# Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry of Dissolved Organic Phosphorus Species in a Treatment Wetland after Selective Isolation and Concentration

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**A method for the selective concentration of dissolved organic phosphorus (DOP) from complex surface water samples for the first time allows mass spectral characterization of individual DOP compounds in phosphorus-limited ecosystems. The entire dissolved organic matter (DOM) pool is first separated according to molecular weight by tangential cross-flow ultrafiltration (CFF). DOP is selectively isolated and concentrated from CFF fractions by a barium precipitation procedure. The DOP precipitate is then reconstituted in distilled water and excess barium, and other cations are removed with an ion-exchange resin. The DOP isolation/concentration step can provide up to 15-fold concentration and 300-fold concentration of high molecular weight DOP when combined with the inherent concentration provided by CFF. The procedure also removes cations and most of the background DOM, leaving DOP in a matrix suitable for electrospray ionization and mass spectral characterization. Model organic phosphate standards representative of DOP species expected in aquatic environments were used to evaluate the technique. It was then applied to a series of high molecular weight (>1000) CFF retentates isolated from sites within the Everglades Nutrient Removal (ENR) treatment wetland. The elemental compositions of several individual DOP compounds observed at different sites within the ENR were determined by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry.**

Nutrient loading from anthropogenic sources has become a significant problem in many ecosystems, and especially in the Florida Everglades. Increases in phosphorus have been linked to alterations in this naturally oligotrophic ecosystem, including taxonomic shifts and eutrophication.[1–3] Large areas of the Everglades once covered by marsh grasses have been replaced by dense cattail stands that dominate the once diverse wetland ecosystem. Many studies of other impacted ecosystems have also linked similar effects to increases in total phosphorus (TP) concentrations.[4,5]

Total phosphorus is generally reported as the sum of various classes of chemically distinct P, including total inorganic, dissolved inorganic, soluble reactive, particulate inorganic, total organic, dissolved organic, and particulate organic.[6] Although such speciation information can provide some useful insights into the environmental chemistry of phosphorus, it does not address the chemical reactivity and biological availability (bioavailability) of specific forms of P. These fundamental chemical and biological issues must be addressed with analytical techniques that provide qualitative and quantitative information on molecular speciation of phosphorus. However, understanding the speciation of organic phosphorus in an oligotrophic, phosphorus-limited environment such as the Everglades is a challenging analytical problem because total phosphorus concentrations are inherently low. In the Everglades, for example, "background" natural surface water levels of total phosphorus in unimpacted, pristine areas are ~10 ppb. Dissolved organic phosphorus (DOP) usually represents 10–50% of this total P, and thus, the concentration of any single DOP compound is well below 1 ppb.

The Everglades Nutrient Removal (ENR) project is a major component of the Everglades Restoration Project. The ENR is the largest constructed treatment wetland in North America, covering a total of 3813 acres, and it is now a fully functional Stormwater Treatment Area.[7] In the ENR, bioavailable phosphorus is converted to plant biomass and removed from the water budget.

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Presently, the target concentration at the ENR outflow is 50 ppb total phosphorus. However, this number does not take into account the various phosphorus species in the ENR outflow and their bioavailability.

During its demonstration phase, the ENR achieved up to 80% reduction in total P on inflow primarily from the Everglades Agricultural Area.[7] Total P concentrations of ~75−175 ppb in the ENR inflow were reduced to less than the 50 ppb target level in the outflow. As expected, phosphorus speciation also changed between inflow and outflow. Dissolved inorganic phosphorus (DIP) was the predominant form of phosphorus entering the ENR, whereas DOP dominated the outflow.[8] Furthermore, low molecular weight DOP (<1000) was significantly reduced, while high molecular weight (high-MW) remained fairly constant. Finally, only ~27% of this high-MW DOP was biologically labile to mono- or diester phosphatase enzymes.[39]

Before any molecular identification of individual DOP compounds in an oligotrophic system such as the Everglades can be accomplished, concentration of DOP from sub-ppb levels must be achieved. Tangential cross-flow filtration (CFF) has become a popular method for fractionating natural dissolved organic matter into molecular weight fractions.[10−12] CFF can also concentrate organic species retained by the membrane (i.e., the "retentate") by up to 20-fold. Unfortunately, this improvement is not sufficient to characterize organic phosphorus compounds in oligotrophic systems at sub-ppb levels. Furthermore, we have found that the background organic matter component isolated by CFF is typically 1000-fold higher in concentration than DOP. This background organic matter is a serious interference for direct mass spectral analysis by electrospray ionization. A method that further concentrates DOP and isolates it from the high-level organic matter background is therefore necessary before identification of individual DOP compounds can be accomplished.

