Chapter 9
Role of Phosphatase Enzymes in Soil

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9.1 Introduction

Phosphatases have been extensively studied in soil, as shown by some reviews (Ramirez-Martinez 1968; Speir and Ross 1978; Malcom 1983; Tabatabai 1994), because they catalyse the hydrolysis of ester–phosphate bonds, leading to the release of phosphate (P), which can be taken up by plants or microorganisms (Cosgrove 1967; Halstead and McKercher 1975; Quiquampoix and Mousain 2005). It has been shown that the activities of phosphatases (like those of many hydrolases) depend on several factors such as soil properties, soil organism interactions, plant cover, leachate inputs and the presence of inhibitors and activators (Speir and Ross 1978).

Phosphatases are enzymes catalysing the hydrolysis of both esters and anhydrides of phosphoric acid (Schmidt and Laskowski 1961) and, according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, they can be classified as phosphoric monoester hydrolases or phosphomonoesterases (EC 3.1.3), phosphoric diester hydrolases or phosphodiesterases (EC 3.1.4), triphosphoric monoester hydrolases (EC 3.1.5) and enzymes acting on phosphoryl-containing anhydrides (EC 3.6.1) and on P–N bonds (EC 3.9). Phosphatases can also be subdivided according to their regulation (e.g. calmodulin), the requirements of metal cations for their activity (e.g. Mg^{2+} and Ca^{2+}) and their sensitivity to various phosphatase inhibitors. Phosphomonoesterases include acid and alkaline phosphomonoesterase (which hydrolyse monoester bonds including mononucleotides and sugar phosphates), phosphoprotein phosphatases (which hydrolyse phosphoester bonds of phosphoserines, phosphothreonines or phosphotyrosines), phytases (EC 3.1.3.26 for 4-phytase and EC 3.1.3.8 for 3-phytase, which hydrolyse all six phosphate groups from inositol hexaphosphate) and nucleotidases. Acid and alkaline phosphomonoesterases do not hydrolyse phosphates of phytic

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acid (*myo*-inositol hexaphosphates) but they can hydrolyse lower-order inositol phosphates (Cosgrove 1980). Phosphodiesterases hydrolyse one or two ester bonds in phosphodiester compounds and include nucleases, which catalyse the hydrolysis of phosphodiester bonds of nucleic acids to produce nucleotide units or mononucleotides but not inorganic phosphates. Phospholipases hydrolyse phospholipids. We shall also discuss inorganic pyrophosphatase (pyrophosphate phosphohydrolases, EC 3.6.1.1), the enzyme that hydrolyses pyrophosphate to inorganic P, because pyrophosphate can be used as a fertilizer (Dick and Tabatabai 1978).

The aim of this review is to discuss the role of phosphatases in P mineralisation in soil and the response of these enzyme activities to changes in environmental factors, agricultural management and pollution. Particular attention will be given to phosphomonoesterase activities, which have been studied most among soil phosphatases. The meaning of measuring phosphatase activities and the drawbacks of the current protocols for enzyme assays have a central role because we think that a better understanding of the role of phosphatases (like that of any enzyme activity in soil) depends on improvement of the present enzyme assays by separating the contribution of extracellular stabilised phosphatase activities from the contribution of activities of phosphatases associated with active microbial cells. The effects of organic amendments, fertilizers and pollutants will be discussed by considering the present drawbacks of the currently used enzyme assays rather than listing all reports on the subject and underlining the contradictory data. We suggest that the reader also considers the review by Speir and Ross (1978) because it discusses the first reports and includes an extensive bibliography of the 1950s, 1960s and part of the 1970s on the effects of sterilisation, air drying, storage of soil samples before measurements, pH, temperature, soil properties, soil depth, fertilizers, trace elements, activators and inhibitors. We also refer to the reviews by Malcom (1983) and Tabatabai (1994) for a more detailed discussion of the analytical problems and for the state-of-the-art on kinetic properties and the effects of inhibitors, activators and soil properties on phosphatase activities.

9.2 Determination of Soil Phosphatase Activities

Activities of soil phosphomonoesterases have been the most studied, although phospholipids and nucleic acids, whose degradation is catalysed by phosphodiesterases, are among the major sources of fresh organic P inputs to soil (Cosgrove 1967). Before the advent of the simple, accurate and rapid enzyme assay based on the use of *p*-nitrophenyl phosphate (pNPP) by Tabatabai and Bremner (1969), phosphatase assays used natural substrates such as β-glycerolphosphate and nucleic acids (Speir and Ross 1978; Malcom 1983; Tabatabai 1994). The use of artificial substrates began in the early 1960s with phenyl phosphate (Hofmann 1963), phenolphthalein phosphate (Dubovenko 1964; Geller and Ginzburg 1979), pNPP (Bertrand and de Wolf 1968), α-naphthyl phosphate (Hochstein 1962) and β-naphthyl phosphate (Ramirez-Martinez and McLaren 1966). The choice of artificial

substrates eliminated the determination of released phosphate, which is easily adsorbed by soil particles (Tabatabai 1994). The success of the pNPP assay stems also from the fact that hydrolysis of pNPP is much more rapid than that of natural substrates such as nucleic acids. The pNPP is hydrolysed to *p*-nitrophenol (pNP), which is usually determined spectrophotometrically at 400 nm under alkaline conditions. Soluble organic compounds can interfere with the quantification of the pNP (Vuorinen and Saharinen 1996). For this reason, Gerritse and van Dijk (1978) suggested the separation of pNP from pNPP and other soluble organic compounds, extracted from organic soils or animal wastes, by high pressure liquid chromatography on a cellulose column. They also observed a marked reduction of both acid and alkaline phosphomonoesterase activity by phosphate concentrations greater than 0.1 mM and therefore suggested using pNPP concentrations of 0.01–0.1 mM in the enzyme assay.

Because the phosphate group in pNPP is attached to the aromatic chromophore, hydrolysis may not reflect the activity of alkyl phosphomonoesterases. To determine this enzyme activity in soil, Avidov et al. (1993) proposed an assay based on the hydrolyis of 4-(*p*-nitrophenoxy)-1,2-butanediol phosphate with successive oxidation of the reaction product 4-(*p*-nitrophenoxy)-1,2-butanediol to pNP.

The hydrolysis of 4-methy-umbelliferyl phosphate (MUP) to 4-methylumbelliferone (MU) has also been used to assay phosphomonoesterase activity in soil by determining the fluorescence of the MU. This assay circumvents interferences by soluble organic compounds because fluorescence is measured for emission wavelengths after specific excitation (Marx et al. 2001). The MUP assay gave higher values than the pNPP assay, but the two enzyme activities (measured in modified universal buffer adjusted to the soil pH value) were significantly correlated ($P < 0.001$) when expressed on the basis of C content but not when expressed on the basis of dry soil weight (Drouillon and Merckx 2005). The MUP assay gave lower K_m values than the pNPP assay (Table 9.1) (Marx et al. 2001) and this may suggest that the former substrate mimics the hydrolysis of naturally occurring soil organic phosphate esters more closely (Freeman et al. 1995).

The phosphodiesterase assay is similar to the phosphomonoesterase assays because it is based on the release of pNP from bis-*p*-nitrophenyl phosphate (bpNPP) when the soil slurry is incubated with the substrate at pH 8.0 for 1 h (Browman and Tabatabai 1978). The bpNPP was first used by Ishii and Hayano (1974). Ohmura and Hayano (1986) showed that the optimum pH of phosphodiesterase activity of 15 soils ranged from 4.5 to 9.5, a broader pH optimum than that suggested in the assay by Browman and Tabatabai (1978). In addition, the enzyme activity was significantly correlated with soil pH.

