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Size exclusion chromatography with online ICP-MS enables molecular weight fractionation of dissolved phosphorus species in water samples

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ABSTRACT

Phosphorus (P) is an important and often limiting element in terrestrial and aquatic ecosystem. A lack of understanding of its distribution and structures in the environment limits the design of effective P mitigation and recovery approaches. Here we developed a robust method employing size exclusion chromatography (SEC) coupled to an ICP-MS to determine the molecular weight (MW) distribution of P in environmental samples. The most abundant fraction of P varied widely in different environmental samples: (i) orthophosphate was the dominant fraction (93–100%) in one lake, two aerosols and DOC isolate samples, (ii) species of 400–600 Da range were abundant (74–100%) in two surface waters, and (iii) species of 150–350 Da range were abundant in wastewater effluents. SEC-DOC of the aqueous samples using a similar SEC column showed overlapping peaks for the 400–600 Da species in two surface waters, and for >20 kDa species in the effluents, suggesting that these fractions are likely associated with organic matter. The MW resolution and performance of SEC-ICP-MS agreed well with the time integrated results obtained using conventional ultrafiltration method. Results show that SEC in combination with ICP-MS and DOC has the potential to be a powerful and easy-to-use method in identifying unknown fractions of P in the environment.

1. Introduction

Phosphorus (P) is an important and often limiting element in terrestrial and aquatic ecosystem and is at the center of important environmental challenges from a perspective of excess and scarcity. Phosphorus is essential because of its biochemical role in RNA, DNA and cellular processes (Gifford et al., 2015). Modern agriculture heavily relies on (mineral) phosphate fertilizers to achieve high yields and feed an ever-growing population (Rittmann et al., 2011; Venkatesan et al., 2016). Excess organic and inorganic phosphate, mainly from plant and animal agricultural waste streams, enters waterways and leads to algae blooms in fresh and seawater that threaten ecosystems. While the most advanced wastewater treatment systems remove (and in some cases attempt to capture and reuse) inorganic P reasonably well, they still discharge organic P (OP) into waterways (Mayer et al., 2016). Research has been performed on eutrophication and P cycling in estuaries, wetlands, lakes etc. over the last decade and identified the bioavailability of OP as a major and underestimated issue (Björkman and Karl, 2003; Ni et al., 2016; Pant et al., 2002). OP is known to be a complex mixture of many different species including DNA and RNA fragments, phospholipids, phosphoproteins, and phosphate esters (Baldwin, 2013; Worsfold et al., 2008). In addition to the unknown fractions of OP, total P (TP) in environmental systems is also comprised of condensed inorganic phosphates (polyphosphates) and colloidal P (Moorleghem et al., 2011). Studies have addressed the mineralization of these unknown forms, but all from a rather coarse perspective as chemically the OP and colloidal P remains poorly characterized. The complexity and lack of chemical information on TP (organic and some inorganic) in the environment, hampers our understanding of the mineralization of the material, its dynamic partitioning in water/sediment/particle systems and eventually its lifetime. The lack of understanding of the P structures (molecular weight distribution, functional group composition), also limits the design of P mitigation and recovery approaches.

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The various fractions of P in the environment are often determined via indirect methods. For example, dissolved organic phosphorus (DOP) measurement in the environment is calculated either as the difference between total dissolved P (TDP) and dissolved reactive P (DRP), or measured in terms of DRP after pretreatment or digestion of samples (Worsfold et al., 2008). Such measurements, however, fail to provide fundamental chemical data on the diverse nature of TP (e.g. size distributions, functional groups, etc.). The most common tool used in the study of the speciation of P in terrestrial systems is ^{31}P NMR spectroscopy. The method is well established and showed that typically a large part of P is under the form of orthophosphate and polyphosphate (Cade-Menun, 2005). NMR also allowed demonstrating the presence of phosphate esters, DNA, RNA, phospholipids and glycophosphates, all derived from biological processes (Bell et al., 2017). Advanced (high resolution) mass spectrometry was only attempted in a few studies but allowed for the discovery of select biomarker species. Most of the characterization work focused on soils and sediments, and less research is available for ^{31}P NMR and MS on organic matter extracted from water, in part because isolation and concentration techniques have not been developed to target P like they have been for bulk carbon or nitrogen (Aiken et al., 1992; Chin et al., 1994; Herckes et al., 2007; Leenheer et al., 2007).

Ion/liquid chromatography coupled to inductively coupled plasma mass spectrometry (ICP-MS) have been successfully applied to determine the speciation of P in soils and foodstuffs (Koplík et al., 2002, Persson et al., 2009; Shah and Caruso, 2005). The advantage of using ICP-MS is that it detects TDP (both organic and inorganic) in samples unlike commonly used spectrophotometric and colorimetric P methods that detect only orthophosphates. Chromatographic separation prior to TDP analysis by ICP-MS could serve as a powerful and robust tool to conveniently analyze various fractions of P in the environment. Two studies have successfully applied size exclusion chromatography (SEC)-ICP-MS technique to investigate the soluble P species and other elements in soybean flour, white bean seeds, and barley grain tissues (Koplík et al., 2002, Persson et al., 2009). By using SEC-ICP-MS, the authors were able to determine the molecular weight (MW) distribution of P in food samples in a single run without the need for complex and successive ultrafiltration techniques. Though SEC coupled to an organic carbon detector (SEC-DOC) is a commonly used technique in determining the MW distribution of DOC in waters (Her et al., 2003; Nam et al., 2008; Wang et al., 2013), to the best of our knowledge, a similar approach has not been applied for the determination of size distribution of P in environmental waters.

