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Sample Pretreatment and Phosphorus Speciation in Wetland Soils

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We assessed the influence of sample pretreatment on the amounts and forms of P extracted in NaOH–EDTA (ethylenediamine tetraacetic acid) from a series of contrasting wetland soils from the Florida Everglades. Samples of unconsolidated benthic floc and underlying soil (0–10 cm) were extracted either fresh (overnight refrigeration only), air dried (10 d at ~30°C), or frozen at –80°C and lyophilized (~48 h), before extraction and solution ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Significant differences in total P extraction following pretreatment were detected for one out of four benthic floc samples and three out of four soil samples, although the changes were inconsistent: in two cases the total P extraction increased, while in two others it decreased. Assessment of the P composition by solution ^{31}P NMR spectroscopy revealed differences among treatments, although these were mostly within the range of error associated with replicate analyses; however, DNA was not detected in a fresh sample of calcareous benthic floc, despite representing an important component of the organic P extracted from dried samples. The apparent sample-specific nature of the changes confirms the importance of carefully assessing pretreatment effects in studies of soil organic P in wetlands.

Abbreviations: EDTA, ethylenediamine tetraacetic acid; NMR, nuclear magnetic resonance.

Organic P constitutes a considerable proportion of the total P in wetland soils, so its accurate measurement is the basis for understanding P biogeochemistry in wetland ecosystems. Soil organic P is now commonly assessed by extraction in a solution containing NaOH and ethylenediamine tetraacetic acid (EDTA), with detection by solution ^{31}P NMR spectroscopy (Cade-Menun, 2005; Turner et al., 2005). The single-step NaOH–EDTA extractant was developed to quantitatively recover organic P from mineral soils (Bowman and Moir, 1993), but is particularly effective for organic soils with relatively low pH, from which >90% of the total P can be extracted (e.g., Cade-Menun et al., 2000; Turner et al., 2004). It should therefore be well suited to the analysis of wetland soils and has recently been applied to a series of slightly acidic to alkaline samples from the Florida Everglades (Robinson et al., 1998; Turner and Newman, 2005; Turner et al., 2006a). Most samples analyzed so far contained a range of P compounds, including phosphate, phosphate monoesters (e.g., inositol phosphates, sugar phosphates), phosphate diesters (e.g., nucleic acids, phospholipids), and pyrophosphate (Robinson et al., 1998; Pant et al., 2002; Turner and Newman, 2005; Turner et al., 2006a). Other compounds detected in soils, but not so far in Everglades peats, include phosphonates and polyphosphates (Condron et al., 2005).

An aspect of the procedure that has received little attention is soil pretreatment (Turner et al., 2005). Soils are commonly stabilized by mild drying before P analysis, which is not considered to greatly alter quantitative organic P extraction (defined here as procedures such as NaOH–EDTA extraction that are designed to extract and quantify the total soil organic P). Drying, however, can markedly influence both inorganic and organic P solubility in water and other mild extractants designed to estimate pools of P available to plants or susceptible to transfer in runoff (Sparling et al., 1985; Turner and Haygarth, 2003). For example, increases in water-extractable organic P of between 185 and 1900% were reported following the air drying of temperate pasture soils (Turner and Haygarth, 2001). Similar changes occur after freezing (Ron Vaz et al., 1994) and can even continue during storage of air-dried samples (Turner, 2005). An alternative drying procedure involves freezing and lyophilization. This allows samples to be stabilized quickly and has proven suitable for the analysis of P in animal manures (Leytem et al., 2004; Turner, 2005), although it has been used only rarely for soils (Dai et al., 1996; Turner and Newman, 2005; Turner et al., 2006a).

In contrast to mineral soils, wetland soils are often analyzed fresh. Such soils are notoriously heterogeneous, however, and the high water content of fresh samples (often >90%) complicates the standardization of solid/solution ratios and extractant molar-

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ity. Some form of soil drying is therefore analytically convenient, although there is little information with which to assess its effect on P solubility. A recent study measured differences in EDTA-extractable inorganic phosphate from a seasonally flooded wetland clay soil from Texas following freezing, air drying, or purging with N_2 (Pezzolesi et al., 2000). Greatest concentrations were extracted from air-dried soil for both surface and subsurface samples, although the differences were significant only for subsurface soil. In contrast, dried samples of a nearby upland loam yielded the lowest P extraction of the three pretreatments. Schlichting and Leinweber (2002) reported that both air drying and lyophilization caused a marked reduction in NaOH-extractable P from a German peat using a sequential fractionation procedure. Lyophilization caused the greatest decrease, although even refrigeration caused a detectable decrease in P extraction, indicating the sensitivity of this soil to pretreatment. Also of relevance is a recent study of storage effects on NaOH–EDTA-extractable P in particulate organic material from the deep ocean (Cade-Menun et al., 2005). Fresh samples yielded the lowest P concentrations compared with samples that had been stored either frozen, refrigerated, or oven dried at 40°C. The differences in P composition by solution ^{31}P NMR spectroscopy were relatively small, however, and the researchers suggested they were within the expected error for replicate analyses by the technique.

