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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, hydro-geomorphic and vegetation types. Total P concentrations ranged between 51 and 3516 µg P g$^{-1}$, of which an average of 58 % was extracted in a single-step NaOH–EDTA extraction 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 predicted by 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 wet-





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lands and aquatic ecosystems (Sundareshwar et al., 2003; Turner et al., 2003e). Due to their high productivity and position in the landscape, the P cycle in wetlands is dominated by the input of biological sources (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 the 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; Cade-Menun, 2005a; Turner et al., 2002). In contrast, phosphodiesters such as polymeric nucleotides (i.e. RNA and DNA) are (comparatively) poorly stabilized in the extracellular 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 assessment of the P compounds entering into, and stabilized within, the soil environment (Cade-Menun, 2005b; 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





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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 ~ 20 % of published articles employing $^{31}$P NMR in soils between 2005 and 2013 being in 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., 2010; 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 turn-over 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 hydrogemorphic 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 three years from a diverse range of 28 wetland systems (Fig. 1, Supplement Table S1). Study sites represent 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 based peatlands (sites 1, and 27) and fens (sites





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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 28 wetlands analyzed included a tropical Changuinola peat dome, Panama (Sites 20, 21, and 22) and Houghton Lake treatment wetland, Michigan (sites 4, 5, and 6) in which three separate locations were treated as individual sites. 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 surface cores (diameter 7.5 cm, 10 cm deep) collected from independent sites considered representative of the study wetland. Samples were kept on ice for immediate shipment to the Univ. 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 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 opera-





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tionally determined using anion exchange membranes (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 mol $L^{-1}$ $H_2SO_4$ and the resulting solution was analyzed for molybdate-reactive P using a discrete auto-analyzer (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 correction factor (Bunemann et al., 2008).

Dried and ground soils were analyzed for loss on ignition (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 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 (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 after sampling. Phosphorus was extracted by shaking 1.00 g ± 0.01 g of dried soil with 30 mL of solution containing 0.25 mol $L^{-1}$ NaOH and 50 mmol $L^{-1}$ EDTA in a 50 mL centrifuge tube for 4 h, after which samples were centrifuged at 7000 rpm (maximum RCF ~ 7000 g) (Sorvall RC6, SL600 Rotor; Thermo Fisher Scientific, Waltham, MA)





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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 were combined, spiked with an internal standard methylenediphosphonic acid (MDP), frozen (−80 °C) and lyophilized to await solution $^{31}$P NMR spectroscopy.

### 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 within 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 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 consistent chemical shift (McDowell and Stewart, 2005) and confidence in peak assignment when comparing to existing spectral libraries (Turner et al., 2003d).

Spectra were acquired immediately using an Avance-500 (500.4 MHz $^{-1}$H), 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 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 $T_1$ con-





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stants 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 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).

### 2.4 Data analysis

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), selected given their lack of co-linearity, and the known influence of both parameters on biogeochemical P cycling. Ordination of P composition diversity was performed using principal components analysis (PCA) and compared with fundamental characteristics, including previously defined wetland





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groups. The importance of organic matter content on 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 against major biogenic P groups. Presented values represent arithmetic mean of four field replicates ±1 SD with statistical analysis carried out using R (R Development Core Team, 2012).

## 3 Results

### 3.1 Biogeochemical characteristics

The wetlands studied (Fig. 1, Supplement Table S1) showed a high degree of variation in hydrogeomorphic setting and biogeochemical characteristics (Table 1, and Supplement Table S2). Initial examination of parameter correlations identified organic matter content and pH as useful in typifying wetland "types" given a lack of co-linearity. Wards hierarchical clustering was applied to delineate sites into four broad wetland types (Fig. 1). The first group of 6 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-dominated 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). However, a broadly similar landscape setting resulted in similar biogeochemical characteristics (De Steven and Toner, 2004; Gaiser et al., 2001).





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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 Supplement Table S2). Total P ranged between $51 \pm 35\,\mu g\,P\,g^{-1}$ in Carolina Bay Site 9 and $3516 \pm 442\,\mu g\,P\,g^{-1}$ in the treatment wetland Houghton lake Site 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 $2.2 \pm 0.8\,mg\,N\,g^{-1}$ in Carolina Bay Site 9 and $36.1 \pm 2.0\,mg\,N\,g^{-1}$ in the Everglades National Park Site 24 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 differences among the four wetland groups (Table 1, Kruskal Wallis test $Chi^2 = 12.8$, df = 3, $p < 0.01$), driven





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by the strong positive correlation between organic matter content and the percentage of a total P found as microbial biomass (Spearman's rho = 0.76, $p < 0.001$).