Phosphotriesterase activity of soil has been determined by hydrolysis of tris-*p*-nitrophenyl phosphate, which is insoluble in water, to pNP (Eivazi and Tabatabai 1977).

According to Turner et al. (2002a, b), soil phytase has been poorly studied because it has been determined by the release of phosphate from phytate and not by using suitable artificial substrates (Yadav and Tarafdar 2003). Berry et al. (2007) proposed measuring the phytase activity of soil by using a chromophoric substrate

Table 9.1 Some K_m values of phosphatases

Enzyme	K_m (mM)	Substrate concentration (mM)	Temperature (°C)	Buffer	pH	References
Acid phosphomonoesterase	0.94–1.75	1–20[a]	37	MUB	6.5	Tabatabai and Bremner (1971)
Acid phosphomonoesterase	0.35–5.40[b]	–	37	Acetate	4.7	Cervelli et al. (1973)
Acid phosphomonoesterase	1.11–3.40	1–20[a]	37	MUB	6.5	Eivazi and Tabatabai (1977)
Acid phosphomonoesterase	0.1	0.05–0.50	30	Acetate	5.0	Gerritse and van Dijk (1978)
Acid phosphomonoesterase	1.71–6.99[b]	3.2–23.0[b]	30	MUB	5–6	Trasar-Cepeda and Gil-Sotres (1988)
Alkaline phosphomonoesterase	0.7	0.05–0.50	30	Tris	8.0	Gerritse and van Dijk (1978)
Alkaline phosphomonoesterase	0.44–4.94	1–20[a]	37	MUB	11	Eivazi and Tabatabai (1977)
Phosphodiesterase	0.25–1.25	1–15[a]	37	MUB	10	Eivazi and Tabatabai (1977)
Pyrophosphatase	21–51[c]	10–60[a]	37	MUB	–	Dick and Tabatabai (1978)

MUB modified universal buffer

Substrates were *p*-nitrophenyl phosphate for phosphomonoesterases, bis-*p*-nitrophenyl phosphate for phosphodiesereases, and pyrophosphate for pyrophosphatase

[a]Soil solution bases

[b]Corrected for the adsorption of the substrate

[c]Lineweaver–Burk plot

analogue of phytic acid whose disappearance can be monitored by high-performance liquid chromatography with UV detection. However, the method has not yet been set up for determining enzyme activity in soil.

Dick and Tabatabai (1977, 1978) set up an accurate method for determining inorganic pyrophosphatase activity of soil at pH 8.0 using pyrophosphate as the substrate and an improved determination of the released phosphate. This enzyme activity can be important from an agricultural point of view because pyrophosphate is a fertilizer P. According to Dick and Tabatabai (1978), the previous assays presented various drawbacks such as the adsorption of enzymatically released inorganic P by soil particles, hydrolysis of pyrophosphate to inorganic P after extraction from soil due to other reactions than that catalysed by pyrophosphatase, and interference of pyroposphate on the determination of inorganic P.

The hydrolysis of polyphosphates in soil has been determined by Dick and Tabatabai (1986). One of the polyphosphates used in agriculture is trimetaphosphate, a cyclic polyphosphate (Busman and Tabatabai 1985). The assay for determining trimetaphosphatase (trimetaphosphate hydrolase, EC 3.6.1.2) activity was set up by Busman and Tabatabai (1985). It involves the incubation of soil with trimetaphosphate at pH 8.0 for 5 h, followed by precipitation of residual trimetaphosphate, pyrophosphate and triphosphate. Phosphate is not precipitated and can then be determined. Trimetaphosphate is hydrolysed by trimetaphosphatase to triphosphate, which is then hydrolysed by triphosphatases to pyrophosphate and phosphate (Tabatabai 1994). Finally, pyrophosphate is hydrolysed to phosphate by pyrophosphatase. Therefore, the interpretation of the data obtained by this assay is complicated by the fact that the enzyme assay measures the activity of three enzymes, trimetaphosphatase, triphosphatase and pyrophosphatase (Tabatabai 1994).

9.3 Range and Kinetic Properties

Table 9.2 shows the range of phosphatase activities measured in soil with current assay procedures. Acid phosphomonoesterase activities in soil have been frequently measured at pH 6.5; however, at this pH the measured enzyme activity may include acid and alkaline phosphomonoesterase activity (Malcom 1983). Acid phosphomonoesterase activity generally prevails in acidic soils, whereas alkaline phosphomonoesterase activity prevails in alkaline soils, and for this reason the activities of the two enzymes are negatively correlated (Juma and Tabatabai 1978). Pang and Kolenko (1986) found a pH optimum of 7.0 for phosphomonoesterase activity in two forest soils. In comparing phosphatase activities (as for any other enzyme activity in soil) it is important to consider the period of the year in which soil sampling is done because enzyme activities of soil can change throughout the year (Schneider et al. 2001). Grierson and Adams (2000) observed that acid phosphomonoesterase activity of Jarrah (*Eucalyptus marginata* Donn ex Sm) forest soils

Table 9.2 Range of measured phosphatase activities

Enzyme	Substrate	Substrate concentration (mM)	Temperature (°C)	Duration of assay (h)	Buffer	pH	Range of enzyme activity (μmol product g^{-1} h^{-1})	References
Phosphomonoesterase	pNNP	50	20	1	MUB	At soil pH of 3.2–8.1	0.05–5.22	Drouillon and Merckx (2005)
Phosphomonoesterase	MUP	16 and 25	20	0.25	MUB	At soil pH of 3.2–8.1	0.48–10.41	Drouillon and Merckx (2005)
Acid Phosphomonoesterase	pNPP	10	37	0.5	0.5 M Tris maleate	6.5	10.4–307	Turner et al. (2002b)
Acid Phosphomonoesterase	pNPP	5	37	1	0.5 M Tris maleate	6.5	2.62–12.19	Turner and Haygarth (2005)
Acid Phosphomonoesterase	–	25	37	1	MUB	6.5	1.03–10.38 in air dried soils; 2.11–27.07 in moist soils	Baligar et al. (1988)
Acid phosphomonoesterase	MUP	0.01–0.40	–	–	Water	At soil pH of 4.0–4.3	0.57–1.08	Santruckova et al. (2004)
Acid phosphomonoesterase	pNNP	50	37	1	MUB	6.5	0.85–14.9	Dick et al. (1988)
Acid phosphomonoesterase	pNNP	50	37	1	MUB	6.5	0.31–3.15	Zornoza et al. (2009)
Acid phosphomonoesterase	pNNP	5	37	1	MUB	6.5	0.35–0.88	Eivazi and Tabatabai (1977)
Acid phosphomonoesterase	–	115	37	1	MUB	6.5	0.06–0.13	Ho (1979)
Alkaline phosphomonoesterase	pNNP	5	37	1	MUB	11	0.06–1.60	Eivazi and Tabatabai (1977)
Alkaline phosphomonoesterase	pNNP	50	37	1	MUB	11	0.34–5.50	Dick et al. (1988)
Phosphodiesterase	bpNPP	5	37	1	MUB	9–11	0.10–0.55	Eivazi and Tabatabai (1977)
Phosphotriesterase	tpNPP (insoluble)	5	37	1	MUB	10	0.01–0.08	Eivazi and Tabatabai (1977)

pNPP *p*-nitrophenyl phosphate, *MUP* 4-methyl umbelliferyl phosphate, *MUB* modified universal buffer, *bpNPP* bis-*p*-nitrophenyl phosphate, *tpNPP* tris-*p*-nitrophenyl phosphate

ranged from 30 to 40 μmol pNP g^{-1} h^{-1} in winter and spring when soil was moist, whereas it was below 10 μmol pNP g^{-1} h^{-1} in the dry summer.

