaCl solution reduced the number of detectable bands. The number of detectable bands was significantly higher for the Wnon-treatment than for DNA extracted directly from soil (average = 25.7 bands, SD = 0.58), whereas the number of bands delivered by the other treatments was significantly lower.

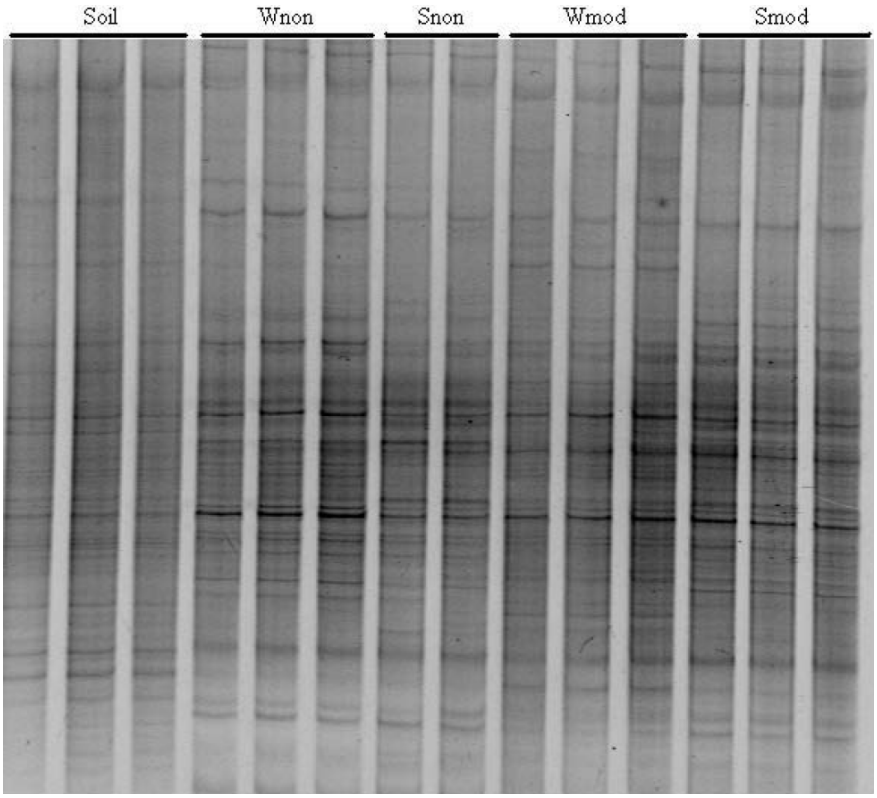

Figure 2.2: Photograph of the DGGE gel, showing three lanes for each treatment except for the Snon treatment (duplicates).

The results of the principal component analysis (PCA) of the DGGE data are shown in Figure 2.3.The first axis, which explained 42.0% of the variation, highlighted the difference between the Wmod treatment and the other treatments. The second axis (25.2% of variation) captured the effect of NaCl in the dispersion liquid (treatments Smod and Snon). The DGGE profile of the Wnon treatment was the one most similar to that of the soil bacterial community.

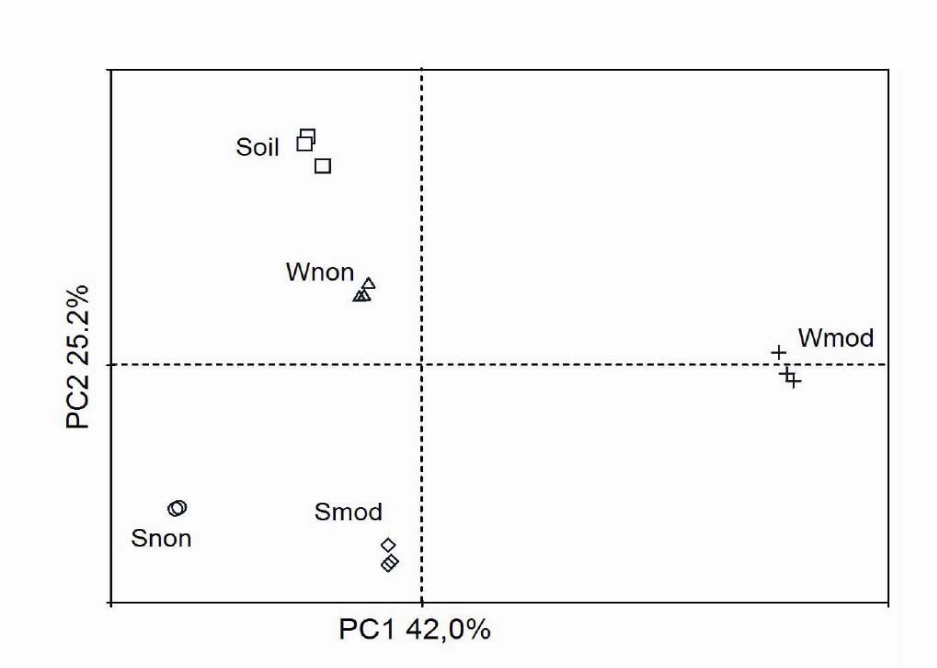

Figure 2.3: PCA generated from DGGE profiles from DNA extracted directly from soil and from extracted bacteria. Profiles were obtained from three replicates except for the Snon treatment (duplicates).

The Bray-Curtis coefficients showed similar trends as the PCA analysis (Table 2.5). The Wnon treatment was most similar to the soil (91%), whereas the Snon treatment was least similar. Nevertheless, similarity between Snon and soil was rather high (86%).

Table 2.5: Bray-Curtis similarity coefficients calculated for the DGGE profiles of DNA extracted directly from soil and of DNA from extracted bacteria (using 4 different extraction treatments). [a]

	Soil	Wnon	Snon	Wmod	Smod
Soil	0.98	0.91	0.86	0.87	0.87
Wnon		0.98	0.85	0.88	0.85
Snon			1.00	0.83	0.91
Wmod				0.97	0.88
Smod					0.98

[a] Coefficients were calculated on the basis of three replicates except for the Snon treatment (duplicates).

4. Discussion

4.1 Yield and purity

Electrostatic attraction caused by surface charges and van der Waals forces play a key role in the attachment of bacteria to soil surfaces. A variable charge on soil particles arises from the presence of amphoteric constituents, like crystalline and amorphous Fe-, Al- and Ti-oxides (Bolan et al., 1999). In the investigated Ferralsol, crystalline minerals containing iron (goethite and hematite), aluminum (kaolinite and crandallite) and titanium (anatase) account for 62% of all soil minerals. Additionally, the soil contained significant amounts of amorphous iron and aluminum oxides and hydroxides, as assessed by oxalate extraction. Amorphous compounds belong to the most reactive oxides in soil due to their high surface area (Loeppert and Inskeep, 1996). Apart from soil particles, bacteria are known to have a variable charge as well (Harden and Harris, 1953). Since the main factor determining the variable charge is the pH (Parfitt, 1980), the significant effect of pH modification on bacterial yield in the present study was probably caused by changing the variable surface charge of interacting particles (soil and bacteria).The PZC for bacteria ranges from pH 1.75 to 4.15 (Harden and Harris, 1953), for quartz it is around pH 2 (Bolan et al., 1999) and for kaolinite it ranges between 4.6 and 4.8 (Kretzschmar, 1997; Bolan et al., 1999). Therefore, it can be assumed that bacteria, quartz and kaolinite had a negative net charge in all treatments, no matter whether the pH was modified or not. Nevertheless the negative charge was most likely increased by raising the pH to 7.5. Parfitt (Parfitt, 1980) reported a PZC of 8.1 for a goethite. Therefore, it can be assumed that goethite had a positive net charge that decreased substantially when the pH was raised to 7.5. A similar effect can be expected for amorphous Fe- and Al-oxides and hydroxides, since Stumm and Morgan (1996) report a PZC of 8.5 for an amorphous $Fe(OH)_3$ and Hendershot and Lavkulich (1983) report a positive surface charge for Al-hydroxides at pH 6.0.

Thus, raising the pH to 7.5 will result in stronger electrostatic repulsion between bacteria and quartz and kaolinite, and in weaker electrostatic attraction between bacteria and goethite and amorphous Fe- and Al-oxides and hydroxides. This would explain why we observed higher cell yields in response to pH 7.5. The same mechanism probably caused

increased electrostatic repulsion between soil particles, which therefore enhanced soil dispersion and hence the detachment of soil bacteria trapped inside soil aggregates. It is thus not surprising that both cell yield and impurities increased after pH modification. Similarly, Frenkel et al. (1992) found an increased dispersion and decreased flocculation of kaolinite clay when increasing the pH.

The addition of NaCl to the dispersion liquid led to a moderate reduction of cell yield and a great reduction in impurities. This was most likely due to the higher ion strength leading to thinner diffuse double layers, hence enhancing flocculation of soil particles as well as reattachment of bacteria due to van der Waals forces (Bolan et al., 1999). During centrifugation these aggregates sank through the Nycodenz layer, sedimented at the bottom of the tube, and led to a decrease in yield and impurity.

Frenkel et al. (1992) observed the same effect of ion concentration on the dispersion and flocculation of clayey soils. Mills et al. (1994) and Gross et al. (1995) found a similar effect of ion density on the attachment of bacteria to particles, whereas Courtois et al. (2001) reported that cell yield was not affected by ion density in their study.

It is remarkable that NaCl addition reduced the contamination of soil particles in the extracted bacterial suspension to a greater extent than it reduced the cell yield. Especially at the high pH, the impurities were practically eliminated (OD reduced by 96%, content of Al and Fe by 99%) by NaCl addition, whereas cell yield was only reduced by 57%. Frenkel et al. (1978) reported that the ion density affects the dispersion and flocculation of different clay minerals differently. In a subsequent study Frenkel et al. (1992) showed that different clay minerals have different critical flocculation concentrations (defined as the NaCl concentration required to sediment 80% of the clay after standing for 24 h). Regarding our results, the physiological NaCl concentration used in the S-treatments caused mineral soil particles to flocculate almost entirely, whereas a considerable amount of soil bacteria evidently remained detached. Thus, in our study, the addition of NaCl promoted a selective removal of soil particles from the extracted bacterial suspension, as hypothesized. This represents a great improvement of the method of cell extraction from the Ferralsol, and is probably applicable for a range of other highly weathered tropical soils.

4.2 Representativeness

The PLFA profile of the Wmod treatment (11% yield) was the one most similar to the PLFA profile of the soil. In contrast, the DGGE profile of the Wnon treatment (5% yield) was the one most similar to the DGGE profile of the soil. Similarly, Maron et al. (2006) found that yield and representativeness are not necessarily related when describing representativeness by automated-ribosomal intergenetic spacer analysis.

One reason for the difference between results based on PLFA and DNA biomarkers could be that PLFA analyses are not specific to bacteria. They rather represent the entire microbial community. This was true also for the present investigation since several of the PLFAs that were included in the PCA analysis exist in both bacteria and fungi. In contrast, 16S rDNA based DGGE analyses target only bacteria. Direct extraction of DNA from soil is however afflicted with several biases, and therefore differences in DGGE profiles between soil DNA and DNA from extracted bacteria do not necessarily reflect a bias of the cell extraction. For example, some bacteria are difficult to lyse either due to morphological characteristics or because they are protected within cavities or soil aggregates. Furthermore, the DNA extracted from intact soil may contain extracellular DNA or be biased by selective adsorption of DNA to soil particles (Bakken and Frostegård, 2006). Drawbacks depend on the soil type and are usually more severe on highly weathered tropical soils with a high clay content (Frostegård et al., 1999; Maron et al., 2006).

Combining all obtained information given by PLFA and DGGE, the S-treatments were the ones with the lowest representativeness according to the PCAs and Bray-Curtis coefficients as well as the information given by the number of detectable bands. The Wmod treatment was the one closest to the soil with regard to the PLFA pattern, yet this was not the case for the DGGE profiles. Therefore, the Wnon treatment seems to be the recommendable treatment regarding representativeness of the bacterial community since its DGGE profile is the one most similar to that of the soil.

DGGE analyses detect only dominating species (i.e. those that represent ≥ 1% of the total community) (Muyzer et al., 1993). A higher number of detected bands for the Wnon treatment than for soil DNA is thus reflecting somewhat higher evenness. Furthermore, amplicons from more than one species may be hidden behind a single band and,

conversely, the same species may be represented by different bands (Heuer et al., 2001). Given these shortcomings of the DGGE analyses, they can only be expected to detect grossly biased cell extraction methods. The fact that neither the present nor previous studies have revealed such biased cell extraction by the Nycodenz methodology (Mayr et al., 1999; Courtois et. al., 2001) suggests a reasonably representative extraction of dominating broad bacterial groups. It 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).

The extraction by density centrifugation focuses on the extraction of bacteria, since recovery of fungal biomass is expected to be rather low (Bakken and Frostegård, 2006). Probably, the fungal hyphal structure is difficult to detach from soil particles. Therefore, the high relative abundance of 18:2ω6 in the extracted bacterial suspensions and the fact that the fungal:bacterial ratio did not differ significantly between the extracted bacterial suspensions and the soil is rather surprising. An explanation could be that significant amounts of single-cell fungal structures like yeasts and spores are recovered from the soil during the extraction. Nevertheless, the relative abundance of 18:2ω6 and the calculated fungal:bacterial ratio in the soil are similar to values found by Frostegård and Bååth (1996) in soils with low organic matter content, and Bünemann et al. (2004) found a comparable relative abundance of 18:2ω6 in a Kenyan Ferralsol.

4.3 Conclusions

Overall, the extraction of bacteria has been successfully adapted to a Ferralsol. However, there was a trade-off between the three main objectives yield, purity and representativeness. The Wmod treatment delivered the highest yield of soil bacteria and was most similar to the PLFA profile of the soil. The Wnon treatment provided a bacterial community with the highest apparent species richness (measured as the number of DGGE bands) and a DGGE profile closest to that of the soil. This treatment would probably be preferred for studies of bacterial community composition. The Smod treatment, however, still provided sufficient yield and representativeness in combination with a great improvement of purity. Accordingly, it might be preferred for studies on

nutrient composition of soil bacteria. The choice of extraction protocol clearly depends on the purpose of the study.

Chapter 2: Phosphorus limitation in a Ferralsol: Impact on microbial activity and cell internal P-pools

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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 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 0-20 cm depth of a field trial site in Nyabeda, Western Kenya (altitude: 1420m, 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 3.1). 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 3.1). 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.

Table 3.1: 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
H20	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 $NaH_2PO_4*2H_2O$ g^{-1} dry soil. All nutrients were added in solution.

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 four hours. The incubation robot also monitors the O_2 concentrations in the headspace which is useful to 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. Figure 3.1 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 17h 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 (Figure 3.2).

