Chapter 1
Soil Organic Phosphorus Speciation Using Spectroscopic Techniques

Ashlea L. Doolette and Ronald J. Smernik

1.1 Introduction

Phosphorus (P) is present in many different forms in soil. At any one time, only a small fraction of total soil P is in a form directly available for plant or microbial uptake. It is generally accepted that this directly available form closely equates to orthophosphate ($H_2PO_4^-$ and HPO_4^{2-}) in soil solution. However, this is not to say that all of the remaining soil P is forever inaccessible to biota. On the contrary, much of it can be converted to the directly available form. For some forms of P, this conversion can be rapid or practically instantaneous – weakly sorbed orthophosphate is constantly coming into and out of soil solution and is in rapid equilibrium with solution orthophosphate. For other forms of P, this conversion can be very slow and these forms of P can remain unavailable for centuries or longer. Therefore, the likelihood that any given P atom in soil is going to be taken up by a plant or a microbe is highly dependent on the form or chemical speciation of that P atom. Consequently, the ability of the soil to provide P to biota depends on what forms of P are present and their relative amounts.

The most commonly used differentiation of soil P is between inorganic and organic forms. This is partly due to the importance of this distinction. Transformations of inorganic forms of soil P are controlled by the processes of precipitation, dissolution and sorption. At the molecular level, nearly all inorganic P in soil is orthophosphate, and its chemistry is determined by the strength of ionic bonds to surrounding atoms. It is the relative strength of these ionic bonds that explains why phosphate behaves so differently to the common mineral forms of other nutrients, e.g. nitrate and sulfate. Whereas phosphate generally has low solubility, is easily fixed and is relatively immobile, nitrate and sulfate have much higher solubility, are not easily fixed and are mobile in soils. What distinguishes organic forms of soil P from inorganic forms is that they contain at least one covalent bond to a carbon

A.L. Doolette and R.J. Smernik (✉)
School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, Urrbrae, SA 5064, Australia
e-mail: ronald.smernik@adelaide.edu.au

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atom, generally via an ester linkage (i.e. through an oxygen atom). Most transformations of organic P, and in particular their conversion to inorganic P, require the breaking of this covalent bond. Precipitation, dissolution and sorption also affect organic forms of P (Berg and Joern 2006).

The other reason that the differentiation between inorganic and organic forms is so fundamental to P speciation is that this distinction has been easy to make using long-established techniques. Inorganic P is traditionally detected spectrophotometrically as a blue-coloured phosphomolybdenum complex formed when free phosphate reacts with an acidified molybdate reagent. Organic P does not form a coloured complex with this reagent, and so can be determined as the difference between total P (usually measured as inorganic P after digestion of the soil extract) and inorganic P. However, there are drawbacks to this method (Turner et al. 2003c, 2006). Organic P is overestimated when inorganic polyphosphates are present because they do not react with the molybdate reagent, and therefore are included in the organic P fraction. Additionally, high organic matter concentrations in alkaline extracts can interfere with the colorimetry, but can be minimised by acidifying the extracts to precipitate organic matter (Tiessen and Moir 1993). There are also several other species that interfere with the formation of the phosphomolybdenum complex. These include silica, arsenic, chromium, nitrite, nitrate and sulfide, though these are more of a concern when determining phosphorus species in water samples (Neal et al. 2000). These interferences are usually negligible in soils (Turner et al. 2006).

The differentiation of inorganic and organic P is only the beginning of soil P speciation, because each of these broad classes encompasses a huge range of chemical forms. The subject of this chapter is speciation of organic P forms. There are three general approaches to detailed soil organic P characterisation: sequential extraction, enzymatic hydrolysis and spectroscopic analysis. Organic P speciation using spectroscopic techniques is the subject of this chapter.

1.1.1 Why Spectroscopy?

Chemical spectroscopy involves the differentiation of species based on their differential absorption or irradiation of electromagnetic radiation. The absorption of radiation by matter results in an increase in its energy. At the atomic and molecular scale, energy levels are quantised, i.e. a material can only absorb radiation if it allows the matter to go from one defined level (usually the "ground state") to another defined level (an "excited state"). Electromagnetic radiation consists of photons, which are tiny "packets" of energy. The amount of energy in any packet is a function of the frequency of the radiation. For example, blue light has a higher energy than red light. Photons with energy less than that of visible light are in the infrared, microwave and radiowave regions (decreasing in that order), whereas photons with energy greater than that of visible light are in the ultraviolet, X-ray and gamma-ray regions (increasing in that order).

Some spectroscopic techniques are based on measuring the decrease in intensity (i.e. the number of photons) observed when radiation impacts on matter, whereas others measure the electromagnetic radiation produced as the excited state returns to a lower energy state (often the ground state). Once again, since energy states are quantised, this involves the release of a photon of a specific energy. The key to chemical spectroscopy is that energy levels of an atom or group of atoms depend on their chemical environment. Therefore, the specific energy absorbed or irradiated conveys information about chemical speciation. There are many types of spectroscopy that differ in the type of radiation involved and in the aspect of chemical structure they probe. For organic P speciation of soils, the main types of spectroscopic techniques that have been used are NMR spectroscopy and X-ray absorption spectroscopy (XAS). The specifics of each of these techniques are considered in the following sections. However, first we will discuss some general aspects of P speciation and spectroscopic techniques.

The history of soil organic P speciation using spectroscopic techniques is as yet a short one, dating back to the first use of NMR spectroscopy to characterise soil organic P (Newman and Tate 1980). Speciation of soil organic P before this (and there is a long history of such studies dating back to at least 1940) relied on "wet" chemical analyses. There are a number of limitations of these methods. For a start, separate analyses are required for each class of organic P compound, the main ones being inositol phosphates, phospholipids and nucleic acids, and these analyses generally involve multiple steps and are time-consuming. The development of these techniques and the understanding they enabled are well documented in the reviews by Dalal (1977) and Anderson (1980).

Another major drawback to these wet chemical techniques is that a large proportion of soil organic P remains unidentified, around 50% according to Dalal (1977). Furthermore, these methods generally require fairly harsh extraction conditions to solubilise the organic P – to "break the links between the [phosphate] esters and other soil components", as described by Anderson (1980) – and consequently they run the risk of "breakdown or alteration of the esters themselves".

Spectroscopic techniques can, to a large extent, overcome these problems. Importantly, the spectroscopic techniques used for soil organic P analysis can identify several organic P species simultaneously, though not always quantitatively. However, some problems are as relevant to spectroscopic techniques as they are to wet chemical techniques, but spectroscopic techniques also have their own unique problems.

The two main measures of the capability and performance of any spectroscopic technique are *resolution* and *sensitivity*. Resolution refers to the ability of the technique to distinguish between species, and depends on both the difference in frequency or energy of radiation absorbed or transmitted between different species and also the line-width or signal broadness. Sensitivity refers to the ability of the technique to detect signal against background signal or noise. The better the sensitivity, the lower the detection limit and the greater the potential for detecting minor components.

For the analysis of complex mixtures such as soils, there is a third key measure of spectroscopic performance, *quantitation,* which is often overlooked. Quantitation refers to the relative amount of signal generated by different species. Clearly, a technique in which the amount of signal produced is the same (or at least predictable) for all species is superior for quantifying the relative amounts of these species.

Another consideration that is especially important for soil analysis is the need for pretreatment. In particular, the problems associated with solubilisation discussed above in relation to wet chemical techniques are equally relevant to solution-based spectroscopic techniques. Some spectroscopic methods require more specific pretreatments, e.g. NMR may require removal of paramagnetic species.

Finally, there are practical considerations, including the cost of analysis and the availability of equipment. A downside of the increasing sophistication of spectroscopic techniques is that the equipment is generally expensive to purchase and therefore is not available in most soil laboratories. For example, most NMR analysis of soils is carried out in a dozen or so specialised facilities worldwide, even though NMR spectrometers are widely available in the chemistry departments of most research institutions. Synchrotron-based analyses require even more specialised and expensive equipment. However, there is an up-side to this specialisation: the expensive and sophisticated instruments usually come with operators who are specialists in the technique and are (or at least should be) keen to collaborate with end-users.

We have organised this chapter primarily by spectroscopic technique and secondarily under the subheadings of "sample preparation", "sensitivity", "resolution" and "quantitation". The intention is to enable end-users of these spectroscopic techniques to compare techniques on the basis of each of these primary measures of analytical performance. Thus, we have approached this subject from a spectroscopist's viewpoint. We have tried to avoid spectroscopists' jargon and unnecessary technical details of the techniques as much as possible but, as pointed out, the trend towards more sophisticated methods of speciation will increasingly mean that end-users will need to interact with specialised spectroscopists to get the best analyses possible.

1.2 Solution ^{31}P NMR Spectroscopy

Solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy is by far the most widely used spectroscopic technique for the speciation of soil organic P. This is partly because it was the first spectroscopic technique used for this purpose (Newman and Tate 1980). However, the main reason is that, of currently available techniques, it provides the most detailed and accurate information (or in spectroscopic terms, the best resolution and quantitation) in most circumstances. Nonetheless, it is has several limitations and these need to be considered when using the technique or interpreting solution ^{31}P NMR data. It is not possible to cover all aspects of the use

of P NMR spectroscopy within this chapter. Further information can be found in numerous helpful reviews (Preston 1996; Condron et al. 1997; Cade-Menun 2005a, b).

Of all the spectroscopic techniques, NMR is the hardest to fully understand. It has a fairly obscure physical basis (a nuclear property called "spin") and can only properly be described using quantum mechanics. Simplified descriptions can be found elsewhere (Veeman 1997; Cade-Menun 2005a) and more complex descriptions in NMR textbooks (Derome 1987; Yoder and Schaeffer 1987). Fortunately, one does not need to completely understand the physical basis of NMR in order to use NMR spectroscopy or to interpret NMR spectra. In fact, one of the great advantages of NMR spectroscopy is that "ordinary" NMR spectra (and most NMR used for the speciation of organic P in soils come under this heading) are very easily interpreted.

