Characterizing the organic phosphorus species in Histosols of the Holland Marsh, Canada

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December 2017

A thesis submitted to McGill University in partial fulfillment of the requirement for the degree of Master of Science

Abstract

Eutrophication of aquatic habitats, due to superfluous algal growth from nutrient pollution, is one of the leading threats to freshwater ecosystems throughout the world. One of the main sectors involved in the release of nutrients to waterways is the agricultural industry, via application of fertilizers to farmland. Various efforts have been made to mitigate this ingress of nutrients, particularly phosphorus (P) in freshwater bodies. However, there is a general lack of knowledge on the specific forms of phosphorus that leads to eutrophication. This is especially true of the organic phosphorus species and attempts to discern this compound group have primarily occurred in mineral soils. This study aimed to quantitatively assess the main organophosphorus functional classes in the Histosols of three farms in the Holland Marsh of Ontario. Through sequential extraction and enzyme hydrolysis, the identification and lability of these compounds were determined for the three Histosol fields as well as in agricultural tile drainage effluent at three relevant agricultural periods: pre-fertilizer, growing season, and post-harvest. Results showed overall low concentrations of organic P forms in available and moderately labile pools: labile phosphomonoesters (0.00 – 14.31mg P/kg), phospholipids (0.00 – 7.99mg P/kg), and phosphodiesters (0 – 23.45mg P/kg). In comparison, inorganic P, which is immediately available to both crop and algae, ranged from 24.62 to 145.83mg P/kg in available and moderately labile pools. In more stable pools, organic P species existed in greater ranges, excluding phospholipids: labile phosphomonoesters (0.00 – 35.34mg P/kg), phospholipids (0.00mg P/kg), and phosphodiesters (0.00 – 140.17mg P/kg). Concentrations of organic P decreased significantly with soil depth. As such, this study demonstrated low concentrations of labile organic P species in available pools in cultivated Histosols in temperate regions. It seems

unlikely that labile P species are a source of plant-available P for crop growth, or contributing to eutrophication of water bodies near the Holland Marsh of Ontario.

Résumé

L'eutrophisation, la dégradation des milieux aquatiques causée par la croissance superflue d'algues provenant de la pollution des éléments nutritifs, constitue l'une des principales menaces pour les écosystèmes d'eau douce dans le monde entier. L'un des principaux secteurs impliqués dans la libération de nutriments dans les cours d'eau est l'industrie agricole, via l'application d'engrais dans les terres arables. Divers efforts ont été faits pour atténuer ces contaminations de phosphore dans nos réserves d'eau douce. Cependant, il y a un manque général de connaissances sur les formes de phosphore dans les milieux agricoles au-delà de leurs phases particulaires et dissoutes dans le drainage. Ceci est particulièrement vrai pour les espèces de phosphore organique et les tentatives pour discerner ce groupe composé se sont principalement produites dans des sols minéraux. Cette étude visait à évaluer qualitativement et quantitativement les principales classes fonctionnelles organophosphorées dans les sols Histosol de trois fermes dans la région agricole Holland Marsh, en Ontario. Grâce à l'extraction séquentielle et à l'hydrolyse enzymatique, l'identification et la stabilité de ces composés ont été déterminées pour les trois champs d'histosol ainsi que dans les effluents de drainage de carreaux agricoles sur trois saisons agricoles: pré-engrais, saison de croissance et post-récolte. L'étude a déterminé des concentrations globales faibles de ces formes de P dans les bassins disponibles et moyennement disponibles: phosphomonoesters labiles (0,00 - 14,31mg P / kg), phospholipides (0,00 - 7,99mg / kg) et phosphodiesters (0 à 23,45mg P / kg). En comparaison, P inorganique (MRP), immédiatement disponible pour les cultures et les algues, variait de 24,62 à 145,83mg P / kg dans les bassins disponibles et moyennement disponibles. Cependant, dans les pools plus récalcitrants, les formulaires P étaient souvent plus importants: phosphomonoesters labiles (0,00 - 35,34mg / kg), phospholipides (0,00mg / kg), phosphodiesters (0,00 - 140,17mg P / kg) et MRP

(233,58 - 1,632.90mg P/ kg). Comme les études antérieures sur la stratification P inorganique, les concentrations organiques de forme de P ont diminué de manière significative avec la profondeur du sol. Les espèces de P organiques n'ont pas été déterminées dans les effluents de drainage, car le MRP a dominé. En tant que tel, cette étude a déterminé que les espèces de P organiques labiles dans les bassins disponibles sont très probablement dans des quantités insatisfaisantes dans les champs d'histosol cultivés tempérés pour soutenir soit la croissance des cultures, soit contribuer à l'eutrophisation.

Acknowledgements

This thesis would not be possible without the invaluable support of the community I have been fortunate to have, both past and present. I am blessed beyond enumeration and I hope that this thesis may represent even just a sliver of my appreciation for you all in my life. First, this study would not even exist without my adviser, Dr. Chandra Madramootoo, who invested his time and energy as well as financially supporting this project. I am grateful for his faith in my abilities as a researcher and granting me the opportunity to join his lab. In addition, I would be at a loss without the guidance and counsel of Dr. Joann Whalen. Her expertise in the field of soil science bolstered the quality of my work and I am forever thankful. I thank the Natural Sciences Engineering Research Council (NSERC) for their generous grant and funding which made this research achievable. I appreciate the generosity and patience of the farmers associated with this project to provide us with space to conduct the research of this thesis. David Meek, Hélène Lalande, Hicham Benslim, and Khosro Mousavi were all critical in providing the proper laboratory environment to conduct my lab analyses and I am grateful for their dedication to my work and those of my colleagues. In addition, Hicham Benslim and Hélène Lalande performed supplementary analyses on my samples, directly contributing to the thoroughness of my research. For their expertise and guidance during my project, thank you to Dr. Barbara Cade-Menun, Dr. Ben Turner, Dr. Klaus Jarosch, Dr. Yuanrong Zhu, and Dr. Zhongqi He. This work would not have been achievable without your consultation. I would also like to graciously thank the members of my lab for their support of my project: Geneviève Grenon, Bhesram Singh, Divya Gupta, Naresh Gaj, Aghil Yari, Kenton Ollivierre, Kaitlin Lloyd, Samuel Ihuoma, Cynthia Crézé, Paddy Enright, Laura Gilbert, Ajay Singh, and Kai Rockafield. As part of the Holland Marsh team, Geneviève Grenon, Bhesram Singh, Naresh Gaj, Kenton Ollivierre, and Paddy

Enright made this project possible whether from assisting in sample collection, organizing logistics, or collecting supplementary data to name only a few of the services they provided. Three undergraduate assistants were essential in the efficiency of the experimental portion of this research: Marjorie Macdonald, Rose Seguin, and Rebecca Seltzer. Their commitment even with the most banal of tasks was truly appreciated. Lastly, I would like to thank Hicham Benslim, Divya Gupta, Zhor Abail, Mariam Sorour, Mitalie Makhani, Marjorie Macdonald, Rose Seguin, and Rebecca Seltzer for creating an enjoyable atmosphere in the laboratory.

I also recognize the extreme privilege of an education, especially at Sparta High School, the College of William and Mary, and McGill University, where I have been fortunate to have an exceptional academic experience. Moreover, I am fortunate to have had outstanding mentors in my life who have been inspired me and shaped my scientific pedagogy. These include Dr. George Schaller, an idol from my childhood when I aspired to be a zoologist and was kind enough to correspond with me since I was 14; Mr. Lawrence Brady, who taught me the fundamentals of chemistry; Mrs. Bernadette Melberger, who in high school was a source of encouragement, counsel, and friendship; Dr. Matthias Leu, who gave me the support to perform independent research for my undergraduate honors thesis and was an outstanding academic adviser; Dr. Charles Munn, who provided me with the most outstanding research experience of my life; and Dr. Gary Rice, who demonstrated proper analytical chemistry techniques which I utilize every day in laboratory. Not only have I had quality mentors but outstanding lab colleagues as well including: Akshay Deverakonda, Alex Chartrain, Ellen Martin, Emil Iqbal, Erin Hills, Gabbie Names, Ingrid Mielke-Maday, Laura Whitworth, Kameron Adams, Kate Gheysens, Kelly O'Toole, Kelsey Miller, Laura Whitworth, Molly Teague, and Mo Shomrat. You all made science fun and enjoyable when at times it was dry and difficult. Many have been

generous in assisting me in my scientific endeavors and I am forever grateful; these include Bernice Verwys, Brittany Jezouit, Daisy Weill, David and Arlene Hennessey, David Logan, Elizabeth Mosolgo-Clark, Emil Iqbal, Erin and Joe Firneno, Hannah Debelius, Jim Plews-Ogan, Katie Boretsky, Kelly O'Toole, Lori Blaisdell, Michael Sullivan, Patricia O'Neill, Seymour Schulich, Susan Harris, Ted Dintersmith, and Tracy and Michael Bollag. Though outside the realm of science, I am grateful to the Office of Community Engagement and WS at the College of William and Mary for instilling other values I hold dear, especially Dr. Drew Stelljes, Dr. Melody Porter, and Elizabeth Miller.

Most of all I am grateful to my friends and family who have made me a better person in all facets of life. I appreciate my Barcelona family for taking me in before graduate school and the everlasting friendships that resulted, especially Chelsea Beller, who unfortunately passed away but who lives on in our memory. Thank you to my friends from afar that despite the distance have been overwhelmingly supportive through texts, phone calls, and visits during my Master's, especially Alicia Moore, Brittany Jezouit, Bryant Silva, Cari Beyrle, Daniel Mills, Erin Hills, Holly Phipps, Jordon VanDyke, Katie Kaugars, Kaya Patel, Kourtenay Plummer, Michael Sullivan, Nate Alston, Sonia Sanghvi, and Yessenia Arias. You may not know it but you kept me going in my most stressful times. Additionally, I am fortunate to have an incredible family of friends here who were always there for me whether to celebrate the highs or commiserate in the lows; thank you Aniket Choudhuri, Brighita Lungu, Divya Gupta, Ekta Amarnani, Jaaved Singh, Kristina Mullahoo, Laura Glenn, Line Dietrich, Mariam Sorour, Mohamed Leila, Roxane Gaff, Swathi Punyamurthula, Tara Gallimore, and Wayne St. Amand. Thank you to Benoit Saint-Hilaire, my boyfriend, who went above and beyond to be there for me during my thesis; I love you. My family has been my foundation and I would not be who I am without them. Thank you

for always being a source of love Erin, Joe, and Joseph Firneno; Stella and Lance Ganney; and Patricia, Tony, and Therese Gadaleta. I am beyond blessed to have my parents, Laura and Fred De Sena, who have always taught be the value of an education, sacrificed so much to provide me with every opportunity, and most of all brought me up in a loving home. I do not know what I would do without my sister, Bronwen De Sena. You are my confidante and inspiration in all things in life; thank you for your love and always being there no matter how far you are.

My thesis is dedicated to Eleanor Hennessey, my grandma. Though you did not get a chance to see me this far, I would not be where I am today without your presence in my life. You shaped me into who I am and I hope every day that I make you proud. I love you always. Thank you, God.

Lastly, I would like to thank the baristas at La Graine Brûlée and the Starbucks in the Village for their warmth and supply of coffee during the writing process. Thank you, as well, to Annie Minoff, Corinne Fisher, Crissle, Dan Savage, Diane Rehm, Elah Feder, Ira Flatow, Ira Glass, Jad Abumrad, Jessica Williams, Joshua Johnson, Judy Gold, Kathy Tu, Kid Fury, Krystyna Hutchinson, Lauren Hennessy, Naiomi Ekperigin, Phoebe Robinson, Robert Krulwich, Terry Gross, and Tobin Low for keeping me company during my research. Of course, thank you to Damien Rice, Elizabeth Warren, Elizabeth Woolridge Grant, Ina Garten, Jessie Ware, Michelle Obama, Simon Green, and Solange Knowles for your constant inspiration and motivation.

Table of Contents

List of Figures

List of Tables

List of Abbreviations and Symbols

Al	Aluminum
AMP	Adenosine 5’ monophosphate
ANOVA	Analysis of variance
AP	Alkaline phosphatase from bovine intestine
B	Boron
BMPs	Best management practices
Ca	Calcium
CD	Color-developing solution
CEC	Cation exchange activity
Co	Cobalt
CsCl	Cesium chloride
Cu	Copper
DH_2O	Deionized water
DNA	Deoxyribonucleic acid
EH	Enzyme-hydrolysable
Fe	Iron
GLM	General linear model
GS	Growing season
HCl	Hydrochloric acid
Hg	Mercury
ICP-OES	Inductively coupled plasma optical emission spectrometry
IP	Inorganic phosphorus

K	Potassium
$LaCl_3$	Lanthanum chloride
LaP	L-α-phosphatidylcholine
LM	Labile phosphomonoesters
Mg	Magnesium
Mn	Manganese
MRP	Molybdate reactive P
MUP	Molybdate unreactive P
NaAc-HAc	Sodium acetate-acetic acid buffer
$NaHCO_3$	Sodium bicarbonate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NIH	National Institute of Health
NMR	Nuclear magnetic resonance
OM	Organic material
OP	Organic phosphorus
OMEE	Ontario Ministry of the Environment and Energy
P	Phosphorus
PC	Phospholipase C
Pd	Phosphodiesters
PDE	Phosphodiesterase
PF	Pre-fertilizer
Ph	Phospholipids

PH	Post-harvest
PO_4^{3-}	Orthophosphate
ppm	Parts per million
PWQOs	Provincial Water Quality Objectives
RNA	Ribonucleic acid
SOC	Soil organic carbon
TOC	Total organic carbon
TP	Total P
UHP	Unhydrolyzable organic P
Zn	Zinc

Preface and Contribution of Authors

The following thesis is composed of eight chapters. The first chapter presents a general introduction to the study, along with its objectives and scope. Following this, the second chapter provides a detailed and comprehensive review of the literature related to the study. The third chapter states the methodology conducted for the specific scientific analyses of the study. Subsequently, the fourth chapter exhibits the results of the scientific analyses while the fifth chapter is the discussion, where the results of the study are described in greater context. The sixth chapter provides the concluding remarks of the study, and the seventh chapter details specific recommendations for future research related to the thesis. Lastly, the eighth chapter catalogues the references utilized to conduct this study.

The first author for each chapter is the Master's candidate, who reviewed all literature, conducted the sample and data collection, performed each scientific and statistical analysis as well as their interpretation, and subsequently wrote the following chapters of this thesis. Dr. Chandra Madramootoo supported this research through his supervision, advisory guidance, and financial contribution. Dr. Joann Whalen also provided consultation throughout this research project. Funding of this study was through a NSERC Strategic Partnership Grant. Abridged manuscripts of this thesis will be submitted for publication.

The chapters of this thesis are in the following order:

Chapter 1 – Introduction – Aidan De Sena, Dr. Chandra Madramootoo, Dr. Joann Whalen

Chapter 2 – Literature Review – Aidan De Sena, Dr. Chandra Madramootoo, Dr. Joann Whalen

Chapter 3 – Methodology – Aidan De Sena, Dr. Chandra Madramootoo, Dr. Joann Whalen

Chapter 4 – Results – Aidan De Sena, Dr. Chandra Madramootoo, Dr. Joann Whalen

Chapter 5 – Discussion – Aidan De Sena, Dr. Chandra Madramootoo, Dr. Joann Whalen

1. Introduction

1.1. Focus of Study

Globally, freshwater ecosystems perform integral functions and possess enormous value. Of these, freshwater lakes serve numerous purposes, both natural and anthrocentric. Ecologically, freshwater lakes provide habitat to a plethora of biota, and consequently, are an important source of food supply and hydration, promoting biodiversity in the aquatic and surrounding terrestrial ecosystem (Perlman, 2017). In addition, freshwater lakes perform vital nutrient cycling which also accounts for their productivity (Adamowski et al., 2013). Society relies on freshwater lakes to supply drinking water, receive treated wastewater, provide recreational activities, and support industries like fishing and tourism (Government of Ontario, 2010; Palmer et al., 2011).

Unfortunately, these water bodies face grave hazards to their security. One of these threats is nonpoint phosphorus (P) loading from agriculture, resulting in eutrophication (Carpenter et al., 1998; Parry, 1998; Sharpley et al., 2001; Ding et al., 2015). Prior to nutrient management programs and regulations, copious amounts of P-rich mineral and manure fertilizer were applied to ensure optimal crop growth as P is often a growth-limiting nutrient for crops (Carpenter, et al., 1998; Kirkby & Johnston, 2008). However, this overapplication of P generates a major hazard for the surrounding environment as studies have exhibited the export of this nutrient from fields via runoff and drainage pathways (Chikhaoui et al., 2008; Whalen & Sampedro, 2010). As such, efforts to mitigate P and reduce eutrophication are in full effect by farmers. Water quality guidelines have been adopted by some government bodies as well as the utilization of P indices to guide farmers in fertilizer application to protect freshwater ecosystems (Slaton et al., 2005; Chambers et al., 2012).

Often attention is only paid to the inorganic form of P, which is immediately available to algae, the catalysts of eutrophication (Zhu et al., 2013). This is because algae, and flora in general, utilize transmembrane symport proteins, known as inorganic P transporters, which are able to overcome the electrochemical gradient across the plant membrane with the co-transport of P and either protons or sodium (Jansa et al., 2011). On the contrary, organic P species, like phosphomonoesters or phosphodiesters, must first undergo hydrolysis by various enzymes to become inorganic P, and as such become available to plant species (Doolette & Smernik, 2011). However, organic P species can be an overlooked hazard if in sufficient labile quantities and in proximity to phosphatase enzymes, therefore warranting further research in conjunction with inorganic P (Turner et al., 2002). In addition, as mineable phosphate stocks are estimated to be depleted during this century, more efficient and effective methods in utilizing all forms of P in soil may be required (Frossard et al., 2011; Deluca et al., 2015). Thus, more comprehensive analysis of organic P species is required to understand how these soil P compounds contribute to the P nutrition of agricultural crops and to the P loading in waterways, which is a trigger for eutrophication.

Few studies report on the concentration of organic P compounds and their lability in agricultural soils and effluent. This is especially true for arable Histosols, organic soils that are often intensively farmed and can yield significant harvests (Planscape, 2009). Histosols represent 1% of the Earth's glacier-free land with 325-375 million hectares found mostly in boreal or temperate areas, but tropical regions as well (FAO 2001; SSSA, 2017). Of these, only 17.9 million hectares are cultivated cropland as many Histosols remain as wetlands; however, this is regionally dependent as over 80% of Histosols are under agricultural management (Terry, 1986; FAO, 2001; Thünen Institute, 2016). The Everglades Agricultural Area in Florida, part of the

largest span of Histosols in the world, produces the USA's largest sugarcane and second largest vegetable crop and yields $1.5million in revenue annually (USGS, 1997; Aillery et al., 2001). Depending on climate and sufficiency of drainage, Histosols can support crops like beans, beets, cabbage, carrots, cauliflower, celery, cereals, choy sum, onions, potatoes, spinach, sugarcane, yow choy (USGS, 1997; Castillo & Wright, 2008; McDonald, 2017). Furthermore, research on the stratification of organic P species through the soil profile is largely absent in current literature. In regards to eutrophication mitigation measures, such as drainage control structures, most studies focus solely on concentrations of inorganic P in drainage effluent. However, such modifications could have a profound effect on P dynamics in the soil.

As such, this study hopes to quantify four organic P form classes (labile phosphomonoesters [LM], phospholipids [Ph], phosphodiesters [Pd], and unhydrolyzable organic P [UHP]), characterize their abiotic lability, and determine their stratification in arable Histosols soils, as well as understand the impact of a drainage control structure on these organic forms. This study occurred in 2016 on three Histosol carrot farms in the Holland Marsh, Ontario, Canada. Each field had subsurface tile drains and were managed with conventional, intensive farming practices. Drainage from these fields were emptied into dykes which upon combining with effluent from neighboring farms would eventually reach the Art Janse Pump, and be transferred to the Holland River. The Holland River flows into Lake Simcoe, thus posing a eutrophication risk. Soils and effluent were sampled during the pre-fertilizer, growing, and post-harvest agricultural periods. As these fields are in Ontario, Provincial Water Quality Objectives (PWQOs) dictate that total phosphorus concentrations of streams and rivers should be no higher than 0.01ppm during the ice-free period in order to afford utmost protection of freshwater bodies and not broach 0.03ppm to avoid rapid plant proliferation (OMEE, 2017). In addition, the

Government of Canada derived a P threshold of ~0.03ppm for southern Ontario with their National Agri-Environmental Standards Initiative (Chambers et al., 2012).

1.2. Objectives

To aid in ameliorating these gaps in knowledge, the study had the following objectives:

i. Compare the concentrations and lability of inorganic P, total organic P, and organic P species in three agricultural Histosol fields and the dynamics of these forms over time;
ii. Determine the concentrations and lability of inorganic P, total organic P, and organic P classes at three different depths of an agricultural Histosol field and the dynamics of these forms over time to understand their stratification in the soil profile;
iii. Examine correlations between P forms and soil characteristics in agricultural Histosols;
iv. Determine the concentrations of inorganic P, total organic P, and organic P species in drainage effluent, to assess the eutrophication potential of the fields.

1.3. Scope

As most farms in the Holland Marsh have organic soils, findings from this study may be applicable to the other fields in this region. However, if management practices and history differ greatly, then these fields may deviate from the results of this analysis. Additionally, temperate agricultural Histosols globally may find this research pertinent.

2. Literature Review

The purpose of this chapter is to facilitate a comprehensive understanding of the current literature relating to this study. First, an overview of eutrophication, its impact, and mitigation is provided. This is followed by an in-depth analysis of the agricultural P cycle. Thirdly the various

reasons for researching organic P are presented. After, the different ways agriculture can impact the organic P pool and its species are described. Then, the gaps in the current research are acknowledged. Lastly, the hypotheses of this study are detailed, based on the review of the literature.

2.1. Eutrophication from P

P derives its hazard from its essentiality. As a limiting nutrient crucial in binding carbon, metabolism, and signaling, its presence in freshwater lake systems can result in accelerated growth and biological productivity, known as eutrophication (Carpenter et al., 1998; McCann & Easter, 1999; Spivakov et al., 1999; Sharpley et al., 2001; Government of Ontario, 2010; Jones & Oburger, 2011). Eutrophication is a natural process where nutrients slowly accumulate in freshwater bodies, which gradually transforms these freshwater habitats into wetlands and eventually prairies (Perlman et al., 2017). However, human activities can vastly expedite this process resulting in exorbitant nutrient levels. Eutrophication from agriculture takes form as either livestock waste, or manure and synthetic fertilizers, both rich in P, entering freshwater bodies (Carpenter et al., 1998; Parry, 1998; Spivakov et al., 1999; Sharpley et al., 2001; Mbonimpa et al., 2014). Subsurface flow, surface runoff, drainage, and erosion are all transport agents of P with irrigation, precipitation, and snowmelt as catalysts (McCann & Easter, 1999; Mbonimpa et al., 2014).

Once introduced into the water column, P has the potential to be a pertinent environmental hazard for the water body. Though P itself is not toxic, its entry into water triggers uninhibited algal growth (Carpenter et al., 1998). As more biomass is formed, there is eventual decay performed by microorganisms that deplete dissolved oxygen. This results in “dead zones” for other organisms, especially fish (Evans et al., 1996; Carpenter et al., 1998; Spivakov et al.,

1999; Government of Ontario, 2010; Palmer et al., 2011; Perlman et al., 2017). Algal blooms also release cyanotoxins and shade the benthic floor vegetation (Carpenter et al., 1998; Sharpley et al., 2001). These effects can profoundly disturb the ecological value of lakes, such as changes in community structure, biodiversity, and habitat (Newman, 2010). As such, eutrophication from nonpoint source agriculture must be taken seriously.

However, anthropogenic eutrophication with its effects on water bodies has been an issue for some time. Consequently, there have been successful improvements such as the adoption of best management practices (BMPs) by farmers to curb nutrient loading. Examples include planting vegetated buffers along agricultural fields (Carpenter et al., 1998; Sharpley et al., 2001; Jeppesen et al., 2009; Mbonimpa et al., 2014), retention ponds with artificial wetlands (Carpenter et al., 1998; Sharpley et al., 2001; Jeppesen et al., 2009; Mbonimpa et al., 2014), and conservation tillage (Carpenter et al., 1998; McCann & Easter, 1999; Sharpley et al., 2001; Government of Ontario, 2010; Mbonimpa et al., 2014; Ding et al., 2015). Most importantly, effective application of P such as by measuring soil P content, balancing crop needs and P already present in soil, and scheduling application around rainfall events have decreased the potential for P export (Parry, 1998; Sharpley et al., 2001; Jeppesen et al., 2009). Through these measures, eutrophication potential of agricultural farms can be mitigated.

Yet, for many freshwater ecosystems whom have reduction initiatives and strategies, plateaus in progress have been encountered towards reduction goals. Dianchi Lake, Chesapeake Bay, Lake Erie and Mississippi River have all had difficulty in meeting targets for P, despite sizeable monetary investments and nutrient management programs (Zhu et al., 2013; Dodd & Sharpley, 2015). Similarly, Lake Simcoe in Ontario has also faced this issue in target improvements as shown in Figure 2.1. Internal loading and diverse non-point sources are

suspected main factors for this strain to meet P goals (Spivakov et al., 1999; Sharpley et al., 2001). However, focus solely on inorganic P ingress could be an additional aspect, underestimating the eutrophication potential of labile organic P species.

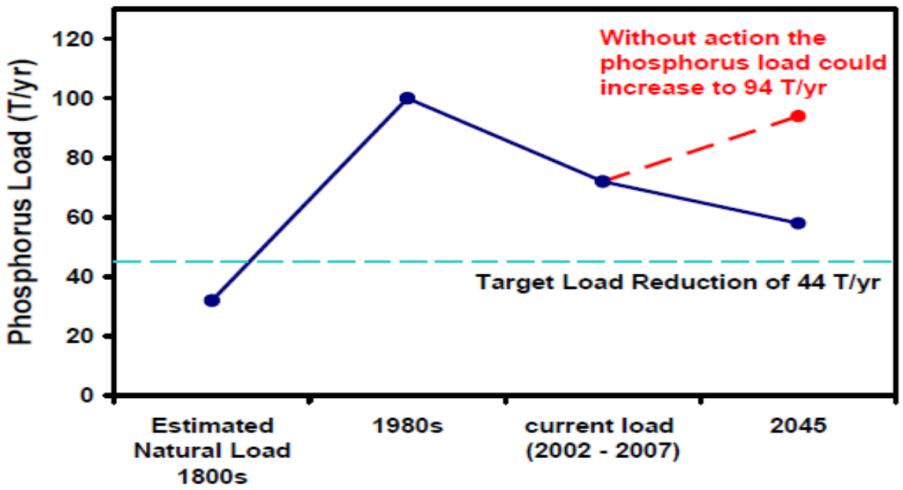

Figure 2.1: The history of phosphorus loading in Lake Simcoe and reduction projections with continued action (Government of Ontario, 2010).

2.2. Agricultural P Nutrient Cycle

In order for freshwater ecosystems, like Lake Simcoe, to reach their target projections, a comprehensive and thorough understanding of P nutrient cycling, especially in the frame of agriculture, is required. Despite the fact that the P cycle has been known for some time, there are many intricacies that are further complexed by different soil qualities, management practices, and more. Consequently, some portions of the cycle have been obscured for simplicity but may play a role in the inability for freshwater bodies to reach target load reductions. The agricultural P cycle is exhibited in Figure 2.2 below, and its components and processes will be discussed throughout this section.

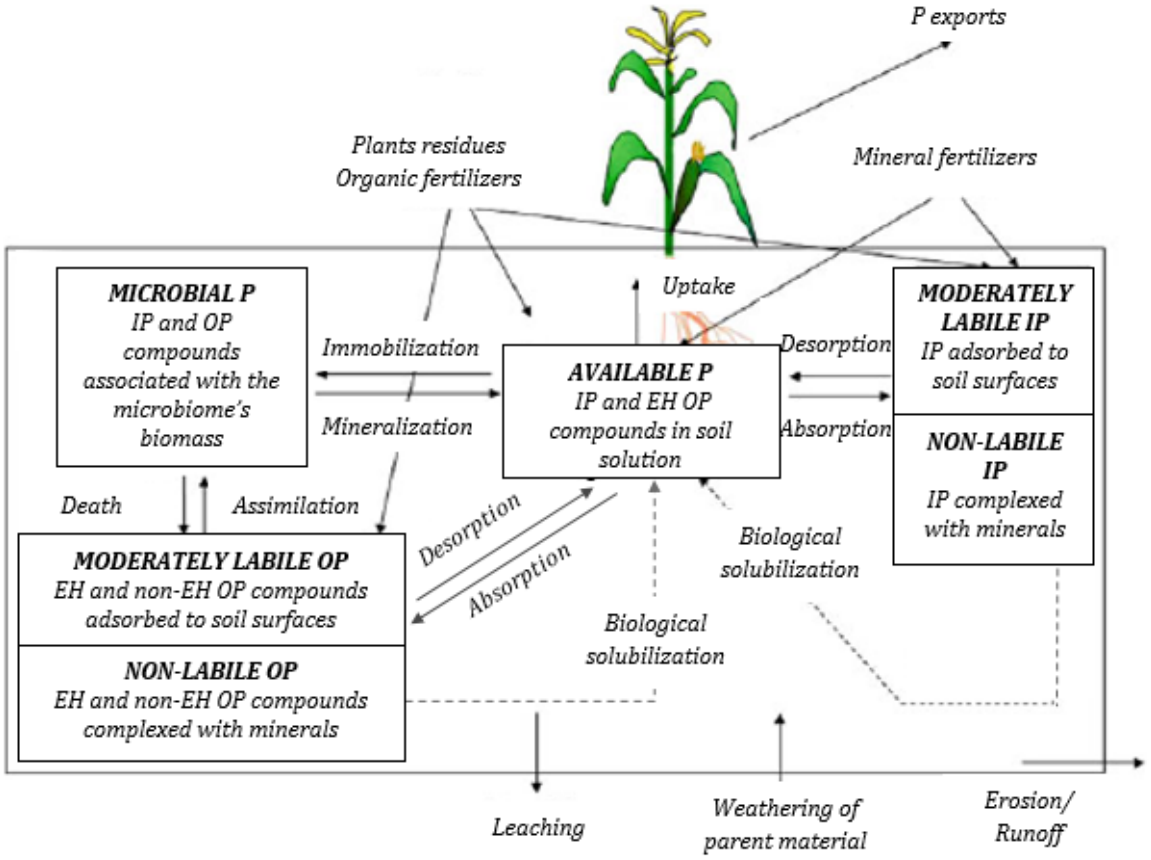

Figure 2.2: The components and processes of the agricultural P cycle (adapted from Frossard et al., 2011). For reference, IP represents inorganic P, OP represents organic P, and EH represents enzyme-hydrolyzable.

Though this nutrient is referred to by its elemental name, P exists in different forms. P in organic form, such as that from plant matter or animal waste, is restricted from plants for uptake, but when inorganic, also known as 'orthophosphate'(PO_4^{3-}), P becomes bioavailable (Zhu et al., 2013). Fertilizers are rich in inorganic P, whether manure or mineral fertilizer, so it is available for uptake by crops immediately (Mbonimpa et al., 2014). Since manure is excrement from mammals, this fertilization method also results in organic P deposition (He et al., 2008). Upon application, fertilizer is subject to the elements and may allow P to immediately enter surface runoff if a precipitation event or irrigation occurs (Carpenter et al., 1998; Mbonimpa et al. 2014). A scenario such as this would pose a serious eutrophication threat.

