A Simplified, Sequential, Phosphorus Fractionation Method.

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ABSTRACT

Hedley *et al.* (1982) developed what has become the most widely used (and modified), phosphorus (P) fractionation technique. It consists of sequential extraction of increasingly less phytoavailable P pools. Extracts are centrifuged at up to 25000 *g* (RCF) and filtered to 0.45 μm to ensure that soil is not lost between extractions. In attempting to transfer this method to laboratories with limited facilities, it was considered that access to high-speed centrifuges, and the cost of frequent filtration may prevent adoption of this P fractionation technique.

The modified method presented here was developed to simplify methodology, reduce cost, and therefore increase accessibility of P fractionation technology. It provides quantitative recovery of soil between extractions, using low speed centrifugation without filtration. This is achieved by increasing the ionic strength of dilute extracts, through the addition of NaCl, to flocculate clay particles. Addition of NaCl does not change the

amount of P extracted. Flocculation with low speed centrifugation produced extracts comparable with those having undergone filtration (0.025 μm).

A malachite green colorimetric method was adopted for inorganic P determination, as this simple manual method provides high sensitivity with negligible interference from other anions. This approach can also be used for total P following digestion, alternatively non-discriminatory methods, such as inductively coupled plasma atomic emission spectroscopy, may be employed.

INTRODUCTION

Phosphorus (P) fractionation is a method developed to estimate both the size of the readily phyto-available P pool and the soils ability to replenish it. Phosphorus fractionation has been used to examine the decline of soil P under cultivation (Tiessen *et al*., 1983; Sharpley and Smith, 1985; Linquist *et al*., 1997); the effect of different management practises on the distribution of soil P (Hedley *et al*., 1982; McKenzie *et al*., 1992; Paniagua *et al*., 1995); the influence of microbiological activity on soil P processes (Lee *et al*., 1990; Oberson *et al*., 1996); and the pathways of P movement and transformation in soil (Tiessen *et al*., 1984; Beck and Sanchez, 1996). The method therefore has many beneficial uses and it is hoped that simplifying it will enable more widespread adoption.

Phosphorus fractionation methodology has changed drastically since the first comprehensive scheme of Chang and Jackson (1956). Moving from essentially inorganic P (P_i) fractionation, through to organic P (P_o) fractionation, and combinations of the two. Hedley *et al*. (1982) developed what has become the most widely used (and modified), P fractionation technique, involving sequential extraction of increasingly less phytoavailable P pools. It consists of sequential extraction (1:60 soil:solution), with first an anion exchange resin (AER), then 0.5 M $NaHCO_3$, 0.1 M NaOH, sonication (2min, 75W) in 0.1 M NaOH, 1 M HCl, and a residual digest in H_2SO_4/H_2O_2. Comparison with a total P determination done separately shows that >95% of P can be extracted using this method.

This basic method has undergone many variations, surprisingly few of which are explicitly detailed. Tiessen and Moir (1993) developed a routine method for use by Canadian soil chemists adding a hot concentrated acid extraction, which removes P_o that may be able to take part in short-term transformations. As this method and some modifications of it involve high-speed centrifugation and filtration, accessibility and use may be restricted. This method was developed to simplify, reduce cost, and increase accessibility of P fractionation technology.

MATERIALS AND METHODS

Soils used

Soils were chosen to provide a range of chemical and physical characteristics. All soils were classified according to the Australian Soil Classification (Isbell, 1996), with U.S Taxonomy following in parentheses. Unless otherwise stated the test soil in each experiment was the top 10cm of a Red Ferrosol (K) (Typic Haplustox) sampled from Kingaroy in south-east Queensland. Other soils used include a Black Vertosol (Typic Haplustert), Red Kandosol (Typic Kanhapludalf), Redoxic Hydrosol (Typic Sulfaquept), Red Ferrosol (C) (Rhodic Haplustox), Brown Chromosol (Typic Paleustalf), Aeric Podosol (Typic Haplorthod), Yellow Kandosol (Typic Paleudalf), and Red Chromosol (Typic Paleustalf). All soils were air-dried and sieved to <2mm prior to use.

