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Identification of inositol hexakisphosphate binding sites in soils by selective extraction and solution ^{31}P NMR spectroscopy

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ABSTRACT

Inositol hexakisphosphate (IP_6) can constitute the majority of the organic phosphorus in soil. Soil IP_6 accumulates through a number of mechanisms, including sorption to metal hydroxides and clays, association with organic matter, and precipitation with cations and on surfaces of metal oxides. However, the relative contributions of these processes remain unknown. We quantified IP_6 stereoisomers by NaOH–EDTA extraction and solution ^{31}P NMR spectroscopy in a series of contrasting soils from natural and agricultural ecosystems, and then used selective extractions to identify associations between IP_6 and soil components. Oxalic acid, which extracts amorphous and organically complexed iron and aluminum oxides, extracted the majority of the IP_6 from temperate grassland and forest soils, but not from strongly weathered tropical rice soils. In contrast, removal of mineral material by pretreatment with hydrofluoric acid completely removed IP_6 from temperate forest soils, but not from temperate grasslands or tropical rice soils. We conclude that the relative importance of IP_6 stabilization on organic and mineral components varies markedly among soils, and that oxalate extraction provides a selective procedure for the quantification of IP_6 associated with amorphous metal oxides and clays.

1. Introduction

Inositol hexakisphosphate (IP_6) constitutes the majority of the organic phosphorus (P) in many soils (Turner et al., 2002). The greatest concentrations appear to occur in grassland soils, but IP_6 has also been detected in crop soils, forest soils, and rice paddies (Turner, 2007). Four stereoisomers of IP_6 have been identified in soils; the most abundant is the *myo* isomer, with smaller amounts of the *scyllo*, *neo* and D-*chiro* isomers (Cosgrove, 1962; Cosgrove and Tate, 1963; Turner, 2007; Turner et al., 2012). *myo*-Inositol hexakisphosphate is the main P compound in seeds and is also present in manure from monogastric animals, which are not able to digest *myo*-IP_6 (Cosgrove, 1980; Leytem and Maguire, 2007). The other three stereoisomers do not occur in plant tissue, so are presumably synthesized by soil microbes, perhaps by epimerization of the *myo* isomer (Turner, 2007).

Inositol hexakisphosphate accumulates in soils through interactions with mineral and organic soil components. This can occur by adsorption to aluminum (Al) and iron (Fe) hydroxides/oxides, clays, or calcite, association with organic matter, or precipitation with cations as phytate (salts of *myo*-IP_6) (Celi and Barberis, 2007; Karathanasis and Shumaker, 2009). Adsorption may occur through a ligand exchange mechanism between the phosphate groups and surface reactive OH^- or H_2O groups on the adsorbents (Celi and Barberis, 2007; Ognalaga et al., 1994), although a recent study indicates that the adsorption to goethite occurs as an outer sphere complexation in which hydrogen bonds between the surface of goethite and IP_6 are formed (Johnson et al., 2012). Furthermore, IP_6 may rapidly form surface precipitates of Al–IP_6 complexes on the surface of Al oxides after a brief initial adsorption phase (Yan et al., 2014a). The adsorption capacity of metal oxides for IP_6 increases as soil pH decreases (Celi et al., 2001), which renders associations with calcite and organic matter less important (Celi and Barberis, 2007). Association between IP_6 and soil organic matter might occur via physical or chemical incorporation within organic matter structures, or through adsorption to organic matter via metal bridges (Celi and Barberis, 2007), although only the latter mechanism has been demonstrated experimentally (Leytem et al., 2002). In acidic soils, associations with amorphous Al and Fe hydroxides are believed to be the most important mechanism of IP_6 stabilization (Celi and Barberis, 2007). This is supported by correlations between amorphous metals and IP_6 across a wide variety of soils (Anderson et al., 1974; McKercher and Anderson, 1968; Turner et al., 2007, 2003; Vincent et al., 2012). However, the relative contribution of these stabilization processes remains poorly understood, in part because most recent studies have extracted IP_6 from soils by a single-step NaOH–EDTA procedure, which is assumed to extract mineral associated IP_6 as well as IP_6 associated with the organic soil matrix (Turner et al., 2005a).

Recently, a procedure using dilute hydrofluoric acid (HF) pretreatment followed by NaOH–EDTA extraction has been used to identify P associated with the organic soil matrix (Dougherty et al., 2007;

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Hamdan et al., 2012). Hydrofluoric acid dissolves the mineral matrix but leaves organic matter largely intact (Dougherty et al., 2007). It will therefore remove all IP_6 associated with the mineral matrix, leaving IP_6 associated with organic matter. This can be used to determine whether IP_6 is associated with mineral or organic material, but cannot indicate whether IP_6 is associated with amorphous or crystalline metal hydroxides. However, extraction in acidic ammonium oxalate is routinely used to extract amorphous Al and Fe hydroxides, as well as associated P (Gleyzes et al., 2002). The oxalate extract acts via a ligand exchange with surface OH^- groups and forms a complex (e.g. Fe(III)–$C_2O_4^{2-}$) that polarizes and weakens the metal–O bonds between metal atoms and the surface of the metal complex, leading to non-reductive dissolution (Gleyzes et al., 2002; Stanjek and Weidler, 1992; Zinder et al., 1986). Thus, the specificity for amorphous metal complexes is due to their relatively high specific surface area (concentration of OH^- per area) leading to a high solubility compared to crystalline forms such as goethite (Karim, 1984; Schwertmann, 1973; Theng et al., 1982). The apparent strong association between amorphous metals and IP_6 in soils suggests that oxalate extraction offers potential as a selective extractant for IP_6 associated with amorphous metals.

