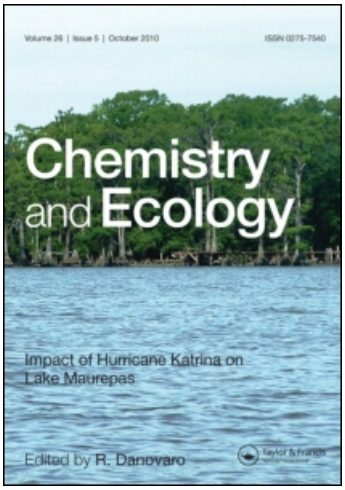

Chemistry and Ecology

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

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To cite this Article Mcdowell, R. W. and Stewart, I.(2005) 'Peak assignments for phosphorus-31 nuclear magnetic resonance spectroscopy in pH range 5-13 and their application in environmental samples', Chemistry and Ecology, 21: 4, 211 — 226
To link to this Article: DOI: 10.1080/02757540500211590
URL: http://dx.doi.org/10.1080/02757540500211590

Chemistry and Ecology
Vol. 21, No. 4, August 2005, 211–226

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Peak assignments for phosphorus-31 nuclear magnetic resonance spectroscopy in pH range 5–13 and their application in environmental samples

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(Received 23 March 2005; in final form 9 May 2005)

Phosphorus-31 nuclear magnetic resonance (NMR) spectroscopy has become popular for the characterization of P species in environmental samples. However, these are commonly made alkaline (pH > 13) to facilitate sample comparison and ease peak identification, but this may cause hydrolysis of some compounds. This study examined the chemical shift of known P compounds and supplemented this with published data to determine the viability of examining samples at their native pH, thereby minimizing sample disturbance. A ^{31}P NMR pH titration of known P compounds resulted in chemical shifts ranging from about −22 to 8 ppm in the pH range 5–13. Categorization and calculation of chemical shifts for over 100 naturally occurring compounds indicated that good distinction between orthophosphate diesters, orthophosphate monoesters, nucleotides, phosphonates, and phosphagens was best at ≥pH 7, but unlikely below this pH. Analysis of several water extracts of soil and dung, overland flow samples, and lake water indicated a wide variety of well-defined peaks that were assigned to orthophosphate, orthophosphate monoesters, orthophosphate diesters, pyrophosphate, polyphosphate, or phosphonates. Changing the sample pH to >13 caused many species (such as phosphonates, orthophosphate diesters, and polyphosphates) to decrease either by hydrolysis or precipitation. Hence, it is recommended that samples be analysed at their native pH but, if poorly resolved, should have their pH raised to ≥7.

Keywords: Orthophosphate; Monoesters; Diesters; Pyrophosphate; Overland flow

1. Introduction

Knowledge of the dynamics and forms of phosphorus (P) in terrestrial and aquatic environments is essential for the maintenance of productive and environmentally sustainable agricultural and undisturbed ecosystems. Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectroscopy is a technique that has been used to characterize P in soil and lake water for many years [1–3]. The method provides the user with a great deal of quantitative information on the form(s) of P. At present, most NMR studies examine P compounds in alkaline solutions usually of 0.1–0.5 M NaOH either with or without EDTA. Several workers have used

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Chemistry and Ecology
ISSN 0275-7540 print/ISSN 1029-0370 online © 2005 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/02757540500211590

known P compounds to determine chemical shifts and the likely origin of peaks in spectra of alkaline extracts of soil. Perhaps the most complete study was that of Turner *et al.* [4]. Later work by Turner [5] also positively identified the occurrence of phytic acid in alkaline extracts of animal manures. Similarly, Hupfer *et al.* [6] identified polyphosphates in alkaline extracts of lake sediments, and were able to calculate chain length.

However, while alkaline extracts aim to extract and emphasize the total organic P fraction, they also present a number of problems. For one, an alkaline extract may not be the most bioavailable fraction to study. It is unlikely that the distribution of P forms extracted from soil by NaOH will yield much quantitative information on the bioavailability of P species in overland flow, even if they are correlated to one another [7]. Furthermore, both alkaline and acid extractions of soil or sediment cause hydrolysis of P compounds and change the distribution in the sample. It is well known that orthophosphate diesters are particularly susceptible to such degradation. For instance, Leinweber *et al.* [8] used degradation to explain the decrease in diester concentration with increasing alkalinity of soil and manure extracts.

With advances in spectrometer technology and sensitivity, the need for vigorous extractants to meet machine detection limits is decreasing. Furthermore, if necessary, pre-concentration techniques now enable changes to be minimized or, if changes occur, then their impact gauged. Consequently, we are now able to routinely examine P in samples such as lake waters and soil solution [3, 9–12]. Unfortunately, most of these studies have altered the pH to >13 following the advice of Crouse *et al.* [13] who recommend that extracts be higher than pH 12.5 to maximize peak delineation, minimize line-broadening, and provide a standard pH so literature values can be used for peak assignments. However, examination of spectra presented by Crouse *et al.* [13] suggests that P compound classes may be sufficiently resolved over a wider range of pH than just >12.5. Indeed, Adams [14] reported sharp peaks and quantitative spectra of neutral extracts of forest soils. Consequently, our objective was to report the chemical shifts of a number of known P compounds at different pH values, to delineate the range(s) for compound classes where peak overlap is unlikely and to demonstrate the utility of the peak assignments with several samples including lake water, overland flow, drainage water, and water extracts of soil and manure.

2. Materials and methods

2.1 *Standard P compounds*

Known P compounds were selected from classes currently identified in ^{31}P NMR spectra. The three inorganic P compounds used were orthophosphate (KH_2PO_4), pyrophosphate – *tetra*-Na salt ($H_4O_7P_2$), and polyphosphate – Na salt ($H_{2n}O_{3n+1}P_n$). The four orthophosphate monoesters used were *myo*-inositol hexakisphosphate (phytic acid, $C_6H_{18}O_{24}P_6$), α-D-glucose 1′ phosphate ($C_6H_{13}O_9P$), D-glucose 6′ phosphate ($C_6H_{13}O_9P$), and guanosine 5′ monophosphate ($C_{10}H_{14}N_5O_8P$). The three orthophosphate diesters used were DNA (from herring sperm), L-α-glycerophosphoserine (from bovine brain, $C_8H_{12}NO_{10}P$ (two variable hydrophobic fatty acyl chains)), and guanosine 3′ 5′ cyclic monophosphate ($C_{10}H_{12}N_5O_7P$); phosphocreatine ($C_4H_{10}N_3O_5P$). The two aromatic esters used were *para*-nitrophenyl-phosphate ($C_6H_6NO_6P$), and bis *para*-nitrophenyl phosphate ($C_{12}H_9N_2O_8P$). All chemicals were sourced from Sigma Chemicals (St. Louis, MO).