Figure 1.1 shows a typical solution ^{31}P NMR spectrum of a soil extract. In general, each peak represents P in a different chemical environment. Some peaks are well separated from all others (e.g. pyrophosphate, polyphosphate and phosphonates) and can easily be assigned without ambiguity. However, other peaks are very close or overlap, as shown in the expanded orthophosphate monoester region, and assigning and quantifying these is more difficult, as discussed in detail in the

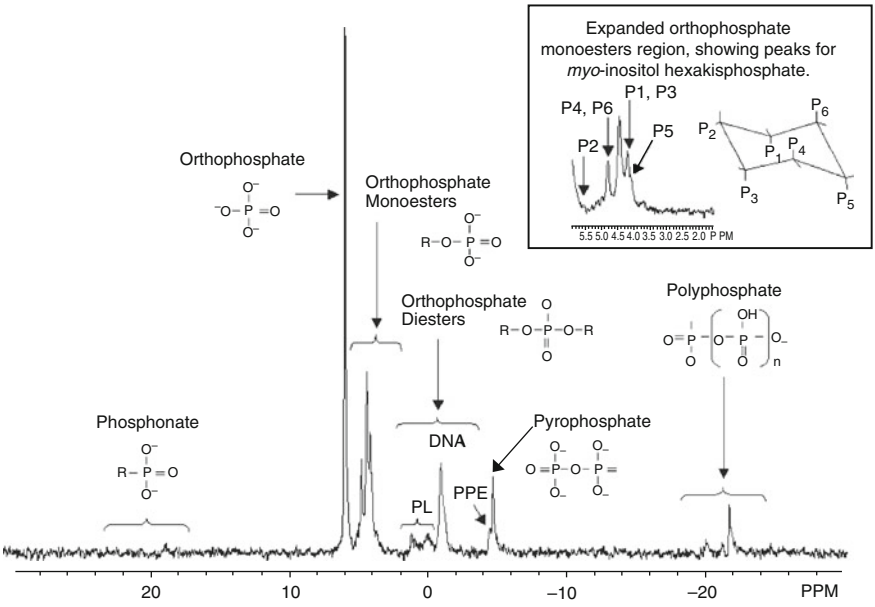

Fig. 1.1 A solution ^{31}P NMR spectrum of a forest floor sample extracted with NaOH–EDTA. This spectrum shows the diversity of P species in natural samples, including phosphonates, orthophosphate, orthophosphate monoesters, orthophosphate diesters such as phospholipids (*PL*) and deoxyribonucleic acid (*DNA*), pyrophosphates and polyphosphate, with the terminal P in the polyphosphate chain indicated by *PPE*. The *inset* shows the expanded orthophosphate monoester region, indicating the peaks and structure for *myo*-inositol hexakisphosphate (phytate). Reprinted from Cade-Menun (2005b), with permission from Elsevier

rest of this section. In general, similar species give rise to peaks in similar parts of the spectrum. As a result, whole regions of the ^{31}P NMR spectrum can be assigned to classes of compound, as shown in Fig. 1.1. Thus NMR can provide both broad and detailed speciation of P types.

1.2.1 Sample Preparation

Being a solution technique, solution ^{31}P NMR spectroscopy has the disadvantage of requiring an extraction step prior to analysis. The aim of such an extraction is to maximise solubilisation of P while minimising alteration of P speciation and optimising the conditions for subsequent NMR analysis. Often these are competing goals, and choices have to be made based on the purpose of the analysis and the nature of the soil. A comprehensive comparison of the performance of different extractants and extraction conditions, and recommendations for optimising these can be found elsewhere (Cade-Menun 2005a, b; Turner et al. 2005). Here, we provide a brief overview of these issues.

Solution ^{31}P NMR is usually carried out on alkaline soil extracts. This is mainly because the solubility of both organic and inorganic P species is maximised at high pH. Most early studies used NaOH as the extractant, usually at a concentration of 0.5 M (Newman and Tate 1980; Tate and Newman 1982; Hawkes et al. 1984). Subsequently, Bowman and Moir (1993) developed a single-step extraction using a mixture of NaOH and EDTA (usually at concentrations of 0.25 and 0.05 M, respectively), and this has now become the most commonly used extractant (Dai et al. 1996; Turner et al. 2003c; Murphy et al. 2009). The inclusion of EDTA, which is a strong chelating ligand, serves two purposes: it complexes paramagnetic cations such as Fe and Mn in the extract and it increases soil P extraction efficiency and the diversity of P compounds extracted (Bowman and Moir 1993). The effectiveness of NaOH–EDTA as an extractant for ^{31}P NMR spectroscopy has been compared to that of other extractants, e.g. 0.25 M NaOH, Chelex (a chelating resin) plus 0.25 M NaOH, and post-extraction treatment with Chelex (Cade-Menun and Preston 1996; Cade-Menun et al. 2002; Briceño et al. 2006; Turner 2008). In general, NaOH–EDTA achieved the highest P extraction efficiency. However, this is dependent on the nature of the soil (Turner et al. 2005). In the four studies mentioned above, NaOH total P extraction efficiencies were 30–60% for volcanic soils (Briceño et al. 2006), 27% for tropical soils (Turner 2008), 22–43% for forest floor samples (Cade-Menun and Preston 1996) and 79% and 43%, respectively, for forest floor and low pH forest soil samples (Cade-Menun et al. 2002). Corresponding extraction efficiencies using NaOH–EDTA were 37–60% for volcanic soils (Briceño et al. 2006), 37% for tropical soils (Turner 2008), 71–91% for forest floor samples (Cade-Menun and Preston 1996; Cade-Menun et al. 2002) and 34% for a forest soil sample (Cade-Menun et al. 2002).

Comparisons cannot be made between Chelex treatments as the techniques differ slightly amongst these studies.

Interestingly, Cade-Menun and Preston (1996) originally advised against the use of NaOH–EDTA because the paramagnetic ions remain in solution (unlike when Chelex is used) and this causes line broadening and overlap of resonances. Their original recommendation was that NaOH–EDTA was only suitable for samples with high P concentrations and low levels of paramagnetic species, unless the metal complexes could be removed prior to ^{31}P NMR analysis. However, their results also showed that NaOH–EDTA extracted a higher concentration and diversity of P compounds. Some P compounds such as polyphosphates were not detected when other extractants were used. Finally, there was less hydrolysis of P compounds when NaOH–EDTA was used (Cade-Menun and Preston 1996). These findings were corroborated by Cade-Menun et al. (2002), Briceño et al. (2006) and Turner (2008) for their soil samples. The fact that paramagnetic ions remain in solution when EDTA is used can also be advantageous because they induce rapid relaxation and this can improve sensitivity (discussed in Sect. 1.2.2). For these reasons, NaOH–EDTA has been generally accepted as the preferred extractant for solution ^{31}P NMR analysis.

Obviously, the nature of the soil P that cannot be extracted cannot be determined using solution ^{31}P NMR spectroscopy and this is one of the major limitations of the technique. There are several reasons why P might not be extractable, and therefore the composition of this non-extractable P fraction can be quite variable: some P may be present in complex polymeric molecules that are alkaline-insoluble (i.e. the "humin" fraction of the classical humic fractionation scheme), some may be strongly complexed to minerals, and some may just be water-insoluble. For example, phospholipids, given their hydrophobic nature, will not be extracted into aqueous extractants. This raises serious doubt on the widespread assignment of signals in ^{31}P NMR spectra of soil extracts to phospholipids, as pointed out by Doolette et al. (2009).

Besides the problem of non-extractable P, the greatest problem with solution ^{31}P NMR analysis of organic P is the potential for hydrolysis. A number of papers have reported the instability of some organic P compounds, particularly orthophosphate diesters, in alkaline solution. Turner et al. (2003b) tested the stability of numerous organic P compounds added to NaOH–EDTA extracts. Most were found to be stable for several days at room temperature. However, ribonucleic acid (RNA) was shown to be very unstable, completely degrading in 24 h to orthophosphate monoesters. This is consistent with other reports (Anderson 1967; Makarov et al. 2002b). By contrast, deoxyribonucleic acid (DNA) is more stable (Turner et al. 2003b) and can remain intact in alkaline solutions for at least 24 h (Makarov et al. 2002a).

Phospholipids are also susceptible to hydrolysis. Turner et al. (2003b) tested the stability of three common phospholipids: phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine. Phosphatidyl ethanolamine showed no degradation, phosphatidyl serine showed minimal degradation after 24 h and partial degradation after 19 days, but phosphatidyl choline was completely degraded within 24 h to two orthophosphate monoester compounds. These compounds have been identified as α- and β-glycerophosphate (Folch 1942; Baer et al. 1953; Doolette et al. 2009).

Since orthophosphate diesters are susceptible to hydrolysis, solution ^{31}P NMR is likely to underestimate the true concentrations of orthophosphate diesters and overestimate the concentrations of orthophosphate monoesters. The pH of the extract can also influence the rate of degradation. Doolette et al. (2009) reported that although phosphatidyl choline degraded over 8 days in 0.25 M NaOH, it completely degraded in just 90 min in 1.16 M NaOH. Although such high NaOH concentrations are not used for soil extractions, freeze-drying and re-dissolving of soil extracts results in these concentrations during solution ^{31}P NMR analysis.

Hydrolysis and modification of native P compounds can be avoided by acquiring NMR spectra at lower pH. McDowell and Stewart (2005a) analysed several water extracts of soil and dung and detected a variety of well-resolved peaks (orthophosphate, orthophosphate monoesters and diesters, pyrophosphate, polyphosphate and phosphonates). Increasing the pH to >13 resulted in a decrease in many P species, and this was attributed to either hydrolysis or precipitation. Adams (1990) analysed neutral soil extracts and found the diester-P concentrations to be greater than those of monoester-P. This technique may have minimised the hydrolysis of some diester-P, but Adams (1990) noted that the extractant conditions would have undoubtedly favoured the soluble P fractions. So, although strong alkaline extractions introduce the risk of hydrolysis they also maximise P extractability. To accurately and quantitatively assess soil organic P using solution ^{31}P NMR, maximum recovery is vital and alkaline reagents are the most effective for this.