The goal of this study was to develop a robust SEC-ICP-MS method to conveniently determine the MW distribution of P in environmental matrices, specifically in waters. The developed method was tested on surface waters, primary and secondary wastewater effluents, aerosols, and wetland samples to identify the abundant MW fraction of P in these environmental matrices. The performance of the SEC-ICP-MS method was compared against a typically used ultrafiltration technique for MW fractionation. SEC-DOC was additionally applied to aqueous samples in order to qualitatively determine the fraction of P associated with organic matter. Though this method does not directly identify OP in samples, it provides critical molecular information and improved understanding of the dynamic nature of P in the environment.

2. Material and methods

2.1. Chemical reagents

All chemicals used were ACS grade and purchased from Sigma-Aldrich (MO, USA) unless specified otherwise. Trace metal grade hydrochloric acid (33–36%) was purchased from J.T. Baker (Ultrex II, JT Baker Inc., NJ, USA). Deionized water (>18.3 MΩ cm, NANOpure Infinity, LA, USA) was used throughout the experiment.

2.2. SEC-ICP-MS

Chromatographic separation was performed using Toyopearl HW-50S resin (20–40 μm size exclusion resin; hydroxylated methacrylate matrix). The resin was gravity packed in a stainless-steel column (21.2 × 250 mm, Hamilton Company, NV, USA). The media and dimension of the column was selected to replicate an existing SEC column in our laboratory that is currently used for SEC-DOC analysis (see section below). The instrumental set-up consisted a Finnigan SpectraSYSTEM high pressure pump (Thermo Fisher, MA, USA), a Rheodyne 7125 sample injector with a 1 mL PEEK sample loop (Rheodyne, CA, USA), the SEC column, and a Thermo Scientific (Waltham, MA, USA) X-Series II ICP-MS as the detector. The column outlet was connected to a T-connector, with one outlet connected to the ICP-MS and the other outlet into a waste-container. An injection volume of 1 mL was used and the flowrate of the mobile phase was set at 1 mL/min.

A set of polyethylene glycol (PEG) standards of molecular weights from 200 to 10,000 g/mol was used as markers to calibrate the column. In addition to PEG, sodium phosphate glass (Type 45; MW 4652 g/mol), L-α-phosphatidylcholine (MW 768 g/mol; Critical Micelle Concentration (CMC): 20–200 μM), myo-inositol hexakis(dihydrogen phosphate) (sodium salt of phytic acid; MW 660 g/mol) and dipotassium phosphate (PO_4^{3-}; MW 94.97 g/mol) were used as P-markers for calibration. For calibration with PEG, an evaporative light scattering detector (Model 2424, Waters, MA, USA) was used. Calibration using P-markers was performed with ICP-MS operating in time-resolved analysis (TRA) mode by monitoring ^{31}P. A 200 ms dwell time was used with a total runtime of 100 min. PlasmaLab software (Thermo Fisher, MA, USA) was used to integrate the area under the peaks. The ICP-MS was tuned in normal mode prior to each day's run. Concentration of various MW fraction of P was calculated using equation (1):

$$C_{MW} = TDP* \left(\frac{Peak\ Area_{MW}}{\sum All\ Peaks}\right)*R \quad (1)$$

Where, C_{MW} is the concentration of specific MW fraction of P in sample, TDP is the total P measured by ICP-MS in normal mode, $Peak\ Area_{MW}$ is the integrated peak area of the specific MW fraction at its corresponding retention time, $\sum All\ Peaks$ is the sum of all P peaks detected in the sample (i.e. total P area), and R is the recovery of the fraction accounting for the analyte loss in the system. When overlapping peaks of P were observed in the analysis, deconvolution of the chromatographic peaks was conducted using the software Peakfit® (Version 4.12) (see supplemental information).

2.3. Environmental samples

Grab samples from three surface waters (Tempe Town Lake, AZ; Tempe Canal, AZ; Lake Alice, Everglades, FL), and primary and secondary effluent from a wastewater treatment plant (WWTP; in Central, AZ) employing a conventional activated sludge process, were collected and analyzed within 48 h of sample collection. Samples were stored at 4 °C prior to analysis. In addition to water samples, a DOC isolate from a wetland in AZ and two archived aerosol samples collected using quartz fiber filters from Whistler, BC, Canada (Wang et al., 2013) were analyzed. The DOC sample was previously isolated using XAD-8 resin from the outlet of a constructed wetland receiving treated wastewater (Pinney et al., 2000).

P from DOC isolate and aerosol filters was extracted by sonication in DI water. All samples were filtered through a 0.2 μm filter (PES, Thermo Scientific), prior to injection into the SEC-ICP-MS system.

