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Complex Forms of Soil Organic Phosphorus—A Major Component of Soil Phosphorus

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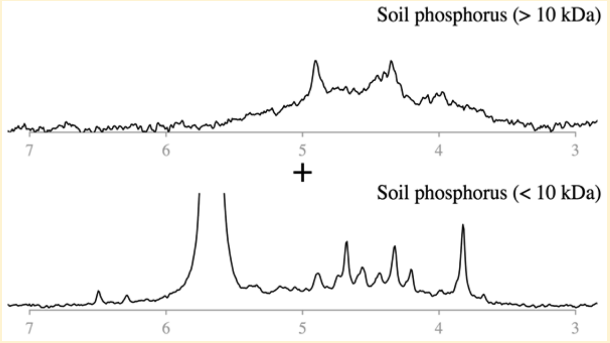
Supporting Information

ABSTRACT: Phosphorus (P) is an essential element for life, an innate constituent of soil organic matter, and a major anthropogenic input to terrestrial ecosystems. The supply of P to living organisms is strongly dependent on the dynamics of soil organic P. However, fluxes of P through soil organic matter remain unclear because only a minority (typically <30%) of soil organic P has been identified as recognizable biomolecules of low molecular weight (e.g., inositol hexakisphosphates). Here, we use ^{31}P nuclear magnetic resonance spectroscopy to determine the speciation of organic P in soil extracts fractionated into two molecular weight ranges. Speciation of organic P in the high molecular weight fraction (>10 kDa) was markedly different to that of the low molecular weight fraction (<10 kDa). The former was dominated by a broad peak, which is consistent with P bound by phosphomonoester linkages of supra-/macro-molecular structures, whereas the latter contained all of the sharp peaks that were present in unfractionated extracts, along with some broad signal. Overall, phosphomonoesters in supra-/macro-molecular structures were found to account for the majority (61% to 73%) of soil organic P across the five diverse soils. These soil phosphomonoesters will need to be integrated within current models of the inorganic–organic P cycle of soil-plant terrestrial ecosystems.



INTRODUCTION

Globally, terrestrial ecosystems contain approximately 50 gigatonnes of phosphorus (P) in the upper 50 cm of the soil profile[1] and up to 80% of P in a soil can be present in an organic form (i.e., in structures that contain covalent bonds to carbon, usually via oxygen through a phosphoester linkage).[2] A large proportion of the P in soils has been known to exist in an organic form since the early 1900s,[3] although its chemical nature has mostly been assumed to be a form similar to that found in living organisms.[4] The most abundant forms of organic P in living cells are phospholipids and nucleic acids.[5−7] However, since phospholipids and nucleic acids undergo rapid microbial degradation in soil,[8,9] inositol phosphates, which are more persistent, are generally assumed to constitute the majority of the stable pool of soil organic P.[4,10] In particular, the *myo* stereoisomer of inositol hexakisphosphate (commonly called phytate), which is mainly found in the seeds of plants, is thought to accumulate in soil due to its ability to bind strongly to soil minerals.[10]

The methods used to characterize organic P in soils have changed over time and can be broadly classified into three eras. In the first era (1900−1950), wet chemical methods were predominant.[3,11] In the second era (1950−1980), chromatography was predominant,[12,13] while in the most recent and current era (1980-present), nuclear magnetic resonance (NMR) spectroscopy is used most widely.[14−16] Throughout each of these eras, organic P characterization has mainly focused on identification and quantification of inositol phosphates.[10] However, there are only a few examples where inositol phosphates have been reported to comprise the majority of organic P in soil extracts,[13,17,18] and even then some of the methods used for its quantification have been questioned.[19−24] Inositol phosphates are more commonly

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Table 1. Basic Chemical Properties of the Soils Used in This Study

soil	depth (cm)	pHw (1:5)	ECw (μS cm^{-1}, 1:5)	total carbon[a] (%)	total nitrogen[a] (%)	oxalate aluminum[b] (mg kg^{-1})	oxalate iron[b] (mg kg^{-1})	total organic phosphorus[c] (mg kg^{-1})	total phosphorus[d] (mg kg^{-1})
Australia	0−10	5.3	68	2.5	0.21	469	1635	194	460
France	0−20	7.7	171	4.1	0.38	2020	6996	665	1448
Germany	0−10	6.7	104	5.1	0.43	4517	3622	707	2444
Sweden	0−20	6.9	170	8.6	0.48	1934	4563	456	1128
U.S.	0−20	6.8	85	2.9	0.26	1138	2650	383	668

