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Phosphorus reactions and fluxes in pasture soils

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Abstract

Pasture growth is often limited by the availability of soil phosphorus (P). Adding P fertiliser can overcome this limitation but the balance efficiency of the fertiliser, i.e. the proportion of fertiliser P that is exported from the paddock in produce in the year of application, is generally low (e.g. < 20%). The objective of this project was to investigate the causes of this low efficiency. Field trials using superphosphate containing a radioactive label indicated relatively high (30-60%) P use efficiency of pasture species (i.e. the proportion of fertiliser P that is taken up by plants). Analysis of soils from long-term field experiments showed that over longer timescales P accumulates in both inorganic and organic forms, with much of the organic P accumulating in these soils apparently produced by a previously unrecognised process. Together these results indicate that an important contributor to low P balance efficiency in grazed pastures are reactions occurring over longer time scales sequestering some of the P cycling in the soil-plant-animal system. Incorporation of this knowledge into fertiliser P management and the selection and breeding of pasture plants holds much promise for reducing P fertiliser requirements in the red meat industry.

Executive summary

Pasture growth is often limited by the availability of soil phosphorus (P) to pasture species, especially legumes. This P limitation is usually overcome by adding P fertiliser (e.g. superphosphate). However, balance efficiency of the fertiliser, i.e. the proportion of fertiliser P that is exported from the paddock in produce in the year of application, is generally low (e.g. < 20%). It is well recognised that most of the added P accumulates in the topsoil in forms that are less available to plants than freshly added fertiliser P. However, until now, our understanding of the processes by which the P becomes less available and the forms in which it accumulates has been limited. The objective of this project was to provide a fresh and detailed insight into the causes of low P balance efficiency through the development and application of radio-labelled P fertiliser and nuclear magnetic resonance (NMR) spectroscopy. These new insights should facilitate decreases in P fertiliser costs to producers.

The incorporation of ^{33}P (radioactive phosphorus) into a fertiliser provided a direct and sensitive way to follow the fate of that fertiliser in the plant-soil system and to measure fertiliser P use efficiency (PUE), i.e. the proportion of fertiliser P that is taken up by plants during the season in which it was applied. A key, initial outcome of this project was the development and validation of a novel method for incorporating ^{33}P radio-label into superphosphate. The radio-labelled superphosphate was then used to follow the fate of fertiliser P in pasture field trials during two growing seasons and at three sites. The central finding in all cases was that a large proportion of fertiliser P (30-60%) was detected in subterranean clover biomass, with the majority of remaining P detected in inorganic forms in the topsoil layer. In cropping systems plant access to fertiliser P, i.e. PUE measured using similar isotopic methods, is usually in the range 10-30%, yet P balance efficiency in cropping systems is much higher than in pasture systems. The implication is that low plant uptake of freshly added fertiliser is probably not the main contributor to low P balance efficiency in pastures. In the second year of field trials, variations of fertiliser timing (autumn versus spring) and placement (surface versus incorporated into the soil) were tested. Overall, differences in the initial uptake of fertiliser P were small. If anything, the standard practice of surface application in autumn was the best by a small margin.

Having established that fixation of fertiliser P in the immediate months after fertiliser addition was not the only cause of low balance efficiency in pastures, we turned our attention to the longer-term processes involving fertiliser P. The radio-tracer approach is not suitable for this because the half-life of ^{33}P is relatively short at 25 days and this limits how long it can be followed. The rule of thumb is that it is impractical to follow the fate of a radio-tracer for longer than ten times its half-life. Thus the radio-tracer approach is only useful for following the fate of fertiliser P for one season and determining the fate of fertiliser P over longer timescales requires a different approach. In this project, we made use of a long-term field experiment at the Ginninderra Experiment Station (Hall, ACT) in which pastures with three different levels of P fertility (low, optimal and supra-optimal) were established and then maintained over 13 years. By comparing the chemical speciation of soil P between these treatments, we were able to deduce the forms of P that had accumulated in the soil and the processes that may be involved. Chemical speciation was assessed

using sequential extraction and solution ^{31}P NMR spectroscopy. Results from the two techniques were quite consistent and pointed to accumulation of P in both inorganic and organic forms. NMR analysis, which provides detailed characterisation of alkaline-extractable organic P forms, provided particular insight into the process of organic P accumulation. A key finding here was that the composition of organic P that accumulated in the fertilised pastures differed little from the composition of organic P originally present. Both were dominated by high molecular weight material. Here we refer to this high molecular weight organic P as “humic P”. It accounted for approximately 75% of the soil organic P in all treatments. This result infers that the majority of organic P present in this soil and that accumulated when P fertiliser was applied, was produced by a previously unrecognised process since high molecular weight humic P is not found in either plant or microbial biomass. The disparity of this finding to the established models of organic P accumulation, which invoke high stability of inositol phosphates either through strong interactions with soil minerals or resistance to most phosphatase enzymes, led us to seek confirmation of the “humic P” interpretation. This was achieved by combining ultrafiltration with NMR analysis, which established beyond doubt the presence of high molecular weight organic P in not only the Ginninderra soil but a range of other soils from around the world.

This research has demonstrated that P uptake from superphosphate applied to the soil surface of an establishing subterranean clover sward was relatively high with up to 60% of the applied P accounted for in the pasture sward. The PBI of the soils examined was in the low range but were typical of very broad areas of Australian farmland. The efficiency of direct fertiliser P uptake by the clover was at least equivalent to that achieved when P fertiliser is applied to a crop. This is particularly interesting because the long-term P efficiency of grazing systems (when measured as P-balance efficiency [100% * $P_{outputs}/P_{inputs}$]) is generally much lower than that of crops. The highest uptake of P directly from the fertiliser was achieved in an optimally-fertilised pasture and when P was applied to an establishing sward soon after the break of season in autumn. These conditions would be regarded as fairly typical and “best practice” in temperate pasture systems (cool autumn/winter; moist autumn, winter and spring). However, the uptake efficiency penalty for delayed application (early spring) or in under- and over-fertilised soils was relatively small and for pragmatic farm-management purposes indicated that a wide window of fertiliser application timing was permissible.

The P-balance efficiency of the “optimal” pasture system used in this work has been reported elsewhere and is very low (~20%). This level of P efficiency is also reasonably close to industry median P-efficiency values. This tells us that over the longer term about 80% of the P applied as fertiliser is accumulated in this fertilised field despite the relatively high immediate P uptake by the clover. Even if we assume that fertiliser P that was not taken up by clover in the first season had entered the accumulating pools of P in the soil, we can deduce that a large proportion of the P was accumulated in the soil as a consequence of its subsequent cycling in the soil-plant-animal system. Recycling of phosphate in the pasture system is likely to re-expose phosphate to soil P-sorption reactions throughout the growing season. This seems to be the main reason why pasture and crop systems differ so much in their P-balance efficiency.

One fertiliser timing issue that could not be examined in the present study due to time limitation was early fertiliser application (i.e. application to dry soil before the break of season). This is also a common practice and deserves further analysis using the novel radio-labelling method developed in the project. Time of application was also examined in a pasture system that experiences a high incidence of later spring-summer rainfall. Unseasonal droughts did not allow the effect of time of application to be resolved in this environment and this also deserves further work.

The majority of P accumulated in fertilised soil was present in inorganic forms and further study of this process is needed. However, accumulation of organic P was also an important sink for P in the soil. The NMR analysis showed that high molecular weight “humic P” represented a large proportion of the accumulated organic P. Little is known about how humic P is formed or how amenable it is to hydrolysis and release of P to plant available pools. This also deserves further study as part of work to find ways to increase the effectiveness of P fertiliser use in pasture systems.

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1 Background

The efficient use of phosphorus (P) in agriculture is essential to increase and maintain productivity on many soils, as well as to safeguard the quality of surface water and to conserve P resources. The application of fertiliser is a significant cost to producers and 64% of Australian producers apply fertiliser. Pasture or livestock production has an average P-balance efficiency of 20% and most of the P applied is either accumulated in soils or lost to the environment (McLaughlin *et al.* 1991; Weaver and Wong 2011). An analysis of cross sector agricultural industries shows that sheepmeat/wool and beef production are amongst the least efficient agronomic enterprises (compared to grain P balance efficiencies of 45-50%).

Improvements in fertiliser management can arguably provide the fastest improvement in P efficiency as uptake and adoption can potentially be very rapid. However, the problems of matching P supply to plant demand and minimising fertiliser P sorption reactions in soil are substantial. There have been very innovative solutions addressing soil-specific P-use problems that have delivered large P-efficiency gains (e.g. fluid P fertiliser for calcareous soils). However, technology change has otherwise been relatively slow, probably reflecting the amount of research effort in this area.

There are still a surprising number of gaps in knowledge about the reactions of phosphate in soil and around fertilisers; especially those leading to P accumulation as sparingly-available phosphate and organic P. Relatively little is known about the P-acquisition mechanisms of P-efficient plants and the role and ecology of soil micro-organisms (including mycorrhizae) in making P available to crops and pastures. Strategic investment in new knowledge is ultimately the only way to develop innovative answers to difficult problems. Innovative fertiliser technologies and the development of novel plants that may access organic P in soil are potentially constrained by a limited understanding of soil P reactions and the chemical forms of organic P in soil. The aim of this project was to develop new knowledge in our understanding of the intractable problems of P supply and P reactions in soil and lay foundations to better manipulate nutrient cycling including P sorption, in order to access the stored P ‘bank’ accumulated in soil.

2 Projective objectives

- Understanding of the rates of transformation of added fertiliser P in pasture production systems. Knowledge of the rates of transfer of P fertiliser on an annual basis to plant shoots, roots and inorganic and organic P pools in the soil. This will be communicated in scientific papers (for scientists) and also in extension material relating to changed fertiliser management (for livestock producers).

- Identification of the major forms of accumulated P in fertilised pasture soils to aid identification of technologies to release P from this pool, or to minimise accumulation in fertilised pastures. This will be communicated in scientific papers (to scientists).

- Fertiliser management strategies to manipulate fluxes of P into plants and minimise transfer to organic and inorganic pools

3 Methodology

3.1 Field trials involving addition of ^{33}P-labelled single superphosphate (SSP)

3.1.1 ^{33}P-labelling of single superphosphate (SSP) granules

A rapid and simple procedure was used to label single superphosphate (SSP) with a ^{33}P radiotracer (half-life 25.4 days), and then 'granulate' (into cubes) using a cut and press technique. In summary, commercial SSP granules were sourced and dried in a laboratory oven at 40°C overnight. The commercial SSP granules were then ground to pass through a 150 µm sieve using a Siebtech© puck mill. Six × 7.0 g (± 0.1 g) aliquots of the SSP powder were then placed into separate 65 mm × 75 mm cylindrical plastic containers prior to incubation with the ^{33}P radiotracer. A 2.0 mL aliquot of solution containing 60 MBq of ^{33}P was added to each of these, resulting in a final moisture content of 22.2 %. The SSP slurry in each container was stirred with a fine needle and then allowed to equilibrate overnight at 40°C in a laboratory oven. Through this treatment, the moisture content of the ^{33}P-labelled slurry was reduced to 19.8%, which was ideal for pressing and cutting based on results of a pilot study. Each batch was worked into a 'bolus' and placed into a 40 mm diameter ring, positioned between two plastic sheets and pressed into a disc at a pressure of 37.9 kPa using a manual hydraulic press (custom built by Templeton, Kenly & Co: Actuant Corporation, Menomonee Falls, WI). The discs were then cut into 3 mm cubes using a knife and dried in a laboratory oven at 40°C overnight. Granules were then weighed and grouped into batches prior to field application.

The P diffusion pattern of the SSP granules made using the press and cut technique and that of the commercial SSP granules was determined using the quantitative visualisation method of Degryse and McLaughlin (2014). The Monarto soil as reported in Degryse and McLaughlin (2014) was used for the test. In summary, eight Petri dishes (55 mm diameter × 10 mm deep) were filled with soil and wetted to field capacity, then sealed with Parafilm and allowed to incubate overnight at 25°C. A hole was made in the soil at the centre of each Petri dish and a single granule was inserted. Each SSP granule weighed 55 – 60 mg and four replicates were used per SSP treatment. The granule was then covered over with soil and the Petri dish re-sealed with Parafilm. Iron-oxide filter papers were prepared as set out by Degryse and McLaughlin (2014). After 7 days incubation, iron-oxide filter papers were deployed for approximately 10 mins on the soil surface of each Petri dish. The filter papers were then removed, stained with malachite green solution, and the stained filter papers were scanned and analysed for the stained area (i.e. area of P diffusion) using imaging software (GNU Image Manipulation Program, v. 2.8.10, Free Software Foundation, Boston, MA). This involved the conversion of the scanned image to a binary image (black-white) using a threshold value of 120 on a scale from 0 – 255, and the stained area quantified (Degryse and McLaughlin 2014).

3.1.2 Field experimentation 2013

Two field sites were selected for field trials in 2013 in the temperate region of south-eastern Australia that were under permanent pasture. A site of high soil P fertility (i.e. predicted to not be fertiliser P responsive) located at the Kybybolite Research Centre, near Naracoorte, South Australia (36°52' S, 140°55' E), and a site of low soil P fertility (i.e. predicted to be fertiliser P responsive) located at the Ginninderra Experiment Station, near Hall, Australian Capital Territory (35°11'S, 149°3'E). The Ginninderra field site was located on the control fields (i.e. an unfertilised treatment; pasture that receives no P fertiliser and referred to here as P0, and a stocking rate of 9 sheep per hectare) of the long-term fertiliser by grazing permanent pasture at the CSIRO Ginninderra Experiment Station (Simpson *et al.* 2015). The Ginninderra field site received no applications of fertiliser P but 4 kg P ha^{-1} as supplementary feed since 1994. The Naracoorte field site received annual applications of 100 kg of SSP ha^{-1} (8 kg P ha^{-1}) since 1935.

Chemical properties were determined on soil collected at pasture establishment and prior to fertiliser application. Six soil samples were collected from four depth intervals (0 – 2.5 cm, 2.5 – 5 cm, 5 – 10 cm and 10 – 20 cm) and bulked within each depth and for each field site location. The soil was then dried in a laboratory oven at 40°C for one week and passed through a 2 mm sieve. Soil pH and electrical conductivity (EC) were measured using a 1:5 soil to solution ratio in deionised water after shaking for 1 hour. A measure of oxalate-extractable aluminium and iron was carried out as described by McKeague and Day (1966). Total soil carbon and total soil nitrogen were determined on a LECO TruSpec CN analyser (LECO Corporation, St Joseph, MI) according to Matejovic (1997). An estimate of total organic P (and inorganic P) was carried out using the ignition-H_2SO_4 method of Walker and Adams (1958). The bicarbonate extraction method of Colwell (1963), which is based on the method of Olsen *et al.* (1954), was used to predict soil P fertility. In summary, 0.5 g (± 0.02) of soil was extracted with 0.5 M $NaHCO_3$ solution adjusted to pH 8.5 at a 1:100 soil to solution ratio and shaken for 16 hours. The Colwell extracts were then centrifuged at 1610 × *g* for 20 minutes, filtered through a Whatman no. 42 filter paper, and concentrations of inorganic P in the filtrate were determined using the molybdenum blue method of Murphy and Riley (1962). All values were blank corrected before standard volume and weight conversions. A measure of P buffering capacity was determined using the single-point method of Burkitt *et al.* (2008), referred to as the P buffering index (PBI).

Total soil P was determined using laboratory X-ray fluorescence carried out at Geoscience Australia laboratories, Canberra, Australia. A Philips PW2404 4 kW sequential wavelength dispersive spectrometer fitted with a rhodium X-ray tube was used to analyse fused beads made from 1.0 g soil / 6.0 g 12:22 flux (35 % lithium tetraborate / 65 % lithium metaborate). Analytical recoveries were calculated using reported values of the Canadian Certified Reference Materials Project (CCRMP) soil standards (Till-1 and Till-4). On average, an analytical recovery of 99 % was obtained using laboratory X-ray fluorescence.

In May 2013, a 5 m × 5 m fence was erected at each field site to prevent outside interference by grazing animals (e.g. sheep, kangaroos and rabbits) and the pasture

within the enclosure was sprayed with knockdown herbicide to remove all vegetation. In June 2013, a subterranean clover sward was established across the enclosure by broadcasting seed to ensure that there was at least 1 seed cm^{-2} and then lightly scratching seed into the soil surface. After clover establishment, 18 'open-ended' cylinders (polyvinyl chloride cylinders – PVC – cores) of 15 cm diameter and 18 cm in height were inserted 15 cm into the soil at each site, so that each core protruded 3 cm above the soil surface, as described by McLaughlin *et al.* (1988b). Basal nutrients were then applied across the field sites in July 2013 to include: 1) Ca as $CaSO_4$ to supply 6.4 kg Ca ha^{-1}; 2) K as K_2SO_4 to supply 44.9 kg K ha^{-1}; 3) Mg as $MgSO_4$ to supply 12.1 kg Mg ha^{-1}; 4) Mo as MoO_3 to supply 0.1 kg Mo ha^{-1}; 5) B as H_3BO_3 to supply 0.3 kg B ha^{-1}; 6) Cu as $CuSO_4$ to supply 0.7 kg Cu ha^{-1}; 7) Zn as $ZnSO_4$ to supply 1.4 kg Zn ha^{-1}, and; 8) S as all previously mentioned sulphate salts to supply 40.5 kg S ha^{-1}.

Each treatment was replicated six times in a randomised block design. Treatments included a control (no added P fertiliser), the addition of commercial SSP granules (8 % P) to supply ~12 kg P ha^{-1}, and the addition of ^{33}P-labelled SSP granules to supply ~12 kg P ha^{-1}. In August 2013, the clover pasture sward was harvested to no less than 3 cm above the soil surface and discarded prior to fertiliser placement. At the Ginninderra site, four granules were used to supply 250 mg (± 5 mg) of SSP $core^{-1}$ (average 62.5 mg of SSP $granule^{-1}$) to the soil surface of each core for the commercial SSP and ^{33}P-labelled SSP treatments. At the Naracoorte site, four granules were used to supply 285 mg (± 5 mg) of SSP $core^{-1}$ (average 71.3 mg of SSP $granule^{-1}$) to the soil surface of each core for the commercial SSP and ^{33}P-labelled SSP treatments. The P rate of the commercial SSP treatment that was applied to the Ginninderra and Naracoorte field sites was 11.4 and 12.9 kg P ha^{-1}, respectively. The P rate and radioactivity of the ^{33}P-labelled SSP treatment that was applied to the Ginninderra and Naracoorte field sites was 11.4 and 13.0 kg P ha^{-1} and 4.2 and 4.8 MBq $core^{-1}$, respectively. In addition, the control cores received a second application of nutrients to balance the S from the SSP treatments, which included Ca and S as $CaSO_4$ to supply 15.6 kg Ca ha^{-1} and 12.5 kg S ha^{-1}. Each site was irrigated to ensure the cumulative rainfall throughout the growing season was close to that of the long-term average. Small amounts of irrigation were mostly associated with watering in applications of basal nutrients to ensure they were accessible to clover roots.

Four clover shoot cuts were collected at the Naracoorte field site (13/09/2013, 01/10/2013, 30/10/2013 and 05/11/2013) and two clover shoot cuts were collected at the Ginninderra field site (24/09/2013 and 14/11/2013). The clover shoots were cut to 3 cm above the soil surface. At the final harvest, the residue of all fertiliser granules was collected from the soil surface, and the cores were removed and sectioned into three layers; the surface layer (0 – 4 cm) and subsurface layer (4 – 8 cm), and a 'buffer' soil layer (8 – 15 cm), which was discarded. At the Ginninderra field site 92 % of the SSP granule residues were recovered from the soil surface, whereas 100 % of the SSP granule residues were recovered from then soil surface at the Naracoorte field site.

3.1.3 Field experimentation 2014

Field trials of two different designs were carried out in 2014. The first design was aimed at confirming the high PUE (P use efficiency) reported for the 2013 field trial and also to investigate how timing and placement of fertiliser affects PUE. The second design was aimed at determining the influence of initial P fertility on PUE

3.1.3.1 Design 1: Effect of timing and placement of fertiliser

Two field sites were selected for field trials in 2014 in the temperate region of south-eastern Australia that were under permanent pasture. Both sites were of low soil P fertility (i.e. predicted to be fertiliser P responsive), located at the Ginninderra Experiment Station, near Hall, ACT (35°11'S, 149°3'E), and near Inman Valley, SA (35°30' S, 138°27' E). In April 2014, a 7 m × 5 m fence was erected at each field site to prevent outside interference by grazing animals (e.g. sheep, kangaroos and rabbits) and the pasture within the enclosure was sprayed with knockdown herbicide (Ginninderra) or removed by hand (Inman Valley) to remove all vegetation.

In April 2014 (Ginninderra site) and May 2014 (Inman Valley site), a subterranean clover sward was established across the enclosure by broadcasting seed to ensure that there was at least 1 seed cm^{-2} and then lightly scratching seed into the soil surface. After clover establishment, 42 'open-ended' polyvinyl chloride (PVC) cylinders (cores) of 15 cm diameter and 18 cm height were inserted 15 cm into the soil at each site, so that each core protruded 3 cm above the soil surface, as described above for the 2013 field trials. Basal nutrients were then applied across the field sites in May 2014 (Ginninderra site) and June 2014 (Inman Valley site) as described above for the 2013 field trials.

Each treatment was replicated six times in a randomised block design (Fig. 1). Treatments are described in Table 1. The fertilised treatments all involved the addition of ^{33}P-labelled SSP granules to supply approximately 20 kg P ha^{-1}. This higher rate of P fertiliser addition compared to the 2013 field trials (12 kg P ha^{-1}) was used to increase the likelihood of an agronomic response to fertiliser management strategies. The early-season application of fertiliser P to clover pastures occurred with the break of the season on the 29th April 2014 at the Ginninderra site and on the 5th of June 2014 at the Inman Valley site. The mid-season application of fertiliser P to clover pastures occurred on the 29th July 2014 at the Ginninderra site and on the 14th of August 2014 at the Inman Valley site. The end of the growing season (last harvest and removal of cores) for treatments A, D, E, F and G (Table 1) was on the 3rd of November 2014 at the Ginninderra site and on the 21st of October 2014 at the Inman Valley site. The end of the growing season (last harvest and removal of cores) for treatments B and C was on the 29th of August at the Ginninderra site and on the 14th of August 2014 at the Inman Valley site.

The early-season application of ^{33}P-labelled SSP to the soil surface was replicated three times (i.e. treatments A, B and C). This was done for two reasons. Firstly, the radioactivity of the ^{33}P radio-label in the soil, and possibly also the root fractions of this treatment, would be too low for detection at the end of the growing season. Therefore, an additional replicate was included (i.e. treatment B) so that the soil

fraction of this treatment could be analysed for the ^{33}P radio-label prior to the favourable growing conditions usually present in spring (mid-season). Secondly, a third replicate was included (i.e. treatment C) so that the root fraction of this treatment could also be analysed for the ^{33}P radio-label. It is not possible to isolate both soil and root fractions from the same core because the isolation of roots by washing results in dispersion of the soil in a large volume of water. Similarly, the mid-season application of ^{33}P-labelled SSP to the soil surface was carried out in duplicate (i.e. treatments E and F) in order for the soil and root fractions of this treatment to be analysed for the ^{33}P radio-label.

Eleven granules were used to supply 408 mg (±5 mg) and 403 mg (±5 mg) of SSP $core^{-1}$ (average 37.1 mg and 36.6 mg of SSP $granule^{-1}$) to the fertilised treatments for the autumn application (A, B, C and D) of the Ginninderra and Inman Valley field sites, respectively. At both sites, nine granules were used to supply 402 mg (± 5 mg) of SSP $core^{-1}$ (average 44.7 mg of SSP $granule^{-1}$) to the fertilised treatments for the spring application (E and F). The P rate of the fertilised treatments for the autumn application was 19.9 kg P ha^{-1} and 19.6 kg P ha^{-1} at the Ginninderra and Inman Valley field sites, respectively. At both sites, the P rate of the fertilised treatments for the spring application was 21.8 kg P ha^{-1}. The radioactivity of ^{33}P added to the fertilised treatments for the autumn application was 4.1 MBq $core^{-1}$ and 4.0 MBq $core^{-1}$ at the Ginninderra and Inman Valley field sites, respectively. At both sites, the radioactivity of ^{33}P added to the fertilised treatments for the spring application was 3.3 MBq $core^{-1}$. In addition, the control cores received a second application of nutrients to balance the S from the SSP treatments, which included Ca and S as $CaSO_4$ to supply 15.6 and 12.5 kg (Ca + S) ha^{-1} respectively. Each site received irrigation to ensure a supply at least equivalent to Decile 5 rainfall.

There were five harvests collected at the Ginninderra site (16/06/2014, 04/08/2014, 29/08/2014, 07/10/2014 and 03/11/2014) and four harvests collected at the Inman Valley site (14/08/2014, 04/09/2014, 24/09/2014 and 21/10/2014). At each harvest, the clover sward was harvested to 3 cm above the soil surface. At the last harvest, the 0 – 3 cm portion of clover shoots was also harvested. In addition, the residue of all fertiliser granules was collected from the fertilised treatments, and the cores were removed and sectioned into three layers: the surface layer (0 – 4 cm), upper subsurface layer (4 – 8 cm), and lower subsurface layer (8 – 15 cm).

For the treatments designated for root analysis, soil was washed from the roots with water in each of the collected layers on a stacked set of sieves. The mesh size of the sieves were 2 mm, 1 mm and 0.075 mm. No root material was observed in the 0.0075 mm sieve. Since the root fraction contained old and fresh roots, the root fraction will also include decaying roots from previous pastures and this may contain some P. After collection, the root material (old and fresh) was then dried, ground and prepared for chemical analysis.

Table 1. A description of the treatments used at the Ginninderra and Inman Valley field sites for the 2014 field trial. The fertilised treatments involved the addition of ^{33}P-labelled single superphosphate (SSP) granules to supply ~ 20 kg P ha^{-1}.

Treatment ID	Placement of fertiliser P added to pastures	Time of fertiliser P added to pastures	Time of treatment finish	Description of core analysis
A	Soil surface	Early-season	End of growing season	Soil fractions were collected but the ^{33}P radio-label was too low for detection
B*	Soil surface	Early-season	Mid-season	Soil fractions were collected and analysed for the ^{33}P radio-label
C†	Soil surface	Early-season	Mid-season	Root fractions were collected and analysed for the ^{33}P radio-label
D	At 6 cm below the soil surface	Early-season	End of growing season	Soil fractions were collected but the ^{33}P radio-label was too low for detection
E	Soil surface	Mid-season	End of growing season	Soil fractions were collected and analysed for the ^{33}P radio-label
F‡	Soil surface	Mid-season	End of growing season	Root fractions were collected and analysed for the ^{33}P radio-label
G	No fertiliser P added	No fertiliser P added	End of growing season	Soil fractions were collected but no ^{33}P radio-label was added

* Treatment B is a duplicate of treatment A. However, this treatment was ended at mid-season (removal of cores and collection of soil fractions) in order to analyse the soil fractions for the ^{33}P radio-label, which would not be possible at the end of the growing season.

† Treatment C is a duplicate of treatment A. However, this treatment was ended at mid-season (removal of cores and collection of root fractions) in order to analyse the root fractions for the ^{33}P radio-label, which may not have been possible at the end of the growing season.

‡ Treatment F is a duplicate of treatment E. At the end of the growing season (removal of cores), the soil fraction was collected in the cores of treatment E and the root fraction in the cores of treatment F in order to analyse both the soil and root fractions for the ^{33}P radio-label, which is not possible in the one replicate treatment.

