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Talanta 66 (2005) 294–306
Talanta
www.elsevier.com/locate/talanta

Extraction of soil organic phosphorus

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Received 15 July 2004; received in revised form 15 November 2004; accepted 17 November 2004
Available online 25 December 2004

Abstract

Organic phosphorus is an important component of soil biogeochemical cycles, but must be extracted from soil prior to analysis. Here we critically review the extraction of soil organic phosphorus, including procedures for quantification, speciation, and assessment of biological availability. Quantitative extraction conventionally requires strong acids and bases, which inevitably alter chemical structure. However, a single-step procedure involving sodium hydroxide and EDTA (ethylenediaminetetraacetate) is suitable for most soils and facilitates subsequent speciation by nuclear magnetic resonance spectroscopy. Analysis of extracts by molybdate colorimetry is a potential source of error in all procedures, because organic phosphorus is overestimated in the presence of inorganic polyphosphates or complexes between inorganic phosphate and humic substances. Sequential extraction schemes fractionate organic phosphorus based on chemical solubility, but the link to potential bioavailability is misleading. Research should be directed urgently towards establishing extractable pools of soil organic phosphorus with ecological relevance.

Keywords: Bioavailability; DNA; Extraction; Inositol phosphate; NMR spectroscopy; Organic phosphorus; Sequential fractionation; Soil

1. Introduction

Organic phosphorus is abundant in soils and is an important source of phosphorus for plants in both natural and managed environments [1]. Information on soil organic phosphorus is essential for understanding biogeochemical cycles and ecosystem ecology, because organisms possess a variety of complex mechanisms to access organic phosphorus in their environment [2]. Organic phosphorus can also be transferred from soil to water bodies and contribute to the nutrition of aquatic organisms, including toxin-producing cyanobacteria [3].

Despite the importance of soil organic phosphorus, its chemical nature and dynamics remain poorly understood. This is due in part to analytical limitations, because there are no direct methods to quantify or speciate soil organic phosphorus. For example, solid-state phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectroscopy cannot detect organic phosphorus in soil due to poor sensitivity and the abundance of paramagnetic ions [4].

Organic phosphorus must therefore be extracted from soil before it can be quantified and identified. Extraction is used to determine the total amount of organic phosphorus in soil, to obtain it in a form suitable for subsequent speciation, or to estimate its mobility, solubility, or biological availability. Numerous procedures exist to achieve each objective, but these can yield profoundly different results.

Here we critically review analytical procedures for the extraction of soil organic phosphorus. We do not address the origins, behaviour, or biological utilisation of soil organic phosphorus, which have been reviewed extensively [5–11]. Nor do we consider the literature on aquatic sediments, which are sufficiently different from soils to warrant separate

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doi:10.1016/j.talanta.2004.11.012

consideration. Our aim is to identify key problems with current methodology that must be addressed to advance research on this important component of soil biogeochemical cycles.

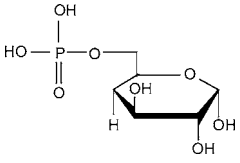
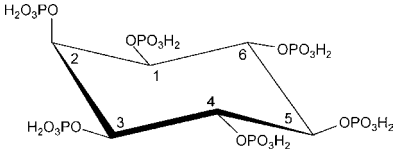
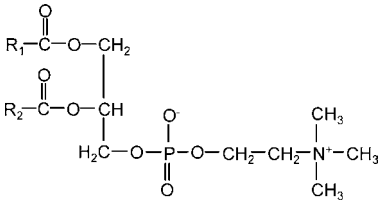
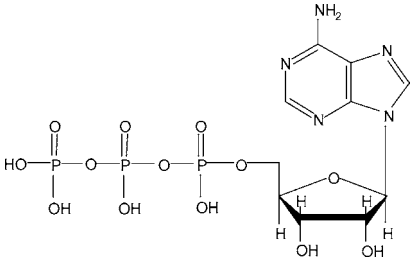
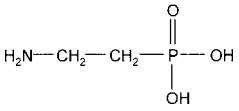
2. Organic phosphorus compounds in soil

Organic phosphorus is defined here as phosphorus present as a constituent of organic compounds (i.e. those containing carbon–hydrogen bonds). Based on the nature of the phosphorus bond, soil organic phosphorus is classified into phosphate esters, phosphonates and phosphoric acid anhydrides. Some common compounds are shown in Table 1. It should be noted that phosphate associated with humic compounds through metal bridges [12] is not classified as organic phosphorus.

Phosphate esters are sub-classified according to the number of ester groups linked to each phosphate. Thus, phosphate monoesters have one carbon moiety per phosphorus, while phosphate diesters have two. Phosphate monoesters are the dominant group of organic phosphorus compounds in most soils [1]. They occur mainly as inositol phosphates, a family of phosphoric esters of hexahydroxy cyclohexane (inositol) [13,14]. There may be one to six phosphate groups linked to the parent inositol. Of the phosphorylated inositols, the *myo* isomer is most common, although other stereoisomeric forms (*scyllo*, D-*chiro*, *neo*) also occur in soil [14]. Other phosphate monoesters present in small amounts in soil include sugar phosphates, phosphoproteins and mononucleotides.

Phosphate diesters include nucleic acids (DNA and RNA), phospholipids and teichoic acids. They typically constitute less than 10% of the soil organic phosphorus [15], although larger proportions are detected in some forest soils [16]. Phospholipids generally constitute a smaller fraction of the soil organic phosphorus than nucleic acids. Teichoic acids are acidic polysaccharides present mainly in the cell walls of Gram-positive bacteria [17]. Small concentrations were reported in alkaline soil extracts using solution ^{31}P NMR spectroscopy,

Table 1
Common soil organic phosphorus compounds

Functional class	Example compound	Structure	Comments
Phosphate monoester	D-Glucose 6-phosphate		Common sugar phosphate. Other sugar phosphates include glucose 1-phosphate and fructose 6-phosphate
Phosphate monoester	*myo*-Inositol hexakisphosphate (phytic acid)		Dominant organic phosphorus compound in plant seeds and most soils, where it is strongly stabilised. Regarded as relatively recalcitrant in the environment [13,14,25]
Phosphate diester	L-α-Phosphatidyl choline (lecithin)		Phospholipid commonly found in plants and microorganisms. One of the two common phospholipids in soil [132]
Organic polyphosphate	Adenosine 5′-triphosphate		Involved in biochemical energy transfer. Uridine, cytidine, guanosine and thymine triphosphates are also common in biological systems [21]
Phosphonate	2-Aminoethyl phosphonic acid		Most common naturally occurring phosphonate, found in a variety of organisms and cold, acidic soils [19,70]

Details of other compounds can be found elsewhere [2,21,55]. ‡R represents hydrophobic fatty acyl chains that may not be identical.

although it now seems that these were identified mistakenly [18].