Given the importance of soil organic P for plant nutrition in both natural and agricultural ecosystems (Richardson et al., 2005), there is an urgent need to develop procedures that provide accurate information on the nature and stabilization of organic P compounds in soils. We used HF pretreatment and oxalate extraction in combination with solution ^{31}P NMR spectroscopy to assess the association of IP_6 stereoisomers with organic matter and metal oxides. Our aim was to separate IP_6 into: (i) IP_6 bound to amorphous Al and Fe, and (ii) IP_6 associated with organic matter. In theory, this would allow calculation of a third group of IP_6 bound to more crystalline metal oxides. The procedure was tested on a series of seven soils known to contain inositol phosphates from three different ecosystems: temperate grasslands, tropical rice fields and lowland temperate rainforest.

2. Methods

2.1. Locations, soil sampling, and preparation

Soil was collected from three ecosystems: tropical rice paddies in Madagascar (Turner, 2006), temperate grasslands in the Falkland Islands (Turner et al., 2012), and temperate rainforest at the Haast chronosequence in New Zealand (Turner et al., 2014). The following labels were used: for the rice paddies (MDG), temperate grassland soils (EAST) and temperate rainforest soils (Dune). The soils had a range of properties, including total P concentrations (Table 1), and were known from previous studies to contain IP_6. Detailed information on the soils is available elsewhere (Turner, 2006; Turner et al., 2012, 2014), although it should be noted that the temperate grassland soils were from slightly different locations from those studied previously (Turner et al., 2012). All samples were surface soils (0–10 cm) and were air dried, screened and sieved (<2 mm) prior to analysis, with storage in sealed plastic bags at ambient laboratory temperature and humidity (22 °C and 55%, respectively).

2.2. NaOH–EDTA extraction

Total IP_6 was extracted by shaking soil (1.00 ± 0.01 g) in 20 mL of a solution containing 0.25 M NaOH and 0.05 M Na_2EDTA (disodium ethylenediaminetetraacetate) for 16 h (Turner et al., 2005a). Extracts were centrifuged at 10,000 *g* for 10 min and the supernatant decanted. Each solution was spiked with 1 mL 50 μg mL^{-1} methylene diphosphonic acid (MDP) as an internal standard, frozen at −40 °C, and lyophilized. We assume that the NaOH–EDTA procedure yields quantitative recovery of organic P and therefore IP_6 from soils, although this remains poorly understood given the lack of a procedure for the direct determination of total soil organic P (Turner et al., 2005a).

2.3. Pretreatment with hydrofluoric acid

To isolate IP_6 associated with soil organic matter, soils were pre-extracted in 10% HF according to the procedure of Hamdan et al. (2012). Briefly, soil (2.0 ± 0.01 g) was extracted four times in 45 mL of 10% HF for 1 h, and then twice for 24 h. The solution was centrifuged at 1790 *g* for 10 min between each step and the supernatant discarded. After the final HF treatment, the soil pellet was rinsed five times in 45 mL distilled water, dried, weighed, and extracted in 30 mL of NaOH–EDTA. The extracts were then frozen, spiked with internal standard, and lyophilized as described above.

2.4. Oxalate extraction

To extract IP_6 associated with amorphous metal oxides, soil (1.00 ± 0.01 g) was extracted in 40 mL of a solution containing 0.2 M ammonium oxalate monohydrate ($(NH_4)_2C_2O_4 \cdot H_2O$)–oxalic acid ($C_2H_2O_4 \cdot 2H_2O$) adjusted to pH 3 (Schwertmann, 1964). The samples were shaken in darkness for 2 h and then centrifuged for 10 min at 2000 *g*. Two milliliters of sample were diluted in 2% HNO_3 and analyzed for Al, Fe, and P by inductively-coupled plasma optical-emission spectroscopy (ICP-OES) (Optima 7300DV, Perkin Elmer, Shelton, CT). The pH of the remaining supernatant was increased to ~8 by addition of NaOH, 20 g of amberlite cation exchange resin (chelex 100 resin; Sigma-Aldrich) was added, and the mixture shaken for 1 h. The supernatant was removed and the resin was washed three times with 10 mL of distilled water. The washings were added to the supernatant and pH was increased to >12 by addition of NaOH. Each solution was spiked with 1 mL of 50 μg P mL^{-1} MDP, frozen at −40 °C, and lyophilized. Samples were kept in darkness throughout the procedure to avoid degradation of the oxalic acid.

Table 1
Locations and soil properties adopted from published papers. Concentrations of Al, Fe, and P (mg kg^{-1}) in the oxalate extracts determined by ICP-OES and P saturation (P_{sat}) = $P_{ox} * 100 / (Al_{ox} + Fe_{ox})$, expressed as molar ratios.

