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INCUBATOR INITIATIVE: PHOSPHORUS SPECIATION IN WASTE BIOMASS STREAMS: WORKING TOWARDS CLOSING THE P LOOP IN WINE PRODUCTION



FINAL REPORT to

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Principal Investigator: **Ashlea Doolette**

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Incubator Initiative: Phosphorus speciation in waste biomass streams: Working towards closing the P loop in wine production

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Author details:
Dr Ashlea Doolette
School of Agriculture, Food and Wine
The University of Adelaide
PMB 1 Glen Osmond
South Australia, 5064

Email: ashlea.doolette@adelaide.edu.au
Phone: +61 8 313 8107

Date: 30 June, 2015

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1. Abstract

Australian vineyard soils are naturally low in phosphorus, an element essential to the functioning of all life-forms. In most vineyards, occasional additions of mineral phosphorus fertilisers are used to enhance grapevine growth and production but a more sustainable approach can be taken through optimal management of leaves, pruning and grape marc, which can result in the majority of P being recycled backed into the vineyard. However, since phosphorus availability is controlled by the forms of phosphorus rather than total amount of P present, this project examined the P forms present and the changes in P composition in the main waste biomass forms over a full season. The amount of P potentially returned to the vineyard was considerable relative to fertilisation rates and was a combination of directly (immediately) plant available and more stable (slow released) forms, which differed between plant part and was also influenced by the phosphorus status of the vine.

2. Executive Summary

Australian vineyard soils are naturally low in phosphorus, an element essential to the functioning of all life-forms. In most vineyards, occasional additions of mineral phosphorus fertilisers are used to enhance vine growth and production. These can be viewed as replacing phosphorus lost from vines in three main ways: pruning of canes in winter, loss of leaves in autumn and removal of fruit at harvest. These losses are in effect incidental to wine production, which itself contains very little phosphorus. This contrasts with the situation for most other agricultural products, especially grain crops, where substantial quantities of phosphorus are contained in the final product. Thus, in theory, it should be possible to "close the phosphorus loop" in wine production systems through careful management of the biomass waste streams that contain substantial quantities of phosphorus. This would provide a direct (though small) economic benefit by removing the need for phosphorus fertiliser. More importantly, closing the phosphorus loop would provide an important demonstration of commitment to sustainability principles and environmental and social stewardship, as the sources of fertiliser phosphorus are non-renewable and their exploitation (e.g. historical mining in Nauru and current mining in Moroccan-occupied Western Sahara) is politically sensitive.

It is well known that grapevines store significant quantities of phosphorus in their roots and trunk to overcome short term deficiencies of soil supply during early canopy development. During later development some phosphorus is lost in senesced leaves and pruned canes. Generally, senesced leaves and prunings are retained in the vineyard, although senesced leaves are often blown around and so phosphorus contained in these leaves is not necessarily recycled efficiently to the soil; management of prunings varies widely. In addition, phosphorus is removed from the vineyard at harvest, at a rate that has been estimated at approximately 0.3-0.6 kg of phosphorus per tonne of grapes. In some management systems, phosphorus removed at harvest is returned into the vineyard as grape marc. Although the total concentration of phosphorus in various parts of grapevines has previously been studied, very little information exists on the chemical forms of phosphorus present. Phosphorus is present in cells in a variety of forms including the simple orthophosphate anion (PO_4^{3-} and its protonated counterparts HPO_4^{2-} and $H_2PO_4^-$) and a range of organic forms including phospholipids, which make up cell membranes, nucleic acids (DNA and RNA), which store and carry genetic information, and inositol phosphates, which act as storage compounds for phosphorus, especially in seeds. The relative quantities of these different chemical forms of phosphorus affect how efficiently and quickly phosphorus in biomass returned to the soil is cycled and, in particular, on the timing of availability of this phosphorus for uptake by the grapevine. In this project, ^{31}P NMR spectroscopy was used to determine the phosphorus speciation in grapevine biomass over a full season, focusing on phosphorus speciation in the major biomass losses of prunings, leaf fall and harvested fruiting bodies (berries and stalks).

The first part of the project involved the collection and analysis of prunings collected from nine Barossa Valley vineyards during vine dormancy in July 2014. The total phosphorus content of these materials ranged from 1040 mg P/kg to 1870 mg P/kg and the total phosphorus in these prunings represented 0.9 kg/ha to 5.6 kg/ha. The overall composition of phosphorus forms was generally similar, with orthophosphate and inositol phosphate predominant. The presence of substantial quantities of inositol phosphate indicates that the dormant canes are acting as phosphorus storage organs; previously only seeds have been reported to contain such high concentrations of inositol phosphates. Of great interest was the fact that two inositol phosphate molecules were detected. The first of these, *myo*-inositol hexakisphosphate (referred to hereon as "IP_6"), commonly known as phytate, is the main phosphorus-storage compound present in seeds and has also been identified in lower concentrations in other plant parts. However, the second inositol phosphate compound, identified through spiking with an authentic sample as D-*myo*-inositol 1,2,4,5,6 pentakisphosphate (referred to hereon as "IP_5"), has not previously been reported in plant tissue. A further interesting observation was that of all of the phosphorus species, only orthophosphate and IP_6 concentrations increased with increasing total phosphorus concentration in the prunings.

These findings establish both the amount and chemical speciation of phosphorus that is removed from vines on pruning. The presence of a mixture of readily available (orthophosphate, RNA and phospholipid) and potentially less labile (inositol phosphate) forms indicates that prunings should be expected to provide a

mixture of fast- and slow-release phosphorus to the growing vines. Further research is needed to determine the exact fate of this phosphorus in subsequent years.

The second part of this project involved the collection and analysis of grapevine biomass throughout the growing season from three sites (representing high, moderate and low phosphorus status, as measured in the first part of the study). Sampling was carried out at flowering, veraison, harvest and leaf fall and plant biomass was divided into shoots, leaves and fruiting bodies. Large and consistent changes in phosphorus speciation were observed. For leaf material, phosphorus concentrations were initially high and dominated by orthophosphate. Over the season, phosphorus concentrations decreased and orthophosphate became less dominant, with phospholipid and RNA representing increasing proportions of leaf phosphorus. Senescent leaves had the lowest phosphorus concentration, but still contained substantial amounts of orthophosphate, phospholipid and RNA, and even some IP_6 in the case of the high phosphorus status grapevines. For fruiting bodies, there was a large difference in phosphorus composition between flowering clusters and fruit at veraison, with the latter much higher in inositol phosphate. This is unsurprising given that inositol-rich seeds develop over this period. The phosphorus composition of the ripe fruit was consistently lower in inositol phosphate than at veraison, with the proportion of phosphorus present as orthophosphate increasing. For shoots, there was a general decrease in phosphorus concentration over the season, along with a change from orthophosphate dominance to an increased proportion of phosphorus present as inositol phosphate. Analysis of several grape marc samples indicated a similar phosphorus composition to that of ripe fruit, as expected. Analysis of prunings and grape marc that had been aged in the field indicated some transformation had occurred over periods up to one year, including the apparent synthesis of new organic phosphorus species. However, insufficient samples were analysed to ascertain the generality of these findings; this aspect was not within the original project scope and further research in this direction is warranted. Finally, analysis of soil samples clearly showed a disconnect between the organic phosphorus in grapevine biomass and that found in the soil, which was dominated by complex forms with little or no inositol phosphate present.

These findings substantially enhance our understanding of phosphorus transformations in grapevines, adding the “meat” of phosphorus speciation to the “bones” of overall phosphorus movement through the plant. Of particular interest in terms of phosphorus cycling in vineyards is the phosphorus composition of the major biomass waste streams of prunings, senescent leaves and harvested fruit. The main messages from this project are:

(1) Harvested fruit represents the largest phosphorus removal pathway, most of which remains in the grape marc and is present as a mixture of immediately available (orthophosphate) and potentially recalcitrant (inositol phosphate) forms. Any effective closing of the phosphorus loop would require return of this grape marc phosphorus to the vineyard.

(2) Prunings represent the second largest phosphorus removal pathway and again phosphorus is present as a mixture of immediately available (orthophosphate) and potentially recalcitrant (inositol phosphate) forms. Analysis of prunings that had been in contact with the soil for one year suggested that substantial changes in composition occur over that timescale. Further research on this aspect is warranted.

(3) Senescent leaves represent the third largest phosphorus removal pathway and contain mostly directly available (orthophosphate) and readily degradable (phospholipid and RNA) forms of phosphorus.

(4) The phosphorus status of grapevines consistently influences not only the phosphorus content of the biomass waste streams, but also its composition, with higher phosphorus status vines generally proportionally richer in both orthophosphate and inositol phosphate and proportionally poorer in phospholipid and RNA.

(5) More research is needed to understand the transformations that occur when plant biomass is added to the soil. Little or no evidence was found to support the proposition that organic phosphorus added in biomass accumulated in the soil.