Phosphonates contain carbon–phosphorus bonds, making them markedly different from other soil organic phosphorus compounds. The predominant phosphonate in nature is 2-aminoethylphosphonic acid, which occurs in a variety of organisms [19]. Phosphonates accumulate in wet, cold, or acidic soils [20].

Phosphoric acid anhydrides (organic condensed phosphates) are involved in biochemical energy transfer and include compounds such as adenosine 5′-triphosphate. They contain phosphate monoester and anhydride bonds [21], but are detected rarely in soil. Condensed inorganic phosphates such as pyrophosphate and polyphosphate are common in soil and constitute a potential source of error in soil organic phosphorus analysis (see below).

Much of the soil organic phosphorus is stabilized by association with mineral components. Negatively charged organic phosphorus compounds attach to minerals such as aluminosilicates (clays) and hydrous iron or aluminium oxides. This can occur directly or through polyvalent bridging cations such as calcium or ferric iron. As a result, much of the soil organic phosphorus is difficult to extract even in strong solvents [22]. In particular, inositol phosphates sorb strongly to clays and react with metals to form insoluble precipitates known as phytates [14]. Calcium phytates are insoluble in alkali, whereas iron and aluminium phytates are insoluble in acid [23]. Phosphate diesters are less strongly sorbed, although DNA can penetrate the interlayer spaces of clays under acidic conditions [24]. Mechanisms involved in the abiotic stabilisation of organic phosphorus in soil are reviewed in detail elsewhere [25].

The various organic phosphorus compounds in soil extracts can be speciated by several techniques. Those involving NMR spectroscopy, mass spectrometry, and various separation procedures were reviewed recently in detail [see chapters in 2]. Phosphatase hydrolysis can also provide information on the composition of extractable organic phosphorus by separating it into functional classes based on susceptibility to enzymatic cleavage [26–28].

3. Extraction of soil organic phosphorus

Procedures to extract organic phosphorus can be separated into five broad categories depending on the objective of the study.

i. Quantitative procedures to extract the total soil organic phosphorus.
ii. Sequential extraction procedures to fractionate organic phosphorus into discrete pools based on relative solubility.
iii. Single-step procedures to extract organic phosphorus in a form suitable for subsequent speciation.
iv. Compound-specific procedures to extract a single form of soil organic phosphorus.
v. Procedures to extract a pool of organic phosphorus with biological or environmental relevance.

Table 2
Quantitative extraction procedures for soil organic phosphorus

Procedure	Extractants
Mehta et al. [30]	i. Hot concentrated HCl/10 min ii. Concentrated HCl at room temperature/1 h iii. 0.5 M NaOH at room temperature/1 h iv. 0.5 M NaOH at 90 °C/8 h
Saunders and Williams [31]	i. 0.1 M HCl/1 h ii. Leached with hot HCl iii. 0.1 M NaOH/16 h (twice)
Anderson [37]	i. 0.3 M NaOH/16 h ii. Hot concentrated HCl/10 min iii. Concentrated HCl at room temperature/1 h iv. 0.5 M NaOH at room temperature/1 h v. 0.5 M NaOH at 90 °C/8 h
Halstead et al. [39]	i. 0.1 M HCl/30 min (twice) ii. Ultrasonic dispersion in 0.2 M aqueous acetylacetone (pH 8.0)/2 h iii. 0.2 M aqueous acetylacetone (pH 8.0)/16 h iv. 0.2 M aqueous acetylacetone (pH 8.0)/24 h (twice) v. Ultrasonic dispersion in 0.2 M aqueous acetylacetone (pH 8.0)/2 h vi. 0.2 M aqueous acetylacetone (pH 8.0)/24 h (twice)
Thomas and Bowman [34]	i. 0.05 M HCl + 0.05 M HF/2 h ii. Cation exchange resin (Na^+ form) in water/4 h
Steward and Oades [41]	i. 1.0 M HCl ii. Ultrasonic dispersion in 0.5 M NaOH (3 min)
Hong and Yamane [38]	i. 0.1 M HCl ii. Cation exchange resin (Na^+ form) in water/10 h iii. 0.2 M aqueous acetylacetone (pH 8.3)/12 h (×4)
Bowman [43]	i. Concentrated H_2SO_4 ii. 0.5 M NaOH at 85 °C/2 h
Bowman and Moir [45]	i. 0.25 M NaOH and 0.05 M EDTA at 85 °C/2 h

3.1. Quantitative extraction

Quantitative extraction is designed to recover all the organic phosphorus from the soil. This is necessary because there are no direct methods to determine total soil organic phosphorus in situ. This also means it is impossible to accurately assess the efficiency of quantitative extraction. Procedures for quantitative extraction are outlined in Table 2.

The most effective extractants for soil organic phosphorus are alkaline solvents such as sodium hydroxide [22]. These create electrostatic repulsion by increasing the negative charge of both organic and mineral components and replace polyvalent bridging cations with markedly less effective monovalent cations such as sodium [29]. It should be noted however that soil organic carbon and nitrogen are not quantitatively recovered by alkaline extraction.

The recovery of organic phosphorus in alkaline solvent may be improved by pretreating the soil with mineral acid

[22]. This removes polyvalent bridging cations and dissolves salts of organic phosphate esters that are relatively insoluble in alkaline solution. Most early methods used a strong mineral acid such as concentrated hydrochloric acid [30], although dilute solutions (e.g. 0.1 M) are also effective [31] (Table 2).