2.2.1. Geochemical Component

However, if proper P application scheduling occurs, this can be avoided and allow for P to enter one of the various soil pools. Inorganic P may immediately join the soil solution where it is directly available for crop and microbial uptake (Available P in Figure 2.2); however, even in fertile agricultural soils, this pool tends to have only a concentration between 0.01mg and 1mg P/L (Whalen & Sampedro 2010; Doolette & Smernik, 2011; George et al. 2011; Jones & Oburger, 2011). This is due to the reactivity of inorganic P's oxygen moieties, distinguishing this nutrient from other more soluble ones (Whalen & Sampedro 2010; Jones & Oburger, 2011). This reactivity also prevents leaching of inorganic P through the horizons, unless the soil is P-saturated (Whalen & Sampedro, 2010; Doolette & Smernik, 2011). As such, remaining in the bioavailable pool is unlikely. Inorganic P is more prone to adsorbing to soil particle surfaces via anion exchange fields where it is in equilibrium with the soil solution pool (Moderately Labile IP in Figure 2.2), replenishing the depleted pool when kinetically favorable (Whalen & Sampedro 2010; Doolette & Smernik, 2011; Jones & Oburger, 2011; Stutter et al., 2015). Depending on the mineral composition of the agricultural soil, inorganic P can also form recalcitrant ionic complexes with aluminum (Al) and iron (Fe), known as sesquisoxides, as well as calcium (Ca). The initial formation of these Al, Fe, and Ca phosphate complexes are amorphous, but become crystalline variscite, strengite, and hydroxyapatite, respectively (Non-Labile IP in Figure 2.2) (Spivakov et al., 1999; Negassa & Leinweber, 2009; Doolette & Smernik, 2011; George et al. 2011; Pagliari et al., 2017). The insolubility or recalcitrance of these formations are due to the chemical bonds formed. For instance, with the interaction between inorganic P and Fe, protonated hydroxyl groups from the iron hydroxide attract phosphate groups which then replace iron's protonated hydroxyl group, forming a covalent bond between the oxygen of phosphate and

the iron atom (Reed et al., 2011; Fink et al., 2016). In addition, multiple bonds can form depending on the number of phosphate hydroxyl groups interacting with iron atoms as shown in Figure 2.3 (Fink et al., 2016). In Figure 2.4, solubility products of inorganic phosphorus compounds in the soil environment are shown (Sanyal & De Datta, 1991).

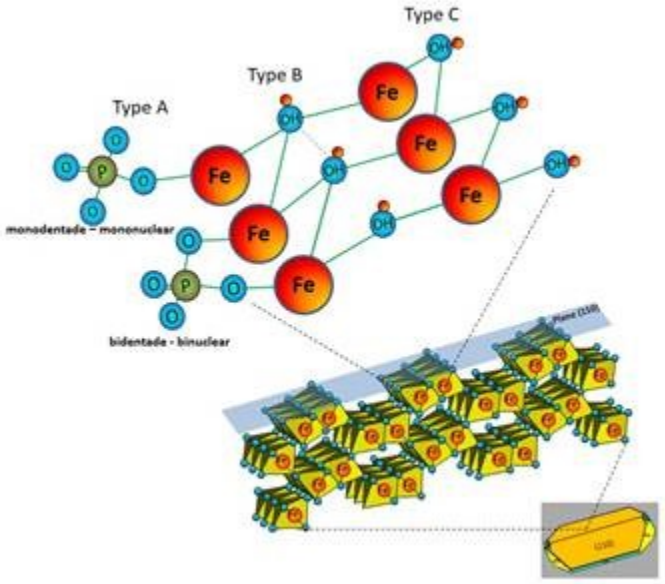

Figure 2.3: Inorganic P interactions with iron hydroxides in the soil environment (Fink et al., 2016).

Compound	Formula	pK_{sp}[a]
Variscite	$AlPO_4 \cdot 2H_2O$	21.5–22.5
Ammonium taranakite	$H_6(NH_4)_3Al_5(PO_4)_8 \cdot 18H_2O$	175.5
Potassium taranakite	$H_6K_3Al_5(PO_4)_8 \cdot 18H_2O$	178.7
Strengite	$FePO_4 \cdot 2H_2O$	35.3
Dicalcium phosphate	$CaHPO_4$	6.66
Dicalcium phosphate dihydrate	$CaHPO_4 \cdot 2H_2O$	6.56
Octacalcium phosphate pentahydrate	$Ca_8H_2(PO_4)_6 \cdot 5H_2O$	93.8
Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	111.8
Flourapatite	$Ca_{10}(PO_4)_6F_2$	120.8
Dimagnesium phosphate trihydrate	$MgHPO_4 \cdot 3H_2O$	5.82
Magnesium ammonium phosphate hexahydrate	$MgNH_4PO_4 \cdot 6H_2O$	13.2

Figure 2.4: Inorganic P compounds in the soil environment and their respective solubility products. The larger the pK_{sp}, or negative logarithm of the solubility product constant, the more insoluble the compound is (Sanyal & De Datta, 1991).

However, these reactions are pH and redox dependent. When soil pH is more acidic, inorganic P will react with present Al and Fe, while when more alkaline, Ca phosphates are formed (Jones & Oburger, 2011; Pagliari et al., 2017). In regards to the soil redox environment, when soils are water-saturated, creating anaerobic reduced conditions, these mineral complexes are more likely to dissolve (Spivakov et al., 1999). While either bound to soil surfaces or complexed with metals, P can be further obscured from availability by soil aggregation or formation of humic complexes (Guérin et al. 2007; Bünemann, 2008). Similarly, organic P can behave like inorganic P, adsorbing to soil particles (Moderately Labile OP in Figure 2.2) or interacting with sesquisoxides, occluding in soil aggregates, and forming cation precipitates (Non-Labile OP in Figure 2.2), as exhibited in Figure 2.5 (Leytem et al., 2002; Quiquampoix & Mousain, 2005; Dodd & Sharpley, 2015; Newcomb et al., 2017). Together these functions form the geochemical arm of the cycle.

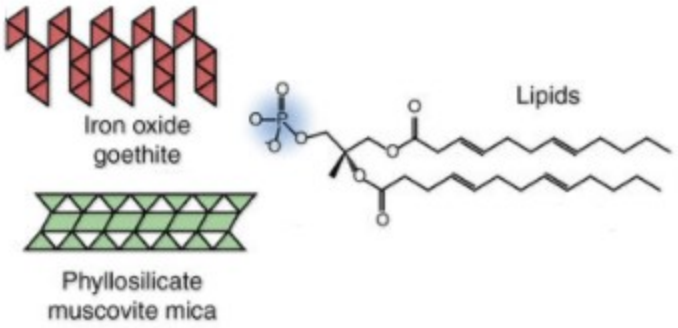

Figure 2.5: Lipids, a form of organic phosphorus found in the soil environment, can interact with soil minerals such as iron oxide goethite and phyllosilicate muscovite mica (adapted from Newcomb et al., 2017).

2.2.2. Biological Component

Yet, this only presents a portion of the cycle as there is a biological component as well. The microbiome's function is crucial in driving this portion of agricultural P dynamics. Though often referred to at large, microorganisms actually represent an immense and diverse group of nematodes, bacteria, fungi, and protozoa, each with a plethora of species (Sakurai et al., 2008; Bünemann et al., 2011; Jones & Oburger, 2011). Though often seen as an agent to plant success, mutualism is not always the reality. As mentioned previously, just as P is available to plants in the soil solution, the same is true for microorganisms, competing directly with plants and crops for limited inorganic P (Microbial P in Figure 2.2) (Jones & Oburger, 2011; Wasaki & Maruyama, 2011; Dodd & Sharpley, 2015). Regardless, their existence together is often intimate and interconnected as they are both critical actors in the management of organic P, an enigmatic subgroup of P still poorly understood (Turner et al., 2002; Wasaki & Maruyama, 2011; Jarosch et al., 2015). Subsequently, a majority of the biological portion of the cycle revolves around organic P.

In contrast to inorganic P, organic P is an umbrella term for a variety of compounds. As an organic P molecule, the P atom(s) is always associated with a carbon group often via an ester linkage (Turner et al., 2005; Doolette & Smernik, 2011). Organic P manifests in agricultural soils either through application of organic fertilizer, decomposition of plant residues, or microfauna release. Manure applied to fields as fertilizer is often rich in organic P, with as much as 50% determined in an analysis by He and colleagues (Johnson & Hill, 2010). Depending on farm management practices, organic residues can be a significant source of organic P to soil. One study determined that 0.1-0.5% of P in organic residues can be released to the soil, often in organic form (Thomas & Sevean, 1985).

Microbial processes resulting in organic P accumulation are more tedious in juxtaposition to organic residues and contributions. While microbes can immobilize inorganic P, the microbiome can also assimilate some organic P compounds, even complex humic compounds in the case of protozoa, further sequestering P forms from the soil environment (Microbial P in Figure 2.2) (Oberson et al., 2001; Jansa et al. 2011; Jones & Oburger, 2011). Once taken up by bacteria, P can be stranded in the microbial pool, known as immobilization, with its turnover dependent upon nutrient ratios (Bünemann et al., 2011; Jones & Oburger, 2011; Dodd & Sharpley, 2015). Soil organic carbon (SOC) is the most pertinent of these, where a ratio of C:P > 300 dictates immobilization of P by microbes (Whalen & Sampedro, 2010; Stutter et al., 2015). While immobilized, both inorganic and organic P undergo modifications and transformations within the membranes of the microbes (Oberson et al. 2011). Yet, this microbial P can be released through a number of events. Just as the C:P ratio determines immobilization, this parameter also determines P release into the soil matrix when organic carbon availability is reduced to C:P < 200 (Whalen & Sampedro, 2010; Jones & Oburger, 2011; Oberson et al. 2011;

Dodd & Sharpley, 2015; Stutter et al., 2015). Sudden fluctuations in environment also have the capability to release organic P into the environment. For example, cell lysis will occur upon desiccation of the soil matrix or upon starvation of the microorganism due to lack of resources (Jones & Oburger, 2011; Oberson et al. 2011; Dodd & Sharpley, 2015). Another possible scenario of organic P release is predation of microbes by other microfauna, such as nematodes or protozoa (Jones & Oburger, 2011; Oberson et al. 2011; Dodd & Sharpley, 2015).

Upon release, much of this microbial P is organic P (Bünemann et al., 2011). Thus, organic P can enter the soil environment. Whether from manure, plant residue decomposition, or microbial release, once present, organic P can accumulate in the soil matrix. This organic P may enter the soil solution (Available P in Figure 2.2). However, organic P can behave similarly to inorganic P, adsorbing to soil particles (Moderately Labile OP) or interacting with sesquisoxides, becoming occluded in soil aggregates, and forming cation precipitates (Non-Labile OP in Figure 2.2) (Leytem et al., 2002; Quiquampoix & Mousain 2005; Dodd & Sharpley, 2015).

Consequently, plants and microbes alike possess methods in which to release P fixed to the soil matrix, whether inorganic or organic. Plants are known to secrete a variety of organic acid anions like citrate which can exchange fields with P in the soil matrix and allows for P to enter the soil solution (Frossard et al., 2011; Jones & Oburger, 2011; Wasaki & Maruyama, 2011). In addition to their chelation abilities, the release of organic acid anions, along with flavonoids, simple sugars and polysaccharides, from the roots into the rhizophere can directly manipulate the make-up of bacterial communities present in the root zone; this changes their environmental conditions as well as supplying them with nutrition (Sakurai et al. 2008; Nannipieri et al., 2011; Oberson et al. 2011; Wasaki & Maruyama, 2011; Dodd & Sharpley, 2015).

Once activated by the plant, certain microbial species will proliferate and also assist in releasing both fixed inorganic and organic P. Similarly to plants, microorganisms can also release a variety of mineral complexing agents, such as organic acid anions and siderophores (Jones & Oburger, 2011). Research studies have confirmed that in soils with overwhelming amounts of Al and Fe hydroxides, organic acid anion ligand exchange is less effective (Jones & Oburger, 2011). Microbes will also release protons, hydroxyl groups, and carbon dioxide (from respiration), which either acidify or basify their immediate environment, regardless of the actual overall soil pH, enhancing the dissolution ability of P (Jones & Oburger, 2011). Acidification is more common than alkalinization, and is often synchronized with ammonium uptake as well, where protons reimburse the positive charge lost by ammonium removal (Jones & Oburger, 2011). Through these mechanisms, P can become more available.

Though these activities release inorganic P into the soil matrix, which is immediately available to the plant or microorganism, organic P is also released. However, this form of P requires an extra step in order to be an accessible resource for plants. Known as mineralization, both plants and microorganisms are capable of performing this critical process of the P cycle through the use of phosphatase enzymes (Pant & Warman, 2000; Bünemann, 2008; Nannipieri et al., 2011; Annaheim et al., 2013; Young et al., 2013; Stutter et al., 2015). These enzymes are able to catalyze the hydrolysis of esters and anhydrides present in organic phosphorus molecules (Jones & Oburger, 2011; Nannipieri et al., 2011). A variety of phosphatase enzymes exist, specialized for a specific organic P substrate, and have been classified by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, including phosphomonoesterases, phosphodiesterases, phytases, and phospholipases (Pant & Warman,

2000; Nannipieri et al., 2011). Figure 2.6, below, exhibits this reaction by both phosphodiesterase and phosphomonoesterase.

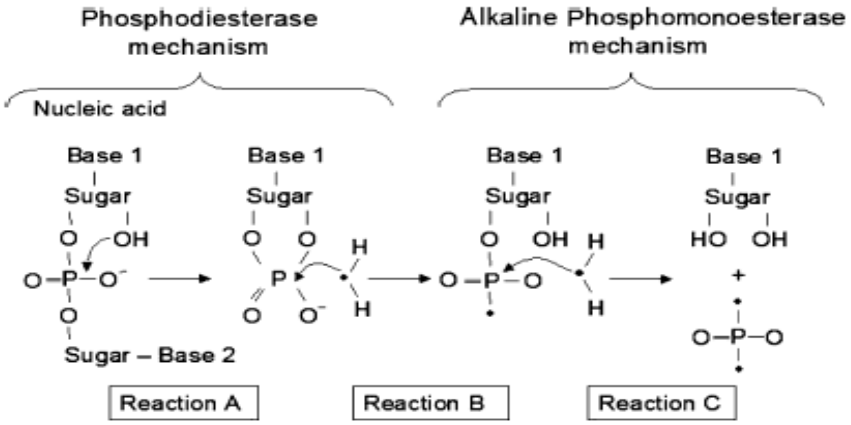

Figure 2.6: Hydrolysis mechanism for the mineralization of an organic phosphorus compound by phosphodiesterase and alkaline phosphomonoesterase (Nannipieri et al., 2011).

These enzymes exist in numerous conditions, occupying different niches to catalyze their specific reaction. Through analysis of enzymes in the soil environment, Burns (1982) enumerated these conditions as enzymes either: 1) contained in active microorganism, 2) affiliated with lysed cells and debris, 3) incorporated with resting cells, 4) released to degrade large humic compounds, 5) complexed with substrates to catalyze their breakdown, 6) existing as free extracellular agents, 7) attached to reactive surfaces in the soil matrix like clay and sesquesoxides, or 8) assembled as humic-enzyme conglomerates (Nannipieri et al., 2011); these scenarios have been substantiated by more recent studies (Pant & Warman, 2000; Bünemann, 2008; Jones & Oburger, 2011). As such, phosphatase enzymes are able to access substrate in a variety of settings, even organic P compounds in mineral complexes (Annaheim et al., 2013).

These enzyme configurations can directly impact their ability to mineralize organic P. Adsorption to soil surfaces may diminished phosphatase activity due to tertiary structure modifications and reduced accessibility to substrate if occluded (Pant & Warman, 2000; Jones & Oburger, 2011; Nannipieri et al., 2011). Early studies by Irving and Cosgrove (1976) found that

certain phosphatase enzymes did not adhere to Michaelis-Menten kinetics, and hypothesized that diffusional effects were at fault (Nannipieri et al., 2011). Yet, research has exhibited that stabilization to soil surfaces preserves the enzyme from pH fluxes, thermal denaturation, and microbial attack compared to its free enzyme counterparts (Pant & Warman, 2000; Jones & Oburger,2011; Nannipieri et al., 2011). Consequently, bound extracellular enzymes are believed to be liable for the majority of organic P mineralization (Jones & Oburger, 2011). With this ability, phosphatase enzymes are necessary for a functioning soil system.

In the soil matrix, the activity of these enzymes can be affected by more than just immobilization to supports. Phosphatase enzymes have a host of activators and inhibitors. Divalent cations such as Ca, Magnesium (Mg), Zinc (Zn), and Cobalt (Co) are known activators, necessary for proper enzyme function (Jones & Oburger, 2011; Nannipieri et al., 2011). However, at higher concentrations, Zn can behave as an inhibitor along with Fe, mercury (Hg), copper (Cu), manganese (Mn), arsenate, and molybdate (Jones & Oburger, 2011). Soil organic matter has also been noted to prevent phosphatase enzymes from functioning properly (Staunton et al., 2012). Due to feedback inhibition mechanisms, inorganic P, the end-product of the catalytic reaction, can inactivate phosphatase enzymes when at elevated concentrations (Jones & Oburger, 2011). For most phosphatase enzymes, an optimal pH range tends to be 6-8 for their proper function (Pant & Warman, 2000). However, there are both acid and alkaline phosphomonesterases which function best in their corresponding soil (Jones & Oburger, 2011). Analyses have determined that moisture is a pertinent factor for the production of phosphatase enzymes, yet this could be an artifact of rather microbial preference for moisture (Nanniperi et al., 2011). In order for optimal performance of enzymes, it is also necessary for some enzymes to work in an orchestrated manner. For example, phosphodiesters compounds are only hydrolyzed

fully into inorganic P with both phosphomonoesterase and phosphodiestderase as shown in Figure 2.6; therefore, it is hypothesized that the enzymes act in sequence (Nanniperi et al., 2011). Indeed, a study by Pant & Warman determined that two phosphomonoesterases were more active in conjunction with a phosphodiesterase as opposed to independently (2000, Nanniperri et al., 2011). Phosphomonoesterases are the most studied as they are the most common phosphatase enzyme found in the soil environment (Jones & Oburger, 2011).

Due to their importance in the soil environment, there have been many studies on their sources, plants and microorganisms. Plant species like white lupin, release acid phosphatase into the rhizosphere and have been documented to be the main source of phosphatase activity in their soil (Frossard et al., 2011; Wasaki & Maruyama, 2011). Logically, soil P deficiency is the main trigger for phosphatase production by plants (George et al. 2011). However, as referenced prior, plants can also initiate production of phosphatase enzymes by stimulating certain microorganism species; consequently, plant species, root exudate, soil type, and plant age are all factors relevant to phosphatase activity (Nannipieri et al., 2011). In addition, some plant species form arbuscular mycorrhizal symbioses, maximizing their P mineralization ability through the release of further phosphatase enzymes (Jansa et al., 2011). Current studies suggest that though plants are capable of producing acid phosphatase enzymes, only microorganisms can also produce alkaline phosphatase (Jones & Oburger, 2011; Nannipieri et al., 2011). There is also research supporting that phosphatase enzymes originating from the microbiome are more compatible with organic P species than those synthesized by plants as shown in a study by Tarafdar and colleagues (2001) where a fungal acid phosphatase was more effective than plant enzymes in mineralization (Jansa et al., 2011; Jones & Oburger, 2011).

Consequently, plants may be more dependent upon microorganisms for organic P mineralization. The microbiome is similar, in that P deficiency is the main trigger in phosphatase production (Oberson et al., 2011). In a study by Renella and colleagues (2007), glucose addition also stimulated phosphatase enzymes, though there was a noted decline over the incubation time (Nannipieri et al., 2011). There is an incredible diversity in of microorganisms able to produce phosphatase enzymes. Some of the main bacterial genii able to mineralize organic P include *Mesorhizobium*, *Pseudomonas*, *Azotobacter*, *Burkholderia*, *Bacillus*, and *Rhizobium*, while fungal genii include *Emericella* and *Penicillium* (Jones & Oburger, 2011). Unfortunately, soil microbial species are difficult to study with less than 99% of the microbiome being successfully cultured, and subsequently there may be even more species performing this pivotal role in the soil ecosystem (Bünemann et al., 2011; Wasaki & Maruyama, 2011). Overall, the role of the microbiome may be more significant as compared to plant contribution. A study by Stutter et al. derived a significant positive relationship between microbial biomass P and organic P species (2015, Dodd & Sharpley, 2015). Researchers have been estimated that between 1-50% and 0.1-0.5% of soil bacteria and fungi, respectively, function in solubilizing P into the available pool (Jones & Oburger, 2011). Regardless both the release of organic acid anions and phosphatase enzymes are crucial for plants and microorganisms to receive proper nutrition as shown below in Figure 2.7.

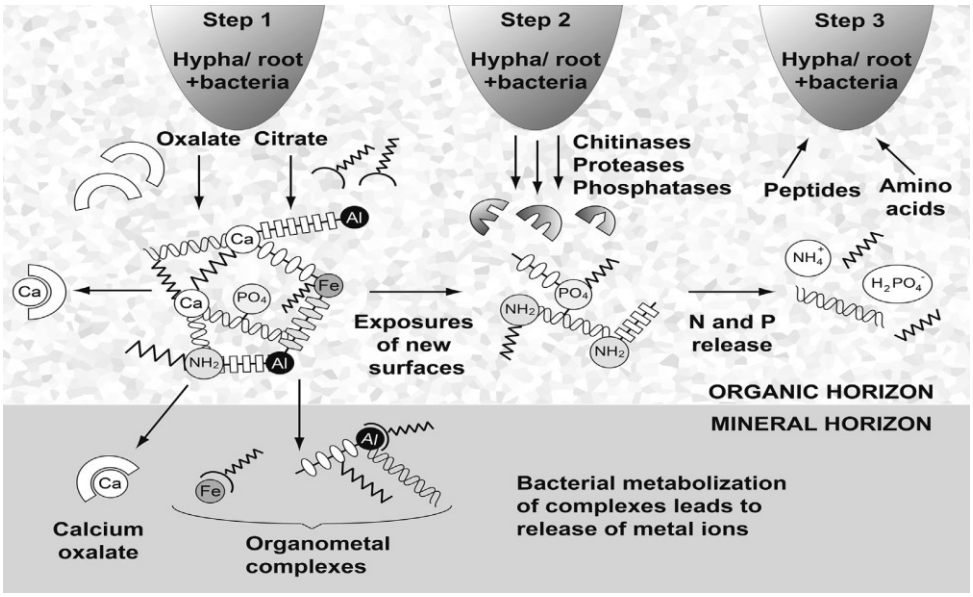

Figure 2.7: Nutrition of plant and microorganism species through the release of organic acid anions (Step 1) and enzymes (Step 2). Note: this diagram includes nitrogen nutrients and enzymes other than phosphatases associated with the general breakdown of organic matter (Clarholm et al., 2015).

However, to further complicate the cycle, organic P exists in a variety of forms, not all of which are hydrolysable. In agricultural soils, organic P is mainly represented by: phosphomonoesters, phosphodiesters, phosphonates, and large P-containing molecules. Examples of phosphomonoesters include inositol hexakisphosphate, otherwise known as phytate, and simple sugars like *D*-glucose-6-phosphate (Turner et al., 2002). Phytates have various stereoisomers which are signatures of its source such as *myo*, found in soils, animals, plants, and microorganisms; *D-chiro*, found in plant matter like seeds and leaves; and *neo* and *scyllo*, both thought to be solely of microbial origin (Quiquampoix & Mousain, 2005; Turner et al., 2005; Whalen & Sampedro, 2010; Jones & Oburger, 2011). Though phosphomonoesters, phytates are more recalcitrant in the soil environment; as such, monoesters are often separated into labile phosphomonoesters and phytates (Jones & Oburger, 2011). Phosphodiesters include phospholipids, like phosphoglycerides, and nucleic acids, such as DNA and RNA (Turner et al., 2002). Phospholipids are important cell membrane components, while nucleic acids form the hereditary material of all organisms (Alberts et al. 2002, NIH, 2017). Phosphonates possess a

bond between carbon and phosphorus, and are found in all organisms (Turner et al., 2005). Large P-containing compounds represent an operational class of high molecular weight humic substances possessing at least one P atom (Turner et al., 2002; He & Honeycutt, 2004, Negassa & Leinweber, 2009). Important to mention is that humic compounds complexed with P do not represent organic P (Turner et al., 2005). Consequently, organic P is divided into either a labile or stable pool based on their susceptibility to enzymatic breakdown (He & Honeycutt, 2004; Whalen & Sampedro, 2010; Deluca et al., 2015). Simple organic P compounds are associated with the labile pool, while large molecular weight humic compounds are categorized as the stable pool (Ivanhoff et al., 1998; Turner et al., 2002; Negassa & Leinweber, 2009).

As can be seen, the agricultural P cycle is convoluted and dependent on a multitude of factors. Though the above description of the agricultural P cycle is extensive, the detail is necessary for full comprehension of P dynamics. Though there are two main components of the cycle, geochemical and biological, separating the cycle into solely these constituents without recognizing their synergy can oversimplify the picture. A myriad of circumstances can occur where P may matriculate in one arm but transfer to the other. For example, inorganic P from fertilizer can enter the available soil P solution, become immobilized in the microbial pool, converted into *myo*-inositol phosphate, an organic P compound, released through microbial cell lysis, and complex with iron (Whalen & Sampedro, 2010; Bünemann et al., 2011; Jones & Oburger, 2011; Oberson et al. 2011; Zhu et al., 2013; Dodd & Sharpley, 2015). Another scenario is presented in Figure 2.8 by Levy-Booth and colleagues, exhibiting the possible fate and transport of organic P compounds, DNA for this situation, in the soil environment (Levy-Booth et al., 2007). However, most prominent in this cycle are the many strategies both plants and

microorganisms have developed to make P available, especially in regards to organic P (Whalen & Sampedro, 2010).

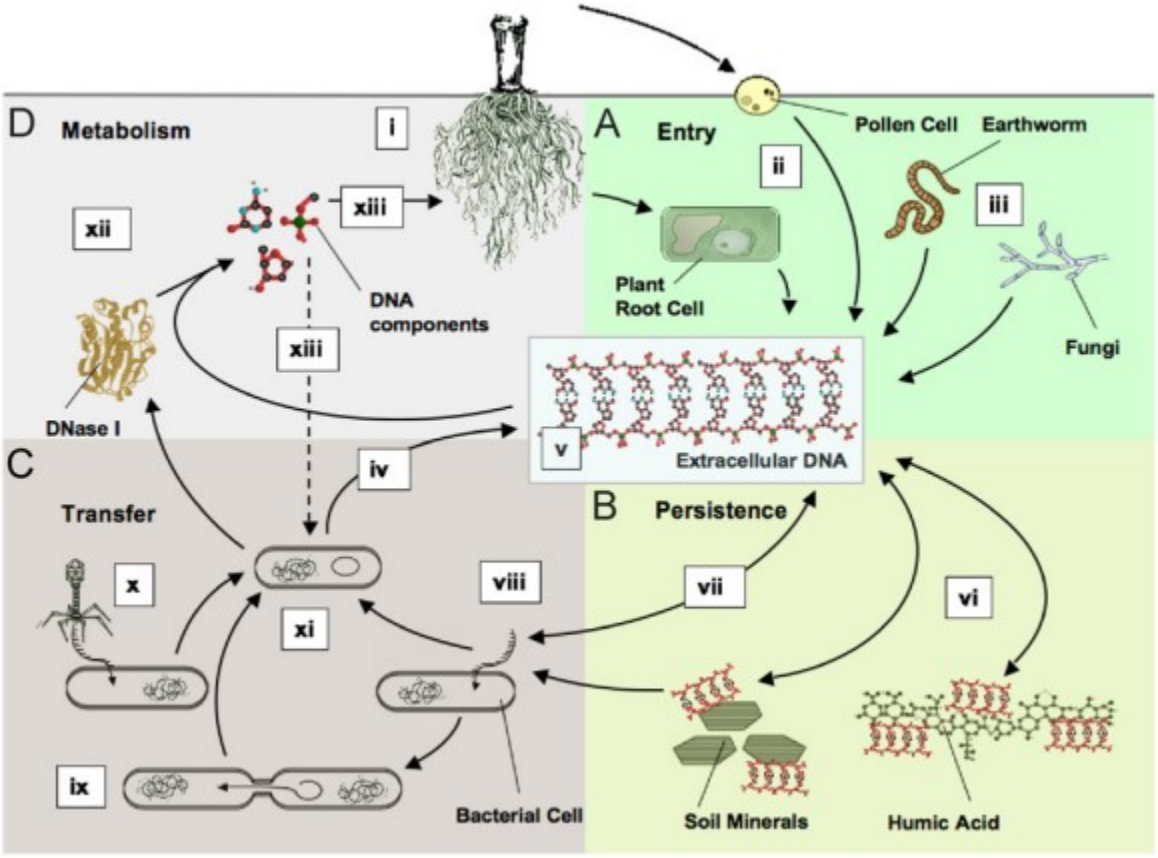

Figure 2.8: DNA is replicated by a plant during growth (i) and may enter the soil environment through the release of pollen, lysis of plant cells, or degradation of plant detritus (ii). Other contributors of DNA to the soil environment include soil meso and microfauna (iii and iv). Once released, this extracellular DNA (v) may remain in the soil environment by binding to humic acid compounds or soil minerals representing non-labile P (vi), but some may remain in the soil solution where it is labile (vii). If present in the soil solution, and therefore available, it can be easily taken up by bacteria and added to their own genome becoming microbial P (viii). Once present in the microbial P pool, the DNA molecule can be transferred to other bacteria through various processes (ix and x) and can be replicated further (xi). This DNA may be released by the living bacteria or upon lysis where it can be degraded in the soil environment by enzymes known as DNases (xii) subsequently releasing organic and inorganic P fragments which are nutrients for both flora and microorganisms (xiii) (Levy-Booth et al., 2007).

2.3. Importance of Organic P

Unfortunately, out of convenience, organic P species are often lumped together despite their diversity. As such, this provides only a one-dimensional understanding of the mechanisms controlling P availability and belittles the contribution organic P can provide. Organic phosphorous can actually comprise of a majority of the total phosphorus identity being 30-65% in most fields, though can represent as little as 10% in mineral soils and as much as 90% in organic soils (Leytem et al., 2002; Dodd & Sharpley, 2015). However, though organic soils are rich in soil organic matter, organic P compounds represents only 1-3% of soil organic matter (Whalen & Sampedro, 2010). Organic P tends to increase in temperate climates due to reduced microbial activity compared to tropical climates (Stutter et al., 2015). In regards to organic P availability, one review found that 5-52% of organic P in agricultural fields had medium to high lability, stressing their possible use by crops (Negassa & Leinweber, 2009). Yet, despite other studies with similar findings, few soil fertility testing methods incorporate labile organic P analysis (Turner et al., 2002; Young et al., 2013; Dodd & Sharpley, 2015).

2.3.1. Phytoavailability

Overall, organic P remains a poorly studied topic in agriculture. Often, if even analyzed for, total organic P is only determined via simple arithmetic and less than 50% of organic P forms have been recognized (Jarosch et al., 2015). Despite this, the studies that have been conducted indicate that organic P is deserving of greater analysis. Phytoavailability of organic P compounds has garnered more attention over past years. As mentioned above, labile organic P is not considered during soil fertility tests. However, with over half of soil microorganisms, whether free or associated with roots, able to mineralize organic P, these forms could be a significant source of nutrients to crops (Pant & Warman, 2000; Sakurai et al., 2008; Johnson &

Hill, 2010; Deluca et al., 2015; Jarosch et al., 2015). Certainly, in natural ecosystems, as well as pasture, plants rely on organic P mineralization for survival (Whalen & Sampedro, 2010; Dodd & Sharpley, 2015). In a study by Olander & Vitousek (2000), P demand was directly synchronized with increased phosphatase activity (Dodd & Sharpley, 2015). This suggests that if crop P demand is high, P nutrition could be satisfied via labile P compounds. Indeed, addition of phosphatase enzymes to soil-water extracts from agricultural fields determined 48% of organic P, itself representing 78% of total P, was potentially available in a study on organic P forms in fertilizer (Young et al., 2013). Annaheim and colleagues found that despite differences in organic P in the fertilizer, over time fields resulted in similar quantities of organic P suggesting modification and mineralization of organic P compounds (2015). Since only 1% of soil P, including inorganic, is utilized by plants during a growing season, a further understanding of labile organic P is worthwhile to manipulate their phytoavailability (Quiquampoix & Mousain, 2005). If able to be tapped, labile organic P compounds have the potential to encourage more sustainable fertilizer application (Dodd & Sharpley, 2015; Stutter et al., 2015).