2.4. SEC-DOC, TOC and orthophosphate measurements

The SEC-DOC consists of High-Performance Liquid Chromatography system (Waters 2695 Separation Module, MA, USA) coupled to an online TOC detector (Sievers Total Organic Carbon Analyzer 800 Turbo) adapted to inline detection using an inorganic carbon remover (900 ICR, GE). The SEC was carried out using the same media used for the SEC-ICP-MS system: TSK 50S column (21.2 mm × 250 mm Tosho Toyopearl HW-50S resin, Japan) and elution with phosphate buffer mobile phase (NaH_2PO_4 and 0.0016 M Na_2HPO_4, pH = 6.8) containing 0.025 M Na_2SO_4 which has ionic strength of 0.1 M (conductivity of 4.57 mS cm^{-1}). Detailed information of the set-up and method performance is provided elsewhere (Allpike et al., 2005; Her et al., 2002). TOC was measured using a Shimadzu TOC-V_{CHS} analyzer. Orthophosphate was measured using ion chromatography (Dionex ICS 5000, AS12A column).

3. Results and discussion

3.1. Mobile phase, calibration, and method optimization of P detection in SEC-ICP-MS

Various mobile phase buffers and eluent pH were tested on the column to optimize for retention time and peak shape of P. Supplemental Fig. S1 shows the peak shapes and retention time of orthophosphate injected into the SEC-ICP-MS system. Similar to the observation made for the SEC-DOC system (Her et al., 2003), increasing the ionic strength of the mobile phase increased the retention of P (orthophosphate) in the column and also improved the peak shape (DI water vs. 20 mM NaCl - conductivity of 2.2 mS/cm). Tris-HCl buffer at pH 7.5 has been successfully used in the past for P separation in SEC systems for speciation analyses in foodstuffs (Koplík et al., 2002, Persson et al., 2009). When using the Tris-HCl buffer in our system, we observed an improved retention time of 64 min, but the chromatogram showed higher background noise (increased baseline interferences) and also tailing of peaks (especially for OP). Other buffers tested (acetate: pH 4; bicarbonate: pH 8.6) showed either poor retention and/or increased background interferences. 50 mM HCl at a pH of 1.3 provided the best retention time (72 min) and peak shape for orthophosphate compared to other buffers. Hydrochloric acid mobile phase has been used in the past for separation of inositol phosphates, an abundant OP form in plant materials, using ion chromatography (IC) (Chen and Li, 2003; Skoglund et al., 1998). Inositol phosphates are highly acidic compounds, and the use of acidic mobile phase has shown to improve retention and separation on IC columns (Chen and Li, 2003). Though SEC separations are dependent on size of the molecules, electrostatic interactions also play a role in the retention of certain charged molecules (Hong et al., 2012; Pujar and Zydney, 1998). Injection of inositol phosphate standard in the SEC-ICP-MS system confirmed this observation by showing improved retention on to the column with 50 mM HCl mobile phase compared to other buffers. Also, it is conventional to acidify samples (~2% HNO_3 or 2% HCl) in ICP-MS systems to avoid loss of analytes due to sorption in the sample introduction system, which may serve as an added benefit for using acidic mobile phase. Though acid hydrolysis could potentially convert some OP to inorganic orthophosphates, this is likely to happen only at strong acidic conditions (12 M HCl; 4 M H_2SO_4) (Bowman, 1989; Turner et al., 2005). To confirm this, we acidified P standards (phytic acid, phosphate glass, and L-α-phosphatidylcholine) for >5 h using 50 mM HCl and analyzed for orthophosphate using IC. No phosphate peak was detected before and after acidification of the standards confirming that there was no concern for hydrolysis of OP during analysis. However, in order to prevent any potential acid hydrolysis of labile OP, the samples were acidified only prior to injection into the SEC-ICP-MS system thus minimizing the contact time with the acid to <100 min.

The column was calibrated using a suite of PEG and P-markers of known MW (Fig. 1). The calibration curve was similar and comparable between both suites of markers. The calibration curve from the P-markers were used for determining the MW distribution of P in environmental samples; but this experiment confirmed that it is feasible to use non-P markers for calibration purposes when ideal standards are not available for speciation analysis. L-α-phosphatidylcholine was not included in the calibration curve as they form micelles at the concentration injected into the SEC-ICP-MS system and exceeded the separation range of the column (>20,000 g/mol). This can be seen in Fig. 1b where the retention time of the phospholipid matches with the injection peak or the void volume of the column (~32 min or 32 mL). Since 1 mL/min was used as the flow-rate in the system, retention time of the various compounds can be interchangeably interpreted as elution volume in mL.

