

PHOSPHORUS SPECIATION IN SURFACE WATERS BY
ION CHROMATOGRAPHY WITH HIGH RESOLUTION
MASS SPECTROMETRY DETECTION

by

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Phosphorus Speciation in Surface Waters by Ion Chromatography with High Resolution Mass Spectrometry Detection

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Ryerson University, Toronto
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ABSTRACT

Phosphorus (P) containing compounds, depending on their origin, can enter freshwater systems in variety of forms. Utilization of bioavailable P, a key nutrient in living organisms, is a crucial factor in biological productivity in aquatic environment¹. An increase in the P concentration in water, resulting from both natural and anthropogenic processes, can lead to eutrophication and an excessive growth of vegetative biomass. Determination of organic and inorganic P species in ground, surface and waste water is an important part of water quality control and monitoring². Analytical determination of P compounds, quantification, identification (Chapter 2) and P speciation process can be problematic due to P adsorption on storage containers, sulfate interference, presence of acid hydrolysable P species and colloidal matter, association of both inorganic and organic phosphates with iron, manganese and aluminum, biological uptake of P. Such processes have to be considered when designing an analytical procedure for analysis of P species. Analytical P compound separation by ion chromatography with mass spectrometry/Orbitrap technology for compound detection (Chapter 3) is a powerful technology with highly sensitive and selective properties capable of performing P speciation in one

analytical run. Selectivity of the Orbitrap detector also allows for utilization of isotope dilution technique which can compensate for loss of P compounds during storage and transportation and thus increase accuracy of measurements and holding time of sample matrices. Analytical techniques used in the past were able to determine broad P-fractions, such as total P, total P or functional groups of inorganic and organic P compounds. Identification of variety of specific P-containing compounds of inorganic and organic origin by the methodology used in this thesis in combination with multivariate statistical analysis can be used to explain the large sets of intercorrelated analytical variables with a smaller set of independent variables (Chapter 4) and help in source identification in water pollution studies³.

FOR

My wife Zuzana, and our daughters Nicole, Victoria and Natalie

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
AES	Atomic Emission Spectrometry
AMP	Adenosine Monophosphate
AOC	Area of Concern
APCI	Atmospheric Pressure Chemical Ionization
APHA	American Public Health Association
API	Atmospheric Pressure Ionization
ATP	Adenosine Triphosphate
CE	Capillary Electrophoresis
CFA	Continuous Flow Analysis
DP	Dissolved Phosphorus
Erythrose-P	D-erythrose 4-phosphate
ESI	Electrospray Ionization
ETV	Electrothermal Vaporization
FIA	Flow Injection Analysis
Fructose-Bi-P	D-fructose 1,6-biphosphate
Fructose-P	D-fructose 6-phosphate
FTMS	Fourier Transform Mass Spectrometry
Galactose-P	α -D-galactose 1-phosphate
GC	Gas Chromatography
Glucose-Bi-P	α -D-glucose 1,6-biphosphate
Glucose-P	D-glucose 6-phosphate
GLWQA	Great Lakes Water Quality Agreement
HAB	Harmful Algal Bloom
HCA	Hierarchical Cluster Analysis
HDPE	High Density Polyethylene
IC	Ion Chromatography
ICP	Inductively Coupled Plasma
ICR	Ion Cyclotron Resonance

IEC	Ion Exclusion Chromatography
IH	Inner Harbor
IJC	International Joint Commission
IS	Internal Standard
ISE	Ion Selective Electrode
ISO	International Organization for Standardization
LC	Liquid Chromatography
MDL	Method Detection Limit
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
OH	Outer Harbor
P	Phosphorus
PC	Principal Components
PCA	Principal Component Analysis
PGA	D-(-)-3-phosphoglyceric acid
Pi	Phosphate Ion
P-metabolites	Phosphate Containing Metabolites
PPi	Pyrophosphate
RAP	Remedial Action Plan
Ribulose-Bis-P	D-ribulose 1,5 – bisphosphate
Ribulose-P	D-ribulose 5-phosphate
RSD	Relative Standard Deviation
SFC	Supercritical Fluid Chromatography
SIM	Selected Ion Monitoring
SPME	Solid Phase Microextraction
SRP	Soluble Reactive Phosphorus
TDP	Total Dissolved Phosphate
TOC	Total Organic Carbon
TP	Total Phosphorus
TRP	Total Reactive Phosphate
UDP-Glucose	Uridine 5' - diphosphoglucose

USEPA

US Environmental Protection Agency

CHAPTER 1

Introduction to Phosphorus Speciation and Environmental Applications of Phosphorus Monitoring

1. INTRODUCTION

1.1 Phosphorus in the Environment

Phosphorus (P) is an element with important biological functions and is found in the nature in the fully oxidized state as phosphate. P is considered as a limiting nutrient for a biological growth primarily in freshwater aquatic environment. Studies of phosphate in the Experimental Lake Area have shown that phytoplankton growth is proportionate to the phosphorus concentration in the water column⁴. P is an essential biological element for animal and plant metabolism and energy conversion. Plants can control phosphate concentration in the cells by regulating phosphate metabolism⁵. P is also an important factor in the cell structure. Covalent ester links formed between phosphate and carbohydrate monomers create a rigid cell structure with organically bound phosphate in the cell walls⁶. Phosphate connects with RNA monomers which form DNA and RNA and thus determining the structure of the nucleic acids. Phospholipids bound together by divalent cations are incorporated in the inner walls of the outer cell membranes⁷. Another important role of P is being an energy carrier. Adenosine triphosphate ATP is the most important energy carrier in the cells⁸. The incorporation of phosphorus into the cell takes place in several steps. Alkaline phosphatase converts organic and inorganic phosphoesters outside the cell, at the cell surface or in the periplasm⁹. Orthophosphate is transported to the cell by the uptake system and converted into ATP. Excess of ortho-phosphate is stored as polyphosphate inside the cell¹⁰. Polyphosphate stored in the cell can serve as a reservoir of ortho-phosphate and function in physiological processes of the cell such as growth, cell development and during events of phosphorus deprivation¹¹. Not all the P in the nature is available for biological utilization. The only immediately bioavailable P sources for phytoplankton are

inorganic phosphate, some phosphorylated sugars and phosphonate¹². Concentrations of these compounds in natural waters are usually very low due to high affinity of phytoplankton to readily available phosphorus sources¹³. Another source of phosphorus for the phytoplankton is phosphorus released from the dead cells including inorganic phosphate, sugar phosphates, glycerophosphate, polynucleotides and phospholipids¹⁴. Accurate determination of P species is crucial for understanding its cycling in the ecosystem and for establishing an effective nutrient management of surface waters. Two main fractions of P species in natural water are dissolved and particulate P with both being in the constant dynamic equilibrium¹⁵. The most frequently determined inorganic forms of P species include phosphate, polyphosphates, cyclic condensed phosphates and also lower oxidation states P compounds such as phosphinate and phosphonate which may be normally detected only in waste waters¹⁶. The main classes of organic P species that can be found in the aquatic environment include nucleic acids, phospholipids, inositol phosphates, phosphoamides, phosphoproteins, sugar phosphates, aminophosphoric acids and organic condensed P species^{17, 18}.

1.2 Eutrophication of Natural Freshwater Sources

P being a part of DNA and RNA is essential for life. Organisms require large amounts of P to grow rapidly. Supply of P from the environment is limited as it can't be easily replenished from the atmosphere such as carbon and nitrogen. Large amounts of P are mined extensively for fertilizer manufacturing to support agricultural crop production¹⁹. Increasing world population and its need for a food supply demands intensified phosphate rock mining and commercial fertilizer application which causes changes in the P pathways and rise of bioavailable P inputs into terrestrial, freshwater and coastal ecosystem²⁰. High nutrient inputs can have very negative environmental consequences on the quality of the receiving waters enhancing the abundance of

algae and aquatic plants²¹. Eutrophication is an accumulation of excessive nutrients such as nitrogen and phosphorus in surface water resulting in changes of water chemical properties. These chemical changes of waters are also a byproduct of light, heat and hydrodynamics resulting from variety of biological, chemical and physical processes²². Deterioration of water quality caused by the rapid production of phytoplankton affects normal functioning of the water bodies and is detrimental to aquatic ecology²³. Original species present in the system can be replaced by opportunistic plant species adapted to the new environmental conditions and thus changing the structure and functioning of the whole aquatic community²⁴. Toxic, hypoxia-generating cyanobacterial harmful blooms, or cyanoHABs, are the most problematic symptoms of nutrient over-enrichment causing serious ecological harm and economic losses. CyanoHABs now affecting ecology of the world's largest water bodies including Lake Victoria (Africa), Lake Erie (US-Canada), Okeechobee (USA), Taihu (China), Kasumigaura (Japan), the Baltic Sea (Europe) and the Caspian Sea (West Asia)^{25, 26}. The main watershed management tool to control freshwater eutrophication in the last 40 years was primarily mitigation of P inputs from waste water and nonpoint sources^{27, 28}. Implementation of P reduction policies have resulted in improvements in aquatic ecology^{29, 30} but many watersheds have reported little or no improvement in P loss reduction³¹ even after extensive best management practices. In some cases, occurrence of nuisance algal growth increased despite a large reduction in P concentrations³²⁻³⁵. It is suggested that a part of the problem is so called "legacy phosphorus" which is a portion of P within a watershed accumulated along transport pathways. Such an accumulated P can be remobilized and become a continuing source of P for years, decades or centuries^{36, 37}. Annually only 20-30% of the P used in agriculture as fertilizer is transported out of the watershed. The remaining 70-80% accumulates in river sediments, groundwater, lakes and

estuaries^{37, 38} contributing to renewing cycles of algal blooms. Preventing such a complex environmental issue as eutrophication and algal blooms requires more systemic interdisciplinary approach including better understanding of phosphorus dynamics in the aquatic environment. One of the possible contributions to such understanding could be phosphorus identification including speciation of organic P compounds in surface water which are currently not fully understood due to lack of sensitive analytical techniques capable of separation and quantification of these species in surface water matrices.

1.3 Eutrophication, Algal Bloom and Environmental Legislation of the Great Lakes

Canada is a country of thousands of lakes. Looking at the map you can't miss magnificent bodies of water located on the US-Canadian border touching 8 US states – Michigan, Wisconsin, Minnesota, Illinois, Indiana Ohio, Pennsylvania and New York and province of Ontario in Canada – The Great Lakes. Lake Superior, Huron, Michigan, Ontario and Erie make up the largest body of fresh water on Earth with the area of 246 463 square kilometres and 6 quadrillion gallons of volume accounting for 20% of the freshwater surface on the planet. The Great Lakes are a home for more than 3500 species of plants and animals including more than 170 species of fish³⁹. In province of Ontario they are a source of drinking water for over 10 million people and help generating 80 per cent of the province's electricity driven by hydro-electric plants. Great Lakes also provide fantastic opportunities for recreation and overall, they are responsible for 40 per cent of Canada's economic activity⁴⁰.

But the Great Lakes are headed for a crisis. Their water quality and ecosystem health are rapidly deteriorating as a result of urban growth, harmful pollutants, aquatic invasive species, climate change and especially nutrient pollution⁴⁰. The problem started during the 1960s when too much

phosphorus in the lakes and especially in Lake Erie caused massive algal blooms, depleted water of oxygen and thus killing fish, plants and many other aquatic animals. By the late 1960s, Canada and the United States came to a conclusion that immediate action is necessary⁴¹. The very first policy tool created as a response to the Great Lakes algal and phosphorus pollution problem was Great Lakes Water Quality Agreement (GLWQA). It was signed by Canadian and United States governments on April 15, 1972. GLWQA has established water quality objectives and included commitments to solve municipal and industrial nutrient pollution. It contains 10 Annexes⁴¹. Annex 4 (Nutrients) is the most relevant part regarding phosphorus management control and algal bloom prevention. As stated in its purpose this annex is supposed “to contribute to the achievement of the general and specific objectives of this agreement by coordinating binational actions to manage phosphorus concentrations and loadings, and other nutrients if warranted, in the Waters of the Great Lakes”⁴¹. The GLWQA also states that both countries have to undertake the necessary research to better understand environmental issues such as nutrient distribution, causes of toxic algal blooms, phosphorus sources and forms and its adverse effects, climate change influence, control methods of phosphorus from non-point sources and research soluble reactive phosphorus.

GLWQA was recognized as a global success story in the mid-1980s by mitigating harmful algal bloom problem (HAB) in the Lake Erie. The goal was achieved by implementing legislature to control point sources of phosphorus at industrial establishments and waste water treatment plants. The great portion of phosphorus reduction which is a major cause of an excessive algal bloom was also achieved by banning phosphates in household detergents. The total phosphorus load to the Lake Erie was reduced by 50% from 29000 MT/year to 14600 MT/year. However, since 1995 the situation has gotten progressively worse. HAB problem is back and it also started affecting

drinking water sources. Lake Erie has been hit by toxic algal bloom every single year since 2008. Severity and toxicity of these occurrences are also very high. Toxicity levels of HAB in August 2014 caused a shutdown of drinking water treatment plant in Toledo, Ohio affecting 500 000 residents. Algal bloom in July 2015 was the largest on the record in terms of affected area in the Lake Erie.

GLWQA and other phosphorus control relevant legislature regulated only total phosphorus in the past. In order to receive a better insight into algal bloom – phosphorus relationship, the bioavailable phosphorus species analysis and monitoring will provide a valuable information. The most recent GLWQA has changed the approach regarding the issue and the agreement has included dissolved reactive phosphorus – DRP monitoring recommendations and has encouraged scientific research of new methods of identification of DRP.

1.4 Analytical Determination of Phosphates

1.4.1 Ion Chromatography

Ion chromatography is a part of a high-performance liquid chromatography which separates ions and polar molecules based on their affinity to the ion exchanger. It uses high performance analytical columns filled with small diameter homogenous particles. Very first commercially available ion chromatograph was available in 1975⁴². Three main separation methods used today in ion chromatography are: Ion chromatography – suppressed or non-suppressed, ion exclusion chromatography (IEC) and ion pair chromatography. The stationary phases or ion exchangers which are used to separate analytes are macromolecular solids with cross-linked space structure. They can be divided into anion exchangers, with functional groups containing cationic

counterion able to exchange anions and cation exchangers with functional groups containing anionic counterion for exchange of cations present in the solution. Functional groups in cation exchangers are acidic: sulfonic ($-\text{SO}_3\text{H}$), carboxylic ($-\text{COOH}$), iminodiacetic ($-\text{n}(\text{CH}_2\text{COOH})_2$), phenolic ($-\text{C}_6\text{H}_4\text{OH}$), phosphonic ($-\text{PO}_3\text{H}_2$) and phosphinic ($-\text{PO}_2\text{H}$). Anion exchange functional groups are alkaline : quarternary ammonium ($-\text{NR}_3^+$), tertiary and secondary protonated amines ($-\text{NR}_2\text{H}^+$, $-\text{NRH}_2^+$) and sulfonic groups ($-\text{SR}_2^+$)⁴³. Parameters of the eluents used in ion chromatography such as type, pH and flow rate are very important factors of the method development. Sodium carbonate and sodium bicarbonate solutions are the most commonly used in the isocratic systems when the concentration of the eluent is constant during the run. In the gradient system when the concentration of the eluent changes during chromatographic separations KOH and NaOH solutions are mostly used⁴⁴. Conductometric detection is the most common detection system in ion chromatography. The biggest challenge in ion chromatography in the past was to determine the concentration of analytes in the presence of high conductivity background of an eluent as the eluent is also an electrolyte. The problem was solved by placing a conductivity suppression column which was later replaced by the suppressor in front of the detector increasing conductivity of the analytes and decreasing the conductivity of the eluent background⁴³.

1.4.2 Mass Spectrometry

Mass spectrometry is an analytical technique of separation of ions based on their (m/z) mass to charge ratio. It requires the conversion of the analytes of interest into positively or negatively charged ions. The ions generated in the ion source are transported into mass analyzer and they sorted out according to their m/z and the signal obtained is recorded by the detector⁴⁵. There are many ionization techniques used in the environmental applications. The most common ones are

electron ionization, chemical ionization, field ionization/field desorption, fast atom bombardment, thermospray ionization, electrospray ionization, atmospheric pressure chemical ionization, inductively coupled plasma and laser desorption⁴³. Electrospray ionization is an atmospheric pressure ionization utilizing a strong electric field generated by applying a high voltage (3-6kV) to the spraying capillary with a counter electrode. Accumulated charge on the sample droplet causes solvent to evaporate and disintegrate with ion desorption⁴⁶. Depending on the conditions used the ions can be protonated $[M+zH]^{z+}$ in the positive mode or deprotonated $[M-zH]^{z-}$ in the negative mode settings. The addition of ammonium hydroxide in the negative mode and formic acid in the positive mode will enhance the ionization process⁴³. Fourier transform mass spectrometry (FTMS) are using analyzers like Ion Cyclotron Resonance (ICR) or Orbitrap, offering a significant increase in mass resolution and accuracy. The FTMS have an ion trap where ions oscillate around a central spindle electrode and m/z ratio is calculated from the frequency of oscillations along the axis of the electric field⁴⁷. The Orbitrap is an ion trap analyzer that consists of a central electrode and two outer electrodes and it can serve as both an analyzer and a detector. Different ions oscillate around the central electrode at different frequencies which are measured and recorded as Fourier transform mass spectra⁴⁸.

1.5 Experimental Setup and Methodology

1.5.1 Sampling

Environmental sampling can be a source of a great variability therefore it is important to consider various aspects where we can see a danger of variances such as sampling location, the number and type of samples and the actual sampling procedure. Lake St. Clair, Thames River, Lake Erie and Lake Ontario were the sampling locations of choice for the phosphorus

speciation⁴⁹. Lake St. Clair is a shallow mesotrophic lake with the maximum depth of less than 6m and recurring instances of a cyanobacterial algal bloom (Fig.1.1). Thames River is the largest Canadian tributary of the Lake St. Clair identified as a priority tributary under Annex 4 – Nutrients of the Great Lakes Water Quality Agreement. The samples were taken from the 96 stations between June and October 2017. The sampling depth was between 1-3m inshore and >3 m nearshore taken by the sampling boats⁴⁹(Fig.1.2).



Fig.1.1 Cyanobacterial bloom in Lake St.Clair Fig.1.2 Great Lakes Guardian sampling vessel

1.5.2 Instrumental Setup – Ion Chromatography

As an instrumental setup the 2-dimensional ion chromatography was used to separate phosphate species from the matrix. Ion Chromatograph: Dionex ICS-5000+ consisted of AS-AP autosampler, 2x DP dual pump modules, 2x EG eluent generators, DC dual column compartment and 2 x AXP-MS auxiliary pumps.

Column assembly 1:

Guard Column: IonPac AG18, 4 mm id x 50 mm

Separation Column: IonPac AS18, 4 mm id x 250 mm

The Dionex™ IonPac™ AS18-4µm Column in combination with the Dionex IonPac AG18- 4µm Guard Column is designed for the analysis of inorganic anions in less than 10 minutes using an isocratic or gradient hydroxide eluent delivered with an Eluent Generator⁵⁰.

Column assembly 2:

Guard Column: IonPac AG24, 2 mm id x 50 mm

Column: IonPac AS24, 2 mm id x 250 mm

Concentrator IonPac TAC-ULPI, 5mm x 23 mm

The Dionex™ IonPac AS-24 – 2mm Analytical and AG24 2 mm Guard Columns are hydroxide selective anion exchange columns designed for separation of environmental ions. The Ionpac AS-24 is a low bleed column designed to be used for ion chromatography separation coupled with Mass Spectrometry (IC/MS/MS)⁵⁰. Dionex IonPac anion concentrator columns are designed primarily for high-purity water analysis. The concentrator column retains anions from a measured volume of aqueous sample matrix, concentrating the analyte species and lowering detection limits by 2-5 orders of magnitude⁵⁰.

DP Dual Pump

Pressure Lower Limit: 200 psi

Pressure Upper Limit: 3000 psi

Pump 1 Flow Rate: 1 mL/min Pump 2 Flow Rate: 300 µL/min

Pump 3 Flow Rate: 200 µL/min

Analytical pumps offer the option of mechanical gradients (proportioning valve) yielding linear, concave and convex gradients. Dual independent seal wash prolongs seal life and removes cross-contamination in dual-channel systems⁵⁰.

AS-AP Autosampler

Injection Volume: 1000 µL

DC Dual Column Compartment

Suppressor 1: AERS-500 4 mm

Suppressor 2: AERS-500 2 mm

Electrolytically Regenerated Suppressors minimize peak dispersion and improve peak efficiency, particularly when coupled with 4µm columns. These suppressors were developed in concert with the high efficiency 4µm ion-exchange columns, which use higher flow rates to achieve faster separations while retaining optimal resolution, or equivalent flow rates to achieve higher resolutions while retaining sample throughput⁵⁰.

1.5.3 Electrospray Ionization Source

All mass spectrometers require an ionization source to generate ions. The ionization setup is based on atmospheric pressure electrospray ionization (API) source IonMax from Thermo-Scientific. The source includes 2 nitrogen inlets, a vaporizer cable for APCI probe heaters and an 8kV cable for the electrospray needle⁵¹.

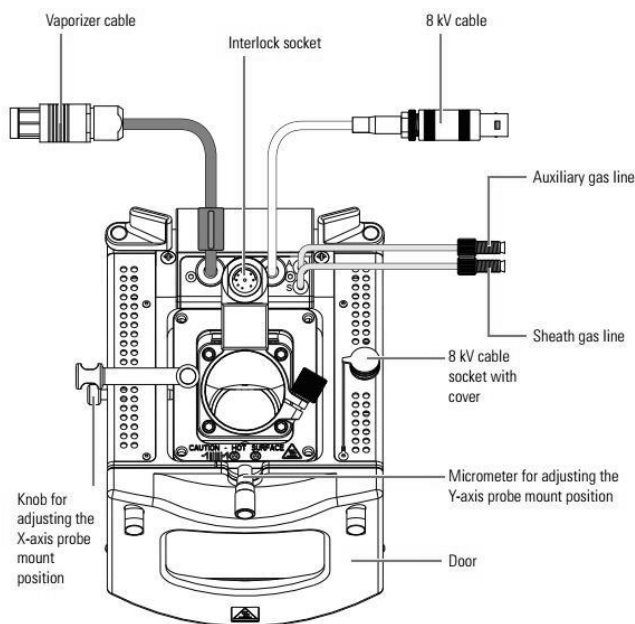


Fig.1.3 Electro Spray Ionization Source IonMax.

In ESI the ions in solution are transferred to gaseous phase as follows:

1. The sample enters the high voltage ESI needle
2. The needle sprays the solution in a form of electrically charged fine droplets
3. As the droplets evaporate their surface electrical charge increases
4. After reaching the Rayleigh stability limit the droplets divide into even smaller droplets due to electrostatic repulsion.
5. The process is repeated many times until the sample ions are changed into a gas phase and enters a mass spectrometer.

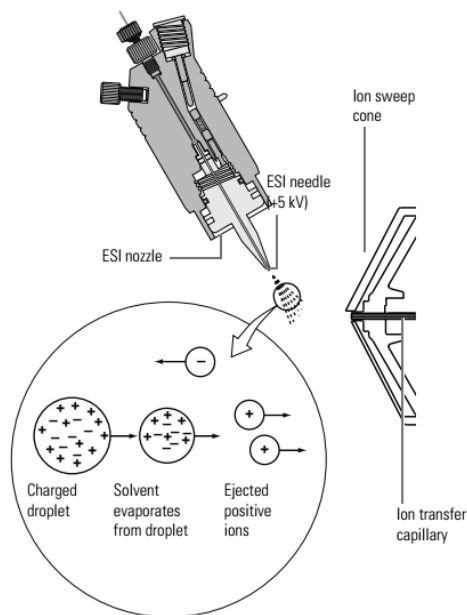


Fig.1.4. Positive mode of ESI process

ESI can be operated in positive or negative polarity mode depending on the ions analyzed.

Acidic molecules form negative ions and basic ions form positive ions in the solution⁵¹.

1.5.4 Mass Spectrometry – Orbitrap Technology

Mass Spectrometer: Q Exactive hybrid quadrupole-Orbitrap mass spectrometer

Ion Source: IonMax, Sheath Gas Flow Rate: 16.0, Auxiliary Gas Flow Rate: 5.0, Spray Voltage:

3.3 kV, Capillary Temperature: 320°C, S-Lens RF Level: 50.0, Auxiliary Gas Heater

Temperature: 200°C, Total Run Time: 40 min, Scan Type: Full MS- SIM (Selected Ion

Monitoring), Polarity: negative, Resolution: 70 000, Scan Range: 50 – 700 m/z, AGC Target:

3e6, Maximum IT: 200 ms

RF lens ion optics contains 18 electrodes. It captures entire expansion from ion transfer tube and focuses ion beam through conductance limiting aperture. In the QExactive the advanced active

beam guide and hyperquad mass filter are a quadrupole assembly with square rods that function as ion guides.

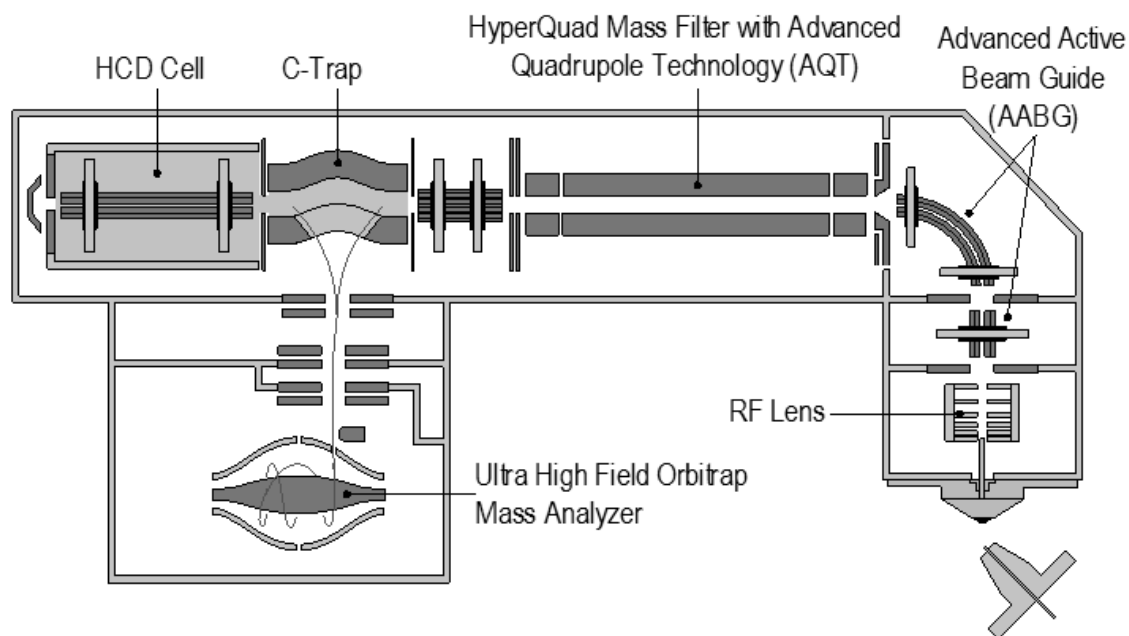


Fig.1.5 Schematics of ThermoScientific Orbitrap Mass Analyzer

RF lens ion optics contains 18 electrodes. It captures entire expansion from ion transfer tube and focuses ion beam through conductance limiting aperture. In the QExactive the advanced active beam guide and hyperquad mass filter are a quadrupole assembly with square rods that function as ion guides. The bent flatapole is curved to prevent neutrals from passing into the Orbitrap. In combination these multipoles focus and transfer the ion beam between the ion source and the orbitrap through successively higher vacuum regions⁵². The ion guides decrease the kinetic energy of the transmitted ions and ensure that ions travel in a stable manner towards the detection region. Ions are stored and cooled in the RF-only C-trap. After trapping the RF is ramped down and DC voltages are applied to the rods creating a field across the trap pushing ions towards the detector. If fragmentation is required the ions are guided into a higher energy

collision induced dissociation HCD cell where precursor ions are fragmented by the gained energy and collisions with nitrogen.

The detector – orbitrap mass analyser consists of a spindle-shape central electrode surrounded by a pair of bell-shaped outer electrodes. The orbitrap employs electric fields to capture and confine ions. The ions oscillating frequencies along the z-axis depend on the m/z and the field curvature. The Fourier transform obtains the frequencies of the axial oscillations and therefore m/z ratios of the ions ⁵².

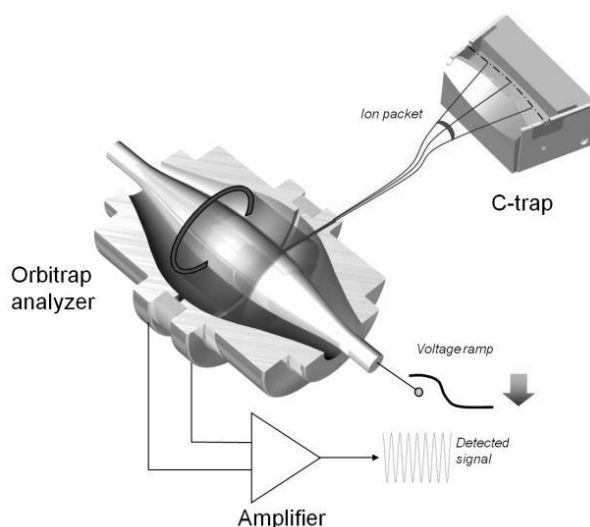


Fig.1.6 Schematics of the Orbitrap and the C-Trap.

1.6 Research Questions and Hypotheses

The main goal of this thesis was development of a methodology for identification and quantification of low-level inorganic and organic species of phosphorus in surface water using Ion Chromatography with Mass Spectrometry/ Orbitrap detection. Currently the most commonly

used analytical approach of phosphorus analysis can mostly identify broader classes of phosphorus species in natural waters.

Quantification of specific trace-level phosphorus metabolites in water column require utilization of novel and more sensitive analytical methodologies. The data obtained by such methodologies including concentrations of bio-available P-containing metabolites would potentially contribute to explaining P utilization in aquatic environment and multivariate statistical analysis of such data could identify sources of P pollution.

Thus, in Chapter 2 past and current analytical techniques of P analysis are reviewed. Advantages and disadvantages of the methods are discussed as well as their utilization for an efficient P analysis and method detection capabilities.

Chapter 3 describes development of a novel method of P-speciation and shows its advantages compared to currently used methods of analysis. Ion chromatography coupled with mass spectrometry approach used in this thesis is superior to other techniques by employing an option of isotope dilution to compensate for P-species adsorption loss after sampling contributing to an accurate trace-level P-measurement.

Chapter 4 shows how the data acquired by this method can be used to statistically evaluate P-sources in the aquatic environment. Multivariate statistical analysis was used to better understand differences between various P-sources including quantification of bioavailable P-metabolites in river and lake samples.

1.7. Contributions of Authors

Chapter 2 was accepted for publication in *Encyclopedia of Water: Science, Technology and Society*, edited by Patricia A. Maurice. Copyright c 2020 JohnWiley & Sons, Inc as Packa, V., Bostan, V., Furdui, V. Analysis of Phosphorus Species in Water. Manuscript was written by VP and edited by VF and VB.

Chapter 3 was published as Packa, V.; Maedler, S.; Howell, T.; Bostan, V.; Diep, N.; Tooley, R.; Furdui, V. I., Unbiased Measurement of Phosphate and Phosphorus Speciation in Surface Waters. *Environmental Science & Technology* 2018.

DOI: 10.1021/acs.est.8b05089

Hypotheses and experimental design were carried out by VP and supervised by VF and SM. Experiments, data analyses and manuscript preparation were carried out by VP and supervised by VF, VB, TH and SM. Sampling schedule was designed and carried out by TH and ND. Manuscript was supervised and edited by VF, VB, SM, TH, ND and RT.

CHAPTER 2

Analysis of Phosphorus Species in Water

Chapter 2 was published in *Encyclopedia of Water: Science, Technology and Society*, edited by Patricia A. Maurice. Copyright c 2020 JohnWiley & Sons, Inc. as Packa, V., Bostan, V., Furdui, V. Analysis of Phosphorus Species in Water.

2.1 Abstract

A limiting nutrient for aquatic ecosystems, the natural and anthropogenic sourced phosphorus is present in surface waters. Monitoring of phosphorus in water samples and reduction procedures were developed to minimize the occurrence of harmful algal blooms. Phosphate is the most frequent phosphorus species in water, being present in anionic form (inorganic phosphate) or attached to a carbon-based molecule (organic phosphate). Although various methods have been developed for phosphate and total phosphorus analysis, the spectrophotometric and ion chromatographic methods remain the most frequently used. Other methods discussed are based on capillary electrophoresis, inductively coupled plasma coupled to atomic emission spectrometry, electrochemical and nuclear magnetic resonance techniques. The simultaneous analysis of various phosphorus species was possible by coupling chromatographic separations with mass spectrometry, using either electrospray or inductively coupled plasma ionization.

2.2 Introduction

2.2.1 Phosphorus: Occurrence, Production and Uses

Phosphorus is an element essential for life. It can exist in three main allotropic forms: white, black and red. A polyatomic nonmetal, phosphorus has a single naturally occurring stable isotope, ^{31}P , and is not found in elemental form in nature due to its high reactivity⁵³. In 1669, searching for the mythical “philosopher’s stone”, the German physician Hennig Brandt discovered elemental phosphorus while carrying out alchemical experiments with urine. The presence of phosphorus in plants was detected for the first time by Albino in 1688⁵⁴. Almost a hundred years later, in 1769, phosphorus was recognized as an essential ingredient in bones of animals and humans by Gahn and Scheele. The first detection of phosphorus in a mineral

(pyromorphite, a lead phosphate) was made by Gahn in 1779 ⁵⁵. The name given to the element was taken from the Greek word phosphorous, or “bearer of light”, from the glow of white phosphorus when exposed to oxygen ⁵⁶. Phosphorus is located in the 15th group of the periodic table (new IUPAC classification, also previously known as group V of main elements) and belongs to the nitrogen family of elements, known also as pnictogens (nitrogen, arsenic, antimony, bismuth and a synthetic element named moscovium). White phosphorus is very poisonous to humans with a lethal oral dose of 1mg/kg of body weight and as little as 0.2 mg/kg may produce adverse effects ⁵⁷. It is absorbed by the liver and chronic exposure can cause necrosis of the teeth and jaw bones ⁵⁸. A characteristic property of phosphorus is the availability of its unfilled 3*d* orbitals (1*s*², 2*s*², 2*p*⁶, 3*s*², 3*p*³) to form p*π* - d*π* bonds with oxygen and nitrogen. Because of its smaller radius, phosphorus forms tetrahedral PO₄³⁻ anions and chain polyacids H_{n+2}P_nO_{3n+1} (n = 2 - 17) ⁵⁹. Phosphorus is the eleventh most abundant element in the earth's crust with the concentrations to the extent of 1120 ppm (mg kg⁻¹). It exists in the terrestrial minerals as orthophosphate with the exception of schreibersite (Fe,Ni)₃P, which is present in meteoric iron ⁶⁰. There are around 200 known crystalline phosphate minerals. The major source of phosphorus in rocks is the apatite mineral family Ca₁₀(PO₄)₆(OH, F, Cl)₂, with common members as fluorapatite Ca₅(PO₄)₃F, chloroapatite Ca₅(PO₄)₃Cl and the most important for industrial applications, hydroxyapatite Ca₅(PO₄)₃OH)⁶¹ used to produce phosphate fertilizers. Phosphoric acid can be obtained using the original Brandt's method, isolation from urine by distillation, process improved by Robert Boyle in 1680, when the fine white sand was added to the mixture to remove volatiles. Later, when the bone ash was used as a raw phosphorus material, sulfuric acid was added to produce phosphoric acid, which was first concentrated and

then heated with coke to produce phosphorus ⁵⁵. In 1890, Readman improved the efficiency of the process by using an electric furnace ⁶² based on the following process:



Most of the industrially produced phosphorus is converted into phosphoric acids and their salts. Phosphoric acid is used in production of superphosphates (phosphorus fertilizers for crops) from phosphate minerals and alternate phosphate sources like guano deposits ⁶³.