The changes in organic P solubility following drying have been attributed to two main factors: (i) the release of microbial compounds by lysis on rewetting, and (ii) the disruption of organic matter by the chemical and physical processes involved in drying (Bartlett and James, 1980; Sparling et al., 1985; Turner and Haygarth, 2003). Only the second factor is expected to be important for quantitative organic P extraction and could cause either an increase or decrease in the total organic P concentration. Drying could increase organic P extraction following disruption of organic matter coatings on mineral particles, or decrease it following the formation of insoluble organic complexes. Microbe-mediated changes in the chemical nature of organic P compounds could also occur during the early stages of air drying. This could involve a decrease in organic P by enzymatic hydrolysis, or an increase through the growth of new microbial tissue. Such processes should be minimized by rapid freezing in the lyophilization procedure, as used in recent studies of organic P in Everglades peats (Turner and Newman, 2005; Turner et al., 2006a).

There is no information on the effect of pretreatment on P speciation in wetland soils, yet this may be of significance. For example, an important finding in recent studies of Everglades peats was that higher order inositol phosphates were not detected, despite their abundance in most mineral soils (Turner, 2007). Although in situ anaerobic decomposition seems the most likely explanation (Suzumura and Kamatani, 1995), the possibility remains that lyophilization before NaOH–EDTA extraction and solution ^{31}P NMR spectroscopy caused the formation of insoluble complexes between inositol phosphates and organic matter.

We investigated the effects of soil pretreatment on P characterization in wetland soils by NaOH–EDTA extraction and solution ^{31}P NMR spectroscopy. We compared air drying, lyophilization, and analysis of fresh samples for a series of wetland soils with contrasting P concentrations and chemical properties.

MATERIALS AND METHODS

Sampling and Pretreatment

Samples of unconsolidated benthic floc and underlying soil (0–10 cm) were taken during February 2004 from four contrasting wetland sites in the Florida Everglades. The sites were (i) a nutrient-enriched area of Water Conservation Area 2A (termed F1 in previous publications) supporting a mono-specific cattail (*Typha* spp.) community (DeBusk et al., 2001); (ii) a treatment wetland (Stormwater Treatment Area 1 West, Cell 4) constructed in 1994 on former agricultural land, supporting submerged aquatic vegetation, including coontail (*Ceratophyllum demersum* L.) and naiad [*Najas guadalupensis* (Spreng.) Magnus]; (iii) an open-water slough in a pristine area of softwater marsh in Water Conservation Area 1, supporting emergent macrophytes and a periphyton community comprised of green algae and diatoms (Newman et al., 1997) and (iv) an open-water slough in an unenriched part of Water Conservation Area 2A (termed U3 in previous publications), dominated by calcareous periphyton mats comprised of Ca-precipitating cyanobacteria and diatoms (McCormick and O'Dell, 1996).

At each site, four cores (10-cm diameter) were taken to 10-cm depth in the organic soil layer. The cores were capped and transported on ice to the laboratory (~5 h), where unconsolidated benthic floc, which included plant detritus and algae, was separated from the underlying soil. Material from the four replicate cores from each site was then combined to produce a single bulked sample of benthic floc and one of soil for each location. Recognizable plant matter (stems, roots) and shells were removed by hand.

Each sample was split into four portions. One portion was stored in a plastic bag at 4°C overnight and analyzed the following morning (see below). These samples are termed *fresh*. A second portion was spread on a shallow metal tray and air dried at ~30°C for 10 d. These samples are termed *air dried*. The third portion was frozen at −80°C and lyophilized (~48 h). These samples are termed *lyophilized*. The fourth portion was dried overnight (105°C) to determine water content. The lyophilized and air-dried samples were ground and stored in plastic bags at ambient laboratory temperature until analysis.

Phosphorus Extraction and Analysis

Phosphorus was extracted by shaking 1.00 ± 0.01 g of benthic floc or soil (oven-dry weight basis) with 40 mL of extractant solution for 16 h at ~22°C in a 50-mL centrifuge tube. Each sample was extracted in triplicate. To account for moisture already in the sample, the solid/solution ratio was calculated on the basis of oven-dry soil to give a consistent 1:40 ratio for all extracts (i.e., the volume and molarity of the extractant were adjusted to account for the water already in the sample). This involved adding deionized water to the sample to give a total water content of 20 mL, and then adding 20 mL of a solution containing 0.5 *M* NaOH and 0.1 *M* Na_2EDTA. Thus, the final concentration of extractant solution was 0.25 *M* NaOH and 0.05 *M* Na_2EDTA. The wide solid/solution ratio used here, compared with the narrower ratio used commonly for solution ^{31}P NMR spectroscopy (e.g., 1:10 or 1:20), was necessary due to the low solid/solution ratios of fresh samples, but was lower than the 1:50 ratio used in the development of the NaOH–EDTA procedure (Bowman and Moir, 1993). Extracts were centrifuged at 8000 × *g* for 30 min and an aliquot taken for the determination of total P (see below). Equal volumes of the remaining replicate extracts were then combined, frozen at −80°C, lyophilized, and homogenized by gentle grinding.

Analysis of replicate extracts by solution ^{31}P NMR spectroscopy is prohibitively expensive and was not attempted here for all samples. To assess the error associated with the analytical procedure, we separately ana-

Table 1. Chemical properties of benthic floc and underlying soil (0–10 cm) from four wetland sites with contrasting chemistry in the Florida Everglades (from Turner et al., 2006b).