The peak areas of high MW P-markers (phytic acid, phosphate glass, and L-α-phosphatidylcholine) were 13–16% relative to the peak area of KH_2PO_4 of the same concentration. Through controlled experiments, we found out that this difference in peak height/area and sensitivity between different markers was due to two reasons: (i) adsorption on to SEC column, and (ii) difference in ICP-MS sensitivity to different P-molecules. After several months of running P-samples and standards, we flushed the SEC column using 0.1 M NaOH solution and collected the eluate to check for P adsorption in column. A large amount of total P (sub ppm levels) was detected in the eluate (first 150 mL fraction) when analyzed using ICP-MS in normal mode. This confirmed that a fraction of high MW P compounds was lost due to adsorption to the SEC column. Similar observations were made in the past when analyzing for aggregates of protein molecules (high MW) using SEC (Hong et al., 2012). We additionally analyzed organic P-markers (phytic acid and L-α-phosphatidylcholine) directly by ICP-MS in normal mode and the results revealed concentration dependent recoveries of 43–114% relative to orthophosphate response, suggesting that the difference in peak areas can additionally result from ICP-MS sensitivity to different molecules (organics vs. inorganics). We compared SEC-ICP-MS performance with ultrafiltration method (see supplemental sections S2 & S3) and the results agreed well both with size resolution and concentration (Supplemental Fig. S3).

3.2. MW distribution of P in environmental samples

The chromatograms of four surface water samples are shown in Fig. 2. The figures revealed that the dominant MW fraction of P in most samples was at a retention time of ~73 min. This matched the retention time of KH_2PO_4 standard (Fig. 1), indicating the presence of orthophosphate in the samples. Tempe Town Lake was the only sample in the present study that did not contain any orthophosphate and the dominant P fraction was detected at a retention time of 62 min (Fig. 2a) corresponding to a MW range of 400–600 g/mol. Tempe canal, that conveys Salt River water to potable water treatment plants, showed two peaks at retention times 62 min and 71 min, corresponding to MW range of 400–600 g/mol and <100 g/mol (orthophosphate), respectively. Both surface waters are present in the dry region of the Salt River with minimum input from agricultural areas. Hence the TDP in these samples were low (<14 ppb) with the abundant form of the P (74–100%) within the MW range of 400–600 g/mol. These can likely be attributed to

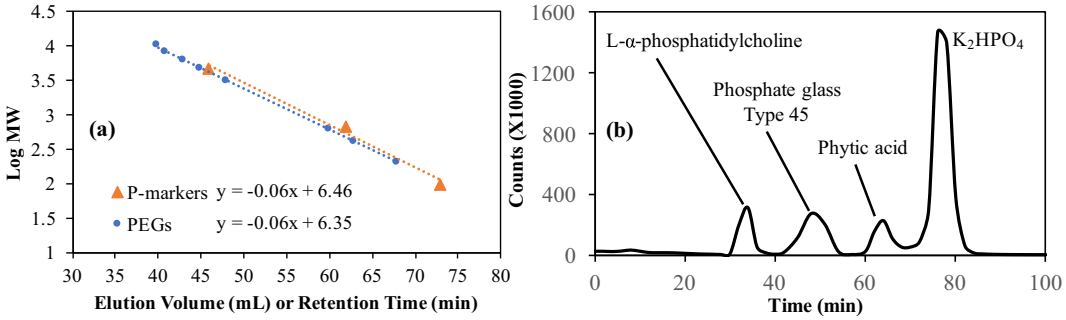

Fig. 1. Molecular weight (MW) calibration of Toyopearl HW50S SEC column using PEG and P-markers of MW ranging from 95 to 10,000 Da (a). Panel (b) shows the chromatogram of the various inorganic and organic P-markers used for calibration.

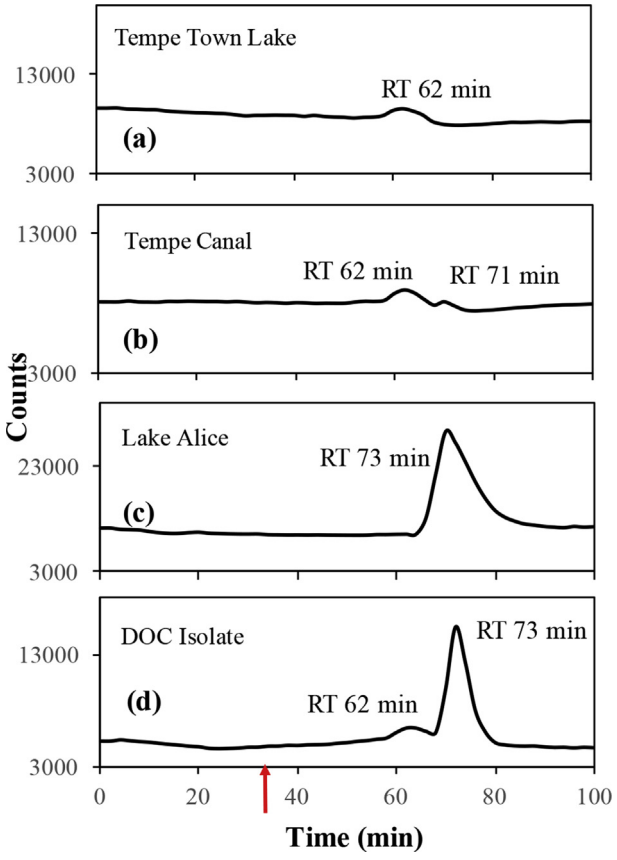

Fig. 2. SEC-ICP-MS chromatograms of ^{31}P for three surface waters and one DOC isolate sample with a flowrate of 1 mL/min and 1 mL sample injection. RT is the retention time of the corresponding peak which is interchangeable with the elution volume in mL. Arrow in the x-axis indicates the void volume of the column at 32 min (32 mL).

