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Forms of organic phosphorus in wetland soils

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Abstract. Phosphorus (P) cycling in freshwater wetlands is dominated by biological mechanisms, yet there has been no comprehensive examination of the forms of biogenic P (i.e., forms derived from biological activity) in wetland soils. We used solution ^{31}P NMR spectroscopy to identify and quantify P forms in surface soils of 28 palustrine wetlands spanning a range of climatic, hydrogeomorphic, and vegetation types. Total P concentrations ranged between 51 and 3516 $\mu g\,P\,g^{-1}$, of which an average of 58 % was extracted in a single-step NaOH–EDTA procedure. The extracts contained a broad range of P forms, including phosphomonoesters (averaging 24 % of the total soil P), phosphodiesters (averaging 10 % of total P), phosphonates (up to 4 % of total P), and both pyrophosphate and long-chain polyphosphates (together averaging 6 % of total P). Soil P composition was found to be dependant upon two key biogeochemical properties: organic matter content and pH. For example, stereoisomers of inositol hexakisphosphate were detected exclusively in acidic soils with high mineral content, while phosphonates were detected in soils from a broad range of vegetation and hydrogeomorphic types but only under acidic conditions. Conversely inorganic polyphosphates occurred in a broad range of wetland soils, and their abundance appears to reflect more broadly that of a "substantial" and presumably active microbial community with a significant relationship between total inorganic polyphosphates and microbial biomass P. We conclude that soil P composition varies markedly among freshwater wetlands but can be predicted by fundamental soil properties.

1 Introduction

Phosphorus constitutes a significant proportion of nucleic acids, lipid membranes, proteins, and phosphorylated metabolic intermediates (Raghothama and Karthikeyan, 2005). It is therefore a vital nutrient for biomass production and often limits primary productivity in freshwater (Reddy et al., 2005; Verhoeven et al., 2006) and coastal wetlands (Sundareshwar et al., 2003; Turner et al., 2003e). The P cycle in wetlands is dominated by the input of biological sources due to their high productivity and position in the landscape (Newman and Robinson, 1999; Reddy et al., 1999, 2005), with organic P accounting for up to 90 % of total soil P in palustrine (marsh- or swamp-like) wetland soils (Reddy et al., 1998).

The functional nature of biologically derived P forms entering into, and found within, wetland soils (i.e., phosphomonoesters, phosphodiesters, phosphonates, and inorganic polyphosphates) influences their fate in the environment (Celi and Barberis, 2005; Condron et al., 2005). For example, inositol phosphates, a ubiquitous component of eukaryotic cells, are assumed to be a significant proportion of P inputs to wetland soils through plant and animal detritus (Weimer and Armstrong, 1979). One specific isomer, *myo*-inositol hexakisphosphate (*myo*-IP_6), has a high pH-dependent charge density, making it likely to interact with mineral and humic substances in the soil matrix (Celi and Barberis, 2007). This reactivity leads to a high degree of recalcitrance in the environment, which is often invoked to explain its dominance in the organic P fraction of upland soils (Harrison, 1987; Celi and Barberis, 2007; Turner et al., 2002). In contrast, phosphodiesters such as polymeric nucleotides (i.e., RNA and DNA) are comparatively poorly stabilized in the extracellular

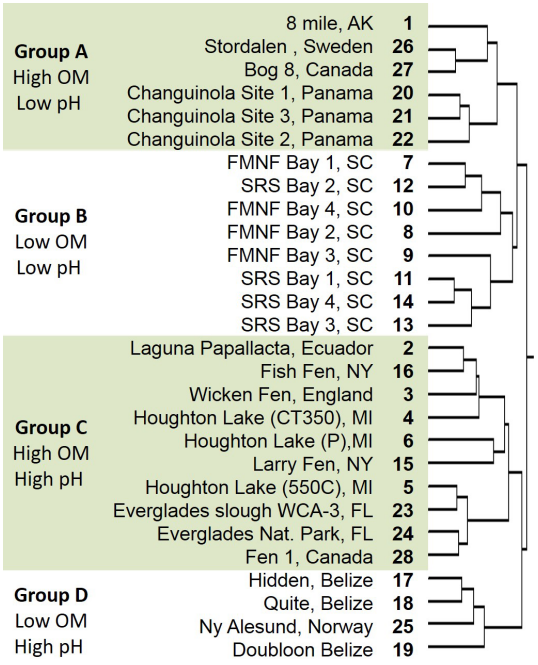

Figure 1. Wards hierarchical classification of 28 palustrine wetlands used to delineate four types of wetland (A–D) based upon organic matter (OM) content and pH. Francis Marion National Forest (FMNF), Savanah River Site (SRS).

environment, leading to a generally greater lability and potential for biological turnover (Niemeyer and Gessler, 2002; Ogram et al., 1988). Information on the chemical composition of soil phosphorus can therefore provide important information on its stability and potential biological availability in a given ecosystem (Condron et al., 2005).

Solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy allows the assessment of P compounds entering into, and stabilized within, the soil environment (Cade-Menun, 2005; Cheesman et al., 2010b; McKelvie, 2005). To date, work in palustrine systems has highlighted the diverse range of biogenic P forms, including inorganic polyphosphates, which occur in wetland soils (Sundareshwar et al., 2009), and how P composition may be fundamentally different to terrestrial systems. Specifically, P in wetland soils studied so far appears to be dominated by phosphodiesters, with a noticeable absence of inositol hexakisphosphate (e.g., Turner and Newman, 2005). However, while solution ^{31}P NMR has been increasingly deployed in wetland systems (Cheesman et al., 2013), with $\sim 20\,\%$ of articles published between 2005 and 2013 and employing ^{31}P NMR in soils focusing on wetlands (Cade-Menun and Liu, 2014), work on palustrine systems has been focused on a relatively narrow range of wetland types. This has included subtropical marshes (Cheesman et al., 2010b; Robinson et al., 1998; Turner and Newman, 2005; Turner et al. 2006, 2007), isolated wetlands (Cheesman et al., 2010a), and constructed wetlands (Turner et al., 2006) of south Florida, USA; blanket bogs of Scotland (Bedrock et al., 1994); Carolina bays in the USA (Sundareshwar et al., 2009); and a tropical peat dome in Panama (Cheesman et al., 2012).

This limited application of solution ^{31}P NMR in the wetland ecotone limits our understanding of the underlying factors controlling the P composition of freshwater wetlands and constrains our ability to predict rates of biological turnover and sequestration. We addressed this fundamental data gap by assessing the chemical nature of P in surface soils of 28 wetlands spanning a broad range of hydrogeomorphic and environmental gradients. Our objectives were (1) to establish an understanding of the nature and diversity of functional P forms found within wetlands soils and (2) to analyze forms identified in the context of ancillary biogeochemical and environmental properties to identify mechanisms regulating the P composition of wetland soils.