Sample	pH	Loss on ignition	Total element			Element ratio (mass)		
			C	N	P	C/N	C/P	N/P
		%	g kg^{-1} dry wt.					
Benthic floc	7.4	84	389	26.8	1.12	15	346	24
Soil	7.6	90	426	30.9	0.27	14	1604	117
Benthic floc	8.0	48	274	16.0	0.79	17	348	20
Soil	7.0	89	444	25.3	0.25	18	1789	102
Benthic floc	5.8	90	440	37.8	0.31	12	1437	123
Soil	5.6	96	468	37.7	0.23	12	2007	162
Benthic floc	7.9	36	222	14.2	0.21	16	1071	69
Soil	7.5	89	431	33.5	0.19	13	2219	173

lyzed each replicate extract of one sample (lyophilized benthic floc from the cattail marsh) by solution ^{31}P NMR spectroscopy.

Solution Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy

Each freeze-dried extract (~100 mg) was redissolved in 0.1 mL of deuterium oxide and 0.9 mL of a solution containing 1.0 *M* NaOH and 0.1 *M* Na_2EDTA and transferred to a 5-mm NMR tube. The deuterium oxide provided an NMR signal lock and the NaOH raised the pH to >13 to ensure consistent chemical shifts and optimum spectral resolution. Solution ^{31}P NMR spectra were obtained using a Bruker Avance DRX 500 MHz spectrometer operating at 202.456 MHz for ^{31}P and 500.134 MHz for ^{1}H. Samples were analyzed using a 6-µs pulse (45°), a delay time of 2.0 s, and an acquisition time of 0.4 s. This delay time is suitable for most soils, although a longer time may be required for samples containing low concentrations of paramagnetic ions (McDowell et al., 2006). Between 24,000 and 38,000 scans were acquired depending on the P concentration of the lyophilized extract, and broadband proton decoupling was used for all samples.

Chemical shifts of signals were determined in parts per million (ppm) relative to an external standard of 85% H_3PO_4. Signals were assigned to individual P compounds or functional groups based on literature reports (Turner et al., 2003) as follows: phosphate (approximately 6.3 ppm), phosphate monoesters (between 3 and 6 ppm), DNA at approximately –0.2 ppm, phospholipids between 0.5 and 2.0 ppm, and pyrophosphate (approximately –4.0 ppm). Neither phosphonates nor long-chain polyphosphates were detected in any sample. Spectra were plotted with a line broadening of 8 or 16 Hz depending on signal strength, and signal areas were calculated by integration. For presentation in figures, the phosphate signal was adjusted to 6.3 ppm. Total organic P was calculated as the sum of phosphate monoesters, DNA, and phospholipids. Spectra were processed using NMR Utility Transform Software (NUTS) for Windows (Acorn NMR Inc., Livermore, CA).

Chemical Analysis

Total C and N were determined by combustion and gas chromatography using a Flash EA1112 elemental analyzer (CE Elantech, Lakewood, NJ). Soil pH was determined in a 1:20 ratio of air-dried soil to deionized water (approximate 1:2 on a wet-weight basis). Loss on ignition was determined by ashing at 550°C for 3 h. Total P was determined on ashed samples following digestion in 6 *M* HCl, with phosphate detection by automated molybdate colorimetry. Total P in NaOH–EDTA extracts was determined by the same procedure, and the mean total P concentration in blank NaOH–EDTA samples (n = 3) was subtracted from each value. We did not attempt to assess inorganic and organic P by molybdate colorimetry in the NaOH–EDTA extracts (i.e., calculation of organic P as the difference between total P and inorganic phosphate), due to the apparent overestimation of organic P in wetland soils using this procedure (Turner et al., 2006b).

Statistics and Data Presentation

Phosphorus concentrations are the mean ± standard deviation of three replicate extracts. Values for solution ^{31}P NMR spectroscopy are presented as a proportion (percentage) of the total extracted P. The statistical significance of pretreatment effects was determined by ANOVA,with treatment, site, and depth as factors. Further means separation among treatments was assessed by least significant differences (5%). All statistical analysis was performed using R (www.r-project.org; verified 28 May 2007).

RESULTS

Soil Properties

Sample properties are shown in Table 1. As reported previously (Turner et al., 2006b), soil pH was neutral or slightly alkaline for all floc and soil samples except those from the softwater marsh (pH <6). Loss on ignition was >80% for all except two calcareous benthic floc samples from the treatment wetland and the calcareous slough. This was reflected in the lower total C concentrations for these two samples, much of which would have been present in carbonates. Phosphorus concentrations in benthic floc were greatest from the enriched cattail marsh (1.12 g P kg^{-1}) and lowest for the calcareous marsh (0.21 g P kg^{-1}). In soil, P concentrations were similar for all samples (0.19–0.27 g P kg^{-1}). Ratios of C/N were relatively similar among samples, whereas C/P and N/P ratios varied markedly. For example, C/P ratios ranged between 346 in benthic floc from the cattail marsh to 2219 in soil from the calcareous slough, while N/P ratios ranged between 20 in benthic floc from the treatment wetland to 173 in soil of the calcareous marsh.

Total Phosphorus Recovery by NaOH–EDTA Extraction

Total P recovery in NaOH–EDTA ranged between 26 and 28% for benthic floc from the calcareous slough and 68 to 87% for benthic floc from the softwater slough (Table 2). Recovery from benthic floc was greater than from soil for both the cattail marsh and softwater slough, while the opposite was true for the two calcareous samples. As the NaOH–EDTA solution was developed to extract soil organic P rather than inorganic phosphate, the low recovery from the calcareous samples was probably due to much of the P occurring as inorganic Ca-phosphate minerals (Dodds, 2003).