Nine replicate samples of each compound were made up to a concentration of about 50 mM of known P compound in reconstituted overland flow from a Waitahuna silt loam soil under pasture (500 mL of overland flow was freeze-dried and reconstituted with 4 mL of distilled water and 1 mL of D_2O; table 1). Just prior to analysis by NMR spectroscopy, the pH of one

Table 1. Concentration (mg L^{-1} in solution before freeze-drying) and percentage in parentheses of P compound classes in the spectra of sheep and cow-dung water extracts and overland flow*.

Sample name	Site description and sampling regime	Total P* (P/[Fe + Mn])	pH (*I*)
Water extracts of dung from animals grazing pasture			
Sheep and deer	Samples were collected fresh within an hour of deposition and 3 g (dry wt. basis) extracted for 30 min in 150 mL water before centrifuging (5000 × *g*, 10 min) and filtering (<0.45 μm) [31]†.	9.815 (2.3) 6.134 (3.0)	9.9 (0.21) 9.2 (0.11)
Cow	Collected fresh samples within an hour of deposition from nearby (1 km) dairy farm and extracted as above.	5.744 (4.7)	9.7 (0.17)
Water extract of soil			
Cargill	Pasture soil (Dystrochrept) grazed by sheep and receiving 30 kg P ha^{-1} yr^{-1} as superphosphate. Soil (0–7.5 cm depth) was air-dried, crushed, and ground (<150 μm) before 1.5 g was extracted in water at the soil-to-water ratio used for estimating P loss in overland flow (1:300), centrifuged (3000 × *g*, 5 min), and filtered (<0.45 μm) [32].	1.508 (2.3)	8.2 (0.10)
Waitahuna—pasture	Five litres of overland flow was collected via simulated rainfall (18 mm h^{-1}) from a Waitahuna silt loam (Haplostoll) that had been sampled intact (0–10 cm depth) and placed in a box. Pasture had not been grazed by dairy cattle for 9 months and soil had received 34 kg P ha^{-1} for the last 3 yr. Sample was filtered (<0.45 μm).	0.174 (0.61)	6.6 (0.18)
Taupo—pasture	Five litres overland flow was collected via simulated rainfall (18 mm h^{-1}) from a Taupo sandy loam (Udand) that had been sampled intact (0–10 cm depth) and placed in a box. Pasture had been grazed 3 weeks prior to sampling, but no dung patches were evident on the soil surface. Soil received about 50 kg P ha^{-1} annually. Sample was filtered (<0.45 μm).	0.128 (0.81)	7.2 (0.23)
Drainage water			
Brighton	Drainage water (1.5 L) was collected from a lysimeter (25 cm diameter × 30 cm deep) in response to a 10 mm rainfall event. The lysimeter contained a Brighton sand soil (Psamment) taken under forest (*Cupressus macrocarpa*). Sample was filtered (<0.45 μm).	0.886 (0.09)	7.0 (0.23)
Lake water			
Rotoiti	Lake water (1.5 L) was taken from the hypolimnion of eutrophic Lake Rotoiti in winter, and filtered (<0.45 μm).	0.051 (0.43)	6.0 (0.05)

*Total P concentration of solution before freeze-drying (mg L^{-1}) and the ratio of P to paramagnetic ions in parentheses. Sample pH and in parentheses, ionic strength (*I*, estimated from electrical conductivity [21], was determined in the re-constituted sample used for NMR analysis.
†All samples were freeze-dried prior to resolubilization and analysis by NMR.

replicate was adjusted to 5, one to 6, and so on up to 13 (as close as possible) with dilute NaOH (0.1–1.0 M) or HCl (0.1–1.0 M) added dropwise. Samples were shaken for 10 min and centrifuged before the supernatent was poured into a 10 mm NMR tube for analysis. After analysis, the pH was re-checked and re-run if it had shifted ±0.1 pH units. (In this paper, pH is presented, although it is recognized that due to the addition of D_2O, the true value lies between pH and pD.)

2.2 *Test samples*

A wide range of freeze-dried samples were reconstituted in 4 mL of distilled water and 1 mL of D_2O, and analysed to demonstrate the application of peak assignments at different pH. These samples were three water extracts of dung from animals grazing pasture (cow, deer and

sheep), two samples of overland flow from two soils under pasture (Taupo and Waitahuna), one drainage water sample from a forested site (Brighton), one water extract of a soil under pasture (Cargill), and one sample of eutrophic lake water (Lake Rotoiti). Site details and the sampling/extraction regime are given in table 1. Total P after digestion with persulphate was determined by colorimetry [15] on samples before freeze-drying. Where poor spectra were obtained due to line-broadening, the concentration of paramagnetics in the sample was decreased by the addition of 0.5 g of Chelating Resin™ (Hampton Research, Aliso Viejo, CA) and total P determined after persulphate digestion.

2.3 *^{31}P NMR*

Solution ^{31}P NMR spectra were obtained using a Varian 500 MHz Inova NMR spectrometer with a 51 mm standard Oxford superconducting magnet, FTS temperature controller, and dual fullband channels. A 10 mm Varian z-axis PFG direct detection probe was used for all samples.

Each reconstituted test sample was ultrasonicated (Crest model 175T) for a minute to maximize dissolution, and centrifuged (Qualitron 6 place mini-centrifuge) for 5 min, the supernatant decanted into a NMR tube, and the pH taken using a pH microprobe just before analysis by NMR. For known compounds and test samples, ^{31}P NMR spectra were obtained at 202.298 MHz at 20 °C. The number of scans accumulated for known compounds varied from 32 to 512 scans, but for test samples, 2571–18 800 scans were accumulated to generate a signal-to-noise (S/N) ratio of >100:1 for the orthophosphate peak. All samples were run using a pulse angle of 45°, a pulse delay of 1 s, and an acquisition time of 1.99 s with 64 K data points. Previous work has shown that while a total delay of 2.99 s was sufficient to meet the spin-lattice relaxation time (T_1) of most peaks (except orthophosphate), the error associated with not meeting T_1 was less than 10% and should not interfere with peak identification or quantification unless in very low concentration (I. Stewart pers. comm.). Poor S/N ratios were obtained for some samples but could not be improved without imposing on the spectrometer time and risking sample hydrolysis. All chemical shifts were recorded relative to an external phosphoric acid standard ($\delta = 0$ ppm) in a capillary tube.

2.4 *Data analysis*

Spectra were processed using a Lorentzian line shape of 5 Hz using Mestre-C software [16]. Peaks were quantified by a combination of manual integration for large obvious peaks such as orthophosphate and deconvolution for regions where peaks overlapped (e.g. for orthophosphate monoesters). Classes of P compounds were made quantitative by combining the percentage spectral area occupied by each class of compound with total P concentration in the sample. Curve-fitting (spline functions) for the variation of chemical shift of known P compounds with pH was performed using SigmaPlot® 2001 [17].