2 Methods

2.1 Study sites and sampling

Surface soil samples (0–10 cm) were collected over the course of 3 years from a diverse range of 28 wetland systems (Fig. 1; Table S1 in the Supplement). Study sites represented a broad range of climatic conditions, landscape positions, dominant vegetation types, and nutrient status. The sites included a tropical ombrotrophic peat dome (sites 20, 21, and 22), high-latitude acidic peatlands (sites 1 and 27) and fens (sites 28), calcareous wetlands (sites 17, 18, 19, 23, 24, and 29), temperate fens (sites 3, 15, and 16), and Carolina bays (sites 7–14). The sites also include those unimpacted by direct anthropogenic pressures and those severely impacted by up to 30 years of nutrient enrichment (Kadlec and Mitsch, 2009; sites 4, 5, and 6). In addition, the study included a number of uncommon wetland types, such as wet tundra (site 25) and high-elevation paramo wetlands (site 2). The wetlands analyzed included two wetland complexes: a tropical peat dome, Changuinola, Panama, and Houghton Lake treatment wetland, Michigan, in which three separate locations were treated as distinct wetlands (sites 20, 21, and 22 and sites 4, 5, and 6, respectively). This was considered appropriate given their physical size (80 and 7 km^2, respectively) and differences in nutrient status and vegetation types across the wetlands (Cheesman et al., 2012; Kadlec and Mitsch, 2009).

Soil sampling consisted of four independent surface cores (7.5 cm diameter, 10 cm deep) collected from an area considered representative of the study wetland and analyzed for biogeochemical characteristics separately. Samples were kept on ice for immediate shipment to the University of Florida or, in two cases, were air-dried on site (sites 25 and 26). Samples were processed by hand, removing coarse inorganic and organic fragments > 2 mm. Homogenized samples were split, with subsamples stored at 4 °C (fresh), and the remainder

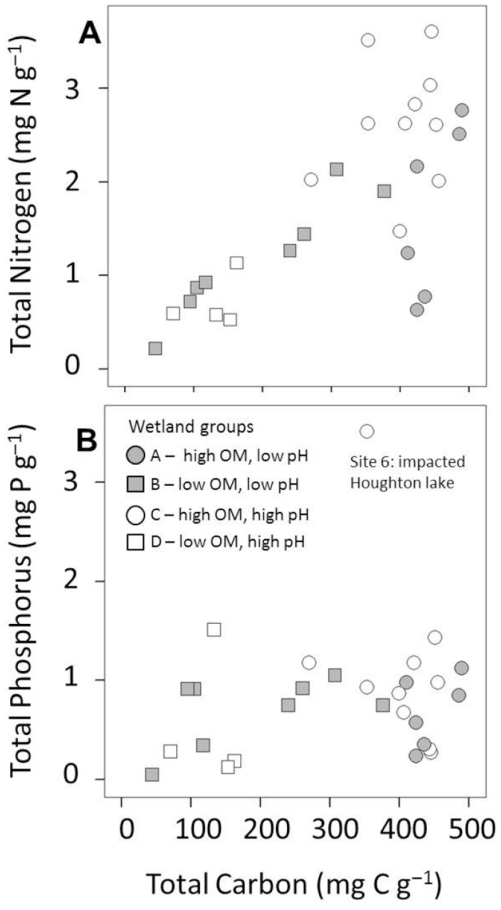

Figure 2. Mean total element concentrations in surface soils of 28 palustrine wetlands. Symbols represent wetland type, grouped by organic matter (OM) content and pH. Total carbon and nitrogen showed significant positive correlation (Spearman's rho = 0.67, $p < 0.0001$), which improved when only considering "low" C ($< 360\,\mathrm{mg\,C\,g^{-1}}$) sites (Spearman's rho = 0.89, $p < 0.0001$). Total carbon and phosphorus showed no significant correlation (Spearman's rho = 0.20, $p = 0.3$). Note the high total P in the highly polluted Houghton Lake (site 6).

air-dried at ambient laboratory temperature for 10 days under conditions of elevated air flow. Fresh samples were analyzed for water content, pH, exchangeable P, and microbial P. Air-dried samples were ground (8000D mixer mill, SPEX SamplePrep, NJ) and sieved (mesh 60, 0.250 mm) prior to analysis for total elemental composition (Al, C, Ca, Fe, N, P) and P composition by solution ^{31}P NMR spectroscopy. Given practical limitations on sample transfer, material from site 26 (Abisko, Sweden) represented a single homogenized and air-dried sample of surface (0–10 cm) soil considered representative of the study site.

2.2 Biogeochemical characterization

Fresh soil samples were analyzed for soil water content by gravimetric loss following drying at 70 °C for 72 h. Sample pH was determined on a 1 : 2 soil-to-water suspension using a glass electrode. Readily exchangeable and microbial P were operationally determined using anion exchange membranes (AEM; BDH Prolabo® Product number: 551642S, VWR International, UK) in a batch method (Kouno et al., 1995; Myers et al., 1999; Thien and Myers, 1992) using a standard 3.5 g dry weight equivalent of soil, total water content of 75 mL, and a single AEM strip (1.5 cm × 6.25 cm) preloaded with HCO_3 counter ions. Membranes were eluted for 3 h in $0.25\,\mathrm{mol\,L^{-1}}$ H_2SO_4, and the resulting solution was analyzed for molybdate-reactive P using a discrete autoanalyzer (AQ2+, SEAL Analytical, UK) and standard molybdate colorimetry (USEPA 1993). The difference between P recovered by AEM with and without hexanol fumigation was attributed to fumigation-released P and is used in this study as a proxy for microbial P without a correction factor (Bunemann et al., 2008).

Dried and ground soils were analyzed for loss on ignition (LOI: an estimate of total organic matter) and elemental concentrations. Total P and metals were determined by combustion of soil at 550 °C in a muffle furnace for 4 h and dissolution of the ash in $6\,\mathrm{mol\,L^{-1}}$ HCl (Andersen, 1976). Acid solutions were analyzed for molybdate-reactive P (as above) and for Al, Ca, and Fe, using ICP–OES (Inductively Coupled Plasma–Optical Emission Spectroscopy) (Thermo Jarrell Ash ICAP 61E, Franklin, MA). Total soil C and N were measured by combustion and gas chromatography using a Flash EA1112 (Thermo Scientific, Waltham, MA).