Of the total metals analyzed, Al ranged from $0.5 \pm 0.2$ to $77.1 \pm 3.3$ mg Al $g^{-1}$ (Table 1 and Supplement Table 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 in site 17 ($232 \pm 52$ mg Ca $g^{-1}$) and 19 ($334 \pm 15$ 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 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 detection limit of 0.2 mg Fe $g^{-1}$ in a large number of wetland sites to a maximum of $18.9 \pm 5.4$ 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 ($51 \pm 35$ 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 that the NaOH–EDTA procedure, designed to





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extract organic P, does not extract acid-soluble inorganic P or other alkali-stable forms (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 Supplement Table S3, Fig. 3 and Supplement Figs. S1–S4). 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 were shown to consist of both pyrophosphate (up to 136 µg P $g^{-1}$) and long-chain polyphosphates (up to 110 µg P $g^{-1}$) (Supplement Table S4).

Given the range of total P between sites, 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 type 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 of P forms found. The proportional loading shows 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 separation of group B wetlands, upon PCA axis 2 appeared to be a result of increased prevalence of phosphonates and phosphomonoesters. Similar examination of P composition with reference to Cowardin "class" and climatic zone (data not shown) failed to show clear clustering. Suggesting that soil P composition is dependent upon basic biogeochemical characteristics including,





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to some degree, both the pH and organic matter content groupings identified 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 just acidic systems, found in 2 of 6 sites within group A, and all but one site in group B. No evidence for phosphonates was found in either group C or D wetlands (Supplement Figs. S3 and S4). When present, total phosphonate concentrations ranged up to 44 $\mu g\,g^{-1}$ 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 known peak assignment 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*-inositol hexakisphosphate ($IP_6$). 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 $\mu g\,g^{-1}$ or 46 % of total phosphomonoesters in site 12 (Table 3, Fig. 5). Two group B wetlands (site 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.

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





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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 (site 25). In addition to PCA results suggesting an influence of organic matter (or its reciprocal mineral content) in delineating high phosphomonoester-containing group B wetlands (Fig. 4) we found LOI significantly predicted the ratio of phosphomonoesters to phosphodiesters ($\beta = 6.1 \times 10^3\,\%^{-1}$, $t_{(23)} = 6.9$, $p < 0.001$) with LOI explain 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 having evidence for at least the 2-phosphate residue pyrophosphate (Table 2, Supplement Table S4) The highly impacted Houghton lake (i.e. total P = 3.5 mg $g^{-1}$) had the highest concentration of pyrophosphate at 136 µg $g^{-1}$ or 4 % 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 were they averaged 10 % and ranged 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 between ~ 0 µg P $g^{-1}$ in two





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of the group D calcareous lagoons (Site 18, and 19) and 267 µg P $g^{-1}$ at Site 21, an acidic highly organic tropical peat dome. Microbial P accounted for up to 31 % of total soil P in Site 21 and was generally found to be significantly higher in the higher organic matter group A and 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 application of Spearman's rank correlation (Supplement 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 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 shape their P composition, independent of vegetation and climatic setting. This has profound implications on researchers interested in P sequestration and wetland productivity.

### 4.1 Biogeochemical characteristics

Wetlands sampled represented a broad range of biogeochemical characteristics highlighting 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 : N across wetland soils. In contrast the decoupling between C and P content, while potentially reflecting the impor-





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tance 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 at 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 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 (c.f. bacterial) at low soil pH it seems likely 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 play an important and highly active component of





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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 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 in-situ stabilization and turnover as a result of biogeochemical conditions that determine the levels of this important phosphomonoesters 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 these factors are further impacted by ambient physicochemical conditions, i.e. anaer-





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obosis 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). Although our findings are coincident with this, with group B wetlands (more mineral acidic pH soils) having notable levels of isomers of $IP_6$ 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 of 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.

#### 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 a prebiotic macromolecule (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 and Noguera, 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





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and 22, see also (Cheesman et al., 2012). Taken in conjunction with additional evidence of polyphosphates in un-impacted Carolina Bays (Sundareshwar et al., 2009) and oligotrophic Swedish lake sediments (Ahlgren et al., 2006) it is clear that polyphosphates play an important role in even 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) and DNA and inorganic polyphosphates would be expected given a standard microbial composition, we are currently able 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 their biological synthesis in response to increased microbial pressure for a critical 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 + longer chain length polyphosphates) and microbial P might reflect the fact that operationally defined microbial P is in a large part due to extraction of polyphosphates from the soil.





