Soil organic phosphorus in tropical forests: an assessment of the NaOH–EDTA extraction procedure for quantitative analysis by solution $^{31}$P NMR spectroscopy

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Summary

The extraction of soil organic phosphorus by the NaOH-EDTA procedure was assessed in detail for a tropical forest soil (clay-loam, pH 4.3, total carbon 2.7%). Optimum conditions for the quantification of soil organic phosphorus and characterization of its composition by solution $^{31}$P NMR spectroscopy were extraction in a solution containing 0.25 M NaOH and 50 mM Na$_2$EDTA in a 1:20 solid to solution ratio for 4 hours at ambient laboratory temperature. Replicate analyses yielded a coefficient of variation of 3% for organic phosphorus as a proportion of the spectral area. There was no significant difference in total phosphorus extraction from fresh and air-dried soil, although slightly more organic phosphorus and less paramagnetic ions were extracted from dried soil. The procedure was not improved by changing the concentration of NaOH or EDTA, extraction time, or solid to solution ratio. Pre-extraction with HCl or Na$_2$EDTA did not increase subsequent organic phosphorus extraction in NaOH-EDTA or improve spectral resolution in solution $^{31}$P NMR spectroscopy. Post-extraction treatment with Chelex resin did not improve spectral resolution, but removed small concentrations of phosphorus from the extracts. Increasing the pH of NaOH-EDTA extracts (up to 1.0 M NaOH) increased the concentration of phosphate monoesters, but decreased DNA to an undetectable level, indicating its hydrolysis in strong alkali. The standardized NaOH-EDTA extraction procedure is therefore recommended for the analysis of organic phosphorus in tropical forest soils.

Introduction

The single-step NaOH–EDTA extraction procedure was developed for the quantitative analysis of soil organic phosphorus and was reported to compare favourably with conventional acid-base procedures for a range of soils (Bowman & Moir, 1993). It was shown subsequently to extract a greater range of phosphorus forms than other extractants such as NaOH alone or NaOH with Chelex resin (Cade-Menun & Preston, 1996) and has been adopted widely for qualitative studies on the chemical nature of soil organic phosphorus by solution $^{31}$P NMR spectroscopy (reviewed in Cade-Menun, 2005). The original procedure involved extraction in a 1:50 solid to solution ratio for 2 hours at 85°C with no shaking (Bowman & Moir, 1993), but has been modified for NMR spectroscopy by reducing the solid to solution ratio to 1:20 (or occasionally 1:10) and using a longer extraction time with shaking at ambient laboratory temperature (e.g. Cade-Menun et al., 2002; Turner et al., 2005b). The influence of these changes on soil organic phosphorus extraction has not been assessed, although some studies using the modified procedure have reported less phosphorus extracted in NaOH-EDTA than in NaOH alone (Cade-Menun et al., 2002; George et al., 2006).

A limitation of the NaOH–EDTA procedure for solution $^{31}$P NMR spectroscopy of some soils, evident in the study of Cade-Menun & Preston (1996), is that spectral resolution can be poor relative to other extractants. This is presumably due to paramagnetic ions such as iron and manganese that are maintained in solution in NaOH-EDTA extracts. Attempts to remove interfering paramagnetic ions have involved either treating NaOH-EDTA extracts with a cation exchange resin (Robinson et al., 1998; McDowell & Stewart, 2005b), or pre-extracting soil with calcium EDTA–dithionite (McDowell & Stewart, 2005a) or salt solutions (Robinson et al., 1998). A sodium EDTA pre-extraction was also recommended to improve organic phosphorus extraction in strong alkali (Harrap, 1998).
and recently to improve polyphosphate extraction from lake sediments in NaOH-EDTA (Hupfer et al., 2004). Pre-extraction with mineral acid has also been widely used to improve subsequent organic phosphorus extraction in alkali (e.g. Mehta et al., 1954; Steward & Oades, 1972), but has not been tested as part of the NaOH-EDTA procedure. Potential problems with pre-treatment prior to alkaline extraction include the risk of hydrolysis of acid-labile organic phosphates and the removal of some organic phosphorus in the pre-extracts (Turner et al., 2005). For example, pre-treatment with mineral acid (Bowman & Cole, 1978; Ivanoff et al., 1998), Na$_2$EDTA (Bowman & Moir, 1993) and salt solutions (Robinson et al., 1998) can all extract considerable concentrations of soil organic phosphorus.

The NaOH-EDTA procedure has not been tested for tropical forest soils, which are typically acidic, strongly weathered, and contain large concentrations of iron oxides. Assessment of organic phosphorus in such soils is problematic due to the difficulty in reconciling values obtained by extraction and ignition, which can vary considerably (Condron et al., 1990). However, it is assumed that values obtained by extraction are more accurate, because ignition causes marked changes in the solubility of organic phosphorus in strongly-weathered soils (Williams et al., 1970). In terms of solution $^{31}$P NMR spectroscopy, the abundant iron oxides in tropical soils would be expected to cause significant line broadening, although in a study of Madagascan rice soils solution $^{31}$P NMR spectra were sufficiently well-resolved in many samples to allow the quantification of inositol phosphates (Turner, 2006). The only work on tropical forest soils using solution $^{31}$P NMR spectroscopy involved the assessment of soil organic phosphorus along a catena (pH in surface soils 3.9–5.0; total carbon 1.1–3.2%) under evergreen forest in Liberia (Forster & Zech, 1993). Organic phosphorus between 46 and 65% of the phosphorus extracted in 0.5 M NaOH (1:20 solid to solution ratio, 16 hours), with phosphate detection by automated molybdate colorimetry using a Lachat Quickchem 8500 (Hach Ltd, Loveland, CO). This procedure gave quantitative recovery of total phosphorus from certified reference soil (Loam Soil D; High Purity Standards, Charleston, SC). Soil pH was determined in a 1:2 soil to deionized water ratio, repeated sonication at 0°C), of which between 74 and 85% was phosphate monoesters and the remainder DNA.

Given that the NaOH–EDTA extraction procedure has not been rigorously tested for tropical soils, it is possible that it could be improved by adjustment of extraction parameters such as the concentration of NaOH or EDTA, or inclusion of a pre-extraction step. This work assessed the NaOH–EDTA extraction procedure in detail for a tropical forest soil. My aim was to determine the precise conditions necessary to maximize the extraction of soil organic phosphorus and simultaneously optimize its subsequent analysis by solution $^{31}$P NMR spectroscopy.

