Carbon, nitrogen and phosphorus retention and dynamics in plant litter, surface and deep soil of intact, restored and drained inland freshwater marshes

Dan Dong

Department of Geography

McGill University, Montréal

November 2024

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy in Geography

Abstract

In response to widespread wetland degradation in the past century, multiple restoration programs are ongoing throughout Canada, aiming to regain important wetland functions. After decades of restoration practices, despite rewetted and revegetated, it is under debate if their key biogeochemical functions and features (e.g., microbial activity, the carbon and nutrients retention in sediment) have recovered successfully. This thesis investigated intact and short and long term restored inland freshwater marshes and their carbon, nutrient retention and dynamics in the ecosystem. I especially focused on the decomposing litter, microbial biomass and enzyme activities in surface soil, and the carbon and nutrients distribution and stoichiometric patterns in the deep soil profile.

Across three wetland restoration sites in Manitoba and Ontario, litter decomposition rate was controlled by litter species and wetland characteristics especially the hydrologic regimes and surrounding land uses, not the intact or restored classes. The nutrients dynamic in litters was determined by both initial litter quality and surrounding human disturbances (e.g., sewage inputs to marshes), and achieved a steady stoichiometric balance for all sites at the end of the decomposition experiment. In a reserve site in Ontario, comparison of an intact and a restored marsh showed consistent spatial variability in microbial biomass and enzyme activities over the landscape transect encompassing the permanently inundated centre, intermittently inundated wet meadow, and never inundated upland. For both marshes, the degree of inundation and dominant plant species mainly determined the surface soil microbial biomass and extracellular enzyme activities, rather than land management or soil properties. Within the soil profiles of thirty-three intact, drained and restored marshes from Manitoba and Alberta, I found the cultivation history and surrounding land uses strongly influenced sediment carbon, nitrogen and phosphorus concentration and stoichiometric features both in surface soils (0-30 cm) and subsoils (30-100 cm). The vertical distribution of elements was highly variable among individual restored marshes and was not distinguished among different restoration age ranges from 2-23 years. Soil stoichiometric features of restored marshes were generally more similar to drained than intact wetlands, indicating a strong cultivation imprint on restored wetland soils.

These findings provided science-grounded evidence on the success or failure of

duplication of ecosystem carbon and nutrients dynamics and retention in decadal restored freshwater marshes to the adjacent intact conditions. They indicate similar environmental controllers on carbon and nutrients dynamic between intact and restored marsh systems in the surface litter and soil where biological processes dominated, however, alterations in deep sediments related to historical land conversions and physiochemical processes might be unrecoverable. Chronosequence (simply grouping wetlands by restoration age) might not be an adequate approach to delineate the recovery states of wetland functions. Instead, wetland structure (e.g., hydrologic regimes, adapted plant species) and interactions with surrounding or onsite anthropogenic activities (e.g., agriculture) are important on the efficacy of marsh biogeochemical functioning recovery, which should be considered in model development.

Résumé

En réponse à la dégradation généralisée des zones humides au cours du siècle dernier, de nombreux programmes de restauration ont été mis en œuvre à travers le Canada pour restaurer les fonctions écologiques essentielles de ces milieux. Malgré des décennies de pratiques de restauration ayant permis la réinondation et la revégétalisation, il reste incertain si leurs principales fonctions biogéochimiques (comme l'activité microbienne et la rétention du carbone et des nutriments dans les sédiments) ont été efficacement restaurées. Cette thèse examine les marais d'eau douce continentaux intacts et ceux restaurés à court et à long terme, ainsi que la rétention et la dynamique des nutriments et du carbone dans ces écosystèmes. Une attention particulière a été accordée à la décomposition de la litière, à la biomasse microbienne et aux activités enzymatiques dans le sol de surface, ainsi qu'à la répartition et aux caractéristiques stœchiométriques du carbone et des nutriments dans le profil de sol profond.

Les résultats montrent qu'à travers trois sites de restauration de zones humides au Manitoba et en Ontario, le taux de décomposition de la litière dépend principalement de l'espèce de litière, indépendamment de la gestion (intacte ou restaurée), bien que les régimes hydrologiques des marais et les utilisations des terres environnantes puissent influencer la décomposition selon les espèces de litière. La dynamique des nutriments dans les litières est déterminée à la fois par la qualité initiale de la litière et par les perturbations humaines environnantes (comme les apports d'eaux usées), atteignant un équilibre stœchiométrique constant à la fin de l'expérience de décomposition dans tous les sites. Dans une réserve en Ontario, la comparaison entre un marais intact et un marais restauré révèle une variabilité spatiale constante dans la biomasse microbienne et les activités enzymatiques le long d'un transect englobant le centre inondé en permanence, la prairie humide inondée par intermittence et les zones élevées non inondées. Pour ces deux marais, le degré d'inondation et les espèces végétales dominantes influencent principalement la biomasse microbienne et les activités enzymatiques dans le sol de surface, plutôt que la gestion des terres ou les propriétés du sol. Dans les profils de sol de trente-trois marais intacts, drainés et restaurés au Manitoba et en Alberta, l'historique de culture et les utilisations des terres avoisinantes influencent fortement la concentration en carbone, azote et phosphore dans les sédiments, ainsi que leurs caractéristiques stœchiométriques, à la fois dans les

sols de surface (0-30 cm) et dans les sous-sols (30-100 cm). La distribution verticale des éléments varie de manière significative entre les différents marais restaurés, sans distinction claire en fonction de l'âge de la restauration (entre 2 et 23 ans). Les caractéristiques stœchiométriques des marais restaurés sont généralement plus proches de celles des zones humides drainées que des zones intactes, ce qui souligne une empreinte agricole persistante sur les sols des zones restaurées.

Ces résultats fournissent des preuves scientifiques concernant le succès ou l'échec de la récupération de la dynamique et de la rétention des nutriments et du carbone dans les marais d'eau douce restaurés depuis plusieurs décennies, par rapport aux conditions intactes adjacentes. Ils indiquent des facteurs environnementaux similaires sur la dynamique des nutriments et du carbone dans les systèmes de marais intacts et restaurés, particulièrement dans les litières de surface et le sol où les processus biologiques dominent. Cependant, des altérations dans les sédiments profonds, liées aux conversions de terres historiques et aux processus physicochimiques, pourraient être irréversibles. Une simple approche chronologique (regroupant les zones humides en fonction de leur âge de restauration) pourrait ne pas suffire pour décrire les états de récupération des fonctions des zones humides. Au contraire, la structure des zones humides (ex. : régimes hydrologiques, espèces végétales adaptées) et leurs interactions avec les activités anthropiques environnantes ou locales (ex. : agriculture) jouent un rôle crucial dans l'efficacité de la récupération biogéochimique des marais et devraient être intégrées aux modèles de développement.

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List of Abbreviations

AP: Acid phosphatase
BG: β-glucosidase
C: Carbon
CH_4: Methane
CO_2: Carbon dioxide
DOC: Dissolved organic carbon
EEAs: Extracellular enzyme activities
Eh: Redox potential
Fe: Iron
LAP: Leucine aminopeptidase
MBC: Microbial biomass carbon
MBN: Microbial biomass nitrogen
Mn: Manganese
NAG: β-N-acetyl-glucosaminidase
NH_4^+: Ammonium
Nitrogen: N
NO: Nitric oxide
N_2O: Nitrous oxide
NO_2^-: Nitrite
NO_3^-: Nitrate
Phosphorus: P
SO_4^{2-}: Sulfate
SOM: Soil organic matter
TDN: Total dissolved nitrogen

Acknowledgements

I sincerely appreciate the presence of numerous people during my PhD journey, without whom I would not be able to grow and achieve as much as I did. First and foremost, I deeply thank my supervisor Dr. Tim Moore for his long-lasting commitment and faith on students' self-growth and high expectations. His rigor in science, broad knowledge in soil, passion on research, inspiring comments and questions, details-enriched emails with sparkling sense of humor enriched my work constantly. Also, his in-time encouragement, long patience and willingness to listen to student's words are fundamental to my mental growth. Overall working with him in the past six years makes my PhD such a meaningful and enjoyable experience.