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## 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 represent a static stabilized component of soil P or one which sees a turnover rate of a similar magnitutde to that seen in organic calcareous systems. Further work identifying the flux rates of particular P forms is therefore needed to put our understanding of static pools in context of the overall P cycle.

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**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 ($\mu g\,g^{-1}$) | Bioavailable 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 | 92 | 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 = below detection limit





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

| | NaOH – EDTA | | | | Organic Phosphorus | | | | | | | | | Inorganic Phosphorus | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TP[a] | | Phosphonate | | Mono-P[b] | | DNA | | Phospholipids | | Total Organic P | | Mono : Dies[c] | Ortho-P[d] | | Total inorganicPolyphosphates | |
| | 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] Phosphomonoestersters.
[c] Ratio of total phosphomonoesters: total phosphodiesters.
[d] Orthophosphate.





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**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 μg 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) |





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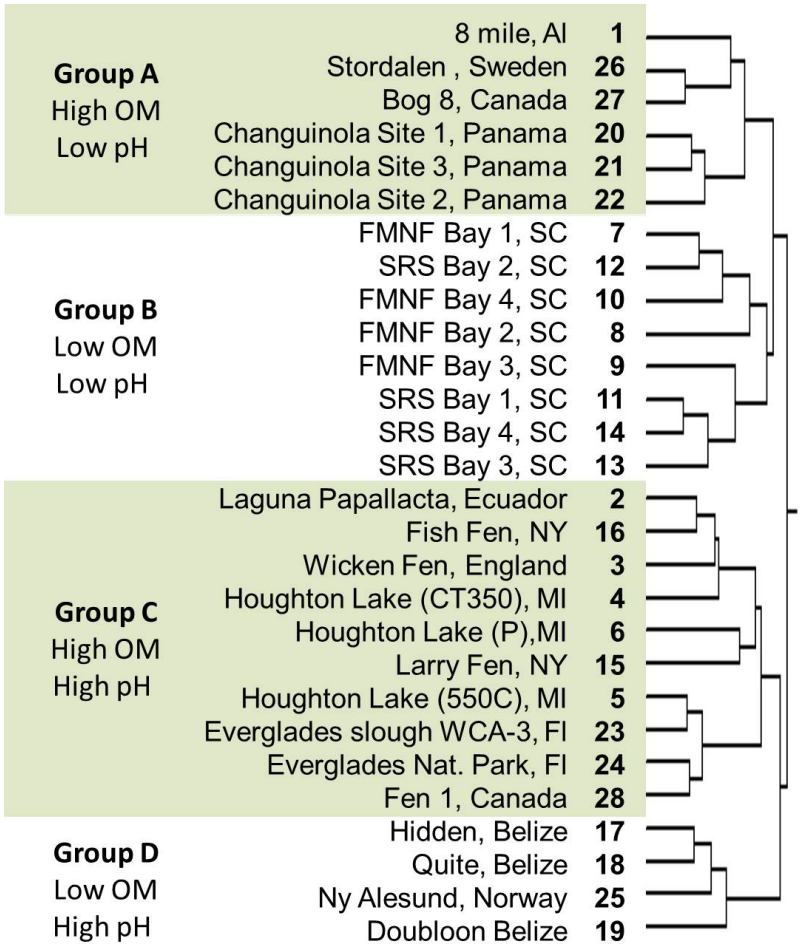


**Figure 1.** Wards hierarchical classification of 28 palustrine wetlands used to delineate four types of wetland A–D based upon organic matter (OM) content and pH.





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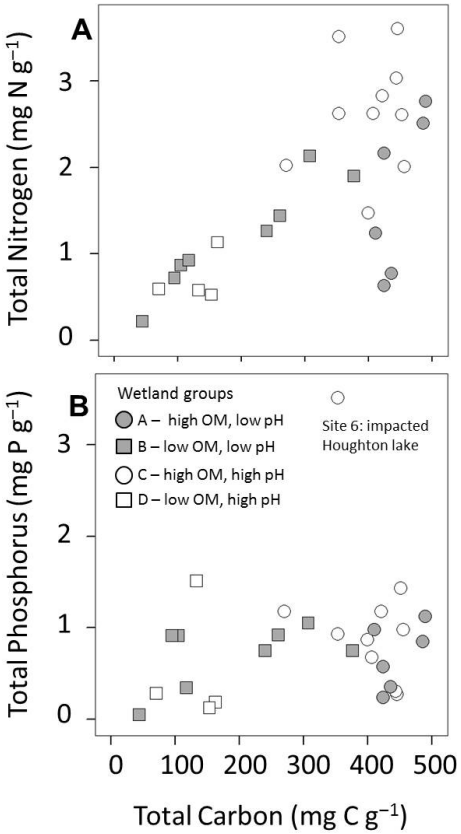