**Methods**

**Soil sampling and preparation**

The soil used throughout this study was sampled from Albrook Hill (8°58′44″N, 79°34′45″W), near Panama City, Republic of Panama. The site receives approximately 1800 mm of rainfall a year, with a 4-month dry season between December and April. This is the site of a 1 ha forest census plot and the soil was considered to be representative of those under lowland tropical forest, being acidic, rich in aluminium and iron oxides, but with relatively low concentrations of carbon and phosphorus. Soil for this study was taken from the surface 10 cm in an area to the north of the census plot on a gentle slope. Fresh soil (24% moisture content) was sieved (< 2 mm) to remove roots and stones, and stored at 4°C for ~ 1 week. It was then spread on plastic plates and air-dried at ~ 22°C to a constant weight (10 days). The dry soil was then ground in a ball mill and stored in a sealed plastic bag at ambient laboratory temperature and humidity (approx. 23°C and 55%). Two additional soils were used to assess the effect of post-extraction treatment with Chelex resin (see below). These were sampled from 1 ha forest census plots in central Panama (0–10 cm) and were prepared as described above.

**Determination of soil chemical properties**

Soil properties are reported in Table 1. The soils have not been formally classified, so no taxonomic classes are given here. Total carbon and nitrogen were determined by combustion and gas chromatography using a Flash NC1112 Soil Analyzer (CE Elantech, Lakewood, NJ). Total phosphorus was determined by ignition (550°C, 1 hour) and extraction in 1 M H$_2$SO$_4$ (1:50 soil to solution ratio, 16 hours), with phosphate detection by automated molybdate colorimetry using a Lachat Quickechem 8500 (Hach Ltd, Loveland, CO). This procedure gave quantitative recovery of total phosphorus from certified reference soil (Loam Soil D; High Purity Standards, Charleston, SC). Soil pH was determined in a 1:2 soil to deionized water ratio using a glass electrode. Plant-available phosphorus was determined by Mehlich extraction (Mehlich, 1984), while amorphous aluminium, iron and manganese were determined by extraction.
in a solution containing ammonium oxalate and oxalic acid (Loepert & Inskeep, 1996), with detection by inductively-coupled plasma optical-emission spectrometry (ICP-OES) using an Optima 2100 (Perkin-Elmer Inc., Shelton, CT). For comparison with the NaOH-EDTA procedure, organic phosphorus was determined by an ignition procedure (Saunders & Williams, 1955) using 1 M H₂SO₄ as the extractant.

**Extraction tests**

A series of experiments were conducted on the Albrook soil to assess the NaOH–EDTA extraction procedure. Unless otherwise stated, the following experiments were conducted by extracting air-dried soil (1.50 ± 0.01 g) in 30 ml of a solution containing 0.25 M NaOH and 50 mM Na₂EDTA (1:20 solid to solution ratio) with a 16 hours shaking time at ambient laboratory temperature. The extracts were analysed for phosphorus fractions, metals and absorbance, with selected extracts also analysed by solution ³¹P NMR spectroscopy (see below).

The precision of the procedure was first assessed by extracting soil in triplicate and analysing each extract separately by solution ³¹P NMR spectroscopy. The effect of soil to solution ratio was assessed by extracting soil in ratios of 1:5, 1:10, 1:20 and 1:40. The effect of shaking time was assessed by extracting soil for 1, 4 and 16 hours in triplicate. The effect of sonication (in an ultrasonic bath) was also tested using 5 minutes of sonication alone, or with a subsequent 1 hour shaking period. The effect of soil drying was assessed by extracting fresh and air-dried soil in triplicate. Fresh soil was extracted on the same day that air-drying of the remaining soil began. The weight of fresh soil and the phosphorus concentrations in the extracts were adjusted to account for soil moisture. The influence of extract pH and the presence of EDTA on organic phosphorus extraction was assessed by extracting soil with solutions containing different concentrations of NaOH (0.05–1.00 M) and Na₂EDTA (0, 25, or 50 mM). Selected extracts were analysed by solution ³¹P NMR spectroscopy. The effect of pre-extraction on subsequent organic phosphorus extraction in NaOH-EDTA and spectral resolution in solution ³¹P NMR spectroscopy was assessed by pre-extracting soil in NaOH-EDTA. The solution was decanted and prepared for NMR spectroscopy as described below.

**Analysis of extracts for phosphorus fractions, metals and absorbance**

NaOH-EDTA extracts were centrifuged at 8000 g for 30 minutes and a 1 ml aliquot was taken for dilution and determination of phosphorus and metals. The aliquot was neutralized using phenolphthalein indicator and 3 M H₂SO₄, and then diluted to 20 ml with deionized water. Reactive phosphorus, which approximates inorganic phosphate, was determined by molybdate colorimetry and flow injection analysis. Interference by organic matter was corrected by analysing samples with acid only (i.e. no reagents). Total phosphorus and metals (aluminium, iron and manganese) were determined by ICP-OES. Unreactive phosphorus, which includes organic phosphorus and inorganic polyphosphates (including pyrophosphate), was calculated as the difference between total and reactive phosphorus. The pH of the remaining extract was measured using a glass electrode (with values converted to hydroxide ion concentration, but not corrected for interference from sodium ions), and absorbance at 550 nm was measured as a proxy for soluble organic matter (Bowman & Moir, 1993). Acid and EDTA pre-extracts were analysed as described above following a suitable dilution.