Location code	Location	Total elements			pH	Oxalate extractable			Psat	Topsoil texture	Taxonomic order	Vegetation	Reference
		P	C	N		Al_{ox}	Fe_{ox}	P_{ox}					
		mg P kg^{-1}	(%)	(%)		g Alkg^{-1}	g Fekg^{-1}	mg P kg^{-1}	%				
MDG 8	Madagascar	828	6.4	0.53	5.0	8.34	6.01	385	3.0	Clay	Oxisol	Tropical rice paddy	Turner (2006)
MDG 10	Madagascar	1128	3.3	0.39	5.0	2.31	4.99	189	3.5	Clay	Oxisol	Tropical rice paddy	Turner (2006)
EAST 46	Falkland Islands	1376	16.0	1.10	5.3	5.91	3.59	952	10.9	n.d.[c]	Spodosol	Temperate grassland	Turner et al. (2012)
EAST 48	Falkland Islands	1213	13.8	0.98	5.2	7.15	4.48	809	7.6	n.d.[c]	Spodosol	Temperate grassland	Turner et al. (2012)
EAST 54	Falkland Islands	995	12.5	0.92	5.5	4.18	4.49	657	9.0	n.d.	Spodosol	Temperate grassland	Turner et al. (2012)
Dune 3[a]	New Zealand	229	3.1	0.16	4.2	0.68	2.37	140	6.7	Loamy sand	Entisol	Temperate rainforest	Turner et al. (2014)
Dune 8[b]	New Zealand	155	1.9	0.09	3.9	1.38	3.00	97	3.0	Loamy sand	Entisol	Temperate rainforest	Turner et al. (2014)

[a] Age: 392 years old.
[b] Age: 1826 years old.
[c] n.d. = not detected.

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2.5. Post-oxalate extraction in NaOH–EDTA

The residual soil from the oxalate extraction was washed twice in distilled water (pH 3), centrifuged (10 min at 10,000 *g*), and the washings discarded. The residue was then extracted in 20 mL of NaOH–EDTA for 16 h, spiked with internal standard, frozen, and lyophilized as described above.

2.6. Total dissolved P and dissolved reactive P in NaOH–EDTA extracts

Subsamples of the NaOH–EDTA extracts were filtered (0.45 μm), diluted 20 times and neutralized with H_2SO_4. Total dissolved P in the extracts (TP_{ex}) was determined by inductive-coupled plasma optical emission spectroscopy (ICP-OES). Reactive P (SRP) was determined by automated molybdate colorimetry on a Lachat Quickchem 8500 (Hach Ltd, Loveland, CO). Unreactive P (UP) was calculated as the difference between TP_{ex} and SRP.

2.7. Solution ^{31}P NMR spectroscopy

The following samples were analyzed by solution ^{31}P NMR spectroscopy: (1) NaOH–EDTA extracts of untreated soils, (2) post-HF NaOH–EDTA extracts, (3) oxalate extracts, (4) post-oxalate NaOH–EDTA extracts. All lyophilized extracts (~500 mg powder) were re-dissolved in 0.1 mL D_2O and 0.9 mL of a solution containing 1 M NaOH and 0.1 M EDTA, centrifuged at 10,000 *g* for 10 min to remove particulates and immediately analyzed by solution ^{31}P NMR spectroscopy.

For the oxalate and the post oxalate extracts, ^{31}P NMR spectra were recorded at 80.9 MHz on a Varian 200 MHz NMR spectrometer (Bruker, Germany) at ambient temperature using a 21.25 μs (45°) observe pulse, 5 s acquisition time, and a relaxation delay of 10 s, acquiring ca. 4000–16,000 transients. Chemical shifts were indirectly referenced to external 85% H_3PO_4 via lock resonance. For the original NaOH–EDTA extracts and the NaOH–EDTA extracts of the soils after HF pretreatment, spectra were obtained on a Bruker Avance DRX 500 MHz spectrometer (Bruker, Germany) operating at 202.456 MHz for ^{31}P. Samples were analyzed using a 6 μs pulse (45°), a delay time of 10 s, an acquisition time of 0.4 s, and broadband proton decoupling. We tested each extract to confirm that this delay time was sufficient to yield full recovery of all P forms. Approximately 30,000 scans were acquired for each sample. Spectra were plotted with a line broadening of 2 Hz and chemical shifts of signals were determined in parts per million (ppm) relative to an external standard of 85% H_3PO_4.

The NMR utility transform software (MestReNova) was used to obtain peak areas from the raw spectrum. Peak areas were determined by integration and deconvolution. From these peak areas, the contribution of the individual P groups was calculated relative to the TP of the extract. Peaks were identified by comparison with literature reports (Turner et al., 2003, 2005b, 2012). Signals in spectra of oxalate extracts were slightly upfield of the corresponding signals in other extracts which may be caused by the high ionic concentration in the oxalate extracts. Hence, the spacing between the signals and the 1:2:2:1 ratio between the four *myo*-IP_6 signals was used to identify the signals.

3. Results

3.1. Original NaOH–EDTA extracts

The NaOH–EDTA extraction of untreated soils extracted 57–94% of total soil P. The highest extraction efficiencies were found in the temperate grassland soil (Table 2). Unreactive P (the difference between total P and reactive P in the extracts) concentrations were generally greater than the concentration of organic P estimated by ^{31}P NMR spectroscopy (Table 3).