Overall, there were significant differences in total P extraction among sites (F = 826, P < 0.001) and depth (F = 1673, P < 0.001), but no significant effect of treatment (F = 0.6, P = 0.55). There were significant differences, however, among treatments for individual samples.

For benthic floc, pretreatment significantly influenced P extraction only in the softwater slough (F = 54.0; P < 0.001), with the lowest recovery from the fresh sample compared with air-dried and lyophilized samples (Table 2). There were no significant differences between treatments for benthic floc from the cattail marsh, treatment wetland, or calcareous slough.

For soil, differences were significant for the cattail marsh (F = 6.1; P < 0.05), treatment wetland (F = 7.7; P < 0.05), and softwater slough (F = 41.0; P < 0.001), although there was no clear trend in the response. For example, the greatest P recovery was obtained from fresh soils for the cattail marsh and softwater slough, but from the air-dried sample for the treatment wetland. The significantly greater recovery for the fresh soil from the softwater slough was also in direct contrast to the much lower total P recovery from the fresh benthic floc for this wetland.

Replicate Nuclear Magnetic Resonance Analysis

The values for replicate analysis shown in Table 3 represent analytical error associated with both NaOH–EDTA extraction and NMR spectroscopy. Variation in total P extraction for three replicate extracts of the lyophilized benthic floc from the cattail marsh was small (CV = 2.0%) and within acceptable limits for replicate extracts of homogenized soil (Table 3). The error associated with total organic P determined by solution ^{31}P NMR spectroscopy was also small (CV = 2.4%; Fig. 1, Table 3). The CVs associated with the two largest (groups of) signals, inorganic phosphate and phosphate monoesters, was <5%, whereas for the smaller signals, the CVs were larger, being around 10% for DNA and phospholipids, and 19% for pyrophosphate.

Table 2. Recovery of total P in NaOH–EDTA from benthic floc and underlying soil (0–10 cm) following three pretreatments. Values are expressed as a proportion of the total soil P to allow comparison among samples containing widely different total P concentrations.

Treatment	Benthic floc	Soil (0–10 cm)
	% of total soil P	
	Cattail marsh	
Fresh	65.5 ± 6.9†	48.5 ± 2.2a‡
Air dried	59.9 ± 5.4	45.7 ± 1.5ab
Lyophilized	66.6 ± 1.3	41.5 ± 3.4b
LSD§	10.2 NS	4.9*
	Treatment wetland	
Fresh	41.8 ± 1.9	63.2 ± 3.7a
Air dried	38.9 ± 1.2	69.6 ± 1.8b
Lyophilized	39.1 ± 1.3	62.8 ± 0.4a
LSD	3.0 NS	4.8*
	Softwater slough	
Fresh	67.9 ± 3.1a	65.8 ± 1.3a
Air dried	86.7 ± 2.7b	54.9 ± 1.1b
Lyophilized	81.6 ± 2.0b	57.6 ± 1.6b
LSD	5.3***	2.7***
	Calcareous slough	
Fresh	25.9 ± 1.9	47.9 ± 1.8
Air dried	28.1 ± 0.8	42.8 ± 2.4
Lyophilized	27.0 ± 0.5	46.6 ± 3.3
LSD	2.5 NS	5.1 NS

* Significant at the 0.05 probability level. NS, not significant at the 0.05 probability level.

*** Significant at the 0.001 probability level.

† Values are means ± standard deviation of three replicate extracts.

‡ Within columns for each sample, means followed by the same letter are not significantly different according to LSD (5%).

§ LSD among treatments at the 5% level.

Table 3. Results from three replicate extracts of the lyophilized benthic floc from the cattail marsh analyzed by solution ^{31}P nuclear magnetic resonance spectroscopy. Values are means ± standard deviation and include error from the extraction and analysis procedures.

Functional group	Phosphorus	CV†
	mg P kg^{-1} soil	%
Total extracted P	748 ± 15	2.0
	% extracted P	
Organic P‡	60 ± 1	2.4
Phosphate	37 ± 2	4.5
Phosphate monoesters	38 ± 1	3.5
Phospholipids	9 ± 1	13.9
DNA	13 ± 1	10.3
Pyrophosphate	3 ± 1	18.9

† Calculated as 100(standard deviation/mean).

‡ Calculated as the sum of phosphate monoesters, DNA, and phospholipids.