2.3.2. Eutrophication Potential

As they are available to crops as a potential source of P, organic P compounds may also be a water quality hazard if removed from agricultural fields. Research suggests that organic P forms, which often have greater sorption capacity than inorganic P, may reduce the risk of eutrophication from farms if most P is in this manner (Turner et al., 2002; Deluca et al., 2015; Dodd & Sharpley, 2015). Despite this, the increased sorption capacity of organic P compounds like phytate may displace inorganic P, posing a greater eutrophication threat (Leytem et al., 2002; Dodd & Sharpley, 2015). Researchers have indicated that labile organic P itself may be an overlooked source of eutrophication (Young et al., 2013; Dodd & Sharpley, 2015). Microbial

biomass, which contains a variety of organic P compounds, is subject to lysis under rapid dry/wet or freeze/thaw events, which could result in a snap of organic P into drainage (Dodd & Sharpley, 2015). Toor and colleagues determined that 85-88% of P leached from pastures was organic P, 50% of which was enzymatically labile (2003; Dodd & Sharpley, 2015). This same study also determined that both phosphomonoesterase and phosphodiesterase were present in drainage, potentially leading to 10-21% organic P hydrolysis during transport if conditions were favorable (Toor et al., 2003). Even if unhydrolyzed, simple organic P compounds such as phosphodiesters and labile phosphomonoesters, can be utilized by blue-green algae (Toor et al., 2003). Of course, organic P's stratification in the soil is important to assess its potential to enter drainage. Unfortunately, there are few studies on this subject but the current body of literature suggests that organic P decreases markedly with depth in cultivated soils (Sanyal & De Datta, 1991). A review of organic P in different soils by Anderson (1980) exhibits the variability possible as shown in Figure 2.9, below. If organic P compounds are able to reach greater depths in the soil profile, then they may be an overlooked hazard in the eutrophication of freshwater bodies.

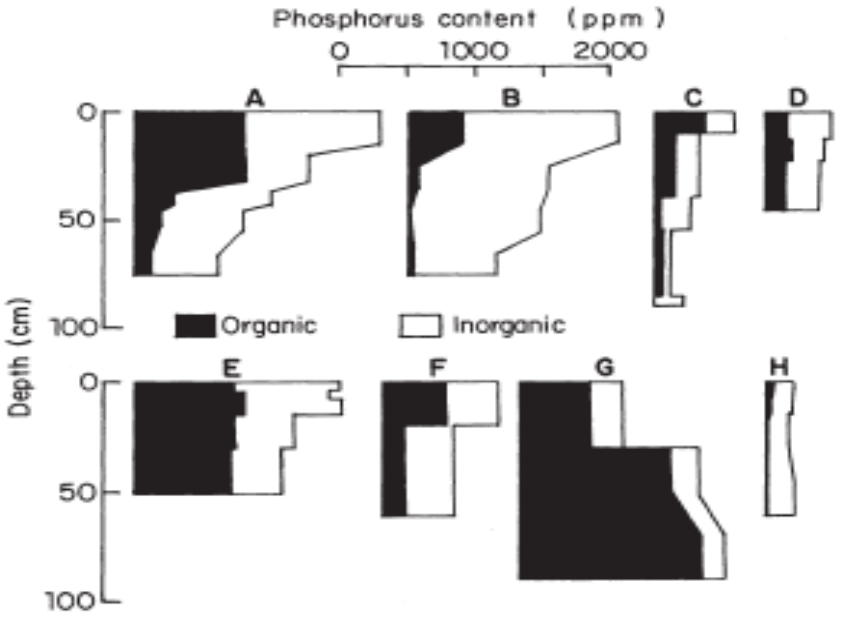

Figure 2.9: Stratification of inorganic and organic P in different soil profiles: freely drained cultivated clay loam in in Insch Association, Scotland (A); poorly drained cultivated clay loam in Insch Association, Scotland (B); uncultivated sandy soil in Koputaroa, New Zealand (C); uncultivated Dawes silt loam in Nebraska, USA (D); uncultivated Pima calcareous clay loam in Arizona, USA (E); cultivated Orthic Deep Black in Melfort, Saskatchewan, Canada (F); uncultivated *Carex globularis* pine bog in Northern Finland (G); and a leached forest soil in Ibadan Nigeria (H). Note that besides the uncultivated soils, organic P decreases with depth in the soil profile (Anderson, 1980; Sanyal & De Datta, 1991).

2.3.3. Microbial Activity Indicator

In addition, organic P forms are worthwhile of study as a fingerprint of microbial activity. Certainly, microbial P forms vary depending on environmental conditions such as presence of soil organic matter of P availability. For example, in a study on *E. coli*, a lower amount of total P was represented by phosphodiesters in inorganic P rich soils (Bünemann et al., 2011). The presence of diesters, like DNA and RNA, and certain phytate stereoisomers suggest a functioning microbial population and consequently P cycling (Whalen & Sampedro, 2010; Dodd & Sharpley, 2015; Stutter et al., 2015). In a study by Turner and colleagues, analyses indicated that greater concentrations of phosphodiesters were associated with the increased microbial activity in a P-deficient grassland (2002). Some studies have led researchers to hypothesize that

organic microbial P is more bioavailable to crops in the long-term rather than mineral-fixed inorganic P (Sakurai et al., 2008; Oberson et al. 2011; Dodd & Sharpley, 2015). However, this requires further research into the dynamics between the microbiome and plants.

2.4. Farm Management Impact on Organic P

In the agricultural setting, practices by farmers can uniquely alter organic P magnitude and diversity. Application of manure and other forms of organic matter were found to be a significant stimulant to phosphatase enzyme and microbial activity, in turn encouraging faster turnover and increased P mineralization (Sakurai et al., 2008; Jones & Oburger, 2011; Oberson et al., 2011; Annaheim et al., 2015). However, a study by Johnson & Hill determined that six weeks after poultry litter application, organic P increased by a factor of seven (2010). This may be due to the type of organic matter applied or the duration of the study as other studies have found that there is an initial increase in organic P, followed by a decline (Jones & Oburger, 2011). In addition, manure itself is high in organic P species like phytic acid, and its application increase their concentrations in arable soils (Dodd & Sharpley, 2015).

Unlike manure, inorganic P fertilizer was found to reduce soil organic P mineralization, possibly inactivating the soil phosphatase enzymes (Annaheim et al., 2015). Inorganic P applied to fields is also available to microbial species, which may immobilize this P into microbial P and release these organic P compounds into the soil environment through lysis or byproducts. Some studies have found increased microbial P with inorganic P fertilizers; soil incubation analyses by Haynes and Swift found that microbial P rose and remained between 30 and 45ppm for 16 weeks after application of 500mg P/g soil (1988). No-till management of fields, which builds organic matter, resulted in greater microbial activity and thus increased mineralization (Oberson et al. 2011). There has been evidence of crops utilizing organic P stocks to replenish the inorganic P

pool. In Mississippi, a study on a cotton plantation with sixty years of growth and no reported fertilizer application revealed that despite no change in inorganic P stores, the proportion of organic P was reduced (Sharpley & Smith, 1983; Dodd & Shapley, 2015). In regards to organic species, for arable fields, labile monoesters tend to be most common, followed by limited amounts of diesters. Stutter et al. (2015) determined in a review of 32 temperate soils that though inorganic P vastly outnumbered organic P species with 276 – 2,520mg P/kg, labile phosphomonoesters existed in sizeable amounts (105 – 446mg P/kg) while phosphodiesters were only 0 – 50mg P/kg. A study by Annaheim et al. (2015) determined that phytate was the most common organic P class in a Swiss agricultural soil, with as much as 61mg P/kg. Research by Sundareshwar and colleagues noticed the stark difference between natural habitats like wetlands to farmland in regards to organic P, with the former hosting a greater diversity compared to the latter; yet this study also found greater bacterial diversity in farmland. This may suggest that with each farm management disturbance, a *tabula rasa* is produced in the soil for all microbial species to pioneer prior to the eventual domination of a few (Sundareshwar et al., 2008). However, microbial activity in general is reduced heavily in cropped fields compared to natural ecosystems (Oberson et al. 2011).

2.5. Gaps in Research

Despite this research, there are still gaps in organic phosphorus research. Organic P is rarely studied below the topsoil, let alone organic P speciation. This is most likely because with increasing depth in the soil profile, there is a reduction in microbial activity and organic matter, especially in arable soils (Jones & Oburger, 2011; Lehmann & Kleber, 2015). Also, as P, including organic P, is quite reactive in the soil matrix, it will most likely remain near the soil surface (Thomas & Sevean, 1985; Doolette & Smernik, 2011). However, if a soil is P saturated,

this may remove binding fields and allow for P to permeate deeper into the soil horizons and stratify. In a study by Nemeth and researchers, inorganic P was determined to be seeping through the soil profile, as deep as 60cm (2012). As shown, in Figure 2.9, studies that have been conducted on organic P in the soil profile have shown a stark decrease in with depth (Anderson, 1980; Sanyal & De Datta, 1991). In addition, very little research has been conducted on organic phosphorus speciation in organic soils, like Histosols. The studies that have been conducted mainly focus on total organic P. In order to be classified as an organic soil, soil organic matter must range between 20 and 30% with a minimum depth of 30cm (Tate, 1980; Jones & Oburger, 2011). Originally, organic soils were wetlands containing slowly decaying plant and animal matter, and must be drained prior to agricultural use (Tate, 1980; McCray et al., 2012). In uncultivated organic peat soils, up to 80% of P can be organic (Thomas & Sevean, 1985). Research on agricultural pocosins, similar to Histosols, has found that there is a high degree of microbial mineralization when compared to unfertilized, natural pocosins (Walbridge & Richardson, 1991). Studies in general have found organic matter content to positively correlate with organic P concentration (Young et al., 2013). The most intensive agricultural activities on Histosols have occurred near the Everglades, Florida, USA, having been drained and cultivated for over a hundred years (Castillo & Wright, 2008). The soils were intensively fertilized with up to 150kg P/ha for vegetable crop production (Castillo & Wright, 2008). In this area, 40-90% of total P is organic, most of which is hypothesized to be labile (Ivanhoff et al., 1998). In a study by Castillo & Wright in the Everglades, the proportion of organic P was less in a cultivated Histosol with 52% of total P, compared to a pasture Histosol with 78% (Castillo & Wright, 2008; Lehmann & Kleber, 2015). This may be due to the oxidation of organic matter and subsequent organic P mineralization common in cultivated Histosols.

In addition, organic P speciation has not been studied in regards to water table management. As agricultural Histosols are often flooded, tile drainage systems made up of a series of clay, concrete, or corrugated plastic pipes are installed below the soil surface to convey water to an outlet ditch, mitigating soil saturation (Hebraud, 2006; Castillo & Wright, 2008; McCray et al., 2012). However, water table control structures, placed before the outlet ditch, may be added to manipulate water table depth (Hebraud, 2006). Subsequently, benefits of water table management have been ease of planting and harvesting, hydration of the root zone, increased nutrient uptake by crop, and greater crop yield (Williams et al., 2015). Water table management has also been found to improve water quality in certain scenarios. In Sweden, Wesström and Messing determined that dissolved P from tile drainage outlets was reduced by between 58 and 95% with tile drainage management (2007; Williams et al., 2015). Similarly, Feser et al. (2010) observed a decrease of 63% in P loading with water table management (Williams et al., 2015). However, results have been mixed, as other studies such as by Stämpfli & Madramootoo (2004) determined a two-fold increase in P concentrations of drainage from water table management fields compared to free drainage fields. These varied results may be due to soil quality, climate, or other factors and require further research. However, there is a dearth of studies on water table management's impact on organic P. As water table management can create saturated conditions, this may result in greater concentrations of organic P species and total organic P in general, as found in poorly drained soils (Young et al., 2013). Yet, those organic P species may have greater solubility if complexed to minerals, due to reduced conditions (Williams et al., 2015).

2.6. Summary

As can be seen from this review, eutrophication from P loading is a serious environmental issue that affects numerous freshwater bodies around the world. To, mitigate

agricultural P pollution, various BMPs have been utilized by farmers, yet numerous freshwater bodies continue to endure excessive algal growth and diminished dissolved oxygen levels. Though complex, a thorough understanding of the geochemical and biological components of the agricultural P cycle allows for insight into their various elements and processes which might be overlooked in their contribution to agricultural P loading. Labile organic P compounds are often disregarded in agricultural P studies, despite their importance as nutrients to crops, potential agents in eutrophication, and a parameter for microbial activity. The current body of literature has exhibited that farm management practices such as type of fertilizer applied, tillage, and cultivation in general can significantly alter organic P compound composition as well as their transport. However, there are significant gaps in the literature such as stratification of organic P compounds in the soil profile, organic P compound composition in Histosols, and impact of water table control structures on the presence of organic P compounds.

Consequently, this study sought to (1) compare the concentrations and lability of inorganic P, total organic P, and three classes of organic P compounds (labile phosphomonoesters [LM], phospholipids [Ph], and phosphodiesters [PD]) in three arable Histosol fields and their dynamics over time, (2) determine the concentrations and lability of inorganic P, total organic P, and three classes of organic P compounds (LM, Ph, and PD) at three different depths of an arable Histosol field, (3) examine correlations between P forms and soil characteristics in agricultural Histosols, and (4) determine the concentrations and lability of inorganic P, total organic P, and three classes of organic P compounds (LM, Ph, and PD) in drainage effluent to assess the eutrophication potential of the fields.

To achieve these objectives, a sequential fractionation analysis was chosen for soil extraction. Most sequential fractionation procedures are based on the Hedley et al. (1982)

fractionation scheme where there are four sequential extracts which represent available P, moderately labile P adsorbed to surfaces, non-labile P complexed with aluminum (Al) or iron (Fe), and non-labile P precipitated with calcium (Ca) (He et al., 2008). This method attempts to better characterize abiotic lability of P in soils (Deluca et al. 2015). For the determination of the three labile organic P compound classes, enzyme hydrolysis was utilized. This is an effective and efficient method of analysis as enzymes specific to a class of organic P compound can hydrolyze this compound releasing inorganic P, which can then be quantified through colorimetry (Annaheim et al., 2013). The colorimetry method used to determine resulting inorganic P concentrations was the molybdate blue – ascorbic acid method. First developed by Murphy and Riley (1962), this analysis is less prone to error than other methods like stannous chloride (O'Halloran & Cade-Menun, 2008). The blue color that results from the reduction of the phosphoantimonylmolybdenum complex by ascorbic acid follows Beer's Law and correlates to inorganic P concentration (O'Halloran & Cade-Menun, 2008).

With the current body of literature and objectives of this study in mind, it is expected that this research will find large concentrations of labile organic P compounds due to microbial cycling inherent in Histosols. However, due to the history of mineral P fertilizer addition at these agricultural fields, inorganic P will be vastly greater than organic P content. In addition, it is expected that both inorganic and organic P species will exist mostly in the labile and moderately-labile fractions, due to lower content of minerals in Histosols. In addition, organic P compound will decrease drastically with depth of the soil profile, due to reduced microbial presence. Consequently, as it is hypothesized that organic P compounds will remain at the topsoil, it is assumed that water table control structures will not have a clear impact on their composition. As Histosols have low mineral concentrations and high organic matter composition, it is

hypothesized that there will be no correlation of P forms and minerals, but that there will be a relationship between P forms and total organic carbon (TOC). Lastly, due to the large application of fertilizer P at these fields, organic P compounds and inorganic P are expected to be present in high concentrations in tile drainage effluent.

3. Methodology

3.1. Field Description

All three fields are located within the Holland Marsh, Ontario, Canada. A Specialty Crop Area located between 44°01'18"N, 79°37'58"W and 44°06'37"N, 79°32'49"W, the Holland Marsh is one of the most productive, cultivated areas in Canada, producing a large portion of the vegetables supplied to the province of Ontario (Planscape, 2009; Google Earth, 2017). It's ~7,000 acres support close to 100 farms which grow mainly onions, carrots, lettuce, celery and Asian vegetables (Bartram et al., 2007; UoG, 2014). Overall, vegetable production is evaluated to be $29 million and gross domestic product of the Holland Marsh is within $35 to $58 million annually (Planscape, 2009). Soils in the Holland Marsh are a Muck soil type and subsequently classified as a Terric Humisol, which are mainly organic and drain poorly (OMAFRA, 2017). They are humic in texture and tend to have a pH between 5.6 and 7.4. Organic carbon content is ~20% and sand, silt, and clay texture are not applicable due to the organic nature of the soil (Agriculture and Agrifood Canada, 2013). Below this, the bedrock is mainly composed of limestone of the Middle Ordovician Simcoe Group and shale of the Upper Ordovician Blue Mountain Group (LSRCA, 2008). The Holland Marsh lies in a broad valley from Cook's Bay and extends southwest to Schomberg (Gerber et al., 2004). The West Holland and East Holland River collectively drain the watershed, approximately 586km^2 in area, into Cook's Bay of Lake Simcoe (Gerber et al., 2004; Planscape, 2009). Though historical weather data is not robust for

this region, average mean temperature for autumn, winter, spring, and summer were 8.4, -5.9, 5.8 and 18.9, respectively, based on the Bradford Muck Research weather station for the period 1974-1998 (Environment Canada, 2017). Average mean snowfall for the period 1974-1998 in autumn, winter spring and summer was 4.2cm, 28.7cm, 8.0cm, and 0cm, respectively, while average precipitation in autumn, winter, spring, and summer was 68.5mm, 21.4mm, 54.2mm, and 83.3mm (Environment Canada, 2017). More recent temperature and precipitation data was provided with the weather station from this study (see Section 4.1.)

The three fields studied are all tile-drained carrot farms. Field A is an agricultural research facility while Field B and C are both commercial. Field A is a small field with a total area of 0.62ha and lies above twelve tiles each with a 150mm diameter. Field B is ~4.05ha with nineteen 100mm diameter tiles, drained by a 150mm diameter collector. Field C is a ~6.07ha farm with 100mm diameter tile lines that are drained by a 150mm diameter collector into a sump. In order to drain this farm, water is pumped from the sump into outlet ditch. Both Field A and B had a drainage control structure installed, which could be set to different water table levels based on a series of gates. An example of a drainage control structure is shown in Figure 3.1. Weather data was collected from an on-field weather station consisting a 0.2mm tipping bucket to measure rainfall and a HC2-S3-L Probe (Campbell Scientific Canada; Edmonton, AB T5L 4X4) to measure air shade temperature.

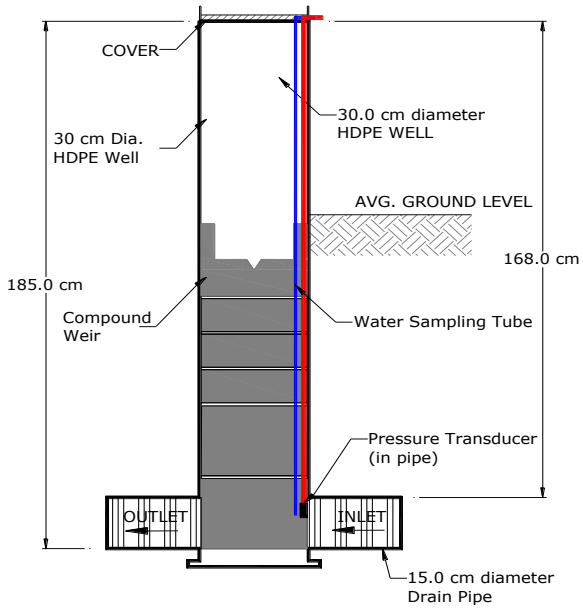

Figure 3.1: Example of a drainage control structure installed in the field.

In order to determine general soil qualities of the fields, two composite soil samples from 0-20cm depth of each field were sent for analysis to Agri Direct (2350, Chemin du Lac, Longueuil, QC J4N 1G8) and were analyzed on 26/11/2015. Determination of cation exchange activity (CEC) was by estimation; pH by pH meter; potassium (K), zinc (Zn), copper (Cu), manganese (Mn), and boron (B) by Mehlich III extraction and inductively coupled plasma optical emission spectrometry (ICP-OES); and organic matter by combustion.

3.2. Sample Collection

3.2.1. Soil

Soil samples were collected on dates to represent three agricultural periods: 3/5/2016 for pre-fertilizer (PF), 11/7/2016 for growing season (GS), and 29/10/16 for post-harvest (PH). For Field B, 30 soil cores were randomly collected in a zig-zag pattern from the 0-20cm, 20-40cm, and 40-60cm depth and removed into a bucket. For Field C, the same soil sampling method was used but 30 soil cores from only the 0-20cm depth. Lastly, for Field A, 20 soil cores were collected at the 0-20cm depth due to the smaller size of the field. Once placed in their respective

bucket, the soil cores were thoroughly mixed in order to achieve an average sample from the field. Once mixed, two Ziploc bags were filled with enough soil for analysis, and then placed in a cooler filled with ice in order to prevent microbial transformations. As soon as samples were brought to the laboratory they were stored in a 4°C refrigerator till analysis.

3.2.2. Drainage

For drainage effluent sample collection, the ISCO 6172 automatic sampler (Teledyne ISCO, Inc.; Lincoln, Nebraska 68504 USA) installed in each field would retrieve the sample from the effluent when the water table reached 34cm below the surface. A CR23X Micrologger (Campbell Scientific Canada; Edmonton, AB T5L 4X4) stored sample collection information. The sample was then stored in a fridge until analysis (4°C). In addition, manual samples were taken in case the water table threshold was not met. Water samples chosen for this analysis were those on or near the date of soil sample collection: 3/5/16, 15/7/16, and 1/11/16.

3.3. Lab Analysis

3.3.1. Sample Preparation

This study chose the fraction scheme as used by Zhu and colleagues, a modification of Hedley et al. (1982) where samples are extracted sequentially with deionized water (DH_2O), 0.5M sodium bicarbonate ($NaHCO_3$) with pH adjusted to 8.5, 0.1M sodium hydroxide (NaOH) and 1M hydrochloric acid (HCl) to represent available, moderately labile, non-labile Al or Fe bound, and non-labile Ca bound P, respectively (2013).

Prior to extraction, soil samples were air-dried for 72 hours. After the drying period, soils were ground and sieved with a 2mm mesh. Soils were then oven dried for 48 hours at 60°C. Centrifuge tubes were weighed and then labeled appropriately so that there were three experimental replicates per each soil sample as well as five blanks. A 2.0g oven-dried soil

sample was then extracted with 40mL respective solvent, shaken in an end-to-end shaker for 30min. After being shaken, extracts were centrifuged at 4,000rpm for 15min with Sorvall RC-SB Refrigerated Superspeed Centrifuge (DuPont Instruments; Thetford Mines, QC G6G 5T1). Centrifuged samples were vacuum-filtered with DUOSEAL Vacuum Pump (Welch; Fürstenfeldbruck, Germany 82256) and a 0.45µm filter (Millipore Canada Ltd.; Etobicoke, ON M9W 6Y1). The extraction steps were adapted from (O'Halloran & Cade-Menun, 2008). After filtration, both filtrate and residues were refrigerated in 4°C until required for further analysis. When ready to proceed with the next sequential fractionation extract, soil residues were dried again for 48 hours at 60°C and the extraction steps were redone in the same manner. The only exception was after both the 0.5M $NaHCO_3$ and 0.1M NaOH extraction, 5mL of DH_2O was added to each soil residue and centrifuged at 4,000rpm for 15min in order to remove any solvent matrix contamination (Zhu et al. 2013). 0.5M $NaHCO_3$ extracts were acidified with 1M HCl to remove carbonates which interfere with analysis (O'Halloran & Cade-Menun, 2008; Zhu et al. 2013). In addition, 0.5M $NaHCO_3$, 0.1M NaOH, and 1M HCl extracts were pH-adjusted to 7 prior to analysis. For 1M HCl extracts, prior to this step, 10mL of 0.1M sodium acetate-acetic acid buffer (NaAc-HAc) with a pH of 5.15 were added to 5mL of 1M HCl extract to prevent precipitate formation in the extract.

Drainage samples required less preparation and were only vacuum-filtered with DUOSEAL Vacuum Pump (Welch; Fürstenfeldbruck, Germany 82256) and a 0.45µm filter (Millipore Canada Ltd.; Etobicoke, ON M9W 6Y1).

3.3.2. Enzyme Hydrolysis Procedure

In order to determine organic P species like labile phosphomonoesters (LM), phospholipids (Ph) and phosphodiesters (Pd), the enzyme hydrolysis procedure was utilized. A

combination of methods utilized by O'Halloran & Cade-Menun (2008) and Zhu et al. (2013) were adapted. For this study, LM was hydrolyzed by 1unit/mL alkaline phosphatase from bovine intestine (AP), Ph was hydrolyzed by a combination of 1unit/mL phospholipase C from *C. perfringens* and 1unit/mL alkaline phosphatase from bovine intestine (PC), and Pd,hydrolyzed by 0.02unit/mL phosphodiesterase from *C. atrox* and and 1u/mL alkaline phosphatase from bovine intestine (PDE). One unit is equal to the liberation of 1μmol/min of product at optimal conditions (Sigma Aldrich, 2017). Alkaline phosphatase was added to phospholipase C and phosphodiesterase, as both only cleave one ester bond in a diester compound; in order to detect released inorganic P, alkaline phosphatase must be added (O'Halloran & Cade-Menun, 2008). All enzymes were sourced from Sigma Aldrich (Oakville, ON L6H 6J8).

Enzyme solutions were made to concentration (unit/mL) in 0.1M Tris HCl. Both AP and PDE were adjusted to pH 9 while PC was adjusted to pH 8. In addition, 2mM $MgCl_2$ was added to the enzyme solutions as Mg is an activator of phosphatase enzymes (Turner et al., 2002). Enzyme solutions were transferred to 1.5mL Eppendorf microcentrifuge tubes (Eppendorf; Mississauga, ON L5N 8L2) and frozen (-20°C) to preserve the solution (Jarosch et al., 2015). The combination of O'Halloran & Cade-Menun (2008) and Zhu et al. (2013) was scaled down to a microplate scale analysis for increased efficiency and optimize enzyme solution use (Jarosch et al. 2015). In order to determine optimum volumes of enzyme to add to extract, model organic P substrates specific to respective enzyme were made to concentrations of 5ppm, 10ppm, 15ppm, 20ppm, and 25ppm. Adenosine 5' monophosphate (AMP) was utilized for AP, L-α-phosphatidylcholine (LaP) was utilized for AP+PC, and deoxyribonucleic acid (DNA) for AP+PDE. All model organic P substrates were acquired from Sigma Aldrich (Sigma Aldrich, Oakville, ON L6H 6J8), besides DNA which was acquired from Fisher Scientific (Fisher

Scientific; Ottawa, ON K2E 7L6). Enzyme solutions were tested in increments of 5µL up to 40µL on model substrate. This assay was conducted with Eppendorf multichannel pipettes (Eppendorf; Mississauga, ON L5N 8L2). with fresh pipette tips for every well and Corning CoStar microplates (Corning; Corning, NY 14831). The following order was followed in the addition of solutions for the volume optimization assay:

- DH_2O: -
- NaN_3: 29µL
- Substrate: 75µL
- Enzyme: 5µL, 10µL, 15µL, 20µL, 25µL, 30µL, 35µL, 40µL

DH_2O volume was added first but calculated by: 150µL – 29µL - 75µL – Enzyme volume (µL). Three replicates were made for each substrate+enzyme combination and the plate was vortexed for 1min with a microplate foam attachment on a Fisher Scientific Vortex Mixer (Fisher Scientific; Ottawa, ON K2E 7L6). The microplate was then sealed with its lid and Parafilm (Bernis Company, Inc.; Oshkosh, WI 54904) along the edge to prevent evaporation and incubated for 16hr at 37°C in a Johns Scientific Incubator Model 1545 (Johns Scientific; Toronto, ON M4C 1A7) (Zhu et al., 2013). After this, the Watanabe & Olsen (1965) molybdate blue-ascorbic acid method was utilized to determine inorganic P present, adding 24µL of the color developing solution. The acidity of the color developing solution prevents further hydrolysis. The plate was vortexed for 1min and after 12min, the plate was read in a µQuant microplate reader (BioTek Canada Industries; Winooski, VT 05404) at 712nm (O'Halloran & Cade-Menun, 2008). Concentrations were determined based on inorganic P standard curves.

From this analysis, it was determined that optimum volumes for organic P hydrolysis were 30µL for AP, 35µL for PC, and 10µL for PDE. As such, the following order of solution additions was followed for experimental analyses of extracts, standards, and model substrates:

- AP:
  - DH_2O: 16µL
  - NaN_3: 29µL
  - Extract/Standard/Substrate (1ppm): 75µL
  - Enzyme: 30µL

- PC:
  - DH_2O: 11µL
  - NaN_3: 29µL
  - Extract/Standard/Substrate (1ppm): 75µL
  - Enzyme: 35µL
- PD:
  - DH_2O: 36µL
  - NaN_3: 29µL
  - Extract/Standard/Substrate (1ppm): 75µL
  - Enzyme: 10µL

- Control:
  - DH_2O: 36µL
  - NaN_3: 29µL
  - Extract/Standard/Substrate (1ppm): 75µL
  - $MgCl_2$: 10µL

Prior to experimental analysis, all extracts and standards were diluted by a factor of four in DH_2O and pH adjusted with *p*-nitrophenol. pH is crucial for the proper analysis of P. For analysis of extracts, four analytical replicates were utilized to increase precision (Jarosch et al., 2015). Microplates were also placed on ice to prevent early hydrolysis of organic P forms before incubation (Jarosch et al., 2015). Sodium azide (NaN_3) is included in the assay to kill any live microbes in the extract that may interfere with the enzyme hydrolysis (O'Halloran & Cade-Menun 2008). A control (2mM $MgCl_2$) is also included to account for any hydrolysis of organic P that is not associated with enzymes. The experimental analysis follows the exact same procedure of vortexing and incubation. 24µL of the color developing solution (CD) is added and vortexed, and after 12 minutes the experimental plate is read at 712nm, selected due to the high organic matter associated with the extracts of this study (O'Halloran & Cade-Menun, 2008).

Inorganic P standards were included on every plate to mitigate any inter-plate differences. In addition, 1ppm model organic P substrate were present on each plate for their respective enzyme to ensure the enzyme was functioning properly (Jarosch et al. 2015). To determine organic P classes LM, Ph, and Pd:

$$LM = Hydrolyzed\ by\ AP - Control \quad (1)$$

$$Ph = Hydrolyzed\ by\ PC - Hydrolyzed\ by\ AP - Control \quad (2)$$

$$Pd = Hydrolyzed\ by\ PDE - Hydrolyzed\ by\ AP - Control \quad (3)$$

If more than one replicate was zero or negative for both analytical and experimental replicates, the entire value was set to zero.

3.3.3. Inorganic and Organic P Determination

The Watanabe & Olsen (1965) version of Murphy and Riley's (1962) molybdate blue-ascorbic acid method was also utilized to determine both inorganic and organic P of soil extracts and drainage. As this reaction is pH dependent, proper pH adjustment, such as with *p*-nitrophenol above, is crucial. However, it must be noted that though inorganic P is defined as molybdate reactive P (MRP), this term is operational as it includes poly-inorganic acids hydrolyzed by the acidic conditions of the assay (Turner et al., 2002). As with enzyme hydrolysis, all extracts and standards were diluted by a factor of four in DH_2O and pH adjusted with *p*-nitrophenol prior to analysis. Drainage effluent was just pH adjusted with *p*-nitrophenol as it was already dilute in concentration. The microplate procedure to determine MRP is:

- DH_2O: 83.6µL
- Sample/Standard: 76µL
- CD: 30.4µL

Plates were then vortexed for 1min, and after 12 minutes were read in the microplate reader at 712nm.

In order to determine organic P, one must determine total P (TP). Extracts and drainage effluent were first digested with an oxidizing solution of 0.74M $K_2S_2O_8$-0.75M NaOH at a 1:1 ratio in digestion tubes (Ebina et al., 1983). An organic P model substrate was also included to ensure digestion was complete. Tubes were sealed tightly and vortexed to ensure thorough mixing, and then autoclaved for 30min at 120°C. After digestion, centrifuge tubes were vortexed once more and were diluted by a factor of four in DH_2O to pH adjust with *p*-nitrophenol prior to analysis. The microplate procedure to determine TP is:

- DH_2O: 83.6µL
- Sample/Standard: 76µL
- CD: 30.4µL

Plates were then vortexed for 1min, and after 12 minutes were read in the microplate reader at 712nm. Then organic P, or known operationally as molybdate unreactive P (MUP) can be determined by:

$$MUP = TP - MRP \qquad (4)$$

As with MRP, MUP is an operational term to recognize the error that may be associated with the value though it largely represents P (Turner et al., 2002). Unhydrolyzable organic P (UHP) can also be determined by:

$$UHP = MUP - LM - Ph - Pd \qquad (5)$$

All standards analyzed concurrent with an extract or drainage sample were made of the same solvent to ensure accuracy. In addition, when extracts were too concentrated several dilutions occurred. All extracts were diluted with DH_2O, besides HCl extracts which were diluted with 0.1NaAc-HAc buffer (pH 5.15). As mentioned previously, due to the dilute nature of the drainage effluent, there was no need to dilute.

