

Chapter 2

Characterization of Phosphorus Forms in Soil Microorganisms

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2.1 Microorganisms as a Pool of Phosphorus in Soil

Soil microorganisms act as sink and source of phosphorus (P) and mediate key processes in the soil P cycle, e.g., P mineralization and immobilization (Oberson and Joner 2005). The role of mycorrhizal fungi in P absorption and transport to plant roots is well documented (Jakobsen et al. 2002; Jansa et al. 2011), but the role of the soil microbial biomass (i.e., soil bacteria, archaea, and mostly saprophytic fungi) in soil P cycling is less well defined. Microbial P immobilization can affect P availability by removing inorganic P from the soil solution, especially when soluble carbon is available for microbial growth (Bünemann et al. 2004a; Olander and Vitousek 2004). In the absence of recent carbon inputs, gross organic P mineralization rates of between 1.4 and 2.5 mg P kg⁻¹ day⁻¹ have been measured for arable soils (Oehl et al. 2004; Frossard et al. 2011). Microbial activity in a grassland soil was also shown to be required to replenish organic P in the soil solution (Seeling and Zasoski 1993). Thus, although the microbial biomass contains only 0.4–2.5% of total P in cropped soils and up to 7.5% in grassland soils, it can play a fundamental role in the soil P cycle, especially when its turnover time is only a few months (Oberson and Joner 2005).

The main forms of P in microorganisms are shown in Table 2.1. Based on values for P-limited aquatic bacteria (Vadstein 2000), the dominant forms of microbial P are nucleic acids and phospholipids (together 60%), cytoplasmic inorganic P (10%), cytoplasmic organic P (10%), and polyphosphate (20%). However, the distribution of P forms in microbial cells changes with environmental conditions, in particular with carbon and nutrient availability (Herbert 1961). Changes may be either qualitative (i.e., some cell components may occur in cells only under certain environmental conditions), or quantitative (i.e., the concentration of cell components that are always present may increase or decrease). This could ultimately affect

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Table 2.1 Quantitatively main forms of P found in microorganisms: chemical groups, NMR regions, examples, location and function

Chemical group	NMR region	Examples	Description
Nucleic acids	Diester	DNA, RNA	Biopolymers present in all living cells; storage of genetic information. About 9% P
Phospholipids	Diester	Phosphatidylglycerol, phosphatidylethanolamine	Major components of cell membranes; ensure semipermeability. Usually one phospholipid molecule consists of glycerol, two saturated or unsaturated fatty acids, and one P atom
Teichoic acids	Diester	Glycerol teichoic acid	P-containing polysaccharides present in cell walls of Gram-positive bacteria
Metabolites	Mostly monoester	AMP, glucose-6-phosphate, phosphoenolpyruvate, ATP	Intermediates and products of all metabolic processes
Phosphonates	Phosphonate	2-Aminoethyl-phosphonic acid	Compounds with direct bonds between carbon and P atoms. Biological function not well known
Organic polyphosphates	Pyro- and polyphosphate	ATP	Produced by ATP synthase. Transfers chemical energy when converted back to AMP or ADP and orthophosphate
Inorganic polyphosphates	Pyrophosphate	Pyrophosphate	P storage compound consisting of two condensed phosphate groups
Inorganic polyphosphates	Polyphosphate	Polyphosphate	P storage compounds consisting of three or more condensed phosphate groups
Orthophosphates	Orthophosphate	HPO_4^{2-} ; H_2PO_4^-	Inorganic anions of phosphoric acid. Used in metabolic processes and for synthesis of complex molecules

DNA deoxyribonucleic acid, RNA ribonucleic acid, AMP adenosine monophosphate, ADP adenosine diphosphate, ATP adenosine triphosphate

the availability of microbial P forms to plants. For example, organic P, but not polyphosphate, contained in filtered extracts of a cultured soil bacterium was shown to be hydrolyzed and taken up by plants growing in a nutrient solution to which the filtrates had been added (Macklon et al. 1997).

Several recent studies have investigated carbon:nitrogen:phosphorus (C:N:P) stoichiometry in aquatic bacteria. Under low P conditions, the P concentration, growth rate, and RNA content were positively related in *Escherichia coli* as well as in lake bacteria, and P in RNA represented 82% of the total bacterial P pool in an oligotrophic lake (Elser et al. 2003). When P was abundant, a lower and more variable proportion of total P was contained in RNA. At constant growth rate of *E. coli* in a chemostat (see Sect. 2.2.1), the proportion of P in RNA varied between 40% and 70% of total P, depending on the temperature (Cotner et al. 2006). From experiments with *E. coli* under controlled conditions and from a literature survey on heterotrophic prokaryotes in freshwater lakes, Makino et al. (2003) concluded that each bacterial species regulates its elemental composition homeostatically within a relatively narrow range of a characteristic biomass C:P ratio, depending mainly on its RNA content, and that shifts in the dominance of different bacterial species in the environment are responsible for the large variation in community C:P ratios.

These examples suggest that the concentration and forms of microbial P are affected by the composition of the microbial community, P availability, and growth stage. To date, however, our knowledge of the variation in microbial P forms has been obtained in aquatic systems or with pure cultures, and methodological constraints have limited our ability to investigate these relationships in soil. The classical approach of measuring the black box “microbial P” by fumigation-extraction methods (Oberson and Joner 2005) has yielded important information, especially in combination with isotopic labeling (Frossard et al. 2011). For example, 2 days after labeling soil with ^{33}P , Oberson et al. (2001) found up to 25% of ^{33}P in microbial P, without a change in the size of the microbial P pool. This recovery of ^{33}P in microbial P was about five times greater than that in available inorganic P and suggests a P flux of about $0.5 \text{ mg P kg}^{-1} \text{ soil day}^{-1}$ through the soil microbial biomass. However, hydrolysis of microbial P during fumigation is an inherent part of the method and thus it provides no information on the chemical forms of soil microbial P present. Therefore, Oberson and Joner (2005) suggested that microorganisms be extracted from soil and their P forms analyzed.