3. Background

Australian vineyard soils are naturally low in phosphorus (P) and in most vineyards mineral P fertiliser is used to enhance vine growth and production. It is well known that grapevines store significant quantities of P in their roots and trunk to overcome short term deficiencies of soil supply during early canopy development, but during later development some P is lost in senesced leaves and pruned canes. Generally, senesced leaves and prunings are retained in the vineyard, although senesced leaves are often blown around and so P contained in these leaves is not necessarily recycled efficiently to the soil and management of prunings varies widely. In addition, P is removed from the vineyard at harvest, at a rate that has been estimated at approximately 0.3-0.6 P kg per tonne of grapes (Robinson 1988). In some management systems, P removed at harvest is returned into the vineyard as grape marc. Total phosphorus levels in grape marc composts range from 0.2-0.3% w/w (Patti, Issa *et al.* 2009). Very little information exists on P chemical speciation of prunings, senescent leaves or grape marc. It is expected that differences in the chemical P species contained in both materials will play an important role in how P will cycle in soils and, in particular, on the timing of availability of this P for uptake by the vine. Recently, in research funded by the Grains Research and Development Cooperation (GRDC), ^{31}P NMR spectroscopy was used to investigate flows of P within cropping systems, focusing on P speciation in crop residues (Noack, McLaughlin *et al.* 2014; Noack, McBeath *et al.* 2014; Noack, McLaughlin *et al.* 2012; Noack, Smernik *et al.* 2014). In this project, ^{31}P NMR spectroscopy was used to determine the P speciation in grapevine biomass over a full season, focusing on P speciation in the major biomass losses of prunings, leaf fall and harvested fruiting bodies (berries and stalks).

The main objective of this project was to establish a thorough understanding of the flux of phosphorus (P) forms in wine production systems. Although little P is contained in the final product (wine), vines require and take up substantial quantities of P, much of which ends up in the waste biomass streams of prunings, senesced leaves and marc. In theory, it should be possible to “close the P loop” in wine production systems. This would provide a direct (though small) economic benefit by removing the need for P fertiliser. More importantly, closing the P loop would provide an important demonstration of commitment to sustainability principles and environmental and social stewardship, as the sources of fertiliser P are non-renewable and their exploitation is politically sensitive. Although the gross fluxes of P (i.e. total amounts of P) in wine production systems are reasonably well established, little is known of the chemical speciation of P in these fluxes and closing this knowledge gap is crucial since soil P fertility is predominantly controlled by P speciation rather than total amounts. In particular, the aims of this project were to twofold: (1) to establish the chemical composition of P in the main waste biomass streams of prunings, senesced leaves and grape marc; and (2) to establish an understanding of the changes in P composition that occur in grapevine biomass over a full season.

4. Project Aims and Performance Targets

The main activity of this project involved collection and chemical analysis of waste biomass streams of wine production, predominantly senescing leaves, prunings and marc, along with grapevine biomass collected at several key points of the season (dormancy, flowering, veraison, harvest and leaf fall). Since the P content and composition of vine biomass was predicted to vary from vineyard to vineyard, material was collected from nine sites across the Barossa Valley region. Since the budget only allowed for analysis of around 50 samples, full analysis of samples from all sites was only carried out for cane material collected at dormancy. Soil collected from each site was also analysed. Analysis of these samples established that P composition varied consistently with the P status of vines, allowing the selection of three of the sites (representing low, moderate and high P status) for full analysis of biomass (shoots, leaves and fruiting bodies) collected at the other four sampling times (a total of 33 further samples: 3 sites x 3 plant parts x 4 sampling times (3 for fruiting bodies)). In addition, four grape marc samples were analysed, along with separated stalks and berries (along with combined stalk + berry) at two sites. Finally, fortuitous circumstances allowed for ad hoc sampling of root and trunk material at one site, where a number of vines had been removed to install frost fans, and aged marc, canes and stalk at other sites. These materials were also analysed. Nine soils were also analysed by ^{31}P NMR. Thus a total of 67 samples were fully analysed, including total P determination using ICP-AES analysis following acid digestion and P speciation using solution ^{31}P NMR spectroscopy; the original aim was to analyse a total of 50 samples.

5. Methods

5.1 Grapevine biomass sample collection and preparation

Grapevine biomass was sampled from nine sites, from five different growers within the Barossa Valley region. Two varieties were selected; *Shiraz* grown at sites 1-5, 7 and 8 and *Viognier* grown at sites 6 and 9. Vines at all sites were greater than 10 years old.

Biomass samples were collected at dormancy (July 23), flowering (September 23), veraison (January 13), harvest (February 12) and leaf fall (May 14) by sampling 5 randomly selected shoots or canes within a single vine row at each site. Samples were collected from within 15 m and within the same vine row at each sampling time.

Shoots and canes were cut back to two buds and carefully removed from the grapevine to ensure all plant material was retained. Trunk and root samples had been destructively removed due to excavation of the site by the grower and were sub-sampled from multiple vines. Grape marc sub-samples (5 L) of Pinot Gris, Chardonnay and Shiraz were collected from the University of Adelaide Winery, Waite Campus and were stored at 4°C until processed (see below). Stockpiled grape marc and stalk was collected from site 7 but the variety is unknown. It was sourced by the grower from Tarac Technologies Pty Ltd, Nuriootpa, SA and had been stockpiled for at least 6-7 months at the time of sampling. Ten sub-samples (approx. 100 mL each) of grape marc and stalk were bulked from the respective stockpiles and mixed thoroughly.

All samples were oven dried at 60°C for five days. Each shoot was then separated into the various vine structures (shoot, leaves, flowers, berries and stalk) weighed individually and ground to <2 mm. The roots were separated into coarse (diameter >4 mm) and fine (diameter <4mm) roots and the trunk into outer (periderm/bark) and inner (vascular tissue) sections. For large woody pieces and such roots and trunk, as well as grape marc and stalk, samples were ground to a fine powder in a coffee mill.

5.2 Soil sample collection and preparation

Bulked samples of 10 cores were collected from each site. Soils were sampled from a depth of 0-10 cm in the vine row over a distance of 15 m, at the same site as vine biomass sampling. Soil samples were air-dried (40°C) and ground to pass through a 2 mm sieve prior to analysis.

5.3 Chemical Analysis

5.3.1 Total phosphorus

Phosphorus concentrations of plant and soil samples were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) following nitric acid and hydrogen peroxide digestion (Wheal, Fowles *et al.* 2011).

5.3.2 NaOH-EDTA extraction

All grapevine biomass, grape marc and soil samples were extracted with NaOH-EDTA based on the method of Cade-Menun and Preston (1996). This involved shaking 2.0 g of dry matter or 4.0g of soil with 40 mL 0.25 M NaOH and 0.05 M Na_2EDTA for 16 hours. The extracts were centrifuged (1400 × g) for 10 minutes and filtered using Whatman No. 42 filter paper. An aliquot (20 mL) from each sample was immediately frozen and freeze-dried for NMR analysis.

5.3.3 ^{31}P NMR analysis of NaOH-EDTA extracts

A 500 mg subsample of freeze-dried extract was ground, re-dissolved in 5 mL of deionised water, and centrifuged at 1400 × *g* for 20 minutes. The supernatant solution (3.5 mL), methylene diphosphonic acid (MDP) (0.1 mL at a concentration of 6 g L^{-1}) and deuterium oxide (D_2O) (0.3 mL) were placed in a 10 mm NMR tube. The resulting pH was >13. Solution ^{31}P NMR spectra were acquired at 24°C on a Varian INOVA400 NMR spectrometer (Varian, Palo Alto, CA) at a ^{31}P frequency of 161.9 MHz. A recovery delay of 7-60 s was used to ensure complete relaxation between scans and avoid saturation effects that can compromise quantitation. We used a 90° pulse of 50 µs, an acquisition time of 1.0 s, and gated broadband

^{1}H decoupling. Between 300 and 1500 scans were acquired for each sample, depending on the P concentration. The spectra presented have a line broadening of 2 Hz.

6. Results and Discussion

6.1 Estimation of phosphorus content and speciation in prunings during dormancy

The total P concentration of cane biomass collected during dormancy in 2014 ranged between 1040 mg P/kg and 1870 mg P/kg (Table 1). The estimated mass of this cane biomass removed by pruning varied widely from 0.89 t/ha to 4.28 t/ha. The P concentrations reported here are similar to those reported elsewhere (700-1800 mg P/kg); however, the cane biomass produced is generally higher than previous studies (0.4-1.4 t/ha) which may be due to differences in grape variety (Conradie 1981b; Pradubsuk and Davenport 2010; Schreiner, Scagel *et al.* 2006). Analysis of the chemical composition of P in this material by ^{31}P NMR spectroscopy requires extraction into an alkaline solution (0.25 M NaOH + 0.05 M Na_2EDTA). When interpreting NMR analysis of P speciation consideration must be given to the extraction efficiency or percentage recovery, as a high recovery ensures that the majority of P in the grapevine material is included in the NMR analysis. A comparison of NMR detected P concentrations against total P concentrations determined following acid digestion and inductively coupled plasma atomic emission spectroscopy (ICP–AES) indicated that a large and consistent portion (77%–87%; Table 1) of total P was indeed detected by NMR. The recoveries reported here are similar to those reported for broad acre crops (average 87%) (Noack, McLaughlin *et al.* 2012).

Table 1. Total phosphorus concentration, content and dry matter of canes collected at dormancy at nine sites in the Barossa Valley.