anthropogenic sources during recreational use of the lake and/or deposits from plant materials (e.g. inositol phosphates, poly/meta-phosphates etc.). Inositol phosphates are produced by plants and are released to the aquatic environments upon plant degradation (Baldwin, 2013). They can also be produced *in situ* by certain species including *Spirodela* sp. (Brearley and Hanke, 1996) and *Azolla* sp. (Benaroya et al., 2004). Similarly, it is known that inorganic poly- and meta-phosphates are produced by biological activity and also introduced from human activities (Persson and Jansson, 2012). The retention time of 62 min observed in these two surface water samples was similar to the inositol phosphate standard (phytic acid: 62 min) used in the study, and thus indicating its presence in these samples.

Everglades is a P sensitive environment where dissolved organic phosphorus (DOP) has been shown to represent up to 56% of TDP, and ~40% of DOP was high MW aromatic-rich compounds (Ged and Boyer, 2013). Sample from Lake Alice from the Everglades analyzed in this study showed a large tailing peak at a RT of 73 min. Though the peak retention time matches with that of the K_2HPO_4 standard, the tailing peak suggests compound interaction with the column. It is known that certain compounds can interact with SEC and gel columns that can result in high retention time and elution volumes (Persson and Jansson, 2012). Comparison of TDP from ICP-MS and orthophosphate from IC showed 61% difference in concentration (~102 ppb) suggesting that there were likely species of P that interacted with the SEC column and co-eluted with orthophosphate resulting in peak tailing. Further research is needed to investigate and identify such interacting species.

Primary and secondary wastewater effluents collected from a WWTP located in Mesa, AZ were filtered and injected into the SEC-ICP-MS system (Fig. 3). Three peaks were initially identified for both primary and secondary effluents at a RT of 32, 63, and 73 min. The peak at 32 min represents the void volume of the column, and hence this fraction of P is associated with high MW (>20 kDa) compounds. Primary and secondary effluents are abundant with microorganisms, and hence it is reasonable to assume that this fraction of P is composed of DNA, RNA, phospholipids and other microbial products containing P. Since the P concentration was high in the effluent samples, we observed overlapping peaks between 60 and 75 min. The peak de-convolution software output suggested that a 3-peak model was the best fit for the observed chromatogram with RT of the peaks at 64, ~69.5, and ~73.5 min (Fig. 3c and f). The peak at 64 min and 69–70 min suggest the presence of P fractions in the 400–450 g/mol and 135–155 g/mol range, respectively. Phosphonates are a group of compounds that have direct C–P bonds, that can occur from natural sources (phosphonolipids) (Clark et al., 1999) and/or from synthetic products (e.g. glyphosate: MW 159 g/mol, an active ingredient used in commercial herbicides) (Nowack, 2003). These are abundantly present in aquatic environment and make up a significant fraction of the dissolved OP (Villarreal-Chiu et al., 2012) suggesting that they likely represent the fraction observed in this study. Other condensed inorganic phosphates (polyphosphate and pyrophosphate) of low MW range can also contribute to these observed peaks, as they are known forms to exist in wastewaters.

We also analyzed an archived DOC isolate obtained from a wetland in Arizona. As seen in Fig. 2d, the majority of the P was detected at the RT of 72 min indicating high levels of orthophosphate in the sample. A peak was also detected at 62 min corresponding to the MW range of 400–600 g/mol as observed for the Tempe surface water samples. A study conducted by Shaw et al.,

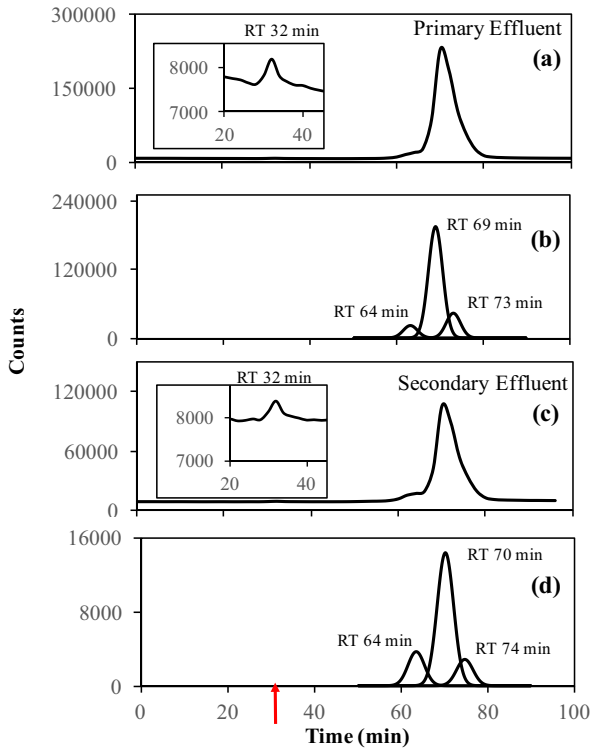

Fig. 3. SEC-ICP-MS chromatograms of ^{31}P for primary effluent (a–b) and secondary effluent (c–d). RT is the retention time of the corresponding peak which is interchangeable with the elution volume in mL. Arrow in the x-axis indicates the void volume of the column at 32 min (32 mL). Panel inserts in (a) and (c) shows zoomed in peaks at 32 min. Panels (b) and (d) represent deconvoluted peaks fitted using Gaussian model.

