Improved Techniques for the Characterisation of Soil Organic Phosphorus Using ^{31}P Nuclear Magnetic Resonance Spectroscopy and Their Application to Australian Soils

A thesis submitted to the University of Adelaide
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I dedicate this thesis to the memory of my Dad, Kevern Doolette, who helped convince me to do a PhD, and who I know would have actually enjoyed reading this thesis, despite probably not completely understanding it.

ABSTRACT

Organic phosphorus is potentially an important source of phosphorus (P) for agriculture, although it is not directly available for plant or microbial uptake. However, organic P can be converted into available inorganic P though hydrolysis or mineralisation. The rate of P release from organic P forms depends partly on the specific organic P compounds present in the soil. Until recently characterising soil organic P has been limited by the lack of appropriate analytic techniques. Consequently, organic P dynamics remains poorly understood.

In this thesis, the focus was on improving techniques for the characterisation of soil organic P using solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy, applying these techniques to characterise a range of Australian soils and developing a better understanding of the cycling and potential bioavailability of soil organic P.

The characterisation of soil organic P relies on the correct identification of resonances. Orthophosphate monoester peaks were identified by spiking model organic P compounds into NaOH-EDTA soil extracts. In this way, seven major resonances that were common to most of the NMR spectra were assigned to adenosine-monophosphate (AMP), *scyllo*-inositol hexakisphosphate, α- and β-glycerophosphate and *myo*-inositol hexakisphosphate (phytate). More importantly, spiking highlighted the similarly in appearance and chemical shift of some of the orthophosphate monoester resonances, particularly those of phytate and α- and β-glycerophosphate. This may have resulted in the misidentification and over-estimation of the concentrations of these species in previous studies.

To provide a detailed quantitative assessment of soil organic P using ^{31}P NMR spectroscopy, a modified method of spectral deconvolution, which included using an internal standard (methylenediphosphonic acid; MDP), was developed. The method of deconvolution implemented in this thesis considered P contained in larger humic molecules. A broad signal, in addition to the routinely fitted sharp peaks, was fitted to the orthophosphate monoester region of the NMR spectrum. A large proportion of monoester P (32–78%) could be assigned to this signal. When the broad signal was not taken into account phytate concentrations were over-estimated by 54%. It is likely that the concentrations of other specific orthophosphate monoester compounds were also over-estimated.

The potential over-estimation of phytate concentrations has implication for the understanding of phytate stability in soils. High phytate concentrations in soils are usually explained by the stability of phytate in soils or the limited presence or activity of specialised enzymes (phytase). Lower phytate concentrations suggest phytate maybe less stable in soils than previously supposed. Therefore, the rate

of phytate degradation in a calcareous soil was investigated. Phytate was applied to a calcareous soil at four different concentrations (ranging from 58–730 mg kg^{-1}) and the effect of wheat straw as an additional source of carbon was also examined. Regardless of treatment, phytate concentrations decreased over the 13-week incubation period and were adequately fitted to a first order decay model. There was no clear trend in the rate of phytate loss with treatment and the half life of phytate ranged from 4 to 8 weeks. The loss of phytate coincided with an increase in orthophosphate concentration, that in some cases more than doubled the native soil P concentrations, and there was very little variation in extraction efficiency. This result provided evidence for the microbial degradation of phytate. It demonstrated that in the calcareous soil examined, phytate was not highly stable, but a bioavailable source of organic P

The composition of soil P in 18 diverse Australian soils was also examined. Across all NaOH-EDTA soil extracts analysed, phytate comprised up to 9%, but averaged only 3% of total extractable P. Two other resonances that were also prominent in all the ^{31}P NMR spectra and comprised a similar proportion of total organic P were due to α- and β-glycerophosphate. By examining the alkaline hydrolysis of a phospholipid (phosphatidlycholine), the potential source of α- and β-glycerophosphate was identified. Although α- and β-glycerophosphate and phyate gave rise to the most intense peaks, the broad signal, which was attributed to humic P, represented the most abundant form of soil organic P (27–72% of total extractable organic P). Therefore, it was suggested that the development of methods that aim to increase the availability of stabilised forms of organic P should give preference to increasing the availability of P contained in humic P complexes.

Understanding P cycling not only relies on analytical methods that enable the accurate identification and quantification of soil organic P but also requires methods that can gauge the susceptibility of different organic P species to enzymatic hydrolysis. Therefore, enzymatic hydrolysis was combined with ^{31}P NMR spectroscopy to identify and compare the specific organic P species in the enzyme labile and non-enzyme labile fractions of a range of NaOH-EDTA soil extracts. Phosphorus-31 NMR analysis of NaOH-EDTA soil extracts treated with active and inactivated phytase enzyme preparations showed that phytase hydrolysed the majority of the small, orthophosphate monoester compounds (α- and β-glycerophosphate, phytate, *scyllo*-inositol hexakisphosphate) and pyrophosphate, but orthophosphate diesters (DNA) and humic P were generally unaffected. The ^{31}P NMR spectra revealed that not only was organic P hydrolysed but new orthophosphate monoester species were formed, possibly as a result of enzymatic phosphorylation. Although combining enzymatic hydrolysis and ^{31}P NMR spectroscopy enabled the identification of individual organic P species that were susceptible or resistant to enzyme hydrolysis, there is still a need for further improvement and refinement of the technique in order to provide an accurate estimate of the potentially available fraction of soil organic P.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Ashlea Doolette and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Ashlea Doolette **Date**

PUBLICATIONS ARISING FROM THIS THESIS

Doolette, A.L., Smernik, R.J. & Dougherty, W.J. 2009. Spiking improved solution phosphorus-31 nuclear magnetic resonance identification of soil phosphorus compounds. *Soil Science Society of America Journal*, **73,** 919–927.

Doolette, A.L., Smernik, R.J. & Dougherty, W.J. 2010. Rapid decomposition of phytate applied to a calcareous soil demonstrated by a solution ^{31}P NMR study. *European Journal of Soil Science*, **61,** 563–575.

Doolette, A.L. & Smernik, R.J. (in press). Soil organic phosphorus speciation by spectroscopic techniques. In: *Phosphorus in action –Biological procesess in soil phosphorus cycling* (eds E.K. Bünemann, A. Oberson & E. Frossard), Springer-Verlag.

Doolette A.L., Smernik R. & Dougherty W.J. (in press). A quantitative assessment of phosphorus forms in some Australian soils. *Australian Journal of Agricultural Research*.

Doolette, A.L., Smernik, R. & Dougherty, W.J. 2008. ^{31}P NMR characterisation of soil organic phosphorus: Have we mistaken phospholipids for phytate? In: *Soils 2008 – The Living Skin of the Earth*, Massey University, Palmerston North, New Zealand.

Doolette, A.L., Smernik, R. & Dougherty, W.J. 2010. A quantitative assessment of phosphorus forms in Australian soils. In: *19th World Congress of Soil Science, Soil Solutions for a Changing World*, Brisbane, Australia.

STATEMENT OF AUTHORSHIP

Components of the research described in this thesis have been published, are in press, or have been submitted for publication (as listed below). The contribution of each author to these works is described below.

Chapter 2: *Soil Science Society of America Journal*; 2009, **73,** 919–927.
Chapter 3: *European Journal of Soil Science*; 2010, **61,** 563–575.
Chapter 5: *Australian Journal of Soil Research*; in press.

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Experimental development, performed analysis on all samples, data analysis and critical interpretation, wrote manuscript.

I hereby certify that the statement of contribution is accurate.

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DOUGHERTY, W.J.
Supervised development of work, data analysis and interpretation, reviewed manuscript.

I hereby certify that the statement of contribution is accurate.

Signed Date 13th July 2010

STRUCTURE OF THIS THESIS

This thesis is presented as a combination of papers that have been published, are in press or have been submitted for publication, as well as chapters that have not been submitted for publication.

Chapter 1 provides an overview of the literature on the chemical nature and dynamics of soil organic P and methods for the determination of soil P. This chapter also includes the proposed objectives of this research. Introductory material relevant to the published and submitted papers is not presented in detail in the literature review because it appears in the introduction of each chapter.

Chapter 2 comprises a paper published in the *Soil Science Society of America Journal*. It describes the application of an improved spiking technique to assign the dominant P resonances in the ^{31}P NMR spectra of NaOH-EDTA soil extracts.

Chapter 3 comprises a paper published in the *European Journal of Soil Science*. It describes an incubation experiment used to determine the course of degradation of *myo*-inositol hexakisphosphate (phytate) applied to an untreated (un-manured) calcareous agricultural soil.

Chapter 4 describes a second incubation experiment that follows on from the incubation experiment described in Chapter 3. It examines the effect of increasing the concentration of phytate on the rate and course of phytate degradation. These results have not been submitted for publication.

Chapter 5 comprises a paper that has been submitted to the *Australian Journal of Soil Research*. It provides a quantitative assessment of phosphorus forms in a range of Australian soils.

Chapter 6 describes attempts to combine enzymatic hydrolysis analysis and ^{31}P NMR spectroscopy to characterise enzyme labile and non-enzyme labile fractions of soil P. These results have not been submitted for publication.

Chapter 7 provides a synthesis of the findings contained in this thesis and includes recommendations for future work.

CHAPTER 1

REVIEW OF THE LITERATURE

Part of the work contained in this chapter has been accepted for publication in a book chapter in *Phosphorus in action –Biological processes in soil phosphorus cycling.*

Doolette, A.L. & Smernik, R.J. (in press). Soil organic phosphorus speciation by spectroscopic techniques. In: *Phosphorus in action –Biological processes in soil phosphorus cycling* (eds E.K. Bünemann, A. Oberson & E. Frossard), Springer-Verlag.

Introduction

Phosphorus (P) is an essential nutrient required for plant growth, although only a small portion exists in soil solution (e.g., 0.01-3.0 mg P L^{-1}) or in forms available to plants at any given time (Frossard *et al.*, 2000). Phosphorus exists naturally as a variety of organic P compounds and phosphate minerals in soils. Despite the possibility of 20 to 80% of P in soils being present in an organic form (Dalal, 1977), P uptake by plants is almost exclusively as orthophosphate in solution. This means that the majority of P in soils is immobile, inaccessible and unavailable to plants. Therefore, the conversion of organic P to orthophosphate though mineralisation and hydrolysis is an important source of orthophosphate in solution.

Studies have demonstrated the importance of organic P to plant nutrition, whereby organic P was at least equal to inorganic P in its ability to supply available P to a variety of crops (Furlani *et al.*, 1987; Tarafdar & Claassen, 1988; Adams & Pate, 1992). Furthermore, the accessibility of organic P may also be increased through the insertion of genes into plants to promote the production of enzymes that hydrolyse organic P to inorganic P (Richardson *et al.*, 2001; George *et al.*, 2004).

As P reserves are finite and the cost of P fertilisers and their application increases, achieving effective and sustainable agricultural production can be difficult. This is particularly so since generally only 10–30% of applied fertilizer is recovered by crops in the year following application (Bolland & Gilkes, 1998; Bünemann *et al.*, 2006). Therefore, it is necessary to understand the biological, chemical and physical factors that control P availability, including the origin of organic P and its potential for mineralisation. These controlling factors include the amount of microbial biomass and microbial cycling within the soil, climate, soil management and agricultural practices, as well as P inputs and outputs.

If organic P is to be further developed as a source of plant P for agricultural production, its chemical nature needs to be well understood. This is a prerequisite to further understand the dynamics of organic P in the soil ecosystem, and its bioavailability to plants and other organisms. This review will summarise our current knowledge of soil organic P compounds, their biochemical cycling, and review the techniques available for their characterisation.

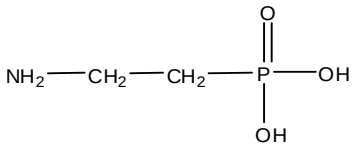
Chemical nature of soil organic phosphorus

Organic P compounds occur in soils as phosphonates, phosphoanhydrides (or condensed phosphates) and orthophosphate monoesters and diesters. Synthetic forms of organic P can also be introduced to soils as insecticides, herbicides, fungicides or plant growth regulators (Condron *et al.*, 2005). However, this review will focus on the natural forms of organic P, that is organic P of plant, animal or microbial origin.

Phosphonates

Phosphonates are different from other soil organic phosphorus compounds because they contain a C-P bond (Turner *et al.*, 2005). The most common naturally occurring phosphonate is 2-aminoethylphosphonic acid (Table 1), which can be produced by a variety of organisms including bacteria, amoeba, fungi and snails (Glonek *et al.*, 1970; Hilderbrand, 1983). Phosphonates are not commonly detected in soils and accumulate mainly in soils in cool, moist, acidic environments, since under these conditions microbial decomposition is reduced (Newman & Tate, 1980; Tate & Newman, 1982). In a series of New Zealand soils, increased soil phosphonate concentrations were attributed to the protozoon *Tetrahymena pyriformis,* in which up to 15% of the total P can be in the form of phosphonates (Hawkes *et al.*, 1984). Phosphonates comprise no more than 2–3% of the total P (Möller *et al.*, 2000; Makarov *et al.*, 2002b; Turner *et al.*, 2003d) and in many soils are below detection limits.

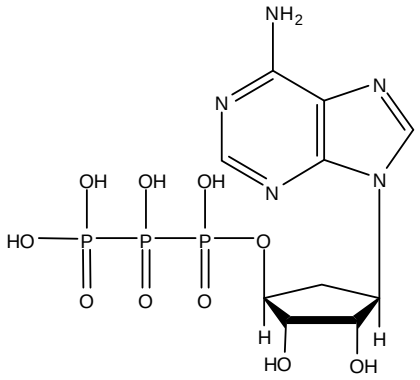
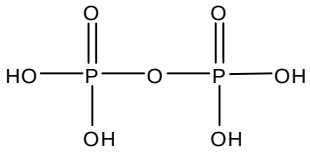
Table 1. Structure of the most common form of soil phosphonate.

Compound	**Structure**
2-aminoethylphosphonic acid	

Polyphosphates

Organic polyphosphates are linear polymers formed through the dehydration of phosphoric acid derivatives and contain energy rich phosphoanhydride bonds (Condron *et al.*, 2005). They are involved in energy transfer in biochemical reactions and include compounds such as adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP) (Table 2). Inorganic polyphosphates are more commonly detected in soils. The simplest polyphosphate is pyrophosphate ($P_2O_7^{2-}$) where two PO_4 groups are bonded through the sharing of one O atom (Table 2). Pyrophosphate is believed to be derived from plants (Dai *et al.*, 1996) and is also thought to accumulate in microbial biomass as a P storage form (Ghonsikar & Miller, 1973). Pyrophosphate and long chain polyphosphates are susceptible to enzymatic hydrolysis in soil, resulting in the conversion of pyrophosphate to orthophosphate. However, the rate of hydrolysis is highly dependent on the level of microbial activity. The hydrolysis of pyrophosphate has been measured in a large number of soils and found to have half lives of 3 to 100 days (Sutton & Larson, 1964; Sutton *et al.*, 1966; Blanchar & Hossner, 1969). Pyrophosphate has been reported to represent up to 19% of total P (Dai *et al.*, 1996), although concentrations are typically much lower. Pyrophosphate concentrations ranged from 1–7% of total extractable P in pasture soils (Turner *et al.*, 2003d), 0.5–4.3 % in cropping soils (Turner *et al.*, 2003b), <1–4.85% in manure treated soils (Dou *et al.*, 2009) and on average 3% in grassland soils (Murphy *et al.*, 2009).

Table 2. Structures of common polyphosphates.

Compound	Structure
Adenosine 5'-triphosphate (ATP)	
Pyrophosphate	

Orthophosphate diesters

Orthophosphate diesters identified in soils include nucleic acids, phospholipids and teichoic acids. Nucleic acids (deoxyribonucleic acid [DNA] and ribonucleic acid [RNA]) (Table 3) are present in every living cell. DNA is confined within the nucleus whereas RNA is found throughout the entire cell (Anderson, 1967). Both DNA and RNA consist of a backbone of alternating sugar and orthophosphate groups connected through ester bonds. DNA and RNA differ in the degree of oxidation of their constituent pentose residues and in the nature of their pyridimine bases. The persistence of DNA in the soil is dependent on its rate of mineralisation. Cold, wet, acidic soils and dry calcareous soils both have relatively low microbial activity and these conditions result in the accumulation of DNA of plant or microbial origin (Makarov *et al.*, 2002b). Several ectomycorrhizal fungi have been identified as utilising nucleic acids as a source of phosphorus (Sawyer *et al.*, 2003; Midgley *et al.*, 2006). Therefore, it would appear that microbial activity, not chemical stabilisation, mostly determines the rate of cycling of nucleic acids within soils.

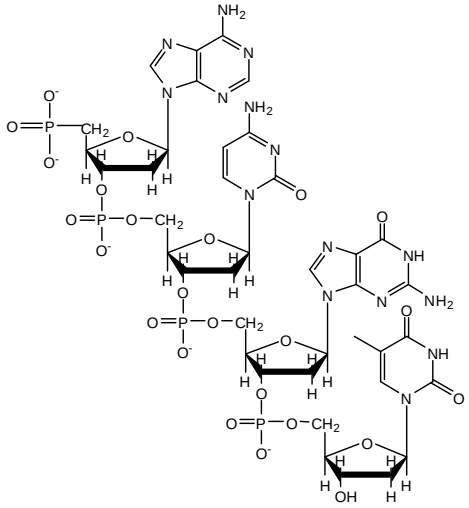
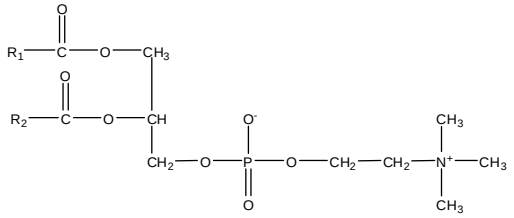
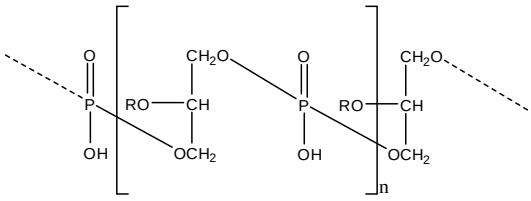
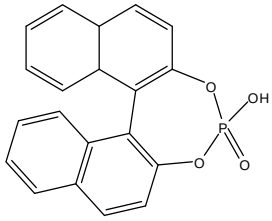
Phospholipids (Table 3) contain a glycerol backbone, with one O-atom attached to a phosphate residue that is esterified with a secondary alcohol group. Phospholipids are important membrane constituents of plants, microorganisms and mammalian tissues. The more common phospholipids are phosphatidyl choline (plants and mammals), phosphatidyl ethanolamine (microorganisms), phosphatidyl inositol (plants) and phosphatidyl serine (mammals) (Hanahan, 1997). The most abundant of the phospholipids in soils is reported to be phosphatidyl choline (Kowalenko & McKercher, 1971). Phospholipids only represent a small fraction of total P in soils (approximately 1%) (Hance & Anderson, 1963; Newman & Tate, 1980; Turner *et al.*, 2003d), however, their concentration can be higher if they become stabilised through adsorption to colloids (Kowalenko & McKercher, 1971).

Teichoic acid is a general term for a range of sugar phosphate structures that can account for up to 85% of total P in the cell walls of Gram-positive bacteria. They are acidic polysaccharides with repeating units of either glycerol or ribitol connected by ester bonds to orthophosphate (Table 3) (Condron *et al.*, 2005). It is proposed that teichoic acids function as a reserve phosphate source when bacteria have a limited inorganic P supply (Grant, 1979; Ward, 1981). Teichoic acids have only been identified in soils using solution ^{31}P NMR [eg. (Tate & Newman, 1982; Condron *et al.*, 1990b; Makarov *et al.*, 2002b)], although it is possible that they have been misidentified (Makarov *et al.*, 2002a).

Aromatic diesters are rarely detected in soil extracts and are thought to be associated with cold wet environments. Recent studies have assigned unidentified peaks in ^{31}P NMR spectra to aromatic diesters such as R-(⁻)-1,1'-binaphthyl-2,2'-diyl hydrogen phosphate (Table 3) (Turner *et al.*, 2003d; Makarov *et al.*, 2004; McDowell *et al.*, 2005). Their assignment should however be regarded with caution until more conclusive results are available.

Organic P from above ground sources, (plants and animals) may be chemically or biochemically modified upon entering the soil. Consequently, the composition of organic P in the soil does not reflect the input to the soil. Phosphate diesters constitute the majority of fresh inputs of organic P, mainly as nucleic acids (approx 60%) and phospholipids (5-30%), but are rapidly degraded upon release and typically represent only a small fraction (approx 10%) of organic P within the soil (Dalal, 1977). Diester forms of soil organic P are more labile and readily mineralised than phosphate monoesters. This has been attributed to their weaker bonds, which are readily broken by soil microorganisms (Tate, 1984; Gatiboni *et al.*, 2005). Orthophosphate diesters therefore not only serve as a source of P but also perform an important role in P transformation.

Table 3. Structures of common orthophosphate diesters.

Functional Class	Compound	Structure
Nucleic Acid	deoxyribonucleic acid (DNA)	
Phospholipid	phosphatidyl choline	
Teichoic Acid	glycerol teichoic acid	
Aromatic	R-(⁻)-1,1'-binaphthyl-2,2'-diyl hydrogen phosphate	

Orthophosphate monoesters

Orthophosphate monoesters have the general structure $ROPO_3^{2-}$, where R is an organic moiety (Table 4). They include sugar phosphates, phosphoproteins, mononucleotides and inositol phosphates (Condron *et al.*, 2005). Inositol phosphates are widely reported to be the most abundant of these (Turner, 2007). There are nine possible stereoisomers of inositol. For each of these stereoisomers between one and six of the hydroxyl groups can be esterified as phosphates (Shears & Turner, 2007).

Monoesters only represent 10–25% of the fresh organic P input from microbial and plant sources but usually comprise the majority of organic P detected in soils (Turner *et al.*, 2005). Monoester concentrations vary widely, ranging from 12–100% of the total P in soils (Williams & Anderson,

1968; Newman & Tate, 1980; Hawkes *et al.*, 1984; Condron *et al.*, 1985; Condron *et al.*, 1990b; Dai *et al.*, 1996; Turner, 2004; McDowell *et al.*, 2005; McDowell & Stewart, 2006). The higher inositol phosphates, such as *myo*-inositol hexakisphosphate (phytate) and *scyllo*-inositol hexakisphosphate are often the most abundant identifiable organic P species (Turner *et al.*, 2002b; Turner & Richardson, 2004). In particular, phytate has been reported to comprise up to 100% of the total organic P pool, although in other soils it accounts for less than 3% of total P (Turner, 2007). Large variations in phytate concentrations are not uncommon in soils, especially in Australian soils (Cosgrove, 1962; Cosgrove, 1963; Cosgrove & Tate, 1963; Irving & Cosgrove, 1982).

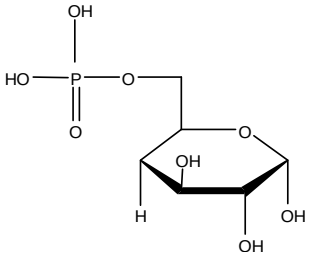
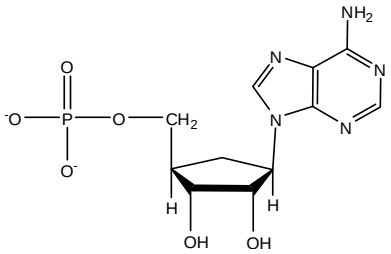
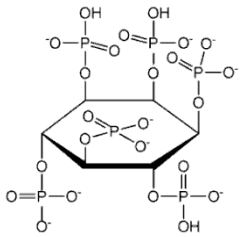
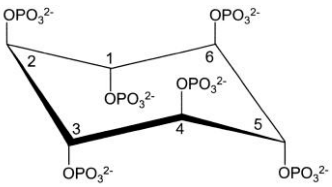
Phytate has been identified in soils from sugar cane fields and coffee orchards (Yoshida, 1940), animal manures (Caldwell & Black, 1958), pastures (Turner *et al.*, 2003c), grasslands (Tate & Newman, 1982), and virgin and cultivated soils (Williams & Anderson, 1968). Phytate functions as the major storage form of P in seeds and is gradually enzymatically hydrolysed to release orthophosphate during germination (Bassiri & Nahapetian, 1977). Williams and Anderson (1968) reported inositol-P in pasture soils accounted for 14–25% of the total organic P, despite the soils being derived from similar parent materials, having similar fertilizer treatments and environmental conditions. The amount of inositol phosphates may vary with the level of soil organic matter, since higher concentrations have been recorded in forest soils (Omotoso & Wild, 1970b) and considerably lower concentrations in sandy soils (Williams & Anderson, 1968). However, some of the earlier measurements of monoester concentrations should be interpreted with caution. Various analytical techniques have been found to overestimate concentrations by incorrectly identifying organic P compounds, or alternatively underestimating concentration as a result of incomplete recovery. This is discussed in greater detail within the section *Methods for the determination of soil phosphorus*.

Monoesters such as phytate, strongly react with clays (Celi *et al.*, 1999; Celi & Barberis, 2007), metals (McDowell *et al.*, 2007; Turner *et al.*, 2007) and humic materials (Anderson & Hance, 1963; Omotoso & Wild, 1970a) due to the high charge density brought about by the six substituted phosphate groups (Turner *et al.*, 2002b). This has been suggested to result in better physical protection from biological attack and therefore explains their higher concentration relative to other organic P compounds, which are poorly sorbed to the soil and therefore readily mineralised (Turner *et al.*, 2003d; Tang *et al.*, 2006).

Enhanced mineralisation of inositol phosphates has been suggested as a potential way to enhance P availability for plants (Chen *et al.*, 2004; Ragon *et al.*, 2008). In particular, genetic manipulation to increase the expression and secretion of phytase has been pursued (Richardson *et al.*, 2001; George *et al.*, 2006; George *et al.*, 2007) in an attempt to make P bound in phytate more available. This assumes that enzymatic activity limits phytate breakdown in soils. It is possible, however, that the solubility of phytate determines its availability, not its susceptibility to phosphatases (Jackman & Black, 1952a; b).

More recently Lung and Lim (2006) suggested that the insolubility of soil phytate was the major limitation for its assimilation, and through the use of citrate secretions, soil phytate assimilation could be improved.

Table 4. Structures of common orthophosphate monoesters.

Functional Class	Compound	Structure
Sugar phosphate	glucose-6-phosphate	
Mononucleotide	adenosine-5'-monophosphate (AMP)	
Inositol phosphate	*myo*-inositol hexakisphosphate (phytate)	
	scyllo-inositol hexakisphosphate	

Phosphorus dynamics in soils

The turnover or cycling of P in soils is determined largely by the rates of immobilisation and mineralisation of both organic and inorganic P. Immobilisation can involve either the biological conversion of inorganic P to organic P or the sorption/precipitation/complexation of inorganic P. Mineralisation is the process whereby inorganic P is released from organic P in the soil. Although the exact mechanisms responsible are debated, it is accepted that phosphatase enzymes produced by plant roots (Tarafdar & Claassen, 1988; Tadano *et al.*, 1993; Richardson *et al.*, 2001) and microorganisms (Tarafdar & Claassen, 1988; Sinsabaugh, 1994) are primarily responsible for mineralisation. Whilst some studies have shown a direct relationship between enzymatic activity and P mineralisation

(Chen *et al.*, 2002; Tarafdar & Claassen, 2005), others have failed to do so (Adams & Pate, 1992). The discrepancy between the various studies, with respect to the relationship between phosphatase activity and mineralisation of organic P, may be attributed to the complex interaction of many factors including plant species, soil properties (pH, soil moisture) and P solubility in soils.

The capacity and affinity of soils to sorb organic P can also influence the amount of organic P in soils. Stabilisation in soil occurs through adsorption onto clay or minerals or by association with humic compounds. Even the type of clay will affect the degree to which the P molecule is sorbed. For example, P compounds are more strongly sorbed to illite than kaolinite (Celi et al 1999). The sorption capacity for organic phosphates increases as the number of phosphate esters on the parent molecule increases (McKercher & Anderson, 1989). Phosphorus stability can also regulate processes other than adsorption, including hydrolysis and leaching, resulting in differing distribution patterns with soil depth (Koopmans *et al.*, 2007; Hill & Cade-Menun, 2009).

Plant uptake and phosphorus availability

In periods of low P supply, the majority of P in the soil is often in an unavailable organic P form. Tate and Newman (1982) suggested that the rate of mineralisation rather than the amount of organic P in the soil would determine plant availability. More specifically, Tarafdar and Claassen (1988) investigated a large number of synthetic organic P compounds and discovered that plant roots hydrolysed more organic P than the plants took up.

The availability of different P sources to plants has been widely studied (McKercher & Tollefson, 1978; Tarafdar & Claassen, 1988; Condron *et al.*, 1990b; Adams *et al.*, 2002; Sawyer *et al.*, 2003). Orthophosphate diesters are readily mineralised in soil (Condron *et al.*, 1985; Condron *et al.*, 1990b). DNA (Tarafdar & Claassen, 1988) and RNA (Adams & Pate, 1992) have both been shown to be an available source of P for barley. Inositol phosphates are considered to be highly resistant to mineralisation and preferentially stabilised compared with other soil P compounds (Berg & Joern, 2006). However, inositol phosphates were identified as a P-source for lupin in sand (Adams & Pate, 1992). Furthermore, in a separate study, legumes grew equally well when supplied with either organic or inorganic sources of phosphorus (Adams *et al.*, 2002). Similarly, a significant amount of phytate was depleted from soil near pine roots, possibly due to increased production and release of acid phosphatase by ecto-mycorrhizae (Chen *et al.*, 2004). The above examples demonstrate that inositol phosphates are potentially an important source of P for plant nutrition. Research into other sources of organic P have demonstrated that gylcerophosphate and lecithin were at least equal to inorganic sources in their availability to barley, clover, oats and wheat (Tarafdar & Claassen, 1988), while Furlani *et al.* (1987) noted that ethylammonium, glycerol and phenyl phosphates were preferable to inorganic phosphates for the growth of sorghum.