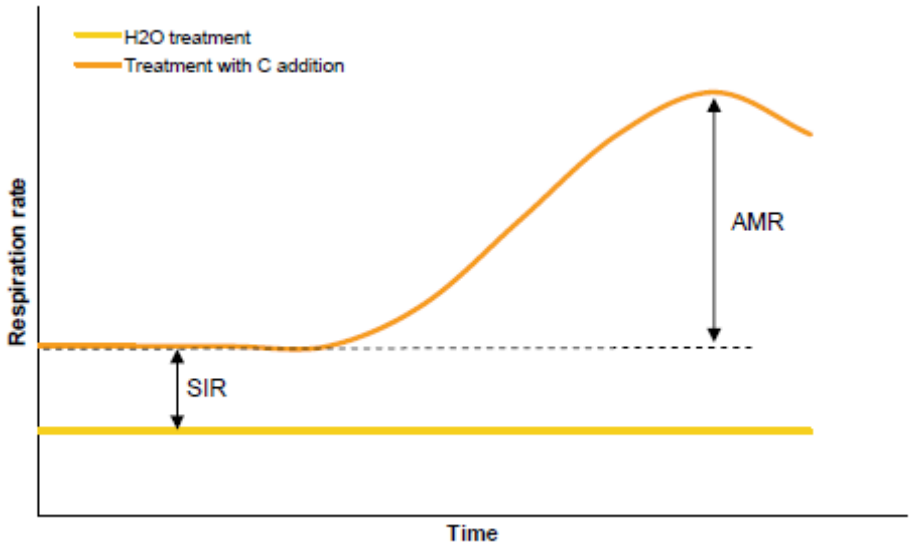

Figure 3.1: Schematic diagram explaining the calculation of substrate induced respiration (SIR) and additional microbial respiration (AMR).

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 3x1 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 10000 *g* (Avanti J-25 Centrifuge, JS-7.5 swinging bucket rotor, Beckmann Coulter International). After centrifugation, we siphoned off and discarded 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 (O´Leary and Wilkinson, 1988). In addition, 10Me17:0 and 10Me18:0 were used as indicators for actinomycetes (Kroppenstedt, 1985; Kroppenstedt, 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 *E.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 moles of PLFA there is 1 mole 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 transesterification of PLFAs, followed by digestion and measurement of P content as described in Chapter 2.7.

2.9. 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 Figure 3.2. Both the P and the H_20 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 3.2). 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.

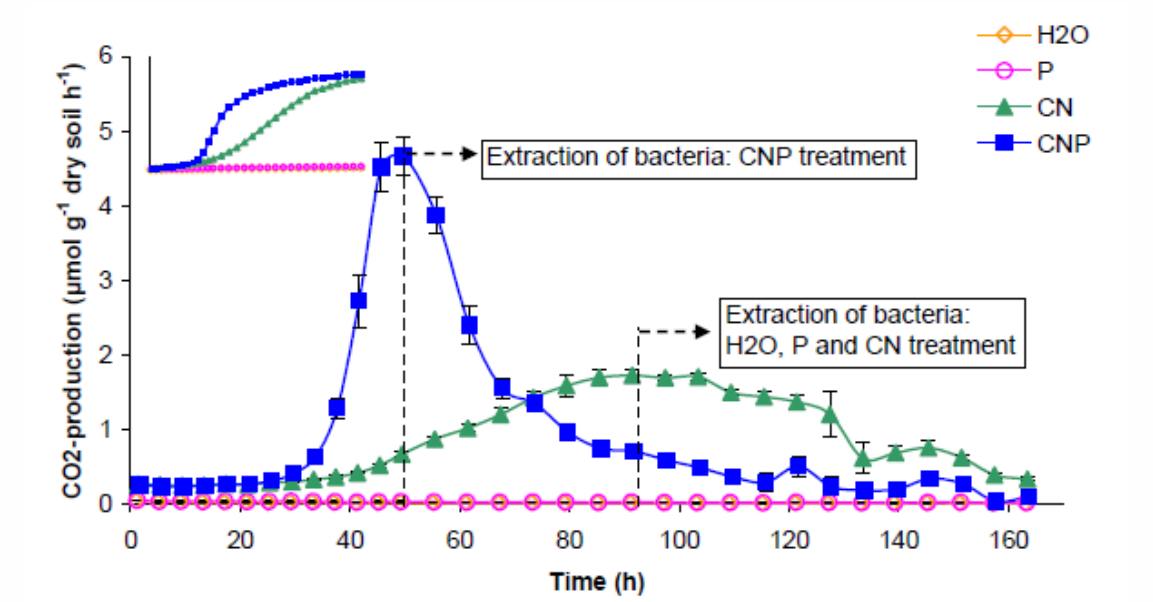

Figure 3.2: Respiration rates for the four treatments during the first 163 h of incubation. Error bars show the SD derived from three independent replicates. Arrows indicate the time of extraction of bacteria from soil for different treatments. The small figure in the upper left corner shows the cumulative CO_2 production.

Table 3.2: SIR, max. AMR, cumulative respiration of added C and the exponential growth rate μ of CN and CNP treatment. [a]

Treatment	SIR	Max. AMR	μ	Cum. respiration of added C until moment of max. AMR
	μmol CO_2 g^{-1} dry soil h^{-1}		h^{-1}	%
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

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 $6.4*10^9$ ($\pm 1.8 *10^9$) bacteria g^{-1} soil for all treatments.

3.3. PLFA analysis

The amounts of total, bacterial, fungal and unspecific PLFA in the soil and in the extracted cells are given in Figure 3.3. 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). Figure 3.3B 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.

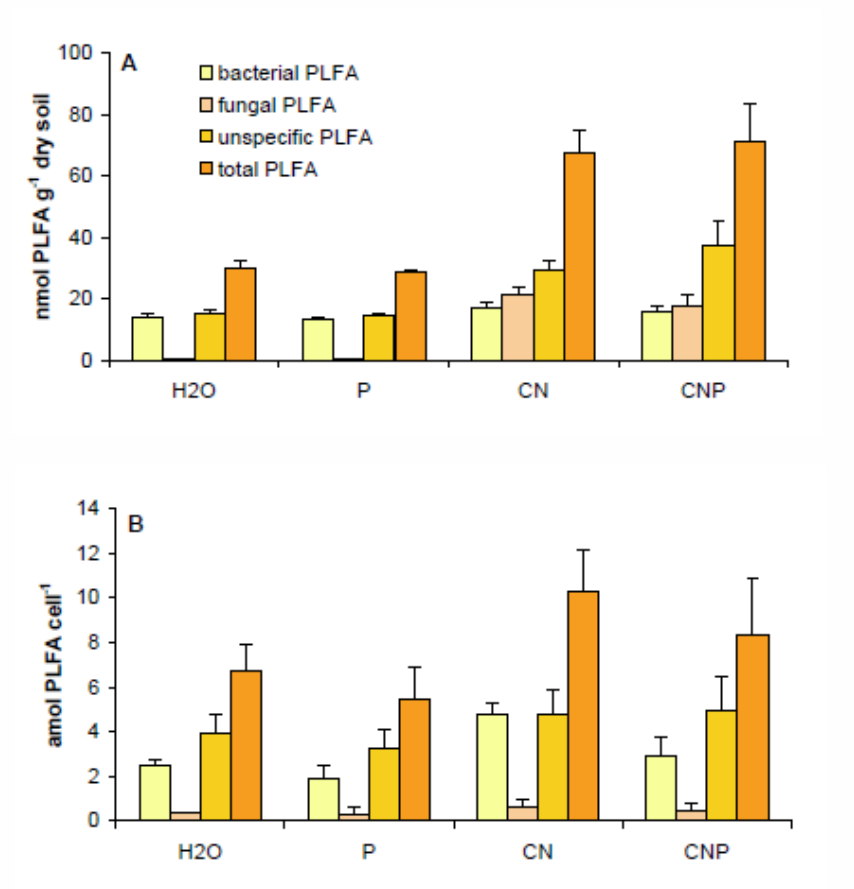

Figure 3.3: PLFA content in soil (A) and extracted cells (B). Error bars show the standard deviation from total amount of PLFA.

In the PCA of bacterial PLFAs (Figure 3.4), 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 (Figure 3.4A). 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 (Figure 3.4B). 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 a 15:0 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.

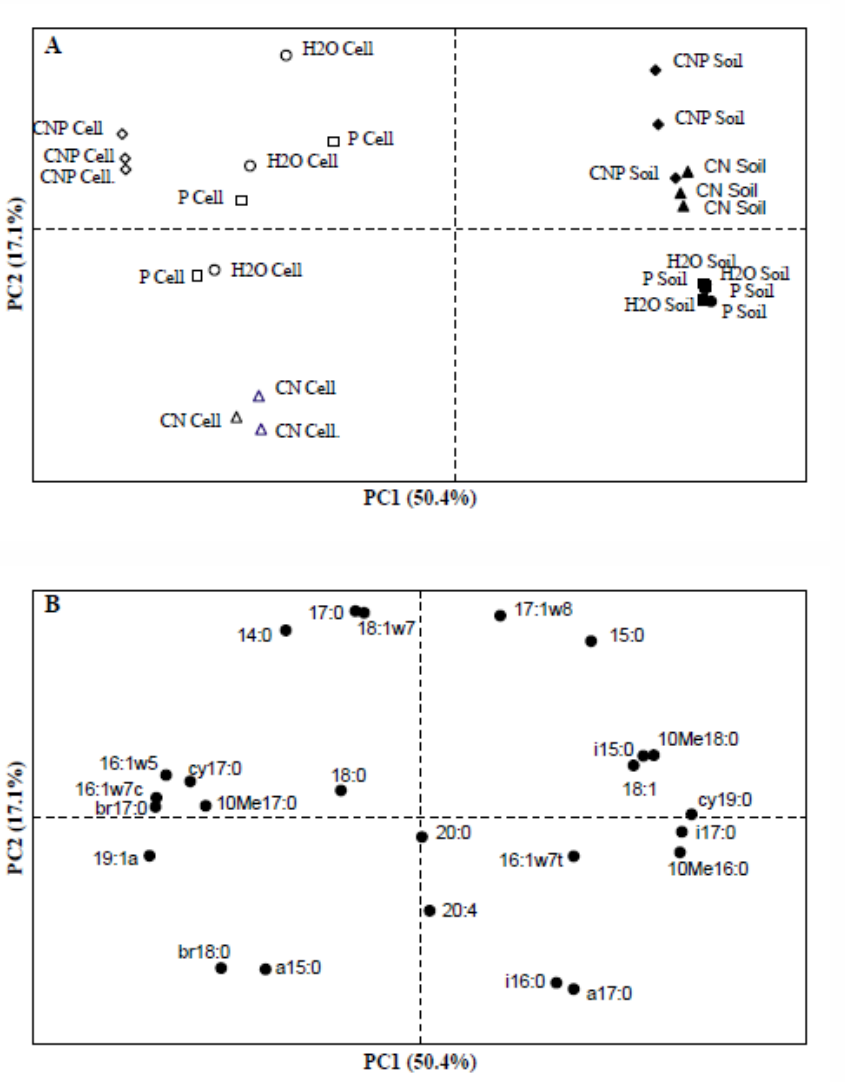

Figure 3.4: PCA for relative abundance of PLFAs in soil and in extracted cells. PLFAs considered to represent mainly fungal biomass (16:0, 18:1ω9 and 18:2ω6,9) were excluded from the analysis.

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 3.3. 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.

Table 3.3: P content, P pools and PLFA:total P ratio in extracted soil microorganisms. [a]

Treatment	total P			DNA-P			RNA-P			PLFA-P (meas.)			PLFA-P (calc.)			PLFA:total P [b]		
	----------			----------			fg cell^{-1}			----------			----------			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)

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 (91h 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 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 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 Frostegård, 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 to 8.9 fg $cell^{-1}$ seem to be within a reasonable range compared to 3.4 to 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; Elser et al., 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 a 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.

Chapter 3: Effect of nitrogen and phosphorus fertilization on microbial nutrient ratios, biomass and activity in a Ferralsol

Abstract

Soil microorganisms act as sink and source of nutrients and mediate key processes in the soil nutrient cycle. As soil microorganisms are affected by nutrient supply knowledge on the effect of agricultural nitrogen (N) and phosphorus (P) fertilization on soil microorganisms is important. We investigated how N and/or P fertilization in the field affects soil microorganisms in a highly weathered Ferralsol in terms of biomass, microbial nutrient pools, microbial nutrient ratios and activity. Soil microbial biomass was measured as microbial C content. The N and P pools in the soil microbial biomass were assessed by two different approaches: Fumigation extraction and cell extraction followed by subsequent analysis of N and P content. Microbial activity was characterized by basal soil respiration, soil respiration after glucose addition and litter degradation. For comparison with soil microorganisms, we analyzed the effect of fertilization on leaf nutrient concentration of maize and common beans. Soil microbial biomass, the microbial P pool, plant litter degradation and soil basal respiration were not influenced by fertilization. This indicates that under the C availability that is likely to occur in the field, soil microorganisms are neither N nor P limited. They seem, however, to be more sensitive to N than to P fertilization in this soil, as glucose induced respiration was accelerated and microbial N content was increased by N application. Maize N and P and bean P concentrations were affected by fertilization with the respective nutrient. Bean N concentration, however, was not affected by fertilization. This is probably due to fixation of atmospheric N. The N:P ratios of both plant species were positively correlated with soil total N:P ratios, while soil microbial N:P ratios were not affected by soil total N:P ratios and averaged around 11 ± 7 and 9 ± 2 for fumigation-extraction and extraction of cells, respectively. These data reinforce the proposition that plant nutrient concentrations depend on the nutrient concentrations in the surrounding environment, whereas microbial nutrient ratios are independent from N:P ratios of the surrounding environment and thus homeostatic. This is the first time that N:P ratios of extracted cells are reported on a per cell basis for microorganisms that actually grew in the soil and not in culture media.

1. Introduction

Soil microorganisms act as sink and source of nutrients and mediate key processes in the biogeochemical nutrient cycle. For a good understanding of microbiologically mediated processes, knowledge about microbial nutrient demand is important as a restricted nutrient supply of soil microorganisms can influence microbial biomass and limit microbial growth. Furthermore, a restricted nutrient supply can affect microbial activities such as litter decomposition (Güsewell and Gessner, 2009) and respiration (Stotzky and Norman, 1961).

As soil microorganisms are mainly heterotrophic, they first of all depend on carbon (C) availability and degradability before any other nutrients may become limiting (Anderson and Domsch, 1985; Wardle, 1992; Tate, 2009). However, exceptions do exist. For instance, Christensen et al. (1996) reported that C addition in form of glucose alone did not seem to induce microbial growth, while a combined C and nitrogen (N) addition did. Several studies have described that under sufficient C supply N and/or phosphorus (P) may become limiting. Whether it is N or P that is the second limiting factor (after C) depends on the microbial demand and the availability of each nutrient in the respective soil. Thus, different soils show different microbial limitation patterns (Nordgren, 1992; Duah-Yentumi et al., 1998; Joergensen and Scheu, 1999; Cleveland et al., 2002; Ilstedt and Singh, 2005; Teklay et al., 2006).