**Solution NMR spectroscopy**

For NMR spectroscopy, a 20 ml aliquot of soil extract was spiked with 1 ml of methylene diphosphonic acid (MDPA) solution as an internal standard (either 50 or 67 mg P kg⁻¹ soil), frozen at −35°C, lyophilized (~ 48 hours), and homogenized by gently crushing to a fine powder. Each lyophilized extract (~ 100 mg) was re-dissolved in 0.1 ml of deuterium oxide and 0.9 ml of a solution containing 1.0 M NaOH and 100 mM Na₂EDTA, and then transferred to a 5-mm NMR tube. Solution ³¹P NMR spectra were obtained using a Bruker Avance DRX 500 MHz spectrometer (Bruker, Billerica, MA, USA) operating at 202.456 MHz for ³¹P. Samples were analysed using a 6 μs pulse (45°), a delay time of 2.0 s, an acquisition time of 0.4 s, and broadband proton decoupling. Approximately 30 000 scans were acquired for each sample. Spectra were plotted with a line broadening of 5 Hz and chemical shifts of signals were determined in parts per million (p.p.m.) relative to an external standard of 85% H₃PO₄. Signals were assigned to phosphorus compounds based on literature reports of model compounds spiked in NaOH-EDTA soil extracts (Turner et al., 2003a). Signal areas were calculated by integration and concentrations of phosphorus compounds were calculated from the integral value of the MDPA internal standard at 17.63 ± 0.09 p.p.m. (n = 17). Chemical shifts of signals were identified as follows: inorganic phosphate, 6.41 ± 0.11 p.p.m. (n = 17); DNA, −0.14 ± 0.07 p.p.m. (n = 15); pyrophosphate, −3.92 ± 0.21 p.p.m. (n = 16);
phosphate monoesters, between 4.0 and 6.0 p.p.m., with prominent signals at 5.70 ± 0.08 p.p.m. (n = 5), 5.44 ± 0.03 p.p.m. (n = 5), 5.35 ± 0.01 p.p.m. (n = 4), 5.30 ± 0.02 p.p.m. (n = 6), 5.25 ± 0.05 p.p.m. (n = 5), 5.07 ± 0.04 p.p.m. (n = 8), 4.96 ± 0.02 p.p.m. (n = 9), 4.87 ± 0.01 p.p.m. (n = 8), 4.73 ± 0.03 p.p.m. (n = 13), 4.67 ± 0.03 p.p.m. (n = 5), 4.28 ± 0.06 p.p.m. (n = 5), 4.28 ± 0.06 p.p.m. (n = 6). A further phosphate monoester signal was detected downfield of the inorganic phosphate signal at 6.95 ± 0.07 p.p.m. (n = 6) in spectra of Soil 1 (Albrook), probably representing an unidentified inositol phosphate (Turner & Richardson, 2004). All spectral processing was done using NMR Utility Transform Software (NUTS) for Windows (Acorn NMR Inc., Livermore, CA). A blank sample containing only 0.25 M NaOH and 50 mM Na₂EDTA taken through the entire procedure contained only 1 mg P kg⁻¹ as phosphate.

Statistics
Differences among means were assessed by single-factor analysis of variance, with further means separation by least significant differences at the 5% level.

Results

Replicate analyses by solution ³¹P NMR spectroscopy

Three replicate NMR spectra of the Albrook soil are shown in Figure 1. The lower signal-to-noise ratio for the lower spectrum was due to the use of a smaller amount of lyophilized powder in the re-dissolved solution. The mean concentration of total phosphorus determined as the sum of all signals based on the MDPA internal standard was 130.3 ± 12.8 mg P kg⁻¹ (Table 2). The coefficient of variation for concentrations of individual compounds was smallest for DNA (2%) and largest for pyrophosphate (29%). When calculated as the proportion (%) of the extracted P, coefficients of variation were generally smaller than for the concentrations, being 2% for phosphate, 3% for total organic phosphorus and 6% for phosphate monoesters. Values were larger for DNA (10%) and pyrophosphate (33%).

Effect of soil drying on phosphorus extraction in NaOH-EDTA

Drying did not significantly influence the extraction of total phosphorus (Table 3). However, there were significant changes in the reactive and unreactive fractions, with less reactive (inorganic) phosphorus and more unreactive (organic) phosphorus extracted from dried soil. There were also significant differences in metal extraction, with more aluminium and manganese, but less iron, extracted from dried soil (Table 3). There was no significant difference in absorbance between extracts of fresh and dried soils.

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Table 2 Replicate analyses of a tropical forest soil (Soil 1, Albrook) extracted in a solution containing 0.25 M NaOH and 50 mM Na₂EDTA, with phosphorus detection by solution \(^{31}\)P NMR spectroscopy. Signals were quantified using an internal MDPA standard (see methods). Values are mean ± standard deviation of three replicate extracts.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration /mg P kg(^{-1})</th>
<th>Proportion of the extracted P /%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± CV(^a)</td>
<td>Mean ± CV(^a)</td>
</tr>
<tr>
<td>Total P</td>
<td>130.3 ± 12.8 ± 10</td>
<td>104.6 ± 1.4 ± 3</td>
</tr>
<tr>
<td>Organic P</td>
<td>71.2 ± 8.9 ± 12</td>
<td>48.4 ± 0.7 ± 3</td>
</tr>
<tr>
<td>Phosphate</td>
<td>56.0 ± 4.4 ± 8</td>
<td>43.0 ± 1.0 ± 0.2</td>
</tr>
<tr>
<td>Phosphate monoesters</td>
<td>54.6 ± 8.9 ± 16</td>
<td>41.7 ± 2.7 ± 6</td>
</tr>
<tr>
<td>DNA</td>
<td>16.6 ± 0.3 ± 2</td>
<td>12.8 ± 1.3 ± 10</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>3.1 ± 0.9 ± 29</td>
<td>2.4 ± 0.8 ± 33</td>
</tr>
</tbody>
</table>

\(^a\)CV, coefficient of variation (standard deviation/mean x 100).

Effect of soil to solution ratio on phosphorus extraction in NaOH-EDTA

Total phosphorus extraction in NaOH-EDTA increased linearly as the soil to solution ratio increased between 1:5 and 1:40 (Figure 3a). This was entirely accounted for by an increase in reactive phosphorus, which occurred in parallel with an increase in aluminium, from 1423 mg Al kg\(^{-1}\) at a 1:5 ratio to 5279 mg Al kg\(^{-1}\) at a 1:40 ratio (data not shown). The relationship between reactive phosphorus and aluminium was described by the equation: \(Y = 0.012x + 10.6, R^2 = 0.99, P < 0.003\). There was no significant change in unreactive phosphorus extraction (Figure 3a). The free hydroxide ion concentration increased as the soil to solution ratio increased (Figure 3b), from 72 mM (pH 12.86) at a 1:5 ratio to 117 mM (pH 13.07) at a 1:40 ratio. The hydroxide ion concentration of the NaOH-EDTA solution alone was 126 mM (pH 13.10).