Here, we argue that a more detailed picture of the P forms within the soil microbial biomass is an important next step in our efforts to understand the role of microorganisms as sink and source of P. In addition, a better understanding of the fate of microbial P forms released into the soil could provide insight into the origin of soil organic P, its availability to mineralization processes and its environmental behavior, e.g., with respect to losses to water courses. Ultimately, such an improved knowledge will contribute to more efficient P use in agroecosystems, which is mandatory because of dwindling mineable P deposits as well as increasing P eutrophication worldwide (Tiessen et al. 2011).

In this chapter, we summarize the current knowledge on P forms in soil microorganisms. We begin with an overview of methods for culturing microorganisms

and for direct extraction of cells from soil. Next, we describe a suite of analytical methods for determining the forms of P in soil microorganisms. Two case studies are presented: one on the use of NMR spectroscopy to analyze P forms in pure cultures of soil microorganisms, and one on the forms of P in microbial cells extracted from soil. We discuss the potential microbial origin of soil organic P, and methodological limitations and future applications of the approach of combining the extraction of microbial cells from soil with chemical analysis.

2.2 Cultivation and Extraction Methods

2.2.1 *Culturing of Microorganisms*

Cultivation of soil microorganisms is important for detailed taxonomic and physiological studies. However, only a small fraction of microorganisms can be cultured (1–5%), even though this percentage can probably be increased with improved cultivation techniques (Janssen et al. 2002).

Batch cultures represent the simplest approach for culturing microorganisms, where microorganisms grow from a small inoculum introduced into a given amount of mineral or complex medium. Typically, after an initial stationary and lag phase, exponential growth at a constant growth rate occurs, which eventually slows down until the maximum stationary phase is reached, followed by a decrease in cell numbers (Herbert 1961). Thus, biomass production stops quickly after depletion of the growth-limiting nutrient, although the number of cells can still increase if initiated cell division is completed. Unlike for carbon, biomass production can continue due to relocation of the nutrient. For example, teichoic acids can be replaced by P-free teichuronic acids in Gram-positive bacteria under P limitation, while in other species the degradation of RNA and polyphosphate can deliver P upon which the cells can continue to grow (Wanner and Egli 1990).

The main disadvantage of batch culture is the highly dynamic nature of the system, which prevents identification of the precise factors that affect the composition of cells (Tempest and Wouters 1981). This is overcome by continuous culture in a chemostat, which is at steady state when the exponential growth rate μ is equal to the dilution rate D (ratio of flow rate to the volume of the culture). Thus, very low growth rates can be achieved at low dilution rates, and low growth rates are likely to be the typical situation in soils. So far, chemostat studies on the chemical forms of P have been done with aquatic microbial communities (Makino and Cotner 2004) but not with soil microbial communities.

Harvesting of cultured cells is done by centrifugation or filtration. For subsequent nutrient analysis, washing steps may be required to obtain cells without contamination by the growth medium. For filtration, various pore sizes may have to be tested to avoid losses of very small cells (Wang et al. 2007). However, there is a trade-off between pore size and duration of filtration.

2.2.2 *Extraction of Microorganisms from Soil*

Ideally, a method for extracting microorganisms from soil in order to analyze microbial P forms would meet the following (often conflicting) conditions:

1. A high percentage of the soil microbial biomass is recovered (high quantitative efficiency).
2. The fraction of the soil microbial biomass that is recovered either represents the soil microbial community well (high qualitative efficiency) or is a proportion that can be regarded as an operational pool (i.e., a proportion that is defined by the extraction procedure and is highly reproducible).
3. The cells are free from adhering soil colloids.
4. The extracted cells are physically intact and their metabolic state is not altered. In particular, microbial P forms are unaffected.

The approach used most often to extract microbial cells from soil is density gradient centrifugation (Bakken and Lindahl 1995). This method consists of mechanical soil dispersion using a blender to destroy aggregates and to detach microbial cells from soil particles, followed by separation of structurally intact microbial cells from soil particles using the nonionic density gradient medium Nycodenz. The quantitative efficiency is assessed by microscopic cell counts after staining with acridin orange or similar DNA stains (Lindahl 1996). The qualitative efficiency is evaluated by comparing the microbial community composition in the soil using either biochemical methods such as phospholipid fatty acid (PLFA) analysis or molecular methods such as denaturing gradient gel electrophoresis (DGGE). The purity of the cells from soil particles can be assessed by determining the loss of cell weight after ignition (Bakken and Lindahl 1995) or by measuring the content of typical soil elements such as iron and aluminum in the extracted fraction (Ehlers et al. 2008). Whether the cells are physically intact or not is seen under the microscope, while changes in the metabolic state can be assessed by measuring adenosine triphosphate (ATP) in the cells (Lindahl and Bakken 1995). Potential changes in the biochemical composition during extraction have to be tested using pure cultures.