[a]Total soil carbon and total soil nitrogen were determined on a LECO TruSpec CN analyzer (LECO Corporation, St Joseph, MI). [b]Measures of oxyhydroxides associated with aluminum and iron were determined using the oxalate extraction technique of McKeague and Day.[34] [c]Total organic P was estimated using the ignition-H_2SO_4 extraction technique of Walker and Adams.[35] However, it is widely known that this extraction technique, which is the most commonly used method for estimating total organic P, is somewhat unreliable and will tend to overestimate total organic P in most soils.[36] [d]Total soil P was determined using laboratory X-ray fluorescence as described in McLaren et al.[37]

reported to comprise less than 30% of soil organic P,[10,14,23,25] and it has often been noted that a substantial portion of the organic P in soils eludes definitive identification as recognizable biomolecules.[2,26]

Recently, debate has arisen regarding the interpretation of the orthophosphate and phosphomonoester region (δ 7.0 to 3.5 ppm) of the ^{31}P NMR spectra of soil extracts.[15,20,24] We have repeatedly observed that this spectral region contains a broad underlying feature that, when not recognized as a separate component of soil organic P, can confound quantification of specific organic P biomolecules that give rise to the sharp peaks present within this region.[20,21,24] We have hypothesized[20,21,27] that this broad feature may be due to organic P species contained in high molecular weight (polymeric) material. The view that there is a component of the soil organic P that exists as large and complex structures is not new,[28,29] although this has remained unresolved due to a lack of direct evidence. In this study, we combine size fractionation of soil extracts with ^{31}P NMR characterization to test the proposition that soil extracts contain organic P in high molecular weight (polymeric) material that gives rise to a broad feature in the NMR spectra.

EXPERIMENTAL SECTION

Soil Information. Soil was obtained from five locations across three continents. These include (1) Australia (Soil ID: P1SR09)—soil collected from the 0−10 cm soil surface layer of a permanent pasture from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Ginninderra Experiment Station, near Hall, Australian Capital Territory, Australia;[30] (2) France (Soil ID: 4850), Germany (Soil ID: 2035) and Sweden (Soil ID: 3359)—surface soils (0−10 or 0−20 cm) were obtained from the Geochemical Mapping of Agricultural Soils and Grazing Land (GEMAS) study;[31] and (3) USA (Soil ID: 1BS102M) − the Elliot reference soil standard was purchased from the International Humic Substances Society.[32] Basic chemical properties of these soils can be found in Table 1.

Soil Characterization. Soil pH and electrical conductivity (EC) were determined in deionized water after shaking for 1 h at a 1:5 soil to solution ratio. Total soil carbon and nitrogen were determined on a LECO TruSpec CN analyzer (LECO Corporation, St Joseph, MI), as described by Matejovic.[33] Oxalate-extractable aluminum and iron were determined in each soil as described by McKeague and Day.[34] Total organic P was estimated in each soil using the ignition-H_2SO_4 extraction technique of Walker and Adams.[35] However, this technique, which is the most commonly used method for estimating total organic P, is considered somewhat unreliable as it tends to overestimate total organic P in most soils.[30,36] Total soil P was determined using laboratory X-ray fluorescence spectroscopy, as described by McLaren et al.[37]

Solution ^{31}P NMR Spectroscopy. Soils were extracted with sodium hydroxide−ethylenediaminetetraacetic acid (0.25 M NaOH + 0.05 M EDTA) at a 1:10 soil to solution ratio and prepared for quantitative NMR analysis as described by Doolette et al.[20] and McLaren et al.[37] In summary, 4 g (±0.10 g) of soil was extracted with 40 mL of NaOH-EDTA, shaken for 16 h, centrifuged at 1400g for 20 min, and then the supernatant passed through a Whatman no. 42 filter paper. A 20 mL aliquot of the filtrate was frozen in liquid nitrogen and freeze-dried; an amount of between 550 mg to 650 mg of freeze-dried solid was generally recovered. For each soil extract, a subsample of 500 mg was dissolved in 5 mL of deionized water, centrifuged at 1400g for 20 min, and a 3.5 mL aliquot of the supernatant were placed in a 10 mm diameter NMR tube. In addition, a 0.3 mL aliquot of deuterium oxide and a 0.1 mL aliquot of methylenediphosphonic acid (Sigma-Aldrich; M9508 - standard solution containing 6.0 g L^{-1}) were then placed in the NMR tube prior to solution ^{31}P NMR spectroscopy. Solution ^{31}P NMR spectra were acquired on a Varian INOVA400 NMR spectrometer at a ^{31}P frequency of 161.9 MHz, with gated broadband proton decoupling, and a 90° pulse of 30 μs. The recycle delay for each sample was determined following a preliminary inversion−recovery experiment, and was set at five times the longest T_1 (spin−lattice relaxation) value.