Inositol hexakisphosphate stereoisomers were identified in all NaOH–EDTA extracts of untreated soils (Figs. 1–2, Table 4). *myo*-Inositol hexakisphosphate was identified as a series of four signals in a 1:2:2:1 ratio at $\delta = 5.94, 5.04, 4.68$ and 4.54 ppm, with small variations among samples (Figs. 1–2). *scyllo*-Inositol hexakisphosphate was identified as a signal at $\delta = 4.30$ ppm (Figs. 1–2) and *neo*-IP_6 as a signal at $\delta = 6.90$ ppm. Both *myo*- and *scyllo*-IP_6 were detected in all untreated soils, whereas the *neo*-isomer was observed in only five of the seven soils. The concentration of *myo*-IP_6 varied between 28 and 386 mg P kg^{-1}, while the concentration of *scyllo*-IP_6 varied between 13 and 178 mg P kg^{-1} (Table 4). *neo*-Inositol hexakisphosphate was not quantified due to the low signal to noise ratio in the spectra. No signals from D-*chiro*-IP_6 were identified in the extracts. We assume that the concentrations of IP_6 in NaOH–EDTA extracts of untreated soils correspond approximately to the total concentration of IP_6 in the soils.

3.2. Post HF NaOH–EDTA extracts

The NaOH–EDTA extraction of the HF treated soils recovered 4–35% of total soil P (Table 2). Organic P detected by ^{31}P NMR spectroscopy was lower than unreactive P in the post-HF NaOH–EDTA extracts (Table 3). In the tropical rice soils (MDG) the post-HF extraction recovered between 56 and 122% of the organic P estimated by NMR in the original NaOH–EDTA extracts (Table 3). The recovery in the temperate grasslands soils (EAST) and the temperate forest soils (Dune) were 25–34% and 0–18%, respectively (Table 3).

Both *myo*- and *scyllo*-IP_6 were observed in NaOH–EDTA extracts of HF treated soils from temperate grassland and rice paddies, but not in extracts of soils from temperate forest (Figs. 1–2, Table 4). Only one of the temperate grassland soils contained a detectable concentration of *neo*-IP_6 in HF pretreated soils (Figs. 1–2, Table 4). In the grassland soils *myo*- and *scyllo*-IP_6 remaining after HF pretreatment accounted for between 20 and 25% of the *myo*-IP_6 in the original NaOH–EDTA extracts and between 15 and 24% and the *scyllo*-IP_6 in the original NaOH–EDTA extracts (Table 4). In the rice paddies between 44 and 154% of the *myo*- and *scyllo*-IP_6 was recovered in extracts of the HF pretreated soils compared to the original NaOH–EDTA extracts.

3.3. Oxalate extracts

The oxalate solution extracted 17–69% of the total soil P and 37–130% of the organic P estimated by ^{31}P NMR spectroscopy in the original NaOH–EDTA extract.

Table 2
Total phosphorus (mg P kg^{-1}) measured by ICP-OES in the extracts and extraction efficiency (% of total soil P).

Location	$TP_{NaOH-EDTA\ original}$	$TP_{oxalate}$	$TP_{post\ oxalate}$	$TP_{ox\ +\ post\ ox}$	$TP_{post\ HF}$
	mg P kg^{-1} (*% of total soil P*)				
MDG 8	662 (*80*)	384 (*46*)	241 (*29*)	628 (*76*)	287 (*35*)
MDG 10	642 (*57*)	189 (*17*)	440 (*39*)	605 (*54*)	109 (*10*)
EAST 46	1296 (*94*)	952 (*69*)	290 (*21*)	1123 (*82*)	365 (*27*)
EAST 48	1088 (*90*)	809 (*67*)	253 (*21*)	961 (*79*)	350 (*29*)
EAST 54	906 (*91*)	657 (*66*)	281 (*28*)	856 (*86*)	223 (*22*)
Dune 3	201 (*88*)	140 (*61*)	45 (*20*)	185 (*81*)	33 (*14*)
Dune 8	103 (*66*)	97 (*63*)	35 (*23*)	132 (*85*)	6 (*4*)

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Table 3
Organic phosphorus (including pyro- and polyphosphates) in the extracts measured by solution ^{31}P NMR spectroscopy (organic P) and unreactive P measured by ICP/colorimetry. Unreactive P was not determined in oxalate extracts due to interference by oxalate in molybdate colorimetry.

Location	NaOH–EDTA		Oxalate	Post oxalate		Oxalate + post oxalate	Post HF	
	Unreactive P	Organic P	Organic P	Unreactive P	Organic P	Organic P	Unreactive P	Organic P
	mg P kg^{-1} (*extraction efficiency of original NMR %*)							
MDG 8	333	163	60 (*37*)	155	61 (*37*)	121 (*74*)	246	199 (*122*)
MDG 10	259	106	– (–)	165	92 (*87*)	92 (*87*)	82	59 (*56*)
EAST 46	1071	943	477 (*51*)	272	238 (*25*)	715 (*76*)	345	319 (34)
EAST 48	965	806	539 (*67*)	237	173 (*21*)	712 (*88*)	333	294 (36)
EAST 54	779	674	326 (*48*)	267	228 (*34*)	554 (*82*)	212	168 (25)
Dune 3	157	102	47 (*46*)	44	21 (*21*)	68 (*66*)	32	18 (18)
Dune 8	84	33	43 (*130*)	33	– (–)	43 (*130*)	3	0 (0)