2.2.2 Phosphorus Biochemistry

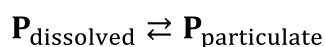
Phosphorus oxidation states range from -3 to +5. However, in nature phosphorus almost exclusively exists in the +5-oxidation state and plays a significant role in living organisms. Prebiotic phosphorus chemistry plays a central role in attempting to shed light on the origin of life on Earth. Phosphorus rich minerals such as brushite [$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$], apatite [$\text{Ca}_5(\text{PO}_4)_3\text{OH}$], struvite [$\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$], lungeburgite [$\text{Mg}_3\text{B}_2(\text{PO}_4)_2(\text{OH})_2 \cdot 8\text{H}_2\text{O}$] and schreibersite [$(\text{Fe},\text{Ni})_3\text{P}$] were sources of ortho[PO_4^{3-}], pyro [$\text{P}_2\text{O}_7^{4-}$] and tripoly [$\text{P}_3\text{O}_{10}^{5-}$] phosphates necessary for incorporation into organic molecules in the early stages of life creation⁶⁴. Westheimer reviewed the role of phosphorus in living systems emphasizing the importance of phosphoric acid and its role in nucleic acids ⁶⁵. Phosphates are ionized at physiological pH due to a low first $\text{pK}_a \sim 2$ which allows conservation of phosphate metabolites within a cell membrane^{65, 66}. Organic phosphate esters are involved in biochemical reactions as energy carriers, coenzymes or as intermediates. They are involved in photosynthesis, carbohydrate and lipid metabolism, the nitrogen cycle and in other biochemical reactions involving energy transfer. Organic phosphate species belong to a few broad compound classes. For example, orthophosphate monoesters, which have one ester linkage, include sugar

phosphates, mononucleotides and inositol phosphates. Adenosine Triphosphate (ATP) is the most important energy carrier and is present in all life forms as a metabolic mediator between high energy phosphate donors and low energy phosphate acceptors. Creatine phosphate and phosphoenol pyruvate also belong to main reservoirs of biochemical energy. Nutrient degradation with highly exergonic phosphoryl-transfer reactions are coupled to the formation of ATP from adenosine diphosphate (ADP) and orthophosphate with the help of enzymes known as kinases. Kinases catalyze the transfer of phosphoryl groups between ATP and other molecules ⁶⁷. Phosphates required for the phosphorylation of adenosine 5'-diphosphate (ADP) to ATP in algae can be stored in the cells by conversion to polyphosphates ⁸. Orthophosphate diesters have two esters per phosphate and are represented by phospholipids, RNA and DNA ⁶⁸. The sugar-phosphate backbone is responsible for the primary structure of the nucleic acids. Phosphate from one nucleotide monomer connected to the sugar (deoxyribose or ribose) of the following nucleotide, continue in this alternating sequence with long chains in DNA and RNA structure. The cell membranes also consist of phospholipids which are bound together by divalent cations ⁷. Animal bone formation, buffering function in urine and blood are also metabolic functions of inorganic phosphates. Catabolic processes in which the nutrients are broken down the released energy is transferred to phosphorus containing energy carriers. The stored energy is subsequently used during anabolic biosynthetic processes where the products from catabolism are reassembled back into proteins, lipids, polysaccharides and other molecules ⁶⁹. Inorganic polyphosphates contain a phosphoanhydride bond which is energetically similar to that of ATP and is used as a phosphate reserve ⁷⁰. Orthophosphate is transported to the cell by the uptake system and converted into ATP by adding a phosphate ion to ADP. The excess of orthophosphate is stored as polyphosphate inside the cell ¹⁰. Microorganisms accumulate reserve phosphorus compounds

in the form of polyphosphates and in some archae and bacteria phosphorus is stored even as magnesium phosphate during phosphorus excess periods in the environment ⁷¹. Some strains of cyanobacteria can store phosphorus as polyphosphates for the events of external phosphorus shortage. Such ability gives the cyanobacteria a competitive advantage over the other phytoplankton when the phosphorus availability is low ¹¹. Phosphorus plays an important factor in structure of certain cells. For instance, carbohydrate monomers and phosphate forms covalent ester links creating a rigid structure of the prokaryotes cells, where the organically bound phosphate is found in the cell walls ⁸. Pyrophosphate is a waste product of the macromolecular biosynthesis ⁷². The incorporation of phosphorus into the cell takes place in several steps. Alkaline phosphatase converts organic and inorganic phosphoesters outside of the cell, at the cell surface or in the periplasm ⁹. Another important role of polyphosphates in living organisms are chelation of metal ions (e.g. Cd^{2+}), decreasing their toxicity and also as a buffer against alkali ions ⁷³.

2.2.3 Phosphorus in the Aquatic Environment

Phosphorus in natural waters is present in a variety of chemical forms that migrate between particulate and dissolved fractions, according to a dynamic equilibrium ¹⁶.



Orthophosphate (H_2PO_4^- and HPO_4^{2-} at normal aquatic pH) is a dominant form of dissolved inorganic phosphorus in natural waters with concentrations often at $1 \mu\text{g L}^{-1}$ as P or below, usually being below detection limits of the most current methods of analysis ⁷⁴. An extensive steady-state phosphorus radiobioassay study of 56 lakes in North America confirmed that orthophosphate was often present in picomolar (pM) concentrations and was order of

magnitudes lower than measured by widely used analytical methods ⁷⁵. Other naturally occurring forms of inorganic phosphorus include condensed phosphates such as polyphosphates (chain phosphates), metaphosphates (cyclic phosphates) and ultraphosphates (branched cyclic structures) ⁷⁶. Polyphosphates are bioavailable and are produced by a wide range of microorganisms and also by human activities. Pyrophosphate and other polyphosphates usually escape detection by standard colorimetric analytical methods but can be quantified by ³¹P nuclear magnetic resonance spectroscopy and enzymatic assay studies⁷⁷. Polyphosphate chain length effect on phosphatase-mediated hydrolysis mechanisms was investigated with the use of ³¹P NMR monitoring the dynamic changes of P molecular configuration during polyphosphate hydrolysis⁷⁸. Determination of linear and cyclic polyphosphates by chromatography and electrophoresis methodologies were reviewed by Rosset et al. ⁷⁹. Inorganic particulate phosphorus can also release orthophosphate in certain conditions. Iron(III) phosphate or Fe(III) complexes have a major role in cycling the phosphorus within the aquatic environment. Hydrated ferric oxides tend to adsorb dissolved phosphates from the water column and co-precipitate in the sediments. During anaerobic conditions, due to iron reduction, phosphorus can be released from the hydrated ferric oxides from sediments into the water column ¹⁶. Absorption of phosphorus on humic-Fe compounds were studied by Franco and Heath ⁸⁰. The research suggested that phosphate adsorbed to oxyhydroxide colloids or ferric-organic colloids can be released and made available to planktonic organisms by photoreduction of iron in the presence of sunlight or by phosphatase activity independently of light intensity. Further studies regarding the biogeochemical cycling of colloidal ferrous oxides in freshwater was investigated by McKnight and Duren ⁸¹. Organic phosphorus species occurring in water due to biological processes include sugar phosphates, phosphate metabolites (AMP, ADP, ATP), phytate and its degradation

products, inositol phosphates and also anthropogenic organophosphorus species such as alkyl substituted phosphonic acids, alkylphosphates and bisphosphonates used as herbicides, pesticides and detergents⁸². Organic and inorganic phosphorus species in natural water samples including phosphate monoesters, phosphate diesters, polyphosphates, phosphonates and myo-inositol hexakisphosphate were detected by ³¹P NMR spectroscopy⁸³⁻⁸⁵. Dissolved organic phosphorus species make up to 15-30% of the total phosphorus in lake waters. During summer periods and high biological activity, these compounds may comprise up to 90 % of the total soluble phosphorus^{86,87}. A diagram of the phosphorus species in natural waters was adapted from Maher and Woo⁸⁸ and presented in Figure 2.1.

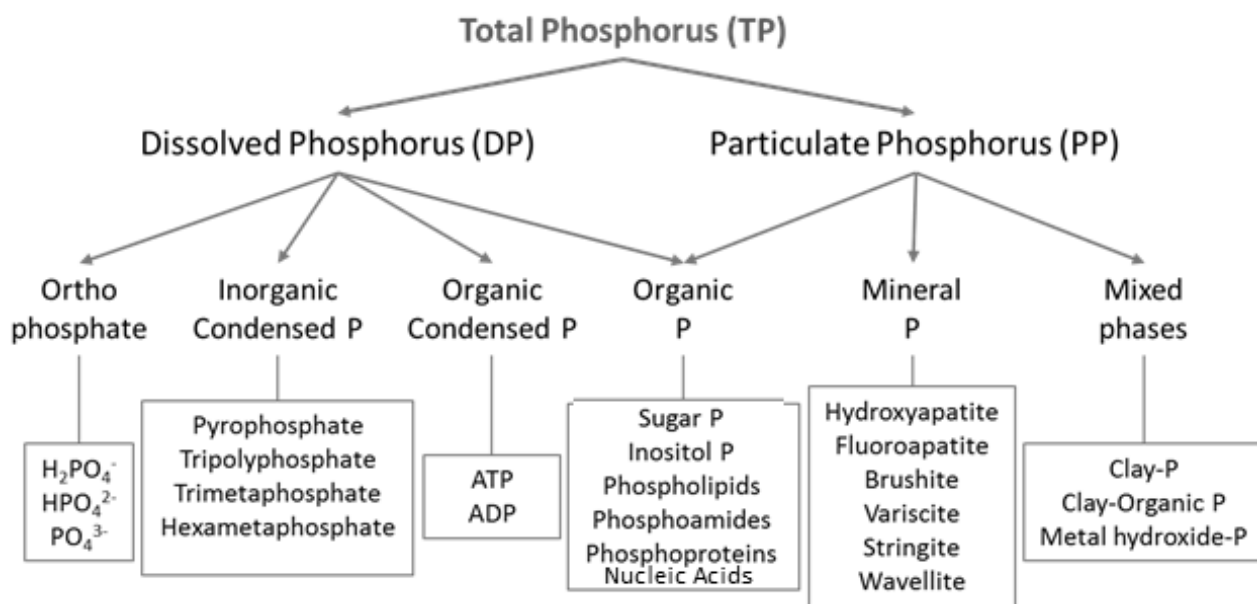


Figure 2.1: Diagram of P species in natural waters adapted from Maher and Woo⁸⁸

2.2.4 Phosphorus and Eutrophication

Eutrophication of freshwater causes excessive algal growth, depletion of oxygen in deeper regions of aquatic systems, loss of aquatic life, deterioration of aquatic habitat and threat of toxin contamination in drinking water supplies caused by algal blooms. Eutrophication of surface

waters is usually related to the transfer of exogenous forms of phosphorus into surface waters. Phosphorus is often a limiting factor for algal growth in surface waters due to fact that it can't be replenished from atmospheric sources like nitrogen and carbon. Studies conducted in the Experimental Lake Area have shown that growth of phytoplankton is proportionate to the concentrations of phosphorus in the water column ^{4, 89}. Further enrichment bioassay studies of phytoplankton growth and its relationship to phosphorus as a limiting factor in freshwater showed that combined nitrogen and phosphorus enrichment enhanced algal growth more substantially than only addition of phosphorus or nitrogen separately ^{90, 91}. Phosphorus is an essential element and is crucial to metabolism and energy conversion in animals and plants. Plants control the phosphate concentration in the cell by regulating phosphate metabolism ⁵. The only immediately available phosphorus sources for phytoplankton are inorganic phosphate, some phosphorylated sugars and phosphonates ¹². Total bio-available phosphorus is a combination of immediately available phosphorus and phosphorus which can be utilized using desorption, dissolution and enzymatic degradation ⁹². Orthophosphate and small organic phosphate esters are readily available for phytoplankton uptake. Concentrations of these compounds in natural waters are usually very low, due to high affinity of phytoplankton to readily available phosphorus sources ¹³. Another source of phosphorus for the phytoplankton is phosphorus released from the dead cells. The main forms of such phosphorus are inorganic phosphate, sugar phosphates, glycerophosphate, polynucleotides and phospholipids ¹⁴.

2.3 Phosphorus Analysis

2.3.1 Sample Collection, Treatment and Stability of the Phosphorus Species

Sampling procedure can be a source of analytical variability and obtaining a representative and stable sample is paramount in analysis of phosphorus species. Sampling from natural aquatic

systems is subjected to seasonal variabilities in phosphorus discharge from anthropogenic sources. The highest phosphorus loadings can correlate with agricultural run-offs during the incidents of increased precipitation, snow melting or irrigation ⁹³. Phosphorus variations can be caused also by phosphorus species leaching from the benthos during anaerobic conditions created in the hypolimnion of a lake. The phosphate is brought to the surface when the surface temperature decreases in the fall and the hypolimnion is mixed with the surface water ⁹⁴. Storing surface water samples for 4 hours at 4 °C, Lambert et al. ⁹⁵ found no significant change in total phosphorus (TP) measurements, but observed 25-54% decreases in total dissolved phosphorus, total reactive phosphorus and dissolved reactive phosphorus. Samples preserved with H₂SO₄ at pH<2 can be stored at 4 °C for up to 28 days ⁹⁶ prior to TP analysis. Preliminary treatment is often necessary for the overall effectiveness of the analyses with dependence on the purpose of the phosphorus species determination and complexity of the sample. Differentiation between dissolved and suspended phosphorus species requires filtration, preferably carried out at the time of the sample collection. Polycarbonate or cellulose acetate membrane filters ⁹⁷ with 0.45 µm or 0.2 µm pore size are recommended. However, 0.2 µm pore size filter is preferred to remove efficiently the bacteria and plankton ⁹⁸. Some bacteria, viruses and colloidal phosphorus can pass through 0.2 µm filter and thus be present in both dissolved and particulate fractions ⁹³. Further studies on filterability of the freshwater bacteria on 0.1 µm filters were conducted by Wang et al. ⁹⁹. “Reactive phosphorus” by definition is the phosphorus measured by colorimetric analyses without previous hydrolysis or digestion of the sample and including dissolved and suspended forms of orthophosphate and also condensed phosphates. The fraction termed “acid-hydrolysable phosphorus” is the portion of organic phosphates converted to orthophosphate by acid hydrolysis at boiling temperatures. “Organically bound phosphorus” is the fraction converted into

orthophosphate by a destructive oxidation ¹⁰⁰. Analysis of the total phosphorus in water samples requires digestion with oxidizing reagents. The digestion procedures will be discussed in the chapter 2.3.4 (Total Phosphorus Analysis). However, it is necessary to point out that persulfate digestion produces high concentrations of sulfate which interferes with ion chromatography measurements of phosphate and further sulfate removal techniques are required. Other sample treatment techniques such as removal of interfering matrix anions (off-line or on-line procedures), column-switching systems and preconcentration are commonly used in chromatographic separations of inorganic non-condensed phosphorus species ⁸².

2.3.2 Spectrophotometric Methods

2.3.2.1 Orthophosphate Analysis

In the early 1900s, Schreiner presented a method of determination of phosphate in waters using ammonium molybdate as a coloring agent. The most common phosphate analysis in water samples is a spectrophotometric (colorimetric) method, a modification of the original Murphy and Riley method using the ascorbic acid as a reducing agent ¹⁰¹. Phosphorus analysis by colorimetry involves two general steps: conversion of the phosphorus species into orthophosphate and subsequent colorimetric determination of the orthophosphate. The International Organization for Standardization (ISO) published method 15681 ^{102, 103} for determination of orthophosphate by flow injection analysis (FIA) and continuous flow analysis (CFA). The methods are suitable for the determination of orthophosphate for concentration ranges between 10 and 1000 $\mu\text{g L}^{-1}$ as P. Samples are mixed with sodium dodecyl sulfate (surfactant) and acidic solution of molybdate and antimony reagents, with molybdenum blue being subsequently produced by the ascorbic acid reduction of a phospho-antimony-molybdate

complex and measured at 880 nm^{103, 104}. Method ISO 15681 suggest that samples with orthophosphate concentrations lower than 100 µg L⁻¹ as P should be stored in glass containers, while samples with higher concentrations can be also stored in plastic containers. Interferences affecting spectrophotometric determination of the orthophosphate include arsenates (100 µg L⁻¹ as As is equivalent to approx. 30 µg L⁻¹ as P), silicates (at 60 times higher concentrations than P), fluoride (above 50 mg L⁻¹), nitrite (above 5 mg L⁻¹ for samples not acidified when collected), suspended particles blocking the light from the lamp¹⁰². Another widely used method for orthophosphate is the vanadomolybdophosphoric acid method. The orthophosphate reacts with ammonium molybdate in the presence of vanadium under acidic conditions and produce a yellow vanadomolybdophosphoric acid, which is measured at 470 nm. The positive interferences for this method are silica and arsenate (when sample is heated) and negative interferences caused by arsenate, fluoride, thorium, bismuth sulfide, thiosulfate, thiocyanate and excess molybdate^{100, 105}. Stannous chloride method is based on reduction of molybdophosphoric acid with SnCl₂, when an intensely colored molybdenum blue complex is produced and measured at 650 nm or 690 nm^{100, 106}. For samples containing less than 10 µg L⁻¹ as P the stannous chloride method provides better sensitivity than the vanadomolybdophosphoric acid method. Ascorbic acid reduction method is recommended for analyzing samples with orthophosphate concentrations between 1 µg L⁻¹ and 100 mg L⁻¹ as P. Using data provided by Standard Methods¹⁰⁰, the performance of the three colorimetric methods in measuring orthophosphate is summarized in Figure 2.2.

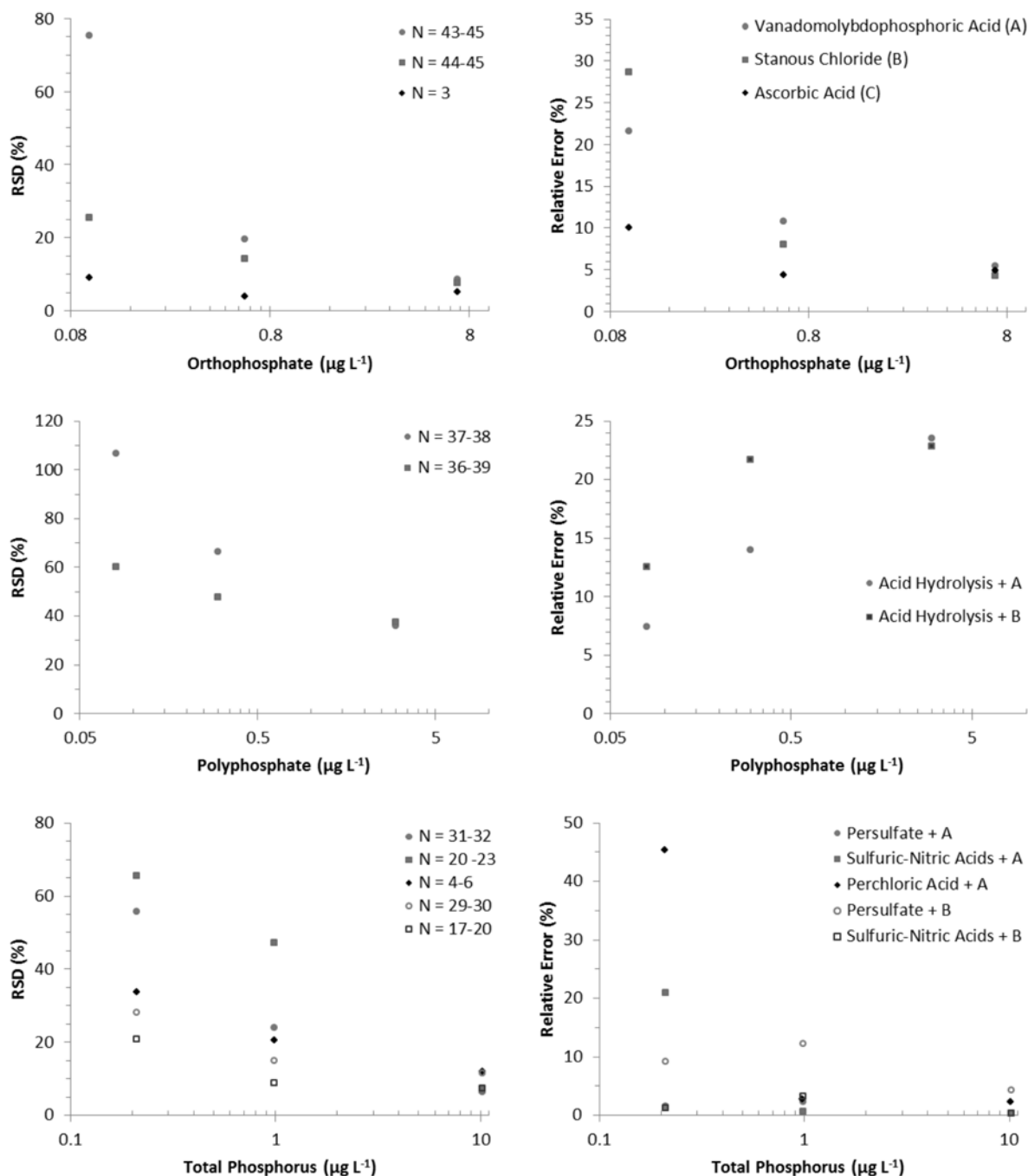


Figure 2.2: Measurements of orthophosphate, acid hydrolyzed polyphosphate and digested total phosphorus using three colorimetric methods (A, B and C). Comparison of the results is based on the relative standard deviation (RSD) and relative error values provided in table 4500-P:I. of Standard Methods, 22nd edition¹⁰⁰, with N being the number of participating laboratories (N).

2.3.2.2 Total Phosphorus Analysis

Digestion of water samples releases phosphorus from all organic phosphorus species and condensed inorganic phosphate. Colorimetric analysis is usually measuring the orthophosphate in the digested samples, being reported as TP. The persulfate digestion by autoclaving at 120 °C was proposed by Menzel and Corwin in 1965¹⁰⁷ and it is still one of the simplest approach. In 1974 US Environmental Protection Agency (US EPA) published method 365.4 for TP analysis¹⁰⁸, with sulfuric acid digestion at 160 and 380 °C in the presence of a mercury catalyst (HgSO₄). If this digestion is still in use, the recent implementation of the Minamata Convention on Mercury¹⁰⁹ encourages its replacement with mercury free alternatives. The 1978 version of the US EPA method (365.3) introduced the acid persulfate digestion. Standard methods published by the American Public Health Association (APHA) provides digestion procedures with nitric acid-sulfuric acid, perchloric acid and persulfate, known also as peroxodisulfate¹⁰⁰. An integrated in-line UV-assisted acid persulfate digestion was also published in ISO method 15681^{102, 103}. Alkaline persulfate digestion was proposed by Valderrama¹¹⁰ in 1981 as a method for simultaneous measurement of TP and total nitrogen. Similar alkaline persulfate digestion methods have been published^{100, 110-113}, with differences in sample volume and in concentrations of persulfate used for digestion (see Table 1). Ormaza-González and Statham¹¹⁴ concluded that alkaline persulfate digestion method following Valderrama's procedure has poor recoveries for polyphosphates, metaphosphates, and ATP, while other similar alkaline digestion methods achieve very good recoveries for ATP (Table 1). The UV based digestion was efficient in releasing phosphorus from sugars, monoesters and phosphonates, but not efficient for the digestion of polyphosphate, metaphosphate and ATP¹¹⁴. The results obtained with an in-line automated system with UV-assisted acid persulfate digestion were about 5% biased low when

compared with manual digestion, mainly due to 90-95% recoveries for pyrophosphate and trimethylphosphate ¹⁰⁰. Due to settling down of the particles in the test tube prior to injection, the method may not be fit also for analyzing samples with high levels of suspended sediments. Lambert and Maher ¹¹⁵ found no significant differences between alkaline persulfate and nitric-sulfuric acid digestion. In a recent study, Dayton et al. ¹¹⁶ confirmed that acid persulfate digestion is much more efficient than alkaline persulfate digestion ¹¹⁴, particularly for samples containing suspended sediments. The perchloric acid digestion was more efficient than acid persulfate digestion for water samples with high-suspended sediment levels ^{117, 118}, but it is labor intensive and require special safety conditions. Using data provided by Standard Methods ¹⁰⁰, the performance of five different digestion methods is summarized in Figure 2.2. Arsenate present at higher concentration than phosphorus and suspended sediments in unfiltered digested solutions can interfere in the colorimetric measurement of the orthophosphate ⁹⁶. In samples with high levels of iron, the phosphorus can be lost by precipitation.

Zhou and Struve found that neutralization and settling time after digestion affect the TP analysis results, especially for samples with very low TP level even in the absence of visible sediments ¹¹⁹. For reduced variability in the interlaboratory studies, they also suggested the need of a standardized method providing digestion solution concentrations (H_2SO_4 and $\text{K}_2\text{S}_2\text{O}_8$) and detailed treatment procedures following the sample digestion until colorimetric analysis.

	Valderrama	Ebina et al.	Hosomi and Sudo	Standard Methods	USGS
Sample Volume	30	5	10	6	10
Digestion Solution Volume	4	5	2	1.25	5
[K₂S₂O₈] (mg/mL)	5.6	10	6.67	11	13.5
[NaOH] (mg/mL)	1.65	1.5	1.5	1.66	2
[H₃BO₃] (mg/mL)	3.53	-	-	-	-
ATP Recovery (%)	≈68	98.1	-	103	-
AMP Recovery (%)	-	100.8	94	-	-

Table 2.1: Summary of published procedures for alkaline digestions (Valderrama ¹¹⁰, Ebina et al. ¹¹³, Hosomi and Sudo ¹¹², Standard Methods ¹⁰⁰ and USGS ¹¹¹). Recoveries for adenosine 5'-triphosphate (ATP) and adenosine 5'-monophosphate (AMP) were provided from the same references, except for Valderrama ¹¹⁰ and Hosomi and Sudo ¹¹² methods.

2.3.3 Ion Chromatography and Hyphenated Methods

Ion chromatography (IC) with conductivity detection was first used to measure inorganic anions and cations by Small et al. in 1975 ⁴⁴. Surface and waste stream waters were analyzed in 15-20 min, measuring anions (F⁻, Cl⁻, Br⁻, I⁻, NO₃⁻, NO₂⁻, SO₄²⁻, SO₃²⁻ and PO₄³⁻) and cations (Li⁺, Na⁺, K⁺, Rb⁺, Cs⁺, NH₄⁺, Ca²⁺, Mg²⁺). The weak acidic character and the complex dissociation equilibrium of phosphate ions make IC analysis more challenging than for other strong acidic anions ¹²⁰. Although phosphate ions were not considered, Gjerde et al. ¹²¹ decreased the analysis time and improved sensitivity by using a macroporous anion exchange column with very low exchange capacity and low conductivity eluents (benzoate, phthalate, sulfobenzoate, perchlorate and malonate). Using IC with conductivity detection, Rosset et al. ⁷⁹ successfully separated a mixture of cyclic and condensed phosphates in less than 30 minutes. Comparing phosphate measurements by suppressed ion chromatography and colorimetry (molybdenum blue complex) Xie et al. ¹²⁰ found that organic phosphorus compounds were less susceptible to hydrolysis when analyzed by IC and the presence of polyvalent metal ions (e.g. Al³⁺ and Fe³⁺) caused lower

phosphate concentrations when measured by IC. US EPA (method 300.1) and Standard Methods (section 4110) published IC analysis procedures for phosphate^{100, 122}. Samples are analyzed after a filtration through 0.45 μm filter¹⁰⁰ and separated on an anion exchange column. Continuous suppression ensures significantly decreased conductivity of the eluent and conversion of the analyzed anions into their acid forms, which have higher conductivity than the eluent. Using a carbonate-bicarbonate eluent, separation of all major anions was achieved in 10 minutes with a method detection limit of orthophosphate at 14 $\mu\text{g L}^{-1}$ as P.

McDowell et al.¹²³ used atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) to analyze hypophosphite ($m/z=65$ for H_2PO_2^-), phosphite ($m/z=81$ for H_2PO_3^-) and phosphate ($m/z=97$ for H_2PO_4^-) in water. Detection limits for hypophosphite was 26 $\mu\text{g L}^{-1}$ as P, for phosphite 12 $\mu\text{g L}^{-1}$ as P and for phosphate 11 $\mu\text{g L}^{-1}$ as P. Development of the high capacity ion chromatography columns allowed the detection and quantification of ultra-low concentrations of phosphorus anions in environmental samples. Ivey and Foster¹²⁴ used IC separation coupled to electrospray ionization (ESI) MS for detection of phosphorus oxyanions. Large volume injection (800 μL) and sample pre-treatment (Dionex Ag OnGuard II cartridges) allowed decreasing the detection limit for hypophosphite (0.34 $\mu\text{g L}^{-1}$ as P) and phosphite (0.62 $\mu\text{g L}^{-1}$ as P), but the Ag cartridge removed also the phosphate ions from the sample. Using a high capacity IC column (Dionex AS-11HC), large injection volume (500 μL) and suppressed conductivity, Han et al.¹²⁵ lowered the detection limit for phosphites to 0.062 $\mu\text{g L}^{-1}$ as P. The results were confirmed using also a two-dimensional IC separation with suppressed conductivity and confirmed ion identities with IC-ESI-MS. In an ESI-MS study of dehydration reactions from inorganic oxyanions with aminopolycarboxylates (e.g. imidodiacetic acid, nitrilotriacetic acid), Kojima et al.¹²⁶ found that a direct measurement ($m/z=97$ for H_2PO_4^-) is the only option

available for phosphate ions. With a combination of two IC columns and a third concentrator column located between the two separation columns, Wang et al.¹²⁷ determined low level anions in seawater, including phosphate at a detection limit of $23\mu\text{g L}^{-1}$ as P.

2.3.4 Capillary Electrophoresis Methods

Capillary electrophoresis (CE) has been used for the analysis of ionic aqueous species including phosphate. It requires only a small sample volume, usually in the picoliter to nanoliter range^{128, 129}. The first electrophoretic separation of inorganic anions was reported in 1967¹³⁰. CE separations are relatively fast, requiring only 10 min¹³¹. Arce et al.¹²⁹ coupled FIA with CE and measured trace level concentrations of phosphate and other inorganic anions and cations. On-line automated monitoring systems for cations and anions were developed for a water purification plant. Using UV detection (214 nm) phosphate was eluted in 9 min and obtained a detection limit of $32\mu\text{g L}^{-1}$ as P¹²⁹. Mayrhofer et al.¹³² used a combination of CE with a contactless conductivity detector, achieving a 3 minutes elution of phosphate with sorbate as a background electrolyte and a detection limit of $23\mu\text{g L}^{-1}$ as P. A similar FIA-CE separation was published by Kuban et al.¹³³ for on-site simultaneous determination of inorganic anions and cations in drainage water from the artificially aerated Lake Sempach (Switzerland). The method achieved peak area repeatability of 1% and a detection limit for phosphate of $65\mu\text{g L}^{-1}$ as P. Gaudry et al.¹³⁴ developed a novel CE method for rapid simultaneous measurement of 23 environmental inorganic cations and anions including phosphate. The method used a sequential injection CE with capacitively-coupled contactless conductivity detector. Oppositely charged ions from a single sample plug were simultaneously injected on two separate capillaries for independent separation and detection. Rapid analysis time was achieved in only 3.5 min with phosphate eluting at 2.4 min, with a detection limit of $20\mu\text{g L}^{-1}$ as P and phosphate analytical range

between 0.5 and 10 ppm¹³⁴. In the mid-2000s, CE had seen significant improvements in the area of indirect photometric detection through the use of dyes as probes and LED light sources and software capable of simulating separations and designing of background electrolytes providing optimal separation conditions and improved sensitivity of analysis¹³⁵.

2.3.5 Inductively Coupled Plasma with Atomic Emission and Mass Spectrometry Methods

Inductively coupled plasma (ICP) either combined with atomic emission spectrometry (AES) or MS have been used to determine TP in water samples. In 1980, Sugimae¹³⁶ analyzed phosphorus in river, sewer and domestic waste water by ICP-AES. Positive interferences produced by argon and NO were minimized by source parameter optimization. The spectral level increase from 1000 mg L⁻¹ Mg was equivalent to 2 mg L⁻¹ P but using a preliminary cation-exchange procedure eliminated the interference. A detection limit of 130 µg P L⁻¹ was achieved and the results were in a good agreement with the colorimetrically obtained results. Fujino et al.¹³⁷ reported difficulties in direct determination of low level phosphorus in natural water, due to Si and Fe spectral interferences. Solvent extraction in 1-octanol with HCl/MoO₄²⁻ complexing agent removed interfering elements and preconcentrated phosphorus, achieving a detection limit of 50 µg L⁻¹ as P. Samples analyzed were from Lake Biwa (Japan) and ranged in concentrations between 12 and 32 mg L⁻¹. Various modes of ICP-AES sampling and influence of anions and metal species in determination of phosphorus were investigated by Wennrich et al.¹³⁸. Using electrothermal vaporization, the method achieved a phosphorus detection limit of 44 µg L⁻¹ as P at 178.27 nm. Pneumatic nebulization and addition of methanol-ethanol mixture into aqueous samples improved detection limits of the studied nonmetals. Using axial determination at 213 nm with Ga as an internal standard and matrix-matched calibration standards (added K, Mg and Ca) reduced the matrix effect of Na and Ca in ground water samples from 20-30% to about 3%¹³⁹. The detection

limit improved from 30 to 12 $\mu\text{g L}^{-1}$ as P by measuring at 213 nm instead of 178 nm, due to a decreased matrix effect and a negligible Cu interference. Hydride generation technique was tried to determine phosphorus by ICP-AES, with phosphate reduced to calcium phosphide by aluminum powder, followed by reaction with HCl in a graphite furnace atomizer. The argon plasma generated phosphine gas (PH_3) was measured at 213.6 nm with an order of magnitude improvement of the detection limit from the direct nebulization approach ¹⁴⁰. Introducing the sample by electrothermal vaporization (ETV), Okamoto et al. ¹⁴¹ lowered the detection limit to 2.6 $\mu\text{g L}^{-1}$ as P measured by ICP-AES. The loss of phosphorus during drying and ashing was suppressed by using a tungsten boat furnace, with phosphate forming stable tungsten phosphate species.