Site	Total P (mg P/kg)[A]	NMR detected total P (mg P/kg)	NMR recovery (%)	Dry matter (t/ha)	Cane P content (kg P/ha)
1	1040	823	79	1.69	1.8
2	1060	737	70	2.16	2.3
3	1110	852	77	0.85	0.9
4	1320	1153	87	4.28	5.6
5	1340	1099	82	2.81	3.8
6	1350	1088	81	1.32	1.8
7	1660	1160	70	1.63	2.7
8	1660	1305	79	0.97	1.6
9	1870	1470	79	2.89	5.4

[A] Determined by acid extraction and ICP-AES

Despite the nearly two-fold difference in P concentration among the cane materials collected from the nine sites, their ^{31}P NMR spectra were on the whole similar (Figure 1). The highest peak in each spectrum (out of scale in Figure 1) appears at ~5.75 ppm and is due to orthophosphate. A series of smaller peaks appears to the right of the orthophosphate peak; these are due to P in organic monoester groups. Specific assignments of the main monoester peaks are shown in Figure 2. These include α- and β-glycerophosphate that are derived from the hydrolysis of phospholipids in the alkaline extractant solution (hence signal due to these peaks is hereafter referred to as "lipid P") and peaks due to two inositol phosphate compounds. The first of these is *myo*-inositol hexakisphosphate (referred to hereon as "IP_6"), commonly known as phytate. Phytate is the main P-storage compound present in seeds (Reddy, Pierson *et al.* 1989) and has also been identified in lower concentrations in other plant parts (Brearley and Hanke 1996; Campbell M, Dunn R *et al.* 1991; Noack, McLaughlin *et al.* 2012). The second inositol phosphate compound was identified (through spiking with an authentic sample of the compound) as D-*myo*-inositol 1,2,4,5,6 pentakisphosphate (referred to hereon as "IP_5"). This compound has not previously been reported in plant material and so it is very interesting that it is present in such high concentrations here. IP_5 differs from IP_6 through the absence of a single, specific phosphate group on the inositol ring (Figure 3). This breaks the symmetry of the molecule and thus its ^{31}P NMR spectrum consists of five peaks of equal intensity, whereas the six peaks of IP_6 appear in a ratio of

1:2:2:1. Two small spectral features appeared outside of the chemical shift region of the ^{31}P NMR spectra shown in Figure 1: a broad feature at ~0 ppm that can be assigned to DNA and a sharp peak at -5.0 ppm that can be assigned to pyrophosphate.

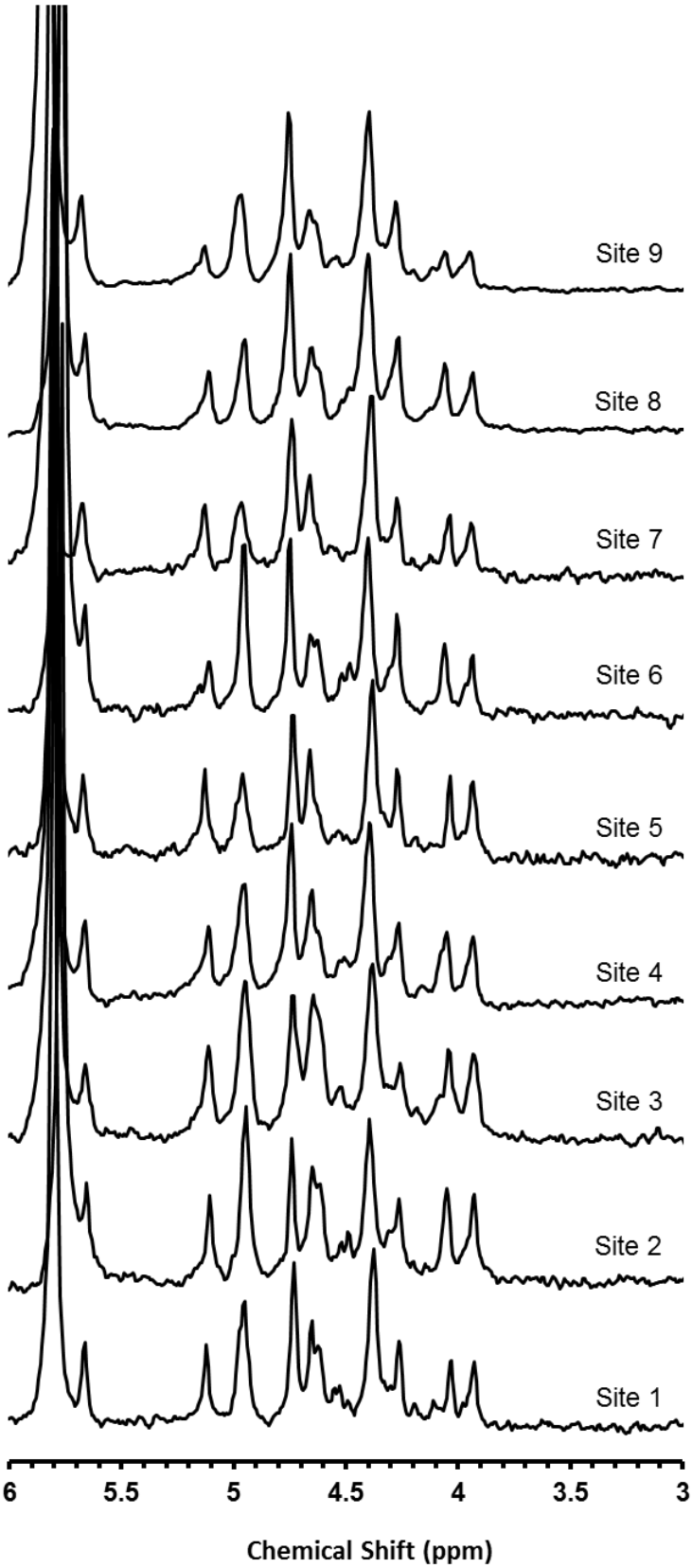


Figure 1. ^{31}P NMR spectra of NaOH-EDTA extracts of canes collected at dormancy from all sites.

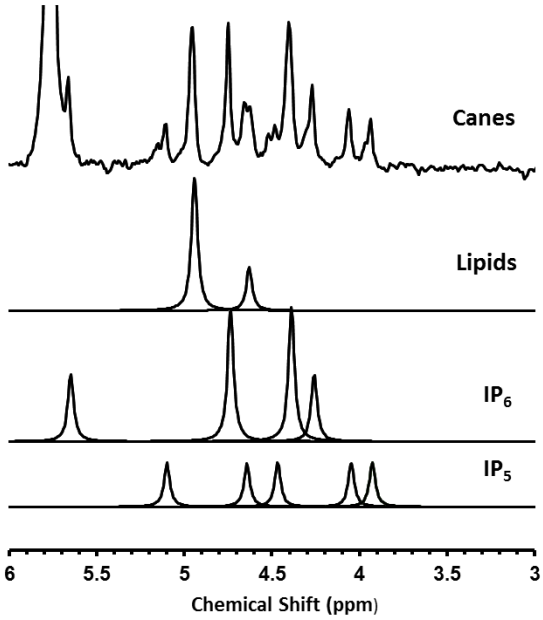


Figure 2. ^{31}P NMR analysis of NaOH-EDTA extracts of canes collected at dormancy from site 6. Lower traces show the spectrum of the compounds assigned as lipids, IP_6 and IP_5 which all correspond to peaks assigned in the original sample.

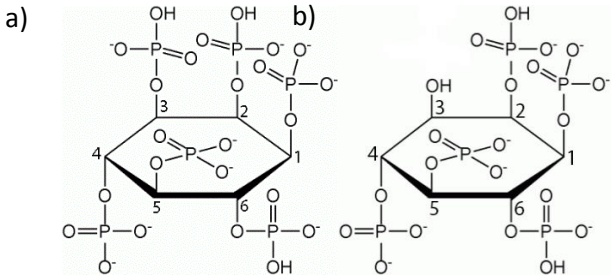


Figure 3. Chemical structures of a) *myo*-inositol hexakisphosphate (IP_6) and b) D-*myo*-inositol 1,2,4,5,6 pentakisphosphate (IP_5).

The concentrations of the six P species detected in the cane samples (orthophosphate, lipid P, IP_6, IP_5, DNA and pyrophosphate) were determined through integration and deconvolution of the spectra and the proportional composition of these materials is presented in Figure 4. Overall, this confirms the general similarity in the composition of cane biomass, with orthophosphate comprising 37-57% of total P, lipids 8-15%, IP_6 20-34% and IP_5 9-25%; DNA and pyrophosphate contribute <1% in all cases. Although differences in P composition among cane samples are relatively subtle, much of the variation that is present appears to be related to the P status of the vines. This is best illustrated in plots of P concentration of the four major components (orthophosphate, lipid P, IP_6 and IP_5) against total P concentration (Figure 5). With increasing total P concentration, the concentrations of orthophosphate and IP_6 increase, while those of lipid P and IP_5 do not. The consistency in lipid P concentration (in terms of absolute concentration in mg P/kg) can be understood in terms of its role in plants: phospholipids are mostly found in cell membranes, which are a vital part of all viable cells, and thus their concentration would be expected to be largely invariant. The other detected components all potentially play a role in P storage in cells. While a baseline concentration of orthophosphate is required for proper cell functioning, additional orthophosphate can be stored in vacuoles, from where it can be readily accessed when required for cellular functions (Mimura 1999). Inositol phosphates are believed to function almost entirely as P storage compounds. Thus it is of no surprise that the concentration of IP_6 increases with increasing total P, while the lack of a similar relationship for IP_5 is perhaps surprising. A possible explanation is that storage of P in canes as inositol phosphate initially occurs as IP_5 but there is limited capacity to store P in this way, resulting in further phosphorylation of IP_5 to IP_6, which builds up most in the highest P status materials. However, it should be noted that the concentration of IP_5 actually does vary widely (> 3-fold among the nine samples), much more so than does the concentration

of lipid P, and this is inconsistent with there being a fixed capacity for storage in this form that is saturated for all of the analysed samples. Clearly further research is needed on the role of this novel and interesting compound.