3. Results and discussion

3.1 *pH titration*

The pH-dependent chemical shift of each P compound re-solubilized in overland flow is given in figure 1. Peaks for known compounds were clearly defined (>80% of the spectral area) from those associated with the Waitahuna overland flow medium. For orthophosphate, the chemical shift varied between 1 and 6 ppm for pH values between 5 and 13. For aromatic esters bis

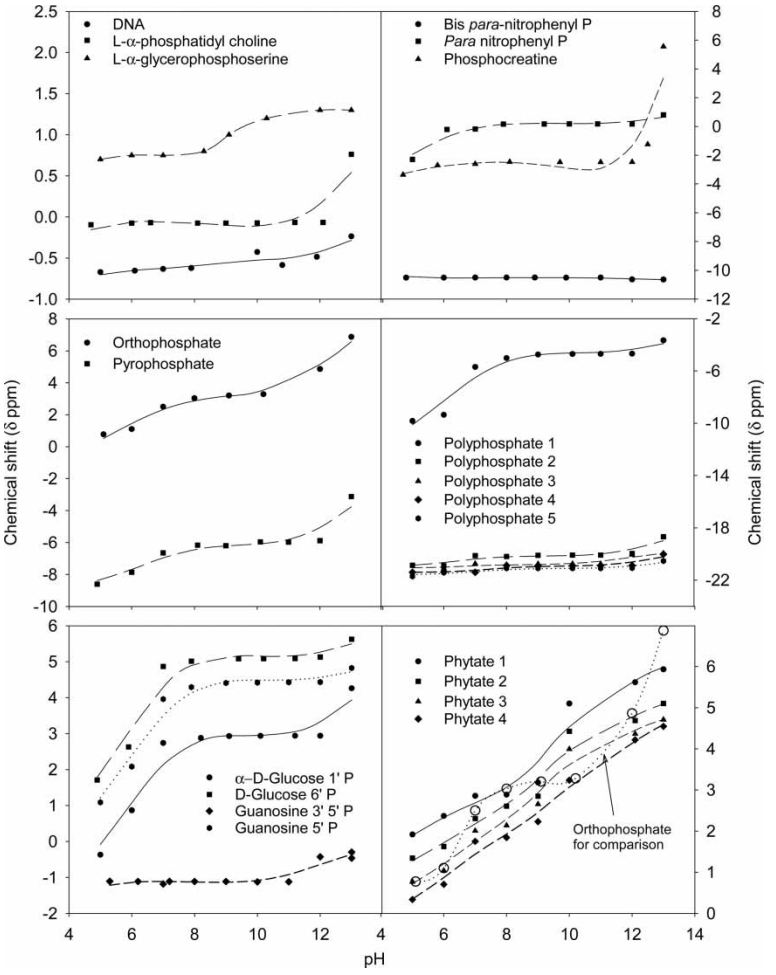

Figure 1. Chemical shift of known P compounds reconstituted in overland flow at pH 5–13. Lines represent data for each compound chemical shift with pH fitted to a spline function.

para-nitrophenyl phosphate and *para*-nitrophenyl phosphate, respective values of −10 ppm and −2 to 1 ppm were found. The consistency in chemical shift for bis *para*-nitrophenyl phosphate reflects the behaviour of most diesters where molecules lack dissociable protons other than those of the phosphate moiety whose pK_a is about 1–2. The exception is L-α-glycerophosphorylserine, whose chemical shift ranged from −3 to 6 ppm, reflecting the presence of other dissociable protons found in the carboxylic acid and amino moeites of the serine molecule [18].

Inorganic polyphosphate exhibited a complex series of peaks reflecting the position of P atoms in the polyphosphate chain. Signals ranging from −22 to about −18 represent the penultimate and mid-chain P groups, while a signal that varied from −10 to about −3 ppm represented the terminal P group [6]. Pyrophosphate is a short-chain polyphosphate ($n = 2$), common in spectra of soils, and can be formed via microbial activity or the degradation of polyphosphate [19]. In the pH range of 5–13, the chemical shift of pyrophosphate varied from about −9 to about −3.5 ppm.

For the orthophosphate monoesters D-glucose 6′ phosphate and α-D-glucose 1′ phosphate, peaks were observed ranging from 1.5 to 5.5 ppm and −0.5 to 4 ppm, respectively. The

nucleotides guanosine 3′ 5′ cyclic monophosphate (a diester) and guanosine 5′ monophosphate resonated from about −1 to 0.5 ppm and from 1 to 4 ppm, respectively. Phosphocreatine, a phosphagen (energy storage compound) made in mammals from adenosine triphosphate via creatine kinase, ranged from −3 to about 6 ppm.

Phytic acid has six separate phosphate groups that, over the course of a pH titration, can yield a complex array of signals usually in the ratio 1:2:2:1 [20]. The range of chemical shifts for the stereo isomer tested here (*myo*-inositol hexakisphosphate) was from near 0 to 6 ppm.

Mathematically, the chemical shift (δ) of a P compound in water can be determined by the Henderson–Hasselbalch equation. Therefore, δ can be calculated at any pH from easily obtained pK_a values, where for one pK_a:

$$\delta = \frac{\delta_1 + (\delta_2 \times 10^{(pH-pK_a)})}{10^{(pH-pK_a)} + 1}, \tag{1}$$

whereas for two dissociable protons the chemical shift is given by:

$$\delta = \frac{\delta_1}{1 + 10^{pH-pK_{a1}} + 10^{2pH-pK_{a1}-pK_{a2}}} + \frac{\delta_2}{1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}}} + \frac{\delta_3}{1 + 10^{pK_{a2}-pH} + 10^{pK_{a1}+pK_{a2}-2pH}}, \tag{2}$$

where δ_1, δ_2, and δ_3 correspond to the chemical shifts of the totally protonated, monoprotonated, and unprotonated forms, respectively, and pK_{a1} and pK_{a2} are the corresponding two pK_a values.

Robitaille *et al.* [18] presented δ_1, δ_2, δ_3, pK_{a1}, and pK_{a2} values for over 100 orthophosphate monoesters, diesters, phosphonates, phosphagens, nucleotides, and inorganic P compounds. However, pK_a and chemical shift values can vary according to the ionic strength and make-up of the solution. Robitaille *et al.* [18] found that pK_a values and chemical shifts of orthophosphate and methyl phosphonates were decreased if measured in 0.53 M NaCl and artificial sea water compared with distilled water or 99.5% D_2O, and concluded that equations such as equations (1) and (2) should be employed with caution. Consequently, we compared the measured chemical shifts of known compounds redissolved in overland flow to those calculated using equations (1) or (2) and data for δ_1, δ_2, δ_3, pK_{a1}, and pK_{a2} given by Robitaille *et al.* [18]. A prior survey of ionic strength (I) in overland flow (determined from electrical conductivity: see Griffin and Jurinak [21]) found that I in overland flow from the Waitahuna silt loam soil, used in this study as the solvent for known P compounds, was near the median value for 15 soils representing a wide range of New Zealand soil types (median $I = 0.2$). Recently grazed or fertilized soils were excluded from this data set. Data in figure 2 show that for five known compounds solubilized in re-constituted overland flow from a Waitahuna soil, the predicted chemical shift was very similar to the measured chemical shift over the pH range 5–13. This infers that data for over 100 naturally occurring P compounds presented by Robitaille *et al.* [18] could be used to calculate the chemical shift of peaks in spectra of reconstituted overland flow. Since other samples such as lake waters and some drainage waters have a similar or lower ionic strength (e.g., those tested in table 1) it is likely these samples would also align to calculated chemical shifts. However, their use in other samples of higher ionic strength is less certain.