The recovery of structurally intact bacterial cells using density gradient centrifugation can be as high as 70–80% when the soil is extracted repeatedly (Lindahl 1996). However, repeated extractions are often impractical and in most studies the recovery is much lower. For example, the recovery was around 20% free from adhering soil colloids (Sitaula et al. 1999). For acid clayey tropical soils, cell yields were as low as 0.5% (Maron et al. 2006). In such instances, soil-specific adaptations might be required, e.g., to obtain enough material for chemical analyses. Working with a tropical Ferralsol, Ehlers et al. (2008) increased cell yield from 4.9% to 10.6% by adjusting the pH to 7.5, but in both cases the extracted fraction was highly contaminated with soil material. Although the addition of 0.8% NaCl solution eliminated this contamination, it also decreased cell yield to 4.6%. However, in the absence of NaCl the extracted microbial communities were more similar to the soil microbial communities as assessed both with PLFA and DGGE.

Using molecular-based methods, Courtois et al. (2001) showed that apart from the preferential extraction of γ -proteobacteria, bacterial diversity was similar for extracted cells and for extraction of DNA from soil after cell lysis. However, there might be a bias for specific groups, as demonstrated for methane-oxidizing (Priemé et al. 1996) and ammonia-oxidizing bacteria (Aakra et al. 2000).

How well the extracted cells represent the soil microbial community always poses a problem because the method of Bakken and Lindahl (1995) was designed to extract bacteria and not fungi. In fact, fungal hyphae are destroyed during mechanical dispersion of soil (Faegri et al. 1977). In addition, they are probably only partly detached from soil particles or become pelleted with soil particles during centrifugation. Nevertheless, the fungal PLFA 18:2 ω 6 was found in the extracted fraction at a similar abundance as in soil (Ehlers et al. 2008). The authors argued that single-cell fungal structures such as yeasts and spores were recovered together with bacteria. In a follow-up study, however, addition of carbon and nitrogen sources led to a great increase in the fungal PLFA in soil, but not in the extracted fraction, suggesting discrimination against fungi during extraction (Ehlers et al. 2010). So far, DNA from the extracted fraction has only been analyzed using primers for prokaryotes. Analysis with eukaryotic primers could yield more information about the composition of the extracted community.

Due to the difficulties of extracting intact and pure fungal hyphae from soil, methods designed to extract mycelium for elemental analysis have used mesh compartments filled with sand (Wallander et al. 2003) or with a mixture of glass beads and soil of <40 μ m particle size (Neumann and George 2005). In the first case (Wallander et al. 2003), mesh bags were buried in spruce forests and recovered after 12–18 months for extraction of ectomycorrhizal mycelia. The C:N ratios in the hyphae were much wider (20) than those found using fumigation-extraction methods (6–14), although the microbial biomass in this soil was dominated by fungi. Presumably, fumigation-extraction methods extract more of the cytoplasm, while cell wall material is likely to remain in the soil. In the second case (Neumann and George 2005), P concentrations in arbuscular mycorrhizal mycelium recovered after 45 days from pots planted with potatoes were lower than values reported for hyphae grown in vitro, suggesting either lack of polyphosphate accumulation due to P limitation or efficient transfer of P to the host plant. Although such approaches deliver useful results, a method to extract fungal hyphae directly from soil so that they can be chemically analyzed is yet to be devised.

2.3 Methods for Analysis of Chemical Forms of Phosphorus

2.3.1 Total P

Most methods for elemental P analysis require liquid samples and, thus, digestion procedures prior to measurements. Acidic or alkaline persulfate digestion, usually in combination with autoclaving (e.g., Ebina et al. 1983), has been applied, as well

as mineralization with single acids such as nitric acid under heating (Danku et al. 2009).

After sample mineralization, total P is often measured photometrically (e.g., Lovdal et al. 2008). The method requires only simple instrumentation, but suffers from severe matrix effects and a short dynamic range. In addition, complete conversion of all P compounds into orthophosphate has to be assured. Alternatively, methods that are based on the atomization and excitation/ionization in an inductively coupled plasma (ICP) torch, with detection by optical emission spectrometry (ICP-OES; e.g., Duboc et al. 1995) or mass spectrometry (ICP-MS; e.g., Danku et al. 2009) can be used. These methods are more resistant to matrix effects and offer broader dynamic ranges, multi-elemental capabilities, and higher sample throughput.

An analytical technique that does not require liquid samples and has been successfully applied to the analysis of total P in single bacteria is X-ray fluorescence (XRF) in combination with scanning electron microscopy (Heldal et al. 1985). In fact, X-ray microanalysis allows measurement of total amounts of all major elements except hydrogen in single microbial cells (Norland et al. 1995). Comparison of native and cultured aquatic bacteria by X-ray microanalysis revealed major differences in size and nutrient contents (Fagerbakke et al. 1996). In particular, the content of P was greatest in cultured cells in the growth phase (17–31 fg P cell⁻¹), intermediate in cultured cells in the stationary phase (3.4–6.7 fg P cell⁻¹) and yet lower in native cells (0.5–1.1 fg P cell⁻¹). Thus, it appears that analyses of the elemental composition of single cells can give information on the physiological status and nutrient limitations. To understand the processes in the soil, such analyses have to be done on extracted native bacteria.