The widely used method developed by Mehta et al. [30] involves two successive extractions in concentrated hydrochloric acid, followed by two extractions in sodium hydroxide. The authors recommended a hot concentrated acid pretreatment because they believed that some soil organic matter was protected by coatings of mineral substances such as iron oxides. However, a later study reported little influence of acid strength on subsequent recovery of organic phosphorus in alkaline solvent. A slight modification of the Mehta et al. [30] method involved acid extraction with 2 M H_2SO_4 for 18 h [32], but proved inferior in subsequent studies [33].

Several early studies assumed that organic phosphorus recovery during acid pretreatment was negligible [e.g. 34], yet acidic solvents can recover considerable amounts of organic phosphorus. For example, acid pretreatment of high organic matter wetland soils recovered between 2 and 9% of the total organic phosphorus [35], while molar sulphuric acid recovered between 25 and 33% of the total organic phosphorus from a low organic matter sandy loam [36]. Quantitative analysis of soil organic phosphorus must therefore include determination of organic phosphorus in acid extracts.

Strong acid or base extraction introduces the risk of organic phosphorus hydrolysis (see Section 4.2), which encouraged development of milder methods for quantitative extraction. Anderson [37] addressed this by including an initial dilute base extraction to recover labile compounds prior to acid pretreatment. The Mehta–Anderson method represents the closest to a 'reference' procedure for quantitative extraction of soil organic phosphorus.

Thomas and Bowman [34] reported that a mildly acidic cation exchange resin extracted similar concentrations of organic phosphorus to a conventional strong acid and alkali procedure. The proportion of high molecular weight organic phosphorus (> 50,000 Da) was greater in the resin extract than in the strong acid and alkali extracts, suggesting that high molecular weight complexes were degraded during conventional acid–base procedures. A similar phenomenon was reported by Hong and Yamane [38], who demonstrated that organic phosphorus associated with humic acids, considered to be high molecular weight compounds, constituted a greater proportion of the soil organic phosphorus extracted by a cation-exchange resin/acetylacetone procedure than by strong sodium hydroxide.

Aqueous acetylacetone had been reported previously to quantitatively extract soil organic phosphorus without causing extensive structural alteration of the extracted compounds [39]. The technique required multiple extraction steps and was not adopted widely, perhaps because it was subsequently shown to be inferior to hot sodium hydroxide in extracting organic phosphorus from some Scottish and Canadian soils [40].

Long analysis times were a clear limitation of the early procedures for quantitative extraction of soil organic phosphorus, due to multiple long extraction steps. For example, the Mehta–Anderson procedure requires at least two days or a 12-h day in the laboratory.

A procedure involving initial acid extraction for one hour followed by a few minutes of ultrasonic dispersion in sodium hydroxide was recommended as a rapid alternative to standard procedures [41]. It recovered more organic phosphorus than the Mehta et al. [30] method for several soils, although more inorganic phosphorus was recovered by the latter procedure, perhaps indicating hydrolysis of organic phosphorus during the strong acid extraction steps. In a later study, ultrasonic dispersion in sodium hydroxide without acid pretreatment gave poor recoveries of organic phosphorus compared to the Mehta–Anderson method [42].

Bowman [43] reported a simplified scheme involving initial treatment with concentrated sulphuric acid followed by extraction in hot 0.5 M NaOH. Most of the organic phosphorus was recovered in the initial acid step. This two-step procedure compared well with the Mehta et al. [30] method for a range of soils from the USA [43] and Nigeria [44], but appeared unsuitable for acidic soils with high organic matter concentrations [43].

Subsequently, Bowman and Moir [45] proposed a solution containing EDTA and sodium hydroxide as a rapid single-step extractant. The presence of EDTA in the alkaline solvent improved soil organic phosphorus recovery by chelating metal cations. The method yielded comparable recoveries to the two-step acid–base procedure [43] and, therefore, the Mehta et al. [30] procedure for a range of soils. The authors' suggestion that NaOH–EDTA extraction was most suitable for high organic matter soils was confirmed by almost quantitative recovery of total phosphorus (i.e. both inorganic and organic) in subsequent studies [16,46]. The procedure is used widely to analyse soil organic phosphorus by solution ^{31}P NMR spectroscopy [47], although such studies generally employ an overnight extraction at room temperature rather than the hot (85 °C for 2 h) conditions proposed originally.

As no method can determine the absolute concentrations of total soil organic phosphorus, it is difficult to assess the efficiency of quantitative extraction. Values for quantitative extraction are therefore compared typically with those determined by an ignition procedure. These involve either low temperature [48] or high temperature [31] ashing to destroy organic matter, with total organic phosphorus calculated as the difference in acid-extractable phosphate between ignited and unignited samples.

The ignition method tends to overestimate organic phosphorus in most soils by increasing the solubility of inorganic phosphate minerals following ignition [49,50], notably in soils that are highly weathered [51] or calcareous [52]. Acid extraction of unignited samples rarely recovers all the inorganic phosphate from soil, which can further overestimate organic phosphorus. Incomplete extraction of phosphate released during ignition may underestimate

organic phosphorus [49], but this is minimal at ignition temperatures <800 °C [31]. Underestimation can also occur if organic phosphorus is hydrolysed to phosphate during the initial acid extraction. However, both factors are likely to be negligible in comparison to those leading to overestimation. Clearly, care must be taken when using ignition to estimate soil organic phosphorus.

3.2. Sequential extraction

Sequential extraction schemes were developed to obtain additional information on the nature of soil phosphorus. A single sample of soil is subjected to increasingly stronger solvents, thus separating the phosphorus into fractions based on chemical solubility. The most common methods for soil are outlined in Table 3. The Chang and Jackson [53] procedure was the first widely used soil fractionation procedure, although it was developed to assess the distribution of inorganic phosphate. We include it in Table 3 because it formed the basis for some of the subsequent fractionation schemes involving organic phosphorus.