Comparison of Cl- and HCO_3-saturated Resin Extraction

Anion exchange resin strips were soaked for at least 24h in either 1 M NaCl or 0.5 M $NaHCO_3$. Two Cl- or HCO_3-saturated resin strips were added with 30 mL triple de-ionised (TDI) water and 0.5 g of air-dried soil in 50 mL centrifuge tubes and shaken overnight. After shaking the resin strips were removed, rinsed briefly, and eluted in 30 mL of 0.7 M NaCl, for at least 1 h. Extracted inorganic and total P (P_t) were determined using malachite green, (method to follow) and inductively coupled plasma atomic emission spectroscopy (ICPAES), respectively.

Examination of Organic P Extraction using Anion Exchange Resins

A synthetic solution consisting of both P_i (60 μM) and phytate (45 μM) was prepared. To 30 mL of this solution an HCO_3-saturated anion exchange resin was added. After shaking overnight the resin strip was removed and eluted in 30 mL of 0.1 to 0.9 M NaCl solutions. Cl-saturated anion exchange resins were eluted with 0.7 M NaCl solution only. Differences between Cl- and HCO_3-saturated resin strips were tested using a Student's *t*-test (Cochran and Cox, 1957).

Comparison of Changing Molarity and Filtration on Soil Flocculation and Recovery

To a 50 mL centrifuge tube, 4 replicates of 1 g of soil and 30 mL of 0.1 M NaOH was added. To adjust the molarity of the final solution, 1 mL of either TDI water, 1 M, 2 M, 3 M, 4 M, or 5 M NaCl was added. The solutions were shaken overnight, then centrifuged for 30 min at 900 *g* (RCF). Supernatant from 2 replicates was analysed for P_i with malachite green, and P_t, Fe and Al by ICPAES. Supernatant from the remaining replicates was ultrafiltered using 0.025 μm filters (Millipore Inc.) under positive pressure using N_2 gas (Menzies *et al*., 1991a), and analysed for P_i and P_t. Differences between the total P contents were tested using analysis of variance with least significant difference (lsd) bars marked ($P<0.01$).

Determination of Optimal Total Soil P Determination

The effect of adding Mg to a soil digest mix was examined on six different soils. Before addition of the acid mixture, some flasks were spiked with 0.5- 0.6 g of anhydrous $MgSO_4$ (optimal amount determined previously, data not shown). To a 75 mL digestion flask, 0.5 g of finely ground soil, approximately 2 mL of TDI water, and 5 mL of H_2SO_4:$HClO_4$ (20:1) acid was added. All samples were then digested in a block digestor for 3 h at 360^{o}C, ramping up to this temperature at 4^{o}C min^{-1}. After cooling, the flasks were made up to 75 mL with TDI water, mixed well, and allowed to settle overnight. Solutions were analysed for P using malachite green and the results compared. Differences between the total P contents were tested using a Student's *t*-test (Cochran and Cox, 1957).

Malachite Green P_i Determination

Analysis with malachite green requires two reagents (A and B), which are mixed in equal proportions just prior to use. To make reagent A, malachite green; add 190 mL concentrated H_2SO_4 to 750 mL TDI water and allow to cool to room temperature. Then add 0.27 g malachite green and stir until dissolved. Add 54 g ammonium molybdate and stir until dissolved. Solution should be orange in colour and stored at 4^{o}C. To make reagent B, polyvinyl alcohol; dissolve 5 g polyvinyl alcohol in 500 mL TDI water,

heating and stirring is required. All solutions analysed have to be diluted to 0 - 12 μM concentration. To 3 mL sample add 1 mL of mixed reagent and read absorbance at 630 nm after 25 min.

Fractionation Method and Recovery

Four soils were extracted using the fractionation method outlined below.
To 0.5 g of finely ground soil in a 50 mL centrifuge tube add 30 mL TDI water and two Cl-saturated anion exchange resin strips. Shake overnight (approx.16 hrs).
Remove resin strips, washing any excess soil off strips back into original tube, and place them in 30 mL of 0.7 M NaCl. Shake for at least 1 h. Remove strips and analyse solution for P_i using malachite green, dilute 1:1 with TDI water for P_t analysis on an ICPAES.
Add 1 mL of 4 M NaCl to original tube, centrifuge 30 mins at approximately 900 *g* (RCF), then decant supernatant.

Add 30 mL of 0.5 M $NaHCO_3$ to retained soil, shake overnight, then centrifuge for 30 minutes, at 900 *g* (RCF) and decant supernatant. Determine P_t using ICPAES. Prepare $NaHCO_3$ solutions for P_i analysis using malachite green by diluting 0.6 mL of supernatant with 2.4 mL TDI water (1:5 dilution) and adding 4 drops of concentrated HCl. Ensure that samples have degassed fully before analysis, (remove bubbles after they develop by tapping tube, or speed process up in sonication bath).