2.3.2 P Speciation Using ³¹P NMR

Speciation analysis of P forms by means of ³¹P NMR is the most promising analytical tool to date (see Doolette and Smernik 2011). However, further developments in instrumentation, spectra analysis, and sample preparation are necessary. The sensitivity is still unsatisfactory for analysis of microorganisms. Up to now, it has been necessary to acquire a large amount of sample, i.e., on the order of 0.1–1 mg P, which is feasible for pure cultures but not when extracting microbial cells from soil. For example, using the cell P content listed in Table 2.2 for the treatment designated H₂O (i.e., without addition of carbon, nitrogen, or P), the total cell count of 4.7×10^9 in this sample and an extraction yield of 5% gives 0.0075 mg P extracted from 10 g of soil, which is 100 times lower than would be required for ³¹P NMR. Other problems include correct identification of resonances and hydrolysis of P compounds during extraction. Nevertheless, the ability to measure the ratio between different P forms (e.g., orthophosphate, monoester, and diester P) can provide very useful information about P species, even at the

current stage of method development. For example, solution ^{31}P NMR has been applied to the analysis of P forms in pure cultures of soil bacteria and fungi (see Sect. 2.4.1).

The application of ^{31}P NMR to living systems is called *in vivo* ^{31}P NMR spectroscopy and has the advantages of being non-invasive and suitable for the study of time-dependent phenomena (Rasmussen et al. 2000). A study of the actinomycete *Corynebacterium glutamicum* used 45 mg dry weight per ml (total volume in the NMR tube was 1.8 ml) to study fluctuations of polyphosphate after additions of glucose, acetate, and phosphate and under changing oxygen supply (Lambert et al. 2002). A study of P forms in arbuscular mycorrhizal hyphae or cucumber roots by *in vivo* ^{31}P NMR distinguished orthophosphate, orthophosphate monoesters, nucleic acid triphosphates, and polyphosphate (Viereck et al. 2004). The fresh weight of hyphae or roots in the NMR tube ranged between 0.03 and 0.14 g.

These studies suggest that *in vivo* ^{31}P NMR has the potential to overcome the degradation problems that occur in the analysis of microbial P forms using solution ^{31}P NMR. However, sufficient material must be available, which again is problematic when studying microorganisms extracted from soil.

2.3.3 P Speciation by Chromatographic, Spectrometric, Staining, and Enzymatic Techniques

2.3.3.1 P in DNA and RNA

Before P analysis, nucleic acids have to be released from the cells. Most often, a combination of physical, chemical, and enzymatic treatment is used (Bakken and Frostegård 2006). Among physical treatments, bead-beating with glass beads or sterilized sand in mini-beadbeaters and grinding in liquid nitrogen are most commonly used. Chemical agents such as EDTA, SDS, and Triton make membranes more permeable, enhancing the effect of the other treatments. Enzymes can destroy bacterial cell walls, e.g., by hydrolyzing glycoside bonds (lysozyme) or peptide bonds (achromopeptidase) in the peptidoglycan layer. However, all treatments are somewhat selective in releasing nucleic acids from different species at different growth stages. For example, growing cells lyse more easily than cells at the stationary stage (Bakken and Frostegård 2006).

Since the concentration of P in DNA and RNA is constant at about 9%, the amount of P in DNA and RNA is usually calculated after quantification of nucleic acids (Makino and Cotner 2004). Methods of DNA and RNA quantification use either spectrophotometry or fluorometry. Photometric methods are based either on the absorbance of the analytes at 260 nm (Sambrook and Russel 2006) or on color development after reaction with orcinol (Endo 1970) or diphenylamine (Burton

1956). Absorbance at 260 nm requires the sample to be protein-free. The purity can be judged from the 260/280 nm absorbance ratio, with a low ratio indicating contamination by protein. If potentially interfering substances have to be removed, every cleaning step introduces the possibility of analyte loss and extends the time of analysis. In contrast, fluorometric measurements based on specific or unspecific fluorescent dyes can be performed on rough cellular extracts, omitting cleaning steps. Their sensitivity is usually higher than spectrophotometric measurements and the time of analysis is considerably shorter. After binding of the dye to the nucleic acids (e.g., of ethidium bromide, Hoechst 33258 or PicoGreen to double-stranded DNA, and of RiboGreen to RNA and DNA), fluorescent yield increases substantially. An example for the application of an unspecific fluorescent dye (RiboGreen) is the analysis of RNA and DNA in rough extracts of *Daphnia* (Gorokhova and Kyle 2002): after addition of the dye, RNase and DNase were subsequently applied and the decrease in the measured fluorescent signals was assigned to the amount of RNA and DNA, respectively.

Introduction of fluorescent dyes has shortened and simplified the quantitative analysis of nucleic acids. However, there are still analytical pitfalls. For example, when enzymes are added, correction has to be made for the increase in fluorescent signal due to the added protein. Some enzymes need specific cofactors, appropriate temperatures, and assay times. Finally, species-specific DNA and RNA structures can produce different spectro- or fluorometric responses. Thus, standard curves using DNA and RNA from the species being assayed assure best quantification (De Mey et al. 2006).

2.3.3.2 P in Lipids

Before analysis of phospholipids, they have to be extracted from the sample. The classical lipid extraction method uses a mixture of chloroform, methanol, and water in the ratio of 1:2:0.8 by volume (Bligh and Dyer 1959). Subsequently, a mixture of chloroform and methanol (2:1 by volume) has been recommended for the quantitative extraction of total phospholipids (Van Der Meeren et al. 1992).

The first analysis of phospholipid classes in microorganisms utilized thin layer chromatography (TLC) with subsequent elution of the phospholipids and analysis of total P in each fraction (Hossack and Rose 1976). This simple method can still provide reliable and relatively sensitive data regarding P distribution among various phospholipid classes. More recently, normal-phase high-performance liquid chromatography (HPLC) with a flame ionization detector (FID) has been applied successfully for analysis of phospholipids in bacteria (Moreau et al. 1995). Alternatively, evaporative light scattering detector (ELSD) and MS are used for analysis of lipids and phospholipids (Arnoldsson and Kaufmann 1994; Valeur et al. 1993).