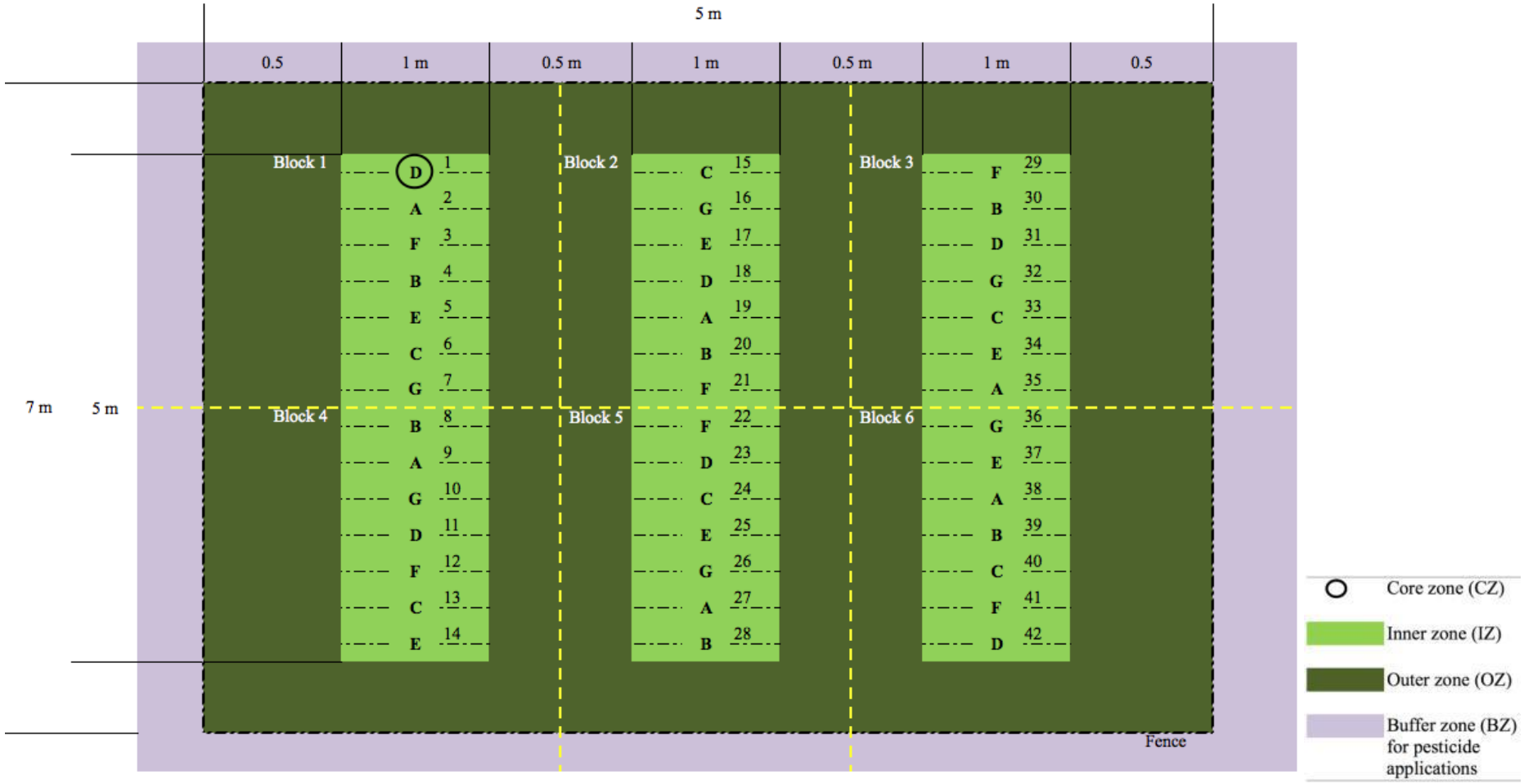

Fig. 1 The treatment layout for the isotopic field experiments (treatments A, B, C, D, E and F) carried out at the Ginninderra and Inman Valley field sites in 2014.

3.1.3.2 Design 2: Effect of initial soil fertility

A second field experiment was established in 2014 at the CSIRO Ginninderra permanent pasture site aimed at determining the influence of soil P fertility on fertiliser PUE. At this site, pastures have been established that are characterised by three levels of extractable P in the surface soil layer (0 – 10 cm): 1) an unfertilised treatment; pasture that receives no P fertiliser (referred to here as P0) resulting in a soil P fertility that has remained in the range 4 – 6 mg of Olsen extractable P kg^{-1}; 2) a fertilised treatment maintained within a target soil P fertility range of 10 – 15 mg Olsen extractable P kg^{-1} (referred to here as P1); and 3) a fertilised treatment to maintain within a target soil P fertility range of 20 – 25 mg Olsen extractable P kg^{-1} (referred to here as P2) (Simpson *et al.* 2015). The pastures were all grazed continuously at a moderate grazing pressure, with 6 to 18 month old merino wether weaners at 9 sheep per hectare (SR09) on 0.66 hectare fields. In the Section 3.1.1.1 above, only the unfertilised (P0) treatment was used.

Each treatment was replicated three times in a randomised block design within each enclosure (Fig. 2) and replicated three times across each level of soil P fertility (Table 2). The fertilised treatments involved the addition of ^{33}P-labelled SSP granules to supply ~ 20 kg P ha^{-1}. An early-season application of fertiliser P to clover pastures occurred on the 29th April 2014 at the Ginninderra site.

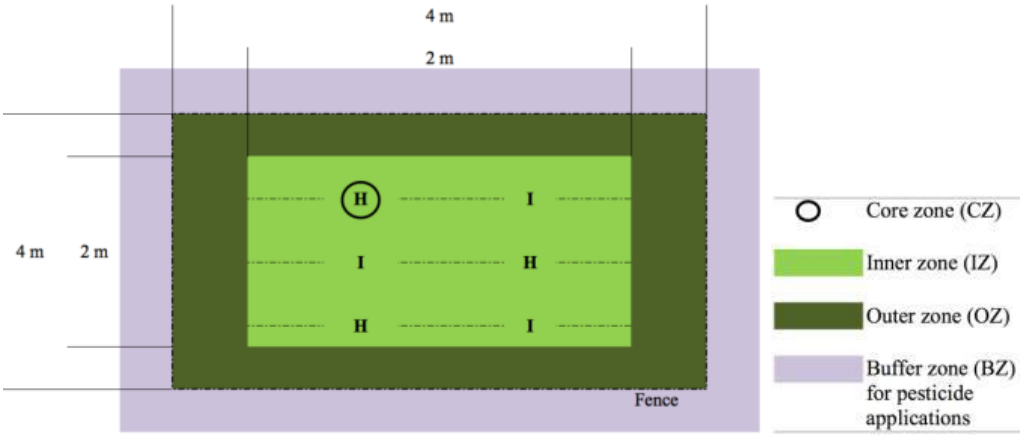

Fig. 2 The treatment layout for the second experimental design of isotopic field experiments (treatments H and I) carried out at the Ginninderra field site.

Table 2. A description of the treatments used at the Ginninderra field site for the second experimental design. The fertilised treatments involved the addition of ^{33}P-labelled single superphosphate (SSP) granules to supply ~ 20 kg P ha^{-1}.

Treatment ID	Placement of fertiliser P added to pastures	Time of fertiliser P added to pastures	Time of treatment finish	Description of core analysis
H	Soil surface	Early-season	End of growing season	Soil fractions were collected but the ^{33}P radio-label was too low for detection
I	No fertiliser P added	No fertiliser P added	End of growing season	Soil fractions were collected but no ^{33}P radio-label was added

3.1.4 Plant digestion and total P analysis

Clover shoots and roots were dried in a laboratory oven at 60°C for seven days. After drying, these samples were weighed and then ground to pass through a 2 mm sieve using a rotor cross beater grinder (Retsch, Haan, Germany) prior to chemical and isotopic analysis. Clover shoots and roots were digested as set out by Zarcinas *et al.* (1987) and subsequently analysed for P by inductively coupled plasma optical emission spectroscopy (ICP-OES). Analytical recovery of P by this method for the National Institute of Standards and Technology (NIST) 1573a plant standard was 89 %, and for the Australasian Soil and Plant Analysis Council (ASPAC) ASPAC-84 plant standard was 95 % (average of nine replicates).

3.1.5 Granule digestion and total Ca, P and S analysis

Concentrations of total P were determined on the commercial SSP granules and ^{33}P-labelled SSP granules prior to field application, and on the granule residues collected from the soil surface after the last harvest. The granules that were collected from the field were dried in a laboratory oven at 60 °C for seven days prior to chemical analysis. All granules were digested as set out by Zarcinas *et al.* (1996) and subsequently analysed for Ca, P and S by ICP-OES. Analytical recoveries of Ca, P and S by this method for the Sigma-Aldrich BCR-032 rock phosphate standard were 97 %, 92 % and 86 % (average of six replicates), respectively.

3.1.6 Soil extraction and P analysis

All soil fractions were extracted with sodium hydroxide–ethylenediaminetetraacetic acid (NaOH-EDTA) at a 1:10 soil to solution ratio as described by Doolette *et al.* (2010). Concentrations of inorganic and total P were determined on the filtrates using the molybdenum blue method of Murphy and Riley (1962) and ICP-OES, respectively. Organic P in the extract was calculated as the difference between total P and inorganic P.

The ignition-H_2SO_4 extraction technique of Saunders and Williams (1955) as modified by Walker and Adams (1958) was carried out on all soil fractions. Concentrations of inorganic P in the filtrates were determined using the molybdenum blue method of Murphy and Riley (1962). Concentrations of inorganic P for the ignited and unignited extracts are referred to as ignition-H_2SO_4 extractable total and inorganic P, respectively. The difference between total and inorganic P determined by ignition-H_2SO_4 extraction is referred to as organic P.

3.1.7 Liquid scintillation counting for ^{33}P analysis

The ^{33}P activity of all plant and granule digests, and all soil extracts was measured using a Rackbeta II Wallac® liquid scintillation counter (LSC). A measure of ^{33}P activity in NaOH-EDTA and H_2SO_4 (ignited and non-ignited) soil extracts was carried out to determine the recovery of fertiliser P as inorganic and total (organic by difference) forms of P in soil fractions. For total P, the ^{33}P activity was determined on an aliquot of all plant and granule digests, and the filtrates of NaOH-EDTA and the

H_2SO_4 (ignited) soil extracts. For inorganic P fractions, the ^{33}P activity was determined on the NaOH-EDTA extracts that had been acidified to flocculate organic P, and on the H_2SO_4 extract of the unignited soil. This involved acidifying 4 mL of the NaOH-EDTA extract with 1 mL of 2.5 M H_2SO_4; the resulting solution was then centrifuged at 1610 × *g* for 20 minutes and the supernatant analysed for ^{33}P activity using LSC. For the 2014 field trials, in some instances, soil extracts contained concentrations of ^{33}P activity that were too low for detection. Therefore, we concentrated soil extracts through freeze-drying prior to LSC. In general, this involved freeze-drying a 15 mL aliquot of extract and reconstituting in 5 mL of water before analysis for ^{33}P activity.

The solution colour of all digests and extracts was examined prior to ^{33}P analysis so that the colour ranges of unknown samples could be adjusted if necessary to be within that of the quench curve established for LSC analysis. The NaOH-EDTA extracts from the total P fraction samples were diluted using a 1:10 ratio of extract to water. The scintillant cocktail was made using 2 mL of sample and 10 mL of scintillant (Perkin Elmer UltimaGold AB). All samples were analysed by LSC for 2 min in duplicate, and the ^{33}P counts were corrected for sample volume, blanks, radioactive decay and dilution. All ^{33}P counts were corrected to the same reference date (T_0), which allows for direct comparison of ^{33}P radioactivity across all samples.

The specific activity of the granules (MBq mg^{-1} water-soluble P – WSP) was calculated using Equation 1 and corrected for WSP as the ^{33}P radiotracer would have only labelled the WSP fraction of the total P in SSP, which was 88 % of total P. The WSP component of the SSP was determined using the recommended procedure of the AOAC (1980) (four replicates). The specific activity of the ^{33}P-labelled SSP granules was 0.2369 MBq mg^{-1} WSP for the 2013 field sites, and for the 2014 field sites this was 0.1315 MBq mg^{-1} of WSP for the early-season fertilised treatments and 0.0996 MBq mg^{-1} of WSP for the mid-season fertilised treatments. The proportion of P in pasture components (samples) that was derived from the fertiliser P was calculated using Equation 2, and the amount of fertiliser P in samples could then be calculated using Equation 3.The recovery of fertiliser P in samples was calculated using Equation 4.

$$(1)\ \text{Specific activity (MBq mg P}^{-1}) = \frac{\text{Radioactivity in sample (MBq kg}^{-1}\text{ of sample)}}{\text{Total P in sample (mg P kg}^{-1}\text{ of plant)}} \times 100$$

$$(2)\ \text{P in sample derived from fertiliser (\%)} = \frac{\text{SA of sample (MBq mg P}^{-1}\text{ of sample)}}{\text{SA of fertiliser (MBq mg WSP}^{-1})} \times 100$$

$$(3)\ \text{Fertiliser P in sample } \left(\text{mg P core}^{-1}\right) = \text{Total P in sample } \left(\text{mg P core}^{-1}\right) \times \frac{\text{P in sample derived from fertiliser (\%)}}{100}$$

$$(4)\ \text{Recovery of fertiliser P (\%)} = \frac{\text{Fertiliser P in sample (mg of fertiliser P core}^{-1})}{\text{Total fertiliser P added (mg of fertiliser P core}^{-1})} \times 100$$

3.2 Sequential extraction on soils from Ginninderra field site

3.2.1 Soil preparation and collection

In 2007, composite soil samples were collected at the Ginninderra experimental field station at two depths (surface layer 0 – 10 cm, and subsurface layer 10 – 20 cm) using a 32 mm diameter soil corer. Soils were collected from paddocks managed by maintaining three levels of extractable P in the surface soil layer (0 – 10 cm) as described in Section 3.1.1.2: 1) unfertilised treatments (P0) resulting in a soil P fertility that remained between 4 – 6 mg of Olsen extractable P kg^{-1}; 2) a fertilised treatment (P1) with target soil P fertility of 10 – 15 mg Olsen extractable P kg^{-1}; and 3) a fertilised treatment (P2) with a target soil P fertility of 20 – 25 mg Olsen extractable P kg^{-1} (Simpson *et al.* 2015). The cumulative P input that was applied to the soil surface over 13 years at stocking rate SR09 was on average 4, 200, and 242 kg P ha^{-1} for the P0, P1 and P2 soil P fertility levels, respectively, and at stocking rate SR18 the cumulative P input was on average 220 and 320 kg P ha^{-1} for the P1 and P2 soil P fertility levels, respectively. The pastures were all grazed continuously at a moderate (9 sheep ha^{-1}, SR09) or high (18 sheep ha^{-1}, SR18) grazing pressure with 6 to 18 month old merino wether weaners on 0.33 or 0.66 hectare fields. Depending on field size (0.33 or 0.66 ha), 15 or 30 cores were collected using a regular grid soil sampling strategy and bulked within each replicate field for each soil depth. Soil samples were air-dried for 7 days, and then sieved to < 2 mm and mixed prior to analysis.

3.2.2 Sequential chemical fractionation

The procedure used for sequential chemical fractionation of soil P was a modification of the Hedley *et al.* (1982) procedure. Briefly, 3.00 g (± 0.01 g) (air-dried equivalent) aliquots of soil that had been incubated at field capacity for two weeks to promote microbial growth was weighed into 250 mL centrifuge tubes. To these, 6 mL of hexanol was added and the tubes incubated for two days to cause microbial lysis, which releases inorganic P of microbial origin; these are referred to as the fumigated soils. In addition, a duplicate set of soils (3 g air-dried) was weighed into 250 mL centrifuge tubes; these are referred to as the untreated soils. The untreated and fumigated soils were extracted for 16 hours with 0.5 M $NaHCO_3$ at a 1:60 soil to solution ratio. The extracts were then centrifuged at 1610 × *g* for 10 minutes and the supernatant passed through a Whatman no. 42 filter paper. Concentrations of inorganic P in the filtrates were determined using the molybdenum blue colorimetric method of Murphy and Riley (1962) and total concentrations of P in filtrates were determined by ICP-OES. Organic P in the filtrates was calculated as the difference between total P and inorganic P. Hexanol released P (or microbial P) was calculated as the difference between total concentrations of P of the $NaHCO_3$ extracts for the untreated and fumigated soil samples.

The residue of the untreated soils after extraction with 0.5 M $NaHCO_3$ was then sequentially extracted with 0.1 M NaOH (providing the NaOH I fraction), 0.1 M NaOH again following three minutes sonication (providing the NaOH II fraction) and finally 1 M HCl; in each case a 1:60 soil to solution ratio was used and samples were shaken for 16 hours. All extracts were prepared and concentrations of inorganic and total P in extracts determined as outlined above, except for the 1 M HCl extract, which was only analyzed for total P (and assumed to be inorganic P (Hedley *et al.* 1982)). The soil residue after the 1 M HCl extraction step was then dried, weighed and the total concentration of P determined using laboratory X-ray fluorescence (LXRF); this is referred to as 'residual P' (i.e. non-extractable P).

All P concentrations were blank and weight corrected, which included correcting for soluble P entrained in the soil residue of the preceding extraction. All P concentrations were calculated on a soil dry weight basis (i.e. mg P kg^{-1}) and these values were used to investigate (i) the relationship between various measures for total, inorganic and organic P in soils under pasture; and (ii) for identifying the major forms of soil P that accumulate using sequential chemical fractionation. To determine the net accumulation of fertiliser P into different pools of soil P, concentrations of soil P determined on a dry weight basis (i.e. mg P kg^{-1} of soil) were converted to an area basis (kg P ha^{-1}) based on a depth of 10 cm for each soil layer. This was calculated using Equation 5. A depth of 10 cm was used because the mass (kg) of soil per unit area (ha) will change with depth; bulk density also needs to be taken into account. Bulk densities of 1320 and 1210 kg m^{-3} were determined for the surface and subsurface layers, respectively.

(5) Soil P (kg ha^{-1}) = (*a* ÷ 1000 ÷ 1000) × (*b* × 10000 × *c*)

where *a* = Soil P (mg kg^{-1}), *b* = Bulk density (kg m^3) and *c* = Depth (m)

3.3 Solution ^{31}P NMR spectroscopic analysis

3.3.1 Method development – comparison of spectra for soils extracted at two different ratios

For this part of the project, the soils used were collected from six sites and two depths (0 – 4 cm and 4 – 10 cm) across the high rainfall zone of eastern Australia (rainfall ranged from 490 to 779 mm yr^{-1}), and one site (Karoonda) from the Mallee low rainfall zone in south-eastern Australia (rainfall 342 mm yr^{-1}). All soils were collected from fields that were under permanent pasture, except for Site 1 (Karoonda; cereal/pasture ley system). Three of the seven sites were experimental plots located at the Ginninderra field site described above (Section 3.2.1). Two of the seven sites were located at the Newholme Field Research Station, near Armidale, New South Wales, Australia. Site details, experiment design and management for Newholme have been described by Flavel *et al.* (2010). At this site, soil was collected from plots at two contrasting levels of soil P fertility: 1) plots receiving no P fertiliser (control); and 2) plots that received P fertiliser to increase soil P fertility for optimum pasture growth (P1; i.e. 10 – 15 mg Olsen P kg^{-1}).

Soils were extracted with NaOH-EDTA at two soil to solution ratios: 1:10 and 1:4, based on the methods of Cade-Menun and Preston (1996) and Turner (2008). Briefly, for the 1:10 extractions 3 g (± 0.10 g) of soil was extracted with 30 mL 0.25 M NaOH + 0.05 M EDTA, whereas for the 1:4 extractions 8 g (± 0.10 g) of soil was extracted with 32 mL 0.25 M NaOH + 0.05 M EDTA. In both cases the mixtures were shaken for 16 hours, centrifuged at 1400 × *g* for 20 minutes and the supernatant collected after filtration using Whatman no. 42 filter paper. A 20 mL aliquot was frozen in liquid nitrogen and freeze-dried; 550 – 650 mg of solid was generally recovered. Concentrations of molybdate reactive P (referred to hereafter as inorganic P) in the remaining supernatant were determined using the molybdenum blue colorimetric method of Murphy and Riley (1962), and total concentrations of Al, Fe, Mg, Mn and P in the extracts were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES). The difference between total and inorganic P determined by NaOH-EDTA extraction is molybdate unreactive P (which is referred to hereafter as organic P).

3.3.2 Solution ^{31}P NMR spectroscopic analysis of soils from Ginninderra field site

The soils used in this part of the project were the same as those used in the sequential study (see Section 3.2.1). All soils were extracted with NaOH-EDTA at a 1:10 ratio as described above (Section 3.3.1).

3.3.3 Solution ^{31}P NMR spectroscopic analysis of soils following size separation

For this part of the project, soil was obtained from five locations across three continents. These include: 1) Australia (Soil ID: P1SR09) – soil from the P0 treatment of the Ginninderra site; 2) France (Soil ID: 4850), Germany (Soil ID: 2035) and Sweden (Soil ID: 3359) – surface soils (0 – 10 or 0 – 20 cm) obtained from the Geochemical Mapping of Agricultural Soils and Grazing Land (GEMAS) study (Reimann *et al.* 2012), and; 3) USA (Soil ID: 1BS102M) – the Elliot reference soil standard was purchased from the International Humic Substances Society. Basic chemical properties of these soils can be found in Table 3.

Table 3. Basic chemical properties of the soils used for NMR spectroscopic analysis of soils following size separation.

Soil	Depth (cm)	pHw (1:5)	ECw (µS cm^{-1}, 1:5)	Total carbon[a] (%)	Total nitrogen* (%)	Oxalate aluminium† (mg kg^{-1})	Oxalate iron† (mg kg^{-1})	Total organic phosphorus‡ (mg kg^{-1})	Total phosphorus§ (mg kg^{-1})
Australia	0 - 10	5.3	68	2.5	0.21	469	1635	194	460
France	0 - 20	7.7	171	4.1	0.38	2020	6996	665	1448
Germany	0 - 10	6.7	104	5.1	0.43	4517	3622	707	2444
Sweden	0 - 20	6.9	170	8.6	0.48	1934	4563	456	1128
USA	0 - 20	6.8	85	2.9	0.26	1138	2650	383	668

*Total soil carbon and total soil nitrogen were determined on a LECO TruSpec CN analyzer (LECO Corporation, St Joseph, MI).

† Measures of oxyhydroxides associated with aluminium and iron were determined using the oxalate extraction technique of McKeague and Day (1966).

‡ Total organic P was estimated using the ignition-H_2SO_4 extraction technique of Walker and Adams (1958). However, it is widely known that this extraction technique, which is the most commonly used method for estimating total organic P, is unreliable and will overestimate total organic P in most soils (Turner *et al.* 2005).

§ Total soil P was determined using laboratory X-ray fluorescence.

Following NMR analysis on the original NaOH-EDTA extracts (unfractionated), carried out as described in Section 3.3.1, the solution was transferred to the 5 mL sample reservoir of an ultrafiltration device containing a 10 kDa filtration membrane made of modified polyethersulfone (PALL Life Sciences©; Microsep™ Advance Centrifugal Devices; Product ID MCP010C41). Solutions were centrifuged at 4000 rpm (1700 × *g*) for 60 minutes and the filtrate (< 10 kDa) and the retentate (> 10 kDa) collected for NMR analysis. The 10 kDa filtrates were transferred to a NMR tube and analyzed as described above. The 10 kDa retentates were collected after washing the retentate with NaOH-EDTA. The washing step involved adding 5 mL of NaOH-EDTA solution to the 5 mL reservoir of the ultrafiltration device (containing the 10 kDa retentate) and centrifuging at 4000 rpm (1700 × *g*) for 60 minutes. This step was repeated twice and the 'wash' filtrate collected each time and stored. After the washing step, 1 mL of NaOH-EDTA solution was added to the 5 mL reservoir of the ultrafiltration device (containing the 10 kDa retentate). The 10 kDa retentate solution (~ 1 mL) was then transferred to a NMR tube using a pipette. This step was repeated three times to give a final volume of ~ 3 mL of solution in the NMR tube. A 0.3 mL aliquot of deuterium oxide and 0.1 mL of a methylenediphosphonic acid (Sigma-Aldrich; M9508) in-house standard solution containing 6.0 g L^{-1} were then placed in the NMR tube containing the 10 kDa retentate for quantitative analysis prior to solution ^{31}P NMR spectroscopy. No further additions of methylenediphosphonic acid and deuterium oxide were required for the 10 kDa filtrate because these were already added to the unfractionated extract prior to NMR analysis and they passed through the 10 kDa filtration membrane.

In order to test the performance of the ultrafiltration devices with known compounds, ten reference materials were added to NaOH-EDTA and analyzed using solution ^{31}P NMR spectroscopy. These included: 1) Glycerol phosphate disodium salt hydrate ($C_3H_7O_6PNa_2.XH_2O$: Sigma-Aldrich, G6501); 2) Glycerol 2-phosphate disodium salt hydrate ($C_3H_7Na_2O_6P.XH_2O$: Sigma-Aldrich, G6251); 3) Adenosine-5'-monophosphate disodium salt ($C_{10}H_{12}N_5Na_2O_7P$: Sigma-Aldrich, 01930); 4) Guanosine 5'-monophosphate disodium salt hydrate ($C_{10}H_{12}N_5Na_2O_8P$: Sigma-Aldrich, G8377); 5) Cytidine 5'-monophosphate disodium salt ($C_9H_{12}N_3Na_2O_8P$: Sigma-Aldrich, C1006); 6) deoxyribonucleic acid sodium salt (Sigma-Aldrich, D1626); 7) D-Glucose 6-phosphate sodium salt ($C_6H_{12}NaO_9P$: Sigma-Aldrich, G7879); 8) α- and β-glycerophosphate ($C_3H_7O_6PCa$: Sigma-Aldrich, G6626); 9) *myo*-inositol hexakisphosphate sodium salt ($C_6H_{18}O_{24}P_6$: Sigma-Aldrich, P8810); and 10) methylenediphosphonic acid (Sigma-Aldrich; M9508). Following NMR analysis of the NaOH-EDTA solutions, each reference sample was fractionated through passage of a 10 kDa ultrafiltration device in the same way as for the soil extracts. Solution ^{31}P NMR spectroscopy was carried out on the 10 kDa filtrates. All organic P species were exclusively recovered in the 10 kDa filtrates, except that of the deoxyribonucleic acid sodium salt, which was exclusively recovered in the 10 kDa retentate.

3.3.4 Acquisition of solution ^{31}P NMR spectra

For all samples analysed by NMR, a 500 mg subsample of each freeze-dried extract was dissolved in 5 mL of deionised water and centrifuged at 1400 × *g* for 20 minutes. A 3.5 mL aliquot of the re-dissolved NaOH-EDTA extract, 0.3 mL of deuterium oxide

and 0.1 mL of a methylenediphosphonic acid (MDP) (Sigma-Aldrich; M9508; ≥ 99 %) in-house standard solution containing 6.0 g of MDP L^{-1} were then placed in a 10 mm diameter NMR tube. Solution ^{31}P NMR spectra were acquired on a Varian INOVA400 NMR spectrometer at a ^{31}P frequency of 161.9 MHz, with gated ^{1}H decoupling. A 90 ° pulse of 30 µs was used. The recycle delay for each sample was measured in a preliminary inversion-recovery experiment, and was set at five times the T_1 value of the orthophosphate resonance.

3.3.5 Quantification of soil P forms in NaOH-EDTA extracts by integration of ^{31}P NMR spectra

For all samples analysed by NMR, quantification of soil P forms in NaOH-EDTA extracts was based on spectral integration against the addition of a known amount of added MDP, which gives a unique spectral signal separate from all other resonances. The peak area of the MDP signal is proportional to the absolute concentration added, which was then quantitatively compared to the peak areas of all other resonances in the solution ^{31}P NMR spectra.

The following broad classes of P species were quantified using spectral integration based on previous studies (Smernik and Dougherty 2007; Doolette *et al.* 2009): orthophosphate (δ 7.0 to 5.4 ppm), orthophosphate monoesters (δ 5.4 to 3.5 ppm), orthophosphate diesters δ 0.5 to -1.0 ppm) and pyrophosphate (δ -4.5 to -5.5 ppm).

To further partition soil P forms contained within the monoester region, spectral deconvolution was carried out on some spectra as described by Doolette *et al.* (2010) and Doolette *et al.* (2011). Deconvolution was carried out in two steps First, the contribution of the broad resonance was modelled as a pair of Gaussian peaks of equal intensity and line-width (δ 0.4 ppm) at 4.7 and 4.3 ppm. A best fit of the profile for the broad resonance was achieved by varying the position of both peaks and the intensity of the pair of peaks (i.e. the line-width was held constant for all soils and the peak intensity was kept the same for both Gaussian peaks but the latter was altered for each soil). The broad resonance was then subtracted from the spectrum, and a best-fit of the remaining signal consisting of the sharp resonances was achieved using a numeric least-squares fit to minimize the sum of the squared residuals by adjusting the frequency and intensity of the Gaussian peak used to fit each resonance (Doolette *et al.* 2011). This was carried out in Microsoft Excel within the Solver numeric methods subroutine. The proportion of *myo*-inositol hexakisphosphate was taken as 6/5 times that of the three observable resonances because the C-2 resonance overlaps with that of orthophosphate. Consequently, 1/5 proportion of signal originally assigned to *myo*-inositol hexakisphosphate was subtracted from the orthophosphate resonance to correct for spectral overlap.

3.4 Statistical analyses

All statistical analyses were carried out using R 3.0.2 (R Core Team 2014). This included linear regression and factorial analysis of variance (ANOVA) analyses, the latter included orthogonal contrasts and the Tukey *post hoc* test of honest significance difference to compare treatment means at the 5 % (P = 0.05) level of

significance. In field experimentation, the blocking factor was not significant and was dropped from the ANOVA model. All regression models were checked for normality of residuals and constant variance using diagnostic plots, the Shapiro-Wilk test, and Levene's test (Levene 1960; Shapiro and Wilk 1965). Outliers were identified using Cook's distance plot (Cook and Weisberg 1982).

4 Results

4.1 Measurement of fertiliser phosphorus use efficiency via radio-labelling

Measurement of P fluxes involved the measurement of fertiliser PUE, i.e. the proportion of added fertiliser P that is detected in pasture biomass using the addition of radio-labelled P fertiliser. These experiments were carried out at three sites over two seasons. The radio-label also allowed us to determine the short-term fate of fertiliser P that is not taken up by plants and makes its way into various soil P pools. A limitation of the radio-label approach is that the short half-life of the ^{33}P isotope (25.4 days) means that the radioactivity quickly decays to below detection limits, which restricts the maximum length of these experiments that directly track the fate of fertiliser P. The rule of thumb is that the label can only be reliably detected for up to ten half-lives (approximately 8 months for ^{33}P).