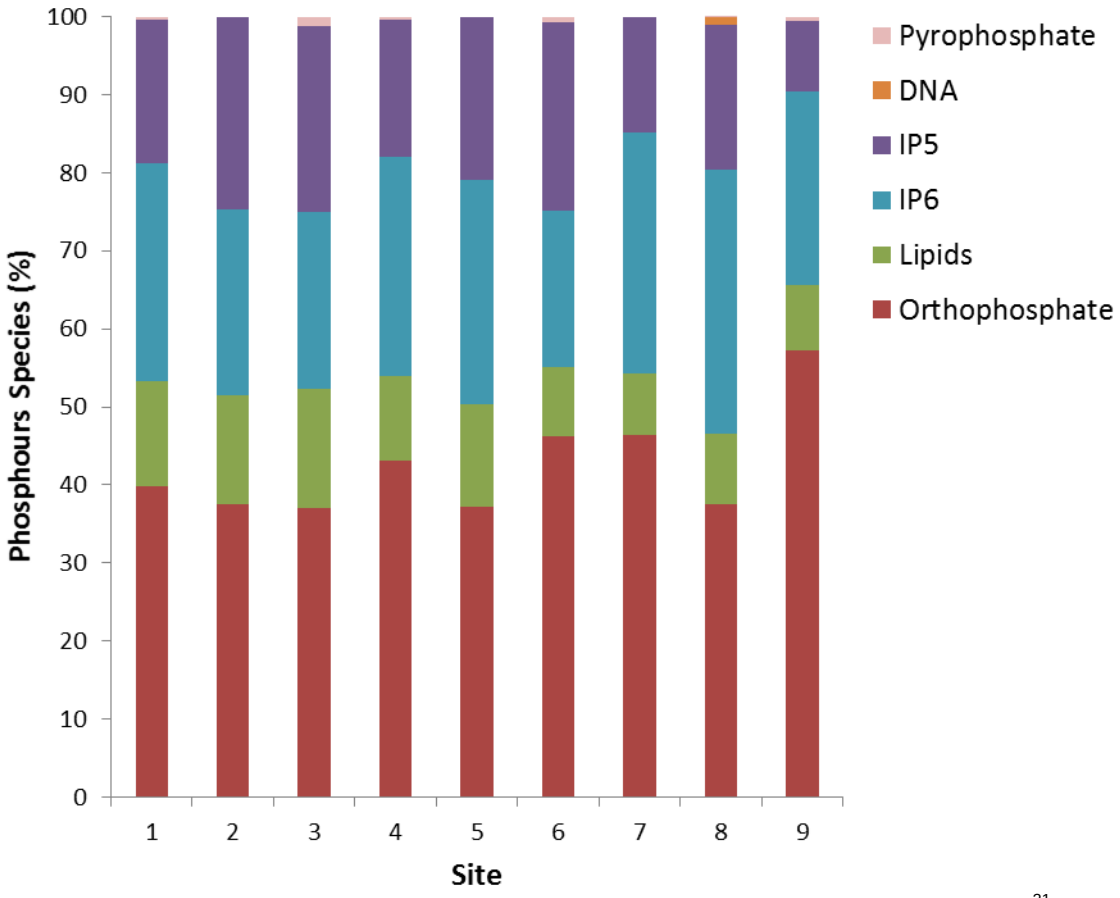


Figure 4. The percentage composition of the six dominant inorganic and organic P species analysed by ^{31}P NMR spectroscopy identified in canes collected at dormancy.

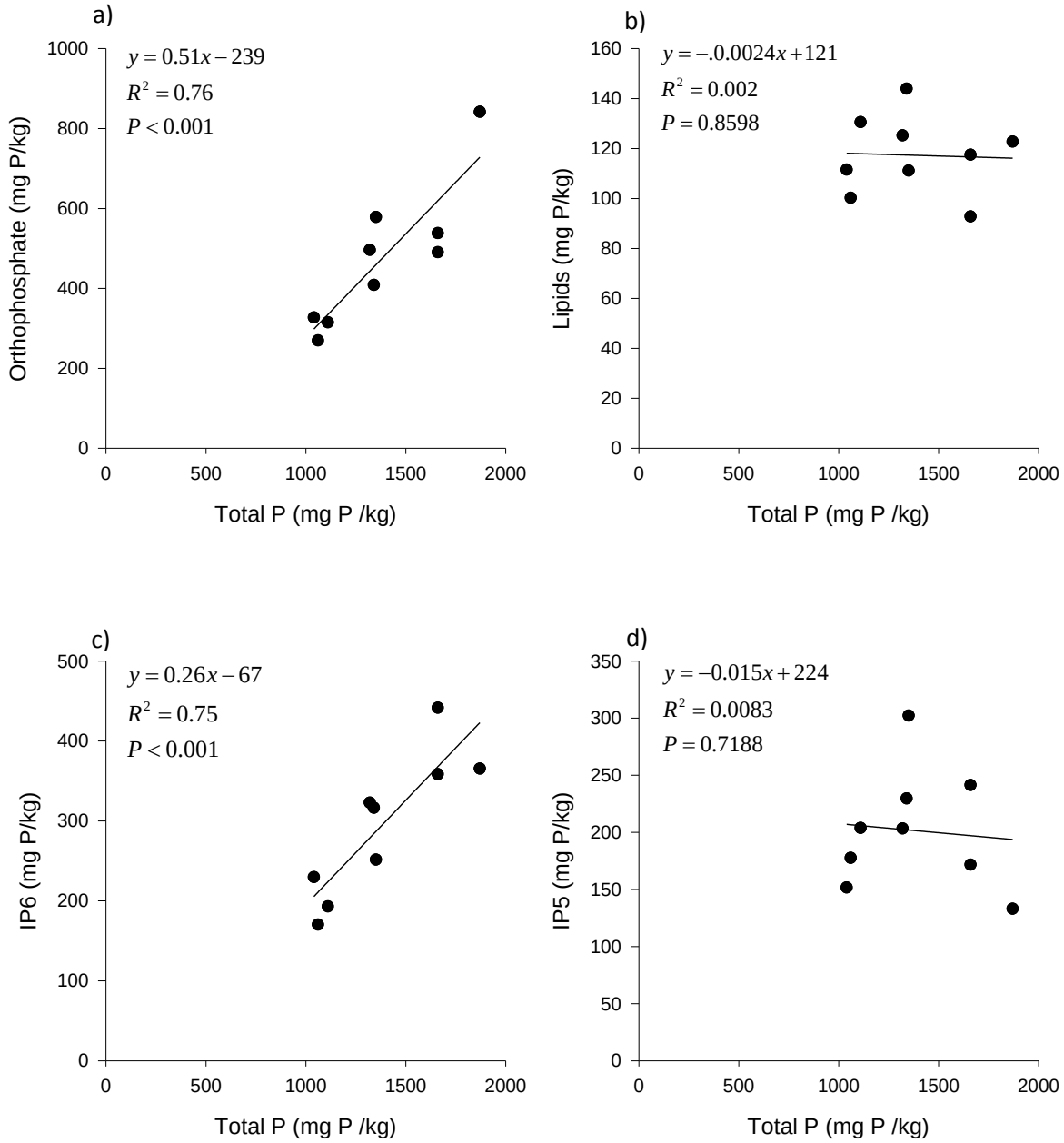


Figure 5. The relationship between total P concentration (plant P status) and the four dominant forms of phosphorus a) orthophosphate, b) lipids c) IP_6 and d) IP_5 in canes collected during dormancy.

There are several implications of these findings on P cycling in vineyards. Of the three main biomass streams (prunings, leaf fall and fruit harvest), prunings are the only one that can be assumed to be returned directly to the adjacent soil in most vineyards. The amount of P involved (~1-6 kg P/ha) is considerable relative to typical rates of P fertilisation (Longbottom 2010; Oag 2001). The chemical composition of this P is roughly a 50:50 combination of directly plant available P (i.e. orthophosphate) and organic P, with the majority of the latter being inositol phosphates, which have the reputation for being relatively persistent in soil. Thus, conventional thinking on P cycling would consider prunings to provide a mix of immediately available P and "slow-release" P, in approximately equal amounts (although the discussion below questions this conventional thinking). The contribution of inositol phosphate (IP_6 + IP_5) to total P (up to 59%) in the dormant cane materials rivals that reported previously for seed (40-80% (Lott, Ockenden *et al.* 2000; Noack, McLaughlin *et al.* 2012; Reddy, Pierson *et al.* 1989)) and contrasts with results for non-seed components of other plant materials (e.g. maximum inositol P content of <45% for crops residues in (Noack, McLaughlin *et al.* 2012)). This is of course is consistent with P being stored in cane biomass over the dormancy period; however, no previous study has reported on P speciation of biomass of deciduous perennials. Finally, the identification of substantial quantities of a specific compound (IP_5) not previously detected in the environment

opens the possibility of using this compound as an indicator of the contribution of grapevine material to soil organic P. This aspect is discussed in detail elsewhere in this report.