Knowing the molar content of P in a given phospholipid, the quantity of P in it can be calculated from the quantitative analysis of the phospholipid. Similarly, P in phospholipids can be calculated from the quantitative analysis of PLFAs. This

method usually consists of phospholipid separation on silica columns, followed by methylation and gas chromatography (GC) analysis with detection by FID or MS (Frostegård et al. 1993). Calculation of the P in phospholipids is based on an assumed molar ratio between fatty acids and P of 2:1. Although this assumption is not always valid (e.g., cytidine diphosphate diacylglycerol and pyrophosphatidic acid have a molar ratio of 1:1), it is legitimate for most fatty acids and, importantly, for the most abundant ones, i.e., phosphatidylglycerol, phosphatidylethanolamine, cardiolipin, phosphatidylcholine, and phosphatidylinositol. Other potential sources of error are losses of phospholipids during the separation step and during methylation.

There are also a few reports on phospholipid analysis without chromatographic separation. Electrospray ionization mass spectrometry was applied to the analysis of crude lipid extracts of two marine bacteria (Mazzella et al. 2005), with proposed fragmentation pathways for the two most abundant phospholipids, i.e., phosphatidylglycerol and phosphatidylethanolamine. Fast atom bombardment with mass spectrometric detection was used for the characterization of bacterial phospholipids (Heller et al. 1988).

2.3.3.3 P in Metabolites

Many metabolites in microbial cells contain P. Examples are P monoesters such as adenosine monophosphate (AMP), glucose-6-phosphate, phosphoenol pyruvate and 3-phosphoglycerate, and ATP as a condensed phosphate.

Extraction of metabolites from bacterial cells is usually done with hot ethanol, pure water, or water solutions containing various additives, commonly acids or bases (Mashego et al. 2007). Because fungal cells are much less susceptible to lysis, extraction with organic solvents is employed, often in several steps (Frisvad and Thrane 1987) or with assistance of sonication (Smedsgaard 1997).

Analysis of ATP extracted from living cells has been used for more than four decades to estimate active biomass in aquatic systems (Holmhans and Booth 1966) and also in soils (Jenkinson and Oades 1979). Many other metabolites have been analyzed, usually by means of enzymatic assays. Analysis of total metabolites (metabolomics) has been gaining importance in recent years but requires highly sophisticated instrumentation, i.e., one or several mass spectrometers coupled with liquid (LC-MS) or gas chromatography (GC-MS) or capillary electrophoresis (CE-MS) (Mashego et al. 2007).

Fortunately, since all P-containing metabolites are ionic molecules their analysis is a much simpler task than analysis of all intracellular metabolites. Thus, there is a broad range of possible extractants to choose from (boiling ethanol, KOH, perchloric acid, hot water, hydrochloric acid, and acetic acid) and ion chromatography can be used, usually with conductometric and UV detection (Bhattacharya et al. 1995) or with pulse amperometric detection for analysis of sugar phosphates (Smits et al. 1998).

2.3.3.4 P in Teichoic Acids and Other Compounds

Teichoic acids can be a significant P pool in Gram-positive bacteria. For example, Ellwood and Tempest (1972) found that the purified cell walls of *Bacillus subtilis* contributed 17–25% of cell dry matter and that the cell walls contained 2.4–6.2% P, mostly in teichoic acids. Due to its complexity, however, quantitative analysis of teichoic acids is rarely performed. The procedure consists of separation of bacterial cell walls by centrifugation, followed by cleaning with chemicals (Ellwood and Tempest 1972) or application of enzymes (Bhavsar et al. 2004), and subsequent analysis of total P. Spectrophotometric and fluorometric titration have also been employed in the analysis of teichoic acids (Pal et al. 1989).

Another P compound in microorganisms is inorganic polyphosphate. Its analysis comprises separation of the inorganic polyphosphate chains on a solid sorbent, application of a polyphosphatase, and subsequent spectrophotometric determination of orthophosphate (Werner et al. 2005).

Analysis of phospho-proteins can be expected to be developed quickly for proteomic studies. A hyphenated quantitative method for the analysis of phospho-proteins has already been developed (Krueger et al. 2009).

Finally, myo-inositol hexakisphosphate (phytate) has been reported to be synthesized by soil microorganisms (Caldwell and Black 1958), although the majority of phytate in soils is thought to be derived from plants (Turner et al. 2002).

2.4 Phosphorus Forms in Cultured and Extracted Soil Microorganisms

2.4.1 Analysis of P Forms in Pure Cultures Using ^{31}P NMR

Bacteria and fungi isolated from soil and cultivated in batch cultures were analyzed by solution ^{31}P NMR in two studies (Bünemann et al. 2008a; Makarov et al. 2005). In both cases, the concentration of P was about twice as high in bacteria (mean values of 18 and 23 mg g⁻¹ dry weight) than in fungi (mean value of 9.5 mg g⁻¹ in both studies). For the fungi, both studies showed quite a similar pattern of P forms (Fig. 2.1): most of the P was more or less equally distributed over the orthophosphate, monoester P, and the pyro- and polyphosphate regions, even though the proportions of orthophosphate and condensed P were highly variable. Very small proportions of P were found in the diester region (mean values of 2% and 6%). In contrast, the diester pool was more significant in bacteria (18% and 38%), whereas very little condensed P was found (1% and 3%). Comprising on average 66% of P, the monoester region was more dominant in the study by Bünemann et al. (2008a) than in that by Makarov et al. (2005) where the average was 34%. This could be due to the difference in bacterial species but more likely due to hydrolysis of diesters during sample preparation and even during NMR analysis, as shown by Turner et al. (2003).