Both *myo*- and *scyllo*-IP_6 were identified in all oxalate extracts except for one of the rice soils. Since, the fourth signal of *myo*-IP_6 at 5.94 ppm in the MDG 8 oxalate extract lacks, the identification is subject to some uncertainty although the position of the other signals provides relatively strong evidences for the identification. *neo*-Inositol hexakisphosphate was identified in the oxalate extracts of two of the temperate grassland soils, but not in extracts of rice soils or forest soils (Figs. 1–2, Table 4). The concentrations of *myo*- and *scyllo*-IP_6 in oxalate extracts corresponded to 55–65% of the original NaOH–EDTA extractable *myo*-IP_6 and 72–78% of the original NaOH–EDTA extractable *scyllo*-IP_6 in the grassland soils (Table 4). In the temperate forest soils, 35–86% and 26–80% of the original NaOH–EDTA extractable *myo*- and *scyllo*-IP_6, respectively, were extracted by oxalate. In the rice paddy soil, 0–86% of the original NaOH–EDTA extractable *myo*-IP_6 and 0–50% of the total *scyllo*-IP_6 were extracted by oxalate (Table 4).

The content of oxalate extractable P, Al and Fe were 0.10–0.95 g P kg^{-1}, 0.67–8.40 g Al kg^{-1} and 2.36–6.01 g Fe kg^{-1} (Table 4). The corresponding P saturation was between 3.0 and 10.7%, with lowest values in rice soils and highest values in grassland soils (Table 4).

3.4. Post oxalate NaOH–EDTA extracts

The NaOH–EDTA of oxalate-pretreated soils recovered 20–39% of the total soil P (Table 2). In total, the oxalate extracts and the post-oxalate extracts contained 54–86% of the total soil P, which were comparable to P extraction from untreated soils by NaOH–EDTA (Table 2). Organic P (estimated by NMR) in post-oxalate extracts constituted 21–87% of the original NaOH–EDTA extractable organic P (Table 3). In total, 66–130% of the original NaOH–EDTA extractable organic P was recovered by the oxalate and the post-oxalate extracts (Table 3).

The NaOH–EDTA extracts of oxalate pretreated soils did not contain identifiable signals from IP_6 stereoisomers (Fig. 1–2), although low IP_6 concentrations and relatively poor spectral resolution in the post-oxalate NaOH–EDTA extracts might have contributed to our inability to identify IP_6 in these extracts.

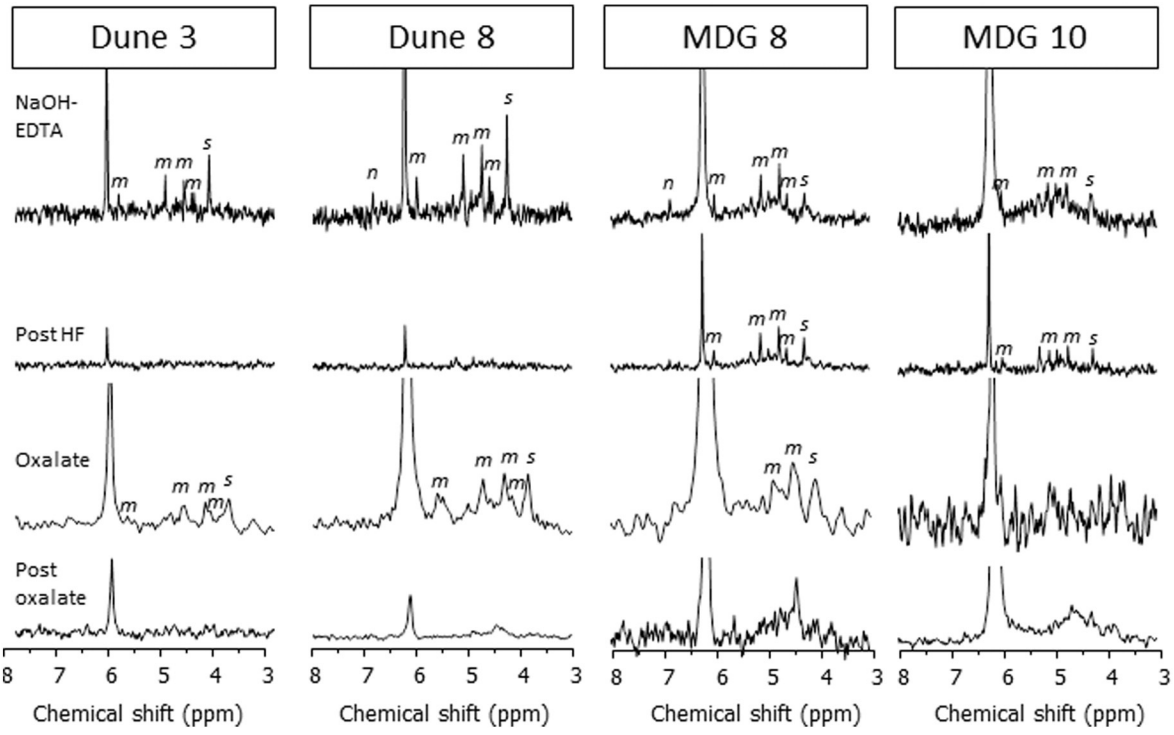

Fig. 1. Solution ^{31}P NMR spectra of extracts of soils from the Haast chronosequence in New Zealand (Dune 3 and 8) and from rice soils in Madagascar (MDG 8 and MDG 10). The spectra show the orthophosphate monoester region, which includes a truncated signal from orthophosphate at approximately 6.2 ppm. All spectra are scaled to the MDP signal height (not shown). For each soil, spectra of four different extracts are shown, from top to bottom: NaOH–EDTA extract of un-pretreated soil, NaOH–EDTA extract after HF pretreatment, oxalate extract, and NaOH–EDTA extract after oxalate extraction.