Nutrient supply may also affect the nutrient ratios within organisms. Plants for instance are known to reflect the nutrient availability in their surrounding environment within a certain range (Koerselman and Meuleman, 1996; Ziadi et al., 2007) and are thus considered to have a resource dependent elemental composition (Sterner and Elser, 2002). Aquatic and cultured microorganisms are however in general homeostatic (Sterner and Elser, 2002; Makino et al., 2003; Makino and Cotner, 2004). This means that their internal element ratios do not depend on the element ratios of the surrounding environment, including their food (Koojiman, 1995). For soil microbial C:N:P ratios as analyzed by fumigation extraction, Cleveland and Liptzin (2007) reported homeostasis. Due to potential errors associated with the fumigation extraction technique, these results need further confirmation.

The aim of this study was to investigate how agricultural N and/or P fertilization in the field affects soil microorganisms in a highly weathered Ferralsol in terms of biomass, microbial nutrient pools, microbial nutrient ratios and activity. The effect of fertilization on the microbial nutrient pools was examined using two approaches. The first one was based on fumigation extraction, a method to determine microbial nutrient contents on a per g of soil basis (Brookes et al., 1982; Hedley and Stewart, 1982; Vance et al., 1987). The second approach was to extract microbial cells from soil by density gradient centrifugation (Bakken and Lindahl, 1995) and to analyze their nutrient contents subsequently. This approach has previously been used to report cellular P contents (Ehlers et al., 2010) and is now used for the first time to report cellular N contents of microbial cells that actually grew in the soil and not in culture media. For comparison we also analyzed the effect of fertilization on nutrient concentration of crops growing in the field. Microbial activity was characterized by basal soil respiration, soil respiration after glucose addition and litter degradation. Basal respiration and respiration after glucose addition were measured in a lab incubation experiment while litter degradation was assessed under field conditions with buried litter bags. Furthermore we investigated the effect of fertilization on soil nutrient pools.

We expected neither microbial biomass nor nutrients concentrations nor nutrient ratios to be affected by N and/or P fertilization. In contrast, we expected plant nutrient concentrations and nutrient ratios to be strongly affected by fertilization. We assumed that N and/or P fertilization would not affect soil basal respiration but hypothesized that after glucose addition differences between treatments would become apparent. For plant litter degradation, we expected a stimulation of microbial activity by enhanced nutrient supply as caused by fertilization.

2. Materials and Methods

2.1. Field trial

The experiments were done on a field trial site in Sidada, Western Kenya. The mean annual rainfall is 1800 mm and occurs in two seasons. The long rainy season is from

March to August and the short rainy season from September to January (Kihara et al., 2010). Our investigations took place under a maize (*Zea mays*) –common bean (*Phaseolus vulgaris*) row intercropping system from September to December (short rainy season) in 2008. The soil is classified as a Ferralsol (FAO/ISRIC/ISSS, 1998), contains 51% clay, 26% silt and 24% sand and has a pH (H_20) of 5.8.

The field trial has already been running under the following conditions for 3.5 years (7 cropping seasons) and has been set up and managed by the African Network for Soil Biology and Fertility (Afnet) in collaboration with the Tropical Soil Biology and Fertility Institute of the International Center for Tropical Agriculture in Nairobi, Kenya.

The field trial contains a two factorial fully randomized design of N and P fertilization with three replicates (plot size 6mx4.5m). Accordingly, we had four treatments (treatment names in brackets): One without P and N fertilization (-P/-N), one with P but without N fertilization (+P/-N), one without P but with N fertilization (-P/+N) and one with both P and N fertilization (+P/+N). Phosphorus fertilization was applied as triple superphosphate at a rate of 60 kg P ha^{-1} per growing season (120 kg ha^{-1} $year^{-1}$). Nitrogen fertilization was applied as urea at a rate of 60 kg ha^{-1} $season^{-1}$. Whereas the P fertilization was applied entirely at planting, the N fertilization was split into two doses. A dose of 20 kg N ha^{-1} was applied at planting and the remaining 40 kg N ha^{-1} five weeks after planting. All treatments received a seasonal potassium fertilization of 60 kg K ha^{-1}. Fertilizer applications were banded in a line right next to the plant rows. Figure 4.1 depicts in a schematic manner the activities that took place in the field during the short rainy season 2008.

2.2. Soil and plant sampling

Soil and plant samples were taken on November 3, 2008 (see Figure 4.1). Maize plants were at the 12^{th} leaf stage (vegetative stage). Beans were about 30-40 cm high and also in vegetative stage. For both species the topmost fully developed leaf was sampled from 5 randomly selected plants per plot. For each species, the leaves from one plot were pooled, dried at 65°C for 72 h and milled. Soil samples were taken from the topsoil (0-15 cm). For each plot, ten samples were randomly taken with an auger and merged to one composite sample per field replicate.

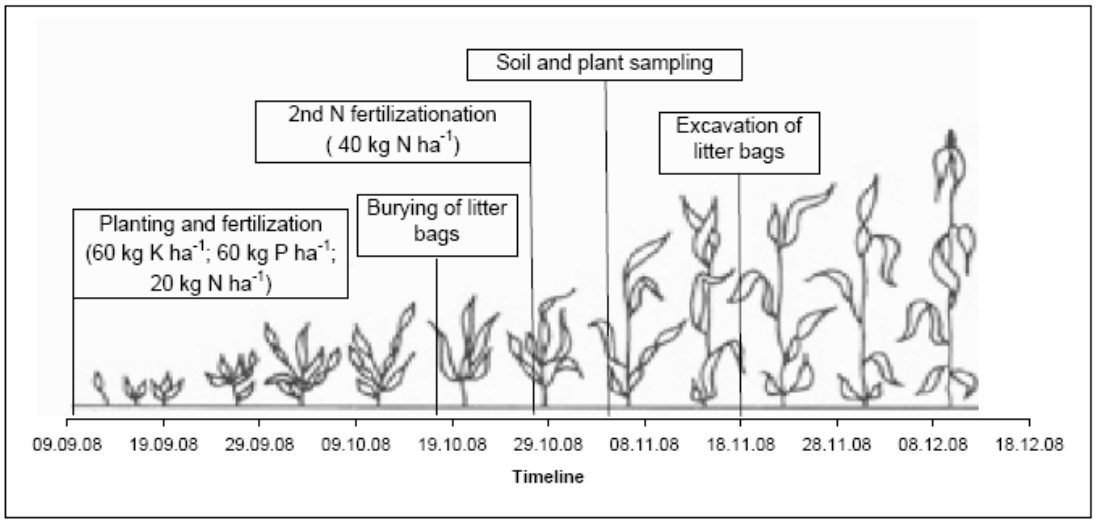

Figure 4.1: Schematic timeline showing the activities taking place in the field.

2.3 Soil and plant C, N and P analysis

Analysis of microbial nutrients, mineral N and $NaHCO_3$-Pt started within 24 h after sampling. For determination of soil total nutrient concentrations samples were frozen (-20°C) and processed within 4 weeks. Soil total C and N (Ctot, Ntot) concentrations as well as plant C and N concentrations were analyzed with a CNS elemental analyzer. Soil total P (Ptot) concentration was determined after H_2SO_4/H_2O_2 digestion (Anderson and Ingram, 1993). Plant P concentration was analysed after ashing plant samples (4h at 550°C) and solubilizing the ash in 20% HCl. Microbial biomass C and N in the soil (Cmic, Nmic) were determined by chloroform fumigation and extraction with 0.5 M K_2SO_4 according to Vance et al. (1987). Soil mineral N (Nmin) was determined colorimetrically as the concentration of NH_4^+ and NO_3^- in the non-fumigated samples were extracted with 0.5 M K_2SO_4. Soil microbial biomass P (Pmic) was measured by chloroform fumigation and extraction as described by Brookes et al. (1982). The extract was digested with ammonium persulphate oxidizing solution (50 g $(NH_4)_2S_2O_8$ to 500 mL of 1.2 M H_2SO_4). Recovery of Pmic was corrected for inorganic P sorption during extraction determined by using an inorganic P spike of 10 mg P kg^{-1} soil. No conversion factor (to account for incomplete cell lysis) was used. $NaHCO_3$-Pt was determined as the amount of P solubilized by 0.5 M $NaHCO_3$ in the non-fumigated samples. Again, the

extract was digested with ammonium persulphate oxidizing solution and thus $NaHCO_3$-Pt reflects the sum of organic and inorganic P solubilized by 0.5 M $NaHCO_3$. P concentrations in the extracts were always determined colorimetrically.

2.4. Respiration measurements

Field moist soils (0.25 g H_2O g^{-1} soil) were stored at -20°C for two weeks before incubation. Freezing of soils is likely to cause lysis of some cells but is nevertheless a recommendable storage form (Stenberg et al., 1998). 24 h prior to incubation the soil was unfrozen. Soil samples were incubated at 60% water holding capacity (0.35 g H_2O g^{-1} soil) at 20°C. Soil respiration was determined by trapping the released CO_2 in NaOH, followed by titration with HCl as described by Alef (1995). The amount of CO_2 evolved was measured after 1, 3, 7 and 14 days. For the substrate induced respiration, 2.5 mg C g^{-1} soil were added to the soil as glucose in liquid form. Whereas soil basal respiration reflects the amount of CO_2 produced without substrate addition, substrate induced respiration (SIR) was calculated as the respiration after addition of glucose minus the basal soil respiration of the respective treatment. Apart from the four field treatments we measured SIR for a fifth treatment called ample NP. For this treatment, soil from the +P/+N field treatment received an addition of 0.5 mg N g^{-1} soil (as NH_4NO_3) and 0.1 mg P g^{-1} soil (as KH_2PO_4) before incubation for basal respiration and SIR, respectively.

2.5. Litter bag experiment

Senescent maize leaves harvested at the end of the previous season (nutrient concentrations per kg dry matter: 411.5 g C; 6.71 g N; 0.51 g P) were chosen as substrate for the litter bags. After air drying for 10 days, the material was cut into pieces of 0.1-0.2 g. Subsamples of 2 g (± 0.01 g) were packed into polyamide litter bags (0.7 x 0.25 mm^2 mesh) with an inner compartment of 9 x 9 cm^2 and sewed with nylon thread. Initial dry mass (1.88 g bag^{-1}) was determined by drying the material at 65°C for 72 h. Four litter bags per plot (12 per treatment) were buried at 5 cm soil depth on October 17, 2008. After 30 days the bags were dug out. Litter bag content was carefully cleaned from extraneous material (e.g. roots and soil particles) and then dried for 72 h at 65°C. As the cleaning of litter bag content took one week, samples were frozen (-20°C) and only

defrosted for the time of cleaning to avoid further litter degradation. Despite cleaning, some smaller soil particles are likely to contaminate the litter bag content. In order to handle contamination with soil particles, weight loss on ignition (4 h at 550°C) was analyzed and the initial and final ash content was used to convert data on dry mass loss into data on organic matter loss in percent (Harmon et al., 1999) as follows:

$$OMloss(\%) = \left[1 - \left(\frac{DWf - AWf}{DWi - AWi}\right)\right] * 100$$

With *OMloss* being the loss of organic matter (%) during the time in the field, *DWi* being the initial dry weight, *AWi* being the initial ash weight and *DWf* being the final dry weight (in the excavated sample) and *AWf* being the final ash weight.

2.6. Extraction of cells from soil

Soil samples were stored for two months at -20°C and were then incubated for 30 days at 25°C and 60% WHC prior to cell analysis. 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 followed Bakken and Lindahl (1995) after increasing the pH in the soil solution to pH 7.5 and dispersing the soil in 0.8% NaCl solution in order to obtain optimal extraction conditions for Ferralsols (Ehlers et al., 2008). Cell counts of extracted cells were performed in order to allow calculation of N and P content per cell basis. Cells counts were done by acridine orange direct counts as described by Hobbie et al. (1977). The extracted cells were digested by autoclaving at 120°C in an oxidizing solution (0.074 M $K_2S_2O_8$; 0.075 M NaOH) (Ebina et al., 1983). Nycodenz contains N and might stick to the filter and cells. This N contamination thus has to be taken into account when measuring microbial N content in extracted cells. As Nycodenz N is combined with iodine in the same molecule, we developed an approach to correct for Nycodenz N contamination by making a standard curve of pure Nycodenz samples and measuring iodine and N. By measuring iodine in the extracted cells, we were thus able to estimate the amount of N derived from Nycodenz. This approach is novel and enables N content measurements of extracted soil microbes for the first time. As a prerequisite, however, we have to assume that iodine content in

extracted microorganisms is negligibly low. Iodine was measured by ICP and N and P were measured colorimetrically.

2.7. Statistical analysis

Data were analyzed by a two way ANOVA with the factors N fertilization, P fertilization and their interaction using SYSTAT 11 (SYSTAT Software Inc. 2004). As we did not have a two factorial design in the SIR measurements, these data were analysed by a one way ANOVA with the factor treatment followed by Tukey's pairwise comparison. In the text data are presented as average ± standard deviation unless otherwise stated.

3. Results

3.1. Soil nutrient pools

Effect of P fertilization on soil total P and C pools was highly significant, while there was no effect on soil Ntot and Nmin (Table 4.1). The $NaHCO_3$-Pt was increased by P fertilization by about one third from around 20 mg P kg^{-1} soil in the –P treatments to more than 30 mg P kg^{-1} soil in the +P treatments. The N fertilization affected Ctot and Ntot pools and but not Ptot and $NaHCO_3$-Pt. The N fertilization more than doubled Nmin concentration from around 6 mg N kg^{-1} soil in the –N treatments to at least 14 mg N kg^{-1} soil in the +N treatments.

3.2. Microbial nutrient pools

Microbial nutrient pools in soil (measured by fumigation-extraction) and per microbial cell (measured after cell extraction and subsequent analysis) are reported in Table 4.2. Nmic and cellular N (cell N) content were the only microbial nutrient pools that were significantly affected by the fertilization regime. Cell N content was doubled by N fertilization but unaffected by P fertilization. Nmic ranged from 15.12 to 38.16 mg N kg^{-1} soil and was significantly affected by N and P fertilization, with a positive significant interaction between the two factors.

Table 4.1: Effect of fertilization treatments on soil nutrient pools. [a]

Treatment	Ctot		Ntot		Ptot		Nmin		$NaHCO_3$-Pt	
	mg kg^{-1} soil									
-P/-N	20230	(135)	1960	(44)	681.1	(29.2)	6.67	(2.29)	18.89	(0.98)
+P/-N	19670	(335)	1935	(40)	913.0	(72.6)	5.64	(0.10)	30.91	(6.21)
-P/+N	20798	(364)	2049	(58)	690.3	(12.8)	17.26	(9.86)	22.60	(0.84)
+P/+N	20131	(205)	1972	(38)	873.3	(72.7)	14.45	(8.41)	31.85	(3.09)
Anova	p	SE	p	SE	p	SE	p	SE	p	SE
N	0.012	112	0.043	18.8	0.635	22.0	0.034	2.69	0.286	1.44
P	0.005	112	0.091	18.8	<0.001	22.0	0.627	2.69	0.001	1.44
N x P	0.745	159	0.357	26.6	0.454	31.1	0.820	3.80	0.516	2.04

[a] All values are means of three replicates. Standard deviation in brackets. SE is the standard error. Nmin is the concentration of NH_4^+ and NO_3^- extracted from soil with 0.5M K_2SO_4. $NaHCO_3$-Pt is the concentration of organic and inorganic P extractable with 0.5 M $NaHCO_3$.