The results described in section 4.1.1 and 4.1.2 have been published in:

McLaren, T.I., McLaughlin, M.J., McBeath, T.M., Simpson, R.J., Smernik, R.J., Guppy, C.N., and Richardson, A.E. (in press) The fate of fertiliser P in soil under pasture and uptake by subterraneum clover - a field study using 33P-labelled single superphosphate, Plant and Soil, accepted July 2015.

4.1.1 Development of a new method for making ^{33}P labelled fertiliser

An important technical development underpinning the radio-labelling experiments was the development of a new method for making ^{33}P labelled single superphosphate (SSP) that is quicker and safer than that previously available, which involved acidulation of rock phosphate. The new method involved incubating ground single superphosphate with the isotopic solution and then forming a 'bolus'. The bolus was then pressed into a pellet and subsequently cut into cubes. The P diffusion pattern of the SSP cubes was similar to that of the commercial SSP granules when incubated in soil at field capacity for one week (Figs 3 and 4). This was determined using the novel visualisation techniques of (Degryse and McLaughlin 2014).

Fig. 3 The single superphosphate cubes made using the 'press and cut' technique.

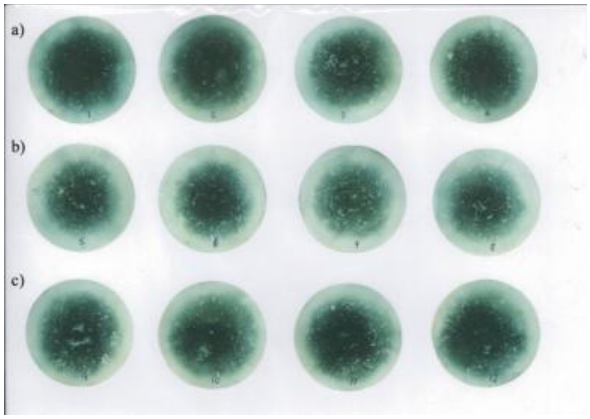

Fig. 4 A visualisation of the P diffusion pattern showing no difference between a 'cut and press' superphosphate cube and commercial superphosphate granules. 'Greenness' represents the location of P after a one week incubation of a single superphosphate cube made using the 'cut and press' technique (a – 1st batch, b – 2nd batch) and that of the commercial single superphosphate granules (c).

4.1.2 First year (2013) field experiments using ^{33}P labelled fertiliser

4.1.2.1 Site characteristics

Soil pH ranged from acidic to slightly acidic for all surface and subsurface layers at the Naracoorte and Ginninderra field sites (Table 4). Concentrations of total soil C, total soil N and total organic P were higher at the Ginninderra site than at the Naracoorte site (Table 1). The concentration of Colwell-extractable P was 12 mg P kg^{-1} when averaged across the 0 – 10 cm layer at the Ginninderra site; this soil would be considered potentially responsive to fertiliser P (Colwell 1963; Reuter *et al.* 1995). Conversely, the concentration of Colwell-extractable P was 57 mg P kg^{-1} (again averaged across the 0 – 10 cm layer) at the Naracoorte site; this soil would be

predicted to be non-responsive to fertiliser P (Colwell 1963; Reuter *et al.* 1995). The soil P sorption capacity at the Ginninderra site, as indicated by PBI, oxalate-extractable aluminium and oxalate-extractable iron, was approximately double that of the Naracoorte site (Table 4).

Table 4. Chemical properties of the soils used for the 2013 field trials. Analyses were carried out on soil samples collected prior to fertiliser addition.

Location	Depth (cm)	pHw (1:5)	ECw (µS cm^{-1}; 1:5)	TC* (%)	TN* (%)	Colwell P (mg kg^{-1})	PBI	Oxalate Al (mg kg^{-1})	Oxalate Fe (mg kg^{-1})	Total P† (mg kg^{-1})	Ignition-H_2SO_4 (mg kg^{-1})		
											total	inorganic	organic
Ginninderra,	0 – 2.5	5.1	74	2.6	0.23	19	31	709	10100	362	213	50	163
ACT	2.5 – 5	4.6	70	1.6	0.15	11	35	773	11600	323	178	34	143
	5 – 10	4.7	64	1.1	0.10	8	33	726	10300	310	156	31	125
	10 – 20	4.7	35	0.6	0.05	5	46	873	11800	301	112	21	90
Naracoorte,	0 – 2.5	5.6	88	1.3	0.11	92	14	435	6260	358	254	159	95
SA	2.5 – 5	5.3	77	1.2	0.12	66	16	427	6740	358	207	123	84
	5 – 10	4.7	51	0.9	0.08	34	23	374	2770	201	140	75	66
	10 – 20	4.7	32	0.4	0.04	23	20	378	3630	148	81	40	41

* TC refers to ‘total soil carbon’ and TN refers to ‘total soil nitrogen’.

† Determined by laboratory X-ray fluorescence

The in-season rainfall at the Ginninderra site was higher than at the Naracoorte field site. The annual rainfall at the Ginninderra (622 mm) and Naracoorte (479 mm) field sites for 2013 were 90 and 98% of the long-term averages, respectively. The cumulative rainfall and irrigation at the Ginninderra (708 mm) and Naracoorte (490 mm) field sites for 2013 were 103 and 100% of the long-term averages, respectively.

4.1.2.2 Dry matter, P uptake and proportion of plant P derived from fertiliser

The cumulative biomass removal for the fertilised treatments was not significantly different ($P < 0.05$) to that for the unfertilised control at both sites (Table 5). Concentrations of P in plant tissue of fertilised treatments at the Ginninderra and Naracoorte field sites were generally higher than in non-fertilised treatments (Fig. 5). The cumulative P uptake for the fertilised treatments was significantly different ($P < 0.05$) to that for the unfertilised control at the Ginninderra site but not at the Naracoorte site (Table 5). The cumulative P removal was approximately three times higher at the Naracoorte site than at the Ginninderra site, despite similar amounts of biomass removal (Table 5).

Table 5. Summary of the cumulative biomass removal (t DM ha^{-1} equivalent) and P uptake (kg P ha^{-1} equivalent) for the control, commercial SSP and ^{33}P-labelled SSP treatments at the Ginninderra and Naracoorte field sites in 2013. Values in parentheses are standard errors.

Measurement	Field site	Control	Commercial SSP	^{33}P-labelled SSP
Cumulative biomass (t DM ha^{-1})	Ginninderra	6.7 (0.5)	7.5 (0.6)	7.3 (0.5)
	Naracoorte	8.0 (0.5)	7.7 (0.3)	6.9 (0.6)
Cumulative P uptake (kg P ha^{-1})	Ginninderra	7.8 (0.7)	10.8 (1.1)	10.5 (0.8)
	Naracoorte	28.4 (1.8)	29.9 (0.7)	28.1 (1.9)

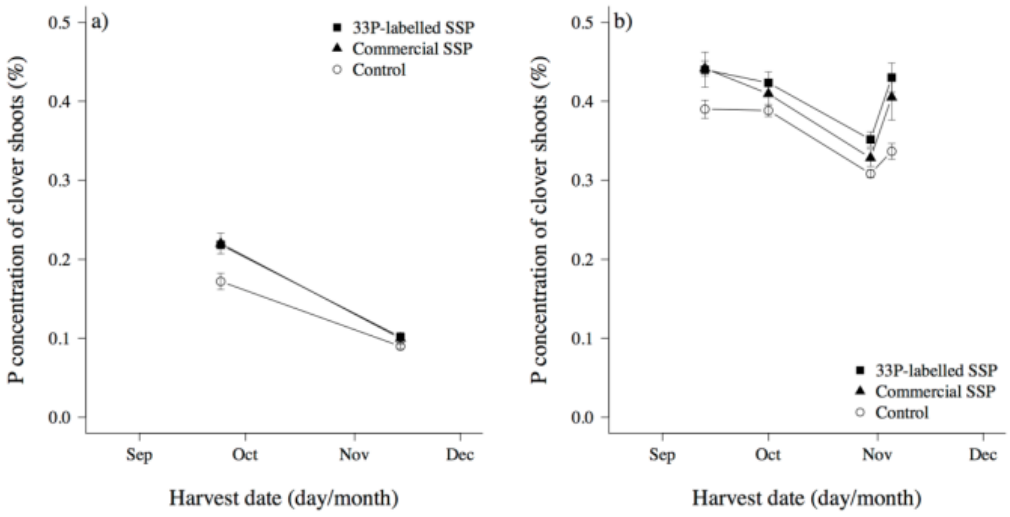

Fig. 5 Tissue P concentrations (%) of clover shoots for each harvest in the control, commercial SSP and ^{33}P-labelled SSP treatments at the a) Ginninderra and b) Naracoorte field sites in 2013. Error bars are one standard error.

The proportion of plant P that was derived from the fertiliser can be calculated by dividing the specific activity of the plant by that of the fertiliser (see Equation 2). The proportion of plant P in clover shoots that was derived from the fertiliser at the Ginninderra site (33%) was approximately double that at the Naracoorte field site (16%) (Fig. 6).

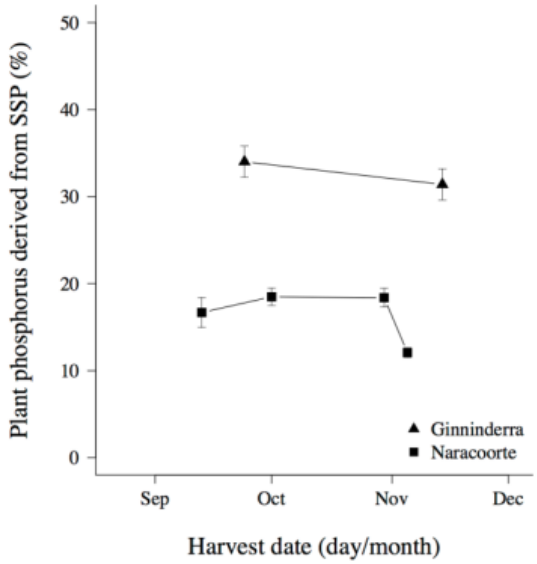

Fig. 6 The proportion of plant P that was derived from the ^{33}P-labelled SSP at each harvest, which was calculated using Equation 2. Standard error bars are displayed for each harvest.

4.1.2.3 Recovery of fertiliser P applied to clover pastures

The proportion of fertiliser P recovered in clover shoots at the Ginninderra field site was 30% and at the Naracoorte field site was 35% (Table 6 and Fig. 7).

Table 5. Concentrations of fertiliser P (mg P core^{-1}) detected in various components of the pasture system at two field sites. Recoveries of fertiliser P in pasture components were calculated using Equation 3 except for that of the applied and unrecovered granule, which was based on total P. Recoveries of fertiliser P in the unrecovered fraction was based on the difference of fertiliser P added and that detected in pasture components. Values in parentheses are standard errors.

Matrix/location	Fertiliser P detected in components of the pasture system (mg P core^{-1})	Ginninderra*	Naracoorte*
Applied granule (soil surface)	Total P applied	20.08	22.89
Clover (shoots)	Cumulative biomass removal	6.05 (0.40)	8.11 (0.42)
Recovered Granule (soil surface)	Total P recovered	2.51 (0.38)	2.67 (0.20)
Soil (0 – 4 cm layer)	Ignition-H_2SO_4 extractable total P	5.57 (0.52)	6.54 (0.27)
	Ignition-H_2SO_4 extractable inorganic P	3.31 (0.41)	6.02 (0.23)
	Ignition-H_2SO_4 extractable organic P	2.25 (1.23)	0.64 (0.19)
	NaOH-EDTA extractable total P	3.37 (0.44)	6.31 (0.42)
	NaOH-EDTA extractable inorganic P	3.35 (0.40)	5.95 (0.39)
	NaOH-EDTA extractable organic P	0.42 (0.14)	0.35 (0.14)

Soil (4 – 8 cm layer)	Ignition-H_2SO_4 extractable total P	BD	0.89 (0.13)
	Ignition-H_2SO_4 extractable inorganic P	BD	0.82 (0.10)
	Ignition-H_2SO_4 extractable organic P	BD	0.09 (0.06)
	NaOH-EDTA extractable total P	BD	BD
	NaOH-EDTA extractable inorganic P	BD	BD
	NaOH-EDTA extractable organic P	BD	BD
Unrecovered	By difference	5.94 (0.50)	4.67 (0.47)

* BD = below detection. Refers to samples that contained ^{33}P activity that was too low for detection by liquid scintillation counting.

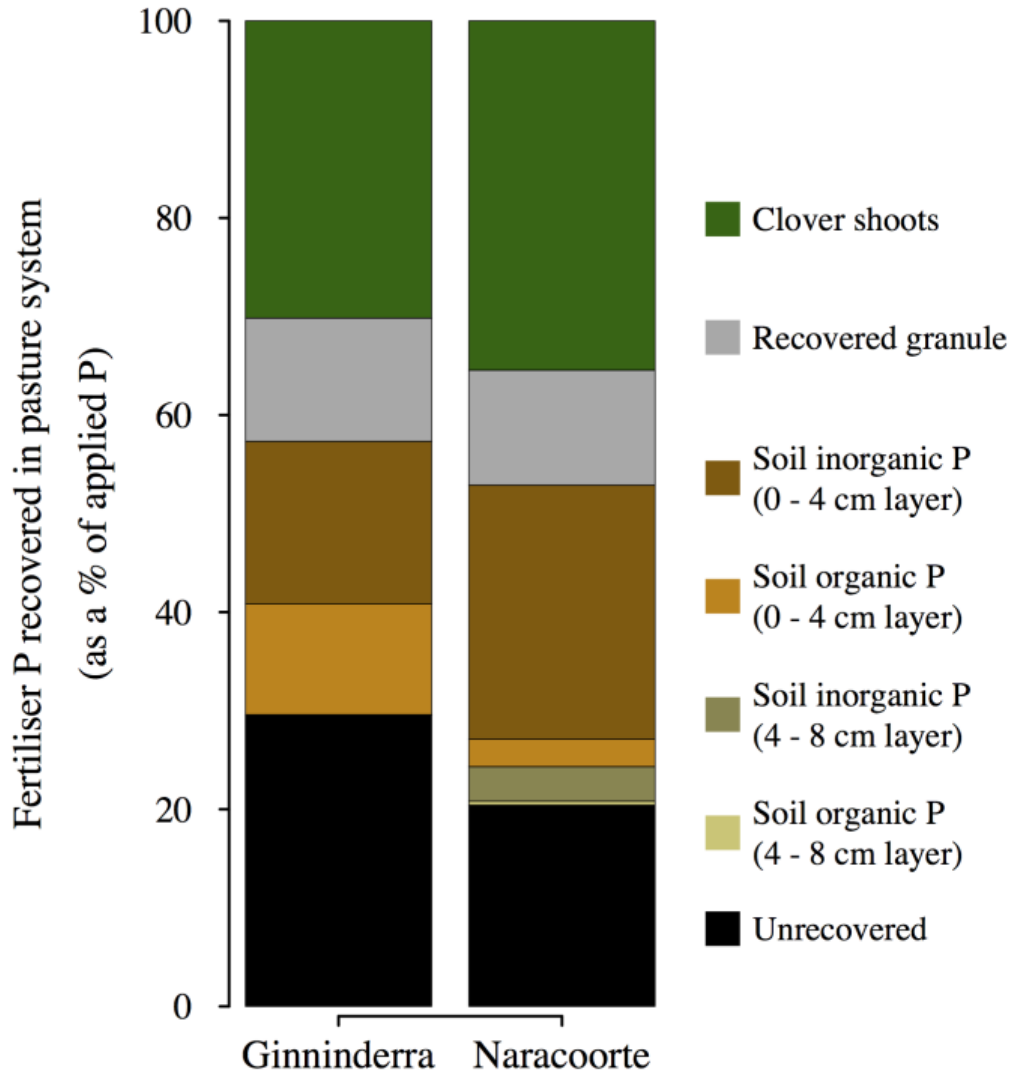

Fig. 7 Recovery of P from the ^{33}P-labelled SSP in various components of the pasture system at two field sites in 2013 (as a % of applied P). Recoveries of fertiliser P in pasture components were calculated using Equation 4 except for that of the unrecovered granule, which was based on total P. The recovery of fertiliser P in soil inorganic and organic P fractions was determined using the ignition-H_2SO_4 extraction technique of Walker and Adams (1958). Recoveries of fertiliser P in the unrecovered fraction was based on the difference of fertiliser P added and that detected in pasture components.

At both sites, approximately 28% of the applied fertiliser P was recovered by H_2SO_4 extraction of ignited soil from the surface (0 – 4 cm) layer of the soil profile (Table 6 and Fig. 7). Similarly, 21 and 28% of the applied fertiliser P was recovered by NaOH-EDTA extraction of the surface (0 – 4 cm) soil layer at the Ginninderra and Naracoorte sites, respectively (Table 6 and Fig. 7). Less than 4% of the applied fertiliser P was recovered from the subsurface layer (4 – 8 cm) at the Naracoorte site using the ignition-H_2SO_4 extraction procedure (Table 6 and Fig. 7). Concentrations of the ^{33}P radionuclide were too low for detection at the Ginninderra site in ignition-

H_2SO_4 extracts, and at both sites in NaOH-EDTA extracts of the 4 – 8 cm layer (Table 6).

The majority of fertiliser P that was recovered from the surface soil layer at both sites using the ignition-H_2SO_4 and NaOH-EDTA extraction techniques was identified as inorganic P, which ranged from 17 to 26% of the applied fertiliser P (Table 6). As a proportion of total extractable P, 89 to 94% of the fertiliser P in soil extracts was identified as inorganic P using the ignition-H_2SO_4 and NaOH-EDTA extraction techniques in all cases, except at the Ginninderra site using the ignition-H_2SO_4 extraction technique, where 60% of the total extractable P was identified as inorganic P (Table 6).

Less than 3% of the applied fertiliser P was recovered as organic P in the surface layer as determined using the NaOH-EDTA extraction technique at the Ginninderra site, and also at the Naracoorte site using both the ignition-H_2SO_4 and NaOH-EDTA extraction techniques (Table 6). The exception to this was at the Ginninderra site using the ignition-H_2SO_4 extraction, where 11% of the fertiliser P was recovered as organic P in the surface layer (Table 6). As a proportion of total extractable P, 5 to 11% of the fertiliser P in soil extracts was identified as organic P using the ignition-H_2SO_4 and NaOH-EDTA extraction techniques in all cases, except at the Ginninderra site using the ignition-H_2SO_4 extraction technique, where 40% of the total extractable P was identified as organic P (Table 6).

Approximately 6% of the applied ^{33}P was recovered in the fertiliser granule residues that were collected from the soil surface at the Ginninderra and Naracoorte field sites (Table 6 and Fig. 7). However, based on concentrations of total P in the granule residue, 13 and 12% of the applied fertiliser P (detected as ^{31}P) was recovered in the granule residues that were collected from the soil surface at the Ginninderra and Naracoorte field sites, respectively.

The proportion of applied fertiliser P that was unaccounted for was determined as the difference in fertiliser P (mg of P $core^{-1}$) added to clover pastures and that recovered in clover shoots, fertiliser granule residues and extracted in soil fractions using the ignition-H_2SO_4 extraction. The proportion of applied fertiliser P that was unaccounted for was 27% at the Ginninderra field site and 20% at the Naracoorte field site (Table 6 and Fig. 7).

4.1.3 Second year (2014) field experiments using ^{33}P labelled fertiliser

The results described in this section are being prepared for publication in Plant and Soil.

The second year field experiments were designed to confirm the high fertiliser P recovery (i.e. PUE) observed in the first year experiments and also to determine the influence on PUE of (i) timing and placement of P fertiliser; and (ii) initial soil P fertility.

4.1.3.1 Design 1: Effect of timing and placement of fertiliser

Field experiments were established in 2014 at Ginninderra, ACT (Fig. 8) and Inman Valley, SA (Fig. 9) to determine the flux of fertiliser P in soil fractions and into above-ground pasture biomass during a single growing season using ^{33}P-labelled SSP.

Fig. 8 One of the enclosures at the Ginninderra field site located at the CSIRO Ginninderra Experiment Station, near Hall, Australian Capital Territory.

Fig. 9 The Inman Valley field site located at a property near Inman Valley, South Australia.

4.1.3.2 Dry matter, P uptake and proportion of plant P derived from fertiliser

The cumulative biomass (> 3 cm) removal and clover P uptake for the fertilised treatments (treatments A, D, E and F) was greater than that for the unfertilised control at both sites (Table 7). Therefore, clover responded at both sites to the application of approximately 20 kg P ha^{-1} as ^{33}P-labelled SSP.

Cumulative P uptake of clover shoots was approximately 60% higher at the Inman Valley site than at the Ginninderra site for the fertilised treatments (Table 7). In general, the highest cumulative biomass and clover P uptake occurred in treatment A (early season surface application) at both sites, although equivalent levels were also achieved for treatment D (early season sub-surface application) at the Ginninderra site (Table 7). Cumulative biomass and clover P uptake in treatments E and F (mid-season surface application) were only slightly (and not always significantly) less than that of treatments A and D (early season surface and deep application) at both sites (Table 7).

Cumulative P uptake of clover shoots was approximately 5 and 12 kg P ha^{-1} for the unfertilised treatment (treatment G) at the Ginninderra and Inman Valley field sites, respectively (Table 7). This would result in a net decline in soil P over time if this material were permanently removed as it is not being replaced by an external source (e.g. fertiliser). Cumulative P uptake of clover shoots was approximately 13 and 20 kg P ha^{-1} for the early-season application of fertiliser P to the soil surface (treatment A) at the Ginninderra and Inman Valley field sites, respectively (Table 7). For the former, this is approximately 7 kg P ha^{-1} less than was added as fertiliser, whereas for the latter this is close to the amount applied as fertiliser P: i.e. ~ 20 kg P ha^{-1} (Table 7). It should be remembered, however, that the majority of P taken up by pasture species is usually returned to the soil surface in the form of animal dung and senesced plant residues or plant trampling.

Table 7. Summary of the cumulative biomass removal (t DM ha^{-1} equivalent) and P uptake (kg P ha^{-1} equivalent) for clover shoots (> 3 cm) of the fertilised treatments (A, B, C, D, E and F) and non-fertilised treatment (G – control) at the Ginninderra and Inman Valley field sites in 2014. Values in parentheses are standard errors.

Treatment ID	Treatment description	Ginninderra	Inman Valley	Ginninderra	Inman Valley
		Cumulative biomass (t DM ha^{-1})		Cumulative P uptake (kg P ha^{-1})	
A	Surface autumn	9.0 (0.7)	8.2 (0.2)	12.8 (1.1)	20.6 (0.6)
B*	Surface autumn (soil)	4.0 (0.2)	4.1 (0.3)	7.6 (0.4)	11.7 (0.3)
C†	Surface autumn (root)	3.9 (0.3)	4.2 (0.3)	7.3 (0.6)	11.5 (0.9)
D	Deep autumn	9.3 (0.3)	6.7 (0.5)	13.3 (0.7)	17.0 (0.9)
E‡	Surface spring (soil)	5.6 (0.4)	7.0 (0.7)	7.9 (0.6)	17.7 (2.1)
F§	Surface spring (root)	7.0 (0.8)	6.7 (0.4)	10.4 (1.3)	18.0 (0.8)
G	Control	3.7 (0.3)	5.9 (0.6)	4.5 (0.7)	12.3 (1.3)

* Only one biomass harvest was collected before the beginning of spring. Cores were removed and soil fractions analysed for the ^{33}P radio-label.

† Only one biomass harvest was collected before the beginning of spring. Cores were removed and root fractions analysed for the ^{33}P radio-label.

‡ The ^{33}P-labelled SSP was applied at the beginning of spring. At the last harvest, cores were removed and soil fractions analysed for the ^{33}P radio-label.

§ The ^{33}P-labelled SSP was applied at the beginning of spring. At the last harvest, cores were removed and root fractions analysed for the ^{33}P radio-label.

The proportion of plant P that was derived from the fertiliser can be calculated by dividing the specific activity of the plant by that of the fertiliser (see Equation 2). This reveals how important the fertiliser was as a source of P to clover plants at each harvest. The proportion of plant P in clover shoots (> 3 cm) that was derived from the fertiliser for treatment A was on average 46% and 33% at the Ginninderra and Inman Valley field sites, respectively. However, at the Ginninderra site this declined from 60% in the first harvest to 35% in the last harvest (Fig. 10). No clear trend was observed in the proportion of plant P derived from fertiliser P between each harvest for treatment A at the Inman Valley site (Fig. 10).

At both sites, the proportion of plant P in clover shoots that was derived from the fertiliser for treatment D (deep placed fertiliser) was higher in the second harvest than in the first harvest (Fig. 10). This proportion then steadily declined at the Ginninderra site from 55% in the second harvest to 44% in the last harvest, whereas no obvious trend was observed after the second harvest at the Inman Valley field site (Fig. 10).

In general, at both sites, the proportion of plant P in clover shoots that was derived from fertiliser P varied by no more than ~25% between surface applications of fertiliser P at mid-season and early-season applications either on the surface or at-depth (Fig. 10). However, at Ginninderra, clover shoots did obtain a greater proportion of their P from fertiliser in the late season (October-November) for spring surface and deep autumn fertiliser application than for surface autumn application.

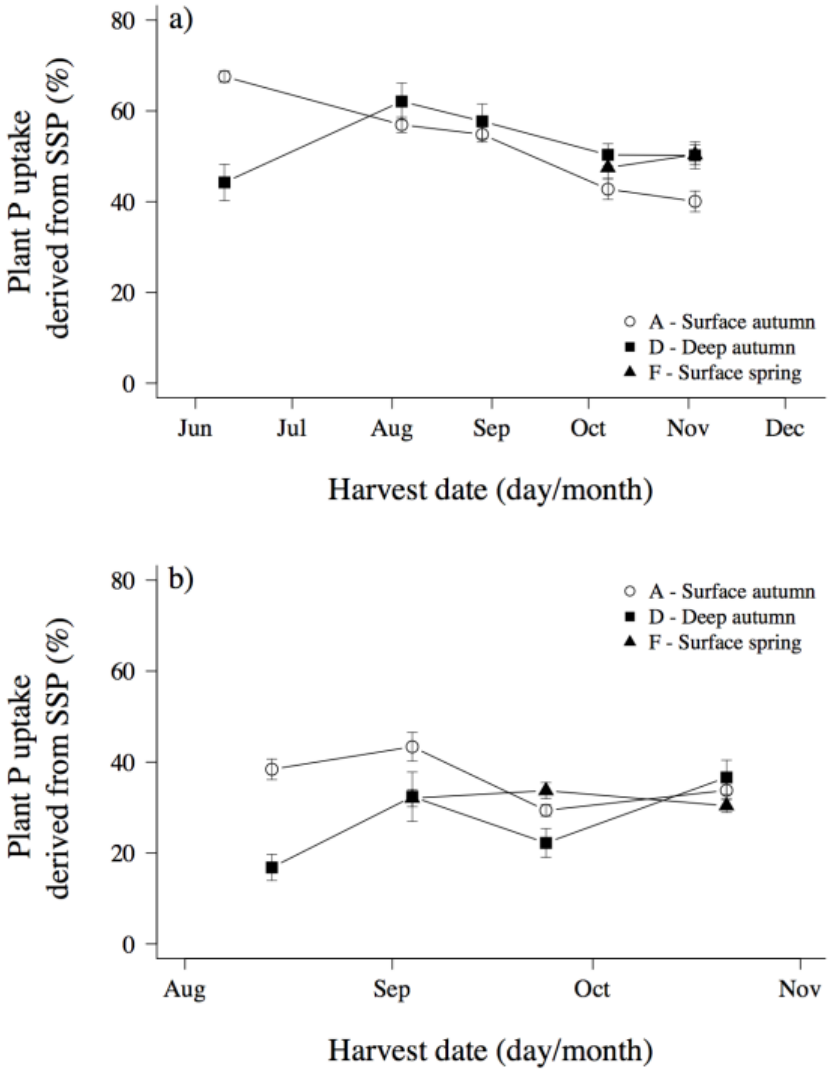

Fig. 10 The proportion of plant P that was derived from the ^{33}P-labelled SSP at each harvest for the Ginninderra field site a) and the Inman Valley field site b). Standard error bars are displayed for each harvest.

Recovery of applied fertiliser P in clover shoots (> 3 cm) was up to 31 and 34% in the year of application at the Ginninderra and Inman Valley field sites, respectively (Table 8). In general, high recoveries of fertiliser P in clover shoots were recorded for the surface application of fertiliser P when applied at early-season (treatment A) for both sites, but also when fertiliser P was applied at depth (treatment D) for the Ginninderra site (Table 8). This clearly shows that a considerable proportion of the fertiliser P was transported to above-ground pasture biomass in the year of application for these treatments and at these sites.

Table 8. Concentrations of ^{33}P radioactivity (MBq core^{-1}) from ^{33}P-labelled SSP detected in clover shoots (> 3 cm) and the cumulative recovery of fertiliser P (as a % of applied ^{33}P-labelled SSP – corrected for WSP) for the fertilised treatments (A, B, C, D, E and F) at the Ginninderra and Inman Valley field sites in 2014. Values in parentheses are standard errors.

