Investigating the Role of Stoichiometry as an Influence on Soil Phosphorus Content and Forms

A thesis submitted to The University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

Melinda R.S. Moata

BSc and MSc (Soil Science)

School of Agriculture, Food and Wine
The University of Adelaide, Australia

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To my husband, Butje A. Louk Fanggi

And my kids, Kayleen and Keanu Louk Fanggi

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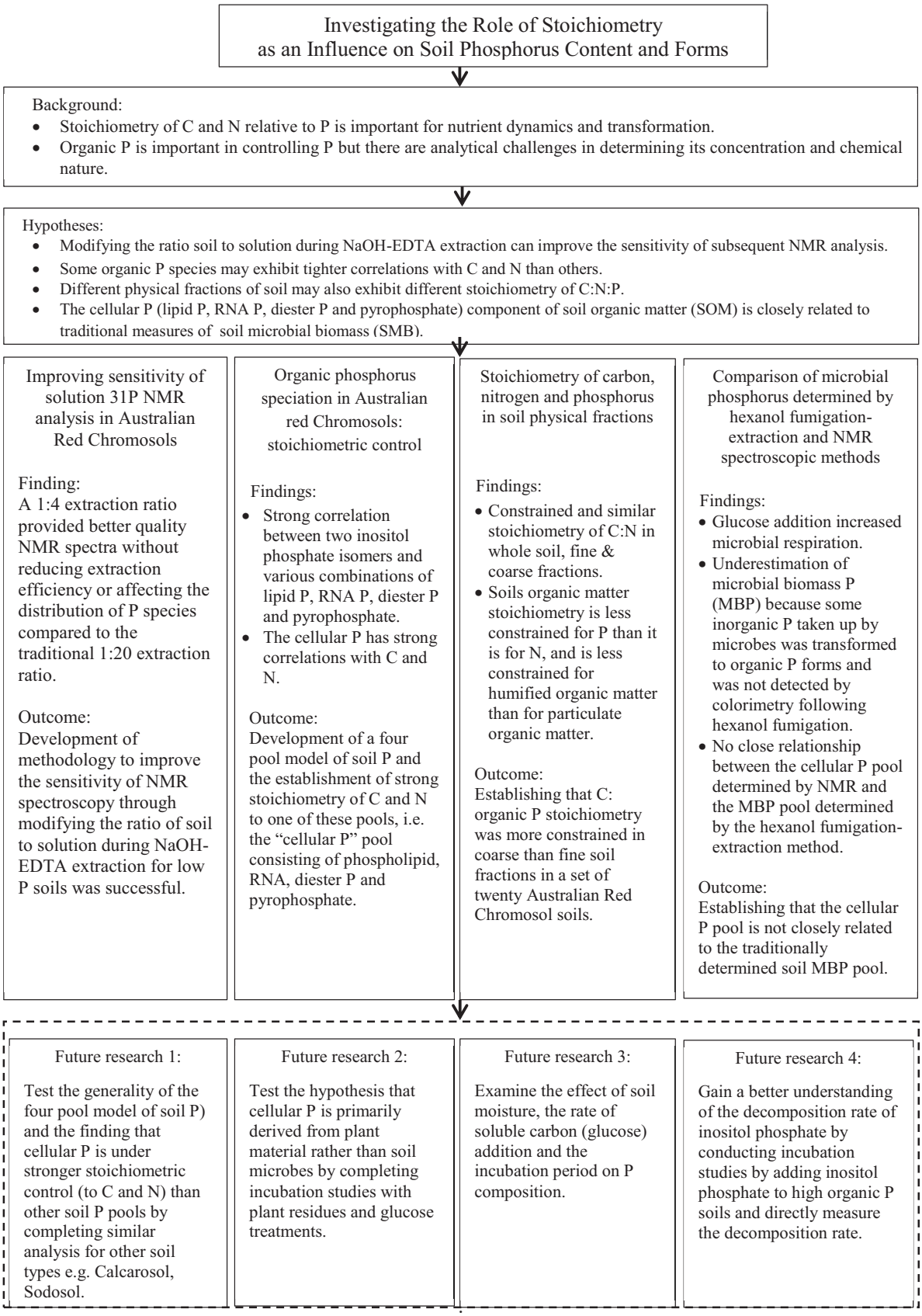
FLOW DIAGRAM OF THESIS



ABSTRACT

The elemental composition (stoichiometry) of soil organic matter (SOM) plays an important role in the dynamics of nutrient transformations in terrestrial ecosystems. Many previous studies have reported several factors affect the stoichiometry of SOM (e.g. climate, land use, soil properties) and confirmed a relatively constrained stoichiometry of carbon (C) and nitrogen (N). On the other hand, correlations of C and N to phosphorus (P) in SOM are usually much weaker. This can partly be attributed to the fact that soils contain substantial quantities of inorganic as well as organic P. Some studies have reported stronger correlations of organic P than total P to the key elements of SOM (i.e. C and N), though are still not as strong as between C and N (mostly present as organic N). There are multiple possible reasons for this, including limitations in the way organic P is measured and the diversity of organic P forms that may be present in soils. Recent advances in solution ^{31}P NMR (nuclear magnetic resonance) analysis provide an opportunity to reassess the stoichiometry of P in SOM, as NMR provides a detailed and quantitative assessment of the various classes of organic P present in soils. Thus the implementation of solution ^{31}P NMR analysis facilitates investigation of whether the weak overall stoichiometric relationships of P in SOM may be masking stronger stoichiometric control of particular SOM components or chemical forms. These issues are agronomic and environmental importance because organic phosphorus represents a substantial pool of P in soils. Although organic P is not directly plant available, it can become available to plants through microbial mineralisation. Understanding the processes involved would be of potential benefit to agricultural producers in assessing P fertility of soils and better estimating P fertiliser requirements and also to environmental managers in assessing the risk of P transfer from soils to water ways where excess P is a major cause of eutrophication. This thesis describes a range of activities aimed at improving understanding the role of stoichiometry of P in SOM.

The main limitation of solution ^{31}P NMR spectroscopy as a method for analysis of soil P is the inherently low sensitivity of NMR. This results in long acquisition times (typically one day per sample) and hence low sample throughput. Furthermore, low sensitivity limits detection and quantification of species present in low concentrations. The first part of this thesis reports on efforts to improve sensitivity by tightening the ratio of soil to solution in the extraction step preceding NMR analysis. The most commonly used procedure involves extraction with a mixture of NaOH and EDTA at a ratio of 1:20. A range of tighter extraction ratios down to 1:4 were tested for a set of four Red Chromosol topsoils with low P contents, and it was shown that at lower extraction ratios the signal to noise ratio of spectra was improved with little or no loss in extraction efficiency, there was no loss in signal resolution and no difference in the distribution of P species detected. Thus it can be confidently claimed that, at least for soils similar to those tested, employing an extraction ratio of 1:4 provides a sensitivity benefit with no detrimental effects on resolution or quantitation.

Using the improved NMR methodology, a wider set of twenty Red Chromosol soils were analysed and a range of P forms quantified in the extracts. A novel P pool structure was proposed based on strong correlations among sets of P species present. A set of four pools was proposed: (i) orthophosphate, (ii) humic P, (iii) cellular organic P (the sum of lipid P, RNA P, diester P and pyrophosphate) and (iv) inositol phosphate P (the sum of *myo*- and *scyllo*-inositol hexakisphosphate). In terms of stoichiometry, it was shown that the cellular organic P pool had closer relationships than other P pools with C and N. These findings support the overall hypothesis that some SOM pools would exhibit stronger P stoichiometry than other pools. These findings also raise the question of whether the cellular P pool is closely related to the microbial biomass P pool or a plant residue P pool.

In part, to address the possible correspondence of the cellular P pool with plant residues, physical fractionation (fine fraction < 50 μm and coarse fraction > 50 μm) of the twenty Red Chromosol soils was carried out and the elemental composition (C, N, P) of the fractions determined. Carbon concentrations were found to correlate strongly with N in both size fractions. In contrast, C concentrations correlated moderately (r^2=0.37, p <0.01) with P in the coarse but not in the fine fractions. Again, these results support the overall hypothesis that some SOM pools would exhibit stronger P stoichiometry than other pools. In particular, since the organic matter in the coarse fraction isolated in this manner, which is often referred to as particulate organic carbon (POC), is usually presumed to be dominated by plant residues, these results suggest stronger stoichiometric constraint of the plant-dominated fraction of soil organic matter. Solution ^{31}P NMR analysis was carried out on a small selection of fractionated soils and this indicated consistent differences in organic P composition between the fractions, with the fine fractions containing a larger proportion of humic P and the coarse fraction containing relatively more cellular P. Unfortunately, practical constraints relating to the amount of soil available and the low sensitivity of NMR analysis restricted the number of soil fractions that could be analysed in this way.

The final set of experiments described in this thesis addressed the potential connection between the cellular organic P pool and the microbial biomass P (MBP) pool. These experiments involved incubation of a pasture soil rich in organic matter with an easily assimilated source of carbon (glucose). Glucose addition resulted in the expected increase in soil respiration, but the effects on measured soil P pools were unexpected. The traditional measure of the MBP pool – the difference in resin P between fumigated and unfumigated soils was only slightly higher for the glucose amended soil, although closer inspection of the components of this measurement indicated a complex response in which resin P was strongly

depleted on glucose amendment but fumigation failed to release all of the extra P that had been taken up. Solution ^{31}P NMR analysis clearly showed that glucose addition did not significantly increase the concentration of cellular organic P. The implication is that the cellular P pool detected by NMR is not closely related to established methods of determining MBP.

The results presented in this thesis provide insight into the role of stoichiometry as a control on the P content of SOM. Overall, the hypothesis that weak overall stoichiometric control of P in SOM masks stronger stoichiometric control of particular components or chemical forms was sustained. In particular, amongst chemical pools, P stoichiometry was strongest for a pool identified as cellular organic P and amongst physical pools, P stoichiometry was stronger for the coarse fraction than the fine fraction. The use of combinations of techniques (e.g. NMR and size separation) and comparison of techniques (e.g. NMR and traditional measures of MBP) was important in providing this new insight and this general approach should be pursued further to better understand the complex transformations that control the fate of P in soils.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution 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. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Melinda R.S. Moata _____ **Date 7th October 2015**

PUBLICATIONS ARISING FROM THIS THESIS

Journal papers

Moata, M. R. S., Smernik, R. J., Doolette, A. L., McNeill, A. M., & Macdonald, L. M. (2015). Improving sensitivity of solution ^{31}P NMR analysis in Australian Xeralfs. *Communications in Soil Science & Plant Analysis,* **46**, 1034–1043.

Moata, M. R. S., Doolette, A. L., Smernik, R. J., McNeill, A. M., & Macdonald, L. M. (2016). Organic phosphorus speciation in Australian Red Chromosols: stoichiometric control. *Soil Research,* **54** (1), 11-19.

Conferences

Melinda R.S. Moata, Ann M. McNeill1, Ronald J. Smernik, Lynne M. Macdonald, Ashlea L. Doolette (2014). Understanding organic phosphorus speciation in agricultural soils: Correlation between P types in relation to carbon (C), nitrogen (N), and organic phosphorus (Po) compounds. *The 20th World Congress of Soil Science (20WCSS)*. Jeju-South Korea, 8-13 June 2014, (Oral presentation).

Melinda R.S. Moata, Ann M. McNeill, Ronald J. Smernik, Lynne M. Macdonald, Ashlea L. Doolette (2014). A three-pool model of soil organic phosphorus. *Australia National Soil Science Conference*. Victoria-Melbourne, 23-27 November 2014, (Oral presentation).

STRUCTURE OF THIS THESIS

This thesis is presented as a combination of papers that have been published and chapters that may be submitted for publication.

Chapter 1 presents an overview of the literature on the concept of stoichiometry in terrestrial ecosystems and soil organic matter pools, especially organic phosphorus pools. This chapter also includes the aim of this research.

Chapter 2 comprises a paper published in *Communications in Soil Science and Plant Analysis*. It describes how solution ^{31}P NMR spectroscopic analysis of soil organic phosphorus can be improved for some soils by tightening the ratio of soil to solution during NaOH-EDTA extraction.

Chapter 3 comprises a paper that has been accepted for publication in *Soil Research*. It describes an investigation into organic P speciation in Australian Red Chromosol soils, with a focus on stoichiometric control of C, N and P in the organic matter of these soils.

Chapter 4 describes an investigation of the stoichiometry of C, N, and P in physical fractions (fine and coarse fractions) of Australian Red Chromosol soils. These results have not been submitted for publication.

Chapter 5 describes an incubation experiment aimed at examining the effect of adding glucose to soil on the rate of soil respiration and the size of the microbial biomass P pool. These results have not been submitted for publication.

Chapter 6 provides a synthesis of the findings contained in this thesis and includes recommendations for future study.

CHAPTER 1

LITERATURE REVIEW

The concept of stoichiometry

Stoichiometry is a term used to describe the relationship between two or more chemical elements. In ecology, stoichiometry is applied at a number of trophic levels from specific organisms to ecosystems. The word stoichiometry comes from the roots *στοιχεα*, which means letter or basic matter, and *μετρν*, which means measure; this conveys the concept that stoichiometry relates to the linkage between chemical formula and reactions between elements (Fink 2009). The concept of stoichiometry can be summarised as a measure of the relative quantities of chemical elements in living or non-living biomass (Schlesinger *et al.* 2011).

It is important to understand stoichiometry at an ecological level because it provides a conceptual basis for understanding how transformations of elements are linked. In an ecosystem, transformations involving one element are likely to affect other elements. Ecological stoichiometry is an approach that conceptualises the constraints and consequences of mass balance of multiple chemical elements in ecological interactions that support the functioning of the ecosystem (Elser *et al.* 1996; Elser and Urabe 1999; Sterner and Elser 2002). Another benefit of understanding stoichiometric relationships is that it allows prediction of changes in one element if we know how another element is changing. In this way, stoichiometry can be used to predict the quantitative relationships involved in nutrient cycling in ecosystems (Macdonald and Baldock 2010), production, and food webs (Hessen *et al.* 2004).

Although conceptually appealing, the idea that changes in one element can be used to estimate changes in other elements (Schlesinger *et al.* 2011) should not be viewed as a fundamental law of nature. Sterner (2004) argued against an assumption of simple

stoichiometric controls. For a start, resource limitations are not always restricted to a single nutrient, or even any nutrient, as other resources such as light or suitable habitat can also be limiting. Furthermore, even when different species are faced with exactly the same limitations, every organism will vary in their use of essential elements, and therefore vary in their element ratios. Sterner (2004) made it clear he was not arguing against the concept of ecological stoichiometry *per se*, but rather against a blanket acceptance of its fundamentality.

In terrestrial ecosystems, and indeed in all ecosystems, carbon (C) is the central element involved in organic matter transformation. There are several reasons for this: (i) C constitutes the majority of the structural skeleton of organic matter; (ii) oxidation of C provides the majority of energy flow; and (iii) the balance between sequestration and release of C is central to impacts on global climate. During microbial processing of soil organic matter (SOM), some C is released to the atmosphere as CO_2, some remains as SOM in a transformed state, while the remainder is incorporated into soil microbial biomass. As part of the process by which C is used as a source of energy for soil microbes, other nutrient elements (e.g. nitrogen-N, phosphorus-P, sulphur-S) contained in organic matter are transformed into inorganic forms that are then available for plant uptake. These nutrients are also required for cell production in plants and microbes.

Sterner & Elser (2002) discuss the range of potential relationships between organism stoichiometry and resource stoichiometry in terms of organism homeostatis, i.e. the ability of organisms to maintain control of their internal conditions, including their chemical composition. Figure 1 shows the extremes of homeostatic control: a complete lack of homeostatic control would result in a 1:1 relationship between organism stoichiometry and resource stoichiometry ("you are what you eat"), while strict homeostatic control would mean

that resource stoichiometry has no effect on organism stoichiometry. In the case of microbial decomposition of SOM, the more strict homeostatic control is, the more likely that microbial àgrowth and SOM decomposition depends on a limiting nutrient. The final possibility illustrated in Figure 1 is variable homeostatic control, in which the degree of homeostasis varies with resource stoichiometry.