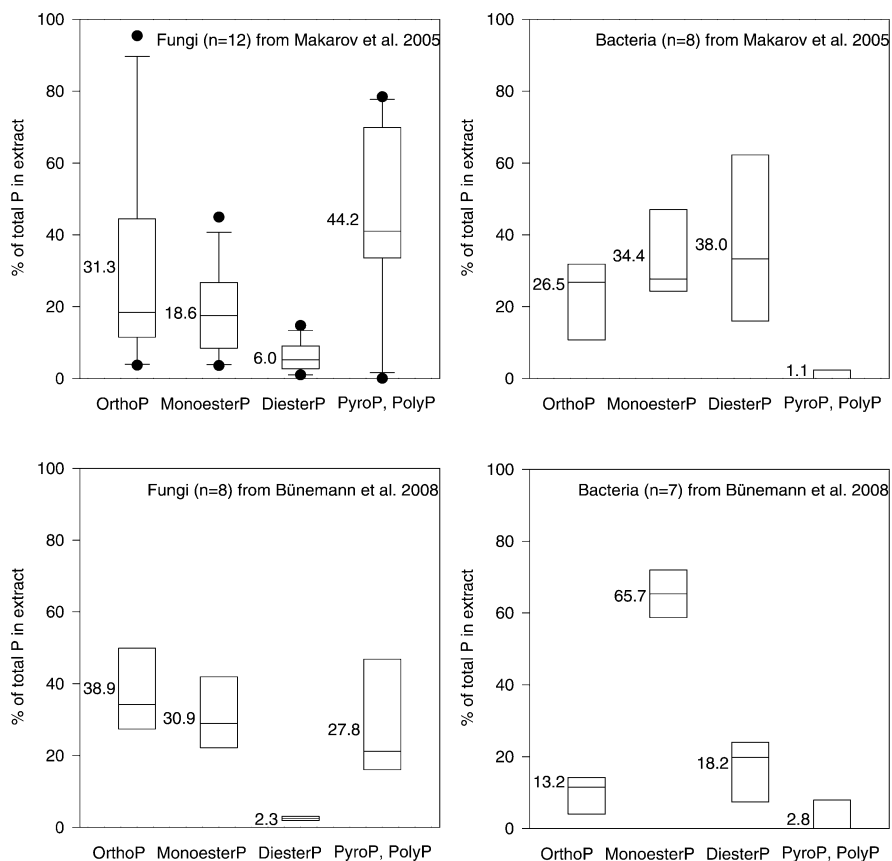


Fig. 2.1 Distribution of P species in pure cultures of fungi and bacteria extracted with 0.5 M NaOH (Makarov et al. 2005) (*upper figures*) or 0.25 M NaOH plus 0.05 M EDTA (Bünemann et al. 2008a) (*lower figures*) as determined by solution ^{31}P NMR. The *lower boundary of each box* indicates the 25th percentile, the *full line within the box* the median, the *dotted line* the mean (with the value given *left of the box*), and the *upper boundary* the 75th percentile. *Error bars* indicate the 90th and 10th percentiles (only if $n > 8$). Outliers are shown as *dots*

Both NMR studies (Bünemann et al. 2008a; Makarov et al. 2005) aimed to elucidate the origin of soil organic P. The spectra from bacteria and plants in the study by Makarov et al. (2005) were rather similar, suggesting that it will be difficult to distinguish between microbial and plant origin of organic P in soils. In their study, the proportions of different diesters in two acid soils corresponded to those in the spectra of bacteria and plants, whereas in a calcareous soil the pattern was more similar to that of fungi. However, fungal growth would be expected to dominate in acid soils (Rousk et al. 2009). In addition, the contribution of bacteria and fungi to soil organic P depends not only on the relative biomasses of the two groups but also on the P concentration, turnover time, and selective stabilization. Thus, the origin of soil organic P cannot be deduced from the broad P forms in

different organisms, with the exception of condensed phosphates. As shown in Fig. 2.1, pyro- and polyphosphates occur in much higher proportions in fungi than in bacteria, and a positive correlation of pyrophosphate and fungal abundance was indeed seen by Bünemann et al. (2008b).

The possibility that specific compounds in the monoester region could be characteristic for either bacteria or fungi was tested by Bünemann et al. (2008a). Of the 15 peaks found by deconvolution of the monoester region, two were unique to but not present in all fungi. For this limited dataset, bacteria and fungi were significantly separated, based on the signal distributions in the monoester region. However, a much more extensive dataset would have to be obtained before any conclusions with respect to the bacterial or fungal origin of soil organic P could be drawn using such an approach.

In contrast to the P forms discussed so far, phosphonates have not been detected in plants. Using ^{31}P NMR, Koukol et al. (2008) found phosphonates in basidiocarps and vegetative mycelia of basidiomycetes. These data support the microbial origin of phosphonates in soils, even though the concentrations ($14\text{--}140\text{ mg kg}^{-1}$ dry matter, equal to $0.1\text{--}4.4\%$ of extracted P) were lower than those of all other P forms, and even though phosphonate production of associated bacteria cannot be excluded because the samples were taken from the field.