Add 30 mL 0.1 M NaOH and 1 mL of 4 M NaCl. Shake overnight, then centrifuge for 30 minutes, at 900 *g* (RCF), then decant supernatant. Determine P_t using ICPAES. Prepare NaOH solutions for P_i analysis using malachite green by adding 3 mL of supernatant to 10 mL tubes with 0.05 mL concentrated HCl. Shake tubes by hand. Allow to settle overnight or sit for at least 1 h and centrifuge to remove flocculated organic matter (2 minutes at 900 *g* (RCF)). Supernatant should be diluted 1:10 with TDI water and analysed for P_i using malachite green.

Add 30 mL of 1 M HCl. Shake overnight. Do not centrifuge HCl extract but allow to settle overnight. This allows easier quantitative transfer of tube contents to a 75 mL digestion tube. After settling remove enough supernatant to dilute 1:10 with TDI water to obtain 3 mL necessary for P analysis using malachite green. Dilution is necessary to obtain correct acid content for malachite green colour development.

Transfer soil quantitatively to a 75 mL digestion tube using TDI water and allow to settle overnight. Remove excess water from 75 mL tube, leaving 2-3 mL in tube. Add 0.5 g anhydrous $MgSO_4$ and 5 mL of H_2SO_4: $HClO_4$ (20:1) acid. Step slowly to 360^{o}C (4^{o}C min^{-1} to boil off $HClO_4$), and digest for 3 hrs. Allow to cool, and dilute to 75 mL with TDI water. Shake well and allow to settle overnight. Prepare standards in 2.4 N H_2SO_4. Dilute extracts and standards 1:30 with TDI water and analyse P with malachite green. Digest unfractionated soil in same way to determine recovery. Differences between the sum and total P contents were tested using a Student's *t*-test (Cochran and Cox, 1957).

RESULTS AND DISCUSSION

Comparison of Cl- and HCO_3-saturated Resin Extraction

The systems used to extract P with resins vary greatly. Different results can be obtained depending on the saturating ion, or whether a cation exchange resin is included (Curtin *et al*., 1987). In this study, there was a significant increase ($P<0.01$) in resin extractable P_i in all soils when HCO_3 was the saturating anion (Table 1). Two of the four soils also demonstrated increase P_o recovery ($P<0.01$). These differences could be due to a number of reasons, the most likely being the relatively greater affinity of HCO_3 than Cl for adsorption sites. This effect is accentuated by the high pH ($\cong 8.5$) produced by a HCO_3-saturated resin strip. Coupled with this is the possibility that an increased anion level in the soil will exchange more HCO_3 off the resin and promote further P removal.

We have chosen a Cl-saturated resin to remove only the most freely exchangeable P in the soil, as the next step is a HCO_3 extraction. More information on readily phytoavailable P may be gained by not modifying the pH of the extracting solution to an unknown degree with a HCO_3-saturated resin.

Examination of Organic P Extraction using Anion Exchange Resins

Elution of phosphate with different molarity NaCl solutions from HCO_3-saturated resin strips resulted in complete recovery of added phosphate at all concentrations (Table 2). Recovery of greater than 100% is attributed to small amounts of phosphate added with the phytate source or to mineralisation of some phytate added. Elution of phytate from HCO_3-saturated resin strips increased with increasing molarity up to 0.4 M NaCl (Table 2). Elution of Cl-saturated resins with 0.7 M NaCl yielded a recovery of 90% (std dev. 9.3), which was not significantly different to HCO_3-saturated resin strips eluted with the same molarity.

Phytate was chosen as the test compound because it is a large, hexa-phosphoric organic compound. It was assumed that if substantial elution could be obtained with this molecule, smaller, less complex organic P compounds could also be eluted. Though phytate could be successfully eluted it is unlikely that compounds such as these will be found in high concentrations in a water extract as they are strongly sorbed (Greaves and Webley, 1969), particularly in highly weathered soils.

The importance of resin P_o determination lies in increasing evidence of the contribution of soil solution P_o to phytoavailabile P through rapid mineralisation (Shand *et al*., 1994). Rubaek and Sibbesen (1995) demonstrated that resin P_i and P_o, and HCO_3-P were good indicators of treatment effects and reactions in the most labile soil P pool.