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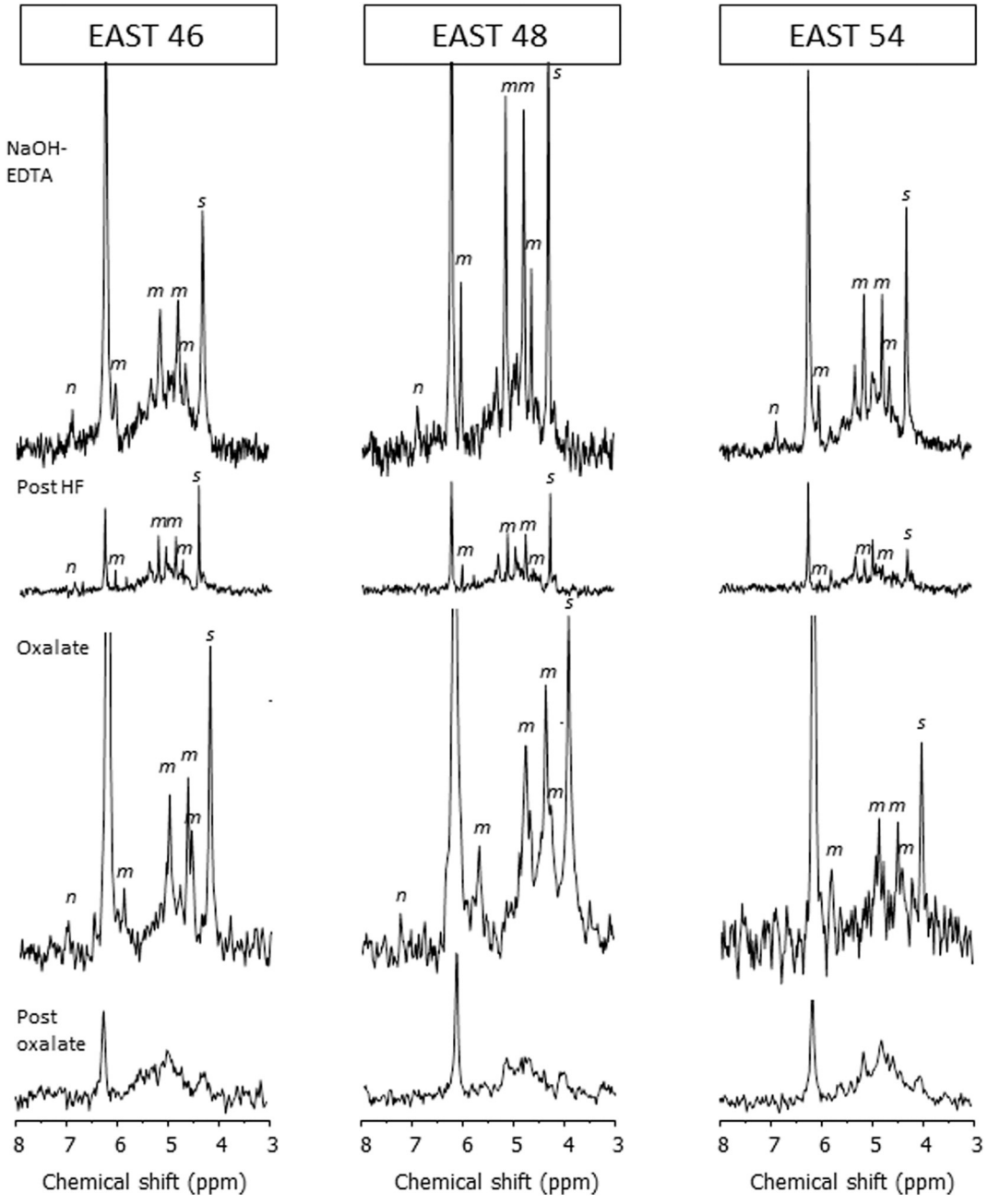

Fig. 2. Solution ^{31}P NMR spectra of extracts of three temperate grassland soils from the Falkland Islands. The spectra show the orthophosphate monoester region, which includes a truncated signal from orthophosphate at approximately 6.2 ppm. All spectra are scaled to the MDP signal (not shown), which is approximately half of the signal height used in Fig. 1. For each soil, spectra of four different extracts are shown, from top to bottom: NaOH–EDTA extract of un-pretreated soil, NaOH–EDTA extract after HF pretreatment, oxalate extract, and NaOH–EDTA extract after oxalate extraction.

4. Discussion

In this study we present a novel procedure combining oxalate and HF extractions to identify and quantify different binding forms of IP_6 in soils. The results are the first direct evidence showing marked variation among soils in the binding sites for IP_6. In the tropical rice paddies a large part of the IP_6 was associated with the organic matrix, whereas IP_6 in the grassland and forest soils were mainly associated with amorphous metal oxides or clays (i.e., oxalate extractable; see detailed discussion of the extractions below).