I highly appreciate the generous financial aids from my supervisor, department of Geography, Faculty of Science and the NSERC program led by Dr. Irena Creed (University of Toronto). These financial supports made my PhD research possible.

Moreover, I deeply thank my committee members Dr. Christian von Sperber, Dr. Pascal Badiou and Dr. Nigel Roulet for their continuous assistance in my PhD work, for example, the research question development, discussions, and laboratory supervision. I am also very grateful to Dr. Cynthia Kallenbach, who contributed fundamentally to my PhD work particularly in writing, experiments and research interest development.

I want to thank a great number of people from the Department of Geography and Department of Natural Resources for their supports on field and laboratory work and the enlightening talks: Paula Kestelman, Mike Dalva, Calder Jones, Dr. Gail Chmura, Dr. Frieda Beauregard, Dr. Joann Whalen, Hicham Benslim, Aidan De Sena, Rachael Harman-Denhoed, Hannah P. Lieberman, Lijun Hou, Dr. Bidhya Sharma, Dr. Tanja Živković, Dr. Siya Shao, Janice Neumann, Camille Jones, Shir Gruber, Dr. Tracy

Rankin, Dr. Hongxing He, Rachel Kendall, Nicolas Adam Perciballi, Alice Watts, and Lucy Lu. I am also grateful for joining the peat meets and peat coffee meets for the broad inputs of ideas, brainstorm discussions and encouragement.

Moreover, I received fundamental aids from Dr. Meng Wang on litter chemistry analysis and from people in the NSERC project and Ducks Unlimited including Brendan Brooks, Purbasha Mistry, Rick Robb, Erling Armson, Joanne Barbazza, Kyle Borrowman, Mallory Carpenter and Tatjana Radulovic on field sampling and site background information collection.

Finally, I deeply thank my beloved mom for her endless love and unconditional supports on every aspects of my life and work and my lovely aunties Yufang, Liu, Huijun, Lili, Zhai who treats me exactly as their own kids, my old teachers Dr. Du, Dr. Kang for their warm encouragements, my amazing friends and roommates Razan, Katrina Kosyk, Wenwen, Aldo, Eduado, Chetna, Danial, Gabielle, Pierrie, Kamina, Julie for the enjoyable accompany, and lastly my forever cherished undergraduate friends Zhengzhen Dai, Yuanyuan Cao, Kun Xu, Weiyuan Zhang for their everlasting trust and mental supports on me.

Contribution to Original Knowledge

In this thesis, my contributions to Original Knowledge included:

Determining the rates of litter decomposition of four common macrophytes across four Ontario and four Manitoba freshwater wetlands, establishing the controls on the rates and examining the fate of original litter nitrogen and phosphorus over two years of decomposition.

In two Ontario freshwater wetlands, examining the relationships between plant species, hydrology and microbial biomass and enzyme activities, which maintains the biogeochemical functioning of these wetlands.

Determination of the concentration and stoichiometric relationships of carbon, nitrogen and phosphorus in soils of three Manitoba wetlands, related to depth and position in the wetland. I then sought broader patterns in the central soils of several Alberta wetlands, ranging from intact to drained and restored. The results showed a close correspondence of carbon and nitrogen stoichiometry and a weaker link with phosphorus with some dependence on wetland history, especially cultivation.

It linked litter decomposition, microbial and enzyme activities and residual soil C, N and P in ecosystems and advanced our understanding on the carbon and nutrient cycling in the restored inland marshes. It also raised the importance of wetland structure recovery (e.g., vegetation communities readaptation, hydrologic regimes reestablishment) on wetland biogeochemical functioning recovery. These findings meanwhile provide empirical suggestions to improve the current land management strategies on restored marshes and facilitate the carbon and nutrient retention in wetland ecosystems.

Contribution of Authors

This thesis contains three main chapters (3-5) formatted as formal manuscripts ready for submission (or already submitted) to scientific journals with Dan Dong as the lead author, together with an introduction (chapter 1), literature review (chapter 2), and a summary (chapter 6). Chapters 1, 2 and 6 were written by Dan Dong, with editorial support from Dr. Tim R. Moore. Detailed contributions of co-authors for each manuscript were listed as below:

Manuscript #1 (Chapter 3): "Litter decomposition and nutrient dynamics of four macrophytes in intact, restored and constructed freshwater marshes of Canada" by Dan Dong, Pascal Badiou, Tim R. Moore, Christian von Sperber (published on *Restoration Ecology*, doi: 10.1111/rec.14135). TRM, PB, DD designed the research; DD, PB, TRM, CVS participated in field and laboratory work and contributed to the writing and editing of the manuscript.

Manuscript # 2 (Chapter 4): "Hydrologic, plant and soil controls on microbial biomass and enzyme activities in a natural and a restored freshwater marsh of eastern Ontario, Canada" by Dan Dong, Cynthia M. Kallenbach, Tim R. Moore (to be submitted). DD designed the research and conducted the laboratory and data analysis work; CMK supervised the laboratory work; DD, CMK and TRM contributed to the writing and editing of the manuscript.

Manuscript # 3 (Chapter 5): "Sediment carbon (C), nitrogen (N), phosphorus (P) concentration and stoichiometry of intact, drained and restored freshwater marshes in the Prairie Pothole Region of Canada" by Dan Dong, Tim R. Moore, Christian von Sperber, Irena F. Creed, Cynthia M. Kallenbach. IFC, TRM and DD designed the research; DD conducted the laboratory and data analysis work; CVS, TRM supervised the laboratory analyses and provided insights on data analysis; CMK contributed to the key explanations on the findings; DD, TRM contributed to the writing and editing of the manuscript.

Chapter 1: Introduction

1.1 Research rationales

In 2019, The United Nations declared 2021-2030 the ‘Decade on Ecosystem Restoration’, highlighting the necessity of ecosystem recovery in the 21st century. Extensive drainage, land conversions upon natural wetlands in the past century has caused severe environmental problems today including significant greenhouse gas emissions, eutrophication in ocean, coastal and inland water bodies, and substantial loss of animal habitats and biodiversity (Dahl and Watmough, 2007; Waltham et al., 2020; Fluet-Chouinard et al., 2023). The recovery of damaged wetland biogeochemical functioning (e.g., water purification, soil carbon (C) accumulation) compared to the intact conditions is an important goal for wetland restoration practices and a main challenge for wetland researchers today, particularly to achieve the continuous burial of C and nutrient retention to the sediments (Loder et al., 2023). Some encouraging results showed increasing C accumulation rates with the age of restoration and comparable C accumulated in restored wetlands with the adjacent intact ones (Ballantine and Schneider, 2009; Guo et al., 2014; Creed et al., 2022). However, other researchers reported no greater C accumulated in hydrologically restored marshes than drained ones (Bansal et al., 2021) and still considerably low C accumulation rate in restored marshes compared to the undisturbed marshes (Moreno-Mateos et al., 2012; Loder et al., 2023). It is pessimistically estimated that the recovery of C accumulation functioning of destroyed wetlands might take a century or never being equivalent to the natural references and largely depends on the seed banks, environmental setting, and wetland intrinsic characteristics such as topography, size, and soil texture (Moreno-Mateos et al., 2012; Loder et al., 2023).

Aside from C, the recovery of nitrogen (N) and phosphorus (P) retention in restored wetland sediment also has not reached a consensus and has evidence of decoupling in response to restoration practices. Audet et al. (2020) found successful N retention in eight marshes restored for 3-to-13 years in Denmark but successful P retention in only five restored marshes with three marshes functioning as P sources. Steinman and Ogdahl (2011) also reported notable P export to downstream lake in rewetted drained wetlands (used to be celery fields) and high P concentration in sediment pore-water samples, suggesting rewetted drained wetlands become P sources, rather than sinks. In contrast, Hogan et al. (2004) found similar total P concentration and greater P-sorption capacity in restored freshwater marshes where P sorption capacity was correlated to various Al and Fe fractions compared to the natural references where P sorption capacity was only correlated to the organically bound aluminum. They explained this unexpected finding by the fundamentally differed soil chemistry between intact and restored wetlands.