Effect of pre-extraction on NaOH-EDTA extraction and solution \(^{31}\)P NMR spectroscopy

Both the acid and sodium EDTA pre-extracts contained small concentrations of phosphorus (Table 4). In the acid extracts this was mainly reactive (inorganic) phosphorus, but the EDTA extract contained more unreactive (organic) phosphorus. Differences among the three pre-extracts were significant for phosphorus compounds, metals and absorbance (Table 4). Only small concentrations of aluminium were detected in the pre-extracts compared with those in the NaOH-EDTA extracts. Some iron and manganese was recovered in the pre-extracts, with maximum concentrations in the 0.5 M HCl extract, but

Table 3 The effect of air-drying on the extraction of phosphorus fractions, metals, and absorbance at 550 nm from a tropical forest soil (Soil 1, Albrook) extracted in a solution containing 0.25 M NaOH and 50 mM Na₂EDTA. RP, reactive P; UP, unreactive P; NS, not significant. Values are mean ± standard deviation of three replicate extracts.

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>Total P</th>
<th>RP</th>
<th>UP</th>
<th>Al</th>
<th>Fe</th>
<th>Mn</th>
<th>Absorbance /x 10(^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>132 ± 3</td>
<td>59 ± 3</td>
<td>73 ± 3</td>
<td>3221 ± 37</td>
<td>74 ± 13</td>
<td>47 ± 1</td>
<td>589 ± 16</td>
</tr>
<tr>
<td>Dried</td>
<td>128 ± 3</td>
<td>50 ± 1</td>
<td>78 ± 1</td>
<td>3554 ± 13</td>
<td>40 ± 3</td>
<td>62 ± 2</td>
<td>598 ± 11</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
<td>&lt; 0.01</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 3 The effect of soil to solution ratio on (a) the extraction of phosphorus fractions and (b) hydroxide ion concentrations for a tropical forest soil (Soil 1, Albrook) extracted in a solution containing 0.25 M NaOH and 50 mM Na$_2$EDTA. TP, total phosphorus; UP, unreactive phosphorus; RP, reactive phosphorus. Regression models in (a) were described by the following equations: Total P, $Y = 1.55x + 99.5$; $R^2 = 0.99$; $P < 0.005$; Reactive P, $Y = 1.28x + 24.2$; $R^2 = 0.99$; $P < 0.007$; Unreactive P, gradient not significantly different from zero.

Effect of post-extraction treatment with Chelex resin on solution $^{31}$P NMR spectral resolution

Post-extraction treatment of NaOH-EDTA extracts with Chelex resin did not visibly improve the resolution of solution $^{31}$P NMR spectra of the Albrook soil, or two additional tropical forest soils (Figure 4). However, total phosphorus concentrations (determined from the MDPA internal standard) were reduced in Chelex-treated extracts of all three soils (Table 6). This was due to a reduction in phosphate monoesters and pyrophosphate for Soil 1 (Albrook), whereas phosphate, DNA and pyrophosphate concentrations were reduced in the Chelex-treated sample of Soil 3 (Laguna). Overall, organic phosphorus concentrations in Chelex-treated extracts were reduced by 6.9% (Soil 1), 10.4% (Soil 2) and 3.6% (Soil 3).

Effect of NaOH and EDTA concentrations on the extraction of phosphorus, metals and organic matter

Reactive phosphorus concentrations increased in a linear manner with increasing hydroxide ion concentrations, although considerably more reactive phosphorus was extracted in the presence of EDTA (Figure 5a). In contrast, the unreactive phosphorus concentration increased to approximately 80 mg P kg$^{-1}$ at a hydroxide ion concentration of around 50 mM and then remained constant (Figure 5b). A slight increase was detected in the two strongest NaOH (0.75 and 1.00 M) plus 50 mM Na$_2$EDTA extracts, although no such increase was detected for the corresponding 25 mM Na$_2$EDTA extracts.

There were striking differences in absorbance among the three EDTA treatments (Figure 5c). For all EDTA treatments, absorbance initially increased with an increase in hydroxide ion concentration to a maximum at approximately 80 mg P kg$^{-1}$ at a hydroxide ion concentration of around 50 mM and then remained constant (Figure 5b). A slight increase was detected in the two strongest NaOH (0.75 and 1.00 M) plus 50 mM Na$_2$EDTA extracts, although no such increase was detected for the corresponding 25 mM Na$_2$EDTA extracts.

There were striking differences in absorbance among the three EDTA treatments (Figure 5c). For all EDTA treatments, absorbance initially increased with an increase in hydroxide ion concentration to a maximum at approximately 20–30 mM. For NaOH plus 50 mM Na$_2$EDTA extracts the absorbance values then remained constant at around $550 \times 10^{-3}$ as hydroxide ion concentrations increased, whereas for NaOH alone and NaOH plus 25 mM Na$_2$EDTA extracts the absorbance values decreased markedly with increasing hydroxide ion concentration.

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although relatively constant values were reached. For the 25 mM Na$_2$EDTA extracts these were around $280 \times 10^{-3}$ (i.e. approximately half of those for 50 mM Na$_2$EDTA extracts), while for NaOH alone the values decreased to $100 \times 10^{-3}$ (i.e. < one-fifth of those for 50 mM Na$_2$EDTA extracts).

Aluminium concentrations increased continuously with increasing hydroxide ion concentration, although the rate of increase was lower for samples that did not contain EDTA (Figure 5d). The concentrations of aluminium and reactive phosphorus were strongly correlated for all extracts, although the relationships were different for those in NaOH alone compared with those with EDTA (Figure 5e). In contrast, iron and manganese concentrations decreased markedly with increasing NaOH concentration and were small at hydroxide ion concentrations $\geq 100$ mM ($\geq$ pH 13.00) (Figure 5c,f). The presence of EDTA reduced iron concentrations to a greater extent than in NaOH alone (Figure 5e). However, the presence of EDTA increased manganese extraction at low hydroxide concentrations compared with extracts in NaOH alone (Figure 5f). Manganese concentrations were < 40 mg Mn kg$^{-1}$ for NaOH alone at all hydroxide concentrations, but only reached these values in the presence of 50 mM EDTA at hydroxide ion concentrations $> 100$ mM. The decreases in iron in NaOH alone and NaOH plus 25 mM Na$_2$EDTA were strongly correlated with the decreases in absorbance, suggesting the precipitation of humic–iron compounds.