2.3 Composition of phosphorus forms

2.3.1 Extraction

Phosphorus forms were characterized via a standard alkaline extract and solution ^{31}P NMR spectroscopy of air-dried soils (Cheesman et al., 2013). Although pretreatment is expected to impact P composition in a sample-specific manner (Turner et al., 2007), the use of air-drying was considered preferable as a means of rapidly stabilizing samples prior to alkaline extraction and solution ^{31}P NMR spectroscopy. Phosphorus was extracted by shaking $1.00\,\mathrm{g} \pm 0.01\,\mathrm{g}$ of dried soil with 30 mL of a solution containing $0.25\,\mathrm{mol\,L^{-1}}$ NaOH and $50\,\mathrm{mmol\,L^{-1}}$ EDTA (Ethylenediaminetetraacetic acid) in a 50 mL centrifuge tube for 4 h, after which samples were centrifuged at a relative centrifugal force ∼ 7000 g (Sorvall RC6, SL600 Rotor; Thermo Fisher Scientific, Waltham, MA) for 10 min. Subsamples of supernatant were analyzed for total P using a double-acid ($HNO_3-H_2SO_4$) digest (Rowland and Haygarth, 1997) and molybdate colorimetry (see above). For each site, an equal volume of each of the four replicate extracts was combined, spiked with an internal standard methylenediphosphonic acid (MDP), frozen (−80 °C), and lyophilized to await solution ^{31}P NMR spectroscopy.

Table 1. Soil biogeochemical characteristics of four wetland groups derived from 28 study sites.

			Phosphorus			C	N	Al	Ca	Fe	Molar ratio	
	pH	Organic matter (%)	Total P ($\mu g\,g^{-1}$)	Exchangeable P (% total P)	Microbial P (% total P)	($mg\,g^{-1}$)					C : P	N : P
Group A												
Mean	4.0	92	689	1.0	22.7	445	16.8	1.6	2.6	4.5	2238	57
Min	3.6	84	238	0.0	16.4	410	6.3	0.4	1.0	0.2	1060	27
Max	4.6	100	1124	2.6	31.3	489	27.7	3.0	4.7	15.0	4596	83
Group B												
Mean	4.0	38	715	0.3	7.0	193	11.9	32.9	0.6	3.6	970	49
Min	3.5	9	51	0.1	0.2	44	2.2	2.9	<DL	0.7	283	18
Max	4.4	69	1056	0.6	18.8	376	21.4	77.1	2.0	6.2	2551	111
Group C												
Mean	6.6	83	1138	3.4	16.4	400	26.4	5.5	29.1	7.2	1550	90
Min	5.9	56	277	0.0	2.0	270	14.8	0.9	8.6	0.2	261	22
Max	7.3	94	3516	13.5	25.7	455	36.1	20.4	96.2	18.9	4170	290
Group D												
Mean	7.4	25	530	0.4	2.8	129	7.1	20.8	148.8	<DL	1776	84
Min	7.0	16	126	0.9	7.7	70	5.3	2.3	14.4		247	9
Max	7.6	30	1513	0.1	0.0	162	11.4	45.8	333.5		3213	137

< DL indicates below detection limit.

2.3.2 Solution ^{31}P spectroscopy

Lyophilized extracts (~ 300 mg) were redissolved in 3 mL of 1 mol L^{-1} NaOH and 0.1 mol L^{-1} EDTA in 15 mL centrifuge tubes and vortexed for 1 min. Samples were subsequently filtered using a prewashed 0.2 µm syringe filter (GF-B) to remove fine particles that may result in poor field homogeneity and thereby cause line broadening. However, comparison of samples with and without filtration suggests that no significant change is associated with the filtration step (data not shown). Subsequently, 2.7 mL of redissolved filtered sample and 0.3 mL D_2O (for signal lock) were loaded into a 10 mm NMR tube for spectra acquisition. It is well known that the use of an alkaline matrix for both P extraction and NMR signal acquisition may result in the degradation of certain phosphodiester functional groups (i.e., RNA and phosphatidyl choline) (Turner et al., 2003d). However, NMR analysis at a final pH > 13 allows for a consistent chemical shift (McDowell and Stewart, 2005) and confidence in peak assignment when comparing it to existing spectral libraries (Turner et al., 2003d).

Spectra were acquired immediately using an Avance III 500 MHz, Magnex 11.8 telsa 54 mm Bore magnet (AMRIS facility, McKinght Brain Institute, University of Florida) at a controlled 25 °C. A simple zgig pulse profile (Berger and Siegmar, 2004) and broad heteronuclear decoupling (waltz 16) were employed, with acquisition parameters including the use of a 30° pulse (calibrated using orthophosphate), 0.4 s acquisition time, and a 2 s pulse delay. Although T_1 constants were not determined on all samples, the conservative use of a 30° pulse and 2.4 s recycle delay ensures quantitative spectra from samples with T_1 constants up to 3.4 s, which is substantially greater than T_1 constants reported previously in similar soil extracts (Cade-Menun et al., 2002; McDowell et al., 2006).

Between 30 000 and 50 000 scans were required to achieve a reasonable signal to noise (S/N) ratio dependent upon sample P concentrations, with a subsequent combination of FIDs using Bruker proprietary software. Spectra were analyzed using wxNUTS vr 1.0.1 for Microsoft Windows (Acorn NMR Inc. 2007). Initially spectra were processed using 15 Hz line broadening, phased and corrected for baseline shift, and referenced using internal standard MDP ($\delta = 17.46$ ppm), established by comparison of MDP within a standard redissolved soil extract with an external standard, 85 % H_3PO_4 (0 ppm). Spectra were integrated over set intervals, corresponding to established bonding environments (Turner et al., 2003d). The region between 3 and 8 ppm was additionally plotted using 2 Hz line broadening and analyzed using spectral deconvolution. Automatic peak-picking parameters were adjusted dependent upon S/N ratio of specific samples but ranged between 1 and 8 % of maximum peak height, with 0.5 for the root-mean-squared noise parameter. The region was split into orthophosphate and phosphomonoesters (all other peaks determined by the algorithm in the region $\delta = 3$ to 8 ppm). Peak proportions from the deconvolution protocol were applied to the integral determined in the 15 Hz spectra. A similar procedure was applied to the region $\delta = -3$ to -5 ppm, to differentiate pyrophosphate ($\delta = -4.37$ ppm) and higher-order polyphosphate groups ($\delta = -3.91$ and $\delta = -4.03$ ppm) based upon comparison with standard biogenic P compounds in the same matrix (data not shown).