These findings from the recent two decades give rise to many new questions after years of restoration practices: Does rewetting and restoration of wetlands really enhance C and nutrients retention or otherwise foster the C and nutrients losses from the system? Will C and nutrients be tightly coupled in the restoration process or decoupled and develop distinct C, N, P states from the intact conditions? What mechanisms and factors affect the accretion of C and nutrients in restored wetlands? Given the science of wetland restoration is generally new, the answer to these questions remains highly obscure to date and can contradict among different study sites, wetland types and restoration scenarios. There still exists a large data gap and lack of science-grounded field observations to better understand the underlying mechanisms of C, N, P dynamics in the restored wetlands before we can achieve the fundamental restoration goals (Creed et al., 2017; Loder et al., 2023).

Among all wetland types, inland freshwater marshes are the least frequently studied in their C, N, P dynamics, accumulation, and the controlling factors (Loder and Finkelstein, 2020), much less compared to peatlands and coastal marshes. Inland freshwater marshes have different ecosystem features from the salt marshes and northern peatlands. They are characterized by high productivity, dominance of herbaceous plants, rich in mineral matter in soil, neutral to basic pH, and under great agricultural impacts with often high potential to receive allochthonous nutrients from surrounding agricultural fields (Burton and Uzarski, 2009). It is of great importance to identify the C, N, P retention states in these inland intact and restored freshwater marshes and examine the controlling mechanisms and influencing environmental parameters (e.g., hydrological regimes, pH, surrounding land uses, restoration age).

1.2 Research objectives

To address this important issue, I conducted field-based studies on restored and intact freshwater marshes in wetland conservation sites in Canada, aiming to determine the potential differences in their C, N, P dynamic and retention status in the ecosystem and the regulating environmental factors. I specifically focused on three ecosystem compartments: the decomposing litters (chapter 3), the microbial biomass and enzyme activities in the surface soil (chapter 4), and the C, N, P vertical profiles in deep sediment (chapter 5).

1.3 Thesis structure

In Chapter 1, the introduction chapter, I present the current debate and knowledge gaps on the still unclear efficacy of biogeochemical functioning recovery of restored wetlands and particularly the lack of data on the degraded and restored inland freshwater marshes. In chapter 2, I introduce what are freshwater marshes and their important biogeochemical functions, the C, N, P storage and cycling processes in marsh ecosystems, and their responses to land use conversions including drainage and restoration. Chapter 3-5 presents the original research findings. In chapter 3, I report the rates of litter decomposition of major plants in eight intact, restored and constructed marshes, and their nutrient dynamic patterns. In chapter 4, I examine the microbial traits (biomass and extracellular enzyme activities) in an intact and a restored freshwater marsh and their relationships with vegetation communities, hydrologic regimes and soil characteristics. In chapter 5, I detect the vertical profiles of C, N, P concentration of three marshes in Manitoba, collated data of 30 additional marshes in Alberta and examined their stoichiometric features. In chapter 6, I summarize these findings, discuss their implications and suggest the avenues for further research and the efficient restoration practices for policy makers.

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Chapter 2: Review of literature

2.1 The restoration of wetlands

The United Nations defines 'ecosystem restoration' as *the process of assisting the recovery of an ecosystem that has been degraded, damaged or destroyed, as well as conserving the ecosystems that are still intact*. The US National Research Council (1992) defines 'restoration' as *the act of bringing an ecosystem back into, as nearly as possible, its original condition.* Restoration practices are increasingly applied to multiple degraded ecosystems (e.g., forest, wetlands) and commonly aim to 1) recover the organisms living in the ecosystem; 2) recover the energy flows, elements cycles and ecosystem functions and 3) re-achieve ecosystem self-sustaining (Palmer and Ruhl, 2015; Craft, 2022).

Wetlands are global biogeochemical hotspots, important C, N, P sinks, breeding habitats for birds and fishes and thus have great values for restoration. The restoration practices upon damaged wetlands are either active or passive, distinguished by additional actions that will potentially accelerate the recovering process is implemented (Trujillo-Miranda et al., 2018). Passive restoration, also regarded as natural restoration, typically only ceased the degradation of wetlands and leaves the ecosystem to self-recover through natural processes. For instance, the agricultural-used marshes in the Canadian Prairie Pothole Region are often passively restored owing to its simpleness, convenience, and low cost. There, the drained and past cultivated wetlands were purchased from private farmers by the government or environmental non-government organizations (e.g., Ducks Unlimited), often on the marginal farmlands, and rewetted through natural processes including precipitation, surface flows and/or groundwater discharges after blocking the ditches with clay-dense soils (Euliss et al., 2006; Anderson, 2017). Active restoration can involve more human interventions such as land construction, seeds and seedling introduction, soil compiling, and wetland reflooding (Hoffmann and Baattrup-Pedersen, 2007; Craft, 2022; Loder et al., 2023). Another type of active restoration practice is constructing artificial wetlands by introducing designed substrates (e.g., gravels, sand, organic materials), emergent macrophytes and water for the use of wastewater treatment (e.g., urban sewage) (Wang, 2020). These marshes are thus called constructed or treatment marshes.

Since 2000, the number of research projects on wetland restoration have increased. There are both promising and frustrating outcomes of wetland restoration. The US Fish and Wildlife Service (2024) reported substantially increased population of waterfowls in North America since the 1990's when inland and coastal prairie marshes were increasingly restored. In Denmark, long-term monitoring showed a range of 26-71 % N removal efficiency in 10 restored sites (Hoffmann and Baattrup-Pedersen, 2007). In China, Yang et al. (2020) revealed surface soil C, N, P concentration of restored saline marsh was resembling the natural reference. Wang et al. (2021) found the microbial biomass C in a 27-year restored marsh was 15 times that in the soybean fields, and comparable to the intact reference. Loder et al. (2023) compared the C accumulation rates in three passive and three active restored freshwater marshes in southern Ontario and found no evidence for faster C accumulation in actively restored wetlands. The rising research from multiple perspectives of ecosystem recovery help set up the scientific basis for wetland restoration practices and draw greater attention on the wetland ecosystems.

2.2 The various forms of wetlands

Wetlands are broadly defined as "transitional lands between terrestrial and aquatic systems where the water table is usually at or near the surface or the land is covered by shallow water" (Cowardin et al., 1979; Mitsch and Gosselink, 2000). Or more specifically as "areas of marsh, fen, peatland or water, whether natural or artificial, permanent or temporary, with water that is static or flowing, fresh, brackish or salt, including areas of marine water the depth of which at low tide does not exceed six metres" (Ramsar Information Paper, 2024).

Wetland habitats are highly variable in their vegetation cover, primary productivity, soil type and hydrogeochemical features and thus are classified into multiple types (Mitsch and Gosselink, 2000). The Canadian wetland classification system categorized wetlands into five main classes including bogs, fens, swamps, marshes and shallow open water (less than 2 m), based on their genetic origins. Wetland surface morphology, underlying soil type, water type, and vegetation further differentiate their forms and types (National Wetlands Working Group 1997). The Cowardin classification system categorized wetlands into five major systems: lacustrine, riverine, palustrine, marine and estuarine according to their landscape positions with further subsystems and classes categorized by the hydrologic regimes, sediment type and vegetation (Cowardin et al., 1979). Based on the water source, transport, geomorphology and hydrodynamics of wetlands, Brinson (1982) developed a hydrogeochemical wetland classification system involving five main classes: depressional, riverine, flat, fringe and slope. These classification systems collectively improve the accuracy in the terminology used for wetlands. Stewart and Kantrud (1971) developed a wetland classification system specifically for the glaciated prairie region and categorized wetlands into permanent, ephemeral, temporary, seasonal, semi-permanent, alkali and fen ponds.

In this thesis, the wetlands studied are inland non-tidal freshwater marshes, isolated basin marsh and isolated basin water (Canadian classification system), palustrine persistent and nonpersistent emergent wetland (Cowardin classification system), vertical fluctuated depressional wetland (Brinson's hydrogeochemical wetland classification system) and permanent, semi-permanent and seasonal pond (Stewart and Kantrud's classification).