**Effect of NaOH and EDTA concentrations on phosphorus composition by solution $^{31}$P NMR spectroscopy**

There was little change in spectral resolution for samples extracted in different concentrations of NaOH alone, although there were obvious differences in signals from DNA and pyrophosphate (Figure 7). A small increase in total phosphorus was detected with increasing NaOH (Table 7), which was accounted for mainly by an increase in organic phosphorus, particularly phosphate monoesters (Table 7). However, there were notable differences between phosphorus concentrations determined by

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**Table 4** Concentrations of phosphorus fractions, metals, and absorbance at 550 nm in pre-extracts and subsequent NaOH–EDTA extracts (0.25 M NaOH + 50 mM Na$_2$EDTA) of a tropical forest soil (Soil 1, Albrook). RP, reactive P; UP, unreactive P; NS, not significant; LSD, least significant difference (5%). Values are mean ± standard deviation of three replicate extracts

<table>
<thead>
<tr>
<th>Pre-extracts</th>
<th>Phosphorus/mg P kg$^{-1}$</th>
<th>Metals/mg kg$^{-1}$</th>
<th>Absorbance /$\times 10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total P</td>
<td>RP</td>
<td>UP</td>
</tr>
<tr>
<td>Pre-treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 M HCl</td>
<td>1.6 ± 1.0</td>
<td>1.0 ± 0.3</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>0.5 M HCl</td>
<td>4.5 ± 4.0</td>
<td>4.0 ± 0.4</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>50 mM Na$_2$EDTA</td>
<td>4.3 ± 2.0</td>
<td>2.0 ± 0.1</td>
<td>2.3 ± 1.0</td>
</tr>
<tr>
<td>$P$ value</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>LSD</td>
<td>1.1</td>
<td>0.6</td>
<td>1.1</td>
</tr>
</tbody>
</table>

**Post-treatment NaOH–EDTA extracts**

| Untreated             | 128 ± 1                   | 50 ± 1               | 78 ± 1                      | 3554 ± 13               | 40 ± 3                 | 62 ± 2         | 598 ± 11        |
| 0.1 M HCl             | 123 ± 9                   | 47 ± 4               | 76 ± 5                      | 2907 ± 102              | 52 ± 5                 | 64 ± 6         | 566 ± 15        |
| 0.5 M HCl             | 119 ± 1                   | 43 ± 1               | 76 ± 1                      | 2297 ± 61               | 50 ± 9                 | 66 ± 10        | 529 ± 2         |
| 50 mM Na$_2$EDTA      | 115 ± 2                   | 47 ± 1               | 68 ± 2                      | 2920 ± 75               | 48 ± 16                | 74 ± 9         | 552 ± 11        |
| $P$ value             | < 0.05                    | < 0.05               | < 0.01                      | < 0.001                 | NS                     | NS             | < 0.001         |
| LSD                   | 8.5                       | 3.9                 | 4.8                         | 133                     | 17.9                   | 13.7           | 20              |

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**Table 5** The effect of soil pre-extraction on concentrations of phosphorus compounds extracted from a tropical forest soil (Soil 1, Albrook) in a solution containing 0.25 M NaOH and 50 mM Na$_2$EDTA, with detection by solution $^{31}$P NMR spectroscopy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total P$^a$</th>
<th>Phosphate$^a$</th>
<th>Monoesters$^a$</th>
<th>DNA$^b$</th>
<th>Pyrophosphate-P$^b$</th>
<th>Organic P$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated$^c$</td>
<td>130.3 (36.4)</td>
<td>56.0 (43.0)</td>
<td>54.5 (41.7)</td>
<td>16.6</td>
<td>3.1</td>
<td>71.2 (54.6)</td>
</tr>
<tr>
<td>0.1 M HCl</td>
<td>140.1 (39.1)</td>
<td>66.1 (47.2)</td>
<td>50.2 (35.8)</td>
<td>16.7</td>
<td>7.2</td>
<td>66.9 (40.9)</td>
</tr>
<tr>
<td>0.5 M HCl</td>
<td>132.1 (36.9)</td>
<td>62.1 (47.0)</td>
<td>49.1 (37.2)</td>
<td>13.3</td>
<td>7.5</td>
<td>62.5 (42.9)</td>
</tr>
<tr>
<td>50 mM EDTA</td>
<td>128.3 (35.7)</td>
<td>53.4 (41.6)</td>
<td>53.4 (41.6)</td>
<td>16.3</td>
<td>5.3</td>
<td>69.7 (45.7)</td>
</tr>
</tbody>
</table>

$^a$Values in parentheses are the proportion (%) of the total soil P.

$^b$Values in parentheses are the proportion (%) of the total extracted P.

$^c$Mean of three replicate extracts (see Table 2).
Figure 4 Solution $^{31}$P NMR spectra of 0.25 M NaOH plus 50 mM Na$_2$EDTA extracts of three tropical forest soils either untreated or treated with Chelex 100 resin; (a) Soil 1 untreated, (b) Soil 1 treated with Chelex, (c) Soil 2 untreated, (d) Soil 2 treated with Chelex, (e) Soil 3 untreated, (f) Soil 3 treated with Chelex. See Table 6 for concentrations of phosphorus compounds. The spectra are truncated vertically to show fine resolution.

NMR spectroscopy and those determined by colorimetry/ICP-OES (see Figure 5). Spectra of NaOH extracts did not contain the signal at 6.8 p.p.m. that was present in NaOH–EDTA extracts (see Figure 1).

There were marked differences in spectra for samples extracted in different concentrations of NaOH with 50 mM Na$_2$EDTA (Figure 8). Clear line broadening was evident for the 0.1 M NaOH plus 50 mM Na$_2$EDTA spectrum (Figure 8, top spectrum), although the other spectra were well resolved. Total phosphorus determined by ICP-OES increased with increasing NaOH concentration up to 0.5 M, and then declined in the 1.0 M NaOH extract (Table 7). Of the total organic phosphorus, phosphate monoesters increased with increasing NaOH concentration (Figure 9, left panel), while DNA concentrations increased to a maximum in the 0.15 M NaOH extract, and then declined to an undetectable level in the 1.0 M NaOH extract (Figure 9, right panel). The presence of EDTA therefore caused clear differences in the organic phosphorus composition, with less DNA extracted in NaOH alone compared with NaOH–EDTA, at least for NaOH concentrations < 0.5 M (Table 7).

**Total organic phosphorus determination by ignition**

Values of total organic phosphorus determined by ignition (the difference in acid-extractable phosphate between ignited and unignited samples) were: Albrook (Soil 1) 306 mg P kg$^{-1}$, Pipeline Road (Soil 2) 236 mg P kg$^{-1}$, and Laguna (Soil 3) 57 mg P kg$^{-1}$. Error in this procedure was < 2% for triplicate extracts.