Much lower detection limits than ICP-AES were achieved with ICP-MS methodology. The advantages of the ICP-MS include a wide dynamic range, specificity, sensitivity and multielement detection capability. A decrease in excitation and ionization efficiencies by the water mist introduced into the nebulizer is responsible for poor sensitivities. Polyatomic interferences on phosphorus ($^{31}\text{P}^+$) include $^{14}\text{N}^{16}\text{OH}^+$, $^{15}\text{N}^{16}\text{O}^+$ and $^{12}\text{C}^1\text{H}_3^{16}\text{O}^+$ ¹⁴¹. Cantarero et al. ¹⁴² measured TP and dissolved phosphorus (DP, filtered through 0.45 μm membrane filter) in agricultural runoffs by ICP-MS, reaching a detection limit of 5 $\mu\text{g L}^{-1}$ as P. Samples were digested with ammonium persulfate and sulfuric acid. The colorimetry and ICP-MS TP results were in good agreement. Speciation techniques require separation, identification and characterization of the phosphorus species. Separation techniques coupled to ICP-MS for phosphorus speciation include chromatographic methods (e.g. liquid chromatography (LC), gas chromatography (GC) and supercritical fluid chromatography (SFC)) and electrophoretic methods (e.g. CE and gel electrophoresis) ^{143, 144}. Hyphenated ICP-MS techniques were effectively used for phosphorus speciation analysis ¹⁴³⁻¹⁴⁵.

Phospholipids, a group of organic phosphorus species, were extracted in organic solvents LC separated and measured using ICP-MS ¹⁴⁶. The optimized method used mobile phase splitting prior to nebulization for reduced solvent load to plasma, spray chamber cooling to -5 °C and carrier gas flow adjusted to maximize condensation of the organic vapors. Helium was used as collision gas to reduce polyatomic interferences and the absolute detection limits ranged between 0.2 and 1.2 ng for the analyzed phospholipids. Bisphosphonates, previously known as biphosphonates (HO₃P-CR₁R₂-PO₃H), are a group of compounds analogues to pyrophosphate. They were initially used as complexing agents in water treatment, detergent additives ¹⁴⁷ and treatment of the osteoporosis ¹⁴⁸. Being water soluble ionic compounds, ion-exchange chromatography is the most suitable separation technique, although challenges still exist due to chelation with metal ions and a loss of column performance ^{149, 150}. Kovacevic et al. ¹⁵⁰ separated bisphosphonates on a Dionex AS-7 IC column using diluted nitric acid as eluent and ICP-MS for detection. Polyatomic interferences at m/z 31, influence of plasma parameters on phosphorus and background signal were investigated. Detection limits achieved for two bisphosphonates, alendronic acid (0.2 mg L⁻¹) and etidronic acid (0.5 mg L⁻¹) were higher than those achieved using pre- or post-column derivatization methods ¹⁵⁰⁻¹⁵².

Following the 1970s ban on toxic and persistent organochlorine pesticides, the agrochemical industry used less persistent but more acutely toxic organophosphorus pesticides. Fidalgo-Used et al. ¹⁵³ developed a method for determination of organophosphorus pesticides in spiked river samples by solid phase microextraction (SPME) coupled to GC-MS and GC-ICP-MS for separation and detection. Their experimental setup proved that SPME is an effective technique for the extraction and preconcentration of trace level organophosphorus pesticides in river water samples. Better detection limits were obtained by SPME-ICP-MS (0.09-143 ng L⁻¹) than by

SPME-GC-MS (0.8-504 ng L⁻¹). The use of GC-ICP-MS offered a higher potential for monitoring organophosphorus pesticides in complex environmental samples ¹⁵³. Glyphosate (N-(Phosphonomethyl)glycine), is a phosphonate compound used as a broad-spectrum herbicide with low toxicity to mammals, but its overall environmental impact is not fully understood yet. Trace levels of glyphosate and phosphate in water were measured by IC coupled to ICP-MS ¹⁵⁴, using an anion exchange column (Dionex AS16) and 20 mM citric acid eluent. The glyphosate and phosphate were detected separately by ICP-MS at m/z=31. This sensitive method had a wide dynamic range and low detection limit (0.7 µg L⁻¹) for both analytes, without using preconcentration or chemical derivatization. Similar detection limits were reported by Wigfield et al. ¹⁵⁵.

2.3.6 Electrochemical Methods

In 2012, Berchmans et al. ¹⁵⁶ reviewed the electroanalytical methods used for inorganic phosphate analysis, which include potentiometry, voltammetry and amperometry. Although detection limits are not as low as for MS and colorimetry methods, the simplicity and capability of miniaturizing the electrochemical instrumentation allowed their use as field and remote sensing instrumentation. However, their low sensitivity, frequent equipment recalibrations and drifts caused by temperature fluctuation are limiting their applications.

In 1947, Flatt and Brunisholz ¹⁵⁷ determined phosphate in aqueous solutions by potentiometric titration with silver nitrate when Ag₃PO₄ precipitate is formed. The silver phosphate ion selective electrode (ISE) developed by Novozamsky and van Riemsdijk ¹⁵⁸ was capable of measuring phosphate with detection limit at 30 µg L⁻¹ as P. Despite the major interference observed from chloride ions, it was one of the first direct potentiometric detection of phosphate. In a

potentiometric flow-through detector with a metallic copper electrode, Alexander et al.¹⁵⁹ investigated the electrode response to phosphates (orthophosphate, pyrophosphate and tripolyphosphate) and inorganic anions. In a similar setup with a lead (II) ISE, orthophosphate and tripolyphosphate were reproducibly measured with non-linear calibration curves¹⁶⁰. Improved selectivity was obtained using FIA with cadmium ISE, when Cd^{2+} from the reagent stream precipitated phosphate as $\text{Cd}_3(\text{PO}_4)_2$ ¹⁶¹. Incorporating bis(p-methylbenzyl)tin dichloride, dibenzyltin dichloride, and bis(p-chlorobenzyl)tin dichloride in plasticized polymer membranes Glazier and Arnold¹⁶² achieved a high level of selectivity for dibasic phosphate, hypothesized as the result of Sn-C hyperconjugation within the organotin compound. Chen et al.¹⁶³ developed a FIA with cobalt wire ISE for potentiometric determination of phosphate ($31 \mu\text{g L}^{-1}$ as P detection limit). Marco et al.¹⁶⁴ measured phosphate in waste waters and fertilizer samples using FIA with cobalt wire ISE, compensating the significant positive chloride interference by independently measuring chloride and using a chloride selectivity coefficient. A low-level detection of dibasic phosphate in wastewater samples ($93 \mu\text{g L}^{-1}$ as P) was achieved with a potentiometric cobalt-based screen-printing sensor fabricated by electroplating cobalt on the surface of a screen-printing electrode. The interferences of pH, dissolved oxygen and other anions were examined¹⁶⁵. A potentiometric detector for phosphate, resistant to turbidity and color interference, was developed by Li et al.¹⁶⁶ using Zn^{2+} and Cu^{2+} complexes with 2,6-bis(bis(2-pyridylmethyl)aminomethyl)-4-methylphenol as a receptor and o-mercaptophenol as an indicator.

Voltametric measurements of orthophosphate can be based on the reduction of the ammonium phosphomolybdate complex, inhibition of the redox reaction of potassium hexacyanoferrate or the inhibition of an enzyme-based glucose electrode. The process is inhibited by the oxygen atoms around the central phosphorus atom¹⁶⁷. Fogg and Bsebsu proposed differential-pulse voltammetric

determination of orthophosphate as molybdovanadophosphate at a glassy carbon electrode ¹⁶⁸ and by direct injection of phosphate into molybdate reagent ¹⁶⁹. Effective blank background elimination was possible with phosphate detection limit at 31 $\mu\text{g L}^{-1}$ as P. A similar analytic procedure with a glassy-carbon electrode was used for the determination of phosphate, silicate, arsenate and germanate as β -heteropolymolybdates ¹⁷⁰. Recently, Barus et al. ¹⁷¹ developed a phosphate sensor using square wave voltammetry. The system does not require addition of any reagents as they are formed in situ (H^+ and MoO_4^{2-}). An optimized prototype electrochemical cell requires only 5 minutes of complexation time and has a concentration range between 15 and 125 $\mu\text{g L}^{-1}$ as P.

Amperometric sensors measure the current produced by the target analyte at an electrode with a constant voltage. The reduction of ferrocene based macrocyclic synthetic receptor and the reduction of a phosphomolybdate complex were used as indirect amperometric measurements of the orthophosphate ¹⁶⁷. Simultaneous determination of phosphate and pyrophosphate by amperometric detection with immobilized enzyme reactors was proposed by Yao and Wasa ¹⁷². The injected sample was split into two channels, with one channel going through the immobilized pyrophosphate reactor prior to a co-immobilized nucleoside-phosphorylase-xanthine oxidase reactor to obtain orthophosphate (31 $\mu\text{g L}^{-1}$ as P detection limit). The second channel passed only through the latter reactor to obtain sum of orthophosphate and pyrophosphate (62 $\mu\text{g L}^{-1}$ as P detection limit). The authors also used a novel bioamperometric flow-injection system with a 16-way switching valve to simultaneously determine orthophosphate and 12 purine phosphate nucleotides. Samples were passed through two channels, acid phosphatase immobilized reactor and a delay coil to separate the peaks corresponding to the two sample portion injected ¹⁷³. Cheng et al. ¹⁷⁴ achieved lower detection limits with amperometric detection of phosphate (0.3 μM). In

this FIA system the adsorption of phosphate at the electrode was triggered by the activation of a barrel plated nickel electrode (Ni-BPE) in alkaline media, forming a $\text{Ni(OH)}_2/\text{NiO(OH)}$ film. Upon the addition of phosphate, the current suppression of the glucose signal at Ni-BPE was observed. The proposed analytical system is an electrochemical sensor that can detect phosphate with good sensitivity and selectivity. A novel phosphate and bicarbonate sensing method was proposed by Honda and Moromitsu ¹⁷⁵ by electrochemical oxidation, with a detecting electrode obtained from amorphous $\text{RuO}_2\text{-Ta}_2\text{O}_5$ catalysts, formed on a titanium substrate.

2.3.7 Nuclear Magnetic Resonance Methods

Nuclear magnetic resonance is one of the most powerful non-invasive experimental techniques available for providing atomic and molecular structure information based on changes in resonance frequency of the intramolecular magnetic field around an atom in a molecule ¹⁷⁶.

Dissolved phosphorus species such as orthophosphate, phosphonates, orthophosphate monoesters and di-esters were detected by ^{31}P -NMR spectroscopy in lake and river water after ultrafiltration and reverse osmosis of 250-500 mL samples. Soluble unreactive phosphorus was analyzed also with the use of praseodymium ethylenediamine tetraacetate, alkaline bromination degradation procedures and adjustment of sample pH ¹⁷⁷. Cade-Menun et al. ¹⁷⁸ developed a procedure for characterization of dissolved and particulate phosphorus species from river waters. Detected phosphorus species included orthophosphate, phosphonates, *myo*-inositol hexakisphosphate and orthophosphate di-esters. Further investigation is required due to possible alteration of the phosphorus forms when using lyophilization of the samples in NaOH-EDTA solutions. A thorough speciation of dissolved and particulate phosphorus forms in the eutrophic Lake Mendota (Wisconsin, USA) by ^{31}P -NMR spectroscopy was performed by Read et al. ¹⁷⁹. The results showed that particulate phosphorus species contained phosphonate, orthophosphate

mono- and di-ester, orthophosphate, pyrophosphate and polyphosphate. Dissolved phosphorus contained all particulate phosphorus forms with orthophosphate being the dominant species. Biochemical regression analysis of the results provided insight into mechanisms of phosphorus dynamics. Similar ^{31}P -NMR study of the dissolved and particulate phosphorus species in an eutrophic lake was done in the shallow Lake Taihu, China ¹⁸⁰. D_2O was added into the supernatant to reach 10% proportion required for signal lock prior to the analysis. The ^{31}P -NMR spectra were measured at 161.84 MHz on an Agilent AV400 spectrometer, with orthophosphate and phosphate monoesters as the dominant phosphorus species in both fractions. While phosphonate was detected in dissolved fraction, pyrophosphate was mainly found in the particulate phosphorus fraction. At this time there is no NMR standard method for phosphorus species analysis. Chemical shifts of phosphorus species are affected by pH and salt concentrations, a challenge for data reproducibility ¹⁸¹. More than 50 phosphorus compounds were analyzed under standardized conditions in order to establish a P-compounds library for easier repeatability of the experiments and ^{31}P -NMR identification of the compounds ¹⁸¹. Forms and lability of phosphorus species in algae and aquatic macrophytes were investigated by ^{31}P -NMR coupled with enzymatic hydrolysis using alkaline phosphatase ¹⁸². Eleven phosphorus species were identified in the mono- and di-ester region as well as orthophosphate, pyrophosphate and phosphonates.

2.3.8 Conclusions

As a direct result of nutrient control policies introduced by many countries, there is a decline in total phosphorus loadings in natural waters, with more than 50% reduction of TP observed in Lake Erie since 1967 ¹⁸³. Similar decreases in TP were reported in a long-term study of Boreal lakes in Sweden ¹⁸⁴. However, algal blooms are still occurring, and the climate changes may

influence the frequency of these events. As the composition of the nutrient pool impacts the harmful algal blooms ¹⁸⁵, phosphorus speciation methods with trace and ultra-trace level detection limits will be required. Currently, FIA spectrophotometric methods are the major analytical technique of choice in the environmental analysis of orthophosphate and total phosphorus. The electrochemical methods support remote sensing with real time results access, but some still require improvements in detection limits to match current environmental levels of phosphate in surface waters.. Most of the developed methods are analyzing orthophosphate and total phosphorus. There is a need for developing new methods able to measure organic phosphate species. ³¹P NMR was capable of analyzing classes of P-species, however there is a lack of methods capable of analyzing specific phosphate compounds. Although not a very popular technique yet, the IC-ESI-MS instrumentation provide the required low-level detections and a possibility to analyze simultaneously a large number of specific phosphorus species. Compared to other analytical methods IC-ESI-MS provides high sensitivity and selectivity required for analysis of both inorganic and organic phosphate species in surface water matrices at various levels. Increasing availability and decreasing cost of instrumentation will support development of highly selective and sensitive analytical methodologies for phosphorus speciation.

CHAPTER 3

Unbiased Measurement of Phosphate and Phosphorus Speciation in Surface Waters

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3.1 Abstract

Trace-level phosphate analysis and phosphorus speciation in surface water remained challenging due to adsorption and phosphate uptake by microorganisms. In this study a two-dimensional ion chromatography separation coupled to electrospray ionization high-resolution mass spectrometry (2D-IC-ESI-MS) allowed isotope dilution quantitation of phosphate with simultaneous analysis of eleven phosphate-containing metabolites and two inorganic condensed phosphates. Samples were collected from Lake St. Clair, Lake Ontario and Georgian Bay (ON, Canada). Comparative experiments showed lower phosphate results for samples not immediately spiked and for external calibration quantitation. Field spiking with ^{18}O -labeled phosphate combined with isotope dilution quantitation allows measurement of the phosphate levels existent at the collection time instead of the phosphate concentrations remaining in the samples at the analysis time. This is a significant advantage against the traditional colorimetric and ion chromatographic (IC) analysis methods, which are unable to compensate for the adsorption loss occurring in standards and samples, especially when phosphate is present at levels below $20\ \mu\text{g L}^{-1}$ as P ($61\ \mu\text{g L}^{-1}$ as PO_4^{3-}). Two phosphate-containing metabolites, adenosine 5'-monophosphate (AMP) and D-glucose 6-phosphate (Glucose-P), were detected in a subset of samples collected from Lake St. Clair, with no statistically significant correlation between them and the simultaneously measured phosphate. Directly bioavailable P (phosphate), indirectly bioavailable P (phosphatase-hydrolyzed P) and non-bioavailable P (non-hydrolyzable P) fractions were quantified by measuring phosphate, phosphate after phosphatase addition and total phosphorus. The proposed 2D-IC-ESI-MS method developed for a QExactive MS instrument with field spiking of the internal standard provides accurate phosphate results and eliminates quantitation errors caused by phosphate adsorption. This

setup allows simultaneous collection of targeted and non-targeted analysis data and thus the detection of trace polar organic phosphorus metabolites as well.

3.2 Introduction

3.2.1 Phosphorus Species in Surface Waters

Phosphate ions (H_2PO_4^- , HPO_4^{2-} , PO_4^{3-}) are a directly bioavailable phosphorus (P) source for biota, including algae. Concentrations of phosphate ions in surface water are relatively low compared to other macronutrients and as such, represent the limiting factor for primary production in freshwater lakes^{186, 187}. Other condensed inorganic phosphate and organic phosphate-containing metabolites (P metabolites) can also be present in surface waters⁷⁶. Species of dissolved inorganic P in natural waters include condensed linear polyphosphates, metaphosphates (cyclic phosphates) and ultraphosphates (branched cyclic structures)⁷⁶. Phosphate containing species can be produced by biological activity and also introduced into natural waters by anthropogenic activities¹⁸⁶. Phosphorus from the inorganic condensed P species is not directly bioavailable to algae⁹², while the organic phosphate-containing species such as sugar phosphates, glycerophosphates, polynucleotides and phospholipids can be released from decomposed cells and utilized by phytoplankton after phosphatase-catalyzed mineralization¹⁴. Phosphatase thus promotes the release of directly bioavailable P from metabolites, contributing to the P recycling dynamics in surface waters^{78, 188}. Alkaline phosphatases are membrane-bound glycoproteins that catalyze the hydrolysis of P metabolites into phosphate and the organic moieties at alkaline pH¹⁸⁹. Phosphatases are also involved in other metabolic processes such as transport of substances across membranes and synthesis of new organic phosphates (transphosphorylation)¹⁹⁰. Phosphatases can be essential for phosphate regeneration and

planktonic nutrition in lakes¹⁸⁸. Lake water can contain alkaline phosphatases, while acid phosphatases play a role in the internal cell metabolism of algae¹⁸⁸. Steiner hypothesized in 1938 the release of enzymes in surface waters from plankton after observing the conversion of organic phosphate compounds into inorganic phosphate in solvent-lysed plankton suspensions¹⁹¹. Huang *et al.* studied phosphatase-mediated hydrolysis mechanisms by alkaline and acid phosphatase using nuclear magnetic resonance (NMR)⁷⁸. Organic phosphate esters are involved in biochemical reactions as energy carriers, coenzymes or as intermediates. They are also involved in photosynthesis, carbohydrate metabolism, lipid metabolism, the nitrogen cycle and in other biochemical reactions involving energy transfer¹⁹². Monitoring the directly bioavailable phosphate and the indirectly bioavailable phosphate species released by phytoplankton can provide important information to the algal bloom research.

3.2.2 Analysis of Phosphorus Species in Surface Waters

Phosphate remains a widely measured parameter in environmental samples and the spectrophotometric parameter measured known as soluble reactive phosphate or soluble reactive phosphorus (SRP), with the values being presented either as phosphate or phosphorus^{100, 193, 194}. In the classic spectrophotometric or colorimetric approach, phosphate is quantified after forming an antimony-phospho-molybdate complex (peak absorption at 880 nm) under acidic conditions (e.g. ascorbic acid)^{100, 193, 194}. The SRP parameter represents the sum of phosphate directly bioavailable to algae⁹² (present in surface water at neutral pH as H_2PO_4^- and HPO_4^{2-} at approximately 1:1 ratio) and non-directly bioavailable phosphate (released from condensed phosphates under acidic conditions required for the antimony-phospho-molybdate complex formation)¹⁹⁵⁻¹⁹⁸. Current environmental standard methods recommend the spectrophotometric method for samples with SRP concentrations higher than $10 \mu\text{g L}^{-1}$ as P^{100, 193, 194}.

In oligotrophic natural waters, phosphate is often present at concentrations below $1 \mu\text{g L}^{-1}$ as P, requiring more sensitive analysis techniques for accurate quantification. Speciation of phosphorus compounds has been demonstrated by NMR^{177, 179, 180}, inductively-coupled plasma mass spectrometry (ICP-MS)¹⁴³⁻¹⁴⁵ and ion chromatography electrospray ionization mass spectrometry (IC-ESI-MS)^{123, 124}. However, phosphorus speciation in surface waters remains problematic due to sub-ppb concentration levels of many P-containing compounds. High concentrations of sulfate and other anions such as chloride and nitrate can affect the separation and interfere with the phosphate measurement by IC¹⁹⁹⁻²⁰¹. In natural waters the sulfate concentration is in general over 1000 times higher than the phosphate concentration²⁰², limiting the anion column capacity for the separation of phosphate, as well as causing signal suppression during the ESI process²⁰³. To increase the analytical signal, some off-line procedures have been described, including the removal of high concentrations of chloride and sulfate with functionalized ion exchange resins (e.g. Ag^+ , Ba^{2+})²⁰⁴ and phosphate pre-concentration with formation of molybdophosphoric acid²⁰⁵. On-line approaches for elimination of interferences include microdialysis²⁰⁶, column-switching systems²⁰⁷ and solid-phase microextraction²⁰⁸.

3.2.3 Phosphate Adsorption on Glassware

Sorption of the phosphate to surfaces of various types of containers and sediment particles has been well documented^{95, 209-214}. Losses of up to 20% of phosphate on acid-washed glass were reported by Ryden et al²¹⁰. Studies with radioactive ^{32}P and colorimetry showed 8% phosphate adsorption on borosilicate glass from a $7.5 \mu\text{g L}^{-1}$ as P solution after 3 days and as much as 31% on polyethylene containers²¹¹. Lambert et al.⁹⁵ measured changes in concentration of the phosphorus fractions in water stored at 4 °C and found within 4 hours of sampling 28-45% decreases in total dissolved phosphorus, 33-40% in total reactive phosphorus and 36-58% in SRP

concentrations. The adsorption of phosphate on porous glass and silica (SiO_2) surfaces is mainly driven by electrostatic forces and strongly influenced by the ionic strength in the solution and temperature. At pH 2 the adsorption was five times higher on porous glass than on silica, either due to increased interaction between the potential fields from opposite walls in micropores or the Al_2O_3 content²¹². The adsorption is pH, solution composition and species dependent, with phosphate being less adsorbed than condensed phosphates, but stronger than the organic phosphate species²¹⁵.

In this study, an IC-ESI-MS instrument was chosen for the simultaneous analysis of phosphate, pyrophosphate, triphosphate and 11 organic P metabolites in surface water samples collected from Lake St. Clair, Lake Ontario and Georgian Bay (ON, Canada). This is the first report of using an isotope dilution quantitation for phosphate measurement in surface water samples. The field spiking with ^{18}O -labeled internal standard (IS) allowed measurement of phosphate existent at collection time, contrasting with existent analytical methods for phosphate analysis that cannot easily compensate for the sample-dependent losses of phosphate by adsorption and biological uptake. Total phosphorus measurements and phosphate reanalysis after phosphatase addition were also used to measure directly bioavailable, indirectly bioavailable (phosphatase-hydrolyzed) and non-bioavailable (non-hydrolyzable) P fractions. A set of organic P metabolites were monitored and identified for the first time in surface water samples.

3.3 Experimental Section

3.3.1 Standards and chemicals

Certified phosphate standards were purchased from Inorganic Ventures (Christiansburg, VA, USA) and NSI Lab Solutions (Raleigh, USA). The isotope-labeled phosphate, $\text{H}_3\text{P}^{18}\text{O}_4$ (IS), the

P metabolite salts, adenosine 5'-monophosphate (AMP) disodium salt, adenosine 5'-diphosphate (ADP) sodium salt, adenosine 5'- triphosphate (ATP) disodium salt hydrate, D-fructose 6-phosphate (Fructose-P) disodium salt hydrate, D-fructose 1,6-biphosphate (Fructose-Bi-P) trisodium salt hydrate, D-glucose 6-phosphate (Glucose-P) sodium salt, α -D-glucose 1,6-biphosphate (Glucose-Bi-P) potassium salt hydrate, α -D-galactose 1-phosphate (Galactose-P) dipotassium salt pentahydrate, D-ribulose 5-phosphate (Ribulose-P) disodium salt, D-ribulose 1,5 – biphosphate (Ribulose-Bis-P) sodium salt hydrate, D-erythrose 4-phosphate (Erythrose-P) sodium salt, D-(-)-3-phosphoglyceric acid (PGA) disodium salt, uridine 5'- diphosphoglucose (UDP-Glucose) disodium salt hydrate and acid phosphatase (from potato) were purchased from Millipore Sigma (Oakville, ON, Canada). Calibration standards were obtained by mixing these 11 P metabolites, containing each analyte at at 0 nM (Blank), 1 nM, 10 nM, 100 nM and 1000 nM concentrations. A second set of calibration standards was prepared for phosphate at 0 $\mu\text{g L}^{-1}$ (Blank), 1 $\mu\text{g L}^{-1}$, 2 $\mu\text{g L}^{-1}$, 5 $\mu\text{g L}^{-1}$, 10 $\mu\text{g L}^{-1}$, 20 $\mu\text{g L}^{-1}$ and 50 $\mu\text{g L}^{-1}$ as P, with each standard containing ^{18}O -labeled phosphate (IS) at 20 $\mu\text{g L}^{-1}$ as P. HPLC grade acetonitrile, sodium pyrophosphate tetrabasic and sodium triphosphate pentabasic were purchased from Millipore Sigma (Oakville, ON, Canada). A third set of calibration standards was prepared containing pyrophosphate and triphosphate, each at 0 nM (Blank), 1 nM, 10 nM, 100 nM and 1000 nM.

3.3.2 Field Sampling and Storage Containers

Surface water samples were collected from coastline of Georgian Bay (including embayments and river mouths, N=51, October 2016), and Lake St. Clair (including river sites, N=1030, May - November 2017). A third set was collected from Lake Ontario adjacent to the cities of Toronto and Mississauga and included sites at the mouths of urban rivers, waste water treatment plant outfalls, in close proximity to the shoreline and in the oligotrophic offshore of the lake during the

late spring and early summer of 2018 (N=203). The water was either pumped by the flow-through system located on the sampling vessel or collected using a Van Dorn beta sampler. After filtering through a Millex-HV syringe driven 0.45 μm Durapore filter (Fisher Scientific Company, Ottawa, ON, Canada) at each sampling station, 10 mL of each sample was immediately pipetted in a 15 mL high density polyethylene (HDPE) amber screw-cap bottle (Thermo Scientific, Mississauga, ON, Canada) and spiked with 100 μL of IS at 2 mg L^{-1} as P, achieving the same final IS concentration as in the calibration standards (20 $\mu\text{g L}^{-1}$ as P). Samples were stored at 5 ± 3 $^{\circ}\text{C}$ until analysis. Additional types of containers were used for a stability study including 15 mL Wheaton polypropylene and 60 mL Nalgene HDPE (Fisher Scientific Company, Ottawa, ON, Canada), 120 mL borosilicate glass (pre-cleaned with 30% H_2SO_4) and 20 mL borosilicate glass (System Plus, Baden, ON, Canada).

3.3.3 Instrumental Analysis

Analysis was performed on an ICS 5000⁺ ion chromatograph (IC) coupled to a QExactive Mass Spectrometer (Thermo Scientific, Mississauga, ON). The IC has a dual configuration with one autosampler, two pumps, two auxiliary pumps, two eluent generators, two ion suppressors (AERS-500 2mm) and two conductivity detectors. The ESI source has a HESI-II probe housed in the IonMax Source. The MS was operated in negative mode, full scan (m/z 50–700) at 70000 resolution with 3×10^6 AGC target and 200 ms maximum injection time. The IC eluent was combined with 0.2 mL min^{-1} acetonitrile prior to entering the MS source, using the following ESI settings: -3.5 kV spray voltage, 12 for sheath gas, 3 for auxiliary gas, 320 $^{\circ}\text{C}$ capillary temperature, 90 for S-lens RF and 200 $^{\circ}\text{C}$ auxiliary gas temperature. XCalibur software version 3.0 was used for data acquisition and TraceFinder version 3.2 (Thermo Scientific, Mississauga, ON) for quantitation.

3.3.4 Method A (IC-ESI-MS analysis)

Separation was achieved at a flow rate of 0.3 mL min^{-1} with $100 \text{ }\mu\text{L}$ injection volume, AG24 Dionex guard column (2 mm I.D. x 50 mm) and AS24 Dionex separation column (2 mm I.D. x 250 mm). The 30 min gradient separation was started at 20 mM OH^- for the first 6.5 min, increased to 45 mM during the next 3 min, then further increased to 60 mM for the next 6 min, followed by a decrease to 20 mM during the last 5 min of the run. The highest calibration standard for phosphate was $20 \text{ }\mu\text{g L}^{-1}$ as P, $R^2 \geq 0.9995$ and method detection limit (MDL) of $0.15 \text{ }\mu\text{g L}^{-1}$ as P.

3.3.5 Method B (2D-IC-ESI-MS analysis)

The two-dimensional chromatographic separation was used to divert to waste sulfate and prior eluting anions at a flow rate of 1 mL min^{-1} . For the first dimensional separation, $1000 \text{ }\mu\text{L}$ from each sample was injected onto an AG18 Dionex guard column (4 mm I.D. x 50 mm) and AS18 Dionex analytical separation column (4 mm I.D. x 250 mm). The 45 min gradient separation on the first column was obtained by increasing from 30 to 60 mM OH^- during the first 8 minutes. The hydroxide concentration remained constant for the following 32 min, then decreased to 30 mM during the last 5 min of the run. The second dimensional separation was achieved with AS24 separation column (2 mm I.D. x 250 mm) at a 0.3 mL min^{-1} flow rate. The 45 min gradient separation on the second dimension started with 60 mM OH^- for 15 min, decreased to 30 mM for the following 5 min, then increased to 60 mM for the remaining 25 min (see Supporting Information (SI)). During the first 7 min of the run, eluent passing through the first column dimension and subsequent suppressor was diverted to waste in order to remove sulfate and other earlier eluting anions. During the following 13 min, the anions present in the eluent

were loaded onto an IonPac TAC-ULP1 Anion Concentrator (5 mm I.D. x 23 mm). 20 min after the initial sample injection, the anions were eluted from the concentrator column and separated by the second-dimension column during the last 25 min of the run. The column switching time window was determined from the retention times by injecting a standard solution containing phosphate and sulfate at 100 $\mu\text{g L}^{-1}$. The highest calibration phosphate standard was 50 $\mu\text{g L}^{-1}$ as P, $R^2 \geq 0.9995$ and MDL of 0.06 $\mu\text{g L}^{-1}$ as P.

3.4 Results and Discussions

3.4.1 Field Spiking and Isotope Dilution Measurement of Phosphate

The phosphate analysis was initially done with Method A by IC-ESI-MS for 51 samples collected from Georgian Bay. A subset of 11 samples were collected as replicates, with the first set spiked immediately with IS, the second set spiked after 24 h and the third set spiked after 120 h. All samples contained trace-level phosphate between 0.7 and 1.8 $\mu\text{g L}^{-1}$ as P. The set of samples immediately spiked were considered as free of any phosphate loss by adsorption and their concentrations were compared with the phosphate concentrations measured in the delayed-spiked sets to identify the adsorbed fractions. Samples spiked after 24 h had phosphate results lower by an average of 30.2 %, while the samples spiked after 120 h had lower results by an average of 52.6 % (Figure 3.1). Similarly, another test on 8 samples identified an average phosphate loss of 33.7% for the set spiked after 48h from collection time (Figure S1, SI). Studies of phosphate adsorption on porous glass and SiO_2 showed that adsorption equilibrium was established within 10 minutes, regardless of the initial phosphate concentration in the solution²¹⁶. Increases in ionic strength of the adsorption medium resulted in suppression of the phosphate uptake, suggesting a significant contribution of electrostatic forces in the adsorption process.

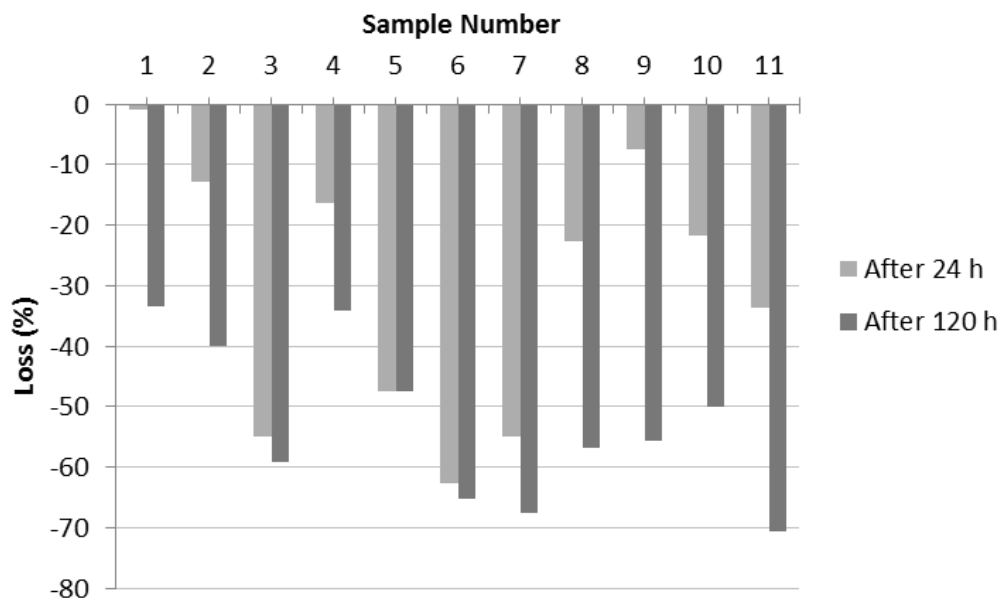
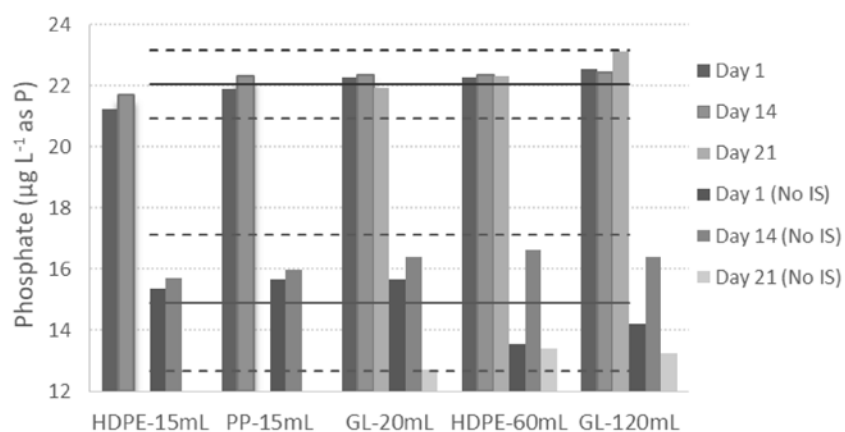


Figure 3.1. Phosphate loss (%) calculated from single measured concentrations in samples spiked 24 h and 120 h after collection. The phosphate content in samples spiked immediately after collection varied between 0.7 and 1.8 $\mu\text{g L}^{-1}$ as P.