2.3 Freshwater marshes

Freshwater marshes are non-forested freshwater wetlands except peatlands, with permanent or seasonal inundations, characterized by shallow-water regimes, hydric soils, and dominated by graminoids, shrubs, forbs and/or emergent plants (National Wetlands Working Group 1997; Burton and Uzarski, 2009). They exist as shallow water bodies near rivers (riverine), lakes (lacustrine) or as millions of inland depressional basins (palustrine) formed by glacial retreat (e.g., prairie pothole wetlands, example see Fig.2.2.1). Marshes can be recharged by runoff, precipitation, and groundwater.

A typical freshwater marsh habitat encompasses a depressional zone (flooded and dominated by floating, submergent and emergent plants) together with the surrounding wet meadow and upland zones (less frequently or never flooded and dominated by grasses, sedges and forbs). Shrubs and trees can also occur in freshwater marsh systems (van der Valk, 2012). Along the hydrological transect, often more than two zones are developed where distinct plant communities can be found along the topographic gradient (Craft, 2015, 2022). Typical plant genus in freshwater marshes involve *Carex*, *Cyperus*, Cladium, *Eleocharis*, *Podostemaceae, Polygonum*, *Potamogeton*, *Sagittaria*, *Salix*, *Typha*, *Utricularis*, and *Vallisneria* (van der Valk, 2012). The water level of marshes often fluctuates which boost the primary productivity at the fringe of ponds both aboveground and belowground (e.g., developed rhizomes in marsh plants). The rich nutrients, freshwater and mild pH also favor the plant and microbial biomass production. According to Mitsch and Gosselink (2000), the net primary productivity of mineral freshwater marshes ranged from 1000 to 6000 g m^{-2} yr^{-1} and is among the most productive ecosystems. Microbial biomass carbon in undisturbed temperate freshwater marshes can range from 2000-5000 mg kg^{-1} in 0-10 cm soil (Wang et al., 2021). Inland freshwater marshes are also exceptional in belowground biomass production, even producing greater biomass than salt marshes. In a mesocosm experiment, the root and shoot biomass of five common wetland species (e.g., *Phragmites australis*) were consistently higher under freshwater treatments than saltwater treatments (Sanicola et al., 2019). Cole et al. (2001) measured average belowground biomass as 1028-3658 g m^{-2} in the natural freshwater marshes in Pennsylvania.

Fig. 2.1 An example of *Typha* spp. dominated freshwater marsh in the Broughton's Creek, Manitoba, Canada. Photo by Dan Dong in 2019.

There is no accurate inventory of currently existing freshwater marshes globally. This is because many existing marshes are unmapped and unclear on their presence and land use changes over years (Creed et al., 2017). Existing literature mostly reported freshwater marshes from the large wetland complexes such as the Prairie Pothole Region in North America, Everglades watershed in Florida, Sanjiang Plain in China and Pantanal in South America (Loder and Finkelstein, 2020). Meanwhile, there exists some long-term monitored marsh conservation sites in Europe (e.g., Denmark where 3060 ha freshwater wetlands have been restored since 1998) (Davidsson et al., 2000; Hoffmann and Baattrup-Pedersen, 2007). In Canada, freshwater marshes can be tidal, occurring along the Atlantic and Pacific coastline and the shores of St. Lawrence River or non-tidal in the Great Lakes Region and the Prairie Pothole Region across southern Alberta, Saskatchewan and Manitoba (Leck et al., 2009). The Canadian Prairie Pothole Region alone contains millions of isolated freshwater marshes in the 467, 000 km^2 landscape (Doherty et al., 2017). Apart from these documented marshes, there are expected to be wide presence of freshwater marshes near rivers, lakes and coasts globally, although not published.

Although covering a small amount of land surface, marshes are biogeochemical hotspots and provide critical biogeochemical functions. For instance, the poorly aerated soil inhibits decomposition and fosters C accumulation (Mitsch and Gosselink, 2000). Despite higher methane emission rates in freshwater wetlands than terrestrial ecosystems, the high primary productivity and low carbon dioxide (CO_2) emission rates yields strong C sequestration capacity and can achieve long-term net C sequestration under appropriate land management (Kayranli et al., 2010). Loder and Finkelstein (2020) synthesized data from 29 freshwater marshes in North America and reported the average C accumulation rates of 0.5-1.5 Mg C ha^{-1} yr^{-1}, comparable to that in the salt marshes in North America (0.1-3.2 Mg C ha^{-1} yr^{-1}; Miller et al., 2022), and temperate peatlands. They suggested the significant C stock in these mineral marshes might be dissembled by the term ‘mineral’. Creed et al. (2022) reported the C accumulation rates of restored freshwater marshes in southern Ontario as 0.51-0.89 Mg C ha^{-1} yr^{-1}, higher than the adjacent cropped wetlands which averaged as 0.49 Mg C ha^{-1} yr^{-1}. Nahlik and Fennessy (2016) estimated a total amount of 9.63 Pg C stored in 0-120 cm depth from 357,000 km^2 inland freshwater marshes in the US, which is twice to three times the C stored in coastal marshes, mainly due to its larger areal extent. The US portion of the Prairie Pothole Region alone is estimated to store 319 Tg C (0.3 Pg C) in the surface 15 cm depth (Tangen and Bansal, 2020). The C storage potential in freshwater marshes vary greatly over the landscapes. Tangen and Bansal (2020) revealed the greatest C stored at the centre of marshes (82 Mg C ha^{-1}) with declining C storage approaching the upland fringe in the prairie marshes. However, despite of lower C accumulation rates than centre, the surrounding fringe areas also stored significant amounts of C (58-66 Mg C ha^{-1}).

Given the often-lower C:N ratio in mineral SOM than organic SOM as ~8:1 (Kirkby et al.2011; Tipping et al., 2016), freshwater marshes also have great potential to store large amount of organic N in their sediment. Lane and Autrey (2017) reported an average annual rate of 3.1 g N m^{-2} year^{-1} in 43 depressional marshes in US. The high plant uptake, rich mineral matter, neutral to basic pH and rich cations (calcium, iron, aluminum, magnesium) also support considerable amount of P storage and adsorption in the sediment of freshwater marshes, reducing the regional eutrophication. These unique features make freshwater marshes exceptional carbon, N and P sinks (Reddy et al., 1999; Mitsch et al., 2005). Lane and Autrey (2017) revealed an annual P accumulation rate between 0.04-0.10 g P m^{-2} year^{-1} in US depressional marshes and found substantially higher P accumulation rates in agricultural sites than natural and urban sites. Other than the C, N, P storage, freshwater marshes also contribute to nitrate and contaminant removal (e.g., toxic metals) through denitrification, enzymatic degradation, oxidation and sedimentation (Marton et al., 2015).

2.3 Biogeochemical processes related to C, N, P dynamics in freshwater marshes

2.3.1 Decomposition of organic materials

In undisturbed marshes, except for a few millimetres in the surface soil where aerobic decomposition prevails, sediments are mostly under reduced environments where redox potentials (Eh) range from +400 to -100 mV, making anaerobic decomposition (or fermentation) and microbial anaerobic respiration the dominant pathway of organic compounds degradation and mineralization (Boyd, 1995). Anaerobic decomposition and respiration are much slower and less efficient than aerobic processes and thus support greater net organic matter accumulation under the certain time period. For example, Kristensen et al. (1995) measured rates of labeled C (^{14}C) losses of wetland sediments under aerobic and anaerobic conditions and found ~10 times faster C losses under aerobic decomposition relative to anaerobic decomposition. Under anoxia, long-term preservation of organic C can be achieved because the rate of organic materials input easily exceeds the slow decomposition rates (Mitsch and Gosselink, 2000; Chapin et al., 2002; Kayranli et al., 2010). The decomposition rates in marshes depend on the quality of organic inputs and the environmental conditions such as wetland hydrological conditions, soil temperature, moisture, and the nutrient status (van der Valk et al., 1991; Qualls et al., 2000; Kayranli et al., 2010).