Bowman and Cole [54] developed a sequential extraction scheme for fractionating soil organic phosphorus based on chemical solubility. This involved sequential extraction of soil with 0.5 M $NaHCO_3$ at pH 8.5, 1.0 M H_2SO_4, and 0.5 M NaOH. Organic phosphorus in the sodium bicarbonate extract was designated as labile, while organic phosphorus extracted in sulphuric acid was termed moderately labile. Organic phosphorus in the sodium hydroxide fraction was separated by acid precipitation (pH 1.0–1.5) into a moderately resistant fraction (fulvic acid, soluble in acid and alkali) and a highly resistant fraction (humic acid, soluble in alkali but insoluble in acid). Inorganic phosphate extracted in sodium hydroxide was also included in the moderately labile organic phosphorus fraction, because it was assumed to originate from degradation of alkali-labile organic phosphorus. This is an error in this scheme, because the main alkali-labile compounds are phosphate diesters, which degrade to phosphate monoesters rather than free phosphate [55].

In the Bowman and Cole [54] scheme the labile and moderately labile fractions were considered to be more readily plant-available than the moderately resistant and highly resistant humic and fulvic acid fractions. However, there was limited information to confirm this. It is also interesting to note that phytic acid added to the soils used in the development of this scheme was not recovered in any fraction. Given the large concentrations of phytic acid in most soils [14], this strongly suggests that organic phosphorus recovery was incomplete, yet organic phosphorus concentrations determined by extraction were similar to those determined by ignition. Possible explanations for this apparent paradox include the inclusion of alkali-extractable inorganic phosphate in the moderately labile organic phosphorus fraction and the

Table 3
Sequential fractionation procedures for soil organic phosphorus

Procedure	Extractants	Designation
Chang and Jackson [53]	i. 1.0 M NH_4Cl	Labile
	ii. 0.5 M NH_4F	Aluminium-bound
	iii. 0.1 M NaOH	Iron-bound
	iv. 0.25 M H_2SO_4	Calcium-bound
	v. Citrate-dithionite	Reductant-soluble iron-bound
	vi. 0.1 M NaOH	Occluded iron and aluminium bound
Bowman and Cole [36]	i. 0.5 M $NaHCO_3$	Labile
	ii. 1.0 M H_2SO_4[a]	Moderately labile
	iii. 0.5 M NaOH[b]	Moderately resistant and highly resistant
Hedley et al. [56]	i. Anion exchange resin	Labile
	ii. 0.5 M $NaHCO_3$	Labile
	iii. Fumigation, 0.5 M $NaHCO_3$	Microbial
	iv. 0.1 M NaOH	Iron- and aluminium-bound
	v. 0.1 M NaOH + sonication	Inter-aggregate
	vi. 0.1 M HCl	Calcium-bound
	vii. Digestion, concentrated H_2SO_4 and H_2O_2	Residual
Ivanoff et al. [35]	i. 0.5 M $NaHCO_3$	Labile
	ii. Fumigation, 0.5 M $NaHCO_3$	Microbial
	iii. 1.0 M HCl	Moderately labile
	iv. 0.5 M NaOH[c]	Moderately labile and nonlabile
	v. Ignition, 1.0 M H_2SO_4 extraction	Residual

[a] Moderately labile organic phosphorus included inorganic phosphate in the NaOH extract, based on the assumption (now known to be in error) that this represented degradation of alkali-labile organic phosphorus.

[b] Moderately resistant (fulvic acid associated) and highly resistant (humic acid associated) organic phosphorus fractions were separated by acid precipitation at pH 1.0–1.5.

[c] Moderately labile (fulvic acid associated) and nonlabile (humic acid associated) organic phosphorus fractions were separated by acid precipitation at pH 0.2.

presence of humic-metal-phosphate complexes in alkali extracts (see Section 4.3).

Hedley et al. [56] proposed a comprehensive scheme to fractionate soil inorganic and organic phosphorus based on chemical solubility. The scheme was based in part on the earlier work of Chang and Jackson [53] and involved sequential extraction of soil with anion-exchange resin, 0.5 M $NaHCO_3$ at pH 8.5, 0.1 M NaOH, 0.1 M NaOH with ultrasonic dispersion, and 1.0 M HCl. A 'residual' fraction was determined by digestion of the residue in hydrogen peroxide and sulphuric acid. Plant availability was inferred on the basis of chemical stability. Thus, the weakly held organic phosphorus extracted in sodium bicarbonate was assumed to be more bioavailable than the strongly held organic phosphorus extracted by sodium hydroxide. The fraction extracted in sodium hydroxide after sonication was considered to be organic phosphorus held within soil aggregates, while the most stable forms of organic phosphorus were assumed to remain in the residual (unextracted) fraction. The original Hedley fractionation scheme has been extensively modified for use in different soils [10], but has been criticised for application to organic phosphorus (see below).

A scheme involving aspects of both the Bowman and Cole [54] and the Hedley et al. [56] procedures was developed for analysis of organic phosphorus in wetland soils [35]. This involved initial extraction of labile compounds in 0.5 M $NaHCO_3$ at pH 8.5, fumigation to estimate microbial phosphorus, an acid extraction in 1.0 M HCl, and finally extraction of stable organic phosphorus in 0.5 M NaOH. Organic phosphorus extracted in hydrochloric acid was included in the moderately labile pool. Residual phosphorus was determined by ignition and acid extraction.

Organic phosphorus extracted in sodium hydroxide was separated into fulvic acid associated (moderately labile) and humic acid associated (nonlabile) fractions, although the precipitation of humic acids was achieved in more acidic conditions (pH 0.2) than in the Bowman and Cole [54] scheme (pH 1.0–1.5). This is significant because it can influence the apparent organic phosphorus content of the humic and fulvic fractions [57]. For example, phytic acid remains in solution at pH 0.2, but is precipitated with humic substances in less acidic conditions [37].

In addition to the procedures developed for soils, several fractionation schemes were developed for phosphorus in aquatic sediments [58–62]. Most estimate organic phosphorus by some form of ignition following sequential extraction, although the procedures of Baldwin [58] and Golterman [59] involve the extraction of organic phosphorus and may be applicable to soils.