1.2.2 Sensitivity

Sensitivity is often the limiting factor in solution ^{31}P NMR analysis of soil organic P. NMR is an inherently insensitive technique. The main reason for this is that the difference in energy levels between the ground and excited spin states is very small and at room temperature the energy levels are nearly equally populated. NMR signal is only generated by the difference in these populations, which is typically around 1 in 10,000.

There are several factors that contribute to NMR sensitivity. Each different type of nucleus has an inherent sensitivity related to a property called its "magnetogyric ratio". Fortunately, the ^{31}P nucleus is one of the more sensitive nuclei; it is less sensitive than ^{1}H, but more sensitive than ^{13}C or ^{15}N. Sensitivity is also linearly related to the number of nuclei in the sample. This depends on the isotopic abundance of the NMR-active nucleus, and the concentration of the element. Again it is fortunate that ^{31}P has 100% isotopic abundance. By comparison, ^{13}C and ^{15}N are 1% and 0.4% abundant, respectively. Against this, P usually has a much lower abundance than C or N in soil. Sensitivity also increases more than linearly with magnetic field strength, so there is a clear benefit in acquiring spectra on high field instruments.

There are two main ways to combat the inherently low sensitivity of ^{31}P NMR. One is to maximise the amount of P in the sample analysed and the other is to

acquire and average a large number of scans. Soil extracts are usually concentrated prior to NMR analysis in order to improve sensitivity. This can be achieved by lyophilisation (freeze drying), rotary evaporation or evaporating under a stream of nitrogen at 40°C (Cade-Menun 2005b). Lyophilisation is the most widely used technique because it avoids an increase in temperature that might degrade the sample (Cade-Menun et al. 2002; Turner et al. 2003b). Dried extracts are re-dissolved immediately prior to ^{31}P NMR analysis in order to minimise hydrolysis. The amount of sample re-dissolved needs to be sufficient to obtain an optimum P concentration but not so great as to increase the viscosity of the sample, which can cause line broadening.

Compared with other spectroscopic techniques, NMR generally requires the collection of many more (often orders of magnitude more) scans. Sensitivity improves as the square root of the number of scans. Solution ^{31}P NMR spectra of soil extracts are acquired using as few as 500 or as many as 110,000 scans (Cade-Menun 2005b) but typically <10,000 scans. However, collecting a large number of scans is not always practical due to the cost involved and the increased risk of hydrolysis. Sensitivity can also be improved by using a 10 mm rather than a 5 mm probe, or by using a higher field spectrometer. Of more importance than the number of scans is the acquisition time, which is the product of the number of scans times the recycle time, i.e. the time between consecutive scans. Acquisition of a ^{31}P NMR spectrum of a soil extract typically takes only a few tenths of a second. However, it may take much longer than this for the nuclei to regain their equilibrium magnetisation. If insufficient time is allowed for this to occur, the result is signal saturation, or a decrease in the amount of signal obtained per scan. The process by which equilibrium magnetisation is regained is called relaxation, and the parameter that describes it is the spin-lattice relaxation time constant (T_1). Recycle times of five times T_1 are required to ensure saturation losses are <1% (Yoder and Schaeffer 1987). Shorter recycle delays can be used to give higher sensitivity (per unit time), by trading off some loss of signal per scan through saturation for running more scans per unit time. However, this can be at the expense of quantitation, as discussed in Sect. 1.2.4.

A wide range of recycle times have been used to acquire solution ^{31}P NMR spectra of alkaline extracts: 10–30 s (Doolette et al. 2009), 15–20 s (Smernik and Dougherty 2007), 1–20 s (Newman and Tate 1980; Tate and Newman 1982), 4.32 s (He et al. 2007a; McDowell et al. 2007), 2 s (Turner 2008), 1.5 s (Dai et al. 1996), 1 s (Koopmans et al. 2007), 0.2 s (Bedrock et al. 1994; Guggenberger et al. 1996) and 0.808 s (Turner et al. 2003a). Given that T_1 varies between P species (Newman and Tate 1980; Cade-Menun et al. 2002; McDowell et al. 2006) and with sample temperature (Crouse et al. 2000; Turner et al. 2003b; Puppato et al. 2007) and concentration of paramagnetic ions (McDowell et al. 2006), it makes sense to measure T_1 for every sample. This can be achieved with either saturation-recovery or inversion-recovery experiments, as first discussed by Newman and Tate (1980) and later strongly recommended by Cade-Menun et al. (2002). However, few researchers explicitly state that they implement this experiment prior to running their samples. As an alternative to measuring T_1 values, McDowell et al. (2006) have suggested that these can be estimated from the P/(Fe + Mn) ratio. This may

have some merit, but more research is warranted before this technique can be used with certainty.

1.2.3 Resolution

Solution ^{31}P NMR spectroscopy provides better resolution of organic P species than any other technique currently available. The resolving power of solution ^{31}P NMR spectroscopy resides in the simple Gaussian–Lorentzian peak shape and the narrow peak width compared to peak spread. Despite this, there is inevitably some signal overlap, especially in the orthophosphate monoester region. However, a further advantage of solution ^{31}P NMR spectroscopy is that similar species appear in similar parts of the spectrum, so even when individual organic P compounds cannot be resolved, groups of peaks can still be assigned to the broad compound classes.

The resolution of solution ^{31}P NMR spectra of soil extracts varies considerably. A major influence is the concentration of paramagnetic ions, the presence of which decreases resolution by increasing line broadening. For tropical soils, Turner (2008) found that maintaining pH >13 can prevent the extraction of excess paramagnetic ions. Poor resolution was apparent when a lower concentration of NaOH was used for the extraction. Resolution was most strongly influenced by Mn. Iron had relatively little influence on line broadening and even at low Mn concentration, Mn was the main paramagnetic ion responsible for reduced resolution. Cade-Menun et al. (2002) reported that the removal of paramagnetic ions from soil extracts reduced line widths and improved resolution in the monoester region. The effect of paramagnetic ions is also evident when pretreatment techniques are used to specifically remove paramagnetic ions. McDowell and Stewart (2005b) pretreated soil with Ca–EDTA–dithionite before extracting with NaOH–EDTA. The pretreatment successfully diminished Fe and Mn concentrations and this decreased the line widths of NMR signals by up to 46%, while having little effect on the forms of P detected.

Resolution is also affected by solution pH. Crouse et al. (2000) evaluated the effects of pH ranging from 4.0 to 13.2 on the solution ^{31}P NMR spectrum of a turkey litter extract composed mainly of orthophosphate and phytate. The appearance and position of the orthophosphate peak in particular was very variable. This peak was sharpest at the highest pH (13.2), and was also clear of all but one of the phytate peaks at this pH. This provides a further reason to acquire solution ^{31}P NMR spectra at pH >13. Resolution can also be affected by the presence of suspended particles. Solution-state NMR spectroscopy will only detect those P nuclei in solution, but often small amounts of dried extract fail to redissolve. Thus it is often beneficial to filter or centrifuge the sample prior to analysis.

1.2.3.1 Identification of P Species

Good spectral resolution is a necessary but not sufficient condition for achieving detailed speciation. The other necessary condition is accurate assignment of peaks.

Many studies rely on comparison to reported literature values to assign peaks to P compounds (Dai et al. 1996; Cade-Menun et al. 2002; Makarov et al. 2002b; Briceño et al. 2006; McDowell and Stewart 2006). These chemical shifts, however, can vary with parameters such as pH, temperature, the concentration of paramagnetic ions and ionic strength (Costello et al. 1976; Derome 1987; Crouse et al. 2000; McDowell and Stewart 2005a; Puppato et al. 2007; Smernik and Dougherty 2007). Some species, such as pyrophosphate and polyphosphate, resonate at very distinct chemical shifts (Turner et al. 2003b). These species can be identified without fear of misassignment, since they do not overlap with other resonances. Other compounds, particularly the orthophosphate monoesters, appear in crowded regions of the NMR spectrum and are much more difficult to identify.

Turner et al. (2003b) approached the problem of extract composition affecting peak positions by acquiring spectra of standard P compounds added to an NaOH–EDTA soil extract. These chemical shifts are likely to be more accurate than those determined in NaOH–EDTA alone. However, the concentrations of many species vary from soil extract to soil extract, and so these should still be taken as a guide only. Smernik and Dougherty (2007) introduced a spiking procedure using low concentrations of added model compounds. Even this resulted in slight changes in the chemical shift of peaks, but because the peaks of the native organic P compounds were still visible, the identity of the spiked species could be determined with high precision.

Spiking soils to assign P species has only been undertaken in a small number of studies (e.g. Adams and Byrne 1989; McDowell and Stewart 2005a; McDowell et al. 2007; Smernik and Dougherty 2007; Doolette et al. 2009). One reason for this is a misplaced belief that maintaining a pH >13 will not only ensure optimal resolution but also consistent chemical shifts. Although the largest changes in chemical shift occur below pH 13 (Costello et al. 1976; Crouse et al. 2000; Puppato et al. 2007), there can still be small variations (up to 0.3 ppm) in the chemical shifts of P compounds between different soils when pH >13, these variations generally being largest for the orthophosphate resonance (Turner et al. 2003c; Doolette et al. 2009). This can become problematic in both the orthophosphate and orthophosphate monoester regions, due to the number and close separation of peaks. Even spiking might not enable definitive identification of some peaks. For example, Doolette et al. (2009) noted that the chemical shifts of ethanolamine phosphate and β-glycerophosphate in NaOH–EDTA extracts can be indistinguishable.