It is recommended that eluting in 0.7 M NaCl to recover a large proportion of P_o extracted will improve the possible use of fractionation techniques in examining the most labile soil P fractions.

Comparison of Changing Molarity and Filtration on Soil Flocculation and Recovery

According to the Gouy-Chapman equation, increasing the ionic strength of a colloidal suspension results in flocculation of clay particles, whilst decreasing it promotes dispersion (Van Olphen, 1977). Particles that are dispersed are less likely to come under the influence of centrifugal force and will tend to remain in suspension despite centrifugation. To ensure quantitative recovery of soil, it is necessary to filter such dispersed extracts. Once flocculated though, their aggregated mass will be greater. They can then be successfully removed from suspension without filtration. This experiment sought to determine if increasing the molarity of a highly dispersive extract, (0.1 M NaOH; pH ≅13), could result in quantitative removal of flocculated clay particles, thus obviating the need for expensive filtration steps.

Raising the molarity of the extractant by the addition of NaCl solutions decreased the concentration of Fe and Al in the extracts (Figure 1). The decrease in Fe and Al concentration is quite rapid with increasing molarity, approaching a constant concentration when 4-5 M NaCl is added. With increasing molarity the supernatants were visibly less turbid and less colloidal particles were caught on the filters. Iron and Al are major constituents of soil colloids (McLaren and Cameron, 1996), and are removed

rapidly from soil solution extracts filtered through decreasing pore size membranes (Menzies *et al*., 1991b). The decreased Fe and Al contents may be due to colloidal particles flocculating at increased molarity and dropping out of solution under low-speed centrifugation.

The effect of increasing molarity on P extracted was slightly different and better illustrated in comparison with filtered extracts (Figure 2). When filtering all solutions to 0.025 μm, the total concentration of P extracted is consistently ≅ 125 μM. However, total P concentration in unfiltered extracts declined until ≥ 3M NaCl was added. The difference between addition of 3-5 M NaCl and ultrafiltration to 0.025 μm is not significant. There was a significant difference between phosphate when filtered and no NaCl addition was made. This could be due to the presence of greater amounts of clay particles in the solution and sorption occurring as the organic matter is flocculated and centrifuged out of solution.

Filtration to 0.025 μm is more efficient than filtration to 0.45 μm as outlined in other fractionation methodologies (Hedley *et al*. 1982). No difference was observed between ultrafiltration and addition of >4 M NaCl spiked unfiltered extracts. Therefore it is reasonable to conclude that addition of NaCl at this concentration can effectively replace the need for filtration. Although it may be necessary to test this in other soils, the Red Ferrosol (K) used for this experiment is naturally dispersive and we expect that in most instances salt addition will be effective.

Determination of Optimal Total Soil P Determination

It has been observed that when quantifying P_t by nitric: perchloric digestion of plant material that significant losses of P can occur through volatilisation (Brookes and Powlson, 1981). They found that addition of saturated $MgCl_2$ to the sample prevented this volatilisation and retained more P in the digest. The addition of Mg (as anhydrous $MgSO_4$) to similar digests in this experiment increased P recovery in 3 of the 5 soils tested (Table 3). It is recommended that small amounts of Mg are added to soil digests.

Comments on Malachite Green Determination

Malachite green was successfully used to determine the P_i content of the different extracts produced in this fractionation. If using malachite green the following factors must be taken into account. It is important in each extract to match sample and standard matrices closely. Dilution of samples and analysis with water standards will yield erroneous results. In 0.7 M NaCl determination, changing the molarity affects the standard curve, absorbance at 630 nm is decreased with increasing P concentration and molarity. It is recommended that at least 1:5 dilution of HCO_3 extracts be undertaken prior to P_i determination. Larger proportions of HCO_3 in the sample decrease the molar absorptivity as most HCO_3 sources have considerable P contamination. In acid extracts, (HCl and residual/total), it is important to use the dilution factors suggested as the malachite green colour reaction is sensitive to pH, (Motomizu *et al*., 1983). Although

malachite green is advocated in this paper molybdate methods (eg. Murphy and Riley) could also be used with appropriate modifications.

Fractionation Method and Recovery

The modified fractionation method was examined to determine if greater than 95% recovery of soil P could be achieved. The method has not been compared with the Hedley (1982) fractionation method, as the extracts are substantially different and it was thought that recovery of total soil P was sufficient to test the modifications made. Recovery of P using this method was greater than 95% and there was no significant difference ($P<0.05$) between the sum of the fractions and the total soil P content (Table 4).