Ultrafiltration. Following NMR analysis on the original NaOH-EDTA extracts (unfractionated), the solution was transferred to the 5 mL sample reservoir of an ultrafiltration device containing a 10 kDa filtration membrane made of modified poly(ether sulfone) (PALL Life Sciences©; Microsep Advance Centrifugal Devices; Product ID MCP010C41). Solutions were centrifuged at 4000 rpm (1700g) for 60 min and the filtrate (<10 kDa) and the retentate (>10 kDa) collected for NMR analysis. The 10 kDa filtrates were transferred to a NMR tube and analyzed as described above. The 10 kDa retentates were collected after washing the retentate with NaOH-EDTA. The washing step involved adding 5 mL of NaOH−EDTA solution to the 5 mL reservoir of the ultrafiltration device (containing the 10 kDa retentate) and centrifuging at 4000 rpm (1700g) for 60 min. This step was repeated twice and the "wash" filtrate collected each time and stored. After the washing step, 1 mL of NaOH−EDTA solution was added to the 5 mL reservoir of the ultrafiltration device (containing the 10 kDa retentate: ∼0.1 mL in volume). The 10

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kDa retentate solution (~1 mL) was then transferred to a NMR tube using a pipet. This step was repeated three times to give a final volume of ~3 mL of solution in the NMR tube. A 0.3 mL aliquot of deuterium oxide and a 0.1 mL aliquot of methylenediphosphonic acid (Sigma-Aldrich; M9508, standard solution containing 6.0 g L^{-1}) were then placed in the NMR tube containing the 10 kDa retentate for quantitative analysis prior to solution ^{31}P NMR spectroscopy. No further additions of methylenediphosphonic acid and deuterium oxide were required for the 10 kDa filtrate because these were already added to the unfractionated extract prior to NMR analysis and they passed through the 10 kDa filtration membrane.

In order to test the performance of the ultrafiltration devices with known compounds, 10 reference materials were added to NaOH-EDTA and analyzed using solution ^{31}P NMR spectroscopy. These included (1) glycerol phosphate disodium salt hydrate ($C_3H_7O_6PNa_2.XH_2O$: Sigma-Aldrich, G6501); (2) glycerol 2-phosphate disodium salt hydrate ($C_3H_7Na_2O_6P.XH_2O$: Sigma-Aldrich, G6251); (3) adenosine-5′-monophosphate disodium salt ($C_{10}H_{12}N_5Na_2O_7P$: Sigma-Aldrich, 01930); (4) guanosine 5′-monophosphate disodium salt hydrate ($C_{10}H_{12}N_5Na_2O_8P$: Sigma-Aldrich, G8377); (5) cytidine 5′-monophosphate disodium salt ($C_9H_{12}N_3Na_2O_8P$: Sigma-Aldrich, C1006); (6) DNA sodium salt (Sigma-Aldrich, D1626); (7) D-glucose 6-phosphate sodium salt ($C_6H_{12}NaO_9P$: Sigma-Aldrich, G7879); (8) α- and β-glycerophosphate ($C_3H_7O_6PCa$: Sigma-Aldrich, G6626); (9) *myo*-inositol hexakisphosphate sodium salt ($C_6H_{18}O_{24}P_6$: Sigma-Aldrich, P8810); and (10) methylenediphosphonic acid (Sigma-Aldrich; M9508). Solution ^{31}P NMR spectroscopy was used for quantitative analysis of organic P species on prepared NaOH-EDTA solutions containing the aforementioned reference materials (unfractionated). Following NMR analysis of the NaOH-EDTA solutions containing the reference materials, each NaOH-EDTA solution was fractionated through passage of a 10 kDa ultrafiltration device in the same way as for the soil extracts. Solution ^{31}P NMR spectroscopy was then used for quantitative analysis of organic P species on the 10 kDa filtrates. All organic P species were exclusively recovered in the 10 kDa filtrates, except that of the DNA sodium salt, which was exclusively recovered in the 10 kDa retentate.

Quantification of P Species. Quantification of P species in NaOH–EDTA extracts was based on spectral integration against the addition of a known amount of added methylenediphosphonic acid, which gives a unique spectral signal separate from all other resonances for the samples tested in this study.[20,37] The peak area of the methylenediphosphonic acid signal is proportional to the absolute concentration added, which was then quantitatively compared to the peak areas of all other resonances in the solution ^{31}P NMR spectra.[20,37]