2.4.2 Analysis of P Forms in Microbial Cells Extracted from a Ferralsol

Highly weathered, acidic tropical Ferralsols are usually characterized by strong P sorption capacities, leading to low amounts of available P. Under these conditions, the role of microbial P as a highly dynamic P pool could be of particular importance.

An incubation experiment was conducted to study the effect of carbon and nutrient additions on microbial activity and cell internal P pools in a Ferralsol (Ehlers et al. 2010). Four treatments were chosen:

1. Without C, N, or P addition (H_2O)
2. With P addition (P)
3. With C and N addition (CN)
4. With C, N, and P addition (CNP)

Microbial cells were extracted from soil by density gradient centrifugation (Bakken and Lindahl 1995), as adapted for highly weathered tropical soils (Ehlers et al. 2008). Extracted cells were analyzed for total P content and P contained in PLFA by using extraction and total P determination, and for P in DNA and RNA by using fluorescent dye. Microbial communities were analyzed in their most active state, as given by the peak of the respiration rates (Fig. 2.2). Since the H_2O and P treatments did not show a peak in the respiration curves, the microbial community was analyzed at the same time as in the CN treatment.

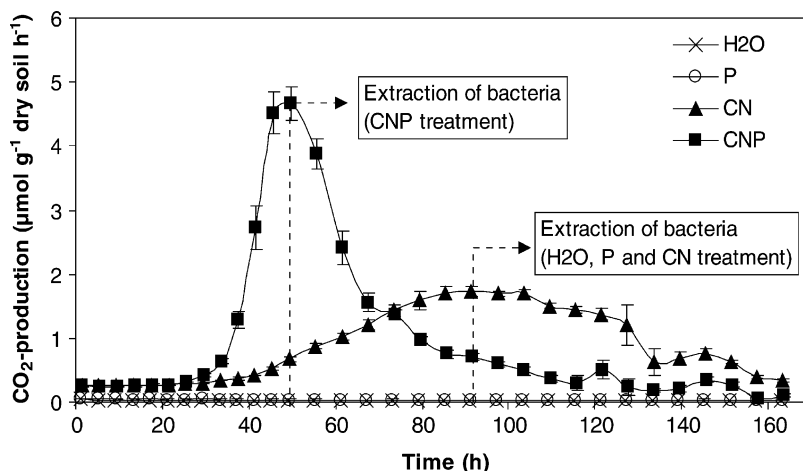


Fig. 2.2 Respiration rates in a Ferralsol from Kenya as affected by addition of P alone (*P*), carbon and nitrogen (*CN*), or carbon, nitrogen, and P (*CNP*) during the first 163 h of incubation (Ehlers et al. 2010). H_2O signifies no additions of C, N or P. Error bars show the standard deviation derived from three independent replicates. Arrows indicate the time of extraction of bacteria from soil for different treatments. Reprinted from Ehlers et al. (2010) with permission from Elsevier

Table 2.2 P content and P pools in soil microorganisms extracted from a Ferralsol^a

Treatment ^a	Total P		DNA-P		RNA-P		PLFA-P	
	fg P cell ⁻¹		fg P cell ⁻¹		fg P cell ⁻¹		fg P cell ⁻¹	
H_2O	3.22 (0.83)	ab	0.52 (0.08)	a	0.06 (0.01)	b	0.15 (0.07)	a
P	2.70 (0.60)	b	0.56 (0.08)	a	0.09 (0.03)	b	0.05 (0.04)	a
CN	4.60 (1.13)	ab	0.74 (0.17)	a	0.28 (0.01)	a	0.15 (0.11)	a
CNP	6.53 (2.46)	a	0.57 (0.16)	a	0.09 (0.03)	b	0.13 (0.08)	a

H_2O no additions; *P* added P; *CN* added carbon and nitrogen; *CNP* added carbon, nitrogen, and P. All values are means of three replicates (standard deviations in brackets). Values within a column followed by the same letter do not differ significantly ($P < 0.05$) according to Tukey's test. Modified from Ehlers et al. (2010). Reprinted with permission from Elsevier

^aSee text for treatment details

Total P contents in extracted cells ranged from 2.1 to 8.9 fg P cell⁻¹, with a tendency for higher contents in treatments CN and CNP (Table 2.2). These observed total P contents (Ehlers et al. 2010) seem to be within a reasonable range compared to 3.4–31 fg P cell⁻¹ for cultured aquatic bacteria (Fagerbakke et al. 1996), or values between 0.01 and 10 fg P cell⁻¹ for in situ analysis of marine bacteria (Gundersen et al. 2002). We found a positive correlation between maximum respiration rates and total P content per cell ($r = 0.59$, $P = 0.003$). This is in accordance with the growth rate hypothesis, which states that biomass P content increases with growth rate due to increased P allocation to P-rich ribosomal RNA (Elser et al. 2000, 2003).

However, the proportion of RNA-P to total P in our study ranged only between 1% and 6%. There are several potential explanations, including:

1. Incomplete cell lysis and RNA extraction
2. Degradation of RNA during extraction and filtration
3. Low growth rates
4. Extraction of a mixture of active and dormant cells from soil with high and low proportions of RNA, respectively
5. Absence of P limitation

These points are discussed in more detail in the following paragraphs.