Fractionation schemes use only small soil samples ($\leq$0.5 g), are relatively simple to perform, and require only basic laboratory equipment. However, they may be unreliable for the analysis of organic phosphorus. The various extractants are unlikely to be either exhaustive or unique with respect to the target compounds. The chemical nature of the organic phosphorus within the operationally defined factions is understood poorly, and specific groups of compounds are probably present in more than one fraction. Fractions that are bioavailable in one soil may not be so in others [10,63] and although the inorganic phosphate in a fraction may be readily bioavailable, the organic phosphorus may not [64].

Importantly, conventional classification of organic phosphorus bioavailability based on chemical solubility is misleading, because plants can obtain phosphorus from supposedly 'stable' fractions of the soil organic phosphorus [65,66]. Furthermore, the unextractable fraction is often assumed to be organic phosphorus, yet there is no direct evidence for this. This can be investigated in future studies by subjecting the residual fraction to hypobromite oxidation and solution ^{31}P NMR spectroscopy to determine the presence of higher-order inositol phosphates [67,68], or by solid-state ^{31}P NMR to determine the possible presence of phosphonates [69].

Information on organic phosphorus obtained by sequential fractionation should clearly be interpreted with caution. Our understanding of the bioavailability of organic phosphorus in the various fractions will be enhanced by studies that link speciation of extracted compounds (e.g. by a secondary technique such as phosphatase hydrolysis or solution ^{31}P NMR spectroscopy) with evidence of uptake by plants and microbes.

3.3. Single-step extraction for subsequent organic phosphorus speciation

The ideal extractant for chemical characterisation of soil organic phosphorus should maximise recovery yet minimise alteration of chemical structure. In reality much of the soil organic phosphorus is extracted only in strong acids or bases, which inevitably alter the chemical structure of at least some compounds.

The choice of post-extraction speciation technique may influence the choice of extractant. For example, solution ^{31}P NMR spectroscopy requires an alkaline solution for optimum spectral resolution. Various extractants have been employed for phosphorus speciation by ^{31}P NMR spectroscopy, including 0.5 M NaOH [70], the cation exchange resin Chelex in water [71], Chelex in 0.5 M NaOH [72], 0.5 M NaOH plus 0.4 M NaF [73], and 0.25 M NaOH plus 0.05 M EDTA [47]. The choice of extractant not only influences the recovery of organic phosphorus from soil, but also the composition of the extracted compounds [47].

As discussed in Section 3.1, NaOH–EDTA is a single-step extractant for the quantitative recovery of soil organic phosphorus [45]. This makes it particularly suitable for analysis of soil phosphorus composition by ^{31}P NMR spectroscopy [74]. Chelex and EDTA both release phosphorus from complexation with paramagnetic ions, although EDTA maintains paramagnetic ions in solution, which allows pulse delay times in solution ^{31}P NMR spectroscopy to be minimised [75]. However, extractants that include EDTA are unsuitable for co-analysis of carbon or nitrogen, for which sodium hydrox-

ide alone or in combination with sodium fluoride may be preferable [72].

As in quantitative extraction procedures, pretreatment of soil by dilute acid removes polyvalent cations that can interfere with alkaline extraction and subsequent speciation of soil organic phosphorus [36]. However, there is a risk of hydrolysis of some compounds to phosphate (see Section 4.2). In a solution ^{31}P NMR spectroscopy study of wetland soils, alternative mild pre-extractants, including sodium bicarbonate and potassium chloride, improved spectral resolution of subsequent alkaline extracts [76]. Sodium EDTA has also been used to remove cations prior to alkaline extraction [77], but like strong acid extractants it also recovers some organic phosphorus [45].

3.4. Extraction of specific compounds

Numerous techniques are available for the examination of specific organic phosphorus compounds in soil, which generally involve extraction with a reagent specific to the recovery of a particular class of compound [7]. A detailed assessment of such techniques is outside the scope of this review, but procedures have been developed to extract phospholipids [78,79], sugar phosphates [80], inositol phosphates [68,81], adenosine triphosphate [82], nucleic acids, and nucleotides [22,83,84]. They are generally laborious and often limited by incomplete extraction or difficulties with post-extraction analysis [14].

Inositol phosphates are used here as an example of the potential problems involved with the extraction of specific organic phosphorus compounds from soil. The inositol phosphates are of particular interest due to their ubiquity in soils, and the presence of stereoisomeric forms that occur rarely elsewhere in nature [14]. The extraction of inositol phosphates from soil is complicated by their strong sorption to clays and their propensity to form insoluble complexes with polyvalent cations [13,14,81].

The conventional extraction scheme was developed initially by McKercher and Anderson [81] and later refined by Anderson [85]. Soil was first treated with a dilute mineral acid to remove carbonates, and then extracted with hot 3 M NaOH to recover the strongly bound inositol phosphates. Sesquioxides were removed as precipitates from the alkali extract and the inositol phosphates precipitated as barium salts in the presence of ethanol. They were then separated by ion-exchange chromatography [81].

Despite its widespread use, the McKercher and Anderson [81] method tended to overestimate the concentration of inositol phosphates, because other organic phosphorus compounds were present in the chromatographic fractions that supposedly contained only inositol phosphate [68]. This can be overcome using hypobromite oxidation [86], which oxidises soil organic matter without degrading inositol phosphates [87,88]. The brominated extract can then be analysed without interference. Other organic phosphates are completely oxidised by hypobromite treatment [87], although it was subsequently reported that DNA was not oxidised [89]. Inorganic pyrophosphate also resists oxidation, which precludes the simple estimation of inositol phosphates in brominated soil extracts by molybdate colorimetry [67]. However, *myo*- and *scyllo*-inositol hexakisphosphate can be quantified by solution ^{31}P NMR spectroscopy [67,90].