The following broad classes of P species were quantified using spectral integration based on previous studies:[20] orthophosphate (δ 7.0 to 5.4 ppm), phosphomonoesters (δ 5.4 to 3.5 ppm), phosphodiesters (δ 0.5 to −1.0 ppm) and pyrophosphate (δ −4.5 to −5.5 ppm). Since demonstrating the presence of large phosphomonoesters in NaOH-EDTA extracts was the purpose of this study, we quantified the P forms arising from both sharp and broad resonances within the orthophosphate and phosphomonoester region (δ 7.0 to 3.5 ppm) using spectral deconvolution fitting procedures,[20,37] as recommended by Doolette and Smernik.[24] The proportion of the broad resonance to that of the total phosphomonoester signal was calculated by (1) determining the combined concentration of orthophosphate and phosphomonoesters (δ 7.0 to 3.5 ppm) using spectral integration; (2) partitioning the NMR signal (broad and sharp P species) within this region using spectral deconvolution fitting procedures, as described by Doolette et al.;[20] (3) multiplying the combined concentration of orthophosphate and phosphomonoesters with that of the percentage of each component (broad and sharp P species) determined from spectral deconvolution fitting;[20] (4) subtracting the concentration of orthophosphate from the combined concentrations of orthophosphate and phosphomonoesters; and (5) then dividing the concentration of broad phosphomonoesters with that of the total phosphomonoesters and converting to a percentage. Spectral deconvolution was used to partition the orthophosphate peak from that of the phosphomonoesters due to overlap within this region.[20] Details of the deconvolution fitting procedure can be found in Doolette et al.[20] and McLaren et al.[37]

RESULTS AND DISCUSSION

Solution ^{31}P NMR spectra were obtained for five diverse soils sourced from Australia, France, Germany, Sweden, and the United States of America. An example spectrum including assignment of sharp peaks is shown in Figure 1. As expected,

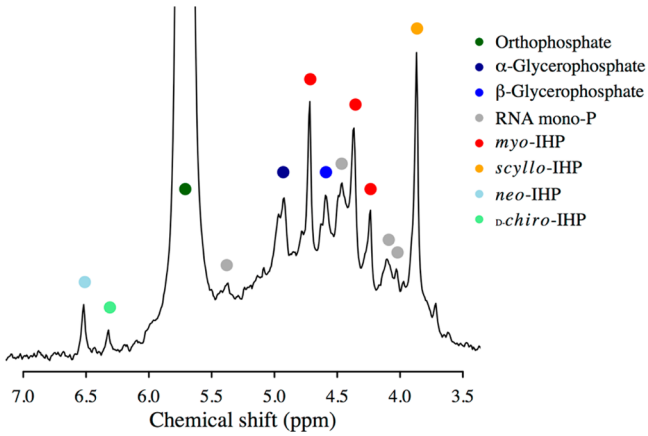

Figure 1. An example solution ^{31}P NMR spectrum (unfractionated extract of soil from Sweden) showing the assignments of sharp peaks typically present in the orthophosphate and phosphomonoester region (δ 7.0 to 3.5 ppm). Peaks identified include orthophosphate (δ 5.7 ppm), α-glycerophosphate (δ 4.9 ppm), β-glycerophosphate (δ 4.6 ppm), *myo*-inositol hexakisphosphate (*myo*-IHP − δ 4.7, 4.4, and 4.2 ppm), *scyllo*-inositol hexakisphosphate (*scyllo*-IHP, δ 3.9 ppm), RNA mononucleotides (RNA mono-P, δ 5.4, 4.5, 4.1, and 4.0 ppm), *neo*-inositol hexakisphosphate (*neo*-IHP, δ 6.5 ppm) and D-*chiro*-inositol hexakisphosphate (D-*chiro*-IHP, δ 6.3 ppm).

the spectra showed large variation within the orthophosphate and phosphomonoester region (δ 7.0 to 3.5 ppm) (Figure 2a) in which the majority of the NMR signal is found (NMR spectra encompassing a wider chemical shift window of δ 7 to −7 ppm are presented in Figure S1 of the Supporting Information). The spectral variability among unfractionated extracts from all five soils tested in this study (Figure 2a) mainly involves differences in the relative amounts of (i) a series of prominent sharp peaks and (ii) a broad resonance spanning from δ 3.5 to 6.0 ppm.[20−22] All of the sharp peaks have previously been identified as low molecular weight organic P compounds that originate from living organisms (Figures 1 and 2a).[5−7] The sharp peaks identified in the unfractionated