The utilisation of organic P as the primary source of P has been reported to occur only when the concentration of inorganic P is extremely low and plants are showing signs of severe stress (Gatiboni *et al.*, 2005). Under these conditions, if hydrolysable organic P is present, the activity of the phosphatases in the soil system may be able to hydrolyse sufficient P to meet and even surpass the P demand of the plant (Tarafdar & Claassen, 1988).

Microbial activity

Pyrophosphate (Condron *et al.*, 1985) and diester-P, in particular DNA (Turner *et al.*, 2003d), are believed to be actively involved in biological P cycling as their presence in the soil is commonly associated with a higher degree of microbial activity. Fungi such as the Chytridiomycota and *Amanita muscaria spp.* contain significant amounts of orthophosphate monoesters including phytic acid, as well as orthophosphate diesters and polyphosphates that they can utilise as a source of P (Sawyer *et al.*, 2003; Midgley *et al.*, 2006).

Biological activity may also regulate the concentration of some P forms, including phytate and other monoesters. Under conditions of P limitation, it has been reported that microbes may be able to utilise the otherwise recalcitrant inositol phosphate. This was evident in a study undertaken by Turner *et al.* (2003c) who found that phytate concentrations were minimal where soil P was scarce but the demand was great. This is further supported in the study of Tarafdar and Claassen (1988) where bacterial and fungal populations increased in soils as a result of the addition of phytin and lecithin. This suggests that phytin and lecithin were the preferred substrates of the two microorganisms.

Climatic and environmental influences

The rate of mineralisation of organic P is also a function of soil moisture and temperature. Under cold, wet conditions, low decomposition rates result in the persistence and accumulation of labile compounds such as orthophosphate diesters, notably DNA (Makarov *et al.*, 2002b) and phosphonates (Tate & Newman, 1982). This is a result of reduced microbial activity. Similarly, research undertaken by Turner *et al.* (2006a) into the organic P composition of subtropical wetland soils showed that inositol phosphates did not accumulate and instead phosphate diesters were the dominant P species. They suggested anaerobic conditions led to the rapid hydrolysis of phytate by reducing iron complexes that protect the phytate molecule from enzymatic attack.

Seasonal differences have also been observed by Sharpley (1985), who found that organic P levels in the soils were higher in winter than in spring, which he attributed to the rate of mineralisation and removal of P from the surface through plant uptake.

Acidic soils have been found to not only accumulate more organic P than alkaline soils, but in addition favour a greater variety of P species (Turner *et al.*, 2003e). This has been ascribed to a decreased rate of decomposition. Furthermore, the strong adsorption of monoesters by clay minerals (Celi *et al.*, 1999) and finely divided iron and aluminium oxides can also increase soil organic P concentrations (McDowell *et al.*, 2007; Turner *et al.*, 2007).

Land use and soil management

Soil management, land use and agricultural practices can all impact upon soil P levels and P availability. Under cultivated soils, Condron *et al.* (1990b) reported a decrease in soil organic P due to mineralisation and the removal of soil P by crops. Their results showed that orthophosphate diester and teichoic acid P were detected in native soil but were not present in cultivated soils. Similarly, Hedley *et al.* (1982) reported that the total P content of cultivated soil was 29% lower than on adjacent permanent pastures, and that the majority of this difference was in the organic and residual P fraction. More specifically, in intensively cultivated soil Williams and Anderson (1968) found that the inositol phosphate content of the soil decreased, but the decrease was much smaller than the overall decrease in total organic P. Therefore cultivation increased the proportion of inositol phosphate in the total organic phosphorus pool. However, Gatiboni *et al.* (2005) reported that successive cropping of soils decreased the total P content but did not change the percentage of each P form, that is, there was equal depletion of the different P forms.

The conversion from grassland to coniferous forest resulted in a substantial reduction in orthophosphate monoesters (Condron *et al.*, 1996). Similarly, a comparison of two unfertilised grasslands, where one was ploughed 20 years prior to the study taking place, indicated the ploughed soil contained lower concentrations and fewer forms of organic P (Hawkes *et al.*, 1984). In the same study, a grassland soil that had received P fertiliser for 121 years showed an increase in orthophosphate monoester P and a small addition of polyphosphate compared to a grassland soils that have received no P fertiliser over the same period. Guggenberger *et al.* (1996) examined the land use effects on P forms in 4 soils: permanent grassland, arable rotation, spruce forest and mixed deciduous forest. The spruce and mixed deciduous forests had the largest portions of organic P with the arable rotation having the lowest organic P contents. The permanent grassland had high concentrations of diester-P as a result of a build up of labile microbial P and the spruce forest soil was the only soil to contain polyphosphates.

Overall, these results provide further evidence of the recalcitrant nature of inositol phosphate and suggest it represents a fraction of soil organic P that is of low availability to plants. It also confirms that orthophosphate diester-P is more readily mineralised than monoester-P.

Methods for the determination of soil phosphorus

The ability of soil to provide P to biota depends on what forms of P are present and their relative amounts. The most commonly used differentiation of soil P is between inorganic and organic forms. The reason that the differentiation between inorganic and organic forms is so fundamental to P speciation is that this distinction has been easy to make using long-established techniques. However, the differentiation of inorganic and organic P is only the beginning of soil P speciation, as each of these broad classes encompasses a huge range of chemical forms. There has been a substantial effort to develop more powerful methods for identifying and quantifying organic P species in soils.

Ignition technique

The ignition method was first used by Saunders and Williams (1955) to quantitatively determine total soil P. At high temperatures, typically >500ºC, soil organic P is oxidised to form inorganic P. Organic P can then be calculated as the difference between inorganic P in acid extracts of ignited and unignited soil samples. The ignition technique is less laborious than other methods but may be prone to a number of sources of error. Ignition tends to overestimate organic P by increasing the solubility of inorganic P minerals in highly weathered (Condron *et al.*, 1990a) or calcareous soils (Williams *et al.*, 1960). This technique may also underestimate organic P as a result of incomplete P extraction during the ignition process or as a result of hydrolysis during acid extraction of the unignited soil (Turner *et al.*, 2005). It is more likely that organic P will be overestimated than underestimated as organic P was shown to be on average 8% greater than when estimated by H_2SO_4-NaOH extraction (Dougherty *et al.*, 2005).

Molybdate Colorimetry

Inorganic P is traditionally detected spectrophotometrically [e.g., (Neal *et al.*, 2000; Makarov *et al.*, 2002a; Turner *et al.*, 2003e; Turner *et al.*, 2003a; d; McDowell & Stewart, 2005b)] as a blue-coloured phosphomolybdenum complex formed when free phosphate reacts with an acidified molybdate reagent (Murphy & Riley, 1962). Organic P does not form a coloured complex with this reagent, and so can be determined as the difference between total P (usually measured as inorganic P after digestion of the soil extract) and inorganic P. However, there are drawbacks to this method. Organic P concentrations have consistently been recorded higher when calculated by molybdate colorimetry than when analysed using alternative techniques such as ^{31}P NMR spectroscopy. In a wetland soil, organic P was overestimated by between 30 and 54% (Turner *et al.*, 2006b). Similarly, in 24 pasture soils, organic P concentrations were greater when analysed using molybdate colorimetry compared to ^{31}P NMR (Turner *et al.*, 2003d). Organic P is overestimated when inorganic polyphosphates are present because they do not react with the molybdate reagent, and therefore are included in the organic P fraction. Additionally, high organic matter concentrations in alkaline extracts can interfere with the colorimetry but this can be minimised by acidifying the extracts to precipitate organic matter

(Tiessen & Moir, 1993). There are also several other species that interfere in the formation of the phosphomolybdenum complex. These include silica, arsenic, chromium, nitrite, nitrate and sulphide, though these are more of a concern when determining phosphorus species in water samples (Neal *et al.*, 2000). These interferences are usually negligible in soils (Turner *et al.*, 2006b).

Sequential Extraction

Sequential fractionation methods were developed to obtain information on the nature of soil P by separating P fractions based upon the extent of their availability to plants (Hedley *et al.*, 1982) or with respect to their chemical bonding (Chang & Jackson, 1957). In all sequential fractionation schemes, a single soil sample is subjected to increasingly strong solvents. This provides separation of P into different fractions based upon solubility. The most commonly used fractionation procedures are those of Chang and Jackson (1957) and Hedley *et al.* (1982) (Table 5). The original method of Hedley *et al.* (1982) was based upon the earlier work of Chang and Jackson (1957) who extracted between 40 and 80% of the P in the soils. These methods have since been modified to improve P recovery [eg. (Bowman & Cole, 1978; Tiessen & Moir, 1993; Turner & Leytem, 2004)].

Table 5. Sequential extraction procedures for soil phosphorus fractionation.

Procedure	**Extractant**	**Fraction**
(Chang & Jackson, 1957)	1 M NH_4Cl	Labile P
	0.5 M NH_4F	Aluminium (Al) bound phosphate
	0.1 M NaOH	Iron (Fe) bound phosphate
	Dithionite-citrate	Calcium bound phosphate
	0.5 M H_2SO_4	Reductant soluble Fe bound phosphate
	0.1 M NaOH	Occluded Al and Fe bound phosphate
(Hedley et al., 1982)	Anion exchange resin	Biologically available inorganic P
	0.5 M $NaHCO_3$	Inorganic and organic P sorbed to soil surface
	Fumigation, 0.5 M $NaHCO_3$	Microbial
	0.1 M NaOH	Fe and Al bound phosphate
	0.1 M NaOH + Ultrasonication	P_i and P_o of internal surfaces of soil aggregates
	1M HCl	Calcium bound
	Oxidation and acid digestion (H_2SO_4, H_2O_2)	Residual P

The advantages of fractionation schemes are that they require only a small soil sample ($\leq$ 0.5g), follow a simple procedure and use basic laboratory equipment. Despite the convenience of this method, it may be unreliable for the determination for organic P. Extractants are not necessarily specific for any particular group of organic P and therefore some P compounds will be present in one or more of the fractions (Condron *et al.*, 2005). Furthermore, fractions that are bioavaliable in one soil may not be in others (Frossard *et al.*, 2000) as enzyme substrate specificity can differ (Hayes *et al.*, 2000; He & Honeycutt, 2001). Strongly acidic solutions can cause hydrolysis of organic P. However, it is also possible that some of the native P minerals within the soil are resistant to an acid treatment resulting in

small amounts of inorganic P remaining in the sample. This would underestimate inorganic P and overestimate organic P (Dougherty *et al.*, 2005). Orthophosphate concentrations can also be underestimated due to their precipitation in alkaline extracts (Makarov *et al.*, 1997). Finally, classifying the form of plant available P based upon extractability is misleading, as plants have been shown to access P from otherwise stable fractions of soil organic P (Chen *et al.*, 2002).

Chromatographic techniques

Size exclusion chromatography/Gel filtration

Size exclusion chromatography or gel filtration discriminates between high and low molecular weight P fractions. The larger molecules are excluded to a greater extent from the inner spaces of a porous column packing material than smaller molecules, which can penetrate the openings of the small pores (Cooper *et al.*, 2007).

Earlier work in this area was focused on discriminating between the high molecular weight (e.g., phytate/humic P) and the more bioavailable low molecular weight (e.g., orthophosphate) fractions (Steward & Tate, 1971). Gel filtration chromatography has since been used to examine the combined effects of superphosphate fertilizer application on the molecular weight distribution of organic phosphorus (P) extracted from soils under irrigated pasture (Condron & Goh, 1989). It has also been used to study the partitioning of phosphate between the aqueous and colloidal phases in soil solutions (Hens & Merckx, 2001).

One of the problems with gel filtration is anion exclusion and adsorption of P species, which depends on elution conditions but can be minimised using alkaline soil extracts with an ionic strength > 0.05 M (Steward & Tate, 1969; McKelvie *et al.*, 1993). McKelvie *et al.* (1993) also reported poor detection of P compounds and the inability of the gel to resolve the small differences in molecular weight as limitations to the technique. More recently, gel filtration has been used as a "clean up technique" prior to NMR analysis. Labile phosphomonoesters are rarely characterised in ^{31}P NMR studies due to spectral signal overlap, but using gel filtration prior to ^{31}P NMR spectroscopy has been reported to enable their quantification (Turner & McKelvie, 2002). Alternatively, gel filtration can remove NaOH from alkaline soil extracts and so prevent the hydrolysis of organic P prior to NMR analysis (Pant *et al.*, 1999).

Ion exchange chromatography

The ionisable phosphate groups in organic P compounds provide the basis for ion exchange chromatography (IEC), whereby the stationary phase surface contains ionic functional groups that interact with analyte ions of opposite charge. This allows separation of P species based on the strength of ionic interactions (McKelvie, 2005). The ability of IEC to isolate individual P species has led to its

widespread use not only for soil analysis (Ruiz-Calero & Galceran, 2005), but also to isolate soil nucleotides (Anderson, 1970). More commonly, it has been used to separate inositol phosphates (Cosgrove, 1962; Cosgrove, 1963; Williams & Anderson, 1968; Irving & Cosgrove, 1981). Many of these early studies used large columns which required long elution times that increased the potential for on-column hydrolysis. Recently, the combined use of high performance liquid chromatography (HPLC) and inductively coupled plasma mass spectrometry (ICP-MS) or electrospray ionisation mass spectrometry (ESI-MS), has in one step provided the possibility of separating and characterising organic P, therefore avoiding the need for time consuming fraction collection (Collins, 2004).

Gas Chromatography

Gas chromatography plays an important role in the detection and quantification of organophosphorus pesticides in soil residues (Perez *et al.*, 1998; Fenoll *et al.*, 2005) by separating and analysing P compounds that can be volatised without decomposing. Gas chromatography can also be used to detect inositol phosphates, but only after derivatization (Dost & Tokul, 2006) and separation by ion-exchange chromatography (Turner *et al.*, 2002b). Irving and Cosgrove (1982) isolated inositol phosphates using HPLC and then identified the various isomers by gas chromatography using flame ionisation detection (GC-FID). Heathers *et al.* (1989) used a more complicated technique, first isolating inositol tetrakis-, tris- bis- and monosphosphates by HPLC, which were subsequently dephosphorylated using magnesium and alkaline phosphatase and finally analysed by GC-FID.

Chromatography can be a rapid technique compared to some spectroscopic analyses but errors are particularly common through poor recoveries from chromatography columns, matrix interferences with standards and misidentification of compounds. Furthermore some chromatographic techniques are laborious, involve complicated post column reactions or complex gradient separations may be necessary.

Nuclear magnetic resonance spectroscopy

The history of using NMR spectroscopy to characterise soil P speciation is as yet a short one, dating back to its first use in 1980 (Newman & Tate, 1980). Its popularity has increased in recent years. NMR spectroscopy has a fairly obscure physical basis (a nuclear property called *spin*) and can only be fully described using quantum mechanics. However, simplified descriptions can be found in several reviews (e.g. Veeman, 1997; Cade-Menun, 2005b) and more complex descriptions in NMR textbooks (Derome, 1987; Yoder & Schaeffer, 1987). Briefly, ^{31}P nuclei have a spin of 1/2, which results in them behaving as magnetic dipoles. When the nuclei are placed in a magnetic field they align their magnetic dipole either parallel or anti parallel to the applied magnetic field. When a radio-frequency pulse of the correct frequency (the so-called “Larmor” frequency at which the nuclei precess about the applied field) is applied, the nuclei can absorb energy. After the radio-frequency pulse stops, the nuclei relax

back to equilibrium, emitting energy that is detected as an emission signal or "free induction decay" (FID). This is then Fourier transformed, to convert from a signal with a time domain to one with a frequency domain, using NMR processing software. The resultant spectrum shows signal intensity (y-axis) as a function of frequency (x-axis). Nuclei will emit at slightly different frequencies depending on their chemical environment, with the largest effect being a slight "shielding" of the magnetic field by electron clouds in the chemical bonds surrounding the nuclei. Following Fourier transformation this results in peaks at different frequencies or "chemical shifts" in the spectrum. Importantly, under ideal circumstances, the area under each peak is proportional to the number of P nuclei that give rise to it and thus NMR is innately quantitative.

The main limitation of NMR is that it is innately insensitive, so multiple excitation-detection-relaxation cycles are usually required to provide sufficient signal so that all P species can be detected above background noise, i.e. to produce a clear spectrum. This requires that between cycles or "scans" the nuclei regain their equilibrium magnetization. In order for the nuclei to do this (a process termed "relaxation"), they must exchange energy with their surroundings (spin-lattice relaxation, T_1) or with each other (spin-spin relaxation, T_2). In solution, the spin-lattice relaxation dominates and spin-spin relaxation is negligible. T_1 varies between P species and with sample temperature and concentration of paramagnetic ions (e.g. iron and manganese) (discussed below). Therefore, the amount of time the nuclei are given to relax is important if quantitative analysis is required. If the nuclei do not fully relax back to equilibrium, signal is reduced in subsequent scans, an effect known as "signal saturation". If nuclei that give rise to different peaks relax at different rates, and insufficient time is provided for all nuclei to fully relax, peak intensities will not reflect the relative abundance of the different types of nuclei, i.e. the spectrum will not be quantitative. Recycle times of five times T_1 are required to ensure saturation losses are <1% (Yoder & Schaeffer, 1987). Shorter recycle delays can be used to give higher sensitivity (per unit time), by trading off some loss of signal per scan through saturation for running more scans per unit time.

Solution ^{31}P NMR spectroscopy

Solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy is by far the most widely-used spectroscopic technique for the speciation of soil organic P. This technique has been used to differentiate between inorganic and organic P as well as broader organic P classes including phosphonates, orthophosphate monoester and diesters, pyrophosphate and polyphosphate (Newman & Tate, 1980; Cade-Menun & Preston, 1996; Dai *et al.*, 1996; Makarov *et al.*, 2002b; Turner, 2004; Turner & Richardson, 2004; He *et al.*, 2006; McDowell & Stewart, 2006). Being a solution technique, solution ^{31}P NMR spectroscopy has the disadvantage of requiring an extraction step prior to analysis. The aim of such an extraction is to maximise solubilisation of P while minimising alteration of P speciation and optimising the conditions for subsequent NMR analysis.

Solution ^{31}P NMR is usually carried out on alkaline soil extracts. This is mainly because solubility of both organic and inorganic P species is maximised at high pH. Most early studies used NaOH as the extractant, usually at a concentration of 0.5 M (Newman & Tate, 1980; Tate & Newman, 1982; Hawkes *et al.*, 1984). Subsequently, Bowman and Moir (1993) developed a single step extraction using a mixture of NaOH and EDTA (usually at concentrations of 0.25 M and 0.05 M, respectively), and this has now become the most commonly-used extractant (Dai *et al.*, 1996; Turner *et al.*, 2003d; Murphy *et al.*, 2009). The inclusion of EDTA, which is a strong chelating ligand, serves two purposes. It complexes paramagnetic cations such as Fe and Mn in the extract and it increases soil P extraction efficiency and the diversity of P compounds extracted (Bowman & Moir, 1993). The fact that paramagnetic ions remain in solution when EDTA is used (unlike when Chelex is used) can also be advantageous because they induce rapid relaxation and this can improve sensitivity (discussed below). The effectiveness of NaOH-EDTA as an extractant for ^{31}P NMR spectroscopy has been compared to that of other extractants, e.g., 0.25 M NaOH, Chelex (a chelating resin) + 0.25 M NaOH, and post-extraction treatment with Chelex (Cade-Menun & Preston, 1996; Cade-Menun *et al.*, 2002; Briceño *et al.*, 2006; Turner, 2008). In general, NaOH-EDTA achieved the highest P extraction efficiency. However, this is dependent on the nature of the soil (Turner *et al.*, 2005). In the four studies mentioned above, corresponding extraction efficiencies using NaOH-EDTA were 37–60% for volcanic soils (Briceño *et al.*, 2006), 37% for tropical soils (Turner, 2008), 71–91% for forest floor samples (Cade-Menun & Preston, 1996; Cade-Menun *et al.*, 2002), and 34% for a forest soil sample (Cade-Menun *et al.*, 2002). NaOH-EDTA also extracted a greater diversity of P compounds and there was less hydrolysis of P compounds compared to other extractants. For these reasons, NaOH-EDTA has been generally accepted as the preferred extractant for solution ^{31}P NMR analysis.

Obviously, the nature of the soil P that cannot be extracted cannot be determined using solution ^{31}P NMR spectroscopy and this is one of the limitations of the technique. Besides the problem of non-extractable P, the greatest problem with solution ^{31}P analysis of organic P is the potential for hydrolysis. A number of papers have reported the instability of some organic P compounds, particularly orthophosphate diesters, in alkaline solution (Anderson, 1967; Makarov *et al.*, 2002a; Turner *et al.*, 2003a). Hydrolysis and modification of native P compounds can be avoided by acquiring NMR spectra at lower pH, at which a variety of well-resolved peaks can still be detected (McDowell & Stewart, 2005b). However, this is likely to favour the extraction of soluble P fractions (Adams, 1990). So although strong alkaline extractions introduce the risk of hydrolysis they also maximise P extractability. To accurately and quantitatively assess soil organic P using solution ^{31}P NMR, maximum recovery is vital and alkaline reagents are the most effective for this.

Sensitivity is often the limiting factor in solution ^{31}P NMR analysis of soil organic P. There are two main ways to combat the inherently low sensitivity of ^{31}P NMR. One is to maximise the amount of P

in the sample analysed and the other is to acquire and average a large number of scans. Soil extracts are usually concentrated prior to NMR analysis in order to improve sensitivity. This can be achieved by lyophilisation (freeze-drying), rotary evaporation or evaporating under a stream of nitrogen at 40ºC (Cade-Menun, 2005a). Lyophilisation is the most widely used technique as it avoids an increase in temperature that may degrade the sample (Cade-Menun *et al.*, 2002; Turner *et al.*, 2003a). Dried extracts are re-dissolved immediately prior to ^{31}P NMR analysis in order to minimise hydrolysis. The amount of sample re-dissolved needs to be sufficient to obtain enough signal but not so great as to increase the viscosity of the sample, which can cause line broadening. Performing ^{31}P NMR spectroscopy at temperatures of 20–25 ºC will not only help to prevent sample degradation but will also shorten relaxation times (Cade-Menun *et al.*, 2002), allowing more scans to be collected per until time. Care must be taken to leave sufficient time between scans, otherwise signal saturation can occur. As general rule, ensuring the total delay time between pulses is five times the spin-lattice relaxation time (T_1) will ensure that 99% of the nuclei are fully relaxed (Yoder & Schaeffer, 1987). T_1 values can be determined from inversion-recovery or saturation-recovery experiments. Alternatively, McDowell *et al.* (2006) have suggested that T_1 can be estimated from the P/(Fe + Mn) ratio. More research is warranted before this technique can be used with certainty.

As mentioned above, the presence of paramagnetic ions in NaOH-EDTA soil extracts causes line broadening. This decreases the resolution of solution ^{31}P NMR spectra. Resolution is most strongly influenced by Mn and Fe (Cade-Menun *et al.*, 2002; Turner, 2008). Pre-treatment of the soil with Ca-EDTA-dithionite before extracting with NaOH-EDTA to diminish Fe and Mn concentrations has been shown to decreased line widths of NMR signals by up to 46%, while having little effect on the forms of P detected (McDowell & Stewart, 2005a). Resolution is also affected by solution pH and the presence of suspended particles (Crouse *et al.*, 2000). Solution NMR spectroscopy will only detect those P nuclei in solution, but often small amounts of dried extract fail to redissolve. Thus it is often beneficial to filter or centrifuge the sample prior to analysis. Good spectral resolution is a necessary but not sufficient condition for achieving detailed speciation. The other necessary condition is accurate assignment of peaks. Many studies rely on comparison to reported literature values to assign peaks to P compounds (Dai *et al.*, 1996; Cade-Menun *et al.*, 2002; Makarov *et al.*, 2002a; Briceño *et al.*, 2006; McDowell & Stewart, 2006). These chemical shifts, however, can vary with parameters such as pH, temperature, the concentration of paramagnetic ions and ionic strength (Costello *et al.*, 1976; Derome, 1987; Crouse *et al.*, 2000; McDowell & Stewart, 2005b; Puppato *et al.*, 2007; Smernik & Dougherty, 2007).

Solid-state ^{31}P NMR spectroscopy

Most soil P NMR analysis is done in solution mode, but NMR analysis can also be carried out on solid samples (Frossard *et al.*, 1994; Condron *et al.*, 1997; McDowell *et al.*, 2002a; Benitez-Nelson *et al.*,

2004). Minimal sample preparation is required for solid-state ^{31}P NMR spectroscopy and this is its main benefit over solution ^{31}P NMR. Another advantage of the solid-state technique is that the amount of sample that can be analysed is not restricted by solubility. Condron *et al.* (2005) suggested that concentrations of around 1 mg P per gram of soil are needed to produce high-quality ^{31}P NMR spectra but the low natural abundance of P in some soils may result in signal being below detection limits.

The greatest disadvantage of solid-state compared to solution ^{31}P NMR is the poorer resolution of the former. Rarely have solid-state ^{31}P NMR studies focused on determining organic P concentrations or identifying individual P compounds in whole soils (Newman & Condron, 1995; Condron *et al.*, 1997; McBeath *et al.*, 2006; Conte *et al.*, 2008). Instead, much research has dealt with the application of solid-state NMR in differentiating between inorganic metal-P species and determining the relative amount of whole soil inorganic and organic P (Hinedi & Chang, 1989; Frossard *et al.*, 1994; Frossard *et al.*, 2002; McDowell *et al.*, 2002b; McDowell *et al.*, 2002a). As is the case for solution-state ^{31}P NMR spectra, poor spectral resolution can also be caused by the presence of paramagnetic ions, but this does not always appear to be the case (Hinedi & Chang, 1989; Shand *et al.*, 1999; McDowell *et al.*, 2003a). Other factors which may affect resolution have not been widely examined, although He *et al.* (2007b) and Conte *et al.* (2008) both recommend analysis of only completely dry samples. They reported that moisture in the soil sample at the time of analysis altered the intensity and position of peaks.

Quantification of soil P using NMR spectroscopy

In most cases where speciation of organic P is being sought, it is not just identification of the species that is important, but also their quantification. Quantification of P species from ^{31}P NMR spectra is usually carried out by multiplying fraction of total peak area by the total P concentration of the extract. This assumes that all the P in the reconstituted NaOH-EDTA extract is soluble and is observed with equal sensitivity. This may not always be the case, and more attention should be paid to this issue.

The other major limitation to accurate quantitation is poor resolution, especially in regions of ^{31}P NMR spectra where there is considerable overlap of resonances. This problem is usually addressed using spectral deconvolution, which can be used to quantify signal in overlapping resonances. Spectral deconvolution involves a numeric least-squares fit of the spectrum as the sum of multiple peaks of standard shape (Lorentzian or Gaussian). Spectral deconvolution has been used to identify the complex signals associated with phytate (Turner *et al.*, 2003c; McDowell & Stewart, 2006) and orthophosphate monoesters (McDowell & Stewart, 2005b) using solution ^{31}P NMR spectroscopy. Although the mathematical process of spectral deconvolution is quite straight-forward (and is included in standard NMR software), special care and consideration is required to produce accurate and reliable results. The main issue of deconvolution which affects solution NMR analysis, but more so solid-state

NMR analysis, is the degree of overlap of resonances in the spectra. Although spectral deconvolution can provide an adequate fit, quantifying individual species from these fits would appear optimistic, as acknowledged in the paper by McDowell *et al.* (2003b). There is quite some way to go before deconvolution of solid-state ^{31}P NMR spectra of soils can be considered a reliable technique for quantification of P species.

Phosphorus X-ray absorption near-edge structure (XANES) spectroscopy

XANES (X-ray absorption near-edge structure) spectroscopy, which is synonymous with NEXAFS (near-edge X-ray fine structure) spectroscopy, is a technique that can be used for speciation of numerous elements in soils. XANES requires an energy-tuneable source of X-rays that is currently only possible with a synchrotron. The use of synchrotron-based techniques for soil analyses is a recent development, coinciding with the rapid and continuing increase in the number of synchrotron facilities world-wide since the 1990s (Lombi & Susini, 2009).

The use of XANES spectroscopy for P speciation in soils is still in its infancy. Hesterberg *et al.* (1999) published the first P XANES spectrum of a soil. Since then there has been a handful of studies using P XANES for speciation of P in soils (Beauchemin *et al.*, 2003; Sato *et al.*, 2005; Lombi *et al.*, 2006; Ajiboye *et al.*, 2008; Kruse & Leinweber, 2008), some related studies on organic amendments (Peak *et al.*, 2002; Toor *et al.*, 2005; Shober *et al.*, 2006; Ajiboye *et al.*, 2007; Gungor *et al.*, 2007) and one on marine sediments (Brandes *et al.*, 2007). In most cases, the focus has been on identifying and quantifying inorganic P species. However, the potential for P XANES spectroscopy for organic P speciation has been raised.

Minimal sample preparation is required for XANES analysis and this is one of its great advantages. Soil samples are usually sieved and ground to improve homogeneity and either placed in a sample holder (e.g., Beauchemin *et al.* (2003)) or spread onto adhesive tape (Sato *et al.*, 2005; Lombi *et al.*, 2006; Ajiboye *et al.*, 2007; Ajiboye *et al.*, 2008; Kruse & Leinweber, 2008).

The ability to differentiate species is the main limitation of P XANES analysis. Phosphorus speciation in soils is particularly difficult to resolve using XANES spectroscopy because all P species have the same oxidation state (+V) and the P atom is nearly always surrounded by four O atoms. The P XANES spectra of organic P compounds are generally similar, contain little in the way of diagnostic features and are difficult to distinguish from aqueous or weakly bound phosphate (Peak *et al.*, 2002; Beauchemin *et al.*, 2003; Shober *et al.*, 2006; Kruse & Leinweber, 2008). He *et al.* (2007a) have shown that the P XANES spectra of pure phytate salts are distinguishable. However, their lack of prominent spectral features, mutual similarity and similarity to P XANES spectra of soluble and

sorbed orthophosphate would make it impossible to distinguish different phytate salts in heterogeneous matrices such as soils.