By fortuitous circumstance, several grapevines were being removed to install frost fans at site 5 while sampling in late July 2014 and this provided an opportunity to collect samples of root and trunk material. The trunk material was divided radially into inner (vascular tissue) and outer (periderm or bark) sections and analysed for total P by elemental analysis and P composition by ^{31}P NMR analysis. The P concentration was 230 mg P/kg for the inner section and 720 mg /kg for the outer section. The P composition of the inner trunk material was quite similar to that of the dormant canes, with the dominant species being orthophosphate (47%) and inositol phosphate (IP_5 11% and IP_6 30%), with phospholipid comprising 12% of total P (Figure 5a). Interestingly, this material contained considerably more IP_5 than IP_6, in contrast to the cane material for which IP_6 was always the more abundant inositol phosphate (Figure 4). The P composition of the outer trunk material was quite unlike that of the inner trunk material in that in contained no detectable inositol phosphate; phospholipid was the most abundant P species detected (42%), followed by orthophosphate (30%), pyrophosphate (15%) and RNA (13%). This makes the P composition of the outer trunk material most similar to that of leaves (see below). The root material was divided into coarse and fine fractions, with the fine root material having a lower P concentration (1970 mg P/kg) than the coarse root material (2400 mg P/kg). The P composition of the coarse roots was quite similar to that of the inner trunk, with orthophosphate (48%) and inositol phosphate (IP_5 14% and IP_6 34%) dominant and phospholipid comprising 4% of total P (Figure 6). As for the inner trunk, the coarse roots contained considerably more IP_5 than IP_6. The composition of the fine root material differed from that of the coarse root and was mainly richer in orthophosphate and poorer in inositol phosphate, especially IP_5. Based on analysis of material collected from this one site, it would appear that the composition of P in the trunk and roots of grapevines differs from that in other parts. Intriguingly, IP_5 seems particularly prevalent in woody material. However, further research is needed to confirm these findings.

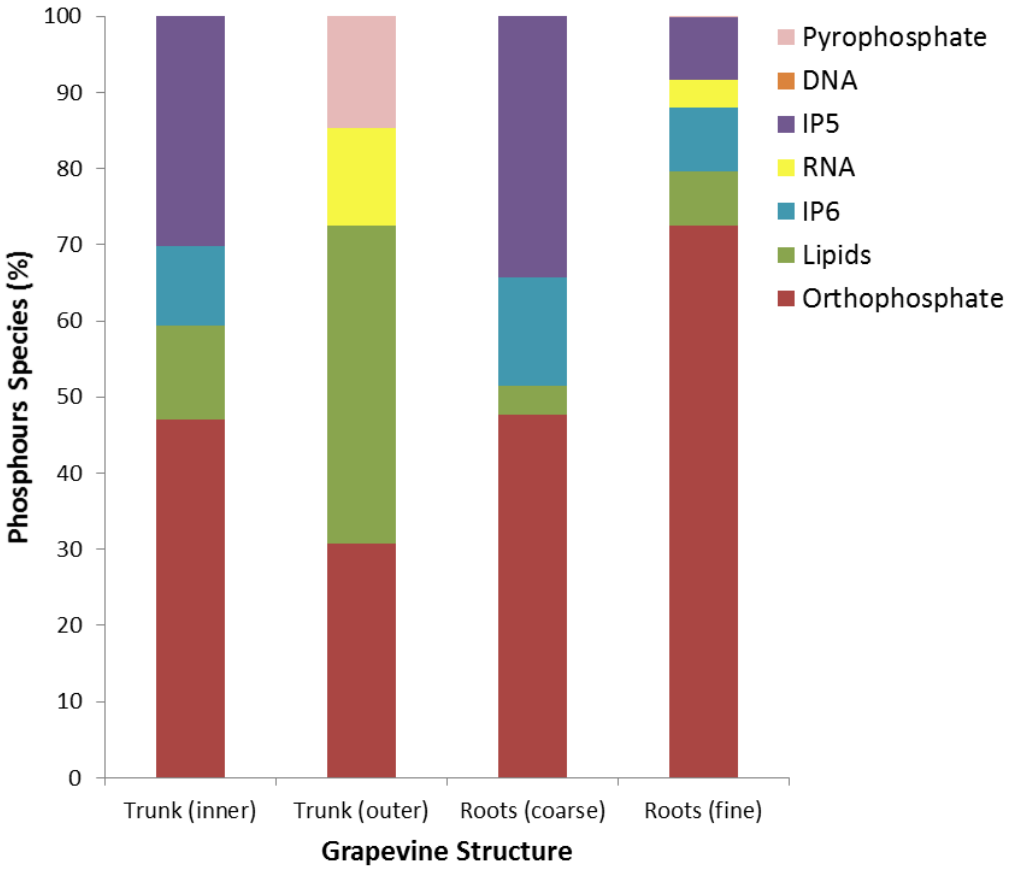


Figure 6. The percentage composition of the seven dominant inorganic and organic P species analysed by ^{31}P NMR spectroscopy identified in grapevine trunk and roots collected at dormancy from site 5.

6.2 Seasonal partitioning of P within the grapevine structure

The seasonal cycle of grapevine growth involves the shooting of new growth in spring, followed by flowering, fruit development and ripening through summer and finally senescence in autumn. During this cycle, three distinct aboveground plant parts develop and mature: shoots, leaves and fruiting bodies. Although previous studies have investigated the flow of P to and among these plant parts (Conradie 1981a; Conradie 1981b; Pradubsuk and Davenport 2010; Schreiner, Scagel *et al.* 2006), until now no study has linked these flows of total P to changes in P speciation. Analysis by ^{31}P NMR spectroscopy of materials collected at five sampling times (dormancy, flowering, veraison, harvest and leaf fall) and divided into three plant parts (shoots, leaves and fruiting bodies) provides this extra dimension to understanding P transformation in vines over a season. Vines from three sites (Sites 1, 5 and 8) were selected for this part of the study. These were chosen from the nine sites originally sampled to represent, respectively, sites of low, moderate and high P status.

The total P concentration of the biomass samples varied greatly according to plant part, sampling time and vine P status (Table 2). The P concentration in shoot material was highest at flowering, when the new shoots were green and supple. By veraison, the concentration of P in the now woodier shoots had more than halved, after which further small decreases in the P concentration were recorded. These decreases in the P concentration of shoots were more than matched by increases in biomass of this plant part, at least until harvest, resulting in increases in the total quantity of P in shoots until this time. The P concentration in leaves followed a similar pattern, being greatest at flowering and generally decreasing through the season. However, for the leaves, there was also a substantial decrease in P concentration at senescence, resulting in a net movement of P out of leaves by the final sampling time. Nonetheless, an estimated 0.9-2.5 t/ha of P was lost from the vines in senesced leaves in autumn. Interestingly, the same pattern of P concentration was also recorded for fruiting bodies, with much higher values for flowering parts than the immature fruit (i.e. at veraison) or mature fruit (i.e. at harvest). Again, it should be noted that these decreases in concentration are more than compensated for by increases in biomass. Removal of the fruiting bodies at harvest resulted in removal of 3.5-8.0 t/ha of total P.

Table 2. The seasonal variation in P concentration, biomass and P content of shoots, leaves and fruiting bodies throughout the 2014-15 growing season at three sites chosen to represent low, moderate and high P status.

Plant Material	Sample timing	Total P (mg P/kg)			Biomass (t/ha)			Plant P content (kg P/ha)		
		Low	Moderate	High	Low	Moderate	High	Low	Moderate	High
Canes/Shoots	Dormancy	1040	1340	1660	2.0	2.8	1.0	2.1	3.8	1.6
	Flowering	2670	4770	5230	0.4	0.2	0.4	1.0	1.0	2.2
	Veraison	881	1380	2220	1.1	1.8	1.0	1.0	2.5	2.2
	Harvest	758	1294	2678	2.5	2.7	1.4	1.9	3.5	3.6
	Leaf Fall	868	1248	1571	3.1	2.3	2.3	2.7	2.8	3.6
Leaves	Flowering	3240	5450	7290	0.5	0.3	0.5	1.7	1.7	3.9
	Veraison	1570	2000	3290	1.4	2.1	1.4	2.1	4.2	4.5
	Harvest	1343	1841	4561	1.4	2.1	1.1	1.9	3.9	4.9
	Leaf Fall	639	1187	1496	1.4	2.1	1.1	0.9	2.5	1.6
Flowers/fruit	Flowering	4400	5340	7160	0.2	0.1	0.1	0.7	0.6	0.7
	Veraison	1510	2060	2930	1.6	1.0	0.7	2.3	2.0	2.1
	Harvest	921	1547	2670	3.8	3.4	3.0	3.5	5.3	8.0

On the whole, ^{31}P NMR spectra of the various plant parts (not shown) identified similar P species as reported for the cane material sampled at dormancy. However, in some cases, little or no inositol phosphate (IP_6 and IP_5) was detected and this enabled clear identification of peaks derived from RNA that was otherwise obscured. This is illustrated in Figure 7, which shows the ^{31}P spectrum of leaves sampled at harvest at site 1. The assignment of six peaks as RNA-derived compounds (mononucleotides) is based on a previous study of

the alkaline decomposition of RNA and pea straw (Smernik, Doolette *et al.* in press). Some of these peaks can be seen in the ^{31}P NMR spectra of the dormant cane material (Figure 1), but severe overlap with more intense inositol phosphate peaks made it impossible to quantify RNA in those samples.