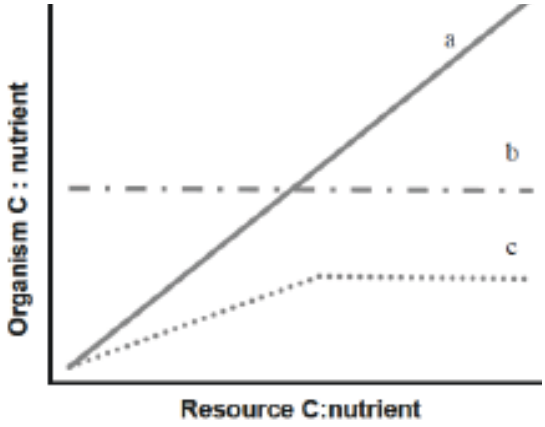

Figure 1. Stoichiometric homeostatis: a) no homeostatic control, b) strict homeostatic control, and c) variable homeostatic control (Macdonald and Baldock 2010)

Stoichiometry in terrestrial systems

The concept of ecological stoichiometry was originally developed for describing aquatic systems, for which Redfield (1958) famously reported a constrained ratio C:N:P for planktonic biomass and marine water that has become known as the Redfield ratio. Subsequently, similar patterns have been sought for terrestrial ecosystems, including plants and soil. McGroddy *et al*. (2004) investigated the elemental stoichiometry of foliar and litter biomass of forest species. They found that these were more variable than for marine systems – and also more C-rich, reflecting the predominance of C-rich cellulose and lignin in plants – but when different forest types (temperate broadleaf, temperate coniferous and tropical) were considered separately, C:N:P ratios were quite tightly constrained. They also reported litter

biomass to be more C-rich than foliar biomass, resulting from substantial resorption of nutrients from senescent leaves. Cleveland and Liptzin (2007) investigated the C:N:P stoichiometry of soil microbial biomass (SMB) and whole soil under different land uses (grassland and forest). They reported that SMB has a lower C:N:P ratio (60:7:1) than whole soil (186:13:1) and that SMB in grassland systems tend to be more P-rich. Similar soil microbial biomass (SMB) C:N:P stoichiometry has been reported for a large set of soils (woodland and cropping) from subtropical southern China (70.2:6:1), but with a less C-rich C:N:P stoichiometry for whole soils (80:7.9:1) (Li *et al.* 2012). In another, more wide-ranging study of soils from across China, Tian *et al*. (2010) reported that C:N stoichiometry was tightly constrained, while C:P and N:P ratios varied quite widely.

Stoichiometry of soil organic matter (SOM)

In terrestrial systems, SOM plays a significant role in biochemical processes and is a vital component of productive soils, where it enhances chemical, physical and biological properties (Baldock and Skjemstad 1999; Macdonald and Baldock 2010; Tate 1987). Along with the "structural" elements of C, hydrogen (H) and oxygen (O), SOM contains substantial quantities of the essential plant macronutrient elements N, P and S. In addition, key chemical properties of soil, such as cation exchange capacity and pH buffering capacity can be provided by SOM. In terms of physical properties, SOM improves soil aggregate stability, soil structure and water retention. Biologically, the activity and population of the microbial community in the soil are influenced by SOM as it provides a source of energy. Although ecological stoichiometry was principally established to understand the living part of the ecosystem (i.e. the living organisms), it is also useful for understanding the non-living component.

The key elements involved in nutrient cycling are C, as the energy driver for soil microbes, and the macro-nutrients N and P, which are considered the most important limiting elements for vegetation in terrestrial ecosystems (Manzoni *et al.* 2008) due to their high concentration in plants and relatively low concentration and/or availability in soil. The stoichiometry of C:N is widely used as a predictor for N mineralization and immobilization in soil. In general, for residues characterised by C:N < 25 net mineralization will occur, whereas a C:N > 40 tends to promote immobilization (Macdonald and Baldock 2010; Manzoni *et al.* 2010; Martens 2000). Corresponding critical values of C:P ratios have been suggested, with C:P < 200 resulting in net mineralisation and C:P > 300 resulting in net immobilisation (Manzoni *et al.* 2010; Stevenson and Cole 1999).

A number of studies have investigated the effect of the stoichiometry of residues on their decomposition rate in soil and have shown the influence of several factors. One finding is the C:N:P ratio of plant litter influences decomposer activity (Manzoni *et al.* 2010) and another is that the C:N ratio is influenced by the biochemical quality of crop residues, especially the amount of C-rich components, such as cellulose, hemicellulose, lignin, relative to nutrient-rich components, such as protein, phospholipid and nucleic acids (Manzoni *et al.* 2010; Trinsoutrot *et al.* 2000). Conversely, other studies have found that the C:N ratio of plant residues was not a significant predictor of decomposition rate, which was more influenced by plant species (Gill *et al.* 2006) and soil fractions (Ostrowska and Porębska 2015). Together, these studies show that while stoichiometry can influence biochemical reactions of nutrients and in some instances can be used to predict the quality and function of SOM, other situations appear to be more complex. A likely contributor to this complexity is the heterogeneity of SOM, which in most cases is not a single homogeneous material, but rather a mixture of components or fractions.

Soil organic matter pools

Soil organic matter consists of multiple pools of different composition. What constitutes SOM is a matter of some debate. It has been defined as 'whole organic matter in soils, including the litter, the light fraction, the microbial biomass, the water-soluble organics, and stabilized organic matter (humus)'(Clapp *et al.* 2005). Others have defined SOM as organic materials found in the soil or on the surface soil that can be divided into living organic matter and non-living organic matter (Baldock and Skjemstad 1999). Living OM consists of phytomass, microbial biomass (MB), and faunal biomass, while non-living OM is often classified as dissolved OM (DOM), particulate OM (POM), humus and inert OM (IOM) (Figure 2). In this scheme, organic litter on the surface soil is considered a part of POM. In this context, humus comprises amorphous organic materials and includes materials traditionally identified as non-humic (polysaccharides, protein, waxes, and lignin) as well as humic substances (humic acid, fulvic acid, and humin) < 53μm (Baldock and Skjemstad 1999). POM is any organic fragment with recognizable cellular structure of plant or animal debris, including partially decomposed material > 53 μm. Some researchers further divide POM into three sub-fractions, namely: free light (1.85 g cm^{-3}), coarse (250-2000 μm), and fine (53-250 μm) fractions (Six *et al.* 2001). DOM is defined as < 0.45 μm diameter organic materials in solution.

These pools vary in size and decomposability. Humus is often the largest pool, typically comprising over 50% of total SOM, followed by POM (typically 25%), DOM (typically 15%), and inert OM (typically 10%). The size of each pool depends on the relative rates of addition and decomposition, and is controlled by numerous factors including residue type, climate, soil and edaphic factors. Transfer between pools is mostly mediated by microbial activity. In general, pool size has a negative correlation with decomposition rate, thus the

largest pools tend to be those that decompose most slowly (McLauchlan and Hobbie 2004). Dissolved OM has higher decomposability compared to other pools. Finally, inert OM is distinguished by its low decomposability. It includes highly carbonized organic materials, such as charcoal, and geologic forms of C. Inert OM constitutes the most variable proportion of SOM as its relative size is more influenced by the size of inputs than its rate of decomposition. Thus in some soils it is the smallest pool, despite being the least decomposable.

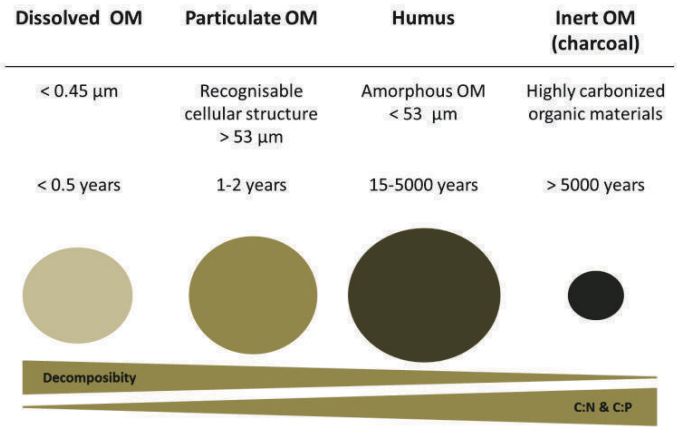

Figure 2. Soil organic matter pools (*Marschner personal communication*)

Each SOM pool has a different ability for C storage in the short and long-term and so the pool structure of a soil has complex effects on how total soil organic carbon (SOC) is impacted on when soil conditions or management change. Figure 3 shows a situation where total SOC has changed due to land use conversion from native land to pasture and intensive cultivation. Pasture increased SOC in whole soil, humus, and POM fractions, while IOM was not affected. On the other hand, conversion of the land to intensive cultivation decreased SOC in whole soil but especially in the rapidly cycling fractions.

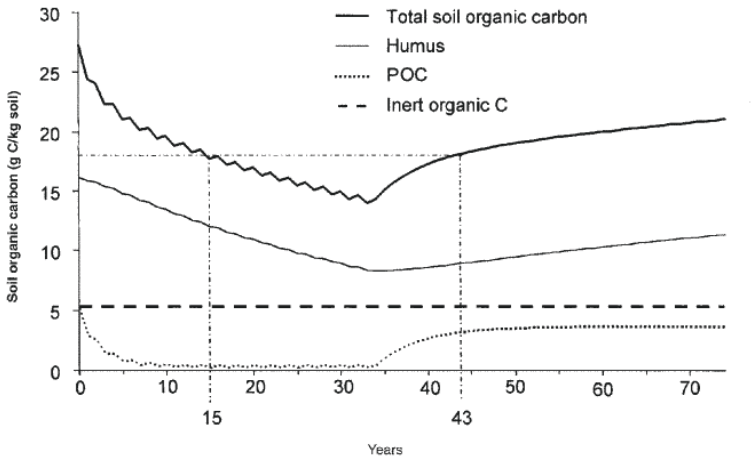

Figure 3. Soil organic carbon (SOC) in different pools (Baldock and Skjemstad 1999)

Release of nutrients and sequestration of C is a consequence of processes that involve soil microbes. Even though the SMB pool is small in size, it plays vital role in decomposition (as a decomposer) and other biochemical reactions in the soil (as a regulator/ mediator), such as nitrification, denitrification, and organic P mineralization. Mineralization releases nutrients from the organic pool to inorganic forms, such as ammonium ($NH4^{+}$), nitrate (NO_3^{-}), phosphate (PO_4^{3-}) and sulphate (SO_4^{2-}) that can be taken up by plants. However, at the same time, some of these species might be lost from the soil due to volatilization, erosion, leaching, mineral fixation, and plant and livestock removal. Inorganic forms can also be immobilized by soil microbes (i.e. converted to organic forms). The balance between organic and inorganic pools of nutrients can influence whether there is sequestration of C or release of nutrients. Detailed understanding about how C is coupled to nutrient fluxes that regulate sequestration and net balance of C in ecosystems is still lacking.

Factors affecting the stoichiometry of SOM

Numerous previous studies have identified factors that can influence the stoichiometry of SOM. These include climate, land use (vegetation), soil type, and the relative proportions of SOM fractions.

a. Climate (rainfall and temperature)

A study on the C:N:P stoichiometry of Chinese soils from different climate zones suggested that soils from tropical and subtropical areas, which have high temperatures and precipitation and thus are characterised by high rates of production of vegetation biomass with high C input and return to soil, have higher C:P and N:P ratios but C:N ratios comparable to other climatic zones (frigid, temperate, cool temperate and warm temperate) that have low C input and less P loss (Tian *et al.* 2010).

b. Land use (forest, grassland, cultivated and native land)

In general, more vegetation production results in greater input of C to soils. However, in addition, C:N varies with the vegetation type, which in turn influences SOM stoichiometry and nutrient cycling (Knops and Tilman 2000). In comparing forest and grassland soils, Cleveland & Liptzin (2007) found a relatively fixed C:N stoichiometry across land uses but varying C:P and N:P ratios. The uniformity of C:N ratios was attributed to their being presumably derived solely from plants, while P was suggested to be derived from both mineralisation of SOM and weathering of primary minerals.

c. Soil factors (type, depth and other factors)

The ratio of C: nutrient has been reported to vary according to soil type, development and depth. A study of the fine fraction (<0.4 mm) of Australian soils from different agro-ecological areas found heavy clay soils had C:N (10.6) and C: P (40) ratios similar to those of sandy loams (11.9 and 40 respectively) but lower than those of sandy soils (13.3 and 60, respectively); sandy clay loams had the highest C:ntutrient ratios (12.3 for C:N and 97 for C:P) (Kirkby *et al.* 2011; Kirkby *et al.* 2013). It should be noted that this variation could also be influenced by land use and climate, which co-vary with texture. Elemental stoichiometry has also been found to vary with soil development in chronosequences (Tian *et al.* 2010). Amongst soil types in a study of Chinese soils, Histosols had higher C:N (17.4), C:P (340) and N:P (17.8) ratios than other soil orders including Andisols, Mollisols, and Alfisols (Tian *et al.* 2010). Histosol soils are comprised of extensively humified litter layers rich organic C and are characterised by long weathering times. This study also suggested that topsoils with high organic matter and microbial activity have a fixed stoichiometry of C:N:P similar to that reported by Cleveland and Liptzin (2007). Soil from 0-10 cm in depth had average C:N:P ratios of 134:9:1, while soils from a depth of 250 cm had lower average C:N:P ratios of 60:5:1. It appears that in general there is little variation of C:N ratios between soils differing in climate, soil order, soil depth, or weathering period, but greater variation in C:P and N:P ratios (Cleveland and Liptzin 2007).

The age of soils and other factors can also influence C:N:P stoichiometry, though not in all cases. One study found that the C:N ratio of litter, forest floor soils, and mineral soils are constant over stand age, even though plant tissue composition varies with stand age (Yang and Luo 2011). However, another study in the McMurdo Dry valleys ecosystem of Antarctica found that N and P significantly varied in aquatic and terrestrial ecosystems. Carbon

concentration was high and N and P low in younger soils; however, across a wide range of ages, N concentrations increased relative to C and P in older soils (Barrett *et al.* 2007). In addition, the ratio of C:N and N:P tended to decrease as soil aged, but the ratio of C:P tended to increase. Thus, landscape position affects the stoichiometry of nutrients and can impact upon availability of N and P in terrestrial ecosystems.

In summary, organic matter and C: nutrient ratios are vital factors affecting the amount and balance of mineralisation and immobilisation. Carbon almost always has a strong correlation with N (e.g Li *et al.* (2012); Kirkby *et al*. (2011); Vitousek (2004)) but not always with P. This results in C:N ratios that are quite uniform (6.9-14.6; r^2=0.98), while C:organic P ratios are more variable (20-259; r^2=0.40) (Li *et al.* 2012).

Phosphorus pools and fluxes

The particular focus of this thesis is the stoichiometry of P in soils. Phosphorus is an essential nutrient and exists in soil in both inorganic and organic forms. Only a small portion of inorganic P, in the form of orthophosphate in soil solution, is directly available to plants. By contrast, organic P species are not directly plant available, whether they are in soil solution or not. However, organic P can become available through mineralisation (Figure 4). Transformation of P between organic and mineral forms is widely believed to always involve microorganisms. As suggested by Stevenson and Cole (1999), microbial activity releases P to solution from labile and stable organic P forms. Rapid transformation of dissolved organic P to orthophosphate is mediated by microbial hydrolysis (Sims and Pierzynski 2005). On the other hand, microbes can also compete with plants by assimilating orthophosphate from soil solution, as well accessing P from plant residues and the stable organic P pool. In the P transformation process, a decrease in mineral nutrient levels with time indicates net

immobilisation and an increase indicates net mineralisation; if levels remain unchanged, it means that rates of immobilisation and mineralisation are equal but does not mean that the internal cycle is not operative (Stevenson 1994). Quantification of various pools is most commonly carried out through sequential extraction (Hedley and Stewart 1982).

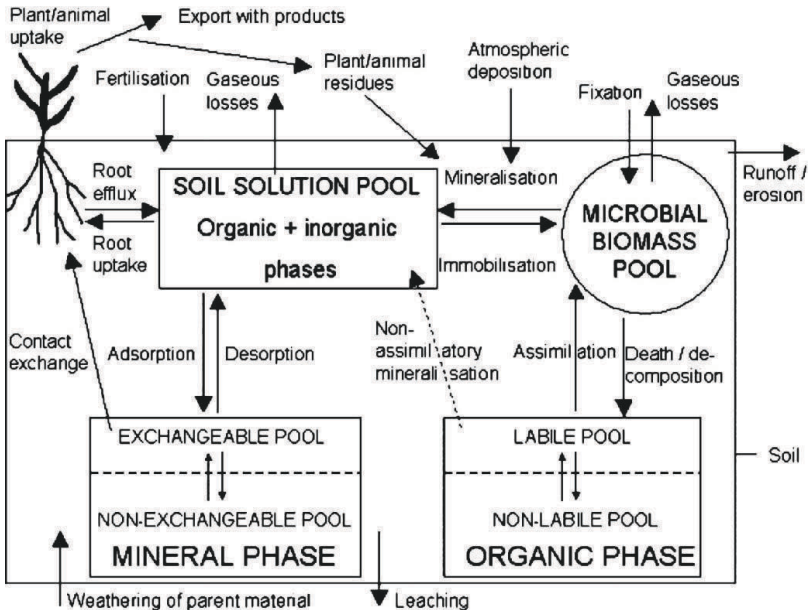

Figure 4. P cycling in terrestrial ecosystem, reproduced with permission from Springer (Bünemann and Condron 2007)

The soil microbial population can act as both a sink and source of nutrients. The amount of P held in microbial biomass (i.e. microbial biomass P or MBP) is usually measured by fumigation-extraction (FE) methods using chloroform or hexanol to kill and lyse the microbial cells prior to extraction. The difference between fumigated and non-fumigated samples is identified as MBP (Brookes *et al.* 1982). Corrections based on recovery of an added P spike are used to correct for sorption, incomplete release and extractability of the soil, with recoveries typically in the range 0.32-0.57 (Brookes *et al.* 1982; Hedley and Stewart 1982; McLaughlin *et al.* 1986). The correction factor may vary among soils and microbial communities (Achat *et al.* 2010; McLaughlin *et al.* 1986). The mostly common used method to measure MBP is liquid hexanol fumigation-extraction (McLaughlin *et al.*

1986). Hexanol fumigation provides similar results to chloroform fumigation (Brookes *et al.* 1982; Hedley and Stewart 1982) but hexanol is less harmful and reportedly more effective (McLaughlin *et al.* 1986). Following extraction P sorbed to the anion-exchange resin membrane (Kouno *et al.* 1995) is eluted with 0.1 M NaCl/HCl and P measured by colorimetry.