Since P XANES signals for each species overlap over virtually the whole spectral width, quantitative analysis of P XANES spectra of soils is usually achieved by fitting the experimental spectrum to a linear combination of XANES spectra of model materials. This is the equivalent of the deconvolution process used in NMR spectroscopy.

Enzymatic hydrolysis

Enzymatic reactions can be used to estimate the potential availability of P forms by quantifying the release of orthophosphate following enzymatic hydrolysis. A recent review by Bünemann (2008) showed that up to 99% of organic P can be identified in this way. Enzymatic hydrolysis has been used to determine the concentration of the labile organic P fraction (Pant *et al.*, 1994; Shand & Smith, 1997; Hayes *et al.*, 2000), but also more specifically P functional groups such as labile monoester-P, phytate-like P, DNA-like P and polynucleotide P (He *et al.*, 2004a; He *et al.*, 2004b). Turner *et al.* (2003b) were reportedly able to identify inositol hexakisphosphate (phytate) using a phytase enzyme, one of a class of phosphatase enzymes with a high specific activity for phytate (Mullaney & Ullah, 2007). However, the subsequent testing of substrate specificies of numerous different phytase enzymes suggested it was likely that the phytase enzyme also hydrolysed other P species (Shand & Smith, 1997; Hayes *et al.*, 2000; Turner *et al.*, 2002a).

Perhaps the greatest limitation of the enzymatic hydrolysis analysis of soil organic P is that although it is possible to determine the potential bioavailability of organic P, it is not possible to identify the individual organic P species susceptible to hydrolysis and there may be some overlap between P groups. He *et al.* (2004b) suggested that pyrophosphate could be incorrectly identified and grouped with orthophosphate monoester P due to its high liability to monophosphatases. Enzymatic hydrolysis has been coupled with ^{31}P NMR spectroscopy to characterise manure but there has been little success in characterising soil extracts (He *et al.*, 2008). Enzymatic hydrolysis is however a relativity new technique and there are many methodological aspects still to be considered before it is applicable to a wide range of samples.

Objectives of this research

Organic phosphorus plays a vital role in soil biochemical cycling. If organic P is to be better managed as a source of plant P for agricultural production and continued emphasis is placed upon the efficient use of applied and existing soil P reserves, there is a need to gain a more comprehensive understanding of soil organic P. Our current understanding of the origins, bioavailability and mobility of organic P in soil systems is inadequate. In order to improve this understanding it is essential to have techniques that

accurately and reliably identify and quantify organic P. Many of the current analytical techniques are subject to problems with extraction methodologies, poor detection, misidentification and over- or underestimations of specific organic P species. Some techniques are also very time consuming and provide limited structural information.

Spectroscopic techniques appear to offer the best potential for determining the speciation of organic P in soils. Phosphorus-31 NMR spectroscopy has become increasingly popular for the analysis of soils and manures to examine the distribution of organic P compounds. However, like any of the other analytical techniques, has its own unique problems. The potential sources of error in the established ^{31}P NMR technique are the incorrect assignment of peaks and lack of accurate quantitation. To properly utilise the technique and generate reproducible results, consideration needs to be given to minimising or eliminating these sources of error. This may therefore enable the accurate characterisation of soil organic P and develop our understanding of P cycling in soils. The main objectives of this research were to:

- Refine and develop an improved spiking procedure and method of spectral deconvolution for NMR analysis, and to apply these techniques to a range of Australian soils.

- Identify and quantify the P compounds responsible for the prominent peaks in the NMR spectra, in order to characterise the organic P speciation in a range of Australian soils.

- Determine the fate of phytate when applied to a calcareous soil and its potential role in P cycling.

- Identify and quantify the potentially bioavailable organic P forms in a range of Australian, Swiss and Kenyan soil extracts by combining enzymatic hydrolysis and solution ^{31}P NMR spectroscopy.

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CHAPTER 2

SPIKING IMPROVED SOLUTION PHOSPHOURS-31 NUCLEAR MAGNETIC RESONANCE IDENTIFICATION OF SOIL PHOSPHORUS COMPOUNDS

The work contained in this chapter has been published in *Soil Science Society of America Journal*.

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Doolette, A.L., Smernik, R.J. & Dougherty, W.J. (2009). Spiking improved solution phosphorus-31 nuclear magnetic resonance identification of soil phosphorus compounds.
Soil Science Society of America Journal, v. 73(3), pp. 919 -927.

CHAPTER 3

RAPID DECOMPOSITION OF PHYTATE APPLIED TO A CALCAREOUS SOIL DEMONSTRATED BY A SOLUTION ^{31}P NMR STUDY

The work contained in this chapter has been published in *European Journal of Soil Science.*

Doolette, A.L., Smernik, R.J. & Dougherty, W.J. 2010. Rapid decomposition of phytate applied to a calcareous soil demonstrated by a solution ^{31}P NMR study. *European Journal of Soil Science*, **61,** 563–575.

Doolette, A.L., Smernik, R.J. & Dougherty, W.J. (2010). Rapid decomposition of phytate applied to a calcareous soil demonstrated by a solution ^{31}P NMR study.
European Journal of Soil Science, v. 61(4), pp. 563 - 575.

CHAPTER 4

CONFIRMATION OF THE MICROBIAL DEGRADATION OF PHYTATE IN A CALCAREOUS SOIL

Introduction

In the previous chapter (Chapter 3), the stability of phytate in soil was investigated in an incubation of a single calcareous soil. The concentration of phytate in NaOH-EDTA extracts was found to decrease over a number of weeks and this was attributed to microbial degradation. An alternative explanation, that the decrease in extractable phytate resulted from increasing interactions of phytate with cations and soil minerals, was considered unlikely for a number of reasons, including the kinetics of phytate loss. A consequence of microbial degradation is that the decrease in phytate concentration should be accompanied by an increase in orthophosphate. However, in the incubation experiment described in Chapter 3, the phytate applied to the soils represented only a small increase (15%) in total P and because the total P in sub-samples varied to a similar degree, the anticipated smooth increase in orthophosphate concentration with increasing incubation time was somewhat obscured. Nonetheless, a clear increase in orthophosphate concentration was evident when expressed relative to total NaOH-EDTA extractable P. The objective of this study was to further investigate these possible mechanisms by applying higher concentrations of phytate that should result in a clearer increase in orthophosphate concentration and thereby confirm the microbial degradation of phytate.

Materials and Methods

Site description and soil sampling

The soil used in this study was a calcareous soil collected from Hart, South Australia, from the 0–10 cm layer of no-till plots under wheat in a field experiment. This is the same soil that was used in the previous incubation study examining the decomposition of phytate (Chapter 3) and the microbial synthesis of organic and condensed forms of phosphorus in acidic and calcareous soils (Bünemann *et al.*, 2008).

Soil amendment and incubation experiment

The design of this incubation experiment was similar to that of the previous incubation experiment (Chapter 3), the only differences being (i) the concentration of phytate added and (ii) that wheat straw was not added to any of the treatments in this study. Briefly, the experimental treatments were a control and the addition of a phytate solution at four different concentrations and were carried out in duplicate. The treated soils received 452 mg kg^{-1}, 904 mg kg^{-1}, 1803 mg kg^{-1} and 4507 mg kg^{-1} of phytate (Na salt from corn [*Zea mays*,], Sigma-Aldrich P8810) in solution, with the nutrient solution (described below) added separately. Solution ^{31}P NMR analysis of the phytate material (Figure 1) indicated it was impure. Spectral deconvolution was used to quantify the distribution of P species from the ^{31}P spectra and involved fitting 17 resonances to the spectrum. The strongest resonances were easily identified as phytate. The remaining resonances were assigned to orthophosphate and other P-impurities, possibly lower inositol phosphate and/or stereoisomers of phytate. The concentrations of P species were determined by integrating the relative intensities of the P species against that of the

methylenediphosphonic acid (MDP standard). The concentration of phytate P in solution was 16.3% and the concentration of other P species (orthophosphate and P-impurities) was 5.9%.

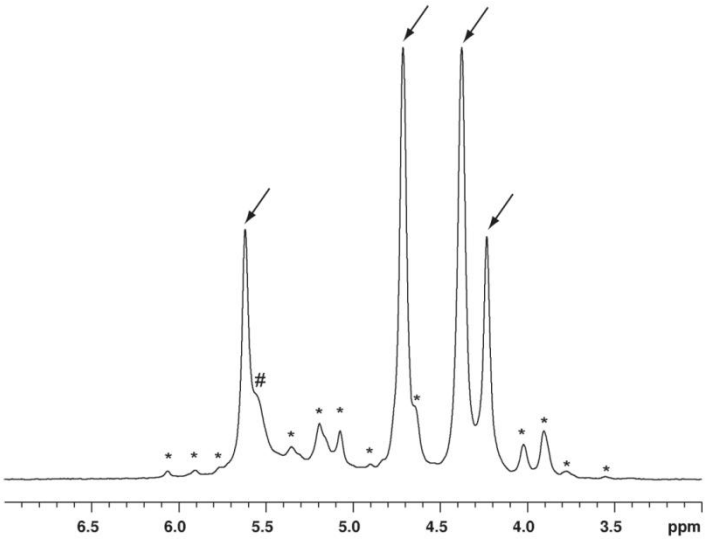

Figure 1. Solution ^{31}P NMR spectrum of phytate dissolved in NaOH-EDTA. The following resonances were identified: phytate ←, orthophosphate #, P-impurities *.

Based on the above analysis, phytate P additions for the four phytate treatments were 74 mg kg^{-1}, 147 mg kg^{-1}, 293 mg kg^{-1} and 733 mg kg^{-1} (hereafter referred to as75P, 150P, 290P and 730P). All treatments received 0.45 ml of a nutrient solution (Bünemann *et al.*, 2008), which added the following nutrients (mg kg^{-1} of soil): N (125), K (25), S (20.1), Ca (5), Mg (5), Fe (0.35), Mn (0.1), B (0.05), Cu (0.01), Zn (0.01) and Mo (0.002). The water content of each soil was then adjusted to 70% of the maximum water holding capacity (19% gravimetric water content). Soils were sampled at 0, 1, 4, 7 and 13 weeks (T0, T1, T4, T7, T13).

NaOH-EDTA extraction and NMR analysis of NaOH-EDTA extracts

For solution ^{31}P NMR analysis, soils were extracted, prepared and the ^{31}P NMR spectra acquired as described in Chapter 3. Spectra were acquired with the addition of an internal standard (0.1 ml) of 6.0 g l^{-1} methylenediphosphonic acid solution (MDP) (Sigma Aldrich; M9508; ≥99%), a recovery delay of 40 s and a 90° pulse of 35–40 µs. Between 1904 and 3740 scans were acquired for each sample. Chemical shifts were referenced to β-glycerophosphate at 4.63 ppm according to Doolette *et al.* (2009).

Quantification of P species from ^{31}P NMR spectra

Signal areas of classes of P compounds were determined by integration and concentrations calculated by integrating against the signal from the MDP standard. Spectral deconvolution was used to quantify the spectra as described in Chapter 3. This involved fitting up to 6 sharp resonances (due to orthophosphate, phytate and α- and β-glycerophosphate) and one broad humic P signal to each ^{31}P

NMR spectrum. The phytate and orthophosphate concentrations were adjusted to account for the overlap of the phytate C-2 peak with the larger orthophosphate peak. Total phytate concentration was calculated as 6/5 the concentration of the three observable peaks and 1/5 of this value was subtracted from the orthophosphate concentration (Doolette *et al.*, 2009).

Results and Discussion

The ^{31}P NMR spectra of the NaOH-EDTA soil extracts show a clear difference between spectra over time and between different phytate treatments (Figure 2). The vertical scale of each spectrum was normalised using the MDP reference signal (17–18 ppm, outside the spectral range shown). Therefore the differences in peak height in Figure 2 approximately reflect differences in the concentration of P species that give rise to each peak. No signal was detected in the orthophosphate diester (2 to -1 ppm) or pyrophosphate (-4.5 to -5.5 ppm) regions (not shown). The quantitative distributions of P species identified in the ^{31}P NMR spectra are presented in Table 1.

Total NaOH-EDTA extractable P concentrations of the control soil ranged from 345–384 mg kg^{-1} and were considerably less than the total NaOH-EDTA extractable P concentrations observed in the treated soils (409–1362 mg kg^{-1}), since the application of the phytate solution to the soils added up to an additional 1000 mg P kg^{-1}, which included phytate-P and P impurities. Total soil P extraction efficiencies for all soils (treated and untreated) were quite high, ranging from 80–83% in the control soil and 60–93% in the treated soils (Table 1). In general, there was a slight decrease in extraction efficiency over time, especially for the phytate amended soils.

For all five treatments, α- and β-glycerophosphate were minor components in the soil extracts, comprising up to 2% of total NaOH-EDTA extractable P (7–10 mg kg^{-1}) in the control soil and up to 3% of NaOH-EDTA extractable P (7–37 mg kg^{-1}) in the treated soils. Despite comprising a similar percentage of total NaOH-EDTA extractable P across all soils, on average the concentration of α- and β-glycerophosphate in the 290P and 730P treated soils were greater than in the control, 75P and130P treatments. In the previous incubation experiment (Chapter 3), the treatments that contained wheat had consistently higher concentrations of α- and β-glycerophosphate, presumably because the wheat provided a readily degradable source of C which increased the microbial population (Bünemann *et al.*, 2008). Therefore, since the addition of phytate not only adds P but also carbon (C) to the soil, it raises the possibility that the microbial community may also be utilising phytate as a source of C in the same way that C is utilised as an energy source in other organic inputs. However, at the highest rate of phytate addition this would have only added approximately 490 mg kg^{-1} of C. This represents a small C addition (~4%) relative to the organic C already present in the soil (13.5 g kg^{-1}). Nevertheless the phytate molecules and their C are bioavailable and so the possibility that they provide an energy source cannot be definitively ruled out.

The highest α- and β-glycerophosphate concentrations occurred at T0 in the 150P and 290P treatments (11 mg kg^{-1} and 23 mg kg^{-1}, respectively) but in the case of the control, 75P and 730P treatments, there was an increase in concentration followed by a slow decay. The highest α- and β-glycerophosphate concentrations occurred at T1 in the 75P and 730P treatments (27 mg kg^{-1} and 37 mg kg^{-1}, respectively) and at T4 in the control. These results confirm the previous hypothesis (Chapter 3), that microbial growth was likely nutrient limited during pre-incubation.

Orthophosphate was the most abundant P species in the control soil regardless of incubation time, ranging in concentration from 273–308 mg kg^{-1} and representing 65–83% of total NaOH-EDTA extractable P (Table 1). In all P treated soils, with the exception of the 730P treatment, orthophosphate was the most abundant P species, comprising 51–83% total NaOH-EDTA extractable P. The addition of phytate to the soils resulted in a large component of extractable P being phytate. Phytate comprised up to 38% total NaOH-EDTA extractable P in the 75P, 150P and 290P treatments and in the case of the 730P treatment was the most abundant species at T0 and T1 (53 and 52%, respectively). The control soil contained no detectable phytate at any stage of the incubation.

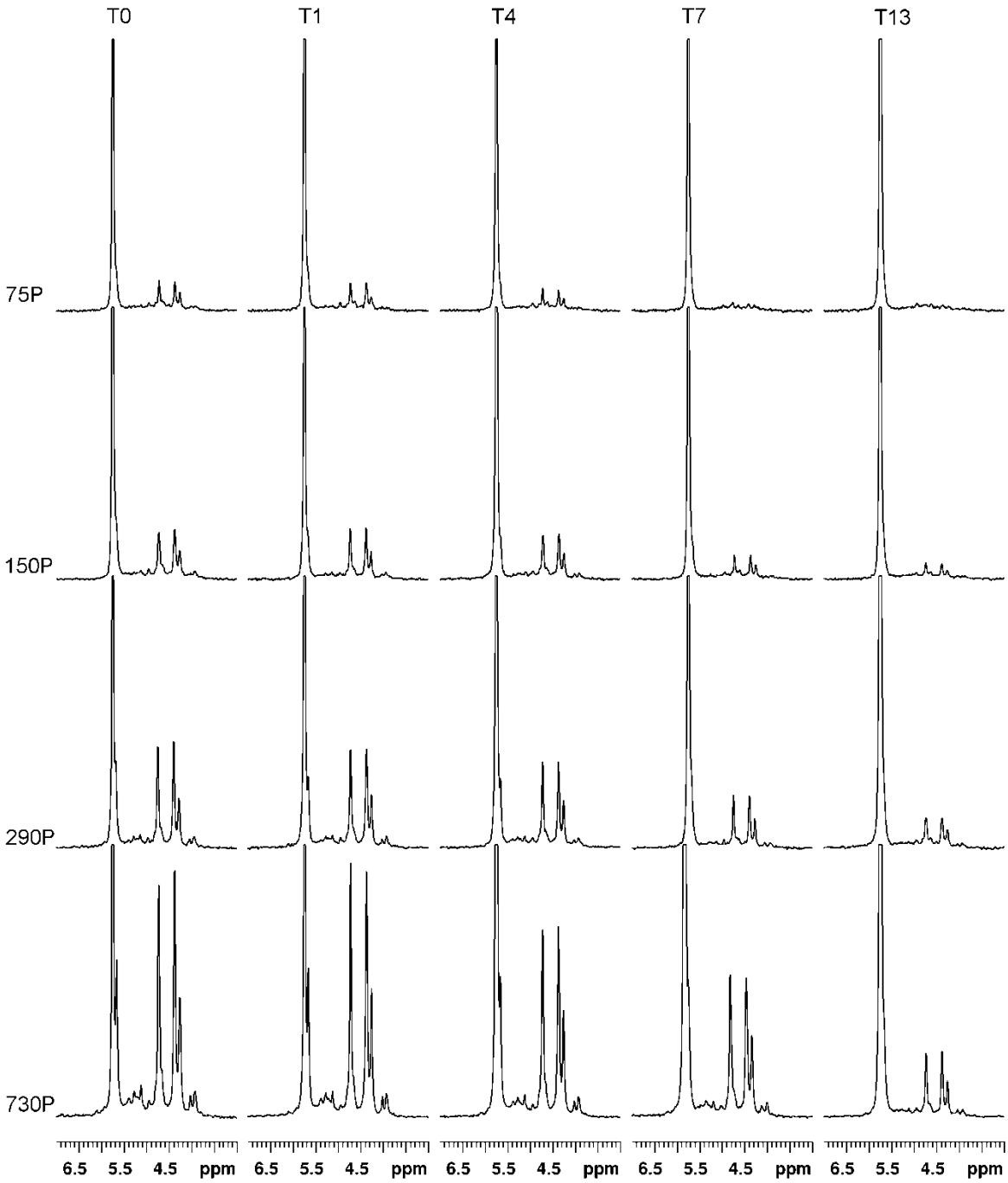

Figure 2. Solution ^{31}P NMR spectra of NaOH-EDTA soil extracts of a calcareous soil from South Australia (0-10 cm) which received four treatments of different phytate concentration (75, 150, 290, 730 mg kg^{-1}) and were incubated for up to 13 weeks (i.e. T13).

Table 1. Orthophosphate monoester and total extractable P concentration of a calcareous soil from South Australia (0-10 cm) which received five treatments of different phytate concentration (0, 75, 150, 290, 730 mg kg^{-1} i.e. 0P, 75P) and was incubated for up to 13 weeks (i.e. T13). Concentrations were calculated by integrating against the signal from the MDP standard and spectral deconvolution was applied to quantify the spectra. Extraction efficiency is defined as total NaOH-EDTA extractable P as a percent of total soil P + spiked P solution (phytate-P + P-impurities).

Treatment		Orthophosphate	Phytate	α– & β– glycerophosphates	Humic P	P impurities[a]	Total NaOH-EDTA Extractable P[b]	Extraction efficiency
		mg kg^{-1}						%
Control								
	T0	281	ND[c]	7	74	0	362	78
	T1	287	ND	9	75	0	371	80
	T4	304	ND	10	70	0	384	82
	T7	308	ND	8	66	0	382	83
	T13	273	ND	4	68	0	345	75
75P								
	T0	296(15)[d]	74(10)	8(2)	66(25)	12(1)	456(33)	81(6)
	T1	288(30)	59(2)	27(1)	65(22)	11(2)	450(47)	80(8)
	T4	331(17)	39(3)	10(1)	80(19)	8(1)	467(44)	83(8)
	T7	325(19)	22(1)	7(1)	54(6)	0	409(34)	72(6)
	T13	351(29)	12(0.03)	9(1)	52(7)	0	424(47)	75(8)
150P								
	T0	313(23)	116(12)	11(0.1)	75(1)	18(1)	533(33)	80(5)
	T1	270(37)	98(25)	8(1)	70(24)	14(0)	460(85)	69(13)
	T4	364(46)	89(23)	8(1)	86(4)	12(1)	559(74)	84(11)
	T7	372(33)	50(2)	9(1)	70(18)	7(2)	508(53)	76(8)
	T13	406(36)	33(9)	9(1)	47(23)	6(1)	501(24)	75(4)
290P								
	T0	367(22)	226(26)	23(4)	ND	57(15)	673(67)	78(8)
	T1	364(24)	269(20)	22(0.2)	ND	57(7)	711(51)	82(6)
	T4	455(21)	202(27)	21(1)	ND	46(9)	729(58)	84(7)
	T7	518(9)	137(8)	19(1)	ND	32(2)	706(26)	82(2)
	T13	512(86)	79(10)	12(4)	ND	19(3)	622(104)	72(12)
730P								
	T0	409(20)	672(81)	34(4)	ND	153(17)	1268(114)	87(8)
	T1	459(1)	704(26)	37(1)	ND	163(6)	1362(30)	93(2)
	T4	634(116)	472(6)	22(6)	ND	111(11)	1238(116)	85(8)
	T7	776(37)	337(28)	25(2)	ND	63(4)	1201(60)	82(5)
	T13	931(70)	115(20)	14(1)	ND	22(8)	1082(42)	74(3)

[a] P impurities from the phytate source used
[b] Total NaOH-EDTA extractable P is the total of extractable native soil P and extractable phytate
[c] ND; not detected
[d] Data are mean (± standard deviation) of analytical replicates except for the control (no replicates analysed)

For all treated soils (75P, 150P, 290P, 730P), there was a decrease in intensity of the phytate peaks with time (Figure 2), which corresponds to a decrease in phytate concentration (Table 1). The decrease in phytate was adequately fitted to a first order decay that indicated similar half-lives observed for each P treatment (Figure 3). There was no clear trend in the rate of degradation with phytate concentration. The half-life of phytate was 4 weeks for the 75P treatment, 7 weeks for the 150P treatment, 8 weeks for the 290P treatment and 6 weeks for the 730P treatment.

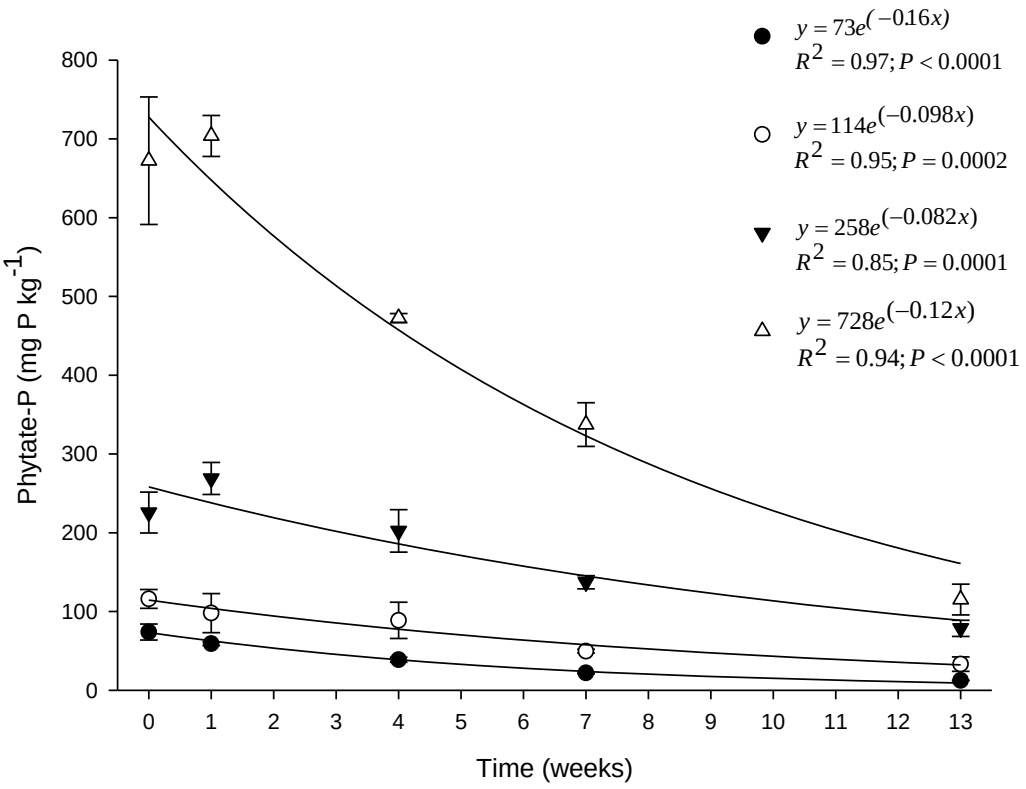

Figure 3. First-order decay model for the four incubated phytate treatments which received the addition of a phytate-P solution; ●75 mg kg^{-1}, ○150 mg kg^{-1}, ▼290 mg kg^{-1}, △730 mg kg^{-1}. The calculated half–life of phytate ranges from 4 to 8 weeks. Data are mean ± standard deviation (n=2).

In the previous incubation experiment, the expected increases in orthophosphate concentration over the incubation were obscured by the fact that phytate represented only about a 15% increase in total P and total P concentrations for sub-samples of the soil varied in a similar range. Expressing the orthophosphate concentration as a percentage relative to total NaOH-EDTA extractable P did provide some evidence that there was an increase in orthophosphate. In this second incubation experiment, in which the additions of phytate P were larger, the increase in orthophosphate concentration over time is more obvious (Figure 4). It is difficult to observe the relatively small increase in orthophosphate concentration for the 75P treatment, because the phytate concentration only represents an increase of 15% in total P. However, as the phytate concentrations increases from 150 mg kg^{-1} to 730 mg kg^{-1}, equivalent to an increase in total P of 32–158%, the difference in orthophosphate concentrations between T0 to T13 is more evident. Between T0 and T13, the orthophosphate concentrations increased

by 55, 93, 145 and 522 mg kg^{-1}, which represents an increase in orthophosphate concentration of 18%, 30%, 40% and 127%, respectively.

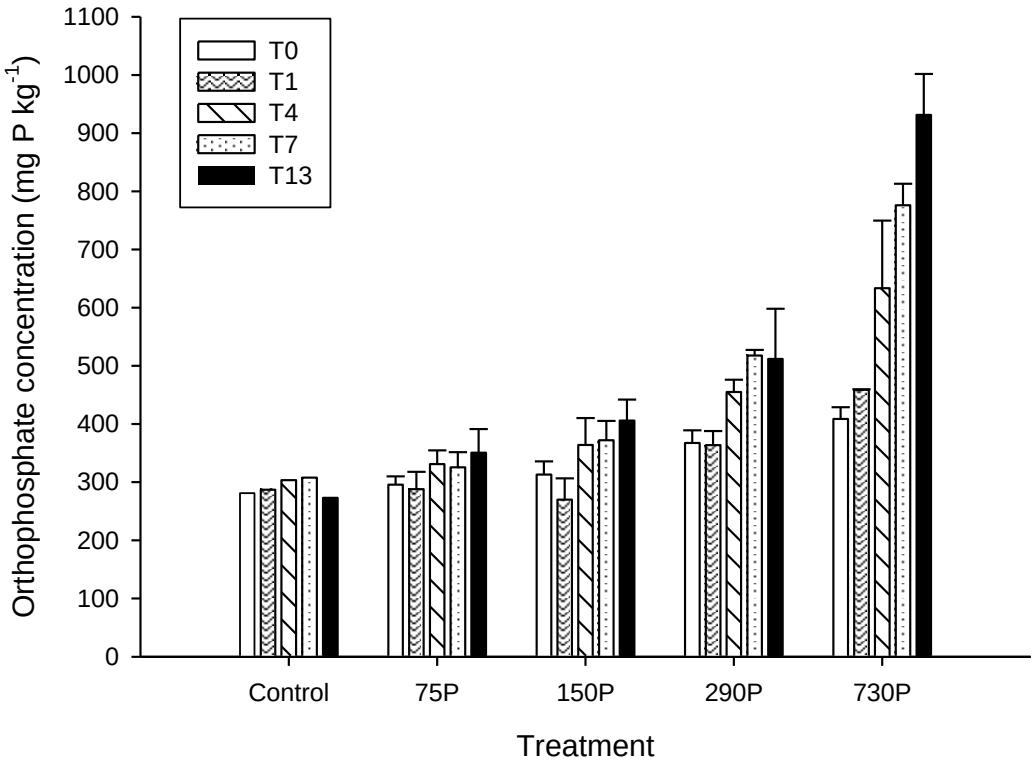

Figure 4. Orthophosphate concentrations of NaOH-EDTA soil extracts over a period of 13 weeks (i.e. T13) determined for a calcareous soil which received five different phytate treatments (0, 75, 150, 290, 730 mg kg^{-1}). Data are mean ± standard deviation (*n*=2). Duplicate analyses were not performed on the control.

Figure 5 shows a strong linear relationship exists between the changes in orthophosphate and phytate concentration, conforming to almost a 1:1 replacement. However, the average decrease in phytate concentration is 9% greater than the average increase in orthophosphate concentration. This is most likely explained by a slight overall decrease in P extractability through the incubation, as discussed above. There is also a clear decrease in the concentration of P impurities and other monoester P, albeit to a lesser extent, which suggest the hydrolysis of other P compounds is likely to have occurred. The signals assigned to P impurities in the ^{31}P NMR spectra of the 75P treatment could not be detected after T7 (Figure 2). In the 150P and 290P treatments, the hydrolysis of P impurities accounted for 2% and 5% of total extractable P, respectively. The intensity of peaks assigned to the P impurities is clearest at T0 in the 730P treatment and decreases rapidly, being approximately halved by T7 and by T13 only 14% of the original P (equating to a 9% reduction in total extractable P) remained.