3.3.4. Mineral Analysis

In order to determine the mineral pool of the soil samples, fresh sample were extracted with 1M HCl as in *3.3.3.1*. As such, they were not sequentially fractionated; this was done to determine the largest pool of aluminum (Al), calcium (Ca), iron (Fe), and magnesium (Mg) present in the soil. Al, Ca, Fe, and Mg in 1M HCl extracts were determined by flame spectrometry (Varian 220FS; Palo Alto, CA 94304) with three replicates. Standards for these analyses were prepared in 1M HCl as well. Matrix modifiers (CsCl-$LaCl_3$) were added for Ca and Mg determination.

3.3.5. Total Organic Carbon

Total organic carbon (TOC) was determined in the same sequentially fractionated extracts utilized in the enzyme hydrolysis and inorganic and organic P determination. Appropriate dilutions were made in order to achieve robust analysis. TOC analysis was conducted by a General Electric Sievers InnovOx Laboratory TOC analyzer Autosampler (General Electric, Boulder, CO 80301)with three replicates of every sample.

3.4. Statistical Analysis

To determine significant differences in fields, seasons, and their interaction for the various P forms, as well as depths, seasons, and their interaction, a multivariate general linear model was used (ANOVA). Significant differences were determined at $p= 0.05$. If the interaction was not significant, the model was rerun for just the two factors. When there was a significant difference determined, a Student t's test was utilized to compare least square means of the fields, seasons, or depths. Normality was determined for all parameters before analysis; if not normal, the log (x+1) was taken. The reason for the (x+1) was that there were frequent zeros values in data. Correlation analyses were also conducted between parameters and mineral forms, as well as

TOC. If one parameter was not normal, the Spearman correlation coefficient was observed. JMP 13 software (JMP; Cary, NC 27513) was utilized to conduct these analyses.

4. Results

4.1. Climate Data

4.1.1. Rainfall

Daily precipitation for 2016 in the Holland Marsh, as determined by the on-field weather station, is presented in Figure 4.1, below. Overall, 2016 was dry where average total rainfall was 1.5mm and average snowfall (01/01/2016 – 10/04/2016) was 0.6cm. Pluviosity was much less than the 30-year monthly averages. The greatest precipitation event occurred on 07/09/2016.

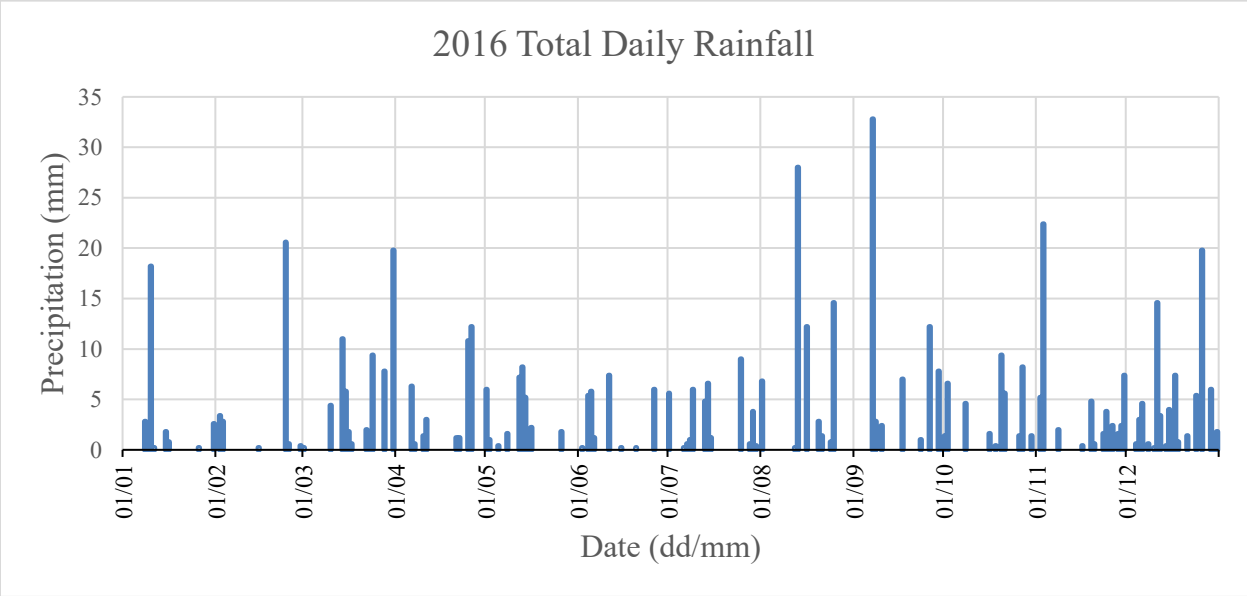

Figure 4.1: Daily total rainfall (mm) for 2016 in Holland Marsh as determined by an on-field weather station.

4.1.2. Air Temperature

Daily air temperature recorded by the on-field weather station for 2016 is displayed in Figure 4.2. The mean maximum temperature was 13.9°C while the mean minimum temperature was 3.2°C. The greatest maximum was on 13/07/16 with 33.3°C while the lowest maximum was

-17.2°C on 13/02/16. The greatest minimum was 22.5°C on 12/08/16, whereas the lowest minimum temperature was -29.0°C on 14/02/16.

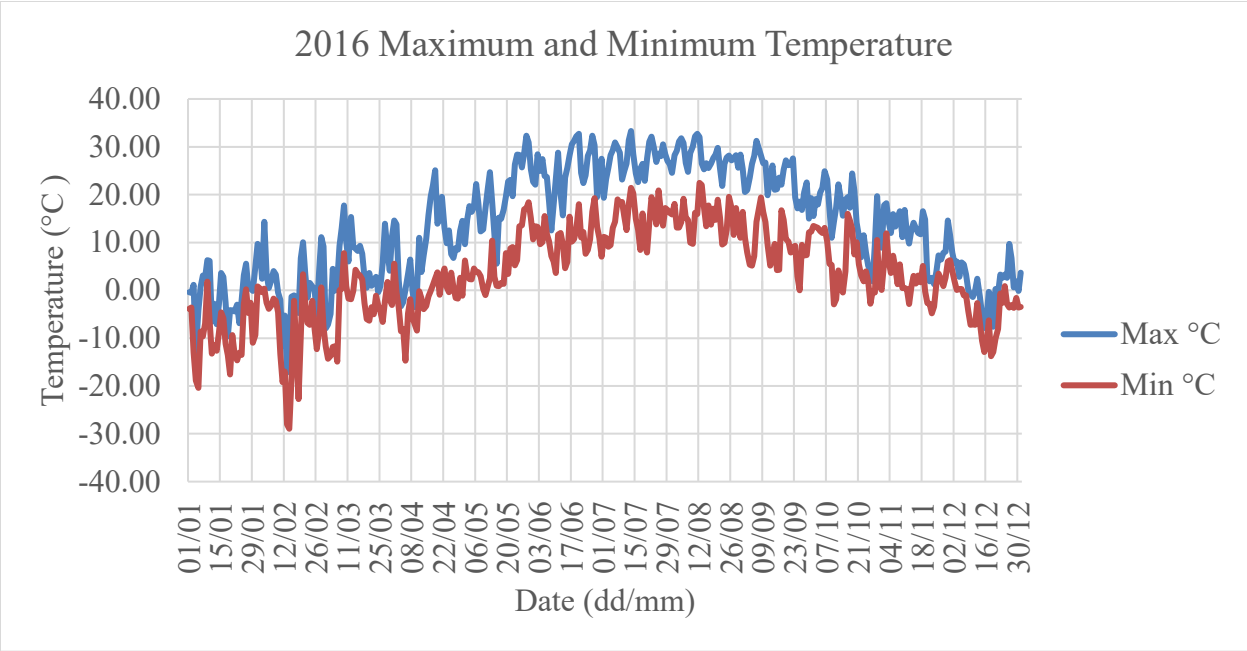

Figure 4.2. Daily maximum and minimum temperature (°C) for 2016 in the Holland Marsh as determined by an on-field weather station.

4.2. Farm Management

All three fields were fertilized as detailed in Table 4.1 for 2015 and 2016. Year 2015 was included to show fertilization history of each field. Field C was the only field to apply an additional side dress. All bulk applications were applied before seeding the fields while the side dress for Field C was applied during the growing season.

Drainage of Field A and B was accomplished with the drainage control structure. However, in Field A there was high soil porosity which led to lateral seepage instead of maintaining a water table in the field. With drainage control, Field B was able to have a water table maintained at 30-40cm below the soil surface, just under the root zone of the crop. Field C did not possess a drainage control structure and therefore water was pumped from the field to a ditch outside the farm.

Sprinkler irrigation was utilized in each field. For Field B, irrigation occurred on August 6th with 25mm of water applied and again on August 29th with 32mm applied. Unfortunately, Field A and C were irrigated on an as-needed basis without accurate recordings.

Marketable carrot yields for each field are presented in Table 4.2. All fields had considerably large yields that were greater than the Ontario average. Field A had the greatest yield with 73,072kg/ha, followed by Field B with 61,417kg/ha and Field C with 59,167kg/ha.

Table 4.1: Fertilization application rates for Field A, B, and C for both 2015 and 2016.

Fertilizer Application	
Field A	
2015	Bulk application of 40kg/ha N; 75kg/ha P; 150kg/ha K; 35kg/ha Mn; and 3.5kg/ha B
2016	Bulk Application of 40kg/ha N; 50kg/ha P; 100kg/ha K; 35kg/ha Mn; and 3.5kg/ha B
Field B	
2015	Bulk application of 10kg/ha N; 45kg/ha P; and 255kg/ha K
2016	Bulk Application of 5kg/ha N; 25kg/ha P; 135kg/ha K; 20 kg/ha S; 90kg/ha Mg; and 1kg/ha B
Field C	
2015	Bulk Application of 30kg/ha N; 65kg/ha P; 250kg/ha Potash; 105kg/ha S; 35kg/ha Mg; 5kg/ha Mn and 3kg/ha B
	Side dress application of 10kg/ha N; 45kg/ha Potash; 40kg/ha S and 10kg/ha Mg
2016	Bulk Application of 35kg/ha N; 80kg/ha P; 290kg/ha Potash; 140kg/ha S; 25kg/ha Mg; 45kg/ha Ca and 5kg/ha Mn
	Side dress application of 10kg/ha N; 45kg/ha Potash; 45kg/ha S and 10kg/ha Mg

Table 4.2: Marketable carrot yields (kg/ha) for Field A, B, and C in 2016.

2016 Marketable Carrot Yields (kg/ha)			
Field A	Field B	Field C	Ontario Average
73,072	61,417	59,167	50,804

4.3. Soil Characteristics

Analysis results by Agri Direct in Longueuil, QC for general soil characteristics are displayed in Table 4.3. Typical of organic soils, the cation exchange capacity (CEC) was quite high. pH values for fields were mildly acidic, ranging from 5.9 to 6.6. Soils were rich in potassium (K), zinc (Zn), copper (Cu), boron (B), and organic material (OM). Field C was rich in manganese (Mn), whereas Field A and B were suitable in concentrations.

4.4. Enzyme Optimization Assays

Recoveries from various substrate concentrations during enzyme optimization assays are exhibited in Table 4.4. For alkaline phosphatase (AP), a volume of 30µL of 1unit/mL AP in 0.1M Tris-HCl (pH 9) was determined to be the most effective for hydrolysis of model substrate adenosine 5' monophosphate (AMP). Phospholipase C (PC) hydrolyzed the greatest amount of substrate L-α-phosphatidylcholine (LaP) with 35µL of 1unit/mL PC and 1unit/mL AP in 0.1M Tris-HCl (pH 8). Lastly, Phosphodiesterase (PDE) had an optimum hydrolysis of deoxyribonucleic acid (DNA) with 10µL of a mixture of 0.02unit/mL PD and 1unit/mL AP in 0.1M Tris-HCl (pH 9). AP was included in the PC and PDE mixtures so that AP could cleave the phosphomonoesters released by both enzymes. Thus, these respective volumes were utilized for enzyme hydrolysis assays of sample extracts.

Table 4.3: Mean values and respective standard deviations (σ) of soil characteristics determined by Agri Direct (Longueuil, Québec) for two experimental replicates of soil from 0-20cm depth in Field A, B, and C.

Field	CEC* (meq/100g)	σ	pH†	σ	K‡ (kg/ha)	σ	Zn‡ (ppm)	σ	Cu‡ (ppm)	σ	Mn‡ (ppm)	σ	B‡ (ppm)	σ	OM^ (%)	σ
A	53.3	2.3	5.9	0.0	999	34	19.7	0.7	38.64	1.10	7.5	0.2	7.6	0.1	81.2	0.2
B	59.3	1.1	6.6	0.0	694	12	29.8	0.5	34.72	0.71	5.6	0.4	9.7	0.3	67.7	1.6
C	54.5	2.1	6.2	0.1	892	31	33.2	3.3	55.88	7.79	14.2	0.6	11.5	0.6	77.7	0.0

* Cation exchange capacity (CEC) was determined by estimation.
† pH was determined by pH meter.
‡ Potassium (K), zinc (Zn), copper (Cu), manganese (Mn), and boron (B) were determined by Mehlich III extraction and inductively coupled plasma optical emission spectrometry (ICP-OES).
^ Organic matter (ON) was determined by combustion.

Table 4.4: Recovery of MRP from enzyme volume optimization assays with, enzyme alkaline phosphatase (AP) and substrate adenosine 5' monophosphate (AMP), enzyme phosphodiesterase (AP/PDE) and substrate deoxyribonucleic acid (DNA), and enzyme phospholipase C (AP/PC) and substrate L-α-phosphatidylcholine (LaP).

	Enzyme Hydrolysis Recovery		
Substrate (µg/mL)	30µL AP + AMP (%)	10µL AP/PD + DNA* (%)	35µL AP/PC + LaP* (%)
5	88	75	84
10	104	86	95
15	99	100	92
20	90	99	106
25	73	101	86

*AP was included in the PD and PC mixtures in order to cleave the phosphomonoesters released from PD and PC.

4.5. Comparison of P Forms Among Fields

4.5.1. Distribution of P in fractions

Distribution of molybdate reactive P (MRP) and molybdate unreactive P (MUP) among deionized water (DH_2O), 0.5M sodium bicarbonate ($NaHCO_3$), 0.1M sodium hydroxide (NaOH), and 1M hydrochloric acid (HCl) extracts for Field A, B, and C during pre-fertilizer (PF), growing season (GS), and post-harvest (PH) agricultural periods are exhibited in Figure 4.3. Across all fractions, MRP dominated for each field across all agricultural periods. DH_2O MRP ranged from 3 – 6% of total P for each field in PF and decreased to between 2% and 4% in the GS, which remained into PH. Field C had the overall largest percentage of DH_2O MRP while Field B had the lowest. Field A had the greatest decrease from 6% in PF to 2% in GS and PH. For all fields across all seasons, DH_2O MUP was virtually 0%. With the $NaHCO_3$ fractions, MRP was relatively the same for all fields ranging from 7 – 8%, decreasing to 4 – 5% for GS, and stayed at this level (5 – 6%) during PH. MUP of the $NaHCO_3$ for all fields during all seasons ranged from 0 – 1%. Though larger in proportion than DH_2O and $NaHCO_3$ MRP, NaOH MRP had a similar trend over seasons. Beginning between 22 and 37% in PF, NaOH MRP reduced to 10 – 22% in GS and remained at this proportion (11 – 21%) into PH. Field B had the largest proportion of NaOH MRP, followed by Field A, and then Field C. NaOH MUP existed in larger proportions compared to the DH_2O and $NaHCO_3$ MUP fractions, ranging from 7 – 10% in PF, 7 – 12% in GS, and 4 – 9% in PH. Levels remained similar across seasons for each field, except Field C which had 7% of its total P as NaOH MUP for PF and GS, but reduced to 4% in PH. Field A had the greatest amount of NaOH MUP (9 – 12%), followed by Field B (6 – 8%), and Field C (4 – 7%). By far, HCl MRP was the greatest fraction of total P for all fields and increased with each season. HCl MRP was 41 – 54% in PF, 53 – 66% in GS, and 63 – 74% in

PH. Of the fields, Field C had the greatest proportion (54 – 74%), while Field A and B had similar proportions with 42 – 64% and 41 – 63%, respectively. Lastly, fields experienced similar trends in HCl MUP, remaining low in PF (2 – 3%), rose in GS (6 – 10%), and reduced back to 2 – 3% in PH.

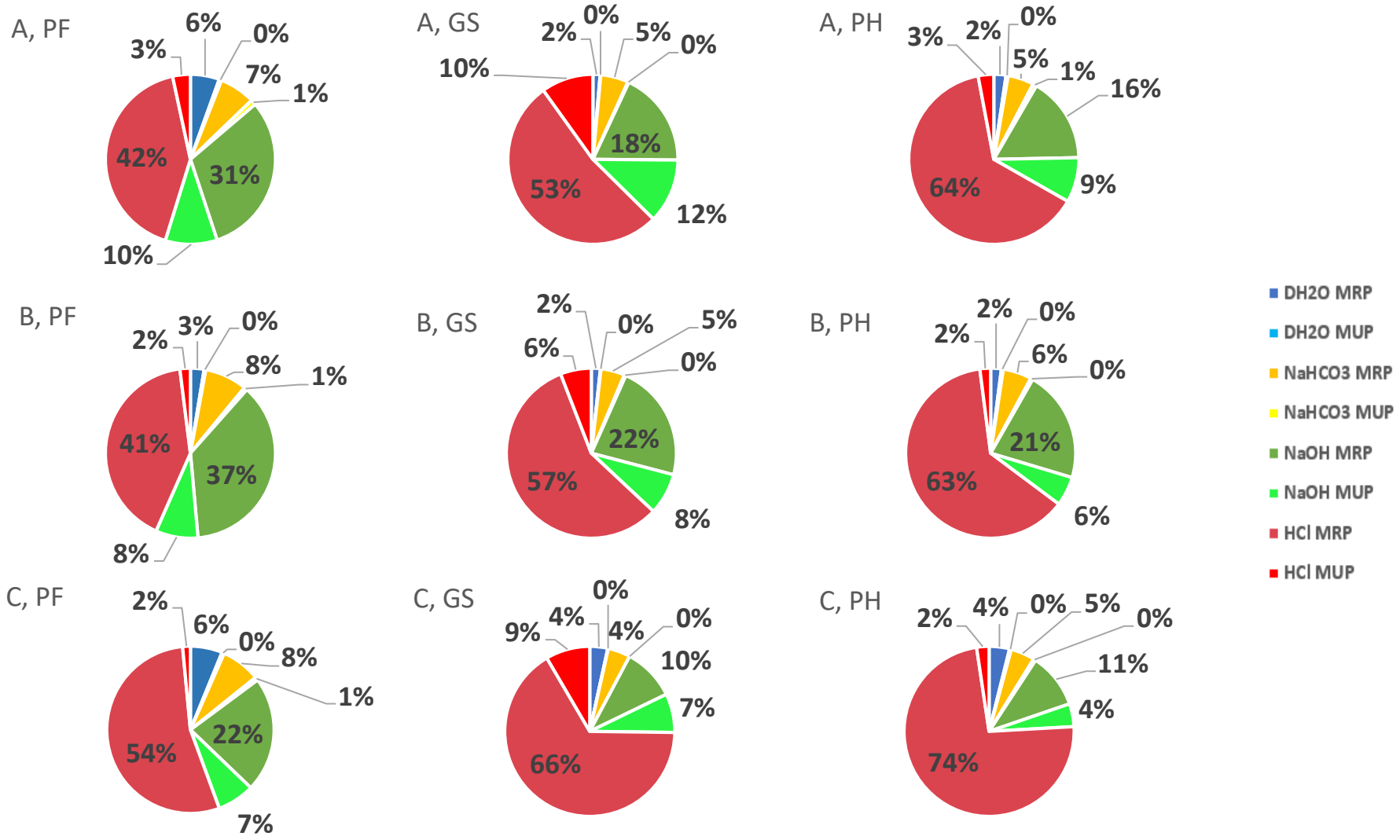

Figure 4.3: Distribution (%) of molybdate reactive P (MRP) and molybdate unreactive P (MUP) among deionized water (DH_2O), 0.5M sodium bicarbonate ($NaHCO_3$), 0.1M sodium hydroxide (NaOH), and 1M hydrochloric acid (HCl) extracts for Field A, B, and C during pre-fertilizer (PF), growing season (GS), and post-harvest (PH) agricultural periods.

4.5.2. Concentrations of P forms

4.5.2.1. DH_2O

Mean concentrations and standard deviations of total P (TP), molybdate reactive P (MRP), molybdate unreactive P (MUP), labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable P (UHP) for deionized water (DH_2O) soil extracts of fields across agricultural periods are presented in Table 4.5. For TP, field, season, and their interaction were determined to be significant. Overall, Field C was greatest with 83.12 – 93.67mg P/kg while Field B was lowest with 47.65 – 57.79mg P/kg. Field A was less stable in concentrations over seasons, beginning with 77.76mg P/kg in PF, dipping to 26.90mg P/kg in GS, and rising back to 50.32mg P/kg. With MRP, field, season, and their interaction were significant as well. Levels were similar to TP as MRP made much of TP. Field C remained statistically similar across all seasons, while Field B was mostly similar over time. Field A like in TP, fluctuated between seasons, dipping in GS. Statistical analysis determined that season was a significant main effect for MUP. For all fields, MUP dipped in GS and rose again in PH. These trends of MRP and MUP for fields in DH_2O extracts over agricultural periods can be seen in Figure 4.4.

In regards to organic P characterization, organic P species existed in minute concentrations with large standard deviations, and were quite variable. In certain scenarios, organic P species may have been over-predicted, as some species had larger concentrations than MUP determined. Enzyme assays on organic P model substrates at 1µg/mL supported this. There were no significant effects determined for LM between fields over time. LM concentrations ranged between 0.26 and 2.58mg P/kg for all three fields over the three agricultural periods. Only season had a significant effect on Ph where there was an absence of any Ph in PF, but then

concentrations determined in GS (2.79 – 9.73mg P/kg) and PH (8.71 – 10.48mg P/kg). In DH_2O extracts, there were no concentrations of Pd found for all fields over the agricultural periods. With UHP, there was a significant effect of season, where concentrations ranged between 3.53 and 3.72mg P/kg in PF, diminished to 0mg P/kg in GS, and remained as such in PH besides for Field B which had a concentration of 0.73mg P/kg. Organic P species in DH_2O extracts for fields over the agricultural periods are exhibited in Figure 4.5.

Table 4.5: Mean concentration values and their respective standard deviation (σ) for total phosphorus (TP), molybdate reactive phosphorus (MRP), molybdate unreactive phosphorus (MUP), labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable organic phosphorus (UHP) determined in deionized water (DH_2O) soil extracts of fields for the pre-fertilizer (PF), growing (GS), and post-harvest (PH) agricultural periods. Field A, B, and C were statistically compared through a GLM ANOVA ($p < 0.05$) for field and season main effects, as well as their interaction. If more than one replicate was zero or negative for both analytical and experimental replicates, the entire value was set to zero. If MUP was zero, all organophosphorus classes (LM, Ph, Pd, and UHP) were set to zero, regardless of experimental results.

DH_2O - Fields

Season	Field	TP (mg/kg)	σ	MRP (mg/kg)	σ	MUP (mg/kg)	σ	LM (mg/kg)	σ	Ph (mg/kg)	σ	Pd (mg/kg)	σ	UHP (mg/kg)	σ
PF	**A**	77.76 [c]	1.79	71.63 [b]	2.35	6.13	2.02	2.56	0.59	0.00	0.00	0.00	0.00	3.57	1.63
	B	52.70 [e]	1.65	46.94 [c,d]	0.57	5.76	1.28	2.04	2.43	0.00	0.00	0.00	0.00	3.72	2.56
	C	87.54 [a,b]	0.68	81.60 [a]	0.47	5.94	0.85	2.41	2.29	0.00	0.00	0.00	0.00	3.53	1.61
GS	**A**	26.90 [g]	2.51	24.62 [f]	1.76	2.28	2.10	0.26	0.23	7.12*	6.69	0.00	0.00	0.00	0.00
	B	47.65 [f]	0.53	44.65 [d,e]	1.51	3.00	1.66	1.60	0.36	9.73*	8.50	0.00	0.00	0.00	0.00
	C	83.12 [b]	8.55	80.77 [a]	7.87	2.35	1.35	2.58*	1.27	2.79*	1.65	0.00	0.00	0.00	0.00
PH	**A**	50.32 [e,f]	0.62	42.90 [e]	0.46	7.42	0.32	1.78	0.71	8.71*	2.54	0.00	0.00	0.00	0.00
	B	57.79 [d]	0.82	49.13 [c]	0.36	8.66	0.50	0.84	0.84	8.74*	3.56	0.00	0.00	0.73	0.63
	C	93.67 [a]	1.35	86.12 [a]	0.88	7.55	0.64	2.58	1.83	10.48*	9.11	0.00	0.00	0.00	0.00

* enzyme hydrolysis would intermittently overestimate organophosphorus classes, possibly due to the overall dilute concentrations of organic phosphorus present in the soil samples. This was supported by enzyme hydrolysis assays on organic phosphorus substrates at 1µg/mL concentration.

[a-h] field concentrations within a column labeled with a different letter superscript represent a significant difference between the values based on a Student's t test when the field*season interaction was significant ($p < 0.05$)

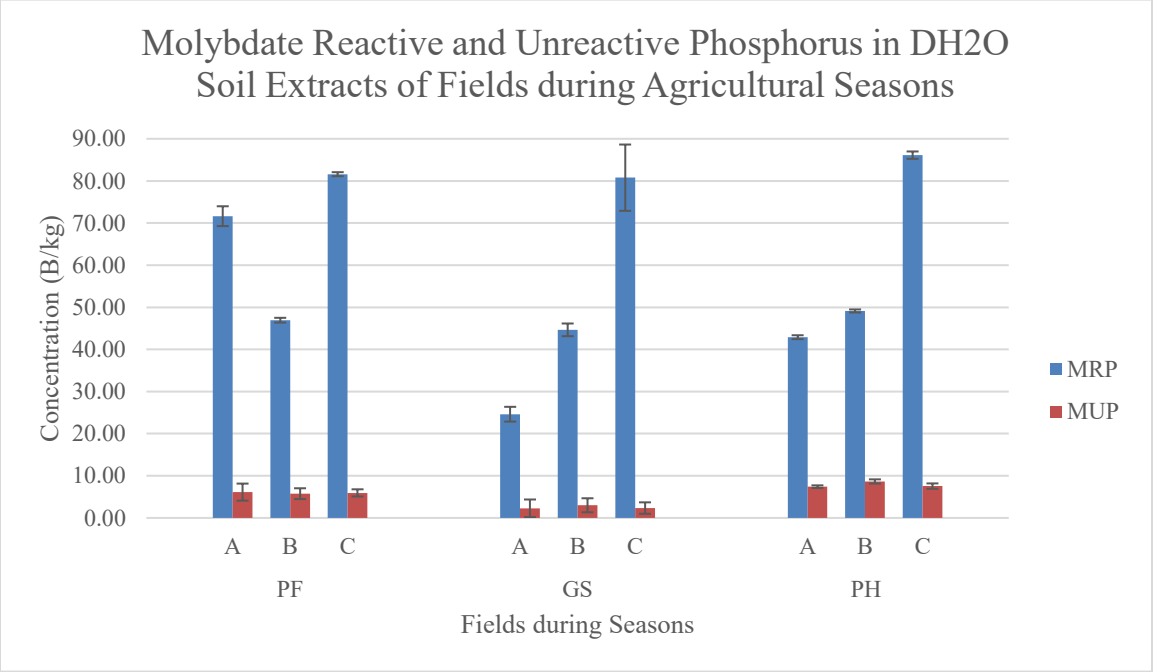

Figure 4.4: Mean concentrations of molybdate reactive P (MRP) and molybdate unreactive P (MUP) in deionized water (DH_2O) soil extracts from Field A, B, and C over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

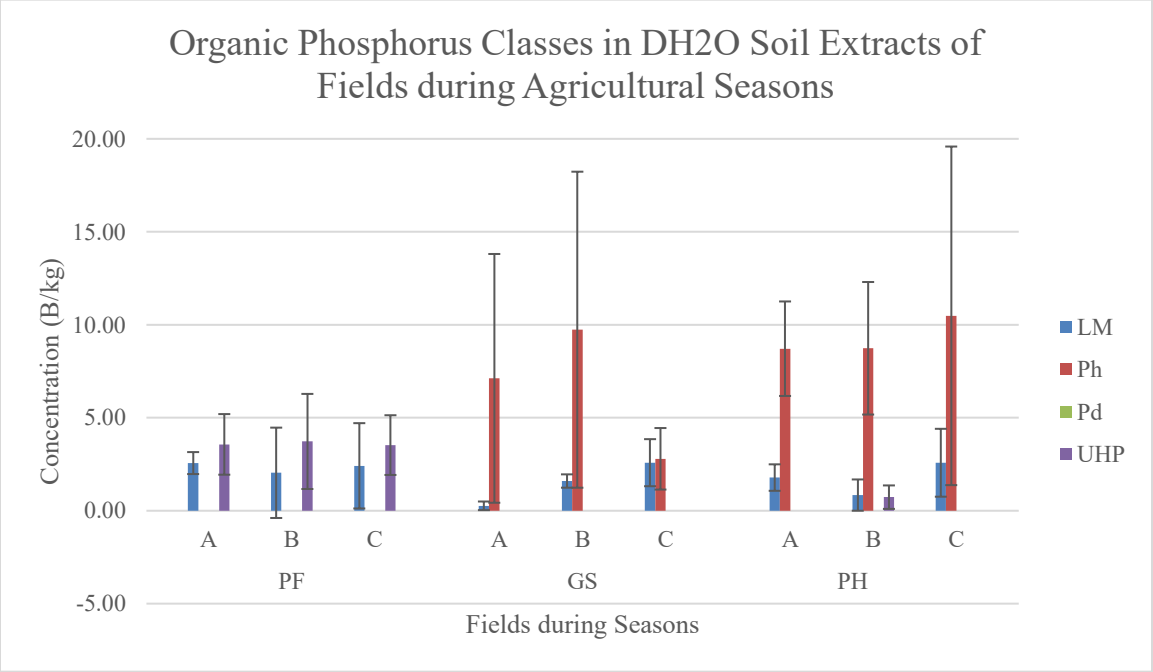

Figure 4.5: Mean concentration of organic P species [labile phosphomonoesters (LM), phospholipids (PC), phosphodiesters (PD) and unhydrolyzable P(UHP)] in deionized water (DH_2O) soil extracts from Field A, B, and C over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

4.5.2.2. $NaHCO_3$

For 0.5M sodium bicarbonate ($NaHCO_3$) extracts, mean concentrations and standard deviations for TP, MRP, MUP, LM, Ph, Pd, and UHP of fields across the PF, GS, and PH agricultural periods are shown in Table 4.6. Overall, concentrations of TP were larger in $NaHCO_3$ extracts than in DH_2O. There was a significant effect of both field and season, but not their interaction. Field B was significantly greater in concentration ranging 125.69 – 156.75mg P/kg over the agricultural periods, compared to Field A (98.47 – 104.88mg P/kg) and Field C (100.93 – 111.24mg P/kg). Unlike DH_2O extracts, concentrations of TP were determined to be significantly reduced in the GS with 98.47 – 125.69mg P/kg, whereas PF and PH were between 101.87 – 156.75mg P/kg and 104.88 – 142.48mg P/kg, respectively. All fields were found to be significantly different for MRP, where Field B was greatest in concentration with 117.60 – 145.83mg P/kg, followed by Field C with 100.40 – 101.13mg P/kg and Field A with 88.68 – 94.81mg P/kg. Like DH_2O extracts, $NaHCO_3$ MUP was also affected significantly by season, with a decline in concentrations from 8.31 – 13.19mg P/kg for PF to 0 – 8.10mg P/kg for GS, and rising again in PH to 10.11 – 13.39mg P/kg. The variations in $NaHCO_3$ MRP and MUP across agricultural periods are demonstrated in Figure 4.6.