3.5. Extraction of organic phosphorus pools with biological or environmental relevance

Various extractants are used to determine pools of organic phosphorus with potential availability to plants or mobility in the environment. Organic phosphorus that is likely to be transferred in runoff to water bodies can be estimated by a simple water extraction [91], although there is little information on the organic phosphorus composition of drainage water with which to validate such procedures [92]. Pore water that moves slowly by matrix flow to depth can be obtained by centrifugation, although it may more closely approximate organic phosphorus available to plants rather than that transferred in runoff [93,94]. The small concentrations of organic phosphorus in both water extracts and soil solution can be further characterised by phosphatase hydrolysis [26,95,96] or solution ^{31}P NMR spectroscopy [97].

Organic phosphorus that is potentially available to plants is often estimated by extraction in sodium bicarbonate. This is based in part on the widespread use of bicarbonate extraction to estimate plant-available phosphate [98] and the fact that compounds which degrade rapidly in soils (e.g. ribonucleic acid) can be recovered in bicarbonate extracts [36]. A potentially useful index of bioavailable phosphorus included the increase in bicarbonate-extractable organic phosphorus that followed stimulation of the microbial biomass by addition of labile carbon substrate [99], but has not been adopted widely. It is also worth noting that organic phosphorus extracted from soil by a macroporous anion exchange resin (Lewatit MP500a, Bayer Corp., Pittsburgh, USA) was reported to represent a pool of potentially mineralisable organic phosphorus, apparently of microbial origin [103–105].

Unfortunately, information confirming bicarbonate-extractable organic phosphorus as a readily plant available fraction is scarce. Assessment of the potential bioavailability of bicarbonate-extractable organic phosphorus based on its susceptibility to hydrolysis by phosphatase enzymes has revealed both large [27] and small [28] proportions of hydrolysable compounds. Bicarbonate-extractable organic phosphorus is also sensitive to soil preparation [100,101], because concentrations can increase markedly following soil drying (see Section 4.1). Importantly, plants and algae access organic phosphorus compounds from soil pools with a range of chemical solubility rather than just the bicarbonate-extractable fraction [65,66,102].

An alternative approach to estimating bioavailable organic phosphorus is to simulate the secretion of organic anions such as citrate, malate, or oxalate by plant roots. These compounds chelate metals like aluminium in soil [106] and can

therefore simultaneously solubilise associated organic phosphorus. Citrate (50 mM at a pH of approximately 2.3) was used to estimate plant-available organic phosphorus in Australian soils, with speciation of the extracted compounds by phosphatase hydrolysis [28]. A large proportion of the extracted organic phosphorus was hydrolysed by phosphomonoesterase and phytase, in contrast to only small amounts of hydrolysable compounds in bicarbonate and water extracts.

Estimation of bioavailable soil organic phosphorus is important for studies of plant nutrition in both natural and managed environments, yet current procedures lack experimental validation. A key research priority is therefore the development of methodology to estimate pools of organic phosphorus with biological relevance. Development of such procedures should include information on the chemical forms of extracted organic phosphorus, as well as direct evidence of uptake by plants.

4. Methodological limitations

There are various limitations on the accuracy of procedures for extracting soil organic phosphorus. These range from artifacts induced by soil preparation to problems with the determination of organic phosphorus in the extracts. These issues have almost certainly influenced the accuracy of most studies, although it is often difficult to assess this, especially for some of the older literature.

4.1. Soil preparation

Soils are commonly dried, sieved, and stored prior to analysis, but this can alter the solubility of soil organic phosphorus. This is unlikely to influence quantitative analysis, but can profoundly affect the distribution of organic phosphorus in fractionation schemes and the extraction of organic phosphorus pools with biological or environmental relevance. In particular, drying can increase the amount of organic phosphorus extracted in water [91] and bicarbonate [101]. Water-extractable organic phosphorus is markedly influenced by even mild drying [91], because rewetting releases compounds from microbial cells that lyse during rapid rehydration [107,108]. A similar mechanism probably occurs following freezing and thawing [109].

The physical stresses induced by soil drying also disrupt organic matter coatings on clay and mineral surfaces [110], which may contribute to organic phosphorus solubilisation. Functional classification of organic phosphorus in water extracts of some dried Australian pasture soils revealed similar amounts of microbially derived phosphate diesters and phytic acid from the non-biomass soil organic matter [96]. However, it should be noted that the phytic acid fraction may have included small amounts of phospholipids not hydrolysed by the phosphodiesterase used in that study [27]. Physical disruption of organic matter coatings probably accounts for most of the increase in bicarbonate-extractable organic phosphorus following soil drying [101], because the high ionic strength of the bicarbonate solution reduces osmotic stress and associated lysis of viable cells compared to water extraction [111]. This hypothesis is supported by evidence that the composition of organic phosphorus in bicarbonate extracts is similar to that in strong alkaline extracts [27,112].

The impact of preparation is less clear for wetland soils. For example, Schlichting et al. [113] reported that extractable phosphorus concentrations in a peat decreased following drying, freezing, or cold storage. In contrast, Pezzolesi et al. [114] observed no significant differences in extractable phosphorus concentrations in wetland soils that were dried, frozen or stored under nitrogen gas. The differences may be linked to redox status, because oxidation of anaerobic soils, including those in rice paddy or wetlands, can induce marked changes in chemistry and microbiology [115]. However, there is little information on the effect of changing oxidation status on the composition and solubility of soil organic phosphorus.

The effects of soil pretreatment on the solubility of organic phosphorus are potentially important, but rarely assessed. As the effects appear to vary among soils, pretreatment methodology should be considered carefully before any study of soil organic phosphorus based on solubility.

4.2. Alteration of the chemical structure of organic phosphorus during extraction

Any procedure for extracting soil organic phosphorus must minimise degradation of the compounds of interest. This is difficult because the strong solvents necessary to extract organic phosphorus from soil inevitably alter the chemical structure of some compounds. The extent and nature of the degradation differs between acidic and alkaline solvents, while the importance of the respective effects varies with the objective of the study.