We have modified and added to a method originally developed by McKercher and Anderson[13,14] for the extraction of inositol penta- and hexaphosphates from soil samples. Our method both concentrates DOP from water and isolates it from most of the background organic matter. The new method also includes a cation removal step resulting in a DOP concentrate suitable for electrospray ionization (ESI).

Electrospray ionization offers two great advantages over other commonly used mass spectral ionization techniques.[15−17] First, ESI is very efficient at ionizing large, charged solutes in aqueous samples and introducing them into the gas phase. Second, it is a very "soft" ionization technique because little if any energy is imparted to solutes. Thus, parent ions remain intact and can be identified.

ESI coupled to Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) is a powerful tool for determining the elemental compositions (chemical formulas) and molecular structures of large organic molecules.[18] In recent years, the ability of FTICR MS to identify molecules in complex mixtures has been realized and applications in biological and environmental mass spectrometry have become commonplace.[19−23] The soft ionization capabilities of ESI allow exploitation of high-mass resolving power (up to $m/\Delta m_{50\%} > 8\,000\,000$ at 9 kDa),[24] high-mass resolution (less than 0.0005 Da at $m/z$ 904),[25] and high-mass accuracy (error associated with mass determination better than 1 ppm) of FTICR MS.[26]

## EXPERIMENTAL SECTION

The systematic process of sample collection, treatment, and analysis is described in detail in the following paragraphs. This process is schematically represented by the flowchart in Figure 1.

**Sample Collection.** Surface water samples were obtained at four sites within the ENR (Figure 2), the sites representing a gradation in residence time. It would normally be difficult to obtain representative samples from an area as large as the ENR. However, the control and conveyance structures in place there allow water representative of different treatment stages to be sampled very efficiently. We sampled the following conveyances: G 303 (inflow; no treatment), G 253 (conveyance linking cells 1 and 3; one stage of biological treatment), G 256 (cell 4 outflow; one stage of biological treatment with additional physical and biological "polishing"), and ENR 012 (total ENR outflow that includes completely treated water from two separate hydraulic flow paths). Samples were collected with a peristaltic pump and filtered through a 0.2-$\mu$m Gelman SuporCap (polyethersulfone) cartridge filter. Samples were immediately put on ice and kept in the dark until their return to the laboratory.

Organic matter in these samples was then separated according to molecular weight by tangential cross-flow filtration (CFF). Other groups have used molecular filtration methods as well to determine which fractions of phosphorus are present as high or low molecular weight species or to preconcentrate the sample prior to analysis.[27] It is important to note that sample alterations can occur during the filtration process. Tangential flow helps eliminate this problem. The CFF system used in this experiment was a Filtron Centrasette system, comprising four stacked 0.5-m$^2$