Several studies have reported correlations between IP_6 and amorphous metal oxides in acidic soils (Anderson et al., 1974; Giaveno et al., 2008; McKercher and Anderson, 1968; Turner et al., 2003). In addition, IP_6 concentrations have been shown to fluctuate in parallel with changes in amorphous Al and Fe hydroxides during long-term pedogenesis (Turner et al., 2007). Here we provide direct evidence for the importance of associations to amorphous metal oxides and/or clays in acidic soils. Most of the total IP_6 in forest and grassland soils was associated with amorphous metal oxides and/or clays rather than with crystalline oxides or organic matter. In the strongly weathered tropical Oxisols (MDG) more than half of the *myo*- and *scyllo*-IP_6 were associated with organic matter. Also up to 25% of the IP_6 in the organic rich grassland soils was associated with organic matter, demonstrating that associations between IP_6 and organic matter may be important even in acidic soils with abundant amorphous Al and Fe oxides. Association with organic matter can be through physical or chemical incorporation in the organic matter, direct adsorption to the surface of the organic matter or adsorption to metal bridges associated with

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Table 4
Concentrations of *myo*-IP_6 and *scyllo*-IP_6 (mg P kg^{-1}) determined by solution ^{31}P NMR spectroscopy after different soil treatments. Values in parentheses are IP_6 extracted in the treatment as percentage of total IP_6 extracted in a single-step NaOH–EDTA extract. The presence of *neo*-IP_6 is indicated by +, although this stereoisomer was not quantified. IP_6 in NaOH–EDTA extracts is assumed to represent the total IP_6; IP_6 in the post-HF NaOH–EDTA extracts represents organically bound IP_6; IP_6 in the oxalate extracts represents IP_6 bound to amorphous Al and Fe hydroxides (we assume that Ca bound IP_6 is not important in these acid soils). n.d. not detected.

Location code	NaOH–EDTA (original)[a]			Post HF[b]			Oxalate[c]		
	myo	*scyllo*	*neo*	*myo*	*scyllo*	*neo*	*myo*	*scyllo*	*neo*
	mg P kg^{-1}			mg P kg^{-1} (*% of original*)					
MDG 8	29	16	+	20 (*69*)	7 (*44*)	n.d.	25 (*86*)	8 (*50*)	n.d.
MDG 10	59	13	n.d.	77 (*131*)	20 (*154*)	n.d.	n.d.	n.d.	n.d.
EAST 46	354	167	+	70 (*20*)	40 (*24*)	+	230 (*65*)	131 (*78*)	+
EAST 48	386	178	+	83 (*22*)	27 (*15*)	n.d.	239 (*62*)	136 (*76*)	+
EAST 54	230	92	+	58 (*25*)	18 (*20*)	n.d.	126 (*55*)	66 (*72*)	n.d.
Dune 3	43	19	+	n.d.	n.d.	n.d.	15 (*35*)	5 (*26*)	n.d.
Dune 8	28	15	n.d.	n.d.	n.d.	n.d.	24 (*86*)	12 (*80*)	n.d.

[a] Untreated soil extracted in NaOH–EDTA.
[b] HF pretreated soil extracted in NaOH–EDTA.
[c] Untreated soil extracted in oxalate.

organic matter (Celi and Barberis, 2007). Since both the oxalic acid (García-Rodeja et al., 2007) and the HF pretreatment should have interrupted the associations between the metal bridges and organic matter (Hamdan et al., 2012) we assume that the IP_6 is either directly adsorbed to the organic matter or incorporated in it.

Degradation of IP_6 is catalyzed by phytases which are produced by some plants and many microorganisms (Greiner, 2007; Hill and Richardson, 2007; Richardson, 2007). Several plants, especially transgenic plants, are able to grow on IP_6 as their sole P source in low P sorbing media, but in high P fixing media the growth ceases due to the inaccessibility of strongly-fixed IP_6 for enzymatic degradation (Richardson, 2007). Hence, it is likely that oxalate extractable IP_6 is potentially bioavailable, but utilization will require desorption/dissolution and enzymatic hydrolysis (Giles et al., 2012; Hayes et al., 2000). As this is costly for plants and microbes, degradation of IP_6 in high P fixing soils has been suggested primarily to take place under P or carbon limitation (Greiner, 2007), which might explain the accumulation of high concentrations of IP_6 in temperate soils rich in P and Al/Fe (Celi and Barberis, 2007; Turner, 2007). Even less is known about the stabilization of organic bound IP_6, but the fate of this IP_6 pool is probably controlled by the same factors that control organic matter dynamics. This may also explain why *scyllo*-IP_6 concentrations in Madagascan rice soils correlate positively with total carbon, total nitrogen and organic P (Turner, 2006).

Oxalic acid extracted at least some of the IP_6 in all seven soils. The oxalate solution is used to extract amorphous Al and Fe oxides as well as P bound to those oxides, but does not extract more crystalline metals (García-Rodeja et al., 2007; Gleyzes et al., 2002; Jan et al., 2013; McKeague et al., 1971a; McKeague and Day, 1966). It also extracts amorphous Al and Fe associated with organic matter (McKeague et al., 1971b) and calcium-bound P due to pH dependent dissolution of Ca compounds (Lookman et al., 1997; Uusitalo and Tuhkanen, 2000), although the acidic pH in the soils used in this study suggests that Ca compounds are unimportant as sorption sites for P in these soils. The identification of IP_6 in oxalate extracts of soils by solution ^{31}P NMR spectroscopy is to our knowledge the first evidence for the chemical nature of P in oxalate extracts of soils and shows that oxalate extractable P can contain considerable concentrations of IP_6.