As with DH_2O samples, organic P species in $NaHCO_3$ extracts varied greatly, possessed large standard deviations, and in some situations seemed to overestimate the actual concentration when compared to actual MUP determined via digestion. With LM, there were significant effects for field, season, and their interaction. In both PF and PH, no concentration of LM was determined; in GS 14.31mg P/kg and 11.53mg P/kg were determined for Field A and B, respectively. Like LM, Ph had significant effects for field, season, and their interaction. Field C was the only field to have a concentration of Ph, 2.07mg P/kg, in PF, while in GS there were no

determined concentrations in fields. In PH, there were significantly larger concentrations of Ph with 7.21mg P/kg and 7.99mg P/kg for Field A and C, respectively. Pd was much larger compared to the other organic P species and determined erroneously to be even more than MUP. As mentioned previously, this is most likely due to overestimation of dilute concentrations. For PF, concentrations of Pd were significantly higher, ranging from 14.84mg P/kg to 23.45mg P/kg, as opposed to 0.00 – 12.71mg P/kg in GS and and 4.31 – 14.80mg P/kg in PH. For UHP there were significant effects of field, season, and their interaction as Field B during PH was the only field to have any concentration of MUP (9.02mg P/kg). The reader may refer to Figure 4.7 to observe the concentration fluctuations of organic P species over time.

Table 4.6: Mean concentration values and their respective standard deviation (σ) for total phosphorus (TP), molybdate reactive phosphorus (MRP), molybdate unreactive phosphorus (MUP), labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable organic phosphorus (UHP) determined in 0.5M sodium bicarbonate ($NaHCO_3$) soil extracts of fields for the pre-fertilizer (PF), growing (GS), and post-harvest (PH) agricultural periods. Field A, B, and C were statistically compared through a GLM ANOVA ($p < 0.05$) for field and season main effects, as well as their interaction. If more than one replicate was zero or negative for both analytical and experimental replicates, the entire value was set to zero. If MUP was zero, all organophosphorus classes (LM, Ph, Pd, and UHP) were set to zero, regardless of experimental results.

0.5M $NaHCO_3$ - Fields

Season	Field	TP (mg/kg)	σ	MRP (mg/kg)	σ	MUP (mg/kg)	σ	LM (mg/kg)	σ	Ph (mg/kg)	σ	Pd (mg/kg)	σ	UHP (mg/kg)	σ
PF	**A**	101.87	6.72	88.68	5.99	13.19	11.37	0.00 [b]	0.00	0.00 [c]	0.00	14.84*	9.87	0.00 [b]	0.00
	B	156.75	0.85	145.83	6.17	10.91	5.32	0.00 [b]	0.00	0.00 [c]	0.00	16.34*	2.79	0.00 [b]	0.00
	C	108.71	12.30	100.40	9.36	8.31	3.01	0.00 [b]	0.00	2.07 [b]	2.61	23.45*	9.07	0.00 [b]	0.00
GS	**A**	98.47	3.93	94.81	0.73	3.67	3.41	14.31* [a]	4.13	0.00 [c]	0.00	12.71*	6.61	0.00 [b]	0.00
	B	125.69	6.70	117.60	3.37	8.10	3.38	11.53* [a]	3.30	0.00 [c]	0.00	7.61*	4.38	0.00 [b]	0.00
	C	100.93	12.00	100.93	7.86	0.00	0.00	0.00 [b]	0.00	0.00 [c]	0.00	0.00	0.00	0.00 [b]	0.00
PH	**A**	104.88	4.24	91.49	0.43	13.39	3.81	0.00 [b]	0.00	7.21* [a]	1.00	14.80*	6.51	0.00 [b]	0.00
	B	142.48	6.70	132.02	2.60	10.46	7.77	0.00 [b]	0.00	0.00 [c]	0.00	4.31	6.65	9.02 [a]	9.42
	C	111.24	3.69	101.13	4.22	10.11	3.49	0.00 [b]	0.00	7.99* [a]	2.84	11.19*	8.24	0.00 [b]	0.00

* enzyme hydrolysis would intermittently overestimate organophosphorus classes, possibly due to the overall dilute concentrations of organic phosphorus present in the soil samples. This was supported by enzyme hydrolysis assays on organic phosphorus substrates at 1µg/mL concentration.

[a-c] field concentrations within a column labeled with a different letter superscript represent a significant difference between the values based on a Student's t test when the field*season interaction was significant ($p < 0.05$)

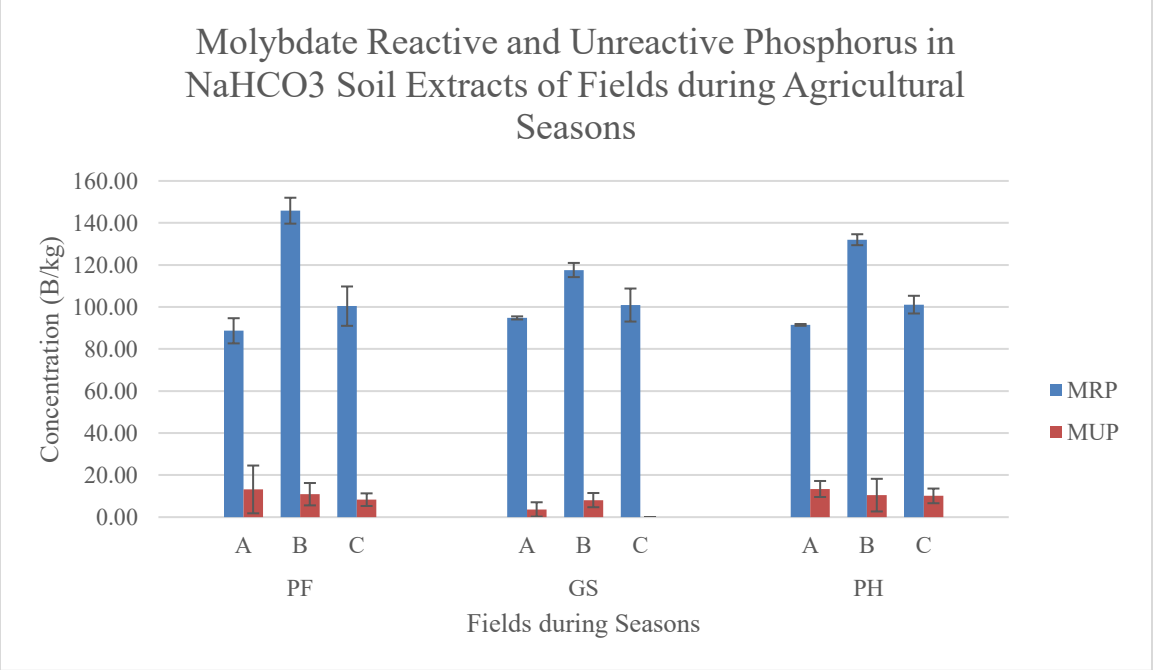

Figure 4.6: Mean concentrations of molybdate reactive P (MRP) and molybdate unreactive P (MUP) in 0.5M sodium bicarbonate ($NaHCO_3$) soil extracts from Field A, B, and C over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

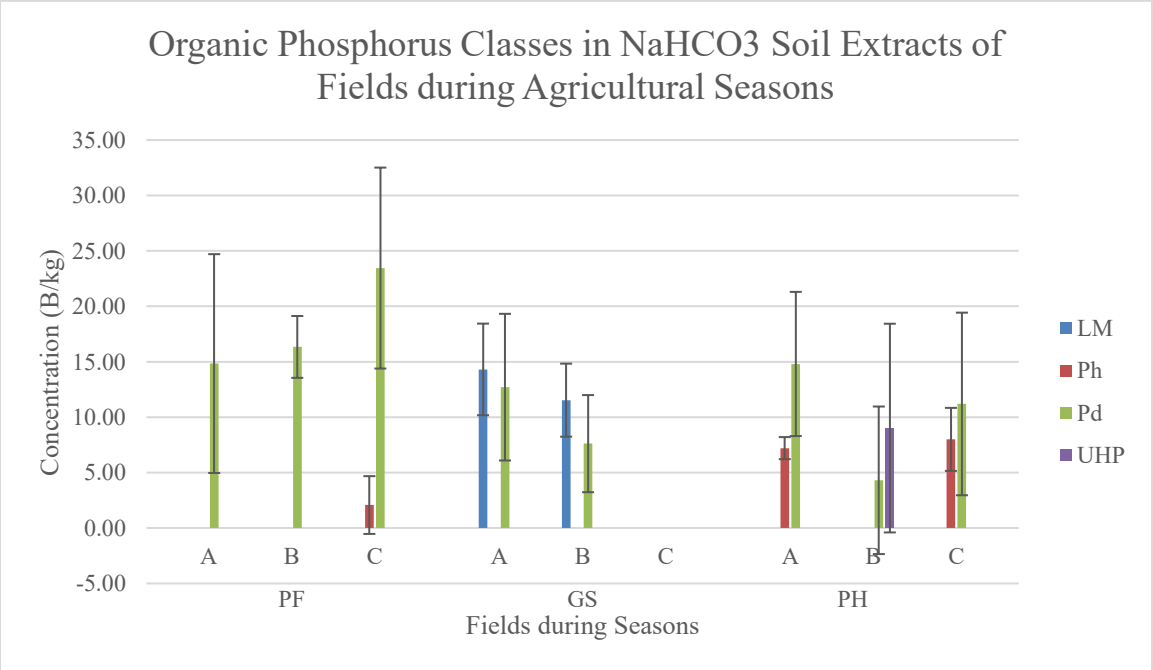

Figure 4.7: Mean concentrations of organic P species [labile phosphomonoesters (LM), phospholipids (PC), phosphodiesters (PD) and unhydrolyzable P(UHP)] in 0.5M sodium bicarbonate ($NaHCO_3$) soil extracts from Field A, B, and C over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

4.5.2.3. NaOH

The mean concentration values with their standard deviations for 0.1M sodium hydroxide (NaOH) TP, MRP, MUP, LM, Ph, Pd, and UHP in PF, GS, and PH are displayed in Table 4.7. Values for each parameter, besides Ph, were much higher for NaOH extracts in comparison to DH_2O and $NaHCO_3$ extracts. However, there was no significant effect of the interaction of field and seasons for all P forms in NaOH extracts. Unfortunately, NaOH extracts of organic soils produced an opaque solution, most likely the result of organic matter. This color may have interfered with the development of the molybdate blue color. TP was statistically greater in Field B (654.42 – 816.98mg P/kg) followed by Field A (461.15 – 548.46mg P/kg), and Field C (330.26 – 412.80mg P/kg). In regards to seasons, PF (390.78 – 816.98mg P/kg) and GS (412.80 – 789.35mg P/kg) were similar in concentration, but PH was significantly less with concentrations ranging between 330.26 – 654.42mg P/kg. For NaOH MRP, there was both a significant field and season main effect. Like TP, MRP was greatest in Field B with a range of 518.95 – 671.03mg P/kg, while Field A had 302.63 – 405.44mg P/kg and Field C had the lowest concentrations (233.58 – 295.33mg P/kg). NaOH MRP decreased over time beginning with 295.33 – 671.03mg P/kg for PF, then 237.92 – 583.63mg P/kg for GS, and lastly 233.58 – 518.95mg P/kg in PH. With MUP in NaOH extracts there was a significant effect of both field and season. Field A and B were most abundant in NaOH MUP having concentrations of 128.51 – 221.19mg P/kg and 135.47 – 205.73mg P/kg, respectively. Field C had lower concentrations ranging between 95.44 and 174.88mg P/kg. In comparing seasons, GS had statistically greater NaOH MUP concentrations with 174.88 – 221.19mg P/kg, as opposed to PF (95.44 – 145.96mg P/kg) and PH (96.68 – 158.52mg P/kg). Results for MRP and MUP in NaOH extracts over the agricultural periods can be visualized in Figure 4.8.

Like TP, MRP, and MUP, organic P species were much greater in NaOH extracts in comparison to DH_2O and $NaHCO_3$ extracts. However, results were still accompanied by large standard deviations. The effect of season was significant for NaOH LM with PF having lesser concentrations (0.00 – 2.07mg P/kg) in contrast to GS (15.76 – 26.33mg P/kg) and PH (16.18 – 35.34mg P/kg). There was a complete absence of Ph concentrations in extracts of fields across all seasons. This was contrary to Pd, where concentrations across all fields and seasons were variable, ranging from 0.00 to 140.17mg P/kg. However, there was no statistically significant effect of field or season. For Pd of Field B in PH season, there may have been an overestimation as the concentration of Pd determined was 132.55mg P/kg while MUP was 135.47mg P/kg. UHP was greatest in NaOH extracts, while UHP concentrations in DH_2O and $NaHCO_3$ extracts were virtually none. Yet, there were no significant main effects of field or seasons, and concentrations were variable, ranging from 0.00 – 145.96mg P/kg. Figure 4.9 illustrates the concentrations of organic P species in NaOH extracts across seasons.

Table 4.7: Mean concentration values and their respective standard deviation (σ) for total phosphorus (TP), molybdate reactive phosphorus (MRP), molybdate unreactive phosphorus (MUP), labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable organic phosphorus (UHP) determined in 0.1M sodium hydroxide (NaOH) soil extracts of fields for the pre-fertilizer (PF), growing (GS), and post-harvest (PH) agricultural periods. Field A, B, and C were statistically compared through a GLM ANOVA ($p < 0.05$) for field and season main effects, as well as their interaction. If more than one replicate was zero or negative for both analytical and experimental replicates, the entire value was set to zero. If MUP was zero, all organophosphorus classes (LM, Ph, Pd, and UHP) were set to zero, regardless of experimental results.

0.1M NaOH - Fields

Season	Field	TP (mg/kg)	σ	MRP (mg/kg)	σ	MUP (mg/kg)	σ	LM (mg/kg)	σ	Ph (mg/kg)	σ	Pd (mg/kg)	σ	UHP (mg/kg)	σ
PF	**A**	533.95	55.82	405.44	30.75	128.51	25.42	2.07	1.79	0.00	0.00	86.07	35.34	40.38	10.39
	B	816.98	74.11	671.03	36.62	145.96	37.59	0.00	0.00	0.00	0.00	0.00	0.00	145.96	37.59
	C	390.78	32.28	295.33	28.70	95.44	24.98	1.93	2.25	0.00	0.00	73.87	26.13	20.83	19.73
GS	**A**	548.46	17.95	327.28	2.47	221.19	20.15	15.76	3.77	0.00	0.00	129.47	67.70	79.95	70.23
	B	789.35	33.42	583.63	26.24	205.73	15.54	19.89	28.17	0.00	0.00	140.17	99.47	68.51	73.69
	C	412.80	25.01	237.92	5.29	174.88	24.17	26.33	7.69	0.00	0.00	89.01	50.10	59.54	64.93
PH	**A**	461.15	46.78	302.63	37.29	158.52	12.37	35.34	9.60	0.00	0.00	79.79	22.02	43.38	36.26
	B	654.42	59.80	518.95	37.42	135.47	22.38	21.32*	7.73	0.00	0.00	132.55*	45.12	0.00	0.00
	C	330.26	5.61	233.58	6.80	96.68	5.75	16.18	3.41	0.00	0.00	80.03	28.43	9.88	10.08

* enzyme hydrolysis would intermittently overestimate organophosphorus classes, possibly due to the overall dilute concentrations of organic phosphorus present in the soil samples. This was supported by enzyme hydrolysis assays on organic phosphorus substrates at 1µg/mL concentration.

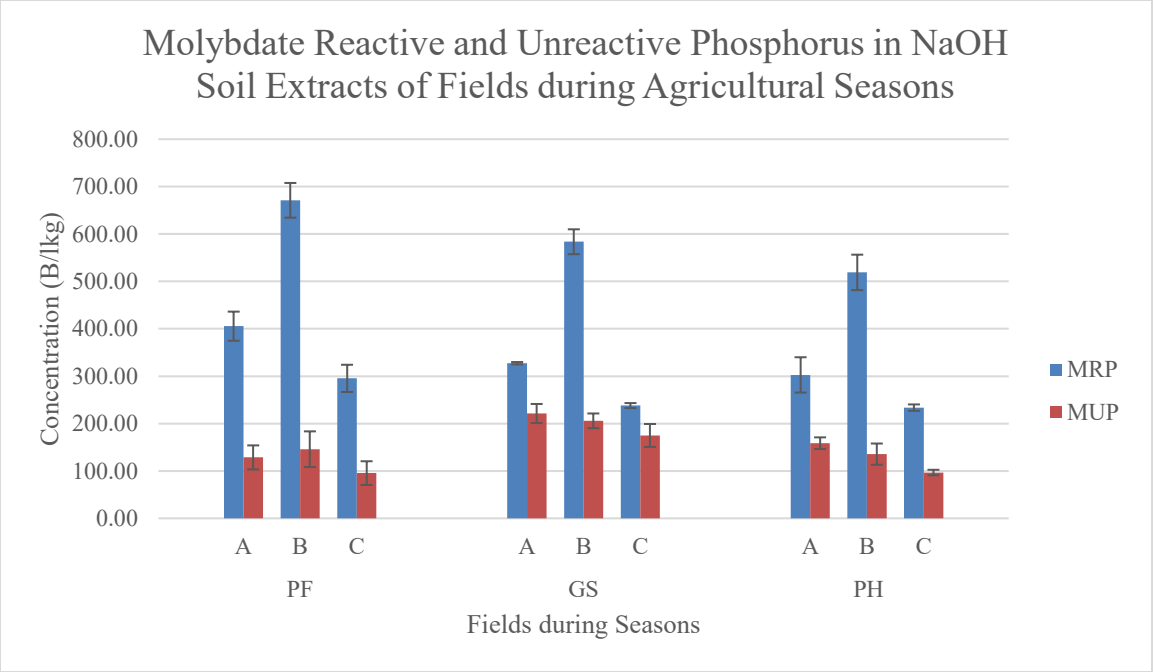

Figure 4.8: Mean concentrations of molybdate reactive P (MRP) and molybdate unreactive P (MUP) in 0.1M sodium hydroxide (NaOH) soil extracts from Field A, B, and C over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

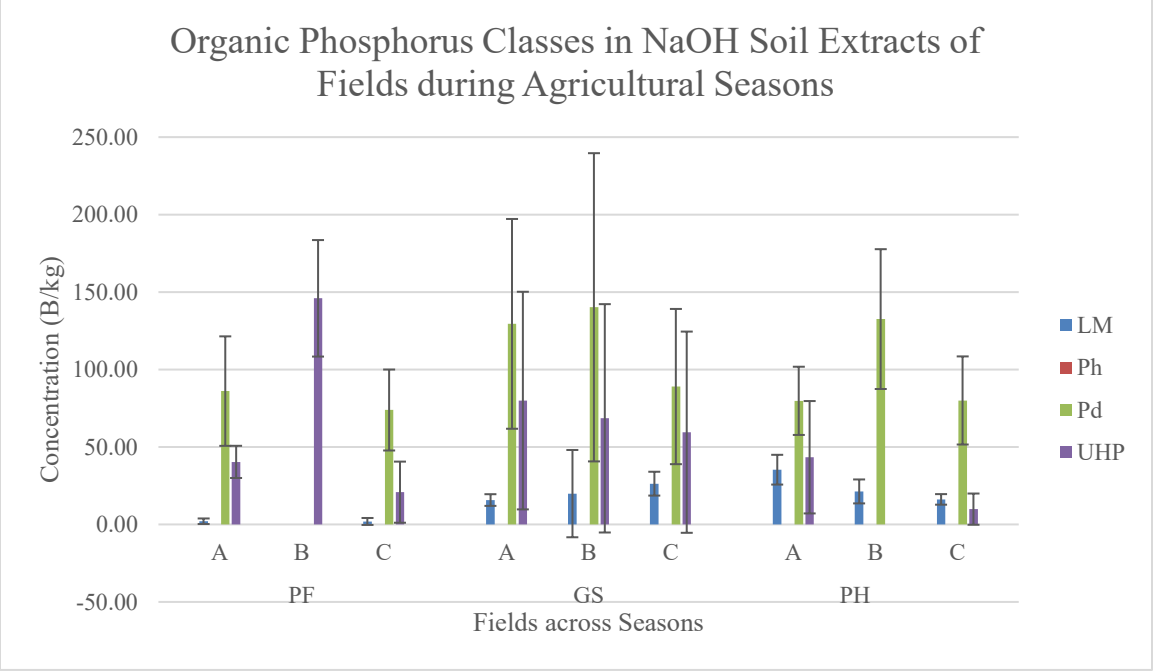

Figure 4.9: Mean concentrations of organic P species [labile phosphomonoesters (LM), phospholipids (PC), phosphodiesters (PD) and unhydrolyzable P(UHP)] in 0.1M sodium hydroxide (NaOH) soil extracts from Field A, B, and C over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

4.5.2.4. HCl

As mentioned previously, 1M hydrochloric acid (HCl) was the largest fraction. Unfortunately, organic P species were not characterized for this fraction as model organic P substrates were insoluble. Therefore could not accurate determine if enzymes were functioning. Mean concentrations for HCl TP, MRP and MUP, and standard deviations of these values are presented in Table 4.8. For HCl TP there was a significant effect of both field and season. Field B and C both had similarly high concentrations of TP with 786.18 – 1,640.62mg P/kg for the former and 735.26 – 1,771.57mg P/kg for the latter; Field A was significantly less with 588.82 – 1,238.58mg P/kg. In regards to seasonal effect on TP concentrations, PF had much lower concentrations (588.82 – 786.18mg P/kg) as opposed to GS (1,127.29 – 1,771.57mg P/kg) and PH (1,238.58 – 1,685.20mg P/kg). Likewise, MRP had a significant field and season main effect. MRP was greatest in concentration for both Field B and C with 750.12 – 1,522.95mg P/kg and 715.49 – 1,632.90mg P/kg, respectively. MRP for Field A was lower having concentrations ranging between 544.88 and 1,183.54mg P/kg. GS and PH seasons had the most abundant concentrations of HCl MRP with 948.88 – 1,572.12mg P/kg and 1,183.54 – 1,632.90mg P/kg, respectively. PF had lower concentrations of 544.88 – 750.12mg P/kg. Only the seasonal main effect was significant for HCl MUP where GS had much greater concentrations of MUP (153.05 – 199.45mg P/kg). In contrast, PF and PH were significantly less with 19.77 – 43.94mg P/kg and 49.47 – 55.03mg P/kg. However, MUP concentration values had large standard deviations. Figure 4.10 exhibits the concentrations of MRP and MUP in HCl extracts across seasons.

Table 4.8: Mean concentration values and their respective standard deviation (σ) for total phosphorus (TP), molybdate reactive phosphorus (MRP), and molybdate unreactive phosphorus (MUP) determined in 1M hydrochloric acid (HCl) soil extracts of fields for the pre-fertilizer (PF), growing (GS), and post-harvest (PH) agricultural periods. Field A, B, and C were statistically compared through a GLM ANOVA ($p < 0.05$) for field and season main effects, as well as their interaction. If more than one replicate was zero or negative for both analytical and experimental replicates, the entire value was set to zero. If MUP was zero, all organophosphorus classes (LM, Ph, Pd, and UHP) were set to zero, regardless of experimental results. Enzyme hydrolysis assays were not conducted on HCl extracts as organophosphorus model substrates were insoluble in 1M HCl and therefore could not determine if enzymes were functioning properly to hydrolyze organophosphorus compounds in HCl extracts.

1M HCl - Fields

Season	Field	TP (mg/kg)	σ	MRP (mg/kg)	σ	MUP (mg/kg)	σ
PF	**A**	588.82	65.42	544.88	25.58	43.94	40.22
	B	786.18	25.76	750.12	12.12	36.06	19.39
	C	735.26	66.87	715.49	68.71	19.77	15.06
GS	**A**	1127.29	73.10	948.88	53.50	178.41	56.93
	B	1640.62	211.29	1487.57	323.84	153.05	129.36
	C	1771.57	60.11	1572.12	5.98	199.45	54.13
PH	**A**	1238.58	55.42	1183.54	128.37	55.03	78.13
	B	1572.42	316.25	1522.95	275.08	49.47	41.77
	C	1685.20	72.98	1632.90	55.87	52.29	66.25

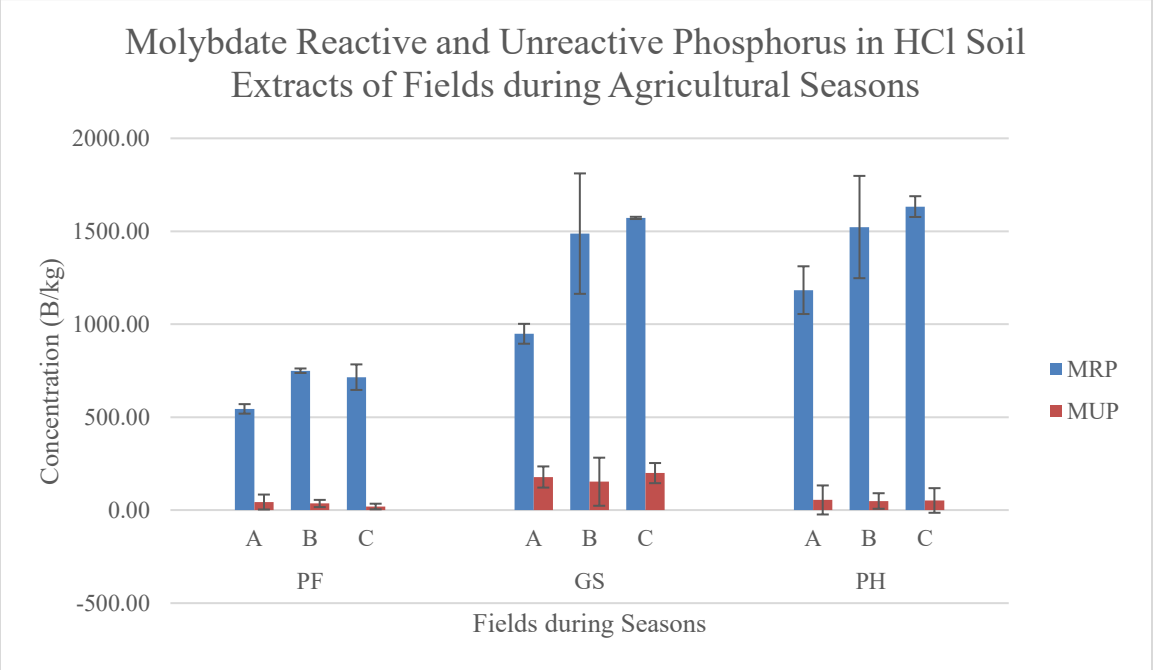

Figure 4.10: Mean concentrations of molybdate reactive P (MRP) and molybdate unreactive P (MUP) in 1M hydrochloric acid (HCl) soil extracts from Field A, B, and C over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

4.6. Comparison of P Forms Among Depths

4.6.1. Distribution of P in fractions

The partitioning of MRP and MUP among extracts of soil from Depth B (0-20cm), B2 (20-40cm) and B3 (40-60cm) during PF, GS, and PH seasons is illustrated in Figure 4.11. As with the distribution of P in fields, MRP was the dominant P form at all depths for all seasons. For DH_2O MRP, percentage of total P at each depth ranged from 0-3% in PF, 1-2% in GS, and 1-2% in PH. In comparing depths, Depth B had the greatest proportion of DH_2O MRP beginning with 3% in PF and reducing to 2% in GS and remained at this proportion in PH. Throughout all the agricultural periods, DH_2O MRP remained at 1% for Depth B2. Unlike, Depth B and B2, DH_2O MRP actually increased for Depth B3, beginning at 0% in PF, and rising to 1% for GS, where it stabilized into PH. DH_2O MUP was 0% for all depths and seasons, besides Depth B3, PF where it was 1%. $NaHCO_3$ MRP for Depth B and B2 decreased between PF and GS, with 8% to 5% for the former and 6% to 4% for the latter. Subsequently, both rose in PH with Depth B to 6% and Depth B2 to 5%. In contrast, Depth B3 remained at 5% in PF and GS, but decreased to 4% for $NaHCO_3$ MRP. All depths had 1% of their total P as $NaHCO_3$ MUP in PF but diminished to 0% for GS and PH. In regards to NaOH MRP, Depth B and B2 decreased in DH_2O concentration between PF and GS and remained at similar proportions in PH. However, Depth B had a greater proportion with 37% in PF which diminished to 22% in GS and 21% in PH, while Depth B2 was 20% in PF which reduced to 16% in GS and 13% in PH. On the contrary, Depth B3 was relatively stable between PF (17%) and GS (18%), but reduced to 13% in PH. NaOH MUP remained relatively constant across agricultural periods at all depths ranging from 6-8% for Depth B, 3-6% for Depth B2, and 5-8% for Depth B3. As for fields, HCl MRP was the largest fraction but varied in fluctuations over time at each depth. HCl MRP rose in each season for

Depth B, from 41% (GS) to 63% (PH). For Depth B2, HCl MRP was stable between PF (65%) and GS (66%), but rose to 76% for PH. The proportion of HCl MRP in Depth B3 subsided from PF (68%) to GS (63%) but rose again in PH (77%). For all depths, HCl MUP peaked in GS ranging between 5 and 7% but was relatively low for PF (2-4%) and virtually nonexistent in PH (0-2%).

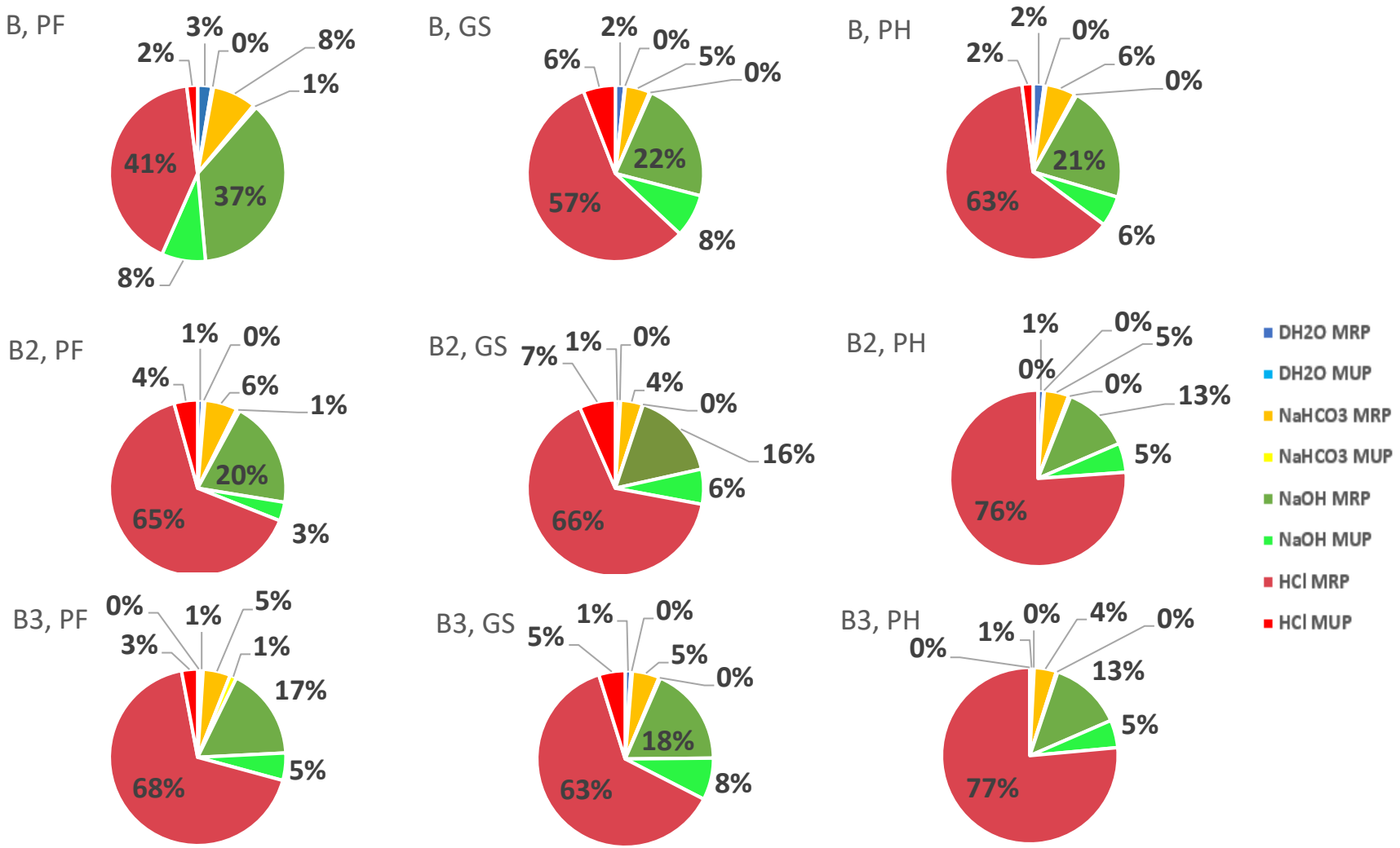

Figure 4.11: Distribution (%) of molybdate reactive P (MRP) and molybdate unreactive P (MUP) among deionized water (DH_2O), 0.5M sodium bicarbonate ($NaHCO_3$), 0.1M sodium hydroxide (NaOH), and 1M hydrochloric acid (HCl) extracts for Depth B (0-20cm), B2 (20-40cm), and B3 (40-60cm) during pre-fertilizer (PF), growing season (GS), and post-harvest (PH) agricultural periods.