Organic P speciation using solution ^{31}P NMR spectroscopy

Solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy is widely used to provide detailed chemical speciation of organic P (Cade-Menun and Liu 2014; Cade-Menun and Preston 1996; Doolette and Smernik 2011; Doolette *et al.* 2011; Makarov *et al.* 2002; Newman and Tate 1980; Turner 2004; Turner *et al.* 2003). This method usually involves an initial NaOH-EDTA extraction (Bowman and Moir 1993), which has been found to optimise solubilisation of organic P and facilitate the best quality NMR spectra (Cade-Menun and Liu 2014; Doolette and Smernik 2011; Turner *et al.* 2003). A typical solution ^{31}P NMR spectrum of a soil extract is shown in Figure 5. Several inorganic P species are identified, with orthophosphate the predominant species, but pyrophosphate and polyphosphate also detected. Three classes of organic P species are identified based on their appearance in distinct regions of the spectrum, with orthophosphate monoester (~3-6 ppm) and diesters (-2 to 2 ppm) the most abundant and phosphonates (15-30 ppm) present in low concentrations.

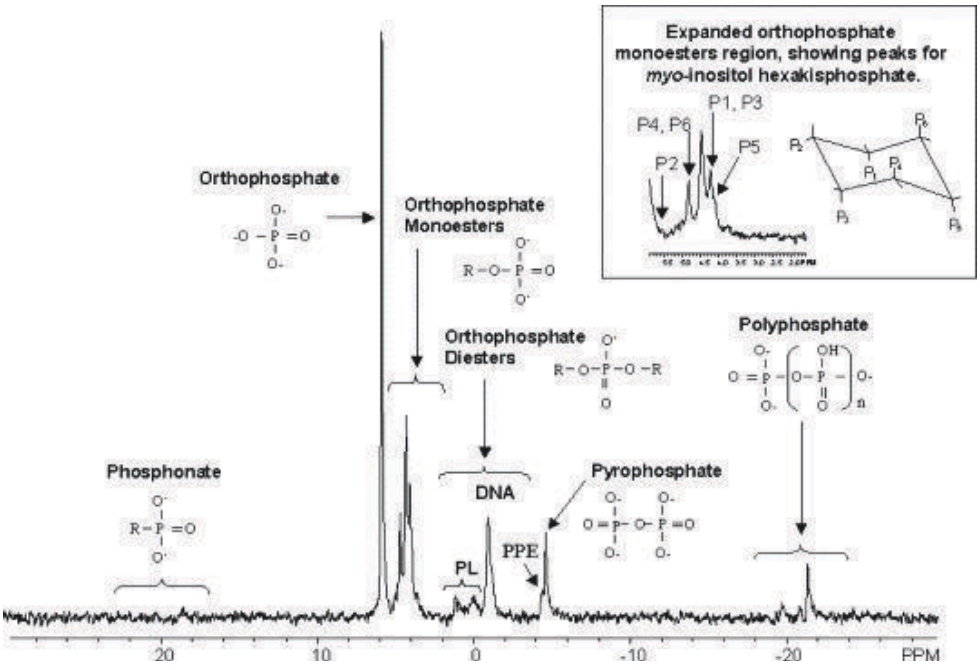

Figure 5. Solution ^{31}P NMR spectrum shows diversity of P species; PL (phospholipid), DNA (deoxyribonucleic acids), reproduced with permission from Elsevier (Cade-Menun 2005)

Numerous ^{31}P NMR studies have shown organic P is present in a wide range of chemical species in soils (Cade-Menun 2005; Condron *et al.* 2005; Turner *et al.* 2003). In alkaline extracts, most organic P is detected in monoester form; however, as discussed below, this is partly due to rapid alkaline hydrolysis of some diester species, including phospholipids and RNA, to monoester products (Turner *et al*. 2003b; Doolette *et al.* 2009; Smernik *et al.* 2015). Inositol phosphates are an important class of monoester compounds in soils. The most abundant of these is usually *myo*-inositol hexakisphosphate, otherwise known as phytate. Phytate is the main P storage compound in plant seeds (Bassiri and Nahapetian 1977; Noack *et al.* 2012; Raboy 2007) and has also been found in other plant parts (Noack *et al.* 2012). Isomers of phytate that differ in the arrangement of phosphate groups around the base inositol ring have also been reported in soils, the most abundant of which is *scyllo*-inositol hexakisphosphate (Condron *et al.* 2005; Turner 2007; Turner *et al.* 2005; Turner *et al.* 2002).

The most abundant forms of organic P in living cells are diesters in the form of phospholipids and the nucleic acids RNA and DNA. Under the high pH conditions of NaOH-EDTA extraction, phospholipids rapidly degrade to the monoester compounds α- and β-glycerophosphate (Baer and Kates 1950; Doolette *et al.* 2009), and RNA is depolymerised to 2'- and 3'- mononucleotides (Makarov *et al.* 2002). On the other hand, DNA is relatively stable in alkaline conditions and remains as a diester and in polymeric form. Thus DNA is assumed to account for most diester P identified by solution ^{31}P NMR spectroscopy of NaOH-EDTA extracts.

The distribution and composition of the various organic P species present in soil have been found to vary with geographical region (Stevenson and Cole 1999), pedogenesis (McDowell *et al.* 2007), soil type (Cade-Menun 2005; McLaren *et al.* 2014) and land use (Ahlgren *et al.* 2013). Besides soil, plant residues (Noack *et al.* 2012) (Figure 6) and microbes (Bünemann *et al.* 2008) (Figure 7) have also been shown to exhibit variation in P species composition using ^{31}P NMR analysis.

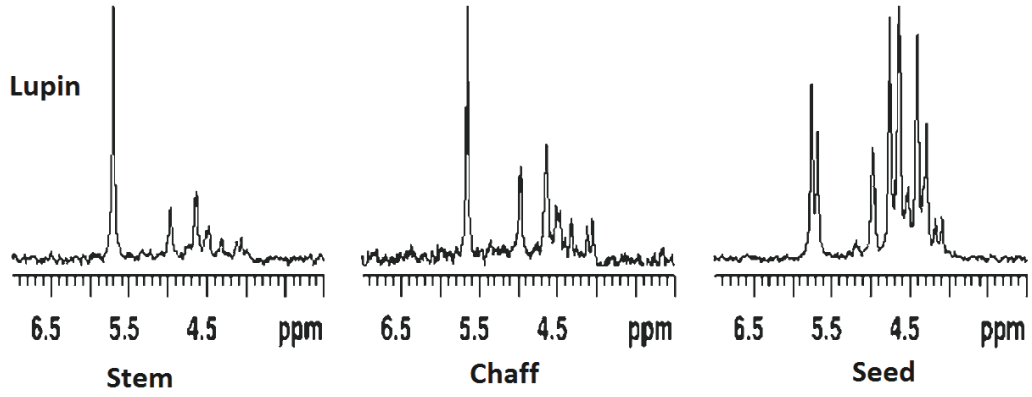

Figure 6. ^{31}P NMR spectra of NaOH-EDTA extract of plant, reproduced with permission from Springer (Noack *et al.* 2012)

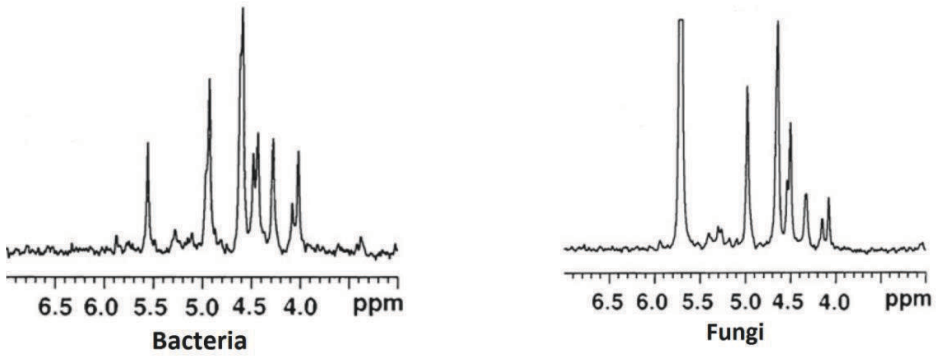

Figure 7. ^{31}P NMR spectra of NaOH-EDTA extract of bacteria and fungi and soil microbes, reproduced with permission from Elsevier (Bünemann *et al.* 2008)

An important difference in the organic P composition of soils compared to that of plant and microbial biomass is the presence of high molecular weight organic P, which has been termed "humic P" (Bünemann *et al.* 2008; Doolette *et al.* 2009; Doolette *et al.* 2010; Doolette *et al.* 2011; McLaren *et al.* 2014; Smernik and Dougherty 2007). Humic-P appears as a broad signal in orthophosphate monoester region at NMR spectra (Figure 8).

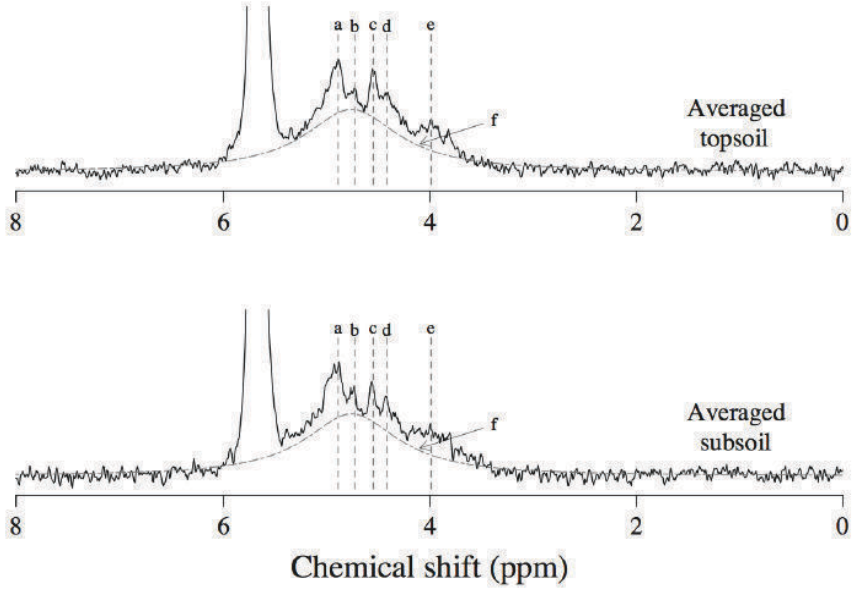

Figure 8. ^{31}P NMR spectra of NaOH-EDTA extract of topsoil and subsoil layers. Organic P species: a) α-glycerophosphate, c) β-glycerophosphate, b & d) *myo*-inositol hexakisphosphate, e) *scyllo*-inositol hexakisphosphate, and f) humic-P, reproduced with permission from Soil Science Society of America (McLaren *et al.* 2014)

Objectives of this research

A stoichiometric understanding of SOM could potentially provide significant insights into the dynamics and chemical balance of the key elements C, N and P in the soil. The elemental ratios could be used for predicting nutrient mineralization and immobilization, and the quality and function of SOM. It is clear that this understanding is much more fully developed for C:N stoichiometry than for stoichiometry involving P (i.e. C:P and N:P ratios). There are several factors that contribute to the lack of current understanding around P, but foremost is the complexity of soil P chemistry and methodological limitations of P analysis. Improvements in ^{31}P NMR spectroscopic analysis provide opportunities to address these limitations. Solution ^{31}P NMR spectroscopy enables substantial differentiation of organic P species, where traditional soil P analysis only allows determination of total organic P concentration. This development has provided new insight into the nature and complexity of organic P in soils; the recognition of humic P as a major and often dominant component of soils being one example of this. Thus far, there has been no attempt to combine detailed organic P speciation with the concept of ecological stoichiometry; this thesis addresses this gap.

The approach taken in this research was to seek further improvements in solution ^{31}P NMR spectroscopic analysis of soils and to apply this methodology to improving the understanding of P stoichiometry in SOM. The specific objectives to this research were to:

1. Investigate the potential to improve the sensitivity of NMR spectroscopy through modifying the ratio of soil to solution during NaOH-EDTA extractions;

2. Investigate the degree of stoichiometric control of P in a restricted set of agricultural soils (Red Chromosols soils from the mid-north region of South Australia);

3. Investigate whether different physical fractions of soil (coarse and fine) express more or less constrained P stoichiometry than whole soils; and

4. Investigate whether a specific P pool identified by solution ^{31}P NMR spectroscopy (cellular organic P) is closely correlates with microbial biomass P (as determined using the hexanol fumigation-extraction method).

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CHAPTER 2

IMPROVING SENSITIVITY OF SOLUTION ^{31}P NMR ANALYSIS IN AUSTRALIAN XERALFS

The work contained in this chapter has been published in Communications in Soil Science & Plant Analysis.

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Principal Author

Name of Principal Author (Candidate)	Melinda R.S. Moata		
Contribution to the Paper	Experimental development, performed analysis on all samples, data analysis and critical interpretation, wrote the manuscript		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Ronald J. Smernik		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Ashlea L. Doolette		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Ann M. McNeill		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Lynne M. Macdonald		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

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CHAPTER 3

ORGANIC PHOSPHORUS SPECIATION IN AUSTRALIAN RED CHROMOSOLS: STOICHIOMETRIC CONTROL

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Principal Author

Name of Principal Author (Candidate)	Melinda R.S. Moata		
Contribution to the Paper	Experimental development, performed analysis on all samples, data analysis and critical interpretation, wrote the manuscript		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Ashlea L. Doolette		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Ronald J. Smernik		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Ann M. McNeill		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Lynne M. Macdonald		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
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CHAPTER 4

STOICHIOMETRY OF CARBON, NITROGEN AND PHOSPHORUS IN SOIL PHYSICAL FRACTIONS

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Principal Author

Name of Principal Author (Candidate)	Melinda R.S. Moata		
Contribution to the Paper	Experimental development, performed analysis on all samples, data analysis and critical interpretation, wrote the manuscript		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Ashlea L. Doolette		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Ronald J. Smernik		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Ann M. McNeill		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Lynne M. Macdonald		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Introduction

Soil organic matter (SOM) is not a single homogeneous material, but rather must be considered as a mixture of many individual components. Some of these components are more similar to each other than others; in other words SOM differentiation is hierarchical. There are multiple different ways in which to divide or partition SOM. This division may be conceptual, in which case the components are usually referred to as "pools", or it may involve physical division, in which case the components are usually referred to as "fractions". The results presented in Chapter 3 addressed one aspect of the influence of SOM heterogeneity on SOM stoichiometry. In short, some chemical forms of P, notably phospholipid and RNA, appeared to be under stoichiometric control, whereas other chemical forms of P, notably orthophosphate and humic P did not appear to be under stoichiometric control.

The four pool conceptual model of organic P suggested in Chapter 3 is novel and was developed specifically based on the correlations observed for the P types identified using ^{31}P NMR analysis of the soils analysed. An alternative approach to investigating the influence of SOM heterogeneity on C:P stoichiometry is to appropriate fractionation schemes previously devised for other purposes. A common way to divide SOM is into living organic matter and non-living organic matter. In this case, the division is usually conceptual, although some fractionation schemes may seek to approximate this division. Further sub-divisions are possible, e.g. living organic matter can be divided into phytomass, microbial biomass (MB), and faunal biomass. Non-living organic matter, which usually comprises the majority of organic matter in soils, is usually sub-divided along different lines into dissolved organic matter (DOM), particulate organic matter (POM), humus and inert organic matter (IOM)

(Baldock and Skjemstad 1999). In such a division, organic litter on the soil surface can be considered a part of POM.

Physical fractionation usually involves separation based on particle-size and/or density. In general, these two approaches result in similar divisions, although it has been shown they are not identical (Gregorich *et al.* 2006). Physical fractionation usually involves some degree of dispersion of the soil to disrupt soil structure. By varying the level of disruption it may be possible to separate plant litter, un-complexed organic matter (a transitory pool between litter and particle-associated OM) and organoparticle complexes (Christensen 2001). When fractionated according to the method of Baldock *et al.* (2013) into DOM, POM (> 50 μm), humus (< 50 μm) and inert organic matter, most SOM is usually found in two of these: POM and humus. These fractions have different chemical properties. POM has low cation exchange and water holding capacities, and a low affinity towards complexing polyvalent metal cations (e.g. Al^{3+}) and anions (e.g. phosphate), but is a readily accessible and a rich energy source for microbial populations. This accessibility to microbes means that POM can be considered a labile pool of SOM because it is relatively quickly decomposed. Conversely, relative to POM, humus has high water holding capacity and affinity for metals, and is a less available energy source for microbial populations (Murphy 2014). Humus is thus more stable, has a slower turnover rate and can be considered a relatively stable SOM pool.