The broad resonance in the monoester region, previously assigned to humic P compounds (Bünemann *et al.*, 2008) (Chapter 3), could only be identified in the control soils and the soils incubated with the lowest two phytate additions (75P and 150P). This resonance could not be distinguished from the

sharp monoester resonances at the higher phytate additions (Figure 2). Similar humic P concentrations were observed in all soils (where humic P could be detected), with concentrations ranging from 66–75 mg kg^{-1} in the control soil, 52–80 mg kg^{-1} in the 75P treatment and 47–86 mg kg^{-1} in the 150P treatment. It appears that there may have been a small decrease in humic P over the 13 week incubation period because the humic P concentration at T13 is consistently lower than at T0. The decrease in humic P concentration equates to a difference of 3% of total extractable P in the 75P treatment and 5% of total extractable P in the 150P treatment, but is likely to also be affected by variations in sub-samples.

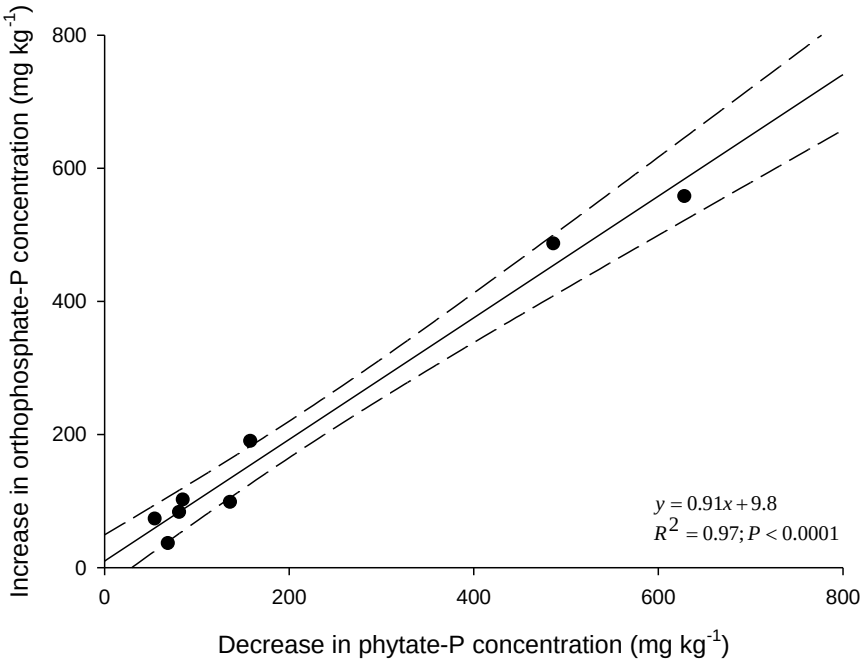

Figure 5. The relationship between the decrease in phytate-P concentration and increase in orthophosphate-P calculated as the difference in concentrations between T0 and T13 for the four incubated phytate treatments which received the addition of a phytate solution (75, 150, 300, 730 mg kg^{-1}). Data are mean ± standard deviation (n=2). Dashed lines represent the upper and lower 95% confidence limits.

Although the phytate and orthophosphate concentrations were much higher in this experiment than in the initial incubation experiment (Chapter 3), variability in extraction efficiencies still causes problems when comparing absolute changes in the concentration of P species. Expressing changes in concentration of orthophosphate, phytate and the P impurities relative to total extractable P provides a clearer illustration of trends (Figure 6). The decrease in phytate concentration accounts for the majority of the increase in monoester P (13–42% of total extractable P), with decreases in the P impurities accounting for the rest.

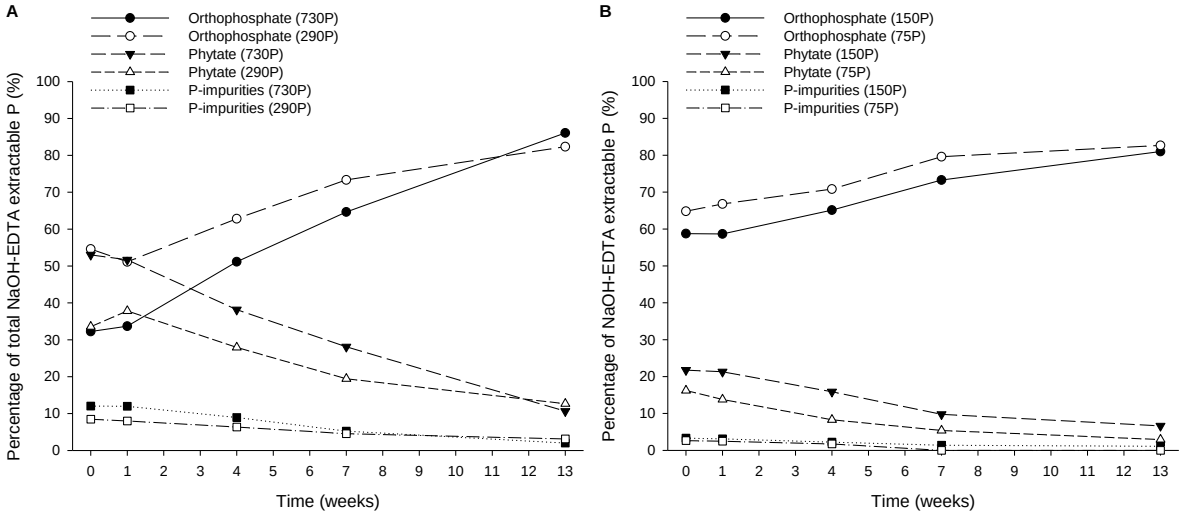

Figure 6. The changes in concentration of orthophosphate, phytate and P impurities relative to total extractable P between T0 and T13 for the four incubated phytate treatments which received the addition of a phytate solution (75, 150, 300, 730 mg kg^{-1}).

This study confirms that for the calcareous soil examined, added phytate is lost relatively rapidly via microbial degradation and that the loss of phytate cannot be attributed to the sorption of phytate to minerals or precipitation with soil cations to form insoluble salts. The strong relationship between orthophosphate gain and phytate loss eliminates the possibility that fixation/precipitation of phytate is occurring, since orthophosphate is not released when phytate is fixed. As was the case in our previous incubation study, there is insufficient variation in the P extraction efficiencies to account for the substantial decrease in phytate concentration and increase in orthophosphate concentration. Furthermore, if phytate was being sorbed while orthophosphate was simultaneously desorbed, although more likely to occur at low phytate concentrations (Berg & Joern, 2006), orthophosphate concentrations could not increase above the total native soil P concentrations. This was observed for the 730P treatment where the orthophosphate concentration increased by 522 mg kg^{-1} in a soil with a native P concentration of only 463 mg kg^{-1}.

It is interesting to note that the rate of phytate degradation was not influenced by the rate of phytate addition. It may have been expected that increasing the concentration of phytate P could affect its rate of degradation as phytate has been shown to be the preferred substrate of some bacteria, with bacterial population increasing as a result of phytate addition (Tarafdar & Claassen, 1988). It has also been proposed that phytate P is only utilised under P limited conditions (Turner *et al.*, 2003). At the highest rates of P addition in this experiment, there is clearly no P limitation, so presumably phytate is being used by the microbial community as a carbon (energy) source, rather than as a P source.

Several previous studies have focused on the fate of phytate added to soils (Jackman & Black, 1952b; a; Crouse *et al.*, 2002; Leytem *et al.*, 2006) but not all the analytical techniques used have allowed for a fully quantitative assessment. Some of the earliest work that examined the fate and

stability of phytate was undertaken by Jackman and Black (1952a; b). Jackman and Black (1952a) added phytate to soil at rates of 400, 800, 1200, 1600 and 2000 mg P kg^{-1} and were able to show that phytate could be readily hydrolysed in soil after 85 hours of incubation. However, direct measurements of changes in phytate concentrations were not made. Rather, phytate hydrolysis was measured as an increase in inorganic P concentration. Crouse *et al.* (2002) conducted a 20-week laboratory incubation to monitor phosphatase activity and P mineralisation, and used ^{31}P NMR spectroscopy to measure the relative concentration of soil organic P forms of previously manured and non-manured soils, which were subsequently amended with poultry manure. They reported a decrease in organic P, which could be identified as phytate from the spectra presented, from 23 to 15% in the previously manured soils and from 13 to 6% in the previously unmanured soil. These results suggest a half life of phytate, when added as a component of poultry manure, of approximately 20 weeks. In a similar study, Leytem *et al.* (2006) applied two different phytate P sources: poultry manure and reagent grade inositol hexakisphosphate (phytate), to two calcareous soils, at three P based application rates (10, 20 and 40 mg kg^{-1}). They used ^{31}P NMR spectroscopy to show a loss of phytate over 12 weeks from the poultry manure treated soils and found the change in Olsen P concentration over time was related to the amount of phytate present in the treatment. However, when the reagent grade phytate was applied to the soil, the increases in Olsen P appeared to be minimal (< 5 mg kg^{-1}). The results of Leytem *et al.* (2006) are consistent with the breakdown of phytate in soil over time. However, similar to the study of Jackman and Black (1952a), the results of Leytem *et al.* (2006) lack the direct quantitative analysis of phytate degradation.

The rapid rate of phytate degradation observed in this study is in good agreement with the previous studies that have indirectly examined phytate degradation in non-calcareous and manure-treated agricultural soils. Given the similarity in half-lives (4–8 weeks) for the different P concentrations applied to the soil, this suggests that the amount of P in solution was still less that that required to limit the maximum rate of hydrolysis. Based on the reasoning of Jackman and Black (1952a), the soil in this current study may have contained enough phytase to hydrolyse the added phytate. This could also explain why our control soil had no detectable levels of phytate. This is consistent with the hypothesis presented in Chapter 3, that in the calcareous soil examined, phytate is not highly stable. More generally, it suggests that the high calcium availability and moderately alkaline pH of calcareous soils do not necessarily result in phytate stabilisation.

Conclusions

By applying a high concentration of phytate to an agricultural calcareous soil we were able to confirm that microbial processes were responsible for the loss of added phytate. The existence of a strong linear relationship between decreasing phytate concentration and increasing orthophosphate concentration, and that the orthophosphate concentration more than doubled the native soil P

concentration at the highest phytate treatment concentration, allows for the possibility of fixation/sorption of phytate P to be eliminated. Although, more broadly, this suggests that phytate stabilisation is not necessarily associated with calcium availability and moderately alkaline soil pH, the generality of these findings requires further testing, since this study considers a single calcareous soil only.

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CHAPTER 5

A QUANTITATIVE ASSESSMENT OF PHOSPHORUS FORMS IN SOME AUSTRALIAN SOILS

The work contained in this chapter has been accepted for publication in *Australian Journal of Soil Research.*

Doolette A.L., Smernik R. & Dougherty W.J. (in press). A quantitative assessment of phosphorus forms in some Australian soils *Australian Journal of Agricultural Research*.

Abstract

Solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy is the most common technique for the detailed characterisation of soil organic P, but is yet to be applied widely to Australian soils. Therefore we investigated the composition of soil P in 18 diverse Australian soils using this technique. Soils were treated with a mixture of sodium hydroxide-ethylenediaminetetra-acetic acid (NaOH-EDTA), which resulted in the extraction of up to 89% of total soil P. It was possible to identify up to 15 well-resolved resonances and one broad signal in each ^{31}P NMR spectrum. The well-resolved resonances included those of orthophosphate, α– and β–glycerophosphate, phytate, adenosine-5´-monosphosphate (AMP) and *scyllo*-inositol hexakisphosphate, as well as five unassigned resonances in the monoester region and two unassigned resonances downfield (higher ppm values) of orthophosphate. The majority of ^{31}P NMR signal in the NaOH-EDTA extracts was assigned to orthophosphate, representing 37 to 90% of extractable P. Orthophosphate monoesters comprised the next largest pool of extractable P (7–55%). The most prominent resonances were due to phytate, which comprised up to 9% of total NaOH-EDTA extractable P and α- & β-glycerophosphate which comprised 1–5% of total NaOH-EDTA extractable P. A substantially greater portion of organic P (8–39% of total NaOH-EDTA extractable P) appeared as a broad peak in the monoester P region that we propose is due to P found in large, "humic" molecules. Orthophosphate diesters (1-5% of total NaOH-EDTA extractable P) and pyrophosphate (0-5% of total NaOH-EDTA extractable P) were minor components of P in all soil extracts. These results suggest that organic P in large humic molecules represents the second most abundant form of NaOH-EDTA extractable soil P (behind orthophosphate) and that small, P-containing compounds, such as phytate, represent a much smaller proportion of soil P than is commonly assumed.

Introduction

Australian soils are generally characterised by low phosphorus (P) concentrations by world standards, primarily because they are generally highly weathered and many are derived from sedimentary rocks, including sandstone, which are generally low in P (Beadle 1962; Holford 1997). Plant available P in soil is an important determinant of soil fertility (Norrish and Rosser 1983), consequently the application of P fertilisers is usually required to ensure adequate crop production in Australian soils (Cornish 2009a).

The long-term application of P fertilisers in southern Australia has exceeded P removal such that substantial quantities of P have accumulated in some soils (McLaughlin *et al.* 1991). This not only represents an inefficient and unsustainable use of P fertilisers but increases the potential for nutrient leaching to the off-farm environment and can cause eutrophication of waterways (Sharpley *et al.* 1994).

The ineffectiveness of P fertilisers is due to the fixation of P by sorption, complexation and precipitation reactions with soil constituents. Generally, only 10–30% of applied fertiliser P is recovered by crops in the year following application, with the remainder of P necessary for plant growth obtained from residual inorganic P and recalcitrant forms of organic P or 'unavailable' P forms (Bolland and Gilkes 1998; Bünemann *et al.* 2006). Recent research has examined ways to reduce the input of soluble P fertilisers and better use existing soil P (e.g., Conyers and Moody 2009; Cornish 2009b; Evans and Condon 2009; Guppy and McLaughlin 2009; Richardson *et al.* 2009). Potential strategies to increase plant available P include the selection or modification of plants with favourable root architecture (fine roots, lots of root hairs) and effective root exploration (Richardson *et al.* 2009), the mutualistic association of plant roots with mycorrhizal fungi (Smith *et al.* 2003) and the direct application of manufactured extracellular enzymes (Conyers and Moody 2009; Evans and Condon 2009). However, the success of any technique that aims to increase the availability of soil P and implement a more sustainable approach to P management in Australian soils will be reliant on an understanding of the P forms (including organic P forms) that exist within the soil.

Early work on the characterisation of soil organic P used extraction and chromatographic techniques. These techniques indicated *myo*-inositol hexakisphosphate (phytate), a P-rich compound derived predominantly from plant seeds (Reddy *et al.* 1989), to be a dominant form of soil organic P. Initial investigations of soil 'phytate fractions' in three Australian soils were undertaken by Cosgrove (1962; 1963) and Cosgrove and Tate (1963), where they were able to show the presence of *myo*-+ DL, *neo*- and *scyllo*-inositol hexaphosphates. Cosgrove (1963) provided approximate concentrations of some inositol phosphate components, with estimates of *myo*- +DL-inositol hexaphosphate equating to 10–13% of total organic phosphate and *scyllo*-inositol hexaphosphate ranging from 2–3%. Williams and Anderson (1968) undertook the first comprehensive characterisation of Australian soils and focused on identifying the inositol-phosphate concentrations of 47 surface soils from eastern Australia. The concentrations varied widely, ranging from 0.4–38% of total organic P, but they did not identify other forms of organic P. Irving and Cosgrove (1982) detected *chiro*-, *neo*-, *myo*- and *scyllo*-inositol pentakis- and hexakisphosphates in four soils from New South Wales. The two most abundant species being the *myo*- and *scyllo*- hexakisphosphates, with concentrations ranging from 12 to 167 mg P kg^{-1}. Similarly, Steward and Tate (1971) reported that inositol polyphosphates comprised 2–31% of soil organic P in four South Australian (Urrbrae) and four eastern Australian soils.

More recently, solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy has become the most widely-used technique for the speciation of soil P (McDowell *et al.* 2005; Turner *et al.* 2005; Murphy *et al.* 2009). However, the application of solution ^{31}P NMR spectroscopy to Australian soils and therefore a more detailed characterisation of P forms has been limited to a few recent studies (Smernik and Dougherty 2007; Bünemann *et al.* 2008b; Doolette *et al.* 2009). In one of these studies, it was

suggested that phytate concentrations can be overestimated in ^{31}P NMR analyses through mis-assignment of phytate peaks (Smernik and Dougherty 2007). This is consistent with the earlier findings of McLaughlin *et al.* (1988), who suggested that a major portion of organic P that accumulated in cropping soils was of microbial, rather than plant origin.

In this study we apply solution ^{31}P NMR spectroscopy to a diverse range of Australian soils with varying chemical and physical properties to provide a detailed characterisation and quantitative assessment of the forms of NaOH-EDTA extractable P.

Materials and methods

Soil chemical and physical analysis

Eighteen topsoils were collected from New South Wales, South Australia and Tasmania. Eight of these soils (Nowra, Casino, Collector, Coonabarabran, Wollongbar, Tocal and Somersby A & B) were included in a previous study (Doolette *et al.* 2009). Of the extra ten soils included in this study, two are from South Australia and the remaining eight comprise four pairs of soils with contrasting P fertiliser histories from Tasmania. All sites had been under their current management regime for at least 4 years. All soils were sampled, after the removal of the litter layer, to a depth of 10 cm and air-dried.

Total soil P, inorganic and organic P contents were determined using the acid extraction and ignition methods of Saunders and Williams (1955). Soil pH was determined on 1:5 soil:water extracts. Organic C was determined using the method of Walkley (1947). Soils have been classified according to the Australian Soil Classification (ASC) (Isbell 1996).

NaOH-EDTA extraction

Samples were ground to pass through a 2-mm sieve prior to extraction. Soils were extracted in triplicate using methods based on those of Cade-Menun and Preston (1996). For all but three soils, 2.0 g of soil was shaken with 40 mL of 0.25 M NaOH and 0.05 M Na_2EDTA for 16 h. For three low-P soils (Somersby A & B and Coonabarabran), 6.0 g rather than 2.0 g of soil was used in the extraction to provide acceptable signal-to-noise ratios in the NMR spectra. All extracts were centrifuged ($1400 \times g$) for 10 min and filtered using Whatman no. 42 filter paper. A 15 mL aliquot was immediately frozen and freeze-dried for NMR analysis. Triplicate sub-samples of the supernatant were also taken to determine the total P concentrations using nitric acid digestion and subsequent analysis by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The determination of inorganic P in the NaOH-EDTA soil extracts was based on colorimetry using the molybdenum blue method of Murphy and Riley (1962). Inorganic P in the NaOH-EDTA extracts was also quantified using deconvolution of ^{31}P NMR spectra (detailed below).

NMR analysis of NaOH-EDTA extracts

Triplicate freeze-dried NaOH-EDTA extracts for each soil were combined for NMR analysis. A 500 mg sub-sample of each composite extract was ground, re-dissolved in 5 mL of deionised water and centrifuged (1400 × *g*) for 20 min. The supernatant solution (3.5 mL) and deuterium oxide (D_2O, 0.3 mL) were placed in a 10 mm diameter NMR tube. Solution ^{31}P NMR spectra were acquired at 24ºC on a Varian INOVA400 NMR spectrometer (Varian, Palo Alto, CA) at a ^{31}P frequency of 161.9 MHz. Recovery delays ranged from 10 s to 30 s and were set to at least five times the T_1 value of the orthophosphate resonance determined in preliminary inversion-recovery experiments for each extract (data not presented). We used a 90º pulse of 32 to 45 µs, an acquisition time of 1.0 s and broadband ^{1}H decoupling. Between 3224 and 49000 scans were acquired for each sample, depending on the P concentration of the freeze-dried extract. Chemical shifts were referenced to β-glycerophosphate at 4.63 ppm according to Doolette *et al.* (2009). The spectra presented have a line broadening of 2 Hz.

Quantification of P species from ^{31}P NMR spectra

The relative concentrations of P species in the NaOH-EDTA extracts were determined from ^{31}P NMR spectra using a combination of integration and deconvolution. Orthophosphate diester (2.0 to -1.0 ppm) and pyrophosphate (-4.5 to -5.5 ppm) concentrations were determined using integration alone. Integration was used to determine the combined concentration of inorganic orthophosphate and orthophosphate monoeseter P (6.2–2.6 ppm). The relative concentrations of P species giving rise to the numerous individual peaks in this region of the spectrum were quantified by spectral deconvolution, using a method similar to that of Bünemann *et al.* (2008b). Each spectrum was fitted with up to 15 sharp peaks as well as a broad signal that spanned most of the monoester region. The sharp peaks included those of orthophosphate, α– and β–glycerophosphate, phytate, adenosine-5´-monosphosphate (AMP), *scyllo*-inositol hexakisphosphate, five unknown peaks in the monoester region and two unknown peaks downfield (higher ppm values) of orthophosphate. Each peak was defined by three parameters: the chemical shift (frequency), intensity and line width. The degrees of freedom for the fit were limited by fixing the line widths of the sharp peaks at 10 Hz (0.06 ppm), and the broad resonance at 200 Hz (1.24 ppm). The line width of the well-defined inorganic orthophosphate resonance was allowed to vary. The absolute concentration of each P species (including those determined using integration alone and those determined using integration and deconvolution combined) was calculated by multiplying its relative contribution to total NMR signal by the total NaOH-EDTA extractable P concentration determined by ICP-AES.

A minor correction was used in the determination of phytate and orthophosphate concentrations due to the overlap of the phytate C-2 peak with the much larger orthophosphate peak. Total phytate was

calculated as 6/5 times the total concentration of the three observable resonances and 1/5 of this value was subtracted from the total orthophosphate concentration (Doolette *et al.* 2009).

Spiking experiments

Spiking experiments were carried out on two soil extracts to confirm the accuracy of the spectral deconvolution technique used for quantifying P species. This enabled an independent measure of the concentration of one of the species (β–glycerophosphate) using the method of Smernik and Dougherty (2007). A 0.1 mL aqueous solution of β–glycerophosphate was spiked into the Parkham +P (0.74 g L^{-1}) and Elizabeth Town control (1.4 g L^{-1}) redissolved NaOH-EDTA soil extracts (3.5 mL and 0.3 mL D_2O). The ^{31}P NMR spectrum of the unspiked NaOH-EDTA extract was subtracted from the β–glycerophosphate-spiked NaOH–EDTA spectrum, resulting in a spectrum of the β–glycerophosphate spike alone. Increasing proportions of the β–glycerophosphate spectrum were then subtracted from the unspiked NaOH-EDTA spectrum until the β–glycerophosphate resonance was null. The lower limit of β–glycerophosphate was defined by the maximum proportion of the β–glycerophosphate spectrum which resulted in the appearance of a positive β–glycerophosphate resonance in the unspiked NaOH-EDTA spectrum. The upper limit was defined by the minimum proportion of the β–glycerophosphate spectrum which resulted in the appearance of a negative β–glycerophosphate resonance in the unspiked NaOH-EDTA spectrum.

Results and discussion

The eighteen Australian soils selected for this study span a wide range of soil management, climate, chemical and physical properties and soil orders (Table 1). Mean annual temperature ranged from 15 to 23ºC and mean annual precipitation from 387 to 1318 mm (www.bom.gov.au). Soil pH (H_2O) varied from moderately acidic (4.5) to moderately alkaline (8.3). Soil organic carbon (OC) ranged from 10 to 95 g kg^{-1}. Soils were used for a range of agricultural practices including pasture production (dairy and sheep), horticulture (conventional and organic), cropping and a cropping / grazing rotation. Four pairs of soils differ in P fertilisation history, with each pair having one unfertilised soil and one with a known history of inorganic P fertilisation (Togari, Parkham, Elliot Research Station and Elizabeth Town).

Table 1. Selected physical, climatic and chemical properties of the 18 Australian soils used in this study.

Soil	Land use/treatment	MAT[b] (°C)	MAP[c] (mm)	pH[d]	OC (g kg^{-1})[e]	Texture	Soil Classification
Nowra [a]	Dairy pasture + effluent	16	1110	8.1	38	Clay loam	Chromosol
Casino [a]	Dairy pasture	20	1096	6.5	42	Silty clay	Vertosol
Collector [a]	Sheep pasture	14	644	5.0	27	Loam	Chromosol
Coonabarabran [a]	Horticulture	16	748	4.5	16	Loamy sand	Podosol
Wollongbar [a]	Dairy pasture	19	1793	5.2	38	Clay	Ferrosol
Tocal [a]	Dairy pasture + poultry litter	18	922	6.1	43	Loamy sand	Chromosol
Somersby A [a]	Organic vegetable	17	1318	6.7	11	Sandy loam	Tenosol
Somersby B [a]	Conventional vegetable	17	1318	5.9	10	Loamy sand	Tenosol
Kadina	Cropping	23	387	8.3	19	Loamy sand	Calcarosol
Crystal Brook	Cropping/grazing rotation	23	471	7.9	17	Sandy loam	Calcarosol
Togari control	Dairy pasture	15	1243	5.5	87	Loamy Sand	Hydrosol
Togari + P	Dairy pasture	15	1243	5.3	87	Loamy Sand	Hydrosol
Parkham control	Dairy pasture	16	947	5.7	28	Clay Loam/Loam	Vertosol
Parkham + P	Dairy pasture	16	947	5.9	28	Clay Loam/Loam	Vertosol
Elliot control	Dairy pasture	16	1188	5.2	95	Silty Loam	Ferrosol
Elliot + P	Dairy pasture	16	1188	5.3	95	Silty Loam	Ferrosol
Elizabeth Town control	Dairy pasture	16	947	5.4	60	Silty Clay Loam	Ferrosol
Elizabeth Town + P	Dairy pasture	16	947	5.3	60	Silty Clay Loam	Ferrosol

[a] Data reproduced from Doolette *et al*. (2009)
[b] MAT; Mean annual temperature
[c] MAP; Mean annual precipitation
[d] pH in water (1:5 soil/water ratio)
[e] OC; Organic Carbon

Table 2 shows the variation in the total P and organic P concentrations of the whole soils. Total soil P concentration across all soils ranged from 239 mg kg^{-1} to 15111 mg kg^{-1}, of which inorganic P comprised 25–90% (84 to 12559 mg kg^{-1}) and organic P 10–75% (72–2552 mg kg^{-1}). Comparisons of the total inorganic and organic P fractions of each soil are shown in Fig. 1*a*. In all cases, the P treated soils (+P) had a higher total phosphorus concentration of which the majority of the extra P was inorganic P, except Togari, where +P treatment was higher in organic P. This is consistent with the results of McLaughlin *et al.* (1988) that showed, using isotope tracer experiments, fertiliser was not quickly incorporated into organic P forms.

Table 2. Phosphorus characteristics of the whole soil and NaOH-EDTA extractable P fractions.

Soil	Whole soil P		NaOH-EDTA extractable P
	Total	Organic	
	(mg kg^{-1})		
Nowra [a]	15111	2552	6222 (41)[b]
Casino [a]	1576	423	1184 (75)
Collector [a]	257	172	166 (64)
Coonabarabran [a]	239	144	149 (63)
Wollongbar [a]	1113	650	882 (79)
Tocal [a]	1214	564	887 (73)
Somersby A [a]	743	90	542 (73)
Somersby B [a]	702	72	627 (89)
Kadina	844	200	178 (21)
Crystal Brook	571	214	335 (59)
Togari control	465	220	340 (73)
Togari +P	665	490	336 (51)
Parkham control	627	470	355 (60)
Parkham +P	1386	465	950 (69)
Elliot control	1106	816	677 (61)
Elliot +P	2526	776	1912 (76)
Elizabeth Town control	1910	1094	1066 (56)
Elizabeth Town +P	2293	963	1443 (63)

[a] Data reproduced from Doolette *et al*. (2009)
[b] Figures in parentheses are percentages of total NaOH-EDTA extractable P

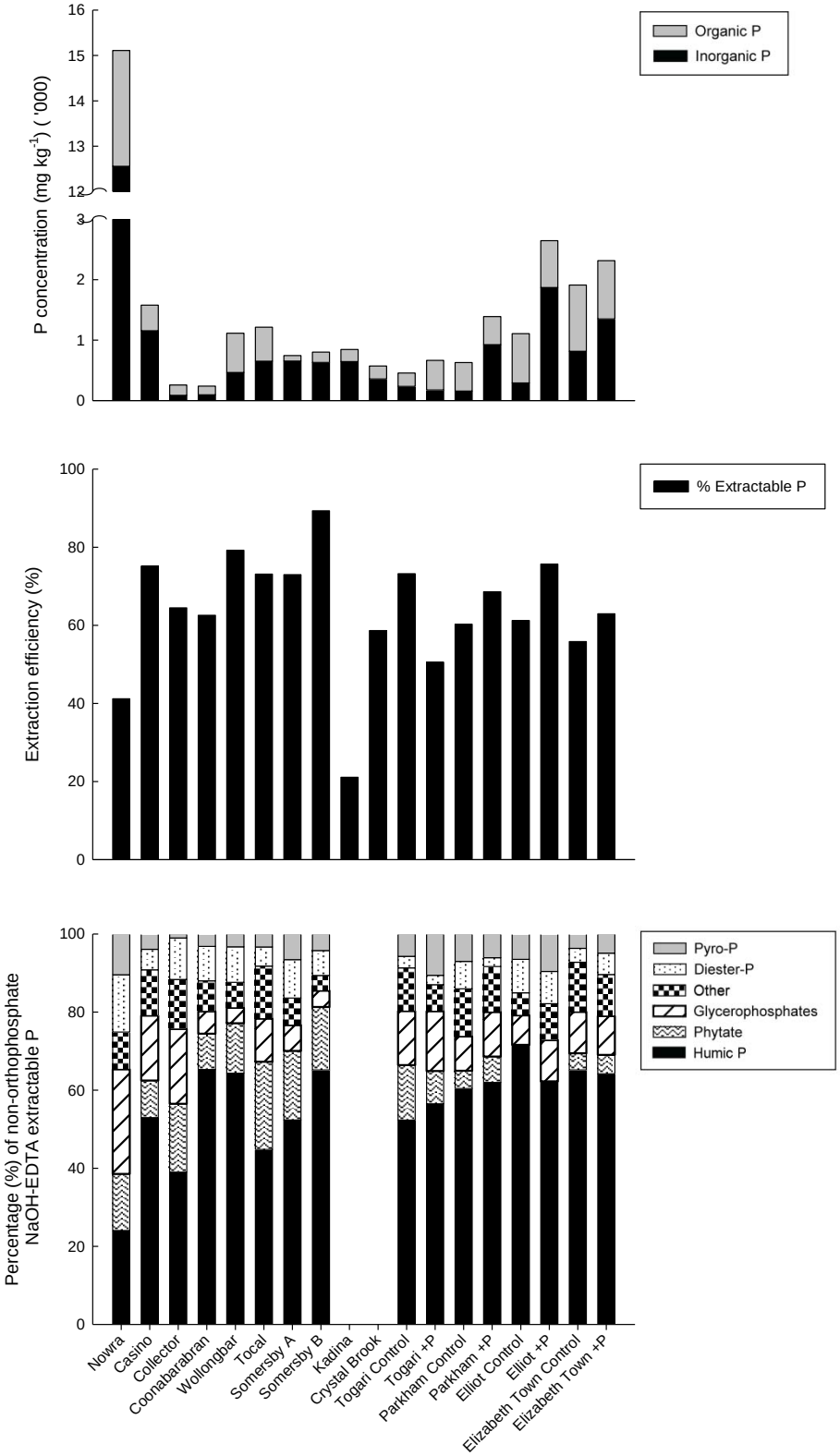

Figure 1. Comparison of whole soil total, inorganic and organic P (A), total NaOH-EDTA extractable P expressed as a percentage of whole soil total P (B) and the percentage composition of the 6 dominant organic P groups in each NaOH-EDTA extract analysed by solution ^{31}P NMR spectroscopy. The groups include; pyrophosphate, diester-P, "other P" (unknown monoesters with the following chemical shift ranges (ppm); 6.53-6.58, 6.34-6.36, 5.23-5.24; 5.10-5.11; 4.06-4.17; 3.04; 2.89), adenosine -5´-monophosphate and *scyllo*-inositol hexakisphosphate), glycerophosphates (α- and β- forms), phytate and P assigned to the broad signal (humic P) (C).