Identification of P species, particularly in the orthophosphate monoester region, is further complicated by the fact that, besides specific small P-containing molecules, soils also contain much larger "humic" molecules. These also contain P, but since the P is in a variety of slightly different chemical environments, these do not produce sharp resonances, but rather a broad signal that is often overlooked. Figure 1.2 shows the ^{31}P NMR spectra of NaOH–EDTA soil extracts of a calcareous soil (Hart), an acidic soil (Wagga) and of acid and calcareous model soils (mixtures of pure clay + sand). All soils were incubated with the addition of cellulose for 25 weeks. Whilst all spectra contain sharp resonances that can be attributed to specific small P-containing molecules, resulting from microbial

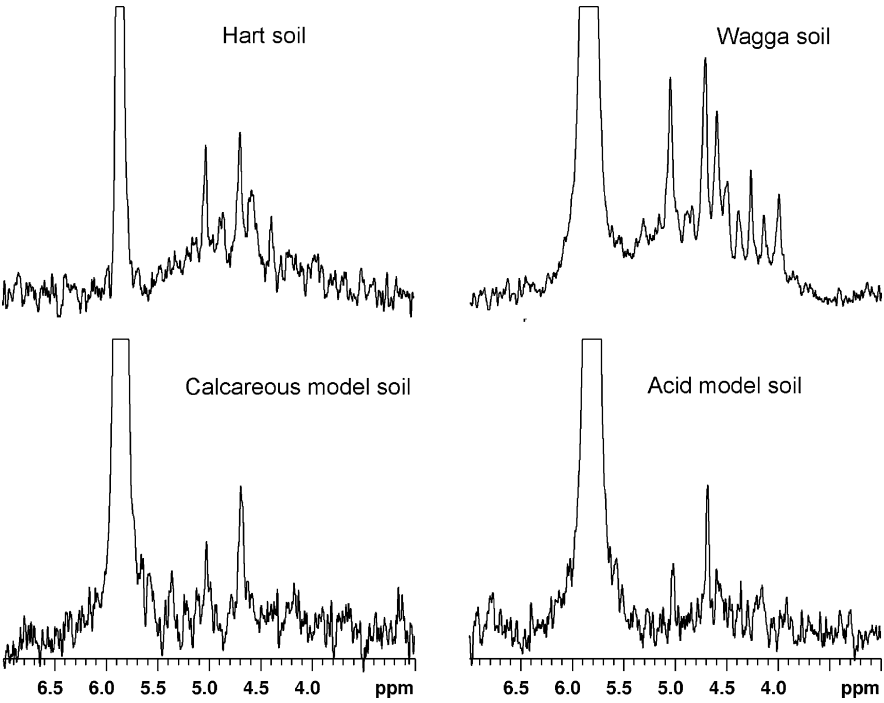

Fig. 1.2 Expansion of the monoester region of ^{31}P solution NMR spectra of NaOH–EDTA extracts of a calcareous soil (Hart), an acidic soil (Wagga) and acidic and calcareous model soils following a 25-week incubation with cellulose addition. Reprinted from Bünemann et al. (2008), with permission from Elsevier

P immobilisation after carbon addition, there is clearly an underlying broad signal for the "real" soils that is absent for the model soils.

Support for this interpretation comes from humic fractionations that show that P associated with humic and fulvic acid in volcanic soils can account for 32–75% and 51–68% of organic P, respectively (Borie et al. 1989; Escudey et al. 2001; Borie and Rubio 2003). Using ^{31}P NMR, He et al. (2006) examined the spectral characteristics of P in humic substances and concluded that the organic compounds had molecular weights greater than 3,000. Furthermore, the ^{31}P NMR spectra they presented of the humic acid fraction contained a broad resonance in the orthophosphate monoester region. Similar spectra were presented by Makarov et al. (1997), who also found that orthophosphate monoesters were the dominant alkali-extractable P species associated with humic acid fractions. However, the poor resolution of their spectra could also be due to the use of NaOH alone as the extractant.

The presence of a broad underlying signal in the monoester region of solution ^{31}P NMR spectra of soil extracts warrants further consideration when attempting to identify and quantify P compounds. Although most of the focus has been on accurately identifying the sharp resonances in the monoester region, the

identification and characterisation of the broader resonance has been limited to a small number of samples (Bünemann et al. 2008).

1.2.4 Quantitation

In most cases where speciation of organic P is being sought, it is not just identification of the species that is important, but also their quantification. This is a considerably more difficult task for a spectroscopic technique. Many studies equate quantification of spectral signal with quantification of the species that give rise to them. Although this may be true for solution ^{31}P NMR, it is by no means assured. Quantification of P species from ^{31}P NMR spectra is usually carried out by multiplying peak areas by the total P concentration of the extract. This assumes that all the P in the reconstituted NaOH–EDTA extract is soluble and is observed with equal sensitivity. This may not always be the case, and more attention should be paid to this issue.

As discussed in Sect. 1.2.2, when recycle times are insufficient to ensure complete relaxation between scans, signal saturation occurs, i.e. less signal is produced by the affected nuclei. This decreases sensitivity, but more importantly it compromises quantitation if not all P species relax at the same rate. Therefore, there can be a trade-off between sensitivity and quantitation. For samples where identification is more important than quantification, shorter relaxation times may be appropriate; but, for any sample where quantification is sought, sufficient time for complete relaxation of *all* species must be allowed for or a bias will result.

A useful method for dealing with quantitation issues is spin counting, which involves the use of a signal intensity standard to gauge the overall NMR observability of a sample. This has been used extensively in solid-state NMR analysis of soil C (Smernik and Oades 2000a, b), N (Smernik and Baldock 2005) and P (Dougherty et al. 2005; McBeath et al. 2006). Since most quantitation problems in NMR involve the under-detection of nuclei (e.g. through saturation), showing that NMR observability for a sample is close to 100% is usually sufficient to prove that the NMR spectrum is quantitative.

The use of an internal standard, although not commonly implemented, will help to overcome the problems associated with reduced and variable observability of different P species. For the successful use of an internal standard, the standard should be chemically inert, soluble in the extract, and produce peaks that do not overlap with those of the sample (Metz and Dunphy 1996; Al Deen et al. 2002). Methylenediphosphonic acid (MDP) appears to satisfy these criteria and has been used in a couple of soil studies (Bedrock et al. 1994; Turner 2008). Other compounds, such as trimethyl phosphate, sodium phosphate (Al Deen et al. 2002) and triphenyl phosphate (Maniara et al. 1998), have been used for the quantitative analysis of P-containing agricultural chemicals, but their suitability for soil studies is yet to be determined.

The other major limitation to accurate quantitation is poor resolution, especially in regions of ^{31}P NMR spectra where there is considerable overlap of resonances. This problem is usually addressed using spectral deconvolution, which can be used to quantify signal in overlapping resonances. Spectral deconvolution involves a numeric least-squares fit of the spectrum as the sum of multiple peaks of standard shape (Lorentzian or Gaussian). Spectral deconvolution has been used to identify the complex signals associated with *myo*-inositol hexakisphosphate (phytic acid), as shown in Fig. 1.3 (Turner et al. 2003a; McDowell and Stewart 2006) and with orthophosphate monoesters (McDowell and Stewart 2005a).

Although the mathematical process of spectral deconvolution is quite straightforward (and is included in standard NMR software), special care and consideration is required to produce accurate and reliable results. To date, these problems appear to have not been fully appreciated and understood within the soil science community. By contrast, researchers in the biomedical sciences community, who experience similar problems with solution ^{31}P NMR analyses, have studied the problems with spectral deconvolution in quite some detail, and their findings are worthy of discussion here. They have adopted different approaches, including the generation of model test and simulated spectra (Corbett 1993) and the incorporation of prior knowledge, which takes into account what is known about the sample and the spectrum (Changani et al. 1999). The aim of these approaches is to account for the overlap of peaks and improve quantitation of spectra with poor resolution and sensitivity that occur due to the complexity and heterogeneity of samples and limited time available to obtain a sufficient number of scans (Šárka and Mika 2001).

The incorporation of prior knowledge takes into account baseline adjustment (essential for estimation of accurate peak areas), as well as the selection of the

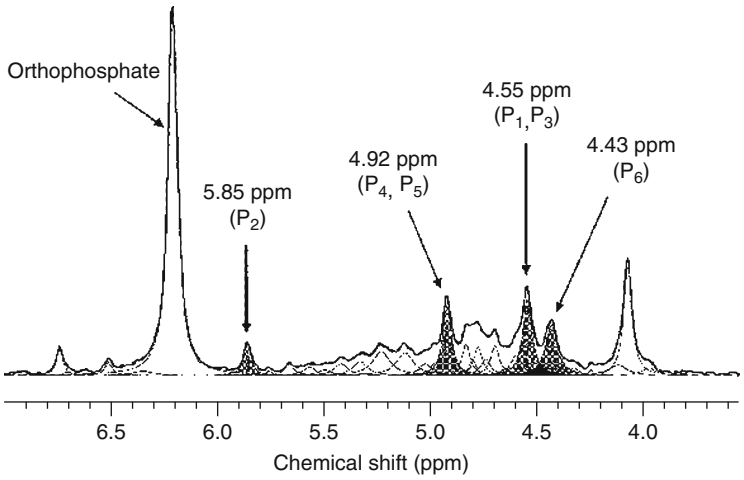

Fig. 1.3 ^{31}P NMR spectrum of a NaOH–EDTA extract of a lowland permanent pasture soil. The *shaded peaks* are phytate resonances that have been identified using spectral deconvolution. Reprinted from Turner et al. (2003a) with permission from Soil Science

correct line shape (Gaussian or Lorentzian) and line width (Corbett 1993; Šárka and Mika 2001). The researcher inputs or fixes these values using what they know about the sample and the peaks they can identify. Inclusion of this prior knowledge in the analysis enables the generation of more information with increased accuracy and reliability (Changani et al. 1999). When analysing soil extracts, for example, this can include fixing line widths of the "sharp" and "broad" peaks where this is appropriate (Dougherty et al. 2007; Smernik and Dougherty 2007).

Corbett (1993) showed that as signal-to-noise decreased, deconvolution produced larger linewidths as it attempted to fit the local noise in the vicinity of the peak. As a consequence, deconvolution tended to overestimate the true area of small or noisy peaks. In other spectra, where there was a sharp noise peak located at the same chemical shift as a true signal, deconvolution attempted to fit the peak shape to the noise. This usually resulted in the true peak area being underestimated. Their analysis also showed that when deconvolution is applied to regions with substantial overlap of peaks, the predicted areas for adjacent peaks are interrelated (i.e. when one is overestimated the other is underestimated) and this error increases in proportion to the degree of overlap. Both of these errors can be minimised by fixing line widths, provided that information about the true line widths is known.