Of course physical fractionation by size has similarities to methods used to determine soil texture, in which soil is divided into clay (< 2μm), silt (2-20 μm) and sand (20 μm-2 mm). Thus, the POM fraction of the method of Baldock *et al.*(2013) consists of most sand-sized particles, while the humus consists of silt and clay, along with the finest sand particles.

The experiments described in this chapter investigate the C:N:P stoichiometry of SOM in physical fractions of the twenty Red Chromosol soils described in Chapters 2 and 3. The chemical composition of P in these fractions was determined using the ignition method of Saunders and Williams (1955) and a small detect we analysed using solution ^{31}P NMR spectroscopy.

Materials and Methods

Soil organic matter fractionation

This part of the study was carried out on the twenty Red Chromosols described in Chapters 2 and 3 which contain full descriptions of the soil locations, sample collection and preparation. Soil fractionation was carried out using a vibratory sieve shaker (Fritsch Analysette 3 Pro) according to the method of Baldock *et al.* (2013). Soil samples (10 g sieved to 2 mm) were suspended in 45 mL of 2 M NaCl (sodium chloride) in 50 mL centrifuge tubes and shaken overnight on a reciprocal shaker. Separation on the sieve shaker (50 μm size) was carried out using the following settings: 3 minutes sieve time, 20 s interval time, 2.5 mm amplitude and 1.5 modular controller (water pump). Two size fractions were thus isolated: a coarse > 50 μm fraction in which SOM was assumed to be mostly POM and a fine < 50 μm fraction in which the major SOM fraction was assumed to be humus. Fractions were collected and transferred into 500 mL bottles, frozen and freeze-dried prior to analysis. This procedure resulted in the fine fraction containing a substantial amount (approximately 5.3 g) of NaCl. Therefore, for the subsequent analysis using NMR spectroscopy, the samples were rinsed 4 times with 45 mL deionised water to remove salt.

C, N, P analyses

Total soil carbon (C) and nitrogen (N) of the soil fractions were determined using a dry combustion analyser (LECO CNS 2000). Total soil P, organic P and inorganic P concentrations were determined using the ignition method of Saunders and Williams (1955), as described in Chapter 3. The C, N and P contents of the fine fractions were corrected for the NaCl contained in this fraction.

NaOH-EDTA extracts and NMR spectroscopic analysis

Duplicate sub-samples of 8 g dry and ground (<200 μm) soil fractions were extracted by shaking end-over-end with 32 mL of NaOH-EDTA solution (0.25 *M* NaOH and 0.05 *M* Na_2EDTA) for 16 hours. A 1:4 soil to solution ratio was used, as described in Chapter 2 (Moata *et al.* 2015), because these soil fractions have low organic P contents.

Statistical analysis

Data was subjected to one way analysis of variance (ANOVA) with least significant different (LSD) $p<0.05$ to determine the effect of physical fractions on elemental (C, N, P) concentrations of SOM. Multivariate regression among C, N, and P within each physical soil fraction was carried out using SigmaPlot 12.0.

Results and Discussion

C, N and P concentrations in whole soil, coarse and fine fractions

Separation of the soils into fine (<50 μm) and coarse (> 50 μm) fractions was achieved with an average mass recovery of 99.7%. Elemental (C, N and P) recoveries were also high,

averaging 99.5, 99.9 and 98.4%. On average, 60% of soil mass was isolated in the fine fraction, with 40% in the coarse fraction (Table 1). However, there was considerable variation in the ratio of fine: coarse fractions, which ranged from 83:17 to 37:63. Unsurprisingly, the proportion of fine fraction was closely correlated with the clay content (r^2 =0.56 $p < 0.001$).

Table 1. Mass of soil fractions and the concentration of C,N, P in coarse (> 50 μm) and fine fractions (50 μm) for 20 Red Chromosol soils.

Soil ID	Fraction mass of whole soil		Coarse fraction (> 50 μm)					Fine fraction (< 50 μm)				
	Coarse	Fine	C	N	Pt	Pi	Po	C	N	Pt	Pi	Po
	%	%	mg/g	mg/g	mg/kg	mg/kg	mg/kg	mg/g	mg/g	mg/kg	mg/kg	mg/kg
1	24.0	76.0	2.8	0.3	25.0	12.5	12.5	15.6	1.6	357.9	134.0	223.9
2	56.8	43.2	3.3	0.2	50.0	24.9	25.0	18.0	1.6	509.1	304.6	204.4
3	37.5	62.5	6.9	0.9	149.9	74.8	75.1	16.3	1.6	420.1	209.7	210.3
4	45.4	54.6	3.1	0.2	49.9	25.0	24.9	23.2	2.2	417.0	245.6	171.3
5	29.1	70.9	2.5	0.2	60.9	40.1	20.8	15.4	1.3	502.2	225.5	276.7
6	56.7	43.3	8.0	0.5	100.0	50.0	50.0	12.4	1.1	296.9	118.5	178.3
7	24.0	76.0	15.9	0.9	149.9	59.6	90.3	22.9	2.2	291.5	116.5	175.0
8	35.4	64.6	9.5	0.7	137.4	87.3	50.0	19.0	2.1	463.5	273.6	190.0
9	54.6	45.4	32.1	1.9	N/A	N/A	N/A	18.5	2.0	400.5	209.1	191.4
10	47.0	53.0	7.6	0.5	62.5	49.9	12.5	17.4	1.6	306.7	122.7	184.0
11	23.6	76.4	18.6	1.3	174.7	118.1	56.7	18.8	1.9	299.4	159.4	140.0
12	23.6	76.4	3.7	0.2	175.4	158.9	16.5	12.9	1.0	429.0	240.7	188.3
13	47.8	52.2	12.4	0.9	112.4	37.4	74.9	15.3	1.5	265.1	81.6	183.5
14	24.0	76.0	8.0	0.5	99.9	75.1	24.8	11.4	1.3	355.5	196.7	158.7
15	47.8	52.2	8.6	0.7	112.4	49.9	62.6	19.3	2.0	364.2	181.9	182.3
16	37.8	62.2	12.4	0.8	125.0	74.9	50.1	22.1	2.0	354.0	166.5	187.5
17	56.9	43.1	4.4	0.2	37.4	12.4	25.0	18.5	1.6	252.0	75.5	176.5
18	62.6	37.4	11.2	0.9	249.9	149.2	100.7	14.1	1.6	774.7	630.4	144.3
19	45.0	55.0	5.6	0.4	137.5	99.9	37.6	24.7	2.1	409.4	169.5	239.8
20	54.6	45.4	4.7	0.3	50.0	25.0	25.0	12.8	1.5	271.1	73.9	197.2
Average	**41.7**	**58.3**	**9.0**	**0.6**	**108.4**	**64.5**	**44.0**	**17.4**	**1.7**	**387.0**	**196.8**	**190.2**
Max	**62.6**	**76.4**	**32.1**	**1.9**	**249.9**	**158.9**	**100.7**	**24.7**	**2.2**	**774.7**	**630.4**	**276.7**
Min	**23.6**	**37.4**	**2.5**	**0.2**	**25.0**	**12.4**	**12.5**	**11.4**	**1.0**	**252.0**	**73.9**	**140.0**
SD	**13.4**	**13.4**	**7.0**	**0.4**	**58.3**	**42.9**	**26.9**	**3.8**	**0.4**	**119.2**	**121.3**	**31.1**

N/A : Not-available due to limited materials

The concentrations of C and N were generally lower and more variable in the coarse fractions than in the fine fractions (Table 1). For all the soils, the coarse fraction was predominantly sand particles (based on observation). The organic component of this fraction (i.e. POM) was minor, as demonstrated by an average C content of only 0.9% and a maximum C content of 3.2%. Much of the variation in C content can be attributed to differences in texture among the soils, with the sandier soils containing a greater mass of sand particles, which dilutes POM to a greater degree. Thus there was a negative correlation (r^2 =0.58 $p < 0.001$) between the mass proportion of coarse fraction and the C content of this fraction. The organic component of the fine fraction was also minor (average C content 1.7%, maximum 2.5%), but much less variable. This is consistent with the finer soil fractions (i.e. silt and clay particles) interacting with (and perhaps stabilising) organic matter at a relatively consistent mass ratio. Similar relationships with N content are apparent; this reflects the generally strong stoichiometric relationships between C and N in both fractions, as discussed below.

The partitioning of total P between the fine and coarse fractions is complicated by the existence of P in both inorganic and organic forms (whereas C and N are almost exclusively present in organic forms). The total P content was much lower in the coarse fraction (average 108 mg/kg) than in the fine fraction (average 387 mg/kg). This likely reflects differences in mineralogy of the two fractions, with the coarse fraction presumably dominated by quartz particles, which not only have low specific surface area but also little capacity to sorb inorganic P. On the other hand, the fine fraction is presumably dominated by clay minerals, which have both a higher specific surface area and greater P sorption capacity.

In addition to total P content, the ignition technique of Saunders and Williams (1955) was employed to provide estimates of organic and inorganic P contents. However, it should be

noted that a number of studies have shown that this technique can overestimate the organic P concentration (Oniani *et al.* 1973; Williams *et al.* 1970), particularly for highly weathered soils (Condron *et al.* 1990).

Stoichiometry of C, N, P in different physical fractions

Figure 1 shows that carbon (C) exhibits a tightly constrained stoichiometry with nitrogen (N) in whole soil (r^2=0.90, p<0.001), the coarse fraction (r^2=0.91, p <0.001) and the fine fraction (r^2=0.81, p <0.001). A strong correlation between C and N has been widely reported for other soils, both in Australia and elsewhere (Kirkby *et al.* 2011). The wider C:N ratio for the coarse fraction (average C:N=14) relative to that of the fine fraction (average C:N=10) is consistent with the coarse fraction being derived mainly from plants (Six *et al.* 2001), as expected. The C:N ratio of the fine fraction was close to that of the whole soil (average C:N=11). Organic matter in the fine fraction is presumably mostly associated with clay and silt particles, and is likely a mix of humified plant residues and microbial residues. Similar findings were reported for a long-term study on European agricultural soils (Courtier-Murias *et al.* 2013) in which the C:N ratio of whole soils (C:N=7.9) was close to that of a heavy fraction identified as mineral-associated organic matter (C:N=6.9). This study reported the light fraction (intra-aggregate OM) and free OM had wider C:N ratios (C:N=12-24).

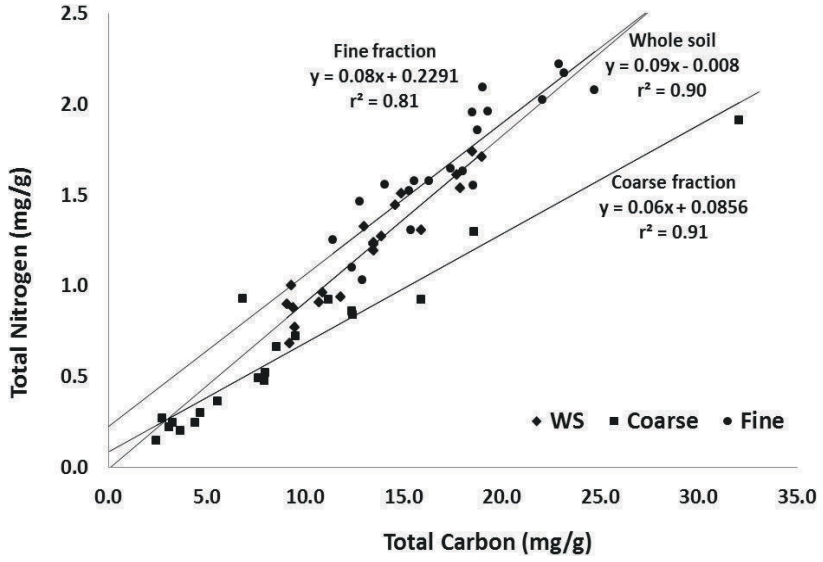

Figure 1. Correlation between total carbon and total nitrogen in the whole soil, coarse and fine fractions of twenty Red Chromosol soils.

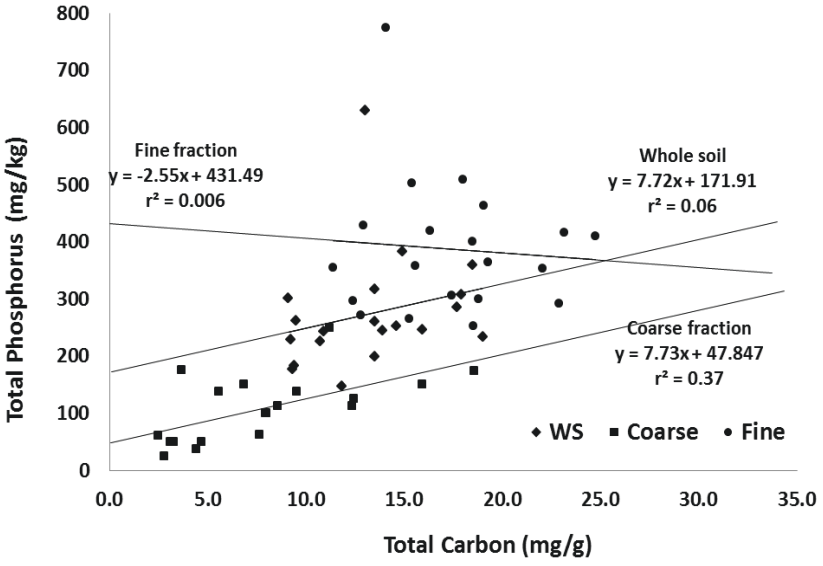

Figure 2. Correlation between total carbon and total phosphorus (as determined by the ignition method of Saunders and Williams (1955)) in whole soil, coarse and fine fractions of twenty Red Chromosol soils.

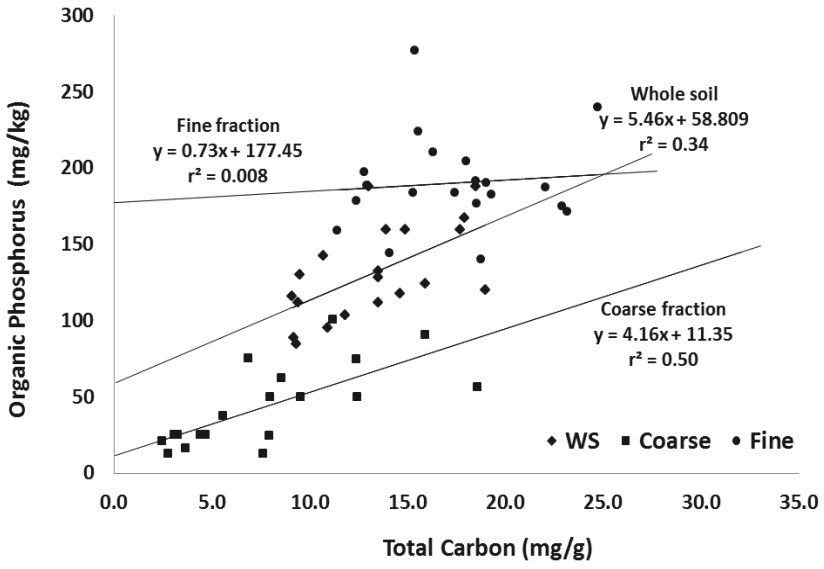

Figure 3. Correlation between total carbon and organic phosphorus (as determined by the ignition method of Saunders and Williams (1955)) in whole soil, coarse and fine fractions of twenty Red Chromosol soils.

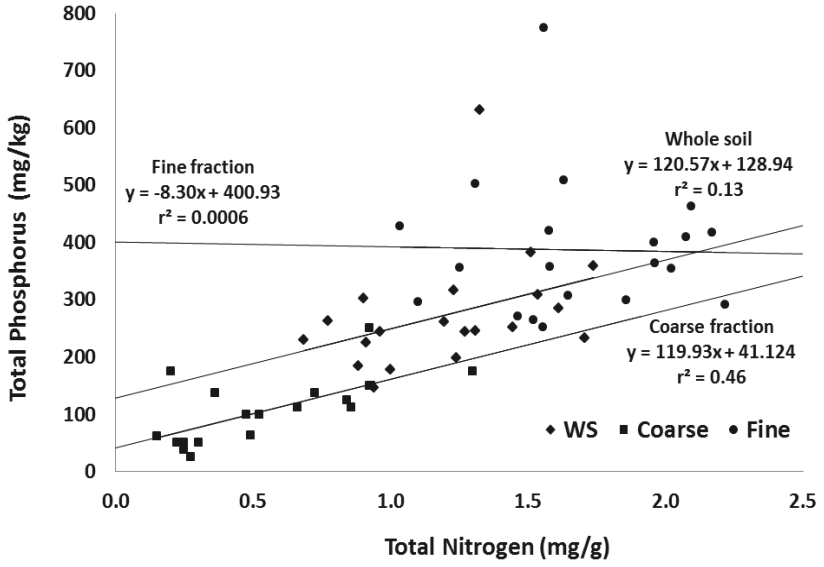

Figure 4. Correlation between total nitrogen and total phosphorus (as determined by the ignition method of Saunders and Williams (1955)) in whole soil, coarse and fine fractions.