investigated the influence of humic substances on the MW distribution of inorganic phosphate by adding radioisotope $^{32}PO_4^{3-}$ to isolated high MW humic substances (10 kDa to >50 kDa) in lake waters (Shaw et al., 2000). They observed complexation of added $^{32}PO_4^{3-}$ to high MW humic substances, but only at specific pH (pH 4 for >50 kDa; pH 5.5 and 7 for 10 kDa–50 kDa) and ionic strength of the lake water samples. Hence, the large orthophosphate peak observed for the DOC isolate in our method was likely due to the dissociation of inorganic phosphates from high MW colloidal materials when the isolate was re-suspended in a DI water matrix. We confirmed that PO_4^{3-} did not form from acid hydrolysis from acidic mobile phase (50 mM HCl) used for chromatography by measuring for PO_4^{3-} using IC in non-acidified water samples (Table 1). The orthophosphate concentration detected in IC was comparable (relative percentage difference (RPD) < 33%) to the concentration determined from integration of the peak. This suggests that the inorganic phosphate more likely resulted due to dissociation from colloidal DOC isolates during sonication.

While atmospheric P has been found to be a very small reservoir compared to water or sediments, it might still be important in terms of transport. The chromatograms from the two aerosol samples show a large (over-lapping) peak between 62 and 75 min (Supplemental Fig. S4). Peak deconvolution suggested three peaks at a RT of 65, 70, and 73 min, as observed for the wastewater effluents. Phosphate associated with mineral dust and fuel emissions are abundant in aerosols (Anderson et al., 2010) and may have caused the large peak at the RT of 71–73 min suggesting a significant fraction of inorganic phosphates in the samples. Very little is known about atmospheric OP despite recent studies showing the ubiquity of biological material in aerosols and clouds and significant contributions to atmospheric organic matter (Herckes et al., 2013). One study showed that the occurrence of OP in aerosols was seasonal and increases during warm periods when biological activity increase (Chen et al., 2006).

3.3. Fraction of orthophosphate in environmental samples

Table 1 summarizes the estimated concentration of P fractions with different MW ranges in the tested samples. TDP as measured by ICP-MS in normal mode was higher than the concentration of PO_4^{3-} measured by IC in all aqueous samples (surface waters and wastewater effluents). The integrated peak area for PO_4^{3-} at RT ~73 min was comparable to IC measurements for all aqueous samples, except for Lake Alice. Results showed that the percentage of P as orthophosphate varied highly with an average of 55 ± 49% ($n = 3$) of TDP in surface water samples. In wastewater effluents, the fraction of orthophosphate was lower and represented only 20 ± 1.2% ($n = 2$) of TDP. Though the recovery of orthophosphate peaks in the SEC column was satisfactory, varying recovery of higher MW compounds through the column made it difficult for accurate quantification of various fractions of P in the samples. As described in the methods section, the high MW P-markers used for calibration purpose showed a recovery of 13–16% relative to K_2HPO_4 standard. Assuming a 15% recovery of the different MW fraction of P, the fraction of orthophosphate in surface waters was estimated at 42 ± 48%, which was similar to the uncorrected value estimated above (55 ± 49% of TDP). Similar results were observed in a study conducted by Ged and Boyer, where the percentage of orthophosphate varied seasonally in the Everglades between 44 and 87% of TDP (Ged and Boyer, 2013). For wastewater effluent

Table 1
Summary of concentrations of various MW fractions of environmental P determined by SEC-ICP-MS.

Sample		TDP[a] by ICP-MS (ppb)	MW Range (g/mol)				Orthophosphate	
			<100	120–350	400–600	>20,000	Conc. by IC (ppb)	% of TDP
Surface Waters	Tempe Town Lake	12 ± 2	0	0	12 (90)[b]	0	<DL[c]	0
	Tempe Canal	14 ± 3	3.6	0	11 (80)	0	<DL	26 (4)
	Lake Alice	268 ± 61	268	0	0	0	166	100
Wastewaters	WWTP Primary	2462 ± 166	475	1812 (14,000)	172 (1300)	3.3 (25)	562	19 (3)
	WWTP Secondary	1080 ± 109	227	783 (6000)	68 (520)	2 (15)	221	21 (3)
	DOC Isolate [d]	220 ± 75	204	0	16 (120)	0	146	93 (62)
Aerosols	Aerosol 1 [d]	1564 ± 565	1501	63 (480)	0	0	1550	96 (76)
	Aerosol 2 [d]	810 ± 42	790	20 (150)	0	0	950	98 (84)

[a] TDP: total dissolved phosphorus.
[b] Values in parenthesis are recovery corrected estimated concentration of P-fractions. An average of 15% recovery was used to account for analyte losses in the system.
[c] DL: detection limit.
[d] Concentrations reported are for the water extract and not in the original matrix.