As a general guide, values for δ_1, δ_2, δ_3, pK_{a1}, and pK_{a2} presented by Robitaille *et al.* [18] were used to calculate the range and mean chemical shift of orthophosphate monoesters, orthophosphate diesters, nucleotides, phosphonates, and phosphagens (figure 3). As expected, the least variation in chemical shift was evident for orthophosphate diesters. In contrast, the greatest variation was found for phosphonates. However, calculation of a standard error for

each class of compound shows that variation among compounds within a class is generally small (commonly <1–2 ppm). In general, all classes of compounds except phosphonates exhibited a similar and smaller range of chemical shifts at pH $\leq$ 6. Conversely, the distinction between the mean chemical shift for each compound class was better when pH > 6 and optimal when pH $\geq$ 7. This opposes the view of Crouse *et al.* [13], who concluded that a good spectral definition of P compounds was best at pH > 12.5. However, both Adams [14] and Koopmans *et al.* [11] have successfully assigned peaks to P compounds in near neutral pH solutions.

3.2 *Determination of P compounds at native pH*

Using data in figures 2 and 3, peaks were assigned to spectra in figure 4 according to sample pH. During assignment, P species were split into their likely compound classes by first identifying orthophosphate (usually the largest peak), followed by phosphonates and polyphosphates, which exist at opposite and extreme ends of the spectrum, orthophosphate diesters at around −0.5 to 0.5 ppm, and finally other peaks that were assumed to be largely orthophosphate monoesters but may also contain unidentified compounds.

Of the four samples initially analysed (figure 4), all contained orthophosphate and orthophosphate diesters, while orthophosphate monoesters and pyrophosphate were found in all but the Rotoiti lake water and deer dung water extract samples, respectively (figure 4, table 2). During the analysis of the Brighton drainage water, line-broadening caused peaks to overlap. This made accurate peak assignment difficult, even with the aid of deconvolution software (figure 4, table 2). Line-broadening of peaks occurs via paramagnetics, ansiotropic effects, and viscosity, and, as demonstrated in the previous section, this would be more likely

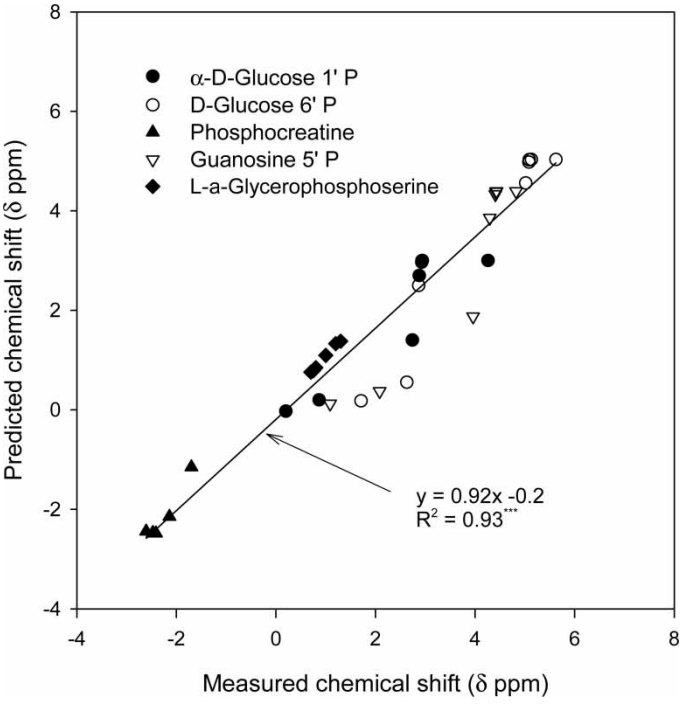

Figure 2. Relationship between the measured chemical shift for compounds reconstituted in overland flow at pH 5–13 and the calculated chemical shift using equations (1) and (2) and data from Robitaille *et al.* [18].