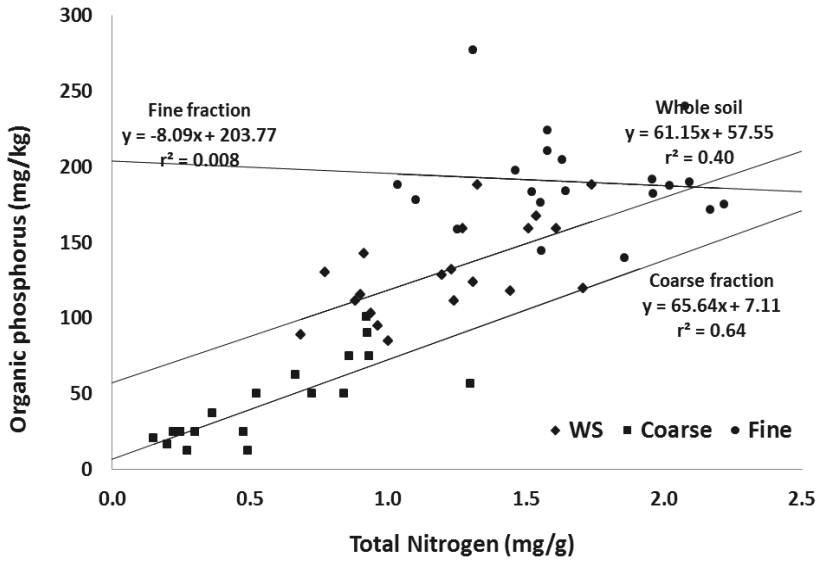

Figure 5. Correlation between total nitrogen and organic P (as determined by the ignition method of Saunders and Williams (1955)) in whole soil, coarse and fine fractions.

In contrast to the strong correlation found between C and N for both whole soils and fractions, correlations between C and P tended to be weak. There was no significant correlation between C and total P for either the whole soils or the fine fractions, but there was a weak but significant correlation between C and total P for the coarse fractions (r^2= 0.37, p <0.01; Figure 2). Correlations between C and organic P (as determined by the Saunders and Williams (1955)) method were generally stronger than between C and total P, with r^2= 0.34 for the whole soils and with r^2= 0.50 for the coarse fractions; however, there was no significant correlation between C and organic P for the fine fractions (Figure 3). Correlations between N and P (both total P and organic P) (Figures 4 and 5) were similar to, but generally slightly stronger than the corresponding correlations between C and P (Figure 2).

Figures 2-5 show also that for the correlations between C or N to total P and organic P, there were similar slopes for whole soils and coarse fractions, but whereas the intercept was close to zero for the coarse fractions, there was a substantial positive intercept for the whole soils.

A positive intercept for total P is consistent with the presence of a stable pool of inorganic P in these soils. On the other hand, a positive intercept for organic P seems incongruous, as one can't have organic P in the absence of soil carbon. A likely explanation is that the Saunders and Williams (1955) method consistently overestimates organic P (i.e. Oniani *et al.* 1973; Williams *et al.* 1970), by around this amount (i.e. ~60 mg/kg).

Overall, the elemental relationships presented in Figures 1-5 confirm that there are different stoichiometric relationships for the coarse and fine fractions of this set of soils and this provides insight for understanding the stoichiometric relationships apparent for the whole soils. These soils confirm a familiar story for C:N stoichiometry, i.e. strong linear relationships for all fractions, with consistently small intercepts but different slopes (a higher slope for the fine fraction indicating a lower average C:N ratio and a lower slope for the coarse fraction indicating a higher average C:N ratio). This is entirely consistent with well-established mechanisms (Manzoni *et al.* 2010) whereby large plant residues with high C:N undergo a degradation process that reduces particle size at the same time as increasing relative N content. The situation for C:P stoichiometry is more complex for a number of reasons. First, there is the complication posed by the presence of inorganic P. As previously noted by Kirkby *et al.* (2011), this tends to diminish the strength of C:P stoichiometric relationships and here no correlation was found between total P and C for the whole soils or fine fractions. The fact that a moderate correlation was found for the coarse fractions can in part be attributed to the lower capacity of sand particles to contain or sorb inorganic P. The use of the Saunders and Williams (1955) method to differentiate inorganic and organic P helped somewhat, with stronger relationships found between organic P and C than between total P and C for the whole soils and the coarse fractions. However, there was evidence that the Saunders and Williams (1955) method tended to overestimate organic P and this is likely

to have impacted on the strength of correlations, especially for the whole soils and fine fractions. In any case, one consistent finding throughout was the absence of evidence for stoichiometric control of C:P in the fine fractions. Although the limitations of the Saunders and Williams (1955) method are likely to have contributed to this, it is also possible that C: organic P ratios in these fractions do truly vary widely.

Comparison of ^{31}P NMR spectra of whole soil, coarse and fine fractions

Analysis of the elemental contents of the size fractions, as discussed above, clearly suggests that the organic matter in these fractions differs in a consistent pattern. As demonstrated in Chapters 2 and 3, ^{31}P NMR spectroscopy is a powerful way to gauge differences in organic P composition. Therefore ^{31}P NMR analysis of the size fractions would appear to be a potentially fruitful extension of the studies. Unfortunately, technical limitations made this extension difficult in practice for several reasons, including insufficient quantities of coarse and fine fractions after fractionation and the long NMR acquisition times involved. As a result of these limitations, it was not possible to obtain NMR spectra of the size fractions of all soils. Instead, two soils were chosen based on the availability of sufficient quantities of materials with higher concentrations of organic P, and the results of ^{31}P NMR analysis of the size fractions of these two soils are discussed below.

Figure 6 shows that the ^{31}P NMR spectra of the coarse fractions are relatively richer in the "sharp" peaks that can be assigned to specific organic P molecules, including α- and β-glycerophosphate, RNA-P, and phytate, as discussed in Chapters 2 and 3. On the other hand, the ^{31}P NMR spectra of the fine fractions are richer in the broad feature identified as "humic-P", as also discussed in Chapters 2 and 3. The ^{31}P NMR spectra of the whole soils are more similar to those of the fine fractions than those of the coarse fractions. These findings are

generally consistent with the elemental analyses discussed earlier in this chapter. In particular, the relatively low contribution of humic P in the coarse fractions is consistent with the absence of humic P in ^{31}P NMR spectra of plant residues (Noack *et al.* 2012), i.e. it is consistent with the assumption that the coarse fractions represent partially humified plant residues while the fine fractions represent more fully humified organic matter.

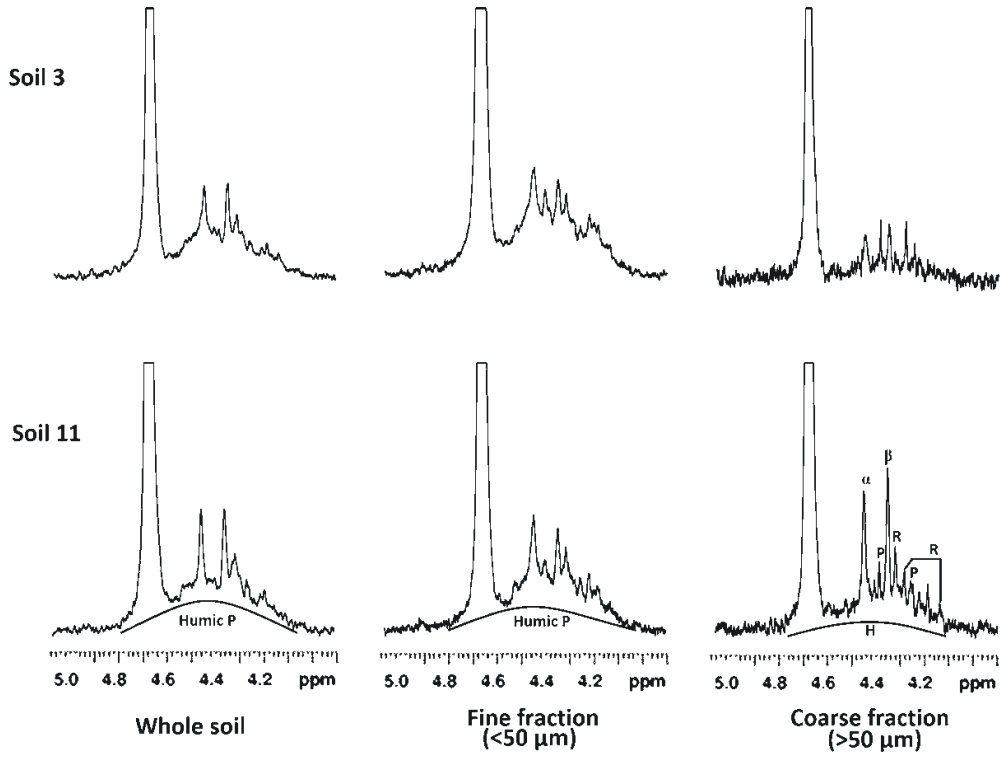

Figure 6. NaOH-EDTA extractable P forms in whole soil, fine fraction (<50 µm) and coarse fraction (> 50 µm) as determined by ^{31}P NMR spectroscopy. Ortho-P– Orthophosphate; Lipid – α-glycerophosphate & β- glycerophosphate; mononucleotides-RNA (R); phytate (P); and humic-P (H).

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Comparison of P contents determined by elemental analysis and NMR analysis

One final comparison can be made from the results presented here. Total phosphorus and organic phosphorus were measured by two methods (Figure 7-8): the ignition method of Saunders and Williams (1955) and NMR analysis. This enabled determination of the proportion of soil P that was detected by NMR following NaOH-EDTA extraction; average

recoveries were 50% for total P and 33% for organic P (although this second value is likely to be an underestimate due to the potential overestimation of organic P by the Saunders and Williams (1955) method. Figure 7 shows a strong correlation for total P (r^2=0.71) and Figure 8 shows a weaker correlation for organic P (r^2=0.42), with a 'tell-tale' positive intercept. Thus it is likely that the weakness of the correlation for organic P is at least partially due to overestimation of organic P using the Saunders and Williams (1955) method.

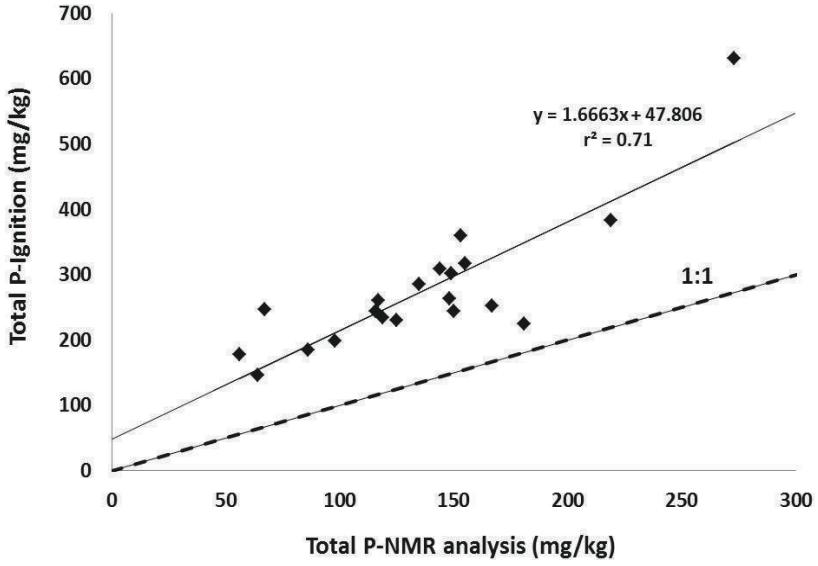

Figure 7. Correlation between total phosphorus determined by NMR analysis and the ignition method of Saunders and Williams (1955).

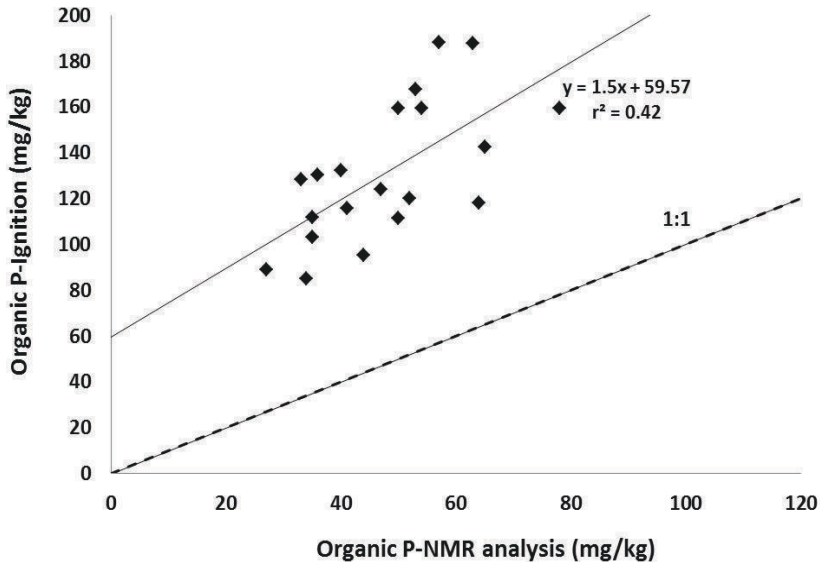

Figure 8. Correlation between organic phosphorus determined by NMR analysis and the ignition method of Saunders and Williams (1955).

Conclusions

This study demonstrated C:N:P stoichiometry differs between physical fractions of soil organic matter. The twenty soils used in this study on average comprised by mass 60% fine fraction (<50 μm) and 40% coarse fraction (>50 μm). The fine fraction had higher concentrations of C, N, and total, inorganic and organic P, the latter presumably due to stronger interactions of organic matter and phosphate with clay particles than sand particles. Strong linear relationships were found between C and N for whole soils and both fine and coarse fractions, with C:N ratios increasing in the order fine fractions < whole soils < coarse fractions, as expected. Correlations of P (both total P and organic P) with C and N were generally weaker, but stronger for coarse fractions than whole soils, and non-significant for fine fractions. Thus it can be concluded that in these soils organic matter stoichiometry is less constrained for P than it is for N, and is less constrained for humified OM than for POM. Limited ^{31}P NMR analysis of soil fractions supported the proposition that the composition of

organic P differs between fine and coarse fractions, with “humic P” comprising a greater proportion of organic P in the fine fraction.

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CHAPTER 5

COMPARISON OF MICROBIAL PHOSPHORUS DETERMINED BY HEXANOL FUMIGATION-EXTRACTION AND NMR SPECTROSCOPIC METHODS

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Principal Author

Name of Principal Author (Candidate)	Melinda R.S. Moata		
Contribution to the Paper	Experimental development, performed analysis on all samples, data analysis and critical interpretation, wrote the manuscript		
Overall percentage (%)	85		
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Signature		Date	

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Ashlea L. Doolette		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Ronald J. Smernik		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Ann M. McNeill		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Lynne M. Macdonald		
Contribution to the Paper	Supervised development of work, data analysis and interpretation, reviewed and edited the manuscript.		
Signature		Date	

Introduction

In Chapter 3, a novel model for the pool structure of soil organic P was proposed based on NMR characterisation of twenty Red Chromosol soils. This model consists of four chemically distinct pools: orthophosphate, humic P, cellular organic P and inositol phosphate P. This chapter explores the potential correspondence of the cellular organic P pool with traditionally determined microbial P. This is interesting because as shown in Chapter 3, the "cellular organic P" components like phospholipid P and RNA P and diester P have closer correlation to key element of SOM, C and N compared to other species of P in the soil.

Soil microbial biomass represents a relatively small pool of organic matter in the soil, but one that plays a vital role in the dynamics and transformations of organic matter. In particular, the microbial population is thought to be primarily responsible for conversion of organic P to inorganic P and can also (along with plants) be able to convert inorganic P to organic P. Thus through microbial mineralisation organic P can become available as inorganic P in soluble form and hence be an important source of P to plants, especially in areas where there is less intensive agriculture and a low input of fertilizer. Approximately 2% of the dry weight of microbial biomass is P, and it has been estimated microbial biomass represents a reserve of 2.7×10^9 tonnes P globally (Stevenson and Cole 1999).

In contrast to C and N, which exist almost exclusively in organic forms in soil, P exists as a mix of inorganic and organic forms. This is true both for the total soil P pool and the soil microbial P pool. It has been estimated that in a soil with 2-4% organic matter about 5-10% of total organic phosphorus is present in living microbial tissue (Stevenson 1994) or about 1.5-2.5% in bacteria and 4.8% in fungi (Stevenson and Cole 1999) . Similar estimates have been made by other researchers, e.g. Brookes *et al.* (1984) suggested microbial biomass in

European soils represents about 3% of organic P in agricultural soils and about 5-24% of organic P in grassland soil. Oberson *et al.* (2005) provided estimates of 0.4-2.5% of total P in arable soils and up to 7.5% of total P in grassland soils. Some estimates have also been made for Australian arable and pasture soils. A study of 32 soils from agricultural areas of southern Australia, including Western Australia, reported that microbial P accounted for 2-13% of organic P; these soils had total P concentrations of 40-678 mg P kg^{-1} and organic P concentrations of 34-332 mg P kg^{-1} (Butterly *et al.* 2010).