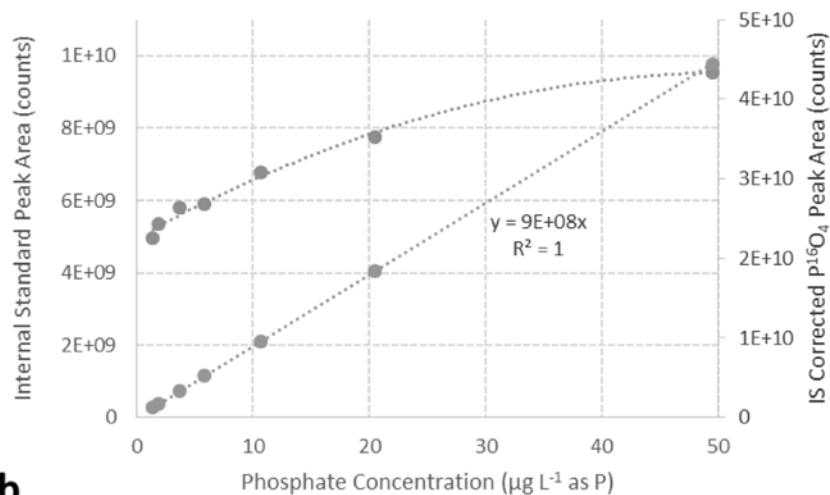
Similarly, Lambert et al.⁹⁵ showed a fast phosphate adsorption with concentration decreases within 4 hours from sampling for total dissolved phosphate (TDP) (28-45%), total reactive phosphate (TRP) (33-40%) and soluble reactive phosphate (SRP) (36-58%). The results from this study confirmed previous findings about adsorption loss and suggest that accurate phosphate results (e.g. phosphate, SRP, TRP) can be obtained only if samples are analyzed immediately on-site. For most studies such an approach would be difficult. US EPA method ¹²² for IC analysis allows holding times of up to 48 h for phosphate, but based on this study a 30% average phosphate loss was observed within 24 h. The percentage absorption loss of phosphate is a function of the total phosphate concentration in a sample, with appreciably lower absorption losses from high concentration samples (e.g. urban river) than lower concentration samples (e.g. oligotrophic lake).

The influence of different sample containers on the adsorption of phosphate has been previously studied²¹¹. To investigate the container influence on phosphate stability during storage, a larger volume (500 mL) surface water sample was filtered in a PET container, spiked immediately with concentrated IS (1 % of the sample volume), then subsamples were transferred in five different plastic and glass containers, then stored at 4 °C for up to 21 days. The results showed that the phosphate concentrations could be accurately quantified with isotope dilution for up to 21 days of storage. Overall the phosphate concentration measured by isotope dilution in HDPE and glass containers remained stable within $\pm 5\%$ of the initial values (Figure 3.2a). The variation is within the uncertainty of the phosphate results of $\pm 6.3\%$ at $20 \mu\text{g L}^{-1}$ as P. Ignoring the internal standard values and quantifying phosphate peaks by external calibration, the phosphate results obtained were 32.5% lower for a sample containing $20 \mu\text{g/L}$ phosphate as P, with the results within $\pm 15\%$ of the initial values. The third set of values (Day 21) is not available for the 15 mL containers due to insufficient sample volume. The relatively high charge density of the phosphate ions at neutral pH explains their higher adsorption affinity compared to the other major ions present in natural water. The adsorption rate and mechanism depends on the type of the container material, pre-treatment of sample containers, surface area to volume ratio, matrix composition and concentration of phosphate in the sample²¹¹. Sample containers such as plastic and glass have positively charged ion-exchange sites, which become neutral after sorption of anions from the aqueous sample²¹⁷. The isotope dilution quantitation with field-spiked IS allowed measurement of the initial phosphate concentration even after 21 days, because the ratio between phosphate and IS (^{18}O -labeled phosphate) remained the same in the sample even after phosphate adsorption occurred, as both phosphate and IS are adsorbed at the same rate. The phosphate concentration dependence of the IS adsorption was observed also in the calibration standards

(Figure 3.2b). A 40-50% loss of the internal standard was observed when spiked at $20 \mu\text{g L}^{-1}$ in calibration standard solutions containing less than $10 \mu\text{g L}^{-1}$ phosphate. A much smaller loss (0-20%) was observed in solutions containing 20-50 $\mu\text{g/L}$ phosphate. The age of the solutions and also instrumental factors can influence the measured peak areas of the phosphate and the internal standard, with the good linearity of the calibration curve achieved by the new isotope dilution (internal standard) method as a proof of compensating for all these factors.



a



b

Figure 3.2. a) Phosphate concentration in a surface water sample (initial values mean of $22.05 \pm 1.10 \mu\text{g L}^{-1}$) remained stable (within $\pm 5\%$) when stored for up to 21 days in high density polyethylene (HDPE, 15 and 60 mL capacity), polypropylene (PP, 15 mL) and borosilicate glass (GL, 20 and 120 mL). External calibration (set of smaller bars in brown shades) provided 32.5% lower results from Day 0 (initial values mean of $14.89 \pm 2.23 \mu\text{g L}^{-1}$), with values varying within $\pm 15\%$. b) Variation of the internal standard peak area with phosphate concentration ($\text{H}_2\text{P}^{16}\text{O}_4^-$) in the calibration standard set and a linear calibration curve obtained with $\text{H}_2\text{P}^{18}\text{O}_4^-$ as internal standard.

3.4.2 Isotope Dilution, External Calibration and Colorimetric Measurement of Phosphate

A set of 217 samples collected from Lake St. Clair in 2017 were quantified by isotope dilution with Method B, a 2D-IC-ESI-MS method. The same set of samples was quantified by external calibration and also analyzed by a classical colorimetric method (see SI) to demonstrate the advantage of using the isotope dilution quantification against the traditional phosphate measurements. The external calibration quantitation is similar to a simple IC separation with conductivity detection, although the mass spectrometric detection without internal standard is more prone to be affected by matrix effects. As observed in Figure 3.3a, the phosphate results obtained by both, external calibration and colorimetric analysis (SRP) were lower than the isotope dilution results for the entire concentration range, with the exception of some colorimetric results for samples containing less than $3 \mu\text{g L}^{-1}$ as P. The two data sets, external calibration by 2D-IC-ESI-MS and colorimetric SRP, present similar bias trends (see Figure 3.3b). A positive bias is present for the SRP results below $3 \mu\text{g L}^{-1}$ as P, as previously presented by Hudson et al.⁷⁵. This is caused by the adsorption of phosphate from the unpreserved calibration standards used by colorimetry and quantification errors inherent to the low end of the

calibration curve, resulting in an overestimation of the data at low phosphate levels, even in the presence of the adsorption loss in the samples. The same trend is confirmed when the phosphate results were quantified by external calibration using the 2D-IC-ESI-MS generated data. For phosphate levels above $3 \mu\text{g L}^{-1}$ as P, a negative bias was observed in comparison to the isotope dilution approach for both, external calibration and colorimetry data, most likely caused by phosphate adsorption in the analyzed samples. The relatively lower external calibration results in comparison to the SRP results could be the result of matrix effects during the mass spectrometric analysis, but are more likely the consequence of increased phosphate concentrations by colorimetry caused by acid-driven hydrolysis of condensed inorganic and organic phosphates^{197, 218, 219} as well as release of phosphate from colloids^{120, 220-222} during the analysis. These results demonstrate the significant advantage of the isotope dilution quantitation against the classical colorimetric and IC methods, being able to provide the phosphate results at the time of collection instead of the phosphate concentrations present in samples at the analysis time.

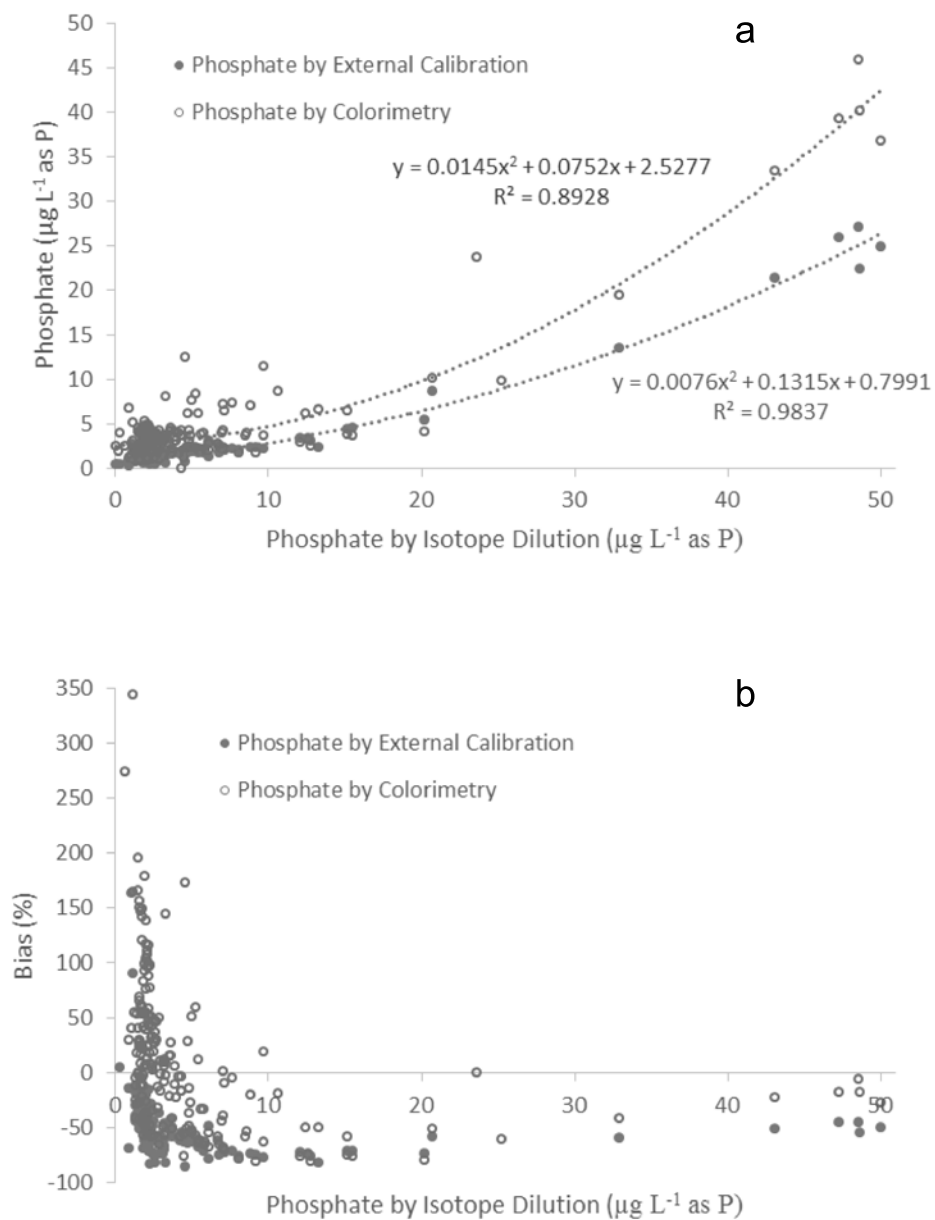


Figure 3.3. Comparison of results (a) and bias (b) for Method B (2D-IC-ESI-MS) phosphate results (N=217, collected from Lake St. Clair in July 2017) obtained by isotope dilution quantification with the results obtained by Method B with external calibration (filled markers) and the colorimetrically measured phosphate, SRP (empty markers).

The two-dimensional IC configuration allowed removal of sulfate from the sample matrix prior to the chromatographic separation, eliminating signal suppression and column overloading issues. A “heart-cutting” column-switching setup was used to redirect the sulfate and less retained anions to waste and the rest of the anions to the concentrator column. The 2D-IC-ESI-MS method allowed lowering the MDL to $0.06 \mu\text{g L}^{-1}$ as P from $0.15 \mu\text{g L}^{-1}$ as P achieved with Method A (IC-ESI-MS). The procedure did not require sample pre-treatment or sample dilution, minimizing also the risks for phosphate loss and contamination.

Samples collected from Georgian Bay (2016, N=51) had lower levels of sulfate and were analyzed by Method A (IC-ESI-MS), while samples collected from Lake St. Clair (2017, N=1030) were analyzed by Method B (2D-IC-ESI-MS). All samples were spiked in the field with IS. In Georgian Bay samples, the phosphate concentrations were between 0.20 and $1.87 \mu\text{g L}^{-1}$ as P with sulfate present at $5\text{-}10 \text{ mg L}^{-1}$ (see SI for sulfate analysis method). The phosphate concentration in Lake St. Clair samples ranged between 0.06 (MDL) and $50 \mu\text{g L}^{-1}$ as P, with sulfate present at $15\text{-}30 \text{ mg L}^{-1}$. The typical environmental sample concentration ranges can be observed in Figure 3.4, for samples collected from the Georgian Bay, Lake St. Clair and Lake Ontario. The Lake St. Clair data set includes inshore, open-water near-shore and tributaries (Thames River and Detroit River). The Lake Ontario samples were collected adjacent to the cities of Toronto and Mississauga, including sites at the mouths of urban rivers, waste water treatment plant outfalls, in close proximity to the shoreline and in the oligotrophic offshore of the lake. Phosphate from surface water samples provides an indication of the immediately bioavailable P to algae and can be a predictor of the algal growth²²³⁻²²⁶. The ability of the new isotope dilution method to accurately and reliably measure phosphate across a broad range of concentrations is useful for environmental monitoring programs, particularly those in the Great

Lakes with a variety of habitats including the oligotrophic offshore waters and the nutrient-rich river mouth areas and tributaries waters.

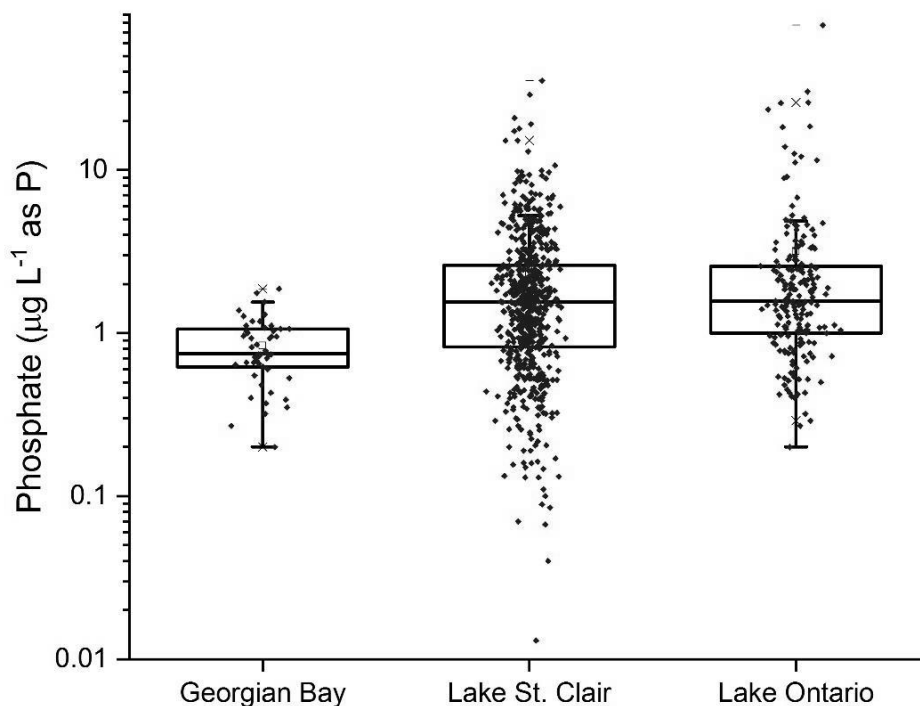


Figure 3.4. Summary of the phosphate concentrations measured in samples collected from: Georgian Bay (N=51), , Lake St. Clair (N=1030) and Lake Ontario (N=203). Samples from Georgian Bay were analyzed by Method A (IC-ESI-MS), while the rest of the samples were analyzed by Method B (2D-IC-ESI-MS).

3.4.3 Comparison of Phosphate and Total Phosphorus Results

The most common phosphate analysis method in water samples currently used in analytical laboratories is the spectrophotometric approach modified from the original Murphy and Riley method using ascorbic acid as a reducing agent, adapted to colorimetric and continuous flow analyzers¹⁰¹. The methods 15681 from the International Organization for Standardization (ISO)^{193, 194} that determine the phosphate by flow injection analysis (FIA) and continuous flow analysis (CFA) are suitable for SRP determination and total phosphorus (oxidative-acid digestion prior to analysis) at concentrations between 10 and 1000 $\mu\text{g L}^{-1}$ as P. Colorimetric methods, especially for SRP determination, can be affected by spectrophotometric interferences (e.g. arsenates, silicates, fluoride, nitrite and suspended particles)¹⁹³. A set of 217 samples with SRP concentrations between 0.2 and 45.9 $\mu\text{g L}^{-1}$ as P and total phosphorus concentrations between 0.3 and 57.6 $\mu\text{g L}^{-1}$ as P was used to compare isotope dilution measured phosphate (2D-IC-ESI-MS) with colorimetric SRP and total phosphorus results (Figure 3.5). This set of samples was collected in August 2017 from Lake St. Clair (ON, Canada). The advantage of using an IC-ESI-MS instrument with isotope dilution instead of a spectrophotometric analyzer is the avoidance of interferences. Graphical representation of the results showed a linear relationship between the 2D-IC-ESI-MS method and the colorimetric total phosphorus results (Pearson correlation coefficient $R^2=0.9049$), although such a relationship is not expected because the degree to which biological utilization draws down phosphate or breakdown introduces phosphate in the lake water at the sample collection time is variable and not dependent on TP. Colorimetric SRP and total phosphorus results do not show a linear relationship and no correlation below total phosphorus concentrations of 10 $\mu\text{g L}^{-1}$ P. Such non-linear relationship between SRP and TP may be caused also by uncompensated phosphate adsorption losses during storage of samples

and calibration standards. The field-spiking of the samples with IS and also at calibration standard preparation allowed to compensate for phosphate losses and allowed reliable measurement of the phosphate existent in the samples at the collection time.

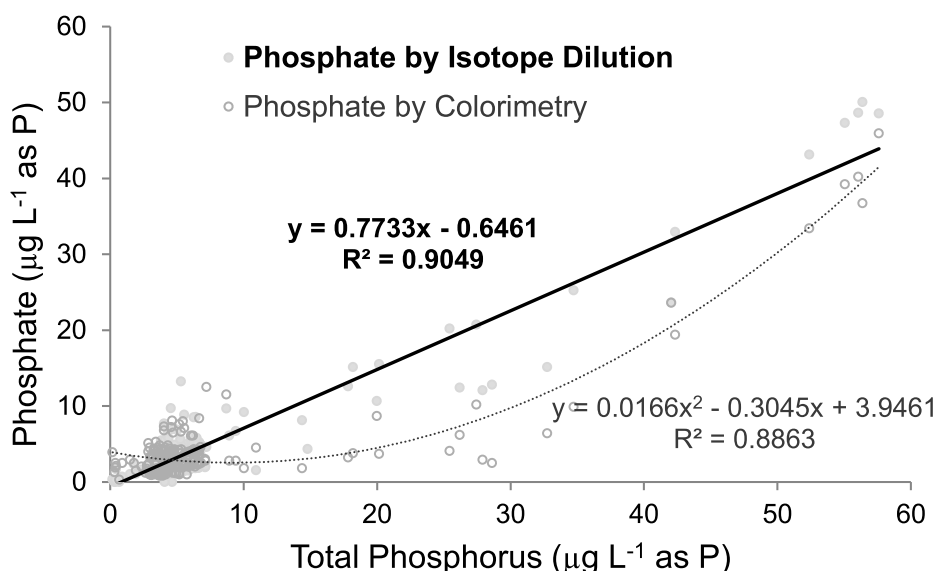


Figure 3.5. Correlations between phosphate concentrations measured by 2D-IC-ESI-MS (Method B) and colorimetry with total phosphorus (N=217).

3.4.4 Bioavailable Phosphorus Measured in Surface Waters

Isotope dilution measurement with Method B (2D-IC-ESI-MS) was used to measure the bioavailable phosphorus, which is the sum of free dissolved phosphate and phosphatase-hydrolyzed phosphate expressed as P units. A set of 10 surface water samples with phosphate concentrations between 0.25 and 0.77 $\mu\text{g L}^{-1}$ as P was selected from the samples collected in late spring from Lake St. Clair. After analyzing for phosphate, the remaining 7 mL samples were spiked with 100 μL of acid phosphatase (0.5 unit mL^{-1} enzyme concentration in the sample) and reanalyzed for phosphate after 8 h. The new measured phosphate values, expressed as $\mu\text{g L}^{-1}$ P, represent the total bioavailable phosphorus. For a complete phosphorus mass balance, the total

phosphorus was also measured. The total bioavailable P results are presented in Figure 3.6 as the two bottom bar fractions representing the directly bioavailable P (phosphate) and indirectly bioavailable P (phosphatase-hydrolyzed phosphate). The top bar fraction represents the difference between the total and total bioavailable phosphorus (non-hydrolyzable P). For the studied samples, the total bioavailable P averaged at 36 % (RSD=12 %), three times higher than the directly bioavailable P (phosphate, 12 %, RSD=4 %). The selected samples had relatively low P levels with total bioavailable P concentrations between 1.14 and 2.55 $\mu\text{g L}^{-1}$ as P.

Dissolved organic P species, the precursors of the indirectly bioavailable P (phosphatase-released P), typically represent 15-30% of the total phosphorus in lake water, but may increase up to 90 % of the total soluble phosphorus during periods of high biological activity^{86, 87}. The majority of the samples analyzed in this study had 60-80% P as phosphate, with the remaining 20-40% represented by P from the dissolved organic species and from non-hydrolyzable by phosphatase P species (non-bioavailable).

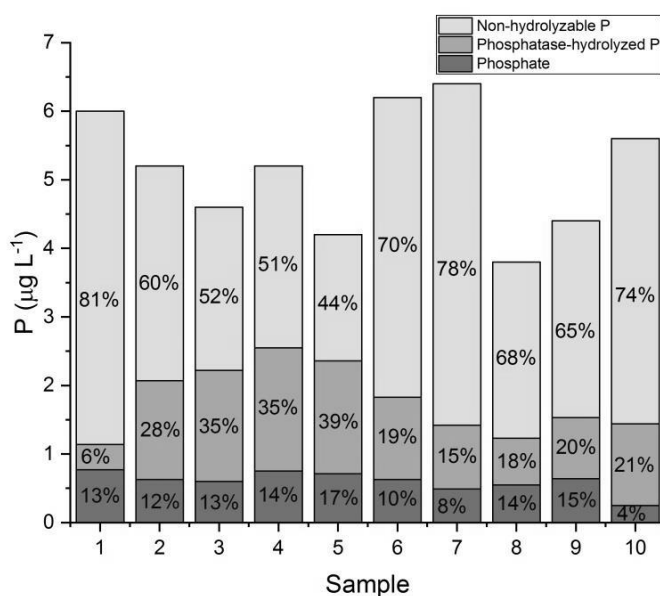


Figure 3.6. Concentrations of phosphate (bottom values), phosphatase-hydrolyzed phosphate (middle values) and non-hydrolyzable phosphorus (top values), all expressed as $\mu\text{g L}^{-1}$ P with the percentage values representing the fraction from total P for each sample. The phosphatase–hydrolyzed P values were calculated as difference between the total bioavailable phosphorus and phosphate concentrations.

The phosphate released from phosphate monoesters in the presence of the alkaline phosphatase incorporates one oxygen atom from water²²⁷. The result of these enzyme-specific isotopic fractionations at one of the four O sites in PO_4 is an increase in the oxygen isotope ratio of phosphate ($^{18}\text{O}/^{16}\text{O}$), $\delta^{18}\text{O}_\text{P}$, with 82.6‰ as the highest $\delta^{18}\text{O}_\text{P}$ observed in microbial cell cultures at 25 °C²²⁸. Measured $\delta^{18}\text{O}_\text{P}$ in Lake Erie ranged from +10‰ to +17‰²²⁹. Under these conditions the net calculated effect on the IS (P^{18}O_4) is negligible. Stability of the oxygen labeled phosphate in aqueous solution (Figure S4) was also confirmed by Melby et al.²³⁰ with a slow half-life period of 100 months.

3.4.5 Analysis of Phosphate-Containing Metabolites and Condensed Phosphate Species

Various organic phosphorus species (P metabolites) resulting from biological processes may be present in surface waters. P metabolites contain bound phosphate and include species like sugar phosphates, nucleoside phosphates, phytic acid (inositol polyphosphate), phytate and degradation products. Anthropogenic organophosphorus species such as alkyl substituted phosphonic acids, alkylphosphates, bisphosphonates used as herbicides, pesticides and detergents can also be present in surface waters⁸². Other P metabolites were previously quantified by NMR in surface water as functional group classes, measuring phosphate monoesters, phosphate diesters and polyphosphates, mainly in the particulate fraction and only in one lake water sample in the

dissolved fraction¹⁷⁹. Method B (2D-IC-ESI-MS) was used for inorganic and organic phosphate speciation in surface water samples, quantitating eleven anionic P metabolites (AMP, Fructose-P, Fructose-Bi-P, Glucose-P, Glucose-Bi-P, Galactose-P, Ribulose-P, Ribulose-Bis-P, Erythrose-P, PGA and UDP-Glucose) and two inorganic condensed phosphate species (pyrophosphate and triphosphate). A direct comparison between method B (2D-IC-ESI-MS) and NMR is not possible as NMR measures functional group classes of compounds, while new method measures specific metabolites. Separation method B was not optimized to chromatographically separate adenosine diphosphate (ADP) from adenosine triphosphate (ATP) and their in-source fragmentation prevented their separate quantitation. A chromatogram obtained from a 10 nM standard containing the 11 P metabolites is shown in Figure 3.7. Even if the chromatographic separation was not achieved for all species, the high-resolution mass spectrometer used can easily distinguish between these particular species.

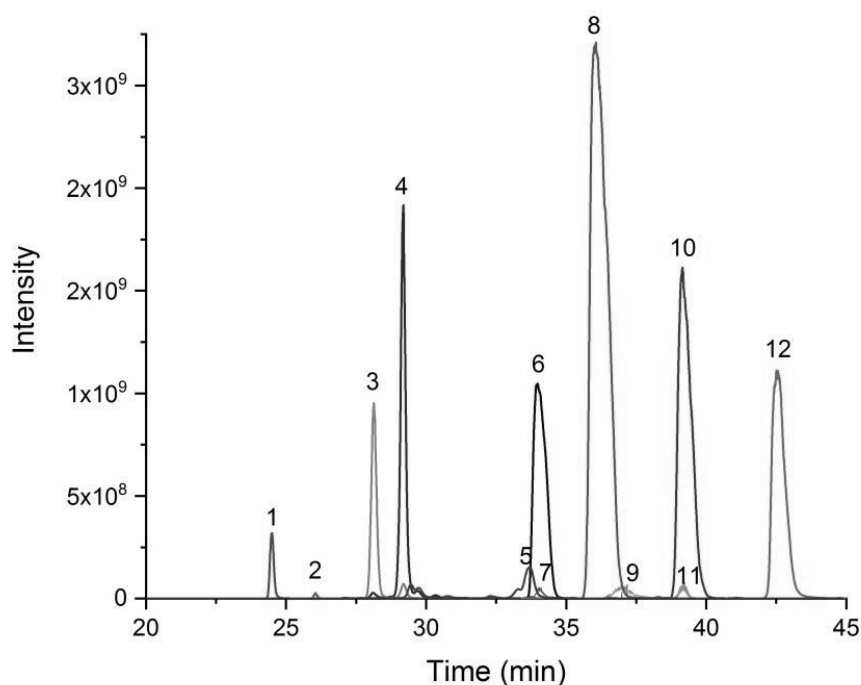


Figure 3.7. Chromatograms of phosphate and phosphate-containing metabolites in a standard mixture analyzed by Method B (2D-IC-ESI-MS) : phosphate (1), Galactose-P (2), Glucose-P (3), Fructose-P (4), Ribulose-P(5), PGA (6), Erythrose-P (7), Glucose-Bi-P (8), AMP (9), Fructose-Bi-P (10), Ribulose-Bis-P (11) and UDP-Glucose (12).

From the analyzed Lake St. Clair samples, only two P metabolites, AMP and Glucose-P, were present (N=70) at levels higher than the method detection limit (between MDL=0.1 nM and 2 nM) (Figure 3.8), with traces of Glucose-Bi-P below MDL (<0.25 nM). For easy comparison the corresponding phosphate and TP concentrations were also included with log y axis required to observe the much lower P metabolite concentrations. The 11 P metabolites were not identified in the Georgian Bay samples. While phosphate may be stronger adsorbed on solid surfaces than the organic phosphate species²¹⁵, the current method cannot confirm if any loss of the P metabolites occurred during storage. However, the added IS was present at higher concentrations (20 $\mu\text{g L}^{-1}$) than the P metabolites and phosphate from the sample and the IS would be predominantly adsorbed during storage, minimizing the loss of P metabolites.

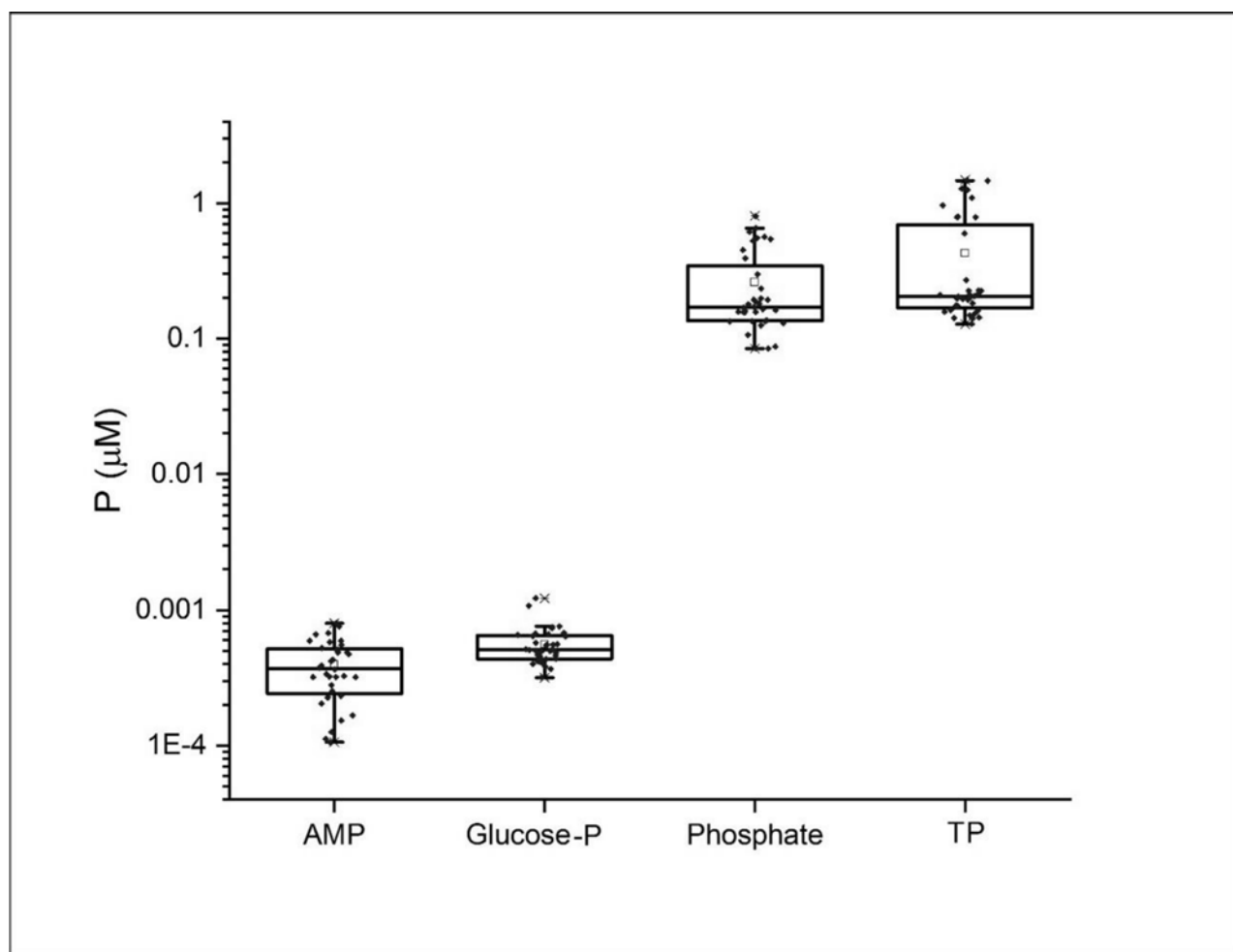


Figure 3.8. Concentration ranges of adenosine 5'-monophosphate (AMP) and D-glucose 6-phosphate (Glucose-P) in surface water samples collected in late fall from Lake St. Clair (N=70) and analyzed by Method B (2D-IC-ESI-MS). Concentrations ranges of the phosphate and total phosphorus (TP) from the same samples.

The field-spiked isotope dilution measurement by IC-ESI-MS demonstrated its advantages for measuring unbiased trace levels of phosphate in surface water samples. Due to immediate field spiking the new proposed IC-ESI-MS method measures the phosphate concentration present at the collection time, instead of the concentration measured at the analysis time by the existent colorimetric and IC methods. Phosphate stability was observed for up to 21 days of storage, a significant increase from the 48 hours holding time, currently recommended by US EPA 300.1

for phosphate measurement by IC¹²². In addition, the IC-ESI-MS method allowed quantitation of P species and precise measurement of total bioavailable phosphorus. By implementing a 2D IC separation in the analytical method, lower detection limits were obtained which allowed for reliable quantification of phosphate at trace and ultra-trace levels. The unbiased measurement of phosphate will allow to better estimate P loadings in surface waters. The 2D-IC-ESI-MS method developed on the QExactive MS instrument setup allows simultaneous collection of targeted and non-targeted data and thus the detection of trace polar organic phosphorus metabolites.

3.4.6 Associated Content

Additional information about analytical methods for colorimetric measurement of phosphate, total phosphorus, sulfate by IC, MDLs for P-containing metabolites, schematic of the 2D-IC configuration and phosphate loss (%) in samples spiked 48h after collection are included in the Supporting Information file.

3.4.7 Acknowledgements

Hany Eskander from the Ontario Ministry of the Environment, Conservation and Parks (OMECP) is thanked for the total phosphorus analysis of the samples. Shelly Chan and Carlos Jurado (OMECP) are thanked for the colorimetric analysis of phosphate.