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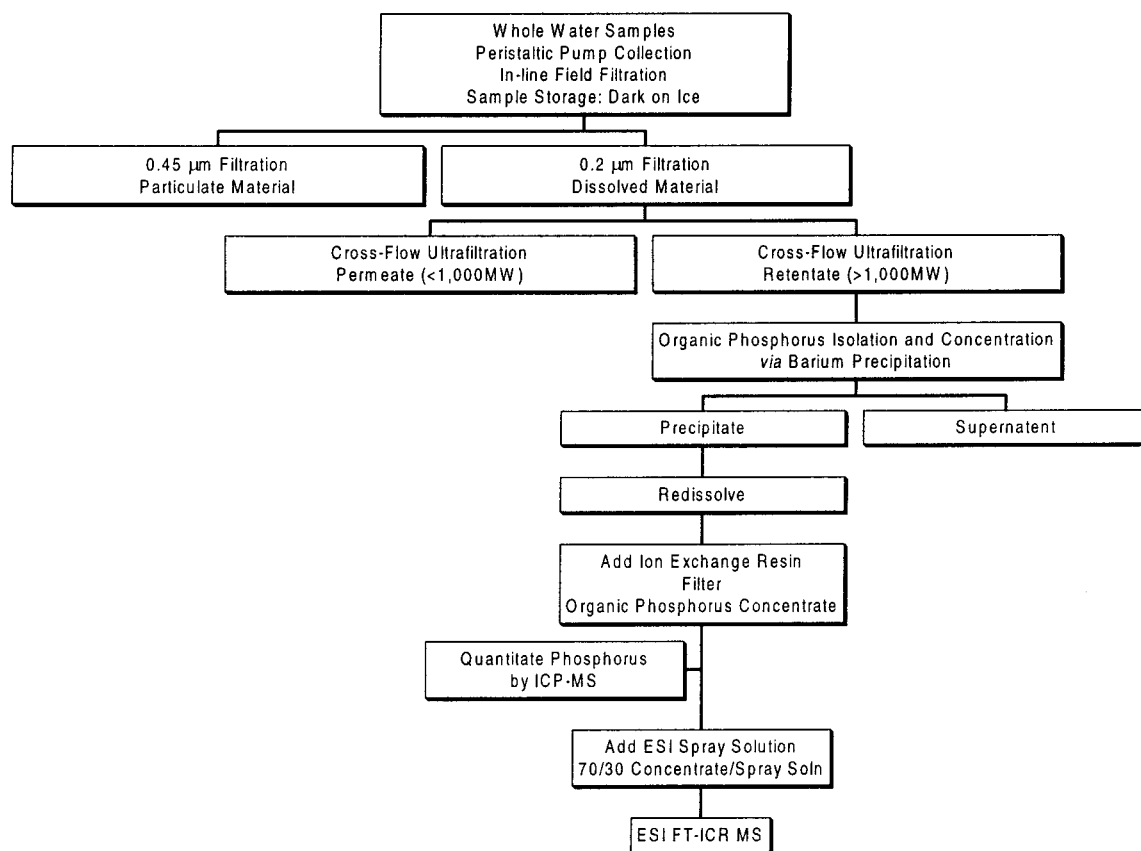
**Figure 1.** Flowchart illustrating sample treatment from collection through ESI FTICR MS analysis.

polysulfone membranes with nominal molecular weight cutoff of 1000. The retentate portion of sample (>1000 Da) was passed repeatedly across the membrane and concentrated approximately 10–15-fold. Retentate was collected and retained for further analysis.

**Phosphate Isolation Procedure.** Organic phosphate compounds were isolated from the complex surface water mixture by formation of an insoluble barium–phosphate complex. A 30% (w/v) solution of barium acetate (Fisher Scientific, Fairlawn, NJ) was prepared with pure (18 M$\Omega$) Millipore water (Millipore, Bedford, MA). The filtered surface water sample was brought to a pH between 8 and 9 and allowed to equilibrate for 15 min. Ethanol was added to the sample at a volume of 10% relative to the sample volume. The barium acetate solution was added in excess of the volume of the ethanol. The sample was covered with aluminum foil to reduce evaporation and then placed in a boiling water bath for 10 min to induce flocculation, after which the sample was removed from the water bath and allowed to sit overnight (~16 h). The precipitate was collected on 0.45-$\mu$m filter paper and then redissolved with pure water containing a strongly acidic (H$^+$ form) cation-exchange resin and the mixture shaken until completely dissolved. The resin removes barium as well as other cations from the organic species and is itself removed from the final concentrated solution by filtration. The resulting solution contains the isolated dissolved organic phosphates and little of the background dissolved organic matter (DOM), reducing interferences in FTICR MS analyses. Pure water was reintroduced to the sample to adjust the concentration. For example, if a sample was originally 70 mL, 7 mL of water can be used to redissolve the precipitate, effectively concentrating the sample 10-fold.
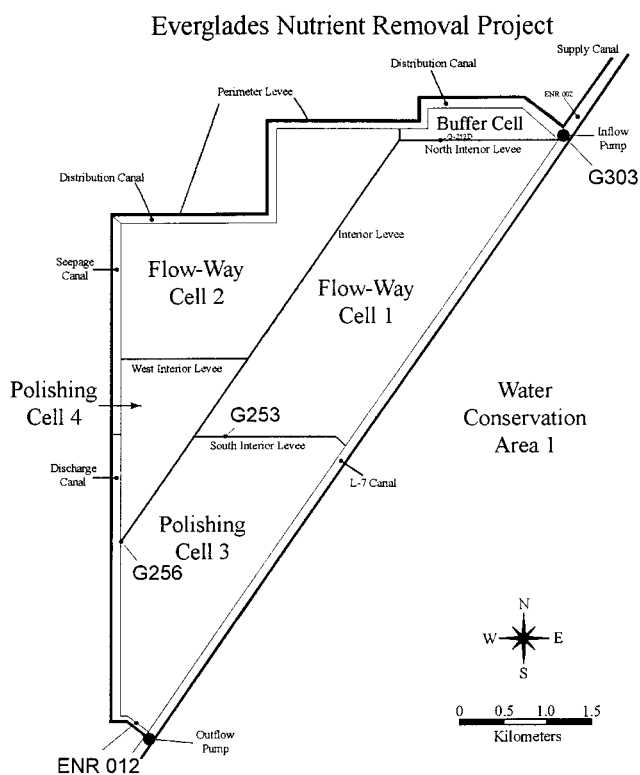