Treatment ID	Treatment description	Ginninderra	Inman Valley	Ginninderra	Inman Valley
		Cumulative biomass (MBq core^{-1})		Recovery of fertiliser P (as a % of applied SSP)	
A	Surface autumn	1.46 (0.12)	1.55 (0.04)	31.5 (2.1)	34.0 (0.8)
B*	Surface autumn (soil)	0.99 (0.03)	0.86 (0.08)	21.4 (0.7)	18.9 (1.7)
C†	Surface autumn (root)	0.95 (0.05)	0.91 (0.04)	20.6 (1.1)	19.9 (0.9)
D	Deep autumn	1.45 (0.06)	0.83 (0.14)	31.3 (1.4)	18.3 (3.0)
E‡	Surface spring (soil)	0.68 (0.05)	0.67 (0.10)	17.8 (1.2)	17.5 (2.5)
F§	Surface spring (root)	0.62 (0.04)	0.60 (0.04)	16.2 (1.0)	15.6 (1.0)

* Only one biomass harvest was collected before the beginning of spring. Cores were removed and soil fractions analysed for the ^{33}P radio-label.

† Only one biomass harvest was collected before the beginning of spring. Cores were removed and root fractions analysed for the ^{33}P radio-label.

‡ The ^{33}P-labelled SSP was applied at the beginning of spring. At the last harvest, cores were removed and soil fractions analysed for the ^{33}P radio-label.

§ The ^{33}P-labelled SSP was applied at the beginning of spring. At the last harvest, cores were removed and root fractions analysed for the ^{33}P radio-label.

In Tables 9 and 10, concentrations of the ^{33}P radio-label and the recovery of applied fertiliser P in all above ground biomass (> 0 cm) is reported; this includes measures of the ^{33}P radio-label in clover shoots collected at each harvest (> 3 cm) and also the clover shoots collected from the soil surface to 3 cm above the soil surface (i.e. 0 – 3 cm) at the last harvest.

Recoveries of applied fertiliser P in all above ground biomass was up to 40 and 42% in the year of application at the Ginninderra and Inman Valley field sites, respectively (Tables 9 and 10). In general, high recoveries of fertiliser P in clover shoots (~ 40%) were obtained for all early season applications of fertiliser P regardless of fertiliser P placement (treatments A, B, C and D), whereas this was slightly lower (~ 30%) for all mid-season applications of fertiliser P at the Ginninderra site (Tables 9 and 10). At the Inman Valley site, recoveries of applied fertiliser P in all above ground biomass was highest for the surface application of fertiliser P at early-season, and lower (~ 27%) for all other treatments (treatments B, C, D, E and F) (Tables 9 and 10).

Treatments A, B and C all involved surface application of fertiliser P to clover pastures at early-season; however, treatments B and C were ended (i.e. cores removed) at the same time as the mid-season application of fertiliser P to treatments E and F. Treatments B and C were designed to determine the fate of fertiliser P in clover pastures just prior to the spring growth phase (mid-season). At the Ginninderra field site, 3 out of the 5 harvests were collected prior to the mid-season application of fertiliser P, whereas at the Inman Valley field site only 1 of the 4 harvests had been collected. Approximately 97% of the fertiliser P recovered in above ground biomass across all harvests (treatment A) was recovered in the above ground biomass in treatments B and C at the Ginninderra site. At the Inman Valley field site, ~ 67% of the fertiliser P recovered in above ground biomass across all harvests (treatment A) was recovered in the above ground biomass in treatments B and C. It thus appears that much of the fertiliser P was taken up by clover pastures soon after application.

The majority of roots collected in treatments C and F were in the 0 – 4 cm layer below the soil surface at both sites. Recoveries of fertiliser P in root fractions from the 0 – 15 cm layer below the soil surface were 7 and 10% at the Ginninderra and Inman Valley field sites across both treatments, respectively (Tables 9 and 10). Adding recoveries of fertiliser P in root fractions (7 and 10% at the Ginninderra and Inman Valley field sites, respectively) to that of above ground biomass in treatment A results in total recovery of fertiliser P in whole clover plants of approximately 47 and 52% at the Ginninderra and Inman Valley field sites, respectively.

Table 9. Concentrations of ^{33}P radioactivity (MBq core^{-1}) from ^{33}P-labelled SSP detected in various components of the pasture system at the Ginninderra and Inman Valley field sites in 2014. Values in parentheses are standard errors.

Field site location	Treatment ID	Placement and timing of fertiliser P	Clover shoots (> 0 cm)	Clover roots (0 - 15 cm)	Soil (0 - 15 cm)[#]	Granule residues
Ginninderra	A	Surface, early-season	1.69 (0.12)			
	B*	Surface, early-season (soil)	1.64 (0.04)		0.70 (0.08)	0.08 (0.01)
	C†	Surface, early-season (root)	1.65 (0.04)	0.32 (0.01)		
	D	Deep, early-season	1.85 (0.07)			
	E‡	Surface, mid-season (soil)	1.13 (0.04)		0.79 (0.05)	0.49 (0.06)
	F§	Surface, mid-season (root)	1.20 (0.04)	0.26 (0.03)		0.47 (0.02)
Inman Valley	A	Surface, early-season	1.94 (0.05)			0.36 (0.03)
	B*	Surface, early-season (soil)	1.30 (0.13)		0.77 (0.05)	0.38 (0.02)
	C†	Surface, early-season (root)	1.29 (0.08)	0.52 (0.03)		0.10 (0.03) ‖
	D	Deep, early-season	1.13 (0.15)			0.31 (0.02)
	E‡	Surface, mid-season (soil)	1.00 (0.09)		0.67 (0.05)	0.49 (0.01)
	F§	Surface, mid-season (root)	1.10 (0.05)	0.38 (0.02)		0.50 (0.02)

* Three biomass harvests were collected at the Ginninderra field site and one biomass harvest at the Inman Valley field site before the mid-season application of fertiliser P to treatments E and F. At this time, cores were removed and soil fractions analysed for the ^{33}P radio-label.

† Three biomass harvests were collected at the Ginninderra field site and one biomass harvest at the Inman Valley field site before the mid-season application of fertiliser P to treatments E and F. At this time, cores were removed and root fractions analysed for the ^{33}P radio-label.

‡ The ^{33}P-labelled SSP was applied at mid-season. At the last harvest, cores were removed and soil fractions analysed for the ^{33}P radio-label.

§ The ^{33}P-labelled SSP was applied at mid-season. At the last harvest, cores were removed and root fractions analysed for the ^{33}P radio-label.

‖ Only 27% of the applied granules was recovered from this treatment.

Total NaOH-EDTA extractable P

Table 10. Recovery of fertiliser P (as a % of applied – corrected for WSP) from ^{33}P-labelled SSP detected in various components of the pasture system at the Ginninderra and Inman Valley field sites in 2014. Values in parentheses are standard errors.

Field site location	Treatment ID	Placement and timing of fertiliser P	Clover shoots (> 0 cm)	Clover roots (0 - 15 cm)	Soil (0 - 15 cm)[#]	Granule residues
Ginninderra	A	Surface, early-season	38.4 (2.1)			
	B*	Surface, early-season (soil)	35.4 (0.9)		15.2 (1.7)	1.9 (0.2)
	C[†]	Surface, early-season (root)	35.6 (0.9)	6.9 (0.3)		
	D	Deep, early-season	40.0 (1.5)			
	E[‡]	Surface, mid-season (soil)	29.4 (0.9)		20.5 (1.4)	12.2 (0.4)
	F[§]	Surface, mid-season (root)	31.2 (1.0)	6.9 (0.9)		14.0 (1.0)
Inman Valley	A	Surface, early-season	42.4 (1.1)			8.0 (0.7)
	B*	Surface, early-season (soil)	25.4 (2.0)		16.8 (1.1)	8.3 (0.4)
	C[†]	Surface, early-season (root)	31.3 (0.8)	11.5 (0.7)		2.1 (0.6)[II]
	D	Deep, early-season	24.7 (3.2)			6.7 (0.4)
	E[‡]	Surface, mid-season (soil)	26.0 (2.4)		17.5 (1.4)	12.8 (0.4)
	F[§]	Surface, mid-season (root)	28.6 (1.3)	10.0 (0.6)		13.1 (0.5)

* Three biomass harvests were collected at the Ginninderra field site and one biomass harvest at the Inman Valley field site before the mid-season application of fertiliser P to treatments E and F. At this time, cores were removed and soil fractions analysed for the ^{33}P radio-label.

† Three biomass harvests were collected at the Ginninderra field site and one biomass harvest at the Inman Valley field site before the mid-season application of fertiliser P to treatments E and F. At this time, cores were removed and root fractions analysed for the ^{33}P radio-label.

‡ The ^{33}P-labelled SSP was applied at mid-season. At the last harvest, cores were removed and soil fractions analysed for the ^{33}P radio-label.

§ The ^{33}P-labelled SSP was applied at mid-season. At the last harvest, cores were removed and root fractions analysed for the ^{33}P radio-label.

II Only 27% of the applied granules was recovered from this treatment.

Total NaOH-EDTA extractable P

A large proportion of the fertiliser P in soil fractions was recovered in the 0 – 4 cm layer below the soil surface at both sites. Recoveries of fertiliser P in NaOH-EDTA extracts of soil fractions from the 0 – 15 cm layer below the soil surface were 17 and 18% at the Inman Valley field site from treatments B (early-season applied, mid-season detected) and E (mid-season applied, late-season detected), respectively (Tables 9 and 10).

No more than 14% of the applied fertiliser P was recovered in the granule residues collected at the last harvest for each treatment (Tables 9 and 10). Considerably less of the fertiliser P was recovered in the granule residues for treatments when fertiliser P was applied at early-season compared to that at mid-season (Tables 9 and 10).

Two extraction procedures were used to determine the recovery of fertiliser P in forms of soil P (inorganic and organic) in soil fractions under clover. Both the NaOH-EDTA and ignition-H_2SO_4 extraction techniques provide estimates of total inorganic and total organic P in soil. At each site, concentrations of the ^{33}P radio-label were higher in the surface 0 – 4 cm layer than in the 4 – 8 cm layer. No ^{33}P radio-label was detected in the 8 – 15 cm layer. In general, the inorganic pool appeared to be the most important sink of fertiliser P at the Inman Valley field site, although there was evidence that organic P was an important sink of fertiliser P at mid-season (Tables 11 and 12). It is likely that this reflects favourable conditions for microbial growth at mid-season.

Table 11. The fate of ^{33}P-labelled SSP (MBq core^{-1}) in soil P fractions collected after at the beginning (B) and end (E) of spring at the Ginninderra and Inman Valley field sites. Values in parentheses are standard errors.

Treatment ID	Placement and timing of fertiliser P	Soil layer (cm)*	Field site	NaOH-EDTA extractable P			H_2SO_4-ignition extractable P		
				total	inorganic	organic	total	inorganic	organic
B	Surface, early-season (soil)	0 – 4	Ginninderra	0.70 (0.08)	0.84 (0.04)	0.00 (0.00)	1.19 (0.06)	0.86 (0.09)	0.36 (0.10)
			Inman Valley	0.71 (0.02)	0.37 (0.02)	0.34 (0.03)	0.74 (0.07)	0.61 (0.04)	0.15 (0.04)
		4 – 8	Ginninderra	MDL	MDL	MDL	MDL	MDL	MDL
			Inman Valley	0.31 (0.04)	0.17 (0.02)	0.14 (0.03)	MDL	MDL	MDL
E	Surface, mid-season (soil)	0 – 4	Ginninderra	0.79 (0.05)	0.72 (0.05)	0.06 (0.01)	0.89 (0.06)	0.53 (0.06)	0.37 (0.05)
			Inman Valley	0.59 (0.03)	0.55 (0.03)	0.04 (0.01)	0.74 (0.04)	0.48 (0.04)	0.26 (0.03)
		4 – 8	Ginninderra	MDL	MDL	MDL	MDL	MDL	MDL
			Inman Valley	0.11 (0.02)	0.10 (0.02)	0.00 (0.00)	MDL	MDL	MDL

* All measures of radioactivity for the ^{33}P radio-label were below the method detection limit of analysis in the 8 – 15 cm layer, and in some cases in the 4 – 8 cm layer (indicated as "MDL").

Table 12. Recovery of ^{33}P-labelled SSP (as a % of applied SSP – corrected for WSP) in soil P fractions collected after at the beginning (B) and end (E) of spring at the Ginninderra and Inman Valley field sites. Values in parentheses are standard errors.

Treatment ID	Placement and timing of fertiliser P	Soil layer (cm)*	Field site	NaOH-EDTA extractable P			H_2SO_4-ignition extractable P		
				total	inorganic	organic	total	inorganic	organic
B	Surface, early-season (soil)	0 – 4	Ginninderra	13.9 (0.7)	18.1 (1.0)	0.0 (0.0)	25.7 (1.2)	18.7 (1.9)	7.9 (2.2)
			Inman Valley	15.5 (0.5)	8.2 (0.5)	7.4 (0.7)	16.2 (1.6)	13.3 (0.9)	3.3 (0.8)
		4 – 8	Ginninderra	MDL	MDL	MDL	MDL	MDL	MDL
			Inman Valley	MDL	MDL	MDL	MDL	MDL	MDL
E	Surface, mid-season (soil)	0 – 4	Ginninderra	20.3 (1.4)	18.8 (1.3)	1.5 (0.3)	23.2 (1.5)	13.7 (1.5)	9.5 (1.3)
			Inman Valley	15.3 (0.9)	14.3 (0.9)	0.9 (0.2)	19.3 (0.9)	12.5 (0.9)	6.7 (0.8)
		4 – 8	Ginninderra	MDL	MDL	MDL	MDL	MDL	MDL
			Inman Valley	2.7 (0.6)	2.6 (0.6)	0.1 (0.0)	MDL	MDL	MDL

* All measures of radioactivity for the ^{33}P radio-label were below the method detection limit of analysis in the 8 – 15 cm layer, and in some cases in the 4 – 8 cm layer (indicated as “MDL”).

4.1.3.3 Design 2: Effect of initial soil fertility

The cumulative biomass (> 3 cm) removal and clover P uptake for the fertilised treatment (treatment H) was greater than that of the unfertilised control at the P0 level of soil P fertility, whereas these were similar at both the P1 and P2 levels of soil P fertility (Table 13). Therefore, an application of approximately 20 kg P ha^{-1} as ^{33}P-labelled SSP resulted in a clear clover response to fertiliser P, which was expected based on pre-determined levels of soil P fertility at this site. In general, cumulative biomass (> 3 cm) removal and clover P uptake increased from P0 < P1 < P2 for both unfertilised and fertilised treatments (Table 13).

Across all harvests, the proportion of plant P in clover shoots (> 3 cm) that was derived from the fertiliser for treatment H was on average 45, 24 and 19% at the P0, P1 and P2 levels of soil P fertility, respectively. For all levels of soil P fertility, the proportion of plant P in clover shoots (> 3 cm) that was derived from the fertiliser declined with each biomass harvest (Fig. 11).

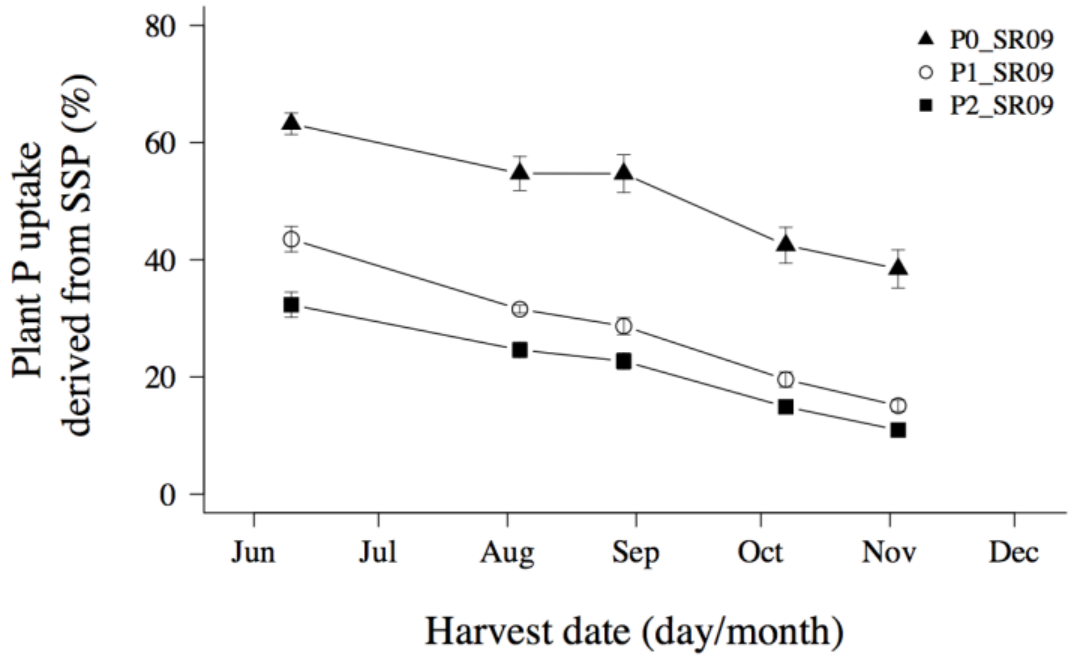

Fig. 11 The proportion of plant P that was derived from the ^{33}P-labelled SSP at each harvest for the Ginninderra field site across three levels of soil P fertility and one stocking rate (P0_SR09, P1_SR09 and P2_SR09). Standard error bars are displayed for each harvest.

Recovery of applied fertiliser P in clover shoots (> 3 cm) was approximately 40% across all levels of soil P fertility in the year of application at the Ginninderra field site (Table 14). As plants at lower levels of P fertility were smaller but the fertiliser P was of relatively higher importance (proportion of plant P from fertiliser), the recovery of applied fertiliser P in all above ground biomass was similar at the 3 levels of soil fertility. Phosphorus fertiliser recovery was approximately 45% at the P0 level of soil P fertility and approximately 50% at the P1 and P2 levels of soil P fertility (Table 15).

Table 13. Summary of the cumulative biomass removal (t DM ha^{-1} equivalent) and P uptake (kg P ha^{-1} equivalent) of clover shoots (> 3 cm) for the fertilised treatment of adding ^{33}P-labelled SSP to the soil surface at the end of autumn (H) across three different levels of soil P fertility and one stocking rate (P0_SR09, P1_SR09 and P2_SR09) at the Ginninderra field site. Values in parentheses are standard errors.

Treatment ID	Treatment description	P0_SR09	P1_SR09	P2_SR09	P0_SR09	P1_SR09	P2_SR09
		Cumulative biomass (t DM ha^{-1})			Cumulative P uptake (kg P ha^{-1})		
H	Surface autumn	9.8 (0.5)	13.5 (0.5)	16.0 (0.9)	15.1 (1.6)	32.7 (1.7)	42.7 (2.4)
I	Control	4.1 (0.6)	12.1 (0.4)	15.4 (1.0)	5.4 (1.1)	26.7 (1.4)	38.1 (2.7)

Table 14. Concentrations of ^{33}P radioactivity (MBq core^{-1}) from ^{33}P-labelled SSP detected in clover shoots (> 3 cm) and the cumulative recovery of fertiliser P (as a % of applied ^{33}P-labelled SSP – corrected for WSP) of the fertilised treatment of adding ^{33}P-labelled SSP to the soil surface at the end of autumn (H) across three levels of soil P fertility and one stocking rate (P0_SR09, P1_SR09 and P2_SR09) at the Ginninderra field site. Values in parentheses are standard errors.

Treatment ID	Treatment description	P0_SR09	P1_SR09	P2_SR09	P0_SR09	P1_SR09	P2_SR09
		Cumulative biomass (MBq core^{-1})			Recovery of fertiliser P (as a % of applied SSP)		
H	Surface autumn	1.54 (0.07)	1.80 (0.05)	1.71 (0.05)	33.3 (1.6)	39.0 (1.0)	37.0 (1.1)

Table 15. Concentrations of ^{33}P radioactivity (MBq core^{-1}) from ^{33}P-labelled SSP detected in clover shoots (> 0 cm) and the cumulative recovery of fertiliser P (as a % of applied ^{33}P-labelled SSP – corrected for WSP) of the fertilised treatment of adding ^{33}P-labelled SSP to the soil surface at the end of autumn (H) across three levels of soil P fertility (P0_SR09, P1_SR09 and P2_SR09) at the Ginninderra field site. Values in parentheses are standard errors.

Treatment ID	Treatment description	P0_SR09	P1_SR09	P2_SR09	P0_SR09	P1_SR09	P2_SR09
		Cumulative biomass (MBq core^{-1})			Recovery of fertiliser P (as a % of applied SSP)		
H	Surface autumn	1.86 (0.06)	2.10 (0.04)	1.96 (0.07)	40.3 (1.3)	45.5 (0.8)	42.5 (1.5)

4.2 Determination of the fate of fertiliser phosphorus via sequential chemical fractionation

The results described in this section have been published in:

McLaren, T.I., Simpson, R., McLaughlin, M., Smernik, R.J., McBeath, T., Guppy, C.N., and Richardson, A. (2015). An assessment of various measures of soil P and the net accumulation of P in fertilised soils under pasture. Journal of Plant Nutrition and Soil Science, ***178****, 543–554.*

4.2.1 Concentration, composition and extractability of soil P

Several different methods have been used to estimate the concentrations of total, inorganic and organic P of soils. In general, total P is determined by ICP-OES following acid digestion or by LXRF in the solid-state, and inorganic P is usually determined by the summation of extractable P using sequential chemical fractionation. Organic P can be determined in several ways: (1) by the summation of extractable organic P fractions using sequential chemical fractionation; (2) as the difference in inorganic P extractable with H_2SO_4 between ignited and unignited soils; or (3) as the difference between total and inorganic P in an alkaline extract (e.g. NaOH-EDTA) (Turner *et al.* 2005; Cade-Menun and Liu 2014). The relationships among these measures have not been widely investigated for soils under pasture.

Concentrations of total P in soil determined by LXRF ranged from 236 to 550 mg P kg^{-1} in surface and subsurface soils across all treatments, and these were strongly correlated with those determined by sequential chemical fractionation (summation of all P fractions). Sequential chemical fractionation recovered on average 90% of the total soil P determined by LXRF (Fig. 12). Concentrations of total P in soil determined by LXRF were also strongly correlated with those measured after *aqua regia* digestion, after ignition and extraction with H_2SO_4 and by extraction with NaOH-EDTA extractions (Fig. 12). However, concentrations of total P determined by *aqua regia* digestion, by ignition-H_2SO_4 extraction and by NaOH-EDTA extraction were considerably lower than those determined by LXRF (Fig. 12).

Interestingly, there were substantial negative intercepts for the correlations between total P determined by LXRF and P determined following ignition-H_2SO_4 extraction and also between total P determined by LXRF and P determined following NaOH-EDTA extraction (Fig. 12). This is consistent with the native soil containing a fraction of strongly-held mineral P that is neither acid nor alkali extractable. The fact that the slopes for these correlations are close to unity indicates that little of the P added during the experiment is converted to this form.

When expressed in terms of recovery relative to the LXRF values, average recoveries of total P were 59, 59 and 38%, for ICP-OES following *aqua regia* digestion, and total extractable P using the ignition-H_2SO_4 and NaOH-EDTA extractions, respectively. We consider the LXRF method to be the most reliable for determination of total P concentrations in soils because the digestion and extraction techniques only detect P that can be brought into solution, whereas LXRF, being a solid-state technique, does not have this limitation. The chemical composition of soil

P forms that are unaccounted for by the two extraction techniques is not known. However, it is interesting to note that when the 'residual P' pool of the chemical fractionation procedure is added to the estimate of total soil P using *aqua regia* digestion, and to that of the total extractable P measures using the ignition-H_2SO_4 and NaOH-EDTA extractions, average recoveries of 103, 103 and 82%, respectively, are obtained relative to total soil P determined by LXRF.

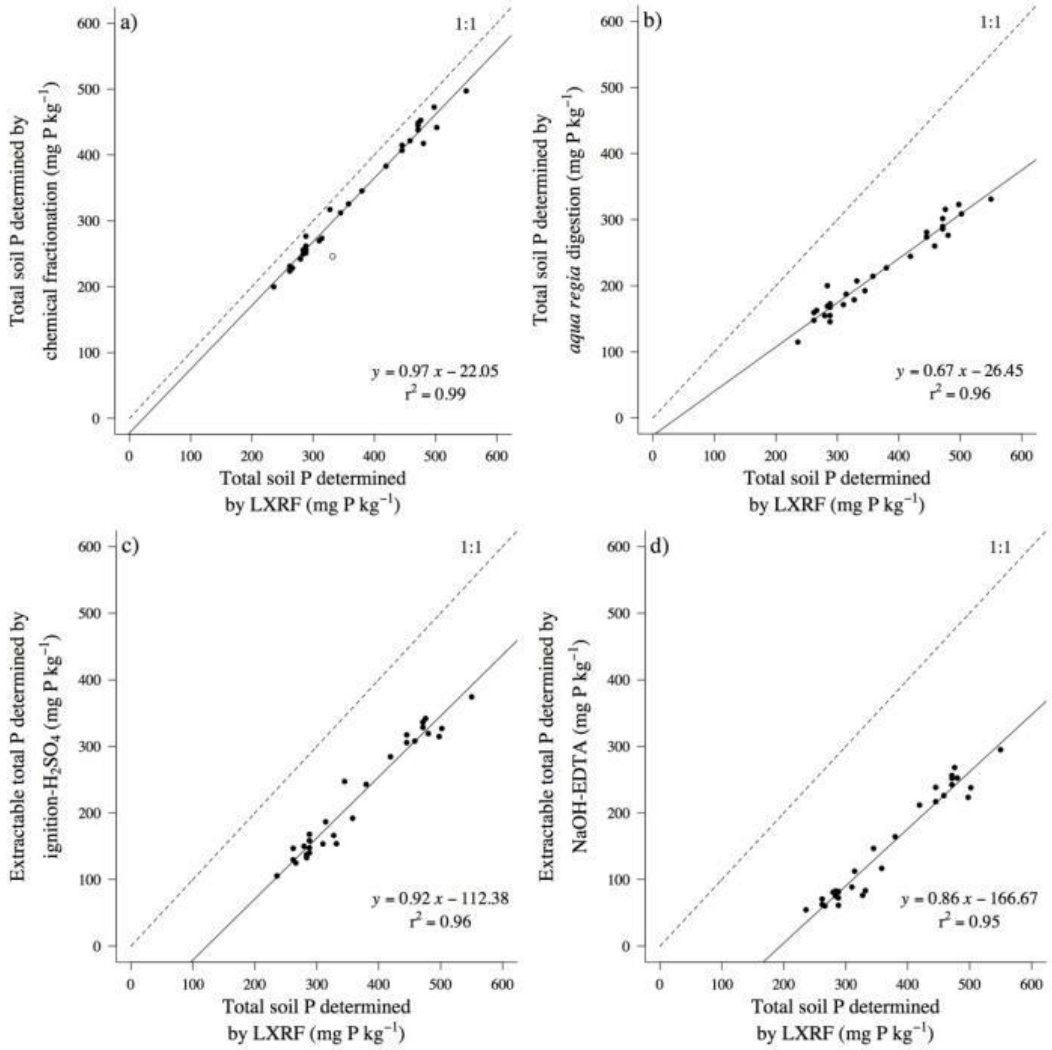

Fig. 12 Relationships between concentrations (mg P kg^{-1}) of total P in soil determined by laboratory X-ray fluorescence (LXRF) and sequential chemical fractionation (based on the method of Hedley *et al.* (1982)) (a), *aqua regia* digestion (b), the ignition-H_2SO_4 extraction technique of Walker and Adams (1958) (c), and the NaOH-EDTA extraction technique (based on Cade-Menun and Preston (1996) and Turner (2008)) (d). All regression models were significant ($P < 0.05$). The identified outlier in Fig. 12a is shown as an open circle and was not included in the regression analysis.

Summed concentrations of extractable inorganic P determined by sequential chemical fractionation ranged from 20 to 142 mg P kg^{-1} in surface and subsurface soils across all treatments. Total inorganic P determined by sequential chemical

fractionation was strongly correlated with inorganic P determined by the ignition-H_2SO_4 extraction and NaOH-EDTA extractable inorganic P (Fig. 13). The average recoveries of inorganic P determined by ignition-H_2SO_4 extraction and NaOH-EDTA extraction (relative to that of sequential chemical fractionation) were 102 and 84%, respectively. Whilst the ignition-H_2SO_4 and NaOH-EDTA extractions were originally designed to estimate total organic P, it appears they also provide reliable estimates of total inorganic P in these soils. It is not uncommon for studies to use these techniques to provide an estimate of both total inorganic and organic P (Bünemann *et al.* 2008a; Cade-Menun *et al.* 2010; Turner and Blackwell 2013).