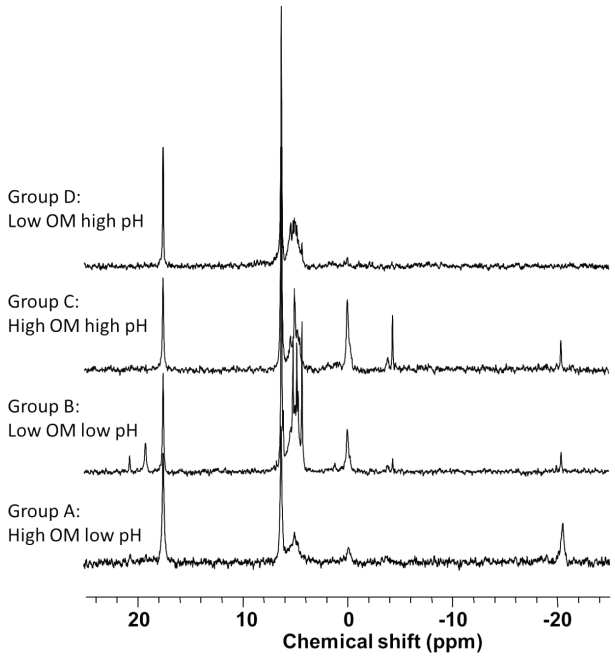

Figure 3. Solution ^{31}P NMR spectra of wetland soils representative of four wetland types identified in this study; see Figs. S1–S4 in the Supplement. Spectra acquired using an Avance III 500, Magnex 11.8 tesla 54 mm Bore, at pH > 13, using a simple zgig pulse program and calibrated 30° pulse angle. Spectra presented here, using 15 Hz line broadening, scaled and referenced to internal standard methylenediphosphonic acid ($\delta = 17.46$ ppm).

2.4 Data analysis

The exploration of emergent patterns in P composition was carried out by delineating wetland sites into four (A–D) fundamental groups, using Wards hierarchical clustering for organic matter content and pH (Fig. 1); these parameters were selected given their lack of colinearity and their known influence upon biogeochemical P cycling. The ordination of P composition diversity was performed using principal components analysis (PCA) and compared with fundamental characteristics, including previously defined wetland groups. The importance of organic matter content to the ratio of phosphomonoesters and phosphodiesters in wetland soils was tested using simple linear regression, while relationships between microbial biomass P and P composition were explored using Spearman's rank correlation with major biogenic P groups. Where reported, site-specific values represent the arithmetic mean of four field replicates ± one standard deviation. Statistical analysis was carried out using R (R Development Core Team, 2014).

3 Results

3.1 Biogeochemical characteristics

The wetlands studied (Fig. 1; Table S1) showed a high degree of variation in hydrogeomorphic setting and biogeochemical characteristics (Table 1, and Table S2 in the Supplement). The initial examination of parameter correlations identified organic matter content and pH as useful in typifying wetland "types", given a lack of colinearity. Wards hierarchical clustering was applied to delineate sites into four broad wetland types (Fig. 1). The first group of six wetlands (group A) consists of highly organic (84 to 100 % loss on ignition), acidic (pH 3.6 to 4.6) systems. Typified by *Sphagnum* sp.-dominated, high-latitude bogs and mires (i.e., sites 1, 26, and 27), this group also included tropical ombrotrophic systems with a range of vegetation types (i.e., mono-dominant palm swamp, mixed tropical forest, and herbaceous vegetation at sites 20, 21, and 22).

The second grouping of eight wetlands (group B) represents those with an acidic (3.5 to 4.4) pH and lower organic matter content (9 to 69 % loss on ignition) than group A. This group consisted of Carolina bay wetlands from the Southeast Coastal Plain, US, and included a broad range of vegetation types, including both cypress-dominated forested systems (e.g., site 8) and herbaceous open-water systems (e.g., site 13). (De Steven and Toner, 2004; Gaiser et al., 2001).

The third group (group C) represents 10 wetlands with moderately/slightly acid to neutral pH (5.9 to 7.3) and high organic matter content (56 to 94 % loss on ignition). It included calcareous fens from England (site 3), New York (sites 15, 16), Canada (site 28), and south Florida (sites 23, 24), plus wet paramo of Ecuador (site 2) and the Houghton Lake treatment wetland (sites 5, 6, and 7).

The final group of wetlands (group D) represented those with a neutral to slightly alkaline pH (pH 7.0 to 7.6) and relatively low organic matter content (16 to 30 % loss on ignition). This group was dominated by calcareous fens (Macek and Rejmánková, 2007) situated near the coast of northern Belize (sites 17, 18 and 19), but also included an arctic tundra system (site 25) that has experienced heavy grazing by migrating pink-footed geese (Wal et al., 2007).

The macronutrients P and N varied markedly among wetland sites (Table 1 and S2). Total P ranged between 51 ± 35 μg P g^{-1} in a Carolina bay (site 9) and 3516 ± 442 μg P g^{-1} in a Houghton Lake treatment wetland (site 6) and showed no significant difference among the four wetland groups (Kruskal–Wallis test chi$^2 = 3.5$, df $= 3$, $p = 0.32$). Total N ranged between 2.2 ± 0.8 mg N g^{-1} in a Carolina bay (site 9) and 36.1 ± 2.0 mg N g^{-1} in the Everglades National Park (site 23) and varied significantly among wetland groups (Kruskal–Wallis test chi$^2 = 15.9$, df $= 3$, $p < 0.005$). The variation in N, but not in P, was likely due to the close coupling of N with organic matter, used to delineate the original groups. Biplots of wetland soil nutrient concentrations (Fig. 2) highlight the

difference in relationship between N, P, and organic matter, with a close coupling of total C and N across all 28 sites (Spearman's rho = 0.67, $p < 0.001$) and no correlation seen between total P and total C (Spearman's rho = 0.20, $p = 0.3$).

There was no significant difference in exchangeable P as a percentage of total P between the four wetland groups (Table 1, Kruskal–Wallis test chi$^2 = 1.2$, df = 3, $p = 0.74$), with values generally less than 4 % of total soil P. Fumigation-released P (i.e., microbial P) as a percentage of total soil P showed a significant difference among the four wetland groups (Table 1, Kruskal–Wallis test chi$^2 = 12.8$, df = 3, $p < 0.01$), driven by the strong positive correlation between organic matter content and the percentage of a total P found in microbial biomass (Spearman's rho = 0.76, $p < 0.001$).