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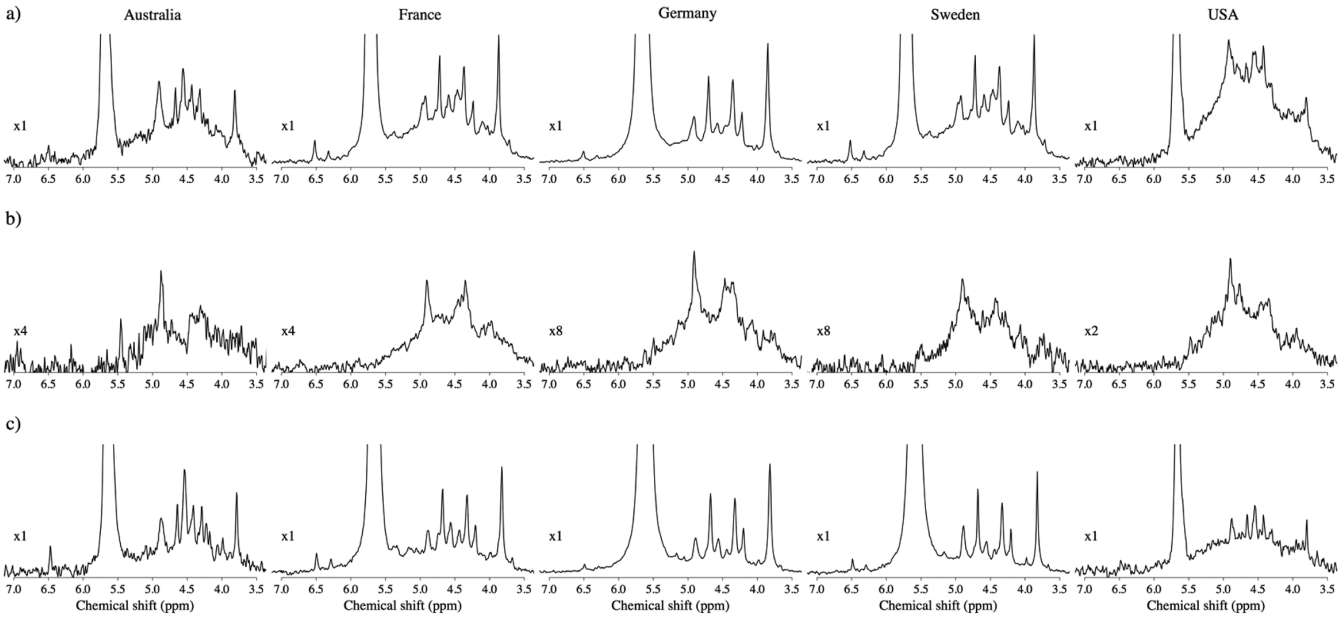

Figure 2. Solution ^{31}P NMR spectra of the orthophosphate and phosphomonoester region (δ 7.0 to 3.5 ppm) on (a) the original (unfractionated) soil extracts, (b) 10 kDa retentates, and (c) 10 kDa filtrates for each soil. For each soil the vertical scale of each spectrum was normalized to the methylenediphosphonic acid peak at δ 16.9 ppm (beyond the limits of the spectrum shown), and the vertical scale of the spectrum for the 10 kDa retentates was increased by a factor of 2−8, as indicated in the figure. Spectra for different soils are not on the same scale.

extracts include α- and β-glycerophosphate (δ 4.9 and 4.6 ppm, respectively), which are most likely primarily derived from the alkaline hydrolysis of phospholipids originally present in the soil,[22] three peaks due to *myo*-inositol hexakisphosphate (δ 4.7, 4.4, and 4.2 ppm), a peak due to *scyllo*-inositol hexakisphosphate (δ 3.9 ppm), and up to four peaks that are attributed to mononucleotides which are most likely primarily derived from the alkaline hydrolysis of ribonucleic acid (δ 5.4, 4.5, 4.1, and 4.0 ppm) (Figures 1 and 2a).[38,39] The broad feature is unique to the NMR spectra of soil extracts and is not found in extracts of plants, bacteria or fungi.[5−7,20] Therefore, it appears that there are forms of organic P in soil that are not directly produced in living organisms."

Soil extracts were separated into high and low molecular weight fractions by passage through ultrafiltration devices with a nominal cutoff of 10 kDa. Solution ^{31}P NMR spectra of the 10 kDa retentates (Figure 2b) were markedly different to those of the corresponding 10 kDa filtrates (Figure 2c). For all soils, the NMR spectra of the high molecular weight fraction (10 kDa retentates) were dominated by a broad resonance that was devoid of an orthophosphate peak, which indicates the absence of inorganic P (Figure 2b). In addition, these NMR spectra did not contain any of the sharp peaks attributable to recognizable forms of organic P commonly found in living organisms, although some distinct peaks were present (see below) (Figure 2b). These results confirm the existence of high molecular weight organic P species with phosphomonoester linkages, and demonstrate that this material predominantly gives rise to a broad NMR signal (Figure 2b).

The broadness of the NMR signal in the 10 kDa retentates is entirely consistent with P being a component of polymeric (humic) organic material containing phosphomonoester linkages.[20,40] In such heterogeneous polymeric materials, P nuclei would occupy a range of unique, but broadly similar chemical environments. The overall effect is a broad envelope of signal that differs from the much sharper signals of specific organic P containing molecules, for which the local chemical environment is identical. A similar effect has been noted for the DNA resonance that appears in the phosphodiester region (δ 0.5 to −1.0 ppm) of ^{31}P NMR spectra of most soil extracts including those investigated here (Figure S1 of the Supporting Information). The broad peak arising from DNAs in the phosphodiester region indicates that DNAs remain polymeric in alkaline extracts, in contrast to ribonucleic acids, which are hydrolyzed to monomeric units in alkaline extracts (δ 0.5 to −1.0 ppm).[38,39]