**Figure 2.** Map of the Everglades Nutrient Removal project. Samples were obtained from four ENR sites: G 303 (inflow), G 253 (cells 1–3), G 256 (cell 4 outflow), and ENR 012 (total ENR outflow).

A solution of model dissolved organic phosphates (all from Sigma, St. Louis, MO) was used to test the reliability of the barium

**Table 1. Elemental Compositions and Monoisotopic Masses of Standard Organic Phosphates Used to Test the Barium Precipitation Procedure.**

| compound | elemental composition | monoisotopic mass (Da) |
|---|---|---|
| o-phospho-DL-tyrosine | $C_9H_{12}NO_6P$ | 261.0402 |
| phosphoenolpyruvate | $C_3H_5O_6P$ | 167.9824 |
| D-ribose 5-phosphate | $C_5H_9O_8PNa_2$ | 273.9831 |
| adenosine monophosphate | $C_{10}H_{14}N_5O_7P$ | 347.0631 |

precipitation method for providing phosphates in a matrix suitable for mass spectral analysis by ESI FTICR MS. The phosphate standards were chosen so that they would model the behavior of a broad spectrum of DOP compounds that have been previously identified in surface water samples.[6,28] The compositions and exact masses of the standards used are summarized in Table 1. Unfortunately, because so few organophosphorus compounds have actually been identified in natural samples, we were unable to develop a standard mixture that matched the molecular weight range we targeted with our sampling procedure.

**Phosphorus Quantitation.** The total phosphorus concentration of the ENR samples was measured by high-resolution inductively coupled plasma mass spectrometry (ICPMS), (Element, Finnigan MAT, Bremen, Germany). The instrument is located in the Geochemistry Laboratory at the National High Magnetic Field Laboratory (NHMFL) and has been described in detail elsewhere.[29] Briefly, instrument design is a reversed Nier−Johnson geometry that utilizes magnetic and electrostatic sectors to achieve mass resolving power up to 8000. For phosphorus measurements, the instrument is operated in the medium-resolution mode ($m/\Delta m = 3000$), which is sufficient to resolve elemental P ions from interfering oxides [NO, O$_2$] that are present at high concentrations. Samples were prepared for ICPMS analysis by adding 1−2% quartz-distilled nitric acid (Seastar, Sidney, BC, Canada) for improved nebulization. An indium ($^{115}$In) standard was added to each sample at a concentration of 1 ppb for internal calibration. Phosphorus standards (10 ppm in 2% HNO$_3$) (High Purity Standards, Charleston, SC) and a blank were prepared in the same manner as the samples.

**Mass Spectral Characterization.** Mass spectral characterization of model organic phosphates as well as DOP isolated from ENR samples was carried out by positive-ion ESI FTICR MS. ESI FTICR mass spectra were collected on a home-built mass spectrometer located in the Center for Interdisciplinary Magnetic Resonance at the NHMFL.[30] The instrument is equipped with a 9.4-T superconducting magnet. Ions are produced externally in the electrospray source and accumulated in a 45-cm octopole before reaching a second 200-cm-long octopole which transfers ions into an open cylindrical Penning trap.[31]

Ionization was achieved by an electrospray ionization source with a voltage of between 1800 and 2200 V (to optimize ionization efficiency) applied to the needle, and a counter voltage of 450 V

applied to the capillary. Samples were prepared by adding a spray solution of 49:49:2 (v/v/v) water/methanol/acetic acid to the sample at a v/v ratio of 30% to give the best signal-to-noise ratio.

Internal calibration was achieved by use of poly(ethylene glycol) 400 (PEG) with a number-average molecular weight $\overline{M}_n = 400$.[32,33] PEG ions span the mass window of interest such that all ions in a spectrum can be calibrated. Internal calibration reduces errors associated with measured mass to better than 1 ppm. Thus, unambiguous elemental compositions can be assigned.