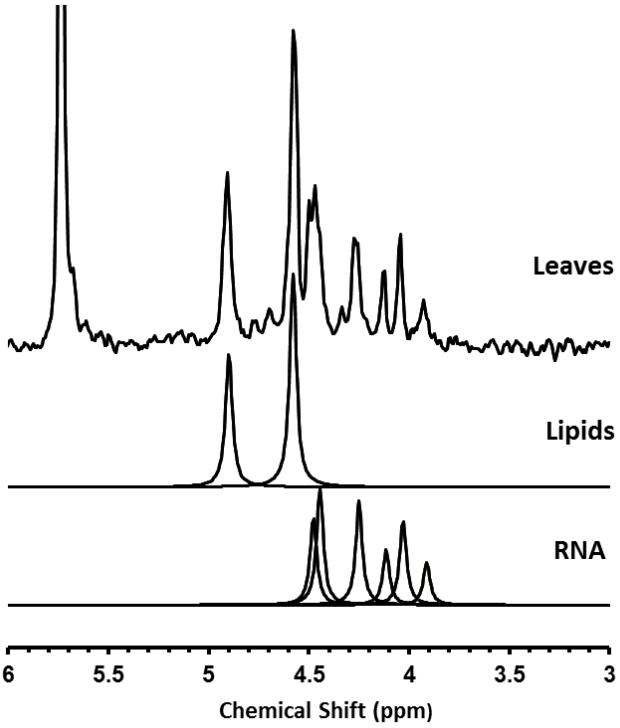


Figure 7. ^{31}P NMR analysis of leaves collected at harvest from site 1. Lower traces show the spectra of the compounds assigned to lipids and RNA which corresponds to peaks assigned in the original sample.

The P composition of the three classes of grapevine biomass over a growing season, as determined by quantification of their ^{31}P NMR spectra using integration and deconvolution, varied greatly (Figure 8). For shoot material, orthophosphate was the dominant component at flowering (80-90% of total P), but decreased through the season (37-51% of total P at leaf fall), with the concentration of inositol phosphate species (IP_6 and IP_5) exhibiting the opposite trend (i.e. increasing from very low levels at flowering to representing approximately 50% of P in shoots at leaf fall). In contrast, the P composition of leaf material changed less over the season. The majority of P in leaves was identified as orthophosphate, lipid and RNA, with IP_6 comprising <10% of total P in almost all cases and IP_5 only detected in one sample. This is generally consistent with expectations, as P in leaves would be expected to be present in forms with a biochemical role rather than a storage role. In fact, it could be argued that the presence of any inositol phosphate in leaves is unexpected; in particular it seems peculiar to find IP_6 in leaf material collected at senescence, as one might have expected the plant to withdraw all available P resources from leaves before they are senesced. The P in the fruiting bodies was dominated by two species in all samples analysed: orthophosphate and IP_6. The proportion of total P present as IP_6 was consistently highest at veraison, i.e. it was higher in immature fruit than flowers or mature fruit. This may be because seeds contain the highest proportions of IP_6 and seed development precedes fruit ripening. Overall, the influence of P status on P composition is minor relative to the differences between plant plants and sampling times discussed above. Nonetheless, in general, the proportion of P present as orthophosphate appears to be slightly higher in plants with higher P status.

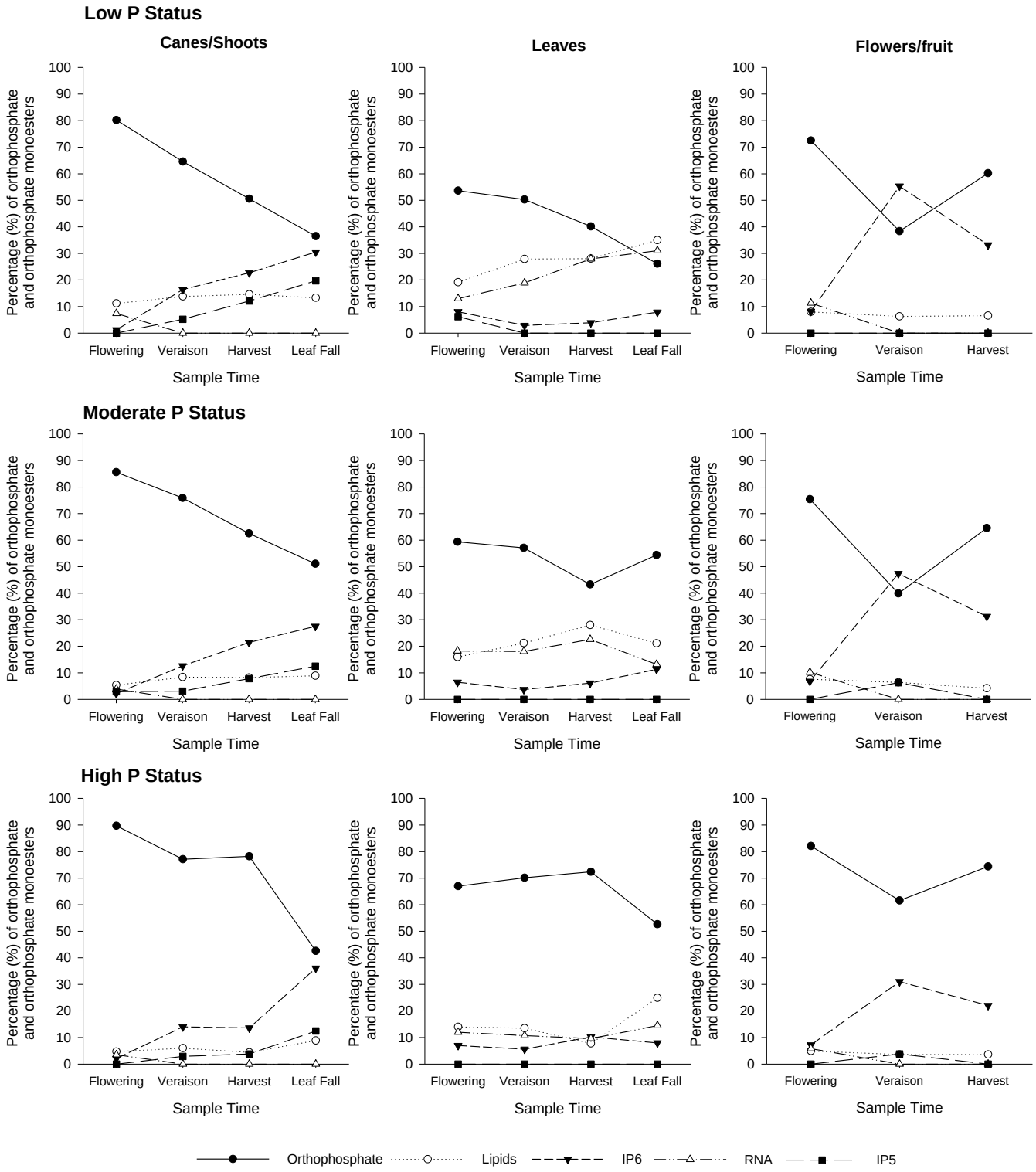


Figure 8. The percentage composition and seasonal partitioning of the dominant forms of P identified in the canes/shoots, leaves and flowers/fruit at three sites representing low, moderate and high P status during the 2014-15 growing season.

Several key implications can be drawn from this part of the study. First, the composition of P varies considerably between plant parts and over the season. The results reported here provide the most detailed and comprehensive picture of P transformations in any plant system and should facilitate a more detailed understanding of this aspect of plant physiology not only in grapevines, but, if applied more broadly, to other plant species. More specifically, the results demonstrate a dominance of “biochemical P” species (orthophosphate, lipid P and RNA) in leaves that contrasts with a dominance of “storage P” species (orthophosphate, IP_6) in fruiting bodies, with shoot material transitioning from “biochemical P” dominance to

“storage P” dominance through the season. This emphasises the flexibility of grapevines to manage their P resources. Applying these ideas to a consideration of the three main biomass streams in vineyards (prunings, leaf fall and fruit removal) provides several key insights. Of these streams, leaf fall represents the smallest losses (13-24%) and produces a relatively high C:P material in which the majority of P is present as orthophosphate and very labile organic P species (phospholipid and RNA). Although in theory P present in leaf fall is returned to the vineyard soil, in practice senesced leaves tend to be blown around the vineyard so the rate of return of this P to the soil is variable. Prunings represent a bigger proportion of P loss (27-38%) that is richer in potentially less labile organic P (i.e. inositol phosphates) and which is much more likely to remain close to the plants from which it is removed, although this will depend on management. Harvest of fruit represents the largest P loss (49-61%), the composition of which is richest in potentially less labile organic P. Removal of this component is essentially permanent, but may be offset by the return of marc to the vineyard. This aspect is addressed further below.