Although these more sophisticated methods of deconvolution and curve fitting are likely to be time-consuming, the adoption of these techniques will result in more accurate and reliable quantification of ^{31}P NMR soil spectra.

An alternative approach to quantification using spectral deconvolution is to use a spiking procedure, but to date this method has only been used by Smernik and Dougherty (2007). By directly spiking a known concentration of phytate into soil extracts immediately prior to analysis, they confirmed the identification of the peaks and were also able to quantify the native phytate concentration.

1.3 Solid-State ^{31}P NMR Spectroscopy

Although most soil P NMR analysis is done in solution mode, NMR analysis can also be carried out on solid samples. Acquiring NMR spectra on solid-state samples introduces some additional problems and requires the use of substantially different and specialised equipment and techniques. The differences between solution and solid-state NMR modes have important consequences for sample preparation, sensitivity, resolution and quantitation, as detailed below.

A complete description of the differences between solution and solid-state NMR can be found in NMR textbooks (e.g. Fyfe 1983). In essence, the two modes differ mainly because molecules in solution are in constant, rapid motion. This constant movement averages out all aspects of the chemical environment of a given nucleus (e.g. a ^{31}P nucleus) except those associated with chemical bonding. Furthermore, even though at any instant a molecule will have a particular orientation with respect to the applied magnetic field, this orientation is constantly changing, and on average all orientations are equally likely. As a consequence, the frequency or chemical

shift of a solution NMR peak is the isotropic value averaged over all orientations. On the other hand, in solid samples, the orientations of molecules with respect to the magnetic field are fixed. In this case, chemically equivalent nuclei can have different chemical shifts depending on their orientation and so signals are much broader. The extent of this broadening depends on the chemical shift anisotropy (CSA), i.e. how much the chemical shift varies with orientation, and this varies substantially among molecules.

In general, for ^{31}P NMR of soils, CSA is much greater (~10–200 ppm) than differences in chemical shift between species (generally <10 ppm) and so unless steps are taken to counter CSA, solid-state ^{31}P NMR analysis of soils would result in one broad lump and no speciation information could be gained. Fortunately, there is a way to overcome CSA broadening, known as magic angle spinning (MAS). MAS involves rapidly spinning (in the kHz range) a sample at 54.7° (the "magic angle") to the applied magnetic field. A description of the physical basis for the technique can be found elsewhere (Fyfe 1983). When MAS is used, nuclei produce relatively sharp signals, mainly at their isotropic chemical shift (i.e. equivalent to the solution NMR shifts). However, solid-state NMR signals are still nearly always 1–2 orders of magnitude broader than for solution NMR, even when MAS is used. Furthermore, if MAS is not fast enough, artefacts called "spinning sidebands" (SSBs) appear. The intensity of SSBs increases with the CSA of the nucleus, increases with increasing magnetic field strength and decreases with increasing MAS rate.

One advantage of acquiring NMR spectra in the solid-state is that one can take advantage of cross polarisation (CP), a technique in which magnetisation is transferred from ^{1}H to ^{31}P nuclei (Fyfe 1983). In solid-state NMR, the "normal" mode of signal acquisition in which ^{31}P nuclei are directly irradiated is usually called direct polarisation (DP). The CP technique relies on strong dipolar coupling between ^{31}P and ^{1}H nuclei that only occurs in the solid-state. CP provides a direct signal enhancement and also allows for a shorter recycle time that is controlled by relaxation of ^{1}H nuclei rather than ^{31}P nuclei (the former generally being 1–2 orders of magnitude faster). Thus, CP offers a substantial improvement in sensitivity over DP. Unfortunately, this comes at a cost of poorer quantitation, because the transfer of magnetisation to ^{31}P nuclei can be adversely affected by a number of factors including a lack of nearby ^{1}H nuclei, the presence of paramagnetic species, and molecular motion (McDowell et al. 2003a; Benitez-Nelson et al. 2004; Dougherty et al. 2005; He et al. 2007b). These issues are discussed in detail in Sect. 1.3.4.

1.3.1 Sample Preparation

Minimal sample preparation is required for solid-state ^{31}P NMR spectroscopy and this is its main benefit over solution ^{31}P NMR. It is best to grind soil samples to a fine powder, as this improves homogeneity and also facilitates sample spinning. Samples should be as dry as possible for acquisition of CP spectra because

molecular motion affects this technique. Only a small amount of soil is required (approximately 0.2 g) for analysis.

1.3.2 Sensitivity

Solid-state ^{31}P NMR suffers the same sensitivity problem as solution ^{31}P NMR, and generally a large number of scans needs to be acquired to produce a good quality spectrum. One advantage of the solid-state technique is that the amount of sample that can be analysed is not restricted by solubility. Sensitivity is thus influenced by (a) the P content of the soil, (b) the number of scans that can be acquired and (c) the relative "NMR observability" of P species present. Condron et al. (2005) suggested that concentrations of around 1 mg P/gram of soil are needed to produce high-quality ^{31}P NMR spectra and that the low natural abundance of P in some soils may result in the signal being below detection limits.

As is the case for solution ^{31}P NMR, sensitivity per unit time is strongly influenced by the time required between scans and, as discussed in Sect. 1.3, there are large differences between CP and DP techniques in this respect. For example, for the same samples, Frossard et al. (2002) used a 3 s relaxation delay for CP spectra and 20 s for DP, whilst McBeath et al. (2006) used relaxation delays of 4 and 100 s for CP and DP, respectively. A range of recycle times have been used for the acquisition of solid-state ^{31}P NMR spectra: e.g. 0.2 s (Shand et al. 1999) and 10 s (Williams et al. 1981; Frossard et al. 1994) for CP; and 1 s (Benitez-Nelson et al. 2004), 1.5 s (He et al. 2009) and 10 s (Conte et al. 2008) for DP. However, in most cases the reasons for these choices of recycle times are not made clear. Dougherty et al. (2005) used saturation-recovery to show that, for their samples, a recycle delay of at least 20 s was required to detect at least 80% of potential signal intensity in DP spectra and that the use of 5 s recycle times resulted in the detection of around 50% of signal intensity.

NMR observability in solid-state ^{31}P NMR spectra of soils is greatly influenced by the presence of paramagnetic species. Manganese and Fe phytate salts are reported as being undetectable by solid-state NMR (He et al. 2007c), as are other Fe-associated P species (Hinedi and Chang 1989; McDowell et al. 2002b). Using spin counting, Dougherty et al. (2005) found that the majority of potential NMR signal for their soils was undetected, and attributed this to the effect of paramagnetic species.

Dougherty et al. (2005) also found that the ^{31}P NMR observability of P in DP spectra was 2–2.7 times greater than for CP spectra. Similarly, Benitez-Nelson et al. (2004) found that the CP spectra of marine particulates required over four times more scans to achieve the same signal-to-noise ratio as corresponding DP spectra. McDowell et al. (2002b) reported similar findings for cropping soils. Consequently, the use of DP alone and CP with DP is becoming more common due to the low sensitivity of CP alone (McDowell et al. 2002b; Benitez-Nelson et al. 2004; Conte et al. 2008).

1.3.3 Resolution

The greatest disadvantage of solid-state compared to solution ^{31}P NMR is the poorer resolution of the former. Rarely have solid-state ^{31}P NMR studies focused on determining organic P concentrations or identifying individual P compounds in whole soils (Newman and Condron 1995; Condron et al. 1997; McBeath et al. 2006; Conte et al. 2008). Instead, much research has dealt with the application of solid-state NMR in differentiating between inorganic metal–P species and determining the relative amount of whole-soil inorganic and organic P (Hinedi and Chang 1989; Frossard et al. 1994, 2002; McDowell et al. 2002a, b).

Identifying Na, K, Ca and Mn phytate salts (He et al. 2007c) and their corresponding inorganic phosphate salts (Turner et al. 1986) in reference materials can be undertaken with relative ease because a linear correlation exists between the chemical shift and electronegativity of the cation. That is, the chemical shift decreases with increasing metal ion valence. The major peaks for phytate and other reference organic P compounds have been shown to be broader than those of the corresponding inorganic orthophosphate compounds (He et al. 2007b).

In addition to differences in the average (isotropic) chemical shift, strong SSBs, can be used to aid P characterisation. Variations in the intensity of SSBs have been noted by Frossard et al. (1994), Williams et al. (1981) and Hinedi and Chang (1989), but they were not used to identify specific P pools or compounds in soils. Dougherty et al. (2005) used a combination of CP and DP with selective extractions, and reported that organic P could be identified by its broad resonance and prominent SSBs, in comparison with the considerably sharper resonances and smaller SSBs of inorganic P. It has been proposed that SSBs can also be used to distinguish between phytate and inorganic orthophosphate species when the major resonances of both compounds are similar (He et al. 2007c). In particular, the intensity of the SSBs was greatest in the spectra of phytate compounds, minor SSBs were present in the spectra of inorganic hydrogen phosphate compounds, and SSBs were absent in tribasic phosphate compounds (He et al. 2007b).