**Discussion**

The error associated with replicate analyses reported here, which includes error associated with both extraction and solution $^{31}$P NMR spectroscopy, agrees broadly with results from the analysis of manures (Kemme et al., 1999), high organic matter wetland soils (Turner et al., 2007), and marine particulate material (Cade-Menun et al., 2005). The somewhat lower coefficients of variation for proportions of compounds, as compared with concentrations, suggests that the MDPA internal standard introduces an additional source of error, although this should be no greater than that derived from calculation of concentrations based on determination of total phosphorus in the extract by ICP-OES.

Although air-drying can induce marked changes in organic phosphorus solubility in mild extractants such as water or sodium bicarbonate (Turner & Haygarth, 2001, 2003), the difference in unreactive phosphorus (organic phosphorus plus pyrophosphate) extracted from fresh and air-dried samples was relatively small for the soil analysed here, as expected for quantitative organic phosphorus extraction (Turner et al., 2005). However, the difference was significant, with slightly greater unreactive phosphorus extracted from dried soil, and is most likely due to the release of organic phosphorus from aggregates disrupted by grinding.

The original NaOH EDTA extraction procedure involved a 1:50 solid to solution ratio and a 2-hour extraction at 85°C with no shaking. The extract ratio is typically reduced for qualitative studies with solution $^{31}$P NMR spectroscopy, although the results presented here indicate that this can also reduce the pH of the extract solution due to buffering by the soil. This did not influence unreactive phosphorus extraction, although...
Table 6: The effect of post-extraction treatment with Chelex-100 resin on concentrations of phosphorus compounds in three tropical forest soils extracted in a solution containing 0.25 M NaOH and 50 mM Na$_2$EDTA, with phosphorus detection by solution $^{31}$P NMR spectroscopy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total P$^b$</th>
<th>Phosphate$^b$</th>
<th>Monoesters$^b$</th>
<th>DNA$^b$</th>
<th>Pyrophosphate-P$^b$</th>
<th>Organic P$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil 1. Albrook</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>130.3 (36.4)</td>
<td>56.0 (43.0)</td>
<td>54.5 (41.7)</td>
<td>16.6 (12.8)</td>
<td>3.1 (2.4)</td>
<td>71.2 (54.6)</td>
</tr>
<tr>
<td>Chelex treated</td>
<td>127.8 (35.7)</td>
<td>59.1 (46.2)</td>
<td>49.4 (38.6)</td>
<td>16.9 (13.2)</td>
<td>2.4 (1.9)</td>
<td>66.3 (40.5)</td>
</tr>
<tr>
<td>Soil 2. Pipeline Road</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>129.4 (46.6)</td>
<td>42.5 (32.9)</td>
<td>60.5 (46.7)</td>
<td>18.4 (14.2)</td>
<td>8.0 (6.2)</td>
<td>78.9 (32.9)</td>
</tr>
<tr>
<td>Chelex treated</td>
<td>119.4 (42.9)</td>
<td>40.6 (34.0)</td>
<td>52.4 (43.9)</td>
<td>18.3 (15.3)</td>
<td>8.0 (6.7)</td>
<td>70.7 (30.6)</td>
</tr>
<tr>
<td>Soil 3. Laguna</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>47.4 (64.1)</td>
<td>18.0 (38.0)</td>
<td>18.2 (38.3)</td>
<td>9.5 (20.1)</td>
<td>1.7 (3.6)</td>
<td>27.7 (41.9)</td>
</tr>
<tr>
<td>Chelex treated</td>
<td>42.7 (57.8)</td>
<td>15.2 (35.6)</td>
<td>18.0 (42.0)</td>
<td>8.7 (20.4)</td>
<td>0.8 (2.0)</td>
<td>26.7 (44.0)</td>
</tr>
</tbody>
</table>

$^a$Values in parentheses are the proportion (%) of the total soil P.  
$^b$Values in parentheses are the proportion (%) of the total extracted P.  
$^c$Mean values of three replicate extracts (see Table 2).  

it could alter the phosphorus composition determined by solution $^{31}$P NMR spectroscopy (Figure 9) and increase the concentration of paramagnetic ions, introducing the risk of line broadening. Care is therefore required with low extraction ratios to ensure that solution pH is $\geq 13$. A 1:20 ratio seems likely to ensure that the pH remains at or above this value for mineral soils.

Subsequent studies have also used a longer extraction at ambient laboratory temperature, either with or without shaking. The results presented here suggest that a 4-hour extraction period with shaking at ambient temperature is adequate for quantitative organic phosphorus extraction, although a benefit of a longer (16 hour) extraction period is a reduction in the concentrations of paramagnetic ions in solution. Interestingly, this indicates that iron and manganese are initially solubilized in NaOH-EDTA and then precipitated, rather than not extracted at all. This may explain the discrepancies found here between phosphorus fractions determined by ICP-OES and NMR spectroscopy for extracts containing high concentrations of NaOH. The influence of elevated temperature on organic phosphorus extraction was not assessed here, but for practical reasons (i.e. the requirement for a shaking water bath and the use of elevated temperature) and because temperatures as low as 30°C can accelerate the hydrolysis of some organic phosphorus compounds in alkali extracts (e.g. Cade-Menun et al., 2002).

In contrast to previous studies using NaOH alone, pre-extraction of soil with mineral acid did not improve subsequent organic phosphorus extraction in NaOH-EDTA or improve resolution in solution $^{31}$P NMR spectroscopy. This suggests that inclusion of EDTA in the extractant chelates polyvalent cations that were the target of mineral pre-extraction in previous studies. Several organic phosphates are unstable in mineral acid (Anderson, 1960; Ivanoff et al., 1998), although Bowman (1989) suggested that hydrolysis of soil organic phosphorus was negligible during extraction with an acid-base procedure involving concentrated sulfuric acid. However, acid pre-extraction appeared to extract and/or hydrolyze small amounts of organic phosphorus in the soil analysed here, including both phosphate monoesters and DNA.

Pre-extraction with EDTA has been suggested to improve the subsequent extraction of organic phosphorus in NaOH alone (Harrap, 1963), while the pre-treatment of aquatic sediments with 67 mM Na$_2$EDTA was recently recommended to improve subsequent extraction of polyphosphate in NaOH–EDTA (Hupfer et al., 2004). Similarly, McDowell & Stewart (2005b) recommended a calcium EDTA-dithionite pre-extraction to improve spectral resolution of subsequent NaOH–EDTA extracts in solution $^{31}$P NMR spectroscopy. Robinson et al. (1998) reported an improvement in spectral resolution following pre-extraction of Histosols with 0.5 M NaHCO$_3$ or 1 M KCl, although resolution appeared no better than in the extracts of untreated soils. For the soil analysed here, pre-extraction with Na$_2$EDTA did not increase organic phosphorus extraction in NaOH–EDTA or improve spectral resolution. It did, however, improve the extraction of pyrophosphate, as suggested by Hupfer et al. (2004) for aquatic sediments. The NaOH–EDTA procedure may therefore underestimate the concentration of pyrophosphate (and polyphosphate if present) in soil, due to hydrolysis by, or precipitation with, free iron.