CHAPTER 4

Phosphorus speciation in Toronto Harbour and Rivers

4.1 Abstract

Environmental phosphorus is commonly analyzed as soluble reactive phosphorus and total phosphorus based on colorimetric methods. Quantification and detection of other organic and inorganic phosphorus (P) species in Lake Ontario and some of its tributaries is demonstrated using a two-dimensional ion-chromatography mass spectrometer system. In addition to dissolved phosphate, other P-metabolites such as pyrophosphate, adenosine 5'-monophosphate, glucose 6-phosphate, α -D-glucose 1,6-biphosphate and D-(-)-3-phosphoglyceric acid were detected and quantified and the results were used to compare the differences between lake and river samples. Multivariate statistical analysis was used to identify potential correlations across analytical data sets and analyze spatial differences in sample matrices.

4.2 Introduction

The decline in water quality of Lake Ontario waterfront ranging across nine watersheds can be attributed to the intensive urbanization including shoreline/stream channel alteration and land clearing. These activities have resulted in increased sewage and runoff entering local rivers and subsequently the lake causing increased concentrations of phosphorus and nitrogen-based compounds in Lake Ontario and its tributaries. Eutrophication and nuisance algal bloom due to nutrient enrichment caused by agricultural and urban activities pose a threat to an overall freshwater quality globally.

In surface waters phosphorus is mostly present as phosphate ion (Pi). Pi can precipitate in the presence of Ca^{2+} or Fe^{3+} and is upon biological activation involved in metabolic pathways of fish and algae, producing various phosphate containing metabolites (P-metabolites). Pyrophosphate (PPi) presence can result from anthropogenic uses or as a by-product from biota metabolic

processes. While majority of the analyses are focused on Pi and total phosphorus (TP) measurements, PPi and other species measurements can provide valuable information in better understanding sources of increased phosphorus loadings and algal blooms, particularly the harmful algal blooms (HABs)^{17, 226, 231-234}. Algae and aquatic macrophytes characterization studies by ³¹NMR showed that organic-P compounds accounted for up to 80% of TP. Organic P species identified in algal biomass extracts included P monoesters and P diesters²³⁵. In addition to directly bioavailable inorganic phosphate, pyrophosphate and up to 40% percent of the organic phosphate from decaying algal biomass can be hydrolyzed to inorganic phosphate and released to water column supporting further growth of aquatic organism and leading to algal blooms^{236, 237}.

Adsorption on analytical containers and a subsequent loss of Pi when using colorimetric and other common analytical techniques have been reported. Lambert et al.⁹⁵ confirmed that a fast Pi adsorption caused 28-45% decrease in total dissolved phosphate (TDP), 33-40% decrease in total phosphate (TP) and 36-58% decrease in soluble reactive phosphate (SRP) within first 4 hours of sampling. Two-dimensional electrospray mass spectrometry (2D-ESI-MS) and was recently demonstrated as a reliable methodology for trace and ultra-trace measurements of Pi, mainly because the immediate field spiking with ¹⁸O-labelled phosphate allowed obtaining results unbiased by the inherent adsorption losses during storage and analysis²³⁸.

The most common multivariate statistical analysis method, the principal component analysis (PCA), has been widely used to evaluate natural environment and eutrophication²³⁹ and to classify water sources based on seasonal and spatial criteria. Previous studies showed that performing PCA can identify correlated patterns from a set of multiple analytes and eliminate redundant tracers or the ones with a poor contribution to total variance. Identification and

assessment of pollution sources as well as seasonal and climatic sources of variations was also successfully achieved by the application of multivariate analysis to analytical datasets²⁴⁰⁻²⁴⁴.

The Toronto and Region Area of Concern (AOC) as identified by the International Joint Commission (IJC) in 1985²⁴⁵, has been impacted by industrialization and urbanization, being one of the 43 areas in the Great Lakes with badly impaired water quality and ecosystems. The six major watersheds draining into Lake Ontario from Toronto and Region AOC (Etobicoke Creek, Mimico Creek, the Humber River, the Don River, Highland Creek and the Rouge River) cover an area of 2000 square kilometers. They include 42 km of waterfront, shared by eleven municipal jurisdictions with over 4 million inhabitants²⁴⁵.

In this study samples from Lake Ontario (Inner Toronto Harbour area) and from the Lake Ontario tributaries (Humber River, Don River, Rouge River) and Highland Creek were collected during summer and fall months. Concentration of P-containing metabolites, PPi, Pi, TP, major ions, pH, alkalinity and conductivity was analyzed and PCA was applied to test its applicability in source identification and better understanding of the spatial variability in water chemistry and risks associated with development of eutrophic conditions and algal blooms. The study was conducted to identify sources of phosphate and other P species.

4.3 Study Area

Water samples for analysis of phosphorus-containing compounds were collected from the nearshore and tributaries of Lake Ontario in the Laurentian Great Lakes within the City of Toronto, Canada (Fig 4.1). The lake samples were collected over six locations within Toronto Harbour, an embayment bordered by the heavily urbanized waterfront of city. This mesotrophic embayment, with a maximum depth of 14 m, exchanges water with oligotrophic Lake Ontario by

channels at the east and west extents of the harbour, respectively²⁴⁶. The harbour receives discharge from numerous storm and combined storm sewers outfalls and runoff from the Don River, one of the four tributaries examined here. In 1987, Toronto and Region was designated as one of 42 locations around the Great Lakes where environmental degradation have been adversely affecting the Great Lakes system. These locations are referred to as Areas of Concern (AOC). AOCs are required to implement a Remedial Action Plan (RAP) that improves the aquatic conditions in their area. Nutrient enrichment and eutrophication were identified as a water quality concern. The decline of water quality in the Toronto Harbour has occurred over 200 years of development in this large urban center bordering, however, there have been improvements in recent years⁴⁰.

The study tributaries vary in size and extent to which urbanized lands makeup the drainage sheds of the rivers. The watershed of Hyland Creek, the smallest of the rivers, is approximately 102 km² in extent and is fully urbanized falling almost entirely within the City of Toronto. There is a high proportion of paved, impermeable surfaces, such as roads, parking lots and roofs. Approximately 6% of the Highland Creek watershed is forested, making it the most extensively urbanized watershed in the Toronto region. The larger Don River with a drainage area of approximately 360 km² stretching its headwaters on the Oak Ridges Moraine to the Keating Channel, where it empties into Toronto Harbour is also highly urbanized. With 1.4 million residents, the watershed is one of the most heavily urbanized in Canada. Despite the challenges associated with this urbanization, the landscape of the Don River is slowly being regenerated and revitalized²⁴⁷. The river receives treated wastewater from the Lower Don Wastewater Treatment Plant.

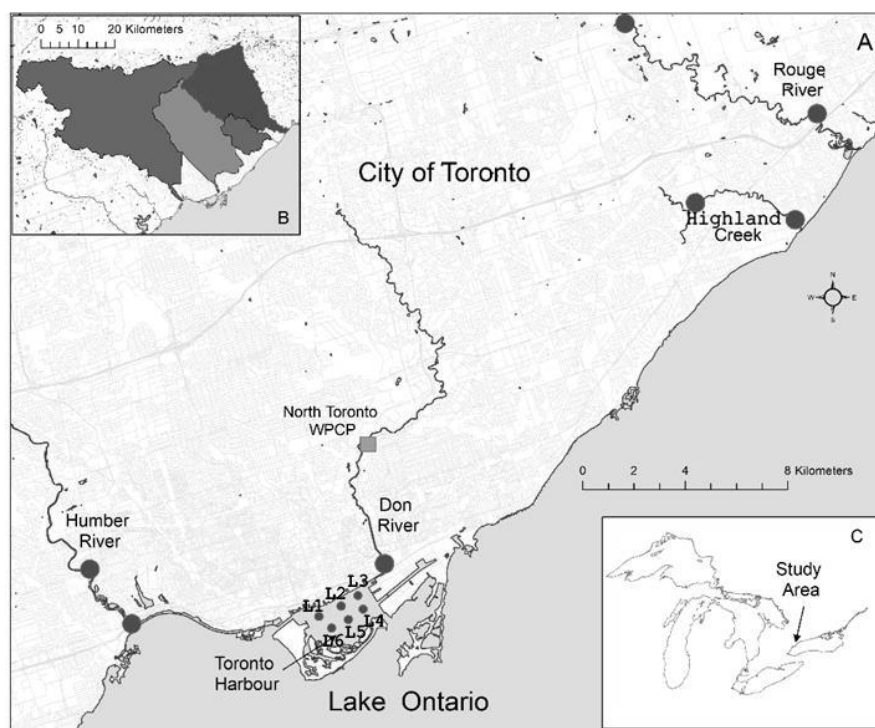


Figure 4.1 Sampling locations in Lake Ontario, Don River, Humber River, Rouge River and Highland Creek.

The Humber River and Rouge River are river systems with appreciable proportions of the drainage area made up of urbanized land, particularly in the reaches where water samples were collected for this study, however, there are also large portions of the drainage area with more mixed character, notably upstream areas with lands used for farming, lower density rural development and parklands. The Humber River, the largest river in study has a watershed of 911 km² with > 850,000 residents. The Rouge River watershed with an area 336 km² is 40% rural, 35% urban, 24% a mix of forest, wetlands and meadows. The southern third of the watershed is protected by Rouge National Urban Park, the middle and western sections of the watershed are urban with sparse natural cover outside of the park. The northeastern areas of the watershed are primarily rural and agricultural with small towns and villages²⁴⁷.

4.4 Experimental Section

4.4.1 Standards and chemicals. Certified Pi standards were purchased from Inorganic Ventures (Christiansburg, VA, USA) and NSI Lab Solutions (Raleigh, USA) and the isotope-labeled phosphate, $\text{H}_3\text{P}^{18}\text{O}_4$ (IS), HPLC grade acetonitrile, alkaline phosphatase, sodium pyrophosphate tetrabasic and sodium triphosphate pentabasic were purchased from Millipore Sigma (Oakville, ON, Canada). Also 11 P-metabolites were purchased from Millipore Sigma as salt species: adenosine 5'-monophosphate (AMP) disodium salt, adenosine 5'-diphosphate (ADP) sodium salt, adenosine 5'-triphosphate (ATP) disodium salt hydrate, D-fructose 6-phosphate (Fructose-P) disodium salt hydrate, D-fructose 1,6-biphosphate (Fructose-Bi-P) trisodium salt hydrate, D-glucose 6-phosphate (Glucose-P) sodium salt, α -D-glucose 1,6-biphosphate (Glucose-2P) potassium salt hydrate, α -D-galactose 1-phosphate (Galactose-P) dipotassium salt pentahydrate, D-ribulose 5-phosphate (Ribulose-P) disodium salt, D-ribulose 1,5 – biphosphate (Ribulose-Bis-P) sodium salt hydrate, D-erythrose 4-phosphate (Erythrose-P) sodium salt, D-(-)-3-phosphoglyceric acid (PGA) disodium salt, uridine 5'-diphosphoglucose (UDP-Glucose) disodium salt hydrate. A first set of 5 calibration standards contained the 11 P-metabolites, each at concentrations between 0 (Blank) and 20 nM. A second set of 7 calibration standards was prepared for Pi at concentrations between 0 (Blank) and $50\ \mu\text{g L}^{-1}$ as P, with each standard containing IS at $20\ \mu\text{g L}^{-1}$ as P. A third set of 5 calibration standards contained pyrophosphate and triphosphate, each at concentrations between 0 and $50\ \mu\text{g L}^{-1}$ as P.

4.4.2 Field Sampling and Storage Containers. Surface water samples were collected from 14 sampling sites in Lake Ontario (6 samples from Toronto Harbourfront) and the 4 tributaries Rouge River (upstream, midstream and downstream), Highland Creek (upstream and downstream), Don River (downstream) and Humber River (upstream and downstream). The first

set of samples were collected in July 2018 and the second set was collected from the same locations in October 2018, with locations presented in Figure 1. Samples were obtained as grab samples (0.5 m depth), except for Lake Ontario samples collected in October 2018, which were collected using a flow-through pumping system located on a sampling vessel. Aliquots collected for the analysis of Pi and P-metabolites were filtered through Fisherbrand 0.45 μm Durapore filters (Fisher Scientific Company, Ottawa, ON, Canada), with 10 mL immediately pipetted in a 15 mL HDPE amber screw-cap bottle (Thermo Scientific, Mississauga, ON, Canada) and spiked with 100 μL IS at 2 mg L^{-1} as P. Additional 500 mL of unfiltered samples were collected in PET bottles from each sampling site for additional analytical parameters described in the instrumental analysis section. Samples were stored at 5 ± 3 $^{\circ}\text{C}$ until analysis.

4.4.3 2D-IC-ESI-MS analysis: The method used for P-metabolite analyses was published²³⁸ with following P species being analyzed: Pi, P-metabolites (AMP, Fructose-P, Fructose-Bi-P, Glucose-P, Glucose-Bi-P, Galactose-P, Ribulose-P, Ribulose-Bis-P, Erythrose-P, PGA, UDP-Glucose), PPi and triphosphate. The system consisted of an ICS 5000⁺ ion chromatograph (IC) coupled to a QExactive Mass Spectrometer (Thermo Scientific, Mississauga, ON). In brief, the first dimension of the dual system was used to separate sulfate and less retained anions from the sample and divert these analytes to waste. The second dimension was used to separate the P species.

Three sets of calibration standards for Pi, organic and inorganic P species, were run to establish calibration curves. The method detection limits (MDLs) and m/z ratios of the analytes can be found in the SI. XCalibur software version 3.0 was used for data acquisition and TraceFinder version 3.2 (Thermo Scientific, Mississauga, ON) for quantitation.

4.4.4 Additional Instrumental Analysis. SRP and TP, chloride, sulfate, bromide, sodium, potassium, magnesium, calcium, nitrate, nitrite and ammonia were measured by capillary IC. Total organic carbon (TOC) was measured by combustion. Also, pH, alkalinity and conductivity were measured for all samples. Additional information regarding these separation methods is included in the SI.

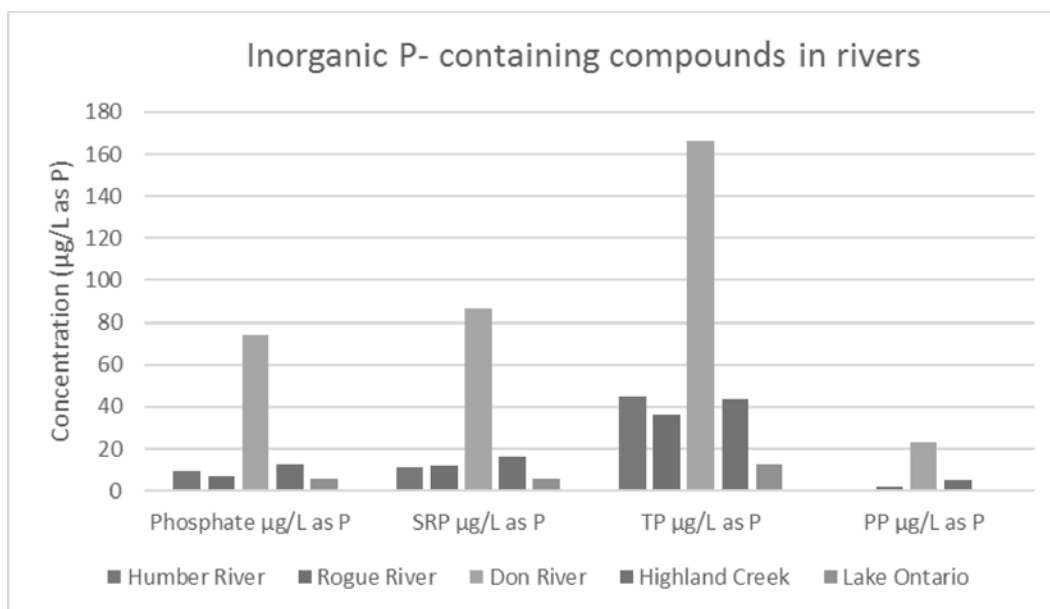
4.4.5 Multivariate Statistical Analysis: PCA and hierarchical cluster analysis (HCA) in this study was applied to 23 analytes in total. The number of meaningful principal components (PC) was determined by eigenvalues ≥ 1 . Calculations and graphical representations of PCA and HCA were performed using Origin Pro 2018. Concentrations of parameters below MDLs were replaced with randomly generated values between 0 and corresponding MDL value for each measured parameter.

4.5 Results and discussions

4.5.1 Inorganic and organic P-species in river and lake water

Samples from 14 locations were collected in July and October of 2018. The Don River watershed, covering the area of 36000 hectares and 1.4 million residents, is one of the most urbanized in Canada. Increase in paved surfaces and combine sewers, carrying both stormwater and sanitary sewage can cause nutrient pollution due to sewer overflow during large storm events²⁴⁷. The Don River, connected to Lake Ontario via Keating Channel, has the greatest effect on Inner Harbour (IH) water quality²⁴⁸. Samples collected near the mouth of the Don River contained 74 $\mu\text{g L}^{-1}$ of Pi as P, 87 $\mu\text{g L}^{-1}$ of SRP as P, 166 $\mu\text{g L}^{-1}$ of TP as P and 23 $\mu\text{g L}^{-1}$ of PPi as P. In comparison, other 3 tributaries contained on average 10 $\mu\text{g L}^{-1}$ of Pi as P, 13 $\mu\text{g L}^{-1}$ of SRP as P, 41 $\mu\text{g L}^{-1}$ of TP as P and 2.5 $\mu\text{g L}^{-1}$ of PPi as P. Lake samples collected from six

Toronto Harbourfront locations contained on average $6 \mu\text{g L}^{-1}$ of Pi as P, $5.9 \mu\text{g L}^{-1}$ of SRP as P, $13 \mu\text{g L}^{-1}$ of TP as P and $0.35 \mu\text{g L}^{-1}$ of PPi as P. Within harbor analysis of six locations L1-L6 with different proximities to a Don River sampling site confirmed increased concentrations of inorganic and organic P-compounds in locations closer to the Don River sampling site with an average concentration increase of 67% for inorganic P-compounds and almost the same increase of 69% in organic P-compounds between the furthest and closest sampling location from the Don River. Concentrations of inorganic and organic P-compounds in Lake Ontario and the tributaries are shown in Figure 4.2. Previously measured TP values showed the highest P concentrations in the northeast corner of Inner Harbor (IH) by the Don River and gradually decreasing through zones to the Outer Harbor (OH)²⁴⁸. Howell et al. found nearshore TP values in IH higher than ambient oligotrophic nearshore levels in Lake Ontario²⁴⁹, with levels between 8 and $11 \mu\text{g L}^{-1}$.



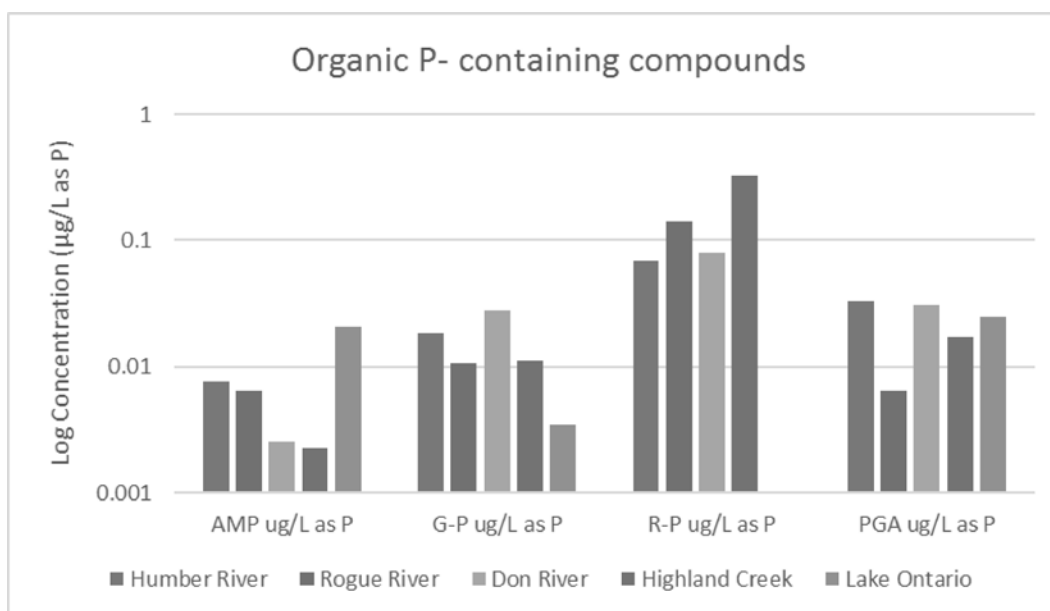


Figure 4.2 Average concentrations of inorganic (above) and organic P-containing compounds (below) in tributaries and Lake Ontario

The shortest chain polyphosphate, PPi, is bioavailable, can function as a phosphate reservoir for aquatic microorganisms as it can be present at higher concentrations than SRP⁷⁷. The main classes of P-species observed by ³¹P NMR in sewage sludge were Pi, PPi, longer chain polyphosphates, mono and di-esters²⁵⁰. Average amount of PPi detected in Lake Ontario tributaries varied between 0.8 and 23 µg L⁻¹ as P, with Don River containing the highest PPi levels. The PPi can have an anthropogenic source or be a by-product of biological activity. The most urbanized watersheds, Highland Creek and Don River contained 5 and 23 µg L⁻¹ PPi as P, respectively, while partially urbanized watersheds such as Rouge River and Humber River contained only 1.7 and 0.8 µg L⁻¹ PPi as P, respectively, suggesting an anthropogenic source of PPi in these watersheds. The lake water contained 0.35 µg L⁻¹ of PPi as P, lower level than in the tributaries, mainly due to lake diluting effect and biological uptake. The concentration ranges of P-containing inorganic and organic compounds quantified in Lake Ontario Harbourfront and

tributaries are shown in Figure 4.3. The dilution factor of P-containing inorganic compounds and sugar phosphates such as G-P and G-2P in the lake as compared to tributaries ranged from 2.0 – 10.7, with 10.7 PPI dilution factor (Figure 4.5). The higher concentration of other organic P-containing compounds such as PGA and AMP in lake samples than in river samples was probably due to the difference in the biological activity of these two sources.

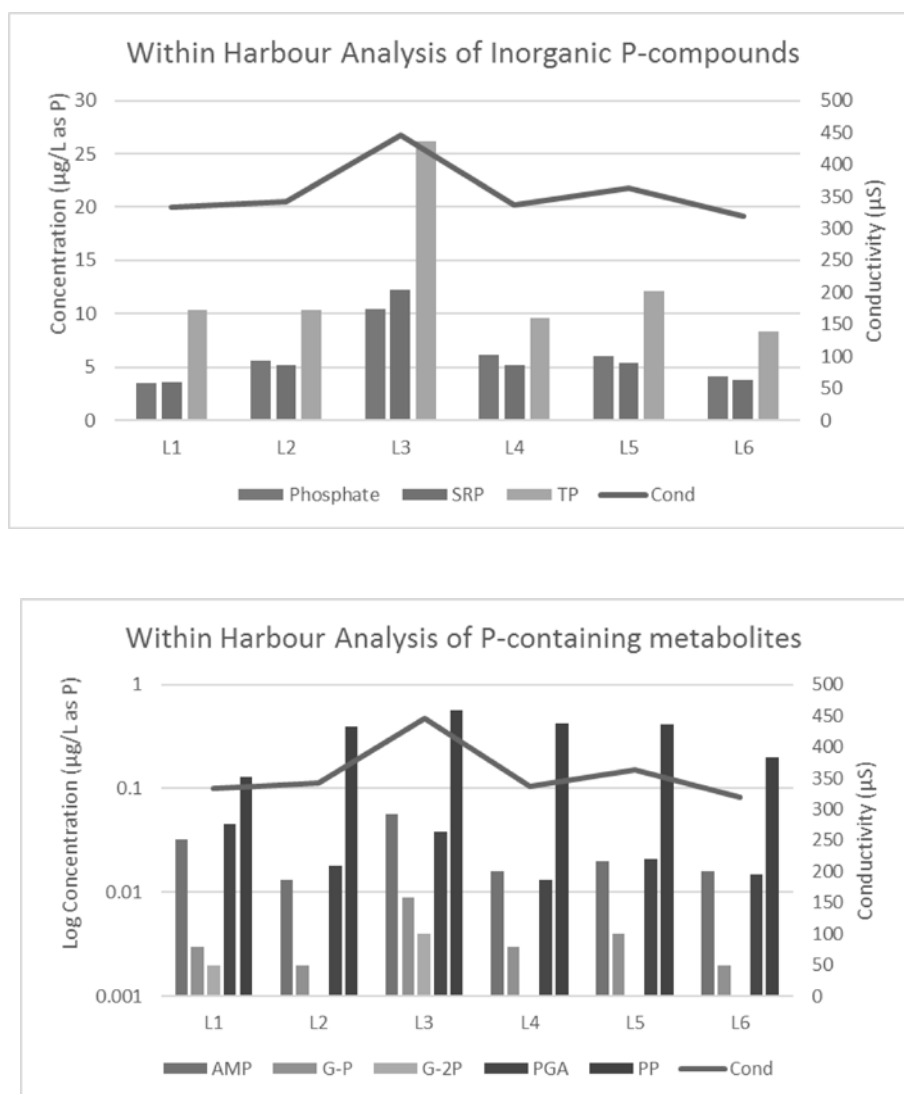


Figure 4.3 Concentrations of inorganic P-compounds (above) and P-containing metabolites (below) per Lake Ontario Inner Harbour location compared with conductivity.

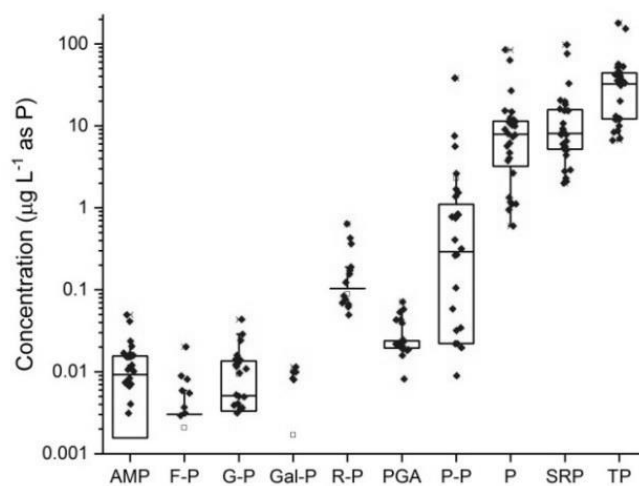


Figure 4.4 Log concentration ranges of P-metabolites and P_{Pi} in river and lake samples including SRP and TP.

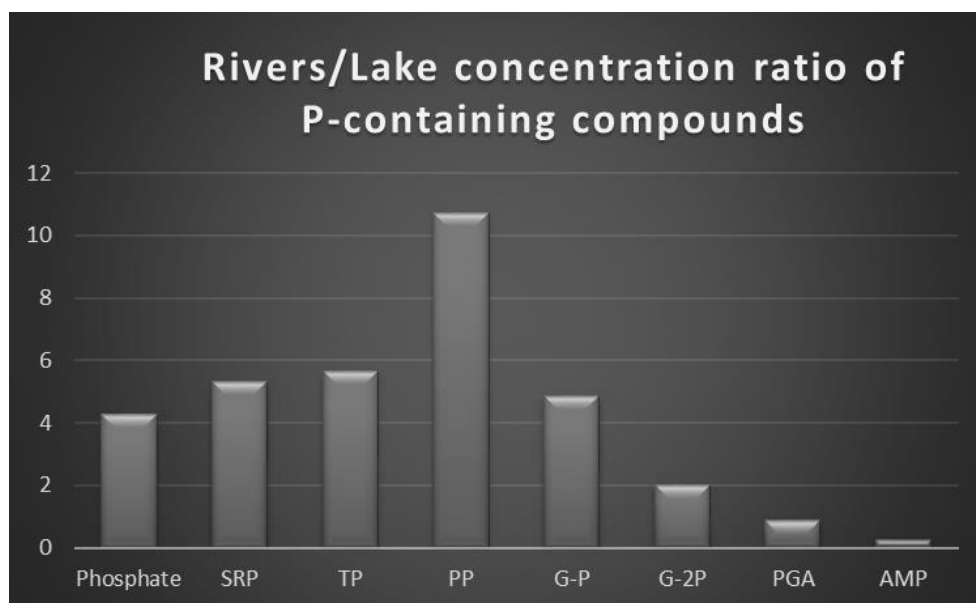


Figure 4.5 Concentration ratio of P-containing compounds between rivers and lake water.

4.5.2 PCA and analysis of P-containing metabolites and condensed phosphate species

Inorganic and organic P compounds such as phosphonates, Pi, P monoesters, P diesters, PPi and longer chain polyphosphates in soil, animal manure and algal biomass were previously identified and quantified by ^{31}P NMR²⁵¹⁻²⁵⁴. However, its application in surface water environmental samples remained challenging until recently²³⁸, due to trace concentrations of P species in rivers and lakes. The same IC-ESI-MS method was used in this study to quantify P-metabolites in surface water. down to nM levels. Previous ^{31}P NMR measurements of surface water samples were based on quantification of functional groups¹⁷⁹, while IC-ESI-MS measures separate P species present at much lower concentrations. The analyzed river samples contained AMP up to 8 ng L⁻¹ as P in July and 12 ng/L in October, F-P below MDL = 2.8 ng L⁻¹ as P in July and up to 20 ng L⁻¹ as P in October, G-P up to 44 ng L⁻¹ as P in July and 14 ng L⁻¹ as P in October, Gal-P below MDL = 5.6 ng L⁻¹ as P in July and up to 11 ng L⁻¹ as P in October, R-P up to 360 ng L⁻¹ as P in July and 640 ng L⁻¹ as P in October, PGA up to 53 ng L⁻¹ as P in July and 43 ng L⁻¹ as P in October and PP up to 7.5 $\mu\text{g L}^{-1}$ as P in July and 38 $\mu\text{g L}^{-1}$ as P in October. Concentrations of 16 most commonly analyzed inorganic parameters, isotope dilution Pi and 11 P-metabolites were used for PCA. Figure 4.6 shows corresponding loading graphs, scree diagrams and PCA of the 28 parameters. More information on the analytical procedures used in this study can be found in the SI file. From the 616 parameters used for PCA 65 (10.5%) were below MDL. The values below MDL were replaced with randomly generated values between 0 and corresponding MDLs for each measured parameter. Separate PCA was done using only P species (Figure 4.7), with 252 parameters with the same 65 values below MDL replaced by randomly generated values between 0 and corresponding MDLs Concentration units were normalized from data distribution using standardization (z-score) based on mean and standard deviation prior to PCA.

4.5.3 Statistical analysis based on all measured analytical parameters

For July samples, two PCs (1 and 2) accounted for 85% of the total variance and three PCs (1, 2 and 3) accounted for 92% of the total variance. The most significant PC1 components were TP, TOC, Pi, SRP, G-P and PPI (0.28 to 0.31), AMP (-0.14) and pH (-0.19) and the most significant components of PC2 were sulfate, Mg, Ca, Cond, Na, Cl (0.29 to 0.31), PGA, AMP and nitrate (-0.20 to -0.24). The number of significant PCs for all PCA calculations was selected based on eigenvalues > 1 (Figure 4.6). For October samples, PC1 and PC2 accounted for 84% of the total variance, three PCs (1, 2 and 3) accounted for 92% and four PCs (1, 2, 3 and 4) accounted for 96% of the total variance. The most significant components of PC1 were K, F-P, Na, Cl (all 0.25) and AMP (-0.24). The most significant components of PC2 were nitrite, ammonia, PPI, Pi (0.3 to 0.34) and G-P, SO₄, R-P and pH (-0.20 to -0.25). PC1 / PC2 relationship is shown in Figure 4.7. The figure shows a spatial evaluation and clear distinction between lake samples (L1-L6) and river samples (R1-R8), with each group being correlated to the particular analytes. Lake samples collected in July were correlated with AMP and the river samples (except R3) were correlated with major ions, pH, alkalinity, conductivity, sulfate, chloride and R-P. River sample R3 contained higher concentration of nutrients than the other river samples and was correlated with inorganic nitrogen and P species. Similar PC1/PC2 distribution and spatial separation of lake and river samples was observed in October samples (Fig.4.7).

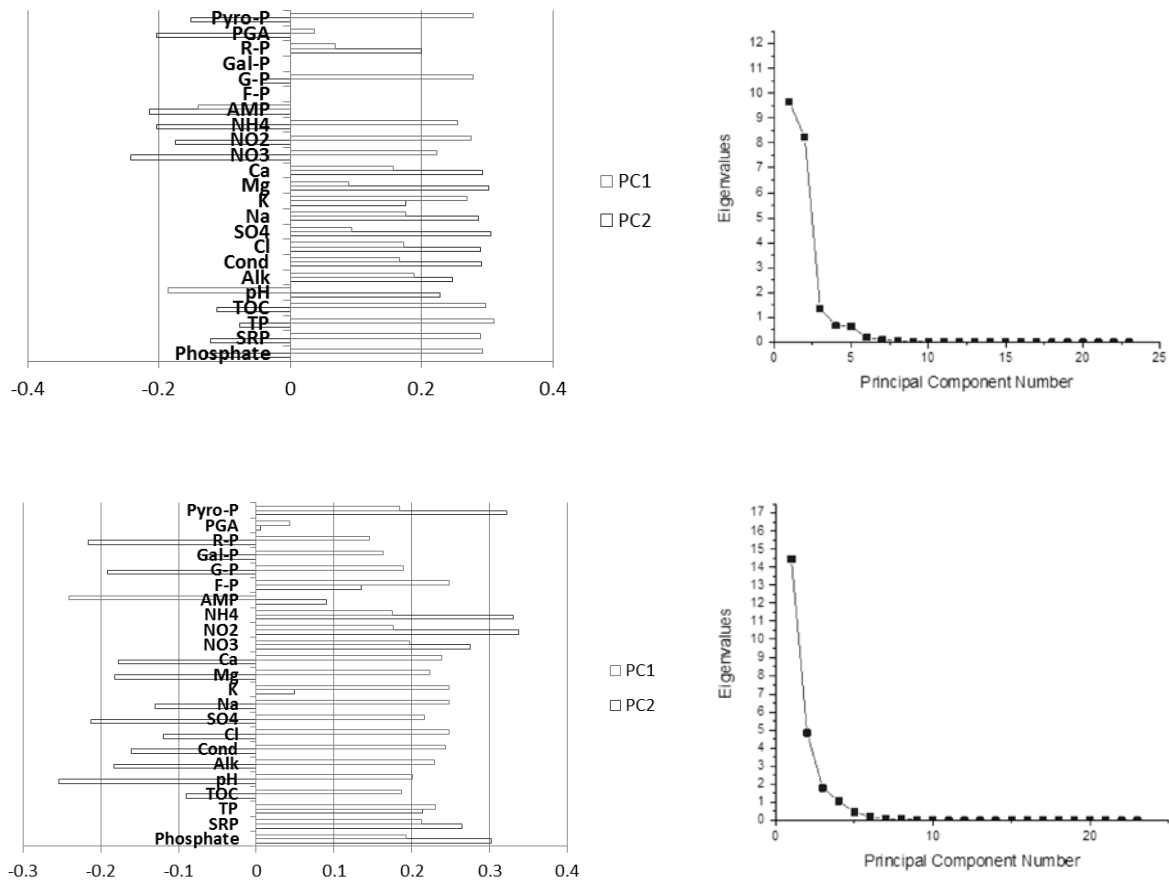


Figure 4.6 Loading graphs and Scree diagrams based on all analyzed variables in July (top) and October (bottom).