The chemical composition of P in microorganisms has been reported to vary depending on the environment, microbial community composition, nutrient availability, and growth stage (Bünemann *et al.* 2011). For example, aquatic bacteria mainly consist of nucleic acids and phospholipids (60%), cytoplasmic inorganic and organic P (each 10%), and polyphosphate (20%) (Vadstein 2000). A variety of methods have been used to determine the chemical composition of P in soil microbial biomass. Bünemann *et al.* (2011) used ^{31}P NMR to classify microbial P into nine classes: nucleic acid, phospholipid, teichoic acids, metabolites, phosphonates, organic polyphosphates, pyrophosphate, polyphosphate and orthophosphate. Wanner *et al*. (1990) measured teichoic acid, RNA and polyphosphate using a cultivation method. DNA (Lindahl 1996) and adenosine triphosphate (ATP) (Lindahl and Bakken 1995) can be determined using extraction methods, while phospholipid fatty acid (PLFA) analysis provides a measure of total phospholipids even though it is mainly designed for another purpose (determining the composition of microbial communities based on the different fatty acids contained in their phospholipids). Using solution ^{31}P NMR spectroscopy, Makarov *et al*. (2005) reported that fungi and bacteria from soil consist mainly of monoester and diester P with smaller pools of inorganic orthophosphate and inorganic pyro- and polyphosphates also present. With the same method, Bünemann *et al*. (2008a) identified that soil bacteria have

more monoester and diester P, while fungi have more ortho-, pyro- and polyphosphate. Other methods used to determine soil microbial P, including chromatographic, spectrometric, staining and enzymatic techniques, have been reviewed by Bünemann *et al*. (2011).

The microbial biomass P (MBP) content of soil has traditionally been determined using chloroform fumigation (Brookes *et al.* 1982) and extraction with a water solution containing anion-exchange membranes that absorb orthophosphate (Kouno *et al.* 1995). Although numerous recent studies still use chloroform as the fumigant (Ahmed *et al.* 2008; Dodd *et al.* 2014; Gichangi *et al.* 2009; Khan and Joergensen 2012; Malik *et al.* 2013; Oberson *et al.* 1997), others have used hexanol as an alternative (Bünemann *et al.* 2012; Bünemann *et al.* 2008b; Hassan *et al.* 2013; Sugihara *et al.* 2015) which is reportedly more effective and less harmful, since chloroform is a recognised carcinogen (McLaughlin *et al.* 1986). In either case, the concentration of inorganic P in the extracts following fumigation is determined colorimetrically after releasing orthophosphate from the exchange resin.

It should be noted that this method only measures soluble orthophosphate (inorganic P) released from lysed microbial cells. A correction factor (K_p) can be applied when calculating the microbial biomass to correct for incomplete release of P from the cells due to resistance of some microbes to lysis and mineral adsorption of inorganic P released (Brookes *et al.* 1982; Hedley and Stewart 1982; McLaughlin *et al.* 1986). Typical correction factors are K_{pi} = 0.32-0.38; K_{pt} = 0.37-0.49 for chloroform fumigation (Brookes *et al.* 1982; Hedley and Stewart 1982) and K_p = 0.33, 0.40 and 0.57 for hexanol fumigation (McLaughlin *et al.* 1986). However, some researchers have advised against the use of a correction factor (Bünemann *et al.* 2012; Bünemann *et al.* 2008b; Selles *et al.* 1995) because of large variations due to low microbial protection in certain types of soil (Achat *et al.* 2010) and uncertainty among soils

and microbial communities (McLaughlin *et al.* 1986). In addition to soluble inorganic P, as discussed above, soil microbes can contain a high proportion of organic P. It is possible that organic P may be converted to orthophosphate through enzymatic hydrolysis by phosphatases released from the lysed cells of the microorganisms or dead roots (Blackwell *et al.* 2009; Hassan *et al.* 2012; Oberson *et al.* 1997; Sparling *et al.* 1985).

In this study, the traditional measure of microbial biomass P determined using hexanol fumigation extraction (FE) is compared against the ^{31}P NMR determined 'cellular P' pool in a soil incubated with a labile organic C substrate (glucose). The hypothesis was that both measures should rapidly increase as the microbial population increases rapidly. Therefore the aim was to compare concentrations of microbial and cellular P on soils incubated with and without glucose addition. This experiment was carried out on a different soil to those used in the previous experiments (Chapters 2-4); the soil chosen for the experiment described here has much higher concentrations of organic C and organic P, which was hoped, would increase the ability to detect any changes to the "cellular P" pool by NMR spectroscopy.

Materials and methods

Soil sampling and basic properties

This study was carried out using a sandy loam soil collected near Mount Gambier in south-east South Australia (37°55'36"S, 140°42'21"E). The soil was collected to a depth of 10 cm, dried at 20°C for 4 days, sieved to <2 mm to remove stones and large plant debris, then mixed in a cement mixer to ensure homogeneity. Total P, inorganic and organic P were determined using the ignition method of Saunders & Williams (1955) followed by

colorimetry according to Murphy & Riley (1962). Total C and N were determined using a dry combustion analyser (LECO CNS 2000).

Soil incubation

Subsamples of soil (100 g) were placed into 100 mL plastic containers and pre-incubated in a dark room at 25°C for 14 days at 55% of the maximum water holding capacity (WHC) to re-initiate microbial activity following the disturbance due to sieving and drying. Half of the samples were amended with 2.5 g kg^{-1} C as glucose as a readily available C source and the other half were used as unamended soil control samples. A total of 70 samples were prepared to enable seven sampling times (0, 3, 7, 10, 13, 21, 31 days) and five replicates of each treatment at each time. Soil moisture was adjusted at day 0 together with adding glucose in glucose-amended soils. All soils were maintained at 70% of WHC for the duration of the incubation of 31 days. After thorough mixing of the soil and glucose solution (or water for the control soils), the containers were placed individually into 1 L glass incubation jars. The jars were sealed with gas-tight lids equipped with septa to allow headspace sampling. Respiration was quantified daily over 31 days by measuring headspace CO_2 concentrations every 24 h using a Servomex 1450 infra-red gas analyser (Servomex Group, Crowborough, England). After each measurement, the jars were opened and refreshed to equilibrate the CO_2 to ambient concentration and then resealed. The soil water content was adjusted every three days. At each sampling time, the soils were analysed for resin P and microbial P, and prepared for solution ^{31}P NMR spectroscopic analysis.

Resin and microbial P were determined using a fumigation-extraction method as described by Kouno *et al.* (1995) but using hexanol as the fumigant instead of chloroform (McLaughlin *et al.* 1986). Soil samples were shaken at a 15:1 ratio with resin membrane strips (BDH no.

551642S, cut to 61 mm × 13 mm strips in bicarbonate form) in: (1) water; (2) water + hexanol (1 mL), and (3) water + a P spike (20 μgP/mL) to test for sorption of P released during fumigation-extraction as recommended by Bünemann *et al.* (2004). The resin strips were eluted with 30 mL 0.1 M NaCl/HCl and the concentration of inorganic P was determined colorimetrically (Murphy & Riley (1962). Microbial P (hexanol-released P) is reported as the difference in resin P between fumigated and non-fumigated subsamples (McLaughlin *et al.* 1986). Phosphorus extracted from non-fumigated subsamples is reported as resin-extractable P (P_{resin}), an indicator of labile P. Sorption correction was not applied due to high (>90%) recovery of the P spike for all soils.

Solution ^{31}P NMR spectroscopic analysis of NaOH-EDTA extracts

Sub-samples of moist soils equivalent to 3 g dry weight were extracted by shaking end-over-end with 30 mL of NaOH-EDTA solution (0.25 M NaOH and 0.05 M Na_2EDTA) for 16 h. Solution ^{31}P NMR spectra were obtained on a Varian INOVA400 NMR spectrometer (Varian, Palo Alto, CA). Detailed methods for extraction and analysis can be found in Chapter 4. Note that the soil:extract ratio used here was 1:10 rather than the 1:4 ratio used in Chapters 3 and 4; this reflects the higher P concentration of the soil used in this study.

Statistical analysis

Statistical analyses were performed with SigmaPlot 12.0. Soil respiration, resin P and microbial biomass P were analysed in 5 replications but the NMR analysis was only performed on one replicate per treatment and per harvest time due to the high cost and long acquisition times required. All the data was subjected to two-way ANOVA (treatment x harvest time) where $P \leq 0.05$. Treatments were compared by taking the average of a given treatment for a given sampling time.

Results and Discussion

Basic chemical properties of the soil used in this experiment

A different soil was chosen for this experiment to those used in previous experiments (Chapters 2-4). Basic chemical properties of the soil are shown in Table 1. It was collected from a dairy farm and has much higher total C (5.3%) and N (0.5%) concentrations than the Red Chromosol soils described in Chapters 2-4, which had average C and N concentrations of 1.3% and 0.12%, respectively. The soil used here also has much higher concentrations of total, inorganic and organic P (1734 mg/kg, 1164 mg/kg and 570 mg/kg) than the Red Chromosol soils described in Chapters 2-4, which had average total, inorganic and organic P concentrations of 275 mg/kg, 143 mg/kg and 131 mg/kg , respectively. The composition of P of the soil used here also differed to that of the Red Chromosol soils described in Chapters 2-4 (see below).

Table 1. Soil physico-chemical properties.

Total C (%)	Total N (%)	P_t (mg/kg)	P_i (mg/kg)	P_o (mg/kg)	C/N	C/P	Clay (%)	Silt (%)	Sand (%)
5.3	0.5	1734	1164	570	11	31	8	29	63

P_t: Whole soil total P determined by the method of Saunders and Williams (1955)
P_i: Whole soil inorganic P determined by the methods of Saunders and Williams (1955) and Murphy and Riley (1962)
P_o: Whole soil organic P determined by the method of Saunders and Williams (1955)

Effect of glucose addition on soil microbial respiration

Glucose amendment rapidly increased microbial respiration. Figure 1 shows the ratio of soil respiration between glucose-amended and unamended treatments increased sharply from day

0 to day 3 (26-fold increase) then slowly decreased to day 13. From day 14 to day 31, this ratio remained steady at 7: 1 (Figure 1).

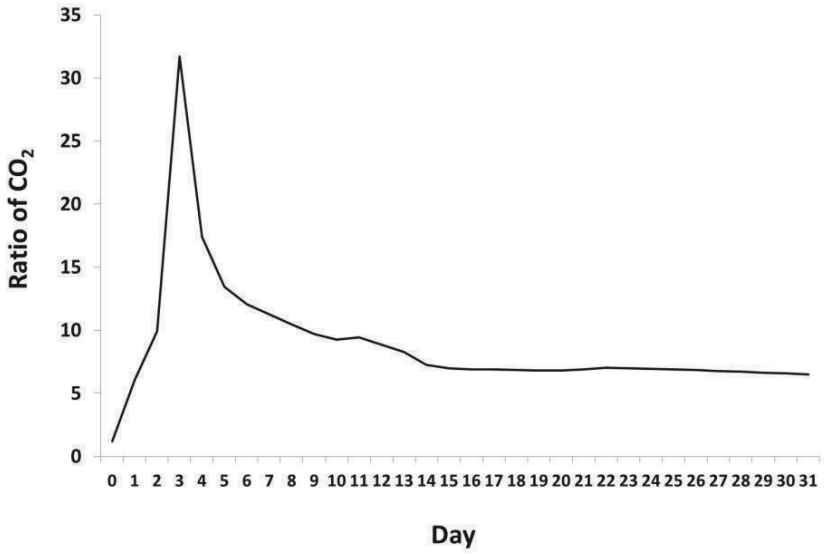

Figure 1. Ratio of soil respiration for glucose amended soil relative to the unamended soil over 31 days of incubation

Effect of glucose addition on resin P and microbial P

Figure 2 shows that resin P was significantly lower ($p<0.001$) for the glucose amended soil (average 31 mg/kg) than for the unamended soil (average 40 mg/kg) throughout the incubation. Resin P for both treatments showed similar patterns of variation throughout the incubation, with an initial sharp decrease between day 0 and day 3 followed by a slow increase up to day 31.

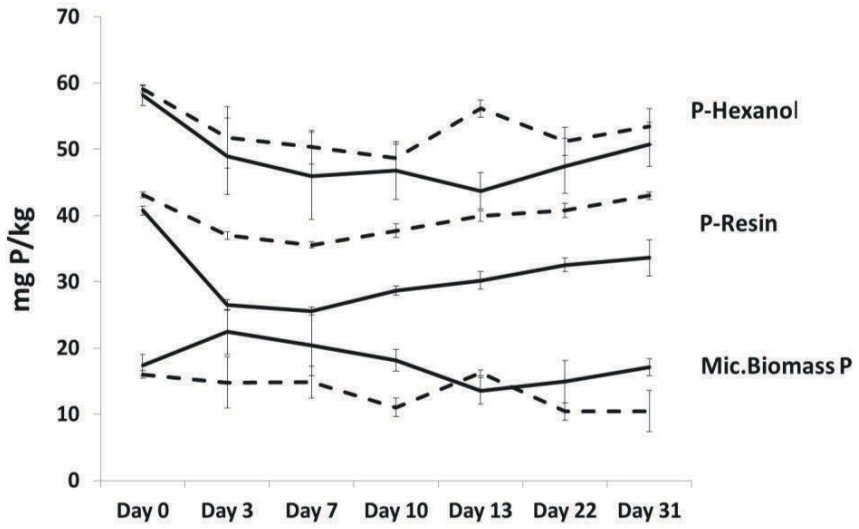

Figure 2. Resin P and microbial biomass P determined by the hexanol fumigation-extraction method; **(** __ **:** glucose-amended soils and - - - -: unamended soils**)**

Resin P following fumigation should be the sum of resin P of the soil prior to fumigation plus extractable P released on the lysis of microbial cells. This would include orthophosphate that was present in the microbial cells as well as any organic P that is hydrolysed by phosphatase enzymes released from the microbial cells. Figure 2 shows that resin P following fumigation tended to be lower for the glucose amended soil than for the unamended soil throughout the incubation. However, the difference between glucose amended and unamended soils was smaller than for resin P. There was also greater variability between replicates for resin P following fumigation (than for resin P) resulting in there only being a significant difference in resin P following fumigation between glucose amended and unamended soils at one sampling time (day 13). Over the course of the incubation there was an average 4 mg/kg difference in resin P following fumigation between unamended soils (average 53 mg/kg) and glucose amended soils (average 49 mg/kg). Resin P following fumigation varied significantly over the course of the incubation ($p<0.05$). For both glucose-amended and unamended soils resin P following fumigation decreased from day 0 until day 13 (24% and 5% decrease,

respectively) and increased slowly from that point. By day 31, the unamended soil had 10% less resin P following fumigation than at day 0; the corresponding loss for the glucose amended soil was 16%.

Traditionally, the difference in resin P with and without hexanol treatment is regarded as MBP. According to this methodology, MBP was generally higher for the glucose-amended soil, but the difference was only significant at day 10 and day 31. There was little variation in MBP over the course of the incubation, with average values of 17.7 mg/kg for the glucose-amended soil and 13.4 mg/kg for the unamended soil.

The lower resin P values for the glucose-amended soil can be attributed to orthophosphate taken up by microbes from soil solution. The addition of available C as glucose stimulated microbial catabolic and metabolic activities (as demonstrated by the increase in respiration), and the consequent increase in microbial biomass would result in increased nutrient uptake and rapid depletion of available N and P from solution, i.e. net immobilisation (Qiu *et al.* 2008) . The difference in resin P between glucose amended and unamended soils provides one estimate (~10 mg/kg) of the amount of P contained in the additional microbial biomass stimulated by the addition of glucose. However, this will be an underestimate if the microbial population can access pools other than the soluble orthophosphate pool represented by resin P. As discussed above, the more usual way to estimate the amount of P contained in the additional microbial biomass stimulated by the addition of glucose is the difference in MBP between glucose amended and unamended soils. However, this actually results in a lower estimate (5 mg/kg), as resin P following fumigation tended to be lower for the glucose amended soil than for the unamended soil. The most likely explanation is that some of the

orthophosphate taken up by microbes was converted to organic P that was not detected as resin P following fumigation.

These results indicate that adding glucose increased microbial P by at least 10 mg/kg and suggest that at least some of this microbial P was present in organic forms. This estimate is greater than the difference in MBP (average 5 mg/kg) as determined in the standard way (i.e. as the difference between resin P with and without hexanol fumigation). In any case, it must be kept in mind that all estimates of microbial P are small in comparison to the total P in the soil (1734 mg/kg) and the estimate of organic P provided by the Saunders and Williams (1955) technique (570 mg/kg).

Solution ^{31}P NMR spectroscopy

Solution ^{31}P NMR spectroscopy was used to determine the composition of organic P in this soil and also to gauge changes in the composition of organic P caused by the addition of glucose. In Figure 3 the orthophosphate and monoester region of the ^{31}P NMR spectra of the soil used here is shown along with those of two Red Chromosol soils described in Chapters 2-4. All three spectra share the same features: (i) an intense peak at ~5.75 ppm (off scale in this representation) due to orthophosphate; (ii) a broad peak that extends from 6.0 to 3.5 ppm that can be attributed to humic P; and (iii) a series of sharp peaks in the range 5.5-3.5 ppm that are due to specific compounds with monoester groups. In Chapter 3, these specific compounds were partitioned into two groups: inositol phosphates and "cellular" P species (derivatives of phospholipids and RNA). The main difference in P composition between the two Red Chromosol soils shown is in the relative amounts of these groups of compounds, with one (Soil 12) containing virtually no inositol phosphate, while the other (Soil 6) contains

moderate amounts of inositol phosphate. In comparison, the Mt Gambier sandy loam contains more inositol phosphate than even Soil 6.