Activities of phosphodiesterases are lower than acid and alkaline phosphomonoesterase activities (Criquet et al. 2007) because the production of P monoesters from P diesters may stimulate the microbial synthesis of phosphomonoesterases (Turner and Haygarth 2005). It is reasonable to hypothesise that phosphodiesterase and phosphomonoesterase activities act sequentially in soil (Fig. 9.1). Phosphotriesterase activity was also lower than acid and alkaline phosphomonoesterase activities of soil (Eivazi and Tabatabai 1977).

Table 9.1 shows the K_m (the Michaelis–Menten constant) values of phosphatases in soil. Although phosphatases, like other hydrolases in soil, can derive from different sources and thus have different kinetic constants, the K_m value of phosphatase activity of a soil can be calculated. As discussed by Nannipieri and Gianfreda (1998), the calculated values probably represent a weighted average of the various constants of enzymes involved in the measured enzyme activity, with an unknown weighting factor. However, in the case of acid phosphomonoesterases, at least two enzymes with markedly different K_m values were found in pyrophosphate extracts from two soils by applying the Eadie–Scatchard plot (rate of reaction V vs. the substrate concentration S) (Nannipieri et al. 1982).

Brams and McLaren (1974) observed a marked deviation from linearity at higher substrate concentration for soil phosphomonoesterase (pH 6.90), and Irving and Cosgrove (1976) suggested that diffusional effects and adsorption of substrate by soil colloids were responsible for the fact that acid phosphomonoesterase of a Krasnozem did not follow Michaelis–Menten kinetics. Cervelli et al. (1973) proposed calculating the K_m value by considering the adsorption of the substrate (pNPP) by the Freundlich law; the corrected K_m value of acid phosphomonoesterase was lower than

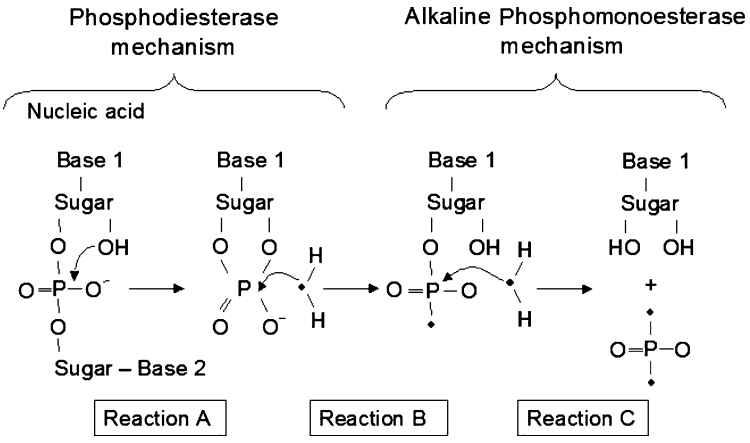

Fig. 9.1 Mechanisms of phosphodiesterase and alkaline phosphomonoesterase reaction involving molecular rearrangement (*Reaction A*) and incorporation of oxygen atoms (•) in the phosphate molecule (*Reactions B* and *C*). Redrawn from Blake et al. (2005)

the uncorrected value. The same was observed for the K_m values of acid phosphomonoesterase in acid and organic soils from Galicia, Spain (Trasar-Cepeda and Gil-Sotres 1988). The shaking of soil slurries can accelerate the diffusion of the substrate towards the enzymes. Indeed, K_m values of phosphomonoesterases measured at pH 6.9 were 2.5 times greater when measured in soil columns than when measured in a batch-type system with shaking (Brams and McLaren 1974).

9.4 Limitations of the Present Enzyme Assays

Although the drawbacks of the currently used enzyme assays have been extensively discussed (Skujins 1978; Burns 1978, 1982; Nannipieri 1994; Tabatabai 1994; Nannipieri et al. 2002; Gianfreda and Ruggiero 2006), they are still frequently neglected when soil enzyme activities are interpreted. Firstly, the present enzyme assays measure potential and not real enzyme activities (Fig. 9.2) because the assay conditions are different (optimal pH, optimal temperature, substrate present at saturating concentration, presence of buffer to control pH during the assay, soil slurry, shaking) from those occurring in situ (fluctuations of temperature and moisture of the soil; pH and substrate concentration are rarely at the optimum for enzyme activity, etc.). Secondly, we do not know which enzymes contributed to the

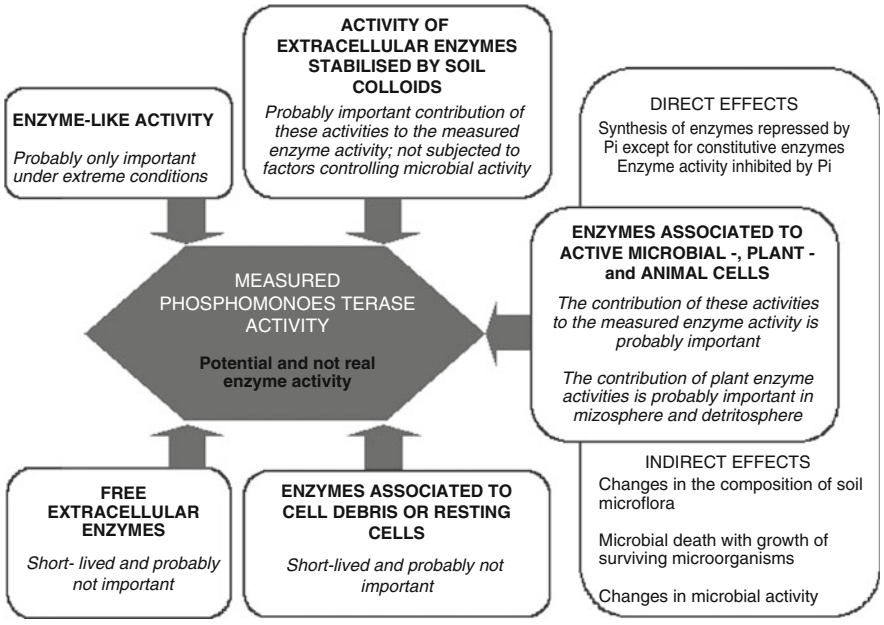

Fig. 9.2 Contribution of activities of phosphomonoesterase differently located in the soil matrix to the measured enzyme activity, and drawbacks of the currently used assays

measured enzyme activity. According to Burns (1982), enzymes catalysing the measured reaction can be:

1. Associated with active microbial cells, either intracellular or attached to the outer cell surface
2. Associated with cell debris or dead cells
3. Associated with resting cells, such as bacterial spores
4. Released as truly extracellular enzymes to degrade high molecular weight or insoluble substrates
5. Present as extracellular enzymes of enzyme–substrates complexes
6. Present as free extracellular enzymes
7. Present as extracellular enzymes stabilised by their association with surface-reactive particles (e.g. clay minerals, iron oxides and hydroxides)
8. Entrapped by humic matter (the humus–enzyme complexes)

The activity of enzyme-like catalysts is probably significant under extreme environmental conditions where these catalysts are present, whereas activities of free extracellular enzymes (6), enzymes associated with substrates (5), and enzymes of cell debris and dead cells (2) are probably short-lived because they can be rapidly degraded unless they are adsorbed by soil particles. The contribution of enzymes associated with resting cells is probably insignificant (Nannipieri et al. 2002). Therefore, it is reasonable to hypothesise that the measured enzyme activity depends on the activity of enzymes associated with active microbial cells, including enzyme activities of plant cells in the rhizosphere and detritosphere soil, and on the activity of extracellular enzymes stabilised by soil colloids (Fig. 9.2) (Nannipieri 1994; Nannipieri et al. 2002). The extracellular stabilised enzyme activity is not affected by changes in composition, abundance or activity of the soil microflora; thus the intracelluler enzyme activity of active cells should be used as an indicator of nutrient dynamics and changes in soil functioning due to agricultural management and ecological factors because it is well established that microbial activities of soil are more sensitive to these changes than other soil properties (Nannipieri et al. 2003). However, the separation of stabilised extracellular enzyme activity and enzyme activity associated with active microbial cells and plant cells is not possible with the present enzyme assays (Nannipieri et al. 2002). Most reports on soil enzymes assume that the present short assays only determine extracellular and stabilised enzyme activity. Despite this assumption, the measured enzyme activities are often taken as indicators of soil quality, which is strictly related to microbial activity and thus to intracellular enzyme activity. In addition, it is often assumed that changes in enzyme activities only reflect the response of microbiota to environmental factors, neglecting the fact that the measured enzyme activity also depends on the activity of stabilised extracellular enzymes.