The relative sizes of SSBs of inorganic and organic P species can be explained by differences in their electronic structures (Fig. 1.4). As discussed earlier, the size of SSBs is proportional to the CSA of the species, which is a measure of how symmetric the chemical environment is around the P nucleus. In both inorganic and the vast majority of organic P species, the P atom is surrounded by four O atoms arranged in a symmetric tetrahedral fashion. However, these O atoms are not equivalent, in that one has a double-bond to P, whereas the rest have a single bond to P. In orthophosphate itself, the remaining three O atoms each carry a negative charge, resulting in a polar (and hence non-symmetric) environment around the P nucleus. However, in the case of orthophosphate, the distinction between these two types of O environments is illusory, because there are four equivalent resonant electronic structures that in fact make these O atoms chemically equivalent. In contrast, for organic P molecules, at least one of the oxygen atoms is distinguished by being bound through a covalent bond to an organic residue

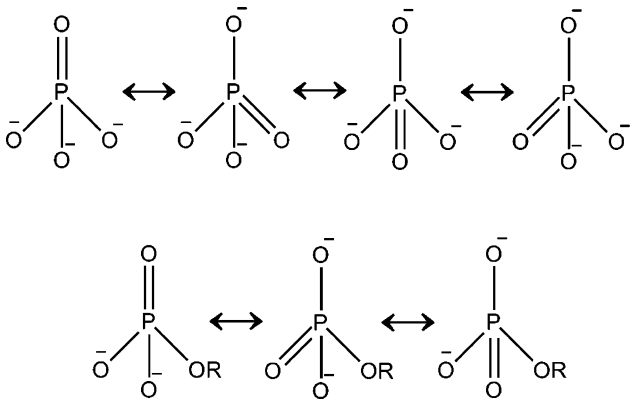

Fig. 1.4 The four resonance structures of orthophosphate (*above*) and three resonance structures of organic phosphate molecules (*below*). The organic residue is denoted as *R*

(denoted R in Fig. 1.4). Therefore, the P in organic P species is always in an unsymmetrical environment and would be expected to have larger SSBs than orthophosphate.

As is the case for solution ^{31}P NMR spectra, poor spectral resolution can also be caused by the presence of paramagnetic ions, but this does not always appear to be the case. Increased line broadening (McDowell et al. 2003a) and high intensity SSBs (Hinedi and Chang 1989) have been ascribed to the presence of paramagnetic species (Fe and Mn) in soil samples. However, Shand et al. (1999) decreased the Fe and Mn concentrations in soil humic acid samples and found there to be no improvement in spectral resolution. Instead, the authors attributed the poor spectral resolution to the various ways in which the P species were associated with the organic phase.

Other factors that may affect resolution have not been widely examined. He et al. (2007b) and Conte et al. (2008) both recommend analysis of only completely dry samples. They reported that moisture in the soil sample at the time of analysis altered the intensity and position of peaks and the size of SSBs.

1.3.4 Quantitation

Quantification of P species using solid-state ^{31}P NMR spectra is hampered by low observability and poor resolution. The low P observabilities reported by Dougherty et al. (2005) for whole soils (an average of 9% for CP and 22% for DP) give a pessimistic outlook for using solid-state ^{31}P NMR for quantitative P speciation in soils. On the other hand, much higher values of 71–88% for DP spectra have been reported for low-Fe calcareous soils (McBeath et al. 2006). At the very least, there is a clear need to address the issue of selective observation of P species and the bias this is likely to create, and spin counting appears to be best way to do this. It may be

that the Dougherty et al. (2005) soils were particularly prone to paramagnetic interferences, but only further studies will make this clear.

The poor resolution in solid-state ^{31}P NMR spectra of soils makes deconvolution necessary to quantify signals in almost all cases (McDowell et al. 2002b; Hunger et al. 2004; Conte et al. 2008). One exception is where species with very distinctive chemical shifts, such as pyrophosphate, are of interest (McBeath et al. 2006). As is the case for solution ^{31}P NMR, the results of deconvolution will be dependent on the way in which the resonances are selected by the user. Peak assignments can be made using an automatic peak-picking routine in the processing software (He et al. 2009), through visual identification by comparing the sample with standard P compounds acquired prior to running the sample (Shand et al. 1999; Frossard et al. 2002; Conte et al. 2008) or by directly spiking into the soil sample (Benitez-Nelson et al. 2004). Usually, assignments are based on previous literature assignments (McDowell et al. 2002b, 2003b). Directly spiking the soil sample would provide the most accurate results, but Benitez-Nelson et al. (2004) reported that the peaks broadened when the standards were mixed into the sample matrix. Therefore, for reliable quantitation, careful consideration of peak selection is required when fitting peaks.

The main issues of deconvolution that affect solution NMR analysis also affect solid-state NMR analysis. However, the greater degree of overlap in solid-state spectra makes these problems greater. For example, Fig. 1.5 shows solid-state ^{31}P NMR spectra of two soils and the fits of these spectra to seven components using deconvolution. Although a good fit is obtained in each case, quantifying individual species from these fits would appear optimistic, as acknowledged in the paper (McDowell et al. 2003b). The incorporation of prior knowledge has been attempted by Hunger et al. (2004), who used a minimum set number of peaks, a Lorentzian lineshape for the narrower peaks and a Gaussian lineshape for the broad peaks, and in doing so improved the quality of the fit. There is quite some way to go before deconvolution of solid-state ^{31}P NMR spectra of soils can be considered a reliable technique for quantification of P species.

1.4 P XANES Spectroscopy

XANES (X-ray absorption near-edge structure) spectroscopy, which is synonymous with NEXAFS (near-edge X-ray fine structure) spectroscopy, is a technique that can be used for speciation of numerous elements in soils. XANES requires an energy-tunable source of X-rays that is currently only possible with a synchrotron. The use of synchrotron-based techniques for soil analyses is a recent development, coinciding with the rapid and continuing increase in the number of synchrotron facilities worldwide since the 1990s (Lombi and Susini 2009).

XANES is a type of XAS and is based on the photoelectric effect. When materials are irradiated with high-energy electromagnetic radiation, electrons (photoelectrons) are released. In XAS, the energy of the incident radiation is varied close to the threshold required to excite strongly bound (core) electrons of an atom to produce

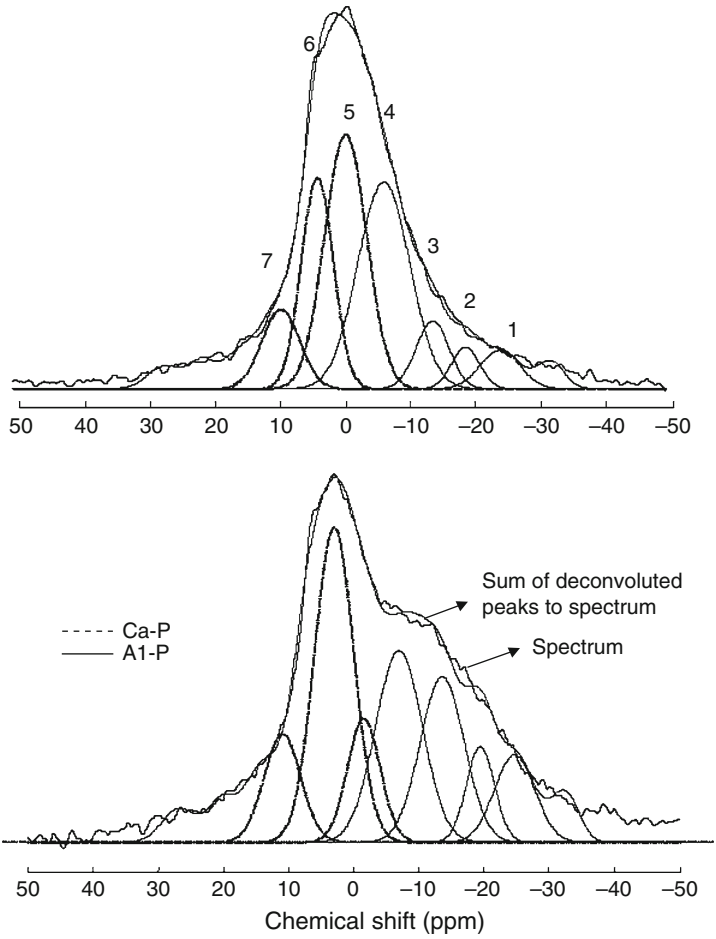

Fig. 1.5 ^{31}P HP/Dec MAS NMR spectra of soils with pH of 5.91 (*top*) and 4.48 (*bottom*) showing the deconvolution of the NMR signal into peaks assigned to Al–P and Ca–P species. The *numbers* in the upper spectrum refer to assignments made to individual P species. Reprinted from McDowell et al. (2003b), with permission from Elsevier

photoelectrons. This energy is quite specific for each different element, ensuring that in most cases a spectrum can be generated for a given element free of interferences from other elements that may be present. Below the threshold energy for photoelectron production there may be some absorption that corresponds to excitation of core electrons into high-energy excited states (Ajiboye et al. 2008). The main feature of the XAS spectrum is the "absorption edge", which basically corresponds to the threshold energy of photoelectron release. The position (in terms of frequency or energy) of the absorption edge is strongly affected by the oxidation state of the atom, since the removal of electrons from an atom results in the remaining electrons being held more strongly. The electronegativity of neighbouring atoms will have a similar, but smaller effect. At energies beyond the absorption edge there are features that

relate to interactions of the outgoing photoelectron wave with neighbouring atoms (Lombi and Susini 2009). These features are usually divided into the XANES spectra region (which includes pre-edge and post-edge features as well as the absorption edge itself) and the EXAFS (extended X-ray fine structure) region, which extends beyond the XANES region on the high-energy side.

The use of XANES for P speciation in soils is still in its infancy. Hesterberg et al. (1999) published the first P XANES spectrum of a soil. Since then, there has been a handful of studies using P XANES for speciation of P in soils (Beauchemin et al. 2003; Sato et al. 2005; Lombi et al. 2006; Ajiboye et al. 2008; Kruse and Leinweber 2008), some related studies on organic amendments (Peak et al. 2002; Toor et al. 2005; Shober et al. 2006; Ajiboye et al. 2007a; Gungor et al. 2007) and one on marine sediments (Brandes et al. 2007). In most cases, the focus has been on identifying and quantifying inorganic P species. However, the potential for P XANES for organic P speciation has been raised and so is relevant to the discussion here.

1.4.1 Sample Preparation

Minimal sample preparation is required for XANES analysis and this is one of its great advantages. Soil samples are usually sieved and ground to improve homogeneity and either placed in a sample holder (e.g. Beauchemin et al. 2003) or spread onto adhesive tape (Sato et al. 2005; Lombi et al. 2006; Ajiboye et al. 2007a, 2008; Kruse and Leinweber 2008). Samples can be analysed at any water content, including as pastes (Khare et al. 2004). P XANES analysis can be combined with other analytical techniques including sequential extraction (Beauchemin et al. 2003; Ajiboye et al. 2007a; Kruse and Leinweber 2008).