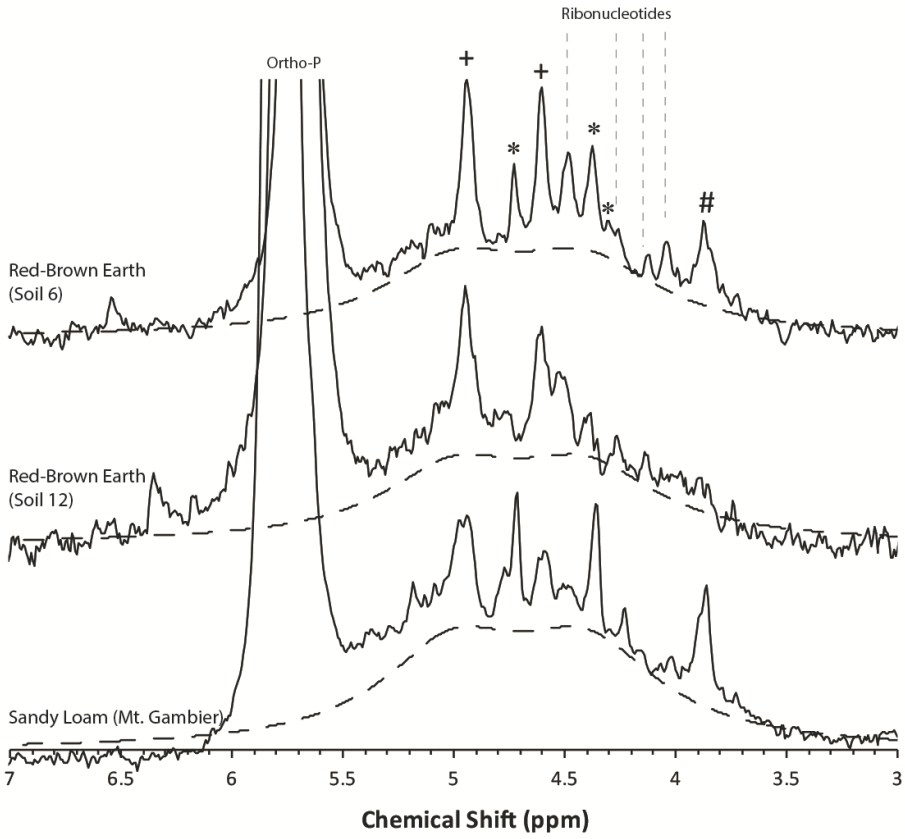

Figure 3. Orthophosphate and monoester region of the ^{31}P NMR spectrum of the soil used here along with those of two Red Chromosol soils described in Chapters 2-4. The vertical scale of each spectrum has been adjusted so that the peak of maximum intensity in the orthophosphate monoester region in each spectrum is the same height. The symbols identify common resonances among spectra: Ortho-P orthophosphate; + α- and β-glycerophosphate; * *myo*-IP_6 (*myo*-inositol hexakisphosphate); ribonucleotides are identified by the four vertical dashed lines; # *scyllo*-IP_6; the dashed line extending from 7 to 3 ppm identifies the approximate shape and position of the humic P signal in each spectrum.

Figure 4 shows the orthophosphate and monoester region of solution ^{31}P NMR spectra of glucose amended and unamended soils at four points during the incubation. All spectra are very similar in appearance, suggesting there was no major influence of glucose addition on organic P speciation as determined by ^{31}P NMR spectroscopy. In particular, glucose addition did not appear to increase the size of peaks assigned to phospholipid or RNA (highlighted in

Figure 4). This was unexpected, as it was hypothesised that their concentrations should increase in response to the addition of labile carbon in the form of glucose, which clearly increased microbial activity as demonstrated by an increase in respiration (see above).

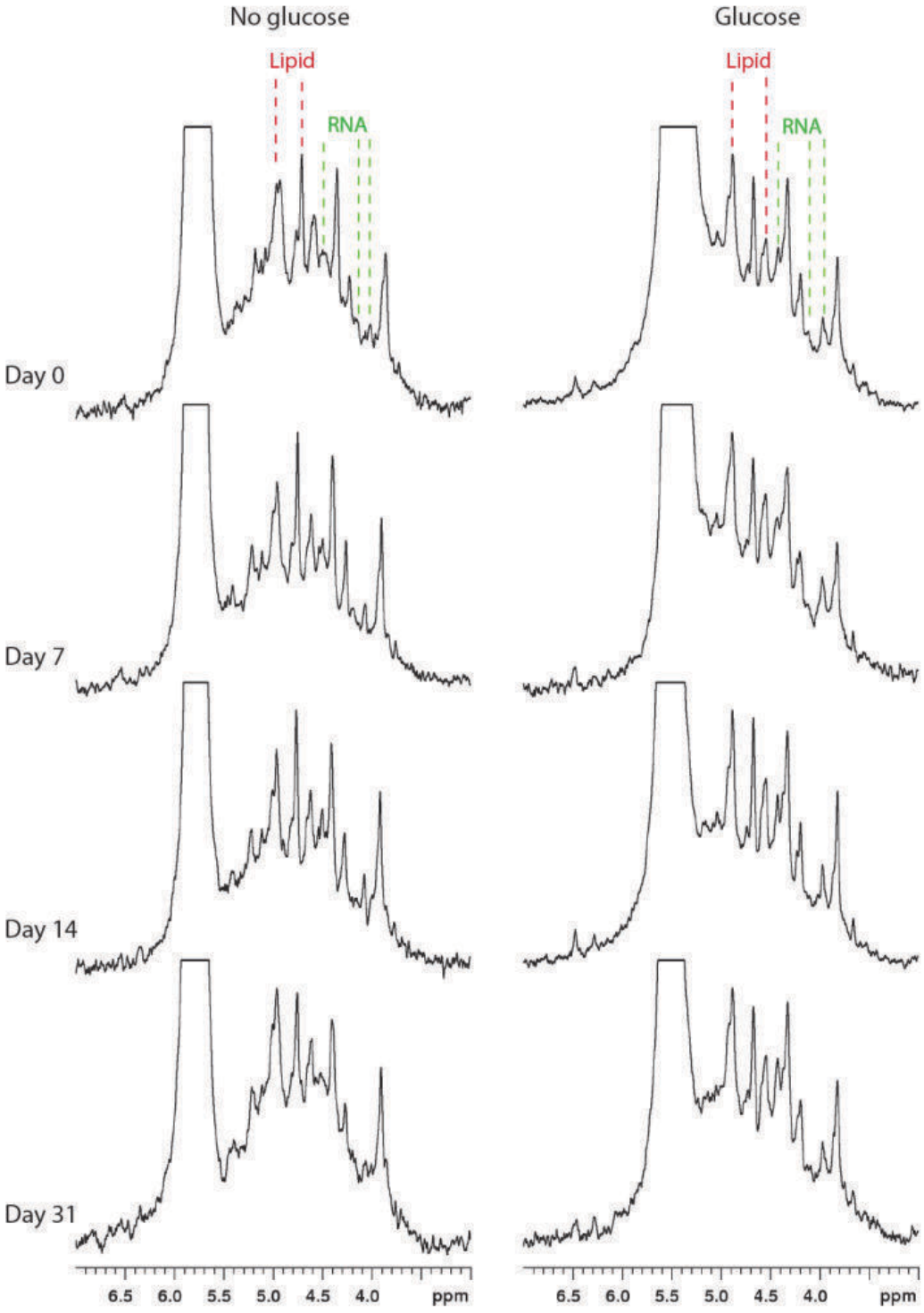

Figure 4. Orthophosphate and monoester region of the ^{31}P NMR spectra of the glucose-amended and unamended soil at four times during the incubation. Red dashed lines indicate the peaks assigned to lipids (α- glycerophosphate & β- glycerophosphate); green dashed lines indicate the peaks assigned to RNA (ribonucleotides).

The concentration of P species in the extracts calculated from the ^{31}P NMR spectra using a combination of integration and deconvolution are shown in Table 2. This confirms the high degree of similarity across all spectra and in particular the lack an effect of glucose addition. Note that Table 2 also includes the concentrations of diester P and pyrophosphate, whose signals appear outside the chemical shift range presented in Figure 4. Importantly, the concentration of these species, which were classified in Chapter 3 as being part of the cellular P pool, also did not increase in response to glucose addition.

Table 2. Concentrations (mg/kg) of phosphorus forms and their percentages (in parentheses) of NaOH-EDTA extracts determined by ^{31}P NMR spectroscopy and quantified using spectral deconvolution. Ortho-P– Orthophosphate; Lipid – α- glycerophosphate & β- glycerophosphate; RNA– sum of up to three individual resonances in the chemical shift range 3.95–4.52 ppm; *scyllo*-IP_6 – *scyllo*-Inositol hexakisphosphate; Diester – Orthophosphate diester; Pyro-P – pyrophosphate.

Treatment	Total P [A]	Ortho-P	Humic-P	Lipid	Phytate	RNA	*scyllo*-IP_6	Diester	Pyro-P
Unamended soil									
Day 1	814 (44)	579 (71)	175 (22)	10 (1)	20 (2)	6 (1)	7 (1)	14 (2)	4 (1)
Day 7	842 (45)	601 (71)	176 (21)	11 (1)	22 (3)	6 (1)	7 (1)	15 (2)	3 (<1)
Day 13	813 (44)	590 (73)	156 (19)	13 (2)	23 (3)	7 (1)	8 (1)	15 (2)	2 (<1)
Day 31	763 (41)	548 (72)	162 (21)	10 (1)	15 (2)	5 (1)	6 (1)	15 (2)	2 (<1)
Glucose									
Day 1	798 (43)	582 (73)	151 (19)	13 (2)	23 (3)	7 (1)	7 (1)	11 (1)	4 (<1)
Day 7	797 (43)	570 (71)	151 (19)	18 (2)	26 (3)	10 (1)	8 (1)	11 (1)	4 (<1)
Day 13	758 (41)	541 (71)	153 (20)	14 (2)	22 (3)	7 (1)	8 (1)	11 (1)	2 (<1)
Day 31	856 (46)	613 (72)	171 (20)	12 (1)	25 (3)	7 (1)	7 (1)	15 (2)	5 (1)

[A] Values in parentheses for Total P are the extraction efficiencies

There are several possible reasons why the expected increase in cellular P components following glucose addition was not observed. Since NMR analysis is carried out on soil extracts, only extractable P species are detected. Comparison of the sum of NMR-detected P species to the total P content of the soil indicated an extraction efficiency of 41-46% (Table 2), so it is clear that a large proportion of soil P was not detected by ^{31}P NMR. However, previous studies have shown good extraction efficiency for microbial P (Bünemann *et al.* 2008a), so it is unlikely that cellular P would be biased against in NMR analysis.

It is important to consider how large the expected increase in cellular P is relative to the size of the other organic P components present. Besides confirming the lack of a clear influence of glucose addition on P composition, Table 2 emphasises the fact that the magnitude of the increase in the size microbial P pool indicated by the fumigation method of ~10 mg/kg is small relative to the amount of P detected by NMR (~800 mg/kg). Some of the MBP will be present as orthophosphate, which is indistinguishable by NMR from the much larger orthophosphate pool present in the soil (~600 mg/kg detected by NMR). The rationale of this experiment was that some of the MBP would be the "cellular organic P" components of phospholipid, RNA, diester P and pyrophosphate and that the relatively small concentrations of these components in soil would facilitate identification of the increase brought about by the addition of glucose. Table 2 indicates that the unamended soil contains approximately 35 mg/kg of cellular P (10 mg/kg of phospholipid, 6 mg/kg of RNA, 15 mg/kg of diester P and 3 mg/kg of pyrophosphate). Against this background, it is perhaps not surprising that an addition of 10 mg/kg of cellular P on addition of glucose would not be reliably detected.

One final opportunity this experiment provided was to combine the traditional (hexanol fumigation-extraction) method of measuring MBP with ^{31}P NMR analysis. This was achieved

by carrying out NaOH-EDTA extraction and NMR analysis on subsamples of a soil (glucose-amended soil after 7 days incubation) that had been subjected to the two treatments used in the fumigation-extraction method (i.e. water extraction with and without hexanol fumigation). Figure 5 indicates little difference in composition between these two soils. In particular, there is no indication of a decrease in cellular P components for the hexanol-treated soil. This suggests that any phosphatase enzymes released by cell lysis on hexanol treatment do not noticeably act upon the organic P compounds that are subsequently detected by ^{31}P NMR following NaOH-EDTA extraction.

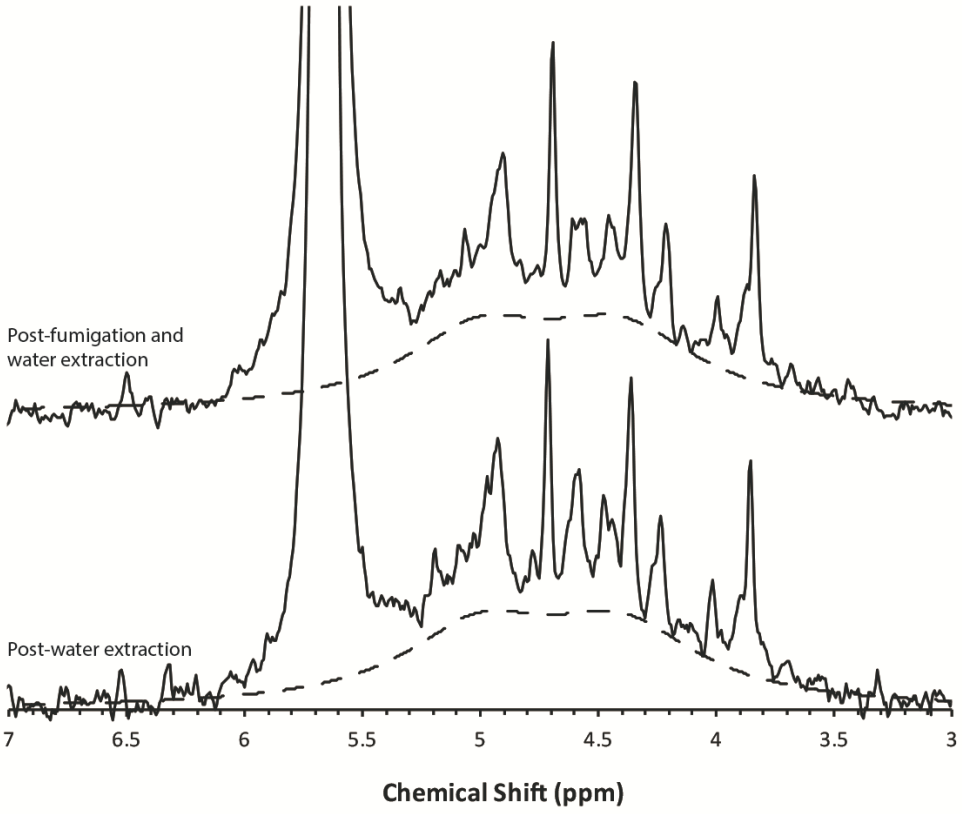

Figure 5. A comparison of ^{31}P NMR spectra of NaOH-EDTA soil extracts following hexanol fumigation and water extraction and water extraction (resin –P) only.

Conclusions

This experiment showed that the cellular P component identified in Chapter 3 has little or no relationship to the traditional standard measure of MBP determined as an increase in water soluble orthophosphate following fumigation. Glucose addition to a sandy loam soil had a large impact on the rate of microbial respiration, increasing it by a factor of 26 relative to the unamended soil by day 3, and by a factor of 7 by the end of the 31-day incubation. A modest decrease in resin P (from 40 mg/kg down to 30 mg/kg) on glucose addition was taken to reflect microbial immobilisation of at least this much P (since it is possible microbes may have taken up additional P from less soluble P pools); however, the standard measure of MBP was less than this (around 5 mg/kg on average over the course of the incubation). A likely explanation is that much of the P taken up by the microbes was converted to organic P forms that were not subsequently detected following lysis of cells with hexanol. The cellular organic P content of the unamended soil, determined as the sum of phospholipid, RNA, diester P and pyrophosphate detected by solution ^{31}P NMR spectroscopy following NaOH-EDTA extraction, was around 35 mg/kg and was not influenced noticeably by glucose addition or incubation. This rules out cellular P being a useful measure of MBP and perhaps suggests it may be more closely related to organic P in plant residues than to microbial biomass. The implications of this experiment are: 1) microbial biomass P determined by fumigation-extraction does not detect organic P forms; and 2) cellular P is not particularly labile and may include substantial amounts of stabilised phospholipid and RNA.