CONCLUSIONS

Despite the number and variety of modifications to the P fractionation method, this substantially simpler method results in quantitative recovery of total soil P. This fractionation method is easily transferable and robust, allowing laboratories that may not be able to afford filtration or high speed centrifugation equipment to utilise the benefits of fractionation methodology.

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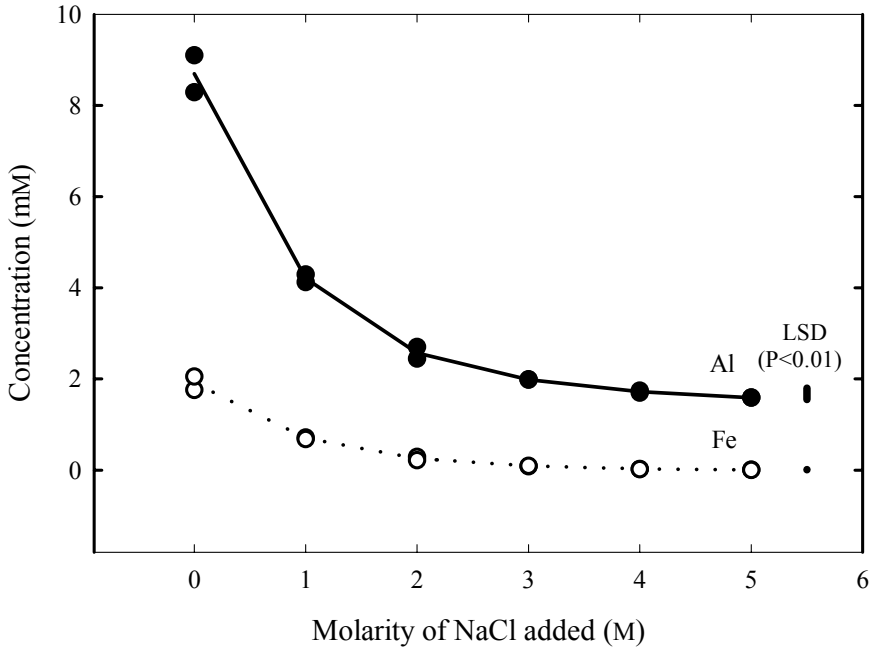

FIGURE 1. Effect of increasing molarity of NaCl solution added on amount of Fe and Al in 0.1 M NaOH extracts of a Red Ferrosol (K).

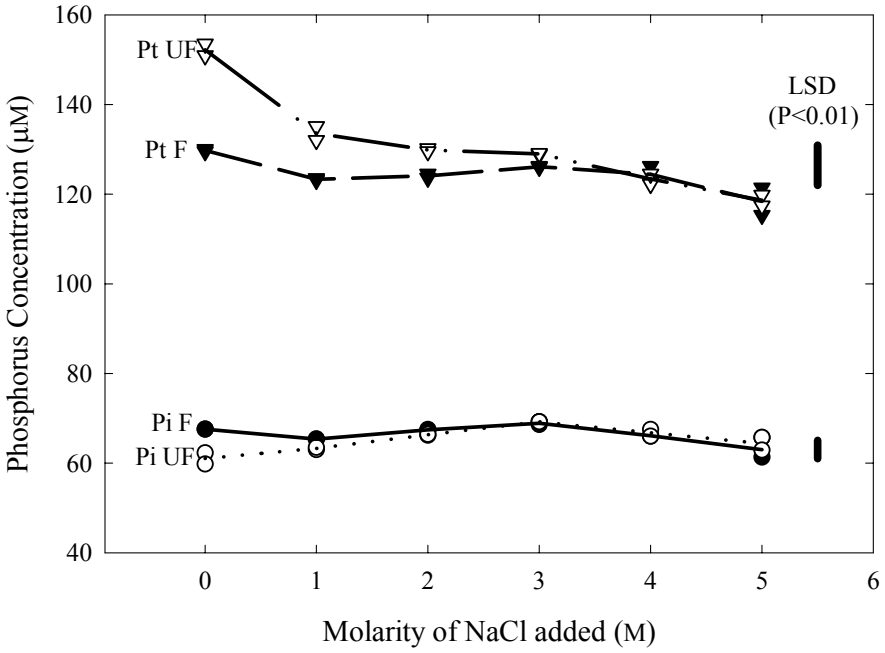

FIGURE 2. Effect of increasing molarity of NaCl solution added on changes in phosphate (P_i)(Unfiltered (UF) ○ ; Filtered (F) ●), and total phosphorus (P_t) (Unfiltered ∇ ; Filtered ▼) concentrations in 0.1 M NaOH extracts of a Red Ferrosol.