## RESULTS AND DISCUSSION

**Mass Spectral Characterization of Standards before and after Barium Precipitation.** Since the primary goal of the barium precipitation isolation step is to produce a concentrated DOP solution that can be effectively electrosprayed and characterized by FTICR MS, the first evaluation involved comparison of mass spectra before and after the standard mixture was carried through the isolation procedure (Figure 3). For this evaluation, the mixture was reconstituted to the same volume as the original sample and thus no concentration is involved.

The mass spectrum of the mixture before precipitation (Figure 3, top) shows that D-ribose 5-phosphate was not detected as the monomer but rather as a dimer in the gas phase [2RP + H]$^+$. Adenosine monophosphate [AMP] was seen as both the protonated [AMP + H]$^+$ form and a sodiated, [AMP + Na]$^+$ form. Phosphoenolpyruvate was detected only as the sodiated form [PEP + Na]$^+$, whereas tyrosine phosphate was observed as the protonated form [TP + H]$^+$. In addition, electrospray ionization efficiencies of organophosphates do not appear to be particularly high. With the exception of AMP, the relative abundances of ions associated with the standard compounds are generally less than the relative abundances of peaks associated with degradation products or other organic contamination in the mixture. These observations further reinforce the need for selective concentration of DOP. Fortunately, inspection of the bottom spectrum in Figure 3 suggests that the barium precipitation procedure does not alter the electrospray ionization characteristics of these compounds. Thus, comparison of spectra of natural DOP isolated by this technique with spectra of standard compounds for unequivocal molecular identifications should be possible.

**Quantitative Concentration and Isolation of High Molecular Weight DOP from ENR Retentates.** Retentate samples from CFF processing of water at site ENR 012 were used to probe the quantitative reliability of concentrating DOP by barium precipitation. Figure 4 is a plot of total P concentration in the final solution as a function of concentration factor, namely, the volume of original solution divided by volume of final solution. Barium precipitation appears to produce a quantitatively reliable concentration factor of at least 15-fold for DOP in these natural, complex samples. Concentration beyond this factor of 15 is difficult because redissolution of the precipitate is not always possible.

It should be noted that the data presented in Figure 4 reflect only the inherent concentration effect on DOP provided by the

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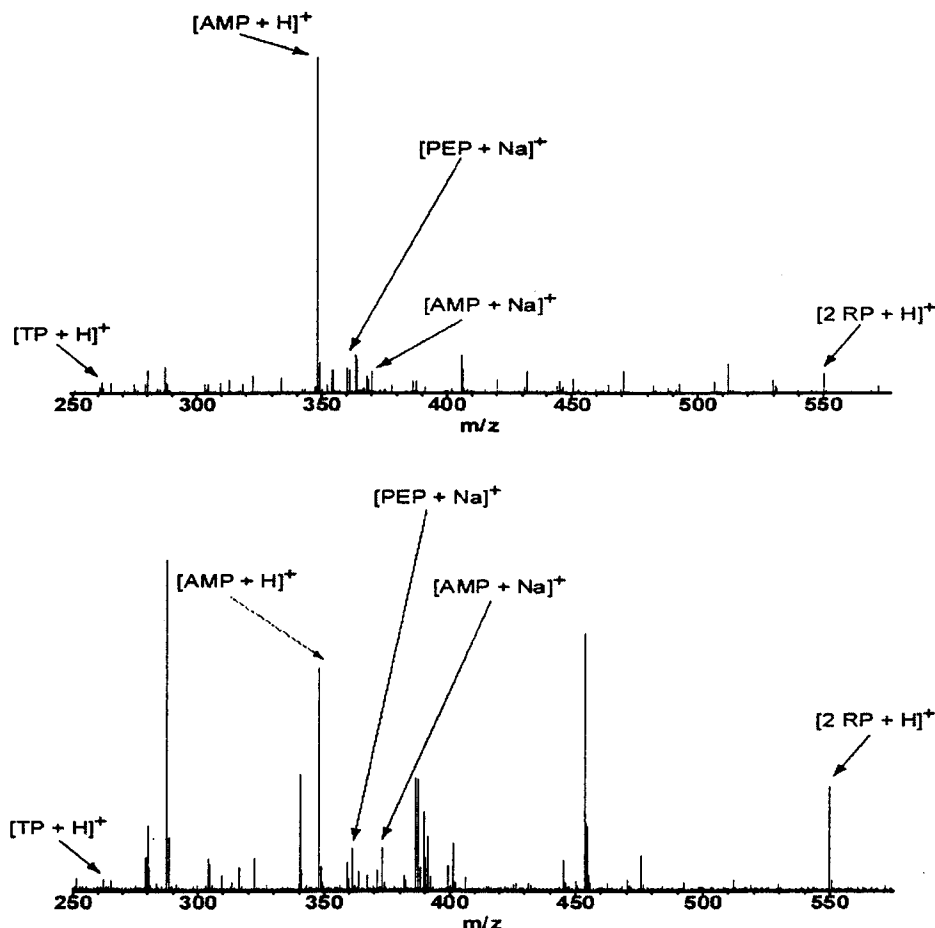
**Figure 3.** Positive ion electrospray ionization 9.4-T FTICR mass spectra showing the standard DOP mixture [tyrosine phosphate (TP), adenosine monophosphate (AMP), phosphoenolpyruvate (PEP), and ribose phosphate (RP)] prior to barium precipitation (top) and following barium precipitation (bottom).
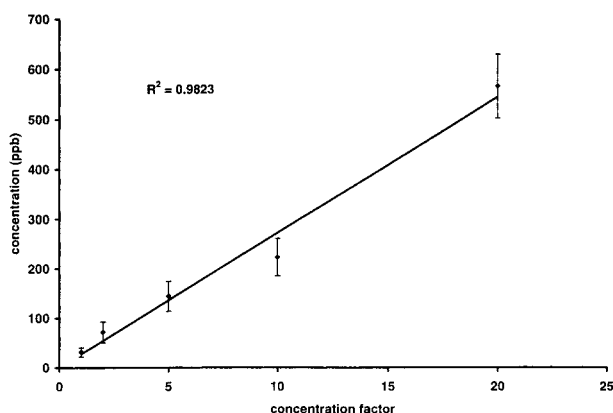