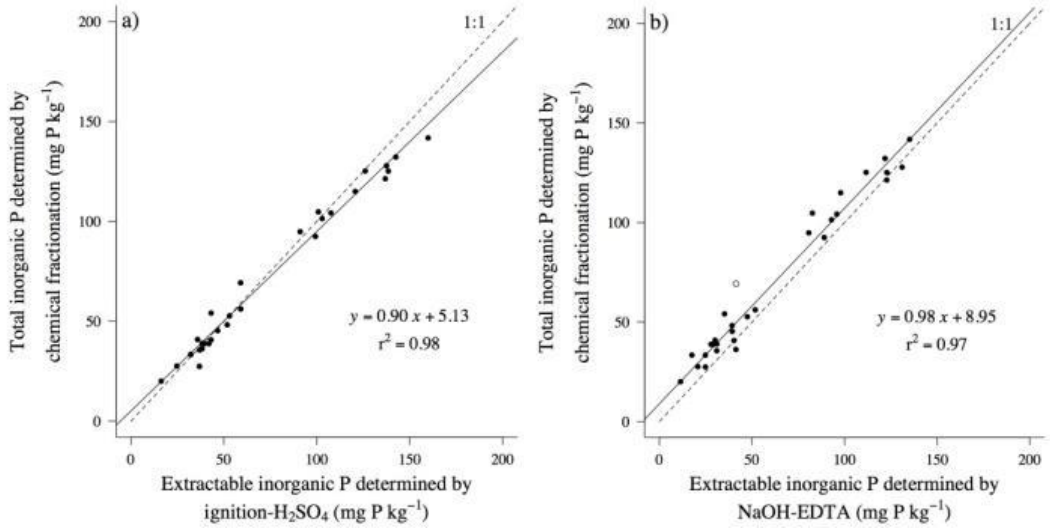

Fig. 13 Relationships between concentrations of total inorganic P determined by sequential chemical fractionation (based on the method of Hedley *et al.* (1982)) and those determined by the ignition-H_2SO_4 extraction technique of Walker and Adams (1958) (a), and the NaOH-EDTA extraction (based on Cade-Menun and Preston (1996) and Turner (2008)) (b). All regression models were significant ($P < 0.05$). The identified outlier in b) is shown as an open circle and was not included in the regression analysis.

Summed concentrations of extractable organic P determined by sequential chemical fractionation ranged from 48 to 174 mg P kg^{-1} in surface and subsurface soils across all treatments. Total organic P concentrations determined by sequential chemical fractionation were strongly correlated with those determined by the NaOH-EDTA and ignition-H_2SO_4 extraction methods (Fig. 14). Again, these correlations appear to involve non-zero intercepts. The small negative intercept for the correlation between summed concentrations of extractable organic P determined by sequential chemical fractionation and that determined by ignition-H_2SO_4 extraction (Fig. 14a) most likely represents organic P in the native soil that escapes extraction through the sequential chemical fractionation process. However, there is also a possibility that ignition-H_2SO_4 extraction overestimates organic P. The small positive intercept for the correlation between summed concentrations of extractable organic P determined by

sequential chemical fractionation and that determined by NaOH-EDTA extraction indicates that the native soil contains a small amount of organic P that is soluble in alkaline solution, but is not extracted by NaOH-EDTA; this likely includes organic P released by sonication in the sequential chemical fractionation.

In general, there is strong agreement between all three measures for total organic P (and inorganic P); r^2 values are high, slopes are close to unity and intercepts are small (Figs 13 and 14), notwithstanding the issues discussed above. This suggests that the fraction of soil P characterized using sequential chemical fractionation (i.e. the sum of all fractions extracted during the sequential procedure) is closely aligned with that detected by these alternative techniques. This is an important finding given that detailed characterization of organic P forms using solution ^{31}P NMR spectroscopy (see Section 4.3) can only be carried out on alkaline extracts (e.g. NaOH-EDTA) (Bowman and Moir 1993; Turner *et al.* 2003b).

The average recovery of organic P determined by the ignition-H_2SO_4 extraction technique was higher (149%) than that for the NaOH-EDTA technique (78%), when expressed relative to the measure of total organic P obtained from sequential chemical fractionation. Ignition-H_2SO_4 extraction has been reported to overestimate concentrations of organic P in soil (Williams *et al.* 1970; Oniani *et al.* 1973), and this would contribute to the higher estimates of organic P compared to those determined by sequential chemical fractionation (Cade-Menun and Lavkulich 1997).

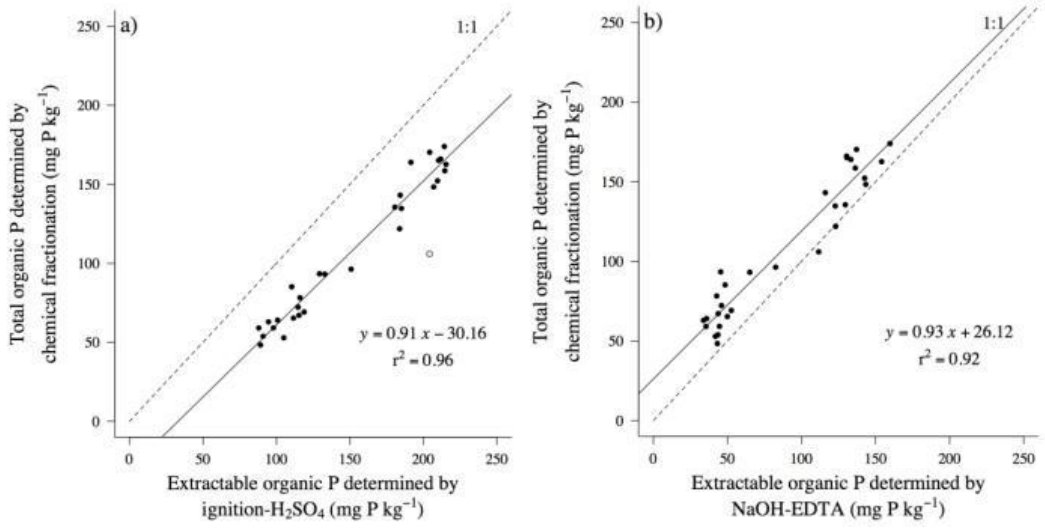

Fig. 14 Relationships between concentrations of organic P determined by sequential chemical fractionation (based on the method of Hedley *et al.* (1982)) and organic P determined using the ignition-H_2SO_4 extraction of Walker and Adams (1958) (a) and the NaOH-EDTA extraction (based on Cade-Menun and Preston (1996) and Turner (2008)) (b). The identified outlier in a) is shown as an open circle. All regression models were significant ($P < 0.05$).

4.2.2 Distribution of soil P forms in Ginninderra soils as determined by sequential fractionation

On average, total extractable inorganic, organic and residual P (i.e. non-extractable P) fractions accounted for 20, 31 and 49% of total soil P, respectively, as determined by sequential chemical fractionation in surface and subsurface soils across all treatments. Residual P did not significantly increase with the addition of fertiliser P (Table 16), consistent with the understanding that residual P is a relatively stable form of soil P that is likely to contribute little to pasture growth.

The majority of inorganic and organic P was detected in the $NaHCO_3$ and NaOH I pools across all treatments and at both soil depths (Table 16). Organic P forms accounted for the majority of P extracted in the $NaHCO_3$ and NaOH I pools, representing 80 and 62% of the total extractable P, respectively (Table 16).

Table 16. Concentrations of P in soil fractions (mg P kg^{-1}) from the Ginninderra medium-term field experiment measured by sequential chemical fractionation. The cumulative P input that was applied to the soil surface over 13 years was on average 4, 200, and 242 kg P ha^{-1} for the P0, P1 and P2 soil P fertility levels at stocking rate SR09, respectively, and at stocking rate SR18 the cumulative P input was on average 221 and 320 kg P ha^{-1} for the P1 and P2 soil P fertility levels, respectively.

Soil Depth	Stocking rate	Soil P fertility	Inorganic P*				Organic P *				Total sums*,†		Residual soil P *‡	Total soil P *,§
			$NaHCO_3$	NaOH I	NaOH II	HCl	$NaHCO_3$	Hexanol	NaOH I	NaOH II	inorganic P	organic P		
0 – 10	SR09	P0	6.0 a	27.1 a	4.8 a	16.9 a	39.1 a	1.3 a	59.8 a	7.9 a	54.7 a	108.1 a	147.5 a	346.3 a
(cm)		P1	15.7 b	52.0 b	8.0 b	25.6 a	63.3 bc	2.3 a	77.4 a	10.1 a	101.2 b	153.1 b	160.2 a	449.6 b
		P2	21.1 c	68.1 c	8.0 b	26.7 a	70.7 c	1.3 a	72.7 a	10.1 a	123.9 bc	154.8 b	156.0 a	474.3 b
	SR18	P1	16.4 b	55.1 b	8.0 b	23.5 a	57.3 b	1.8 a	82.7 a	12.6 a	102.9 b	154.5 b	176.1 a	462.7 b
		P2	22.7 c	73.4 c	9.0 b	28.7 a	75.9 c	1.8 a	73.5 a	11.3 a	133.9 c	162.4 b	167.5 a	509.2 b
10 – 20	SR09	P0	1.8 a	13.5 a	3.3 a	8.4 a	12.2 a	0.3 a	39.1 a	4.5 a	27.0 a	56.1 a	144.1 a	261.9 a
(cm)		P1	4.4 b	20.9 b	3.8 a	7.8 a	18.6 bc	0.2 a	42.6 a	5.5 a	37.0 ac	66.8 a	143.3 a	279.3 ab
		P2	5.8 bc	25.3 bc	4.0 a	8.1 a	20.7 bc	0.2 a	38.4 a	5.6 a	43.2 bc	65.0 a	152.4 a	285.2 ab
	SR18	P1	4.4 b	21.2 b	3.8 a	4.6 a	16.5 ab	0.3 a	47.5 a	8.2 a	34.0 ac	72.6 a	156.0 a	291.0 ab
		P2	7.0 c	29.0 c	4.4 a	9.5 a	24.0 c	0.3 a	49.6 a	6.9 a	49.8 bc	80.8 a	149.8 a	333.2 b

* Means of soil P fractions within each column and depth followed by the same letter are not significantly different according to a one-way ANOVA ($P > 0.05$).

† The total sums of extractable inorganic and organic P were calculated by summing the inorganic or organic P fractions of each extract of the sequential chemical fractionation.

‡ Residual P (i.e. non-extractable P) was determined using LXRF on the soil residue after sequential chemical fractionation.

§ Total soil P was determined using LXRF.

The largest increases in soil P concentrations between the unfertilised and fertilised treatments were in the $NaHCO_3$ and NaOH I fractions. In the $NaHCO_3$ fraction, the accumulation of organic P was greater than that of inorganic P, whereas in the NaOH I fraction, the opposite occurred, with the accumulation of inorganic P being greater than that of organic P (Table 16). There was no significant increase for organic P in the NaOH I fraction. There was generally a small increase in organic P in the $NaHCO_3$ fraction going from the P1 to the P2 treatment (Table 16).

Fertiliser P tended to accumulate preferentially in the NaOH I fraction. Consequently, it would appear that the accumulation of inorganic P is mostly found in fractions associated with more strongly bound pools of P (i.e. NaOH I fractions) rather than that of weakly sorbed pools of P (i.e. $NaHCO_3$ fractions) based on chemical solubility. Only small quantities of HCl-extractable P were detected across all soils (Table 16).

In the subsurface layer, the accumulation of soil P with the addition of fertiliser P followed a similar trend to that seen for the surface soil layer, but in most instances was not significantly different across P fertiliser treatments (Table 16). Where differences were evident (e.g. organic P soluble in $NaHCO_3$), the amounts of accumulated P were generally smaller than those observed in the surface soil layer (Table 16).

There was no significant difference in the accumulation of soil P forms with the addition of fertiliser P between the moderate (SR09) and high (SR18) stocking rate at each soil P fertility level (Table 16). The cumulative P input was similar for the P1 treatment at the SR09 and SR18 stocking rates (Table 16). On the other hand, for the P2 treatment, the cumulative P input was substantially higher (~ 78 kg P ha^{-1}) for the SR18 stocking rate (Table 16). The additional fertiliser P required to maintain the P2 soil P fertility at the SR18 stocking rate appears to be spread throughout most of the measured P fractions.

The main aim of this study was to determine the net accumulation of fertiliser P into soil P forms under pasture. This is best illustrated by considering differences in soil P fractions between the unfertilised and fertilised treatments. In Table 17, the sums of inorganic and organic P fractions, residual P and total soil P in the P0SR09 treatment were subtracted from those for the P1SR09, P1SR18, P2SR09 and P2SR18 treatments, to provide a measure of the amount of soil P that had accumulated due to fertiliser P at both stocking rates and both soil depths. This difference, which represents soil P that had accumulated with the addition of fertiliser P, was then divided by the cumulative P input and converted to a percentage to provide a measure of the proportion of fertiliser P that had accumulated in various soil P forms. Similarly, in Table 18, these results are presented for the individual inorganic and organic P fractions of the sequential chemical fractionation for the surface soil, but only at stocking rate SR09.

Approximately 87% of the cumulative P input can be accounted for in the increase of total soil P in the surface and subsurface layers (0 – 20 cm), with most of this increase occurring in the surface soil layer (0 – 10 cm) (Table 17).

Table 17. Summed concentrations (kg P ha^{-1} equivalents) of extractable inorganic and organic P, and residual P determined by sequential chemical fractionation and total soil P pools determined by LXRF of P in soil fractions of the Ginninderra medium-term field experiment. The cumulative P inputs, soil P concentrations and recoveries are expressed relative to the unfertilised (P0SR09) treatment. This equated to total P inputs of on average 196 and 234 kg P ha^{-1} at stocking rate SR09 for the P1 and P2 soil P fertility levels, respectively, and at stocking rate SR18 this was on average 216 and 316 kg P ha^{-1} for the P1 and P2 soil P fertility levels, respectively. Recovery of the cumulative P input was calculated as the increase in soil P (relative to the unfertilised treatment; P0SR09), divided by the cumulative P input, and converted to a percentage.

Soil Depth	Stocking rate	Cumulative P input	Total sums inorganic P (kg P ha^{-1}; relative to P0SR09)	Recovery of cumulative P input (%)	Total sums organic P (kg P ha^{-1}; relative to P0SR09)	Recovery of cumulative P input (%)	Residual P (kg P ha^{-1}; relative to P0SR09)	Recovery of cumulative P input (%)	Total soil P (kg P ha^{-1}; relative to P0SR09)	Recovery of cumulative P input (%)
0 – 10 (cm)	SR09	P1	61.4	31	59.4	30	16.7	9	137.5	70
		P2	91.3	38	61.8	26	11.3	5	164.3	69
	SR18	P1	63.6	29	61.3	28	37.8	18	162.6	75
		P2	104.5	33	71.8	23	26.4	8	202.7	64
		Average		*33*		*27*		*10*		*70*
10 – 20 (cm)	SR09	P1	12.0	6	13.0	7	-1.1	-1	24.0	12
		P2	19.6	8	10.8	5	9.9	4	40.4	17
	SR18	P1	8.5	4	19.9	9	14.3	7	42.7	20
		P2	27.6	9	29.8	9	6.9	2	64.4	20
		Average		*7*		*8*		*3*		*17*

Table 18 summarizes the accumulation of P in the various inorganic and organic P fractions in the surface soil layer after 13 years of P fertilisation at stocking rate SR09. Since soil P concentrations varied little between this and the higher stocking rate (SR18), it can be assumed that similar accumulations would have occurred in that case as well (note that for the higher stocking rate the unfertilised treatment was discontinued due to overstocking which caused soil erosion). Approximately 70 and 69% of the cumulative P input could be accounted for in the surface soil layer of the P1 and P2 treatments, respectively, at stocking rate SR09 (Table 18). For the P1 treatment, 31 and 30% of the cumulative P input could be accounted for in the total inorganic and organic P fractions of the sequential fractionation scheme, respectively (Table 18). For the P2 treatment, a larger proportion of the cumulative P input (38%) was present as inorganic P, and hence a smaller proportion (26%) accumulated as organic P (Table 18). Soil P pools associated with $NaHCO_3$ and NaOH I fractions were the most important sinks of fertiliser P, accounting for over half of the cumulative P at both soil P fertility levels (Table 18).

Table 18. Distribution across fractions of accumulated fertiliser P after 13 years continuous P fertilisation to maintain a target soil P fertility of 10 – 15 mg Olsen P kg^{-1} (P1) and 20 – 25 mg Olsen P kg^{-1} (P2) in the surface soil layer (0 – 10 cm) at stocking rate SR09.

Form	Soil P fraction	P1; Recovery of cumulative P input (%)	P2; Recovery of cumulative P input (%)
Inorganic	$NaHCO_3$	7	8
	NaOH I	17	23
	NaOH II	2	2
	HCl	6	5
Organic	$NaHCO_3$	16	18
	NaOH I	12	7
	NaOH II	1	1
Unknown	Residual	9	5
	Total sum inorganic	31	38
	Total sum organic	30	26
	Total soil P	70	69

4.3 Determination of the fate of fertiliser phosphorus via nuclear magnetic resonance (NMR) spectroscopy

4.3.1 Modification of extraction conditions to improve NMR characterisation of P in subsoils

The results described in this section have been published in:

McLaren, T.I., Smernik, R.J., Simpson, R., McLaughlin, M., McBeath, T., Guppy, C.N., and Richardson, A. (2015) Spectral sensitivity of solution ^{31}P NMR spectroscopy is improved by narrowing the soil to solution ratio to 1:4 for pasture soils of low organic P content. Geoderma, **257-258**, 48–57 .

The main limitation of NMR analysis is low sensitivity, which results in low sample throughput, high cost and/or a limited ability to detect and quantify species present at low concentrations. These issues are particularly acute for low P soils, including low fertility topsoils and most subsoils. Modifying extraction conditions, in particular by narrowing the soil:solution ratioduring extraction was investigated as a way to improve sensitivity.

4.3.1.1 Concentration, composition and extractability of soil P

Concentrations of total soil P ranged from 153 to 650 mg P kg^{-1} (Table 19). On average, 68% of total P was extracted by H_2SO_4 following ignition (Table 19). The proportion of organic and inorganic P that comprised total extractable P varied widely: 32 – 92% for organic P and 8 – 68% for inorganic P. The efficiency with which NaOH-EDTA extracted total P (determined by LXRF) at the 1:10 soil to solution ratio ranged from 30 to 78% (47% on average), and at the 1:4 soil to solution ratio ranged from 23 to 58% (41% on average) (Table 19).

The concentrations of organic P extracted by NaOH-EDTA (1:10) ranged from 40 to 150 mg P kg^{-1} (average 78 mg P kg^{-1}) (Table 19). The concentrations of organic P extracted by NaOH-EDTA when the soil to solution ratio was reduced to 1:4 ranged from 23 to 156 mg P kg^{-1} (average 66 mg P kg^{-1}) (Table 19). The concentrations of organic P extracted by NaOH-EDTA at both soil to solution ratios were strongly correlated with, but consistently lower than, concentrations of organic P determined by ignition-H_2SO_4 extraction.

Table 19. The concentrations (mg kg^{-1}) of total soil P determined by X-ray fluorescence; total, inorganic and organic P contents determined by ignition-H_2SO_4 extraction; and concentrations of total, inorganic and organic P extractable in NaOH-EDTA at 1:10 and 1:4 soil: solution extraction ratios. Values in parentheses are extraction efficiencies relative to total soil P.

Site ID *	Total soil P† (mg kg^{-1})	Ignition-H_2SO_4 (mg kg^{-1})			NaOH-EDTA 1:10 (mg kg^{-1})			NaOH-EDTA 1:4 (mg kg^{-1})		
		total P	inorganic P	organic P	total P	inorganic P	organic P	total P	inorganic P	organic P
1a	162	121 (75)	82	39	126 (78)	55	72	93 (58)	67	27
1b	166	123 (74)	65	58	88 (53)	48	40	78 (47)	55	23
2a	292	247 (85)	147	100	181 (62)	127	55	167 (57)	123	44
2b	240	196 (82)	106	91	131 (54)	82	49	119 (50)	79	40
3a	362	234 (65)	57	177	149 (41)	39	110	144 (40)	40	104
3b	292	185 (63)	59	126	110 (38)	30	80	102 (35)	30	73
4a	506	345 (68)	125	221	225 (44)	95	129	229 (45)	91	137
4b	388	253 (65)	78	175	169 (43)	60	109	156 (40)	60	95
5a	650	471 (72)	223	249	331 (51)	181	150	329 (51)	173	156
5b	485	307 (63)	137	171	222 (46)	113	109	206 (42)	108	98
6a	183	95 (52)	11	84	60 (33)	10	51	48 (26)	11	37
6b	157	78 (50)	6	72	47 (30)	3	45	36 (23)	6	30
7a	201	144 (72)	44	100	93 (46)	39	54	72 (36)	36	35
7b	153	97 (64)	26	71	64 (42)	23	41	46 (30)	23	23

* The same number denotes a soil sample obtained from the same site, and the letter “a” and “b” refers to the topsoil (0 – 4 cm) and subsurface (4 – 10 cm) layers respectively.

† Determined by laboratory X-ray fluorescence.

Concentrations of organic P extracted by NaOH-EDTA at the 1:4 ratio were strongly correlated with those extracted at the 1:10 ratio and for most soils similar values were obtained at both ratios (Fig. 15), although extractability was consistently higher at the 1:10 ratio for the low P soils (soils 1, 6 and 7; Table 19). Concentrations of inorganic P extracted by NaOH-EDTA at the two soil to solution ratios (1:4 and 1:10) were also highly correlated, and this relationship was close to 1:1 (Fig. 15). It is thus clear that for most of the soils of the current study, the 1:10 and 1:4 soil to solution NaOH-EDTA extracts are solubilising the same fraction of soil P and hence a quantitative comparison of the NMR spectra for these extracts is valid.

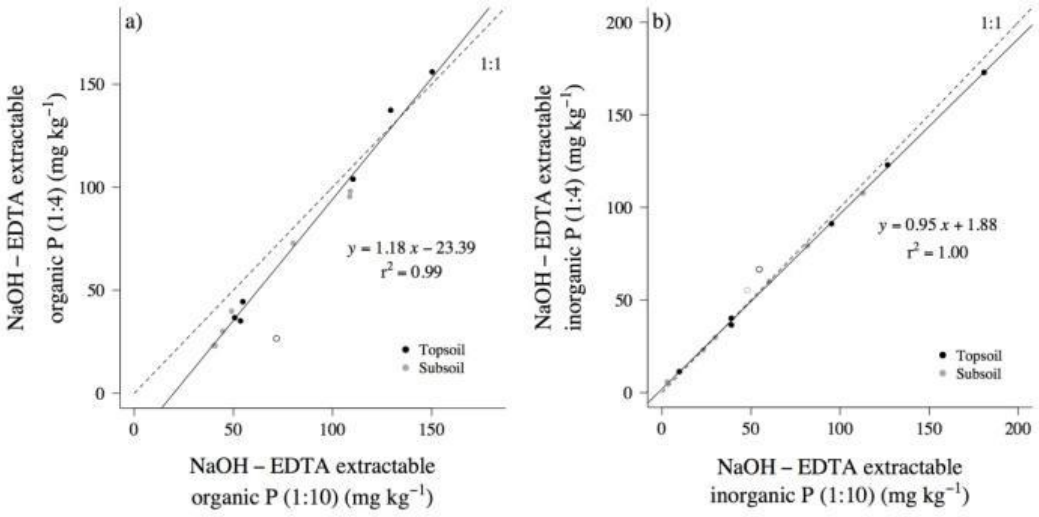

Fig. 15 Relationships between concentrations of organic (a) and inorganic (b) P determined with NaOH-EDTA using a 1:10 and 1:4 soil to solution ratio. The identified outliers in a) and b) are shown an open circle. In a) this is soil 1a (Table 19) and those in b) are soils 1a and 1b (Table 19), which were excluded from the regression model.

4.3.1.2 Concentrations and extractability of metals

The influence of soil to extractant ratio on extractability was also determined for four metals. This is of interest for two reasons. First, narrowing the extraction ratio may result in an exhaustion of hydroxide ion as reported by Turner (2008). This would be evidenced by a decrease in Al and Fe extractability at the narrower ratio. Second, it is of interest to establish whether narrowing the extraction ratio results in a proportional increase in the concentration of paramagnetic ions in the extract and subsequently to determine whether this adversely influences the quality of the NMR spectra.

Concentrations of all metals extracted by NaOH-EDTA at the 1:4 and 1:10 soil to extract ratios were highly correlated, and the slopes of relationships varied from 0.77 to 1.30 (Fig. 16). For Fe and Mn, which are both paramagnetic, extractability was

slightly higher at the 1:4 ratio, whereas the opposite occurred for Al and Mg, which are not paramagnetic and of no direct concern for NMR analysis (Fig. 16).

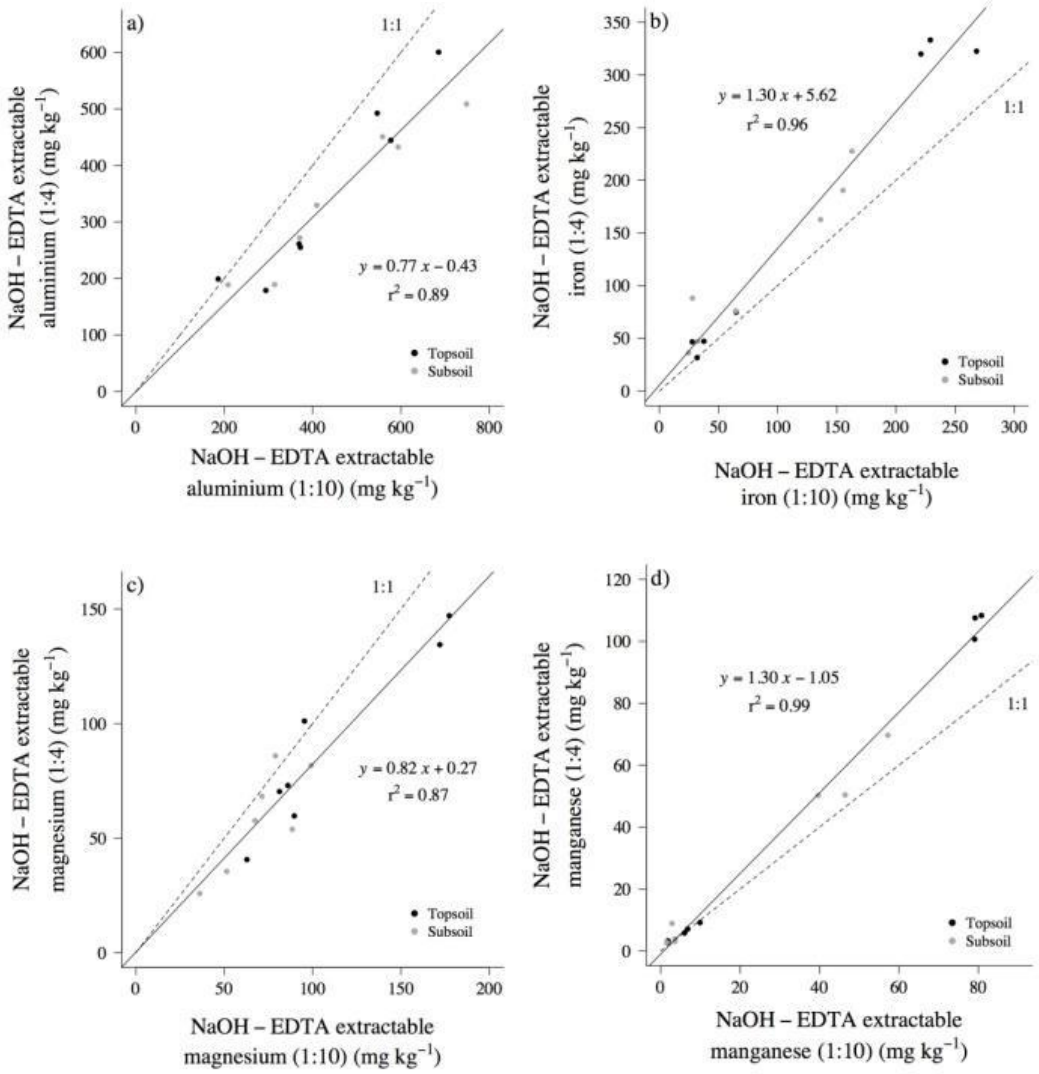

Fig. 16 A linear regression between aluminium (a), iron (b), magnesium (c) and manganese (d) in NaOH-EDTA extracts using a 1:10 and 1:4 soil to solution ratio.

4.3.1.3 Solution phosphorus-31 nuclear magnetic resonance spectra

Orthophosphate provided the most intense peak in the majority of spectra (Figs 17 and 18). Integration of the NMR spectra indicated orthophosphate accounted for 26 to 76% (53% on average) of total P in the NaOH-EDTA(1:4) extracts, equating to NaOH-EDTA extractable concentrations of orthophosphate between 9 and 180 mg P kg^{-1}. Similarly, integration of the NMR spectra indicated orthophosphate accounted for 23 to 68% (47% on average) of total P in the NaOH-EDTA(1:10) extracts, equating to NaOH-EDTA extractable concentrations of orthophosphate between 11

and 177 mg P kg^{-1}. Most of the remaining P was detected as monoester P; only low concentrations of diesters (δ 0.5 to -1.0 ppm) and pyrophosphate were detected (< 9 and < 5 mg P kg^{-1}, respectively).