6.3 Phosphorus speciation and composition of grape marc and stalk

As discussed above, removal of fruit at harvest represents the largest loss of P from the vine system. However, it should be noted that the discussion above relates to removal of stalk as well as fruit, as would occur with manual picking. During mechanical picking, only the fruit is removed, while the stalk remains on the vine. This distinction is also important when considering the return of grape marc to vineyards, as marc is derived from the fruit and not the stalk component of the fruiting bodies; the stalk material of manually harvested grapes is separated before pressing and this is generally not returned to vineyards.

Analyses were carried out on separated fruit and stalk material collected at harvest from sites 1 (low P status) and 8 (high P status). The P concentration of the berry and stalk were similar at the low P site, equating to 984 mg P/kg and 1055 mg P/kg for the berry and stalk respectively. The P concentration in berries at the high P sites was two times greater (2258 mg P/kg), and the stalk P concentration six times greater (6252 mg P/kg) than at the low P status site. The P composition of the berries and stalks differed, but overall composition for each individual vine structure was similar regardless of concentration, with 27-30% of the P in the berries present as inositol phosphate (primarily IP_6). The stalks contained no inositol phosphate at the low P site and little inositol P (<5% of total P) at the high P site (Figure 9). Overall, the P composition of the whole fruiting bodies (berry + stalk) more closely reflected the composition of the berries than that of the stalk; this is consistent with the fact that the berries represent the majority of biomass in the fruiting bodies.

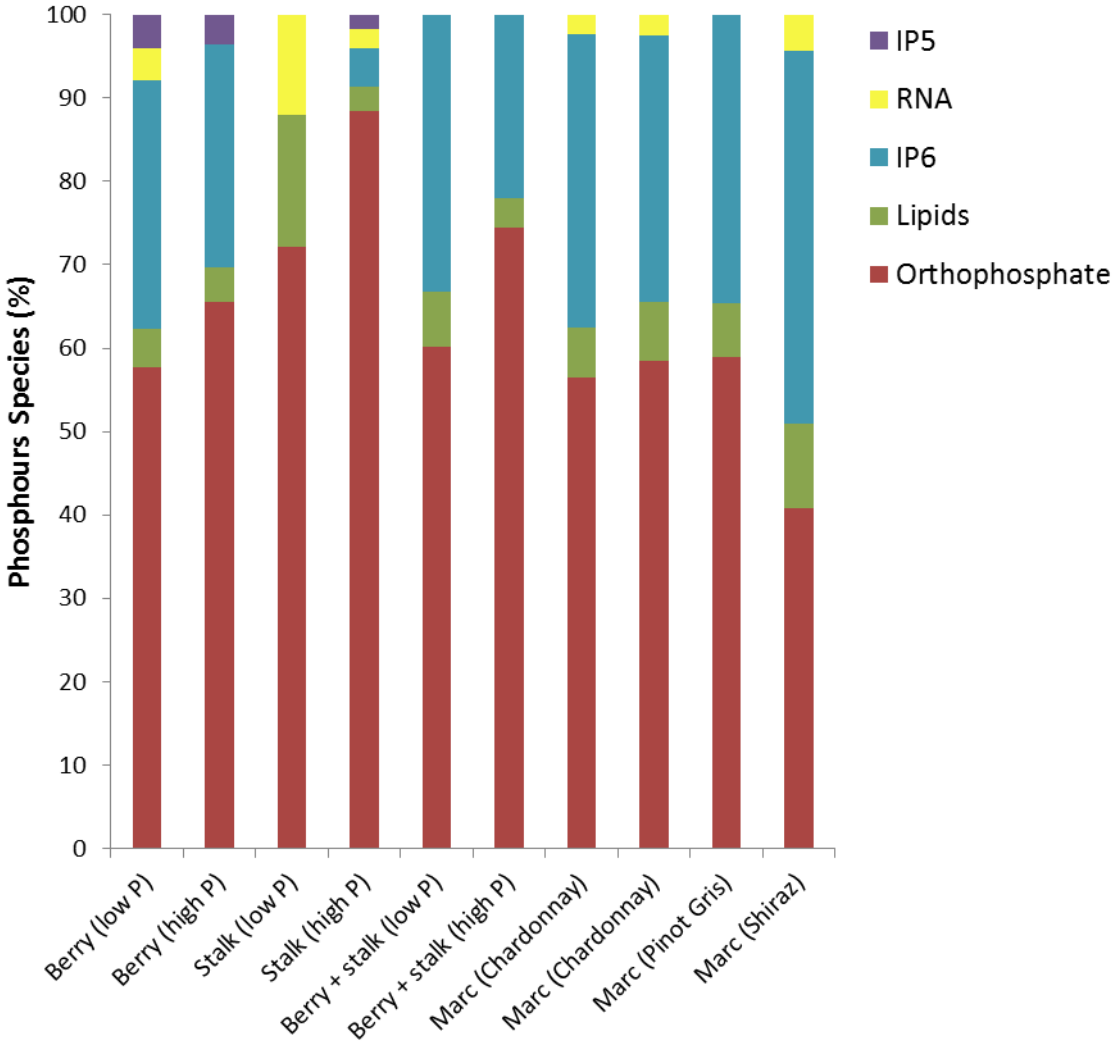


Figure 9. A comparison of the percentage distribution of P species in the fruiting bodies with separated into individual structures.

The composition of P in grape marc is expected to reflect that of the berries since grape juice contains relatively little phosphorus. It was not possible to collect and analyse the grape marc from each site following pressing. However, four fresh grape marc samples from the Barossa Valley and McLaren Vale grape growing regions were analysed, and the P composition of these grape marcs was in general consistent with that of the berries analysed, with orthophosphate (37-56%) and IP_6 (31-41%) the dominant P species present (Figure 9).

6.4 Effect of aging on P composition of grapevine biomass streams

While the main purpose of this study was to determine the composition of P in the key biomass waste streams (prunings, leaf fall and harvested fruiting bodies), the ultimate goal is to understand how management of these streams influences the recycling of P in vineyard systems. Thus while it is crucial to understand the P composition of the fresh waste streams, it is also important to understand the way in which P is transformed in these materials as they age. A number of opportunities arose during this study to start to address this aspect and the results are addressed briefly below.

At site 7, a stockpile of grape marc and stalks from the previous season, which had been aging in the field for approximately 6-7 months, was sampled in July 2014. Comparison of the ^{31}P NMR spectrum of this material to that of fresh marc suggests some changes on aging, with peaks for RNA and phospholipid more prominent in the aged material. This is consistent with microbial decomposition of IP_6, as has been reported previously both on stockpiling of chicken manure (Peirce, Smernik *et al.* 2013) and on the addition of chicken manure (Hill and Cade-Menun 2009) and IP_6 directly to soil (Doolette, Smernik *et al.* 2010). Similarly, a

comparison of the fresh stalk with aged stalk also suggests some changes, namely an increase in RNA on aging, but these changes appear to be minimal compared to those of the grape marc. A greater degree of change in P composition is seen when comparing fresh prunings (i.e. freshly sampled from the vine) collected at Site 1 with prunings collected from the ground at the same site that had presumably been there for one year (Figure 10). The year-old prunings contained much less IP_6 and no detectable IP_5. In contrast, peaks for phospholipids and RNA were larger for the aged prunings, again indicating microbial decomposition of inositol phosphate and synthesis of phospholipid and RNA. Most intriguing is the appearance of new peaks at 5.2-5.4 ppm in the ^{31}P NMR spectrum of the year-old prunings; these species were not detected in any of the fresh vine biomass samples. The presence of these new P species along with a greater degree of change may reflect a longer residence time in the vineyard compared to the 6-7 months the grape marc and stalk were stockpiled. It may also reflect more specific interactions when in contact with the soil.

