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

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

ABSTRACT

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

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

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

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

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

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

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

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

DECLARATION

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

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Ashlea Doolette **Date**

PUBLICATIONS ARISING FROM THIS THESIS

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

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

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

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

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

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

STATEMENT OF AUTHORSHIP

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

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

DOOLETTE, A.L. (Candidate)
Experimental development, performed analysis on all samples, data analysis and critical interpretation, wrote manuscript.

I hereby certify that the statement of contribution is accurate.

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SMERNIK, R.J.
Supervised development of work, data analysis and interpretation, reviewed manuscript.

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

I hereby certify that the statement of contribution is accurate.

Signed Date 13th July 2010

STRUCTURE OF THIS THESIS

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

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

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

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

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

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

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

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