Decomposition in marshes is mediated by the bacteria, fungi and archaea and their microbial products such as extracellular enzymes which aids the depolymerization of complex biopolymers to bioavailable monomers (Boyd, 1995; Lehmann and Keeler, 2015). Along with litter decomposition, plant and microbial residues can chemically bind to the iron (Fe) and/or aluminum oxyhydrates and silt or clay coatings of mineral soils (Cui et al., 2014) or being physically protected by microaggregates and macroaggregates occlusion and becoming progressively resistant to biodegradation (Six et al., 2002; Kögel-Knabner et al., 2008).

2.3.2 Gaseous emissions of carbon and nitrogen

Despite considerable C and N retention in soil, undisturbed marshes can emit various forms of C and N gases to the atmosphere with some being potent greenhouse gases. In reduced sediments, fermentation oxidizes carbohydrates incompletely, usually producing alcohols, lactic acids and carbon dioxide (CO_2) at a very slow rate (Kayranli et al., 2010). These small organic compounds can be further reduced by other reducing bacteria such as manganese Mn (IV)-, iron Fe (III)- and sulfate (SO_4^{2-})- reducing bacteria. Mn (IV) and Fe (III) are prevalent electron accepters in the rhizosphere of mineral marshes (Yarwood, 2018). The presence of oxidized forms of Mn (IV) and Fe (III) within reduced soil matrix is because some plants (e.g., *Typha* spp.) release O_2 around their roots creating independent oxic zones (Caffrey and Kemp, 1991). Nitrate (NO_3^-) and SO_4^{2-} are also important electron accepter when present. The overall sequence of oxidants utilised to oxidize organic compounds from high to low soil Eh is $O_2 > NO_3^- >$ Mn (IV) > Fe (III) > $SO_4^{2-} > CO_2 > H^+$ (Yarwood, 2018). The by-products of these oxidants through reduction reactions include Mn (II), Fe (II), hydrogen sulfide, nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2). Meanwhile, CO_2 is released through the respiration of these reducing microorganisms (e.g., sulfate reducers) (Lovley et al., 1991; Boyd, 1995). When soil Eh is below -200 mV and NO_3^-, Mn (IV), Fe (III) and SO_4^{2-} are all depleted, CO_2 and H^+ are the predominant electron accepters and methanogenesis becomes the dominant anaerobic decomposition pathway (Kayranli et al., 2010; Bhaduri et al., 2017). Methanogenesis is mediated by methanogenic archaea who utilise a small number of simple substrates, e.g., H_2, CO_2 and acetate, for reduction-oxidation reactions which generates methane

(CH_4) (Zehnder and Stumm, 1988). In most reduced soils, CH_4 is the most important end-product of organic matter decomposition (Kayranli et al., 2010), although the rich SO_4^{2-} can reduce CH_4 production and enhance CO_2 production instead (Pester et al., 2012; Zak et al., 2021).

At the surface oxic layer, some CH_4 can be oxidized to CO_2 by methanotrophs, which is known as methane oxidation (Bürgmann, 2011). Meanwhile, the reduced forms of N (e.g., ammonium (NH_4^+)) can be oxidized to nitrite (NO_2^-) and NO_3^-, which is referred to as nitrification. Through the nitrification, a small amount of N_2O gas can also be emitted. Below the soil surface, reduced environments favor denitrification, through which NO_3^- can be reduced to NO_2^-, NO, N_2O or more thoroughly to N_2 depending on abundance of reducers and reductase in soil (e.g., more N_2O reductase supports greater N_2O reduction) (Hagemann et al., 2016). Denitrification is an important process for nitrate removal and N_2O reduction in wetlands. The overall N_2O emissions measured from undisturbed marshes are often very low and can even be a net sink for N_2O due to the strong N_2O uptake and consumption (e.g., -0.25 to 0.50 g N_2O-N m^{-2} yr^{-1} reported from undisturbed riparian marshes; Audet et al., 2014). Besides the NO, N_2O and N_2 emissions, ammonia can be another important N gas emitted from marshes through volatilization.

2.3.3 Nutrient mineralization and immobilization

Along with the decomposition, organic nutrients are also mineralized to inorganic forms such as NH_4^+ and phosphate (PO_4^{3-}) which are available for direct uptake by plants and microbes. Opposite to mineralization, the process of inorganic nutrients being incorporated into microbial cells forming organic compounds is immobilization. Strong microbial immobilization often occurs on N- and P- poor organic materials. The relative strengths of mineralization and immobilization of N and P can be controlled by the litter stoichiometry, microbial stoichiometry and the availability of soil substrate nutrients (Mooshammer et al., 2012). Soil nutrient can affect microbial mineralization and immobilization because microbes can incorporate the dissolved inorganic and organic N and P from soil solution and depolymerize the surrounding organic substrates to relax their nutrient limitation induced by poor quality litters (Mooshammer et al., 2012, 2014).

Through mineralization, because N and P are often more limiting than C in natural ecosystems (Elser et al., 2007), nutrients tend to accumulate more significantly than C in decomposing compounds due to the preferable immobilization in microorganisms than C. Typically, the C to N (C:N) and C to P (C:P) ratios decrease in the progressively decomposed litters (Moore et al., 2006; Manzoni et al., 2011). Accordingly, the C:N and C:P ratios of microbial biomass and SOM are much smaller than the plant litter tissues (Manzoni et al., 2011).

Extracellular enzymes are closely linked with the microbial C and nutrient mineralization and assimilation. There exists a wide spectrum of extracellular enzymes that participate in organic C-, N-, and P- degradation such as phenol oxidase, β-glucosidase, chitinase, leucine aminopeptidase, β-N-acetyl-glucosaminidase and phosphatase (Burns et al., 2013). It has been suggested that microbes have to allocate at least 2% of assimilated carbon on extracellular enzymes production for their biomass sustainability (Schimel and Weintraub, 2003). Some extracellular enzymes are effective indicator on the soil mineralization potential and nutrient limitation. For instance, greater microbial alkaline phosphatase secretion was observed under low soil P concentration (Ogram et al., 2011; Luo et al., 2017). In P-enriched freshwater marshes of Everglades, Wright and Reddy (2001) reported a dramatic decline in alkaline phosphatase activity in comparison with the unimpacted wetlands. The secretion of extracellular enzymes can be determined by both the soil and litter substrate quality and microbial demand. A ‘foraging strategy’ was proposed on microbial C, N, P acquisition based on the findings that microbes tend to allocate more energy and resource to the synthesis and secretion of enzymes which aids the acquisition of certain element that is under more severe restriction and vice versa (Burns et al., 2013).

2.3.4 P weathering and adsorption

In contrast to C and N that almost exclusively store in organic forms in soil organic matter (SOM) and microbial biomass, soil P occur in more various pools including microbial biomass P, litter organic P, dissolved P and precipitate P and can be absorbed to a wide spectrum of complexes including SOM, reactive secondary minerals and SOM-mineral complexes (Reddy et al., 1999; Brödlin et al., 2019). The formation of these complexes is dependent on a variety of regulators such as soil age, pH, soil moisture and contents of clay and mineral oxides. In mineral marshes, P is originated from rock weathering and primarily released as inorganic P as phosphate (Walker and Syers,1976; Turner et al., 2007), while through pedogenesis the component of inorganic P generally declined (Reich and Oleksyn, 2004) and the released P were either lost through intense leaching or being absorbed as organic forms (Brödlin et al., 2019). Due to the often-strong absorption of P by clay minerals or Fe/Al oxides, soil P concentration can be poorly related to organic C in deep mineral soils indicated by no clear constraints and large variability in global soil C:P quotients (Yang and Post, 2011; Hartman et al., 2011; Tipping et al., 2016). Instead, soil P abundance can exhibit strong relationships with extractable Al concentrations in wetlands (Richardson et al., 1985), SOM, clay content (Gérard, 2016) and the phosphatase activities (Burns et al., 2013).