4.6.2. Concentrations of P forms

4.6.2.1. DH_2O

Table 4.9 displays the mean concentrations and standard deviations of TP, MRP, MUP, LM, Ph, Pd, and UHP across agricultural periods for DH_2O extracts of soil from three different depths of Field B: B (0-20cm), B2 (20-40cm), and B3 (40-60cm). With TP, depth, season and their interaction were determined to be significant main effects. Depth B was greatest in concentration ranging from 47.65 to 57.79mg P/kg, followed by Depth B2 with 11.68 – 16.24mg P/kg, and Depth B3 with 6.03 – 15.15mg P/kg. Depth B and B2 were similar across all seasons while in GS, Depth B3 rose significantly from 6.03mg P/kg in PF to 15.15mg P/kg in GS, and subsided down to 8.08mg P/kg. Likewise, MRP had a significant effect for depth, season and their interaction. Depth B had the most abundant MRP concentrations ranging between 44.65 to 49.13mg P/kg and similar across all agricultural periods. In contrast, Depth B2 and B3 were much less with 8.23 – 14.58mg P/kg and 2.61 – 11.99mg P/kg, respectively. Trends were also different, where Depth B2 rose in value for MRP across agricultural periods while Depth B3 grew from PF (2.61mg P/kg) to GS (11.99mg P/kg) and diminished to 6.51mg P/kg in PH. In regards to MUP, there was a significant effect of depth and the interaction between season and depth. Both Depth B2 (1.66 – 3.45mg P/kg) and Depth B3 (1.57 – 3.42mg P/kg) had significantly less MUP than Depth B (3.00 – 8.66mg P/kg). A graph of the concentrations of MRP and MUP over the agricultural periods is shown in Figure 4.12.

As with fields, soil samples from the different depths had minute concentrations of organic P species with large standard deviations and may have been overestimated, on occasion. There were no significant effects for LM, with 0.84 – 2.04mg P/kg, 0.42 – 0.50mg P/kg, and 0.26 – 1.32mg P/kg for Depth B, B2, and B3, respectively. Ph had a significant effect for season

and the interaction of depth and season. No Ph concentrations were determined for PF, but were found in GS (7.30 – 21.06mg P/kg) and PH (0.97 – 8.74mg P/kg). With Pd, there was a significant effect of depth, season, and their interaction. Pd was zero for all extracts except Depth B3 in PF (1.33mg P/kg) and PH (0.15mg P/kg). UHP only had a significant effect for season with PF possessing greater concentrations (2.15 – 3.72mg P/kg) than GS (0.00mg P/kg) and PH (0.00 – 0.73mg P/kg). Trends in organic P species over the agricultural periods are shown in Figure 4.13.

Table 4.9: Mean concentration values and their respective standard deviation (σ) for total phosphorus (TP), molybdate reactive phosphorus (MRP), molybdate unreactive phosphorus (MUP), labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable organic phosphorus (UHP) determined in deionized water (DH_2O) soil extracts of Field B at three different depths for the pre-fertilizer (PF), growing (GS), and post-harvest (PH) agricultural periods. Depth B (0-20cm), B2 (20-40cm), and B3 (40-60cm) were statistically compared through a GLM ANOVA ($p < 0.05$) for depth and season main effects, as well as their interaction. If more than one replicate was zero or negative for both analytical and experimental replicates, the entire value was set to zero. If MUP was zero, all organophosphorus classes (LM, Ph, Pd, and UHP) were set to zero, regardless of experimental results.

DH_2O - Depths

Season	Field	TP (mg/kg)	σ	MRP (mg/kg)	σ	MUP (mg/kg)	σ	LM (mg/kg)	σ	Ph (mg/kg)	σ	Pd (mg/kg)	σ	UHP (mg/kg)	σ
PF	**B**	52.70 [a]	1.65	46.94 [a]	0.57	5.76 [a,b]	1.28	2.04	2.43	0.00 [b]	0.00	0.00 [c]	0.00	3.72	2.56
	B2	11.68 [b]	1.95	8.23 [d,e]	1.02	3.45 [b,c]	0.94	0.50	0.57	0.00 [b]	0.00	0.00 [c]	0.00	2.95	0.56
	B3	6.03 [c]	2.75	2.61 [f]	0.12	3.42 [b,c]	2.65	0.26	0.24	0.00 [b]	0.00	1.33 [a]	0.10	2.15	2.18
GS	**B**	47.65 [a]	0.53	44.65 [a]	1.51	3.00 [b,c]	1.66	1.60	0.36	9.73* [a]	8.50	0.00 [c]	0.00	0.00	0.00
	B2	12.43 [b]	2.14	9.24 [c,d]	2.77	3.19 [b,c]	2.79	0.48	0.43	21.06* [a]	20.50	0.00 [c]	0.00	0.00	0.00
	B3	15.15 [b]	2.62	11.99 [c]	0.95	3.16 [b,c]	2.18	1.32	1.19	7.30* [a]	0.60	0.00 [c]	0.00	0.00	0.00
PH	**B**	57.79 [a]	0.82	49.13 [a]	0.36	8.66 [a]	0.50	0.84	0.84	8.74* [a]	3.56	0.00 [c]	0.00	0.73	0.63
	B2	16.24 [b]	1.86	14.58 [b]	2.20	1.66 [c]	1.94	0.42	0.73	0.97 [b]	1.10	0.00 [c]	0.00	0.00	0.00
	B3	8.08 [c]	1.33	6.51 [e]	2.61	1.57 [c]	1.52	0.68	0.64	2.28* [b]	1.99	0.15 [b]	0.16	0.00	0.00

* enzyme hydrolysis would intermittently overestimate organophosphorus classes, possibly due to the overall dilute concentrations of organic phosphorus present in the soil samples. This was supported by enzyme hydrolysis assays on organic phosphorus substrates at 1µg/mL concentration.

[a-f] depth concentrations within a column labeled with a different letter superscript represent a significant difference between the values based on a Student's t test when the depth*season interaction was significant ($p < 0.05$)

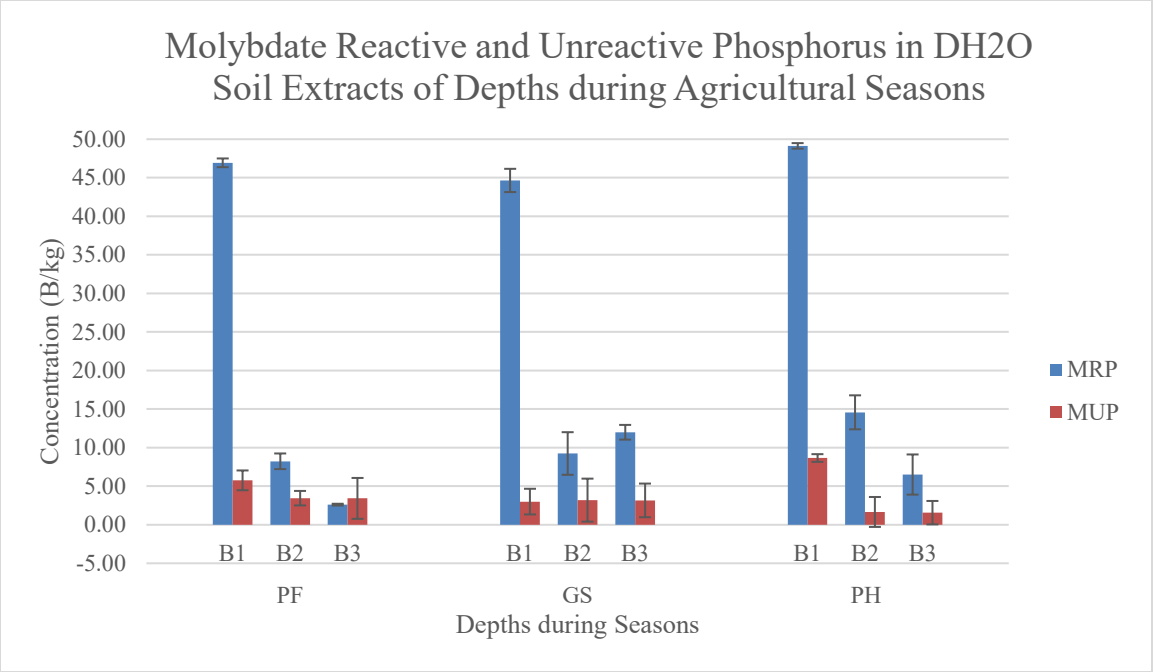

Figure 4.12: Mean concentrations of molybdate reactive P (MRP) and molybdate unreactive P (MUP) in deionized water (DH_2O) soil extracts from Depth B (0-20cm), B2 (20-40cm), and B3 (40-60cm) over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

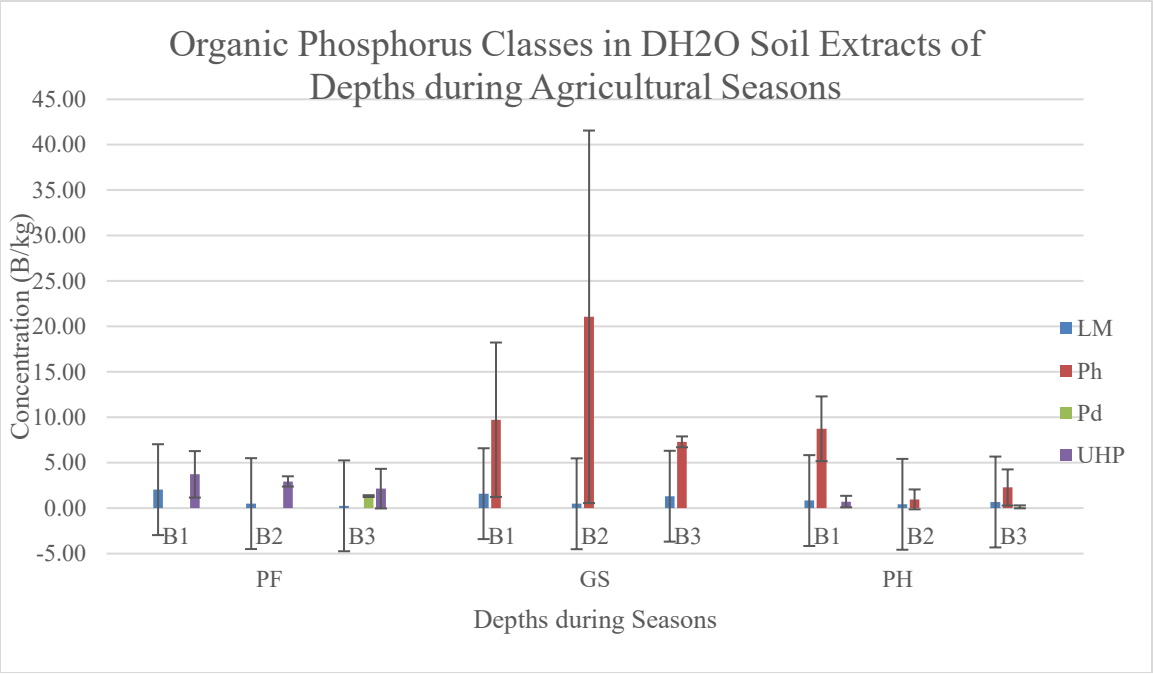

Figure 4.13: Mean concentrations of organic P species [labile phosphomonoesters (LM), phospholipids (PC), phosphodiesters (PD) and unhydrolyzable P(UHP)] in deionized water (DH_2O) soil extracts from Depth B (0-20cm), B2 (20-40cm), and B3 (40-60cm) over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

4.6.2.2. $NaHCO_3$

Mean concentrations for TP, MRP, MUP, LM, Ph, Pd, and UHP across agricultural periods for $NaHCO_3$ extracts of Depth B, B2 and B3, and standard deviations of these values are presented in Table 4.10. With TP, depth and the interaction of depth and season were determined to be significant. Depth B had the greatest concentrations of TP with 125.69 – 156.75mg P/kg, followed by Depth B2 with 54.95 – 68.62mg P/kg, and then Depth B3 with 37.13 – 59.89mg P/kg. For the most part, concentrations remained stable over time for each soil depth. A significant effect for depth, season and the interaction of were determine for MRP. Depth B was greatest in concentration for MRP ranging from 117.60 to 145.83mg P/kg and diminished significantly in concentration from PF (145.83mg P/kg) to GS (117.60mg P/kg). Depth B2 followed in concentration with 52.47 – 63.08mg P/kg, rising significantly from GS (52.94mg P/kg) to PH (63.08mg P/kg). Depth B3 had the lowest concentrations (29.79 – 56.61mg P/kg) but the greatest variation, rising from PF (29.79mg P/kg) to GS (56.61mg P/kg), and reducing in PH (41.09mg P/kg). MUP only had a significant effect of depth with Depth B (8.10 – 10.91mg P/kg) statistically greater in concentration than Depth B2 (2.00 – 5.54mg P/kg) and Depth B3 (2.79 – 7.34mg P/kg). Figure 4.14 exhibits the dynamics of MRP and MUP in $NaHCO_3$ extracts of Depth B, B2, and B3 across agricultural periods.

Organic P speciation analyses in $NaHCO_3$ extracts determined dilute concentrations with large standard deviations, as with DH_2O extracts. Occasionally, organic P species were overestimated. For LM, there were significant effects for season and the interaction of depth and season. PF and PH concentrations of LM were low with 0.00 – 2.67mg P/kg and 0.00mg P/kg, respectively, while GS was significantly greater with 11.24 – 13.54mg P/kg. Ph had significant main effects for both depth and season. Depth B (0.00mg P/kg) and B2 (0.00 – 3.36mg P/kg)

were significantly different from each other but Depth B3 was not. Similarly, with seasonal main effects, GS (0.00mg P/kg) and PH (0.00 – 3.67mg P/kg) were significantly different but PF was not. Season was the only significant main effect for Pd concentrations, where PF and PH were statistically different, with 9.52 – 19.04mg P/kg and 4.31 – 8.57mg P/kg, respectively. UHP was significant for depth, season, and their interaction. This was due to only Depth B in PH possessing any UHP (9.02mg P/kg), while the rest of the depths across agricultural periods were zero. Trends of these P species for $NaHCO_3$ extracts of depths are displayed in Figure 4.15.

Table 4.10: Mean concentration values and their respective standard deviation (σ) for total phosphorus (TP), molybdate reactive phosphorus (MRP), molybdate unreactive phosphorus (MUP), labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable organic phosphorus (UHP) determined in 0.5M sodium bicarbonate ($NaHCO_3$) soil extracts of Field B at three different depths for the pre-fertilizer (PF), growing (GS), and post-harvest (PH) agricultural periods. Depth B (0-20cm), B2 (20-40cm), and B3 (40-60cm) were statistically compared through a GLM ANOVA ($p < 0.05$) for depth and season main effects, as well as their interaction. If more than one replicate was zero or negative for both analytical and experimental replicates, the entire value was set to zero. If MUP was zero, all organophosphorus classes (LM, Ph, Pd, and UHP) were set to zero, regardless of experimental results.

0.5M $NaHCO_3$ - Depths

Season	Field	TP (mg/kg)	σ	MRP (mg/kg)	σ	MUP (mg/kg)	σ	LM (mg/kg)	σ	Ph (mg/kg)	σ	Pd (mg/kg)	σ	UHP (mg/kg)	σ
PF	**B**	156.75 [a]	0.85	145.83 [a]	6.17	10.91	5.32	0.00 [c]	0.00	0.00	0.00	16.34*	2.79	0.00 [b]	0.00
	B2	58.01 [d]	5.26	52.47 [d]	7.15	5.54	3.26	0.00 [c]	0.00	2.96	2.50	19.04*	8.89	0.00 [b]	0.00
	B3	37.13 [f]	2.23	29.79 [f]	2.37	7.34	2.65	2.67 [b]	3.06	1.30	1.75	9.52*	4.72	0.00 [b]	0.00
GS	**B**	125.69 [b]	6.70	117.60 [b]	3.37	8.10	3.38	11.53* [a]	3.30	0.00	0.00	7.61*	4.38	0.00 [b]	0.00
	B2	54.95 [d]	5.21	52.94 [d]	6.61	2.00	1.48	13.54* [a]	3.70	0.00	0.00	11.01*	7.17	0.00 [b]	0.00
	B3	59.89 [d]	10.38	56.61 [c,d]	7.77	3.28	2.65	11.24* [a]	3.52	0.00	0.00	11.20*	0.94	0.00 [b]	0.00
PH	**B**	142.48 [a,b]	6.70	132.02 [a,b]	2.60	10.46	7.77	0.00 [c]	0.00	0.00	0.00	4.31	6.65	9.02 [a]	9.42
	B2	68.62 [c]	1.70	63.08 [c]	3.00	5.54	4.12	0.00 [c]	0.00	3.36	2.96	8.53*	7.65	0.00 [b]	0.00
	B3	43.89 [e]	3.71	41.09 [e]	2.99	2.79	2.84	0.00 [c]	0.00	3.67	4.09	8.57*	7.42	0.00 [b]	0.00

* enzyme hydrolysis would intermittently overestimate organophosphorus classes, possibly due to the overall dilute concentrations of organic phosphorus present in the soil samples. This was supported by enzyme hydrolysis assays on organic phosphorus substrates at 1µg/mL concentration.

[a-f] depth concentrations within a column labeled with a different letter superscript represent a significant difference between the values based on a Student's t test when the depth*season interaction was significant ($p < 0.05$)

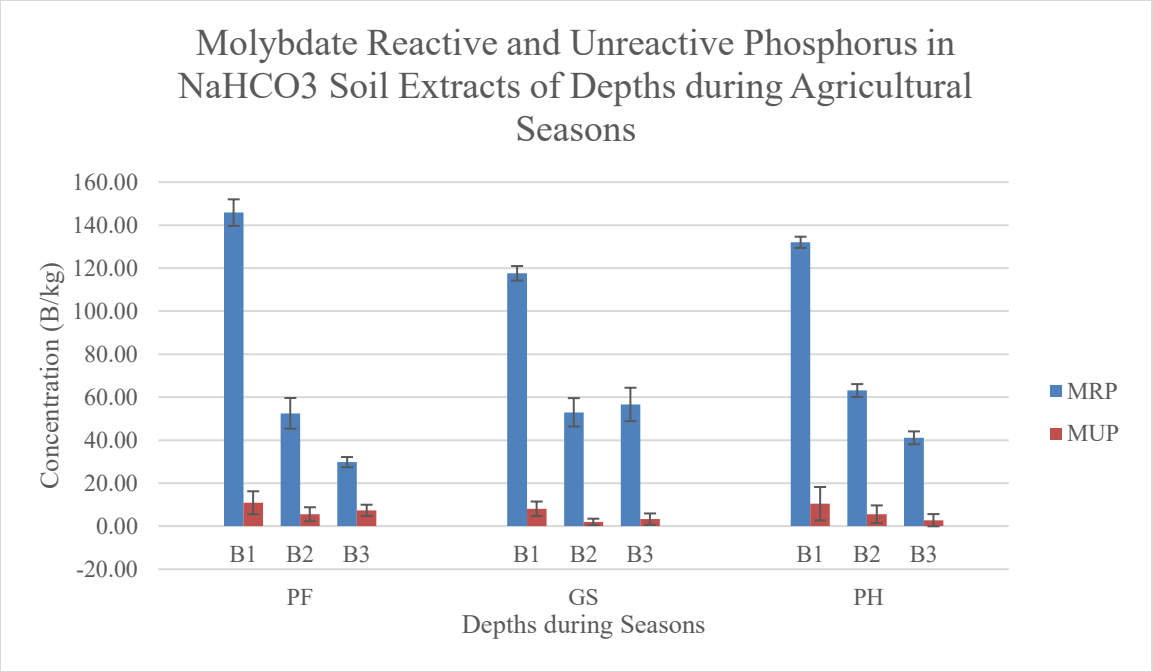

Figure 4.14: Mean concentrations of molybdate reactive P (MRP) and molybdate unreactive P (MUP) in 0.5M sodium bicarbonate ($NaHCO_3$) soil extracts from Depth B (0-20cm), B2 (20-40cm), and B3 (40-60cm) over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

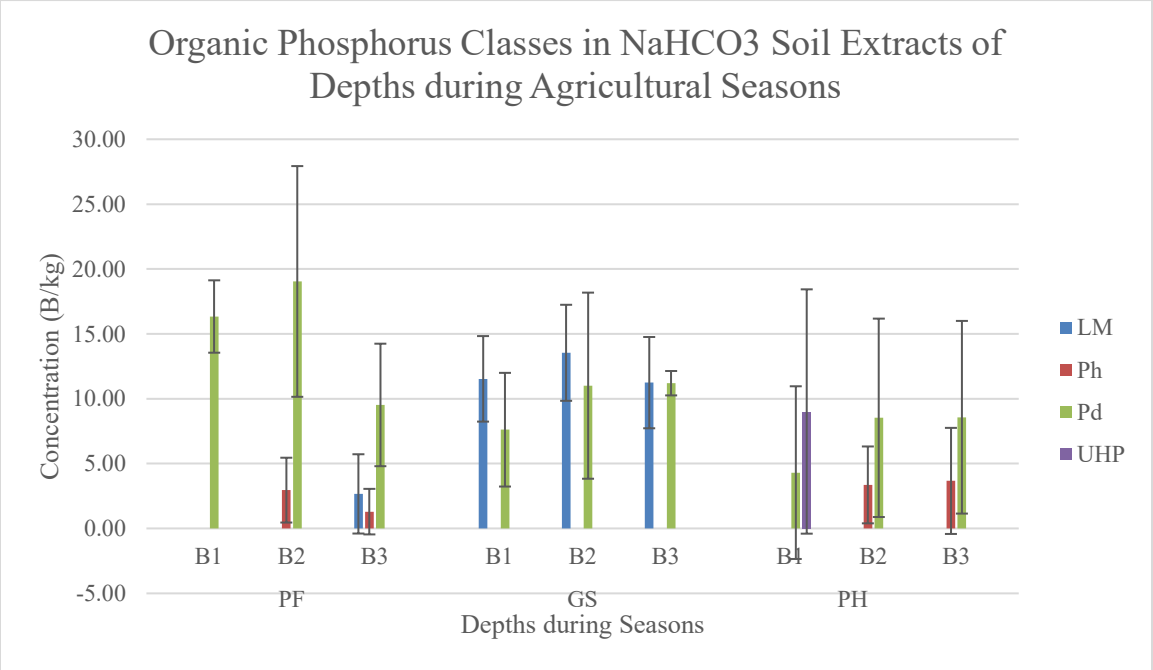

Figure 4.15: Mean concentrations of organic P species [labile phosphomonoesters (LM), phospholipids (PC), phosphodiesters (PD) and unhydrolyzable P(UHP)] in 0.5M sodium bicarbonate ($NaHCO_3$) soil extracts from Depth B (0-20cm), B2 (20-40cm), and B3 (40-60cm) over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

4.6.2.3. NaOH

In Table 4.11, mean concentrations and standard deviations for TP, MRP, MUP, LM, Ph, Pd, and UHP in NaOH extracts of Depth B, B2 and B3 across agricultural periods are shown. There was a significant effect for depth, season, and their interaction for TP. Depth B had the highest concentrations (654.42 – 816.98mg P/kg), followed by Depth B2 (202.81 – 299.05mg P/kg) and Depth B3 (131.00 – 297.29mg P/kg). While TP in Depth B decreased from 816.98 to 654.42mg P/kg during PF to PH, Depth B2 and B3 both grew in GS and diminished in concentration by PH. MRP was similar to TP with significant effects of depth, season, and their interaction. Depth B possessed the largest concentrations (518.95 – 671.03mg P/kg) while Depth B3 had the least (101.08 – 209.36mg P/kg). Depth B2 was intermediate in concentration ranging between 172.62 and 214.76mg P/kg. While for Depth B, concentrations across agricultural periods were relatively stable, both Depth B2 and B3 grew from 172.62 and 101.08mg P/kg in PF to 214.76 and 209.36mg P/kg in GS, respectively. Both depths then reduced in concentration in PH to 174.77 and 134.69mg P/kg, respectively. Depth, season, and their interaction were also significant effects for MUP. Depth B was significantly greater in concentration ranging between 135.47 and 205.73mg P/kg, as compared to Depth B2 (30.20 – 84.29mg P/kg) and Depth B3 (29.92 – 87.94mg P/kg). Depth B2 and B3 were similar in trends besides for in PH where Depth B3 decreased from GS (87.94mg P/kg) to PH (51.15mg P/kg), while Depth B2 remained relatively stable between GS (84.29mg P/kg) and PH (75.05mg P/kg). Concentrations of MRP and MUP over seasons for depths are illustrated in Figure 4.16.

For organic P species in NaOH extracts, dilute concentrations were determined with larger standard deviations, except for Pd which were exceptionally high. As with other extracts, there was an issue of overestimation for some organic P species on occasion. LM only had a

significant effect of season where PF (0.00 – 9.53mg P/kg) was statistically less than GS (18.25 – 19.90mg P/kg) and PH (10.13 – 21.32mg P/kg). There was also a significant effect for season with Ph concentrations, where PF (0.00 – 15.44mg P/kg) and GS (0.00mg P/kg) were significantly different, but not to PH (0.00 – 2.02mg P/kg). Pd had a significant effect of both season and the interaction of depth and season. GS was significantly greater with concentrations ranging from 36.52 to 140.17mg P/kg, followed by PH (6.88 – 132.55mg P/kg), and then PF (0.00 – 24.38mg P/kg). Depth B2 remained relatively constant over all seasons with 22.60 – 49.49mg P/kg while Depth B3 was statistically similar in PF (24.38mg P/kg) and GS (36.52mg P/kg) but diminished to 6.88mg P/kg in PH. In contrast, Depth B rose in concentration significantly from PF (0.00mg P/kg) to GS (140.17mg P/kg) and remained at a similar concentration in PH (132.55mg P/kg). Season and the interaction of season and depth were determined to be significant for UHP, as well. B reduced in UHP significantly over seasons from 145.96mg P/kg in PF to 68.51mg P/kg in GS, and to 0.00mg P/kg in PH. Both Depth B2 and B3 had similar trends, increasing in UHP from 0.00mg P/kg in PF to 16.55mg P/kg for Depth B2 and 31.55mg P/kg for Depth B3 in GS. These concentrations remained statistically similar into PH for Depth B2 (17.45mg P/kg) and Depth B3 (34.14mg P/kg). The patterns for all organic P species at each depth over the agricultural periods are displayed in Figure 4.17.

Table 4.11: Mean concentration values and their respective standard deviation (σ) for total phosphorus (TP), molybdate reactive phosphorus (MRP), molybdate unreactive phosphorus (MUP), labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable organic phosphorus (UHP) determined in 0.1M sodium hydroxide (NaOH) soil extracts of Field B at three different depths for the pre-fertilizer (PF), growing (GS), and post-harvest (PH) agricultural periods. Depth B (0-20cm), B2 (20-40cm), and B3 (40-60cm) were statistically compared through a GLM ANOVA ($p < 0.05$) for depth and season main effects, as well as their interaction. If more than one replicate was zero or negative for both analytical and experimental replicates, the entire value was set to zero. If MUP was zero, all organophosphorus classes (LM, Ph, Pd, and UHP) were set to zero, regardless of experimental results.

0.1M NaOH - Depths

Season	Field	TP (mg/kg)	σ	MRP (mg/kg)	σ	MUP (mg/kg)	σ	LM (mg/kg)	σ	Ph (mg/kg)	σ	Pd (mg/kg)	σ	UHP (mg/kg)	σ
PF	**B**	816.98 [a]	74.11	671.03 [a]	36.62	145.96 [b]	37.59	0.00	0.00	0.00	0.00	0.00 [e]	0.00	145.96 [a]	37.59
	B2	202.81 [e]	24.88	172.62 [d]	23.07	30.20 [e]	5.22	7.13*	9.41	15.44*	14.07	22.60* [c]	12.86	0.00 [c]	0.00
	B3	131.00 [f]	12.91	101.08 [f]	16.58	29.92 [e]	7.46	9.53*	5.33	11.92*	20.20	24.38 *[c]	2.80	0.00 [c]	0.00
GS	**B**	789.35 [a]	33.42	583.63 [a,b]	26.24	205.73 [a]	15.54	19.89	28.17	0.00	0.00	140.17 [a,b]	99.47	68.51 [b]	73.69
	B2	299.05 [c]	23.34	214.76 [c]	22.75	84.29 [c]	10.95	18.25	4.19	0.00	0.00	49.49 [a,b,c]	24.88	16.55 [b]	14.73
	B3	297.29 [c]	15.92	209.36 [c]	15.16	87.94 [c]	3.42	19.90	8.54	0.00	0.00	36.52 [b,c]	2.34	31.51 [a,b]	11.02
PH	**B**	654.42 [b]	59.80	518.95 [b]	37.42	135.47 [b]	22.38	21.32*	7.73	0.00	0.00	132.55* [a]	45.12	0.00 [c]	0.00
	B2	249.82 [d]	26.77	174.77 [d]	10.30	75.05 [c]	16.83	15.17	9.04	2.02	1.75	40.41 [b,c]	21.06	17.45 [b]	14.48
	B3	185.84 [e]	14.85	134.69 [e]	10.65	51.15 [d]	7.10	10.13	3.13	0.00	0.00	6.88 [d]	6.21	34.14 [a,b]	2.59

* enzyme hydrolysis would intermittently overestimate organophosphorus classes, possibly due to the overall dilute concentrations of organic phosphorus present in the soil samples. This was supported by enzyme hydrolysis assays on organic phosphorus substrates at 1µg/mL concentration.