TABLE 1. Difference in extractable P in four soils using Cl- or HCO_3-saturated resin strips. Values are the mean of 3 replicates, with standard deviations provided in parentheses.

Soil	Cl- saturated resin		HCO_3-saturated Resin	
	Pi (μg/g)	Po (μg/g)	Pi (μg/g)	Po (μg/g)
Red Ferrosol (K)	0.5 (0.03)	7.1 (0.4)	1.7 (0.02)*[a]	10.4 (0.3)*
Redoxic Hydrosol	0.5 (0.02)	7.4 (0.5)	10.7 (0.3)*	8.4 (0.5)
Red Kandosol	0.7 (0.07)	7.8 (0.6)	16.2 (1.4)*	6.8 (1.1)
Red Ferrosol (C)	1.8 (0.5)	8.4 (0.2)	16.7 (0.6)*	13.5 (0.3)*

[a]For each soil, results followed by * are significantly different ($P<0.01$) to Cl-saturated resin values.

TABLE 2. Elution of P_i and phytate from anion exchange resin strips by different molarity NaCl solutions. Values are the mean of 3 replicates, with standard deviations provided in parentheses.

Molarity (M)	Recovery of added P_i (%)	Recovery of added phytate (%)
0.1	106.6 (0.1)[a]	0.4 (0.3)[a]
0.2	102.9 (0.7)[a]	42.2 (3.9)[b]
0.4	105.6 (0.4)[a]	84.8 (0.3)[c]
0.7	102.1 (1.7)[a]	91.5 (1.9)[c]
0.9	100.8 (1.4)[a]	82.7 (1.5)[c]

[a]Means followed by the same letter are not significantly different ($P<0.01$)

TABLE 3. Effect of Mg addition on the recovery of P from high temperature soil digestion in a variety of soil types. Values are the mean of 3 replicates, with standard deviations provided in parentheses.

Soil	Without Mg (μg P/g)	With Mg (μg P/g)
Brown Chromosol	454.8 (1.7)	466.7 (3.4)**[a]
Black Vertosol	266.8 (5.4)	290.5 (4.1)**
Redoxic Hydrosol	207.5 (7)	226.7 (2.5)*
Yellow Kandosol	112.9 (4.9)	117.3 (4.2)
Aeric Podosol	33.3 (0.8)	33.5 (0.4)

[a]For each soil, results followed by * ($P<0.05$) or ** ($P<0.01$) indicate significant effect of Mg.

TABLE 4. Phosphorus fractions and recovery in four different soils. Values are the mean of 3 replicates, with standard deviations provided in parentheses.

Soil	Resin		HCO_3		OH		HCl	Residual	Sum	Total[a]	R'v'ry
	µg Pi/g	µg Po/g	µg Pi/g	µg Po/g	µg Pi/g	µg Po/g	µg Pi/g	µg Pt/g	µg Pt/g	µg Pt/g	%
Red Ferrosol (K)	0.5	7.1	1.3	11.3	22.6	60.3	2.4	140.7	246.1	250.7	98.2
	(0.03)	(0.4)	(0.2)	(1.6)	(1.7)	(0.8)	(0.3)	(2.8)	(4.1)	(15.5)	
Redoxic Hydrosol	0.5	7.4	22.3	90.7	220.2	370.6	17.3	152.4	881.4	856.8	102.9
	(0.02)	(0.5)	(1.6)	(5)	(4.5)	(14.1)	(1.1)	(7.1)	(30)	(24)	
Red Kandosol	0.7	7.8	34.1	10.2	131.4	75.4	9	119	385.7	349.7	110.3
	(0.07)	(0.6)	(1.7)	(0.2)	(2.3)	(2.3)	(0.7)	(2.7)	(5.7)	(27)	
Red Ferrosol (C)	1.8	8.4	34.8	9.7	88.6	193.6	24.2	384.1	745.2	731.9	101.8
	(0.5)	(0.2)	(1.9)	(0.2)	(6.7)	(11.4)	(2.7)	(12)	(13)	(13)	

[a]For each soil, no significant difference ($P<0.05$) observed between the sum of the fractions and total P.