It is noteworthy that the solution ^{31}P NMR spectra of the 10 kDa retentates are similar across all soils (Figure 2b), both in their general broadness and by the presence of some distinct peaks. In particular, a sharper peak is evident at δ 4.91 ppm and other peaks are distinguishable around δ 4.47 to 4.34 ppm (Figure 2b). The chemical shift of the former is close (but not identical) to that of the α-glycerophosphate peak, which could indicate that it is due to phosphate esters of primary alcohols. The presence of this peak in soil extracts may result in an overestimation of α-glycerophosphate due to peak overlap using current methods of analysis. In any case, the similarity of solution ^{31}P NMR spectra of the 10 kDa retentates across all soils suggests that this type of organic P is an intrinsic component of the soil (e.g., soil organic matter) and/or its dynamics are governed by common mechanisms.

All of the sharp peaks detected in the NMR spectra of the unfractionated soil extracts were present in the spectra of the low molecular weight fraction (10 kDa filtrates), including those due to α- and β-glycerophosphate, *myo*- and *scyllo*-inositol hexakisphosphate, and most of the ribonucleic acid mononucleotides (Figure 2a and c). Therefore, all of the sharp peaks that are considered "recognizable biomolecules of low molecular weight" in unfractionated soil extracts were indeed accounted for in the "low molecular weight" fraction (i.e., the 10 kDa filtrates). This confirms that when quantifying forms of organic P in the phosphomonoester region using deconvolution fitting procedures, the NMR signal underneath the sharp peaks (such as that detected in the 10 kDa retentates) must be

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Table 2. Concentrations (mg kg^{-1}) of P Species As Determined from Solution ^{31}P NMR Spectra on the Original NaOH–EDTA Extracts (Unfractionated), and Following Size Separation into <10 kDa and >10 kDa Fractions[a]

			phosphomonoesters[c]								
soil	treatment	orthophosphate[b]	total phosphorus	humic phosphorus[d]	α- and β-glycerophosphate	*myo*-inositol hexakisphosphate	*scyllo*-inositol hexakisphosphate	RNA mononucleotides	phosphodiesters	pyrophosphate	total phosphorus[b]
Australia	unfractionated	73	84	57	9	8	4	5	8	6	171
	> 10 kDa	0	22	22	0	0	0	0	4	0	26
	< 10 kDa	77	55	31	8	8	4	4	0	5	137
	recovery (%)	106									95
France	unfractionated	487	378	275	17	43	27	16	14	12	890
	> 10 kDa	0	93	93	0	0	0	0	10	0	103
	< 10 kDa	527	191	115	10	37	22	6	0	12	729
	recovery (%)	108									94
Germany	unfractionated	1082	351	215	15	72	43	6	19	17	1470
	> 10 kDa	0	64	64	0	0	0	0	10	0	73
	< 10 kDa	1115	232	118	12	63	38	1	0	16	1363
	recovery (%)	103									98
Sweden	unfractionated	574	447	324	21	52	32	19	18	15	1055
	> 10 kDa	0	40	40	0	0	0	0	10	0	50
	< 10 kDa	608	150	80	11	39	20	0	0	8	766
	recovery (%)	106									77
U.S.	unfractionated	68	162						13	3	246
	> 10 kDa	0	66	66	0	0	0	0	9	0	76
	< 10 kDa	58	101	80	7	5	4	5	0	6	166
	recovery (%)	85									98

[a]Quantification of P species involved spectral integration of the whole spectrum, and spectral deconvolution within the orthophosphate and phosphomonoester region (δ 7.0 to 3.5 ppm).[20,37]. [b]Recovery values were calculated for orthophosphate and total P as the sum of the concentrations in the <10 kDa and >10 kDa fractions relative to the concentration in the unfractionated extract. [c]Deconvolution was not possible on the USA soil due to a lack of prominent sharp peaks within the phosphomonoester region. [d]Humic P refers to the broad peak that underlies the sharp peaks within the orthophosphate and phosphomonoester region.[20,37] Concentrations of humic P in the 10 kDa retentate was taken as that of the total phosphomonoester P for this fraction because of the predominantly broad signal in this fraction.[24]

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separately quantified as it is due to a different form of organic P (i.e., phosphomonoesters in supra-/macro-molecular structures).[24]