2.3.5 Leaching of C, N, and P

Due to the intense water flows both vertical and lateral within and among wetlands such as precipitation, storm, surface and subsurface flow, groundwater recharge and discharge (Todd et al., 2006), marshes have great potential to leach and release dissolved labile C and nutrients to deep depth and surrounding stream channels (Reddy and Rao, 1983). Major forms of C, N and P in the leachate of the organic surface layer are dissolved organic C, dissolved organic N, dissolved organic P either derived from organic matter decomposition or desorption from sediments (Kalbitz et al., 2000; Qualls and Richardson, 2008). Inorganic N and P releases (e.g., nitrate and phosphate) also exist and are intimately associated with the rates of mineralization, nitrification and denitrification. The NO_3^- and PO_4^{3-} leaching rates can be insignificant when N and P mineralization rate is low.

2.4 Land conversion upon freshwater marshes and its ecological implications

Since 1900s, wetlands have long been regarded as wastelands and in need of development (Tiner, 1984). Freshwater marshes are originally very productive and nutrient-rich ecosystems and thus have great potential for agricultural activities. In the past centaury, intensive conversion from marshes to agricultural lands was recorded globally (Rubec et al., 1994; Junk et al., 2013). For instance, in Canada, over half of the intact freshwater marshes have been drained, filled or reclaimed since European settlement (Rubec et al., 1994; Dahl and Watmough, 2007). The remaining marshes, although not being directly drained, can be surrounded by agricultural fields and under the threats of nutrients loadings of nitrate and phosphate (Reddy et al., 1993).

Alterations on wetland hydrologic regimes through drainage and restoration can induce significant physical and chemical changes in soils such as bulk density, macroaggregates content, moisture, pH and redox potentials (Faulkner and

Richardson,1989; Werkmeister et al., 2018), together with a range of biogeochemical features and processes including rates of C and nutrient mineralization, C and N gaseous emissions, SOM content, enzymatic activities, cations forms and P adsorption and desorption (Bowden, 1987; Venterink et al., 2002). For example, elevated N mineralization rates and soil inorganic N and available phosphate concentrations have been widely reported in drained wetlands (Bridgham et al., 1998; Regina et al., 1998; Badiou et al., 2018). Significant increases in N-, P- and S- acquiring enzymatic activities were also recorded (e.g., 31-67% increases of β-glucosidase, phosphatase and sulphatase activities after peatland water table drawdown; Freeman et al., 1996). In general, drainage is regarded as an effective approach to increase the soil nutrient availability in wetlands. The cultivation activities after wetland drainage often introduces fertilizer and additional nutrients to marshes, further deviate the ecosystem C, N, P status and stoichiometry. For example, Wang et al. (2014) noted substantially decreased soil C and C:P ratio after converting wetlands to croplands. Moreover, animal excrement inputs from grazing activities following drainage can reduce soil C:P and N:P ratios as manure are generally P-rich with low N:P ratios, e.g. pig slurry N:P ratio as 2.0-3.8 (Peñuelas et al., 2009; Sardans et al., 2012).

Soil P dynamics through drainage and rewetting can be distinct from C and N due to the lack of gaseous phase as well as the significant involvement of abiotic processes on top of the biotic microbial responses. The behavior of P after drainage is intimately related to P adsorption and desorption processes, which depends on the changes in pH, metal (e.g., aluminium, iron, and calcium) contents and organic matter content over land conversions.

In mineral marshes which contain decreasing organic matter and increasing mineral matter with depth in the profile, total abundance and fractions of P (including dissolved organic and inorganic P and precipitate organic and inorganic P) vary greatly in surface organic and deep mineral soils and can respond differently to drainage and restoration (Dunne and Reddy, 2005). P at surface litter and organic matter layer mainly exists as organic P and the proportion of inorganic P in the total P pool is mainly biologically controlled indicated by the P mineralization rates. Drainage and restoration greatly disturb the aboveground vegetation and the organic layer which subsequently alter the organic C, N and P contents and proportions in soil. In response to the aeration of SOM, studies showed a significant decline in total P accompanied with increased proportion of inorganic P owing to the elevated P mineralization rates (Olila et al., 1997). However, experimental evidence did not support significant release of P upon wetland drainage (Venterink et al., 2002; Dieter et al., 2015). It seems that only when drying/drainage is followed by rewetting can result in P release (Ardón et al., 2010; Schönbrunner et al., 2012; Bai et al., 2019).

In mineral-rich layer, P dynamic is mainly regulated by soil physiochemical characteristics and processes (e.g., adsorption, weathering). P generally precipitates as aluminum-phosphates and Fe^{3+}- phosphates or calcium-phosphates, depending on the soil pH (Qualls and Richardson, 1995). Among the three major P-precipitates, Fe^{3+}-phosphates is most sensitive to drainage and restoration as drying and rewetting greatly alter the redox potential in soils which regulates the abundance of Fe^{3+} (>250mV) and Fe^{2+} (<250mV) and subsequently determines net absorption or net release of P. Noticeable P release from Fe^{3+}- phosphates into soluble phosphate was reported as a result of rewetting (Aldous et al. 2007). In contrast, aluminum-phosphates, which generally dominate in acidic wetland soils, only transforms between oxidic and amorphous forms with no significant net loss or gain of P.

Richardson (1985) pointed that the amorphous Al content was the best predictor of adsorbed P in freshwater wetlands.

2.5 References

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Chapter 3: Litter decomposition and nutrient dynamics of four macrophytes in intact, restored and constructed freshwater marshes of Canada

Bridging statement to Chapter 3

Restoration of the drained freshwater marshes rewets soils, inhibits aerobic decomposition, nitrification and is expected to promote the accretion of C, N, P in organic compounds. However, the rates of decomposition and nutrient retention under various restoration scenarios (e.g., different restoration age and surrounding land uses) are rarely examined. In this chapter, I determined the rates of decomposition of four major plants in eight intact, restored and constructed marshes, and their nutrient dynamic patterns, to see whether the decomposition rates and nutrients loss and retention are at similar levels. We found no consistent pattern in litter decomposition rate among wetland classes (intact versus restored). Instead, litter quality and wetland characteristics, especially the inundation periods and surrounding land uses, significantly affected the decomposition rates. Litter quality and wetland surrounding land uses (e.g., sewage inputs) also significantly affected nutrient retention in decomposing litters.

© Dan Dong, Pascal Badiou, Tim R. Moore, Christian von Sperber (2024) Restoration Ecology, 32: e14135. https://doi.org/10.1111/rec.14135

3.1 Abstract

The restoration and construction of wetlands offers opportunities to rewet soils, inhibit decomposition and enhance nutrient retention in decomposing litters. Here we report the decomposition rates and nutrient dynamics of macrophyte litters in intact, restored, and constructed wetlands. A 2.1-yr litterbag experiment of four common freshwater macrophytes (*Phalaris arundinacea, Phragmites australis, Scirpus cyperinus* and *Typha latifolia)* was conducted in 8 freshwater marshes (3 intact, 4 restored and 1 constructed) within three sites in Manitoba and Ontario, Canada, which varied in restoration age, inundation periods and surrounding land uses. Litter mass loss and N and P dynamics were measured. Litter decomposition rates (*k*) followed the order of *P. arundinacea* (0.42 ± 0.03 yr^{-1}) > *T. latifolia* (0.31 ± 0.03 yr^{-1}) > *P. australis* (0.19 ± 0.01 yr^{-1}) > *S. cyperinus* (0.13 ± 0.01 yr^{-1}) in most wetlands and were positively correlated to the initial litter N concentration. Litters decomposed fastest under seasonally inundated conditions, rather than permanent inundation. N and P retention in litters were significantly affected by both initial litter N and P concentration and wetland surrounding land uses. After 2.1 yr of decomposition, the N:P ratio of all litters converged to 20 to 28:1 regardless of initial litter N:P ratio or N or P concentrations. The effectiveness of wetland restoration on slowing decomposition and enhancing nutrient accumulation depends on the quality of the input litters and wetland characteristics including inundated periods and surrounding anthropogenic disturbances.