The monoester regions of the ^{31}P NMR spectra of the NaOH-EDTA (1:4) and NaOH-EDTA (1:10) extracts are shown in Fig. 17 for the topsoil layers and Fig. 18 for the subsurface layers. In general, the spectra contain two distinctive features in the monoester region: 1) a broad feature centred around δ 4.7 ppm and spanning from approximately δ 3.5 to 6.0 ppm that has been attributed to high molecular weight compounds containing P in monoester linkages and termed humic P (Doolette *et al.* 2011); and 2) a series of prominent sharp peaks. The latter include peaks that can be assigned to α- and β-glycerophosphate (δ 4.90 and 4.56 ppm, respectively), which are most likely derived from alkaline hydrolysis of phospholipids (Doolette *et al.* 2009), three peaks due to *myo*-inositol hexakisphosphate (δ 4.69, 4.34 and 4.25 ppm), a peak due to *scyllo*-inositol hexakisphosphate (δ 3.84 ppm) and up to three peaks from mononucleotides (δ 4.44, 4.10 and 4.01 ppm) derived from the alkaline hydrolysis of RNA (Makarov *et al.* 2002; Turner *et al.* 2003a; Turner and Richardson 2004).

For all soils, the signal-to-noise ratio was considerably better for the 1:4 extracts than for the 1:10 extracts (Figs 17 and 18). This can be attributed to the greater density of P in the NMR tube at the 1:4 ratio than at the 1:10 ratio (Doolette and Smernik 2011). Furthermore, the signal resolution (peak width) appears identical for corresponding 1:4 and 1:10 extracts, clearly indicating that the higher paramagnetic metal concentrations in the 1:4 extracts are not causing additional line broadening for these soils (Figs 17 and 18). Enhanced signal-to-noise ratios are particularly evident for the low P soils, including the subsurface layers, for which only the largest peaks can be clearly identified. As a result, quantification of species contributing signal to the monoester region using deconvolution was not attempted for 4 of the 14 NaOH-EDTA (1:10) extracts because the relatively low signal-to-noise ratio makes deconvolution highly subjective and prone to error. The improved spectral sensitivity of ^{31}P NMR spectra using the lower soil to solution ratio generally enabled us to better judge the position, extent and lineshape of peaks (for example, Fig. 17; Soil 1a, and Fig. 18; Soil 6b). Consequently, the identification and quantification of organic P forms in the monoester region of the 1:4 extracts was more reliable than for the 1:10 extracts, particularly in the subsurface layer.

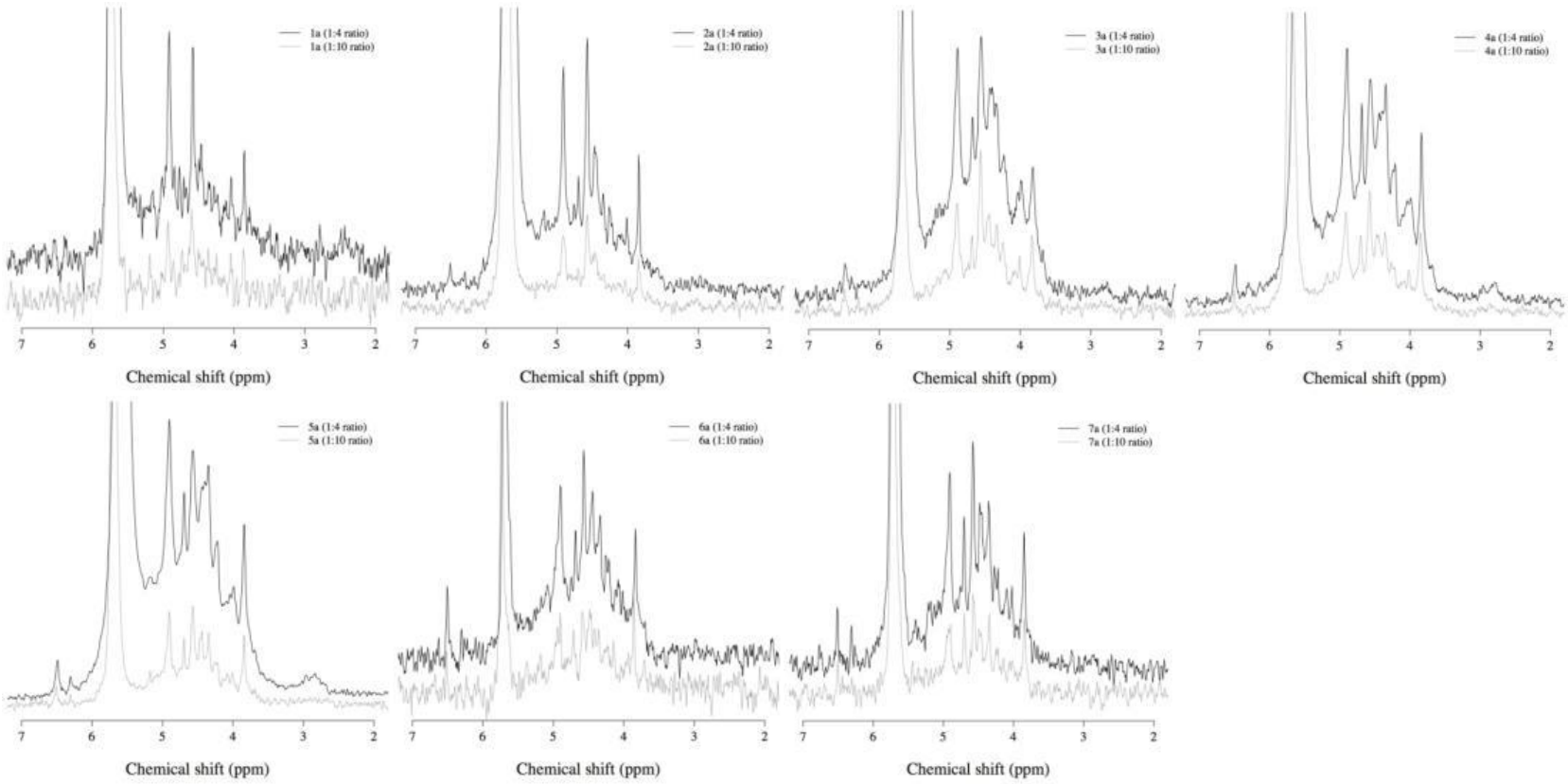

Fig. 17 Orthophosphate and monoester region of solution ^{31}P NMR spectra of the topsoil layer on NaOH-EDTA extracts at a 1:10 and 1:4 soil to solution ratios. The vertical scale of each spectrum has been normalised to the methylenediphosphonic acid peak at δ 17.01 ppm.

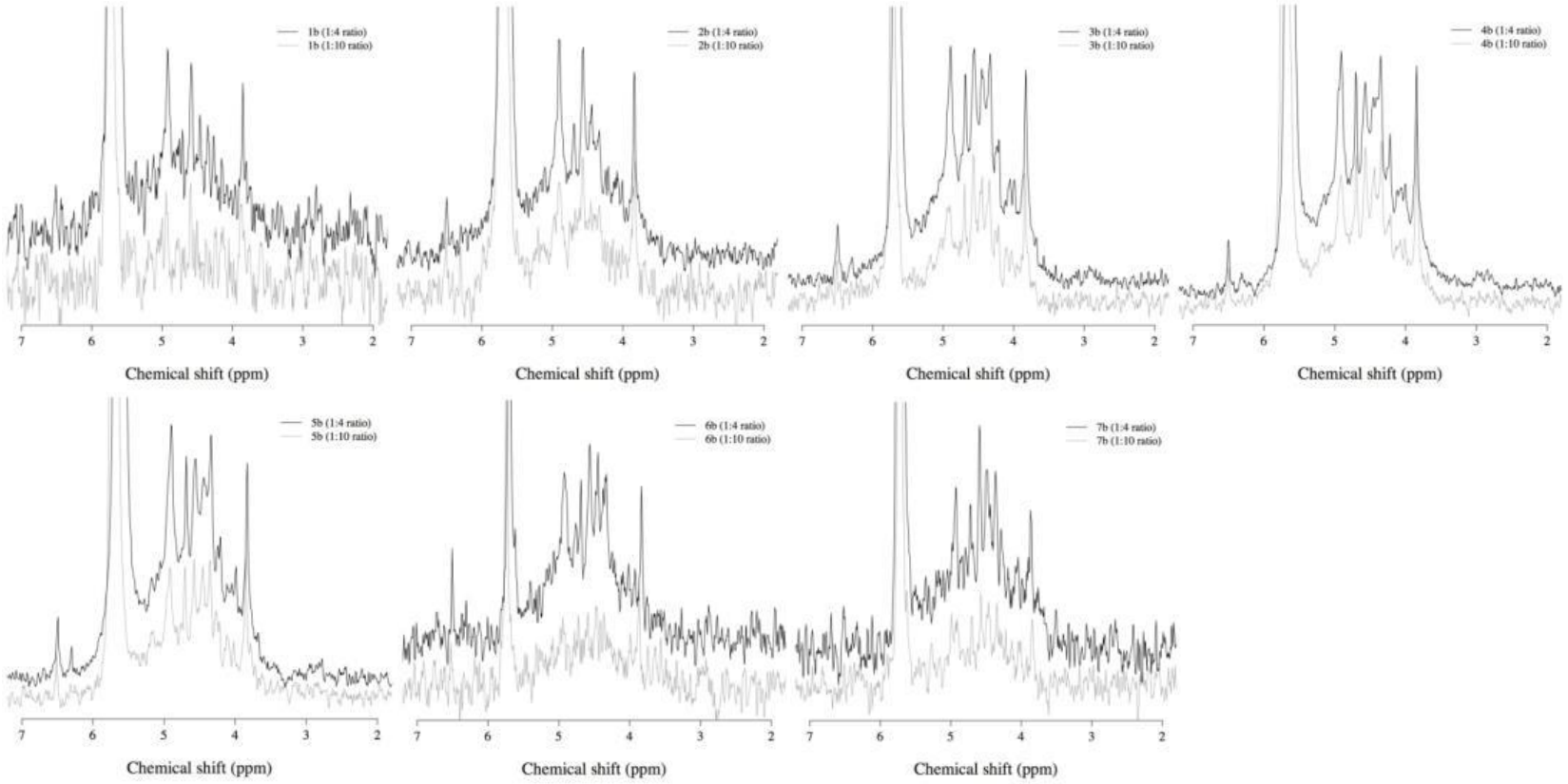

Fig. 18 Orthophosphate and monoester region of solution ^{31}P NMR spectra of the subsurface layer on NaOH-EDTA extracts at a 1:10 and 1:4 soil to solution ratios. The vertical scale of each spectrum has been normalised to the methylenediphosphonic acid peak at δ 17.01 ppm.

There was a strong correlation between all broad P types determined by integration of the NMR spectra of the 1:4 and 1:10 extracts, and these relationships were generally close to 1:1 in all cases (Fig. 19). Similarly, strong correlations were found between all specific P types determined using deconvolution of the orthophosphate and monoester region of the NMR spectrum at the 1:4 and 1:10 soil to solution ratios (data not shown). The quantity of NaOH-EDTA extractable diesters (δ 0.5 to -1.0 ppm) and pyrophosphate determined for the 1:4 extracts was slightly higher than that determined for the 1:10 extracts (Fig. 19). This likely reflects difficulties in detecting these P types at the wider soil to solution ratio. The significance of this is that narrowing the soil to solution ratio may improve detection of some organic P species that are typically found at low concentrations.

We recommend that the narrower 1:4 soil to solution ratio be used to characterise organic P forms after initially extracting with NaOH-EDTA(1:10) and ascertaining if the signal to noise ratio of the subsequent NMR spectrum would hinder spectral deconvolution (Doolette *et al.* 2010).

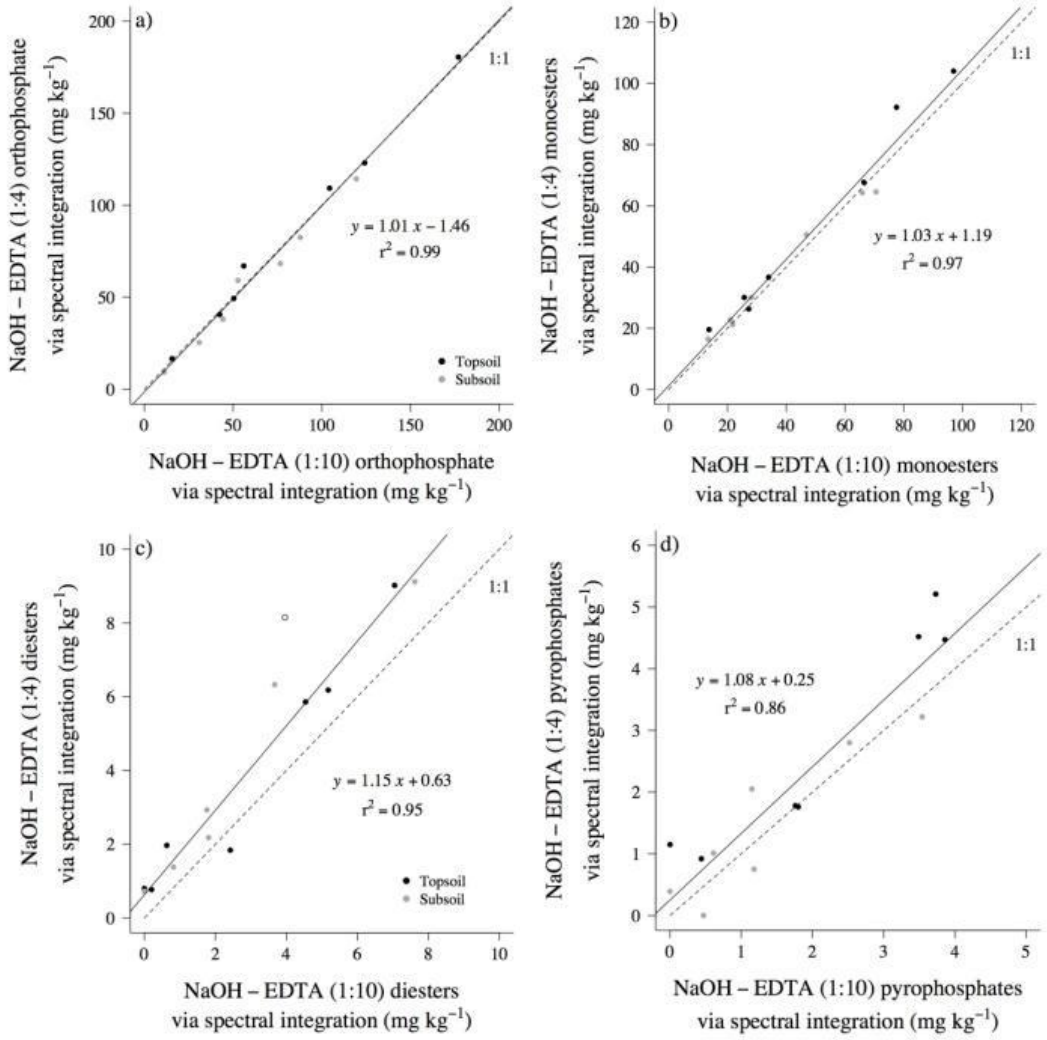

Fig. 19 Relationship between orthophosphate (a), monoesters (b), diesters (c) and pyrophosphates (d) determined by solution ^{31}P NMR spectral integration of NaOH-EDTA extracts using a 1:10 and a 1:4 soil to solution ratio.

4.3.2 Distribution of soil P forms in Gininderra soils as determined by NMR

The results described in this section are being prepared for publication in Journal of Plant Nutrition and Soil Science.

Since NMR analysis of P is carried out on NaOH-EDTA extracts, an important consideration is the extraction efficiency of soil P in NaOH-EDTA. NaOH-EDTA extracted between 21 and 56% (average 38%) of total soil P as determined by LXRF, the concentration of which ranged from 236 to 550 mg P kg^{-1} to a depth of 20 cm from the soil surface across all treatments. The extraction efficiency of NaOH-EDTA was greater for the surface layers than in subsurface layers; the former ranged from 36 to 56% (average 49%) and the latter ranged from 21 to 33% (average 26%). Concentrations of inorganic and organic P in NaOH-EDTA extracts to a depth of 20

cm from the soil surface across all treatments was on average 40 and 60% of total NaOH-EDTA extractable P, respectively. Concentrations of inorganic P in NaOH-EDTA extracts was strongly correlated with that determined by ignition-H_2SO_4 extraction. Similarly, concentrations of organic P in NaOH-EDTA extracts were strongly correlated with those determined by ignition-H_2SO_4 extraction. There were also strong correlations between concentrations of inorganic and organic P in NaOH-EDTA extracts and the summed concentrations of inorganic and organic P as determined by sequential chemical fractionation.

Solution ^{31}P NMR analysis of the NaOH-EDTA extracts confirmed the presence of a range of inorganic and organic P forms in all soils. Integration of the NMR spectra revealed that concentrations of orthophosphate, organic P forms (i.e. monoesters and diesters), and pyrophosphate comprised on average 51, 46 and 3%, respectively, of the total concentrations of NaOH-EDTA extracts to a depth of 20 cm from the soil surface across all treatments. Monoesters were the most abundant class of organic P detected in NaOH-EDTA extracts, comprising between 87 and 100% (average 93%) of NaOH-EDTA extractable organic P.

The orthophosphate and monoester region (δ 6.0 to 3.0 ppm) of one replicate for each soil P fertility level at stocking rate SR09 is shown in Fig. 20. Spectra for both surface and subsurface layers are shown and the vertical scale has been set to allow direct comparison of the intensity of features between spectra (i.e. normalised to the MDP peak at δ 16.9 ppm). In general, the monoester signal increased with fertiliser level (P0 < P1 < P2), which was more evident for the surface layers than the subsurface layers (Fig. 20). Spectral sensitivity (the ability to detect signal against background noise) is good for all spectra of the surface layers (Fig. 20), which facilitates identification of individual peaks that correspond to specific organic P compounds. In contrast, spectral sensitivity was relatively poor for all spectra in the subsurface layers (Fig. 20).

Quantitative analysis of the NMR spectra via integration was carried out to quantify the differences in the chemical nature of the extracted organic P. The largest increase in concentrations of NaOH-EDTA extractable organic P between the unfertilised and fertilised treatments was in the monoester fraction, which increased on average from approximately 60 mg P kg^{-1} in the unfertilised treatment to 109 mg P kg^{-1} in the fertilised treatments (when averaged across soil P fertility (P1 and P2) and stocking rates) of the surface layer following 13 years of P fertilisation (Table 20). In contrast, concentrations of diester only increased on average from approximately 4 mg P kg^{-1} in the unfertilised treatment to 7 mg P kg^{-1} in the fertilised treatments (Table 20).

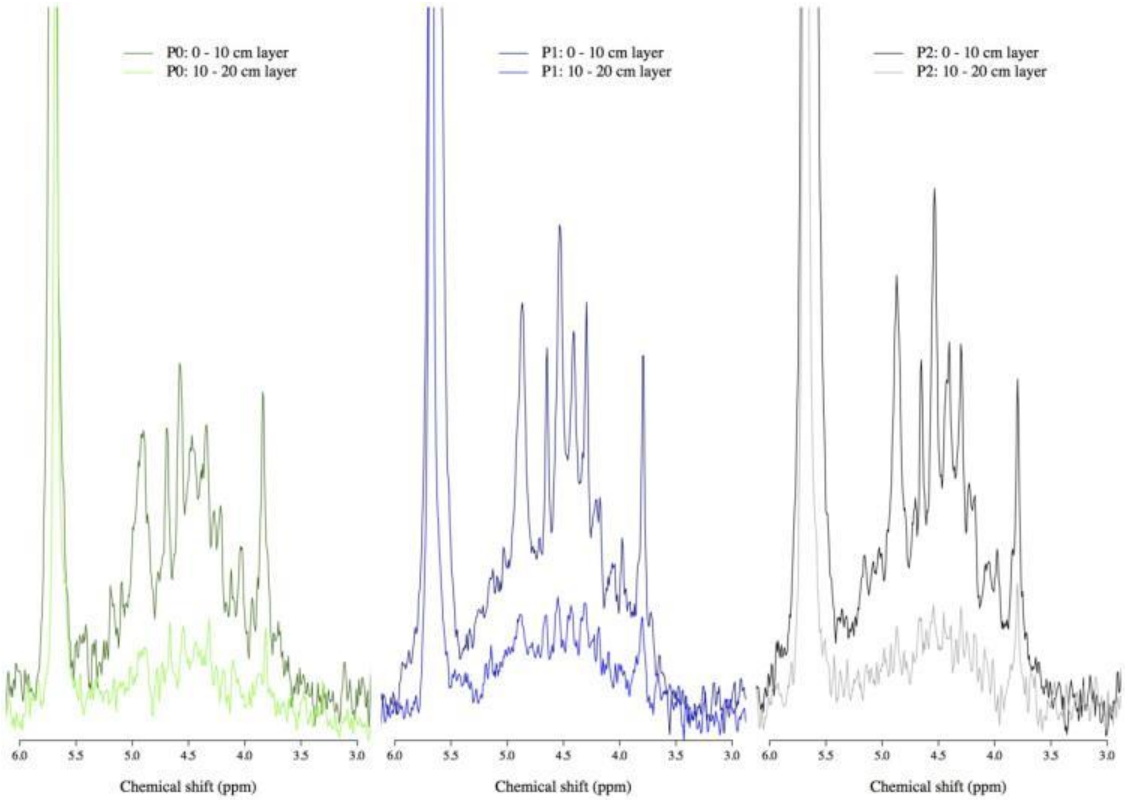

Fig. 20 Orthophosphate and monoester region of solution ^{31}P NMR spectra of the surface (0 – 10 cm) and subsurface (10 – 20 cm) layers at the SR09 stocking rate for the three soil P fertility levels (P0, P1 and P2). One representative spectrum (of the three replicates) is shown from the 0 – 10 cm and 10 – 20 cm layers. The vertical scale of each spectrum has been normalised to the MDP peak at δ 16.93 ppm (beyond the limits of the spectrum shown).

Figure 21 shows an overlay trace of the orthophosphate and monoester region of one representative replicate from each level of soil P fertility at stocking rate SR09. All three spectra exhibit the same two main features: 1) a broad feature centred around δ 4.6 ppm and spanning from δ 3.5 to 6.0 ppm; and 2) a series of prominent sharp peaks. The broad feature has previously been attributed to high molecular weight compounds containing P in monoester linkages and has been termed humic P (Bünemann *et al.* 2008c; Doolette *et al.* 2011). The series of prominent peaks at diagnostic chemical shifts (ppm) can be assigned to the following specific organic P species as discussed in Section 4.3.1.3.: α- and β-glycerophosphate, three peaks due to *myo*-inositol hexakisphosphate, a peak due to *scyllo*-inositol hexakisphosphate, and up to three peaks of RNA mononucleotides. Most notably, Fig. 21 suggests that the relative composition of organic P forms do not differ with the addition of fertiliser P, rather all forms increase to much the same extent (Fig. 21). Therefore, based on the visual appearance of the NMR spectra, it appears that no specific form of soil organic P is preferentially accumulating in these soils under pasture in response to P fertiliser addition.

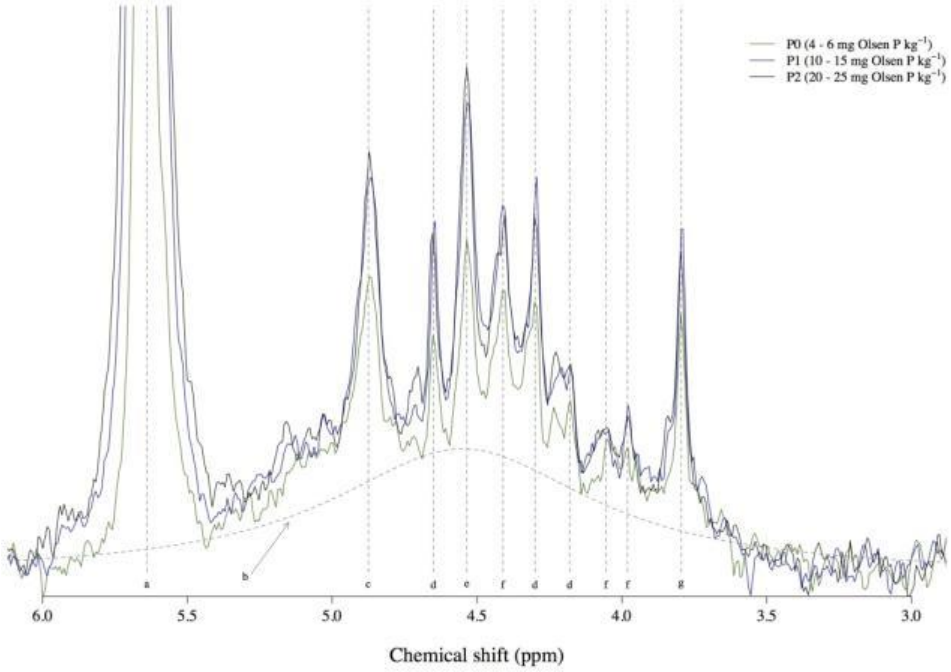

Fig. 21 An overlay of the orthophosphate and monoester region of solution ^{31}P NMR spectra of the surface (0 – 10 cm) layers at the SR09 stocking rate for the three soil P fertility levels (P0, P1 and P2). One representative spectrum for each fertility level is shown. The vertical scale of each spectrum has been normalised to the MDP peak at δ 16.93 ppm (beyond the limits of the spectrum shown). Phosphate species were assigned as follows: 'a' – orthophosphate; 'b' – humic P; 'c' – α-glycerophosphate; 'd' – *myo*-inositol hexakisphosphate; 'e' – β-glycerophosphate; 'f' – RNA mononucleotides, and; 'g' – *scyllo*-inositol hexakisphosphate.

The ^{31}P NMR spectra on NaOH-EDTA extracts of the surface layers were of sufficient quality to enable further quantification of spectral features in the monoester region through deconvolution. On the other hand, since the ^{31}P NMR spectra for the subsurface layers were relatively noisy, quantification of the spectral features in the subsurface layer through deconvolution was not attempted. A large proportion of monoester P (71 – 82%, average 76%) was identified as humic P across all surface soils (Table 20). Humic P also significantly increased with the addition of fertiliser P, increasing from approximately 46 mg P kg^{-1} in the unfertilised treatment to 61 mg P kg^{-1} in the fertilised treatments (when averaged across soil P fertility (P1 and P2) and stocking rates) of the surface layer following 13 years of P fertilisation (Table 20).

Table 20. Concentrations of P in soil fractions (mg P kg^{-1}) determined from solution ^{31}P nuclear magnetic resonance (NMR) spectra on the NaOH-EDTA extracts using spectral integration of the whole spectrum, and deconvolution within the orthophosphate and monoester region (δ 7.0 to 3.5 ppm). The cumulative P input that was applied to the soil surface over 13 years was on average 4, 200, and 242 kg P ha^{-1} for the P0, P1 and P2 soil P fertility levels at stocking rate SR09, respectively, and at stocking rate SR18 the cumulative P input was on average 221 and 320 kg P ha^{-1} for the P1 and P2 soil P fertility levels, respectively. Ortho-P, orthophosphate; Lipid-P, α- and β-glycerophosphate; *myo*-IHP, *myo*-inositol hexakisphosphate, *scyllo*-IHP, *scyllo*-inositol hexakisphosphate; RNA, RNA mononucleotides.

Soil depth (cm)	Stocking rate	P fertility level	Ortho-P*	Monoesters* †						Diesters*	Pyrophosphate*
				Total	Humic P	Lipid-P	*myo*-IHP	*scyllo*-IHP	RNA		
0 – 10	SR9	P0	36.0 a	66.0 a	46.1 a	7.2 a	5.1 a	3.9 a	3.7 a	3.6 a	3.2 a
		P1	84.1 b	90.1 b	63.6 b	10.7 ab	6.5 a	4.9 b	4.5 a	7.5 b	6.0 b
		P2	113.3 c	83.4 b	59.6 b	10.1 ab	6.1 a	4.2 ab	3.2 a	6.4 b	4.2 ab
	SR18	P1	89.7 b	84.6 b	57.2 ab	11.4 b	6.8 a	4.3 ab	4.9 a	6.8 b	5.0 ab
		P2	122.0 c	88.5 b	62.5 b	10.4 ab	6.9 a	5.1 b	3.7 a	6.4 b	3.9 ab
10 – 20	SR9	P0	22.0 a	28.9 a						1.1 a	2.1 a
		P1	34.2 ab	38.7 a						2.9 a	2.1 a
		P2	41.6 b	34.5 a						1.7 a	2.0 a
	SR18	P1	32.4 ab	26.8 a						2.6 a	1.5 a
		P2	49.2 b	38.1 a						1.7 a	1.8 a

* Means of soil P fractions within each column and depth followed by the same letter are not significantly different according to a one-way ANOVA ($P > 0.05$) where $n=3$.

† Deconvolution is not reported on the ^{31}P NMR spectra for the subsurface layers (10 – 20 cm) due to poor spectral quality that would make quantification of specific forms of organic P unreliable.