Many of the original extraction schemes for soil organic phosphorus were compromised by the inclusion of a strong acid extraction step, because several organic phosphates are unstable in acid solution [21]. These are likely to be quantitatively negligible when determining total organic phosphorus, but may have ecological relevance. Bowman [43] reported negligible acid hydrolysis for several compounds (glucose phosphate, phytic acid, *para*-nitrophenyl phosphate and bis-*para*-nitrophenyl phosphate) during his extraction procedure that involved addition of concentrated sulphuric acid. In contrast, Anderson [37] showed that strong acid pretreatment (4 M H_2SO_4, 12 M HCl) hydrolysed between 5 and 100% of some organic phosphorus esters (glucose 1-phosphate, RNA and DNA) added to soil. Mehta et al. [30] suggested that the strong acid extraction steps in their procedure did not cause significant hydrolysis of organic phosphorus, but could not rule this out completely. Acid pretreatment remains useful for the extraction of compounds such as inositol phosphates, where there is no risk of hydrolysis of the target compounds [14,22].

The effects of alkaline hydrolysis were reported recently for a wide range of soil organic phosphorus compounds [18,55]. Phosphate monoesters were stable, but some purified phospholipids added to soil extracts were hydrolysed to phosphate monoesters. Of the three phospholipids commonly found in soils, phosphatidyl choline was degraded within hours, phosphatidyl serine degraded more slowly over days, while phosphatidyl ethanolamine was relatively resistant to hydrolysis within the timeframe of the experiment. Of the nucleic acids, DNA was stable in alkaline solution, but RNA hydrolysed rapidly to its constituent mononucleotides.

Some phosphonates also degrade in alkaline solution. The most common phosphonate, 2-aminoethylphosphonic acid, is stable [55], but phosphonolipids degrade relatively rapidly, especially at elevated temperature [75]. Organic polyphosphates appear to be relatively stable in alkaline solution [55], but may precipitate with metals at high pH [116].

The risk of organic phosphorus hydrolysis in strong acid or alkaline solutions can be partly overcome by initially extracting labile compounds in a mild solvent such as sodium bicarbonate or dilute sodium hydroxide [35,37]. This recovers labile compounds that may be degraded by strong acid or base extraction, but which are likely to have ecological relevance. They can then be quantified and speciated by a suitable technique. It might be possible to use alternative extractants to avoid hydrolysis such as dimethylsulphoxide [1], while chelators such as EDTA at neutral pH might also be appropriate [77].

While some degradation of soil organic phosphorus during extraction and analysis is inevitable, understanding the process can minimise misinterpretation of results. As an example, phosphatidyl choline degrades in alkaline solution to two phosphate monoesters, glycerophosphate and phosphatidic acid (Fig. 1). This means that hydrolysis of phospholipids does not compromise the estimation of total organic phosphorus in alkaline extracts, because the hydrolysis products are other organic phosphorus compounds rather than free phosphate. In contrast, acid hydrolysis releases phosphate, leading to an underestimation of the organic phosphorus content. The major pathway of phosphatidyl choline hydrolysis in nature is by phospholipase C to yield choline phosphate. As this compound appears at a different chemical shift to glycerophosphate and phosphatidic acid in solution ^{31}P NMR spectroscopy, it is possible to differentiate analytical and enzymatic breakdown products of phosphatidyl choline in well-resolved spectra [46].

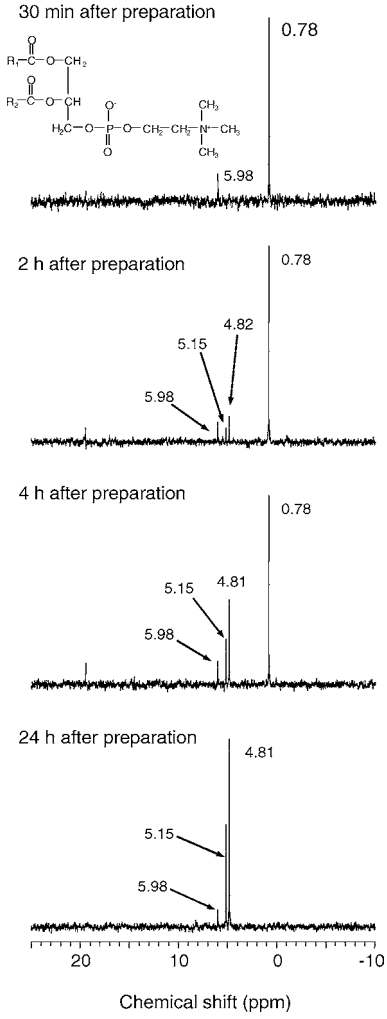


Fig. 1. The breakdown of phosphatidyl choline in NaOH assessed by solution ^{31}P NMR spectroscopy [55]. The strong signal from phosphatidyl choline at 0.78 ppm transforms relatively rapidly into two signals at 4.80 ppm and 5.15 ppm, representing glycerophosphate and phosphatidic acid, respectively. As degradation does not release free phosphate it does not compromise assessment of total organic phosphorus. It should be noted that choline phosphate, the natural product of enzymatic hydrolysis of phosphatidyl choline, appears close to 4.0 ppm in alkaline solution. This means that compounds originating from alkaline hydrolysis during extraction and analysis can be differentiated from the natural product of phospholipase C hydrolysis. R = fatty acyl chains.

4.3. Analysis of extracted organic phosphorus

Almost all procedures for the extraction of soil organic phosphorus use the same method to measure organic phosphorus in the extracts. Organic phosphorus cannot be quantified directly, because most solvents extract both organic and inorganic phosphorus from soil. Organic phosphorus is therefore determined colorimetrically as the difference between total phosphorus and inorganic phosphate.

The most common colorimetric detection procedure for inorganic phosphate is the molybdate blue method [117]. Total phosphorus is measured by the same procedure following a suitable digestion step to convert organic phosphorus to phosphate, although it can also be measured by inductively coupled plasma optical-emission spectroscopy (ICP–OES). Issues involved in the estimation of organic phosphorus by molybdate colorimetry are described in detail elsewhere in this issue [118]. For example, care must be taken to ensure that extracts are neutralised prior to analysis to ensure that colour development proceeds normally, while extracts containing EDTA must be sufficiently diluted to avoid interference with

colour development. However, some important issues specific to the analysis of soil extracts are described here.