Extractability of inorganic and organic P

Total NaOH-EDTA extractable P ranged from 149 to 6222 mg kg^{-1} (Table 2) representing an extraction efficiency of 21 to 89%, with an average of 64% (Fig. 1*b*). Comparable extraction efficiencies have been reported for other ^{31}P NMR studies. Dou *et al.* (2009) extracted 67–97% of total P from 8–10 year manured soils, whilst Turner *et al.* (2003a) has reported considerably lower extraction efficiencies (12–45%) for a range of cropped soils. Plots of whole soil and NaOH-EDTA extractable inorganic and organic P are shown in Figs. 2*a-b*. These exclude data for the Nowra sample (Pt; 12558 mg kg^{-1}, Pt_{ex}; 4893 mg kg^{-1}) as they were several times higher than for any of the other soils presented in Fig. 2*a*.

A 1:1 line is shown in Figs. 2*a-b*, which indicates the potential maximum (100%) extraction efficiency. It is clear that the average extraction efficiency of inorganic P was greater (82%) than for organic P (47%). This implies that, on average, around half of the organic P in these soils is unextractable and therefore cannot be identified by solution ^{31}P NMR analysis. However, it is likely that hydrolysis of organic P in the alkaline extract results in an overestimation of the extraction efficiency of inorganic P and consequently an underestimation of the extraction efficiency of organic P. Indeed, for four soils (Tocal, Wollongbar, Coonabarabran and Collector) the apparent extraction efficiency of inorganic P was greater than 100%. It should also be noted that the ashing technique that was used to determine the organic P content of the whole soils can be unreliable (Condron *et al.* 1990b) and this represents a second possible source of bias in the reported inorganic and organic P extraction efficiencies.

The Kadina soil had a total P extraction efficiency of only 21% (Fig. 1*b*), with the extraction efficiency of inorganic P particularly low (16%) compared to the other soils. The low extractability may be due to the calcareous nature of the Kadina soil, as high soil pH and the presence of Ca minerals has potential to complex P rendering it hard to extract from soil (Cole *et al.* 1953; Celi *et al.* 2000). Across all soils there was no noticeable relationship between P extractability and soil pH. The only other sample with a high soil pH was Nowra and it too had a comparatively low P extraction efficiency (41%). Differences in extraction efficiency are also likely to have affected inorganic and organic P concentrations of the P treated and untreated paired soil samples. The extraction efficiency of organic P tended to be greater in the treated soils compared to the untreated soils.

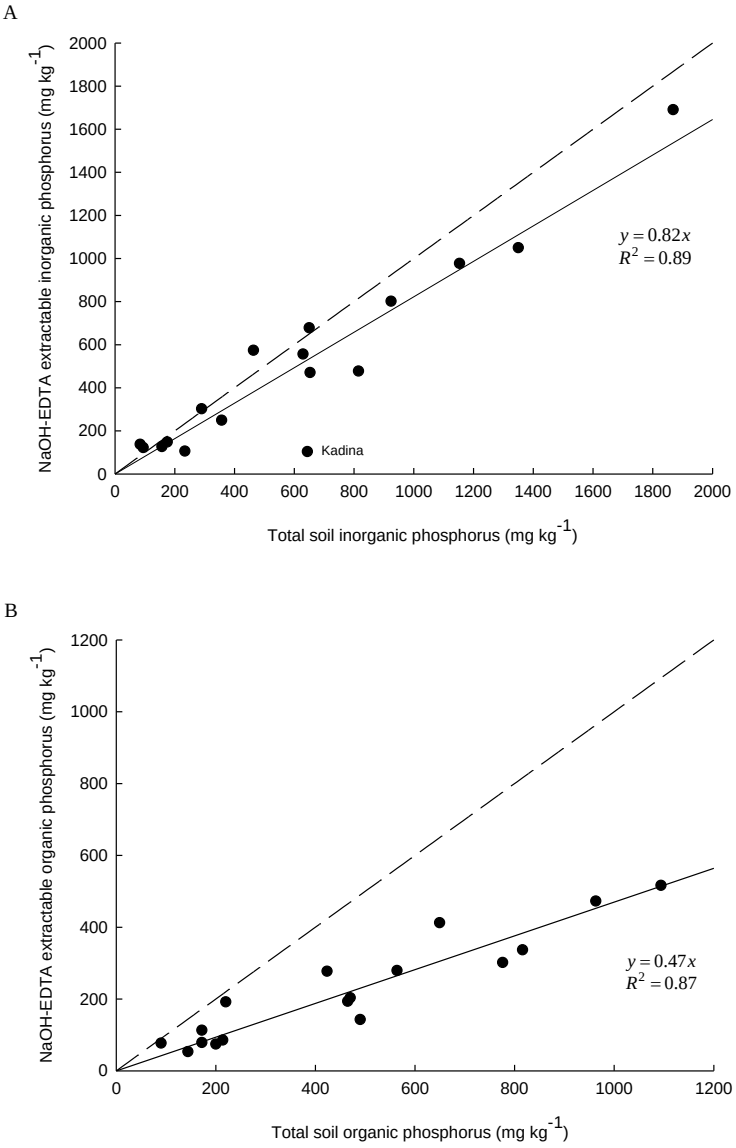

Figure 2. The relationship between total NaOH-EDTA extractable and total soil inorganic (A) and organic (B) P. Regression analysis shows the average extraction efficiencies of both inorganic and organic P. The broken line is the 1:1 line. All samples (*n*=17), excluding Nowra, were included in the regression analysis of inorganic and organic P. Nowra was excluded from the regression analysis because it had a much higher P content than the remaining soils and its inclusion would skew the data.

Assessment of the reliability of spectral deconvolution

Overlap of resonances in the 2–7 ppm region of ^{31}P NMR spectra of soil extracts, the region that encompasses the orthophosphate peak and numerous monoester peaks, hampers quantitative assessment of these P types. This problem can be overcome by using spectral deconvolution, which involves a numerical least squares fit of the spectrum as the sum of multiple peaks of standard shape. Spectral deconvolution has been used in several ^{31}P NMR soil studies (Turner *et al.* 2003d; McDowell *et al.* 2005; McDowell and Stewart 2005). In this study we use a modified version (see Materials and Methods for description) that has only been used once before (Bünemann *et al.* 2008b). The main modification is the inclusion of a broad signal (3.95–5.45 ppm) in the fit. In a study that compared

extracts of "real" soils and "model" soils (mixtures of pure sand and clay that were incubated with pure organic substrates e.g. cellulose, starch and glucose), Bünemann *et al.* (2008b) observed that the extracts of the model soils contained only sharp resonances. These resonances could be assigned to specific, simple organic P molecules. In contrast, the spectra of the real soils contained a broad signal in addition to the sharp resonances. This modified technique is yet to be applied to a variety of soils with considerably different inorganic and organic P concentrations.

The reliability of our methods for quantifying P species was tested by (i) comparing orthophosphate concentrations determined using NMR with those determined colorimetrically, and (ii) by using the spiking method of Smernik and Dougherty (2007) to provide an independent measure of the concentration of the monoester compound β-glycerophosphate in two soil extracts.

Fig. 3 shows the close relationship between NaOH-EDTA extractable orthophosphate concentrations measured using spectral deconvolution and those measured using molybdate colorimetry. Both methods gave very similar values (i.e. the line of regression is close to the 1:1 line in Fig. 3), but concentrations determined by colorimetry were generally slightly higher (on average 8%) than those determined using spectral deconvolution.

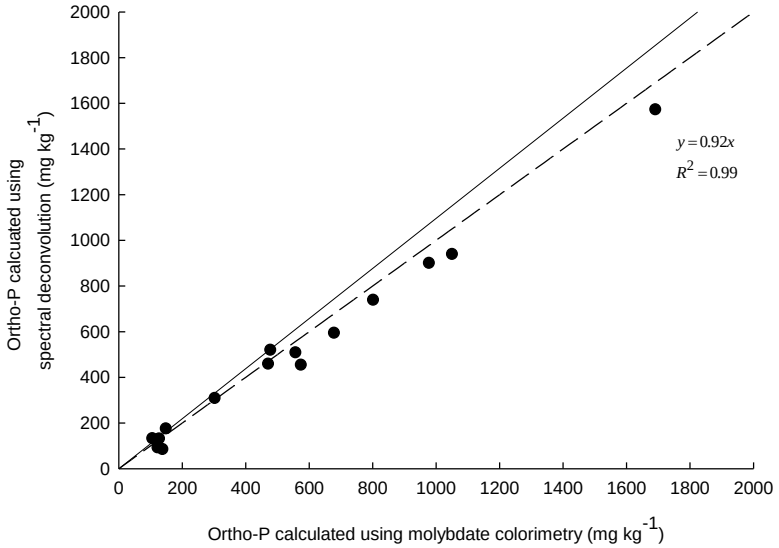

Figure 3. The relationship between inorganic orthophosphate measured by spectral deconvolution and molybdate colorimetry. The broken line is the 1:1 line. Nowra was excluded from the regression analysis as its values were markedly different from those of the other soils and did not satisfactorily fit with the remaining data, thus appearing as an outlier. Kadina and Crystal Brook samples are not included because of their poor signal to noise ratios, which was the likely result of a low extraction efficiency and presence of paramagnetic species.

Spiking experiments were carried out on Elizabeth Town control and Parkham +P extracts. The calculated β-glycerophosphate concentration in the Elizabeth Town control sample was 22 ± 1.9 mg P kg^{-1} using the spiking method and 21 mg P kg^{-1} using spectral deconvolution. Spiking the Parkham +P

NaOH-EDTA soil extract revealed a β-glycerophosphate concentration of 5 ± 1.8 mg P kg^{-1} which was slightly less than that determined using deconvolution (10 mg P kg^{-1}). This suggests that deconvolution may overestimate the concentration of species with low abundances, although the practical implication of such errors is relatively small.

Quantitative analysis and interpretation of ^{31}P NMR spectra of NaOH-EDTA soil extracts

Spectra of eight of the soil extracts are not presented here as they have been reported elsewhere (Doolette *et al.* 2009) but have not been quantitatively analysed. For two of the soils (Kadina and Crystal Brook), we were unable to obtain an adequate ^{31}P NMR spectrum. As discussed, the extraction efficiency was very low for the Kadina soil and this resulted in a low concentration of P in the NaOH-EDTA extract. This was also the case, but to a lesser extent, for the Crystal Brook soil, which suffered from the combination of a relatively low P concentration and relatively low extraction efficiency. However, it should be noted that we were able to obtain adequate NMR spectra on other soil extracts with similar or even lower P contents (e.g. Togari and Collector). This shows that the difficulties encountered in the analysis of these two soil extracts is not entirely explained by their low P content. The Kadina and Crystal Brook soils were also the only alkaline soils analysed other than the Nowra soil which had an extremely high P content (Table 1). The potential link between difficulties in NMR analysis and high soil pH requires further investigation. It is likely that the presence of paramagnetic species (iron and manganese), which have been shown to influence line broadening (Cade-Menun *et al.* 2002), could have contributed to the poor quality of these spectra.

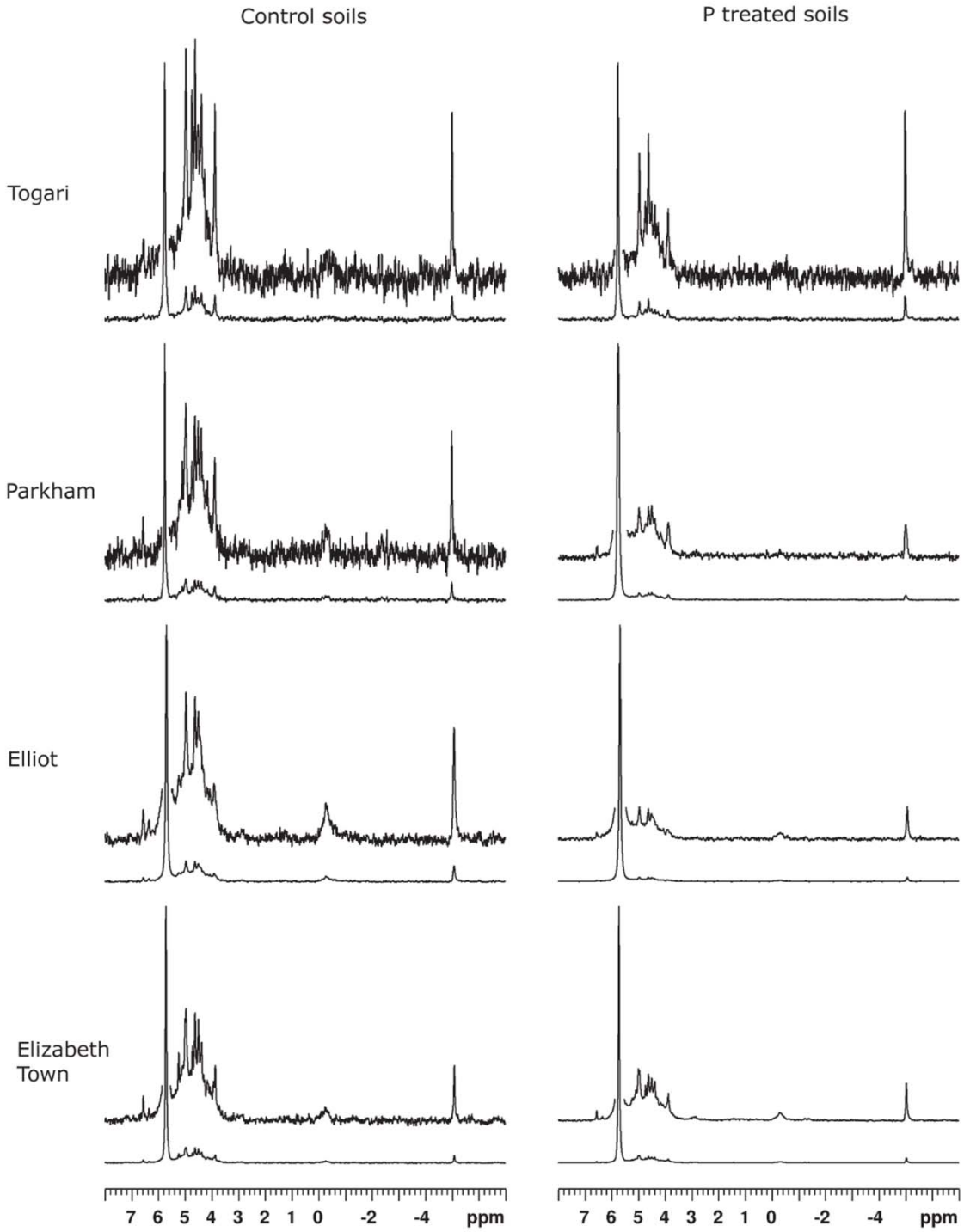

Figure 4. Solution ^{31}P NMR spectra of NaOH-EDTA soil extracts from the untreated and P treated Australian soils from Tasmania. Lower traces are vertically scaled to the maximum intenisty of the orthophosphate peak (5.75 ppm). The vertical scale has been increased by a factor of 7 in the upper traces of each pair.

The ^{31}P NMR spectra of the other NaOH-EDTA soil extracts, which consist of four pairs of control and P treated soils, contain signals in four diagnostic chemical shift regions, orthophosphate (6.5–5.3 ppm), orthophosphate monesters (5.3–2.6 ppm), orthophosphate diesters (2 to -1 ppm) and pyrophosphate (-4.5 to -5.5 ppm) (Fig. 4). An expansion of the orthophosphate and orthophosphate monoester regions is shown in Fig. 5. Equivalent spectra for the other soils can be found in Doolette *et al.* (2009). The two regions that comprised the majority of signal across all soils were orthophosphate and orthophosphate monoester, together equating to 94–98% of total NaOH-EDTA extractable P. Orthophosphate diesters and pyrophosphate were minor components, comprising only 2–6% of total NaOH-EDTA extractable P. When comparing the control and treated soils, the only noticeable differences were in the orthophosphate region. The differences between the control and treated soils in the orthophosphate monoester, orthophosphate diester and pyrophosphate chemical shift regions were relatively small. However, it might be expected that with continued P fertilizer application soil organic P could increase (Condron *et al.* 1985; McLaughlin *et al.* 1988).

Quantitative distributions of P forms determined for all soil extracts are presented in Table 3. The percentage distribution of each P species excluding orthophosphate in relation to total non-orthophosphate P is presented in Fig. 1*c*.

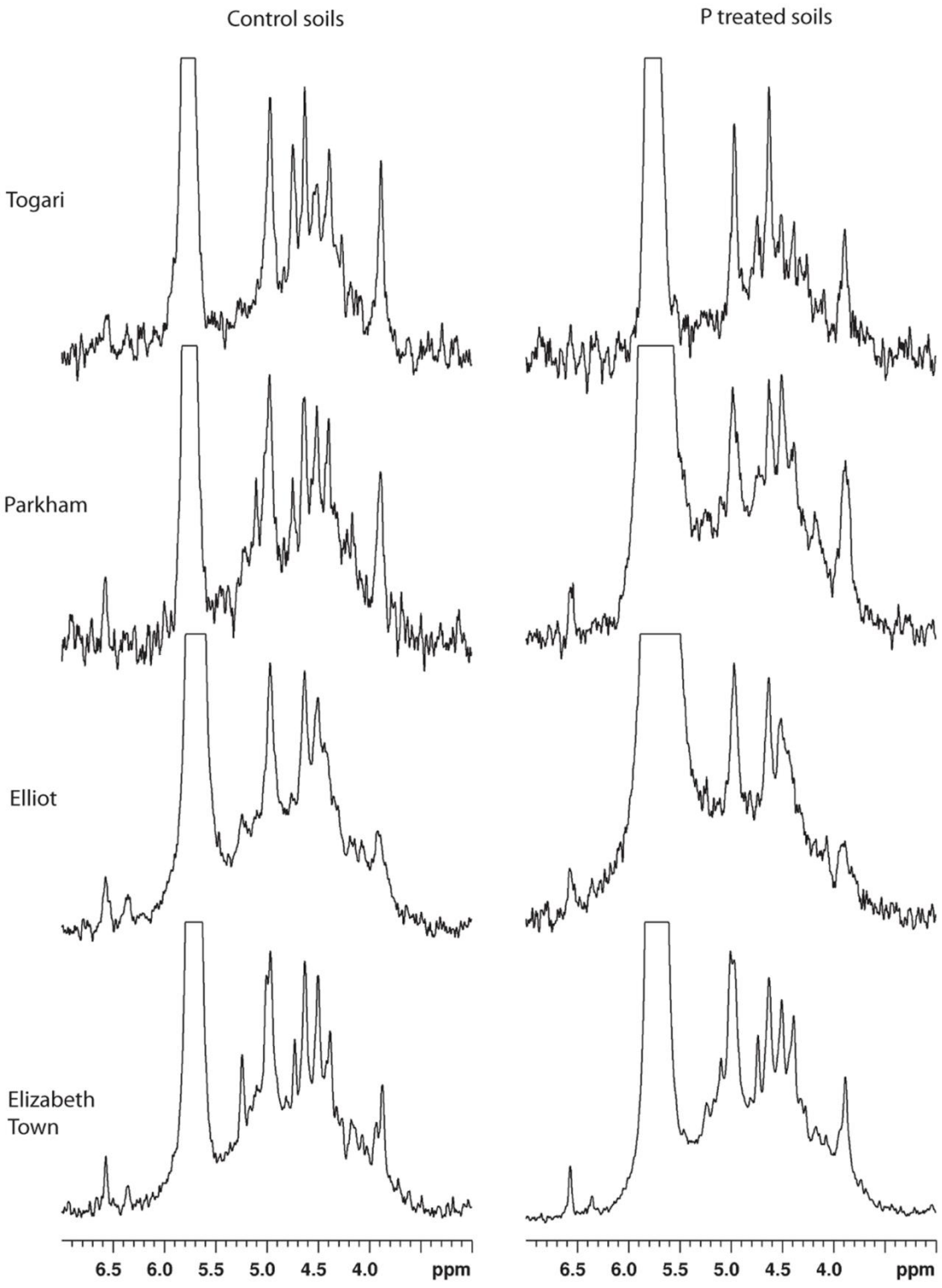

Figure 5. Solution ^{31}P NMR spectra of the NaOH-EDTA soil extracts from the untreated and P treated Australian soils from Tasmania. Each spectrum has been individually vertically expanded to show in detail the orthophosphate (6.5-5.3 ppm) and orthophosphate monoester (5.3-2.6 ppm) regions.

Table 3. Concentration and percentage (in parentheses) of NaOH-EDTA extractable total P of P forms as detected by ^{31}P NMR and quantified using spectral deconvolution.

Soil Location	Unknown P	Ortho-P[a]	α-glycerophosphate	Humic P	Phytate	β-glycerophosphate	AMP[b]	*scyllo*-Inositol hexakisphosphate	Unknown monoesters[c]	Diester	Pyro-P[d]
	(6.53-6.58)[e] (6.34-6.36)	(5.69-5.78)	(4.95-4.98)	(3.95-5.45)	(4.17-4.28) (4.35-4.40) (4.71-4.82)	(4.59-4.64)	(4.47-4.52)	(3.85-3.91)	A-E	(2 to -1)	(-4.5 to -5.5)
	(mg kg^{-1})										
Nowra	0 (0)	5622 (90)	80 (1)	143 (2)	86 (1)	80 (1)	41 (<1)	5 (<1)	11 (<1)	87 (1)	62 (1)
Casino	0 (0)	904 (76)	22 (2)	143 (12)	26 (2)	23 (2)	15 (1)	11 (1)	6 (<1)	14 (1)	11 (1)
Collector	0 (0)	86 (52)	8 (5)	31 (19)	14 (8)	8 (5)	3 (2)	5 (3)	2 (1)	8 (1)	1 (1)
Coonabarabran	1 (<1)	94 (63)	3 (2)	37 (24)	5 (3)	1 (<1)	1 (<1)	2 (1)	0.4 (<1)	5 (1)	2 (1)
Wollongbar	0 (0)	455 (52)	6 (<1)	274 (31)	55 (6)	10 (1)	7 (<1)	14 (2)	6 (<1)	39 (2)	14 (2)
Tocal	2 (<1)	595 (67)	17 (2)	131 (15)	65 (7)	15 (2)	11 (1)	20 (2)	6 (<1)	14 (1)	10 (1)
Somersby A	0 (0)	460 (85)	2 (<1)	43 (8)	15 (3)	3 (<1)	2 (<1)	4 (<1)	0 (0)	8 (1)	5 (1)
Somersby B	0 (0)	509 (81)	1 (<1)	77 (12)	19 (3)	4 (<1)	0 (0)	5 (<1)	0 (0)	8 (1)	5 (1)
Kadina	N.D.[f]	104 (58)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Crystal Brook	N.D.	249 (74)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Togari control	3 (<1)	134 (39)	16 (5)	108 (32)	29 (9)	12 (4)	8 (2)	12 (4)	0 (0)	6 (3)	12 (3)
Togari +P	0 (0)	176 (52)	13 (4)	90 (27)	13 (4)	12 (4)	4 (1)	7 (2)	0 (0)	4 (5)	17 (5)
Parkham control	4 (1)	132 (37)	12 (4)	135 (38)	10 (3)	7 (2)	7 (2)	8 (2)	9 (3)	16 (4)	16 (4)
Parkham +P	4 (<1)	739 (78)	14 (2)	130 (14)	14 (1)	10 (1)	10 (1)	9 (1)	2 (<1)	5 (1)	13 (1)
Elliot control	6 (<1)	310 (46)	17 (3)	263 (39)	0 (0)	10 (2)	10 (2)	5 (<1)	0 (0)	31 (4)	24 (4)
Elliot +P	4 (<1)	1574 (82)	18 (1)	210 (11)	0 (0)	18 (1)	19 (1)	9 (<1)	0 (0)	28 (2)	33 (2)
Elizabeth Town control	9 (1)	520 (49)	37 (4)	355 (33)	23 (2)	21 (2)	19 (2)	14 (1)	27 (3)	19 (2)	20 (2)
Elizabeth Town +P	6 (<1)	939 (65)	34 (2)	323 (22)	24 (2)	17 (1)	13 (1)	13 (1)	22 (2)	28 (2)	25 (2)

[a] Ortho-P, Orthophosphate
[b] AMP, adenosine-5´-monophosphate;
[c] Unknown monoesters (A-E) are the sum of five individual resonances with the following chemical shift ranges (ppm); (A) 5.23-5.24; (B) 5.10-5.11; (C) 4.06-4.17; (D) 3.04; (E) 2.89,
[d] Pyro-P, pyrophosphate
[e] Chemical shift ranges (ppm)
[f] N.D., not detected.

Orthophosphate (5.69–5.78 ppm) produced the most intense resonance in each spectrum and is the most abundant P species detected in every soil extract. Orthophosphate concentrations ranged from 104 to 5622 mg kg $^{-1}$, equivalent to 37–90% of total NaOH-EDTA extractable P.

Orthophosphate monoesters represented the next largest P class and accounted for 7–55% of total NaOH-EDTA extractable P. The majority of the orthophosphate monoester was assigned to a broad signal, centred at 3.95–5.45 ppm, which is present in all spectra but is more easily discerned in the spectra that contained less intense sharp resonances e.g., Parkham, Elliot and Elizabeth Town. The broad monoester signal represented 31 to 355 mg P kg^{-1} (equivalent to 2–39% of total extractable P or 32–86% of total orthophosphate monoesters). The absolute P concentration and proportion of organic or total extractable P of the broad resonance did not appear to be associated with any of the climatic, chemical or physical properties measured, but more soils need to be examined to further examine this possibility.

Most previous attempts at quantifying monoester P species using deconvolution analysis of ^{31}P NMR spectra of soil extracts have not included a broad peak in the fit. Consequently, signal from this broad signal is assigned to species that give rise to the sharper peaks in the spectrum. More specifically, we have previously tested this method of deconvolution and shown that by not accounting for the broad peak phytate concentrations were overestimated by 54% (Doolette *et al.* 2010). Dougherty *et al.* (2007) suggested that this broad signal is due to large, complex, humic molecules, as opposed to smaller, specific monoester-P compounds that produce sharp resonances. This explanation is supported by Bünemann *et al.* (2008b), who found this broad signal to be absent in model soils that have had insufficient time for extensive humification to have occurred. Earlier evidence for the dominance of humic P can be found in Anderson and Hance (1963), who found evidence of phytate and other organic phosphate molecules bound in a complex or compound that contained appreciable amounts of carbohydrates and proteins. Omotoso and Wild (1970) also considered that phytate existed in an organic complex combined with other organic P compounds.

The concentrations of humic organic P reported here are consistent with those determined by different methods in previous studies of volcanic and alpine soils. The humic fractionation of volcanic soils showed P associated with humic and fulvic acids accounted for 32–75% and 51–68% of organic P, respectively (Borie *et al.* 1989; Escudey *et al.* 2001; Borie and Rubio 2003) and 52–90% of alkali-extractable organic P in alpine soils was associated with humic acids with phosphate monoesters the dominant P species (Makarov *et al.* 1997). Furthermore, using ^{31}P NMR, He *et al.* (2006) examined the spectral and chemical characteristics of humic substances and also found phosphate monoesters to be the dominant P species. More importantly, they were unable to identify individual monoester-P compounds as the spectra had no sharp resonances but instead a broad signal in the monoester region.

We conclude that failing to include the broad signal in the deconvolution procedure would result in the overestimation of the concentration of species that give rise to the sharp monoester resonances.