We used cells of *E. coli* to test the protocol for cell lysis and RNA extraction. Because we found a proportion of RNA-P in *E. coli* of 53%, the protocol was deemed to be efficient. Nevertheless, cell lysis might still have been incomplete for the microorganisms extracted from soil. Degradation of RNA during extraction and filtration was tested by subjecting a pure culture of *Arthrobacter* sp. to the same procedure as soil bacteria, i.e., centrifugation over Nycodenz, sampling of the bacterial layer, and filtration. Compared to samples that were only filtered, centrifugation over Nycodenz decreased the proportion of RNA-P to total cell P by about 18%, whereas total cell P and P in DNA were unaffected (Prusisz and Bünemann unpublished results). However, RNA losses during filtration may have occurred in both cases. Since the filtration of cells extracted from soil usually takes longer than the filtration of pure cultures, this could be an important factor contributing to low RNA recovery.

Apart from methodological problems, the low proportions of RNA-P in cells extracted from soil could point to low growth rates. For example, Makino et al. (2003) found that RNA-P in *E. coli* grown in a chemostat contributed about 40% of total P at low growth rates and 80% at high growth rates. In carbon-amended soils, growth rates were rather high (Fig. 2.2). However, the extracted cells were probably a mixture of active and dormant cells. In that case, the higher RNA-P content in activated cells would have been diluted by the low RNA-P content in dormant cells.

Finally, P availability can affect the proportion of RNA-P in microbial cells. In a study on a mixed bacterial community from a lake, RNA-P contained 25–43% of total P when grown in P-sufficient culture, compared with 79–93% under P limitation (Makino and Cotner 2004). Since the formation of microbial biomass was similar for the treatments CN and CNP (Ehlers et al. 2010), limitation of the microorganisms by carbon rather than P was indeed indicated. This is in agreement with an earlier study on the same soil type (Bünemann et al. 2004b).

Cell contents of DNA and PLFA were similar to those reported in other studies. The DNA content of 6.6 fg cell^{-1} was higher than the $1.6\text{--}2.4 \text{ fg cell}^{-1}$ reported by Bakken and Olsen (1989) for indigenous soil bacteria, but well within the range of $2\text{--}9 \text{ fg DNA cell}^{-1}$ that the same authors found for cultured soil bacteria. Similarly, for soil bacteria Torsvik and Goksoyr (1978) and Sandaa et al. (1998) found values of $8.4 \text{ fg DNA cell}^{-1}$ and $8.8\text{--}11.5 \text{ fg DNA cell}^{-1}$, respectively. For PLFA, the overall average of $0.77 \times 10^{-17} \text{ mol PLFA cell}^{-1}$ for all treatments is similar to PLFA values of $0.62 \times 10^{-17}\text{--}2.35 \times 10^{-17} \text{ mol cell}^{-1}$ for 15 Swedish soils

covering a wide range of pH and organic matter contents (Frostegård and Bååth 1996).

Together, the investigated P pools explained between 10% (CNP) and 25% (P) of the total P content per cell. Due to the low recovery of RNA discussed above, these numbers are low compared to the results of, e.g., Vadstein (1998) who found that about 60% of the total P in heterotrophic planktonic bacteria cultured under P limitation was bound in DNA, RNA, and phospholipids. Other P compounds that were not measured in this study include metabolites and teichoic acids. For a complete understanding of carbon and P effects it would be important to analyze all P pools in the extracted cells and to overcome the problem of degradation of compounds during the procedure.

2.5 Conclusions and Outlook

This chapter has provided some evidence that characterization of P forms in microorganisms can contribute to an improved understanding of the role of bacteria and fungi in soil P cycling. Importantly, this approach delivers for the first time data on P forms in soil microorganisms that actually grew in the soil and not in batch culture or chemostat. Thus, unculturable microorganisms are likely to be included in the data. The presence of dormant as well as active cells might partly explain the low proportions of RNA in extracted cells, although incomplete extraction or degradation during the procedure are likely to contribute as well.

In addition, a predominantly fungal origin of condensed phosphates as well as phosphonates in soils was shown. To identify the origin of monoester and diester P in soils, specific compounds of unequivocal microbial origin would have to be found. For microbial communities extracted from a Ferralsol, carbon addition rather than changes in P supply affected the chemical composition of the microbial P pool. This points to the lack of P limitation of microorganisms in high-P-sorbing tropical soils.

However, there are two main problems with this approach of characterizing P forms in soil microorganisms growing in situ:

1. Different methods have to be applied for extraction of bacteria and fungi, and they are not exclusive, i.e., fungal structures can be extracted together with bacterial cells and vice versa. In addition, available methods for extraction of hyphae are not suitable for most soils except possibly those with a high sand content. Future work should therefore focus on improving methods for extraction of fungal hyphae.
2. Changes in cell internal P forms during extraction and sample preparation can occur, and the sensitivity of comprehensive analytical techniques such as ^{31}P NMR is generally too low. When different methods are applied to quantify different P species, there is potential for overlap between P pools, and many different methods have to be applied for a complete speciation analysis, including a range of extraction protocols. Direct comparison of P speciation by ^{31}P

NMR and by a combination of chromatographic, fluorometric, and colorimetric techniques would reveal the scope and limits of each approach.

Recent analytical developments in metabolomics and single-cell ecophysiology (Wagner 2009) hold great promise, and the combination of cell extraction with molecular analysis of microbial communities and functions will eventually give further insight into the role of specific groups of microorganisms in soil P cycling.

Acknowledgements We would like to thank Astrid Oberson for initiating this work and the entire Group of Plant Nutrition for fruitful discussions on the topic. We are grateful to Cory Cleveland, Astrid Oberson, and Emmanuel Frossard for helping to improve the manuscript.

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