**Figure 2.** Mean total element concentrations in surface soils of 28 palustrine wetlands. Symbols represent wetland type, grouped by organic matter (OM) content and pH. Total carbon and nitrogen showed significant positive correlation (Spearmans rho = 0.67, $p < 0.0001$) which improved when considering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





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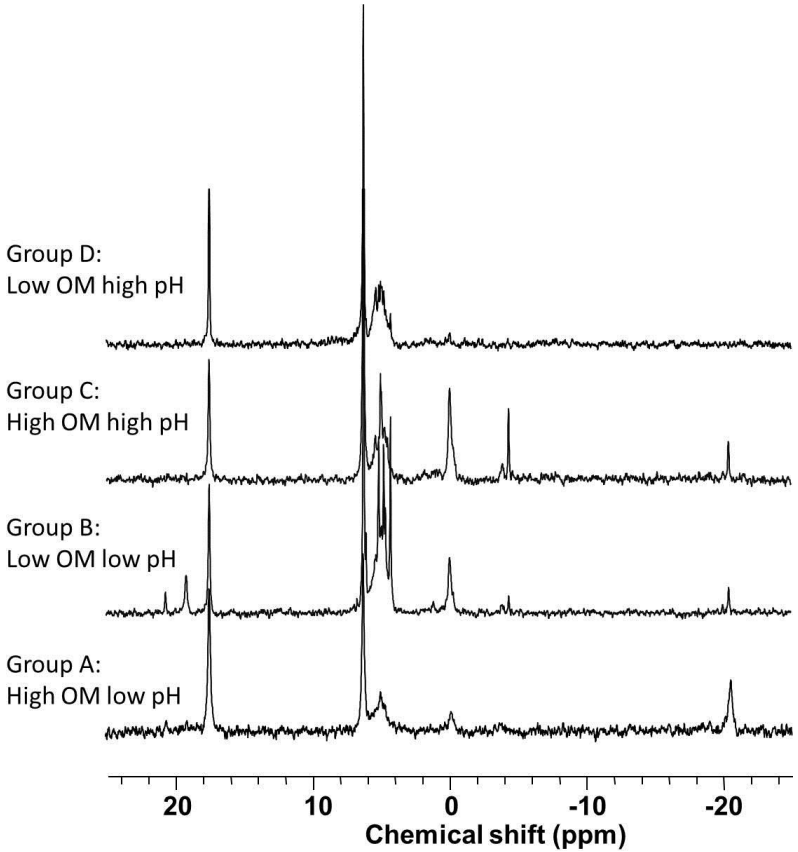


**Figure 3.** Solution $^{31}$P NMR spectra of biogenic P composition of wetland soils representative of 4 wetland types identified in this study, see Supplement Figs. S1–S4. Spectra acquired using an Avance-500 (500.4 MHz 1H), Magnex 11.8 Tesla/54 mm Bore, at pH > 13 using a simple zgig pulse program 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).





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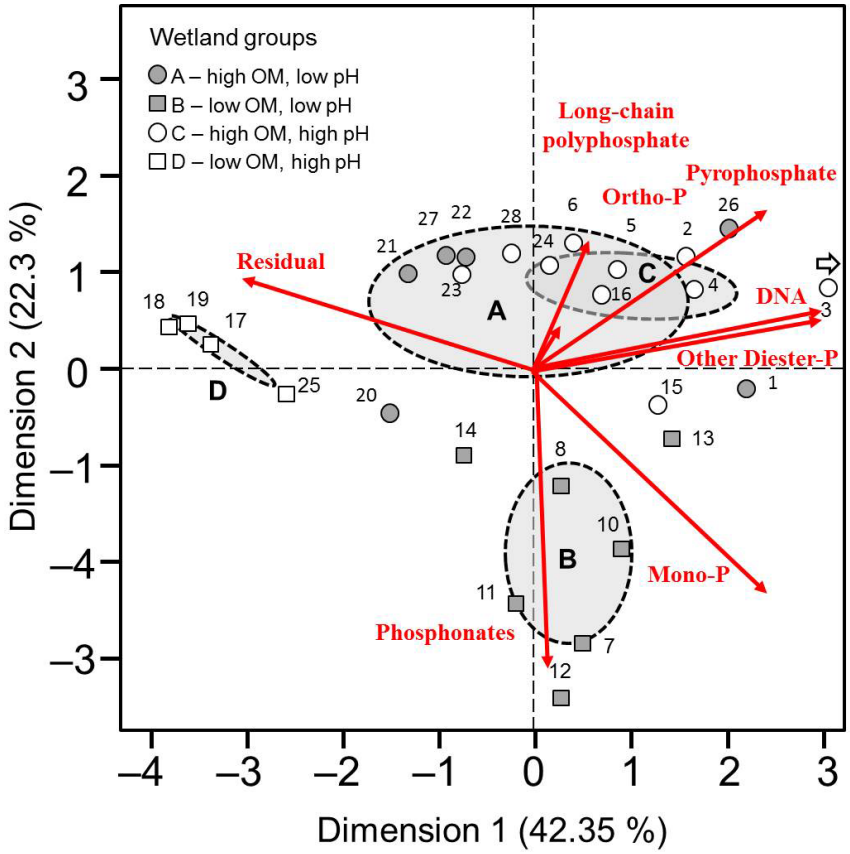