Phosphorus Composition by Solution Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy

For the cattail marsh (Fig. 2), pretreatment effects on P composition were relatively small, being <3% for larger signals contributing >30% of the extracted P (phosphate, phosphate monoesters,

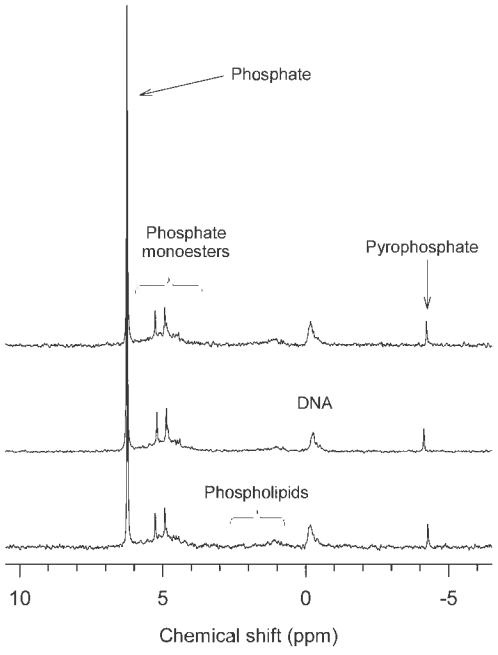

Fig. 1. Solution ^{31}P nuclear magnetic resonance spectra of triplicate extracts of a lyophilized benthic floc sample of a cattail marsh. The spectra are plotted with 1 Hz line broadening to show fine resolution and the phosphate signal adjusted to 6.3 ppm.

and total organic P), 10 to 12% for signals contributing around 10% of the extracted P (DNA and phospholipids) and 41% for the smallest signal (pyrophosphate) (Table 4). These values were comparable to those for the triplicate extracts of the lyophilized benthic floc sample. Pyrophosphate was greater in the fresh sample of benthic floc than the air-dried or lyophilized treatments, while DNA was greater in the lyophilized treatment. Results for the soil were similar, except for a larger difference in phospholipids (CV = 18%) and a much smaller difference for pyrophosphate (CV = 1%; Table 4). The cattail marsh samples were the only ones that contained quantifiable concentrations of phospholipids.

For the treatment wetland, spectra of the benthic floc were well resolved in comparison to those of the soil (Fig. 3), due largely to the marked difference in P concentrations. In the benthic floc, the differences in phosphate, phosphate monoesters, and total organic P were relatively small (CV < 10%), although phosphate was a greater proportion of the extracted P from the lyophilized sample. The greatest differences were for DNA, which ranged from 8% of the extracted P in the fresh sample to 13% in the air-dried sample. In soil extracts, the proportion of DNA was similar among pretreatments, but there were marked differences in the proportions of phosphate, which varied from 37% for the air-dried sample to 54% for the fresh sample. Similarly, phosphate monoesters varied from 29% of the extracted P in the fresh sample to 45% in the air-dried sample. The soil extracts did not contain detectable concentrations of phospholipids or pyrophosphate.

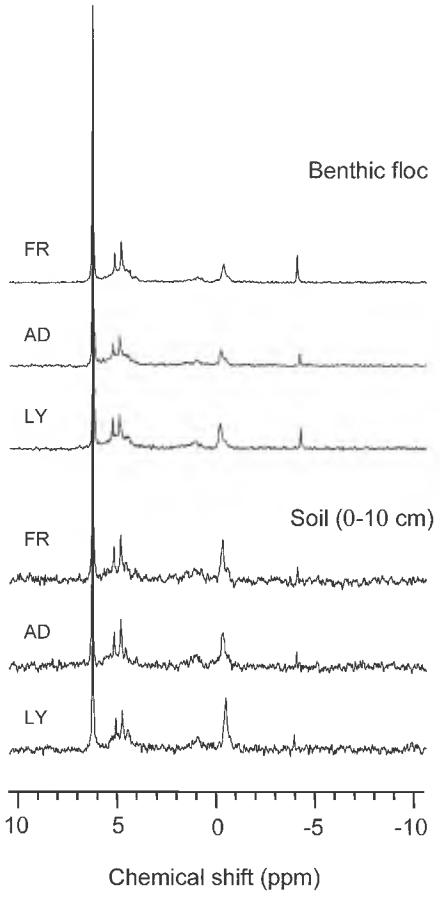

Fig. 2. Solution ^{31}P nuclear magnetic resonance spectra of NaOH–EDTA extracts of benthic floc and soil (0–10 cm) from an enriched cattail marsh in Water Conservation Area 2A of the Florida Everglades, subjected to three different pretreatments: FR, fresh sample (overnight refrigeration at 4°C); AD, air dried for 10 d at 30°C; LY, frozen at –80°C and lyophilized. The spectra are plotted with 8 Hz line broadening and the phosphate signal adjusted to 6.3 ppm.

The softwater slough samples (Fig. 4) contained the greatest proportions of DNA of the four sites (23–29% of extracted P). For the benthic floc, the main pretreatment differences were a greater proportion of phosphate in the lyophilized sample (34% of extracted P) than the fresh or air-dried samples (27%) and a greater proportion of pyrophosphate in the air-dried sample (19%) than the fresh or lyophilized samples (13%). For soil, there were major differences among pretreatments, with more organic P and less phosphate in the fresh sample than the air-dried or lyophilized samples (Table 4). This was in direct contrast to the differences observed for the treatment wetland soil (see above). The differences for the softwater slough soil were due mainly to the greater concentration of total P extracted from the fresh sample (Table 2). The spectrum of the fresh sample showed a lower signal-to-noise ratio than the dried samples, however, despite containing more total P (Fig. 4). The reason for this is unclear, but may be due to a difference in the extraction of paramagnetic ions between fresh and dried soil.

The spectra of the calcareous slough samples were the least well resolved of the four sites (Fig. 5), although an effect of pretreatment was apparent for both the benthic floc and soil (Table 4). Pyrophosphate was not detected in the fresh samples or the air-dried benthic floc; DNA was also not detected in the fresh floc sample, yet constituted 16–17% of the extracted P in the air-dried and lyophilized samples. Phosphate monoesters were a greater proportion of the extracted P in the fresh samples of both benthic floc and soil, although differences in phosphate and total organic P were not consistent.