Table 4.2: Effect of fertilization treatments on microbial nutrient pools.[a]

Treatment	Cmic		Nmic		Pmic		Cell N		Cell P	
	mg kg^{-1} soil						fg $cell^{-1}$			
-P/-N	181.0	(11.5)	17.7	(2.3)	4.5	(1.4)	6.5	(2.1)	1.9	(0.1)
+P/-N	185.8	(11.0)	19.2	(0.5)	6.4	(2.7)	5.9	(2.5)	1.8	(0.9)
-P/+N	189.4	(4.9)	19.6	(2.3)	3.4	(0.8)	10.7	(3.3)	2.6	(0.6)
+P/+N	174.6	(13.7)	31.7	(5.8)	5.2	(3.3)	12.1	(5.4)	2.2	(0.9)
Anova	p	SE	p	SE	p	SE	p	SE	p	SE
N	0.829	4.4	0.006	1.4	0.415	0.8	0.035	1.45	0.200	0.28
P	0.442	4.4	0.008	1.4	0.211	0.8	0.847	1.45	0.495	0.28
N x P	0.153	6.2	0.025	1.9	0.952	1.2	0.651	2.05	0.645	0.39

[a] All values are means of three replicates. Standard deviation in brackets. SE is the standard error. Cmic, Nmic and Pmic were obtained using fumigation extraction with chloroform. Cell N and cell P were determined on microbial cells extracted from soil.

3.3. Litter degradation, basal respiration and SIR

Around 40% of the organic material in the litter bags was mineralized within 30 days in the field, with no significant differences between treatments (Table 4.3). Similarly, fertilization did not significantly affect basal soil respiration.

Table 4.3: Effect of fertilization treatments on litter degradation and soil basal respiration. [a]

Treatment	Litter degradation		Basal respiration	
	% dry weight loss of added org. matter		µmol CO_2 g^{-1} soil day^{-1}	
-P/-N	39.1	(1.6)	0.277	(0.126)
+P/-N	42.2	(9.3)	0.329	(0.066)
-P/+N	42.3	(1.9)	0.273	(0.053)
+P/+N	41.0	(1.6)	0.368	(0.088)
Anova	p	SE	p	SE
N	0.729	2.0	0.734	0.084
P	0.757	2.0	0.185	0.084
N x P	0.473	2.8	0.691	0.118

[a] All values are means of three replicates. Standard deviation in brackets. SE is the standard error. Litter degradation was measured with litter bags filled in the field. Basal soil respiration was measured in a lab incubation experiment over a period of 14 days.

One day after incubating the soil samples with glucose, all treatments showed the same SIR (Figure 4.2). After 3 and after 7 days, though, SIR was significantly higher with than without N fertilization. Furthermore, at these two dates the addition of ample amounts of N and P additionally increased SIR. For instance, after 3 days of incubation SIR in the ample NP treatment was almost twice as high as in field treatments. After 14 days, however, differences in SIR had disappeared and 30.8% (±0.6%) of added glucose-C had been respired in all treatments.

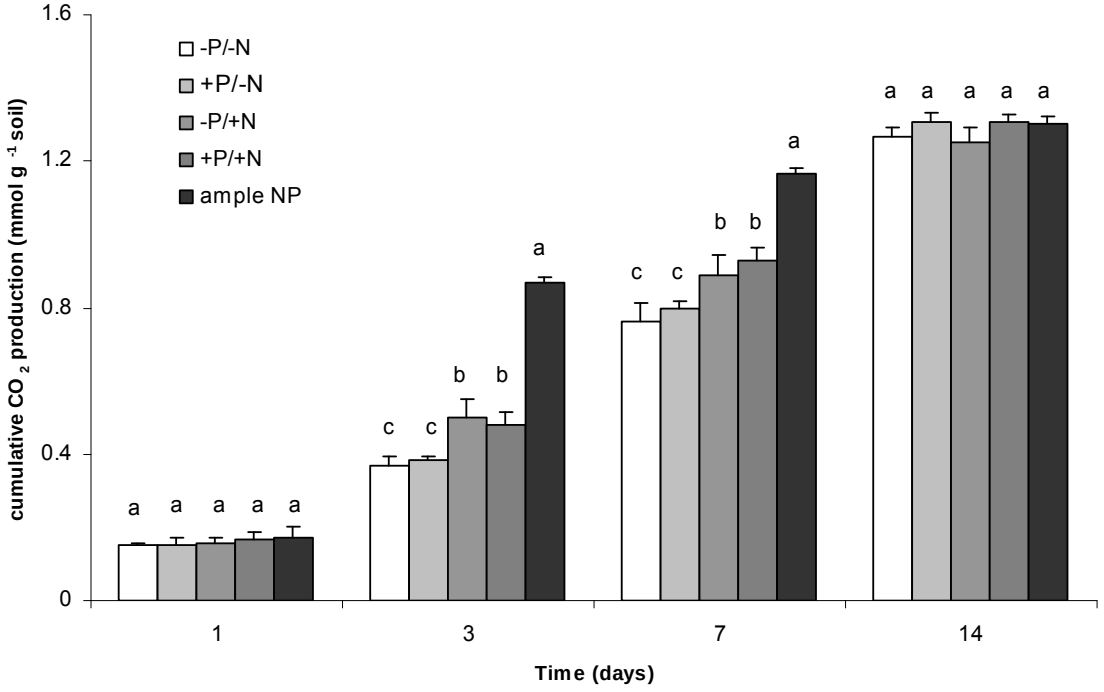

Figure 4.2: Substrate induced respiration due to glucose addition (2.5 mg^{-1} C g soil) in the four field treatments (-P/-N,+P/-N,-P/+N, +P/+N) and a fifth treatment (ample NP) with soil from the +P/+N treatment after additional amendment of 0.5 mg^{-1} N g soil and 0.1 mg^{-1} P g soil. Within each day, respiration bars with the same letter do not differ significantly (p>0.05) according to Tukey's test.

3.4. Nutrient concentrations in plant tissue

Nitrogen fertilization increased N concentration in maize leaves from 17 to 26 g N kg^{-1} leaf dry matter (Table 4.4). In contrast, N concentration in bean was 28 g N kg^{-1} leaf dry matter or more, irrespective of the N treatment. Phosphorus fertilization increased P concentrations of both plants by at least 20%.

Table 4.4: Effect of fertilization treatments on plant N and P concentration.[a]

Treatment	Maize N		Maize P		Bean N		Bean P	
	g^{-1} kg plant dry matter							
-P/-N	17.6	(1.4)	1.6	(0.1)	28.5	(4.3)	2.0	(0.1)
+P/-N	16.0	(1.0)	2.8	(0.2)	31.5	(1.7)	2.6	(0.1)
-P/+N	28.8	(0.8)	1.7	(0.1)	37.4	(4.3)	2.2	(0.4)
+P/+N	24.2	(6.1)	2.4	(0.5)	31.2	(7.8)	2.6	(0.3)
Anova	p	SE	p	SE	p	SE	p	SE
N	0.001	1.3	0.517	0.1	0.177	2.0	0.586	0.1
P	0.134	1.3	0.001	0.1	0.590	2.0	0.013	0.1
N x P	0.440	1.9	0.208	0.2	0.152	2.9	0.764	0.2

[a] All values are means of three replicates. Standard deviation in brackets. SE is the standard error. Leaf tissue was sampled 5 weeks after planting. Both plants were still in vegetative stage.

4. Discussion

4.2 Fertilization effects on soil microorganisms

Microbial N was sensitive to the soil nutrient status as affected by N and P fertilization, yet microbial C and P contents were not influenced. Microbial activity as characterized by basal soil respiration and litter degradation was not affected by fertilization treatments. Similarly, fertilization did not influence other eco-physiological indicators like the metabolic quotient, calculated as the amount of CO_2-C evolved per mg Cmic and hour (Anderson and Domsch, 1985) or the microbial quotient, calculated as the ratio of Cmic to Ctot (Anderson and Domsch, 1989) (data not shown). Accordingly, it seems that under the C availability given in the field soil microorganisms face neither N nor P limitation in this Ferralsol.

The SIR was always one order of magnitude higher than soil basal respiration, showing that regardless of the fertilization treatment the first limiting factor for microbial activity was C availability. After overcoming C limitation by glucose addition, however, other nutrient limitations became obvious after three and seven days of incubation. The higher

respiration in the treatment with addition of ample amounts of N and P shows that in all field treatments SIR was still limited by N and/or P. Obviously the dose of N and P fertilization in any field treatment was not high enough to meet microbial requirements after glucose addition. It has to be kept in mind, however, that the glucose addition increased soil total C content by more than 12% and that glucose represents a high quality C source that is immediately available and is thus not representative for C sources soil microbes are likely to face in the soil. Within the four field treatments, SIR was increased only by N and not by P fertilization. Thus, it seems that for all field treatments N was the first limiting factor for SIR and that the N fertilizer rates being applied were not high enough to fully overcome this limitation. Ferralsols often have strong P sorption capacities due to their rather low pH and high amount of iron and aluminum oxides (Parfitt, 1980). One could thus argue that the P fertilization might not show a positive effect because the added P was quickly sorbed and did not remain available for microorganisms. However, our results show that P fertilization increased $NaHCO_3$-Pt content by more than 30% but this was not relevant for soil microbial respiration.
After 14 days, the same amount of added glucose–C had been respired in all treatments, which implies that microbial respiration was rather decelerated by N limitation but not absolutely limited.
The fact that we were able to detect changes in SIR between –N and +N treatments shows that SIR is an appropriate method to detect differences in nutrient availability as caused by fertilization in the field or by nutrient addition applied right before respiration measurements start. However, as respiration rates and growth of soil microbes are not necessarily linked (Iovieno and Bååth, 2008; Ehlers et al., 2010) respiration kinetics do not necessarily give an indication on microbial growth.

4.3 Fertilization effect on maize and beans

Bergmann (1993) considers a nutrient concentration of 35 – 50 g N kg^{-1} plant dry matter and 3.5 – 6 g P kg^{-1} plant dry matter as adequate in mature maize leaves at a plant height of 40-60 cm. Accordingly, the measured concentrations of N and P in maize where rather low when the respective fertilizer is missing (Table 4.4). With fertilization maize nutrient

concentration was increased by at least 50% but remained well below the optimum given by Bergmann (1993) .

For a range of different grain legumes, Bergmann (1993) considers a N concentration of 28 – 60 g N kg^{-1} plant dry matter and a P concentration of 2.5 – 6 g P kg^{-1} plant dry matter in the mature leaves as adequate. Thus bean P concentration was rather low in the minus P treatments. Bean N concentrations were always within the range given by Bergmann (1993) and were not affected by N fertilization. Supposedly, the beans were able to compensate for the low soil N supply in the minus N treatments by N_2 fixation.

4.4. Resource dependent stoichiometry of soil microorganisms

In the soil, fertilization treatments modified not only N and P pools (Table 4.1) but also N:P ratios. Plant N:P ratios were significantly (maize: p=0.001; beans: p=0.003) correlated with total N:P ratios in the soil (Figure 4.3A). Likewise, positive correlations of plant N:P ratios with ratios of available N and $NaHCO_3$-Pt were observed, although they were not significant in all cases (data not shown). In contrast, no significant correlations of N:P ratios in soil microorganisms to total soil N:P ratios (Figure 4.3B) or available N: $NaHCO_3$-Pt ratios (data not shown) were found, regardless of whether microbial N and P were quantified by fumigation-extraction (p=0.834) or by extraction of cells from soil by density gradient centrifugation and subsequent analysis (p=0.57). This outcome was supported by the fact that cellular N:P ratios of extracted soil microorganisms were not even affected by a 30 day incubation of soil samples which were previously enriched with an extra addition of 0.5 mg N g^{-1} soil (as NH_4NO_3) and/or 0.1 mg P g^{-1} soil (as KH_2PO_4) (data not shown).

N:P ratios determined using fumigation-extraction or cell extraction and subsequent analysis agreed well, averaging 11 ± 7 and 9 ± 2 for fumigation-extraction and extraction of cells, respectively. Cleveland and Liptzin (2007) reported an average N:P ratio of 7 in a metaanalysis of soil microorganisms analyzed by fumigation extraction. Makino et al. (2003) reported N:P ratios of 13 – 16.5 for cultured *E.coli.* Thus, our results seem to be within a reasonable range. Our data support the assumption that soil microorganisms are homeostatic while plant N:P ratios reflect soil N:P ratios and are thus rather resource dependent (Sterner and Elser, 2002; Cleveland and Liptzin, 2007). A special emphasis

has to be put on the N:P ratios on a per cell basis, as this is the first time that N:P ratios in microbial cells are reported for microbes that actually grew in the soil and not in culture media. It has to be kept in mind though that the soil microbial biomass is not one homogeneous pool. For instance, in vitro experiments with cultured bacteria and fungi showed that the homeostatic regulation in bacteria is very strict while homeostasis in fungi may be weaker (Sterner and Elser, 2002). Accordingly, differences in homeostatic regulation within the soil microbial community may occur. Fumigation extraction and extraction of cells from soil, however, both characterize a mixture of bacteria and fungi (Ehlers et al., 2008; Ehlers et al., 2010).

4.4 Conclusions

The N:P ratio in soil microorganisms were not affected by soil N and P content. This clearly supports the assumption that soil microorganisms are homeostatic (Cleveland and Liptzin, 2007). In spite of the fact that microorganisms cannot adapt their N:P ratio to nutrient availability, they faced neither N nor P limitation under the C availability that is likely to occur in the field. The glucose induced respiration data and the fact that Nmic and cell N were affected by N fertilization, however, show that microorganisms in this soil are rather sensitive to N fertilization than to P fertilization.