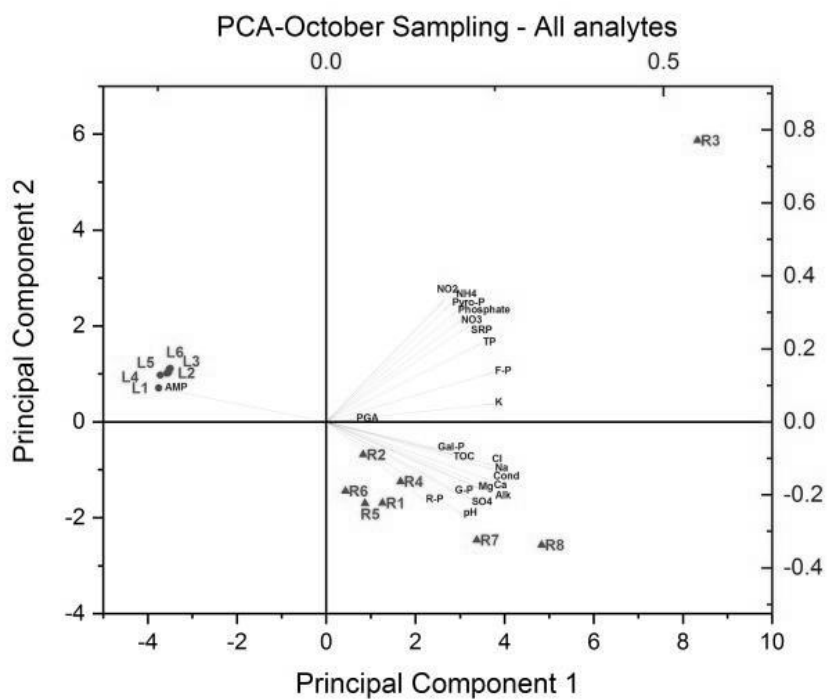
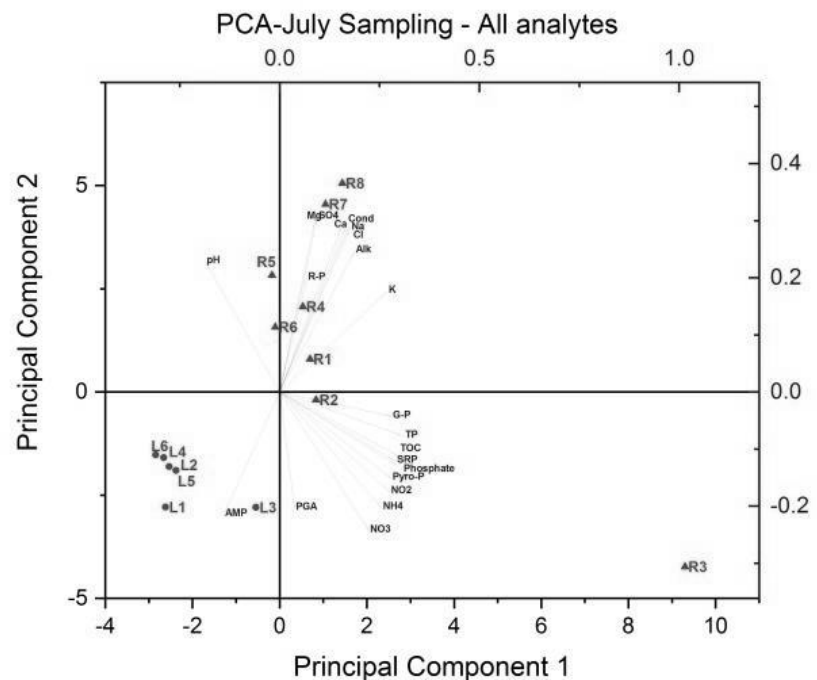


Figure 4.7 PCA based on all analyzed variables in July (top) and October (bottom).

4.5.4 Statistical analysis based on measured P-species.

Two PCs (1 and 2) accounted for 85% of the total variance in July sampling, the same as when all the analytes were used. Three PCs (1, 2 and 3) accounted for 92% of the total variance (Figure 4.8). The most significant PC1 components were TP, Pi, SRP, PPI and G-P (0.40 to 0.46) and the most significant components of PC2 were AMP, PGA (both 0.60) and R-P (-0.53). In October sampling PC1 and PC2 accounted for 77% and three PCs (1, 2 and 3) accounted for 89% of the total variance (Figure 4.8). The most significant components of PC1 were TP, Pi, SRP, PPI and F-P (0.37 to 0.41) and the most significant components of PC were G-P (0.5), R-P (0.53) and Pi, AMP and pyro-P (-0.30 to -0.34). Spatial distribution of river and lake samples based on PCA of P species in July and October are showed in Figure 4.9. In July, lake and river samples are grouped vertically along the PC2 axis with AMP, PGA and R-P being the most significant components. In October, lake samples and river samples are separated along PC2 with lake samples correlated with AMP and river samples correlated with G-P, R-P, Gal-P and PGA. Successful PCA separation of the river and lake matrix based only on P species reduced number of analytes needed for PCA by 13 (from 23 to 10) or 56%. Sample source hierarchical cluster analysis (HCA) dendrograms are shown in Figure 4.10 (using 23 analytes) and Figure 4.11 (P species only). HCA yielded two statistically significant clusters separating river and lake samples when all analytes were used for calculation. When using HCA based on P-species, the dendrogram selected 3 statistically significant clusters separating river and lake samples and separating highly polluted R3 sample from other two clusters. July dendrogram failed to assign L3 and R5 to the proper clusters.

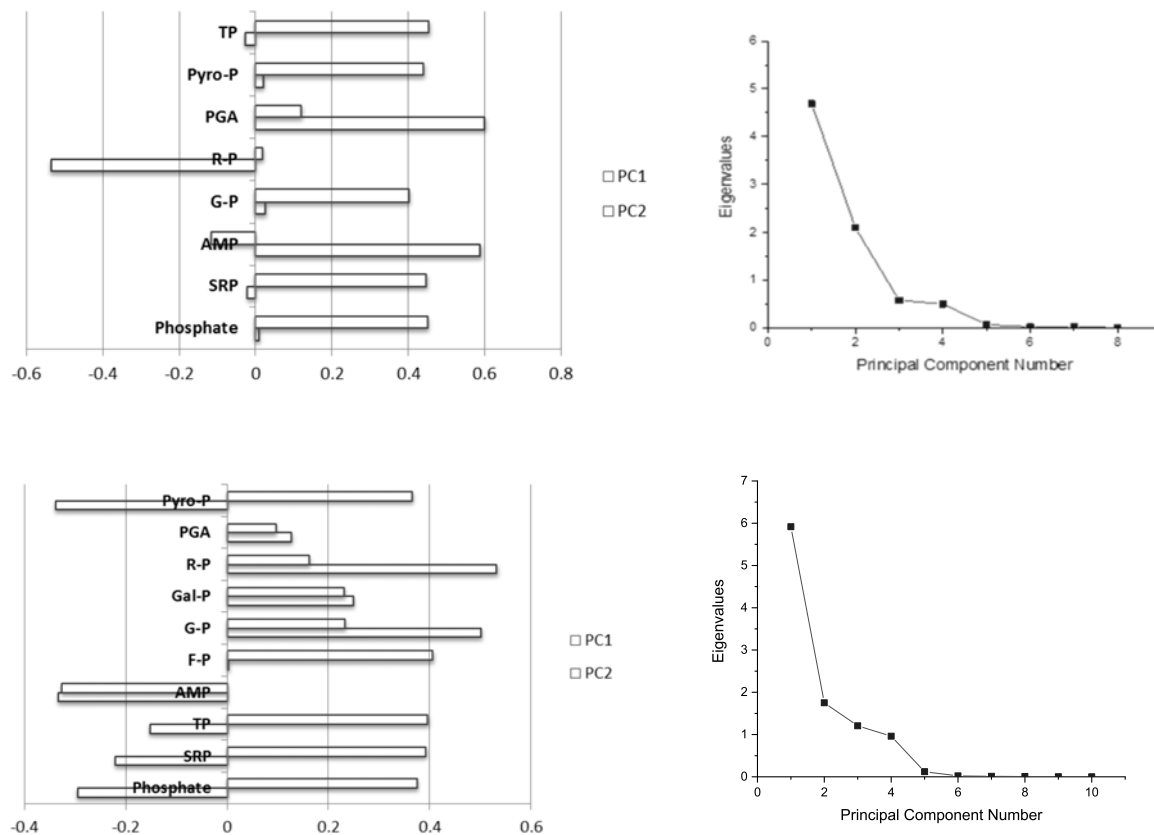


Figure 4.8 Loading and Scree diagrams using P species sampled in July (top) and October (bottom).

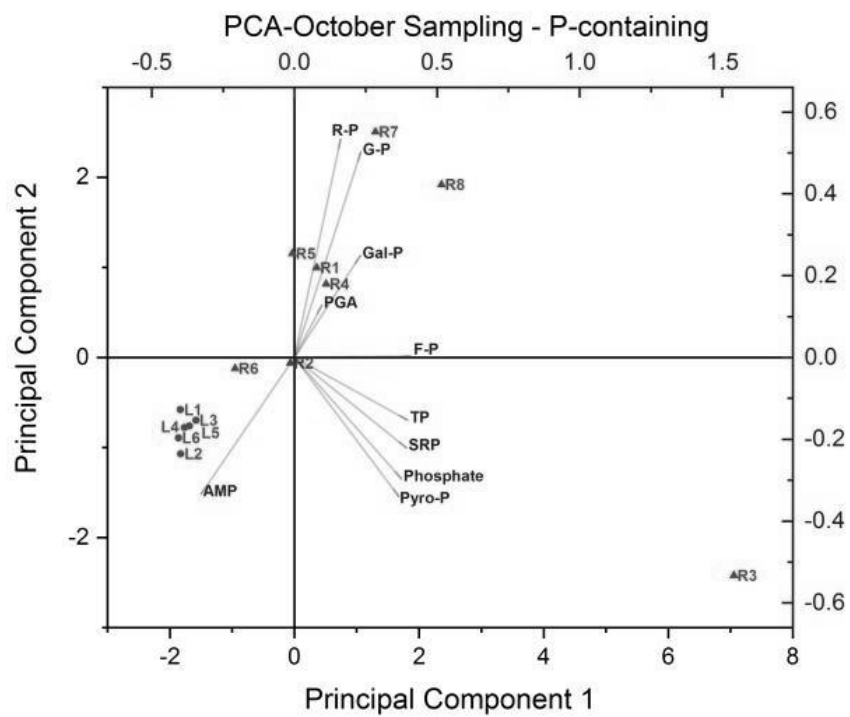
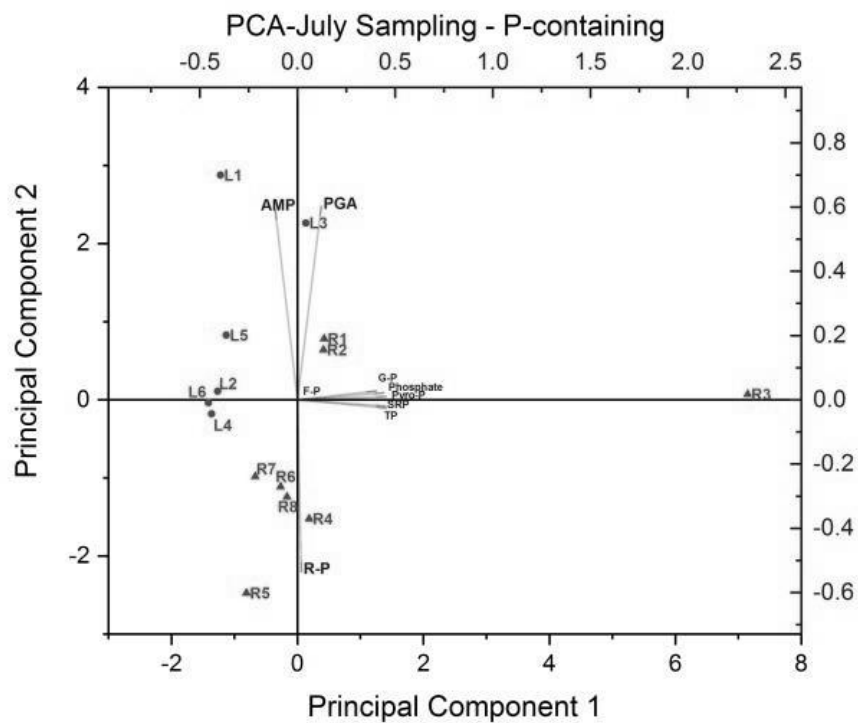


Figure 4.9 PCA based on P-containing compounds in July (top) and October (bottom).

4.5.6 Correlation between inorganic and organic P-species

Inorganic P parameters such as Pi, SRP, TP and PPi were highly correlated in both sampling sets (early summer and fall samples) with correlation coefficients over 0.90. Organic P-species were not well correlated between each other or with other parameters, most correlation coefficients being below 0.5. Due to low correlation of these species with other analytical parameters it is therefore useful to analyze organic P-species as separate entities in addition to total organic P to better understand their dynamics in the aquatic environment.

4.6 Conclusions

Soluble reactive phosphorus and total phosphorus analysis and data provide useful information of phosphorus interaction with aquatic environment. The data has been widely used for decades for monitoring and prevention of an excessive nutrient loadings into the Great Lakes. With the contribution of many other environmental variables the eutrophication of freshwater is still a major threat to the aquatic ecology. Nuisance algal blooms is still a widespread phenomenon causing a vast economic and environmental damage. A large variety of P-containing organic metabolite species can be released into the aquatic environment during periods of a high biological activity. The identification and quantification of these species continue to be problematic and their functions and interactions in the water column remains not fully understood. Quantification of biologically available phosphate species which can greatly contribute to algal growth provide an additional valuable information for better understanding of phosphorus dynamics and its biological utilization. However, the analysis requires highly sensitive and selective analytical methods. Two-dimensional ion chromatography with tandem mass spectrometry confirmed its suitability for low-level phosphate quantification and ultra-trace

analysis of P-containing organic and inorganic compounds present in freshwater samples. The large injection volume and the concentrator used in the second dimension of the analytical separation eliminated the need for off-line pre-concentration or other sample pre-treatment methods. Various P-containing metabolites including pyrophosphate, adenosine 5'-monophosphate, glucose 6-phosphate, α -D-glucose 1,6-biphosphate, D-ribulose 5-phosphate and D-(-)-3-phosphoglyceric acid were identified and quantified for the first time in Lake Ontario and its tributaries. Multivariate statistical analysis of lake and river samples separated matrices into distinct groups even when using only P-containing analytical variables as inputs for a PCA analysis suggesting that P-containing compounds could be used for an identification of pollution sources.

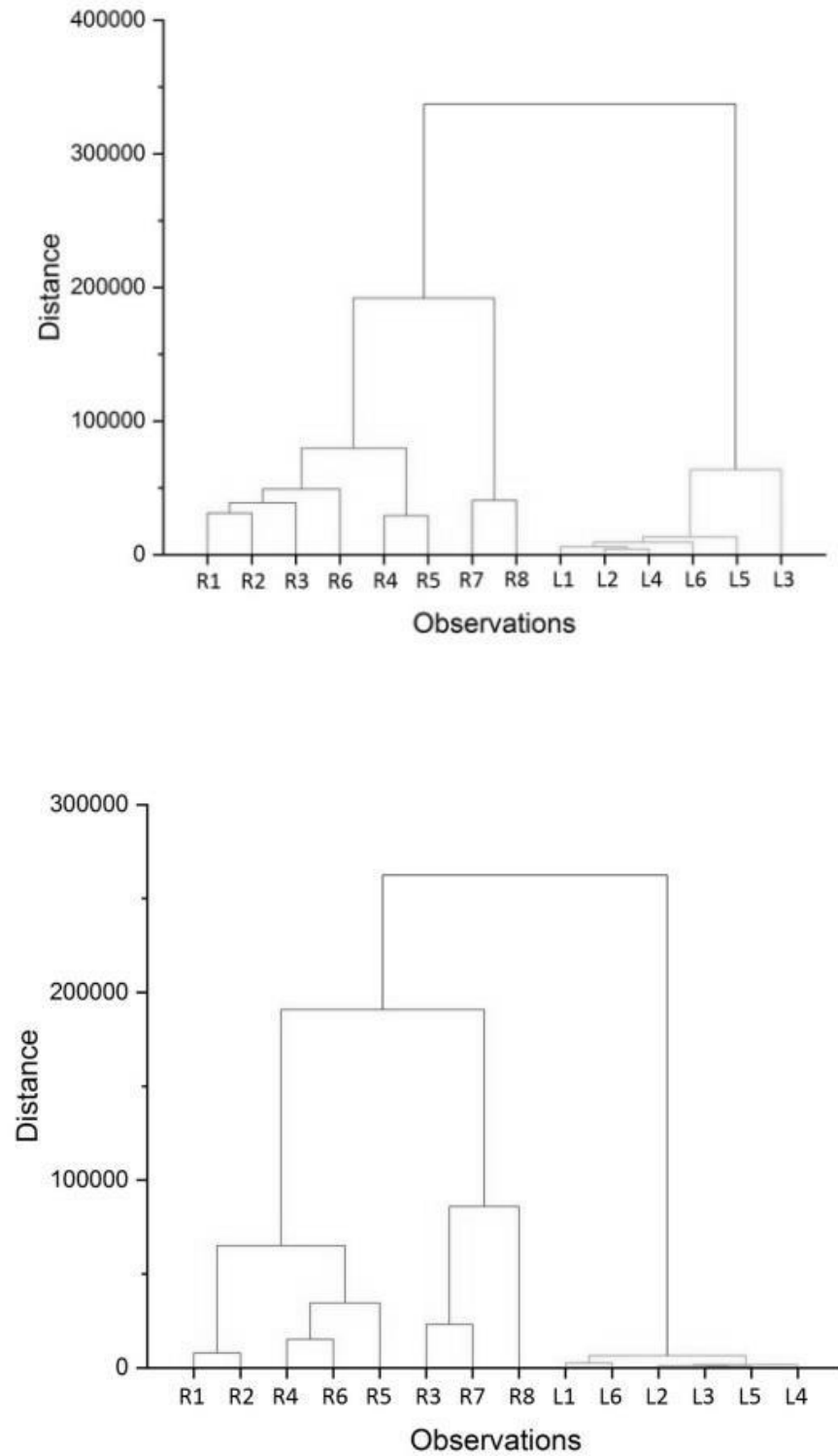


Figure 4.10 Dendrograms using all analytes (top) July sampling (bottom) October sampling

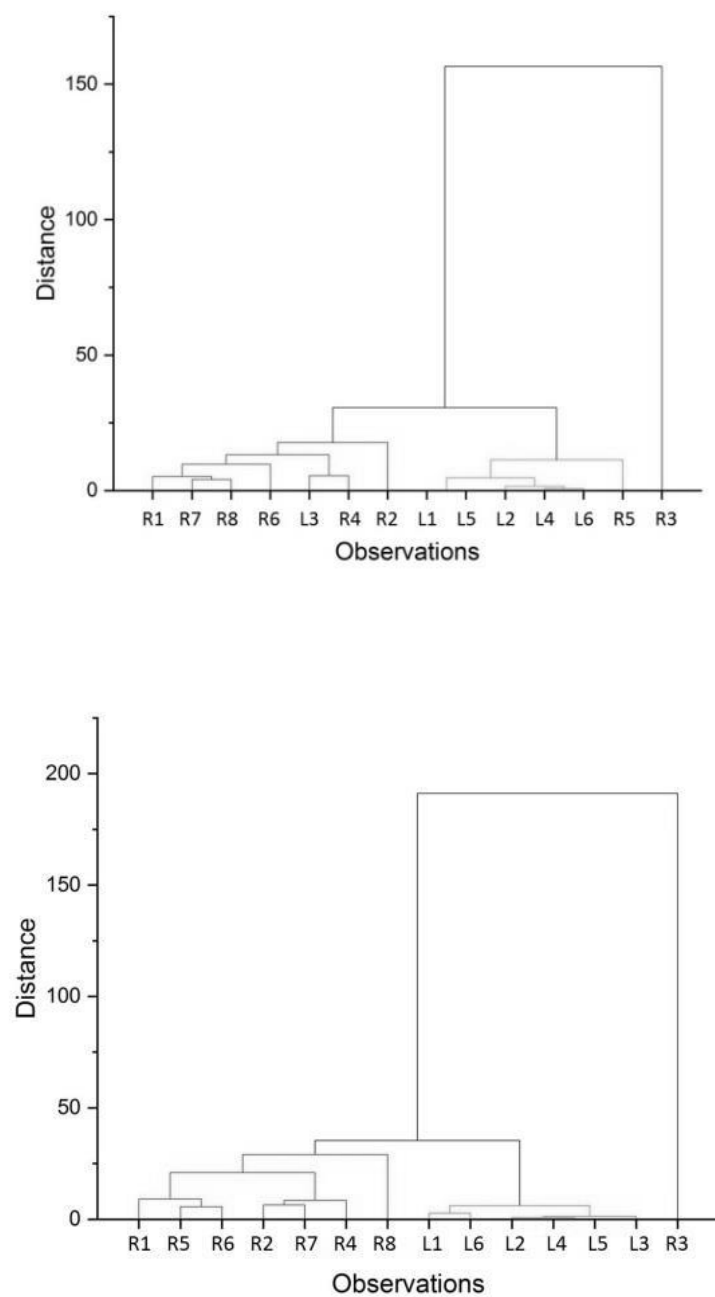


Figure 4.11 Dendrograms using P-metabolites only (top) July sampling (bottom) October sampling

4.7 Associated Content

Additional information about analytical methods for colorimetric measurement of Pi, TP, sulfate by IC, MDLs for species, schematic of the 2D-IC configuration and Pi loss (%) in samples spiked 48h after collection are included in the Supporting Information file.

5.0 Summary and Future Work

Incorporating P-speciation data in the environmental monitoring programs can contribute to better understanding of phosphorus dynamics in aquatic environment and prevent potential large-scale ecological and economic losses to the society. The Ontario Ministry of the Environment, Conservation and Parks has been using this new method to evaluate the advantages in measuring phosphate in surface water samples. There was a limited number of inorganic and organic P-species present in natural waters covered in our experimental work. Other species may be identified by unknown identification software. Utilization of such software including analysis of MS-MS data can give us information on presence of up to hundreds of P-containing metabolites in the samples. The newly identified species can be quantified once their standards become available.

APPENDIX A

SUPPORTING INFORMATION

Unbiased Measurement of Phosphate and Phosphorus Speciation in Surface Waters

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SUPPORTING INFORMATION

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Total phosphorus (TP) measurement	SI-2
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Schematic of the 2D-IC configuration (Figure S2)	SI-5
Phosphate loss (%) in samples spiked 48h after collection (Figure S3)	SI-5
Distribution of the ¹⁸ O labeled H ₂ PO ₄ ⁻ species during storage (Figure S4)	SI-6

Phosphate measurement by colorimetry

Soluble reactive phosphorus (SRP) was measured using a Skalar continuous flow analyzer (Breda, Netherlands). SRP was measured after formation of a phospho-antimonyl-molybdate complex in the presence of the ascorbic acid as a reducing agent. The absorbance was measured at 880 nm with quantitation based on external calibration. The method detection limit (MDL) was $3 \mu\text{g L}^{-1}$ as P. This method is slightly modified (see Table S1) from the reference method 4500-P G from the Standard Methods for the Examination of Water and Wastewater¹⁰⁰.

Table S1. Differences between Standard Method 4500-P G and SRP method

Analysis Modification	Standard Method 4500-P G	SRP Method
Antimony potassium tartrate solution	1.37 g to 500 mL water	5 g to 4000 mL water
Ammonium molybdate solution	20 g to 500 mL water	240 g to 4000 mL water
Ascorbic acid solution	1.76 g to 100 mL	10 g to 500 mL water
Wetting agent	Not used/needed	FDD6 soap

Total phosphorus (TP) measurement

Samples were preserved with sulfuric acid and 10 mL aliquots were digested at 121°C for 30 min after adding potassium persulfate using a Tuttnauer 2540 EK-B/L autoclave (Heidolph Brinkmann, Hauppauge, NY, USA). After digestion TP was measured as phosphate using a Skalar continuous flow analyzer (Breda, Netherlands) with MDL = $0.1 \mu\text{g L}^{-1}$ as P. This method is slightly modified (see Table S2) from the reference method 4500-P (section B5 for digestion and section F for analysis)¹⁰⁰.

Table S2. Differences between Standard Method 4500-P B5 and TP method

Digestion Modification	Standard Method 4500- P B5	TP Method
Indicator solution	Phenolphthalein	Not used
Persulfate	Add 0.5 g solid K ₂ S ₂ O ₈ to each sample	Add 150 µL of saturated K ₂ S ₂ O ₈ solution to 10 mL of sample
Analysis Modification	Standard Method 4500-P F	TP Method
Flow cell	15 or 50 mm	500 mm
Antimony potassium tartrate solution	0.3 g to 100 mL water	2.3 g to 100 mL water
Ammonium molybdate solution	4 g to 100 mL water	3 g to 1000 mL water
Ascorbic acid solution	1.76 g to 100 mL water	0.2 g to 100 mL water
Wetting agent	Not used/needed	FDD6 soap

Sulfate measurement by ion chromatography (IC)

Sulfate was analyzed using capillary ion chromatography (ICS-5000⁺, Thermo Fisher Scientific, Mississauga, ON, Canada) containing AS-AP, DP, EG and DC modules. Separation of all major anions, including sulfate was obtained in 25 minutes using an AS18 column and conductivity detector (see Figure S1). The method is similar to US EPA Method 300.1 (Determination of Inorganic Anions in Drinking Water by Ion Chromatography PART A. Common Anions in EPA 300.1)²⁵⁵, with differences summarized in Table S3. The phosphate was simultaneously measured, but it was below MDL for the majority of the analyzed surface water samples.

Table S3. Differences between Standard Method EPA 300.1 and IC method used

Analysis Modification	EPA Method 300.1	IC Method
IC Column	AS9-HC	AS18
Eluent	9 mM carbonate	10-34 mM hydroxide
Injection Volume	40 µL	0.4 µL
Analysis Time	15 min	25 min
Column type	4 mm	capillary

1. *Standard methods for the examination of water and waste water, 23rd ed.* American Public Health Association, American Water Works Association, Water Environment Federation, Washington, DC: 2017.
2. Hautman, D. P.; Munch, D. J., Method 300.1: Determination of inorganic anions in drinking water by ion chromatography. *EPA: Ohio* 1997.

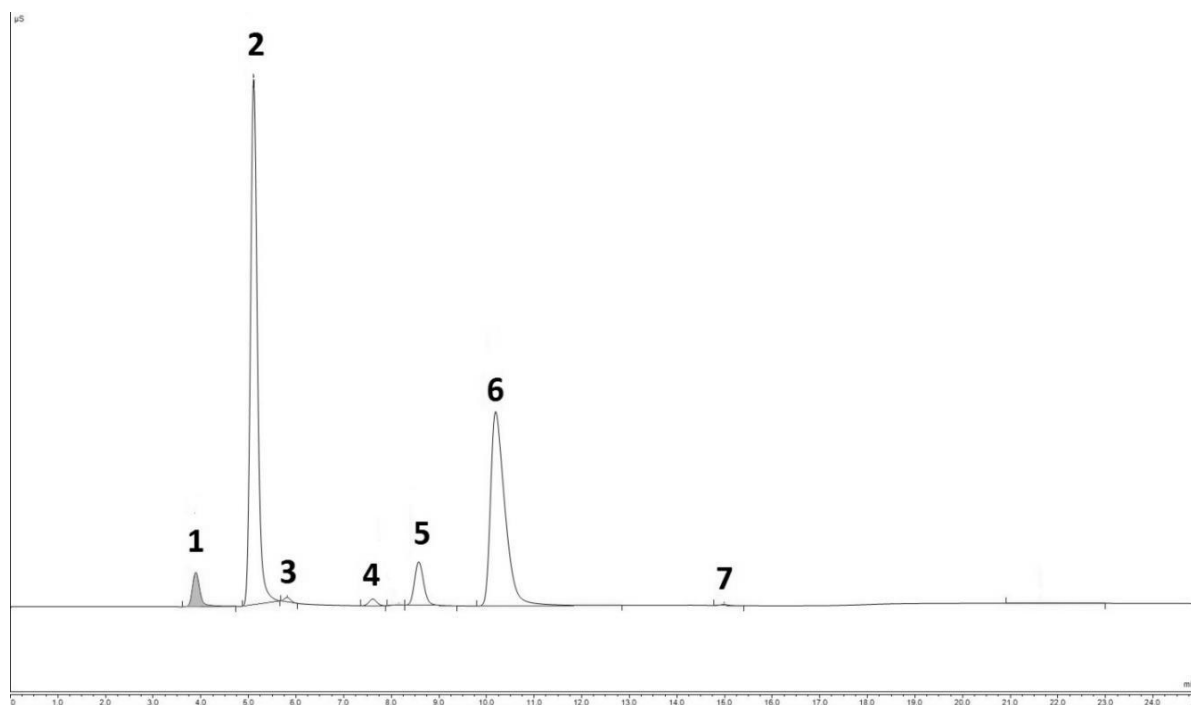


Figure S1. Anion separation by capillary IC from a standard mixture containing: (1) fluoride, (2) chloride, (3) nitrite, (4) bromide, (5) nitrate, (6) sulfate, (7) phosphate.

Table S4. List of phosphate and P-containing metabolites with their MDLs by IC-Q Exactive MS (Method B):

Peak No.	Name	Formula	[M-H] ⁻	MDL
1	Phosphoric Acid	H ₃ PO ₄	96.969	0.06 µg L ⁻¹ as P
2	Galactose-P	C ₆ H ₁₃ O ₉ P	259.022	0.18 nM
3	Glucose-P	C ₆ H ₁₃ O ₉ P	259.022	0.10 nM
4	Fructose-P	C ₆ H ₁₃ O ₉ P	259.022	0.09 nM
5	Ribulose-P	C ₅ H ₁₁ O ₈ P	229.012	0.27 nM
6	PGA	C ₃ H ₇ O ₇ P	184.986	0.36 nM
7	Erythrose-P	C ₄ H ₉ O ₇ P	199.001	0.09 nM
8	Glucose-Bi-P	C ₆ H ₁₄ O ₁₂ P ₂	338.989	0.25 nM
9	AMP	C ₁₀ H ₁₄ N ₅ O ₇ P	346.056	0.10 nM
10	Fructose-Bi-P	C ₆ H ₁₄ O ₁₂ P ₂	338.989	0.14 nM
11	Ribulose-Bis-P	C ₅ H ₁₂ O ₁₁ P ₂	308.978	0.31 nM
12	UDP-Glucose	C ₁₅ H ₂₄ N ₂ O ₁₇ P ₂	565.048	0.23 nM

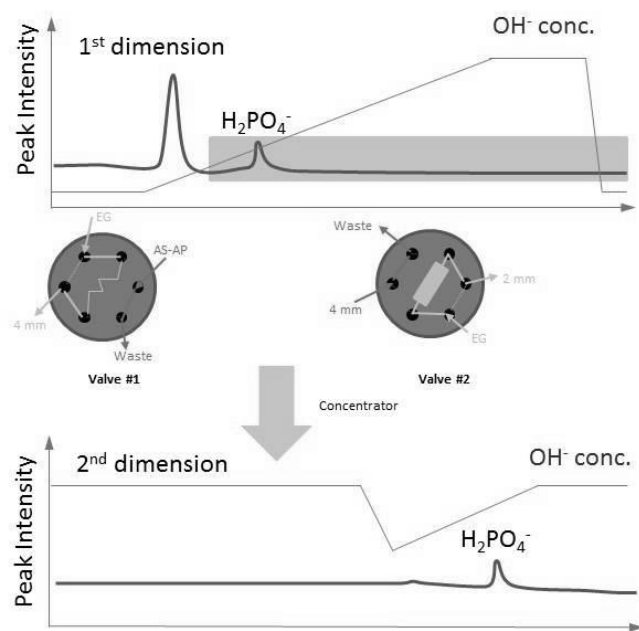


Figure S2. Schematic of the 2D-IC configuration

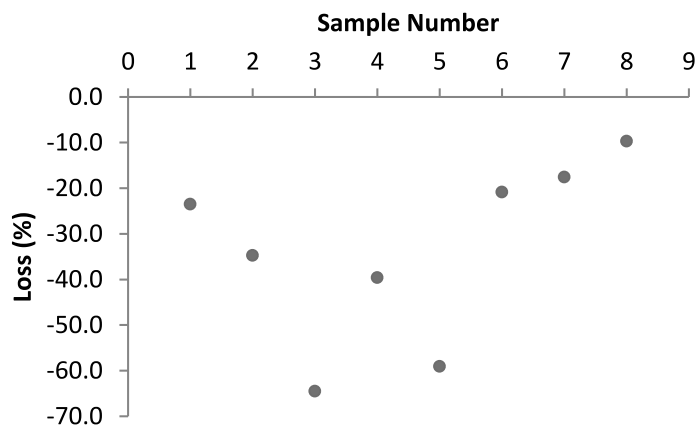


Figure S3: Phosphate loss (%) in samples spiked 48h after collection calculated by comparing with the phosphate in corresponding replicates spiked immediately when collected.

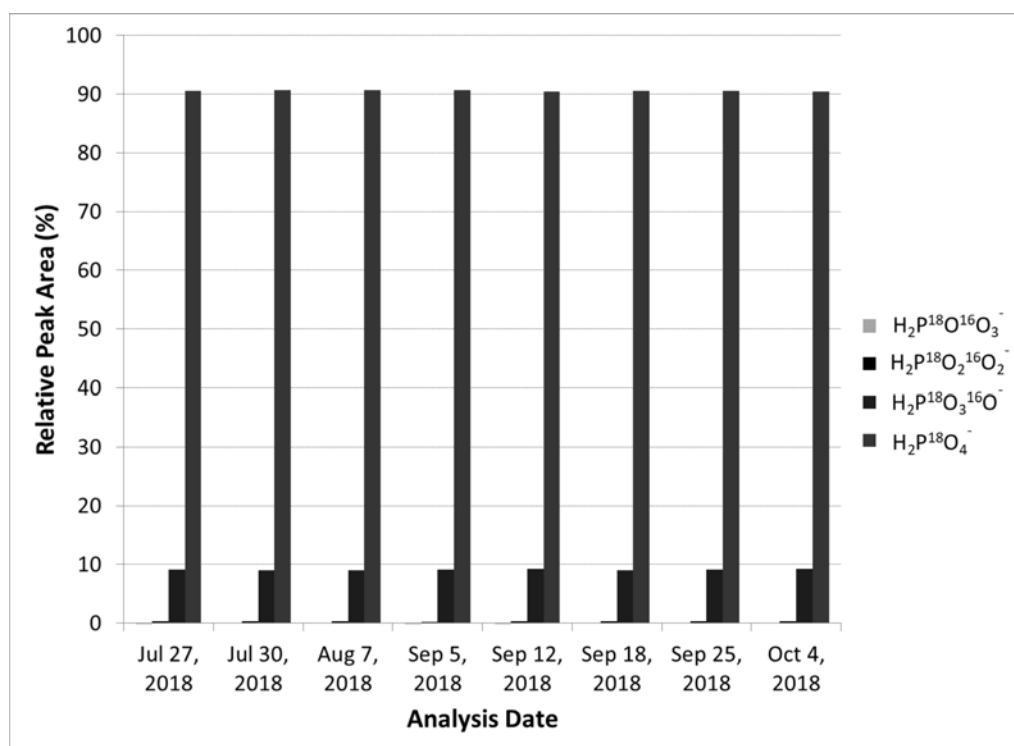


Figure S4: Distribution of the ^{18}O labeled H_2PO_4^- species remained unchanged over a two months storage (pure water sample spiked with ^{18}O - H_2PO_4^- at same level as all samples at $20\ \mu\text{g}\ \text{L}^{-1}$ as P).