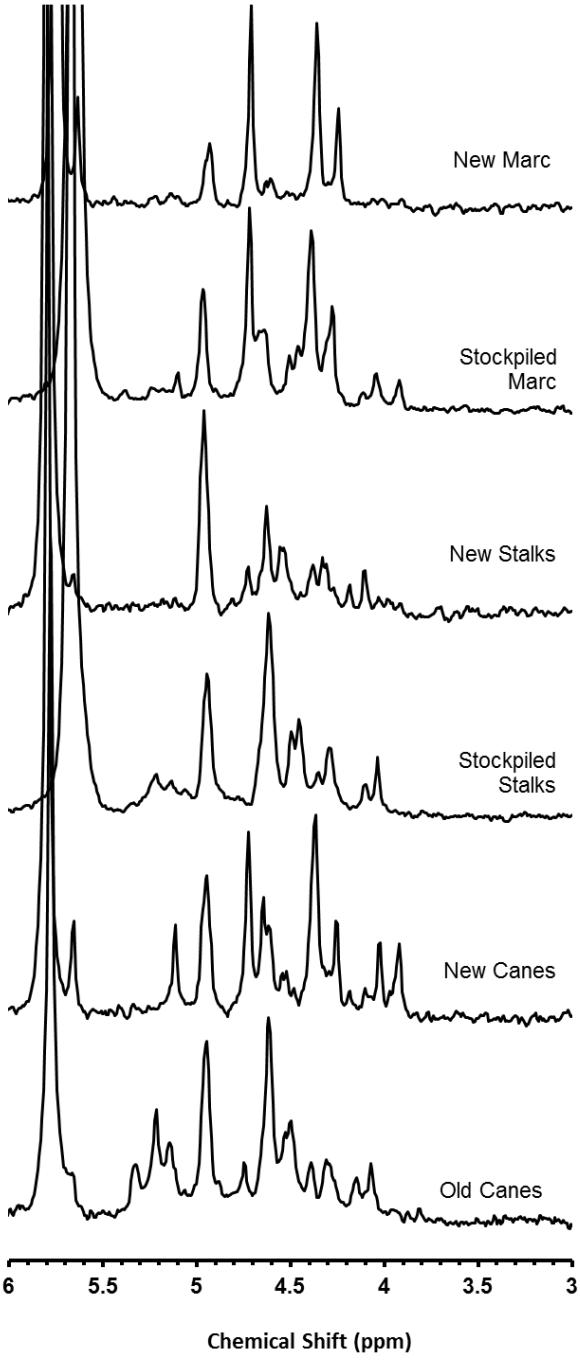


Figure 10. ^{31}P NMR spectra comparing fresh and aged materials of grape marc, stalk and canes.

Ultimately, when biomass is returned to the soil it is added to the soil P already there. On an annual basis, this contribution is small relative to size of the soil P pool. For example, the sum of P in prunings, leaf fall and harvested fruit estimated in this study (5.2-9.36 kg/ha, Table 1) represents around 0.1% of total P in the top 10 cm of a typical soil with a total P concentration of 500 mg P/kg and a bulk density of 1.5 t/m^3. However, given that vines have been present at each of these sites for decades, one would predict that if any of the specific P forms present in vine tissue are resistant to microbial degradation and hence contribute substantially to the organic P pool in soil, these should be detectable in the ^{31}P NMR spectra of the soils themselves. However, this appears not to be the case, with the ^{31}P NMR spectra of the grapevine soils (Figure 11) being very similar in appearance to the ^{31}P NMR spectra soils under other land uses, including cropping and pasture (Doolette, Smernik *et al.* 2011; McLaren, Smernik *et al.* 2014; Moata, Smernik *et al.* 2015). In particular, the main organic P species in the soils appears to be "humic P", a term used to describe organic P in large, polymeric organic structures and which gives rise to the broad "lump" that extends between about 3.5 ppm and 5.5 ppm in the soil spectra (Figure 10). The only "sharp" spectral features that can be attributed to organic P in all of the soil spectra are those of α- and β-glycerophosphate (5.0 ppm and 4.6 ppm, respectively). These indicate the presence of phospholipid at relatively low concentrations; microbial biomass is a likely source of this phospholipid. Very low amounts of inositol phosphate, in the form of IP_6, are indicated at Sites 1 and 2. Thus it would appear that the specific organic P compounds found in vine biomass, especially IP_5, which has not been reported previously, are efficiently recycled in these soils and that the large stores of organic P in these soils are therefore synthesised by a process unrelated to the vines themselves.

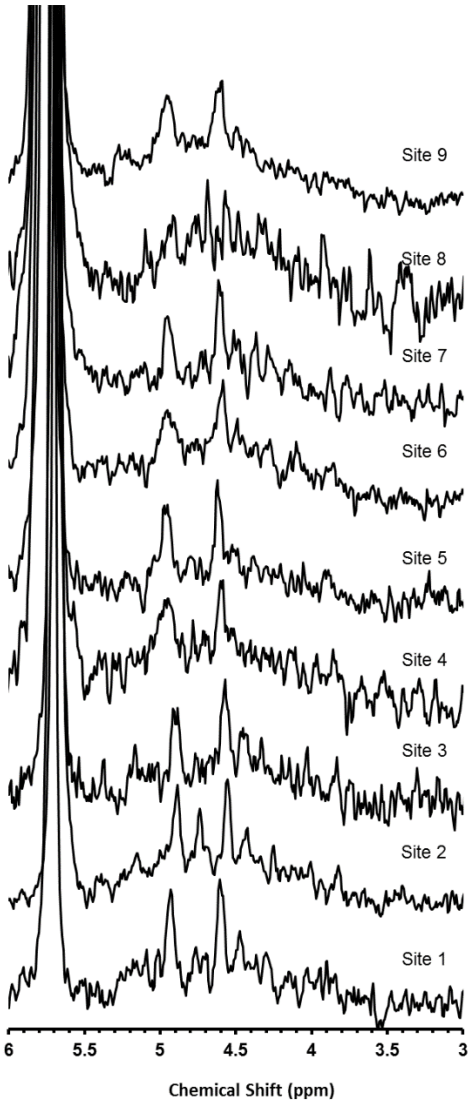


Figure 11. ^{31}P NMR spectra of NaOH-EDTA soil extracts collected from sites 1-9, corresponding with the same sites selected to sample canes at dormancy. Soil samples were also collected at dormancy 2014.

7. Conclusions

This project substantially enhances our understanding of phosphorus transformations in grapevines, and is one of the first of its kind to examine the phosphorus composition of the major biomass waste streams of pruning, senescent leaves and harvest fruits.

Of the three waste biomass streams, the harvested fruit represented the largest P removal pathway, most of which was contained in the grape marc, and was a mixture of immediately available (orthophosphate) and potentially recalcitrant (inositol phosphate) forms. Since the majority of vineyards don't currently return the marc to the vineyard this pathway represents the only permanent loss of P from the system and any potential P benefit is not currently being realised. Prunings represent the second largest phosphorus removal pathway and again P is present as a mixture of immediately available (orthophosphate) and potentially recalcitrant (inositol phosphate) forms. Analysis of prunings that had been in contact with the soil for one year provided evidence for microbial decomposition and that substantial changes in P composition occur over that timescale. Further, prunings are more likely to remain close to the vine from which it is removed so any P recycled is likely to provide a direct benefit to the same vine. Senesced leaves represented the smallest P removal pathway and in contrast to the harvested berries and prunings, contained mostly immediately plant available forms of phosphorus. However, since leaves are often blown around the vineyard, the phosphorus contained in these leaves is not necessarily recycled efficiently to the soil, thus management of this pathway is complicated.

The phosphorus status of the grapevines is also an important consideration because it was shown to consistently influence not only the phosphorus content of the biomass waste streams, but also its composition, with higher phosphorus status vines generally proportionally richer in both orthophosphate (immediately available) and inositol phosphate (stable/slow released P) and proportionally poorer in phospholipid and RNA (readily available/labile P).

Overall, the amount of P that can be potentially returned to the vineyard was considerable relative to the current fertilisation rates and could be a direct benefit to growers, but will be high highly dependent on management. However, more research is required to understand the transformations that occur when the grapevine biomass is added to the soils to enable a better understanding of both the economic and environmental benefits.

8. Recommendations

The research undertaken in this study was a part of the AGWA Incubator Initiative, a scheme for short-term projects (12 months) in which the aim was to generate fundamental knowledge and research capacity that could be to the long-term benefit of the industry. This project has substantially increased our understanding of phosphorus transformations in grapevines and provided growers with an indication of the potential phosphorus benefits that could arise through optimal management of the main waste biomass streams. However, further research will be required to test the generality of these findings across other regions and grapevine varieties. In addition, to continue to develop our understanding of phosphorus cycling in vineyards and wine production, further research into the two following areas is recommended.

- The presence of IP_5, a compound that has not been widely reported in any other plant material, clearly requires further research. In particular the role of this compound within the grapevine and the reason for its prevalence in woody materials needs to be investigated.

- While this study has demonstrated the importance of understanding the P composition of the fresh and aged biomass streams, future research will also be required to understand the way in which P is transformed when the plant biomass is added to the soil. While it appeared that plant biomass was efficiently recycled in the soil, the results showed little or no evidence to support the proposition that organic phosphorus added in biomass accumulated in the soil. Thus more research is required to improve the prediction of the fate of biomass P within the soil and the mechanisms controlling this process.

Appendix 1: Communication

On two occasions the project proposal and preliminary results and final project overview have been presented to the Wine Future Advisory Board. Preliminary results were presented at the University of Adelaide School of Agriculture, Food and Wine Research Day.

A “Researcher in Focus” article for *Grapegrower and Winemaker* (*Issue: October 2014*) magazine has been published highlighting the objectives of this project.

Due to the short term nature of this project (12 months) it is anticipated that at least two papers arising from this project will be published in peer-reviewed journals in the following months as well as future presentations focusing on engagement with the industry. Further, extension materials such as facts sheets could be developed from this project and made publically available.

Appendix 2: Intellectual Property

No intellectual property has been identified for this project. Much of the work contained in this report is currently being prepared for publication, thus future use of tables and figures may require copyright permission to use.

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Appendix 4: Associated Staff and Acknowledgements

Dr Ashlea Doolette
School of Agriculture, Food and Wine
The University of Adelaide
SA 5005

Dr Ronald Smernik
School of Agriculture, Food and Wine
The University of Adelaide
SA 5005

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