The sharp peaks arising from *myo*- and *scyllo*-inositol hexakisphosphate were particularly prominent in the 10 kDa filtrates of the soils from France, Germany and Sweden (Figure 2c), reflecting their higher abundance in the corresponding unfractionated soil extracts (Figure 2a). However, it is clear that for all five soils, the sharp peaks were more prominent in the 10 kDa filtrates than in the whole soil extracts. The reason is that the sharp peaks in the 10 kDa filtrates represent a greater proportion of the total NMR signal than they do in the unfractionated soil extracts. The NMR spectra of the 10 kDa filtrates contained considerably less of the broad peak that is detected in the unfractionated soil extracts because it had been removed by size separation (i.e., the 10 kDa retentates). This is especially evident for the soil from the U.S. (Figure 2a and c) for which the sharp peaks are difficult to distinguish in the unfractionated soil extracts, but are more clearly visible in the 10 kDa filtrate. The sharp peaks that are more clearly visible in this soil include: α- and β-glycerophosphate, RNA mononucleotide, and *myo*- and *scyllo*-inositol hexakisphosphate (Figures 1 and 2c).

Importantly, a broad resonance is still present in the ^{31}P NMR spectra of the 10 kDa filtrates for all soils (Figure 2c), although the broad resonance in the NMR spectra of the 10 kDa filtrates contributed less to total signal than in the unfractionated soil extracts (Figure 2a and c). The presence of a broad underlying signal in low molecular weight fractions of soil extracts suggests this fraction also includes a complex mixture of organic molecules containing P. It should be noted that the 10 kDa cutoff implies that "low molecular weight" includes organic molecules containing up to 300 carbon atoms, which is still considerably higher than those of the sharp peaks arising from known forms of organic P (e.g., *myo*-inositol hexakisphosphate has a molecular weight of <1 kDa). In any case, the identification of these soil phosphomonoesters in supra-/macro-molecular structures appears to complement the prevailing models of organic carbon and nitrogen dynamics carried out on humic substances.

Using solution ^{31}P NMR spectroscopy, only phosphomonoesters of recognizable biomolecules have been detected in substantial quantities in NaOH-EDTA extracts of living organisms: these biomolecules are predominantly nucleic acids and phospholipids in most instances, although *myo*-inositol hexakisphosphate can be predominant in seeds.[5,7] However, these forms of organic P represented a relatively minor component of the total phosphomonoesters in the soils analyzed in this study. The abundance of broad phosphomonoesters in soil suggests that their synthesis may involve slow and "soil-based" processes, which could involve substantial alteration of recognizable biomolecules that are added to soils (e.g., via a "humification" process). Consequently, the cycling of organic P in terrestrial ecosystems likely involves two parallel processes affecting different substrates that are characterized by different rates of turnover: (1) one that is fast and largely biologically mediated (predominantly involving recognizable biomolecules) and (2) one that is slow due to the difficultly of microbial hydrolysis of complex structures and/or involving abiotic processes (predominantly involving broad phosphomonoesters).

The NaOH−EDTA extraction technique recovered between 37% and 94% (on average 58%) of the total soil P as determined by laboratory X-ray fluorescence, which is consistent with previous studies.[15,20,37] It is not clear what forms of soil P were nonextractable with the NaOH−EDTA extraction technique but we suggest that this is comprised of largely inorganic forms. This is because the NaOH−EDTA extraction technique has been shown to provide a close estimate to that of the total organic P in soil. McLaren et al.[30] reported that the magnitude of total organic P and inorganic P in soil measured using the NaOH−EDTA extraction technique were similar to that determined using sequential chemical fractionation of soils (sandy loam, acid to slightly acid in soil pH, and low to moderate soil carbon in the 0−20 cm soil layer) collected from a permanent pasture field site located in Australia. In addition, the difference between P extracted with NaOH−EDTA and the total soil P (determined by laboratory X-ray fluorescence) was similar in magnitude to the pool of "residual P" determined by sequential chemical fractionation, which comprises soil P that is not easily extracted and is likely inorganic P strongly held within mineral silicates. Therefore, the NaOH−EDTA extraction technique can generally be considered a valid approach for estimating total organic P, which has the benefit of facilitating detailed characterization of organic P using solution ^{31}P NMR spectroscopy.[36]

Concentrations of inorganic, organic and total P in soil extracts, as determined by NMR spectroscopy, varied widely among the soils (Table 2). The proportion of organic P in whole soil extracts ranged from 25% to 71% (Table 2). Concentrations of P were also determined on the 10 kDa retentates (>10 kDa) and 10 kDa filtrates (<10 kDa) of the unfractionated soil extracts. For all soils, 94% to 98% of the total P in unfractionated extracts was recovered in the combined 10 kDa retentates and filtrates, except for the soil from Sweden, where 77% was recovered in the combined retentate and filtrate (Table 2). The reason for this lower recovery is unclear but a possible explanation is the incomplete recovery of the retentate material when transferring this to the NMR tube, due to material being trapped at the bottom of the 10 kDa ultrafiltration device, possibly caused by the high concentration of total carbon in this soil (8.6%). For all soils, inorganic P (orthophosphate and pyrophosphate) was only detected in the 10 kDa filtrates, whereas organic P was detected in both the 10 kDa retentates and 10 kDa filtrates (Table 2 and Figure 2). Concentrations of organic P ranged from 26 to 103 mg kg^{-1} in the 10 kDa retentates and from 55 to 232 mg kg^{-1} in the 10 kDa filtrates (Table 2).