Post-extraction treatment of alkaline extracts with cation exchange resin has also been reported to improve spectral resolution for some samples (McDowell & Stewart, 2005a), but there was no such improvement here for NaOH–EDTA extracts of three tropical forest soils treated with Chelex-100 resin. Chelex-100 has a high affinity for polyvalent cations such as iron, although this is less than the affinity of EDTA for the same cations (Cade-Menun et al., 2002). Importantly, Chelex-treated extracts contained lower concentrations of organic phosphorus than untreated extracts, indicating that some compounds were removed by binding to the resin, as suggested previously (Cade-Menun et al., 2002; McDowell & Stewart, 2005a).
As pre- or post-extraction treatment did not influence organic phosphorus extraction, nor improve resolution in solution $^{31}$P NMR spectroscopy, such treatments seem unnecessary for mineral soils using the standard NaOH-EDTA procedure. However, they may be useful for extracts containing large concentrations of paramagnetic ions, or for samples extracted using a low concentration of NaOH (Solomon & Lehmann, 2000; see below). Inclusion of EDTA in the NMR tube may also help to minimize line broadening (Turner & Richardson, 2004).

On the basis of analysis by colorimetry and ICP-OES there was little effect of NaOH or EDTA concentrations on unreactive phosphorus extraction, with maximum concentrations occurring at hydroxide concentrations $\geq 40$ mM (pH $\geq 12.86$). This is a strong indication that organic phosphorus extraction was complete for this soil and also suggests that the inclusion of EDTA is relatively unimportant for quantitative organic phosphorus analysis. However, there were important differences in the concentrations and composition of organic phosphorus as determined by solution $^{31}$P NMR spectroscopy for samples extracted with varying concentrations of NaOH and EDTA. Most notably, NaOH alone extracted less DNA than similar strength solutions containing EDTA. This was also reported in a comparison of 0.25 M NaOH extracts of a temperate forest soil either with or without 50 mM EDTA (Cade-Menun & Preston, 1996). The inclusion of EDTA in the extraction solution is therefore essential for the accurate assessment of soil organic phosphorus composition.

The marked decline in iron and manganese concentrations with increasing pH indicates that maintaining solution pH $\geq 13$ is necessary to prevent the extraction of excess paramagnetic ions that could cause line broadening. Of all the extracts analysed by solution $^{31}$P NMR spectroscopy, significant line broadening was evident only for the 0.10 M NaOH plus 50 mM Na$_2$EDTA extract. A similar problem with line
Soil organic phosphorus in tropical forests

Figure 6 The relationship between aluminium and reactive phosphorus in extracts of a tropical forest soil (Soil 1, Albrook) containing varying concentrations of NaOH, either alone or with Na₂EDTA (25 or 50 mM). The regression lines are described by the following equations: NaOH alone, \( Y = 0.006x + 11.4; R^2 = 0.90; P < 0.0001; n = 10 \), NaOH + EDTA, \( Y = 0.016x - 9.9; R^2 = 0.98; P < 0.0001; n = 20 \).

broadening was encountered by Solomon & Lehmann (2000), who reduced the NaOH concentration in NaOH–NaF extracts from 0.5 to 0.10 M NaOH to minimize alkaline degradation. The line broadening at low NaOH concentration in the current study was presumably due to the large concentrations of iron and manganese in this sample. However, a similar iron concentration was present in the 0.10 M NaOH extractant (i.e. with no EDTA), yet its spectrum was well resolved and there was little qualitative difference in resolution between this and the other stronger NaOH concentrations tested, despite marked differences in iron concentrations. This suggests that iron was a relatively unimportant influence on line broadening.

Figure 7 Solution \(^{31}\)P NMR spectra of phosphorus compounds in a tropical forest soil (Soil 1, Albrook) extracted in solutions containing varying concentrations of NaOH. The spectra are scaled to the height of the internal MDPA standard (50 mg P kg\(^{-1}\)) at 17.6 p.p.m. See Table 7 for concentrations of phosphorus compounds.

Table 7 Concentrations of phosphorus compounds in a tropical forest soil (Soil 1, Albrook) extracted in solutions containing varying concentrations of NaOH either with or without 50 mM Na₂EDTA, with detection by solution \(^{31}\)P NMR spectroscopy. ND, not detected.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Total P (^{a})</th>
<th>Phosphate (^{b})</th>
<th>Monoesters (^{b})</th>
<th>DNA (^{b})</th>
<th>Pyrophosphate-P (^{b})</th>
<th>Organic P (^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>/mg P kg(^{-1})</td>
<td>/mg P kg(^{-1})</td>
<td>/mg P kg(^{-1})</td>
<td>/mg P kg(^{-1})</td>
<td>/mg P kg(^{-1})</td>
<td>/mg P kg(^{-1})</td>
</tr>
<tr>
<td>NaOH alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.10 M</td>
<td>93.6 (26.1)</td>
<td>47.0 (50.3)</td>
<td>38.5 (41.1)</td>
<td>3.7 (3.9)</td>
<td>4.4 (4.7)</td>
<td>42.2 (45.8)</td>
</tr>
<tr>
<td>0.25 M</td>
<td>98.0 (27.4)</td>
<td>46.2 (47.1)</td>
<td>46.8 (47.8)</td>
<td>2.9 (2.9)</td>
<td>2.1 (2.2)</td>
<td>49.7 (49.9)</td>
</tr>
<tr>
<td>0.50 M</td>
<td>110.9 (31.0)</td>
<td>42.9 (38.6)</td>
<td>55.9 (50.4)</td>
<td>7.1 (6.4)</td>
<td>5.0 (4.5)</td>
<td>64.5 (54.9)</td>
</tr>
<tr>
<td>NaOH + 50 mM Na₂EDTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.10 M</td>
<td>54.7 (15.3)</td>
<td>16.1 (29.4)</td>
<td>24.1 (44.0)</td>
<td>14.6 (26.7)</td>
<td>0.0 (0.0)</td>
<td>38.7 (44.0)</td>
</tr>
<tr>
<td>0.15 M</td>
<td>88.2 (24.6)</td>
<td>26.9 (30.5)</td>
<td>40.4 (45.9)</td>
<td>18.1 (20.6)</td>
<td>2.7 (3.0)</td>
<td>58.6 (48.9)</td>
</tr>
<tr>
<td>0.25 M(^{c})</td>
<td>130.3 (36.4)</td>
<td>56.0 (43.0)</td>
<td>54.6 (41.9)</td>
<td>16.6 (12.8)</td>
<td>3.1 (2.4)</td>
<td>71.2 (44.3)</td>
</tr>
<tr>
<td>0.50 M</td>
<td>165.3 (46.2)</td>
<td>92.9 (56.2)</td>
<td>63.4 (38.3)</td>
<td>9.0 (5.4)</td>
<td>ND (0)</td>
<td>72.4 (38.3)</td>
</tr>
<tr>
<td>1.00 M</td>
<td>133.5 (37.3)</td>
<td>67.1 (50.3)</td>
<td>63.8 (47.8)</td>
<td>ND (0)</td>
<td>2.6 (1.9)</td>
<td>63.8 (49.7)</td>
</tr>
</tbody>
</table>