samples, assuming a 15% recovery of various MW fractions of P suggests that orthophosphate can be as low as 3.2 ± 0.2% of TP in the samples. Though this is an extreme-case estimate of orthophosphate fraction, dissolved OP in wastewater effluents can be as high as 81% of the TP (Gu et al., 2011) since the majority of the P is stored within microbial cells after secondary biological treatment (Qin et al., 2015). Of these fractions, Qin et al. determined that 61.4–80.7% are hydrophobic in nature and exist predominantly in the forms of phosphate monoesters and phosphate diesters. For aerosol samples, the concentration determined by ICP-MS and IC was comparable indicating >90% of TDP is in the form of orthophosphate. We observed multiple peaks between 62 and 75 min in aerosol samples; this could be due to the presence of labile forms of low MW condensed phosphates and/or particle-adsorbed phosphate forms that could have released orthophosphate in the alkaline mobile phase of the IC system.

3.4. Qualitative determination of organic fraction of P using SEC-DOC

The MW distribution of dissolved organic matter (DOM) in surface water and wastewater effluent samples was determined by using an SEC-DOC system equipped with the same SEC media (hydroxylated methacrylate) and column dimension as the SEC-ICP-MS system. An overlay of both SEC-ICP-MS and SEC-DOC chromatograms of the samples is shown in Fig. 4. A large peak is observed for both chromatograms at ~100 g/mol MW range. This peak represents small MW compounds that feature an elution volume greater than the total volume of the packed bed (i.e. outside the separation range of the column). Inorganic orthophosphate falls in this region of the SEC-ICP-MS chromatogram, and hence the peak overlap observed with the SEC-DOC chromatogram does not imply organic fraction of P. However, for higher MW compounds, overlapping peaks between the chromatograms can be interpreted as the fraction of P associated with organic matter. This can be observed for the primary effluent (Fig. 4b), secondary effluent (Fig. 4e), Tempe Town Lake (Fig. 4g), and Tempe Canal (Fig. 4h) showing peaks at same MW fraction in both chromatograms (highlighted by the arrow mark). The primary and secondary effluent peaks overlap at a MW of ~30 kDa, and is likely comprised of large biopolymers and protein-like molecules (e.g. phospholipids and nucleotides). The peak at ~470 g/mol (RT of 63–65 min) determined by the deconvolution of peak do not coincide with any DOC peaks (highlighted by the circle: Fig. 4c and f), suggesting that these are likely to be inorganic P (e.g. polyphosphates). The Tempe Town Lake and Tempe Canal samples show overlapping peaks with SEC-DOC chromatograms at ~500 g/mol indicating the presence of OP fraction in the samples. The RT of these peaks is very close to the RT of phytic acid standard used in the calibration. Phytic acids or inositol phosphates are present in large quantities in aquatic environment, where they may contribute to eutrophication (Turner et al., 2005). Though this is not an ideal method to quantify the OP fraction of samples, the qualitative results provided here is critical in understanding the distribution of various species of P in the environment that cannot be obtained by other traditional methods such as ultrafiltration and chemical extractions.

3.5. Advantages and limitations of SEC-ICP-MS method

Like SEC-DOC, the developed SEC-ICP-MS method has its advantages and limitations in its applications. The most important advantage of SEC-ICP-MS over other MW characterization techniques is the ability to obtain information of various P-species as a function of MW in a single 100 min run. After the initial set-up of the instrument, minimal sample preparation was required prior to obtaining the chromatograms. In ultrafiltration, a series of membranes with different MW cut-off, a time-consuming experimental set-up, and extended experimental runtime (>5 h) is required in order to obtain similar MW distribution results. Also, the SEC-ICP-MS required much less volume (1 mL) to characterize samples; whereas in membrane techniques >100 mL of samples is needed. Using ICP-MS as the detector provides the opportunity to detect for multiple elements simultaneously. This way various metal-complexes of P-species in the environment can additionally be