Of the total metals analyzed, Al ranged from 0.5 ± 0.2 to 77.1 ± 3.3 mg Al g^{-1} (Table 1 and S2), with a significant difference among the four wetland groups (Kruskal–Wallis test chi$^2 = 13.8$, df = 3, $p < 0.005$) driven by its significant negative correlation with organic matter (Spearman's rho = −0.73, $p < 0.001$). Calcium content also varied significantly among the four wetland groups (Kruskal–Wallis test chi$^2 = 22.1$, df = 3, $p < 0.0001$), ranging from barely detectable in group B wetlands to a group D average of 149 mg Ca g^{-1}. The very high Ca concentrations at sites 17 (232 ± 52 mg Ca g^{-1}) and 19 (334 ± 15 mg Ca g^{-1}) probably reflect the presence of shell fragments and calcareous cyanobacterial mats within surface samples collected from these sites (Macek and Rejmánková, 2007). Even if these sites were considered outliers and excluded from analysis, there is still a clear correlation between Ca concentration and site pH (Spearman's rho = 0.70, $p < 0.001$). The redox-sensitive metal, Fe, showed no apparent correlation with other basic biogeochemical characteristics and ranged from the detection limit of 0.2 mg Fe g^{-1} in a large number of wetland sites to a maximum of 18.9 ± 5.4 mg Fe g^{-1} within the heavily impacted portion of the Houghton Lake treatment wetland (site 6).

3.2 Phosphorus composition

3.2.1 Extraction of total phosphorus

Phosphorus extracted in NaOH–EDTA ranged from 25 to 84 % of the total soil P, with one site (9) calculated to have an extraction efficiency of 125 % due to very low soil total-P concentrations (51 ± 35 µg P g^{-1}). This site was therefore removed from further consideration of P composition. Extraction efficiencies varied significantly among wetland groups (Kruskal–Wallis test chi$^2 = 8.2$, df = 3, $p < 0.05$), reflecting the known influence of calcareous soils on the standard NaOH–EDTA extraction (McDowell and Stewart, 2006; Turner et al., 2003a), in particular the fact that the NaOH–EDTA procedure, designed to extract organic P, does not extract acid-soluble inorganic P or other alkali-stable forms

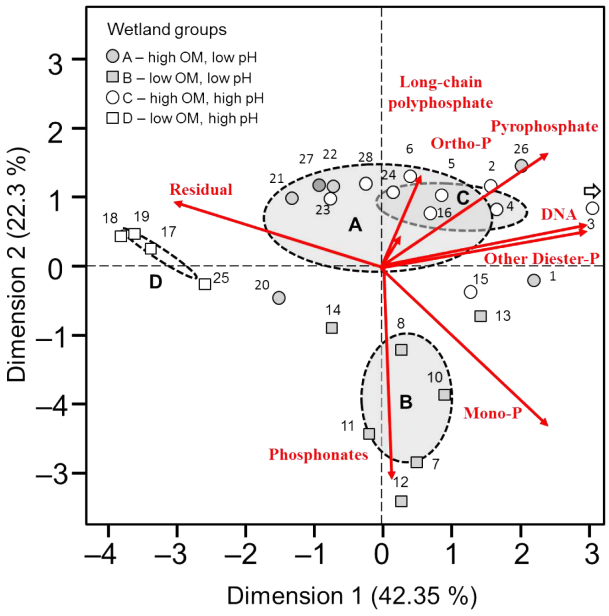

Figure 4. Biplot of the scaled first two principal components of phosphorus composition in wetland soils. Ellipses represent 95 % confidence interval surrounding barycenter of four wetland groupings based upon pH and organic matter content. Proportional loading of P composition superimposed in red.

(Turner et al., 2005). Therefore, the operationally defined "residual-P" is considered a distinct P type, mainly consisting of alkali-stable inorganic P, and is included here when considering patterns in P composition between sites.

3.2.2 Phosphorus composition

Solution ^{31}P NMR spectroscopy of alkaline extracts identified a diverse range of P forms within wetland soils (Table 2 and S3, Fig. 3 and Figs. S1–S4 in the Supplement). Two calcareous, low-P sites from Belize (sites 18 and 19) showed no evidence of biogenic P, with only orthophosphate identified. The remaining sites contained phosphonates (up to 44 µg P g^{-1}), phosphomonoesters (8 to 461 µg P g^{-1}), DNA (3 to 144 µg P g^{-1}), other phosphodiesters (6 to 67 µg P g^{-1}), and inorganic polyphosphates (up to 197 µg P g^{-1}). Total inorganic polyphosphates contained both pyrophosphate (up to 136 µg P g^{-1}) and long-chain polyphosphates (up to 110 µg P g^{-1}) (Table S4 in the Supplement).

Given the range in total P between sites, the analysis of P composition was based upon forms as a percentage of total P. Ordination using PCA, produced two axes which together accounted for 65 % of the observed variance in P composition of wetland soils. Superimposing the biplot of the first two dimensions with fundamental wetland types, i.e., A–D (Fig. 4), clearly demonstrates the significant separation of wetland groups B and D, while groups A and C (both high organic matter) fail to show any clear distinction in the composition

Table 2. Phosphorus composition of surface soils as determined by solution ^{31}P NMR spectroscopy.

	NaOH–EDTA		Organic phosphorus											Inorganic phosphorus			
	TP[a]		Phos-P		Mono-P[b]		DNA		P-lipids[c]		Organic P		Mono : Dies[d]	Ortho-P[e]		Poly-P[f]	
	μg g^{-1} (% total P)													μg g^{-1} (% total P)			
Group A																	
Mean	364	(54)	14	(2)	116	(16)	51	(8)	14	(2)	186	(26)	1.7	105	(16)	74	(12)
Min	138	(38)	8	(1)	31	(6)	23	(3)	3	(1)	64	(13)	0.9	36	(9)	38	(4)
Max	758	(80)	20	(2)	337	(34)	135	(14)	42	(4)	514	(52)	3.9	201	(29)	123	(17)
Group B																	
Mean	526	(65)	20	(2)	273	(33)	50	(6)	16	(2)	358	(44)	4.6	146	(18)	22	(3)
Min	219	(58)	4	(1)	110	(21)	25	(3)	10	(1)	154	(28)	2.4	56	(14)	4	(1)
Max	722	(69)	44	(4)	408	(44)	67	(9)	24	(4)	518	(51)	9.3	267	(29)	50	(7)
Group C																	
Mean	733	(59)	nd		229	(20)	90	(9)	36	(3)	354	(32)	1.7	308	(20)	72	(6)
Min	102	(37)	nd		28	(10)	18	(4)	6	(2)	53	(17)	1.0	38	(8)	10	(3)
Max	2569	(84)	nd		461	(39)	144	(15)	67	(7)	612	(59)	3.7	1759	(50)	197	(11)
Group D																	
Mean	167	(33)	nd		57	(5)	4	(1)	3	(0)	127	(11)	6	104	(28)	nd	
Min	33	(25)	nd		nd		nd		nd		11	(6)	2	33	(19)	nd	
Max	534	(46)	nd		221	(15)	11	(2)	10	(1)	242	(16)	11	292	(46)	nd	