Microbial inhibitors such as toluene have been used to inhibit the enzyme activity associated with active microbial cells, but this can create artefacts; for example, toluene can increase the permeability of cell membranes and thus the access of the urea substrate to intracellular ureases (Nannipieri et al. 2002). In addition, microorganisms can also use toluene as a substrate (Kaplan and

Hartenstein 1979). Toluene did not affect phosphodiesterase or acid and alkaline phosphomonoesterase activities but increased the phosphotriesterase activity of soil (Eivazi and Tabatabai 1977).

The so-called physiological response method is based on the measurements of enzyme activities and microbial biomass of soil during the period when microbial growth is stimulated by adding glucose and a nitrogen source to soil (Nannipieri et al. 2002). If enzyme activity is plotted against biomass, there is generally a significant and positive correlation between the enzyme activity (*y*-axis) and microbial biomass (*x*-axis). The extrapolation to zero of microbial biomass gives a positive intercept of the plot on the *y*-axis, which is the extracellular stabilised enzyme activity. This approach has been used to calculate extracellular acid phosphomonoesterase activity of a moist soil treated with different rates of sewage sludges, with measurement of microbial biomass by measuring ATP. Extracellular phosphomonoesterase activity of soil was 14.9, 5.3 and 4.3 μmol pNPP g^{-1} h^{-1} after addition of 0, 50 and 100 tons of sewage sludge per hectare, respectively (Nannipieri et al. 1996a). This approach can only work for constitutive but not for inducible or repressible enzymes, such as phosphomonoesterase, whose synthesis is generally repressed by inorganic phosphate (Nannipieri 1994). Indeed, changes in inducible or repressible enzyme activities are not related to changes in microbial biomass. In addition, the percentage of glucose-utilising microorganisms depends on soil type, management and pollution (Nannipieri et al. 2002). Acid phosphomonoesterase activity of two eucalypt forest soils was significantly correlated with ergosterol content and microbial P when all these properties were measured throughout the year, the relative plots giving a positive intercept on the *y*-axis (Grierson and Adams 2000). Obviously, the intercept obtained with the first correlation cannot represent the extracellular acid phosphomonoesterase activity because ergosterol content only determines the fungal biomass. However, the approach by Grierson and Adams (2000) does not involve the stimulation of microbial growth by adding easily degradable organic compounds to soil, and thus does not present the above-mentioned drawbacks (Nannipieri et al. 2002).

Chloroform fumigation has also been used to distinguish enzyme activity associated with active microbial and plant cells from the extracellular enzyme activity stabilised in soil (Klose and Tabatabai 1999). This method assumes that the present short-term enzyme assays measure the stabilised extracellular enzyme activity and that the increase in enzyme activity after $CHCl_3$ fumigation is due to the intracellular enzyme activity. Therefore, the intracellular enzyme activity of soil can be calculated by subtracting the enzyme activity before fumigation from that after fumigation. As discussed by Nannipieri et al. (2002), this approach presents the following problems: (1) $CHCl_3$ fumigation does not kill all microbial cells and the efficiency of cell lysis depends on soil structure (Arnebrant and Schnurer 1990), and (2) the assumption that the present short-term enzyme assays determine only the extracellular enzyme activity has never been proven. The fact that enzyme activities, including acid and alkaline phosphomonoesterase activities, can increase with microbial biomass when easily degradable organic compounds, such as glucose, are added to soil (Nannipieri et al. 1978, 1979, 1983; Renella et al. 2006a, b, 2007b)

suggests that the present enzyme assays also measure the contribution of enzyme activities associated with active microbial cells of soil, and (3) proteases are active during the $CHCl_3$ fumigation period and degrade urease and both phosphomonoesterase enzymes (Renella et al. 2002); thus, protease activity needs to be inhibited during soil fumigation.

The use of sonication can increase the enzyme activity of soil. Indeed, the activity of acid phosphomonoesterase was 156% higher with soil sonication than without it (De Cesare et al. 2000). The increase probably depended on the release of extracellular enzymes stabilised by soil colloids and not on cell lysis, because the release of enzymes by sonication was not related to the release of ATP.

9.5 Role of Phosphatase in Organic P Mineralisation in Soil and the Effect of Inorganic P

As already mentioned, phosphodiesterase and phosphomonoesterase activities may act sequentially (Fig. 9.1). Pant and Warman (2000) observed that acid phosphomonoesterase (from wheat germ), alkaline phosphomonoesterase (from calf intestinal mucosa), phospholipase (from *Clostridium perfingrens*) and nuclease (from *Staphylococcus aureus*), all immobilised on positively charged supports, were able to mineralise (at pH 7.0) organic P extracted from different soils by water or NaOH. The activities of both phosphomonoesterases were generally increased when these enzymes were used with one of the two phosphodiesterases.

Soil acid phosphomonoesterase activity was higher at low inorganic P content of soil than at high content, and the enzyme activity of the low-P soil was significantly correlated with herbage yield, probably due to the importance of organic P mineralisation for plant P nutrition (Speir and Cowling 1991). Santruckova et al. (2004) found that higher enzymatic hydrolysis of organic P depended on the higher microbial P immobilisation but not on the higher mineralisation of organic P compounds.

Application of inorganic P can repress the synthesis of phosphomonoesterases in soil because it inhibits the expression of *PHO* genes (Oshima et al. 1996) and, indeed, phosphate inhibits the phosphatase activities of soil (Halstead 1964; Juma and Tabatabai 1977, 1978; Lima et al. 1996; Moscatelli et al. 2005; Nannipieri et al. 1978; Olander and Vitousek 2000; Spiers and McGill 1979). However, the absence of a response of phosphatase activities to P addition has also been reported. For example, the application of triple superphosphate to an oak soil in 1992 did not affect acid phosphomonoesterase activity of soil samples taken in 1993 and 1994 (Schneider et al. 2001). Addition of phosphate with glucose and inorganic N did not stimulate the phosphomonoesterase activity (pH 6.5) of soil, whereas the stimulation occurred in the respective soil treated only with glucose and inorganic N (Nannipieri et al. 1978). Presumably, the enzyme activity was not decreased by phosphate due to the presence of extracellular phosphomonoesterases stabilised by

soil colloids or due to the presence of constitutive microbial phosphomonoesterase in soil. Enzyme assays discriminating the activities of extracellular stabilised enzymes from activities of enzymes associated with soil microorganisms would permit an understanding of the underlying mechanisms (Fig. 9.2).