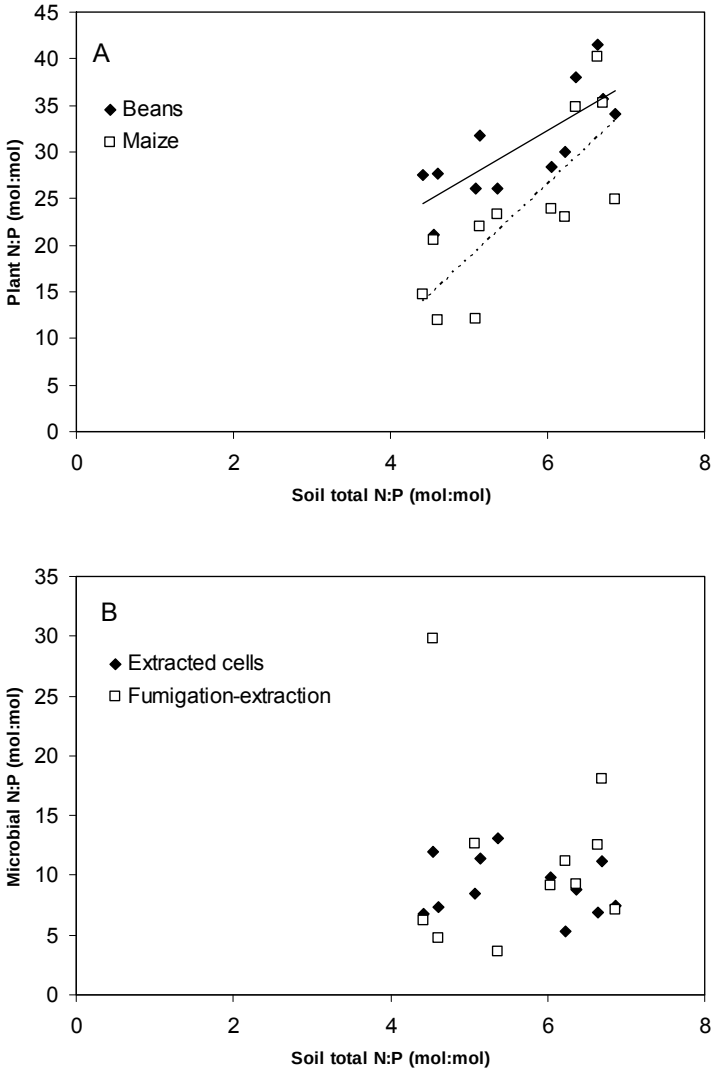

Figure 4.3: Plant N:P ratios (A) and microbial N:P ratios (B) in relation to total N:P ratios in the soil

Chapter 4: The source of the phosphorus flush after soil drying and rewetting in a Ferralsol as revealed by ^{33}P labeling

Abstract

Drying and rewetting (DRW) of soil is an important factor influencing soil phosphorus (P) dynamics and might be especially relevant in the tropics as climatic conditions are likely to favor DRW events. We investigated the impact of DRW on three soil P pools (available inorganic P, microbial P, and NaOH extractable inorganic P) in a Kenyan Ferralsol as affected by amendments with inorganic P or carbon substrates with ample nitrogen (CN). Soil was carrier free labeled with ^{33}P in treatments without nutrient addition and with CN addition. In the third treatment the ^{33}P was added together with the inorganic P addition. Soils were analyzed at three points in time (before drying, 1 h after rewetting, 1 week after rewetting). While P addition mainly increased the inorganic P status of the soil, CN addition led to an increase in microbial P and a decrease in available inorganic P. A P flush as indicated by an increase in available inorganic P appeared 1 h after rewetting in conjunction with a decrease in microbial P. The isotopic composition of available inorganic P and microbial P was similar but differed from the isotopic composition in the NaOH extractable inorganic P pool. As the isotopic composition in available inorganic P was not affected by DRW, the source of the P flush must have had a similar isotopic composition. Altogether, our findings suggest that the P flush after DRW was derived from a release of microbial P after rewetting. One week after rewetting the P flush had disappeared while the microbial P pool had not recovered. We assume that the P flush was sorbed to soil particles. Overall, DRW led to a significant short term increase in available P and might be an important factor for the redistribution of immobilized microbial P into inorganic P.

1. Introduction

Drying and rewetting (DRW) of soil can influence soil nutrient availability and dynamics. Flushes of carbon (C), nitrogen (N) and phosphorus (P) in form of increased CO_2 evolution and increased N and P availability have been reported in several studies as a result of soil rewetting following a period of drying. Three explanations for such nutrient

flushes have been proposed: (1) increased accessibility of nutrients due to physical processes such as disruption of soil aggregates (Utomo and Dexter, 1982; Appel, 1998; Denef et al., 2001), (2) release of nutrients from microbial cells due to cell lysis following an osmotic shock due to rapid rewetting (Sørensen, 1974; Turner and Haygarth, 2001; Turner et al., 2003; Mikha et al., 2005) and (3) exudation of osmolytes from microbial cells which allow the cells to adjust to the change in water potential (Kieft et al., 1987; Halverson et al., 2000; Fierer and Schimel, 2003). The latter, however, could explain flushes in C and N but not in P as none of the typically released osmolytes (amino compounds for bacteria and polyols for fungi) contain P (Schimel et al., 2007). This leaves disrupted soil aggregates and lysed cells as potential sources for flushes in available P caused by drying and rewetting.

Unlike soil C and N which occur mainly in organic form, inorganically bound P is usually the dominating P form in soil (Cross and Schlesinger, 1995). Accordingly, not only mineralization of organic P but also the release of inorganic P could contribute to P flushes after DRW.

Surviving soil microbes have been postulated as a possible sink for DRW P flushes (Blackwell et al., 2009). Until today, sinks of DRW P flushes have not yet been investigated in detail. Knowing the sinks of P flushes, however, would improve the understanding of DRW as a process influencing soil P dynamics.

Depending on soil properties, soils from temperate regions do not necessarily dry out sufficiently to show flush effects after rewetting (Appel, 1998). In contrast, fluctuations in soil water content due to seasonal rainfall pattern may be quite common in agricultural tropical soils. Furthermore, high temperature and insolation coupled with high rainfall intensity might cause strong daily fluctuations in soil water content. Inspite of this, to our knowledge no investigations of DRW effects on tropical soils exist.

Ferralsols are an especially interesting tropical soil type for the investigation of DRW P flushes not only because of their high abundance (approx. 7% of the nonpolar land area) but also because P is often a major constraint for plant growth on this soil type and an improved knowledge about P dynamics is therefore important (Friesen et al., 1997).

Even though the use of labeled C sources has proven to be very useful to analyze the source of C flushes after DRW (Sørensen, 1974; Van Gestel et al., 1993; Fierer and

Schimel, 2003; Wu and Brookes, 2005) labeling techniques have not yet been used to investigate soil P flushes after DRW.

In this study we used soil ^{33}P labeling in order to investigate the effect of soil DRW on three soil P pools in a Ferralsol with low amounts of available P and a high P sorption capacity. Our objectives were to assess the relevance of DRW as a driving force for P dynamics in this soil and to reveal sources and sinks of DRW P flushes. We expected ^{33}P to be relatively enriched in easily accessible and dynamic soil P pools compared to inactive and recalcitrant ones (Oehl et al., 2001; Buehler et al., 2002). We hypothesized that DRW would trigger P fluxes between the investigated P pools causing changes in pool size and possibly changes in their isotopic composition.

2. Materials and Methods

2.1. Soil

Soil was collected in December 2008 from 0-20 cm depth of a field trial site in Nyabeda, Western Kenya (altitude: 1420m, latitude: 0° 06' N, longitude 34° 34' E) under a maize-soybean crop rotation without P fertilization. The field trial is managed by the African Network for Soil Biology and Fertility (Afnet) in collaboration with the Tropical Soil Biology and Fertility Institute of the International Center for Tropical Agriculture in Nairobi, Kenya.

The soil was stored at 4°C in moist condition (0.16 g water g^{-1} soil dry weight, equal to 26% water holding capacity) for four months 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 mineralogical composition of the soil showing a high content of Fe and Al oxides is given in Ehlers et al. (2008). The concentration of organic P as measured by ignition and extraction with 0.5 M H_2SO_4 (Saunders and Williams, 1955) is 323 mg kg^{-1}. Total P content measured by H_2SO_4/H_2O_2 digestion (Anderson and Ingram, 1993) is 636 mg kg^{-1}. Isotopic exchange kinetics with ^{33}P (Frossard et al., 1993) revealed that according to Tran et al.(1988) the soil has a high P

sorption capacity (r_1/R=0.009; n=0.426). Organic C content measured with a CNS Analyzer (FlashEA 1112, Thermo Electron Corporation) is 22 g kg^{-1} (Ehlers et al., 2010).

2.2. Experimental design

Six different treatments with two factors (nutrient addition and soil moisture regime) were chosen (Table 5.1). Whereas the soil P status was not modified in the H_2O-treatment, the aim of the CN addition was to induce microbial growth and increase the microbial P pool. The aim of the P treatment was to increase the size of the inorganic soil P pools. The constantly moist treatments served as comparisons for the DRW-treatments.

Table 5.1: Description of incubation treatments

Treatment name	Nutrient addition	Soil moisture regime	Labeled P source
H_2O-DRW	None	DRW [b]	Soil IEP [a]
H_2O-M	None	M [c]	Soil IEP
P-DRW	P	DRW	Added Pi
P-M	P	M	Added Pi
CN-DRW	CN	DRW	Soil IEP
CN-M	CN	M	Soil IEP

[a] Soil IEP = isotopically exchangeable soil phosphorus.

[b] DRW = drying and rewetting

[c] M = constantly moist

Figure 5.1 depicts in a schematic manner the experimental setup for soil moisture dynamics and sampling dates. Three grams of labeled and amended soil were incubated at 25° C with a water content of 0.38 g water g^{-1} soil (equal to 60% water holding capacity) in 50 ml tubes for 30 days. During this period samples were aerated and if necessary soil water content was adjusted to 60% water holding capacity (WHC) twice a week. The 30 day incubation period before the first analyses was chosen to give the soil microbial community enough time to react to the CN addition and equilibrate the soil. After 30 days of incubation at constant soil moisture, the first set of samples was

analyzed (Date I). Half of the remaining samples subjected to drying and rewetting (DRW), whereas the other half remained at constant soil moisture (M). The DRW samples were dried down to a water content of 0.06 g water g^{-1} soil (equal to 10% WHC) over a three day period and kept at this level for four more days. To impose drying, open tubes were placed in boxes together with perforated plastic bags filled with self indicating silica gel. Silica gel bags were changed on a daily basis. After this week of drying the samples were rewetted to 0.38 g water g^{-1} soil (60% WHC) by adding 0.32 ml water g^{-1} soil and analyzed together with the M samples one hour after rewetting (Date II). Subsequently all remaining samples were kept at 60% WHC for another week and then analyzed for the last time (Date III).

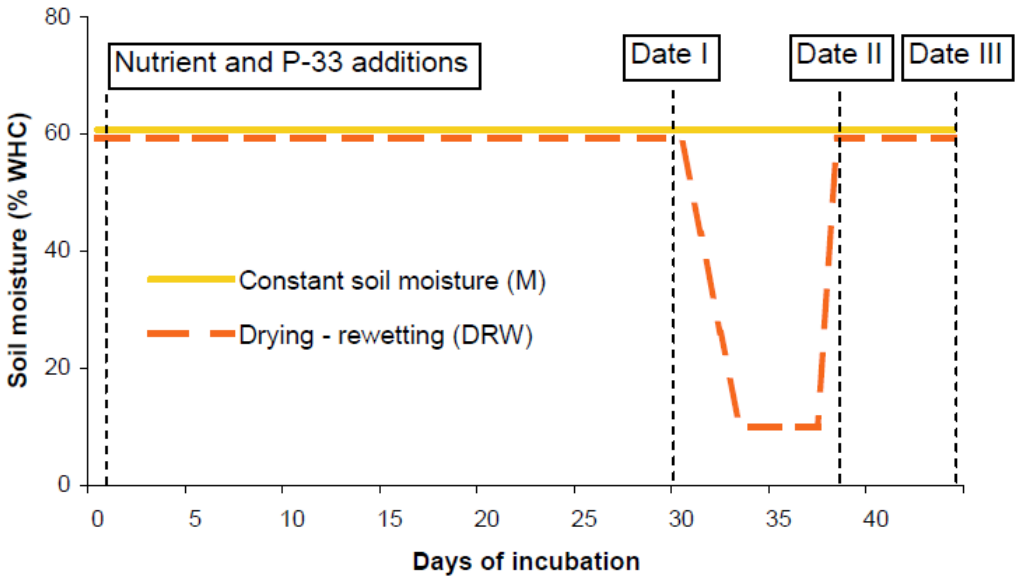

Figure 5.1: Schematic diagram explaining the soil water regime and sampling dates.

2.3. Nutrient additions and ^{33}P labeling

Nutrients and ^{33}P were added at the start of the incubation (Figure 5.1). The field moist soil was brought to 60% water holding capacity (WHC) by addition of 0.21 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 KH_2PO_4-solution was used to add 0.04 g P kg^{-1} soil. In the CN treatment, 2.5 g C as microcrystalline cellulose and 0.3 g N as NH_4NO_3 were added per kg of soil. The

dissolved N and P sources were applied in solution together with a fraction of the added water. The insoluble cellulose was added as powder.

Soil was labeled with ^{33}P in the form of phosphoric acid at the start of the incubation. The isotopically exchangeable soil phosphorus (soil IEP) was carrier-free labeled with 15.9 kBq g^{-1} soil in treatments that received H_2O or CN addition. For treatments with P addition a dose of 16.4 kBq ^{33}P g^{-1} soil was given together with the KH_2PO_4-solution. During nutrient addition and ^{33}P labeling the soil was carefully mixed in order to reach an even distribution.

2.4. Phosphorus analyses

Three different P pools were analyzed at each point of time: resin extractable inorganic P (Presin), P that was additionally resin extractable in the presence of a fumigant (Pfum) and NaOH extractable inorganic P (NaOH-Pi). Presin and Pfum were determined according to Kouno et al. (1995) except that hexanol was used as fumigant (McLaughlin et al., 1986) and resins were eluted in 0.1 M NaCl/HCl. Presin is the amount of P that has sorbed to anion-exchange resin membranes after shaking 3 g of incubated soil in 30 ml of water for 16h. It reflects the amount of P which can be rapidly transferred to the soil solution. Pfum is the additional amount of P sorbed to the anion-exchange resins during simultaneous fumigation and extraction with hexanol and is calculated as the difference between fumigated and non-fumigated samples. Pfum is an indicator for the amount of P bound in microbes but underestimates this pool as cell lysis after fumigation and hydrolysis of the released organic P are not complete and the released microbial P gets partly readsorbed to soil particles during the extraction procedure (Brookes et al., 1982; Hedley and Stewart, 1982).

The procedure for NaOH-Pi analyses was based on the protocol for sequential P fractionation by Tiessen and Moir (1993). Resin strips were rinsed above the tube and the total volume of water in the soil solution was made up to 44ml (by weight). Then, 1 ml of 4.5 M NaOH was added and the soil in the resulting 45 ml of 0.1 M NaOH was shaken overnight. Samples were then centrifuged at 10000 *g* and an aliquot of the supernatant was filtered (0.2 µm pore size). For determination of NaOH-Pi 5 ml of the filtered extract were acidified to pH 1.5 by adding 0.6 ml of 0.9 M H_2SO_4. Acidified extracts were kept

at 4°C for 30 min and centrifuged in order to separate precipitated organic matter from the solution. The NaOH-Pi extracts were neutralized before ^{31}P and ^{33}P determination. According to McDowell et al. (2003), NaOH-Pi represents inorganic P associated with Al and Fe.