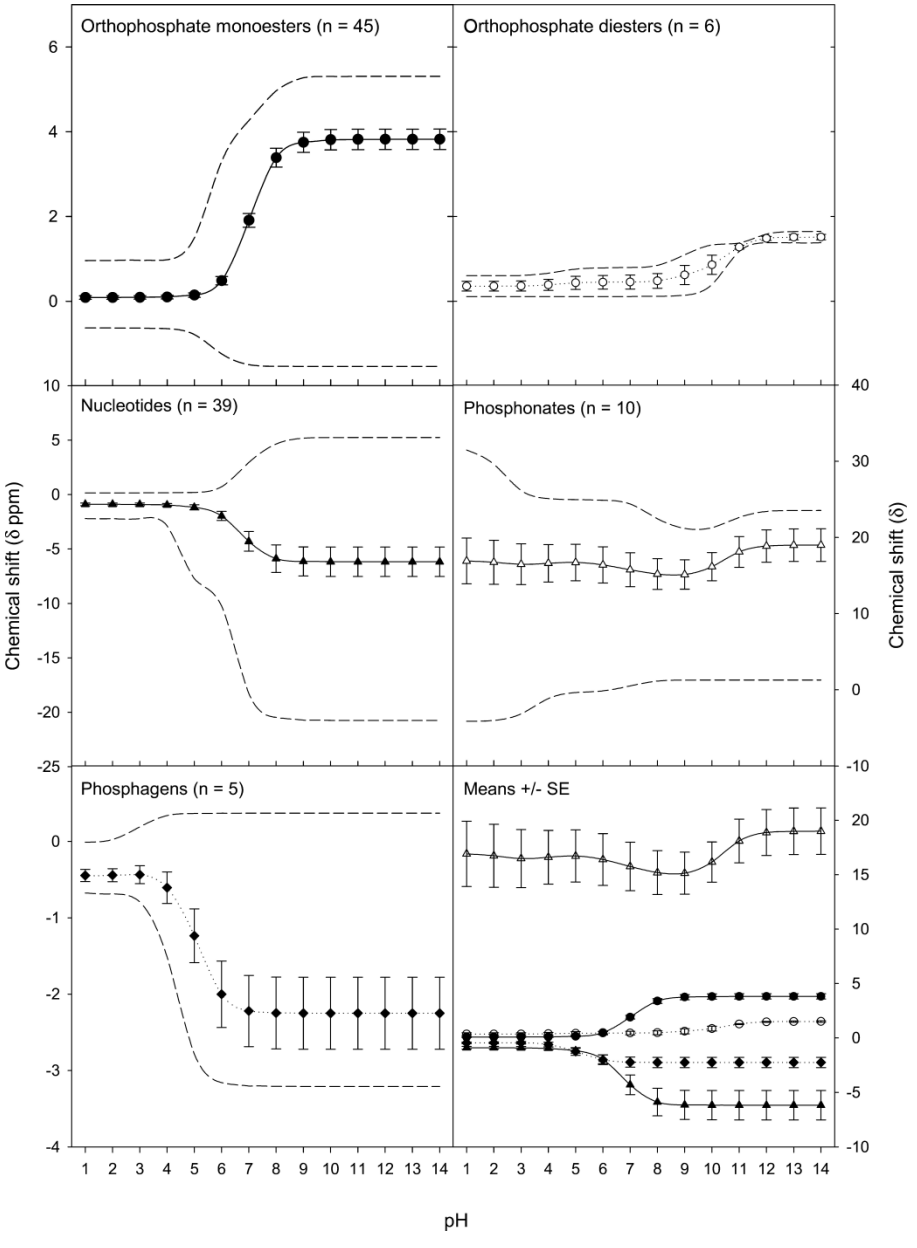

Figure 3. Variation (error bars are ± standard error) in the mean chemical shift of P compound classes with pH. The dashed lines are the maximum and minimum values for each compound class (data calculated from equations (1) and (2); Robitaille *et al.* [18]). Lines represent data for each compound chemical shift with pH fitted to a spline function.

in a sample with an acidic pH (figure 3). Consequently, to remove paramagnetics, Chelating Resin™ was used. This resin was chosen for its ability to function over pH 2–14, its high selectivity for transition metals especially Co, Fe(III), and Mn over P, and its ability to remove paramagnetics without greatly altering the sample pH. For the Brighton drainage water sample treated with Chelating Resin™, the pH only increased by 0.1 units. However, by including the resin, there is potential for the removal of some P compounds if still associated with paramagnetics. As this was the only sample the resin was used on, we did not exhaustively

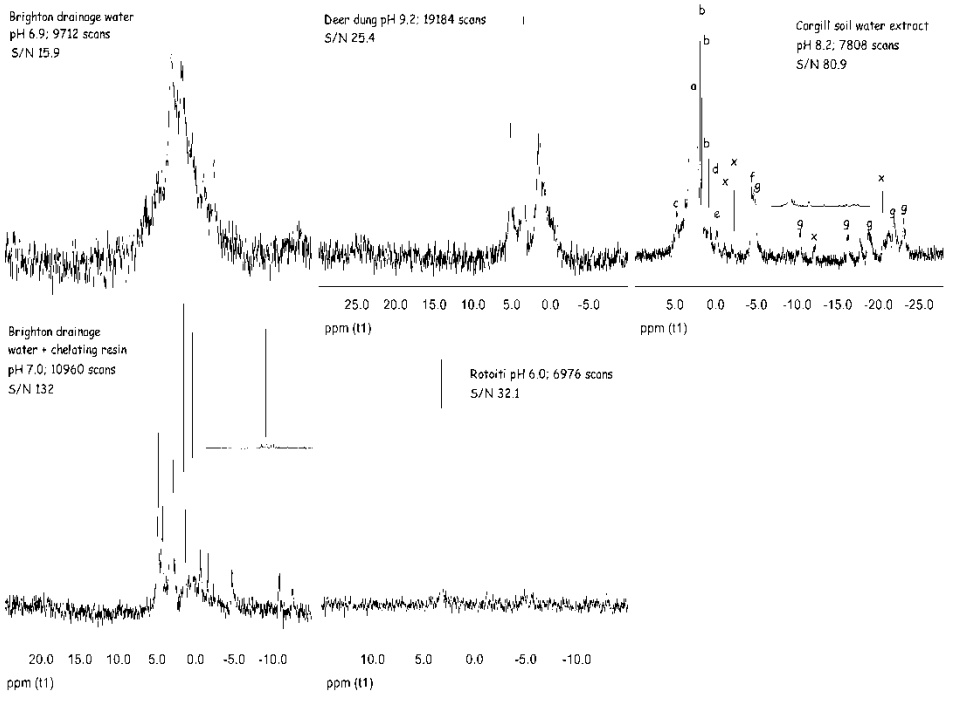

Figure 4. ^{31}P NMR spectra of deer dung, Rotoiti lake water, Brighton drainage water, and Brighton drainage water, and a soil water extract of a Cargill pasture soil treated with chelating resin. Miniature spectra show the full height of the orthophosphate peak vis-à-vis other peaks. S/N: signal-to-noise ratio. An example of peak assignment is given for the Cargill soil water extract where a = orthophosphate, b = phytate (fourth peak obscured by orthophosphate), c = orthophosphate monoesters, d = phospholipids, e = DNA, f = pyrophosphate, g = polyphosphates, h = phosphonates, and x = unknown but most likely either monoester or polyphosphate.

test which P compounds would have been susceptible to removal with resin. Despite some uncertainty, the resulting spectrum exhibited a much improved S/N ratio and decreased peak width (figure 4; without resin, the width at half peak height was 195 Hz (0.96 ppm), and with resin, it was 14 Hz (0.07 ppm)). The number of peaks (>15) was greater than many alkaline extractions which commonly show large peaks of only orthophosphate monoesters and diesters, pyrophosphate, and orthophosphate. Of note in both the soil drainage water and water

Table 2. Concentration (mg l^{-1} in solution before freeze-drying) and percentage in parentheses of P compound classes in the spectra of drainage waters, water extracts of deer dung and a soil and a lake water.

Sample	Phosphonates	Orthophosphate	Orthophosphate monoesters	Orthophosphate diesters	Pyrophosphate	Polyphosphate
Brighton drainage water	–*	0.608 (68.6)	0.210 (23.8)	0.106 (12.1)	0.038 (4.4)	–
Brighton drainage water-resin treated	–	0.622 (70.2)	0.136 (15.3)	0.089 (10.0)	0.013 (1.5)	0.026 (2.9)
Deer dung water extract	0.325 (5.3)	1.736 (28.3)	2.061 (33.6)	2.012 (32.8)	–	–
Cargill soil water extract	–	1.222 (81.0)	0.053 (3.4)	0.024 (1.7)	0.071 (4.7)	0.138 (9.2)
Rotoiti lake water	–	0.045 (89.0)	–	0.001 (2.0)	0.005 (9.0)	–

*Not detected or assigned.

extract of the Cargill soil was the detection of polyphosphates. Polyphosphates are a storage product made when P is in good supply and are common in aerobic sediments [22]. While concentrations of up to 11% of total P have been detected in NaOH extracts of lake sediments [23], these compounds are not detected in spectra of many alkaline soil extracts. The Cargill soil water extract contained nearly 10% of P as polyphosphates, whereas previous examination of an NaOH-EDTA extract of the same soil found no polyphosphates [24].