1.4.2 Sensitivity

The sensitivity of P XANES analysis is generally very high. This can be attributed to the high energy of the transitions involved (especially compared to NMR) and also to the high intensity radiation produced by synchrotrons. Most published P XANES spectra were acquired in two to ten scans and have good signal-to-noise ratios, although Ajiboye et al. (2008) have commented on the low signal-to-noise ratios for low P soils.

1.4.3 Resolution

The ability to differentiate species is the main limitation of P XANES analysis. This is basically a resolution issue in spectroscopic terms. The nature of a XANES signal (e.g. Fig. 1.6), which is complex and extends across most of the spectral range for every species, is very different to that of an NMR signal, which is a simple Gaussian–Lorentzian "peak". This complicates the issue of resolution, and greatly

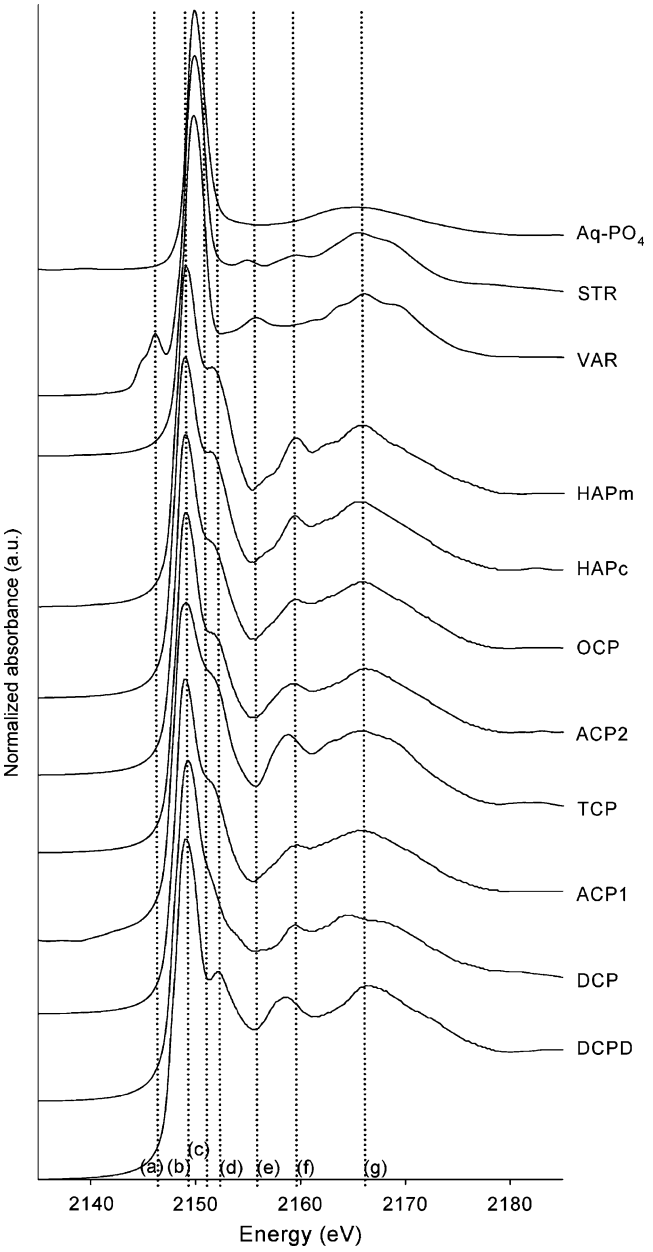

Fig. 1.6 Phosphorus *K*-edge XANES spectra for different inorganic phosphate standard species: $H_2PO_4^-$ in 0.1 M NaCl solution (*Aq-PO₄*); strengite (*STR*; $FePO_4{\cdot}2H_2O$); variscite (*VAR*; $AlPO_4{\cdot}2H_2O$); synthesised hydroxyapatite [*HAPc*; $Ca_5(PO_4)_3OH$]; hydroxyapatite in mineral form (*HAPm*); octacalcium phosphate [*OCP*; $Ca_4H(PO_4)_3{\cdot}2.5H_2O$]; β-tricalcium calcium phosphate [*TCP*; $Ca_3(PO_4)_2$]; amorphous calcium phosphate phase 1 (*ACP1*) and amorphous calcium

reduces the ability to detect multiple components in a mixture. Phosphorus speciation in soils is particularly difficult to resolve using XANES because all P species have the same oxidation state (+V) and the P atom is nearly always surrounded by four O atoms. Therefore, the spectral position of the major XANES feature – the absorption edge – varies little between species. This is in contrast to sulfur and many metals, which exist in multiple oxidation states.

Some features of P XANES spectra are diagnostic. Figure 1.6 shows P XANES spectra of several P minerals and compounds. A pre-edge feature is characteristic of iron phosphates (Hesterberg et al. 1999; Beauchemin et al. 2003; Lombi et al. 2006; Ajiboye et al. 2008), although it is more prominent in crystalline than non-crystalline minerals (Hesterberg et al. 1999; Kruse and Leinweber 2008). Calcium phosphates usually have a distinct shoulder on the high-energy side of the absorption edge (Hesterberg et al. 1999; Beauchemin et al. 2003; Lombi et al. 2006; Ajiboye et al. 2008; Kruse and Leinweber 2008), which is again more distinct for some minerals than others (Hesterberg et al. 1999; Ajiboye et al. 2008). The P XANES spectra of organic P compounds are generally similar, contain little in the way of diagnostic features and are difficult to distinguish from aqueous or weakly bound phosphate (Peak et al. 2002; Beauchemin et al. 2003; Shober et al. 2006; Kruse and Leinweber 2008).

An important distinction needs to be made between the ability to distinguish between the XANES spectra of pure P-containing compounds and minerals and the ability to resolve these species in a mixture. As discussed by Peak et al. (2002), the former may be possible on the basis of subtle features such as small differences in peak position and peak broadness, but these differences cannot be used for resolving mixtures. This is particularly important with regard to organic P speciation. For example, He et al. (2007c) have shown that the P XANES spectra of pure phytate salts are distinguishable. However, their lack of prominent spectral features, mutual similarity and similarity to P XANES spectra of soluble and sorbed orthophosphate would make it impossible to distinguish different phytate salts in heterogeneous matrices such as soils.

1.4.4 Quantitation

Quantitation in XANES analysis of soil P is severely limited by the issues of resolution discussed above. Since P XANES signals for each species overlap over

Fig. 1.6 (Continued) phosphate phase 2 (*ACP2*) [both $Ca_3(PO_4)_2{\cdot}xH_2O$, where x depends on the phase]; dibasic calcium phosphate [*DCP*; ($CaHPO_4$)]; and dibasic calcium phosphate dihydrate [*DCPD*; ($CaHPO_4{\cdot}2H_2O$)]. The *dashed lines* show energy levels of importance to indicate unique spectra features for different species: (**a**) STR; (**b**) absorption edge; (**c, d**) CaP species related to their solubility; (**e**) STR and VAR; (**f**) CaP species; (**g**) oxygen oscillation. Reprinted from Sato et al. (2005) with permission. Copyright 2005 American Chemical Society

virtually the whole spectral width, quantitative analysis of P XANES spectra of soils is usually achieved by fitting the experimental spectrum to a linear combination of XANES spectra of model materials. This is the equivalent of the deconvolution process used in NMR spectroscopy. There are inherent problems with this process. The main one is that even if a good fit is achieved, one cannot determine whether or not an equally good or even better fit could be achieved with a different combination of input spectra. This fundamental problem appears to be unappreciated in some studies, where the results of "best-fits" to XANES spectra are reported as actual compositions.

A key step in any linear combination fit (LCF) to a XANES spectrum is choosing the number and identity of component spectra. Beauchemin et al. (2003) tackled the first issue, choosing the number of components for the LCF by using principle component analysis (PCA). They followed a similar approach to one they employed to analyse sulfur XANES spectra of soils (Beauchemin et al. 2002), which involves using PCA to identify how many independent (orthogonal) components are required to explain the variance of P XANES spectra of a set of soils. In their case, they found that just two components were required for their set of five soils. The second step of this approach is "target transformation" (TT), which identifies the likely nature of these components through comparison of spectra generated for each component with those of model materials. Beauchemin et al. (2003) found that of their 11 model materials, eight were identified as likely component species. They then carried out LCFs for each soil using two to three component spectra (Fig. 1.7). Importantly, similar quality fits were achieved using different input spectra. This highlights the non-uniqueness of the LCF procedure. However, when components were considered as belonging to two general classes (calcium phosphate and phosphate sorbed to iron or aluminium oxide), rather than as specific minerals, there was good consistency in the fits.

It should be noted that the P compositions of soils derived from P XANES spectra reported by Beauchemin et al. (2003) and in subsequent studies (Sato et al. 2005; Lombi et al. 2006; Gungor et al. 2007) that used the same method identify no organic P forms. This is clearly not reasonable, and highlights the limitations of the method. Other studies (Toor et al. 2005; Shober et al. 2006; Ajiboye et al. 2007a, 2008), again using the same method for analysis of P XANES spectra, do include an organic P component (invariably phytate). However, given the lack of features in P XANES spectra of organic P compounds and their similarity to some inorganic P forms, this method of quantifying organic P, let alone organic P speciation does not appear promising.