In all cases, P was measured colorimetrically using malachite green at a wavelength of 610 nm (Ohno and Zibilske, 1991). Radioactivity was determined using a liquid scintillation counter (2500 TR, Packard Bioscience) with 5 ml Packard Ultima Gold scintillation liquid per ml of sample and corrected for radioactive decay. No quenching effects were detectable in the extracts.

2.6. Calculations

The size of the P flush after DRW was calculated as the increase in Presin in the DRW treatments compared to the P resin in the M treatments. Similarly, the decrease in microbial biomass P after DRW is calculated as the difference between Pfum in the M-treatments and Pfum in the DRW-treatments.

As the amount of radioactivity applied differed slightly between treatments the recovery of ^{33}P (in %) in each investigated P pool is calculated as

$$\text{recovery} = (r/R)*100$$

where r is the radioactivity in the investigated P pool and R is the total amount of applied radioactivity (both in kBq kg^{-1} soil).

Recoveries of ^{33}P are only presented for date I to give an impression of the fate of the label. The ratio of ^{33}P to ^{31}P in each P pool is presented as specific activity (SA) calculated as

$$SA = (r/R)/Q_P$$

where Q_P is the amount of P (in mg kg^{-1} soil) in each pool.

2.7. Statistics

Statistical analyses were done with Systat (SYSTAT Software INC. 2004). For each sampling date data were analyzed with a two-way ANOVA with the factors nutrient addition and moisture regime, and the interaction of nutrient addition and moisture regime. We did not use the sampling date as a third factor as the values differed strongly

between sampling dates under constant conditios. For example, Presin values for H_2O-M went from 1.65 ± 0.11 mg kg^{-1} soil on date I to 2.26 ± 0.03 mg kg^{-1} soil on date II and back to 1.74 ± 0.12 mg kg^{-1} soil on date III. After incubation for 30 days under constant conditions, those changes within one week are uncommon and we attribute them to slightly different extraction and determination conditions (e.g. room temperature, freshly prepared chemicals for each date etc.). Within each sampling date, however, the comparison of data is valid as conditions were the same for all samples.
In the text all data are presented as average ± standard deviation and are the results of 4 replicates.

3. Results

3.1. P-pools before DRW (date I)

Nutrient additions significantly affected all measured P pools (Table 5.2). The addition of 40 mg inorganic P kg^{-1} soil (P treatment) led to an overall increase of 34 mg P kg^{-1} in the investigated P pools as compared to the H_2O treatment. Especially, Presin increased more than 4-fold to 7.2 ± 0.4 mg P g^{-1} soil and NaOH Pi increased by 30% to 110.8 ± 0.6 mg P g^{-1} soil. The addition of C and N led to a decrease in Presin by 72% and an increase in Pfum by more than 100%.
Altogether, 55.0 ± 5.6% of the applied ^{33}P were found in the investigated P pools with no significant differences between treatments. Table 5.2 shows that around half of the added label was found in NaOH-Pi while recoveries in each of the other P-pools were below 10%.
SAs were similar for the H_2O and CN treatments and were highest in Presin followed by Pfum pools. For the P treatment, SAs in Presin, Pfum and NaOH-Pi were lower than in the other treatments, with Presin having the highest SA, followed by NaOH-Pi and Pfum.

Table 5.2: P content, ^{33}P recovery and SA on Date I (before DRW). [a]

Parameter	Treatment	Unit	Presin		Pfum		NaOH-Pi	
P content	H_2O	mg P kg^{-1} soil	1.65	(0.11)	0.88	(0.26)	83.1	(12.1)
	P		7.19	(0.36)	1.68	(0.47)	110.8	(0.6)
	CN		0.72	(0.08)	1.91	(0.15)	86.5	(0.8)
	Anova		p	SE	p	SE	p	SE
	Nutrient add.		<0.001	0.11	0.004	0.16	0.001	3.5
^{33}P recovery	H_2O	%	3.88	(0.14)	1.51	(0.76)	47.58	(7.45)
	P		8.58	(0.44)	1.20	(0.65)	50.37	(0.57)
	CN		1.65	(0.15)	3.07	(0.13)	47.98	(2.07)
	Anova		p	SE	p	SE	p	SE
	Nutrient add.		<0.001	0.14	0.003	0.29	0.649	2.24
SA	H_2O	(r/R)/mg P kg^{-1} soil	0.024	(0.001)	0.016	(0.005)	0.008	(0.000)
	P		0.012	(0.000)	0.007	(0.002)	0.006	(0.000)
	CN		0.023	(0.001)	0.016	(0.001)	0.007	(0.000)
	Anova		p	SE	p	SE	p	SE
	Nutrient add		<0.001	0.000	0.004	0.002	<0.001	0.000

[a] All values are means of four replicates, SD in brackets.

3.2. P-pools 1h after rewetting (date II)

For all nutrient additions, DRW led to an increase in Presin and a decrease in Pfum, while NaOH-Pi remained unaffected (Table 5.3). The increase in Presin averaged 0.29 ± 0.12 with no significant differences between treatments. Similarly, the decrease in Pfum by 0.62 ± 0.15 mg P kg^{-1} was similar in all treatments.

The SAs remained unaffected by soil moisture regime. The effects of nutrient additions on the size of the investigated P pools and their SAs were similar to date I.

Table 5.3: P content and SA on Date II (1h after rewetting). [a]

Parameter	Treatment	Unit	Presin		Pfum		NaOH-Pi	
P content	H2O-DRW	mg P kg^{-1} soil	2.48	(0.03)	0.81	(0.09)	91.8	(2.1)
	H2O-M		2.26	(0.03)	1.47	(0.20)	89.4	(2.0)
	P-DRW		8.96	(0.15)	1.13	(0.25)	113.6	(3.3)
	P-M		8.67	(0.18)	1.73	(0.42)	116.6	(1.5)
	CN-DRW		1.45	(0.12)	1.50	(0.06)	85.8	(0.7)
	CN-M		1.10	(0.09)	2.11	(0.06)	85.8	(1.4)
	Anova		p	SE	p	SE	p	SE
	Nutrient add.		<0.001	0.04	<0.001	0.08	<0.001	0.7
	Moisture		<0.001	0.03	<0.001	0.06	0.831	0.6
	Nutrient x moisture		0.539	0.06	0.948	0.11	0.045	1.0
SA	H2O-DRW	(r/R)/mg P kg^{-1} soil	0.020	(0.001)	0.018	(0.003)	0.006	(0.001)
	H2O-M		0.020	(0.001)	0.019	(0.003)	0.006	(0.000)
	P-DRW		0.012	(0.000)	0.009	(0.001)	0.005	(0.000)
	P-M		0.012	(0.000)	0.011	(0.003)	0.005	(0.000)
	CN-DRW		0.019	(0.002)	0.020	(0.002)	0.006	(0.000)
	CN-M		0.019	(0.001)	0.016	(0.001)	0.006	(0.000)
	Anova		p	SE	p	SE	p	SE
	Nutrient add.		<0.001	0.000	<0.001	0.001	<0.001	0.000
	Moisture		0.884	0.000	0.927	0.001	0.510	0.000
	Nutrient x moisture		0.956	0.001	0.041	0.001	0.086	0.000

[a] All values are means of four replicates, SD in brackets

3.3. P-pools 1 week after rewetting (date III)

One week after rewetting the differences in Presin between DRW and the corresponding M treatments had disappeared and only the decrease in Pfum remained (Table 5.4). Similar to date II, NaOH-Pi was not affected by soil moisture regime. As reported for date I and II, soil moisture regime had no effect on SAs and the only significant differences were due to the nutrient addition.

Table 5.4: P content and SA on Date III (1 week after rewetting). [a]

Parameter	Treatment	Unit	Presin		Pfum		NaOH-Pi	
P-content	H2O-DRW	mg P kg^{-1} soil	1.74	(0.12)	1.13	(0.05)	89.0	(1.8)
	H2O-M		1.74	(0.02)	1.61	(0.25)	89.5	(5.4)
	P-DRW		7.41	(0.28)	1.44	(0.33)	116.7	(6.8)
	P-M		7.28	(0.44)	2.33	(0.30)	119.6	(11.7)
	CN-DRW		0.87	(0.03)	1.63	(0.08)	86.6	(2.4)
	CN-M		0.86	(0.08)	2.21	(0.20)	87.3	(1.3)
	Anova		p	SE	p	SE	p	SE
	Nutrient add.		<0.001	0.08	<0.001	0.08	<0.001	2.2
	Moisture		0.591	0.06	<0.001	0.07	0.588	1.8
	Nutrient x moisture		0.811	0.11	0.209	0.11	0.907	3.1
SA	H2O-DRW	(r/R)/mg P kg^{-1} soil	0.016	(0.001)	0.024	(0.003)	0.006	(0.000)
	H2O-M		0.014	(0.001)	0.026	(0.001)	0.006	(0.000)
	P-DRW		0.009	(0.001)	0.017	(0.001)	0.005	(0.000)
	P-M		0.011	(0.000)	0.012	(0.005)	0.005	(0.000)
	CN-DRW		0.016	(0.002)	0.021	(0.002)	0.005	(0.001)
	CN-M		0.021	(0.004)	0.020	(0.004)	0.005	(0.000)
	Anova		p	SE	p	SE	p	SE
	Nutrient add.		<0.001	0.001	<0.001	0.001	<0.001	0.000
	Moisture		0.065	0.001	0.514	0.001	0.117	0.000
	Nutrient x moisture		0.033	0.001	0.074	0.001	0.546	0.000

[a] All values are means of four replicates, SD in brackets.

4. Discussion

4.1. Effect of nutrient additions under constantly moist conditions

The investigated P pools represent about 14% of total soil P, with NaOH-Pi being the largest. Furthermore, NaOH-Pi was by far the most important P pool for isotopic exchange. The fact that we recovered in total around 55% of the added ^{33}P, shows that some important pools of IEP remained uninvestigated. Nevertheless, the size of the

investigated P pools and the recovery of ^{33}P in these pools are within the range of earlier reports for Ferralsols (Buehler et al., 2002; Bünemann et al., 2004). The addition of inorganic P strongly increased soil inorganic P status as indicated by Presin and NaOH-Pi. The fact that 50% of the ^{33}P went into the NaOH-Pi pool stresses the importance of this pool as a major geochemical sink for inorganic P in this soil type. Apart from an increase in the inorganic P pools, P addition also led to an increase in Pfum.
The addition of C and N led to an increase in Pfum and a decrease in Presin. Both changes were of about the same size: 0.75 mg kg^{-1} and 0.99 mg kg^{-1}, respectively. Similar effects have been reported by Bünemann et al. (2004) after C and N additions to a Ferralsol and by Butterly et al. (2009) after C addition to a chromic Luvisol. Preliminary experiments showed that during the first 30 days after amendment 40% of the added cellulose-C was respired in the CN treatment and microbial respiration was about ten times higher in the CN treatment than in the H_2O and P treatments. Thus, the addition of C and N increased microbial activity and is likely to have induced microbial growth, which led to an increased P uptake from the Presin pool and incorporation into Pfum. On date II Presin and Pfum were the two pools with the highest SAs for all treatments. Both SAs were roughly similar which is in accordance with the findings of Oehl et al. (2001) that the isotopic compositions in the available and microbial P pools converge with time. Overall, the added nutrient additions fulfilled their purpose. While P addition mainly led to an increase in soil inorganic P pools (Presin and NaOH-Pi), CN addition lead to an increase in microbial P and a decrease in available P.

4.2. Effect of DRW

In all treatments, DRW led to a significant flush of resin extractable P within one hour after rewetting. An increase in Presin after DRW always occurred together with a decrease in microbial P. We found no significant relationship between the size of the P flush in relation to the size of the microbial P pool before DRW (Figure 5.2). Other studies reported a positive correlation between the size of the P flush and the size if the microbial P pool (Sparling et al., 1985; Turner and Haygarth, 2001). Our outcome is possibly due to an overriding effect of P sorption during the extraction procedure. This P

sorption during the extraction is known to lead to an underestimation of the microbial P pool (Brookes et al., 1982; Hedley and Stewart, 1982).

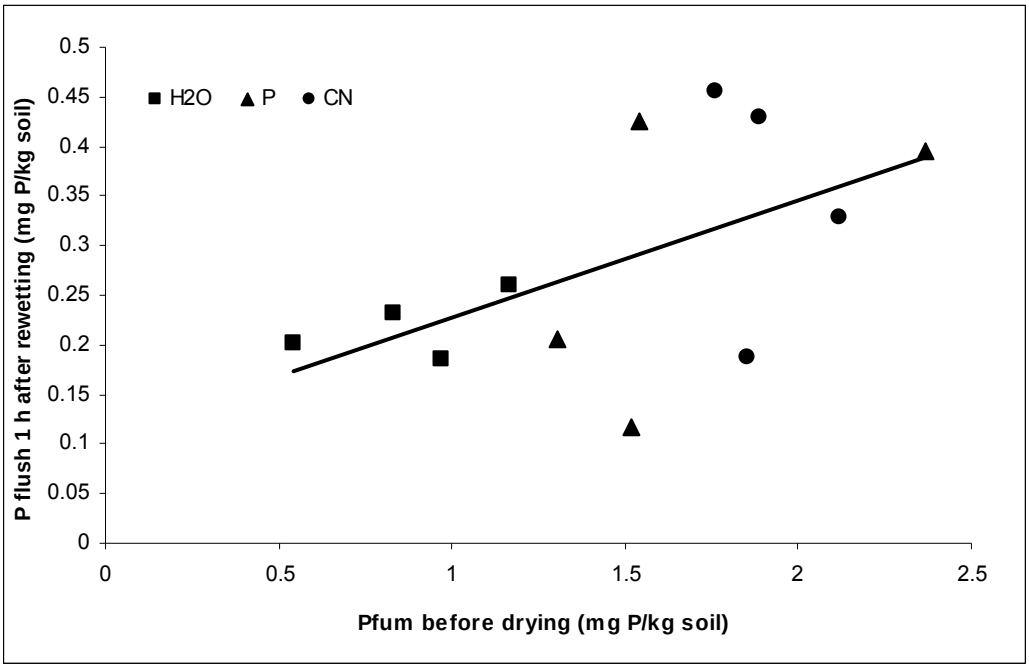

Figure 5.2: P flush due to DRW 1h after rewetting (Date II) in relation to the size of the Pfum pool before DRW (Date I). Correlation coefficient = 0.552; p = 0.063.

Similarly, the P flush one hour after DRW is likely to become partially sorbed as well during the extraction. P sorption during the extraction plays an important role on this soil type. Tests revealed that during the extraction procedure 44-70% of an added inorganic P spike (6.7 mg kg^{-1} soil) were sorbed. P sorption was decreased by P and increased by CN addition. For H_2O and P sorption was higher in DRW compared to M treatments, while for CN the spike recovery was not affected by DRW (data not shown).