[a] Total P recovered by alkaline extraction. [b] Phosphomonoesters. [c] Phospholipids.
[d] Ratio of total phosphomonoesters : total phosphodiesters. [e] Orthophosphate. [f] Total inorganic polyphosphates.

of P forms found. The proportional loading shows the separation of group D wetlands upon axis 1 to be the result of a greater predominance of residual P as compared to the major biogenic P groups (phosphomonoesters, DNA, phosphodiesters, and pyrophosphate) identified, while the separation of group B wetlands upon PCA axis 2 appeared to be a result of the increased prevalence of phosphonates and phosphomonoesters. A similar examination of P composition with reference to Cowardin "class" and climatic zone (data not shown) failed to show clear clustering. This suggests that soil P composition is dependent upon basic biogeochemical characteristics, including, to some degree, both the pH and organic matter content used in this study.

3.2.3 Phosphonates

Two peaks, approximately 20.6 and 19.1 ppm, were attributed to C–P-bonded, phosphonate groups, most likely 2-aminoethyl phosphonic acid and its associated congeners and metabolic precursors (Ternan et al., 1998). These peaks were found to be restricted to acidic systems, being found in two of six sites within group A and all but one site in group B. Phosphonates were not found in either group C or D wetlands (Figs. S3 and S4). When present, total phosphonate concentrations ranged up to 44 μg P g^{-1} or 4 % of total soil P in the *Panicum hemitomon*-dominated site 12 (Carolina bay).

3.2.4 Presence of inositol hexakisphosphate

Spectral deconvolution of the 8 to 3 ppm region revealed that, in some samples, a substantial portion of phosphomonoesters corresponded with the known peak assignments of higher-order inositol phosphates (Turner et al., 2003c, 2012; Turner and Richardson, 2004). The use of a standard preparation and spectra acquisition protocol in conjunction with a stable internal standard (MDP) provided confidence in the assignments of both *myo*- and *scyllo*-IP_6. Inositol groups appeared particularly prevalent in group B wetlands, with *myo*-IP_6 and *scyllo*-IP_6 found in all eight Carolina bays accounting up to 187 μg P g^{-1} or 46 % of total phosphomonoesters in site 12 (Table 3, Fig. 5). Two group B wetlands (sites 7 and 11) also showed evidence of phosphomonoester peaks (6.7 and 6.9 ppm) known to correspond with *neo* and d-*chiro*-IP_6 (Turner et al., 2012) although low concentrations precluded accurate quantification.

The determination of IP_6 within wetlands other than group B systems proved problematic, given the degree of peak overlap within the phosphomonoester region. However, peaks coincident with that attributed to *scyllo*-IP_6 in group B wetlands (4.2 ± 0.02) were found in a broad range of wetland sites (i.e., sites 1, 2, 6, 15, 16, and 25).

3.2.5 Ratio of phosphomonoesters and phosphodiesters in wetland soils

The ratio of phosphomonoesters to total alkaline-stable phosphodiesters in the palustrine wetlands shown to contain both forms averaged 2.8, ranging from 0.9 in an ombrotrophic Canadian bog (site 27) to 10.6 in Norwegian wet tundra

Table 3. Concentrations of two inositol hexakisphosphate isomers as determined by solution ^{31}P NMR spectroscopy within group B (low-pH, low-organic-matter) wetlands. Values represent concentrations $\mu g\,P\,g^{-1}$ (% of phosphomonoesters).

Site	*myo*-IP_6		*scyllo*-IP_6	
7	96.1	(26.1)	56.1	(15.2)
8	63.8	(16.3)	48.1	(12.3)
9	trace		36.3	(14.4)
10	40.7	(21.2)	37.9	(19.7)
11	14.8	(38.9)	2.5	(6.7)
12	131.6	(32.2)	55.4	(13.6)
13	50.1	(26.5)	19.1	(10.1)
14	20.6	(18.7)	7.2	(6.6)

(site 25). In addition to PCA results suggesting an influence of organic matter (or its reciprocal mineral content) on delineating high phosphomonoester-containing group B wetlands (Fig. 4), we found LOI significantly predicted the ratio of phosphomonoesters to phosphodiesters, with LOI explaining a significant proportion of variance seen in the ratio of P forms in wetland soils ($R^2 = 0.46$, $F_{(1,23)} = 19.8$, $p < 0.001$).

3.2.6 Inorganic polyphosphates

This study identified substantial polyphosphate pools within a broad range of wetland sites, with all sites except group D wetlands containing at least the 2-phosphate residue pyrophosphate (Table 2 and S4). The highly impacted Houghton Lake (i.e., total $P = 3.5\,mg\,P\,g^{-1}$) had the highest concentration of pyrophosphate, at $136\,\mu g\,P\,g^{-1}$ or 4 % of total P, with no wetland having greater than 4.4 % of total P. Longer-chain inorganic polyphosphates (residue number > 3) were found in groups A, B, and C wetlands but were more prevalent in group A (acidic, high-organic-matter) systems, where they averaged 10 % and constituted up to 15 % of total soil P.

3.2.7 Microbial biomass

Microbial P, determined by fumigation extraction with AEM strips and without correction for unrecovered biomass, occurred at concentrations below the detection limit in two of the group D calcareous lagoons (site 18, and 19) and up to $267\,\mu g\,P\,g^{-1}$ at site 21, an acidic, highly organic tropical peat dome. Microbial P accounted for up to 31 % of total soil P at site 21 and was generally found to be significantly higher in the higher-organic-matter group A and in C wetlands (Kruskal–Wallis test $chi^2 = 12.8$, $df = 3$, $p < 0.01$), averaging 23 ± 7 % and 16 ± 8 in group A and C wetlands, respectively, as compared to 7 ± 6 % in group B and 3 ± 4 % in group D wetlands. The relationship between microbial P and P composition was explored by the application of Spearman's rank correlation (Table S4). Both DNA (Spearman's rho $= 0.57$, $p < 0.01$) and total inorganic polyphosphates (Spearman's rho $= 0.78$, $p < 0.001$) were correlated positively with the uncorrected measure of microbial P (Fig. 6).

4 Discussion

Sources and pools of P found in wetland soils are often dominated by material of biological origin. Determining the functional nature of this P is critical to predicting both its stability in the environment and its potential for biological turnover. Our unique data set demonstrates both the diverse range of biogenic P forms found within wetlands and how soil biogeochemical characteristics appear to be fundamental in determining their P composition, independent of vegetation and climatic setting. This has profound implications for researchers interested in P sequestration and wetland productivity.