3.2 Introduction

Widespread drainage of wetlands has caused a degradation of wetland biogeochemical functions globally resulting in significant net loss of carbon (C) to the atmosphere and nutrient release especially of nitrogen (N) and phosphorus (P) to the downstream water bodies (Zedler 2003; Blann et al. 2009; Davidson 2014). An important attempt to regain the biogeochemical functions of drained wetlands is through restoration (Moreno-Mateos et al. 2012). In North America, multiple restoration programs have been undertaken since the 1980s and among them significant restoration efforts have been devoted to freshwater marshes in the Canadian Prairie and Great Lakes regions (Bedford 1999; Rubec and Hanson 2009). Assessing the reestablishment of wetland ecological functions has increasingly become a topic of interest (Zedler 2000; Kollmann et al. 2016; Creed et al. 2022) and the rates of plant production, litter decomposition and the ability of decomposing litter to retain N and P are important.

Litter decomposition processes contribute greatly to the regaining of wetland functions such as the nutrient cycling, ecosystem primary productivity and rates and components of sediment accumulation in restored wetlands (Gingerich and Anderson 2011). The restoration and construction of wetlands offers opportunities to rewet soils, inhibit decomposition and enhance the organic nutrient retention in decomposing litters. Yet, the rates of decomposition and nutrient dynamics of macrophyte litters under multiple restoration scenarios such as different water levels and inundation periods, restored ages, with/without substantial surrounding human impacts (e.g., agricultural activities) have not been well addressed.

The fluctuating hydrology of wetlands and surrounding anthropogenic disturbances clearly add uncertainties to the recovery of restored systems. Freshwater marshes with highly variable hydrological conditions can result in alternation between anaerobic and aerobic conditions depending on the position of water level at different times of the year (Craft 2015). This also frames the highly variable soil physiochemical properties and the decomposer communities which greatly affect litter decomposition (Yang et al. 2006; Fennessy et al. 2008; Yarwood 2018). In the Delta Marsh of Manitoba, for instance, van der Valk et al. (1991) and Neckles and Neill (1994) both reported significantly faster litter decomposition of macrophytes under flooding treatments compared to sites which were never flooded. The decomposition rates in marshes also vary significantly with different water depths and frequency and periods of inundation (Neckles and Neill 1994; Sun et al. 2012). One explanation for this phenomenon is that flooding boosts the fragmentation of litters as well as the leaching of dissolved carbon and nutrients into the soil solution which favours litter breakdown (Dolinar et al. 2016). Alternatively, flooding also increases soil moisture and dissolved nutrients which favours the activities of enzymes associated with litter depolymerization (Neckles and Neill 1994; Borowik and Wyszkowska 2016). Apart from hydrology, substantial anthropogenic disturbances around restored sites, such as nutrient enrichment due to the runoff from the surrounding agricultural lands (Qualls and Richardson 2000; Badiou et al. 2018), also have great potential to affect decomposition rates, as they could greatly alter the microbial nutrient use efficiencies and enzymes secretion during litter decomposition (Wright and Reddy 2001; Corstanje et al. 2006).

To meet the nutritional demands of microorganisms, the net retention or loss of nutrients in litters are usually controlled by two major pathways: either from the often-limited initial N and P content of litter or the alternative nutrient supplies from soil substrates or runoff from catchment. Litter tissues with higher concentration of N and P together with lower lignin content tend to lose N and P more readily and are characterized by fast nutrient mineralization and slow immobilization rates (Yang et al. 2006). Critical C:N and C:P ratios were reported in both terrestrial and aquatic ecosystems (e.g., 37-70 and 700-900 for Canadian forests; <58 and 820 for northern peatlands) below which mineralization exceeded immobilization when nutrients tend to be lost and above which immobilization exceeded mineralization when nutrient retention usually occurred (Bridgham and Richardson 2003; Moore et al. 2006; Manzoni et al. 2010). Hydrological and anthropogenic disturbances can greatly affect the nutrient dynamics in wetlands (Rejmánková and Houdková 2006; Corstanje et al. 2007). Flooding often amplifies the nutrients released from litters and at the same time modifies the pH and the oxygen availability that selects the size and groups of decomposers and thus their nutrient demands (van der Valk et al. 1991; Yarwood 2018). Anthropogenic disturbances can directly affect the catchment nutrient contents which alter N and P mineralization and immobilization rates as well (e.g., Rejmánková and Houdková 2006; Fellman and D'Amore 2007).

To better establish rates of litter decomposition and N and P dynamics in restored and constructed wetlands, which is critical to assess the re-establishment of wetland biogeochemical functions, we conducted a 2.1-yr litterbag experiment of four common freshwater macrophytes (*Phalaris arundinacea, Phragmites australis, Scirpus cyperinus and Typha latifolia)* in 8 freshwater marshes (3 intact, 4 restored and 1 constructed) at 3 sites in Manitoba and Ontario, Canada. We aimed to determine the litter decomposition rates and the N and P dynamic patterns - loss, retention or gain over the 2.1 years. Two hypotheses were tested in this study: 1) Litter decomposition will be faster in restored marshes with lower water levels and fewer inundation periods than the adjacent intact wetland; 2) Substantial surrounding human disturbances, specifically, agricultural activities and sewage inputs will affect both the decomposition rates and the N and P dynamics. Our study aims to provide practical restoration suggestions (e.g., wetting strategies and location choices) to best re-establish the lost ecosystem biogeochemical functions.

3.3 Methods

3.3.1 Study areas and wetland characteristics

The litterbag experiment was conducted from May 2019 to June 2021 (2.1 year) in eight depressional-isolated marshes at three sites in Canada (Figure 1; Table 1): four marshes (1 intact, 3 restored) in Atocas Bay Reserve (45°37’N, 74°50’W; 647 ha; ABR), Ontario; three marshes (2 intact, 1 restored) in Broughton’s Creek watershed (50°06’N, 100°13’W; 26,034 ha; BC) and one constructed marsh that receives sewage from the Interpretive Center in Oak Hammock Marsh (50°10’N, 97°08’W; 3,600 ha; OHM), Manitoba, which serves for public education on wetland conservation.

The ABR is located in eastern Ontario with a mean annual temperature and precipitation of 6.7 °C and 895 mm, respectively (2012-2021 average for station OTTAWA INTL A, Environment Canada 2021). It has a history of drainage for agricultural uses but was restored between 2000 and 2010 with land purchased by Ducks Unlimited Canada (Ducks Unlimited 2016). Old ditches were blocked so that wetlands could be rewetted by precipitation and the spring snowmelt. After around 10-20 years of restoration, the drained ponds are wet again although the water levels are not as deep as the intact ones and can sometimes fall to the soil surface in summer (Figure S3.1). The vegetation in restored marshes is similar to the intact ones after over 10-year succession, and both are dominated by perennial grasses and sedges, providing nesting sites for the waterfowl. Dominant species include *Carex* spp., *Eleocharis* spp., *Juncus effusus, Phalaris arundinacea, Phragmites australis, Scirpus cyperinus, Typha* spp. and *Solidago rugosa*. This study selected three representative marshes ABR-R10, ABR-R17 and ABR-R18, with seasonally inundation that were restored in 2010, 2003 and 2002, respectively, together with a permanently inundated intact marsh as reference to determine the impact of the wetland inundation periods and restoration age on litter decomposition processes (Table 3.1).

BC also has a history of drainage and subsequent restoration led by the Government of Manitoba and Ducks Unlimited in the 1960s but unlike ABR, not all drained marshes were restored. Today, the landscape contains a mosaic of intact, drained and restored freshwater marshes (Mitsch and Hernandez 2013; Penfound and Vaz 2022) with 71.8% agriculture fields and 9.8% wetlands (Yang et al. 2010). Wetlands were surrounded by either grassland or conventional cropland in a standard canola-wheat rotation (Agriculture and Agri-Food Canada 2020, 2021). The OHM is close to BC located in western Manitoba within the transitional grassland zone where the mean annual temperature and precipitation are 3.2 °C and 400 mm, respectively (2012-2021 average for station WINNIPEG A CS, Environment Canada 2021). Wetlands in BC and OHM in this study were all permanently inundated (Figure S2) dominated by prairie grasses in uplands (e.g., *Agropyron* spp*., Elymus* spp*., Poa* spp*., Andropogon* spp*., Festuca* spp.) and reeds (*Typha* spp.) and sedges (*Carex* spp.) in the depressional watersheds. We selected two intact wetlands surrounded by grasslands and croplands, respectively, together with a 10-year restored wetland (in 2019) surrounded by croplands in BC and a constructed sewage treatment wetland in OHM to detect the impact of wetland surrounding land uses on litter decomposition (Figure 3.1; Table 3.1).