Also evident in the Cargill soil water extract was the good definition of orthophosphate diesters (around 0 to $-0.5\ \delta$ ppm) and the possibility of some peak overlapping of monoesters. For instance, one of the peaks assigned to phytate was obscured by the large orthophosphate peak. Crans *et al.* [25] and Crouse *et al.* [13] have noted that peak overlapping and assignment are difficult at near neutral pH and below. However, surprisingly little peak overlapping was evident in the Brighton drainage water once paramagnetic concentration was decreased. The corresponding P-to-paramagnetic ratio (P/[Fe + Mn]) increased from 0.09 to 0.49. In addition to decreasing line-broadening, removing paramagnetics also impairs the spin-lattice (T_1) relaxation pathway of P species between pulses. However, prior work established a relationship between T_1 times for quantitative spectra and the P-to-paramagnetic ratio (McDowell, unpublished data). For orthophosphate, the required delay was the greatest of all peak classes and equated to $T_1 = 0.28(\mathrm{P}/[\mathrm{Fe} + \mathrm{Mn}]) + 0.3$. For all samples, except the water extract of cattle dung, the delay on the spectrometer was more than three times greater than T_1, meaning that the conditions for quantitative spectra were met. Figure 3 also indicated that overall, peak assignments should be possible at near neutral pH. Similarly, Adams (1990) used Chelex chelating resin in a neutral form to create extracts that had a pH of 7–7.5. Separation and identification of peaks in their spectra were possible and orthophosphate, orthophosphate monoesters, and orthophosphate diesters were detected. Similarly, in soil water extracts of near neutral to slightly acid pH, Koopmans *et al.* [11] identified orthophosphate and orthophosphate monoesters. Clearly, there is potential for overlap of some species, but this is dependent on sample pH and the concentration and number of compounds present. For many soil extracts, orthophosphate is generally richest, followed by orthophosphate monoesters, orthophosphate diesters, pyrophosphate, polyphosphates and phosphonates. Data in figures 1, 3, and 4 indicate that confusion over the last three compound classes is unlikely. However, depending on pH, there is scope for orthophosphate monoesters to appear either downfield or upfield of orthophosphate or where orthophosphate diesters appear at about 0 ppm. If phytate, the major orthophosphate monoester species, is taken as an example, the major area of overlap is with orthophosphate around pH 7.5–8.2 (figure 1). Although other areas of overlap exist, these areas plus the area at pH 7.5–8.2 should not inhibit peak assignment if deconvolution software is good enough and the degree of line broadening minimal. Furthermore, the concentration of phytate can probably be determined from other phytate peaks. Turner *et al.* [4] noted that the most common isomer *myo*-inositolhexakisphosphate has four signals in the ratio of 1:2:2:1.

For other samples, only orthophosphate, orthophosphate monoesters, and pyrophosphate were detected in the Rotoiti lake water (figure 4). Unusually, no orthophosphate monoesters were detected, whereas other studies such as Nanny and Minear [26], orthophosphate was missing in a sample of mesotrophic lake water. The dominance of orthophosphate in our sample could be related to either the sampling of a eutrophic lake in winter, overlapping of monoester peaks, or decomposition of the sample. The later two possibilities are unlikely, given the narrow peak width of orthophosphate (width at half peak height was 16 Hz) and the neutral sample pH.

In the deer dung sample, phosphonates were detected in good quantity (nearly 5% of total P), while other organic species, orthophosphate monoesters and orthophosphate diesters together constituted most of the total P in the sample. Phosphonates, like polyphosphates, are not common in spectra of soil samples, but have been detected in soils that are cool and moist (e.g. [27]).

It has been hypothesized that phosphonates are the product of microbes, and their presence is facilitated by the cool moist conditions that decrease the potential for decomposition.

3.3 *Comparison of native and alkaline sample pH*

Examination of water extracts, soil solutions, drainage waters, and lake waters is not new. For instance, Nanny and Minear [26] examined lake waters, while Toor *et al.* [12] and Kaiser *et al.* [28] examined P in leachate of pasture and forested soils, respectively. Soil water extracts and soil solutions have been studied by Koopmans *et al.* [11] and McDowell *et al.* [9], and McDowell [10]. However, common among these studies, except Koopmans *et al.* [11], is the use of NaOH to adjust the pH to >13 before analysis by NMR spectroscopy. Both Leinweber *et al.* [8] and Turner [5] have noted that strongly alkaline conditions cause decomposition of susceptible species. Consequently, one major advantage of analysing a sample under its native pH is the minimization of potential hydrolysis and the maintenance of sample integrity.

To test this hypothesis, we took water extracts of two dung samples, one cow and one sheep, and two overland flow samples from soils, and analysed them at their native pH before introducing a small volume of 10 M NaOH to bring the pH to >13, centrifuging, and re-analysing the samples. For the overland flow samples, comparative spectra of NaOH-EDTA extracts of soils were also generated (see [24] for NaOH-EDTA extraction and sample preparation). The results of these analyses are given in figures 5 and 6 and table 3. Unfortunately, the S/R ratio and number of easily identified species in the cow dung water extract were poor under both native and alkaline pH. The poor resolution is probably due to the viscosity of this extract. Although the ionic strength of the cow dung sample was no greater than other dung samples (table 1), and the sample was filtered and centrifuged the same, the suspension was still cloudy. The presence of colloidal material will have contributed to the increased viscosity of the suspension compared with others and inhibited tumbling of P species in the NMR resulting in broad peaks. As a consequence, the orthophosphate monoester and diester regions overlapped, and the spectrum was hard to resolve (figure 6). Line-broadening of a NaOH-EDTA cattle manure extract due to viscosity was also noted by Turner [5]. In contrast, the spectrum of the water extract of sheep dung under native pH (9.9) showed a large number of well-resolved peaks (figure 6) including all classes of P compounds except polyphosphates (table 3). However, when made alkaline and re-analysed, orthophosphate diesters and phosphonates disappeared, orthophosphate monoesters decreased, pyrophosphate was relatively unchanged, and orthophosphate increased. For the Taupo overland flow sample, orthophosphate diesters, orthophosphate monoesters and polyphosphates decreased when NaOH was added, but pyrophosphate and orthophosphate increased. Following pH adjustment in the Waitahuna overland flow sample, orthophosphate diesters, polyphosphates and phosphonates decreased or disappeared, while orthophosphate monoesters and orthophosphate increased.