Since oxalic acid works as a ligand, we suggest that IP_6 was replaced by the oxalate ion leading to desorption of IP_6. Desorption of IP_6 in different solutions depends generally on the number of phosphate moieties of the IP_6 involved in the stabilization and charge of the surface of the IP_6-adsorbent complex, since negatively charged surfaces can hamper ligand exchange (Celi and Barberis, 2007; Celi et al., 2003; Martin et al., 2004). For instance, there is limited desorption of IP_6 from goethite, in which four phosphate groups are involved, whereas desorption from ferrihydrite–kaolinite, which involves only one phosphate group, is relatively easy (Celi and Barberis, 2007). Furthermore, a recent study on *myo*-IP_6 adsorbed to different minerals demonstrated that oxalate effectively extracts IP_6 from IP_6-saturated kaolinite (poorly crystalline) and montmorillonite, whereas extraction of IP_6 from more crystalline metal compounds such as goethite and gibbsite is much lower (Shang et al., 2013). This suggests that oxalate can extract IP_6 associated with some types of clay minerals, in addition to IP_6 bound to amorphous Al and Fe oxides whereas most IP_6 associated with crystalline minerals will not be extracted. Overall, we suggest that oxalate extraction of acidic soils provides a selective extraction method for IP_6 bound to amorphous Al and Fe oxides and possible IP_6 associated with clay minerals.

Recently it has also been demonstrated that IP_6 adsorbed to amorphous Al oxides by ligand exchange rapidly transforms into precipitates on the mineral surface, whereas this transformation is limited when IP_6 is adsorbed to crystalline Al oxides (Yan et al., 2014a, b). Most Fe–IP_6 and Al–IP_6 precipitates are soluble in 0.05 M EDTA (Degroot and Golterman, 1993), so it is likely that oxalate also dissolves these precipitates. However, the high recoveries of IP_6 in the oxalate extracts of the forest and grassland soils suggest that IP_6 from Al precipitates is extracted by oxalate, or that the precipitation process is less pronounced in soils as it is in sorption experiments (Yan et al., 2014a).

In comparison, the HF pretreatment procedure dissolves all minerals and therefore removes all P chelated to metals or sorbed to anionic sorption sites (Hamdan et al., 2012). Hence, pretreatment of the soils with HF should remove the amorphous as well as more crystalline minerals. In theory the combination of these two methods should then allow calculation of three pools of IP_6 dependent on the binding sites: (1) IP_6 bound to amorphous metal hydroxides and possibly clay minerals (oxalate extract), (2) IP_6 associated with organic matter (IP_6 in the post-HF NaOH–EDTA extract), and (3) the IP_6 bound to other inorganic binding sites such as those on crystalline minerals (i.e., the difference between the content of IP_6 lost after HF pretreatment and the content of IP_6 recovered in the oxalate extracts). However, our data was not sufficiently precise for quantification of the three IP_6 pools, since we found recoveries of organic P less than 100% for the summed organic P content in the oxalate and post-oxalate extracts.

Further, the concentrations of organic P estimated by ^{31}P NMR spectroscopy in most of the extracts were significantly less than were measured as unreactive P by ICP/colorimetry. This might be explained by the presence of inorganic polyphosphates, orthophosphate associated with humic or fulvic acids (Turner et al. 2005a), or errors in the quantification of P pools by either procedure. Hence, it is possible that we underestimated oxalate extractable IP_6.

Despite the uncertainties, a large proportion of the inorganically bound IP_6 (difference between IP_6 extracted by NaOH–EDTA before and after HF treatment) was associated with amorphous Fe and Al oxides and possibly also with silicate clay (IP_6 extracted by oxalate) rather than with crystalline metal oxides. The strong association to amorphous Fe

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and Al over association to more crystalline Fe and Al compounds is probably due to the relatively greater affinity for IP_6 and the larger surface area, which provides a high concentration of binding sites (Celi and Barberis, 2007).

Despite the abundance of IP_6 in many soils, our understanding of the factors contributing to its dynamics under different conditions remains limited. We show here that coupling simple extractions with solution ^{31}P NMR spectroscopy can yield important information on the nature of IP_6 in soils. Our results demonstrate that the binding sites for IP_6 varies markedly among soils, which might help to explain why IP_6 is completely absent in some systems (Turner and Engelbrecht, 2011; Turner and Newman, 2005) and yet accumulates in others (Turner, 2007). As shown in this study the association of IP_6 with organic matter can be important even in acidic soils rich in amorphous Al and Fe, but whether organic matter stabilizes IP_6 to the same degree as associations with metal oxides remains unclear. Future studies that combine IP_6 fractionation and mineralization experiments may therefore greatly increase our knowledge on the transformation processes of IP_6 in different soils. Hence, we suggest the single oxalate–oxalic acid extraction and the HF pre-extraction to be extremely useful tools in future studies focusing on the cycle of IP_6 in different environments.

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