As NaOH-Pi pool size was not affected by DRW, this pool does not seem to play a role as source for DRW P flushes. Changes in the NaOH-Pi pool size in the range of the P flush, however, would not have been detectable against the variation.

The SA in Presin was not influenced by DRW. This suggests that Presin and the source of the P flush must have been approximately equally labelled. The SA in NaOH-Pi, thus, seems too low to be the origin of the P flush. The Pfum, however, representing a living P pool has been rather intensely labelled and its SA on Date II is similar to the SA in

Presin. Accordingly, of the investigated P pools, only the soil microbial P pool seems to be plausible as a source for the P flush based on its isotopic composition.

We can not exclude that P pools other than the microbial P pool contributed to the P flush. For instance the NaOH-Pi pool may not have been homogeneously labelled. Therefore, the stronger labelled parts of this pool might have contributed to the P flush, while the changes in pool size and SA would not have been detectable against the variation. However, if NaOH-Pi was the source of the P flush, the flush should have been affected by the strong increase in the size of this P pool in the P treatment. Finally, by the chosen extraction techniques, we only accessed 14% of soil total P and 55% of added ^{33}P. Accordingly, P pools that lie beyond those which we investigated might also contribute to DRW P flushes, even though they are considered to be more recalcitrant and less active (Tiessen and Moir, 1993; Buehler et al., 2002).

The SA in the microbial P pool was not affected by DRW. Assuming that active soil microbes are more intensely labelled than dormant ones (Oehl et al., 2001), this indicates that both active and dormant soil microbes contributed evenly to the decrease in Pfum. In contrast, Van Gestel et al. (1993) reported that after addition of ^{14}C labelled plant material, labelled microbial C decreased relatively more than unlabelled microbial C. Based on this observation, they concluded that active microorganisms are more prone to DRW than dormant ones.

One week after rewetting only the Pfum pool was still significantly affected by DRW, suggesting that the microbial community was not able to fully recover within one week. Similarly, Butterly et al. (2009) reported that microbial biomass P had not returned to the levels prior to DRW after one week of moist incubation. In agreement with our observations, they also reported that the P flush disappeared within one week after rewetting.

Preliminary experiments on this Ferralsol showed that already 24h after rewetting no significant P flush was detectable (data not shown). Even though we were not able to follow the fate of the P flush, it is likely that the NaOH-Pi pool was one of its major sinks. Even if the entire P flush had been sorbed to the NaOH-Pi pool, we would not have been able to detect this against the variation. Microbial P uptake as a possible sink for the

DRW P flush as proposed by Blackwell et al. (2009) seems to play a minor role in this case, as the negative effect on microbial P remained while the flush disappeared.
Our findings raise the question if plants growing under P limiting conditions would be able to benefit from a DRW P flush or whether the time span between P flush and adsorption is too short for the plant to benefit from the P flush. Therefore, we conducted a split root experiment with maize, with half of the roots growing in a nutrient solution (providing plants with water and all nutrients except P) and the other half growing in the soil. We were not able to detect differences in P uptake between plants growing in pots exposed to DRW compared to those growing under constantly moist conditions (data not shown). A possible explanation is that plant roots in a dry soil need some time to re-establish their function after rewetting and therefore cannot benefit from a short lived P flush. However the findings of the plant experiment need to be further investigated and the sensitivity of the system needs to be improved before explicit statements about plant P uptake after DRW can be made.

4.3 Conclusions and Outlook

DRW led to a significant increase in available P and is therefore likely to play an important role for P dynamics in Ferralsols. Such P flushes, however, are rather short lived. Nevertheless, they might be key factors in redistributing P within soil P pools (e.g. from microbial P into sorbed inorganic P).
We found strong circumstantial evidence that the microbial P pool is the source of the P flush. The sink of the P flush, however, could not be determined. Having shown the importance of the NaOH-Pi pool for inorganic P sorption, we expect that this pool absorbs a good portion of the P flush.
Using ^{33}P labeling was a helpful approach in identifying the source of DRW-P flushes; yet more work needs to be done in order to specify our findings. For instance, the use of other soil P extraction steps could be helpful to obtain a clearer picture. Examining the effect of DRW P flushes on plant P uptake remains an especially interesting task.

General discussion

General discussion

This thesis investigates the composition and dynamics of the microbial P pool in a Ferralsol low in available P and with a strong P sorption capacity. In chapter 1, the method for cell extraction was adapted to this soil. In chapter 2, the microbial activity, growth and community composition as well as the composition and dynamics of microbial P as affected by amendments with C and N substrates with and without extra P were investigated. In chapter 3, I analyzed the effect of N and P fertilization in the field on microbial biomass, microbial activity and microbial nutrient ratios. Chapter 4 dealt with the release of microbial P due to soil drying and rewetting.
The collected results were previously discussed separately, in the following they are linked and discussed in a broader context.

Extraction of microbial cells from soil for determination of microbial P forms and N:P ratios

Chapter 1 of this thesis showed that the adaptation of the method for cell extraction by density centrifugation as described by Bakken (1985) was successful in terms of cell yield and removal of soil particles. However, chapter 2 revealed that measured RNA contents in extracted microbial cells were unexpectedly low. It thus seems that over the period of time that is needed for the cell extraction, degradation of RNA in microbial cells might occur. Those processes are likely to bias the information given on the cell internal P forms. Accordingly, the method might not be suitable to detect very labile compounds in soil microorganisms. However, the results on total N and P content of extracted microbial cells seem valid.
Chapter 2 showed that the concentration of P in microbial cells depends on the microbial growth rate as proposed by Elser et al. (2003) but not on the availability of P in the soil. Chapter 3 showed that microbial nutrient ratios did not change with nutrient ratios in the soil. Together these findings confirm the assumption that soil microorganisms are homeostatic. Homeostasis has been previously described for aquatic and cultured microorganisms (Sterner and Elser, 2002; Makino et al., 2003; Makino and Cotner, 2004), and Cleveland and Liptzin (2007) reported the same for soil microbial C:N:P

ratios as analyzed by fumigation-extraction. Due to potential errors associated with the fumigation-extraction technique (Jenkinson et al., 2004; Oberson and Joner, 2005) these results needed further confirmation. The confirmation of homeostasis in soil microorganisms is an important finding as homeostasis on the consumer level compared to producers (plants) without a strict homeostatic regulation can have important consequences for nutrient recycling within ecosystems (Elser and Urabe, 1999; Sterner and Elser, 2002). Based on these findings I will later on discuss a stoichiometrical example how microorganisms might act as a source and sink for available P.

The N:P ratios determined in chapter 3 using fumigation-extraction or cell extraction and subsequent analysis agreed well, averaging 9 ± 2 (for extraction of cells) and 11 ± 7 (for fumigation-extraction). The N:P ratio of 7 as reported by Cleveland and Liptzin (2007) is also similar.

However, it has to be kept in mind that the soil microbial community is complex and nutrient ratios as well as homeostasis might not apply similarly for all microbial groups. For instance, Wallander et al. (2003) reported a C:N ratio of 20 for ectomycorrhizal mycelia while the soil microbial C:N ratio determined by fumigation-extraction ranged between 6 and 13. Sterner and Elser (2002) mention that homeostasis might be very strict for bacteria but less pronounced for fungi.

The main arguments against using cell extraction in order to quantify total nutrient content and nutrient ratios in soil microbial cells is that it is incomplete and not necessarily representative for the soil microbial community (as shown in chapter 1 and 2). Furthermore, the extracted cells are not a 100% free from contamination with soil particles (as shown in chapter 1) and Nycodenz (chapter 3). Hence, nutrient contents are potentially biased. In order to determine total N and P content in the extracted cells we digested the samples as proposed by Ebina et al. (1983). As mentioned in chapter 2, this digestion method is not only very simple and efficient, it also seems to combine a full recovery of microbial P with a very low recovery of soil total P. More aggressive digestion methods might increase overestimation of cellular P content as they might extract more P from contaminating soil particles. Measuring iodine and N in the extracted samples and in a Nycodenz standard curve seems to be a good approach to take the

Nycodenz-N contamination of extracted cells into account. It has to be kept in mind however that any kind of correction is also a potential source of error.
The fumigation-extraction method has partly similar errors. It is also incomplete and thus potentially biased. Further sources for the underestimation of microbial nutrient concentrations by fumigation-extraction are the incomplete hydrolysis of extracted organic nutrients, and the P sorption during the extraction. Results delivered with this method might be furthermore biased because fumigation might extract nutrients from roots contaminating the soil sample and even an extraction of C, N and P from non-living nutrient sources can not be excluded (Jenkinson et al., 2004).
Altogether, nutrient ratios based on cell extraction might be at least as trustworthy as nutrient ratios derived by fumigation-extraction. However, both methods gave similar results and fumigation-extraction is less laborious. Thus, fumigation-extraction may be preferred when a lot of samples need to be processed. Furthermore, fumigation-extraction might be preferred when attempting to measure microbial nutrient concentration per g or kg of soil as such an estimation on the basis of extracted cells would need a further (potentially biased) conversion.
Finally, the cell extraction method can not only be used to investigate cellular nutrient contents and ratios but the extracted cells can be used for a wide range of further studies (Christensen et al., 1994; Prieme et al., 1996; Sitaula et al., 1999; Almas et al., 2004; Almas et al., 2005; Caracciolo et al., 2005; Bakken and Frostegård, 2006; Bertaux et al., 2007).

Assessing nutrient limitation of microbial growth

In chapter 2, respiration kinetics as well as microbial biomass measurements (on the basis of PFLA content) were used to describe P limitation of microbial growth in this soil. Both methods are supposed to reflect microbial growth but gave contradictory results. At the peak of respiration the additional microbial response (measured as respiration rate) to nutrient addition was around three times higher in the CNP than in the CN treatment. As the respiration rate is supposed to reflect the microbial biomass (Anderson and Domsch, 1978) this would suggest a three times higher biomass. However, biomass measurements showed that at the peak of microbial respiration the total amount of microbial growth was

not affected by P availability (as indicated by similar amounts of total PLFA per g soil in the CNP vs. CN treatment).

The problem in interpreting the data is that both methods might deliver biased results. When deducing microbial growth limitation on the basis of biomass measurements, it has to be kept in mind that the soil microbial community involves several trophic levels. Therefore, microbial growth by biomass measurements might be underestimated as growth and biomass decline due to predation are coexisting processes. Thus, growth based on biomass measurements rather represents the net growth, which is the difference between gross growth and predation. The gross growth would be suitable to detect nutrient limitation of microbial growth, but it is impossible to assess by biomass measurements as predation itself is not constant but responds within days to changes in gross growth (Christensen et al., 1996; Rønn et al., 2001).

The growth rate is suitable to determine limitations of microbial growth in an ecosystem involving several trophic levels (Cullen, 1991). For microbial communities growing in soil, the microbial growth rate is commonly deduced from an exponential increase in respiration (Anderson and Domsch, 1978; Nordgren, 1992; Scheu, 1993). It is based on the assumption that an increased microbial respiration rate reflects an increased microbial growth rate. However, it might occur that microbial respiration and microbial growth are decoupled (Schimel and Weintraub, 2003). For instance, Iovieno and Bååth (2008) reported that after soil drying and rewetting respiration rates increased immediately, while bacterial growth recovered only gradually. Accordingly, one can not categorically infer from microbial respiration limitation to microbial growth limitation.

In summary, respiration kinetics are suitable to characterize the speed of the microbial respiratory response to a given substrate as for instance done in Esberg et al. (2010). The speed of the respiratory response may be used to identify situations of nutrient deficiency for soil microorganisms. Yet, no direct conclusions from the respiration kinetics to microbial growth should be drawn. For short term studies, biomass measurements are likely to give more reliable results. The longer the time of growth, however, the more likely is an underestimation of gross microbial growth due to predation.

Measuring both microbial respiration and biomass growth probably allows the most distinctive statement on microbial nutrient limitation. In addition, it gives a good picture on the fate of an added C source.

P release by soil drying and rewetting

The results in chapter 4 showed that microbial P is partly released by drying and rewetting (DRW) of the soil. Chapter 4 showed that soil drying from 0.38 g H_2O g^{-1} soil (60% water holding capacity) to 0.06 g H_2O g^{-1} soil (10% water holding capacity) and subsequent rewetting back to 0.38 g H_2O g^{-1} soil led to a measureable increase in resin extractable P of 0.2 mg P kg^{-1} soil. Assuming a soil density of 1.3 g soil per cm^3 and that the topsoil in a depth from 0-10 cm is prone to such a DRW event this would lead to an approximate P flush of 0.26 kg P ha^{-1}. In comparison, the daily P uptake of growing maize can average around 0.3 kg P ha^{-1} (Sinaj et al., 2009).

Furthermore, the investigated change in water content could be unlikely to occur in the field as drying to 10% water holding capacity reflects a very low water potential in this clay rich soil. Under field conditions drying of topsoil decreases water potential and leads to a comparably higher soil water potential in the subsoil. Along this gradient water will flow from the subsoil to topsoil and thus buffer the water loss.

From the 14th of October 2008 until the 1st of December 2008 we continuously measured soil water content in the field at 5 cm depth. This period represents a part of the short rainy season in 2008. During this time span the lowest water content measured in the field was 0.2 g H_2O g^{-1} soil. This happened at the end of the measurements and we therefore do not know when and to what extent rewetting might have occurred. Within the investigated period soil rewetting from 0.25 g H_2O g^{-1} soil to 0.38 g H_2O g^{-1} soil happened three times (Oct. 17, Nov. 4, Nov 13). Each time the rewetting took place within less than 3h. This indicates that quick soil rewetting can occur in the field. However, it remains open if a rewetting from 0.25 g H_2O g^{-1} soil (around 35% water holding capacity to 0.38 g H_2O g^{-1} soil (around 60% water holding capacity) is sufficient to cause a P flush.

Seasons in the investigated region are characterized by a bimodal rainfall pattern. Two rainy seasons (March-August, September-January) are halted by drought in between

(Kihara et al., 2010). This is likely to affect soil humidity and could possibly lead do microbial P release due to DRW. Soil moisture in the field was however not monitored long enough to confirm this assumption.