Whilst humic P was the main form of organic P detected in all surface soils, there were also important differences in the concentrations of other forms of organic P. Lipid P (detected as α- and β-glycerophosphate) and inositol phosphates (*myo*- and *scyllo*-inositol hexakisphosphate) contributed a small fraction of total NaOH-EDTA extractable organic P, and hence increases in the concentration of these forms on the addition of fertiliser P were relatively small and not always significantly different relative to those of the unfertilised treatment (Table 20 and Fig. 21).

The main aim of this study was to determine the composition of organic P that accumulates when fertiliser P is added to soils under pasture. Table 21 summarises the accumulation of P in various organic P fractions in the surface layer after 13 years of P fertilisation at a moderate stocking rate of SR09, which are expressed as percentages of added P for each treatment. Since concentrations of soil P varied little between the two stocking rates (SR09 and SR18) for the P1 and P2 treatments, it seems reasonable to assume that similar accumulations would have occurred for SR18 treatments.

Approximately 53 and 55% of the cumulative P input could be accounted for in the increase of total NaOH-EDTA extractable P in the P1 and P2 levels of soil P fertility in surface soils, respectively (Table 2). In the surface layers, orthophosphate was the main form of NaOH-EDTA extractable soil P that accumulated with the addition of fertiliser P, comprising on average 32 and 43% of the cumulative P input for the P1 and P2 treatments, respectively (Table 21).

Table 21. Distribution across fractions of accumulated fertiliser P as determined by solution ^{31}P NMR spectroscopy on NaOH-EDTA extracts for a pasture trial following 13 years of continuous P fertilisation to maintain a target soil P fertility of 10 – 15 mg Olsen P kg^{-1} (P1) and 20 – 25 mg Olsen P kg^{-1} (P2) in the surface layer (0 – 10 cm) at stocking rate SR09.

Soil P form	P1; Recovery of cumulative P input (%)	P2; Recovery of cumulative P input (%)
Orthophosphate	32	43
Humic P	12	8
Lipid P	2	2
myo-inositol hexakisphosphate	1	1
scyllo-inositol hexakisphosphate	1	<1
RNA mononucleotides	1	<1
Diesters	3	2
Pyrophosphate	2	1
Total P	53	55

The main form of organic P that accumulated with the addition of fertiliser P was the humic P fraction, which represented approximately 12 and 8% of cumulative P input for the P1 and P2 treatments, respectively (Table 21). The lower net accumulation of humic P as a proportion of the cumulative P input for the P2 treatment than the P1 treatment suggests that the rate of organic P accumulation that occurred was independent of the level of fertiliser P applied in these soils. Hence a greater proportion of the cumulative P input had accumulated as NaOH-EDTA extractable inorganic P at the supra-optimum (P2) level of soil P fertility.

4.3.3 Solution ^{31}P NMR analysis of extracts following size separation

The results described in this section have been submitted to Environmental Science and Technology.

Solution ^{31}P NMR spectra were obtained for five diverse soils sourced from Australia, France, Germany, Sweden and the United States of America (USA). An example spectrum including assignment of sharp peaks is shown in Fig. 22. As expected, the spectra showed large variation within the orthophosphate and monoester region (δ 7 to 3 ppm) (Fig. 23a) in which the majority of the NMR signal is found. The spectral variability among unfractionated extracts from all five soils tested in this study (Fig. 23a) mainly involves differences in the relative amounts of i) a series of prominent sharp peaks and ii) a broad resonance spanning from δ 3.5 to 6.0 ppm (Doolette *et al.* 2009; Doolette *et al.* 2010; Doolette *et al.* 2011). All of the sharp peaks have previously been identified as low molecular weight organic P compounds that originate from living organisms (Figs 22 and 23a).(Bünemann *et al.* 2008b; Bünemann *et al.* 2008c; Noack *et al.* 2012) The sharp peaks identified in the unfractionated extracts include: α- and β-glycerophosphate (δ 4.9 and 4.6 ppm, respectively), three peaks due to *myo*-inositol hexakisphosphate (δ 4.7, 4.4 and 4.2 ppm), a peak due to *scyllo*-inositol hexakisphosphate (δ 3.9 ppm), and up to four peaks that are attributed to mononucleotides (RNA) (δ 5.4, 4.5, 4.1 and 4.0 ppm) (Figs 22 and 23a). The broad feature is unique to the NMR spectra of soil extracts and is not found in extracts of plants, bacteria or fungi (Bünemann *et al.* 2008b; Bünemann *et al.* 2008c; Doolette *et al.* 2011; Noack *et al.* 2012).

Soil extracts were separated into high and low molecular weight fractions by passage through ultrafiltration devices with a nominal cut-off of 10 kDa. Solution ^{31}P NMR spectra of the 10 kDa retentates (Fig. 23b) were markedly different to those of the corresponding 10 kDa filtrates (Fig. 23c). For all soils, the NMR spectra of the high molecular weight fraction (10 kDa retentates) were dominated by a broad resonance that was devoid of an orthophosphate peak, which indicates the absence of inorganic P (Fig. 23b). In addition, these NMR spectra did not contain any of the sharp peaks attributable to recognizable forms of organic P commonly found in living organisms, although some distinct peaks were present (see below) (Fig. 23b). These results confirm the existence of high molecular weight organic P species with single phosphoester linkages, and demonstrate that this material predominantly gives rise to a broad NMR signal (Fig. 23b).

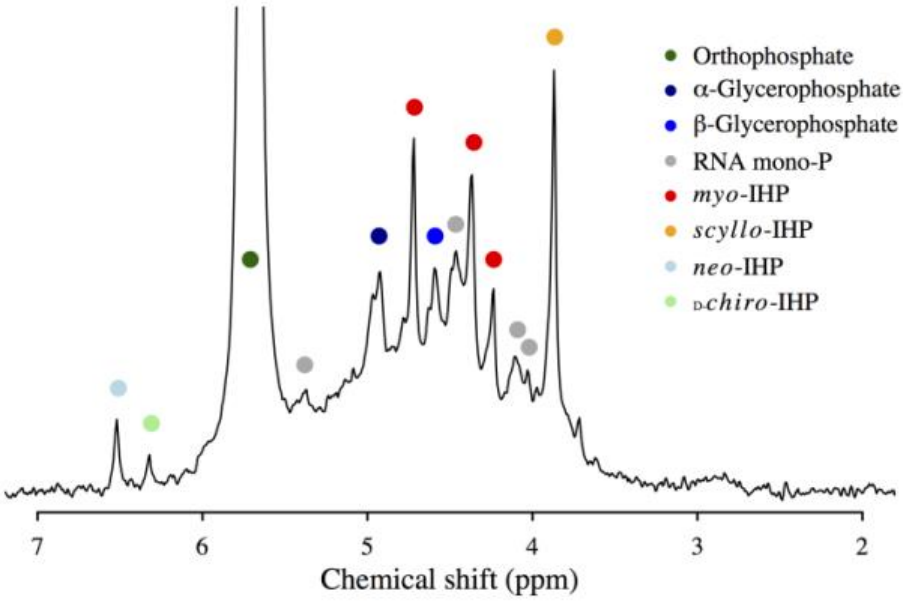

Fig. 22 A representation of the assignments for sharp peaks typically present in the orthophosphate and monoester region (δ 7 to 2 ppm) of solution phosphorus-31 nuclear magnetic resonance spectra on a soil extract (Soil from Sweden) based on current methods of analysis. These include: orthophosphate (δ 5.7 ppm), α-glycerophosphate (δ 4.9 ppm), β-glycerophosphate (δ 4.6 ppm), *myo*-inositol hexakisphosphate (*myo*-IHP – δ 4.7, 4.4 and 4.2 ppm), *scyllo*-inositol hexakisphosphate (*scyllo*-IHP – δ 3.9 ppm), RNA mononucleotides (RNA mono-P – δ 5.4, 4.5, 4.1 and 4.0 ppm), *neo*-inositol hexakisphosphate (*neo*-IHP – δ 6.5 ppm) and D-*chiro*-inositol hexakisphosphate (D-*chiro*-IHP – δ 6.3 ppm).

The broadness of the NMR signal in the 10 kDa retentates is entirely consistent with P being a component of polymeric (humic) organic material containing single phosphoester linkages (Doolette *et al.* 2011; Simpson *et al.* 2011) In such heterogeneous polymeric materials, P nuclei would occupy a range of unique, but broadly similar chemical environments. The overall effect is a broad envelope of signal that differs from the much sharper signals of specific organic P containing molecules, for which the local chemical environment is identical. A similar effect has been noted for the deoxyribonucleic acid resonance that appears in the diester region (δ 0.5 to -1.0 ppm) of ^{31}P NMR spectra.

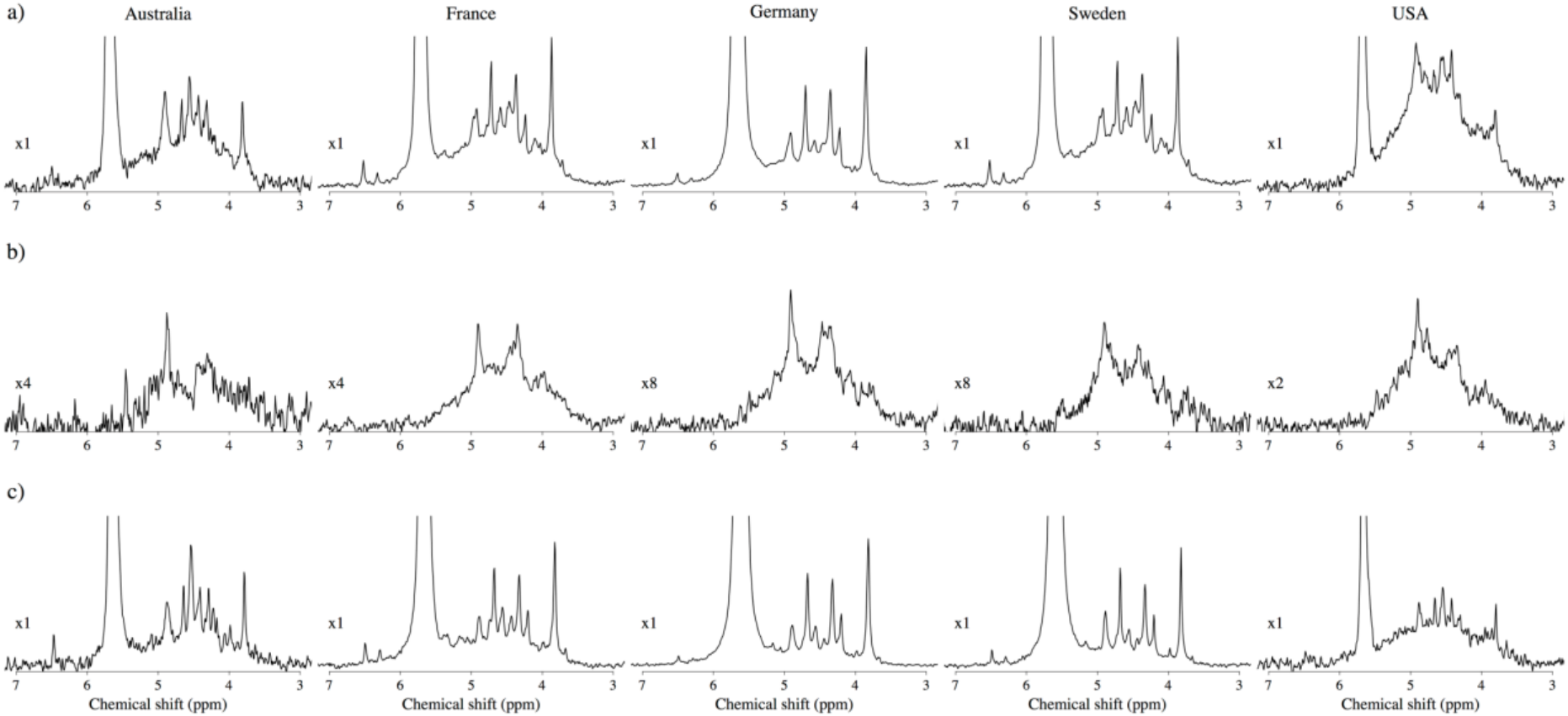

Fig. 23 Solution phosphorus-31 NMR spectra of the orthophosphate and monoester region (δ 7 – 3 ppm) on the (a) original soil extracts, (b) 10 kDa retentates, and (c) 10 kDa filtrates for each soil. The vertical scale of each spectrum and within each soil has been normalized to the methylenediphosphonic acid peak at δ 16.9 ppm (beyond the limits of the spectrum shown), although the spectrum for the 10 kDa retentates within each soil has been magnified.

It is noteworthy that the solution ^{31}P NMR spectra of the 10 kDa retentates are similar across all soils (Fig. 23b), both in their general broadness and by the presence of some distinct peaks. In particular, a sharper peak is evident at δ 4.91 ppm and other peaks are distinguishable around δ 4.47 to 4.34 ppm (Fig. 23b). The chemical shift of the former is close (but not identical) to that of the α-glycerophosphate peak, which could indicate that it is due to phosphate esters of primary alcohols. The presence of this peak in soil extracts may result in an overestimation of α-glycerophosphate due to peak overlap using current methods of analysis.

All of the sharp peaks detected in the NMR spectra of the unfractionated soil extracts were present in the spectra of the low molecular weight fraction (10 kDa filtrates), including those due to α- and β-glycerophosphate, *myo*- and *scyllo*-inositol hexakisphosphate, and most of the ribonucleic acid mononucleotides (Figs 23a and 23c). The sharp peaks arising from *myo*- and *scyllo*-inositol hexakisphosphate were particularly prominent in the 10 kDa filtrates of the soils from France, Germany and Sweden (Fig. 23c), reflecting their higher abundance in the corresponding unfractionated soil extracts (Fig. 23a). However, it is clear that for all five soils, the sharp peaks were more prominent in the 10 kDa filtrates than in the whole soil extracts. This is especially evident for the soil from the USA (Figs 23a and 23c) for which the sharp peaks are difficult to distinguish in the unfractionated soil extracts.

Importantly, a broad resonance is still present in the ^{31}P NMR spectra of the 10 kDa filtrates for all soils (Fig. 23c), although the broad resonance in the NMR spectra of the 10 kDa filtrates contributed less to total signal than in the unfractionated soil extracts (Figs 23a and 23c). The presence of a broad underlying signal in low molecular weight fractions of soil extracts suggests this fraction also includes a complex mixture of organic molecules containing P. It should be noted that the 10 kDa cut-off implies that “low molecular weight” includes organic molecules containing up to 300 carbon atoms, which is still considerably higher than those of the sharp peaks arising from known forms of organic P (e.g. *myo*-inositol hexakisphosphate has a molecular weight of < 1 kDa).

Concentrations of inorganic, organic and total P in soil extracts, as determined by NMR spectroscopy, varied widely among the soils (Table 22). The proportion of organic P in whole soil extracts ranged from 25% to 71% (Table 22). Concentrations of P were also determined on the 10 kDa retentates (> 10 kDa) and 10 kDa filtrates (< 10 kDa) of the unfractionated soil extracts. For all soils, 94% to 98% of the total P in unfractionated extracts was recovered in the combined 10 kDa retentates and filtrates, except for the soil from Sweden, where 77% was recovered in the combined retentate and filtrate (Table 22). For all soils, inorganic P (orthophosphate and pyrophosphate) was only detected in the 10 kDa filtrates, whereas organic P was detected in both the 10 kDa retentates and 10 kDa filtrates (Table 22 and Fig. 23). Concentrations of organic P ranged from 26 to 103 mg kg^{-1} in the 10 kDa retentates and from 55 to 232 mg kg^{-1} in the 10 kDa filtrates (Table 22).

Table 22. Concentrations (mg kg^{-1}) of phosphorus species as determined from solution phosphorus-31 nuclear magnetic resonance spectra on the original NaOH-EDTA extracts (unfractionated), and following size separation with an ultrafiltration device containing a 10 kDa filtration membrane in the 10 kDa retentates (> 10 kDa) and 10 kDa filtrates (< 10 kDa). Quantification of phosphorus species involved spectral integration of the whole spectrum, and spectral deconvolution within the orthophosphate and monoester region (δ 7.0 to 3.5 ppm) as described by Doolette *et al.* (2011). Ortho-P, orthophosphate; Lipid-P, α- and β-glycerophosphate; *myo*-IHP, *myo*-inositol hexakisphosphate, *scyllo*-IHP, *scyllo*-inositol hexakisphosphate; RNA, RNA mononucleotides, Pyro-P, pyrophosphate.

Soil	Treatment	Ortho-P*	Monoesters†						Diesters	Pyro-P	Total P*
			Total P	Humic P‡	Lipid-P	*myo*-IHP	*scyllo*-IHP	RNA			
Australia	Unfractionated	73	84	57	9	8	4	5	8	6	171
	> 10 kDa	0	22	22	0	0	0	0	4	0	26
	< 10 kDa	77	55	31	8	8	4	4	0	5	137
	Recovery (%)	106									95
France	Unfractionated	487	378	275	17	43	27	16	14	12	890
	> 10 kDa	0	93	93	0	0	0	0	10	0	103
	< 10 kDa	527	191	115	10	37	22	6	0	12	729
	Recovery (%)	108									94
Germany	Unfractionated	1082	351	215	15	72	43	6	19	17	1470
	> 10 kDa	0	64	64	0	0	0	0	10	0	73
	< 10 kDa	1115	232	118	12	63	38	1	0	16	1363
	Recovery (%)	103									98
Sweden	Unfractionated	574	447	324	21	52	32	19	18	15	1055
	> 10 kDa	0	40	40	0	0	0	0	10	0	50
	< 10 kDa	608	150	80	11	39	20	0	0	8	766
	Recovery (%)	106									77
USA	Unfractionated	68	162	-	-	-	-	-	13	3	246
	> 10 kDa	0	66	66	0	0	0	0	9	0	166
	< 10 kDa	58	101	80	7	5	4	5	0	6	76
	Recovery (%)	85									98

* Recovery values were calculated for orthophosphate and total phosphorus based on the summation of these concentrations of phosphorus in the 10 kDa filtrate and 10 kDa retentate, to that of the unfractionated soil extracts and converted to a percentage.

† Deconvolution was not possible on the USA soil due to a lack of prominent sharp peaks within the monoester region.

‡ Humic phosphorus refers to the broad peak that underlies the sharp peaks within the orthophosphate and monoester region. Concentrations of humic phosphorus in the 10 kDa retentate was taken as that of the total monoester phosphorus for this fraction.

Concentrations of P species giving rise to both broad and sharp resonances were determined within the monoester region using deconvolution (Table 19). The broad feature (termed here humic P; Table 19) in the unfractionated soil extracts ranged in concentration from 57 to 324 mg kg^{-1} across all soils, and accounted for 61% to 73% of the total monoester signal in the unfractionated soil extracts (Table 19). Deconvolution was not possible for the unfractionated soil extract of the USA soil due to a lack of prominent sharp peaks within the monoester region.

5 Discussion

This section is structured to address each of the three original project objectives.

5.1 Objective 1: Fate of fertiliser P

Original objective:

"Understanding of the rates of transformation of added fertiliser P in pasture production systems. Knowledge of the rates of transfer of P fertiliser on an annual basis to plant shoots, roots and inorganic and organic P pools in the soil. This will be communicated in scientific papers (for scientists) and also in extension material relating to changed fertiliser management (for livestock producers)."

This objective was successfully met via field trials carried out in 2013 and 2014 at three sites: Ginninderra, ACT (2013, 2014a and 2014b), Naracoorte, SA (2013) and Inman Valley, SA (2014) in which radio-labelled P fertiliser was used to follow the fate P fertiliser into plant biomass and soil pools. The relatively short half-life of the radioisotope dictates that this approach can only be used to infer the fate of fertiliser P over one growing season. The sections below provide detailed discussion of how these field trials contributed to meeting this objective.

5.1.1 2013 field trial

5.1.1.1 Validation of isotopically labelled SSP granules

The labelling approach and subsequent granulation using the press and cut technique proved to be an accurate method for radiolabelling SSP granules. Close agreement between the P release behaviour of the ^{33}P-labelled SSP and commercial SSP granules is likely due to the matching chemical composition of the fertiliser material (Hedley *et al.* 1988; Braithwaite *et al.* 1992), and because the majority of P within SSP is water soluble (Williams 1971b; Degryse and McLaughlin 2014). The P diffusion pattern of both granule types is consistent with previous studies that showed the majority of P movement from SSP granules occurs within the first week of application to the soil, and is generally restricted to a few centimetres from the granule (Lawton and Vomocil 1954; Williams 1971b). Indeed, the press and cut technique has successfully been used before in non-isotopic studies for investigating the agronomic effectiveness of various mixtures of fertiliser material and added impurities (Mullins *et al.* 1995; Prochnow *et al.* 2004).

An important consideration of the direct labelling approach is to ensure that the radiotracer is uniformly distributed within the fertiliser source (Frossard *et al.* 2011). A low coefficient of variation for the specific activity (MBq mg WSP^{-1}) of ^{33}P-labelled SSP granules indicates that the ^{33}P radiotracer was homogenously labelled within the SSP. The homogeneous labelling can be attributed to the incubation step where a fraction of the monocalcium phosphate is dissolved, mixed with ^{33}P, and then re-formed on drying, thus incorporating the ^{33}P into the monocalcium phosphate matrix. Consequently, the press and cut technique used to make ^{33}P-labelled SSP granules was successful in producing granules that have equal P release properties to commercial SSP granules.

5.1.1.2 The fate of fertiliser P in pasture systems

A lack of clover response to the application of ~12 kg P ha^{-1} in 2013 was unsurprising at the Naracoorte site given that soil P fertility was well in excess of that required for optimum pasture growth (Reuter *et al.* 1995). The application of ~ 12 kg P ha^{-1} in 2013 at the Ginninderra site was likely too small to result in a significant increase in cumulative dry matter. Simpson *et al.* (2015) have previously shown that the application of ~ 35 kg P ha^{-1} is required to achieve maximum clover growth with a single fertiliser application to low soil P at this site. However, there was a significant increase in the cumulative P uptake of the fertilised treatment relative to that of the non-fertilised treatment at the Ginninderra site. Concentrations of P in plant tissue also appeared higher in fertilised treatments than in non-fertilised treatments at both field sites, which is consistent with an increased supply of P to clover plants in fertilised treatments.

A lower initial soil P status at the Ginninderra site compared to that of the Naracoorte site is likely the main reason for a higher proportion of clover P uptake that is derived from fertiliser sources at the Ginninderra site (Morel and Fardeau 1990; McBeath *et al.* 2012). It has been shown that the relative importance of fertiliser P to plant P uptake is influenced by native soil P fertility (Dean *et al.* 1948; Morel and Fardeau 1990). Morel and Fardeau (1990) reported a decrease in the proportion of plant P derived from fertiliser P (^{32}P-labelled diammonium phosphate) when the quantity of soil P that is predicted to be plant-available increased for ryegrass pastures grown under glasshouse conditions.

The recovery of applied fertiliser P in clover shoots was 30% and 35% at the Ginninderra and Naracoorte field sites, respectively. No published studies have reported the direct recovery of fertiliser P by clover pastures under field conditions, but several have done so for arable crops and medic pasture. Fertiliser recoveries for P by arable crops under field conditions generally range from 5% to 35% (Spinks and Barber 1947; Nelson *et al.* 1948; Dion *et al.* 1949; Mitchell *et al.* 1952; Mattingly and Widdowson 1958a, 1958b; Sharpley 1986; McLaughlin *et al.* 1988b; McBeath *et al.* 2012). Relative to the recovery of fertiliser P reported for arable crops, recoveries of 30% and 35% suggest that clover plants at the Ginninderra and Naracoorte field sites in 2013 were at the upper end of fertiliser P recovery that is typically found for arable crops. Some of the reasons for a relatively high recovery of fertiliser P in clover pastures compared with other systems may include: 1) the root systems of clover pastures were established and concentrated in the surface layer of the soil profile,

which is near to where the fertiliser P is applied (Ozanne *et al.* 1961); 2) a lack of contact between the surface applied fertiliser and the minerals and ions in soil likely to fix P (McLaughlin *et al.* 1990); 3) fertiliser P is unlikely to undergo rapid transformation to less soluble forms of soil P during a single growing season in soils of low to moderate sorption capacity (He *et al.* 2004; Dorahy *et al.* 2007); and 4) moisture conditions were favourable for fertiliser recovery at the Ginninderra and Naracoorte field sites.

At both field sites, most of the fertiliser P in soil fractions was found in the surface layer (0 – 4 cm) of the soil profile in the year of application. Many studies have shown that concentrations of soil P in surface layers of fertilised soils under pasture are enriched relative to those in subsurface layers (Watson 1969; McLaughlin *et al.* 1990; Haynes and Williams 1992; McLaren *et al.* 2015). However, the recovery of fertiliser P in soil fractions reported here over a single growing season is considerably lower than those reported in long-term studies using indirect techniques (soil P audits) (Watson 1969; McCaskill and Cayley 2000). The P balance efficiency of pasture systems is generally considered low due to the accumulation of P in fertilised soil as sparingly-soluble forms of inorganic and organic P (McLaughlin *et al.* 2011). Since a considerable proportion of the fertiliser P was found to be accessible to pasture plants in the year of application, the low P balance efficiency of pastures is only partly due to the transformation of fertiliser P to less soluble forms of soil P in the year of application. Transformations that occur after plant uptake must also therefore contribute substantially to the low P balance efficiency, involving fertiliser P taken up by the plant that is subsequently returned to the soil surface via pasture decay, pasture trampling and/or deposition of faeces and urine from grazing animals (Bromfield 1961; Bircham and Hodgson 1983).

The high recovery of fertiliser P in soil fractions associated with pools of inorganic P is consistent with previous studies for arable crops, which show that there is little conversion of fertiliser P to organic forms in the year of application (Sharpley 1986; Friesen and Blair 1988; McLaughlin *et al.* 1988a). In contrast, long-term studies have reported that concentrations of organic P in fertilised soils under pasture are a major sink of fertiliser P (Oniani *et al.* 1973; Simpson *et al.* 1974; Condron and Goh 1989; McLaren *et al.* 2015).

A small proportion of the fertiliser P was recovered in the granule residues collected from the soil surface at the last harvest, which is likely to consist of water insoluble phosphates (Gilkes and Lim-Nunez 1980; Prochnow *et al.* 2001). The approximately 6% of ^{33}P recovered in the granule residues relative to that originally labelled with the ^{33}P radiotracer is likely to be from subsequently formed precipitates of dicalcium phosphate or dicalcium phosphate dihydrate within the granule on the un-acidulated fraction of the SSP (Lehr *et al.* 1959). Clearly, the majority of fertiliser P is released from the granule during a single growing season upon application to pastures and is potentially available for plant uptake (Williams 1971a).

The unrecovered fertiliser P is likely to be associated with three main components of the pasture system. Possible fates of the unrecovered fertiliser P include: 1) incorporation into clover roots; 2) transport to below 8 cm from the soil surface; and 3) incorporation into pools not extracted by the ignition-H_2SO_4 or NaOH-EDTA

extraction techniques. We consider it unlikely that much fertiliser P was transported below 8 cm from the soil surface because the recovery of fertiliser P in the subsurface layer (4 – 8 cm) was low, and in many cases too low for detection, and the movement of P from SSP granules is generally restricted to a few centimetres from the granule (Lawton and Vomocil 1954; Williams 1971b), except in very sandy soils. We also consider it unlikely that much fertiliser P has been incorporated into pools of soil P not extracted by the ignition-H_2SO_4 extraction and NaOH-EDTA extraction techniques because: 1), the ignition-H_2SO_4 extraction technique provides a close approximation of total soil P as measured using *aqua regia* digestion and; 2) the pools of 'residual P' as determined using sequential chemical fractionation did not significantly accumulate with longer-term addition of fertiliser P under pasture. Consequently, we believe that much of the fertiliser P that was unaccounted for was likely to be in clover roots. This is discussed further in Section 5.1.2 below.

Clover roots contain P and can be a quantitatively important fraction of the total P in clover plants (Biddiscombe *et al.* 1969). In addition, subterranean clover has been reported to translocate less P from their roots to shoots compared to other pasture species, particularly at low levels of P fertility (Barrow 1975; Blair and Cordero 1978; Paynter 1990).

Biddiscombe *et al.* (1969) reported that the proportion of total plant P in the root fraction of subterranean clover after 92 days of growth at low and high levels of P addition was 32% and 22%, respectively. Assuming similar partitioning between roots and shoots occurred in our study, clover roots would account for 4.9 kg P ha^{-1} at the Ginninderra site (based on the portioning value of 32% in the low P soil of Biddiscombe *et al.* (1969)) and 7.9 kg P ha^{-1} at the Naracoorte site (based on the partitioning value of 22% in the high P soil of Biddiscombe *et al.* (1969)). This would account for over half of the unrecovered fertiliser P at each site.

The majority of the fertiliser P remaining in the soil surface layer at the end of the experiment was inorganic, although it was unclear how available this P remained for clover growth. It is likely that the addition of plant residues to the soil surface could also affect the distribution of P pools in soil fractions and possibly the recovery of fertiliser P by clover plants (Friesen and Blair 1988; McLaughlin *et al.* 1988a). However, it is unclear how much of the plant P in clover pastures is returned to the soil surface via plant decay or trampling or when this occurs in its lifecycle.