Mechanisms for these changes with pH adjustment include the decomposition or hydrolysis of species or the precipitation of species out of solution. Hydrolysis of organic P during extraction is inevitable in strongly alkaline solutions. Leinweber *et al.* [8] noted that the concentration of orthophosphate diesters in NaOH extracts of manures and some soil samples decreased with increasing alkalinity. They attributed this to the hydrolysis of orthophosphate diesters or the preferential extraction of orthophosphate diesters in weaker solutions. Similarly, Turner [5] studied the change in P forms in animal manures extracted by NaOH-EDTA solutions of various alkalinity and noted that phospholipids (a subset of orthophosphate diesters) and polyphosphates disappeared with increasing hydroxide strength. In a study of known P compounds, Turner *et al.* [4] also showed that some orthophosphate diesters such as RNA and

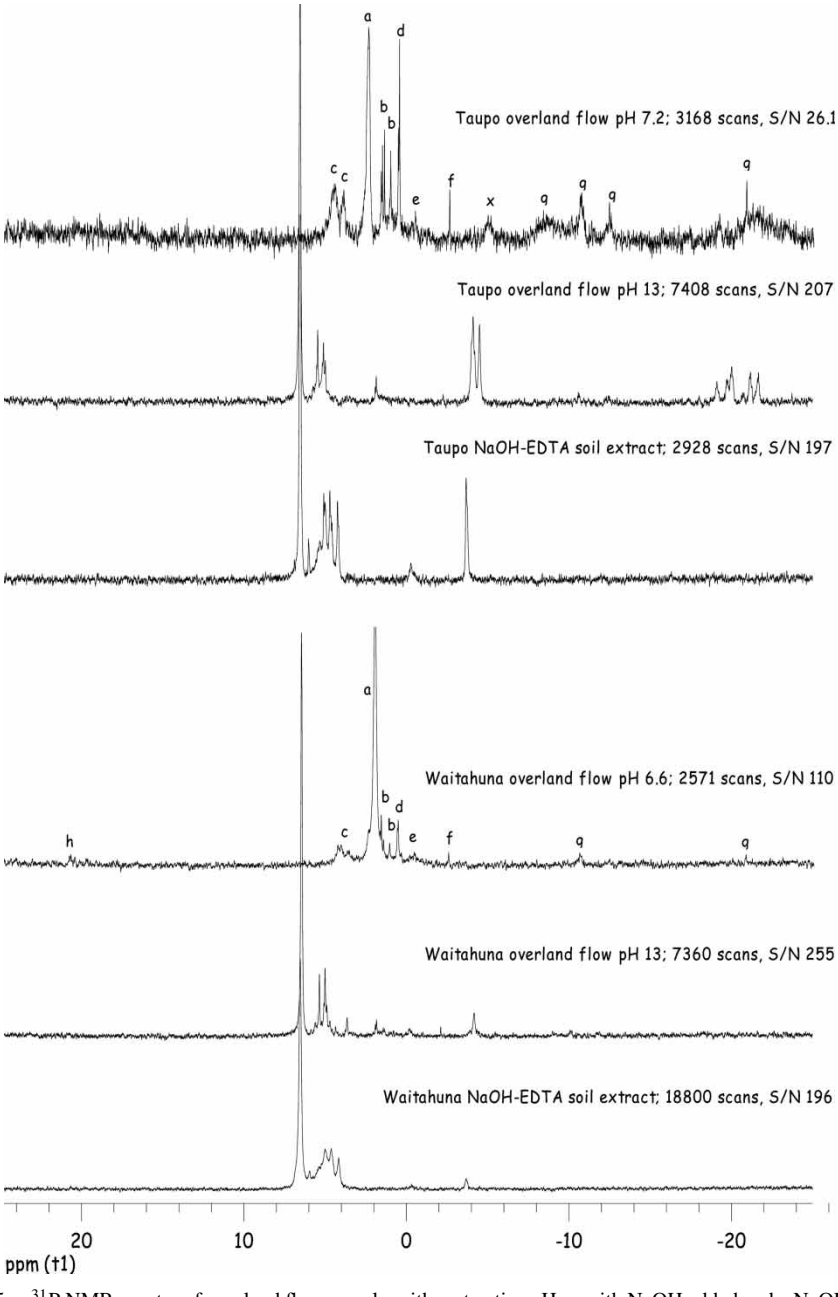

Figure 5. ^{31}P NMR spectra of overland flow samples either at native pH or with NaOH added and a NaOH-EDTA extract of a Taupo sandy silt and a Waitahuna silt loam soil. S/N: signal-to-noise ratio. Letters refer to peak assignments where a = orthophosphate, b = phytate (fourth peak obscured by orthophosphate), c = orthophosphate monoesters, d = phospholipids, e = DNA, f = pyrophosphate, g = polyphosphates, h = phosphonates, and x = unknown but most likely either monoester or polyphosphate.

phosphatidyl choline degraded rapidly to orthophosphate monoesters, while some other phospholipids such as phosphatidyl serine and phosphatidyl ethanolamine degraded more slowly. Phosphonates may also degrade in alkaline solutions, especially some phospholipids at temperatures >30 °C [29]. Hupfer *et al.* [6] and Turner *et al.* [4] argued that chemical hydrolysis

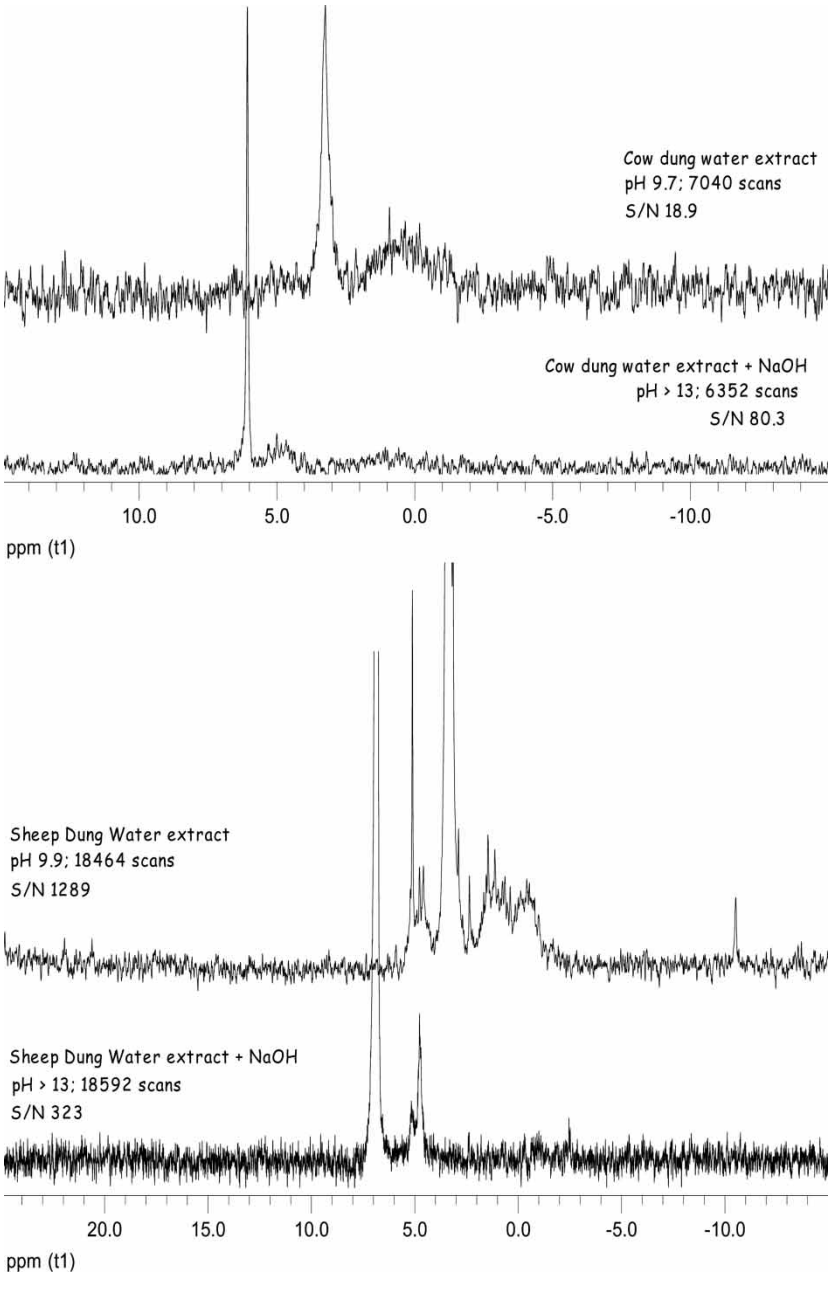

Figure 6. Spectra of cow and sheep dung water extracts at native pH and after the addition of NaOH. S/N: signal-to-noise ratio. Spectra are expanded to emphasize non-orthophosphate species.