DISCUSSION

Most mineral soils are air-dried and ground before extraction, but the effects of sample pretreatment on P recovery in NaOH–EDTA extracts have not been previously investigated for either mineral or organic soils (Turner et al., 2005). Several studies of mineral soils reported increases in organic P solubility following soil drying, attributed to microbial lysis and disruption of organic matter coatings on mineral particles (e.g., Sparling et al., 1985; Turner and Haygarth, 2003). Other studies of wetland soils reported little effect of pretreatment on the extraction of inorganic phosphate in EDTA alone (Pezzolesi et al., 2000), or a reduction in organic P extraction in NaOH alone following air drying or lyophilization (Schlichting and Leinweber, 2002). We found significant changes in the extraction of total P in NaOH–EDTA following air drying or lyophilization in four out of eight wetland samples, but the changes were inconsistent and both increases and decreases in extracted P were detected. The effects of drying on total P extraction in NaOH–EDTA therefore appear to be sample specific, although in general the effects were significant for the more humified soil layers compared with unconsolidated benthic floc.

Differences in P composition determined by solution ^{31}P NMR spectroscopy occurred following sample pretreatment, although as for total P extraction the changes appeared to be sample specific and no clear trends were apparent. Similar results were reported for deep-ocean particulate material, which yielded sample-specific differences among pretreatments that included drying, freezing, and refrigeration (Cade-Menun

Table 4. Phosphorus compounds in NaOH–EDTA extracts of benthic floc and underlying soil (0–10 cm) following different pretreatments determined by solution ^{31}P nuclear magnetic resonance spectroscopy.

Sample	Treatment	Phosphate	Phosphate monoesters	Phospholipids	DNA	Pyrophosphate	Organic P†
		% extracted P					
			Cattail marsh				
Benthic floc	fresh	38	39	8	11	5	58
	air dried	40	37	10	11	2	58
	lyophilized	37	38	9	13	3	60
	CV‡	3	2	12	10	41	2
Soil	fresh	30	35	15	18	1	68
	air dried	31	36	11	20	1	67
	lyophilized	31	34	13	21	1	67
	CV	2	3	18	8	2	1
			Treatment wetland				
Benthic floc	fresh	49	39	ND§	8	3	48
	air dried	49	35	ND	13	3	48
	lyophilized	55	33	ND	10	2	43
	CV	7	9	–	22	32	6
Soil	fresh	54	29	ND	17	ND	46
	air dried	37	45	ND	18	ND	63
	lyophilized	42	41	ND	17	ND	58
	CV	19	22	–	3	–	15
			Softwater slough				
Benthic floc	fresh	27	31	ND	28	13	59
	air dried	27	29	ND	24	19	54
	lyophilized	34	28	ND	25	13	53
	CV	13	5	–	8	23	6
Soil	fresh	20	44	ND	29	7	73
	air dried	32	37	ND	23	8	60
	lyophilized	39	29	ND	27	6	56
	CV	31	21	–	11	15	14
			Calcareous slough				
Benthic floc	fresh	47	53	ND	ND	ND	53
	air dried	35	48	ND	17	ND	65
	lyophilized	39	43	ND	16	2	59
	CV	15	11	–	87	173	10
Soil	fresh	32	43	ND	25	ND	68
	air dried	41	34	ND	23	2	57
	lyophilized	37	34	ND	27	2	61
	CV	13	14	–	7	87	9

† Calculated as the sum of phosphate monoesters, DNA, and phospholipids.

‡ Calculated as 100(standard deviation/mean).

§ ND, not detected.

et al., 2005). The authors suggested that that differences among treatments were relatively small, being within the error expected for replicate analyses by the NMR procedure. There are few published examples of replicated solution ^{31}P NMR spectra of soils, but previous studies of manures, which contain at least an order of magnitude more P than soils, reported that the error for replicate NMR analyses of the same extract (i.e., not replicate extractions) was 5% for smaller signals and 10% for larger signals (Leinweber et al., 1997; Kemme et al., 1999). These values are similar to those reported here for triplicate extracts of the benthic floc from the cattail marsh, which included error in both extraction and NMR analysis. For this sample, which gave well-resolved spectra with good signal-to-noise ratios, the error in replicate analyses was also comparable to the variation due to pretreatment, being small for the larger signals, and large for the smaller signals. Greater variation would therefore be expected for samples with lower P concentrations and poor-quality spectra. This may explain some of the apparent sample-specific pretreatment effects on P composition, because the greatest pretreatment differences occurred for samples with poorly resolved spectra. This cannot, however, explain the fact that less pyrophosphate was extracted from the lyophilized benthic floc from the treatment wetland and the softwater slough (Fig. 3 and 4), as these spectra are well resolved and have good signal-to-noise ratios.