[a-f] depth concentrations within a column labeled with a different letter superscript represent a significant difference between the values based on a Student's t test when the depth*season interaction was significant ($p < 0.05$)

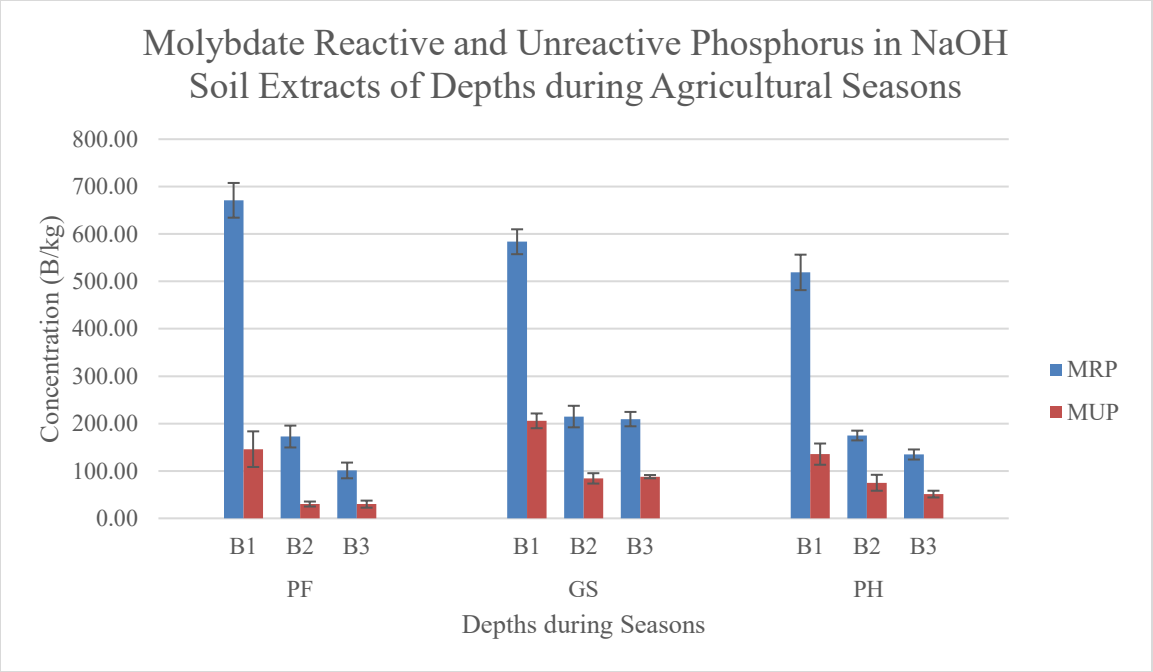

Figure 4.16: Mean concentrations of molybdate reactive P (MRP) and molybdate unreactive P (MUP) in 0.1M sodium hydroxide (NaOH) soil extracts from Depth B (0-20cm), B2 (20-40cm), and B3 (40-60cm) over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

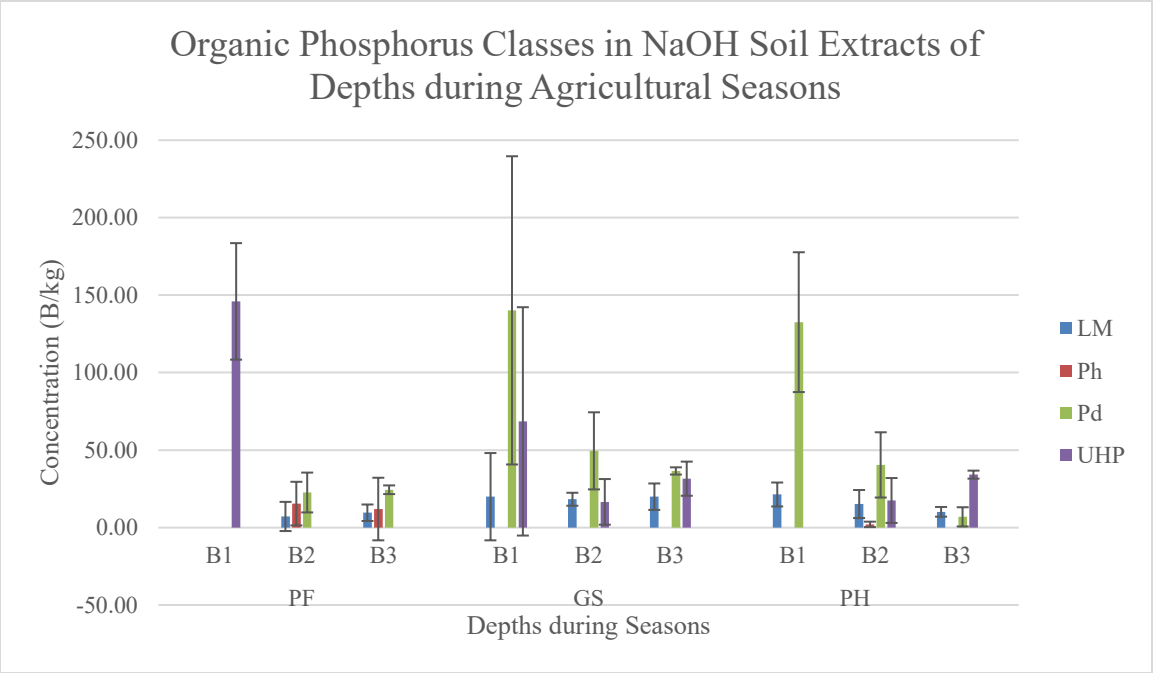

Figure 4.17: Mean concentrations of organic P species [labile phosphomonoesters (LM), phospholipids (PC), phosphodiesters (PD) and unhydrolyzable P(UHP)] in 0.1M sodium hydroxide (NaOH) soil extracts from Depth B (0-20cm), B2 (20-40cm), and B3 (40-60cm) over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

4.6.2.4. HCl

As mentioned for HCl field extracts, organic P species were not characterized for this fraction. Table 4.12 presents mean concentrations and their standard deviations for TP, MRP, and MUP of HCl extracts from Depth B, B2, and B3. Like in the field comparison, HCl was the largest fraction. There were significant effects for both depth and season. Depth B (786.18 – 1,640.62mg P/kg) was significantly greater than Depth B2 (606.45 – 1,066.79mg P/kg), while Depth B2 was significantly greater than Depth B3 (421.73 – 772.64mg P/kg). In regards to season, GS (772.24 – 1,640.62mg P/kg) and PH (772.64 – 1,572.42mg P/kg) were significantly greater than PF (421.73 – 786.18mg P/kg). Likewise, depth and season effects were significant for HCl MRP. Depth B had the most abundant concentrations of MRP with 750.12 – 1,522.95mg P/kg, followed by 568.53 – 1,066.79mg P/kg for Depth B2, and 404.18 – 772.64mg P/kg for Depth B3. For seasons, PF (404.18 – 750.12mg P/kg) was significantly less than GS (717.02 – 1,487.57mg P/kg) and PH (772.64 – 1,522.95mg P/kg). With HCl MUP, depth and season main effects were significant. Depth B (36.06 – 153.05mg P/kg) was significantly greater than Depth B2 (0.00 – 86.52mg P/kg) and Depth B3 (0.00 – 55.22mg P/kg). All seasons were significantly different with GS (55.22 – 153.05mg P/kg) greater than PF (17.55 – 37.92mg P/kg), which was greater than PH (0.00 – 49.47mg P/kg). Figure 4.18 exhibits the trends of MRP and MUP for HCl extracts of Depth B, B2, and B3 for all agricultural periods.

Table 4.12: Mean concentration values and their respective standard deviation (σ) for total phosphorus (TP), molybdate reactive phosphorus (MRP), and molybdate unreactive phosphorus (MUP) determined in 1M hydrochloric acid (HCl) soil extracts of Field B at three different depths for the pre-fertilizer (PF), growing (GS), and post-harvest (PH) agricultural periods. Depth B (0-20cm), B2 (20-40cm), and B3 (40-60cm) were statistically compared through a GLM ANOVA ($p < 0.05$) for depth and season main effects, as well as their interaction. If more than one replicate was zero or negative for both analytical and experimental replicates, the entire value was set to zero. Enzyme hydrolysis assays were not conducted on HCl extracts as organophosphorus model substrates were insoluble in 1M HCl and therefore could not determine if enzymes were functioning properly to hydrolyze organophosphorus compounds in HCl extracts.

1 M HCl - Depths							
Season	Field	TP (mg/kg)	σ	MRP (mg/kg)	σ	MUP (mg/kg)	σ
PF	**B**	786.18	25.76	750.12	12.12	36.06	19.39
	B2	606.45	68.46	568.53	22.92	37.92	49.30
	B3	421.73	15.83	404.18	35.09	17.55	19.26
GS	**B**	1640.62	211.29	1487.57	323.84	153.05	129.36
	B2	945.47	30.33	858.94	29.78	86.52	6.37
	B3	772.24	121.15	717.02	121.18	55.22	4.30
PH	**B**	1572.42	316.25	1522.95	275.08	49.47	41.77
	B2	1066.79	44.55	1066.79	54.72	0.00	0.00
	B3	772.64	40.57	772.64	42.70	0.00	0.00

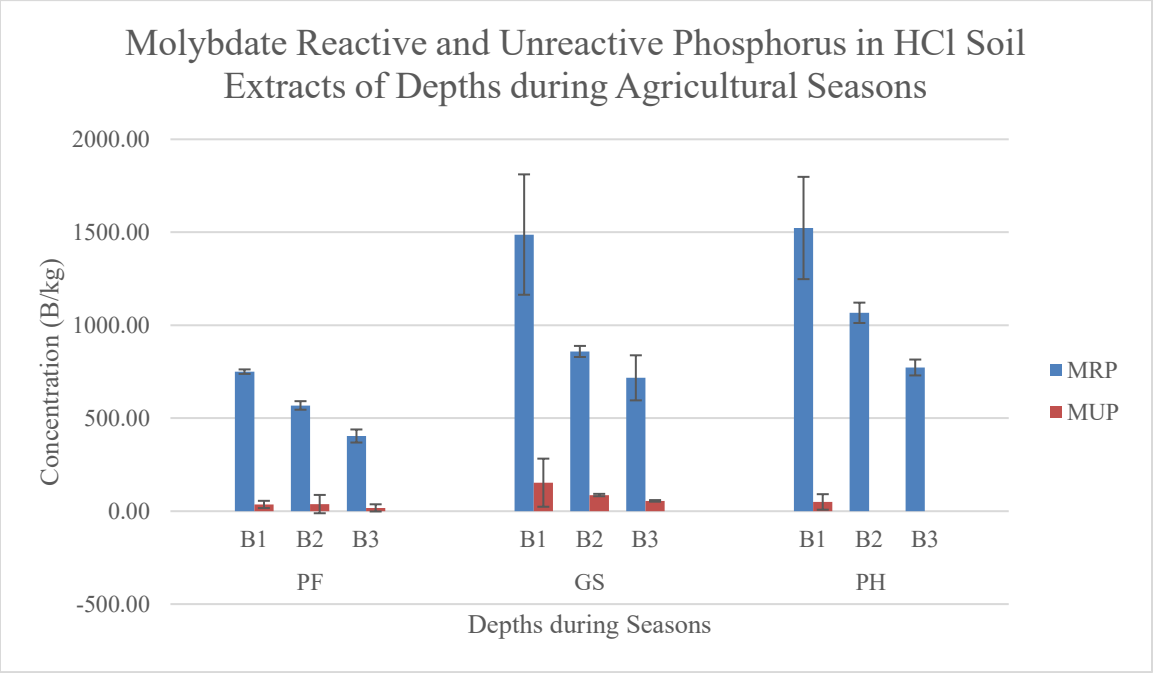

Figure 4.18: Mean concentrations of molybdate reactive P (MRP) and molybdate unreactive P (MUP) in 1M hydrochloric acid (HCl) soil extracts from Depth B (0-20cm), B2 (20-40cm), and B3 (40-60cm) over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

4.7. P Form Correlations

4.7.1. Correlation with Minerals

4.7.1.1. Aluminum (Al)

Mean aluminum (Al) concentrations and their standard deviations determined in non-sequentially fractionated 1M HCl soil extracts for Field A, C, and B (0-20cm), and Depth B2 (20-40cm) and B3 (40-60cm) across agricultural periods are presented in Table 4.13. Al concentrations ranged from 351 to 1,322mg/kg for all samples. For DH_2O extracts there was a significant negative Spearman correlation coefficient for TP (-0.64), MRP (-0.60), MUP (-0.55), LM (-0.33), and Ph (-0.33), and a positive significant correlation for Pd (0.47). In 0.5M $NaHCO_3$ extracts, TP, MRP, and MUP had negative Spearman coefficients of -0.53, -0.49, and -0.32, respectively. 0.1M NaOH extracts had significant negative correlations for TP (-0.57), MRP (-0.59), MUP (-0.56), and Pd (-0.56), while a positive correlation for Ph (0.55). Lastly, in 1M HCl extracts there was a significant negative Spearman coefficient correlation for TP and MRP with -0.39 and -0.36. However, these correlations were relatively weak overall. Correlation coefficients determined between Al and P forms in extracts are shown in Table 4.14.

4.7.1.2. Calcium (Ca)

For calcium (Ca), concentrations and their respective standard deviations for non-sequentially fractionated 1M HCl soil extracts of A, C, B, B2, and B3 across agricultural periods are displayed in Table 4.13. Out of all the minerals, calcium was greatest in concentration with 9,355 – 22,425mg/kg across agricultural periods. In DH_2O extracts, Spearman correlation coefficients were significantly positive for TP (0.43) and MRP (0.46). Pd had a significant negative Spearman correlation coefficient of -0.29. TP had a significant Spearman correlation

coefficient of 0.54 while MRP had a significant Pearson correlation coefficient of 0.60 for 0.5M $NaHCO_3$ extracts. In 0.1M NaOH extracts, a significant positive Spearman correlation coefficient was determined for TP (0.58) and MRP (0.56), while LM had a significant negative Spearman correlation coefficient of -0.39. A significant Pearson correlation coefficient was also found for MUP with 0.55. With 1M HCl, there was a significant positive Spearman correlation coefficient for TP (0.30) and MUP (0.40). As with Al, these significant correlations were not particularly strong. All correlations assessed for Ca are exhibited in Table 4.15.

4.7.1.3. Iron (Fe)

Table 4.13 presents the mean concentrations and standard deviations of Iron (Fe) for non-sequentially fractionated 1M HCl soil extracts of C, A, B, B2, and B3. Fe ranged from 1,652 to 4,085mg/kg for all soil samples across the PF, GS, and PH agricultural periods. Fe was determined to have a significant negative Spearman correlation coefficient of -0.31 for TP and -0.32 for MRP in DH_2O extracts. In contrast, Fe had a significant positive Spearman correlation coefficient with UHP of 0.38. For 0.5M $NaHCO_3$ extracts, there was only a significant Spearman correlation coefficient for Ph (-0.30). Significant positive Spearman correlation coefficients were found for TP (0.31) and MRP (0.33) in 0.1M NaOH extracts. A significant negative Spearman correlation coefficient was determined for LM (-0.38). There were no significant Spearman correlation coefficients for 1M HCl determined. As mentioned previously for Al and Ca, Fe had weak correlations. These correlation coefficients between Fe and P forms of extracts are displayed in Table 4.16.

4.7.1.4. Magnesium (Mg)

Mean concentrations for magnesium (Mg) and their standard deviations for non-sequentially fractionated 1M HCl soil extracts of A, C, B, B2, and B3 are presented in Table

4.13. For PF, GS, and PH agricultural periods, Mg concentrations ranged from 572 to 1,924mg/kg. In DH_2O extracts, a positive significant Spearman correlation coefficient was determined for TP (0.55) and MRP (0.58). Likewise, significant positive Spearman correlation coefficients were found for TP (0.44) and MRP (0.51) in 0.5M $NaHCO_3$ extracts. For 0.1M NaOH extracts, there were significant positive Spearman correlation coefficients for TP (0.48), MRP (0.45), MUP (0.55), and Pd (0.35). Mg was found to have a significant positive Spearman correlation coefficient with TP (0.30) and MUP (0.39) in 1M HCl extracts. Though significant, these correlations were not very strong. Table 4.17 shows the correlation coefficients for Mg and P forms in each extract for soil samples.

Table 4.13: Mean concentration values and their respective standard deviation (σ) for aluminum (Al), calcium (Ca), iron (Fe), and magnesium (Mg) in non-sequentially fractionated 1M hydrochloric acid (HCl) soil extracts of Field A, C, B (0-20cm), and Depth B2 (20-40cm) and B3 (40-60cm).

		Minerals							
Season	Sample	Al (mg/kg)	σ	Ca (mg/kg)	σ	Fe (mg/kg)	σ	Mg (mg/kg)	σ
PF	A	351	23	13255	591	2888	49	1083	83
	C	474	33	19808	955	2188	81	1687	177
	B	521	22	20235	1160	4045	36	1269	84
	B2	1078	62	14902	307	3662	146	872	49
	B3	1322	60	13365	1103	3518	93	914	14
GS	A	409	41	21002	835	3322	85	1924	99
	C	521	6	18738	825	1708	15	1807	62
	B	549	45	22425	1880	4085	166	1316	168
	B2	469	10	10708	1972	2605	356	661	165
	B3	691	7	9718	1937	2778	121	604	64
PH	A	358	120	11435	6002	2858	560	988	505
	C	409	31	15922	1566	1652	85	1303	174
	B	391	71	14952	3054	3272	448	856	178
	B2	863	64	9355	2468	2668	397	572	89
	B3	663	177	10565	4517	1968	248	897	583

Table 4.14: Spearman correlation coefficients and ρ values between P variables [total phosphorus (TP), molybdate reactive phosphorus (MRP), molybdate unreactive phosphorus (MUP), labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable organic phosphorus (UHP)] determined in sequentially fractionated deionized water (DH_2O), 0.5M sodium bicarbonate ($NaHCO_3$), 0.1M sodium hydroxide (NaOH), and 1M hydrochloric acid (HCl) soil extracts, and aluminum (Al) in non-sequentially fractionated 1M hydrochloric acid (HCl) soil extracts for all fields and seasons. $p < 0.05$ were determined to be significant.

	Al							
	DH_2O		$NaHCO_3$		NaOH		HCl	
Variable	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|
TP	-0.64	<.0001	-0.53	0.0002	-0.57	<.0001	-0.39	0.0075
MRP	-0.60	<.0001	-0.49	0.0007	-0.59	<.0001	-0.36	0.0164
MUP	-0.55	<.0001	-0.32	0.035	-0.56	<.0001	-0.23	0.1287
LM	-0.33	0.0268	0.07	0.6319	-0.21	0.1632		
Ph	-0.33	0.0245	0.02	0.8861	0.55	<.0001		
Pd	0.47	0.001	-0.10	0.5134	-0.56	<.0001		
UHP	0.07	0.6292	-0.28	0.0673	-0.18	0.2315		

Table 4.15: Spearman correlation coefficients and ρ values between P variables [total phosphorus (TP), molybdate reactive phosphorus (MRP), molybdate unreactive phosphorus (MUP), labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable organic phosphorus (UHP)] determined in sequentially fractionated deionized water (DH_2O), 0.5M sodium bicarbonate ($NaHCO_3$), 0.1M sodium hydroxide (NaOH), and 1M hydrochloric acid (HCl) soil extracts, and calcium (Ca) in non-sequentially fractionated 1M hydrochloric acid (HCl) soil extracts for all fields and seasons. $p < 0.05$ were determined to be significant.

	Ca							
	DH_2O		$NaHCO_3$		NaOH		HCl	
Variable	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|
TP	0.43	0.0032	0.54	0.0001	0.58	<.0001	0.30	0.042
MRP	0.46	0.0013	0.60*	<.0001	0.56	<.0001	0.24	0.1181
MUP	0.05*	0.7304	0.06	0.6778	0.55*	<.0001	0.40	0.0063
LM	0.12	0.4483	0.10	0.518	-0.39	0.0087		
Ph	-0.15	0.3197	-0.20	0.1829	-0.23	0.1264		
Pd	-0.29	0.0495	0.03	0.8469	0.28	0.0598		
UHP	0.25	0.0982	-0.06	0.6912	0.17	0.2664		

* if data of variable followed a normal distribution the Pearson correlation coefficient was utilized.

Table 4.16: Spearman correlation coefficients and ρ values between P variables [total phosphorus (TP), molybdate reactive phosphorus (MRP), molybdate unreactive phosphorus (MUP), labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable organic phosphorus (UHP)] determined in sequentially fractionated deionized water (DH_2O), 0.5M sodium bicarbonate ($NaHCO_3$), 0.1M sodium hydroxide (NaOH), and 1M hydrochloric acid (HCl) soil extracts, and iron (Fe) in non-sequentially fractionated 1M hydrochloric acid (HCl) soil extracts for all fields and seasons. $p < 0.05$ were determined to be significant.

	Fe							
	DH_2O		$NaHCO_3$		NaOH		HCl	
Variable	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|
TP	-0.31	0.0366	0.17	0.2506	0.31	0.0366	-0.20	0.1784
MRP	-0.32	0.032	0.21	0.1761	0.33	0.0263	-0.24	0.1139
MUP	0.01*	0.9302	0.21	0.1673	0.17	0.2543	0.08	0.5843
LM	-0.26	0.0869	0.26	0.0799	-0.38	0.0111		
Ph	-0.15	0.3183	-0.30	0.0438	0.17	0.2581		
Pd	0.05	0.7233	0.07	0.6456	-0.12	0.4398		
UHP	0.38	0.01	0.09	0.5646	0.01	0.9317		

* if data of variable followed a normal distribution the Pearson correlation coefficient was utilized.

Table 4.17: Spearman correlation coefficients and ρ values between P variables [total phosphorus (TP), molybdate reactive phosphorus (MRP), molybdate unreactive phosphorus (MUP), labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable organic phosphorus (UHP)] determined in sequentially fractionated deionized water (DH_2O), 0.5M sodium bicarbonate ($NaHCO_3$), 0.1M sodium hydroxide (NaOH), and 1M hydrochloric acid (HCl) soil extracts, and magnesium (Mg) in non-sequentially fractionated 1M hydrochloric acid (HCl) soil extracts for all fields and seasons. $p < 0.05$ were determined to be significant.

	Mg							
	DH_2O		$NaHCO_3$		NaOH		HCl	
Variable	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|
TP	0.55	<.0001	0.44	0.0025	0.48	0.0009	0.30	0.0485
MRP	0.58	<.0001	0.51	0.0004	0.45	0.0019	0.22	0.1474
MUP	0.09	0.5624	0.03	0.8443	0.55	<.0001	0.39	0.0081
LM	0.22	0.153	-0.04	0.8121	-0.26	0.0812		
Ph	-0.15	0.314	-0.04	0.776	-0.29	0.0564		
Pd	-0.27	0.0769	0.01	0.9349	0.35	0.0173		
UHP	0.15	0.3149	-0.12	0.4385	0.21	0.1714		

4.7.2. Correlation with Total Organic Carbon (TOC)

Table 4.18 displays total organic carbon (TOC) mean concentrations and standard deviations for sequentially fractionated extracts of soil samples from A, C, B, B2, and B3 across agricultural periods PF, GS, and PH. TOC concentrations in extracts were as follows: DH_2O (623.79 – 1,058.66mg/kg), $NaHCO_3$ (1089.04 – 6,703.94mg/kg), NaOH (3,553.07 – 75,680.00mg/kg), and HCl (3,141.75 – 8,803.54mg/kg). Unfortunately, the samples for HCl GS were contaminated and not included in analysis. TOC had a significant positive Spearman correlation coefficient for TP (0.64), MRP (0.62), MUP (0.69), LM (0.43), and UHP (0.45) in DH_2O extracts. For 0.5M $NaHCO_3$ extracts, there was a significant positive Spearman correlation coefficient for TP (0.36) and MRP (0.39). Ph had a significant negative Spearman correlation coefficient with -0.30. For both 0.1M NaOH and 1M HCl extracts, no significant correlation was determined. Table 4.19 exhibits these correlations between TOC and P forms for extracts of each sample.

Table 4.18: Mean concentration values and their respective standard deviation (σ) for total organic carbon (TOC) determined in sequentially fractionated deionized water (DH_2O), 0.5M sodium bicarbonate ($NaHCO_3$), 0.1M sodium hydroxide (NaOH), and 1M hydrochloric acid (HCl) soil extracts of Field A, C, B (0-20cm), and Depth B2 (20-40cm) and B3 (40-60cm). "-" signifies that the sample was contaminated and therefore excluded from the correlation analysis.

TOC

		DH_2O		$NaHCO_3$		NaOH		HCl	
Season	Sample	TOC (mg/kg)	σ	TOC (mg/kg)	σ	TOC (mg/kg)	σ	TOC (mg/kg)	σ
PF	A	972.30	25.58	3643.20	107.60	13900.00*	2901.97	8435.09	810.88
	C	1053.25	25.52	4777.76	773.42	17608.00	7008.57	6599.15	1867.48
	B	920.47	16.63	6703.94	268.18	53708.00*	2392.85	4693.22	183.53
	B2	718.18	45.76	6024.20	648.02	27206.67*	1517.92	3552.00	502.44
	B3	698.89	51.17	5051.57	470.74	22527.11	6529.46	3821.74*	2257.84
GS	A	623.79	62.27	4762.43	131.86	3712.98*	523.36	-	-
	C	697.13	61.03	4492.88	249.14	42640.00	1808.42	-	-
	B	746.71	29.87	6504.55	731.98	32140.00*	9475.23	-	-
	B2	665.18	52.43	1365.12*	637.57	3553.07*	575.34	-	-
	B3	686.54	61.80	1938.76	276.29	45835.56	4774.49	-	-
PH	A	867.65	28.57	1656.26	24.57	19465.33	9483.36	8803.54	1040.46
	C	960.52	6.27	2019.27	69.42	37608.89	2758.30	6360.00	481.63
	B	1058.66	25.75	2976.44	63.65	75680.00	15519.18	4193.60	1050.75
	B2	748.57	61.21	2167.11	199.61	4970.96	1417.22	3982.35	730.45
	B3	746.59	35.74	1089.04	207.10	40393.33*	6288.54	3141.75	544.96

* mean and standard deviation were calculated from only two replicates as instrument malfunctioned from salt concentration of extracts.

Table 4.19: Spearman correlation coefficients and ρ values between P variables [total phosphorus (TP), molybdate reactive phosphorus (MRP), molybdate unreactive phosphorus (MUP), labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable organic phosphorus (UHP)] determined in sequentially fractionated deionized water (DH_2O), 0.5M sodium bicarbonate ($NaHCO_3$), 0.1M sodium hydroxide (NaOH), and 1M hydrochloric acid (HCl) soil extracts, and total organic carbon (TOC) from same extract. $p < 0.05$ were determined to be significant.

	TOC							
	DH_2O		$NaHCO_3$		NaOH		HCl	
Variable	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|	Correlation Coeff	Prob>|ρ|
TP	0.64	<.0001	0.36	0.016	0.24	0.1391	0.27	0.1646
MRP	0.62	<.0001	0.39	0.0088	0.25	0.1373	0.17	0.3795
MUP	0.69	<.0001	0.15	0.3392	0.10	0.5467	0.36	0.0565
LM	0.43	0.003	0.10	0.5105	0.17	0.3164		
Ph	-0.09	0.5583	-0.30	0.0488	-0.21	0.2126		
Pd	-0.18	0.2377	0.11	0.4855	-0.05	0.7601		
UHP	0.45	0.0021	-0.06	0.7001	-0.09	0.5835		

4.8. Drainage

Mean concentrations and respective standard deviations of TP, MRP, and MUP of drainage effluent across agricultural periods for Field A, B, and C are presented in Table 4.20. Organic P species were not determined as there was no MUP in significant concentrations identified. There was no drainage effluent from Field A in PH and therefore could not be assessed. TP was found to have significant main effects for field, season, and their interaction. Field A was significantly less (0.00 – 0.01mg P/L) than Field C (0.12 – 0.33mg/L) and Field B (0.04 – 0.45mg/L). Over the agricultural periods, Field C decreased from PF (0.33mg P/L) to GS (0.12mg P/L), which stabilized into PH (0.14mg P/L). For Field B, PF and GS were similar with 0.05 and 0.04mg P/L, respectively. However, B rose significantly in PH to 0.45mg P/L. A in PF and GS were not significantly different ranging from 0.00 to 0.01mg P/L. For MRP, there were also significant main effects for field, season, and their interaction. Though MRP made up all of TP, MRP had a slightly different student's t test result in determining significant differences between fields, where B and C were also statistically different. This was due to the difference between TP and MRP in replicate values. As mentioned above, significant concentrations of MUP were absent from effluent. MRP trends over the agricultural periods for drainage are displayed in Figure 4.19.

Table 4.20: Mean concentration values and their respective standard deviation (σ) for total phosphorus (TP), molybdate reactive phosphorus (MRP), and molybdate unreactive phosphorus (MUP), determined in tile drainage effluent from Field A, B, and C for the pre-fertilizer (PF), growing (GS), and post-harvest (PH) agricultural periods. Field A, B, and C were statistically compared through a GLM ANOVA ($p < 0.05$) for field and season main effects, as well as their interaction. If more than one replicate was zero or negative for both analytical and experimental replicates, the entire value was set to zero. If the subtraction of TP and MRP resulted in a negative value, then TP was assumed to be the same as MRP, and set to equal MRP. Enzyme hydrolysis assays were not conducted on drainage effluent as MUP concentrations were zero. Field A was omitted from the PH drainage analysis as there was no drainage from this field during this season.

		Drainage					
Season	Field	TP (mg/L)	σ	MRP (mg/L)	σ	MUP (mg/L)	σ
PF	**A**	0.00 ^{d}	0.03	0.00 ^{e}	0.02	0.00	0.00
	B	0.05 ^{d}	0.02	0.05 ^{d}	0.02	0.00	0.00
	C	0.33 ^{b}	0.01	0.33 ^{b}	0.01	0.00	0.00
GS	**A**	0.01 ^{d}	0.01	0.01 ^{e}	0.00	0.00	0.00
	B	0.04 ^{d}	0.02	0.04 ^{d}	0.02	0.00	0.00
	C	0.12 ^{c}	0.01	0.12 ^{c}	0.01	0.00	0.00
PH	**B**	0.45 ^{a}	0.09	0.45 ^{a}	0.05	0.00	0.00
	C	0.14 ^{c}	0.01	0.14 ^{c}	0.01	0.00	0.00

$^{a\text{-}e}$ field concentrations within a column labeled with a different letter superscript represent a significant difference between the values based on a Student's t test when the field*season interaction was significant ($p < 0.05$)

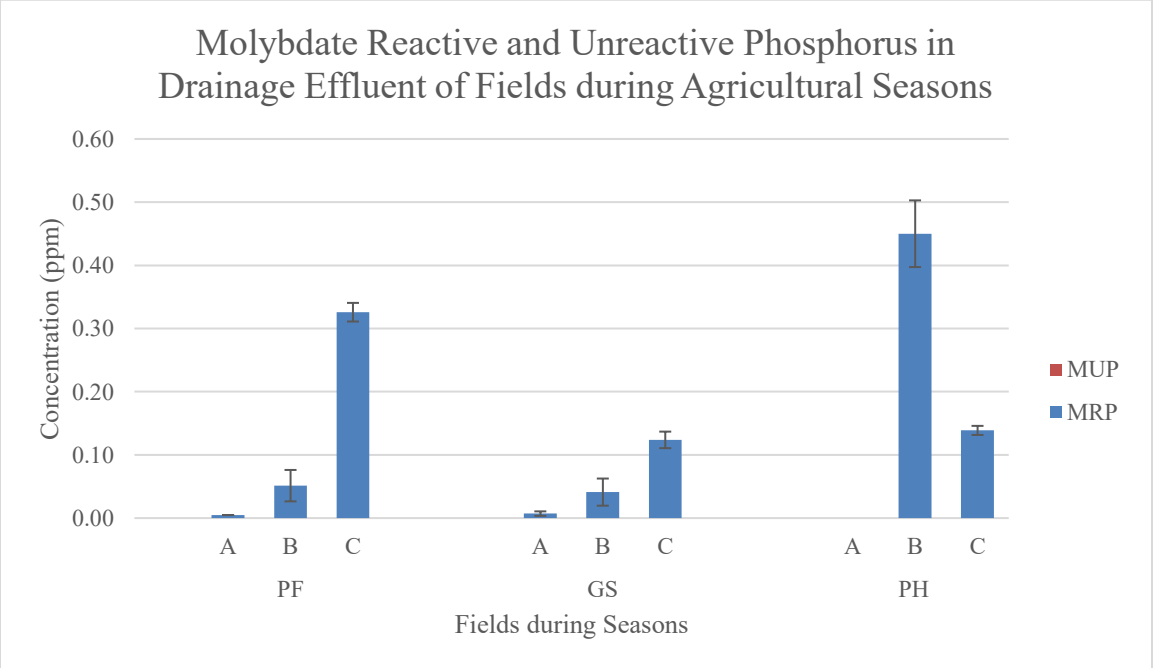

Figure 4.19: Mean concentrations of molybdate reactive P (MRP) and molybdate unreactive P (MUP) in drainage effluent from Field A, B, and C over the agricultural periods pre-fertilizer (PF), growing season (GS), and post-harvest (PH).