**Figure 4.** Phosphorus concentrations (as total P) in high-MW retentates isolated from water samples at ENR site ENR 012 after barium precipitation and redissolution. Concentration factor is the volume of initial sample divided by volume of final concentrate. Total P measurements were made by ICPMS.

barium precipitation step. In addition, CFF processing concentrates the total high-MW DOM fraction by a factor of ~15 so that, when combined with concentration by barium precipitation, high-MW DOP has been effectively concentrated by a factor of 300. Another benefit of CFF processing is that, since the retentate is being continuously rinsed, low molecular weight species that often limit mass spectral signal-to-noise ratio and dynamic range are

greatly reduced in the retentate—particularly important for electrospray ionization, in which salts seriously degrade ionization efficiency.

The advantages of removing DOM in the final concentrate are demonstrated in Figure 5, which shows FTICR mass spectra of a retentate sample obtained in December 1998 from site ENR 012 before and after the DOP was isolated by barium precipitation. Reducing the number of (other) ions in the analyzer cell significantly improves signal-to-noise ratios and mass resolution and allows the determination of unambiguous molecular formulas for many compounds. Table 2 lists a number of phosphorus-containing compounds that could be identified in the ENR 012 retentate after barium isolation and concentration. It should be noted that the phosphorus isolation procedure is not completely selective, and a number of compounds with no phosphorus were also identified in the bottom spectrum of Figure 5.