9.6 Phosphatase Activities of Bulk and Rhizosphere Soil and the Origin of Phosphatases in Soil

It is well established that enzyme activities are higher in rhizosphere than bulk soil (Skujins 1978; Tarafdar and Chhonkar 1978; Dinkelaker and Marschner 1992). Both acid and alkaline phosphomonoesterase activites of soil were increased near the rhizoplane of *Brassica oleracea*, *Allium cepa*, *Triticum aestivum* and *Trifolium alexandrium* and such an increase depended on plant species, soil type and plant age (Tarafdar and Jungk 1987). Probably, the increase with plant age was due to the gradual formation of the rhizosphere microflora and to the release of plant phosphomonoesterases. The distance from the rhizoplane at which the rhizosphere effect on enzyme activities was observed was higher for acid (from 2 to 3.1 mm) than for alkaline (from 1.2 to 1.6 mm) phosphomonoesterase. There was an inverse and significant correlation between the acid or the alkaline phosphomonoesterase activity and the content of organic P of the rhizosphere soil sampled from *Triticum aestivum* and *Trifolium alexandrium,* whereas the content of inorganic P increased towards the rhizoplane. Increases in both acid and alkaline phosphomonoesterase activities near the rhizoplane of maize were accompanied by changes in the composition of bacterial communities as determined by PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) (Kandeler et al. 2002). Some agroforestry species (*Tithonia diversifolia, Tephrosia vogelii* and *Crotalaria grahmiana*) stimulated acid phosphomonoesterase activity of rhizosphere soil, whereas maize stimulated alkaline phosphomonoesterase activity of rhizosphere soil (George et al. 2002). Higher soil phosphomonoesterase activities were found under invader plant species than under grass and forbs, presumably due to the higher P uptake of the invading plants (Neal 1973). Izaguirre-Mayoral et al. (2002) found that nodulated legumes species growing in acid savanna soils stimulated the acid phosphomonoesterase activity of rhizosphere soil.

Interactions between soil microorganisms and plant species can also affect phosphomonoesterase activities of rhizosphere soil. It has been suggested that arbuscular mycorrhizal (AM) fungi stimulate the release of acid phosphomonoesterase from roots of subterranean clover (*Trifolium subterraneum* L.) (Joner and Jakobsen 1995). However, mycorrhizal infection of cucumber (*Cucumus sativus* L.) did not affect acid and alkaline phosphomonoesterase activities of soil (Joner et al. 1995). More information on the relationship between phosphatases and mycorrhizae is given by Jansa et al. (2011).

Obviously, it is difficult to interpret the measurement of phosphomonoesterase activities of rhizosphere soil if the contribution of plant and microbial phosphatases to the measured enzyme activity are not separated. Colvan et al. (2001) suggested that acid phosphomonoesterase activity of hay meadow soils was due to enzyme released by plants, because the enzyme activity was high and microbial P was low in soils never treated with fertilizer or treated with N or K fertilizer for 100 years. However, the measured acid phosphomonoesterase activities could also have been at least partly due to enzymes synthesised by the soil microflora in response to P-deficient conditions (Nannipieri 1994). A significant correlation between acid phosphomonoesterase activity of an oak soil and fine root length density of oak has been found (Schneider et al. 2001). The response of activities of hydrolases, including acid and alkaline phosphomonoesterase and phosphodiesterase, in rhizosphere soil depends on the type of root exudate stimulating microbial activity (Renella et al. 2006b, 2007a), which suggests that active microbial phosphomonoesterases are probably the major contributing enzymes to the measured enzyme activity of the rhizosphere soil.

Phosphorus nutrition of transgenic *Nicotiana tabacum* expressing a chimeric phytase gene (*ex::ph A*) from soil fungus *Aspergillus niger* was better in neutral than in acid soils, because the adsorption by soil of phytase released from roots was lower at neutral than at acid pH values (George et al. 2005a, b). The expression of phytase in the transgenic plant had no effect on the structure of microbial communities of rhizosphere soil compared to the wild type (George et al. 2009).

No correlations have been found between acid phosphomonoesterase activity and acid phosphomonoesterase-active bacterial colonies, and between alkaline phosphomonoesterase activity and alkaline phosphomonoesterase-active bacterial colonies of forest soils (Hysek and Sarapatka 1998), but this is not surprising since culturable bacteria only represent 1–10% of the bacteria inhabiting soil (Torsvik et al. 1996). Acid phosphomonoesterase was secreted by roots of three cereals (wheat, pearl millet and sorghum), three legumes (mung bean, moth bean and cluster bean), and three oil seed crops (groundnut, sesame and mustard) when these plants were grown in culture solution under P-deficient conditions (Yadav and Tarafdar 2001). The secretion pattern depended on the plant species, and the amount of root exudates increased with plant age and was higher with phytin than with leucithin and glycerolphosphate, each present as the sole source of P. Because acid phosphomonoesterases (like any enzyme molecule) diffuse poorly into the soil matrix, plant-released enzymes probably mineralise organic P from sloughed off or damaged cells rather than from native soil organic P (Lefebvre et al. 1990; Yadav and Tarafdar 2001).

Alkaline phosphomonoesterase activity has not been detected in plants (Dick et al. 1983; Juma and Tabatabai 1988a, b, c) and for this reason microbial cells supposedly synthesise most of the soil alkaline phosphomonoesterases (Tabatabai 1994). Both soil bacteria and soil microorganisms other than AM fungi (Joner and Jakobsen 1995) are thought to contribute to the measured soil alkaline phosphomonoesterase activity. Indeed, alkaline phosphatase activity of both rhizosphere and bulk soil depend on the composition of bacterial communities harbouring alkaline phosphatase genes, as determined by DGGE after amplification of extracted DNA by polymerase chain

reaction with specific primers (Sakurai et al. 2008). Increases in acid and alkaline phosphomonoesterase activities due to the addition of ryegrass residues to soils were related to changes in the composition of bacterial communities, as determined by DGGE (Renella et al. 2006a). Changes in the ergosterol content of Jarrah forest soils explained 50% of the changes in acid phosphomonoesterase activity in each season, whereas the ergosterol content of soil sampled under *Banksia grandis*, an understorey dominant plant growing in dense thickets in the absence of fire, explained 74% of the changes in enzyme activity during the dry season (summer) but only 10% in moist soils (Grierson and Adams 2000). Both phytase- and phosphomonoesterase-producing fungi isolated from arid and semiarid soils of India belonged to the genera *Aspergillus*, *Emmericella* and *Penicillum* (Yadav and Tarafdar 2003). In conclusion, the present evidence confirms that bacteria are the main source of alkaline phosphomonoesterase activity in soil, whereas acid phosphomonoesterase and phytase can derive from plants, fungi and bacteria. However, studies such as that by Sakurai et al. (2008) involving detection of genes codifying these enzymes are necessary.

Renella et al. (2007b) estimated the production and persistence of acid and alkaline phosphomonoesterase and phosphodiesterase activities in soils with a wide range of properties, by stimulating microbial growth through the addition of glucose and inorganic N to soil. Phosphatase activities of the soil increased, with microbial biomass reaching a peak value, but then both declined on prolonging the incubation time. Enzyme production (Pr) was calculated by the relationship $Pr = H/t_H$, where H is the peak of the enzyme activity and t_H is the time of the peak after adding glucose plus N to soil. Enzyme persistence (Pe) was calculated by the relationship $Pe = (r/H)\Delta t$, where r indicates the residual enzyme activity at the end of the incubation time and Δt is the time interval between the peak value (H) and the residual activity (r). The Pr values of acid phosphomonoesterase activity were highest in soils under forest and set-aside management, whereas Pr values of alkaline phosphomonoesterase and phosphodiesterase activities were highest in alkaline and neutral soils. The Pe values of acid phosphomonoesterase activity were highest in acid soils, whereas no relationship was found between alkaline phosphomonoesterase or phosphodiesterase activity and the soil pH or management (Renella et al. 2007b).

Both phosphodiesterases and pyrophosphatases are ubiquitous in animal, plant and microbial cells because they are involved in the degradation of nucleic acids and in several basic metabolic pathways of cells, respectively (Browman and Tabatabai 1978; Cooperman et al. 1992; Tabatabai 1994).