The remainder of orthophosphate monoester P was assigned to the ^{31}P NMR signal contained within the sharp resonances. Phytate (4.17–4.28, 4.35–4.40, 4.71–4.82 ppm), α– glycerophosphate (4.95–4.98 ppm) and β–glycerophosphate (4.59–4.64 ppm) were the most prominent sharp resonances. Phytate was present in all soil extracts except for the Elliot control and +P samples. Concentrations in the other soils ranged from 5 to 86 mg P kg^{-1} (equivalent to up to 9% of total NaOH-EDTA extractable P). Phytate concentrations averaged just 3% of total NaOH-EDTA extractable P and 13% of NaOH-EDTA extractable organic P. α– and β–glycerophosphate were present in all ^{31}P NMR spectra but were only a very minor component in some extracts. β–glycerophosphate comprised less than 1% of total NaOH-EDTA extractable P in the Wollongbar extract, α–glycerophosphate comprised less than 1% in the Coonabarabran extracts and combined, α– and β–glycerophosphate comprised less than 1% in both the Somersby A and B samples. In the remaining spectra, α– and β–glycerophosphate resonances were more prominent and α–glycerophosphate concentrations ranged from 3–80 mg P kg^{-1} (1–5% total NaOH-EDTA extracable P) and β–glycerophosphate ranged from 7–80 mg P kg^{-1} (1-5% total NaOH-EDTA extractable P). In total, α– and β–glycerophosphate comprised up to a maximum of 10% of extractable P.

Phytate in soils primarily originates from the decay of crop seeds and fruits (Lott *et al.* 2000). Relatively high phytate concentrations in soils can result from the addition of animal manures, such as poultry and swine manure, as phytate passes though the digestive tracts of these animals as they lack the ability to break it down (Selinger *et al.* 1996). This may explain the relatively high concentration of phytate in the Tocal soil extract, as it had received poultry manure.

The remainder of the soils are predominantly dairy pastures where the strongest monoester resonances have been assigned to α– and β–glycerophosphate, which are most likely products of the alkaline hydrolysis of phospholipids (Doolette *et al.* 2009) rather than being actual soil constituents themselves. They are likely to be derived mainly from microbial biomass, as Bünemann *et al.* (2008a) showed that α– and β–glycerophosphate were the major forms of monoester-P identified in NaOH-EDTA extracts of bacterial and fungal cultures. Microbial biomass is often quite high in dairying soils due to high organic carbon and moisture regimes (Burkitt *et al.* 2007).

Other orthophosphate monoester compounds present in the soil extracts were detected in much smaller quantities. *scyllo*-Inositol hexakisphosphate (3.85–3.91 ppm), a common stereoisomer of phytate, was present in all soil extracts at concentrations ranging from 2 to 20 mg P kg^{-1} or <1–4% of total extractable P. Despite the widespread occurrence of *scyllo*-inositol hexakisphosphate in soils

(McKercher and Anderson 1968; Turner *et al.* 2005; Turner 2007; Murphy *et al.* 2009) its origins are still unknown and there are conflicting reports regarding the bioavailability or susceptibility of *scyllo*-inositol hexakisphosphate to hydrolytic attack by phytase (Cosgrove 1966; 1970; Turner *et al.* 2005).

Trace amounts of AMP (≤ 2% extractable P; 4.47–4.52 ppm) were present in all soil extracts except for Somersby B. This resonance is also likely to include other ribonucleotides (RNA monomers) and deoxyribonucleotides (DNA monomers) as they resonate at very similar chemical shifts (Turner *et al.* 2003b; Bünemann *et al.* 2008a; Doolette *et al.* 2009). However, due to the instability of RNA it is not possible to conclude whether the nucleotides are present in the soil or are products of alkaline hydrolysis during the sample extraction/preparation stages.

Five other well-resolved orthophosphate monoester P resonances (5.23-5.24, 5.10-5.11, 4.06-4.17, 3.04 ppm) were identified and are labelled as unknown compounds *A*-*E* in Table 3. In total these unknown compounds accounted for no more than 3% of total extractable P, but in most cases less than 1%. Two further unidentified resonances between 6.34 and 6.58 ppm (comprising less than 1% of total extractable P) have previously been assigned as aromatic orthophosphate diesters in the NaOH extracts of Scottish mineral soils by Bedrock *et al.* (1994). Aromatic orthophosphate diesters are generally thought to be associated with cold and wet environments. They have been identified in grassland soils from Russia and New Zealand (Amelung *et al.* 2001; McDowell 2003) and pasture soils from New Zealand, England and Wales (Turner *et al.* 2003c; McDowell *et al.* 2005). However, little is known about the possible structures or origins of these compounds and the chemical shifts of aromatic orthophosphate diesters can vary widely (Turner *et al.* 2003b; Makarov *et al.* 2004). It has been proposed that it is more likely that these signals actually represent stereo-isomers of inositol phosphates (Turner *et al.* 2005).

Orthophosphate diesters represent the next largest class of P compounds and were present in all soil extracts, with concentrations ranging from 4 to 87 mg kg^{-1} or 1-5% of total ^{31}P NMR signal. Orthophosphate diesters comprised only of DNA with other potential diester-P forms such as phospholipids and ribonucleic acid (RNA) absent from our ^{31}P NMR spectra. Phospholipids and RNA are rarely detected in soils (Makarov *et al.* 2002; Turner *et al.* 2007) despite their rate of addition to soils being much greater than that of other organic P forms (Anderson 1980). This is likely governed by the low stability of these compounds in the soil and in alkaline solutions (Makarov *et al.* 2002; Turner *et al.* 2003b) and the low aqueous solubility of phospholipids (Doolette et al., 2009).

Low diester-P concentrations reported in some soils have been attributed to the preferential mineralisation of diester-P compared to other more stable organic P forms (Hawkes *et al.* 1984; Condron *et al.* 1985; Condron *et al.* 1990a) and are considered to be linked to nutrient turnover, with

diester P representing a labile pool of soil organic P (Turner *et al.* 2003c). In particular, cell-free RNA and DNA are highly susceptible to degradation due to the presence of active nucleases in soils (Paul and Cark 1996). However, extracellular DNA can strongly sorb to sand, clay minerals and humic substances, which provides protection from degradation by nucleases in comparison to free DNA in solution (Pietramellara *et al.* 2009). This may be one of the factors contributing to the presence of DNA in preference to other diester-P forms.

It should be noted that the concentrations of diester-P in this study (as in other ^{31}P NMR studies) are likely to be underestimated due to its degradation in alkaline solution during extraction. Turner *et al.* (2003b) showed RNA to be highly unstable in NaOH-EDTA, completely degrading to orthophosphate monoesters within 24 h and this is consistent with the findings of Makarov *et al.* (2002).

Phospholipids are also susceptible to hydrolysis, although the rate of degradation varies between phospholipids. One of the most common phospholipids, phosphatidyl choline (lecithin), completely degrades in NaOH-EDTA within 24 h (Turner *et al.* 2003b) to α- and β-glycerophosphate (Doolette *et al.* 2009). In contrast, DNA is more stable (Turner *et al.* 2003b) and can remain intact in alkaline solutions for at least 24 h (Makarov *et al.* 2002). Low diester-P concentrations may also be due to a combination of the low aqueous solubility of phospholipids in NaOH-EDTA (Doolette *et al.* 2009) and low organic P extractability. On average only 47% of total organic P was extracted which suggests that potentially there may be some forms of diester-P that are not extracted from the soil.

Pyrophosphate was present in all soil extracts at concentrations ranging from 1 to 62 mg kg^{-1} (up to 5% of total NaOH-EDTA extractable P). No higher polyphosphates, which produce resonances in the range -18 to -22 ppm, were detected in any of the soil extracts. Pyrophosphate concentrations reported here are similar to those reported elsewhere; 1–7% of total extractable P in pasture soils (Turner *et al.* 2003c), 0.5–4.3% in cropping soils (Turner *et al.* 2003a), < 1–4.85% in manure treated soils (Dou *et al.* 2009) and on average 3% in grassland soils (Murphy *et al.* 2009). Inorganic polyphosphates, of which pyrophosphate is the simplest form, are believed to accumulate in microbial biomass as a P storage form, however, the factors controlling their abundance remain unclear (Blanchar and Hossner 1969; Turner *et al.* 2003a).

The results presented here suggest that the largest portion of organic P in some Australian soils is bound in complex humic P molecules and not in smaller specific compounds such as phytate. This not only challenges our understanding of soil organic P cycling but has implications for the way in which methods are developed to increase P availability in the soil profile. In terms of sustainable P management, it is likely a slightly new approach is required, especially in low-input and organic agricultural systems where access to recalcitrant or poorly available forms of P such as phytate are

seen as potentially available forms of organic P (Guppy and McLaughlin 2009). There is likely to be great promise for plant-based approaches to increase P efficiency in agricultural systems (Richardson *et al.* 2009), although, some of the most effective strategies for increased P availability may not be through techniques that target specific soil organic P compounds such as the genetic modification of plants with increased phytase activity (George *et al.* 2004; George *et al.* 2005) or the direct application of manufactured extracellular enzymes to increase mineralisation of inositol P (Conyers and Moody 2009). Instead, greater success could come from techniques isolating genes in plants that aid or alter root development (Grierson *et al.* 2001; Williamson *et al.* 2001), therefore increasing the chances of P uptake, or focusing on the exudation of organic anions with a known capability of mobilising P bound in humic-metal complexes (Gerke 1993). It is doubtful that a single strategy that increases the availability of organic P would be applicable to all soils. However, we suggest that the development of strategies to increase uptake of P by plants and crops should not only be focused on increasing the availability of P contained in small, supposedly recalcitrant compounds, such as phytate, but consideration should also be given to the availability of P in larger complex molecules.

Conclusions

We have used an improved method for assessing the speciation of soil P using solution ^{31}P NMR and spectral deconvolution. This method not only provided a more detailed characterisation of organic P forms in Australian soils than has hitherto been undertaken, but also provided a more accurate estimation of the quantity of these forms by minimising the potential for overestimation of the P concentrations of simple orthophosphate monoesters. The ^{31}P NMR spectra of 18 NaOH-EDTA soil extracts revealed that a large proportion of organic P is present in large, complex molecules such as humic acids. Phytate was the most abundant identifiable organic P compound, but only comprised a maximum of 10% of total extractable P. In most cases, humic organic P represented more P than all of the small discrete P molecules combined. These findings have implications for attempts to increase the availability of stabilised forms of organic P. Consideration should be given to further investigation of the presence and availability of P in large complex molecules, rather than solely developing methods to increase the availability of individual P compounds (such as phytate) that are present in the soils at much lower concentrations.

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CHAPTER 6

COMBINING ENZYMATIC HYDROLYSIS AND ^{31}P NMR SPECTROSCOPY TO EXAMINE THE POTENTIAL BIOAVAILABLITY OF SOIL ORGANIC PHOSPHORUS

This work was carried out in collaboration with E.K Bünemann [Swiss Federal Institute of Technology Zurich (ETH)]. Soils were sent to ETH for enzymatic hydrolysis and returned for NMR analysis.

Introduction

The management of phosphorus (P) in agricultural systems requires an understanding of P cycling in soils to ensure that adequate P is maintained for plant growth while the impact of adverse environmental consequences, such as the potential for nutrient leaching, are minimised. In order to achieve this, a better understanding of the potential availability of P species is required. The availability of P in soils is not only related to the form and concentration of the P species present, but is also dependant on the interactions that occur within the soil. Plants directly access inorganic P (orthophosphate) from soil solution. Organic P is a potentially available source of P for plants but must first be converted to orthophosphate. This usually occurs through enzymatic hydrolysis. Since soil organic P exists in many different forms, its susceptibility to enzymatic hydrolysis varies (Bünemann, 2008), and therefore its bioavailability to plants also varies. The continued development of strategies for sustainable P management will be dependent on our understanding of P cycling. It will rely on analytical methods that enable the accurate identification and quantification of soil organic P, as well as methods that can gauge the susceptibility of different organic P species to enzymatic hydrolysis.

The most widely used method for soil organic P analysis is solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy. This method has been used to characterise P forms in a diverse range of soils (e.g., volcanic (Briceño *et al.*, 2006), tropical forest (Turner, 2008), pasture (McDowell *et al.*, 2005)). One reason for its widespread application is that it not only enables the characterisation of broad classes of organic P forms (orthophosphate, orthophosphate monoester, orthophosphate diester and polyphosphates), but also enables the identification of specific P compounds, such as phytate in the orthophosphate monoester region and deoxyribonucleic acid (DNA) in the orthophosphate diester region. This technique provides the most detailed and accurate characterisation of soil organic P but cannot determine the potential bioavailability of the different organic P species identified within the soil analysed.

Enzymatic hydrolysis is a technique that has been used to characterise P forms, but, unlike ^{31}P NMR spectroscopy, enzymatic hydrolysis can provide an estimate of the potentially available (labile) organic P fraction. This fraction is defined as the increase in inorganic P on treatment with an enzyme. A number of studies have examined the lability of organic P in soil extracts by phosphatase hydrolysis, as reviewed by Bünemann (2008), with labile organic P fractions reported to comprise up to 93% of the extractable organic P concentration. Many different enzymes, both individually and in combination, have been used in conjunction with numerous different soil extraction and fractionation methods. This can complicate any comparisons that are made between different studies. Therefore the development of a standard protocol would aid the future use of this technique.

Since organic P must be hydrolysed to inorganic P before it is detected colorimetrically, and not all organic P is necessarily hydrolysed, it is not possible to identify every organic P form or species in the soil sample when using enzymatic hydrolysis. Therefore a prerequisite for the interpretation and quantification of organic P is the accurate determination of enzyme substrate specificity. Numerous studies used previously published enzyme substrate specificities as a guide to characterise enzymatically hydrolysable organic P, with the identification of different P forms commonly based on differences in inorganic P released by the addition of different combinations of phosphatase enzymes (Turner *et al.*, 2003; He *et al.*, 2006; He *et al.*, 2007). However, a more accurate method involves characterising enzymatically hydrolysable organic P based on the specificity of enzymes towards model compounds (Hayes *et al.*, 2000; He & Honeycutt, 2001; Turner *et al.*, 2002; He *et al.*, 2004).

Using enzymatic hydrolysis to identify individual organic P species has had mixed results. Turner *et al.* (2003) reportedly identified inositol hexakisphosphate (phytate) by measuring the difference between the P released by a phytase enzyme, one of a class of phosphatase enzymes with a high specific activity for phytate (Mullaney & Ullah, 2007), and P released by all other enzymatic treatments, which included combinations of treatments with alkaline phosphatase, phospholipase and phosphodiesterase. However, testing the substrate specificity of numerous different phytase enzymes has shown that although phytase can completely hydrolyse phytate, it can also partially or completely hydrolyse other condensed phosphates, orthophosphate monoesters and orthophosphate diesters (Shand & Smith, 1997; Hayes *et al.*, 2000; Turner *et al.*, 2002).

Both solution ^{31}P NMR spectroscopy and enzymatic hydrolysis are valuable tools for the characterisation of soil organic P. However, when used in combination it may be possible to identify, quantify and estimate the potential bioavailability of the soil organic P species present. A combination of the two methods has recently been reported for analysing sequentially fractionated extracts of a poultry litter and a soil with and without poultry manure amendment (He *et al.*, 2008). A comparison of the two methods was also undertaken using NaOH-EDTA extracts of dairy and poultry manure (He *et al.*, 2007). He *et al.* (2008) found the distributions of P species in the NaOH extracted fraction of poultry litter determined by the two methods were similar, but ^{31}P NMR spectroscopy detected less organic P in the water, HCO_3 and HCl fractions. Furthermore, He *et al.* (2008) were unable to obtain any conclusive results for the soil or manure amended soil. Conversely, He *et al.* (2007) found that the major P forms in dairy manures determined by the two methods were similar, although ^{31}P NMR spectroscopy identified greater amounts of orthophosphate monoesters and phytate than enzymatic hydrolysis. It appears there is great promise in combining the two methods of characterisation but closer examination of this technique and the expansion of its application, particularly to soils, is required.

The aim of this study was to identify and compare the specific organic P species in the enzyme labile and non-enzyme labile fractions of a range of NaOH-EDTA soil extracts using solution ^{31}P NMR spectroscopy and enzymatic hydrolysis.

Methods and Materials

Site Descriptions

Eight Australian topsoils (0–10 cm) from New South Wales and Tasmania were analysed. These soils were included in a previous study examining the phosphorus forms in NaOH-EDTA extracts by ^{31}P NMR spectroscopy (Chapter 5) and details of their properties can be found there.

In addition, three soils from Switzerland and one from Kenya were included in this study. The Basel soil sample (0–20 cm) is a Haplic Luvisol collected from a long-term organic farming trial near Basel, Switzerland (Mäder *et al.*, 2006). The soil contains 15% sand, 70% silt and 15% clay. The pH (in H_2O) is 6.5. The Regensdorf-Watt soil sample (0–10 cm) is a Cambisol collected from the border strip of a long-term fertilisation experiment on permanent grassland which has not been fertilised since 1992. It was collected near Regensdorf-Watt, Switzerland (Philipp *et al.*, 2004). The soil contains 54% sand, 34% silt and 22% clay, and has a pH (in H_2O) of 6.1. The Damma glacier soil is a Haplic Cambisol (Dystric, Humic, Skeletic) collected near the Damma glacier in the Central Alps of Switzerland, in the canton of Uri. The site has been ice-free for approximately 3000 years and was under grassy vegetation. The soil contains 57% sand, 28% silt and 14% clay. The Western Kenyan soil (0–15 cm) is an Oxisol collected from a plot under maize-crotalaria rotation with P fertilisation (as triple superphosphate, TSP) of 50 kg P ha^{-1} $year^{-1}$ (Bünemann *et al.*, 2004). The soil contains 37% sand, 24% silt and 39% clay and has a pH (in H_2O) of 5.0.

NaOH-EDTA extraction

Samples were ground to pass through a 2-mm sieve prior to extraction and were extracted in duplicate following methods similar to those of Cade-Menun and Preston (1996). Soil samples were extracted using 0.25 M NaOH and 0.05 M Na_2EDTA for 16 h, centrifuged (1400 × *g)* for 10 min and filtered. The soil:solution ratios and filtration methods varied for each sample and are shown in Table 1.

The concentration of inorganic P in the NaOH-EDTA soil extracts was determined by colorimetry on diluted extracts (at least 1:100) using the malachite green method (Ohno & Zibilske, 1991). P concentrations were also determined by ^{31}P NMR spectroscopy and integration (refer to *^{31}P NMR analysis of enzyme treated NaOH-EDTA soil extracts*). Sub-samples (0.1–0.3 ml) of the NaOH-EDTA soil extracts were taken to determine total P concentration by autoclaving with a 2 ml digestion mix (6 g ammonium persulphate in 100 ml 0.9 M H_2SO_4), followed by neutralization and colorimetry. Organic P was calculated as the difference between total and inorganic P.

Enzyme lability of NaOH-EDTA extractable P (enzymatic hydrolysis)

Organic P in NaOH-EDTA extracts was enzymatically hydrolysed using a commercial phytase preparation (Bio-Feed ™ Phytase L, Ronozyme P (L), Novozyme, Denmark). Preliminary experiments were performed to test the susceptibility of various model P compounds to enzymatic hydrolysis. P compounds included orthophosphate monoesters (glucose-6-phosphate, phosphoenolypyruvate and *myo*-inositol hexakisphosphate), condensed phosphates (adenosine triphosphate and pyrophosphate), orthophosphate diesters (ribonucleic acid (RNA) and deoxyribonucleic acid (DNA)) and phosphonate (2-aminoethyl-phosphonic acid). The control treatment for enzyme hydrolysis reactions involved the addition of an equivalent amount of autoclaved phytase preparation in the place of fresh (active) enzyme preparation. Autoclaving the enzyme preparation prevented the hydrolysis of all model P compounds and of P in the NaOH-EDTA soil extracts.

Before treating larger volumes of NaOH-EDTA soil extracts for NMR analysis, the duration of the enzyme treatment and the volume of added enzyme preparation were optimized in a microplate assay. The NaOH-EDTA extracts (20 or 25 µl) were diluted to a final volume of 200 µl using MES-EDTA buffer (0.15 M 2-(N-morpholino)ethanesulfonic acid (MES) and 0.01 M EDTA, adjusted to pH 5.5) followed by the addition of 85 or 30 µl of H_2O. The reaction was initiated by the addition of either 15 or 60 µl of phytase (Ronozyme, ≥5000 FYT/g) preparation (1:10 diluted) and incubated at 37°C with gentle shaking. The pH in the final mixture was approximately 6.5. Following 3, 24, 48 and 96 h of incubation, 25 or 50 µl were transferred to a new plate and the concentration of inorganic P measured using the malachite green method in a microplate reader (EL 800, Biotek). The enzymatic reactions were terminated by the acidic conditions necessary for colorimetry (Annaheim, unpublished). Samples that did not receive the enzyme addition showed no orthophosphate release due to the MES-EDTA buffer or incubation. This pretest showed that after the addition of 60 µl of diluted enzyme preparation, maximum P release was reached after 24 h. The addition of 15 µl of diluted enzyme preparation required 48 h to reach similar levels of orthophosphate release. An addition of diluted enzyme preparation equivalent to 30 µl and an incubation time of 48 h were chosen for the upscaled treatment of extracts for NMR analysis. Upscaled conditions during hydrolysis are shown in Table 1.

To account for background P in the enzyme preparation, as well as potential interference of other components of the enzyme preparation, paired samples with the addition of fresh (active) or autoclaved (deactivated) phytase preparation were prepared. The filtered extracts of two NaOH-EDTA replicate samples were combined before taking 22–30 ml of the total extract for treatment with either active or deactivated enzyme preparation. Orthophosphate concentration was measured colorimetrically (Ohno & Zibilske, 1991) after 48 h incubation on 0.1 ml aliquots of treated NaOH-EDTA soil extracts, and enzyme-labile organic P was calculated as the difference in orthophosphate

concentration between samples incubated with active and deactivated enzyme preparation. Non-enzyme-labile organic P in the NaOH-EDTA extracts was calculated as the difference between total P, enzyme-labile organic P and orthophosphate. The remainder of the enzyme-treated NaOH-EDTA soil extracts was immediately frozen (-20°C) and freeze-dried for NMR analysis.

Table 1. Details of extraction and hydrolysis conditions.

	Extraction details			Enzyme treatment (48 h, 37°C)			
Soil Location	**Soil (g)**	**Extractant[a] (ml)**	**Filtration**	**Buffer[b] (ml)**	**Volume of soil extract treated (ml)**	**Ronozyme preparation[c] (ml)**	**Water (ml)**
Nowra	2	40	Whatman 42	225	25	37.5	87.5
Casino	2	40	Whatman 42	225	22	37.5	87.5
Tocal	2	40	Whatman 42	225	25	37.5	87.5
Elliot	2	40	Whatman 42	225	25	37.5	87.5
Parkham	2	40	Whatman 42	225	25	37.5	87.5
Elizabeth Town	2	40	Whatman 42	225	22	37.5	87.5
Basel	4	40	0.45 um	175	25	30	70
Regensdorf-Watt	4	40	0.45 um	210	30	36	84
Damma glacier	2.7	40	0.8 um	210	30	36	84
Western Kenya	4	40	0.45 um	210	30	36	84

[a] 0.25 M NaOH and 0.05 M EDTA
[b] 0.15 M MES and 0.01 M EDTA
[c] Fresh (active) or autoclaved (deactivated) phytase preparation (1:10 dilution)

^{31}P NMR analysis of enzyme treated NaOH-EDTA soil extracts

A 5 g sub-sample of each freeze-dried extract was ground, re-dissolved in 5 ml of 0.25 M NaOH and 0.05 M Na_2EDTA and centrifuged (1400 × *g*) for 20 min. It was necessary to increase the sub-sample size from the 500 mg used for straight soil extracts (Chapter 5) because the majority of the mass of freeze-dried extract was MES and EDTA from the buffer solution and sorbitol from the phytase preparation (Table 1). The pH of supernatant solution was adjusted using 10 M NaOH to ensure a pH > 12. The resultant supernatant (3.5 ml), D_2O (0.3 ml) and internal standard (0.1 ml) of 6.0 g l^{-1} methylenediphosphonic acid solution (MDP) (Sigma Aldrich; M9508; ≥99%) were placed in a 10 mm NMR tube.

Solution ^{31}P NMR spectra were acquired at 20°C on a Varian INOVA400 NMR spectrometer (Varian, Palo Alto, CA) at a ^{31}P frequency of 161.9 MHz. Recovery delays ranged from 5 s to 30 s and were set to at least five times the T_1 value of the orthophosphate resonance determined in preliminary inversion-recovery experiments. A 90° pulse of 27 to 30 µs, an acquisition time of 1.0 s and broadband ^{1}H decoupling were used. Between 4000 and 18980 scans were acquired for each sample. Chemical shifts were referenced to the internal MDP standard at 17.63 ppm. The spectra presented have a line broadening of 2 Hz.

Quantification of P species from ^{31}P NMR spectra

Signal areas of classes of P compounds were assigned as follows: orthophosphate (7–5.4 ppm), orthophosphate monoester (5.4–3.5 ppm), orthophosphate diester (2 to -1) and pyrophosphate (-3 to -5), and concentrations were calculated by integrating against the signal from the MDP standard (Chapter 3, 4).

Results and Discussion

Phosphorus characterisation by enzymatic hydrolysis and colorimetry

Total P, inorganic P and organic P (enzyme labile and non-enzyme labile) concentrations determined for all the NaOH-EDTA soil extracts are shown in Table 2. Total NaOH-EDTA extractable P concentrations ranged from 307 to 5924 mg kg^{-1} of which organic P comprised 16–87%. In all cases, the addition of the active enzyme preparation decreased the amount of organic P present (Table 2). Enzyme labile organic P, defined as the difference in organic P between active and deactivated treatments, represented 3–53% of total extractable P and non-enzyme labile organic P represented 7–53% of total extractable P. Treatment with active enzyme resulted in the apparent hydrolysis of 22–63% of extractable organic P.

Table 2. Total, inorganic and organic P in NaOH-EDTA soil extracts determined by digestion and colorimetry, and the enzyme labile P, non-enzyme labile P and difference in organic P content between active and deactivated enzyme treatments. Data are means of three analytical replicates. Standard deviations are shown in parentheses.

Soil Location	Total P	Inorganic P	Organic P	Enzyme labile P	Non-enzyme labile P	Change in organic P
	mg kg^{-1}					— % —
Nowra	5924 (69)	4982 (11)	942 (69)	206 (47)	736 (36)	-22 (5)
Casino	929 (21)	753 (21)	176 (21)	111(6)	65 (6)	-63 (3)
Tocal	825 (15)	596 (7)	230 (15)	162 (21)	68 (21)	-70 (9)
Elliot	771 (2)	388 (10)	384 (2)	174 (6)	210 (6)	-45 (1)
Parkham	1018 (4)	819 (5)	199 (4)	115 (19)	84 (19)	-58 (9)
Elizabeth Town	1558 (13)	1116 (8)	441 (13)	207 (20)	234 (20)	-47 (5)
Basel	460 (7)	121 (3)	339 (7)	118 (6)	221 (6)	-35 (2)
Regensdorf-Watt	356 (6)	45 (1)	310 (6)	120 (2)	190 (2)	-39 (1)
Damma glacier	1259 (11)	192 (1)	1067 (11)	671 (16)	396 (16)	-63 (1)
Western Kenya	307 (3)	128 (1)	179 (3)	103 (93)	76 (3)	-57 (2)

Phosphorus forms identified using ^{31}P NMR spectroscopy

Total, inorganic and organic P concentrations of the soil extracts treated with active and deactivated enzyme preparations were also quantified using ^{31}P NMR spectroscopy (Table 3). Total extractable P concentration ranged from 340 to 4621 mg kg^{-1} and the ^{31}P NMR spectra contained signal in all four diagnostic chemical shift regions: orthophosphate, orthophosphate monoesters, orthophosphate diesters and pyrophosphate (Figures 1 and 2). Pyrophosphate was present in all soil extracts treated

with deactivated enzyme but was absent in all extracts treated with active enzyme. This is unsurprising given that preliminary experiments showed the complete hydrolysis of condensed phosphates (adenosine triphosphate and pyrophosphate) by the enzyme preparation. No higher polyphosphates, which produce resonances in the range -18 to -22 ppm, were detected in any of the soil extracts. Orthophosphate diesters were also present in all soil extracts. Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were assigned to a broad resonance at 0 ppm, and a small resonance at 1.06 ppm was assigned to the degradation products of phospholipids due to alkaline hydrolysis (Chapter 2). Preliminary experiments used to determine the susceptibility of diester-P to enzymatic hydrolysis showed complete degradation of ribonucleic acid (RNA) but only 9% hydrolysis of deoxyribonucleic acid (DNA). There was no obvious difference in diester-P concentration between the two treatments, but given the low signal to noise ratio it is difficult to identify minor changes in concentration.

Table 3. Concentrations of P classes of incubated NaOH-EDTA soil extract following the addition of autoclaved (deactivated) or fresh (active) phytate preparation as determined by solution ^{31}P NMR spectroscopy.