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CHAPTER 6

CONCLUSIONS AND FUTURE RESEARCH

Executive summary

Stoichiometry is a term used to describe the balance or relative amounts of elements in an organism or ecosystem (Fink 2009). The concept of stoichiometry is useful in understanding nutrient cycling because it provides a way of predicting how changes in the concentration of one element may affect other elements. In terrestrial ecosystems, stoichiometry is complex because there are interactions between recently added and native plant residues, soil organic matter (SOM) and the soil microbial biomass (Macdonald and Baldock 2010), all of which are likely to have different stoichiometries. The stoichiometry of SOM, which is usually the largest of the organic matter pools in terrestrial ecosystems, is of particular interest as SOM plays a significant role in agricultural management. The main elements of interest in SOM are carbon (C), nitrogen (N) and phosphorus (P). Of these, C plays the central role, as it provides the skeletal basis of SOM and acts as the primary energy source that drives microbial transformations of SOM (Manzoni *et al.* 2008). On the other hand, N and P are essential nutrients for plant growth and hence the main interest is in the dynamics of their conversion from organic forms, which are not directly plant available into mineralised forms that are plant available.

Stoichiometry has proven a very useful concept for understanding N cycling in soils, with the C:N ratio of both plant residues and SOM a sensitive predictor of the potential for and magnitude of N release (Manzoni *et al.* 2008; Martens 2000). On the other hand, the value of the concept of stoichiometry with regards to P transformations in soils is more uncertain. There are several potential reasons for this. Firstly, whereas the vast majority of N in soils exists in organic forms, most soils contain substantial quantities of both inorganic and organic forms of P, and the relative amounts of these two forms varies widely. This compromises the

usefulness of the C:P ratio as a predictor of the potential for and the magnitude of P release from SOM (Manzoni *et al.* 2010). An obvious solution is to use a measure of organic P rather than total P in determining the C:P ratio; however, doubts have been raised as to the reliability of established methods for measuring organic P in soil (McLaren *et al.* 2014; Oniani *et al.* 1973; Williams *et al.* 1970). A further complication is the fact that soils appear to contain a diversity of organic P forms that turn over on very different timescales. The majority of organic P in most cells is present as phospholipid and nucleic acids (Vadstein 2000), both of which are highly labile (i.e. undergo rapid microbial decomposition in the environment). However, organic P can also be present in inositol phosphates, which are principally P storage compounds and found mostly in plant seeds (Noack *et al.* 2012; Raboy 2007) and which can reportedly build up in soils due to their relative stability (especially compared to phospholipids and nucleic acids). Recent research has further complicated this issue by identifying that the majority of organic P in many soils is in fact present as neither of these forms, but rather as high molecular weight "humic P" (Bünemann *et al.* 2008a; Doolette *et al.* 2009; Doolette *et al.* 2011; Smernik and Dougherty 2007), the relative stability of which has not been thoroughly investigated. Given these complications, it is unsurprising that an understanding of the stoichiometry of P in SOM has remained elusive to this point.

The central idea in this thesis is that the best approach to improving our understanding of the stoichiometry of organic P in SOM involves considering organic P as being composed of multiple components, some or all of which may exhibit stronger stoichiometric constraint than does the whole of SOM. A key tool for pursuing this approach is solution ^{31}P NMR spectroscopy, which can provide detailed chemical speciation of organic P. However, other more traditional techniques were also employed in combination with NMR, including

elemental analysis, physical fractionation and established chemical methods for determining organic P and microbial biomass P.

The main outcomes of this thesis were:

1. Development of methodology to improve the sensitivity of NMR spectroscopy through modifying the ratio of soil to solution during NaOH-EDTA extraction for low P soils.

2. Development of a four pool model of P for a set of Australian Red Chromosol soils and the establishment of strong stoichiometry of C and N to one of these pools, i.e. the "cellular P" pool consisting of phospholipid, RNA, diester P and pyrophosphate.

3. Establishing that C: organic P stoichiometry was more constrained in coarse than fine soil fractions in a set of twenty Australian Red Chromosol soils.

4. Establishing that the cellular P pool is not closely related to the traditionally determined soil microbial biomass P (MBP) pool.

Overall, this thesis demonstrates the importance of improving and combining analytical methodologies in order to achieve accurate and detailed characterisation of organic P, particularly for low P soils, as a pre-requisite to the investigation of the stoichiometry of C, N and organic P. The key findings of this thesis are expanded on below and potential priorities for future research on the stoichiometry of SOM are discussed.

Method development for solution ^{31}P NMR spectroscopy

Solution ^{31}P NMR spectroscopy is widely recognised as the most powerful method for determining organic P speciation in soils. The main limitation of this method is its relative insensitivity, which often necessitates compromises between quality, quantity and cost of analysis (Doolette and Smernik 2011). Sensitivity limitations are particularly acute for Australian soils, which tend to have low concentrations of P due to their highly weathered nature (Beadle 1962). Red Chromosols, a soil type widely used for cropping in Australia (McKenzie *et al.* 2005), typify this problem and were the main focus of this thesis.

The total P concentration of the twenty Red Chromosol soils investigated here ranged between 252-631 mg/kg. The first part of this research involved adapting the standard method of soil extraction prior to solution ^{31}P NMR spectroscopy for use with low P soils by modifying the ratio of soil to NaOH-EDTA solution during extraction (Chapter 2). The P concentration in the extract was increased by tightening the extraction ratio from the standard 1:20 down to 1:4. As expected, the 1:4 ratio extracts provided the best NMR sensitivity (highest signal-to-noise ratio spectra) while not substantially affecting extractability; the recovery of extractable P in the wide ratio (1:20) extracts was 56% compared to 51% for the 1:4 ratio extracts. Importantly, the change in extraction ratio did not greatly affect the distribution of P species detected; small differences in minor components, especially lower detected concentrations of diester P at wider ratios, was most likely due to the underestimation of these components at the wider ratio caused by low signal-to-noise. Previous research suggested that increased concentrations of paramagnetic ions (Fe^{3+} and Mn^{2+}) in the lower ratio extracts may impact on spectral resolution. This was not the case for the current study; however, the potential for greater interference by paramagnetic ions for lower ratio extracts still needs to be considered. In other words, the results of this study

should not be taken to mean 1:4 extract ratios should be adopted for all soils, rather adopting a lower ratio should be considered for low P soils and comparison of spectra carried out to test whether extractability or resolution are adversely affected.

In summary, the adoption of a tighter extraction ratio of 1:4 proved useful for improving the determination of P composition using solution ^{31}P NMR spectroscopy for typical Australian soils with low P (Moata *et al.* 2015). This finding has since been reinforced in another study on Australian soils (McLaren *et al.* 2015). The approach developed here could also be implemented for soils from other regions that face a similar problem with low P concentrations.

Stoichiometry of C, N, P with organic P species characterised by solution ^{31}P NMR spectroscopy

After improving the method, the next step was a detailed investigation of the C:N:P stoichiometry of twenty Red Chromosol soils making use of the detailed chemical speciation of P provided by solution ^{31}P NMR spectroscopy. The composition and distribution of P species for the twenty soils were comparable to other Australian soils (Doolette *et al.* 2011; McLaren *et al.* 2014), with the most abundant P species detected being orthophosphate and humic P followed by lipid P, RNA P, inositol hexakisphosphate (*myo* + *scyllo*), diester P and pyrophosphate.

Correlations of varying strength were found between the various P species detected across the twenty soils (Chapter 3). The strongest correlations detected were between the two inositol phosphate isomers and various combinations of lipid P, RNA P, diester P and pyrophosphate. Based on these correlations, a novel four-pool model of soil P was developed, consisting of orthophosphate, humic P, cellular P (lipid P, RNA P, diester P and pyrophosphate) and

inositol phosphate P (*myo*- and *scyllo*-inositol hexakisphosphate). This model takes into account not only the strength of correlations between P species, but also the obvious chemical connection between the inositol phosphate isomers and the functional connection between the cellular P components. These are the main P species (other than orthophosphate) detected in both microbial cells (fungi and bacteria biomass) (Bünemann *et al.* 2008b; Makarov *et al.* 2002) and plant tissue (Noack *et al.* 2012; Noack *et al.* 2014).

The four-pool model was developed primarily in an attempt to better understand C:N:P stoichiometry of soil organic matter. It has previously been reported that correlations of C and N are stronger to organic P than total soil P (Kirkby *et al.* 2011). This is to be expected, as most inorganic P in soils is present in low-solubility minerals and so would not be expected to be strongly coupled to the cycling of organic matter. The four-pool model extends this concept by providing a logical partitioning of organic P into forms that could be expected to cycle rapidly (cellular P) and more slowly (humic P and inositol P). The division of the latter two reflects differences in the likely stabilisation mechanism, with humic P cycling being limited by turnover of stable organic matter and inositol P reportedly being stabilised through direct interaction with soil minerals.

Correlations of the four P pools with C and N were generally consistent with their conceptual basis. Cellular P exhibited strong correlations with C and N, suggesting a strong stoichiometric control for this small and rapidly cycling pool. On the other hand, correlations of the humic and inositol pools with C and N were much weaker.

In summary, the hypothesis that there is stricter C:N:P stoichiometric control of some components of SOM than others was supported. The development of both a methodology and

a conceptual model to prove this hypothesis is perhaps the most important outcome of this thesis and opens the way for future research in this area.

Stoichiometry of C, N, P in fine and coarse soil fractions compared to that of whole soils

Following the development of a novel four pool model of P in soils that facilitated the identification of a strong hidden stoichiometry of C and N with a particular soil P fraction (cellular P), further investigation of the twenty Red Chromosol soils was aimed at determining whether there is any driver of C, N, and P stoichiometry in different physical fractions of soil. The soils were separated based on particle size (fine fraction <50 μm and coarse fraction >50 μm) using the method of Baldock *et al.* (2013). On average, 60% of soil mass was isolated in the fine fraction and the rest in the coarse fraction. The coarse fraction had on average lower C and N contents and these varied greatly among soils. This variation reflected differences in soil texture, as the coarse fraction consisted primarily of particulate organic matter and sand particles. Unsurprisingly, the C and N contents of the lighter textured (sandier) soils were lower. The fine fractions also had relatively low C and N contents, but the variation was smaller, presumably because the clay particles that comprised the majority of this fraction contain a relatively consistent concentration of sorbed organic matter.

The C and N ratios for both fractions and the whole soils were tightly constrained and increased in the order: fine fraction < whole soil < coarse fraction. The C:N ratio of the coarse fraction was presumably wider because the organic matter of this fraction mostly consists of relatively undecomposed plant material (Six *et al.* 2001) while that of the fine fraction is mainly humified plant material and microbial residues.

Interestingly, correlations of C and N to P were stronger for the coarse fractions than the fine fractions. In fact, there were no significant correlations at all for the fine fractions. This was true for both total P and organic P as determined by the Saunders and Williams (1955) method. Positive intercepts for the correlation of organic P with C and N provided some evidence that this method consistently overestimates organic P.

Solution ^{31}P NMR spectroscopy was used to compare the organic P speciation of the two fractions and showed that the fine fractions were comprised mostly of humic P while the coarse fractions contained relatively more cellular P. Unfortunately, it was only possible to analyse the size fractions of two soils in this way due to limitations in the amount of soil available and the low sensitivity of NMR analysis.

Overall, this part of the study further demonstrated the impact of organic matter heterogeneity (in particular the fact that organic matter is a complex mixture of components) on overall C:N:P stoichiometry. Whereas the previous section (Chapters 3) related to components defined by chemical composition, this section extended the concept to physical fractions that are believed to relate closely to the degree of decomposition and microbial processing. Thus, this set of experiments can be summarised as confirming that P stoichiometry is strongest for the plant-dominated and relative undecomposed fractions of organic matter (i.e. the coarse fraction).

Soil microbial biomass P pool in amended and unamended soils

The final part of this study was an incubation experiment conducted to investigate the relationship between the cellular P pool and the traditionally measured microbial biomass P pool. A different soil was used (i.e. not the Red Chromosol soils used in the previous

experiments (Chapters 2-4)). The soil chosen for this part of the study was a sandy loam from near Mt. Gambier. The soil was collected from a dairy pasture soil and is rich in organic matter and nutrients (5.3% total C, 0.5% total N, and 1734 mg/kg total P). Although the Mt Gambier soil contained the same organic P species as the Red Chromosol soils, the relative proportions of these species differed, with the Mt. Gambier soil containing considerably more inositol phosphate than the Red Chromosols.

Samples of the soil were incubated for 31 days with and without the addition of glucose. As expected, glucose addition resulted in higher microbial respiration; an increase in respiration by a factor of around 30 was recorded early in the incubation, decreasing to a factor of around 7 through the second half of the incubation. In contrast, there was relatively little influence of glucose addition on traditionally measured MBP, i.e. the difference in resin-extractable orthophosphate between hexanol fumigated and unfumigated soils. The average MBP values were 17.7 mg/kg for the glucose-amended soil and 13.4 mg/kg for the unamended soil. It was found that resin P values reduced in glucose amended soils and this was interpreted as orthophosphate being taken up by microbes (Qiu *et al.* 2008). It was deemed likely that MBP concentrations were underestimated because some inorganic P taken up by microbes was transformed to organic P forms and was not detected by colorimetry following hexanol fumigation.

Solution ^{31}P NMR spectroscopy was used to compare the P speciation of glucose-amended and unamended soils. This showed no significant changes in organic P speciation on glucose amendment. This was an unexpected result given that soil respiration clearly increased on glucose amendment and it was expected that an increase in microbial biomass would increase cellular P components, especially phospholipid and RNA. There was also no detectable

change in the concentration of these components over the course of the incubation for either amended or unamended soils. The possibility that the lack of an effect could be due to low extractability of cellular P was deemed unlikely. Although overall P extractability for this soil was only moderate (41-46% recovery), previous studies have shown that cellular P components tend to be more extractable than this (Bünemann *et al.* 2008a; Bünemann *et al.* 2008b; Noack *et al.* 2012; Noack *et al.* 2014), and besides, the cellular P components were detected for all soils.

An attempt was made to combine hexanol fumigation with NMR analysis. Crucially, organic P speciation of soil after hexanol fumigation and resin extraction did not differ from that of soil subject only to resin extraction. This demonstrated that hexanol fumigation-extraction did not release sufficient quantities of organic P to be detected by ^{31}P NMR. Overall, this part of the study showed there was no close relationship between the cellular P pool determined by NMR and the MBP pool determined by the hexanol fumigation-extraction method. Together with the results from Chapter 4 on size fractionation, this suggests that cellular P is more closely related to P in plant residues than microbial biomass.

Future research directions and priorities

To address some issues highlighted in this study and in order to continue investigating the stoichiometry of the key elements of SOM (C, N, P) in terrestrial ecosystems, future research in the following areas is recommended.

1. The four pool model of soil P (orthophosphate, humic P, cellular P and inositol phosphate P) was developed from an analysis of a restricted set of soils. Therefore, the finding that the cellular P pool exhibited the strongest stoichiometric control to C and N is thus far only proven for this restricted set of soils. However, it was noted that the

composition of P in these soils was broadly similar to that of other soils (i.e. the P in most soils can be partitioned into these four categories following solution ^{31}P NMR analysis). Therefore, the first recommendation for future research is to test the hypothesis and the generality of the finding that cellular P is under stronger stoichiometric control (to C and N) than other soil P pools by carrying out similar analyses to those described in Chapter 3 for a broader selection of soils (e.g. Calcarosols, Sodosols, etc).

2. Overall, results from all experiments (Chapters 2-5) indicated the main source of "cellular P" (phospholipid, RNA, diester P and pyrophosphate) was plant residues rather than soil microbes. Previous studies have shown that plant residues are rich in cellular P (Noack *et al.* 2012; Noack *et al.* 2014). In particular, cellular P comprised a greater proportion of total P in the coarse fraction of soils, which is mainly derived from plant materials, than in the fine fraction, which is believed to be richer in microbially- derived organic matter. Furthermore, the stimulation of microbial growth through the addition of a labile carbon source (glucose) did not have a measurable effect on the concentration of cellular P. Therefore, the second recommendation for future research is to carry out an incubation study in which plant residues and glucose are added, both separately and together, or growing different types of plants in order to test the hypothesis that cellular P is primarily derived from plant material.
3. Further effort is required to design incubation experiments where changes in P composition are actually observed. It was not clear why no changes were observed for the incubation experiment described in Chapter 5, but some factors that can be investigated are the level of soil moisture, the rate of soluble carbon (glucose) addition and the incubation period.
4. It was noted that the Mt Gambier soil used in Chapter 5 was richer in inositol phosphates than the Red Chromosol soils used in Chapters 2-4. The cause of this difference should

be investigated. The Mt Gambier soil had a much higher total P content (1734 mg/kg) than the Red Chromosols (252-631 mg/kg). This raises two potential causes for the higher relative amount of inositol P in the Mt Gambier soil: (i) the Mt Gambier soil is inherently more fertile and thus the plants that grow on it are P sufficient and as a result their biomass contains more inositol phosphate; or (ii) the Mt Gambier soil contains more minerals that bind strongly to some P forms, in particular, orthophosphate and inositol phosphate. In other words, the two possible reasons for the higher inositol phosphate concentration of the Mt Gambier soil are greater inositol phosphate inputs or slower inositol phosphate decomposition. These two possibilities could be distinguished in an incubation experiment in which inositol phosphate is added and its decomposition rate directly measured.

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