**Identification of Individual DOP Compounds in high-MW Retentates from the ENR.** ESI FTICR mass spectra were obtained on DOP isolates from each of four ENR sites sampled in March 2000. From those spectra, we were able to identify seven phosphorus-containing elemental compositions present at all sites at that time. The exact masses of these species, along with the theoretical masses of the closest elemental compositions, are listed in Table 3. These data demonstrate the utility of high-field FTICR MS for screening individual organophosphorus compounds in
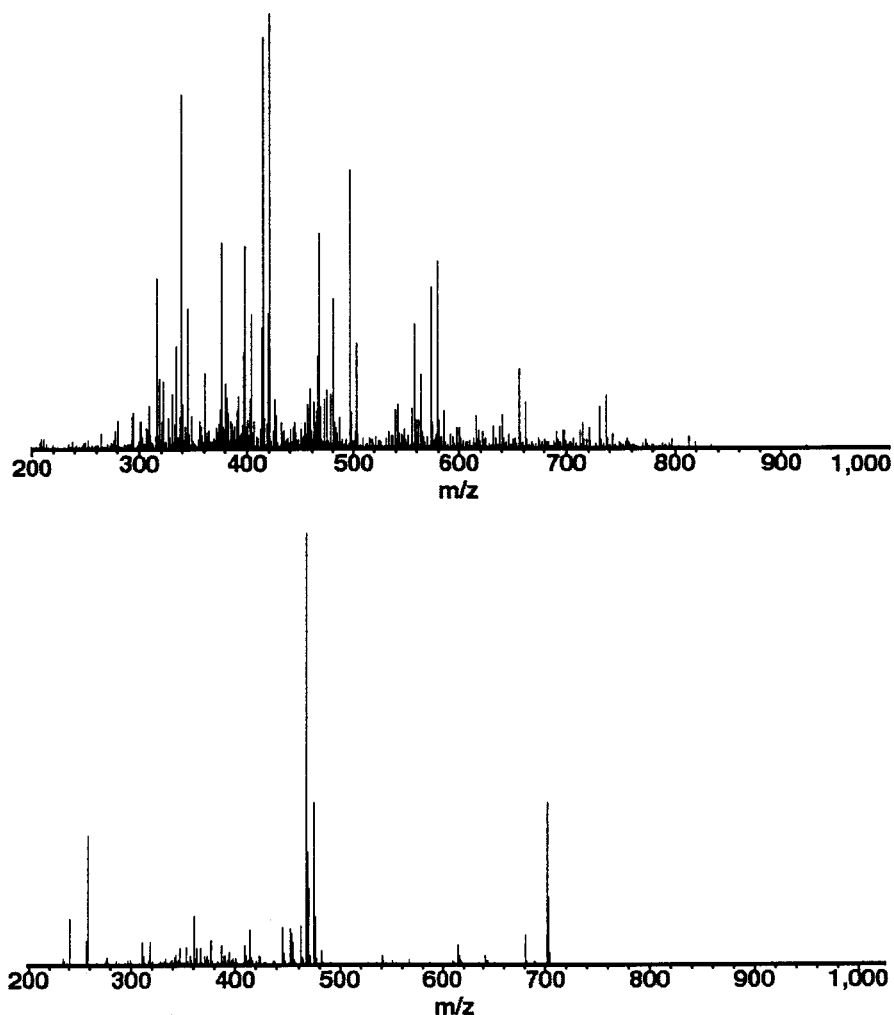
**Figure 5.** ESI FTICR MS spectra of site ENR 012 surface water retentate sample before (top) and after (bottom) barium precipitation and redissolution.

**Table 2. Elemental Compositions of Phosphorus-containing Ions Identified in ENR 012 Retentate Sample after Barium Precipitation Procedure[a]**

| measured mass (Da) | elemental composition |
| --- | --- |
| 626.4261 | $C_{29}H_{60}N_6O_5PNa$ |
| 612.4104 | $C_{28}H_{58}N_6O_5PNa$ |
| 534.2332 | $C_{22}H_{36}N_6O_6PNa$ |
| 478.1189 | $C_{13}H_{24}N_6O_{10}PNa$ |
| 467.1186 | $C_{13}H_{24}N_6O_{10}PNa$ |
| 464.1033 | $C_{12}H_{22}N_6O_{10}PNa$ |
| 450.0876 | $C_{11}H_{20}N_6O_{10}PNa$ |
| 442.1369 | $C_{18}H_{28}O_9PNa$ |
| 436.0712 | $C_{23}H_{17}O_7P$ |
| 434.1090 | $C_{16}H_{23}N_2O_{10}P$ |
| 424.0712 | $C_{22}H_{17}O_7P$ |
| 388.0712 | $C_{19}H_{17}O_7P$ |
| 376.0032 | $C_8H_{10}N_4O_{10}PNa$ |
| 348.0399 | $C_{16}H_{13}O_7P$ |
| 288.0460 | $C_4H_{10}N_8O_4PNa$ |
| 241.0001 | $C_6H_4N_5O_4P$ |

[a] All masses determined with error less than +1 ppm.

**Table 3. Elemental Compositions of Seven Phosphorus-containing Ions Identified in All Four ENR Samples[a]**

| measured mass (Da) | theoretical mass (Da) | elemental composition | error (ppm) |
| --- | --- | --- | --- |
| 592.1506 | 592.1504 | $C_{18}H_{30}N_6O_{13}PNa$ | - 0.3 |
| 577.2025 | 577.2023 | $C_{22}H_{33}N_7O_8PNa$ | - 0.4 |
| 518.1315 | 518.1313 | $C_{21}H_{23}N_6O_8P$ | - 0.2 |
| 451.1053 | 451.1052 | $C_8H_{19}N_{11}O_8PNa$ | - 0.3 |
| 444.1131 | 444.1129 | $C_{11}H_{17}N_{12}O_6P$ | - 0.6 |
| 369.0950 | 369.0950 | $C_{12}H_{16}N_7O_5P$ | 0.0 |
| 364.1287 | 364.1288 | $C_{15}H_{25}O_8P$ | + 0.7 |

[a] All masses determined with error less than +1 ppm.