\(^{a}\)Values in parentheses are the proportion (%) of the total soil P.

\(^{b}\)Values in parentheses are the proportion (%) of the total extracted P.

\(^{c}\)Mean values from three replicate analyses (see Table 2).
Figure 8 Solution $^{31}$P NMR spectra of phosphorus compounds in a tropical forest soil (Soil 1, Albrook) extracted in solutions containing varying concentrations of NaOH plus 50 mM Na$_2$EDTA. The spectra are scaled to the height of the largest signal. The MDPA internal standard was 50 mg P kg$^{-1}$ for all spectra except 0.25 M NaOH + 50 mM Na$_2$EDTA (67 mg P kg$^{-1}$). See Table 7 for concentrations of phosphorus compounds.

and that manganese, present at low concentrations in all NaOH extracts but at a much higher concentration in the 0.1 M NaOH plus 50 mM EDTA extract, was the main paramagnetic ion responsible for reducing spectral resolution.

Figure 9 Changes in phosphate monoesters and DNA determined by solution $^{31}$P NMR spectroscopy in extracts containing varying concentrations of NaOH with constant 50 mM Na$_2$EDTA.

The strong correlation between reactive phosphorus and aluminium in the extracts strongly suggests that much of the NaOH–EDTA extractable phosphate in this soil originated from complexes with aluminium minerals. It is possible that phosphate may also have originated from complexes with iron and manganese, but that these elements were subsequently precipitated after solubilization and therefore showed no correlation with solubilized phosphate. It seems unlikely that organic phosphorus associated with iron minerals is not extracted, because unreactive phosphorus concentrations were similar in all NaOH extracts, despite marked differences in iron concentrations.

For extracts containing 50 mM EDTA, the most striking influence of extract pH was the decline in DNA concentration, which reached undetectable levels in the strongest alkali. DNA is stable in 0.25 M NaOH plus 50 mM EDTA (Turner et al., 2003a), although degradation seems likely in the stronger alkali extracts here, as there was a corresponding increase in phosphate monoesters as DNA concentrations decreased. For extracts with NaOH alone, however, the largest DNA concentration occurred in the strongest alkali solution (although the DNA signal was less clear in this spectrum and its quantification may have been less accurate). Precipitation of DNA with metals is also possible, but if this occurred it could only have accounted for some of the changes, because most iron and manganese was precipitated at NaOH concentrations of ≥ 0.20 M, yet DNA concentrations only declined markedly at ≥ 0.5 M NaOH.

Given that slightly more DNA was extracted by the 0.15 M NaOH plus 50 mM EDTA solution compared with the 0.25 M NaOH plus 50 mM EDTA solution, it is possible that the NaOH concentration could be reduced to 0.20 M to maximize DNA extraction while also minimizing its degradation. In addition, it may be beneficial to reduce the EDTA concentration to 25 mM given that absorbance (and therefore organic matter)
was lower for such extracts at high pH. This warrants further consideration, as it would reduce the total lyophilized powder (~50% less EDTA and organic matter) and therefore increase the phosphorus concentration in the NMR tube, with no apparent effect on unreactive phosphorus.

It was noticeable that total organic phosphorus determined by the NaOH-EDTA procedure was considerably less than that determined using the ignition procedure for all three soils. This is unsurprising, as the ignition procedure is known to overestimate organic phosphorus in strongly-weathered soils (Condron et al., 1990). However, the hot concentrated HCl extraction step of the modified Hedley fractionation procedure has been reported to contain significant quantities of phosphorus that does not react with molybdate (Tiessen & Moir, 1993). Further experiments, such as phytate recovery tests, are therefore required to confirm that significant quantities of organic phosphorus do not remain in well-weathered soils following NaOH-EDTA extraction.

**Recommended procedure**

The standard NaOH-EDTA procedure was not improved by changes in the concentration of NaOH or EDTA, soil to solution ratio, extraction time, or the inclusion of a pre- or post-extraction treatment. Until more soils have been tested in this manner, the following extraction procedure is therefore recommended:

1. Weigh 1.50 ± 0.01 g of air-dried soil into a 50 ml centrifuge tube.
2. Add 30 ml of a solution containing 0.25 M NaOH and 50 mM Na2EDTA (i.e. a 1:20 solid to solution ratio).
3. Shake for 4 hours at ambient laboratory temperature (~22°C).
4. Centrifuge at high speed (~8000 g) for 30 minutes and decant the supernatant (if floating particles are present it may be necessary to filter the supernatant through a qualitative filter paper).
5. Add an internal standard (e.g. 1 ml of 50 µg P ml⁻¹ methylene diphosphonic acid) to a 20 ml aliquot of the extract and mix well (optional).
6. Freeze the sample at ≤-30°C, lyophilize (freeze-dry) the frozen solution, and homogenize the powder.
7. Re-dissolve ~100 mg of the lyophilized powder in 0.9 ml of a solution containing 1.0 M NaOH and 50 mM Na2EDTA, add 0.1 ml of deuterium oxide for signal lock, and analyse by solution 31P NMR spectroscopy. See Cade-Menun (2005) for details of the NMR procedure.

**Acknowledgements**

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**References**