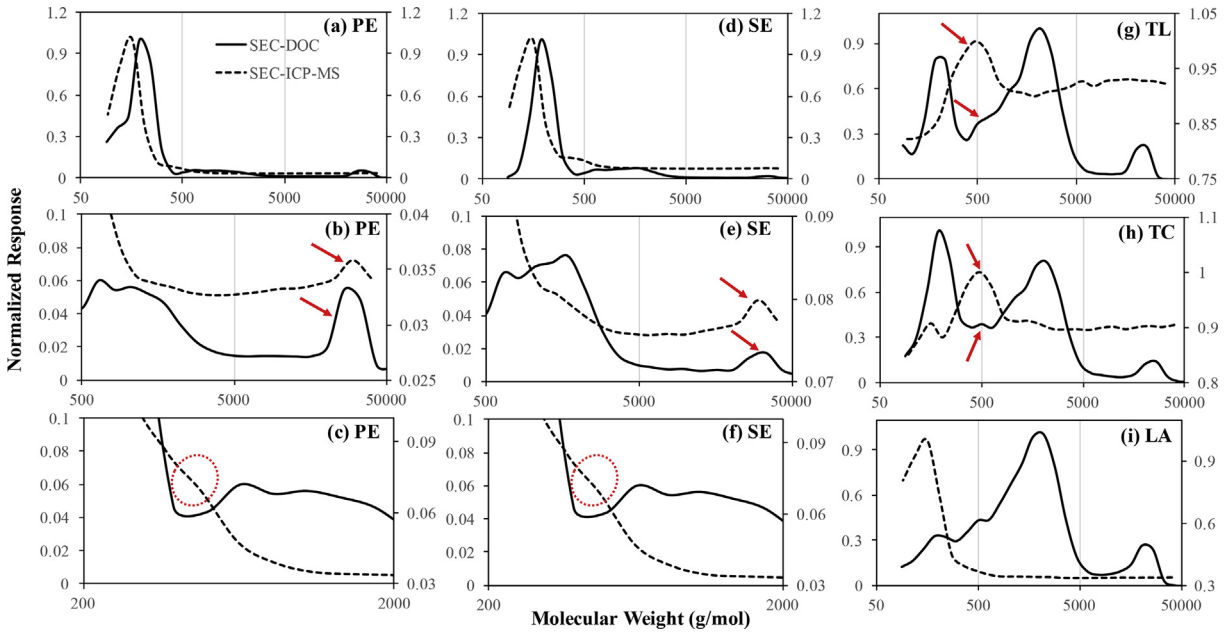

Fig. 4. Overlay of SEC-DOC and SEC-ICP-MS chromatograms for aqueous samples: (a–c) primary effluent: PE, (d–f) secondary effluent: SE, (g) Tempe Town Lake: TL, (h) Tempe Canal: TC, and (i) Lake Alice: LA. Arrows indicate overlapping peaks observed in both the chromatograms suggesting P-associated with organic fractions. Circles indicate P-peaks that do not overlap with any DOC peaks, suggesting P-associated with inorganic fractions in the samples.

determined in a single run. This technique was successfully employed by Persson et al. to determine the association of phytic acid with Fe in barley grain tissue (Persson et al., 2009).

SEC methods are dependent on the use of ideal mobile phase matrix to minimize the interaction of compounds with the media. Though the mobile phase was optimized using various buffers at different pH and ionic strength in the present study, the possibility of compound interactions with the column cannot be completely ignored. This was observed for the Lake Alice sample that showed tailing peak and significant difference in the orthophosphate concentration between IC and ICP-MS data. The present method successfully provided qualitative information of various P-forms in the environment, but accurate quantification relies on using the right markers during calibration. In this study, a suite of inorganic and organic-P markers in addition to non-P (PEG) markers of known MWs, was used to calibrate the column. This enabled in determination of the recovery of different P-markers relative to orthophosphate. The current method featured lower recovery (13–16%) of intermediate and high MW P-compounds relative to orthophosphate, but knowledge of analyte losses by using known P-standards enabled us to perform a mass balance and successfully compare with ultrafiltration method (see supplemental information). However, recovery of unknown P-species can be difficult to estimate without knowing the molecular structure. An important challenge in using this method is that the concentration of intermediate and high MW P-species, especially the organic forms, are very low in the environment for successful detection and quantification using SEC-ICP-MS. This can be noticed by the absence of peaks in the 1000–20,000 g/mol range. Hence preconcentration of samples prior to injection is required in future for successful characterization of trace levels of environmental P.

Though with such optimizations the SEC-ICP-MS can serve to characterize TDP MW fractions, the method does not detect particulate P fractions. The samples were filtered in the present study using 0.2 μm filters to prevent clogging and deterioration of SEC column over time. By filtering the samples, the particulate and colloidal forms of P are removed. Particulate forms of P may constitute a significant fraction of TP in aquatic environment, especially with agricultural and surface runoffs (>30%) to the environment (Grant et al., 1996; Hart et al., 2004; Kronvang, 1992; Uusitalo et al., 2001). Over time these forms can become bioavailable through degradation and hence it is important in understanding their dynamic nature in addition to TDP (Ellison and Brett, 2006; Uusitalo et al., 2003).

4. Conclusions

The present study shows that a combination of SEC and ICP-MS can serve as a powerful and robust tool to characterize unknown fractions of P in the environment to further our understanding of the occurrence and cycling of P in natural and built systems. The study results point out that the abundant fraction of P in the environment is < 600 g/mol in most cases. The average fraction of orthophosphate in various environmental matrices ranged from 21 to 98% of TDP, with the lowest value observed for the secondary effluent from a WWTP. The SEC-ICP-MS method performance was shown to be comparable to conventional ultrafiltration method for determination of MW distribution. Additionally, SEC-DOC measurements of aqueous samples in the present study helped with qualitative determination of OP fractions in the samples. In the future, a combination of detectors in addition to DOC and ICP-MS (UV, fluorescence etc.) can be used in series to better understand the composition of the various organic and inorganic P species. Fraction collectors may be used to obtain specific MW fractions from the column, that can then be used for structural characterization and identification of individual species using NMR and high-resolution mass spectrometry.

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Appendix A. Supplementary data

Included in the Supplementary Material available online is information on de-convolution of chromatographic peaks, ultrafiltration methods and comparison of SEC-ICP-MS performance with ultrafiltration.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.01.048.

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