9.7 Effects of Soil Handling, Soil Properties, Agricultural Management and Pollutants on Soil Phosphatase Activities

Here, we shall only discuss reports after the late 1970s because Speir and Ross (1978) have extensively reviewed the effects of soil sampling, handling and storage, soil

properties, different agricultural managements, forest practices and pollutants on phosphatase activities.

9.7.1 *Effects of Soil Handling and Soil Properties on Phosphatase Activities*

Both air-drying and freeze-drying often decrease acid and alkaline phosphomonoesterase activity of soil (Gerritse and van Dijk 1978; Baligar et al. 1988; Adams 1992). However, Eivazi and Tabatabai (1977) found an increase in acid phosphomonoesterase and phosphotriesterase activities and a decrease in alkaline phosphomonoesterase and phosphodiesterase activites after air-drying of soil. Acid phosphomonoesterase activities in moist soils stored at 4°C and in the respective air-dried soils were significantly correlated (Baligar et al. 1988). Air-drying also decreased pyrophosphatase activity of soil, and the best storage conditions were to keep field-moist soils at 5°C (Tabatabai and Dick 1979). Probably the best strategy is to keep moist soils at 4°C and measure the enzyme activity as soon as possible. Kandeler (2007) suggests that if the determination of the enzyme activity requires storage periods longer than 3 weeks at 4°C, it is better to store the samples at −20°C than at 4°C. At the end of the storage period, soil samples are allowed to thaw at 4°C for about 2 days before the determination of the enzyme activity.

Steam sterilisation at 121°C for 1 h completely inactivated alkaline phosphomonoesterase, phosphodiesterase and phosphotriesterase activity, but increased acid phosphomonoesterase activity (Eivazi and Tabatabai 1977). Heating above 60°C inactivated the pyrophosphatase activity of soil (Tabatabai and Dick 1979).

It is well established that phosphatase activities are correlated with the content of organic matter and decrease with soil depth (Speir and Ross 1978; Tabatabai and Dick 1979; Prado et al. 1982; Pang and Kolenko 1986; Tabatabai 1994). Factors affecting phosphatase activity, measured by using sodium phenyl phosphate as a substrate in different woodland soils, could be ranked as rock type = vegetation type > soil type = season > soil depth (Harrison 1983). Some 20% of the variation of both acid and alkaline phosphomonoesterase activities of semiarid woodland soils depended on soil microclimate and surface depth (0–10 cm); soil temperature together with soil water potential was a better predictor of phosphatase activities than either factor alone (Kramer and Green 2000).

Humic acids competitively inhibited wheat phytase activity measured at 55°C and pH 5.15 (Pereira 1971). For forest soils, phosphomonoesterase activity of litter (pH in water 3.6–3.7), humus (pH in water 3.4–3.5) and mineral layers (pH in water 4.0–4.3), measured with water and MUP as the substrate, were correlated with the contents of organic C, microbial N and microbial P, and with soil respiration (Joergensen and Scheu 1999). Santruckova et al. (2004) observed that changes in soil pH and contents of total organic C, total N, total P, oxalate-soluble reactive and organic P, as well as oxalate-soluble Al and Fe can affect phosphomonoesterase activity and microbial biomass P in various ways, leading either to a surplus or a

deficiency in available soil phosphate. The relationship between phosphomonoesterase activities and the distribution of P forms is not clear. Acid phosphomonoesterase and phosphodiesterase activities of Karri forest soils were not related to soil P fractions (non-occluded Fe- and Al-bound P, P sorbed by carbonates, occluded P, and Ca-bound P) or total P (Adams 1992).

Alkaline phosphomonoesterase activity, measured using disodium phenylphosphate as substrate, was mainly associated with silt and clay fractions of a Haplic Chernozem (Kandeler et al. 1999). By contrast, in a calcareous and in an acid soil, both alkaline and acid phosphomonoesterase activities were associated with larger soil fractions (100–2,000 μm particle diameter) containing plant debris and less humified organic matter and were characterised by the highest mineralisation of organic P among the soil fractions (Rojo et al. 1990).

9.7.2 *Effect of Agricultural Management, Forest Practices and Fire on Soil Phosphatases*

Hay meadow soils treated with farmyard manure for about 100 years had higher acid and alkaline phosphomonoesterase and phosphodiesterase activities and higher NH_4F–HCl-extractable P than those receiving mineral P (Colvan et al. 2001). However, both phosphomonoesterases and phosphodiesterase activities were positively ($P < 0.1$) correlated with extractable P in soils treated with farmyard manure or with phosphate, whereas acid phospomonoesterase activity was negatively ($P < 0.05$) correlated with extractable P. Acid phosphomonoesterase activity was negatively correlated with alkaline phosphomonoesterase ($P < 0.05$) and with phosphodiesterase ($P < 0.05$) activities when considering all treatments, but was always higher than the alkaline phosphomonoesterase activity because all soils were acidic.

Straw burning instead of straw soil incorporation decreased acid but not alkaline phosphomonoesterase activity of the soil (Dick et al. 1988). Sewage sludges applied to soils increased phosphodiesterase and acid and alkaline phosphomonoesterase activities, which subsequently decreased with time (Criquet et al. 2007). Long-term experiments have shown that repeated applications of manure increases both acid and alkaline phosphomonoesterase activities, particularly immediately after manure addition to the soil (Dick et al. 1988; Colvan et al. 2001), due to the stimulation of microbial growth. When the monitoring period is prolonged, stimulation of microbial synthesis of enzymes by easily degradable organic substrates decreases (Garcia et al. 1993; Nannipieri 1994). However, the interpretation of changes in enzyme activity of soils treated with organic materials is difficult because these materials add exogeneous enzymes associated with microorganisms and extracellularly stabilised enzymes to the soil.

No-till systems usually have higher enzyme activities in the surface soils than tilled soils because of the increase in soil organic matter content (Nannipieri 1994). However, this did not occur in an organic soil (Bergstrom et al. 1998).

In forest soils, fertilisation with urea or phosphate reduced acid phosphomonoesterase activity (Pang and Kolenko 1986). Fumigation of forest soils with methylbromide and chloropicrin for 24 h decreased phosphomonoesterase activity (pH 7.0), but there was a recovery when the fumigant was removed and the moist soils were incubated under controlled conditions (Pang and Kolenko 1986).

Controlled fire did not affect acid phosphomonoesterase activity of forest surface (0–5 cm) soil because the temperature never exceeded 50°C, whereas a marked reduction occurred with uncontrolled wildfires (Saa et al. 1993). Also, logging and/or burning operations almost eliminated acid phosphomonoesterase and phosphodiesterase activities of Karri forest soils (Adams 1992). Acid phosphomonoesterase activity did not recover after incubation of a wildfire-affected soil for 11 weeks under controlled conditions, probably because the high inorganic P repressed enzyme synthesis by soil microorganisms (Saa et al. 1998).

9.7.3 Effects of Pollutants on Soil Phosphatase Activities

Both acid and alkaline phosphomonoesterase activities have been monitored to evaluate the effects of several pollutants on organic P mineralisation in soil. The ecological dose (ED_{50}), i.e. the concentration of the pollutant that reduces the enzyme activity by 50%, has been calculated to quantify some of these effects.

Acid phosphomonoesterase activity of a blanket peat, an organic grassland soil and a calcareous grassland soil were high due to the P limitation induced by long-term atmospheric nitrogen deposition (Turner et al. 2002b), whereas sulfur pollution decreased the acid phosphomonoesterase activity of ectomycorrhizal roots in Norway spruce (Rejsek 1991).