Soil Location	Enzyme treatment	Total P	Inorganic P	Organic P	Change in organic P
		mg kg^{-1}			%
Nowra	deactivated (autoclaved)	3690	3450	240	
	active (fresh)	4500	3540	960	+300
Casino	deactivated	760	650	110	
	active	900	700	200	+82
Tocal	deactivated	620	480	140	
	active	590	470	120	-14
Elliot	deactivated	600	380	220	
	active	540	460	80	-64
Parkham	deactivated	790	670	120	
	active	850	790	60	-50
Elizabeth Town	deactivated	1280	970	310	
	active	1120	990	130	-58
Basel	deactivated	340	140	200	
	active	310	190	120	-40
Regensdorf-Watt	deactivated	290	80	210	
	active	440	280	160	-24
Damma glacier	deactivated	940	220	720	
	active	920	680	240	-66
Western Kenya	deactivated	370	200	170	
	active	380	300	80	-53

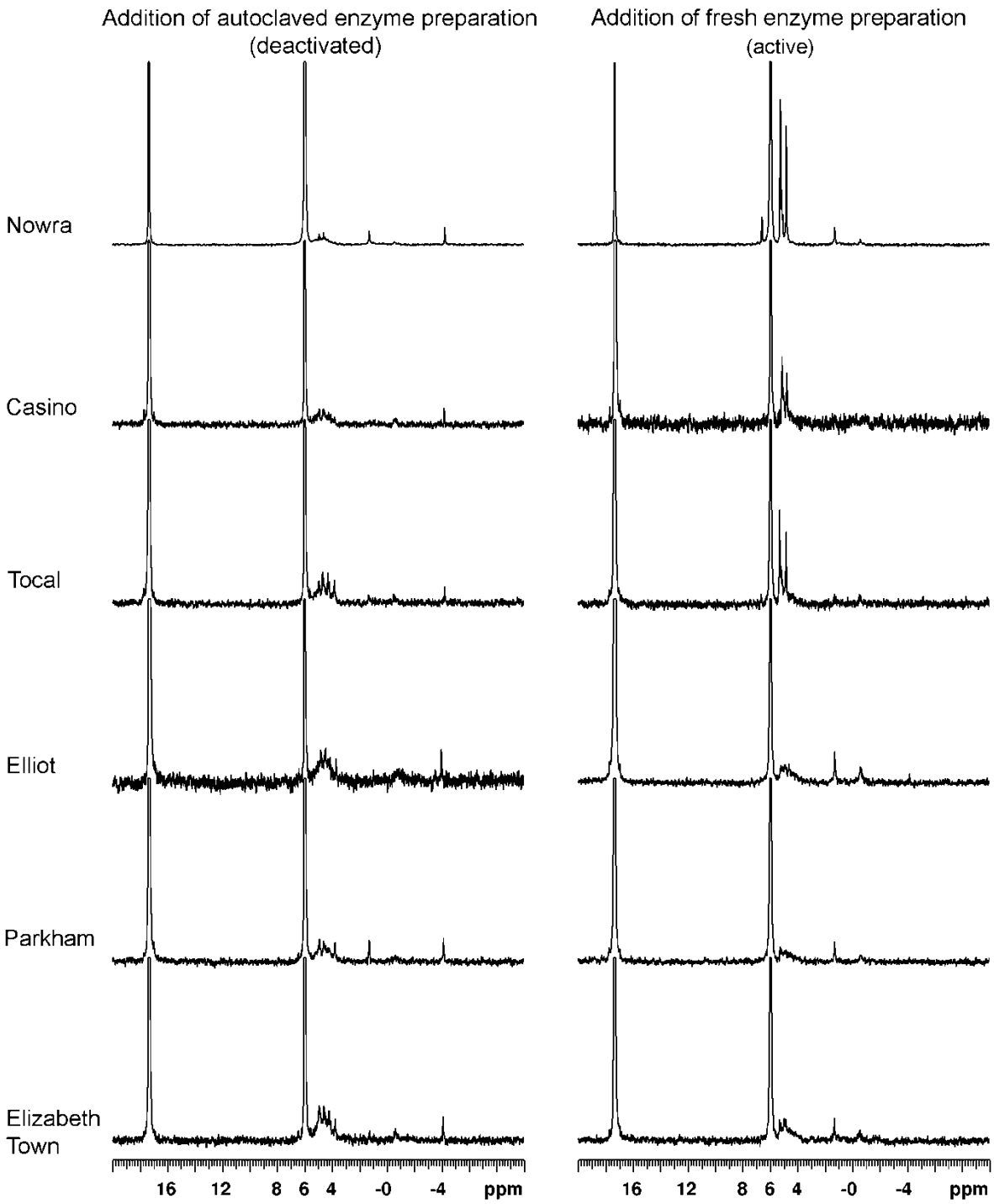

Figure 1. Solution ^{31}P NMR spectra of NaOH-EDTA extracts of Australian soils which received either autoclaved (deactivated) or fresh (active) enzyme preparations. The vertical scale of each spectrum is normalised using the internal reference (MDP) in order to compare the orthophosphate (6.5–5.4 ppm), orthophosphate monoesters (5.4–3.5 ppm), orthophosphate diester (2 to -1) and pyrophosphate (-3 to -5) regions.

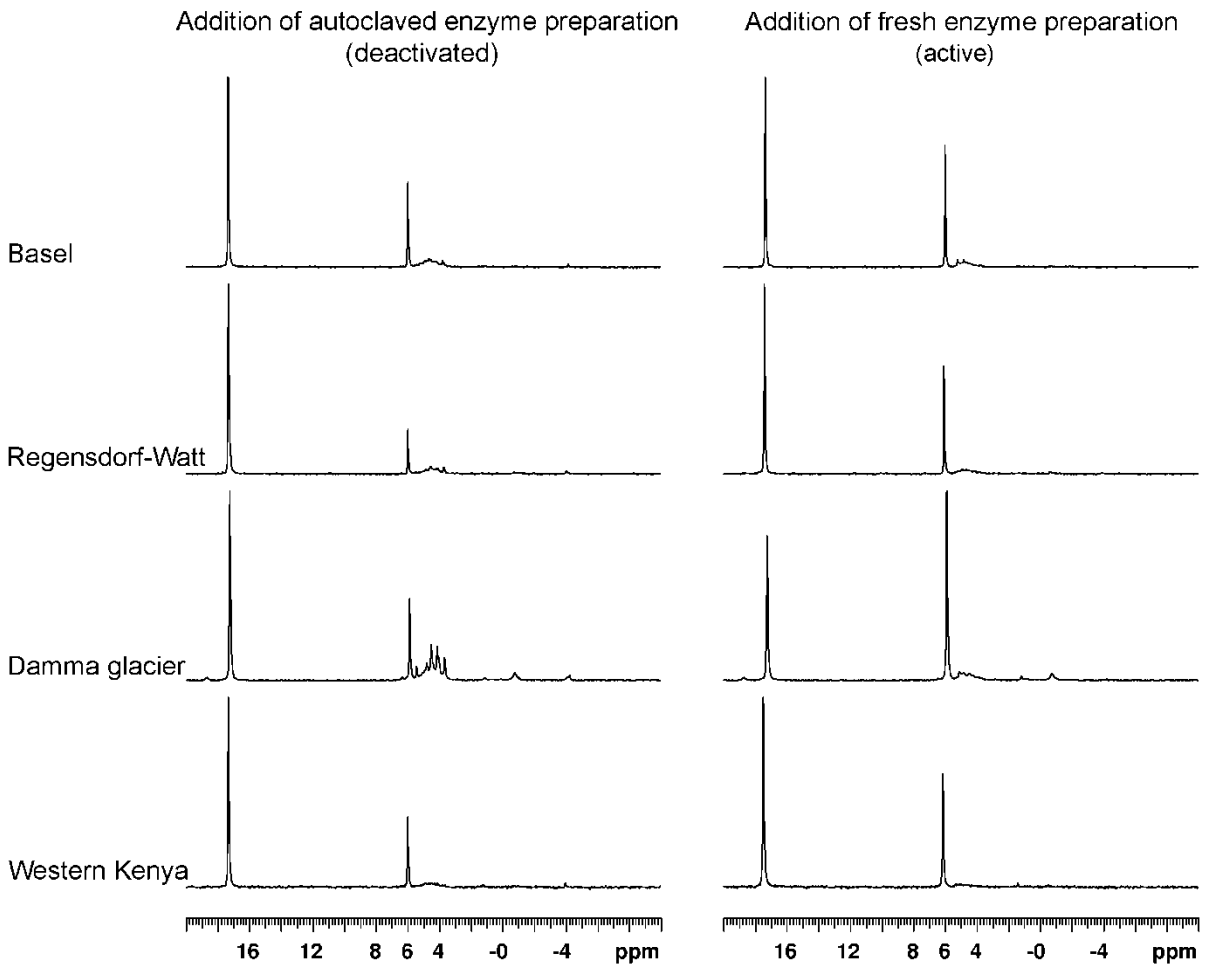

Figure 2. Solution ^{31}P NMR spectra of NaOH-EDTA extracts of three Swiss and a single Kenyan soil which received either autoclaved (deactivated) or fresh (active) enzyme preparations. The vertical scale of each spectrum is normalised using the internal reference (MDP) to compare orthophosphate (6.5–5.4 ppm), orthophosphate monoesters (5.4–3.5 ppm), orthophosphate diester (2 to -1) and pyrophosphate (-3 to -5) regions.

The majority of the ^{31}P NMR signal was due to orthophosphate and orthophosphate monoesters (up to 98%). Expansions of the ^{31}P NMR orthophosphate and orthophosphate monoester chemical shift regions, comparing the active and deactivated treatments, are shown in Figures 3 and 4. Some of the major orthophosphate resonances were identified by spiking one of the samples (Damma, Figure 5). In this way, resonances at 5.60, 4.68, 4.29, 4.19 ppm were assigned to phytate, and those at 4.93 and 4.63 ppm were assigned to α- and β-glycerophosphate, respectively. The resonance at 3.82 ppm was identified as being due to *scyllo*-inositol hexakisphosphate, based on previous spiking experiments (Chapter 2). A broad signal in the orthophosphate monoester region was assigned to humic organic P, based on previous investigations (Chapter 3, 4).

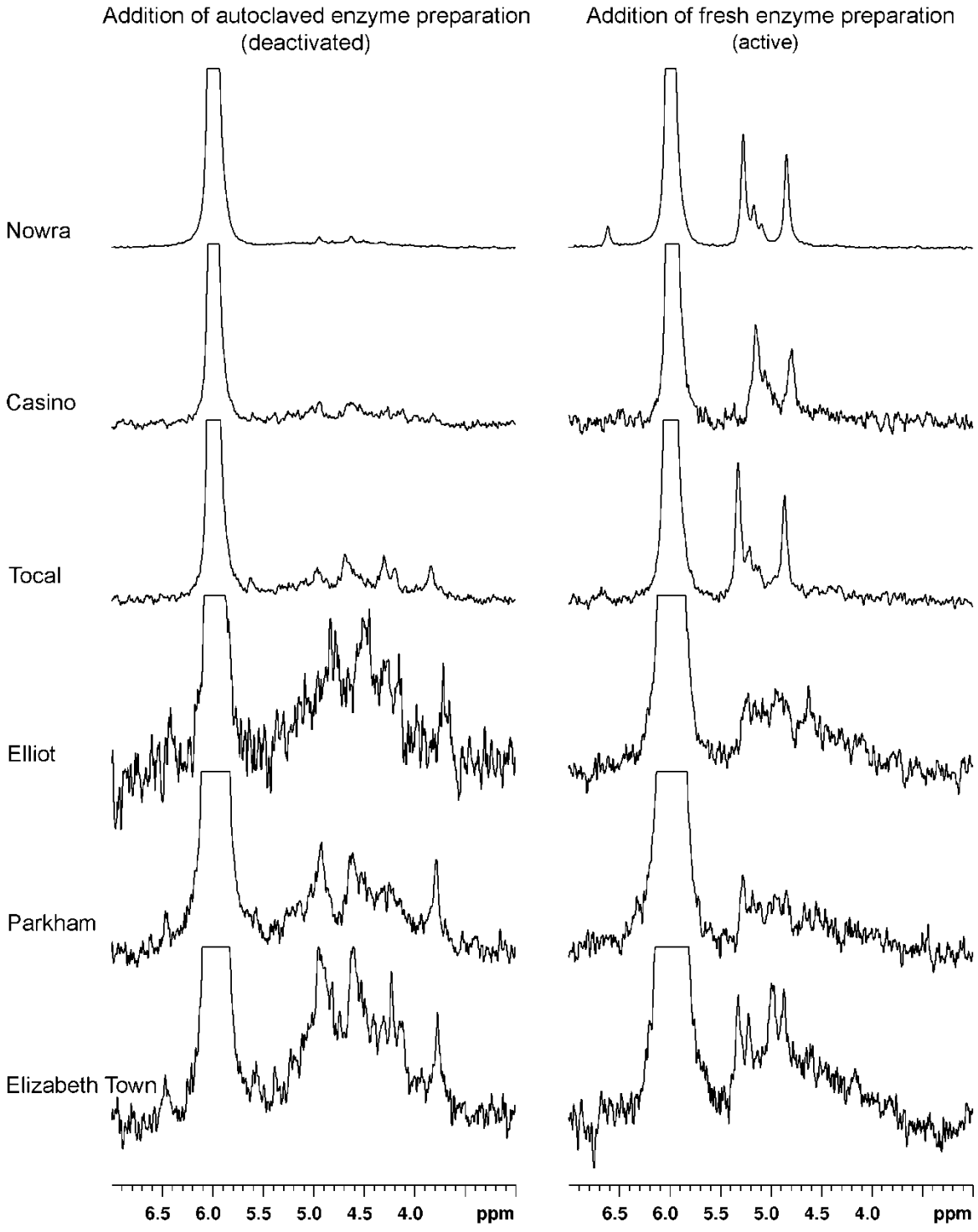

Figure 3. Solution ^{31}P NMR spectra of NaOH-EDTA extracts of Australian soils which received either autoclaved (deactivated) or fresh (active) enzyme preparations. The vertical scale of each spectrum is normalised using the internal reference (MDP) and paired samples are scaled by the same factor. This expansion of the orthophosphate (6.5–5.3 ppm) and orthophosphate monoester (5.3–2.6 ppm) regions shows in detail the enzymatic hydrolysis of orthophosphate monoester species as well as the synthesis of new orthophosphate monoester species.

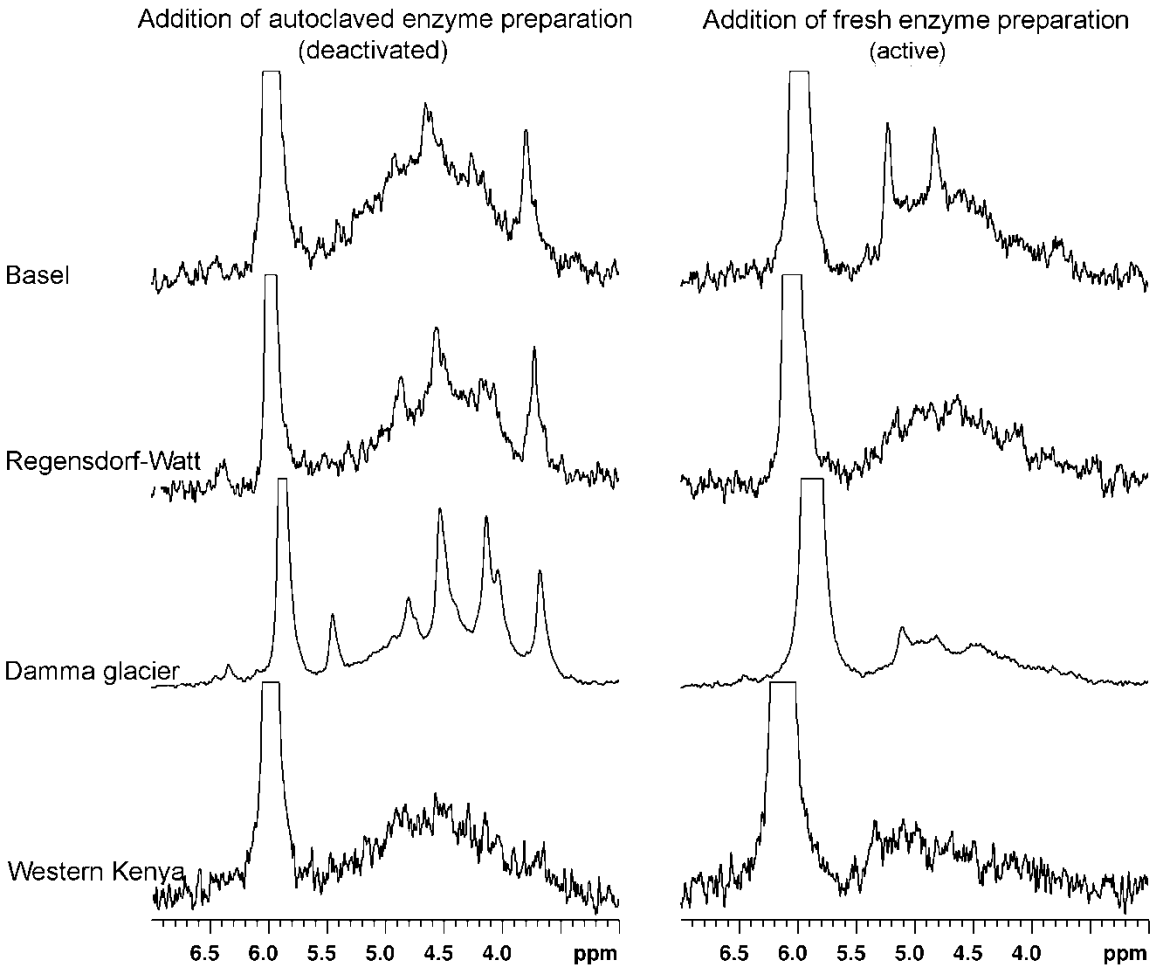

Figure 4. Solution ^{31}P NMR spectra of NaOH-EDTA extracts of three Swiss soils and a single Kenyan soil which received either autoclaved (deactivated) or fresh (active) enzyme preparations. The vertical scale of each spectrum is normalised using the internal reference (MDP) and paired samples are scaled by the same factor. This expansion of the orthophosphate (6.5-5.3 ppm) and orthophosphate monoester (5.3-2.6 ppm) regions shows in detail the enzymatic hydrolysis of orthophosphate monoester species as well as the synthesis of new orthophosphate monoester species.

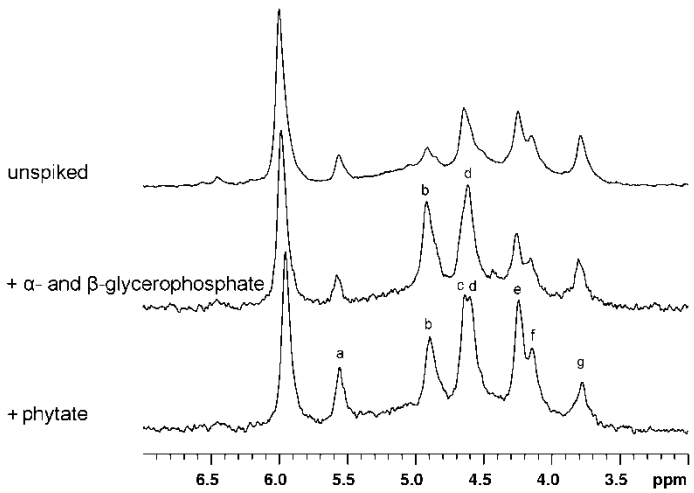

Figure 5. Solution ^{31}P NMR spectra of the unspiked Damma glacier NaOH-EDTA soil extract, and spectra resulting from the consecutive addition of 1:1 mixture of α- and β-glycerophosphate and phytate. The lower case letters identify P species: α- and β-glycerophosphate; b,d; phytate, a,c,e,f; *scyllo*-inositol hexakisphosphate, g.

Close examination of the orthophosphate monoester region (6.5–5.4 ppm) revealed that there was a clear loss of some orthophosphate monoester compounds in the soil extracts, but there also appeared to be the formation of new orthophosphate monoester species. This was most noticeable in the Nowra, Casino, Tocal and Basel activated soil extracts that showed the synthesis of up to five new orthophosphate monoester resonances at 6.62, 5.28, 5.18, 5.09, 4.85 ppm. Due to the low signal-to-noise ratio of our ^{31}P NMR spectra, it was not possible to identify each of the individual orthophosphate monoester species in all the deactivated soil extracts. There was a clear decrease in the intensity of numerous orthophosphate monoester peaks on active enzyme treatment for the Damma glacier sample. This indicated the complete degradation of phytate, α- and β-glycerophosphate and *scyllo*-inositol hexakisphosphate. α- and β-glycerophosphate also appear to have been completely degraded in the Regensdorf-Watt and Nowra extracts and phytate was hydrolysed in the Tocal extract. There was a clear loss of *scyllo*-inositol hexakisphosphate in the Basel, Regensdorf-Watt, Elizabeth Town, Parkham and Elliot extracts. Conversely, there appeared to be very little loss of humic P on active enzyme treatment, indicating that the majority of humic P was resistant to hydrolysis. These results are in good agreement with the preliminary experiments that showed the complete hydrolysis of glucose-6-phosphate, phosphoenolypyruvate and *myo*-inositol hexakisphosphate (phytate). The preliminary experiments aimed to determine the susceptibility of individual P compounds to phytase hydrolysis but did not consider the susceptibility of humic P or soil organic P in complex forms. However, these results are in agreement with the work of Pant *et al.* (1994), He *et al.* (2004) and Tang *et al*. (2006). Pant *et al.* (1994) separated the organic P fraction of soil water extracts on the basis of molecular weight and found that a greater portion of reactive P (enzyme labile P) occurred in the lower molecular weight fractions. He *et al.* (2004) investigated the hydrolysable forms of organic P present in sequentially extracted soils and manured soils, using a combination of acid phosphatases, phytase and nuclease. They observed that a considerable portion of organic P extracted was not enzymatically hydrolysable and suggested this unhydrolysable organic P was in more complex forms, including humic acids. More specifically, Tang *et al.* (2006) has shown phytase is unable to hydrolyse phytate when complexed with Al, Fe, Cu or Zn.

Although there was a clear loss of orthophosphate monoester compounds in the soil extracts, the synthesis of new orthophosphate monoester compounds resulted in an overall increase in organic P on active enzyme treatment for some of the soils. The change in organic P in the Nowra and Casino extracts was + 300% and + 82%, respectively. The change in organic P on active enzyme treatment for the remaining soils ranged from -66 to -14%. Organic P present following active enzyme treatment would usually be interpreted as being organic P that is resistant to enzyme hydrolysis. However, this is misleading, since the actual amount of organic P hydrolysed is underestimated by not accounting for the synthesis of new orthophosphate monoester species.

Attempts to identify newly synthesised orthophosphate monoester species

One possible explanation for the presence of new orthophosphate monoester peaks in the soils treated with active enzyme is that organic P may have been added in the enzyme preparation. Solution ^{31}P NMR spectra of samples of pure freeze-dried active and deactivated enzyme preparation are shown in Figure 6. The main difference between the active and deactivated enzyme preparation was that orthophosphate monoesters were only clearly present in active enzyme preparations. Orthophosphate monoester peaks present in the spectrum of the active enzyme were at chemical shifts similar to those in the soil extracts treated with the active enzyme (4.83, 4.69, 4.53, 6.18 ppm). The difference in chemical shifts between organic P peaks in the enzyme preparation and those in the soil extracts is likely due to the differences in electrolyte concentration, since the enzyme preparation contained no soluble salts and was not combined with NaOH-EDTA, although pH was adjusted with small volume of concentrated NaOH. However, a quantitative assessment of organic P in the active treatment reveals that the orthophosphate monoester species in the enzyme preparation only would only amount to 30–35 mg P kg^{-1} in each soil extract. Therefore, the organic P in the enzyme preparation cannot account for the increase in orthophosphate monoesters, especially in the Nowra sample.

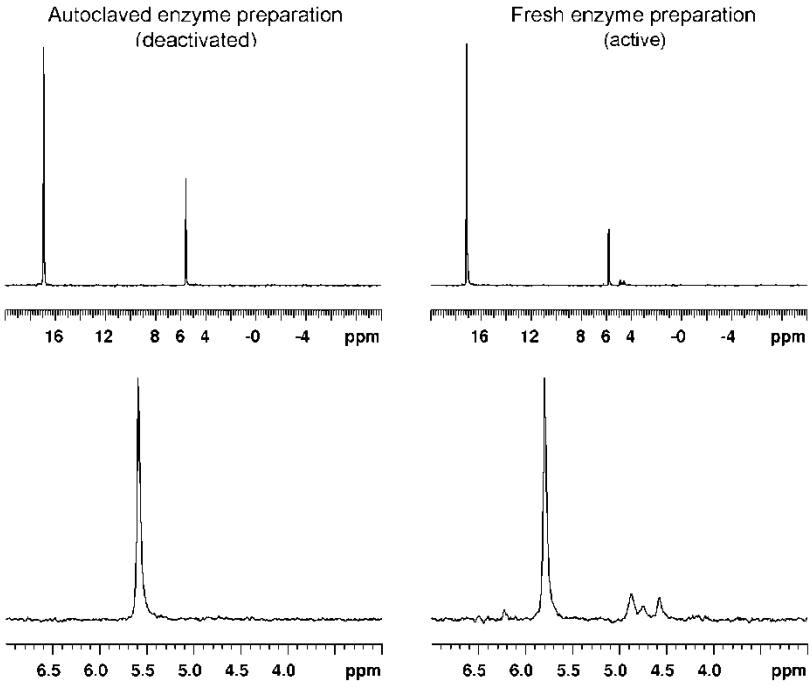

Figure 6. Solution ^{31}P NMR spectra of freeze-dried autoclaved (deactivated) and fresh (active) Ronozyme (phytase) preparation. The vertical scale of the upper spectra is normalised using the internal reference (MDP) and the vertical scale of the lower spectra is normalised using the orthophosphate resonance (5.75ppm).

An alternative explanation, and one that can account for the increase in orthophosphate monoester concentration, is the possibility of enzymatic phosphorylation. Phytase, like all other enzymes, catalyses chemical reactions. We have shown phytase can catalyse the hydrolysis of orthophosphate monoesters, however, like any catalyst it will also catalyse the reverse reaction. Although the

thermodynamics of the reaction is such that the equilibrium is likely to lie towards the hydrolysis of esters (i.e. dephosphorylation), this will also be dependent on the relative concentrations of reactants (orthophosphate and alcohol) and products (phosphate ester). If there is a large concentration of orthophosphate and alcohol present, then the reverse reaction, phosphate esterification, becomes significant. We hypothesise this to be the case here because the enzyme preparation contained a substantial amount of sorbitol buffer (50%) and there was a high concentration of orthophosphate in solution. ^{31}P NMR analysis required that the soil extract be concentrated via freeze-drying and reconstitution into a smaller volume of water. So long as the enzyme remained active, this would have increased the concentration of orthophosphate and sorbitol in solution and shifted the equilibrium towards there being more phosphate monoester (concentrating a solution pushes the equilibrium towards the side with fewer reactants/products, in this case toward the production of orthophosphate monoesters). This explains why the percentage change in organic P (Table 2 and 3) is quite different, and consistently greater by ^{31}P NMR spectroscopy compared to enzymatic hydrolysis analysis. This shows that minimising the effects associated with sample preparation, especially those determining the factors controlling enzymatic phosphorylation, are vital when enzymatic hydrolysis and ^{31}P NMR analysis are combined to characterise soils.

The formation of sorbitol phosphate requires that the enzyme remains active throughout freeze-drying and re-constitution of the sample. However, without buffering the enzyme is unlikely to be able to withstand the highly alkaline conditions since hydrolysis was not observed in preliminary experiments where the pH>12 (result not shown). Differences in the peak intensities of the newly synthesised orthophosphate monoesters between samples suggest that this is not always the case. If the enzyme remained active, the concentration of the new orthophosphate monoester species would be under thermodynamic control, and therefore their concentration should be proportional to the orthophosphate concentration because the sorbitol concentration is similar for all samples. The Nowra soil extract has the greatest orthophosphate concentration of all samples and also the greatest increase in new orthophosphate monoesters. However, other soils, such as Elizabeth Town and Parkham, also had sufficiently high concentrations of orthophosphate that should have resulted in a substantial increase in new orthophosphate monoesters. It is therefore suggested that phytase activity likely varied among soil extracts following freeze-drying. Deactivation of the enzyme following enzymatic hydrolysis and prior to freeze-drying would ensure that no further enzymatic phosphorylation would occur. Clearly this warrants further research.

The fact that enzymatic phosphorylation may occur under the conditions required for ^{31}P NMR analysis raises the possibility that it also occurs under the conditions used for enzymatic hydrolysis. The conditions necessary for enzymatic hydrolysis, namely a near neutral pH (approximately pH 6.5) and a temperature of 37ºC, are different to those required for ^{31}P NMR analysis, and this is likely to

affect where the equilibrium between orthophosphate and orthophosphate monoester species lies. Table 2 shows that in all cases the addition of active enzyme resulted in a net decrease in organic P, i.e. net dephosphorylation. However, the decrease was smallest for the Nowra soil extract (22%), despite the organic P in this soil being predominantly α- and β-glycerophosphate, species that were shown to be enzyme-labile. The high P concentration of the Nowra soil makes it the most likely to undergo enzymatic phosphorylation. This hypothesis, that phosphorylation may occur under enzyme hydrolysis conditions, could be tested by spiking soil extracts with orthophosphate during enzymatic hydrolysis.

Enzymatic phosphorylation and dephosphorylation using an organic P donor and an alcohol or carbohydrate has been reported previously (Axelrod, 1948; van Herk *et al.*, 2005). Enzymatic phosphorylation methods usually utilise kinases that require adenosine triphosphate (ATP) as the phosphate donor. Thermodynamically, this is quite a different reaction as it utilises the potential energy stored in the phosphate-phosphate bonds of ATP. Phosphorylation of glucose has been achieved with various condensed phosphate donors besides ATP (Arion *et al.*, 1972; Tanaka *et al.*, 2003). Importantly, van Herk *et al.* (2005) have shown that the enzymatic phosphorylation of D-sorbitol results in two major products, which they assigned based on their ^{31}P NMR spectra, to the products of phosphorylation of either of the two terminal primary alcohol groups. It is likely that the two most abundant newly formed orthophosphate monoester resonances in our study are these isomers of sorbitol phosphate. This could be confirmed by spiking.

In the small number of studies that have characterised P in manure extracts by enzymatic hydrolysis and ^{31}P NMR spectroscopy, discrepancies in organic P concentration between the analytical techniques have been observed but these have not been attributed to enzymatic phosphorylation. He *et al.* (2007) examined the P forms in poultry and dairy manures and found total organic P in the poultry manure was significantly greater when identified by ^{31}PNMR spectroscopy compared to enzymatic hydrolysis. The authors concluded this was likely due to incomplete hydrolysis with the added enzymes. Conversely, when He *et al*. (2008) characterised P forms in the H_2O fraction of a poultry manure, less organic P was identified by ^{31}P NMR analysis compared to enzymatic hydrolysis, which was attributed to hydrolysis of organic P under the highly alkaline conditions necessary for NMR analysis. This highlights the difficulties in comparing results from different analytical techniques.