Given the variable response among samples, it is difficult to recommend a standardized pretreatment procedure for the analysis of organic P in wetland soils. The extraction of fresh samples would minimize potential artifacts introduced by drying, but could lead to important errors. Of particular concern is that the extract of fresh benthic floc from the calcareous marsh did not contain detectable concentrations of DNA, despite this compound representing 16 to 17% of the extracted P in dried samples. The error is significant given

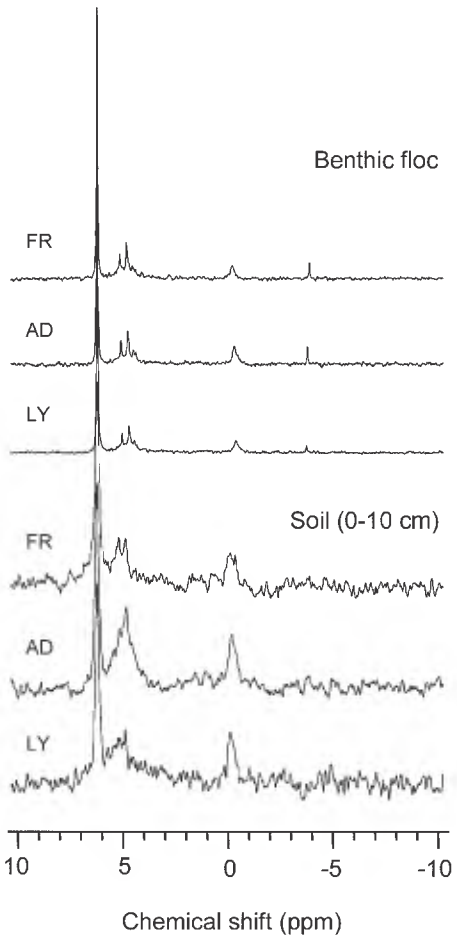

Fig. 3. Solution ^{31}P nuclear magnetic resonance spectra of NaOH–EDTA extracts of benthic floc and soil (0–10 cm) from Stormwater Treatment Area 1, Florida, subjected to three different pretreatments: FR, fresh sample (overnight refrigeration at 4°C); AD, air dried for 10 d at 30°C; LY, frozen at –80°C and lyophilized. The spectra are plotted with a line broadening of 8 Hz (benthic floc) or 16 Hz (soil) and the phosphate signal adjusted to 6.3 ppm.

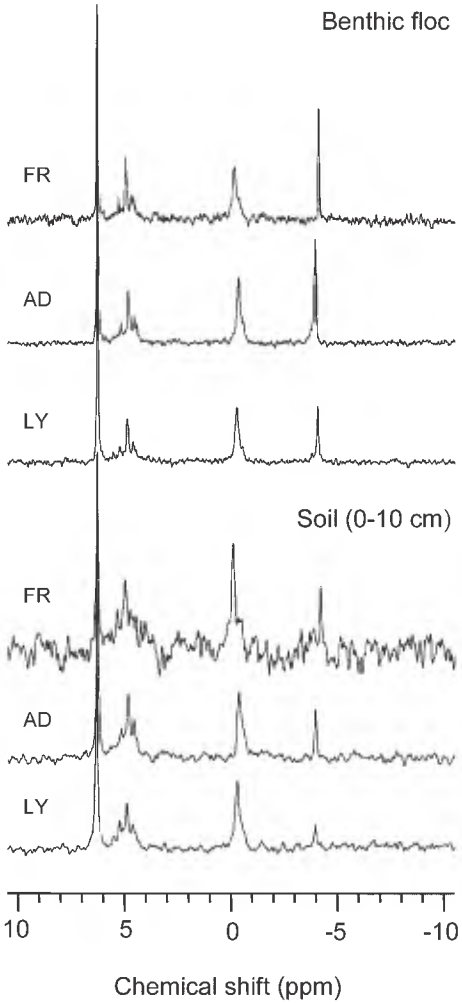

Fig. 4. Solution ^{31}P nuclear magnetic resonance spectra of NaOH–EDTA extracts of benthic floc and soil (0–10 cm) from a soft-water slough in Water Conservation Area 1 in the Florida Everglades, subjected to three different pretreatments: FR, fresh sample (overnight refrigeration at 4°C); AD, air dried for 10 d at 30°C; LY, frozen at –80°C and lyophilized. The spectra are plotted with a line broadening of 8 Hz (benthic floc) or 16 Hz (soil) and the phosphate signal adjusted to 6.3 ppm.

the likely importance of phosphate diesters in the nutrition of organisms in this strongly P-limited wetland (Turner and Newman, 2005). A possible explanation is that microbial cells in the fresh sample were protected from the relatively harsh NaOH–EDTA extractant by occlusion within the calcareous aggregates that occur as periphyton mats in unpolluted hard-water areas of the Everglades (Dodds, 2003). These aggregates would be disrupted by drying and grinding, allowing cellular material to be extracted.

Some organic P compounds, such as RNA and phospholipids, are susceptible to alkaline hydrolysis and degrade to phosphate monoesters during the analytical procedure (Makarov et al., 2002; Turner et al., 2003). No inorganic phosphate is released during this process, so the total organic P pool remains unchanged, even though the relative proportions of phosphate monoesters and diesters may vary. Degradation rates of alkali-labile phosphate diesters vary among the compounds, being in the order of minutes for RNA, but days for some phospholipids (Turner et al., 2003). The degradation of most alkali-labile compounds will therefore be complete following the relatively long extraction and NMR analysis procedures, although it is possible that variations in sample handling (e.g., time in the spectrometer, time between extraction and freezing) may influence the distribution of phosphate monoesters and diesters in samples with large concentrations of phospholipids. Strict standardization of procedures for extraction, lyophilization, and NMR analysis will therefore facilitate comparison among samples (Cade-Menun, 2005). In this respect, it should be noted that we used a wider soil/solution ratio than most studies due to the large water content of the benthic floc samples. Our 1:40 ratio was closer to that used in the development of the NaOH–EDTA extraction procedure (1:50; Bowman and Moir, 1993), so should not influence the extraction of soil organic P. It may, however, reduce the signal-to-noise ratio in solution ^{31}P NMR spectroscopy due to a lower P concentration in the freeze-dried extract.