4.1 Biogeochemical characteristics

Wetlands sampled represented a broad range of biogeochemical characteristics, highlighting the difference in hydrogeomorphic setting and the role of organic matter in accumulating soils. As expected, given the role of N as a component of many structural C forms (McGill and Cole, 1981,) we observed close coupling of C and N across wetland soils. In contrast the decoupling between C and P content, while potentially reflecting the importance of organic P cycling in wetlands (Cleveland and Liptzin, 2007), is more likely to reflect fundamental differences in underlying site mineralogy and anthropogenic inputs of P independent of biological sources.

4.2 Phosphorus composition

Our analysis demonstrates a number of significant distinctions in the phosphorus composition of wetland soils based upon pH and organic matter content. While low-organic-matter (high-mineral) content groups B and D wetlands were the most easily delineated (Fig. 4), high-organic-matter wetlands (groups A and C), while not distinguishable on the broad scale, show subtle distinctions such as the presence of phosphonates and prevalence of long-chain polyphosphates that warrant further study.

4.2.1 Phosphonates

Phosphonates, previously found in Northern Hemisphere blanket bogs (Bedrock et al., 1994; Turner et al., 2003b), were found in this study in both acidic tropical peatlands and in more mineral-dominated Carolina bays. Our results suggest either a greater prevalence in biological sources found at low pH or greater extracellular stability under acidic conditions. Although common to a wide array of organisms, phosphonates within soils are often attributed to in situ

"microbial activity" (Bünemann et al., 2011; Koukol et al., 2008) and, in particular, fungal biosynthesis (Koukol et al., 2006). Given the dominance of fungal biomass at low soil pH, it seems likely that their presence in acidic wetlands reflects a difference in the microbial composition of decomposers. However, the biological role and potential cycling of phosphonates in the soil remains poorly understood (Condron et al., 2005). Research into degradation pathways of the highly resilient phosphonate-containing xenobiotics (i.e., glyphosate, N-(phosphonomethyl)glycine) has identified the potential of certain soil bacteria to utilize phosphonates as a sole P source (Ermakova et al., 2008). Furthermore, recent work has suggested phosphonates may be an important and highly active component of dissolved organic P in the marine water column (Martinez et al., 2010). It is clear that further work is required to investigate the active role phosphonates may play in many natural systems.

4.2.2 Phosphoesters

In terrestrial systems researchers have attributed a proportional increase in phosphodiesters with increasing precipitation to their increased recalcitrance under "wetter" conditions (Condron et al., 1990; Sumann et al., 1998; Tate and Newman, 1982). In addition, the phosphomonoester IP_6, often a major component of organic P in terrestrial soils (Cosgrove, 1966; Murphy et al., 2009; Turner et al., 2002, 2003c), had been thought to be absent from wetlands (Turner and Newman, 2005; Turner et al., 2006), with evidence suggesting rapid degradation under anaerobic conditions, typical of wetland soils (Suzumura and Kamatani, 1995a, b). It has been suggested that these two factors combined could account for the increased prevalence of phosphodiesters in palustrine organic wetland soils studied to date (Turner and Newman, 2005).

In our study we observed a significant negative relationship between organic matter content and the ratio of phosphomonoesters to alkali-stable phosphodiesters. However, as evident from this study, (Fig. 5) and from other recent research on estuarine (Turner and Weckström, 2009), lacustrine (Zhang et al., 2009), and riverine (McDowell, 2009) systems, IP_6 may constitute a substantial proportion of P in anaerobic wetland soils. Taken in conjunction with evidence for low concentrations of IP_6 (i.e., at levels below that detectable by ^{31}P NMR) in calcareous systems (El-Rifai et al., 2008) and the fact that peaks coincident with *scyllo*-IP_6 were found in a substantial range of our wetland sites, it appears that IP_6 is a ubiquitous input into wetland soils, and it is differences in stabilization and turnover as a result of biogeochemical conditions that determine the levels of this important phosphomonoester in the soil.

Calcite, Fe/Al oxides, clay, and organic matter have all been shown to increase terrestrial soil IP_6 sorption capacity (Celi and Barberis, 2007), yet in wetlands it is likely that these factors are further impacted by ambient physicochemical conditions, i.e., anaerobiosis interacting with Fe-oxide sorption. It is also known, from dosing experiments in terrestrial soils, that IP_6 may be rapidly degraded in calcareous systems (Doolette et al., 2010), and our findings are coincident with this, with group B wetlands (more mineral acidic pH soils) having notable levels of isomers of IP_6. However, it is clear that further work, including the use of hypobromite oxidation to hydrolyze non-inositol phosphomonoesters (Irving and Cosgrove, 1981), is needed to elucidate the role that differential sources, i.e., pollen, seeds, and fruiting bodies (Jackson and Linskens, 1982; Lott et al., 2000), and in situ stabilization of IP_6 play in determining the proportion of phosphomonoesters found as IP_6 in particular wetland soils.

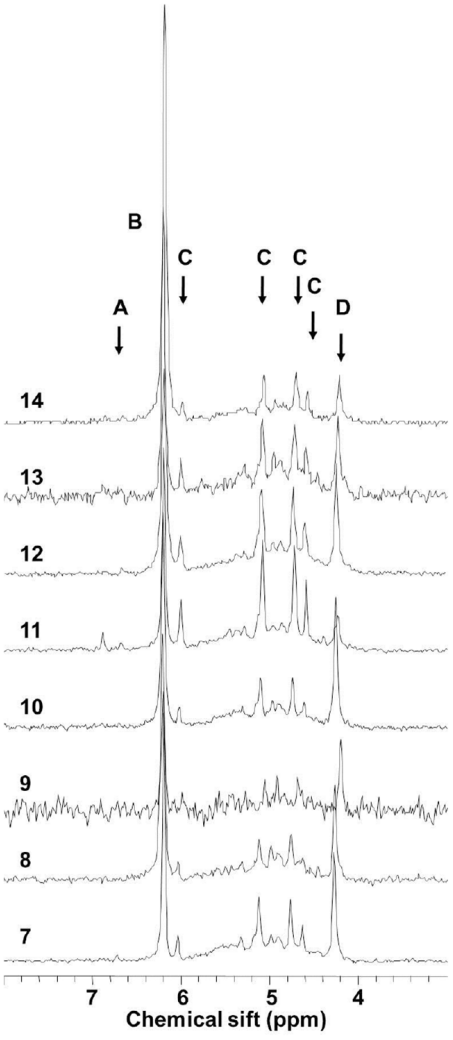

Figure 5. Region 8 to 3 ppm of group B (site 7 to 18) wetlands NMR spectra and peak assignments for **(a)** *neo*- and d-*chiro*-inositol hexakisphosphate, **(b)** orthophosphate, **(c)** *myo*-inositol hexakisphosphate, and **(d)** *scyllo*-inositol hexakisphosphate.