APPENDIX B

Supporting Information

Phosphorus Speciation in Toronto Harbour and Rivers

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Schematic of the 2D-IC configuration (Figure S2)	SI-5

The determination of ammonia, nitrite, nitrate and phosphate by colorimetry

Ammonia, Nitrite, Nitrate plus Nitrite and Phosphorus are determined simultaneously using automated colourimetry on the same aliquot of sample. The ammonia analysis is based on formation of blue complex in buffered alkaline media. Ammonia is converted to a blue complex using sodium nitroprusside as catalyst. The concentration is determined by an absorbance at 630nm against the set of standards (calibration range: 0 - 2 mg/L as N).). The method detection limit (MDL) was 15 $\mu\text{g L}^{-1}$ as N. Nitrite analysis is based on nitrite reaction with sulphanilamide and afterwards coupling with N(1-naphthyl) ethylenediamine dihydrochloride at pH 1 to produce a light red color solution. The absorbance of the solution is measured at 520 nm and concentration is determined by comparison against the set of calibration standards (calibration range: 0 – 0.2 mg/L as N).). MDL of the method was 3 $\mu\text{g L}^{-1}$ as N. Nitrate is determined as a difference between the nitrite + nitrate and nitrite analysis. Nitrate and nitrite are determined simultaneously on the same aliquot sample. Nitrate is reduced to nitrite by heating the aliquote of the sample to 37°C with hydrazine in alkaline media with the addition of cupric ion as a catalyst. Subsequently, an azo dye is formed in acid media by diazotizing sulphanilamide with nitrite and coupling the product with N (1-naphthyl) ethylenediamine dihydrochloride. The absorbance of light red azo dye was measured at 520 nm and quantified against a set of known mixed calibration standards (calibration range nitrite+nitrate: 0 – 12.2 mg/L as N).). MDL was 60 $\mu\text{g L}^{-1}$ as N. Soluble reactive phosphorus (SRP) was measured using a Skalar continuous flow analyzer (Breda, Netherlands). SRP was measured after formation of a phospho-antimonyl-molybdate complex in the presence of the ascorbic acid as a reducing agent. The absorbance was measured at 880 nm with quantitation based on external calibration (calibration range 0 – 0.3 mg/L as P). MDL was

3 $\mu\text{g L}^{-1}$ as P. Quality control of the method was assured by running in-run quality control standards, duplicates, method blanks and reference samples. The phosphate method for was modified (see Table S1) from the reference method 4500-P G from the Standard Methods for the Examination of Water and Wastewater¹⁰⁰.

Table S1. Differences between Standard Method 4500-P G and SRP method

Analysis Modification	Standard Method 4500-P G	SRP Method
Antimony potassium tartrate solution	1.3715 g to 500mL of water	5 g to 4000mL of water
Ammonium molybdate solution	20 g to 500mL of water	240 g to 4000mL water of water
Ascorbic solution	1.76 g to 100 mL	10 g to 500 mL of water
Wetting agent	Not used/needed	FDD6 soap

Total phosphorus (TP) measurement

Samples were preserved with sulfuric acid and 10 mL aliquots were digested at 121°C for 30 min after adding potassium persulfate using a Tuttnauer 2540 EK-B/L autoclave (Heidolph Brinkmann, Hauppauge, NY, USA). After digestion TP was measured as phosphate using a Skalar continuous flow analyzer (Breda, Netherlands) where phosphate reacted in an acidic medium with ammonium molybdate and potassium antimony tartrate forming antimony-phosphomolybdate acid. The complex is reduced by ascorbic acid and forms a molybdenum blue complex. The absorbance is measured at 880 nm and compared with calibration standards (calibration range: 0 – 40 $\mu\text{g L}^{-1}$ as P). MDL of the method was 0.2 $\mu\text{g L}^{-1}$ as P. Quality control of the method was monitored by running quality control standards, duplicates, blanks and reference material. This method is slightly modified (see Table S2) from the reference method 4500-P (section B5 for digestion and section F for analysis)¹⁰⁰.

Table S2. Differences between Standard Method 4500-P B5 and TP method

Digestion Modification	Standard Method 4500- P B5	TP Method
Indicator solution	Phenolphthalein	Not used
Persulfate	Add 0.5 g solid $K_2S_2O_8$ to each sample	Add 150 μ L of saturated $K_2S_2O_8$ solution to 10 mL of sample
Analysis Modification	Standard Method 4500-P F	TP Method
Flow cell	15 or 50 mm	500 mm
Antimony potassium tartrate solution	0.3 g to 100mL of water	2.3 g to 100mL of water
Ammonium molybdate Solution	4 g to 100mL of water	3 g to 1000mL water of water
Ascorbic solution	1.76 g to 100 mL	0.2 g to 100 mL of water
Wetting agent	Not used/needed	FDD6 soap

Analysis of sulfate, chloride, sodium, calcium, potassium and magnesium by capillary ion chromatography (Cap-IC).

Sulfate, chloride, bromide, sodium, calcium, potassium and magnesium were analyzed using capillary ion chromatography (ICS-5000⁺, Thermo Fisher Scientific, Mississauga, ON, Canada) containing AS-AP, DP, EG and DC modules. Separation of major anions was obtained in 25 minutes by using an AS18 column and conductivity detector (see Figure S1). The method is similar to US EPA Method 300.1 (Determination of Inorganic Anions in Drinking Water by Ion Chromatography PART A. Common Anions in EPA 300.1)²⁵⁵, with differences summarized in Table S3. The phosphate was simultaneously measured, but it was below method MDL for the majority of the analyzed surface water samples.

Table S3. Differences between Standard Method EPA 300.1 and IC method used

Analysis Modification	EPA Method 300.1	IC Method
IC Column	AS9-HC	AS18
Eluent	9 mM carbonate	10-34 mM hydroxide
Injection Volume	40 μ L	0.4 μ L
Analysis Time	15 min	24 min
Column type	4 mm	capillary

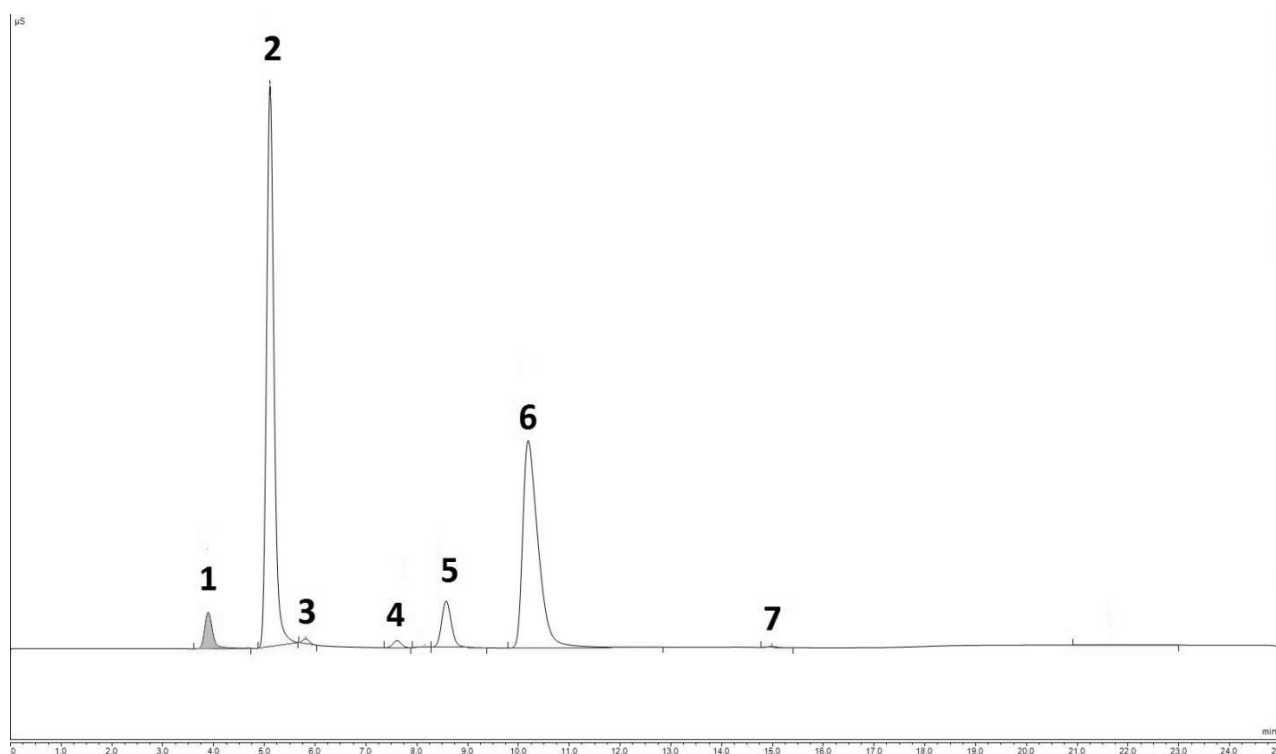


Figure S1. Anion separation by capillary IC from a standard mixture containing: (1) fluoride, (2) chloride, (3) nitrite, (4) bromide, (5) nitrate, (6) sulfate, (7) phosphate.

Table S4. MDLs of P-containing metabolites

Compound	MDL (nM)
AMP	0.10
Erythrose-P	0.09
Fructose-P	0.09
Fructose-Bi-P	0.14
Galactose-P	0.18
Glucose-P	0.10
Glucose-Bi-P	0.25
PGA	0.36
Ribulose-P	0.27
Ribulose-Bis-P	0.31
UDP-Glucose	0.23

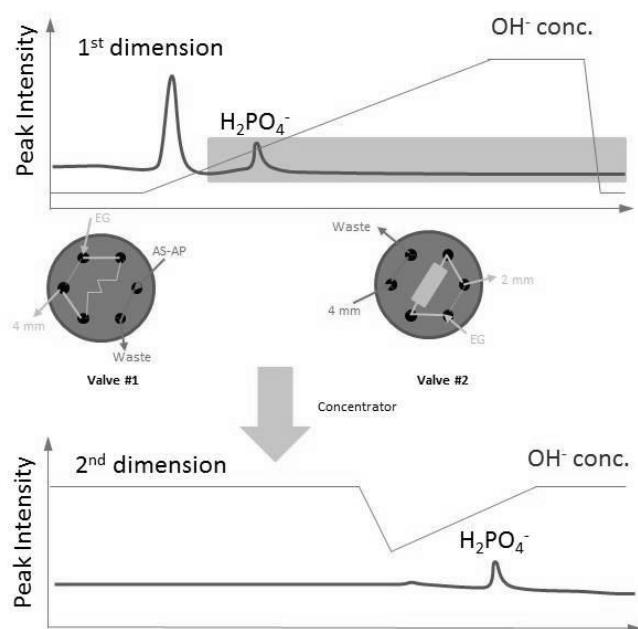


Figure S2. Schematic of the 2D-IC configuration

BIBLIOGRAPHY

1. Hecky, R. E.; Kilham, P., Nutrient limitation of phytoplankton in freshwater and marine environments: A review of recent evidence on the effects of enrichment1: Nutrient enrichment. *Limnology and Oceanography* **1988**, *33*, (4part2), 796-822.
2. Ashraf P, M.; Edwin, L.; Meenakumari, B., Studies on the seasonal changes of phosphorus in the marine environments off Cochin. *Environment International* **2006**, *32*, (2), 159-164.
3. Voutsas, D.; Manoli, E.; Samara, C.; Sofoniou, M.; Stratis, I., A Study of Surface Water Quality in Macedonia, Greece: Speciation of Nitrogen and Phosphorus. *Water, Air, and Soil Pollution* **2001**, *129*, (1), 13-32.
4. Schindler, D. W., Evolution of Phosphorus Limitation in Lakes. *Science* **1977**, *195*, (4275), 260-262.
5. Sekiguchi, Y.; Mitsuhashi, N.; Kokaji, T.; Miyakoda, H.; Mimura, T., Development of a comprehensive analytical method for phosphate metabolites in plants by ion chromatography coupled with tandem mass spectrometry. *Journal of Chromatography A* **2005**, *1085*, (1), 131-136.
6. Jürgens, U. J.; Drews, G.; Weckesser, J., Primary structure of the peptidoglycan from the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6714. *Journal of Bacteriology* **1983**, *154*, (1), 471-478.
7. Hoiczyk, E.; Hansel, A., Cyanobacterial cell walls: news from an unusual prokaryotic envelope. *Journal of bacteriology* **2000**, *182*, (5), 1191-1199.
8. Huisman, J.; Matthijs, H. C. P.; Visser, P. M., *Harmful cyanobacteria*. Springer: 2005; Vol. 3.
9. Aubriot, L.; Bonilla, S.; Falkner, G., Adaptive phosphate uptake behaviour of phytoplankton to environmental phosphate fluctuations. *FEMS microbiology ecology* **2011**, *77*, (1), 1.
10. Kornberg, A., Inorganic polyphosphate: toward making a forgotten polymer unforgettable. *Journal of Bacteriology* **1995**, *177*, (3), 491-496.
11. Ducobu, H.; Jonker, R. R.; Mur, L. R., Competition between a prochlorophyte and a cyanobacterium under various phosphorus regimes: Comparison with the Droop model. *Journal of Phycology* **1998**, *34*, (3), 467-476.
12. Dufresne, A.; Allen, E. E.; Larimer, F. W.; Waterbury, J.; Partensky, F.; Hauser, L.; McCarren, J.; Palenik, B.; Land, M.; Paulsen, I.; Regala, W.; Lamerdin, J.; Webb, E. A.; Chain, P.; Brahamsha, B., The genome of a motile marine *Synechococcus*. *Nature* **2003**, *424*, (6952), 1037-1042.
13. Bentzen, E.; Taylor, W. D.; Millard, E. S., The Importance of Dissolved Organic Phosphorus to Phosphorus Uptake by Limnetic Plankton. *Limnology and Oceanography* **1992**, *37*, (2), 217-231.
14. Pant, H. K.; Reddy, K. R., Hydrologic influence on stability of organic phosphorus in wetland detritus. *Journal of environmental quality* **2001**, *30*, (2), 668.
15. Hayes, F. R.; Phillips, J. E., Lake Water and Sediment IV. Radiophosphorus Equilibrium with Mud, Plants, and Bacteria Under Oxidized and Reduced Conditions. *Limnology and Oceanography* **1958**, *3*, (4), 459-475.
16. Spivakov, B. Y.; Maryutina, T. A.; Muntau, H., Phosphorus Speciation in Water and Sediments. *Pure and Applied Chemistry* **1999**, *71*, (11), 2161-2176.

17. Baldwin, D. S., Organic phosphorus in the aquatic environment. *Environmental Chemistry* **2013**, *10*, (6), 439-454.
18. Worsfold, P. J.; Monbet, P.; Tappin, A. D.; Fitzsimons, M. F.; Stiles, D. A.; McKelvie, I. D., Characterisation and quantification of organic phosphorus and organic nitrogen components in aquatic systems: A Review. *Analytica Chimica Acta* **2008**, *624*, (1), 37-58.
19. Elser, J. J., Phosphorus: a limiting nutrient for humanity? *Current Opinion in Biotechnology* **2012**, *23*, (6), 833-838.
20. Elser, J.; Bennett, E., Phosphorus cycle: A broken biogeochemical cycle. *Nature* U6 - ctx_ver=Z39.88-2004&ctx_enc=info%3Aofi%2Fenc%3AUTF-8&rft_id=info%3Aid%2Fsummon.serialssolutions.com&rft_val_fmt=info%3Aofi%2Ffmt%3Akev%3Amtx%3Ajournal&rft.genre=article&rft.atitle=Phosphorus+cycle%3A+A+broken+biogeochemical+cycle&rft.jtitle=Nature&rft.au=Elser%2C+James&rft.au=Bennett%2C+Elena&rft.date=2011-10-05&rft.eissn=1476-4687&rft.volume=478&rft.issue=7367&rft.spage=29&rft_id=info%3Apmid%2F21979027&rft.externalDocID=21979027¶mdict=en-US U7 - Journal Article **2011**, *478*, (7367), 29.
21. Smith, V. H., Eutrophication of freshwater and coastal marine ecosystems a global problem. *Environmental Science and Pollution Research* **2003**, *10*, (2), 126-139.
22. Le, C.; Zha, Y.; Li, Y.; Sun, D.; Lu, H.; Yin, B., Eutrophication of Lake Waters in China: Cost, Causes, and Control. *Environmental Management* **2010**, *45*, (4), 662-668.
23. Organisation for Economic, C.-o.; Development, *Eutrophication of waters: monitoring, assessment and control*. Organisation for Economic Co-operation and Development: 1982.
24. Le Moal, M.; Gascuel-Oudou, C.; Ménesguen, A.; Souchon, Y.; Étrillard, C.; Levain, A.; Moatar, F.; Pannard, A.; Souchu, P.; Lefebvre, A.; Pinay, G., Eutrophication: A new wine in an old bottle? *Science of the Total Environment* **2019**, *651*, (Pt 1), 1-11.
25. He, X.; Liu, Y.-L.; Conklin, A.; Westrick, J.; Weavers, L. K.; Dionysiou, D. D.; Lenhart, J. J.; Mouser, P. J.; Szlag, D.; Walker, H. W., Toxic cyanobacteria and drinking water: Impacts, detection, and treatment. *Harmful Algae* **2016**, *54*, 174-193.
26. Paerl, H. W.; Hall, N. S.; Calandrino, E. S., Controlling harmful cyanobacterial blooms in a world experiencing anthropogenic and climatic-induced change. *Science of the Total Environment* **2011**, *409*, (10), 1739-1745.
27. Daniel, T. C.; Sharpley, A. N.; Edwards, D. R.; Wedepohl, R.; Lemunyon, J. L., Minimizing surface water eutrophication from agriculture by phosphorus management. *Journal of Soil and Water Conservation* **1994**, *49*, (2), S30.
28. Peter, J. A. K.; Sharpley, A. N.; McDowell, R. W.; Flaten, D. N.; Buda, A. R.; Tao, L.; Bergstrom, L.; Zhu, Q.; Sveriges, I., Managing agricultural phosphorus for water quality protection: principles for progress. *Plant and Soil* **2011**, *349*, (1/2), 169-182.
29. Bowes, M. J.; Smith, J. T.; Neal, C.; Leach, D. V.; Scarlett, P. M.; Wickham, H. D.; Harman, S. A.; Armstrong, L. K.; Davy-Bowker, J.; Haft, M.; Davies, C. E., Changes in water quality of the River Frome (UK) from 1965 to 2009: Is phosphorus mitigation finally working? *Science of the Total Environment* **2011**, *409*, (18), 3418-3430.
30. Kelly, M. G.; Wilson, S., Effect of phosphorus stripping on water chemistry and diatom ecology in an eastern lowland river. *Water Research* **2004**, *38*, (6), 1559-1567.
31. Meals, D. W.; Dressing, S. A.; Davenport, T. E., Lag time in water quality response to best management practices: a review. *Journal of environmental quality* **2010**, *39*, (1), 85.
32. Bowes, M. J.; Ings, N. L.; McCall, S. J.; Warwick, A.; Barrett, C.; Wickham, H. D.; Harman, S. A.; Armstrong, L. K.; Scarlett, P. M.; Roberts, C.; Lehmann, K.; Singer, A. C.,

Nutrient and light limitation of periphyton in the River Thames: Implications for catchment management. *Science of the Total Environment* **2012**, 434, 201-212.

33. Jarvie, H. P.; Neal, C.; Williams, R. J., Assessing Changes in Phosphorus Concentrations in Relation to In-Stream Plant Ecology in Lowland Permeable Catchments: Bringing Ecosystem Functioning Into Water Quality Monitoring. *Water, Air and Soil Pollution: Focus* **2004**, 4, (2), 641-655.

34. Neal, C.; Jarvie, H. P.; Williams, R.; Love, A.; Neal, M.; Wickham, H.; Harman, S.; Armstrong, L., Declines in phosphorus concentration in the upper River Thames (UK): links to sewage effluent cleanup and extended end-member mixing analysis. *The Science of the total environment* **2010**, 408, (6), 1315-1330.

35. Jarvie, H. P.; Sharpley, A. N.; Withers, P. J. A.; Scott, J. T.; Haggard, B. E.; Neal, C., Phosphorus mitigation to control river eutrophication: murky waters, inconvenient truths, and "postnormal" science. *Journal of environmental quality* **2013**, 42, (2), 295.

36. McDowell, R.; Sharpley, A., Availability of residual phosphorus in high phosphorus soils. *Communications in Soil Science and Plant Analysis* **2002**, 33, (7-8), 1235-1246.

37. Sharpley, A.; Jarvie, H. P.; Buda, A.; May, L.; Spears, B.; Kleinman, P., Phosphorus legacy: overcoming the effects of past management practices to mitigate future water quality impairment. *Journal of environmental quality* **2013**, 42, (5), 1308.

38. Jarvie, H. P.; Sharpley, A. N.; Spears, B.; Buda, A. R.; May, L.; Kleinman, P. J. A., Water Quality Remediation Faces Unprecedented Challenges from "Legacy Phosphorus". *Environmental Science & Technology* **2013**, 47, (16), 8997-8998.

39. www.livescience.com/29312-great-lakes.html.

40. *Environmental Commissioner of Ontario Annual Report 2014/2015*, p.69-75.

41. *Environment and Climate Change Canada, 2012 Great Lakes Water Quality Agreement* www.ec.gc.ca/grandslacs-greatlakes/default.asp?lang=En&n=647DC488-1.

42. Small, H., Landmarks in the evolution of ion chromatography. *LC-GC North America* **2013**, 31, (4), S8.

43. Michalski, R., Application of IC-MS and IC-ICP-MS in environmental research. *LC-GC North America* **2012**, 30, (10), S32.

44. Small, H.; Stevens, T. S.; Bauman, W. C., Novel ion exchange chromatographic method using conductimetric detection. *Analytical Chemistry* **1975**, 47, (11), 1801-1809.

45. T. Lebedev, A., *Comprehensive Environmental Mass Spectrometry*. ILM Publications: 2012.

46. Crotti, S.; Seraglia, R.; Traldi, P., Some Thoughts on Electrospray Ionization Mechanisms. In SAGE Publications: London, England, 2011; Vol. 17, pp 85-99.

47. Hu, Q.; Noll, R. J.; Li, H.; Makarov, A.; Hardman, M.; Graham Cooks, R., The Orbitrap: a new mass spectrometer. *Journal of Mass Spectrometry* **2005**, 40, (4), 430-443.

48. <https://www.thermofisher.com/us/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-systems/orbitrap-lc-ms.html>.

49. Ngan Diep, A. D., Todd Howell, Sean Backus *Lake St. Clair-Thames River Water Quality and Harmful Algal Bloom Assessment*; MOECP: 2017.

50. www.thermofisher.com www.thermofisher.com

51. ThermoScientific, IONMax Source Hardware Manual.

52. ThermoScientific, *QExactive Series Operations Manual*. Thermo Scientific: 2014.

53. *The Merck Index*. Fourteenth ed.; Merck and Co., Inc.: Whitehouse Station, NJ, USA, 2006.

54. Mellor, J. W., *A comprehensive treatise on inorganic and theoretical chemistry*. Wiley: 1962.
55. Toy, A. D. F., Elemental Phosphorus. *The Chemistry of Phosphorus* **1973**, 3, 389-406.
56. Stwertka, A., *A Guide to the Elements*. Third ed.; Oxford University Press, Inc: New York, NY, USA, 2012.
57. Bingham, E.; Cohrssen, B.; H Powell, C., *Patty's Toxicology, 5th edition in 9 volumes*. 2001; Vol. 8, p 45-46.
58. Jacobs, M. B., *The analytical toxicology of industrial inorganic poisons*. Interscience Publishers: 1967.
59. Siekierski, S.; Burgess, J., 11 - Group 15. In *Concise Chemistry of the Elements*, Woodhead Publishing: 2002; pp 107-114.
60. Greenwood, A. E. a. N., 12 - Phosphorus. In *Chemistry of the Elements (Second Edition)*, Butterworth-Heinemann: Oxford, 1997; pp 473-546.
61. Theocharis, C. R., Industrial inorganic chemistry By W. Büchner, R. Schliébs, G. Winter and K. H. Bücjel, VCH Verlagsgesellschaft, Weinheim, 1989. pp. 1 + 614, price DM145.00, £51.00. ISBN 3-527-26629-1. *Journal of Chemical Technology & Biotechnology* **1990**, 47, (2), 182-182.
62. Waggaman, W. H., *Phosphoric Acid, Phosphates, and Phosphatic Fertilizers*. LWW: 1952; Vol. 74.
63. Albaigés, J., Stanley E. Manahan, Environmental chemistry: 9th edition, Boca Raton, FL, USA, CRC Press, 2010, 783 pp., 96.70 USD (hardback), ISBN 978-1-4200-5920-5. *International Journal of Environmental Analytical Chemistry* **2013**, 93, (14), 1559-1560.
64. Fernandez-García, C.; Coggins, A. J.; Powner, M. W., A Chemist's Perspective on the Role of Phosphorus at the Origins of Life. *Life* **2017**, 7, (3), 31.
65. Westheimer, F. H., Why Nature Chose Phosphates. *Science* **1987**, 235, (4793), 1173-1178.
66. Davis, B. D., On the importance of being ionized. *Archives of Biochemistry and Biophysics* **1958**, 78, (2), 497-509.
67. Voet, D.; Voet, J. G., *Biochemistry*. J. Wiley & Sons: 1990.
68. Condon, L.; Turner, B.; Cade-Menun, B., *Phosphorus: Agriculture and the Environment*. ASA, CSSA, and SSSA: 2005.
69. Corbridge, D. E. C., *Phosphorus : an outline of its chemistry, biochemistry, and uses*. Fifth edition. Amsterdam ; New York : Elsevier, 1995.: 1995.
70. Wood, H. G.; Clark, J. E., Biological Aspects of Inorganic Polyphosphates. *Annual Review of Biochemistry* **1988**, 57, (1), 235-260.
71. Kulakovskaya, T. V.; Lichko, L. P.; Ryazanova, L. P., Diversity of phosphorus reserves in microorganisms. *Biochemistry (Moscow)* **2014**, 79, (13), 1602-1614.
72. Lahti, R., Microbial inorganic pyrophosphatases. *Microbiological Reviews* **1983**, 47, (2), 169-179.
73. Pick, U.; Weiss, M., Polyphosphate Hydrolysis within Acidic Vacuoles in Response to Amine-Induced Alkaline Stress in the Halotolerant Alga *Dunaliella salina*. *Plant Physiology* **1991**, 97, (3), 1234-1240.
74. Walter Dodds, M., *Freshwater Ecology*. Academic Press: 2010.
75. Hudson, J. J.; Schindler, D. W.; Taylor, W. D., Phosphate concentrations in lakes. *Nature* **2000**, 406, (6791), 54-56.

76. Broberg, O.; Persson, G., Particulate and dissolved phosphorus forms in freshwater: composition and analysis. *Hydrobiologia* **1988**, *170*, (1), 61-90.
77. Sundareshwar, P. V.; Morris, J. T.; Pellechia, P. J.; Cohen, H. J.; Porter, D. E.; Jones, B. C., Occurrence and Ecological Implications of Pyrophosphate in Estuaries. *Limnology and Oceanography* **2001**, *46*, (6), 1570-1577.
78. Huang, R.; Wan, B.; Hultz, M.; Diaz, J. M.; Tang, Y., Phosphatase-Mediated Hydrolysis of Linear Polyphosphates. *Environmental Science & Technology* **2018**, *52*, (3), 1183-1190.
79. Rosset, R.; Hui, F.; Xie, J.; Kolodziejczyk, H.; Bayri, B., Les méthodes chromatographiques et électrophorétiques récentes d'analyse des phosphates inorganiques. *Analisis* **1998**, *26*, (2), 53-60.
80. Francko, D. A.; Heath, R. T., Functionally distinct classes of complex phosphorus compounds in lake water I: Complex phosphorus compounds. *Limnology and Oceanography* **1979**, *24*, (3), 463-473.
81. McKnight, D. M.; Duren, S. M., Biogeochemical processes controlling midday ferrous iron maxima in stream waters affected by acid rock drainage. *Applied Geochemistry* **2004**, *19*, (7), 1075-1084.
82. Ruiz-Calero, V.; Galceran, M. T., Ion chromatographic separations of phosphorus species: a review. *Talanta* **2005**, *66*, (2), 376-410.
83. Cade-Menun, B. J.; Navaratnam, J. A.; Walbridge, M. R., Characterizing dissolved and particulate phosphorus in water with ³¹P nuclear magnetic resonance spectroscopy. *Environmental science & technology* **2006**, *40*, (24), 7874.
84. Nanny, M. A.; Minear, R. A., Characterization of soluble unreactive phosphorus using ³¹P nuclear magnetic resonance spectroscopy. *Marine Geology* **1997**, *139*, (1), 77-94.
85. Read, E. K.; Ivancic, M.; Hanson, P.; Cade-Menun, B. J.; McMahon, K. D., Phosphorus speciation in a eutrophic lake by sup. ³¹P NMR spectroscopy. *Water Research* **2014**, *62*, 229.
86. Minear, R. A., Characterization of naturally occurring dissolved organophosphorus compounds. *Environmental Science & Technology* **1972**, *6*, (5), 431-437.
87. Rigler, F. H., The Phosphorus Fractions and the Turnover Time of Inorganic Phosphorus in Different Types of Lakes. *Limnology and Oceanography* **1964**, *9*, (4), 511-518.
88. Maher, W.; Woo, L., Procedures for the storage and digestion of natural waters for the determination of filterable reactive phosphorus, total filterable phosphorus and total phosphorus. *Analytica Chimica Acta* **1998**, *375*, (1-2), 5-47.
89. Schindler, D. W.; Hecky, R. E.; Findlay, D. L.; Stainton, M. P.; Parker, B. R.; Paterson, M. J.; Beaty, K. G.; Lyng, M.; Kasian, S. E. M., Eutrophication of lakes cannot be controlled by reducing nitrogen input: Results of a 37-year whole-ecosystem experiment. *Proceedings of the National Academy of Sciences* **2008**, *105*, (32), 11254-11258.
90. Elser, J. J.; Marzolf, E. R.; Goldman, C. R., Phosphorus and Nitrogen Limitation of Phytoplankton Growth in the Freshwaters of North America: A Review and Critique of Experimental Enrichments. *Canadian Journal of Fisheries and Aquatic Sciences* **1990**, *47*, (7), 1468-1477.
91. Xu, H.; Paerl, H. W.; Qin, B.; Zhu, G.; Gao, G., Nitrogen and phosphorus inputs control phytoplankton growth in eutrophic Lake Taihu, China. *Limnology and Oceanography* **2010**, *55*, (1), 420-432.
92. Boström, B.; Persson, G.; Broberg, B., Bioavailability of different phosphorus forms in freshwater systems. *Hydrobiologia* **1988**, *170*, (1), 133-155.