Concentrations of P species giving rise to both broad and sharp resonances were determined within the orthophosphate and phosphomonoester region using spectral integration and then deconvolution fitting procedures on the unfractionated extracts (Table 2).[20,37] The broad feature (termed here humic P; Table 2) in the unfractionated soil extracts ranged in concentration from 57 to 324 mg kg^{-1} across all soils, and accounted for 61−73% of the total phosphomonoester signal in the unfractionated soil extracts (Table 2). Deconvolution was not possible for the unfractionated soil extract of the U.S. soil due to a lack of prominent sharp peaks within the phosphomonoester region. Interestingly, while concentrations of phosphomonoesters in supra-/macro-molecular structures varied widely, their proportion to that of the total phosphomonoester signal was relatively consistent across all soils tested for in this study. Although, the proportion of phosphomonoesters in supra-/macro-molecular structure to that of the total phosphomonoester signal in the soil from the

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U.S. appears to be higher. We hypothesize that this might be due to constraints of time and climate (e.g., temperature and rainfall) that govern the synthesis and degradation of these phosphomonoesters.

Concentrations of *myo*- and *scyllo*-inositol hexakisphosphate were determined for four of the five soils and ranged from 12 to 115 mg kg^{-1}. The concentration of these species could not be quantified for the soil extract from the U.S., for which there was a lack of prominent sharp peaks within the phosphomonoester region (Table 2). *Myo*- and *scyllo*-inositol hexakisphosphate accounted for 14−32% of the total phosphomonoester signal in the unfractionated extracts of the four soils where it could be quantified (Table 2). It is unclear exactly why the concentrations of inositol hexakisphosphates were higher for the soils from France, Germany and Sweden compared to those from Australia and the U.S. However, we note that the former also contained higher concentrations of soil organic matter and oxalate extractable aluminum and iron than the latter (Table 1). In any case, it is clear that strategies designed to solubilize *myo*-inositol hexakisphosphate in soil for plant uptake[41] will have limited benefit for soils similar to those collected from Australia and the U.S.[5,7]

This study is the first in which separation of soil extracts into high (nominally >10 kDa) and low (nominally <10 kDa) molecular weight fractions have been combined with ^{31}P NMR characterization. We have confirmed the existence of organic P in the high molecular weight fraction of five diverse soils and demonstrated that the speciation of this organic P is markedly different to that in the low molecular weight fraction. Differences between the ^{31}P NMR spectra of the two fractions were strong and consistent, with the high molecular weight fraction dominated by a broad resonance and the low molecular weight fraction containing the majority of sharp features present in the spectra of the unfractionated extracts, along with some broad signal. We have also demonstrated the absence of signals due to orthophosphate and inositol hexakisphosphates in the high molecular weight fraction and their quantitative recovery in the low molecular weight fraction.

These results have major implications for our understanding of the organic P cycle in terrestrial ecosystems as it indicates that a substantial proportion of organic P in soils is present as phosphomonoesters in supra-/macro-molecular structures. Consequently, based on global estimates of terrestrial P in the upper 50 cm of the soil profile,[1] up to 29 gigatonnes of soil P could be comprised of phosphomonoesters in supra-/macro-molecular structures. Recognition of this is important, and needs to be integrated into current models of the inorganic−organic P cycle in soil-plant terrestrial ecosystems. Specifically, mechanisms need to be identified by which P becomes incorporated into polymeric organic matter and the role of large molecular weight phosphomonoester P in the overall terrestrial P cycle needs to be considered.

The identification of phosphomonoesters in supra-/macro-molecular structures and their abundance in soils suggests that they are an important component of the P cycle in soil-plant systems. Understanding their dynamics will lead to improved outcomes for the environment because (1) pools of soil organic P have been identified as a major sink of fertilizer P in agricultural soils, which results in an inefficiency of finite resources (i.e., rock phosphate);[30,42] (2) these forms could provide a source of P to living organisms, which their supply to crops may be of particular importance in developing countries where there is difficulty in obtaining mineral fertilizers for food production[43]; and (3) pools of soil organic P are known to transfer to aquatic and marine ecosystems where eutrophication can occur.[44] Ultimately, given the importance of the terrestrial P cycle for food production and environmental issues associated with P transport to surface waters, our findings have implications for issues relating to P scarcity, food security and water quality.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b02948.

Figures showing the NMR spectra on all NaOH-EDTA extracts encompassing a wider chemical shift window of δ 7 to −7 ppm for each soil (PDF)

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Notes

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