Tandem FTICR MS/MS experiments are currently underway to confirm the elemental composition assignments and to identify structural groups for these species. However, even preliminary data on elemental compositions reveal some interesting features of DOP cycling in the ENR. First, since the compounds listed in Table 3 were identified in isolates from all four sites within the ENR, they would appear to be a part of the refractory high-MW DOP pool which is nonlabile to mono- and diester phosphatase enzymes.[9]

Second, although all of the phosphorus-containing compounds were isolated from the high-MW (>1000) organic matter fraction,

aquatic environments. However, the ultrahigh resolution capabilities of FTICR MS can be realized only by reducing the total number of ions in the ICR cell, as with the barium precipitation procedure described here.

all have apparent masses less than 1000 Da. While this observation might seem contradictory, it is in agreement with other ESI-MS analyses of humic materials.[19,34−37] One explanation is that these compounds may be linked to larger humic-like substances through ionic interactions that are disrupted during electrospray ionization. Alternatively, it has been suggested that electrospray ionization, even though considered an ideal technique for introducing high molecular weight compounds into a mass spectrometer, may actually produce a low molecular weight bias. This low molecular weight bias has been attributed to inadequate accounting of multiply charged ions; that is, if ions have two or more charges, then the observed $m/z$ is not the actual mass.[35] However, we are able to rule out that possibility because we have sufficient resolution to identify the spacings between monoisotopic M (those with only $^{12}$C atoms) and "heavy" M + 1 peaks (those with $^{13}$C substitutions). Virtually all the peaks we have identified have M + 1 peaks that are located at 1 Da higher mass than the corresponding M peak; if the peaks were doubly charged, these spacings would be $^{1}/_{2}$ Da.

Other sources of a possible low molecular weight bias include charge suppression and molecular fragmentation. Not all ions are ionized with equal efficiency in ESI, resulting in what is termed charge suppression. In positive ion mode ESI, the molecules with the greatest gas-phase basicity are ionized first.[38] Since a limited amount of positive charge is available, there may not be enough charge to ionize all analytes or to provide multiple charges for the heavier ones. This ionization suppression effect has been observed for humics,[34] where the addition of NaCl or NaOH leads to salt−solvent adducts that completely obscured the humic signal.

Molecular fragmentation during electrospray ionization is more difficult to address, since there are essentially no known high molecular weight humic materials whose structures are known unambiguously. We are currently studying this problem by use of high molecular weight, covalently linked polymers such as poly-(ethylene) glycols whose molecular formulas can be obtained from a mass spectrum. Results from those experiments will be forthcoming shortly.

## CONCLUSIONS

A procedure that both concentrates dissolved organic phosphorus from surface waters and isolates it from the background dissolved organic matter has been developed. The result is DOP concentrated up to 15-fold and in a matrix suitable for mass spectral analysis by electrospray ionization. Isolation of DOP from the background dissolved organic matter also greatly simplifies mass spectral identification of individual DOP compounds.

The barium precipitation procedure has been combined with tangential cross-flow ultrafiltration to concentrate and isolate high molecular weight ($>1000$) organic phosphorus compounds by 300-fold. High-MW DOP originally at concentrations of a few ppb P are at ppm P concentrations in the final matrix, allowing mass spectral characterizations of some individual DOP compounds. DOP concentrates from four sites in the Everglades Nutrient Removal project were analyzed by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. The ultrahigh resolving power and mass accuracy of FTICR MS allowed determination of the chemical formulas of several DOP compounds identified at all four sites.

These compounds appear nonreactive during their residence in the ENR, in agreement with recently completed studies of DOP speciation in the ENR.[39] Those studies revealed that the ENR had little if any effect on the concentration of high-MW DOP. Furthermore, most of the high-MW DOP was not hydrolyzable by mono- and/or diester phosphatase enzymes. The significance of the results reported here is that, for the first time, specific organic phosphorus compounds within a pool of nonbiologically available DOP have been identified. The techniques described here should apply to other pools of DOP, including reactive, low molecular weight organic phosphorus, and can play an important role in understanding the molecular basis of phosphorus cycling and bioavailability in the aquatic environments.

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