Implications for combined enzymatic hydrolysis and ^{31}P NMR spectroscopy studies

Using enzymatic hydrolysis, it was possible to quantify the enzyme labile P fraction of the soil extracts. Coupled with ^{31}P NMR spectroscopy we were able to show, more specifically, that phytase hydrolysed the majority of the small orthophosphate monoester compounds (α- and β-

glycerophosphate, phytate, *scyllo*–inositol hexakisphosphate) and pyrophosphate, but orthophosphate diesters (principally DNA) and humic P remained relatively unaltered. This is inconsistent with the phytase preparation used having a high specific activity for phytate. The lack of specificity of commercial phytase preparations, such as the one used here, has been well documented (Hayes *et al.*, 2000; He & Honeycutt, 2001; George *et al.*, 2007).

George *et al.* (2007) compared the specificity of two purified phytases (*Aspergillus niger* and *Peniophora lycii*) with a commercial phytase preparation. All phytases had similar reactivities toward phytate but the purified phytases were less effective in hydrolysing other substrates (ATP, glucose-6-phosphate, phosphoglyceric acid, RNA). Hayes *et al.* (2000) also used two phytase preparations, a commercial and a purified preparation, to characterise the enzyme lability of organic P extracted from soils. The purified phytase showed a specific activity for phytate over 12 times greater than that of the commercial phytase preparation and also showed a narrower substrate specificity. They attributed this result to the contamination of the commercial phytase preparation by phosphatase due to the similarity in behaviour to that of wheat-germ acid phosphatase. He and Honeycutt (2001) tested the substrate specificity of two phytase enzymes (a crude wheat phytase and *Aspergillus ficuum* phytase) and two phosphatase enzymes (acid and alkaline) on 14 phosphorus compounds. The two phytase enzymes showed greater activity toward phytate than the acid or alkaline phosphatase but also showed activity towards twelve of the other P compounds tested including orthophosphate monoesters, orthophosphate diesters and pyrophosphate. In fact, for a majority of the other P compounds tested, the relative amounts of P released by the phytases were greater than that which was released from phytate. He and Honeycutt (2001) also suggested this result reflected the possibility that more than one type of enzyme was present in each of the enzyme preparations.

The poor specificity of the phytase preparation used in this work may also indicate the presence of other enzyme impurities. Alternatively, it may reflect the inherent properties of some phytases. However, confirmation of either one of these possibilities will require further testing. The results presented here highlight the importance of determining substrate specificity prior to enzymatic hydrolysis experiments in order to correctly identify P species that comprise the enzyme labile and non-enzyme labile fractions. Nonetheless, identifying the enzyme labile and non-enzyme labile fractions may be further complicated by the formation of new orthophosphate monoesters. Although phytase hydrolysed a substantial amount of organic P, it may also be responsible for the phosphorylation of orthophosphate. Therefore, the results of this study show that it cannot be assumed that the “non-enzyme labile” P fraction consists entirely of P species that have resisted hydrolysis. It is possible that a majority of organic P in the extract could be hydrolysable and that some of the organic P contained in the “non-enzyme labile” fraction is in fact newly synthesised P. If these results are found to be general, this will limit the extent to which enzymatic hydrolysis alone can be used to

quantify potentially bioavailable organic P. This highlights the importance of determining at which point of analysis phosphorylation occurs and also the necessity of combining multiple analytical techniques to provide an accurate characterisation of soil P.

Finally, it should be noted that besides the differences in organic P concentrations determined by colorimetry in the initial enzyme hydrolysis experiments and subsequent NMR analysis, there were also differences in total P and orthophosphate. For a majority of soil extracts, total P and orthophosphate concentrations were lower when calculated by ^{31}P NMR spectroscopy than by colorimetry and enzymatic hydrolysis. A possible cause is the presence of large amounts of MES-EDTA buffer and sorbitol in the enzyme-treated NaOH-EDTA extracts. Whereas 0.5 g of freeze-dried extract is usually sufficient to provide adequate signal-to-noise ratios, this was increased to 5 g for the enzyme-treated extracts. Consequently, approximately 30% of the freeze-dried material was not dissolved and a small portion of P remained in this undissolved residue. For the Damma sample, this residue was subsequently redissolved in a second aliquot of NaOH-EDTA and analysed by ^{31}P NMR. The main P species present was orthophosphate, but a small amount of organic P was also detected (results not shown).

Conclusions

Phosphorus-31 NMR analysis of NaOH-EDTA soil extracts treated with active and inactivated phytase enzyme preparations showed that phytase hydrolysed the majority of the small orthophosphate monoester compounds (α- and β-glycerophosphate, phytate, *scyllo*-inositol hexakisphosphate) and pyrophosphate, but orthophosphate diesters (DNA) and humic P were generally unaffected. The ^{31}P NMR spectra revealed that not only was organic P hydrolysed but new orthophosphate monoester species were formed, probably as a result of enzymatic phosphorylation.

This study has demonstrated that combining enzymatic hydrolysis with ^{31}P NMR spectroscopy is well-suited to identifying individual organic P species that are susceptible or resistant to enzyme hydrolysis. Although these findings highlight the potential for combining these techniques in future studies to characterise soil organic P, further improvement and refinement of the technique is required. Most importantly, in developing a standard protocol it will be necessary to examine the factors controlling enzymatic dephosphorylation (hydrolysis) and phosphorylation (esterification). This may include implementing conditions that prevent or minimise the synthesis of new organic P compounds by either removing the substrate (sorbitol) or deactivating the enzyme prior to NMR analysis. Consideration should also be given to the specificity of commercially available enzyme preparations, since this current study, as well as previous studies, have shown that the hydrolysed organic P may contain a wide variety of different organic P forms.

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CHAPTER 7

SUMMARY, CONCLUSIONS AND FUTURE RESEARCH PRIORITIES

Phosphorus (P) is present in many different forms in soils. At any one time, only a small fraction of total soil P is in a form directly available for plant or microbial uptake. It is generally accepted that this directly available form equates to orthophosphate (inorganic P) in soil solution. However, a large portion of the remaining soil P is organic P which can be converted into available orthophosphate though hydrolysis or mineralization.

Organic phosphorus therefore plays a vital role in soil biochemical cycling because of its potential availability for plant uptake and role in soil fertility. Developing a better understanding of the forms, origins and bioavailability of organic P in soil systems is of high importance. This is particularly so in Australian soils that are generally characterised by low P concentrations by world standards. However, improving this understanding relies on analytical techniques that enable the accurate characterisation of soil organic P.

Phosphorus-31 NMR spectroscopy has become increasingly popular for the analysis of soils and manures to examine the distribution of organic P compounds. The main reason being that of all the currently available techniques, it provides the most detailed and accurate information. However, potential sources of error in the established solution ^{31}P NMR technique still exist.

The investigations outlined in this thesis were focused on improving techniques for the characterisation of soil organic P using solution ^{31}P NMR spectroscopy, applying these techniques to characterise a range of Australians soils and developing a better understanding of the cycling and potential bioavailability of soil organic P. The main outcomes in this thesis were:

- The expanded application of an improved spiking procedure to assign organic P resonances in the ^{31}P NMR spectra of soil extracts (Appendix 1), the development of an improved method of spectral deconvolution and use of an internal standard to provide an accurate quantitative assessment of P forms in the ^{31}P NMR spectra of soil extracts.

- The identification of the abundant forms of soil organic P in a range of Australian soils.

- The determination of the susceptibility of phytate to microbial degradation when applied to a calcareous soil that has properties reportedly associated with the stabilisation of P.

- The identification of the enzyme labile fractions of several Australian, Swiss and Kenyan NaOH-EDTA soil extracts by using a combination of enzymatic hydrolysis and solution ^{31}P NMR spectroscopy.

As a consequence of the four main findings above, the research contained in this thesis demonstrated the importance of developing suitable analytical techniques for the accurate characterisation and quantification of soil organic P. Furthermore, it questioned our current understanding of the abundant forms of soil organic P and bioavailability of soil organic P forms. Below is a summary of the findings presented in this thesis. It provides a link between the four main outcomes of this thesis to build upon our current understanding of soil organic P as well as highlighting the potential direction and priorities for future P research.

The development and application of improved techniques for the identification and quantification of soil organic P forms

Spiking of NaOH-EDTA soil extracts

The benefit of directly spiking NaOH-EDTA extracts with model compounds of interest is that the potential for the misassignment of resonances is greatly reduced since the problem of variation in chemical shifts of P species (as a result of differences in pH, temperature, concentration of paramagnetic ions and ionic strength) among different soil extracts is eliminated. That is, the spiked solution produces a spectrum of the model compound under the exact conditions in each soil extract. A series of spiking experiments identified α- and β-glycerophosphate, phytate, adenosine-5´-monophosphate (AMP) and *scyllo*-inositol hexakisphosphate as the seven dominant resonances common to all spectra of the Australian soil extracts analysed. The importance of spiking was highlighted when *scyllo*-inositol hexakisphosphate and choline phosphate were spiked into the Tocal extract (Chapter 2) and showed a difference in chemical shift of only 0.12 ppm. Without spiking, those species would have been indistinguishable. Similarly, in the Damma soil extract (Chapter 6) it was only possible to distinguish one of the phytate peaks from the β-glycerophosphate peak by spiking. Therefore, previous ^{31}P NMR studies may have provided erroneous estimates of organic P though the misassignment of peaks, consequently, the spiking technique shown in this thesis should be used to improve the accuracy of peak identification.

Identification and quantification of humic P using ^{31}P NMR spectroscopy

Spectral deconvolution is often used to quantify the concentration of P species from spectra. Spectral deconvolution involves a numeric least-squares fit of the spectrum as the sum of multiple peaks of standard shape. Previous ^{31}P NMR studies that have used deconvolution only attempt to fit P species that give rise to the sharp peaks in the spectrum and therefore only take into account P that can be attributed to specific P-containing compounds. Consideration is not given to P contained in larger humic molecules. Since the P contained in the humic molecules is in a variety of different forms and therefore subtly different chemical environments, these large molecules do not produce sharp resonance but instead a broad signal which is often not accounted for in the deconvolution of spectra. Two methods of spectral deconvolution were applied to quantify the spectrum of a soil spiked with

phytate (Chapter 3, Appendix 2). One method of deconvolution attempted to fit the broad signal to the spectrum in addition to the routinely fitted sharp peaks, whereas the other did not. The results showed that although the fit of both deconvoluted spectra appeared to be superficially similar, the estimates of the concentration of P species were very different. The spectrum that did not fit the broad resonance was found to over-estimate the concentration of phytate by 54% and was also likely to have also over-estimated the concentration of other specific orthophosphate monoester compounds, consequently underestimating humic P forms. In this thesis, it was shown that the presence of a broad signal in the orthophosphate monoester region (3.5–6.5ppm) can complicate the deconvolution procedure but must be accounted for to accurately determine P speciation from NMR spectra.

Using methylenediphosphonic acid (MDP) as an internal standard

Quantification of soil P using solution ^{31}P NMR assumes that all P forms in the freeze-dried NaOH-EDTA soil extracts are soluble and are detected by the spectrometer, although this is unlikely to be the case. As was noted when analysing the enzyme treated soil extracts (Chapter 6), even a small amount of undissolved extract can result in considerable differences in absolute P concentration. Commonly in NMR studies, the absolute concentration of each P species is calculated by multiplying its relative contribution to total NMR signal by the total NaOH-EDTA extractable P concentrations determined by ICP-AES. However, P concentrations of the individual compounds are likely to be over-estimated if all P forms are not soluble. In this thesis, the addition of methylenediphosphonic acid (MDP) provided an absolute observable P concentration. Using spectral deconvolution and integrating the signals from the P species in the spectra against the signal from the MDP standard, the use of an internal standard provided an accurate quantitative analysis of soil P.

The potential for over-estimation of phytate in soil

The refinement of analytical techniques highlighted the potential for the over-estimation of phytate in NMR analysis of soil extracts. This is the result of the potential misassignment of peaks and failure to include the broad signal in the orthophosphate monoester region when using spectral deconvolution to quantify NMR spectra. While it is generally accepted that phytate is an abundant form of phosphate monoester in soils, the intensity of the phytate peaks in the spectra of the 18 Australian soils presented (Chapter 2, 5) showed that phytate was not the dominant form of organic P in all soils. Although phytate may constitute the majority soil organic P in some soils, phytate comprised no more than 10% of total extractable P in any of the soils analysed in this study. Phytate was clearly an important component in some soils (Tocal and Togari), but even in these soils it comprised less than 22% of total extractable organic P. These results contradict the current paradigm of phytate generally being abundant and stable in soils.

An appraisal of published ^{31}P NMR spectra of NaOH-EDTA soil extracts indicated that in numerous cases reportedly high phytate contents may have been over-estimated. It appears likely that in some cases α- and β-glycerophosphate have been misassigned as phytate. Furthermore, the broad signal observed in this study and identified as humic P is also apparent in many published spectra but is never taken account of in quantifying P species via deconvolution. As discussed above, this results in an over-estimation of the concentration of specific organic P compounds, including phytate.

The suggestion that phytate P contents have been over-estimated has implication for our understanding of phytate stability in soils. The belief that phytate contents are high has always required an explanation of why a compound (i.e. phytate) that only represents a minor proportion of fresh organic P input to the soil should constitute a large proportion of organic P in soils. In general, two explanations have been offered: (i) that phytate requires specialised enzymes to be broken down, which most plant and microbes don't possess; and (ii) that phytate is stabilised by interaction with inorganic soil components through sorption and/or precipitation. By bringing into question the abundance of phytate in soils, this study also brings into question the stability and stabilisation of phytate in soils.

To further investigate these issues, two incubation experiments were undertaken (Chapter 3, 4), in which phytate was added to a soil and incubated under ideal conditions for microbial activity for 13 weeks.

The factors controlling stabilisation of phytate in soils are far from clear, although calcareous soils have been reported to have properties conducive to stabilisation of phytate (Jackman & Black, 1951; Celi *et al.*, 2000). Phytate was applied to a calcareous soil at concentrations ranging from 58–730 mg kg^{-1} (Chapter 3, 4) and the effect of wheat straw as an additional source of carbon was also examined (Chapter 3). Regardless of treatment, phytate concentrations decreased over the 13-week incubation period and were adequately fitted to a first order decay model. There was no clear trend in the rate of phytate loss with treatment and the half-life of phytate ranged from 4 to 8 weeks.

There are two possible explanations for the observed decrease in extractable phytate: (i) that the phytate became less extractable over time, and (ii) that the phytate was decomposed or converted into some other species. Two lines of evidence proved the latter to be the case. In particular, this evidence showed that phytate P was microbially degraded and converted to orthophosphate. The first piece of evidence is that the decrease in phytate concentration was accompanied by an increase in orthophosphate concentration, conforming to almost a 1:1 replacement. This was not particularly clear following the addition of low amounts (< 300 mg kg^{-1}) of phytate, because this represented only a small increase in total P (15%) and total P in sub-samples of the soil varied by a similar degree. The

strong linear relationship between phytate and orthophosphate concentrations was much clearer following the addition of larger phytate concentrations (300–730 mg kg^{-1}). The second piece of evidence is that the decrease in phytate concentration was inconsistent with the kinetics of sorption or precipitation of phytate, which would be expected to occur much more rapidly (over hours or days). If phytate was being fixed, there would be very little additional extractable P in the phytate treatments and extraction efficiencies would decline. The results showed this was not the case, since phytate was still detected after 7 weeks, there was an increase in orthophosphate concentration, and there was very little variation in extraction efficiencies.

Interestingly, the addition of wheat straw had little effect on the rate of phytate degradation. The addition of wheat straw may be expected to increase microbial demand for P as previous studies have suggested that phytate degradation can increase in response to increasing P demand. Furthermore, it is clear that phytate degradation was independent of plant P requirements as almost complete phytate degradation occurred without plants extracting P for their growth requirement. This suggests that phytate degradation in this study was not driven by plant/microbial P requirements; rather microbes were utilising phytate as a source of carbon to meet their energy requirements. This is supported by the fact that phytate continued to be degraded even when added at high rates at which P availability could not be limited (Chapter 4).

As a result of the potential over-estimation of phytate in soils, combined with evidence for the microbial degradation of phytate, the research presented in this thesis has demonstrated that phytate may not be a highly stable form of P in all soils and may be a source of bioavailable P.

Forms of soil organic P in Australian soils

A consequence of the finding that phytate represented only a small proportion of organic P in the soils analysed is that the majority of organic P remains unidentified. Two other resonances that were also prominent in all the ^{31}P NMR spectra and comprised a similar proportion of total organic P were due to α- and β-glycerophosphate (Chapter 2, 5). The α- and β-glycerophosphate resonances and the two strongest phytate resonances were deceptively similar in appearance and chemical shift, which is likely to have caused the misidentification of these species in at least some previous studies. The α- and β-glycerophosphate were likely derived from the hydrolysis of phospholipids in alkaline solution (Baer & Kates, 1950). This hypothesis was investigated by examining the degradation of phosphatidlycholine (a phospholipid) in NaOH-EDTA solutions. Due to the low aqueous solubility of phospholipids no signal was detected initially. However, after 1.5 hours, a small signal was detected and assigned to L-α-glycerophosphorylcholine. This species was subsequently hydrolysed to produce α- and β-glycerophosphate. In the starting phospholipid, the P is exclusively on the α-position of the glycerol. Therefore, simple hydrolysis would produce exclusively α-glycerophosphate. The presence

of β-glycerophosphate indicates that isomerisation occurs at some stage during the hydrolysis process. A review of the literature revealed that this had been noted previously. Furthermore, the relative proportion of α- and β-glycerophosphate produced is dependent on reaction conditions (Folch, 1942), with the β form usually being the predominant form under alkaline conditions. These findings showed the previous assignments of phospholipids and their degradation products using ^{31}P NMR spectroscopy to be incorrect; α-glycerophosphate was previously assigned as phosphatidic acid and L-α-glycerophosphorylcholine as the starting phospholipid (Makarov *et al.*, 2002; Turner *et al.*, 2003). This again highlights the importance of spiking experiments in the assignment of peaks.

The presence of α- and β-glycerophosphate in the soil extracts most likely indicates the presence of microbial biomass in soils, since they have been identified as the most prominent resonances in bacteria and fungi (Bünemann *et al.*, 2008). Whether the presence of α- and β-glycerophosphate can be used as a direct indicator of microbial biomass is yet to be determined. However, microbial biomass is often quite high in dairy soils due to high organic C and moisture contents (Burkitt *et al.*, 2007). Perhaps it was not surprising then that the concentrations of α- and β-glycerophosphate were greatest in dairy soils (Nowra, Tocal and Parkham). Similarly, in the incubation study, the soils treated with wheat straw contained marginally higher concentrations of α- and β-glycerophosphate than those in which wheat-straw was not added.

Although α- and β-glycerophosphate gave the most intense peaks in most of the ^{31}P NMR spectra of NaOH-EDTA soil extracts, the majority of signal was not contained in the sharp resonances of specific P compounds such as of α- and β-glycerophosphate or phytate. Rather, the majority of signal was contained in a broad signal in the orthophosphate monoester region (Chapter 5). It was suggested that this broad signal could be attributed to monoester P compounds found in large complex molecules such as humic acids. This humified fraction may include inositol phosphates, sugar phosphates (Omotoso & Wild, 1970) carbohydrates, proteins (Anderson & Hance, 1963), amine and other phosphate ester groups (Brannon & Sommers, 1985), incorporated into organic molecules. In the Australian soils examined, humic P comprised a substantial portion of total P (up to 39% of total extractable P and averaging 21%) compared to phytate (up to 9% of total extractable P and averaging 3%). Humic P is rarely considered in ^{31}P NMR studies (He *et al.*, 2006), although the concentrations were consistent with the concentrations of organic P in the humic and fulvic acid fractions of volcanic soils (Borie *et al.*, 1989; Escudey *et al.*, 2001; Borie & Rubio, 2003) and alpine soils (Makarov *et al.*, 1997). Since humic P potentially represents the most abundant form of soil organic P, the development of methods that aim to increase the availability of stabilised forms of organic P should give preference to increasing the availability of P contained in humic P complexes. Increasing the availability of P in individual compounds (such as phytate) that are not only present in soils at much lower concentrations but appear to be susceptible to microbial degradation, would seem to not be as beneficial.

Characterisation of soil organic P using a combination of enzymatic hydrolysis and ^{31}P NMR spectroscopy

Enzymatic hydrolysis has been used to characterise soil organic P based on its susceptibility to specific and non-specific phosphatase enzymes. Enzymatic hydrolysis relies on the detection of the hydrolysis products of organic P compounds by colorimetrically measuring the increase in inorganic P on treatment with an enzyme. Although the susceptibility of model compounds to enzymatic hydrolysis is commonly determined prior to the addition of the enzyme to the soil (Hayes *et al.*, 2000; He & Honeycutt, 2001; Turner *et al.*, 2002; He *et al.*, 2004), chemical speciation of soil organic P is assumed, rather than actually measured. It was hypothesised that combining enzymatic hydrolysis with ^{31}P NMR spectroscopy should facilitate a more complete understanding of the selectivity of enzymes by enabling the accurate identification and quantification of soil organic P, as well as gauging the susceptibility of different organic P species to enzymatic hydrolysis (Chapter 6). Individually, both techniques are commonly used for the characterisation of soil organic P but have only recently, and to a limited extent, been combined to successfully analyse manure extracts (He *et al.*, 2008).

Phosphours-31 NMR spectroscopy was used to analyse 12 NaOH-EDTA soil extracts (from Australia, Switzerland and Kenya) which had been treated with a commercially available phytase preparation. When measured colorimetrically, the decrease in organic P on treatment with active enzyme (enzyme labile P) ranged from 22 to 63%. Using ^{31}P NMR spectroscopy it was shown that the phytase hydrolysed not only the phytate, but all of the small, specific orthophosphate monoester compounds, including α- and β-glycerophosphate, phytate and *scyllo*–inositol hexakisphosphate, as well as pyrophosphate, but not orthophosphate diester (DNA) or humic P. Previous studies had indicated that commercial phytases, although sold as having a high specific activity for phytate, can have a much broader activity. The results of this study show just how broad that activity is. It is worthwhile to note the lack of susceptibility of humic P to phytase hydrolysis. Clearly, the P species incorporated in the humic fraction are contained in a highly complex and stabilised forms that are resistant to breakdown not only in a NaOH-EDTA solution but are also resistant hydrolysis by phytases and possibly other phosphatases.

Although there was a clear loss of orthophosphate monoester compounds in the soil extracts, the ^{31}P NMR spectra also revealed the formation of new orthophosphate monoester species. The newly formed orthophosphate monoester peaks were present in the enzyme preparation but a quantitative assessment of organic P in the enzyme preparation revealed the concentration of organic P was sufficient to only account for an increase in monoester-P species in the soil extracts of 30–35 mg kg^{-1}. The formation of new orthophosphate monoester species was potentially attributed to enzymatic phosphorylation (phosphate esterification). Like all catalysts, phosphatase enzymes catalyse both forward and reverse reactions, in this case both dephosphorylation of phosphate esters and

phosphorylation of alcohols. Although the thermodynamics of phosphorylation/dephosphorylation are such that the equilibrium lies towards the hydrolysis of esters (dephosphorylation), the large concentration of alcohol (50% sorbitol) in the enzyme preparation and the large concentration of orthophosphate in the soil extracts may result in the formation of a substantial quantity of sorbitol phosphate.

The concentration of sorbitol phosphate appeared to be dependent on solution conditions. Freeze-drying and reconstituting in a smaller volume for NMR analysis concentrated the soil extract, therefore increasing orthophosphate and sorbitol concentrations. This resulted in an increase in sorbitol phosphate and explains why for some soils the quantity of organic P was actually greater for the extract treated with active enzyme, than the corresponding extract treated with deactivated enzyme. Orthophosphate concentrations also differed among soil extracts and could explain some of the variation in concentration of sorbitol phosphate among samples. The formation of sorbitol phosphate was also reliant on the enzyme remaining active after freeze-drying and re-constitution of the samples. There was evidence that enzymatic activity varied among the solutions prepared for NMR analysis because some soil extracts that had sufficiently high concentrations of orthophosphate (Elizabeth Town, Parkham) did not contain substantial quantities of the new peaks. The development of a technique which deactivates the enzyme prior to freeze-drying would eliminate the potential for formation of sorbitol phosphate.

Considering that enzymatic phosphorylation occurred under the conditions required for ^{31}P NMR analysis, it is reasonable to assume that it also occurred under the conditions used for enzymatic hydrolysis. However, the position of the equilibrium would be different due to the different conditions (in terms of solution concentrations, temperature and pH) used for enzymatic hydrolysis. One way to confirm enzymatic phosphorylation using either technique, would be to spike the soil extract with orthophosphate, which should result in an apparent decrease in enzyme labile P.

In conclusion, the combination of enzyme hydrolysis and ^{31}P NMR enabled the identification of the P species susceptible to enzymatic hydrolysis, but further improvement and refinement of the technique is required in order to minimise the potential for enzymatic phosphorylation and to accurately determine the bioavailable (labile) fraction of soil organic P.

Future research directions/priorities

To address some of the issues raised in this study, and in order to continue the development of techniques for the characterisation of soil organic P and develop our understanding of P cycling in soils, further research in the following areas is recommended.

1. This study characterised 18 Australian soils using ^{31}P NMR spectroscopy. While this is the largest number of Australian soils analysed by this method in a single study, it is by no means an exhaustive survey of Australian soils. It would be naïve to assume that all agricultural soils are characterised by the same P forms, and undoubtedly other forms of soil organic P are present in some soils. Further efforts are required to improve our understanding of organic P forms in agricultural and native Australian soils using ^{31}P NMR spectroscopy.

2. The stability of phytate was only examined in a single calcareous soil, although other soil properties are likely to affect its stability also. In particular, soils dominated with metal oxides have been implicated in the stabilisation of phytate in chronosequence studies and it is likely that climate and P status of the soil will also affect the rate of phytate degradation. Of the soils examined in this study, phytate was most abundant in Damma soil from Switzerland. This soil is subject to the cool conditions of the Swiss alpine climate and this may have inhibited microbial activity. The soil used for the two incubation studies contained no native phytate and when phytate was added it rapidly degraded. However, whether the same result would have occurred had the incubated soil naturally contained phytate should also be considered. Clearly some soils contain phytate (e.g. Tocal, Wollongbar). Whether the presence of phytate in these soils is a result of it being stabilised or the input of phytate being greater than what is required for plant P requirements, microbial P requirements or as a source for carbon for microbes, requires further investigation. It is therefore necessary to test the generality of the findings presented in this thesis by examining the stability of phytate in a variety of different soils.

3. Humic P appears to be an abundant yet not fully understood nor utilised component of soil organic P. A closer examination of the forms of orthophosphate monoester contained in the humic P fraction and determining their susceptibility to other phosphatase enzymes and/or their role as a potential source of bioavailable organic P may be one strategy to overcome the P limiting conditions in Australian soils.

4. α- and β-glycerophosphate appear to be indicators of the presence of bacteria and fungi in the soil. The limitation of NMR spectroscopy is that it will not provide information leading to the identification of bacterial species, thus characterising microbial communities in soils in the same way the phospholipid fatty acid (PFLA) and fatty acid methyl ester (FAME) analysis can. If the concentrations of α- and β-glycerophosphate determined by solution ^{31}P NMR spectroscopy can accurately quantify the concentration of microbial biomass it will provide a broad indication of potential expected rates of organic matter turnover. However, whether the

concentrations of α- and β-glycerophosphate are found to reflect microbial biomass is yet to be determined.

5. The identification of soil organic P is the first step to improving our understanding of P cycling. However, combining enzymatic hydrolysis and ^{31}P NMR spectroscopy is a powerful technique since it enables the identification the soil P species and also determines their potential bioavailability. There are many aspects of this technique that must be refined and modified before it can be used with a high level of accuracy. It is necessary to confirm that the two most dominant resonances in the spectra of the active treated soil extracts were due to sorbitol phosphate. This could be confirmed by spiking the soil extract with a sorbitol phosphate solution, although this is complicated by the possibility of numerous isomers. Confirmation that the formation of the newly formed orthophosphate monoesters is a result of enzymatic phosphorylation is also required. The simplest way to test this hypothesis would be to spike the soil extract with orthophosphate which should increase the intensity and therefore concentration of the new orthophosphate monoester species. Provided the above results confirmed the phosphorylation of sorbitol phosphate, understanding the more complex aspects of enzymatic hydrolysis, namely the factors controlling enzymatic dephosphorylation (hydrolysis) and phosphorylation (esterification) would be required. More specifically, a closer examination of the effects of freeze-drying on phytase activity, identifying methods to deactivate the phytase prior to freeze-drying or alternatively undertaking a similar experiment to the one used in this study but eliminating sorbitol from the enzyme preparation could be pursued.

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APPENDICES

Appendix 1 – Assignment of all the resonances in the ^{31}P NMR spectra of the NaOH–ethylenediaminetetraacetic acid (EDTA) soil extracts analysed in this thesis.

Phosphorus Species	Chemical shift range (ppm)
Methylenediphosphonic Acid (MDP)	17–18
Unknown P	6.53–6.58
	6.34–6.36
	5.23–5.24
	5.10–5.11
	4.06–4.17
	3.04
	2.89
Orthophosphate	5.70–5.76
α-glycerophosphate	4.91–4.97
Humic P	3.95–5.45
Phytate	4.69–4.73
	4.33–4.38
	4.22–4.28
β-glycerophosphate	4.58–4.63
adenosine-5´-monophosphate (AMP)	4.47–4.52
scyllo-inositol hexakisphosphate	3.85–3.91
Orthophosphate diesters	2 to -1
Pyrophosphate	-4.5 to -5.5

Appendix 2 – Results of two deconvolution procedures to fit the solution ^{31}P NMR spectrum of a soil NaOH-EDTA extract. In (a), the fit involved seven sharp (small line-width) lines that can be assigned to individual species, whereas in (b) the fit included seven sharp lines and an additional broad peak. In both (a) and (b), individual fitted lines are shown in the upper spectra and the sum of all fitted lines in the lower spectra. In all cases the broken line represents the actual spectrum.