The acid phosphomonoesterase activity of either moist or air-dried acid mesic fibrisols and histosols decreased after addition of copper to soil (Mathur and Rayment 1977; Mathur and Sanderson 1978). Juma and Tabatabai (1977) observed (depending on the soil type) the highest inhibition by Hg(II), As(V), W(VI) and Mo (VI) in the case of the acid phosphomonoesterase activity, and by Ag(I), Cd(II), V(IV) and As(V) in the case of the phosphomonoesterase activity. A negative and significant correlation was found between the sum of Cu and Zn total concentration in the soil and phosphomonoesterase activity determined using phenyl phosphate as the substrate (Tyler 1976).

Short-term laboratory incubations might not reflect the toxic effects in long-term heavy metal polluted soils. Alkaline phosphomonoesterase activity was still reduced in soils contaminated with Cd (concentration ranging from 0 to 0.36 nmol Cd kg^{-1}) in 1988–1990 and sampled in 2001, despite very low Cd availability, as determined either by water extraction or by the BIOMET bacterial biosensor system (Renella et al. 2004). In contrast, acid phosphomonoesterase activity and the composition of the bacterial community, determined either by plate counts or by DGGE, were unaffected, probably because the Cd pollution caused physiological adaptations rather than the selection of metal-resistant culturable bacteria. Addition of dry milled

ryegrass to these long-term Cd-contaminated soils increased both microbial biomass and acid and alkaline phosphomonoesterase activities (Renella et al. 2005a). However, the ratio of alkaline phosphomonoesterase activity to ATP decreased while that of acid phosphomonoesterase activity to ATP was unaffected compared to the respective uncontaminated soils. Both acid and alkaline phosphomonoesterase activities and the respective ratios of hydrolase to microbial biomass C were reduced in soils contaminated on the long term with Ni and Cd, but not in those with a Mn and Zn contamination; the former contamination also changed the composition of the bacterial community, as determined by DGGE (Renella et al. 2005b).

Dose–response curves can combine the effects of pollutants and soil physicochemical properties on soil microflora (Babich et al. 1983) and can be used to calculate the effective ecological dose (ED_{50}) of enzyme activity of soil in response to pollution by heavy metals (Doelman and Haastra 1989). The ED_{50} values for Cd, Cu and Zn were 2.6 mmol kg^{-1} soil in sandy soils and 45 mmol kg^{-1} soil in clayey soils. Generally, the Cd toxicity was higher when observed 1.5 years after addition of heavy metal salts to soils than after 6 weeks. The presence of Cu or Zn increased the toxicity of Cd on acid and alkaline phosphomonoesterase activity of contrasting forest soils, as observed by comparing the relative ED_{50} values determined by the kinetic model (Renella et al. 2003). The toxicity was higher in sandy than in finer textured soils. The sensitivity of acid phosphomonoesterase activity was higher in alkaline than in acid or neutral soils, and the sensitivity of alkaline phosphomonoesterase activity showed an opposite behaviour (Renella et al. 2003).

Recovery of both acid and alkaline phosphomonoesterase activities occurred 7 years after the in situ remediation of soils contaminated with sludge-borne metals. Inorganic amendments were used, such as 5% (w/w) beringite (a coal fly ash) or 1% (w/w) zerovalent iron grid; both treatments reduced the heavy metal availability, whereas the composition of the bacterial community as determined by DGGE was not affected (Mench et al. 2006). The treatment of soils that were vegetated with a grass and herb mixture with alkaline fly ash and peat reduced leaching of Cu and Pb and increased the phosphodiesterase activity and the acid and alkaline phosphomonoesterase activites of the soil (Kumpiene et al. 2009). The treatment of an As-contaminated loamy sand soil with beringite (with or without zerovalent iron grid) reduced the extractable As, uptake of As by lettuce, and acid phosphomonoesterase activity because the treatment increased soil pH, whereas both alkaline phosphomonoesterase and phosphodiesterase activities were increased (Ascher et al. 2009). The composition of bacterial and fungal communities determined by DGGE both changed, with a decrease in microbial diversity induced by the treatments.

The interpretation of the effect of any pollutant on soil phosphatase activities (as for any soil enzyme activity) is problematic because of the limits of the enzyme assays currently used (as already discussed) and the presence of direct and indirect effects on the target enzyme (Nannipieri et al. 2002). For example, inhibition of enzyme activity by a pollutant may be masked by the growth of surviving microorganisms with expression of genes codifying the enzyme; the microbial growth can be caused by the use of microbial debris (derived from microbial cells killed by the pollutant) by the surviving microorganisms.

9.8 Stabilisation of Extracellular Phosphatases in Soil by Interaction with Surface-Reactive Particles or by Entrapment Within Humic Molecules

Three approaches have been followed to study the stabilisation of phosphatases in soil: (1) the use of pure enzymes to create model enzyme complexes, either with inorganic minerals such as clay, or with humus like materials, (2) the extraction and characterisation of phosphatases from soil, and (3) the visualisation of extracellular phosphatases in the soil matrix.

Enzymes, like any protein, are rapidly (within a few hours) adsorbed to clays and can be partially desorbed by washing the clay–protein complex; the molecules that cannot be desorbed by washing are referred to as "bound" proteins (Stotzky 1986; Nielsen et al. 2006). Protein adsorption depends on clay properties such as surface area, cation exchange capacity, charge density, type of saturating cation and degree of clay swelling (Stotzky 1986; Nannipieri et al. 1996b; Nielsen et al. 2006). The type of protein is also important because adsorption is generally maximal at pH values within the range of the protein's isoelectric point and thus involves an ion-exchange mechanism. However, hydrogen bonding, van der Waals forces and hydrophobic effects are also involved in the adsorption of proteins by clay minerals.

Reduced enzyme activity after the adsorption can occur due to modification in the tertiary structure of the protein or due to reduced accessibility of the substrate to the active site. Adsorption of alkaline phosphomonoesterase by illite reduced the enzyme activity more than adsorption of the enzyme by montmorillonite or kaolinite (Makboul and Ottow 1979). Inhibition of enzyme upon adsorption may depend on its function. Intracellular phytase was completely inhibited when adsorbed by clays, whereas extracellular phytase retained its catalytic activity (Quiquampoix and Mousain 2005). Usually, the adsorption of the enzyme by clay minerals increases V_{max} and K_m values (Nannipieri and Gianfreda 1998). Both values were increased when alkaline phosphomonoesterase was adsorbed by Ca-montmorillonite whereas they were decreased when the enzyme was adsorbed by Ca-illite (Makboul and Ottow 1979). The adsorption of acid phosphomonoesterase and pyrophosphatase by Ca-illite and Ca-montmorillonite did not affect the kinetic constants of enzymes (Dick and Tabatabai 1987). The protein adsorption by clays may also improve stability against thermal denaturation, wetting and drying cycles, and proteolysis; the resistance against proteolysis occurs if the protein penetrates the interlayer space of montmorillonite (Stotzky 1986; Nannipieri et al. 1996b; Nielsen et al. 2006). Indeed, acid phosphomonoesterase adsorbed on kaolinite was less resistant to proteolysis than that adsorbed on montmorillonite against proteolysis and thermal denaturation (Sarkar et al. 1989) and alkaline, whereas alkaline phosphomonoesterase adsorbed on Ca-illite was more resistant to proteolysis than the free enzyme (Makboul and Ottow 1979). Both acid and alkaline phosphomonoesterase bound to homo-ionic clays were degraded by soil microorganisms but degradation rates were higher with kaolinite complexes than with bentonite and vermiculate complexes, and higher with Ca-clays than with Al-clays (Chhonkar and Tarafdar 1985).

Acid phosphomonoesterase from sweet potato was more inhibited by tannic acid than urease and invertase (Rao et al. 1998).