Neither long-chain polyphosphates nor phosphonates were detected in the samples analyzed here, so no conclusions can be drawn on the effect of pretreatment on these compounds. Long-chain polyphosphates are common in aquatic sediments, but can be susceptible to decomposition depending on the type

and strength of the extractant, which appears to be linked to the presence of free Fe and is therefore minimized in extracts containing EDTA (Hupfer et al., 1995). Air drying also seems to have a negligible influence on long-chain polyphosphates and phosphonates, given that both compounds have been detected in the solution ^{31}P NMR spectra of high-organic-matter samples that had been previously air dried (Turner et al., 2004), frozen and then air dried (Cade-Menun et al., 2000), or frozen and lyophilized (Dai et al., 1996).

Most mineral soils contain abundant higher-order inositol phosphates, such as *myo*- and *scyllo*-inositol hexakisphosphate (Turner, 2007), but wetland soils from the Florida Everglades analyzed following lyophilization were previously found to contain none of these compounds (Turner and Newman, 2005; Turner et al., 2006a). This raised the possibility that inositol phosphates had formed insoluble complexes during lyophilization, which prevented their extraction in NaOH–EDTA. The results presented here suggest strongly that lyophilization was not a factor, because inositol phosphates were not detected in either fresh or dried samples from any site. A more likely explanation is that anaerobic conditions, which occur at or just below the surface of Everglades peats (Qualls and Richardson, 1995), lead to the rapid decomposition of inositol phosphates, as reported for *myo*-inositol hexakisphosphate in marine sediments (Suzumura and Kamatani, 1995).

The anaerobic nature of the soils at the time of sampling could have influenced subsequent P speciation, given that samples were not extracted under anaerobic conditions; however, sample aeration during extraction does not seem to have a detectable effect on P analysis (Qualls and Richardson, 1995). This is probably because concentrations of exchangeable Fe in Everglades samples are small, being <0.05 mg Fe(III) kg^{-1} and <1.0 mg Fe(II) kg^{-1} for both nutrient-enriched and unenriched areas (Qualls et al., 2001).

CONCLUSIONS

The effects of sample pretreatment by air drying or lyophilization on P extraction in NaOH–EDTA and its subsequent speciation by solution ^{31}P NMR spectroscopy were assessed for a series of contrasting wetland soils from the Florida Everglades. Significant changes in total P extraction occurred following pretreatment, although the changes were inconsistent and both increases and decreases were detected. For high-P samples with well-resolved spectra, differences in P composition determined by solution ^{31}P NMR spectroscopy were within the range of error associated with replicate analyses. The relatively large differences observed for low-P samples may therefore be due in part to poor spectral quality. Given the apparent sample-specific nature of the changes following drying, care is required to ensure that pretreatment artifacts are minimized for the particular samples under investigation.

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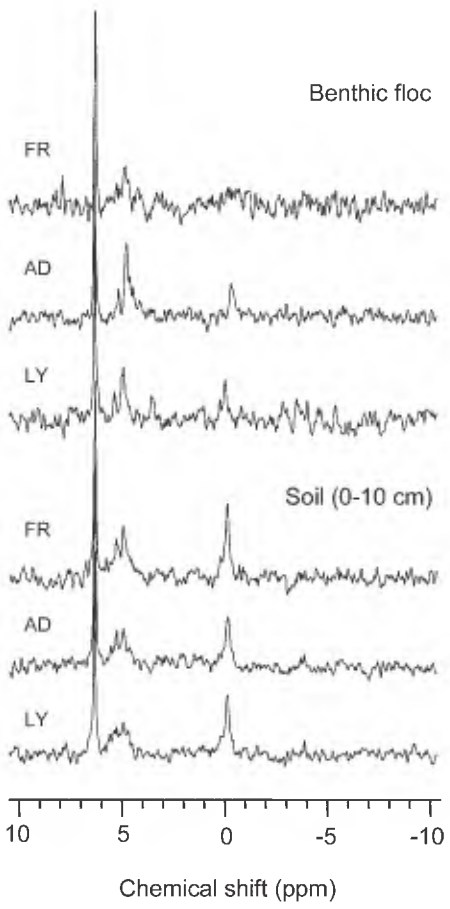

Fig. 5. Solution ^{31}P nuclear magnetic resonance spectra of NaOH–EDTA extracts of benthic floc and soil (0–10 cm) from a calcareous slough in Water Conservation Area 1 of the Florida Everglades, subjected to three different pretreatments: FR, fresh sample (overnight refrigeration at 4°C); AD, air dried for 10 d at 30°C; LY, frozen at –80°C and lyophilized. The spectra are plotted with a line broadening of 16 Hz and the phosphate signal adjusted to 6.3 ppm.

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