In addition to chemical constraints, there also can be physical constraints to accessing the fertiliser P in the surface layer (McBeath *et al.* 2012). Regular drying of surface layers in soils under pasture may restrict the plant's ability to access P in this layer, and subsoil applications of fertiliser P may be beneficial (Scott 1973; Pinkerton and Simpson 1986). In any case, the results of the 2013 field trials indicate that the application of fertiliser P to the soil surface resulted in a relatively high recovery of fertiliser P by clover plants in the year of application.

5.1.2 2014 field trials

The 2014 field trials were primarily designed to address Objective 3 (see section 5.3 below). However, they also provided some information on the fate of fertiliser P more generally. The 2014 field trials were carried out at Ginninderra, ACT (same site used in 2013 field trials) as well as Inman Valley, SA. The Inman Valley site was chosen due to its low P status, which contrasts with the high P status of the Naracoorte, SA site used for the 2013 field trial.

Overall, the 2014 field trials confirmed the relatively high uptake of fertiliser P by clover over a single growing season. For the treatment at Ginninderra that was equivalent to the 2013 field trial at the same site (fertiliser added to the surface in autumn), the recovery of applied fertiliser P in clover shoots was 38% compared with 30% in 2013. A slightly higher recovery of applied fertiliser P in clover shoots (42%) was achieved for the equivalent treatment at Inman Valley. As was the case for the 2013 field trials, the remaining fertiliser P was found mostly in inorganic forms in the upper (0-4 cm) layer.

The inclusion of treatments on soils that had three levels of initial soil P fertility at Ginninderra in 2014 also allowed us to investigate the role of P fertility on PUE. Differential fertiliser management of these soils over the previous 15 years was designed to maintain P fertility at low (P0; 4-6 mg Olsen extractable P kg^{-1}), optimal (P1; 10-15 mg Olsen extractable P kg^{-1}) and supra-optimal (P2; 20-25 mg Olsen extractable P kg^{-1}) levels. Overall, the recovery of applied fertiliser P in clover shoots (> 3 cm) varied little across the three levels: 33% at P0, 39% at P1 and 37% at P2. This was despite substantial effects of initial P fertility on clover biomass yield and especially cumulative P uptake, which both increased with increasing levels of extractable P (i.e. in the order P0<P1<P2).

5.2 Objective 2: Identification of forms of P that accumulate in fertilised pasture soils

Original objective:

"Identification of the major forms of accumulated P in fertilised pasture soils to aid identification of technologies to release P from this pool, or to minimise accumulation in fertilised pastures. This will be communicated in scientific papers (to scientists)."

The short span of the project along with the limitations caused by the relatively short half-life of the radioisotope meant that the field trials using radio-labelled P fertiliser described in the previous section were unsuited to identifying the forms of P that accumulate over the medium to long term (i.e. years to decades) in P fertilised pastures. The field trials demonstrated that direct uptake of P from superphosphate was high during the growing season in which it was applied (most probably up to 50 – 60% of the fertiliser P when an allowance is also made for P uptake into the roots). The fertiliser P that was not been taken up by the clover during the growing season was mainly accounted for as inorganic P near the soil surface. It is likely that some proportion of the P remaining in the soil would be potentially available for subsequent uptake by plants because fresh fertiliser applications are know to have substantial

(albeit declining) residual value. However, even if we assume that all of the P that was not taken up during the growing season had become “fixed”, it is clear from these results that, in the longer term, low efficiency of P fertilisation in pastures could only be partly due to freshly added P being transformed to sparingly-available P forms in the soil during the first growing season. By inference, P inefficiency must also be a result of P being recycling over a longer timeframe in the soil-plant-animal system.

Consequently, the forms of P being accumulated in pasture soils were determined to shed further light on the causes of low P balance efficiency. In this section we discuss the detailed chemical analysis of soils from the Ginninderra site that had been fertilised differentially over 13 years to maintain P fertility at low (P0; 4-6 mg Olsen extractable P kg^{-1}), optimal (P1; 10-15 mg Olsen extractable P kg^{-1}) and supra-optimal (P2; 20-25 mg Olsen extractable P kg^{-1}) levels. Establishing what forms of P have accumulated on this timeframe should provide information of the mechanisms involved. The two characterisation techniques initially employed were sequential fractionation and nuclear magnetic resonance (NMR) spectroscopy. The latter implicated organic P in broad phosphomonoesters (humic P) as the major organic form accumulating. Novel methodology was developed combining size fractionation with NMR analysis in an attempt to confirm this. The sections below provide detailed discussion of each component of the project that contributed to meeting this objective.

5.2.1 Sequential chemical fractionation

Sequential fractionation of the Ginninderra soils provided important information about the fate of fertiliser P on the decadal timescale. Over a 13-year period, the unfertilised (P0) treatment had received <10 kg P ha^{-1} (in the form of supplementary feed), the optimally fertilised (P1) treatment around 200 kg P ha^{-1} and the over-fertilised (P2) treatment around 240 kg P ha^{-1} (Simpson *et al.* 2015). Across all fertilised treatments (P1 and P2 at two different stocking rates), an average of 70% of added P was recovered in the 0-10 cm soil layer and a further 17% was recovered in the 10-20 cm layer. Of the P accumulated in the 0-10 cm layer of the P1 treatment, approximately half was identified as inorganic P and half as organic P; for the P2 treatment the corresponding inorganic:organic spilt was approximately 60:40. This suggests that in contrast to the short (one season) timeframe over which there is little conversion of (radio-labelled) fertiliser P to soil organic P, over the decadal timeframe soil organic P also becomes an important sink for fertiliser P. This is not to discount the importance of inorganic P as a sink for fertiliser P on longer timeframes, especially in the over-fertilised treatment, but rather to emphasise the increasing importance of organic forms over longer timeframes. Both inorganic and organic P accumulated mostly in the more extractable $NaHCO_3$ and NaOH I fractions, rather than the highly insoluble residual P pool that constituted nearly half of the native P in the soil.

5.2.2 Solution ^{31}P NMR

Solution ^{31}P NMR spectroscopy provides a much more detailed description of the chemical forms of organic P than is possible through sequential fractionation. However, solution NMR can only be applied to soluble species and suffers from low sensitivity – as a rule of thumb only one soil extract per day can be analysed using NMR. As part of this project some method development was carried out to improve sensitivity. This was particularly important for analysing subsoils, where inorganic P concentrations are very low. The method development involved using a much lower soil:solution ratio during extraction and it was shown that a sensitivity enhancement was achieved without unduly compromising extraction efficiency or spectral quality.

Solution ^{31}P NMR analysis of the Ginninderra soils provided important information about the fate of fertiliser P on the decadal timescale additional to that obtained from sequential fractionation. As discussed above, extractability of soil P was limiting, with around 50% of P in the topsoils not extractable in the NaOH-EDTA extractant; a similar proportion of soil P was identified as residual P by sequential fractionation (i.e. not extractable at any stage). Of the extractable P, approximately 50% was identified as orthophosphate by NMR. This is generally consistent with the proportion identified as inorganic P through sequential fractionation.

The key result from the NMR analysis of the Ginninderra soils was that most of the organic P was present as “humic P”, a term used to describe organic P present in high molecular weight material and bound through monoester linkages. Across the various treatments at Ginninderra (five combinations of fertility status and stocking rate), humic P accounted for an average of 76% of organic P detected by NMR. The remaining organic P was present as a mixture of phospholipid and nucleic acids, which are the main organic P forms found in most cells, and inositol phosphates, which are key storage P compounds in plants, especially seeds. The clear implication of the dominance of humic P in these soils is that the organic P that builds up in these soils is mostly different to the organic P found in the biota (both plant and microbial). This is indicative of there being an important but unrecognised process at work. Further, it would appear that this process (i.e. the one responsible for the synthesis of humic P) is active on longer timeframes than a single season, as there was little evidence for accumulation of organic P in soil in the radio-labelled P fertiliser experiments described earlier.

As with the sequential extraction experiments, comparison of P composition between the various Ginninderra treatments provides information on the fate of the P fertiliser that had been added over 13 years. Overall, 53% and 55% of that added P was detected in the topsoil as P extractable in NaOH-EDTA for the P1 and P2 treatments, around two-thirds of that as orthophosphate. Of the accumulated organic P, most was again detected as humic P. This confirms that the process by which humic P is synthesised is active on the decadal timescale and was primarily responsible for the accumulation of organic P in the fertilised Ginninderra soils.

It should be noted that the “humic P” interpretation of NMR analysis of soil extracts described here is not universally accepted, with some researchers in the area uncomfortable with the proposition that the majority of organic P in some soils (e.g.

the Ginninderra soils) is present in a form that cannot be defined at a molecular level and that is different to the organic P forms present in biota. These researchers mostly argue that organic P accumulates in soils mostly as inositol phosphate. While the details of this dispute are not of great relevance here, the fundamental difference between the opposing views are important as it goes to the heart of the issue at hand: the chemical composition of the large store of organic P present in most soils, and perhaps even more pertinently in the context of this project, the chemical composition of organic P that accumulates in pasture soils. Since the key distinction between the opposing interpretations is the relative size of the molecules involved, as part of this project we developed methodology to combine size separation through ultrafiltration with NMR analysis and applied it to the Ginninderra soils as well as four other diverse soils from around the world. The findings of this research are wide-reaching, but the discussion below is restricted to implications for this project.

In summary, size separation confirmed our "humic P" interpretation, in that the >10 kDa fraction isolated from each soil contained only the broad monoester signal we assign as humic P and no inorganic P or sharp monoester signals that can be identified as specific organic P molecules, including inositol phosphates. Interestingly, some broad monoester (humic P) signal was also detected in the <10 kDa fraction; this is not surprising given that this fraction should include molecules with up to ~300 carbon atoms. In other words the fractionation was designed to separate out a definitively high molecular weight fraction rather a definitively low molecular weight fraction. In the specific case of the Ginninderra (P0) soil, of the 84 mg P kg^{-1} identified as monoester P in the unfractionated extract, all 22 mg P kg^{-1} of monoester P in the high molecular weight fraction was identified as humic P. A further 31 mg P kg^{-1} was identified as humic P in low molecular weight fraction; this fraction also contained all of the remaining monoester P as specific molecular, including inositol phosphates. In agreement with the literature, our results have confirmed that inositol phosphate accumulates to reach significant concentrations in the European soils we analysed (70-125 mg P kg^{-1}). However, the Ginninderra soil from Australia did not accumulate significant levels of inositol phosphate (12 mg P kg^{-1}) and the more dominant form of organic P was classified as humic P. The emerging hypothesis is that there are climatic differences (predominantly cold and wet in Europe versus seasonally hot and dry in Australia) that are controlling the form of organic P that accumulates and we are exploring this hypothesis in another project.

The finding that organic P accumulates in pasture soils predominantly as high molecular weight compounds indicates that research seeking microbial or plant strategies to "unlock" this organic P needs to target different mechanisms than those sought previously, which have generally targeted inositol phosphates.

5.3 Objective 3: Fertiliser management to minimise loss of fertiliser P to unavailable organic and inorganic pools

Original objective:

"Fertiliser management strategies to manipulate fluxes of P into plants and minimise transfer to organic and inorganic pools."

The prelude to this research was the belief that the efficiency of immediate acquisition of fertiliser P was very low (10-20%) in the year that it was applied reflecting the low P-balance inefficiency of pasture systems (typically over the longer-term only about 10-30% of P applied to pastures is recovered in animal products). As part of the second year field trials we included variations of timing (autumn versus spring), placement (surface versus placement at depth) of fertiliser and cultural management (P applications to soil with differing fertiliser histories) to test whether changes to fertiliser practice could substantially improve the anticipated low P-acquisition efficiency of freshly-applied P.

The rationale for each the fertiliser management options that could be tested within the life of the project was:
(i) application in early spring, when pasture was likely to be growing faster, may improve P uptake because P was being applied to a rapidly growing root system;
(ii) deep placement of fertiliser in early autumn was examined because several reports suggest that P placed below the soil surface may be more effectively taken up, either because it is being placed directly into the path of root growth (akin to banding fertiliser below the seed in cropping) or because it will avoid surface soil drying should it occur. Dry soil is known to severely restrict P availability and topdressed superphosphate would be particularly prone to this restriction;
(iii) fertiliser was also broadcast in autumn into low, optimum and highly-fertilised soil because it was anticipated that the efficiency of P acquisition from the fertiliser would be highest when the soil had a low background concentration of plant-available P.

Typical fertiliser practice (broadcasting superphosphate in early autumn following an autumn break) was used as the control treatment. Right from the earliest results it was clear that the efficiency of direct P acquisition from fertiliser by a clover pasture was considerably better than has been widely believed to be the case. The initial efficiency of P acquisition from fertiliser (50-60%) was similar to or greater than the relatively high P-balance efficiency found in crop systems. Subsequently, only marginal gains, at best, were associated with attempts to optimise typical fertiliser practice:
(a) later application (when pasture was growing faster) did not improve the acquisition of freshly-applied P (it sometimes marginally decreased the efficiency of P acquisition);
(b) deep placement delayed access to freshly applied P (and was less effective at one of the two field sites);
(c) the highest efficiency of P acquisition occurred when fertiliser was applied to optimally-fertilised pasture; it was marginally lower in infertile soil, probably because the plants were growing slowly, and was only marginally lower in over-fertilised soil.

In most cases (except deep placement at one site), the lost efficiency when fertiliser practices were not optimal was relatively small (~5% less efficient). This indicates that there is a reasonably wide window for practical operation on farms within which the penalty for non-optimal placement or timing is small.

The net result is that we should not recommend changes to current practice other than to emphasise that application of fertiliser early in the growing season and to well fertilised soils is relatively effective in the year of

application. The industry can confidently promote a very positive message that current good practice is "best practice" and that broadcasting P fertiliser is, indeed, more effective at directly fertilising pasture than has been thought to be the case.

6 Conclusions/recommendations

6.1 Future research and development

Ways in which the findings generated in this project could be extended in future research include:

1. Benchmarking of the PUE of fertiliser P in a wider range of pasture soils and pasture species would be beneficial. Soils with higher P sorption characteristics than those investigated in this project should be targeted as well as soils subject to different mechanisms of P sequestration e.g. alkaline and calcareous soils. Along with other pasture species already widely utilised (e.g. ryegrass), the PUE of newly-identified, P-efficient legumes (e.g. serradellas) should be determined as this would identify whether their improved performance relative to clover is due to better direct access to fertiliser P or better use of less labile soil pools.
2. A considerable portion of the P accumulated in the Ginninderra soil was inorganic P, yet we did not undertake any characterisation of the chemical forms (speciation) of the inorganic P accumulated. Advanced spectroscopic methods such as synchrotron X-ray spectroscopy could be used to gain more information on the specific forms of P accumulated (similar to the NMR speciation undertaken for organic P).
3. Strategies for pasture plants to access accumulated P in pasture soils (both inorganic and organic forms) should be investigated more broadly – this might involve species with specific root traits (e.g. root architecture, root exudates, etc.) that could allow pasture systems to operate productively at lower levels of available (Olsen) P. Furthermore, the PUE of grass/clover mixed pastures should be examined to determine whether these directly access more fertiliser P than clover alone and if so (i.e. where root systems are complementary), does the choice of grass species make a difference to the outcome.
4. The efficiency of the common practice of applying fertiliser just before the break of season (and sometimes even earlier) should be tested to determine whether there is a penalty in applying fertiliser to dry soil before plants have started growing.

6.2 Practical application of project findings and implications for the red meat industry

The project findings with most practical application require an integrated view of the PUE of fertilisers applied and the outcome at different levels of soil fertility. We now know that fertiliser is utilised by the clover plant at a higher efficiency than represented in the assessment of PBE and that much of the fertiliser P remains in the extractable (i.e. potentially plant available) form at the end of the season in which it is applied. Yet, the results from long term experiment at Ginninderra suggest that frequent inputs of fresh fertiliser are required to maintain a paddock at optimal P status and that the previously applied fertiliser is accumulating in forms of P that are more slowly available to the pasture.

Clearly, the industry can be confident that the current practice of topdressing superphosphate is an effective fertiliser management strategy and growers do not need to drastically modify either placement or timing to try to improve fertiliser efficiency.

While utilisation of fertiliser P by pasture appears to be relatively high compared to crops, efficiency is still only 50-60% and significant amounts of P accumulate in soil in both inorganic and organic forms. Given that fertilizer timing or placement is not going to dramatically improve this efficiency, then alternate strategies need to be considered e.g. development of new pasture species more effective in scavenging fertiliser P from soil or development of fertiliser formulations that minimise soil accumulation and maximise plant acquisition.

It is also important that the P taken up by pastures is effectively utilised given the disparity between the P uptake efficiency measured in this project and the P balance efficiency of grazing systems. Efficiency of pasture utilisation by animals needs to be examined in order to minimise accumulation in soil. Finally, P balance efficiencies are strongly influenced by the rate of fertiliser P applied, and are low in systems where P is supplied in excess of crop requirements. It is therefore important that the industry promotes and adopts diagnostics (soil testing, test strips, etc.) to ensure P is applied at optimal rates to grazing systems.

6.3 Development and adoption activities that would ensure the red meat industry gets full value from the project findings

In terms of development, existing soil testing and fertiliser spreading technology is adequate for growers to achieve current best-practice fertiliser efficiency, but adoption and implementation is not high in the grazing industry. Cheaper soil testing and monitoring would be welcomed and would improve adoption and spatial management of fertiliser inputs, but so far no “remote sensing” technique has been found that can determine soil “extractable-P” concentrations (i.e. plant-available P).

In terms of adoption, there is a need to improve soil testing practices and skill in the grazing industry. Growers cannot achieve the most efficient fertiliser practices until they start soil testing and can manage paddocks at or near their optimum (target) soil

P fertility levels in accordance with the level of pasture productivity that is optimal for their farm.

6.4 List of extension material and communication to target audiences

Over the course of the project, three news articles were published (one each on the MLA website, in 'Beyond the Bale' and in the Stock Journal), 12 oral presentations were given (at a range of scientific conferences and grower meetings) and there were three poster presentations at scientific meetings. Thus far, four papers have been published in peer-review journals and a further six are in preparation or planned. Details are provided below.

6.4.1 Communication of project findings to researchers, growers and industry

1. Meat and Livestock Australia, news article, 28th March 2014 – Where does the P go?: http://www.mla.com.au/News-and-resources/Industry-news/Where-does-the-P-go (not active, attempted to access on the 4th January 2016).
2. Pathways to productivity expo, oral presentation, Bordertown SA, 8th April 2014 – Understanding the fate of P in your pasture.
3. Stock Journal, news article, 23rd April 2014 – Project in hot pursuit of phosphorus, http://www.stockjournal.com.au/news/agriculture/livestock/general-news/project-in-hot-pursuit-of-phosphorus/2695635.aspx (active, accessed on the 4th January 2016)
4. Australian Wool Innovation (Beyond the Bale), news article, June 2014 – Research into phosphorus uptake: http://beyondthebale.wool.com/default.aspx?iid=93078&startpage=page0000041#folio=40 (active, accessed on the 4th January 2016).
5. 20th World Congress of Soil Science, oral presentation, Jeju South Korea, 8th to 13th of June 2014 – The accumulation of inorganic and organic P forms in fertilized pasture soils.
6. Agronomy community forum, oral presentation, Wagga Wagga NSW, 29th July 2014 – The efficiency of fertiliser phosphorus use in legume based pasture.
7. 5th International Symposium of Phosphorus in Soils and Plants, oral and poster presentations, Montpellier France, 26th to 29th August 2014 – 'The fate of fertilizer phosphorus in pastures', and 'Improved detection of organic P forms in pasture subsoils'.
8. National Soil Science Conference, poster presentations, Melbourne Vic, 23rd to 27th November 2014 – 'Tracking fertiliser P in pasture systems using a novel method of creating ^{33}P labelled single superphosphate', and 'The NaOH-EDTA technique provides a simple and rapid measure of total inorganic and organic P in pasture soils'.
9. Visit to ETH Zurich (Switzerland), James Hutton Institute in Dundee (United Kingdom), Lancaster University (United Kingdom) and Teagasc in Johnstown Castle (Ireland), oral presentation, 19th, 27th and 28th January 2015 – Phosphorus use efficiency and cycling in Australian pastures.

10. New Springs Landcare group SA, oral presentation, Mount Torrens SA, 11th March 2015 – Tracking fertiliser P in pastures.
11. Ag Southern Australia Group Meetings, oral presentation, Adelaide SA, 11th May 2015 – Fate of phosphorus in pasture soils.
12. Farm Nutrient Management forum, oral presentation, 'Phosphorus management in Pastures', Bunbury, WA, 14th May 2015. Organised by the South West Catchment Council, WA
13. Grasslands Society of South Australia, oral presentation, Naracoorte SA, 21st to 23rd July 2015 – Improving phosphorus efficiency in pastures.
14. Agronomy community forum, oral presentation, Ballarat Vic 12th August 2015 – The efficiency of fertiliser phosphorus use in legume based pasture.
15. Nuriootpa Grassland Society Pasture Update, oral presentation, Nuriootpa SA, 25th August 2015 – 'Phosphorus efficiency in pastures. Where does all the P go?'.
16. The 17th Australian Agronomy Conference, oral presentation, Hobart Tas, 20th to 24th September 2015 – 'Which fertiliser phosphorus management strategy for maximum clover production and fertiliser phosphorus efficiency?'.
17. The 29th Conference of the Grassland Society of NSW - oral presentation and written paper: Simpson RJ, Sandral GA, Ryan MH, McLaren TI, Smernik RJ, McLaughlin MJ, McBeath TM, Lambers H, Guppy CN, Richardson AE (2015) New insights into phosphorus cycling in pastures: implications for fertiliser management and for closing the phosphorus efficiency gap. *Proceedings of the 29th Conference of the Grassland Society of NSW Inc*. pp 30-40.

6.4.2 Communication of project findings to researchers, growers, and industry as published papers in peer-reviewed scientific journals

1. McLaren TI, Smernik RJ, Simpson RJ, McLaughlin MJ, McBeath TM, Guppy CN, Richardson AE (2015) Spectral sensitivity of solution 31P NMR spectroscopy is improved by narrowing the soil to solution ratio to 1:4 for pasture soils of low organic P content. Geoderma 257-258:48-57.
2. McLaren TI, Simpson RJ, McLaughlin MJ, Smernik RJ, McBeath TM, Guppy CN, Richardson AE (2015) An assessment of various measures of soil P and the net accumulation of P in fertilized soils under pasture. J Plant Nutr Soil Sci 178:543-554.
3. McLaren TI, McLaughlin MJ, McBeath TM, Simpson RJ, Smernik RJ, Guppy CN, Richardson AE (2015) The fate of fertiliser P in soil under pasture and uptake by subterraneum clover - a field study using 33P-labelled single superphosphate. Plant Soil: published online, doi:10.1007/s11104-11015-12610-11106.
4. McLaren TI, Smernik RJ, McLaughlin MJ, McBeath TM, Kirby JK, Simpson RJ, Guppy CN, Doolette AL, Richardson AE (2015) Complex forms of soil organic phosphorus – a major component of soil phosphorus. Environ Sci Technol 49:13238-13245.

7 Key messages

7.1 Key messages for researchers

i) Many important lessons have been learned in this project on how to perform field-based measurement of P use efficiency in pasture systems. The value of this should not be underestimated, nor should the value of having published these experiments in the peer-reviewed literature. The latter is important for two reasons: (i) it provides a strong checking and validation of the techniques used; and (ii) it ensures the widest possible access to other researchers both geographically and over time. This project included the use of multiple innovations of a technical nature, including a novel method for incorporating radiolabel into fertiliser granules, the use of an improved extraction method to facilitate NMR analysis on low P soils, a comparison of multiple methods for determining "total" P concentration in soils and the combination of size separation and NMR spectroscopy for the characterisation of soil organic P. The development of these innovations, and the fact that they have been described and validated in the peer-reviewed literature, builds the capacity for all researchers to carry out subsequent experiments in this area effectively and efficiently.

ii) The pasture species tested (subterranean clover) was clearly shown to be at least as capable of accessing freshly added fertiliser P as grain crop species that had been investigated previously. Thus it is clear that the primary cause of generally lower P balance efficiency for pastures than cropping systems is not due to lower P use efficiency of pasture compared with crop species. Prior to this project it was suspected that low plant P use efficiency may have been a serious issue. Follow-up studies on this point are warranted, as this conclusion is drawn from a very limited set of field experiments, leaving many commercially important combinations of pasture species, soil type and seasonal conditions unstudied. In particular, experiments involving mixed (legume + grass) pasture swards, P-efficient legume species and soils with high P-fixing capacity should be prioritised.

iii) Much of the radio-labelled fertilizer P not taken up by plants appeared to still be in labile soil P fractions at the end of the growing season, suggesting that fertiliser P is likely to have substantial residual value in subsequent years. We invested significant effort towards determining the fate of this fertiliser P not detected in plants in the first growing season as there is a major limitation of the radiolabelling approach associated with the relatively short half-life of P radionuclides, whereby it is practically impossible to detect residual radioactive P a year after it has been added. Chemical fractionation of both topsoil and subsoil fractions indicated the majority of fertiliser P remained in the 0-4 cm layer, remained in an inorganic form and predominated in the more chemically labile fractions (e.g. bicarbonate-soluble). Isolation of root material in the second year field trials clearly indicated root biomass contributed significantly to fertiliser P not detected in above-ground pasture biomass. However, it remains

unclear how to incorporate this information into an estimate of residual fertiliser value in the absence of direct uptake measurements.

iv) Access to the long-term P fertiliser experiment at Hall Experiment Station near Ginninderra allowed us to investigate the longer-term fate of fertiliser P. In this project we generated detailed characterisation through sequential fractionation and ^{31}P NMR spectroscopy to supplement existing data on total soil P and available (Colwell) soil P previously collected. These additional analyses were chosen to shed light on the fate of the fertiliser P not removed in product (wool and meat) and that does not remain in a readily available inorganic form (i.e. Colwell P). This is the fate of the majority of fertiliser P in the mid- to long-term and thus knowing more about what forms it accumulates in is central to understanding low P balance efficiencies in pastures. Detailed P balance calculations on the results of sequential fractionation showed that, for a paddock optimally managed for P availability, approximately 30% of P added as fertiliser over 13 years remained in inorganic forms, with the majority of that being less labile than Colwell P, and a further 30% of added P had been converted to organic forms. For a paddock maintained at higher than optimal available P levels, more P accumulated, and the greater proportion of that was present as inorganic P. Further analysis using ^{31}P NMR spectroscopy showed that the nature of the accumulated organic P differed very little from the native P present in the unfertilised soil, but was predominantly in a form substantially different to that found in plant and microbial biomass. Together, these results point towards a complex set of processes that act on a decadal timescale through which the fertiliser P, originally added in an inorganic and readily plant available form, is converted into a range of less available and chemically complex forms. Although this knowledge does not immediately provide a solution to low P balance efficiency, it does provide much additional information on the nature of the problem.

v) An important development in understanding the nature of organic P was achieved through the combined use of molecular size separation through ultrafiltration with ^{31}P NMR analysis. We have been arguing for a number of years that organic P in Australian soils is chemically very different to that found in living biomass and this development provided direct and convincing evidence. Again, this knowledge does not immediately provide a solution to low P balance efficiency, but it does provide much additional information on the nature of the organic P that builds up over time in fertilised pastures.

7.2 Key messages for producers

i) This project indicates that currently recommended practice (i.e. surface addition in autumn at a rate designed to maintain close to the critical Colwell P value for the legume) is close to being optimal in terms of the manageable factors of P fertiliser use (timing, placement, rate and formulation). The procedures developed and proven in this project will facilitate further assessment over a wider range of soil types, seasons and

management options (including early, dry P application) but we do not expect these will produce any “magic bullets”.

ii) Poor acquisition of P directly after fertiliser has been applied is not as large a factor in the low P balance efficiency of pasture systems as is often thought to be the case. The processes leading to P accumulation in soil are, however, now better understood. The P accumulation problem also stems from the fact that, in pasture systems, P is extensively cycled in the soil–plant-animal-soil cycle and will be exposed on multiple occasions to reactions that may render it less available for plants. It is known that the reactions leading to P accumulation are relatively slow and are related to fertiliser practice because it determines the concentrations of plant-available P in soil.

iii) Losses and accumulation of P can be limited by keeping the available P concentration of the soil at levels that are optimal for pasture production (i.e. by not over-fertilising). The long-term experiments at Ginninderra clearly showed that maintaining soils at supra-optimal Colwell P levels exacerbated P accumulation in the soil and reduced P balance efficiency. We reiterate that producers should test soils for available P and aim to maintain available P at a level appropriate to achieve pasture production targeted to the optimal carrying capacity.

iv) For the same reason, the results of this project highlight the potential benefit of non-traditional legumes (e.g. serradella) that have lower critical Colwell P requirements. At the very least, by enabling producers to maintain soils at lower Colwell P values without adversely affecting N-fixation and hence production, loss of fertiliser P to unavailable forms can be minimised. Further research on why these species can thrive in low Colwell P soils may further show that part of their advantage results from an ability to access “non-Colwell-P” and if so, it may be possible that these species can utilise “legacy P”, i.e. P previously added as fertiliser and considered lost to production.

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