of polyphosphates in NaOH-EDTA extracts was minimal due to metal chelation by EDTA. Moreover, Turner [5] suggested that a decrease could be associated with co-precipitation to metals at alkaline pH. The most likely metal is Fe, which is only sparingly soluble at alkaline pH. This may explain why no polyphosphates were detected in the soil NaOH-EDTA extracts (figure 6, table 3). However, Turner [5] also noted the generation of pyrophosphate attributed to the degradation of a proportion of polyphosphates. In our data, both overland

Table 3. Concentration (mg L^{-1} in solution before freeze-drying) and percentage in parentheses of P compound classes in the spectra of sheep and cow-dung water extracts and overland flow*.

Sample	Phosphonates	Orthophosphate	Orthophosphate monoesters	Orthophosphate diesters	Pyrophosphate	Polyphosphate
Sheep dung extract—D_2O	0.052 (0.6)	7.875 (80.2)	1.019 (10.4)	0.787 (8.0)	0.082 (0.8)	–†
Sheep dung extract—NaOH	–	8.911 (90.8)	0.181 (1.8)	–	0.087 (0.9)	–
Cow dung extract—D_2O	–	2.607 (45.4)	2.973 (51.5)‡	0.183 (3.2)	–	–
Cow dung extract—NaOH	–	4.489 (78.2)	1.255 (21.8)	–	–	–
Taupo overland flow—D_2O	–	0.038 (29.8)	0.028 (21.6)	0.009 (7.5)	0.012 (9.3)	0.041 (32.0)
Taupo overland flow—NaOH	–	0.061 (47.6)	0.016 (12.3)	0.005 (3.7)	0.021 (16.1)	0.025 (19.5)
Taupo soil extract	–	(47.3)	(39.7)	(2.1)	(10.9)	–
Waitahuna overland flow—D_2O	0.001 (0.2)	0.103 (59.4)	0.007 (5.4)	0.011 (8.0)	0.001 (0.5)	0.005 (2.6)
Waitahuna overland flow—NaOH	–	0.110 (63.4)	0.048 (27.6)	0.004 (2.1)	0.010 (5.7)	0.002 (1.0)
Waitahuna soil extract	–	(48.0)	(49.6)	(0.7)	(1.7)	–

*D_2O denotes samples measured at their native pH, while NaOH is the same sample reanalysed after the addition of NaOH to bring the pH > 13. The percentage of each compound class in NaOH-EDTA soil extracts is also given for the Taupo and Waitahuna soils.
†Not detected or assigned.
‡Peak assignment unclear due to the overlap of monoester and diester region via line-broadening.

flow samples exhibited considerable increases in pyrophosphate concentration and decreases in polyphosphates when made alkaline. This suggests that some degradation of polyphosphates does occur in addition to precipitation in alkaline conditions. A slight increase in the pyrophosphate concentration of the sheep manure is probably not significant.

Although the degradation pathways and products of some compounds as a result of hydrolysis are known (e.g. [4]), the majority are not. Furthermore, the exact composition of most P classes remains unknown. Some recent advances have been made in identification of orthophosphate monoesters, especially stereo-isomers of inositol phosphates largely derived from plants (e.g. [30]). However, orthophosphate diesters are thought to constitute the most dynamic pool of soil organic P, and although DNA is largely stable in alkaline solutions, RNA is not. Consequently, minimizing degradation due to hydrolysis is vital if accurate assessment of the role of orthophosphate diesters in bioavailability is sought. Similarly, the potential for precipitation of P species with either Fe or Ca must also be considered. As we have demonstrated by altering sample pH, the chemical shift of P compounds is altered. Lathaninide shift agents such as praseodymium ethylenediaminetetraacetate have been used to alter the chemical shift of dissolved organic P compounds (e.g. [6]), but are expensive and largely untested. Other procedures, such as alkaline bromination to destroy organic matter, will inevitably also destroy some organic P species. We recommend that to determine the nature of P species in environmental samples such as water extracts, overland flow, and leachates, the pH of the solution should not be greatly altered if ≥pH 7 but altered beyond this, if necessary, to prevent major peak overlap. It may also be possible with deconvolution techniques to determine the degradation products of specific species and infer bioavailability from comparison of two spectra at different pH, provided peaks are easily identifiable.

4. Conclusions

The analysis of 14 known P compounds indicated that chemical shifts varied from about −22 to 7 ppm in a pH range of 5–13. Of the compounds tested, orthophosphate diesters changed least. However, the large differences in chemical shift of polyphosphates and pyrophosphates (both common in environmental samples) with changing pH meant that these compounds could be easily separated from other compound classes. Further comparison of five known P compounds suspended in reconstituted overland flow against the chemical shift calculated from published equations and dissociation constants in distilled water indicated that sample ionic strength and composition did not greatly affect chemical shift. Categorization of over 100 naturally occurring P compounds into orthophosphate monoesters, orthophosphate diesters, nucleotides, phosphonates, and phosphagens, and calculation of their chemical shift with changing pH, indicated that the distinction between compound classes was best when the sample pH was ≥ 7. Subsequent analysis of drainage water, a soil water extract, a water extract of deer dung, and a lake water sample indicated a wide range of peaks. Treatment of the drainage water sample with Chelating Resin™ to remove paramagnetics improved the spectrum by decreasing line-broadening without altering sample pH > 0.1 units. Analysis of a water extract of sheep dung indicated that fewer species were present at pH > 13 compared with native pH (9.9). This was confirmed by the analysis of overland flow from two soils under pasture, which indicated that polyphosphates, phosphonates, and orthophosphate diesters decreased, while the effect on orthophosphate monoester and pyrophosphate was variable: decreasing and increasing. Evidence for the loss of P species by either precipitation or degradation is clear and indicates that to minimize this, samples should be run at a pH as near to native pH as possible, but ≥ 7. Peaks assignments can then be made from data presented according to the pH in the NMR tube. If, however, some peaks are unresolved due to line-broadening, then additional treatment such as Chelating Resin™ may be necessary.

Acknowledgements

Funding for this work was provided by the New Zealand Foundation for Research Science and Technology under contract AGRX002.

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