P release via the food chain – a stoichiometric example

Microbial P may be released due to grazing by protozoa (Cole et al., 1977; Bonkowski, 2004). In the following I am discussing in a stoichiometric (i.e. elemental composition) example how microbial P uptake and release via the food chain could look like (Figure 6.1). All data are given on a molar basis and are approximate numbers based on the findings of the previous chapters. The organic matter taken as an example has a C:P ratio of 500:1 (derived from an overall average of freshly harvested maize leaves as described in chapter 3). The C contained in the organic matter is partly respired and partly incorporated in the decomposer biomass. A possible ratio of incorporated C to respired CO_2-C for both decomposers and grazers is around 2:1 (as derived from the calculated growth efficiency mentioned in chapter 2). Furthermore, I assume that decomposers and grazers have the same C:P ratio of 100:1. This is an approximate number derived from microbial C and P data measured by fumigation-extraction in chapter 3. While decomposers are feeding on 500 mol organic matter-C containing 1 mol of P, they would respire 166 mol C as CO_2 and incorporate 334 mol C into their biomass. Being homeostatic they need to balance the low P content of the C source by an uptake of 2.3 mol of P from the soil. Based on the findings in chapter 2, P limitation might decelerate microbial growth but not hamper it in absolute term. Thus, one can expect that decomposers are actually able to use the C supplied by the organic matter. On the next trophic level grazers such as protozoa feed on the 334 mol of decomposer-C. As they respire 111 mol C the decomposer P concentration is too high compared to their needs. In chapter 2 we found no indication that soil microorganisms increase their cell internal P content just because surplus P is available. Thus it seems unlikely that protozoa incorporate the surplus P. In order to stay stoichiometrically balanced, grazers would release 1.1 mol of the decomposer derived P, while incorporating the remaining P together with the decomposer-C.

According to this example, soil microorganisms might be a sink for soil P on the second trophic level (decomposer level) while on the third trophic level (grazer level) microbial P might be released into the soil solution. On subsequent trophic levels a further release of P from soil organisms seems reasonable.

This example, however, represents a simplified model. First of all it assumes that no further nutrients (e.g. N) or environmental conditions (e.g. soil humidity, temperature) limit microbial organic matter degradation. Furthermore, the ratio between incorporated C and respired CO_2-C of 2:1 was derived from the growth efficiency mentioned in Chapter 2. Even though the degradability of the C and N substrates in this experiment might be comparable to that of rhizodeposits (Fischer et al., 2010), Farrar et al. (2003) propose a ratio of incorporated to respired C of 1:2 for soil microbes feeding on root exudates. For microorganisms growing on plant litter the actual ratio of incorporated to respired C might be even lower as microbial degradation of plant litter needs the synthesis of additional enzymes and enzyme production will result in a higher respiration (Schimel and Weintraub, 2003). Apart from that, the example does not take into account C-efflux from soil microorganisms via exoenzyme and organic acid release. For plants the amount of C that is released as rhizodeposits ranges between 7 - 40% of the assimilated C (Haller and Stolp, 1985; Paterson et al., 1997; Singh et al., 2004). To my knowledge no data exist on the C release of soil microorganisms. Finally the C:P ratios in C-substrates and soil microorganisms might vary. For instance, Cleveland and Liptzin reported for soil microorganisms an overall C:P ratio of 60:1 compared to the C:P ratio of 100:1, which I use.

Nevertheless, the general principle of P immobilization on the second trophic level and P release on the third and probably further trophic levels should remain valid.

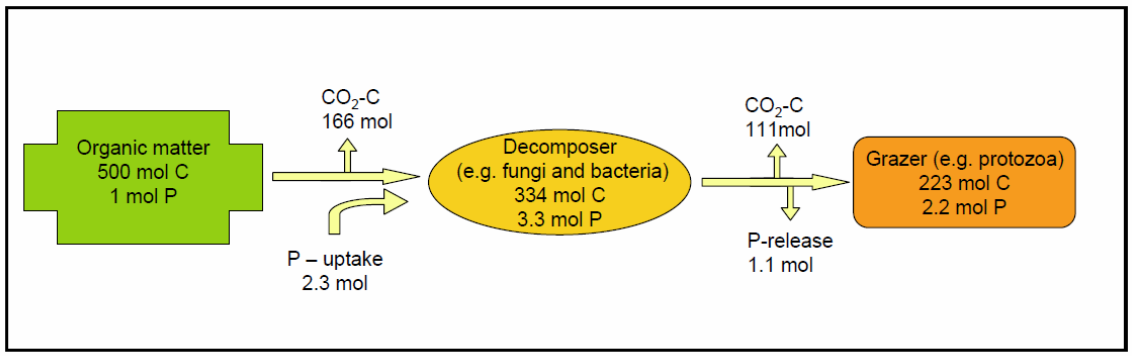

Figure 6.1: Stoichiometric example of potential P release via the food chain

The microbial P pool – beneficial for plant P nutrition?

In chapter 4, available P decreased after C and N addition indicating that growing microorganisms take up P from the available P pool. Similar processes have been observed earlier by Oehl et al. (2001) and Bünemann et al. (2004), suggesting that microorganisms may compete with plants for available P. Such a competition for P between plants and microbes might be especially severe in the rhizosphere as root exudates turn this area into a hotspot for microbial activity and growth (Alphei et al., 1996).

As mentioned earlier, the immobilized P may be partly released by soil DRW or via the food chain. Whether an increase in available P after DRW is reasonable to expect in the field has been discussed above. In the investigated Ferralsol a release of P into the soil solution disappears quickly (Chapter 4). If plant roots themselves are negatively affected by DRW and need some time to recover, a potential P flush could thus be sorbed before the plant may benefit from it. DRW processes could take place especially at the beginning of each rainy season. Such a microbial P release is untimely for plant P uptake as planting takes place after the rainy season has started. Chances for the plant to benefit from the released microbial P via the food chain are probably better as this is a constantly ongoing process and high predation rates in the rhizosphere might lead to a continuous release of microbial P in close proximity of the roots (Bonkowski, 2004).

If soil microorganisms are able to exploit soil P pools which are not plant available, plants could benefit from the loop of microbial P uptake and release as they could

indirectly make use of soil P pools that would otherwise remain inaccessible for them. In chapter 2 I reported that soil microorganisms were able to extract around 20 mg P kg soil^{-1} after C and N addition. In the plant experiment mentioned in chapter 4 maize plants growing on the same soil and under constantly moist conditions had purple leaves and a P concentration in the plant biomass below 0.8 g P kg^{-1}. Both indicate P deficiency. Those plants did not take up statistically significant amounts of P from the soil. Similarly, Bünemann (2003) reported that maize plants growing on a Kenyan Ferralsol low in available P were not able to take up any P in a pot experiment. In contrast, the soil microbial P pool increased its size up to 7fold when readily degradable C was available. These results however do not necessarily prove that soil microorganisms can access soil P pools that are not available to maize plants. The root penetration in our experiment was not very good and most of the plant roots were concentrated on the bottom of the pots. Plants can only access soil P in close proximity of the roots (Claassen et al., 1981). In contrast, soil microorganisms can be found almost everywhere in the soil (Mills, 2003). Thus the proportion of soil volume accessible for microbial P uptake might have been larger than the proportion of soil volume that was accessible for plant P uptake.

As long as plants and soil microorganisms access the same soil P pools, any process of microbial P release would only render P available that has been plant available before microbial P uptake anyway. As soil microbes are considered to prevent P from sorption to soil particles (Olander and Vitousek, 2005) it has been discussed that this mechanisms may improve plant P nutrition (Ayaga et al., 2006). However, this would only work if the release of microbial P is synchronized with the demand of growing plant (Oberson and Joner, 2005). As discussed this may be the case for microbial P release via the food chain, but not necessarily for P release after soil DRW.

Main conclusions in an agronomic perspective

Soil microorganisms represent a very dynamic P pool in Ferralsols with low amounts of available P and high P sorption capacities. The size of this pool and the P concentration in soil microorganisms are dependent on C and N supply but are remarkably little affected by P supply. This evidence suggests that unlike plants soil microorganisms on these soils are not strongly constrained by low P availability.

It remains unclear if the soil microbial P pool itself is beneficial to plant P supply. Organic matter (e.g. by plant residues) input might enhance microbial P uptake, cycling and release of P over the food chain. Furthermore, microbial decomposition can be seen as a requirement to render plant residue P available for crops.
Rhizosphere processes are probably highly important for the plants as these may lead to P immobilization (due to microbial growth on the root exudates) and P mobilization (due to P release via the food chain) in direct proximity of plant roots.
It was shown that soil drying and rewetting can lead to the decline of the microbial biomass and a release of microbial P to the soil solution. Soil humidity in the field is likely to depend on the seasonal rainfall pattern. Probably the first rain at the start of each rainy season causes a rewetting effect that can release microbial P into the soil solution. It was shown that the released P would disappear quickly from the soil solution and be adsorbed to soil particles. As crops are planted after the rainy season has started, it is unlikely that plants can benefit from such an untimely increase in available P. Covering the soil with plant residues over the dry season in order to reduce evaporation could reduce soil drying and thus minimize an untimely release of microbial P due to soil drying and rewetting.
Any kind of agricultural production includes the removal of P from the field - no matter how optimized P cycling in the field may be. Supplying farmers with affordable P fertilizers thus remains the main task in order to fight low yields due to P limitation.

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Appendix

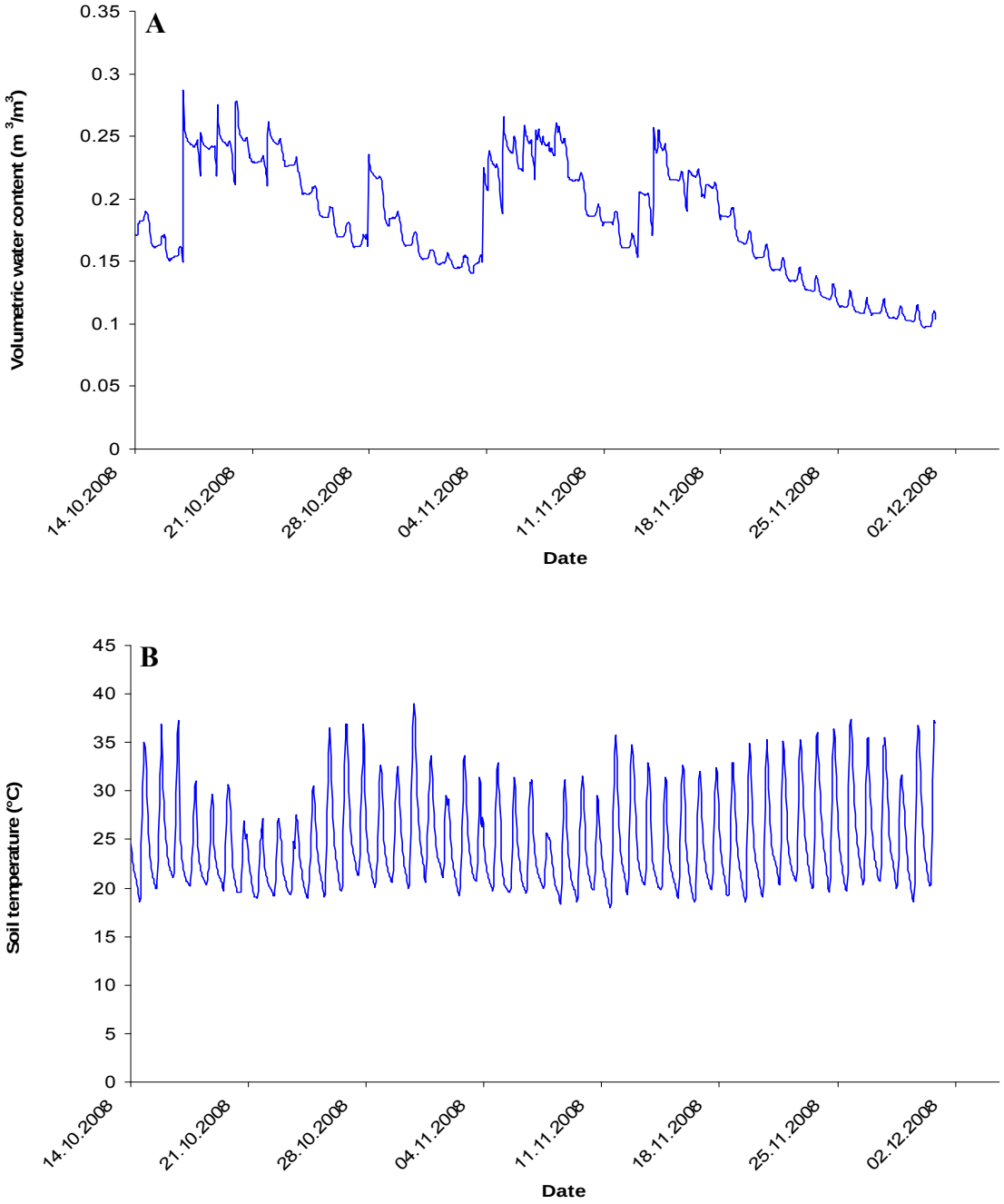

Figure 7.1: Volumetric water content (A) and temperature (B) in the soil at 5 cm depth as measured in the field with probes and data loggers. Temperature fluctuations indicate a strong day/night effect.

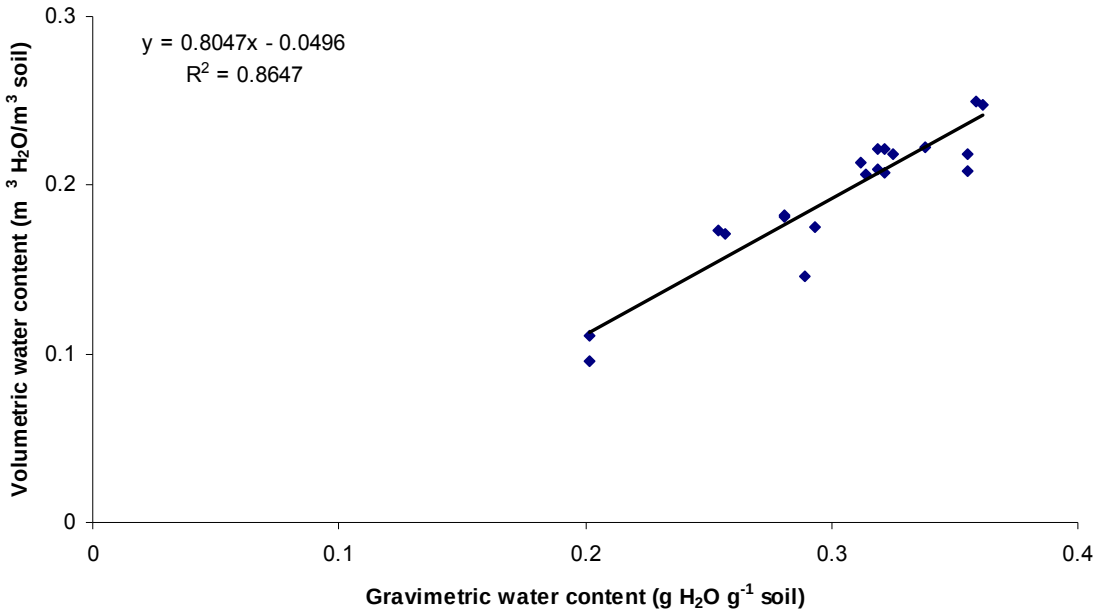

Figure 7.2: Relationship between gravimetric water content and volumetric water content (n=20, CC=0.93, p<0.001).

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