**Figure 4.** Biplot of the scaled first two principal components of phosphorus composition in wetland soils. Ellipse represent 95 % confidence interval surrounding barycenter of four wetland groupings based upon pH and organic matter content. Proportional loading of P composition superimposed in red.





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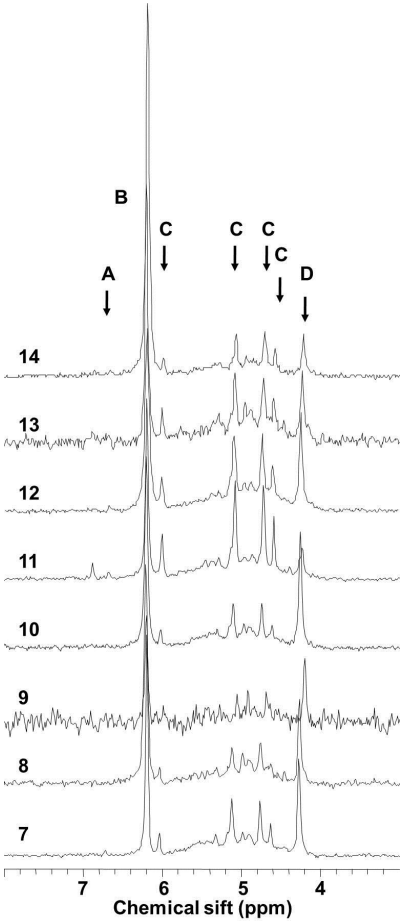


**Figure 5.** Region 8 to 3 ppm of group B wetland NMR spectra and peak assignments for; **(A)** *neo*- and D-*chiro*-inositol hexakisphosphate, **(B)** orthophosphate, **(C)** *myo*-inositol hexakisphosphate, **(D)** *scyllo*-inositol hexakisphosphate.





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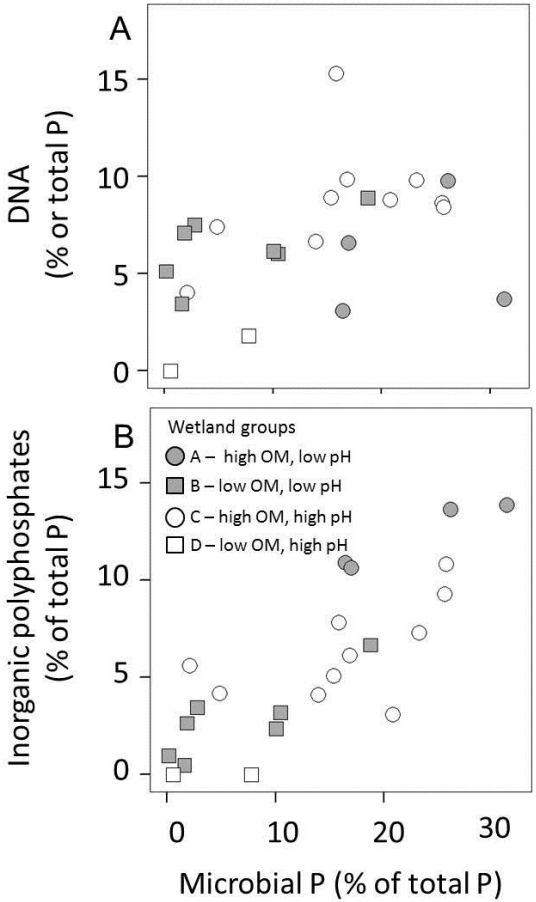


**Figure 6.** Plot of microbial P against **(A)** DNA and **(B)** total inorganic polyphosphates. Both showing significant positive correlation as determined by Spearman's rank correlation ($rho_{(DNA)} = 0.66$, $p < 0.01$, $rho_{(total\ inorganic\ polyphosphates)} = 0.78$, $p < 0.001$).





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