Humus–enzyme complexes have been prepared by oxidative coupling of phenols in the presence of the enzyme to be immobilised (Nannipieri et al. 1996b). Acid phosphomonoesterase was immobilised in a resorcinol polymer synthesised by peroxidase; the enzyme was not linked to the resorcinol moiety by covalent bonds and it was more resistant to denaturation by pH, temperature and proteolysis than the free enzyme (Garzillo et al. 1996).

Acid phosphomonoesterase from potato adsorbed on Ca-polygalacturonate (a polymer simulating mucigel of the root–soil interface) by electrostatic interactions showed increased stability, but a decreased resistance against proteolytic and thermal denaturation (Marzadori et al. 1998).

Phosphatases have been extracted from soil using different solutions (Tabatabai and Fu 1992; Nannipieri et al. 1996b). Both acid and alkaline phosphomonoesterase were extracted by shaking litter with 1 M $CaCl_2$, 0.05% Tween 80 and polyvinylpolypyrrolidone, and the enzyme activities were determined after dialysis and concentration of the extract (Criquet et al. 2004). Soil moisture was the most important factor affecting the production of acid phosphomonoesterase. However, principal component analysis and multiple regressions showed that both temperature and the number of culturable heterotrophic bacteria also affected the dynamics of acid and alkaline phosphomonoesterase activities and organic P mineralisation.

Free extracellular alkaline phosphomonoesterase extracted from two soils by water was less resistant (i.e. had a lower inactivation temperature) and showed lower K_m values than the respective humic–enzyme complexes extracted by the chelating resin Chelex (Kandeler 1990).

Mayaudon (1986) suggested that several enzymes, including phosphomonoesterases and phosphodiesterases extracted from soil by phosphate-EDTA at pH 7–8, are fungal glycoenzymes associated with bacterial lipopolysaccharides, which are linked to humic compounds by Ca bridges. Humus–phosphomonoesterase complexes have been extracted from soil by pyrophosphate (Nannipieri et al. 1996b). Successive exhaustive ultrafiltration divided the soil extract into two fractions: one with molecular weights higher than 100,000 (A_I), and the other with molecular weights between 10,000 and 100,000 (A_{II}). Gel chromatography of the A_I fraction gave three peaks of enzyme activity, whereas gel chromatography of the A_{II} fraction gave only one peak. The kinetic behaviour of some of the fractions showed the existence of two enzymes (or two forms of the same enzyme) catalysing the same reaction with markedly different kinetic properties. In addition, humus–phosphomonoesterase complexes with higher molecular weight were more resistant to thermal and proteolytic denaturation than those with lower molecular weight (Nannipieri et al. 1996b). It was suggested that humus–enzyme complexes of higher molecular weight are likely to possess the molecular arrangement proposed by Burns et al. (1972), in which enzymes are surrounded by a network of humic molecules with pores large enough to permit the passage of substrates and products of the enzyme reaction, but not that of proteolytic enzymes.

The yield of phosphodiesterases extracted from a forest soil using a 0.1 M phosphate buffer was increased when KCl and EDTA were added to the buffer, probably because the extracting solution desorbed extracellular enzymes adsorbed on the surface of soil colloids by ionic bonding (Hayano 1977). The treatment of the soil extract with protamine sulfate removed brown-coloured substances (probably humic molecules), and the partially purified enzyme showed a pH optimum in the range of 5.2–6.0 and hydrolysed either the 3′- or the 5′-phosphodiester bond of deoxythymidine *p*-nitrophenyl phosphate. Two phosphodiesterase fractions extracted from an A horizon of a larch forest Andosol showed high affinity to adenosine 3′- and 5′-mononucleotides (Hayano 1988), whereas a third fraction was 2′,3′-cyclic nucleotide 2′-phosphodiesterase (EC 3.1.4.16) or 2′,3′-cyclic nucleotide 3′-phosphodiesterase (EC 3.1.4.37), with a pH optimum of 5.0 (Hayano 1987). All three phosphodiesterase active fractions were inhibited by Hg^{2+}.

Approaches based on the study of clay–enzyme or humic–enzyme complexes, either prepared with pure enzymes or based on the extraction of humus–enzyme complexes from soil, present several drawbacks. In soil, neither enzymes nor surface-reactive particles, such as clays, are pure; for example, enzymes are released after cell death and lysis together with other cellular remains, which can affect adsorption of proteins to soil particles and the resistance of the formed complexes to thermal denaturation and proteolysis (Nannipieri et al. 1996b; Nielsen et al. 2006). Cell lysis with release of intracellular enzymes and artifacts due to interaction between enzymes and co-extracted soil components can occur during soil extraction.

The visualisation of the stabilised extracellular enzymes in the soil matrix by electron microscopy can give insights into the formation of stabilised enzyme complexes. Ultracytochemical tests have detected acid phosphomonoesterase activity in soil microbial cells and in fragments of microbial membranes as small as 7 × 20 nm, using electron microscopy. However, these tests were not able to locate enzymes in electron-dense minerals such as clays, or in soil components such as humic materials, reacting with counterstains such as OsO_4 (Ladd et al. 1996).

Techniques such as those based on the use of enzyme-labelled fluorescence (ELF)-97 phosphate seem to be promising for localising active phosphatases in the soil matrix because fluorescence is emitted after the enzymatic hydrolysis of the substrate (Wasaki et al. 2008; Wasaki and Maruyama 2011). This technique has detected acid phosphomonoesterase activity in roots of plants grown under P-deficient conditions, but its use in soil needs to be tested.

9.9 Conclusions and Future Research Needs

Probably, phosphodiesterase and phosphomonoesterase activities act sequentially in the mineralisation of organic P to inorganic P, which can be taken up by plant roots or by soil microorganisms (Fig. 9.1). These enzyme activities are higher in rhizosphere than in bulk soil and this suggests an important role for these enzyme activities in plant P nutrition. Acid phosphomonoesterase activity is more important in acid soils than is alkaline phosphomonoesterase activity, and vice versa in

alkaline soils. Effects of soil properties, agrochemicals, soil tillage, forest practices and pollutants have been extensively studied. However, a better understanding of the underlying mechanisms as well as the role of phosphatases (as that of any other enzyme in soil) requires setting up assays that discriminate the activities of enzymes associated with soil microorganisms from those of extracellular stabilised enzymes. A better understanding of the role of phosphatases in soil can be gained from studies on oxygen isotopes (isotope fractionation) of the phosphate group because alkaline phosphomonoesterase, unlike pyrophosphatase, catalyses a unidirectional reaction, producing kinetic isotope effects (Blake et al. 2005; Frossard et al. 2011).

The problem of measuring real rather than soil potential phosphatase activities may be solved by comparing the activities obtained from currently used enzyme assays with the organic P mineralisation (using isotope dilution methods as described by Frossard et al. 2011) in soils with a broad spectrum of properties, and by using the quantitatively most important soil organic P esters as substrates.

Extracellular enzyme activity of soil has been considered in the model describing decomposition of organic matter (Schimel and Weintraub 2003). However, the validation of this model requires determination of soil extracellular enzyme activity.

Phosphatases originate mainly from soil microorganisms, but in the rhizosphere and detritosphere they can also originate from plant cells. Changes in phosphatase activities have been related to changes in the composition of microbial communities, as determined by molecular techniques, in order to better understand the origin of phosphatases in soil. However, further insights into the origin of phosphatases in soil require relating the phosphatase activities to the expression of genes codifying these enzymes. The research carried out by Sakurai et al. (2008) involving the detection of alkaline phosphomonoesterase genes should be extended to soils with a broad spectrum of properties. Detection of the other genes expressing the various phosphatases is needed together with a relative comparison of the activity of these enzymes with the target gene.

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