An important aspect of verifying any quantification method is testing on simple mixes. Ajiboye et al. (2007b) have done this for binary mixes of mineral phosphates. The results were mixed at best, with the quantification of minor components (25%) causing particular difficulties. Khare et al. (2004) successfully used XANES to quantify phosphate sorbed to binary mixtures of iron and aluminium hydroxides; however, it should be emphasised that this is a very simple system compared with soils. Another important prerequisite for quantitation is that the signal produced by all P species is independent of the matrix they are in. This requires validation

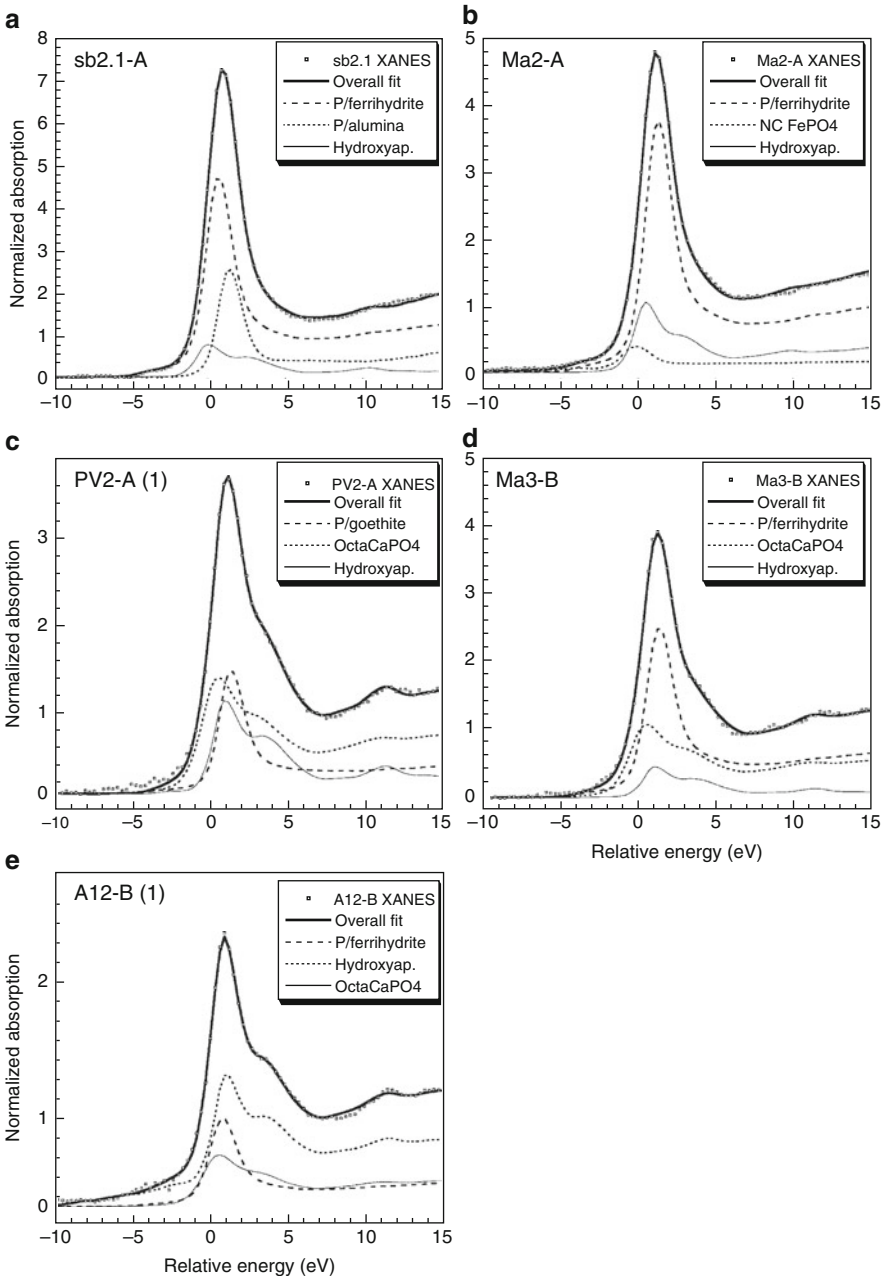

Fig. 1.7 Least-squares fits of the P K-XANES spectra of the five soil samples: (**a**) sb2.1-A, (**b**) Ma2-A, (**c**) PV2-A, (**d**) Ma3-B and (**e**) AI2-B. *P/ferrihydrite*, *P/goethite* and *P/alumina* refer to PO_4 adsorbed on ferrihydrite, goethite or alumina; *octaCaPO*$_4$ octacalcium phosphate; *hydroxyap*. hydroxyapatite; *NC FePO*$_4$ non-crystalline $FePO_4$. Reprinted from Beauchemin et al. (2003), with permission from Journal of Environmental Quality

through spiking experiments analogous to those described above for NMR analysis. Quantitative analysis is certainly made easier if all P species in a sample produce equivalent amounts of signal. Kruse and Leinweber (2008) have tested this for a set of peat soils, with encouraging results.

Finally, it must be remembered that P XANES analysis of soils is still in its infancy and further developments are likely. The identification of only two to four principle components in studies so far (Beauchemin et al. 2003; Sato et al. 2005; Toor et al. 2005; Lombi et al. 2006) partly reflects the small number of spectra analysed. It is likely that the analysis of larger sets of soils would result in the identification of more principle components. P XANES also offers the added ability of mapping distributions of P species at the submicron scale, the utility of which is only just being explored (Lombi et al. 2006; Brandes et al. 2007). Another promising direction involves the use of $L_{2,3}$-edge P XANES spectra. Until now, all P XANES spectra of soils have been acquired at the so-called *K*-edge, which involves excitation of electrons from 1s orbitals. Kruse et al. (2009) have recently shown that $L_{2,3}$-edge P XANES spectra, which involve excitation from p orbitals, are much more information-rich, especially for organic P species. This will not completely overcome the limitations of LCF, but may enable the reliable identification and quantification of more than two to three components in P XANES analysis of soils, possibly including multiple organic P components.

1.5 Conclusions

Spectroscopic techniques offer the best potential for determining the speciation of organic P in soils. The three main spectroscopic techniques that have been used for this purpose are solution ^{31}P NMR spectroscopy, solid-state ^{31}P NMR spectroscopy and P XANES spectroscopy. All techniques have their advantages and their limitations and these need to be understood in order to choose the best technique for any given purpose. All techniques are also highly technical and are best carried out with assistance from experts in the field. To help in deciding between techniques, we summarise the relative merits of each on four criteria (sample preparation, sensitivity, resolution and quantitation) in Table 1.1, on a scale of one to five stars. This is of course a subjective assessment, but we believe it is a useful starting point. It is also based on the techniques as they currently exist and does not take into account the potential for future development.

Table 1.1 indicates that where the main goal is quantification of organic P species, solution ^{31}P NMR is likely to be the best alternative. However, this comes with the proviso that species not soluble in alkaline extracts will be missed, and that hydrolysis is likely to cause some species to be misidentified. Furthermore, solution ^{31}P NMR cannot differentiate between organic P associated with different cations or minerals. The solid-state techniques (solid-state ^{31}P NMR and P XANES) may help here, but detailed identification and quantification is unlikely. Solid-state ^{31}P NMR is particularly unsuited to speciation of Fe-associated organic

Table 1.1 Summary of the relative merits of each spectroscopic technique for organic P speciation

	Solution ^{31}P NMR	Solid-state ^{31}P NMR	P XANES
Sample preparation	⋆ ⋆ ⋆ Requires extraction into alkaline solution. Not all organic P is extracted and some organic P compounds may be hydrolysed under these conditions	⋆ ⋆ ⋆ ⋆ Virtually no sample preparation required. CP spectra can be affected if samples are not dry	⋆ ⋆ ⋆ ⋆ ⋆ Virtually no sample preparation required. Spectra can be acquired at any water content
Sensitivity	⋆ ⋆ ⋆ Sensitivity of NMR is generally poor. Can be overcome by acquiring many scans	⋆ ⋆ ⋆ Sensitivity of NMR is generally poor. Can be overcome by acquiring many scans. Not limited by solubility	⋆ ⋆ ⋆ ⋆ Sensitivity very good
Resolution	⋆ ⋆ ⋆ ⋆ Resolution is very good compared to other techniques. However, not every individual organic P molecule can be resolved	⋆ ⋆ Resolution is quite poor due to broadness of signals. Overlap inevitable	⋆ ⋆ Resolution (of mixtures) is poor due to signal for each species being similar and covering whole spectral range. Overlap inevitable
Quantitation	⋆ ⋆ ⋆ ⋆ Best prospects for quantification of organic P species. Somewhat limited by overlap of signals in crowded regions. Cannot quantify non-extractable species and hydrolysis may cause bias	⋆ Severely limited by poor resolution and also interferences by paramagnetic species	⋆ Severely limited by poor ability to resolve individual organic P signals

P (due to low observability), whereas P XANES is particularly suited (due to a diagnostic pre-edge feature).

1.6 Future Directions

All three spectroscopic techniques can be improved. A particular focus should be the quantitative aspects of each technique, as this is what end-users mostly want from spectroscopic analysis of organic P in soils, and there currently appears to be a lack of understanding of the limitations in this area.

Some issues are common to several techniques, and foremost amongst these is deconvolution and fitting of spectra. There needs to be a wider appreciation amongst both spectroscopists and end-users that a good fit to a spectrum doesn't equate to a definitive composition, and much more can be done to test the validity and identify the limitations of these procedures.

Other issues are specific to each technique. Key areas for improvement for solution ^{31}P NMR include accurate and reliable identification of as many peaks as possible, a quantitative understanding of hydrolysis, and quantification of the apparent broad resonance of large P-containing molecules. A key area for improvement in solid-state ^{31}P NMR analysis would be routine gauging of NMR observability, as this would identify under what circumstances a quantitative assessment is possible. For P XANES analysis, further assessment of the potential of $L_{2,3}$-edge spectra seems warranted, along with development of submicron mapping of P speciation. Understanding how P speciation varies in space would be invaluable, even if the detail of P speciation is limited.

The use of spectroscopic techniques for organic P speciation is still in its infancy. There have been many important advances already and there are surely more to come. However, we believe the time has come for research in this area to mature to a new phase of development, from being dominated by "proof-of-concept" studies, to being dominated by hard-nosed and rigorous assessment of capabilities and limitations. It is only through the feedback of such assessment into further development that accurate, reliable and quantitative methods will emerge that can be applied to all of the areas where organic P speciation is important.

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