The most serious source of error is due to the association of inorganic phosphate with humic substances. This prevents the phosphate from detection by molybdate colorimetry and leads to an overestimation of organic phosphorus, especially when humic material (and associated phosphate) is precipitated by chilling and acidification prior to analysis [119]. The quantitative importance of this is unclear. Craft and Richardson [120] reported no effect of precipitation on phosphate concentrations when analysed by several procedures, but recent studies reported that phosphate determined in alkaline extracts by colorimetry was consistently lower than that determined by solution ^{31}P NMR spectroscopy [121,122]. Underestimations varied among soils, but in a calcareous upland soil, phosphate concentrations determined by colorimetry were 1–4% of the total extracted phosphorus, compared to 20–34% determined by NMR spectroscopy [122]. Smaller errors were reported for extracts of other soils [15,27].

The association between phosphate and humic substances is probably through polyvalent metal bridging cations. This hypothesis is supported by the presence of phosphate in precipitated humic acids [123,124] and dialysed alkaline soil extracts [103]. As almost all studies in the older literature determined organic phosphorus in alkaline extracts by molybdate colorimetry, this effect may be highly significant.

A further important source of error is the presence of complex inorganic phosphates, such as pyrophosphate and polyphosphate, in soil extracts. These are included in the organic phosphorus fraction, because only free phosphate is determined by molybdate colorimetry (notwithstanding possible acid-induced hydrolysis of labile organic phosphorus). This can be overcome for water extracts by using ultraviolet photo-oxidation to determine organic phosphorus [125], but leads to an overestimation of organic phosphorus in other types of extracts. As pyrophosphate is present in almost all soils, typically constituting around 5% of the total phosphorus [15,16,103], it represents a potentially serious error in the estimation of soil organic phosphorus.

Problems of organic phosphorus detection are overcome by solution ^{31}P NMR spectroscopy. This technique avoids problems with complexes between phosphate and humic substances and allows organic phosphorus to be quantified separately from condensed inorganic phosphates. Such analysis is relatively expensive for most studies, but it may be worthwhile to assess the likely error in organic phosphorus determination for one or two representative samples.

4.4. Microbial contribution to extracted organic phosphorus

An aspect of soil organic phosphorus analysis that receives relatively little attention is the contribution of viable microbes to the measured organic phosphorus composition [126]. Soil preparation by drying and grinding combined with strong acid or base extraction is likely to release most of the microbial phosphorus to solution, which is then included in the respective total inorganic and organic phosphorus values.

Of particular relevance is the origin of phosphate diesters in alkaline extracts. These are typically a small proportion of the soil organic phosphorus, but are assumed to represent a labile fraction [103,127]. Microbial phosphorus is mainly phosphate diesters [10,128], yet the contribution of microbes that were alive at the time of extraction is considered rarely. Based on laboratory manipulations, Makarov et al. [126] suggested that much of the DNA in alkaline extracts of mountain soils was stable extracellular material, whereas the phospholipids were extracted mainly from living microbes. Separating cellular and extracellular compounds is necessary to understand soil organic phosphorus dynamics, so the development of a procedure to distinguish these sources of organic phosphorus would be a considerable advance.

5. Conclusions and research priorities

Despite the importance of organic phosphorus in soil biogeochemical cycles, our understanding of its dynamics and ecological function remain unsatisfactory. Progress is limited in part by extraction methodology, because information on organic phosphorus species provided by modern analytical techniques cannot be linked to biological availability with any degree of confidence.

Quantitative extraction of soil organic phosphorus is limited by the absence of a direct method to determine absolute values. It is remarkable that such a method remains elusive, yet it may be appropriate to reassess this given recent advances in analytical instrumentation. In the meantime, a useful alternative may be the adoption of a standard reference soil, as developed recently for analysis of aquatic sediments [62].

Differences in the results obtained using the various quantitative extraction procedures probably depend in part on the properties of the soil being analysed. This means that no single method has evolved as a ‘standard’ procedure, which complicates comparison of literature values. The single-step NaOH–EDTA procedure may be important in this respect, because it extracts similar amounts of organic phosphorus to conventional strong acid–base procedures and facilitates subsequent speciation of the extracted compounds by solution ^{31}P NMR spectroscopy. The method might be further improved by including an acid or chelating pretreatment, but this remains to be assessed.

Perhaps the most important issue is the development of procedures to accurately estimate pools of soil organic phosphorus with ecological relevance. Potential bioavailability is often inferred from chemical solubility, yet there is little empirical evidence to confirm this. Where bioavailability was investigated using sequential extraction [65,66], plant roots depleted all fractions irrespective of apparent stability, indicating clearly that chemical solubility and plant uptake are not coincident. A first step in addressing this will be to

obtain information on organic phosphorus species recovered by the various sequential extracts. Given the changes in organic phosphorus solubility that can result from soil drying, such studies should also assess the effect of soil pretreatment prior to extraction.

It is possible that linking chemical solubility and potential bioavailability may require the development of novel approaches that go beyond conventional schemes designed to assess inorganic phosphate. There are important conceptual problems with measuring static pools of organic phosphorus to assess plant availability, because such pools may not reflect turnover in soil. For example, phosphate diesters are the major inputs of organic phosphorus to soils, but they degrade rapidly and only small concentrations are detected in most soils. In contrast, relatively small amounts of inositol phosphates enter soil, but they accumulate to form the major group of organic phosphorus compounds [10].

Small amounts of some compounds may therefore conceal rapid rates of turnover. This means that the contribution of organic phosphorus to plant nutrition may be more usefully assessed by measuring rates of turnover rather than amounts in static pools. However, methods to determine rates organic phosphorus turnover are lengthy, complex, and difficult to interpret in soils with a high capacity to sorb phosphate [129–131]. Until such methods are suitable for routine use, extraction schemes will remain an important tool for assessing organic phosphorus in soil. In this respect, the development of a simple extraction procedure to estimate plant availability is desirable. This would facilitate a rapid improvement in our understanding of the role of soil organic phosphorus in biogeochemical cycles, crop nutrition, and the functioning of terrestrial ecosystems.

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