93. Worsfold, P. J.; Gimbert, L. J.; Mankasingh, U.; Omaka, O. N.; Hanrahan, G.; Gardolinski, P. C. F. C.; Haygarth, P. M.; Turner, B. L.; Keith-Roach, M. J.; McKelvie, I. D., Sampling, sample treatment and quality assurance issues for the determination of phosphorus species in natural waters and soils. *Talanta* **2005**, *66*, (2), 273-293.
94. Dodds, W. K.; Whiles, M. R., Chapter 14 - Nitrogen, Sulfur, Phosphorus, and Other Nutrients. In *Freshwater Ecology (Second Edition)*, Academic Press: London, 2010; pp 345-373.
95. Lambert, D.; Maher, W.; Hogg, I., Changes in phosphorus fractions during storage of lake water. *Water Research* **1992**, *26*, (5), 645-648.
96. US Environmental Protection Agency, Method 365.1: Determination of Phosphorus by Semi-Automated Colorimetry. https://www.epa.gov/sites/production/files/2015-08/documents/method_365-1_1993.pdf
97. Hall, G. E. M.; Bonham-Carter, G. F.; Horowitz, A. J.; Lum, K.; Lemieux, C.; Quemerais, B.; Garbarino, J. R., The effect of using different 0.45 µm filter membranes on 'dissolved' element concentrations in natural waters. *Applied Geochemistry* **1996**, *11*, (1), 243-249.
98. Horowitz, A. J.; Elrick, K. A.; Colberg, M. R., The effect of membrane filtration artifacts on dissolved trace element concentrations. *Water Research* **1992**, *26*, (6), 753-763.
99. Wang, Y.; Hammes, F.; Boon, N.; Egli, T., Quantification of the Filterability of Freshwater Bacteria through 0.45, 0.22, and 0.1 µm Pore Size Filters and Shape-Dependent Enrichment of Filterable Bacterial Communities. *Environmental Science & Technology* **2007**, *41*, (20), 7080.
100. *Standard methods for the examination of water and waste water, 23rd ed.* American Public Health Association, American Water Works Association, Water Environment Federation, Washington, DC: 2017.
101. Murphy, J.; Riley, J. P., A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta* **1962**, *27*, 31-36.
102. International Organization for Standardization, ISO 15681-1:2003. <https://www.iso.org/standard/35050.html>
103. International Organization for Standardization, ISO 15681-2:2003. <https://www.iso.org/standard/35051.html>
104. Eberlein, K.; Kattner, G., Automatic method for the determination of ortho-phosphate and total dissolved phosphorus in the marine environment. *Fresenius Zeitschrift für Analytische Chemie* **1987**, *326*, (4), 354-357.
105. Kitson, R.; Mellon, M., Colorimetric Determination of Phosphorus as Molybdivanadophosphoric Acid. *Industrial & Engineering Chemistry Analytical Edition* **1944**, *16*, (6), 379-383.
106. Edwards, G. P.; Molof, A. H.; Schneeman, R. W., Determination of Orthophosphate in Fresh and Saline Waters. *Journal - American Water Works Association* **1965**, *57*, (7), 917-925.
107. Menzel, D. W.; Corwin, N., THE MEASUREMENT OF TOTAL PHOSPHORUS IN SEAWATER BASED ON THE LIBERATION OF ORGANICALLY BOUND FRACTIONS BY PERSULFATE OXIDATION. *Limnology and Oceanography* **1965**, *10*, (2), 280-282.
108. US Environmental Protection Agency, Method 365.4: Phosphorus, Total (Colorimetric, Automated, Block Digester AA II). https://www.epa.gov/sites/production/files/2015-08/documents/method_365-4_1974.pdf
109. Minamata Convention on Mercury, United Nations Environment Programme (UNEP). <http://www.mercuryconvention.org/Convention/tabid/3426/language/en-US/Default.aspx>

110. Valderrama, J. C., The simultaneous analysis of total nitrogen and total phosphorus in natural waters. *Marine Chemistry* **1981**, *10*, (2), 109-122.
111. Patton, C. J.; Kryskalla, J. R., Methods of Analysis by the US Geological Survey National Water Quality Laboratory: Evaluation of Alkaline Persulfate Digestion as an Alternative to Kjeldahl Digestion for Determination of Total and Dissolved Nitrogen and Phosphorus in Water. In accessed on October 21, 2017.
112. Hosomi, M.; Sudo, R., Simultaneous determination of total nitrogen and total phosphorus in freshwater samples using persulfate digestion. *International Journal of Environmental Studies* **1986**, *27*, (3-4), 267-275.
113. Ebina, J.; Tsutsui, T.; Shirai, T., Simultaneous determination of total nitrogen and total phosphorus in water using peroxodisulfate oxidation. *Water Research* **1983**, *17*, (12), 1721-1726.
114. Ormaza-González, F. I.; Statham, P. J., A comparison of methods for the determination of dissolved and particulate phosphorus in natural waters. *Water Research* **1996**, *30*, (11), 2739-2747.
115. Lambert, D.; Maher, W., An evaluation of the efficiency of the alkaline persulphate digestion method for the determination of total phosphorus in turbid waters. *Water Research* **1995**, *29*, (1), 7-9.
116. Dayton, E. A.; Whitacre, S.; Holloman, C., Comparison of three persulfate digestion methods for total phosphorus analysis and estimation of suspended sediments. *Applied Geochemistry* **2017**, *78*, (Supplement C), 357-362.
117. O'Connor, P. W.; Syers, J. K., Comparison of methods for the determination of total phosphorus in waters containing particulate material. *Journal of Environmental Quality* **1975**, *4*, (3), 347-350.
118. Logan, T. J.; Oloya, T. O.; Yaksich, S. M., Phosphate Characteristics and Bioavailability of Suspended Sediments from Streams Draining into Lake Erie. *Journal of Great Lakes Research* **1979**, *5*, (2), 112-123.
119. Zhou, M.; Struve, D. M., The effects of post-persulfate-digestion procedures on total phosphorus analysis in water. *Water Research* **2004**, *38*, (18), 3893-3898.
120. Xie, C.; Xu, J.; Tang, J.; Baig, S. A.; Xu, X., Comparison of Phosphorus Determination Methods by Ion Chromatography and Molybdenum Blue Methods. *Communications in Soil Science and Plant Analysis* **2013**, *44*, (17), 2535-2545.
121. Gjerde, D. T.; Fritz, J. S.; Schmuckler, G., Anion chromatography with low-conductivity eluents. *Journal of Chromatography A* **1979**, *186*, 509-519.
122. US Environmental Protection Agency, Method 300.1: Determination of Inorganic Anions in Drinking Water by Ion Chromatography. https://www.epa.gov/sites/production/files/2015-08/documents/method_300-1_1997.pdf
123. McDowell, M. M.; Ivey, M. M.; Lee, M. E.; Firpo, V. V. V. D.; Salmassi, T. M.; Khachikian, C. S.; Foster, K. L., Detection of hypophosphite, phosphite, and orthophosphate in natural geothermal water by ion chromatography. *Journal of Chromatography A* **2004**, *1039*, (1-2), 105-111.
124. Ivey, M. M.; Foster, K. L., Detection of phosphorus oxyanions in synthetic geothermal water using ion chromatography-mass spectrometry techniques. *Journal of Chromatography A* **2005**, *1098*, (1-2), 95-103.
125. Han, C.; Geng, J.; Xie, X.; Wang, X.; Ren, H.; Gao, S., Determination of phosphite in a eutrophic freshwater lake by suppressed conductivity ion chromatography. *Environmental Science and Technology* **2012**, *46*, (19), 10667-10674.

126. Kojima, H.; Kurihara, S.; Watanabe, Y.; Iwamaru, K.; Sato, K.; Tsunoda, K. I.; Hotta, H., A novel method for determination of inorganic oxyanions by electrospray ionization mass spectrometry using dehydration reactions. *Journal of Mass Spectrometry* **2016**, *51*, (2), 123-131.
127. Wang, R.; Wang, N.; Ye, M.; Zhu, Y., Determination of low-level anions in seawater by ion chromatography with cycling-column-switching. *Journal of Chromatography A* **2012**, *1265*, 186-190.
128. Altria, K. D., *Capillary electrophoresis guidebook: principles, operation, and applications*. Humana Press: 1996; Vol. 52.;52;.
129. Arce, L.; Ríos, A.; Valcárcel, M., Flow injection–capillary electrophoresis coupling to automate on-line sample treatment for the determination of inorganic ions in waters. *Journal of Chromatography A* **1997**, *791*, (1), 279-287.
130. Hjertén, S., Free zone electrophoresis. *Chromatographic Reviews* **1967**, *9*, (2), 122-219.
131. Jandik, P.; Jones, W. R., Optimization of detection sensitivity in the capillary electrophoresis of inorganic anions. *Journal of Chromatography A* **1991**, *546*, (C), 431-443.
132. Mayrhofer, K.; Zemmann, A. J.; Schnell, E.; Bonn, G. K., Capillary electrophoresis and contactless conductivity detection of ions in narrow inner diameter capillaries. *Analytical Chemistry* **1999**, *71*, (17), 3828-3833.
133. Kubáň, P.; Reinhardt, M.; Müller, B.; Hauser, P. C., On-site simultaneous determination of anions and cations in drainage water using a flow injection-capillary electrophoresis system with contactless conductivity detection. *Journal of Environmental Monitoring* **2004**, *6*, (3), 169-174.
134. Gaudry, A. J.; Guijt, R. M.; Macka, M.; Hutchinson, J. P.; Johns, C.; Hilder, E. F.; Dicinoski, G. W.; Nesterenko, P. N.; Haddad, P. R.; Breadmore, M. C., On-line simultaneous and rapid separation of anions and cations from a single sample using dual-capillary sequential injection-capillary electrophoresis. *Analytica Chimica Acta* **2013**, *781*, 80-87.
135. Johns, C.; Breadmore, M. C.; Macka, M.; Ryvolová, M.; Haddad, P. R., Recent significant developments in detection and method development for the determination of inorganic ions by CE. *Electrophoresis* **2009**, *30*, (SUPPL. 1), S53-S67.
136. Sugimae, A., Determination of phosphorus in river water, sewer water and domestic waste water by inductively coupled plasma-optical emission spectrometry. *BUNSEKI KAGAKU* **1980**, *29*, (8), 502-508.
137. Fujino, O.; Koga, Y.; Hiraki, K., Determination of phosphate ions in natural water by solvent extraction/ICP-AES. *BUNSEKI KAGAKU* **1991**, *40*, (1), 19-24.
138. Wennrich, R.; Mroczek, A.; Dittrich, K.; Werner, G., Determination of nonmetals using ICP-AES-techniques. *Fresenius' Journal of Analytical Chemistry* **1995**, *352*, (5), 461-469.
139. de Boer, J. L. M.; Kohlmeyer, U.; Breugem, P. M.; van der Velde-Koerts, T., Determination of total dissolved phosphorus in water samples by axial inductively coupled plasma atomic emission spectrometry. *Fresenius' Journal of Analytical Chemistry* **1998**, *360*, (1), 132-136.
140. Fan, Z. F.; Du, L. M., Determination of Phosphorus by Inductively Coupled Plasma Atomic Emission Spectroscopy after Hydride Generation. *Chinese Chemical Letters* **2000**, *11*, (5), 425-426.
141. Okamoto, Y.; Kanda, K.; Kishiwada, S.; Fujiwara, T., Determination of Phosphorous and Sulfur in Environmental Samples by Electrothermal Vaporization Inductively Coupled Plasma Atomic Emission Spectrometry. *Applied Spectroscopy* **2004**, *58*, (1), 105-110.

142. Cantarero, A.; López, M. B.; Mahía, J.; Maestro, M. A.; Paz, A., Determination of total and dissolved phosphorus in agricultural runoff samples by inductively coupled plasma mass spectrometry. *Communications in Soil Science and Plant Analysis* **2002**, 33, (15-18), 3431-3436.
143. Caruso, J. A.; Montes-Bayon, M., Elemental speciation studies - New directions for trace metal analysis. *Ecotoxicology and Environmental Safety* **2003**, 56, (1), 148-163.
144. Shah, M.; Caruso, J. A., Inductively coupled plasma mass spectrometry in separation techniques: Recent trends in phosphorus speciation. *Journal of Separation Science* **2005**, 28, (15), 1969-1984.
145. Olesik, J. W., Elemental analysis using ICP-OES and ICP/MS. *Analytical Chemistry* **1991**, 63, (1), 12-21A.
146. Kovačević, M.; Leber, R.; Kohlwein, S. D.; Goessler, W., Application of inductively coupled plasma mass spectrometry to phospholipid analysis. *Journal of Analytical Atomic Spectrometry* **2004**, 19, (1), 80-84.
147. Lovdahl, M. J.; Pietrzyk, D. J., Anion-exchange separation and determination of bisphosphonates and related analytes by post-column indirect fluorescence detection. *Journal of Chromatography A* **1999**, 850, (1), 143-152.
148. Allgrove, J., Biphosphonates. *Archives of Disease in Childhood* **1997**, 76, (1), 73-75.
149. Daley-Yates, P. T.; Gifford, L. A.; Hoggarth, C. R., Assay of 1-hydroxy-3-aminopropylidene-1,1-bisphosphonate and related bisphosphonates in human urine and plasma by high-performance ion chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications* **1989**, 490, (C), 329-338.
150. Kovačević, M.; Gartner, A.; Novič, M., Determination of bisphosphonates by ion chromatography-inductively coupled plasma mass spectrometry. *Journal of Chromatography A* **2004**, 1039, (1-2), 77-82.
151. Kline, W. F.; Matuszewski, B. K.; Bayne, W. F., Determination of 4-amino-1-hydroxybutane-1,1-bisphosphonic acid in urine by automated pre-column derivatization with 2,3-naphthalene dicarboxyaldehyde and high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography B: Biomedical Sciences and Applications* **1990**, 534, (C), 139-149.
152. Kline, W. F.; Matuszewski, B. K., Improved determination of the bisphosphonate alendronate in human plasma and urine by automated precolumn derivatization and high-performance liquid chromatography with fluorescence and electrochemical detection. *Journal of Chromatography B: Biomedical Sciences and Applications* **1992**, 583, (2), 183-193.
153. Fidalgo-Used, N.; Montes-Bayón, M.; Blanco-González, E.; Sanz-Medel, A., Determination of organophosphorus pesticides in spiked river water samples using solid phase microextraction coupled to gas chromatography with EI-MS and ICP-MS detection. *Journal of Analytical Atomic Spectrometry* **2005**, 20, (9), 876-882.
154. Guo, Z. X.; Cai, Q.; Yang, Z., Determination of glyphosate and phosphate in water by ion chromatography - Inductively coupled plasma mass spectrometry detection. *Journal of Chromatography A* **2005**, 1100, (2), 160-167.
155. Wigfield, Y. Y.; Lanouette, M., Simplified liquid chromatographic determination of glyphosate and metabolite residues in environmental water using post-column fluorogenic labelling. *Analytica Chimica Acta* **1990**, 233, 311-314.
156. Berchmans, S.; Issa, T. B.; Singh, P., Determination of inorganic phosphate by electroanalytical methods: a review. *Analytica chimica acta* **2012**, 729, 7-20.

157. Flatt, R.; Brunisholz, G., Sur le dosage argentométrique de l'anion phosphorique. *Analytica Chimica Acta* **1947**, *1*, (C), 124-134.
158. Novozamsky, I.; van Riemsdijk, W. H., The behaviour of silver phosphate as the electroactive sensor in a phosphate-sensitive electrode. *Analytica Chimica Acta* **1976**, *85*, (1), 41-46.
159. Alexander, P. W.; Haddad, P. R.; Trojanowicz, M., Potentiometric flow-injection determination of copper-complexing inorganic anions with a copper-wire indicator electrode. *Analytical Chemistry* **1984**, *56*, (13), 2417-2422.
160. Coetzee, J. F.; Gardner, C. W., Determination of Sulfate, Orthophosphate, and Triphosphate Ions by Flow Injection Analysis with the Lead Ion Selective Electrode as Detector. *Analytical Chemistry* **1986**, *58*, (3), 608-611.
161. Davey, D. E.; Mulcahy, D. E.; O'Connell, G. R., Flow-injection determination of phosphate with a cadmium ion-selective electrode. *Talanta* **1990**, *37*, (7), 683-687.
162. Glazier, S. A.; Arnold, M. A., Selectivity of Membrane Electrodes Based on Derivatives of Dibenzytin Dichloride. *Analytical Chemistry* **1991**, *63*, (8), 754-759.
163. Chen, Z.; Marco, R. D.; Alexander, P. W., Flow-injection Potentiometric Detection of Phosphates Using a Metallic Cobalt Wire Ion-selective Electrode. *Analytical Communications* **1997**, *34*, (3), 93-95.
164. Marco, R. D.; Pejicic, B.; Chen, Z., Flow injection potentiometric determination of phosphate in waste waters and fertilisers using a cobalt wire ion-selective electrode. *The Analyst* **1998**, *123*, (7), 1635-1640.
165. Zhu, L.; Zhou, X.; Shi, H., A potentiometric cobalt-based phosphate sensor based on screen-printing technology. *Frontiers of Environmental Science & Engineering* **2014**, *8*, (6), 945-951.
166. Li, L.; Shang, G.; Qin, W., Potentiometric sensing of aqueous phosphate by competition assays using ion-exchanger doped-polymeric membrane electrodes as transducers. *Analyst* **2016**, *141*, (15), 4573-4577.
167. Warwick, C.; Guerreiro, A.; Soares, A., Sensing and analysis of soluble phosphates in environmental samples: A review. *Biosensors and Bioelectronics* **2013**, *41*, (1), 1-11.
168. Fogg, A. G.; Bsebsu, N. K., Differential-pulse voltammetric determination of phosphate as molybdovanadophosphate at a glassy carbon electrode and assessment of eluents for the flow injection voltammetric determination of phosphate, silicate, arsenate and germanate. *Analyst* **1981**, *106*, (1269), 1288-1295.
169. Fogg, A. G.; Bsebsu, N. K., Flow injection voltammetric determination of phosphate: direct injection of phosphate into molybdate reagent. *Analyst* **1982**, *107*, (1274), 566-570.
170. Fogg, A. G.; Bsebsu, N. K.; Birch, B. J., Differential-pulse anodic voltammetric determination of phosphate, silicate, arsenate and germanate as β -heteropolymolybdates at a stationary glassy-carbon electrode. *Talanta* **1981**, *28*, (7, Supplement 1), 473-476.
171. Barus, C.; Romanytsia, I.; Striebig, N.; Garçon, V., Toward an in situ phosphate sensor in seawater using Square Wave Voltammetry. *Talanta* **2016**, *160*, 417-424.
172. Yao, T.; Wasa, T., Simultaneous determination of phosphate and pyrophosphate by an amperometric flow-injection system with immobilized enzyme reactors. *Electroanalysis* **1993**, *5*, (9-10), 887-890.
173. Yao, T.; Takashima, K.; Nanjyo, Y., Simultaneous determination of orthophosphate and total phosphates (inorganic phosphates plus purine nucleotides) using a bioamperometric flow-injection system made up by a 16-way switching valve. *Talanta* **2003**, *60*, (4), 845-51.

174. Cheng, W.-L.; Sue, J.-W.; Chen, W.-C.; Chang, J.-L.; Zen, J.-M., Activated nickel platform for electrochemical sensing of phosphate. *Analytical chemistry* **2010**, 82, (3), 1157.
175. Honda, A.; Morimitsu, M., A Novel Sensing Method for HPO_4^{2-} and HCO_3^- by Electrochemical Oxidation with Amorphous Mixed Oxide Catalyst. *Procedia Engineering* **2016**, 168, (Supplement C), 501-505.
176. Nanny, M. A.; Minear, R. A.; Leenheer, J. A., *Nuclear Magnetic Resonance Spectroscopy in Environmental Chemistry*. Oxford University Press: 1997.
177. Cade-Menun, B. J., Characterizing phosphorus in environmental and agricultural samples by ^{31}P nuclear magnetic resonance spectroscopy. *Talanta* **2005**, 66, (2 SPEC. ISS.), 359-371.
178. Cade-Menun, B. J.; Navaratnam, J. A.; Walbridge, M. R., Characterizing dissolved and particulate phosphorus in water with ^{31}P nuclear magnetic resonance spectroscopy. *Environmental Science and Technology* **2006**, 40, (24), 7874-7880.
179. Read, E. K.; Ivancic, M.; Hanson, P.; Cade-Menun, B. J.; McMahon, K. D., Phosphorus speciation in a eutrophic lake by ^{31}P NMR spectroscopy. *Water Research* **2014**, 62, 229-240.
180. Bai, X. L.; Zhou, Y. K.; Sun, J. H.; Ma, J. H.; Zhao, H. Y.; Liu, X. F., Classes of dissolved and particulate phosphorus compounds and their spatial distributions in the water of a eutrophic lake: a ^{31}P NMR study. *Biogeochemistry* **2015**, 126, (1-2), 227-240.
181. Cade-Menun, B. J., Improved peak identification in ^{31}P -NMR spectra of environmental samples with a standardized method and peak library. *Geoderma* **2015**, 257-258, 102-114.
182. Feng, W.; Zhu, Y.; Wu, F.; He, Z.; Zhang, C.; Giesy, J. P., Forms and Lability of Phosphorus in Algae and Aquatic Macrophytes Characterized by Solution ^{31}P NMR Coupled with Enzymatic Hydrolysis. *Scientific Reports* **2016**, 6.
183. Maccoux, M. J.; Dove, A.; Backus, S. M.; Dolan, D. M., Total and soluble reactive phosphorus loadings to Lake Erie. *Journal of Great Lakes Research* **2016**, 42, (6), 1151-1165.
184. Huser, B. J.; Futter, M. N.; Wang, R.; Fölster, J., Persistent and widespread long-term phosphorus declines in Boreal lakes in Sweden. *Science of The Total Environment* **2018**, 613-614, (Supplement C), 240-249.
185. Heisler, J.; Glibert, P. M.; Burkholder, J. M.; Anderson, D. M.; Cochlan, W.; Dennison, W. C.; Dortch, Q.; Gobler, C. J.; Heil, C. A.; Humphries, E.; Lewitus, A.; Magnien, R.; Marshall, H. G.; Sellner, K.; Stockwell, D. A.; Stoecker, D. K.; Suddleson, M., Eutrophication and harmful algal blooms: A scientific consensus. *Harmful Algae* **2008**, 8, (1), 3-13.
186. Correll, D. L., The role of phosphorus in the eutrophication of receiving waters: A review. *Journal of Environmental Quality* **1998**, 27, (2), 261-266.
187. Correll, D. L., Phosphorus: A rate limiting nutrient in surface waters. *Poultry Science* **1999**, 78, (5), 674-682.
188. Jansson, M.; Olsson, H.; Pettersson, K., Phosphatases; origin, characteristics and function in lakes. *Hydrobiologia* **1988**, 170, (1), 157-175.
189. Sharma, U.; Pal, D.; Prasad, R., Alkaline Phosphatase: An Overview. *Indian Journal of Clinical Biochemistry* **2014**, 29, (3), 269-278.
190. Herries, D., Alkaline phosphatase by R B McComb, G N Bowers, Jr and S Posen. pp 986. Plenum Press, New York and London. 1979. \$75 ISBN 0-306-40214-9. *Biochemical Education* **1981**, 9, (2), 76-76.
191. Steiner, M., Zur Kenntnis des Phosphatkreislaufes in Seen. *Die Naturwissenschaften* **1938**, 26, (44), 723-724.
192. Huisman, J.; Matthijs, H.; Visser, P., *Harmful Cyanobacteria*. 2005; Vol. 3.

193. Standardization, I. O. f., ISO 15681-2 Water Quality-determination of orthophosphate and total phosphorus by flow analysis (CFA)-part2. In 2003.
194. Standardization, I. O. f., ISO 15681-1 Water Quality-determination of orthophosphate and total phosphorus by flow analysis (FIA) -part1. In 2005.
195. J. Tarapchak, S.; M. Bigelow, S.; Rubitschun, C., *Overestimation of Orthophosphorus Concentrations in Surface Waters of Southern Lake Michigan: Effects of Acid and Ammonium Molybdate*. 2011; Vol. 39, p 296-304.
196. Tarapchak, S. J., Soluble Reactive Phosphorus Measurements in Lake Water: Evidence for Molybdate-enhanced Hydrolysis. *Journal of Environmental Quality* **1983**, 12, (1), 105-108.
197. Baldwin, D. S., Reactive “organic” phosphorus revisited. *Water Research* **1998**, 32, (8), 2265-2270.
198. Denison, F. H.; Haygarth, P. M.; House, W. A.; Bristow, A. W., The Measurement of Dissolved Phosphorus Compounds: Evidence for Hydrolysis During Storage and Implications for Analytical Definitions in Environmental Analysis. *International Journal of Environmental Analytical Chemistry* **1998**, 69, (2), 111-123.
199. Wahab, M. F.; Anderson, J. K.; Abdelrady, M.; Lucy, C. A., Peak distortion effects in analytical ion chromatography. *Analytical Chemistry* **2014**, 86, (1), 559-566.
200. Zhang, Y.; Lucy, C. A., Effect of injection matrix concentration on peak shape and separation efficiency in ion chromatography. *Journal of Chromatography A* **2014**, 1371, 177-183.
201. Pappoe, M. K.; Naeeni, M. H.; Lucy, C. A., Bromate peak distortion in ion chromatography in samples containing high chloride concentrations. *Journal of Chromatography A* **2016**, 1444, 57-63.
202. Hudak, P. F.; Sanmanee, S., Spatial patterns of nitrate, chloride, sulfate, and fluoride concentrations in the woodbine aquifer of North-Central Texas. *Environmental Monitoring and Assessment* **2003**, 82, (3), 311-320.
203. King, R.; Bonfiglio, R.; Fernandez-Metzler, C.; Miller-Stein, C.; Olah, T., Mechanistic investigation of ionization suppression in electrospray ionization. *Journal of the American Society for Mass Spectrometry* **2000**, 11, (11), 942-950.
204. Slingsby, R. W.; Pohl, C. A., Approaches to sample preparation for ion chromatography sulfate precipitation on barium-form ion exchangers. *Journal of Chromatography A* **1996**, 739, (1), 49-55.
205. Tikhomirova, T. I.; Krokhin, O. V.; Dubovik, D. B.; Ivanov, A. V.; Shpigun, O. A., Chromatographic Determination of Silicon and Phosphorus as Molybdic Heteropoly Acids with Preconcentration. *Journal of Analytical Chemistry* **2002**, 57, (1), 18-23.
206. Buldini, P. L.; Mevoli, A.; Quirini, A., On-line microdialysis-ion chromatographic determination of inorganic anions in olive-oil mill wastewater. *Journal of Chromatography A* **2000**, 882, (1), 321-328.
207. Dahllöf, I.; Svensson, O.; Torstensson, C., Optimising the determination of nitrate and phosphate in sea water with ion chromatography using experimental design. *Journal of Chromatography A* **1997**, 771, (1), 163-168.
208. Wu, J.; Yu, X.; Lord, H.; Pawliszyn, J., Solid phase microextraction of inorganic anions based on polypyrrole film. *The Analyst* **2000**, 125, (3), 391-394.
209. Hassenteufel, W.; Jagitsch, R.; Koczy, F. F., Impregnation of Glass Surface Against Sorption of Phosphate Traces. *Limnology and Oceanography* **1963**, 8, (2), 152-156.

210. Ryden, J. C.; Syers, J. K.; Harris, R. F., Sorption of inorganic phosphate by laboratory ware. Implications in environmental phosphorus techniques. *Analyst* **1972**, 97, (1160), 903-908.
211. Latterell, J. J.; Timmons, D. R.; Holt, R. F.; Sherstad, E. M., Sorption of orthophosphate on the surface of water sample containers. *Water Resources Research* **1974**, 10, (4), 865-869.
212. Dalas, E., Phosphate adsorption at the porous glass/water and SiO₂/water interfaces. *Journal of Colloid and Interface Science* **1990**, 134, (2), 299-304.
213. House, W. A.; Denison, F. H.; Armitage, P. D., Comparison of the uptake of inorganic phosphorus to a suspended and stream bed-sediment. *Water Research* **1995**, 29, (3), 767-779.
214. Chen, J.; Li, Y.; Li, D. P.; Huang, Y.; Zhu, P. Y., [Distribution of Phosphorus Forms in the Overlying Water Under Disturbance with the Addition of Algae]. *Huan jing ke xue= Huanjing kexue* **2016**, 37, (4), 1413-21.
215. Altundoğan, H. S.; Tümen, F., Removal of phosphates from aqueous solutions by using bauxite. I: Effect of pH on the adsorption of various phosphates. *Journal of Chemical Technology & Biotechnology* **2002**, 77, (1), 77-85.
216. Dalas, E.; Koutsoukos, P. G., Phosphate adsorption at the porous glass/water and SiO₂/water interfaces. *Journal of Colloid And Interface Science* **1990**, 134, (2), 299-304.
217. Jarvie, H. P.; Withers, J. A.; Neal, C., Review of robust measurement of phosphorus in river water: Sampling, storage, fractionation and sensitivity. *Hydrology and Earth System Sciences* **2002**, 6, (1), 113-132.
218. Stainton, M., *Errors in Molybdenum Blue Methods for Determining Orthophosphate in Freshwater*. 2011; Vol. 37, p 472-478.
219. Worsfold, P.; McKelvie, I.; Monbet, P., Determination of phosphorus in natural waters: A historical review. *Analytica Chimica Acta* **2016**, 918, 8-20.
220. Baken, S.; Regelink, I. C.; Comans, R. N. J.; Smolders, E.; Koopmans, G. F., Iron-rich colloids as carriers of phosphorus in streams: A field-flow fractionation study. *Water Res* **2016**, 99, 83-90.
221. Sinaj, S.; Mächler, F.; Frossard, E.; Faisse, C.; Oberson, A.; Morel, C., Interference of colloidal particles in the determination of orthophosphate concentrations in soil water extracts. *Communications in Soil Science and Plant Analysis* **1998**, 29, (9-10), 1091-1105.
222. Hens, M.; Merckx, R., The role of colloidal particles in the speciation and analysis of "dissolved" phosphorus. *Water research* **2002**, 36, (6), 1483.
223. Bradford, M. E.; Peters, R. H., The Relationship Between Chemically Analyzed Phosphorus Fractions and Bioavailable Phosphorus. *Limnology and Oceanography* **1987**, 32, (5), 1124-1137.
224. Halmann, M.; Stiller, M., Turnover and Uptake of Dissolved Phosphate in Freshwater. A Study in Lake Kinneret. *Limnology and Oceanography* **1974**, 19, (5), 774-783.
225. Ekholm, P.; Krogerus, K., Determining algal-available phosphorus of differing origin: routine phosphorus analyses versus algal assays. *Hydrobiologia* **2003**, 492, (1), 29-42.
226. Van Moorleghe, C.; De Schutter, N.; Smolders, E.; Merckx, R., The bioavailability of colloidal and dissolved organic phosphorus to the alga *Pseudokirchneriella subcapitata* in relation to analytical phosphorus measurements. *Hydrobiologia* **2013**, 709, (1), 41-53.
227. Liang, Y.; Blake, R. E., Oxygen isotope signature of Pi regeneration from organic compounds by phosphomonoesterases and photooxidation. *Geochimica et Cosmochimica Acta* **2006**, 70, (15), 3957-3969.
228. Blake, R. E.; Alt, J. C.; Martini, A. M., Oxygen isotope ratios of PO₄: An inorganic indicator of enzymatic activity and P metabolism and a new biomarker in the search for life.

- Proceedings of the National Academy of Sciences of the United States of America* **2001**, 98, (5), 2148-2153.
229. Elsbury, K. E.; Paytan, A.; Ostrom, N. E.; Kendall, C.; Young, M. B.; McLaughlin, K.; Rollog, M. E.; Watson, S., Using oxygen isotopes of phosphate to trace phosphorus sources and cycling in lake Erie. *Environmental Science and Technology* **2009**, 43, (9), 3108-3114.
230. Melby, E. S.; Soldat, D. J.; Barak, P., Synthesis and detection of oxygen-18 labeled phosphate. *PLoS ONE* **2011**, 6, (4).
231. Baykov, A. A.; Anashkin, V. A.; Salminen, A.; Lahti, R., Inorganic pyrophosphatases of Family II—two decades after their discovery. *FEBS Letters* **2017**, 591, (20), 3225-3234.
232. Cooper, W. T.; Llewelyn, J. M.; Bennett, G. L.; Salters, V. J. M., Mass spectrometry of natural organic phosphorus. *Talanta* **2005**, 66, (2 SPEC. ISS.), 348-358.
233. Zhao, J.; Liu, X., Organic and inorganic phosphorus uptake by bacteria in a plug-flow microcosm. *Frontiers of Environmental Science and Engineering* **2013**, 7, (2), 173-184.
234. Saiardi, A., How inositol pyrophosphates control cellular phosphate homeostasis? In *Advances in Biological Regulation*, 2012; Vol. 52, pp 351-359.
235. Feng, W.; Li, C.; Zhang, C.; Liu, S.; Song, F.; Guo, W.; He, Z.; Li, T.; Chen, H., Characterization of phosphorus in algae from a eutrophic lake by solution ³¹P nuclear magnetic resonance spectroscopy. *Limnology* **2019**, 20, (2), 163-171.
236. Feng, W.; Zhu, Y.; Wu, F.; He, Z.; Zhang, C.; Giesy, J. P., Forms and Lability of Phosphorus in Algae and Aquatic Macrophytes Characterized by Solution ³¹P NMR Coupled with Enzymatic Hydrolysis. *Scientific Reports* **2016**, 6, (1), 37164.
237. Wenqiang, Z.; Baoqing, S.; Hong, Z.; Wenzhong, T., Characteristics of Phosphorus Compounds and Their Effects in Sediments of an Eutrophic Chaohu Lake, China. *CLEAN – Soil, Air, Water* **2015**, 43, (4), 544-550.
238. Packa, V.; Maedler, S.; Howell, T.; Bostan, V.; Diep, N.; Tooley, R.; Furdui, V. I., Unbiased Measurement of Phosphate and Phosphorus Speciation in Surface Waters. *Environmental Science & Technology* **2018**.
239. de Ceballos, B. S. O.; König, A.; de Oliveira, J. F., Dam reservoir eutrophication: A simplified technique for a fast diagnosis of environmental degradation. *Water Research* **1998**, 32, (11), 3477-3483.
240. Vega, M.; Pardo, R.; Barrado, E.; Debán, L., Assessment of seasonal and polluting effects on the quality of river water by exploratory data analysis. *Water Research* **1998**, 32, (12), 3581-3592.
241. Reisenhofer, E.; Picciotto, A.; Li, D., A factor analysis approach to the study of the eutrophication of a shallow, temperate lake (San Daniele, North Eastern Italy). *Analytica Chimica Acta* **1995**, 306, (1), 99-106.
242. Parinet, B.; Lhote, A.; Legube, B., Principal component analysis: an appropriate tool for water quality evaluation and management—application to a tropical lake system. *Ecological Modelling* **2004**, 178, (3), 295-311.
243. Simeonov, V.; Stratis, J. A.; Samara, C.; Zachariadis, G.; Voutsas, D.; Anthemidis, A.; Sofoniou, M.; Kouimtzis, T., Assessment of the surface water quality in Northern Greece. *Water Research* **2003**, 37, (17), 4119-4124.
244. Olsen, R. L.; Chappell, R. W.; Loftis, J. C., Water quality sample collection, data treatment and results presentation for principal components analysis – literature review and Illinois River watershed case study. *Water Research* **2012**, 46, (9), 3110-3122.
245. Toronto and Region Remedial Action Plan. In Toronto, Ontario: 2015.

246. Estepp, L. R.; Reavie, E. D., The ecological history of Lake Ontario according to phytoplankton. *Journal of Great Lakes Research* **2015**, *41*, (3), 669-687.
247. Don River Watershed Plan. In Conditions, S. W. Q.-R. o. C., Ed. Toronto and Region Conservation.
248. Poulton, D. J.; Griffiths, M.; Ontario. Ministry of the, E., *Toronto waterfront general water quality, 1976-1983*. Ministry of the Environment: 1986.
249. Howell, E. T.; Chomicki, K. M.; Kaltenecker, G., Patterns in water quality on Canadian shores of Lake Ontario: Correspondence with proximity to land and level of urbanization. *Journal of Great Lakes Research* **2012**, *38*, 32-46.
250. Huang, R.; Tang, Y., Speciation Dynamics of Phosphorus during (Hydro)Thermal Treatments of Sewage Sludge. *Environmental Science & Technology* **2015**, *49*, (24), 14466-14474.
251. He, Z.; Cade-Menun, B. J.; Toor, G. S.; Fortuna, A.-M.; Honeycutt, C. W.; Sims, J. T., Comparison of phosphorus forms in wet and dried animal manures by solution phosphorus-31 nuclear magnetic resonance spectroscopy and enzymatic hydrolysis. *Journal of environmental quality* **2007**, *36*, (4), 1086.
252. Turner, B. L.; Cheesman, A. W.; Godage, H. Y.; Riley, A. M.; Potter, B. V. L., Determination of neo- and D-chiro-inositol hexakisphosphate in soils by solution 31P NMR spectroscopy. *Environmental science & technology* **2012**, *46*, (9), 4994-5002.
253. Zhu, Y.; Wu, F.; He, Z.; Guo, J.; Qu, X.; Xie, F.; Giesy, J. P.; Liao, H.; Guo, F., Characterization of organic phosphorus in lake sediments by sequential fractionation and enzymatic hydrolysis. *Environmental science & technology* **2013**, *47*, (14), 7679-7687.
254. Feng, W.; Li, C.; Zhang, C.; Liu, S.; Song, F.; Guo, W.; He, Z.; Li, T.; Chen, H., Characterization of phosphorus in algae from a eutrophic lake by solution 31P nuclear magnetic resonance spectroscopy. *Limnology* **2018**, 1-9.
255. Hautman, D. P.; Munch, D. J., Method 300.1: Determination of inorganic anions in drinking water by ion chromatography. *EPA: Ohio* **1997**.