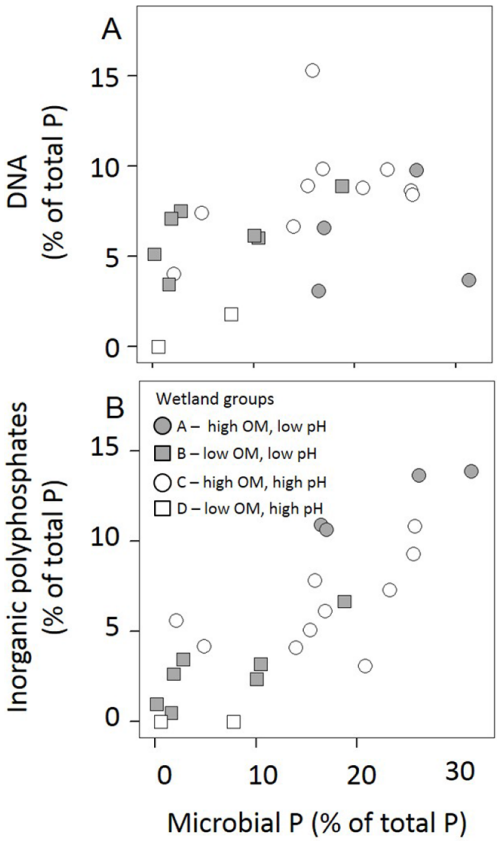

Figure 6. Plot of microbial P against **(a)** DNA and **(b)** total inorganic polyphosphates, both showing significant positive correlation as determined by Spearman's rank correlation ($\text{rho}_{(\text{DNA})} = 0.66$, $p < 0.01$, $\text{rho}_{(\text{total inorganic polyphosphates})} = 0.78$, $p < 0.001$).

4.2.3 Inorganic polyphosphates

Polyphosphates are molecules containing multiple phosphate residues bound by high-energy acid anhydride bonds (Harold, 1966) and are found ubiquitously in both eukaryotic and prokaryotic cells (Kornberg et al., 1999). Potentially prebiotic macromolecules (Brown and Kornberg, 2004), they are now implicated in a range of biochemical functions from phosphate and energy storage to providing biochemical adaptation to extreme environments (Kornberg, 1995; Kornberg et al., 1999; Kulaev and Kulakovskaya, 2000; Seufferheld et al., 2008). The biological accumulation of significant concentrations of polyphosphates was first identified by the isolation of metachromatic granules in yeast cells (Liebrmann, 1890, in Kornberg et al., 1999). Subsequently the identification and isolation of so-called polyphosphate-accumulating organisms (PAO) has been studied as part of enhanced biological P removal (EBPR) within wastewater treatment facilities (Zilles et al., 2002) as well as terrestrial and aquatic environments in which there was a surplus of phosphate (Gachter and Meyer, 1993). The importance of PAO in both biotic and abiotic mediated P flux in lacustrine sediments has been clearly demonstrated (Gachter and Meyer, 1993; Hupfer et al., 2004, 2007; Sannigrahi and Ingall, 2005). However, this study identified substantial polyphosphate pools within a broad range of wetlands (although predominantly acidic high-organic-matter systems), including samples from a low-P tropical ombrotrophic peat dome (sites 20, 21, and 22; see also Cheesman et al. (2012)). Taken in conjunction with additional evidence of polyphosphates in unimpacted Carolina bays (Sundareshwar et al., 2009) and oligotrophic Swedish lake sediments (Ahlgren et al., 2006) it is clear that polyphosphates play an important role even in P-limited wetland systems. It is also interesting to note the role of polyphosphates in fungal biomass (Koukol et al., 2008) while acknowledging the growing recognition of the role that fungal decomposition plays in wetland systems (Joergensen and Wichern, 2008). Polyphosphates appear to represent a dynamic and quantitatively important, yet poorly studied P pool within many wetlands.

4.3 Microbial biomass

Phosphorus forms found within wetland soils include both intracellular P held within viable algal, macrophyte, microbial, and faunal biomass and extracellular P held within the soil matrix. Although a significant positive correlation between microbial P (determined by AEM), DNA, and inorganic polyphosphates would be expected given a standard microbial composition, we are currently unable to distinguish between P forms derived from viable cells and the soil matrix. We are therefore unable to discount confounding factors that may influence the proportion of soil P found as particular functional groups within certain wetlands, including altered microbial P composition between systems (Makarov et al., 2005) and the influence of extracellular stabilization of compounds such as DNA (Celi and Barberis, 2005; Niemeyer and Gessler, 2002). The highly significant correlation between microbial P and long-chain polyphosphates may reflect the biological synthesis of polyphosphate in response to increased microbial demand for a critical and scarce resource (Harold, 1966; Seufferheld et al., 2008). However, the known interaction of anion exchange membranes with certain inorganic P forms (Cheesman et al., 2010c) indicates that causation must be assigned with caution. The strong positive correlation between total inorganic polyphosphates (i.e., pyrophosphate plus longer-chain-length polyphosphates) and microbial P might reflect the fact that operationally defined microbial P is, in large part, due to the extraction of polyphosphates from the soil.

5 Conclusions and implications

We demonstrate that there are significant differences in P composition of wetland soils based upon soil biogeochemical characteristics, irrespective of geographical location or

dominant vegetation type. If we assume that the nature of biological P inputs to wetlands are, broadly, similar between wetlands with similar vegetation/faunal communities, then it becomes apparent that there are fundamental differences in P stabilization and therefore P cycling between wetlands. While confirming the nature of P within highly studied calcareous palustrine systems (Turner and Newman, 2005; Turner et al., 2006), our work also demonstrates how both the nature and prevalence of P forms contributing to total soil P may vary in response to both organic matter content and pH, necessitating caution when extrapolating our understanding of P biogeochemical cycling in novel systems. However, while demonstrating differences in P composition, questions still remain as to the relative flux of P forms into and out of the soil environment. For example, while IP_6 and phosphonates appears to be significant standing pools within acidic mineral-dominated wetlands, we are currently unsure if this represents a static stabilized component of soil P or one which is turned over at a rate similar to the degradation rates seen in calcareous systems. Further work identifying the flux rates of particular P forms is therefore needed to put our understanding of static pools in the context of the overall P cycle.

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