5. Discussion

5.1. Fields

In regards to the distribution of fractions, MRP dominated in all extracts. Thus, as MRP is an operational term for estimated orthophosphate from molybdate blue method, the fields in this study were most abundant in inorganic P. This is contrary to other studies which have determined up to 50% of total P in fields is organic P (George et al., 2011). Young and colleagues identified as much as 75% MUP, the operational term for organic phosphate from the molybdate blue method, in DH_2O soil extracts; however, the research focused on unfertilized pasture, much different from the arable Histosols of this study (2013). However, in agricultural organic soils of North Carolina, inorganic P was determined to be the largest form present (Sundareshwar et al., 2008). Due to this large portion of inorganic P, organic P's role in these agricultural Histosol systems does not seem substantial. Indeed, the portion of organic P in DH_2O, operationally defined as available P in sequential fractionation procedures, and $NaHCO_3$, operationally defined as moderately labile in sequential fractionation procedures, extracts ranges between 0-1% throughout the agricultural periods. Often, there is no growth in organic P portion for fields with large mineral fertilizer inputs unaccompanied by substantial organic matter input (Oberson et al., 2011; Keller et al., 2012). Yet, in regards to total P in fields, inorganic available P (DH_2O inorganic P) was only a slight portion, as found in other studies (McCray et al. 2012). Farming practices often reduce availability of P as determined by Castillo & Wright in comparing arable Histosols to natural wetlands (2008). A larger portion of organic P was determined in NaOH extracts, but these signify minerally-bound P (non-labile organic P) which is occluded from plants and microorganisms alike. Only in situations of high moisture and optimal pH (ph 6-7) would it be likely for sesquisoxides of Al and Fe to dissolve (Walbridge &

Richardson, 1991). Even if released, the organic P would require proximal phosphatase enzymes to be rendered available to both crop and microorganisms. Most apparent in this study was the magnitude of the inorganic P represented in the HCl fraction, operationally defined as unavailable Ca-bound P pool (non-labile inorganic P). In addition, over the course of the agricultural periods, this portion steadily increased. This HCl pool may function as a sink for P in organic soils, like the NaOH pool for mineral soils, as determined in other studies (Oberson et al., 2001; He et al., 2008). Some long-term studies have identified as much as an eight-fold P increase in the HCl portion (He et al., 2008). Similar to this study, Qian and colleagues determined significant proportions of inorganic P in NaOH and HCl fractions for a rice farm on riparian soil with nitrogen phosphorus and potassium fertilizer application (2017). Most likely, these large stores of non-labile P represent "legacy P", or P retained in the soil from historical applications due to its rapid sorption and occlusion over time (Vu et al., 2010; Withers et al., 2014). The original conceptual model of applied P fertilizer in agricultural soils presented by Barrow (1979) hypothesized that applied P would initially adsorb to the soil environment (moderately labile P) and over time become more recalcitrant in stable mineral forms (non-labile P) (Vu et al., 2010). The results of this study agree with this conceptual model as other studies have, specifically ^{32}P and ^{33}P isotope tracer analyses. However, it has been found that the fate of P in agricultural soils is dictated mostly by soil type. For example, with tropical, highly weathered mineral soils like Oxisols, fertilizer P (inorganic and organic) only affected inorganic P stocks in the soil, especially available and non-labile inorganic P, perhaps due to the minor quantities of organic matter present in these soils (Oberson et al., 1993; Buhler et al., 2002; Bünemann et al., 2004). In this study, DH2O inorganic P (available inorganic P) remained relatively constant while organic P was dynamic, such as the increase in HCl organic P (non-

labile organic P) over the agricultural periods. However with ^{32}P and ^{33}P isotope tracer analyses, it must be noted that these studies track the isotope over the half-life of the isotope (14.3 days for ^{32}P and 25.3 days for ^{33}P) and may not represent the dynamics of P over the long term (Frossard et al., 2011). Vu and colleagues found that in ^{32}P isotope experiments in cultivated Calcarosols, Chromosols, and Vertosols, most applied P became minerally-bound (non-labile inorganic P), especially for Chromosols, though it also did appear in the available P portion of the soil to varying degrees (Vu et al., 2014). In addition, path analysis and other ^{32}P isotope studies have suggested that the $NaHCO_3$ pool (moderately labile P) actually represents a transitory pool such as increasing available P concentrations in Mollisols or accumulating non-labile organic P in Ultisols (Tiessen et al., 1984; Zhang & Mackenzie, 1997; Zheng et al., 2002; Zheng et al., 2004). Though the proportion of the $NaHCO_3$ was relatively small and static over the agricultural periods in the study of this thesis, samples were collected only three times and may have not captured $NaHCO_3$ as a transitory pool. However, similar to the results of this research a study utilizing X-ray absorption near-edge structure spectroscopy in combination with chemical fractionation on agricultural Inceptisols of Quebec, determined that a majority of P present in the soil was bound to Ca (non-labile P) regardless of measured soil pH (Beauchemin et al., 2003).

As mentioned previously, though there are few studies on fractionation of P in various pools of Histosols. However, Schlicting and colleagues conducted a thorough fractionation procedure on an arable Histosol field in Germany determining a relatively large proportion of available P in the topsoil (20%) which contrasts the low available P portion in this study; however, they had measured available P with resin and could be a procedural difference (Schlicting et al., 2002). In comparison to mineral soils, Schlicting had found large proportions of NaOH- non-labile organic P (25%), like this study which found 4-12%, and may suggest

organic P exists in sizeable concentrations with mostly organic soils (Schlicting et al., 2002). Although this study had more (43-76%), Schlicting and colleagues also found 34% of P in the Ca-bound (non-labile) pool, which they attributed to precipitation of acid-soluble phosphate and carbonate in their field; however they utilized 1M H_2SO_4, as opposed to 1M HCl in this study to determine Ca-bound P (Schlicting et al., 2002).

With DH_2O extracts, representing the available pool, both Field B and C remained relatively constant in amounts of inorganic P throughout the agricultural periods. This is notable as this is the pool from which both crop and microorganism can most easily assimilate P. In a review by Negassa & Leinweber, analyses suggested that increased fertilizer application leads to greater available P, as much as a four-fold increase with 75kg P/ha in one study (2009). Yet, as concentrations remained relatively constant after fertilization, this effect was not seen. Field A was more erratic over the growing seasons; however, it may not be comparable as Field A is an experimental field while both Field B and C are commercial farms. Field C was significantly larger in inorganic P concentrations than Field B and especially Field A; this is unexpected as one of the studied benefits of drainage control structures is increased P availability in soils (Williams et al., 2015). However, the water table never rose past 20cm so consequently it may not have affected the topsoil. Crop and microbial uptake could be one explanation for depleted available P in Field A and B, which was not assessed by this study. Field A may have also been low in available inorganic P because a proper water table was not able to be maintained by the drainage control structure due to lateral flow from the field. In addition, Field C had greater P fertilizer application, a plausible explanation for larger available inorganic P concentrations. Also noteworthy, total organic P (MUP) decreased in concentration during the growing season,

perhaps suggesting that this available pool of organic P was mineralized during the growing season to maintain the concentrations of available inorganic.

The organic P speciation analysis of the available P pool (DH_2O) determined no trend between fields, proposing that drainage control does not impact these species in the available pool. However, this could simply be due to the fact that the water table never rose above 20cm to affect the topsoil. Labile phosphate monoesters (LM) were in consistent but low concentrations, insufficient as a viable source of crop nutrition. LM concentrations are often sugar phosphates, the byproduct of complex organic material decomposition (Ivanhoff et al., 1998). This may imply microbial activity is occurring in fields, decaying organic matter. However, as concentrations remained relatively constant across agricultural periods, immobilization by the microorganisms may be occurring. Phospholipid (Ph) concentrations swelled in GS and PH, perhaps a result of increased microbial activity due to crop growth. Originating from plants, microorganisms, and animals, Ph are frequently in concentrations ranging from 0.2 to 14mg P/kg in soils, similar to the concentrations determined in this study (Quiquampoix & Mousain, 2005). In the available pool, phosphodiesters (Pd), primary components of microbial biomass, were not detected (Ivanhoff et al., 1998). This diverges from conclusions made by Turner and colleagues, who determined greater concentrations of Pd than LM in DH_2O extracts; however, the analysis by Turner was conducted on grassland soils (2002). Pd is known to degrade rapidly as it has only one ionizable proton and leaves it vulnerable to deterioration (Stutter et al., 2015). This excludes DNA which is a stable molecule (Rastogi, 2005). Unhydrolyzable organic P (UHP) decreased in concentration over the agricultural periods, possibly being decomposed into more labile or inorganic P. These forms of P are thought to consist of complex microbial cell debris and high molecular weight P compounds (Turner et al., 2002).

In $NaHCO_3$ extracts, representing the moderately labile pool, Field B had a greater abundance of inorganic P, perhaps a consequence of the drainage control structure. Field A's concentration of inorganic P was not as large, but could be a difference due to its inability to maintain a constant water table, as mentioned previously. Of course, both Field A and C may have a lower concentration of inorganic P due to crop uptake as well. Inorganic P from this moderately labile pool has been an index of P crop availability for Ontario since 1969, known as Olsen P (Bates, 1990). As in the available pool, inorganic P was relatively constant for fields. The moderately labile and available pool are thought to be in equilibrium with each other, which may maintain their relatively constant inorganic P concentrations across agricultural periods (Negassa & Leinweber, 2009). There was a decline in total organic P (MUP) during the growing season, but then replenished in post-harvest for each field. Organic P from the moderately labile P pool is believed to be mineralized to support crop growth (Ivanhoff et al., 1998). In comparison to the study by Schlicting and colleagues on an arable Histosol field in Germany, the concentrations of inorganic P (MRP) were similar, with this study determining concentrations 88.68 – 145.83mg P/kg while the study by Schlicting and colleagues found a concentration of 123mg P/kg; however, the organic P (MUP) concentrations of this study did not agree with their findings, 0.00 – 13.39mg P/kg and 171mg P/kg, respectively (Schlicting et al., 2002).

For organic P species, LM was shown to increase during GS but only for fields with a drainage control structure, Field A and B. However, further analysis would need to be conducted to relate LM to drainage control. There were large, variable concentrations of Pd in this pool, which conflicts with other studies, where Pd rapidly degrades in soil (Quiquampoix & Mousain, 2005; Jones & Oburger, 2011). However, variable Pd concentrations were identified in a study by Johnson & Hill (2010). There was rarely any UHP determined except in PH for Field B; this

may suggest an active microbial population efficient in decomposition and general subsidence associated with Histosols.

For the nonlabile pool, NaOH, there were large portions of both inorganic and organic P. As in the moderately available pool, B had the greatest concentration of inorganic P, possibly related to the presence of the drainage control structure. This is not expected as a higher water table would create reducing conditions that would dissolve these mineral complexes (Walbridge & Richardson, 1999). Unlike the other pools, there was a reduction in inorganic P for PH, perhaps replenishing the available pools; laboratory studies have given credence to this hypothesis (Oberson et al., 2001; He et al. 2008). With organic P, Field A and B had the greatest concentrations; drainage control structures can create higher water tables which may prevent the oxidation of organic material, preserving organic P (Castillo & Wright, 2008). The most abundant concentrations of organic P in this fraction occurred in the GS. This may imply that residue inputs are greater than mineralization; both Field B and C till in unwanted yield. In addition, mineralization by microbes may not be warranted due to the surplus of inorganic P available (Jones & Oburger, 2011). The study by Schlicting et al. (2002) determined an inorganic P concentration of only 112mg P/kg in their arable Histosol field, while the topsoil of the Histosol fields in this study ranged between 233.58 – 671.03mg P/kg. In contrast, organic P (MUP) in this NaOH-non-labile pool was much greater for the German Histosol field, where a concentration of 481mg P/kg was determined, while the fields in this study had concentrations of only 95.44 – 221.19mg P/kg (Schlicting et al., 2002).

Organic P speciation analyses determined greater concentrations of LM in GS and PH, possibly a result of the organic residue inputs mentioned above or the oxidation of organic matter. Subsidence is common in drained agricultural Histosols and allows for rapid

decomposition of organic matter, which may be the source of this LM (Castillo & Wright, 2008). In NaOH-EDTA extracts for PNMR, Annaheim et al. (2015) determined 11-19mg P/kg for LM in a Luvisol, similar to the concentrations of this study. However, one must note that EDTA was present in the extract and that NaOH had a greater molarity (0.25M). No Ph was present, but may be due to their sporadic dynamics, where they are often rapidly degraded and resynthesized again (Quiquampoix & Mousain, 2005). By far, Pd was greatest and most variable in this fraction. This may represent microbial biomass as cells lyse easily upon rapid rewetting of soil (Turner et al., 2002). However, Pd is a main component of organic inputs and could originate from tilled-in crop residue (Nannipieri et al. 2011). These concentrations are in stark contrast to other studies where either none are detected or concentrations between 1-4mg P/kg (Annaheim et al., 2015; Stutter et al. 2015). Yet, these are P NMR studies that require high pH and alkalinity to be conducted, and therefore may have inherent experimental artifacts. There were consistent concentrations of UHP throughout the seasons without any apparent trend. This is similar to other studies which identify a significant proportion of OP from this fraction to be more complex organic P compounds (Annaheim et al. 2015; Jarosch et al. 2015).

As mentioned above, the HCl fraction of inorganic P was largest, with Field B and C having the most abundant concentrations. This could be attributed to the large, continuous inputs of fertilizer on these commercial farms (Castillo & Wright, 2008). Management techniques such as liming and tilling have been known to increase this fraction due to the presence of Ca, which compounds with P (Castillo & Wright, 2008; Negassa & Leinweber, 2009). A study in the Everglades Agriculture Area, a region of Histosol farms, by Castillo & Wright found that calcium from the underlying limestone bedrock was being brought closer to the surface through tillage and subsidence (2008). Indeed, the bedrock of the Holland Marsh is also limestone; thus,

a similar event could be occurring (Ivanhoff et al. 1998; Gerber et al., 2004). Large concentrations of Ca were detected in this study, but they were determined to not correlate. The growth of inorganic P in this fraction did occur after fertilization and may suggest HCl is a sink for applied fertilizer. Beauchemin and colleagues found a large store of P associated with Ca in their study on Inceptisols, similarly (2003). There was also a build-up of organic P in HCl for the GS, however these concentrations were small in comparison to the inorganic P of this fraction. Though in their study 1M H_2SO_4 was utilized to determine Ca-bound P, Schlicting et al. (2002) determined a large concentration of total P in this fraction (653mg P/kg) which they attributed to the precipitation of acid soluble phosphates. With this study, total P concentration of the 1M HCl fraction were 588.82 – 1,771.57mg P/kg. This study did attempt to analyzed organic P species in this fraction as the analysis encountered difficulties in dissolving model organic P substrates. Therefore, enzyme function could not be properly assessed. However, as organic P was determined in this analysis, its study is worthwhile as He et al. (2006) found. It must be mentioned that the concentrations of P found in the HCl fraction may be subject to experimental artifacts. For example, acid hydrolysis of the organic matter present in these soils by 1M HCl may release P in the organic matter, therefore erroneously increasing P concentrations in this fraction (Rumpel et al., 2008). Alternatively, P concentrations in this fraction may be higher in reality if some P forms become trapped in the aromatic carbons or melanoidins that result from acid hydrolysis (Rumpel et al., 2008). Further research should address the appropriateness of the sequential fractionation method on soils rich in organic matter.

Overall, the Histosol fields of this study had large concentrations of P. This can be attributed to legacy P most likely as well as the high sorption capacity of organic soils due to organic matter (Schlicting et al., 2002; Withers et al., 2014). However organic P concentrations

in the pools were lower than concentrations in the Schlicting et al. (2002) study. This could be due to possible greater subsidence and decay of organic material or heightened microbial mineralization in the Histosols of this study.

5.2. Depths

As with the fields, inorganic P was the dominant form in all fractions assessed for depths. Dynamics among the depths were different. For example, DH_2O MRP increased in Depth B3, possibly suggesting seepage. Yet, 1% of organic P in the available pool disappeared in GS when the 1% increase in inorganic P occurred for this layer, perhaps resulting from organic P mineralization. This could be a chance occurrence, however, and be a shift from either another layer (seepage) or from another pool like the moderately labile pool. Certain portions were stable across seasons such as NaOH-non-labile organic P. For most of the inorganic fractions, Depth B always had a larger proportion, followed by Depth B2 and B3. However, this was not the case in HCl, the unavailable Ca-bound pool, where Depth B2 and B3 had the greatest portion of inorganic P in this fraction, increasing from PF to PH. This could be due to the proximity of the calcium-containing limestone bedrock at these depths.

With the available pool of DH_2O, Depth B had the greatest concentrations of inorganic P followed by Depth B2 and B3. Season played a significant role where for Depth B2 these concentrations grew throughout the season possibly due to seepage. Likewise, Depth B3 grew in concentration for GS but decreased in PH, suggesting seepage out of the layer. Crop uptake could be a possible reason for a decrease in PH for Depth B3 as carrot roots can reach as deep as 60cm (Bruner, 1926). However, the fluctuating water table due to the water table management structure most likely prevented roots from penetrating this deep. In this pool, organic P was much greater in Depth B, but Depth B2 and B3 were almost identical in concentration across

agricultural periods. Though Depth B decreased in GS, both Depth B2 and B3 remained relatively stable, implying immobilization. This could be due to the little soil organic matter available deeper in the soil profile which impedes mineralization (Whalen & Sampedro, 2010).

With organic P speciation, LM was stable and possibly immobilized across all agricultural periods. Interestingly, there were similar concentrations across all depths, which means that there is a greater proportion of available organic P deeper in the soil profile. There was a build-up of Ph for all depths in GS and PH which could be the signature of both microbial breakdown of organic matter and the microbial population itself. For Depth B2 and B3, it is also possible that these OP forms leach from the topsoil (Thomas & Sevean, 1985). It is assumed that there is less microbial activity below topsoil without organic matter and the rhizosphere of crops (Jones & Oburger, 2011). Pd was detected in PF and PH, but only for Depth B3, which was unexpected as there is less microbial activity deeper in the soil profile. UHP present in this available pool diminished with season, perhaps suggesting it was decomposed and mineralized. However, further analysis must be conducted to assess this.

In the moderately labile pool of $NaHCO_3$, inorganic P followed the same pattern of the available pool, with decreasing inorganic P with depth in soil profile. There were also similar dynamics in that Depth B2 grew in inorganic P with season, most likely due to seepage, while Depth B3 increased during GS but decreased in PH, which suggests seepage or exchange across fractions. Organic P was more sporadic in this fraction than in the available pool, and may have been mineralized during GS due to its decrease from PF to GS, while also explaining the increase in inorganic P at these depths. However, microbial activity tends to decrease with depth as mentioned previously.

With organic P species, LM was shown to increase in GS, similar to fields. This could be due to rapid decomposition by the microbiome, as suggested for fields. Ph concentrations were more variable, most likely due to the constant flux of degradation and resynthesis found in soils (Quiquampoix & Mousain, 2005). However, there was none determined for Depth B. Pd was determined for every depth in every agricultural season but was greater in PF, which may indicate microbial biomass increased due to warmer weather and moisture. However, if so, one would expect for the concentrations to remain as high in GS. UHP was only present in Depth B at PH, possibly the result of crop residue remaining at the surface of the field.

The nonlabile pool of NaOH, had large overall concentrations of inorganic P and followed the same decreasing concentration stratification in the soil profile. However, while the concentrations in Depth B decreased over seasons, both Depth B2 and B3 grew in concentration during GS but decreased in PH. Like the other pools, it could indicate seepage from layers. Organic P was greater in concentration for Depth B, while Depth B2 and B3 fluctuated, increasing during GS, but remained relatively equal in concentration.

For organic species, LM was similar in concentration across all depths and more abundant in GS and PH than PF. This may be due to decomposition of organic matter by an active microbial population releasing LM which seeps into lower depths. While there was no Ph in Depth B, there were large quantities initially for Depth B2 and B3, though diminishing in PH. Pd concentrations in this fraction were large and variable, implying microbial turnover. This is distinct compared to studies where Pd is the minority of organic P species, if at all detected (Quiquampoix & Mousain, 2005; Annaheim et al. 2015; Stutter et al., 2015). Each depth followed different dynamics where Depth B2 remained relatively constant whereas Pd in Depth B3 shrunk in PH and Depth B increased in concentration during GS. The source of this Pd could

be microbial populations thriving in warmer temperatures after the winter thaw. However, these organic P species may also be of plant origin, from microbial breakdown of the organic matter. Though larger in concentration than other fractions, this pool of minerally bound P is not readily accessible. Lastly, UHP was greater in this pool than other fractions but variable in fluctuations. Depth B2 had the greatest concentrations but diminished with each agricultural season. This may be due to microbial breakdown. In contrast, UHP increased in Depth B2 and B3 either suggesting seepage or immobilization due to depth in soil profile. UHP from crop residues migrating through the soil layers could also be the source of the increase at these depths.

As with fields, the stable calcium-bound pool (non-labile) possessed the greatest concentration of P for depths. In addition, Depth B, B2, and B3 all increased in concentration post-fertilizer. This suggests that this HCl pool may be a sink not only for fields but also for depths. Though this decreases lability for crops, it reduces the hazard of eutrophication (Castillo & Wright, 2008). As expected, there was more organic P at Depth B, due to greater microbial activity and organic P inputs. Organic P swelled during GS, implying immobilization, but decreased in PH, to zero for Depth B2 and B3, perhaps due to incorporation of organic residues at all depths triggering mineralization, or seepage.

5.3. Correlation of P Forms

Overall, correlations between P forms were quite weak, often with correlation coefficients less than 0.60, though significant. Organic P species seemed to have no relation with either minerals or TOC. All of the correlations with Al were low and negative in correlation. Ca had stronger relationships, such as with moderately labile inorganic P which had a correlation coefficient of 0.60 or NaOH-non-labile total P with a correlation coefficient of 0.58. However, this study expected to find a strong significant correlation between Ca and HCl P forms, due to

the large quantities of P in the HCl fraction and quantities of Ca determined in fields. However, the correlations were weak with a correlation coefficient of 0.40 between Ca and inorganic P of the HCl fraction. This may be due to the fact that minerals were extracted with non-sequentially fractionated 1M HCl soil extracts; other studies have utilized oxalate extractable minerals for correlation studies of minerals (Schlicting et al., 2002). However, Castillo & Wright (2008) did not assess a correlation but assumed that Ca from the limestone bedrock was binding the P in the HCl pool. Indeed, it is worth mentioning that the sequential fractionation procedure provides operational terms for pools and could behave quite differently in organic soils (Castillo & Wright, 2008; Negassa & Leinweber, 2009). With Fe there were no strong correlations either. Though Mg is not usually included in these analyses, Mg is an activator of phosphatase enzymes so was assessed in the correlation analysis (Jones & Oburger, 2011). However, the strongest correlation was 0.58 with inorganic P in DH_2O extracts. TOC seemed to be most related, with a correlation coefficient of 0.64, 0.62, and 0.69 for total P, inorganic P, and total organic P, respectively. Jarosch and colleagues also determined weak correlations between soil characteristics and P forms (2015). Other studies have had strong correlations with TOC, suggesting the importance in its characterization for P analyses (Stutter et al., 2015; Qian et al., 2017). The relationship of P with TOC may be indicative of the complexation of P forms with organic matter. For example, in highly weather tropical soils which have issues in retaining P, the addition of organic matter, like biochar or straw, in conjunction with mineral P fertilizer increased P availability (Goyal et al., 1999; Lehmann & Rondon, 2006). In addition, the relationship may be due to the fact that organic matter is the energy source of the microbiome; therefore, with more organic matter these is an increase in P forms, whether originating from the microbial population itself or the organic matter that they breakdown (Belnap, 2011).

5.4. Drainage Effluent

With drainage, inorganic P was the main P form as negligible concentrations of organic P were found. Though drainage control seemed effective in reducing concentrations of inorganic P in effluent for most of the year, in PH, B had a significant concentration of inorganic P greater than C in all other seasons. As mentioned previously, there has been varied success with drainage control, where significant concentrations of P in effluent have been determined (Stämpfli & Madramootoo, 2004). The concentrations determined from the tile drainage effluent of these fields, besides Field A, are concerning as they are over 0.03ppm, the Provincial Water Quality Objective (PWQO) of Ontario and suggested threshold of the Government of Canada (Chambers et al., 2012; OMEE, 2017). However, as this is not time-series data, it may not be an accurate impression of the actual dynamics in drainage effluent. As negligible amounts of organic P were determined, organic P speciation analysis was not carried out. However, other studies have been able to. Toor et al. (2003) determined that effluent from a grassland soil contained more than 50% enzyme hydrolysable organic P forms of total organic P. Therefore labile organic P species do not seem to be a eutrophication hazard in drainage effluent for this system.

5.5. Limitations of Study

There were limitations encountered in this study. In previous studies on organic P characterization, phytic acid has been determined to be the main component of labile organic P in most soils (Ivanhoff et al., 1998; George et al., 2011; Jones & Oburger, 2011). However, the phytase initially utilized in this study was crude phytase from wheat and contaminated with P species. Despite a standard curve constructed with this enzyme to correct for this interference, phytase addition assays were often imprecise. Therefore, analysis of the extracts with phytase

was ceased. In addition, enzyme hydrolysis analysis was not conducted on HCl extracts. This was due to the insolubility of model organic P substrates in 1M HCl. As such, accurate analysis could not be conducted without assessing the enzymes on model organic P substrates in the solvent being assessed. However, there can be significant organic P fractions determined in 1M HCl extracts and warrant assessment (He et al., 2006). Furthermore, 0.1M NaOH soil extraction resulted in an opaque solution that may have interfered with the color development and therefore accurate assessment of concentrations. This color is most likely due to the organic matter, rich in Histosols (Mitchell & Smith, 1974). Activated charcoal was not used to remove this color as it may have removed organic P species in the process of filtration. As a result, NaOH extracts were diluted as much as possible to develop a more translucent solution. Overestimation of P species occurred as well. Total organic P, as determined from digestion, would occasionally be less than the sum of the organic P species determined by enzyme addition. This may be due to matrix effects of the analytical solution, where enzyme hydrolysis assays had many solutions with different viscosities, increasing error as compared to the rather simple analysis procedure for both inorganic P and total organic P. Enzyme mixtures are also heterogenous solutions, which could add to the interference. When analyzing dilute concentrations of organic P species, such as those in arable soils, these errors can be significant. Lastly, there were large standard deviations associated with organic P species. This could be error from the enzyme hydrolysis procedure as mentioned above. However, the role of microorganisms could be significant if they vary greatly between experimental replicates. Though soil samples were composite, organic soils have great variation and may require more thorough mixing in order to achieve a true average of the soil (Schwärzel et al. 2002).

6. Conclusion

The main purpose of this study was to understand the P dynamics of agricultural Histosols, with particular interest in organic P species. As such, total P, inorganic P, total organic P, and organic P species labile phosphomonoesters (LM), phospholipids (Ph), phosphodiesters (Pd), and unhydrolyzable P (UHP) were determined in three arable tile-drained Histosols of the Holland Marsh, Ontario over the three relevant agricultural periods of pre-fertilizer (PF), growing season (GS), and post-harvest (PH) for 2016. Two of the fields, Field A and B, were modified with a drainage control structure to limit nutrient pollution as well as increase yields. For Field B, three different depths (0-20cm, 20-40cm, and 40-60cm) were assessed for these P forms as well to understand their stratification in the soil profile. Concentrations of minerals Al, Ca, Fe, and Mg as well as TOC were determined to analyze any potential relationships between forms of P and these soil characteristics. Lastly, tile drainage effluent from all three fields were studied for these P forms to understand the eutrophication potential of these arable Histosols. The main purpose of this study was to understand the P dynamics of agricultural Histosols, with particular interest in organic P species. Based on the objectives of this study, the following specific conclusions were drawn:

i. The first objective was to compare the concentrations and lability of inorganic P, total organic P, and organic P species among three agricultural Histosol fields (two of which have a water management structure) and the dynamics of these forms over time. Shifts in fractions seem to be occurring over time, in order to replenish available inorganic P. Overall, inorganic P was the most dominant form of P. Of all fractions, the HCl-non-labile portion of P was foremost in concentration and may be a sink for applied fertilizer. The largest amount of total organic P was found in the NaOH-non-

labile pool, where it is occluded. For specific enzyme species, concentrations were quite variable among the fractions. Available and moderately labile organic P species had minute concentrations and are therefore not a viable source of crop nutrition as well as low in eutrophication potential. However, a majority of the total organic P in non-labile pools was hydrolysable. Larger concentrations existed in this pool, but since occluded, are not a likely source of plant nutrition. Large Pd concentration determination conflict with other studies in the current literature and may be a result of the unique Histosols characteristics. The drainage control structure did not seem to impact organic P species, but may have resulted in less available P, while more P in the moderately available and non-labile pools.

ii. The second objective was to determine the concentrations and lability of inorganic P, total organic P, and organic P classes at three different depths of an agricultural Histosol field and the dynamics of these forms over time to understand their stratification in the soil profile. There was no apparent trend in P forms for depth, besides the expected of more P in the topsoil than in the lower layers. The stable Ca-bound non-labile P pool proportion was larger for lower soil depths than for the topsoil. Increases in P concentration for various P forms in the lower soil depths may suggest seepage from the topsoil or microbial activity. Interestingly, some organic P species like LM would be equal among all soil depths; consequently, organic P species would represent a greater portion in lower soil depths than in the topsoil. Unfortunately, there is little research on organic P species stratification, and the results of this study could not be compared to other analyses.

iii. The third objective was to identify any correlations between P forms and soil characteristics in agricultural Histosols. Though some significant correlations were identified, they were overall loosely correlated. However, the greatest relationship occurred between total organic P in the available P pool and TOC. As such, TOC may play an important role in keeping available organic P in the field. There was no significant relationship determined between stable calcium bound P forms and Ca. This could suggest that the operational name of sequentially fractionated 1M HCl extracts, stable Ca-bound non-labile P pool, is inappropriate for organic soils.

iv. The fourth objective was to determine the concentration of inorganic P, total organic P, and organic P species in drainage effluent from fields to assess their eutrophication potential. Negligible amounts of total organic P were determined and therefore characterization of organic P species could not be conducted. Inorganic P was dominant in tile drainage effluent from fields, besides Field A. Therefore there is a significant eutrophication hazard, with concentrations greater than the Provincial Water Quality Objective (PWQO) of 0.03ppm P. However, the drainage control structure was effective in preventing this effluent from leaving the field, unless the effluent surpassed the set height of the control structure.

7. Recommendations for Future Research

Based on the conclusions of this study, there are evident research gaps that should be addressed as well as additional methods that should be utilized for future studies. As such, a greater understanding of soil P dynamics and organic phosphorus speciation in Histosols would be attained. These include:

i. As phytic acid is the most commonly determined form of organic P species in other agricultural P characterization studies, its analysis is paramount. Commercial purified phytases exist and are guaranteed to function without interference or contamination. An analysis with a phytase component would be beneficial to elucidate phytic acid's presence in Histosols.

ii. Enzyme hydrolysis assays conducted with microplates allow for prolific analysis of many samples, adaptable to most laboratory settings, and are inexpensive as a laboratory experiment overall (Jarsoch et al., 2015). However, these analyses are broad, only reporting enzyme classes and not actual species. In addition, data obtained from these experiments can be quite variable (Jarosch et al., 2015). It would be advantageous to supplement enzyme hydrolysis assays with 31P nuclear magnetic resonance (NMR) analysis. 31P NMR analyses identify actual organic P species based on the absorbance of electromagnetic radiation by P atoms. Organic P characterization studies are frequently utilizing both enzyme hydrolysis and 31P NMR to increase accuracy of characterization.

iii. As opposed to enzyme hydrolysis assays, substrate addition assays exist where model organic P substrates are added to soil extracts. Such a study allows for an understanding of the natural enzymatic activity in a soil. This would supplement an enzyme hydrolysis assay with insight into the capacity the soil matrix has to hydrolyze organic P classes identified.

iv. Though, many organic P classes are cited as being of microbial origin and signifying microbial turnover, assessment of microbial P would be useful. This can be determined via the chloroform fumigation incubation method which would provide

greater understanding on their role in organic P cycling, especially in an arable Histosol with mineral fertilizer application.

v. It is difficult to differentiate direct transfer of applied P to a fraction versus a shift in fractions to accommodate the applied P. As such, utilizing ^{32}P or ^{33}P, isotopes of P, as a tracer would elucidate the dynamics between the available, moderately labile, Al/Fe-bound non-labile, and stable calcium bound non-labile pools. This would also provide farmers with a better understand of how repeated fertilization impacts P availability to crops.

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