Soils are clay rich (>50%) and slightly acidic to neutral (pH value ranges between 5-7) in ABR wetlands, while clay loam and mostly alkaline (pH value ranges between 7-8) in BC wetlands except for restored wetland BC-R10 where soil pH is between 5-6 (Table S1; Badiou et al. 2018). The soil C, N and P concentrations of ABR wetlands range within 4.6-6.7 %, 0.4-0.5 % and 0.06-0.07 %, respectively, and those of BC wetlands range within 7.1-13.2 %, 0.6-1.7 % and 0.04-0.05 %, respectively (Table S3.1).

At each wetland, we installed a continuous water level recorder in the open water section of each wetland close to litterbag emplacement to record changes in water table level during the growing season (June to October).

3.3.2 Litterbag preparation, installment and retrieval

We collected the senescent litter of 4 common macrophytes in October 2018. These were *Typha latifolia* from the peatland margin at Mer Bleue bog near Ottawa, stems of *Phragmites australis* and *Scirpus cyperinus* and leaves of *Phalaris arundinacea* sampled from two wetlands in ABR. Litter was air-dried and subsampled into two groups. One group was used for the litterbag experiment and the other group was to determine the air-dry and oven-dry conversion and the initial litter C, N and P concentrations.

Five grams of air-dried litter, together with individual metal tags (weight excluded) were installed in a 10×10 cm polypropylene fabric litterbag with 2×2 mm mesh. In May 2019, 4 litter types with triplicates for 4 retrievals totalling 384 $(4\ \text{litter types} \times 3\ \text{replicates} \times 4\ \text{retrievals} \times 8\ \text{wetlands})$ litterbags were deployed on the surface soil at the center of the 8 marshes and tied to wooden sticks installed in each wetland.

Litterbags were retrieved four times: September 2019, July 2020, October-November 2020 and June 2021. Each time, triplicate litter bags were collected for calculation of average decomposition rates for each species at each wetland. Retrieved litterbags were cleaned under DI water to remove the attached mineral matter and the fresh plant materials with a 0.5 mm mesh placed underneath to collect residues. Litter was air-dried and oven-dried at 65°C for 24 hr till constant weight and then weighed to calculate the remaining mass relative to the initial litter mass (g) based on a conversion for air-dry to oven-dry mass for each litter type. Initial and remaining litters at the last harvest in June 2021 were both milled and analyzed for the N and P concentrations. The fractions of initial nutrients remaining in litters were calculated by the equation:

$$\text{The fractions of initial nutrients remaining in litters} = \frac{\mathrm{C_{re}} \times \mathrm{M_{re}}}{\mathrm{C_i} \times \mathrm{M_i}} \qquad (1)$$

Where C_{re} and C_i refer to the concentration of N and P from remaining litters and initial litters, respectively, and M_{re} and M_i refer to the mass of the remaining and initial litters, respectively.

3.3.3 Soil sampling and CNP determination

Triplicate soil cores of 0-10 cm depth in three wetlands of BC (BC-IC, BC-IG and BC-R10) and two wetlands of ABR (ABR-Intact and ABR-R10) were sampled around the litterbags in June of 2019 and July of 2021, respectively. Soil C, N and P concentration and pH were then measured. For both soils and litters, the C and N concentrations (%) of were determined by a CHN analyzer (EA 1112, Carlo Erba, Milan, Italy) and the P concentration (%) was determined colorimetrically using the Malachite Green method (Ohno and Zibilske 1991) after hot acid digestion at 360 °C in a H_2SO_4-H_2O_2 mixture following Akinremi et al. (2003). Soil pH was determined in a solution of 1:5 soil/deionized water (m/v) by a pH-meter.

3.3.4 Data analyses

Annual litter decomposition rates were estimated from a modified exponential decay model with an equation of $M_{(t)}/M_0 = e^{-kt+b}$ (Moore et al. 2006) where M(*t*) is the remaining mass (g) at harvest time *t* and *t* is the elapsed time (yr), M_0 is the original mass of litters (g), *k* is the annual decomposition rate constant (yr^{-1}) and b is a fitted parameter. We ln-transformed $M_{(t)}/M_0$ to estimate k with a linear least square model assessed by adjusted R^2 and p-values. All of the mass-loss curves started from 1 (t=0). For each litter species at each wetland, triplicate k values were estimated, and the average k and standard deviation were calculated and presented.

The differences in initial litter chemical properties were assessed by One-way Analysis of Variance (ANOVA). Two-way ANOVA followed by Tukey's test was used to detect the effects of litter species, wetland characteristics (hydrological conditions or surrounding land uses) and their interaction on litter decomposition rates, the fractions of initial nutrient remaining and the remaining N:P quotient within two groups of wetlands. The eight marshes were pooled into two groups for Two-way ANOVA based on their wetland characteristics: four marshes (ABR-Intact, ABR-R10, ABR-R17 and ABR-R18) in ABR and four marshes (BC-IC, BC-IG, BC-R10 and OH) in BC and OHM which vary in their inundation periods and surrounding land uses, respectively (Table 3.1). Data residues were assessed for normality and homoscedasticity by quantile-quantile plot and Shapiro-Wilk test and Levene's test, respectively.

Pearson's correlation analysis was performed to detect the relationships between the averaged k, litter remaining N, P, N:P quotient (mass based) and the initial litter N, P and the stoichiometric characteristics (mass based) of four litter species for each individual wetland. The relationships between restoration age and the decomposition rates of each litter species from three restored marshes in ABR (ABR-R10, ABR-R17 and ABR-R18) (n=9) were determined by Pearson's correlation and linear regression. Prior to Pearson correlations and linear regressions, data normality, homoscedasticity and no autocorrelation assumptions were confirmed by quantile-quantile plot and Shapiro-Wilk test, Levene's test and both Durbin-Watson and Breusch-Godfrey tests, respectively. When normality was not met, ln-transformed data were used. All data analyses were performed in RStudio version 1.1.463. The significance level of this study was at 95% confidence interval (p<0.05). Unless otherwise specified, mean ± standard errors (SE) were presented for all the values.

3.4 Results

3.4.1 Wetland hydrologic regimes

Intact wetlands including ABR-Intact, BC-IC and BC-IG were all permanently inundated and showed higher average water table than the adjacent restored wetlands. The restored wetland in BC (BC-R10) and the treatment marsh in OHM (OH) were also permanently inundated while those in ABR (ABR-R10, R17 and R18) were all small, shallow waterbodies (< 1 ha) that tended to dry out over the course of the growing seasons (Figures S3.1 and S3.2).

3.4.2 Initial litter quality

The four litter species encompassed a narrow range of total C (44.3-48.4 %) but a wide range of total N (0.44-1.01 %) and P (0.018-0.062 %) (Table S3.2). The initial N and P concentrations and C:N, C:P and N:P quotients varied significantly among litters (p<0.05). Leaves of *P. arundinacea* contained the richest N content followed by *T. latifolia* and *P. australis* with *S. cyperinus* the lowest N content. Litter total P was in the order of *P. arundinacea* > *T. latifolia* > *S. cyperinus* > *P. australis*. Among them, *P. arundinacea* had significantly higher P than the other three litters (p<0.05). With respect to the N:P quotient, *P. australis* had a significantly higher N:P quotient than *P. arundinacea* and *S. cyperinus* (p<0.05). Other litter traits such as wax surface was observed on the stems of *S. cyperinus* only.

3.4.3 Decomposition rates

Litter decomposition rates expressed as the exponential decay *k* value varied significantly among the four different macrophytes ($p<0.001$) with average *k* across 8 wetlands in the order of *P. arundinacea* 0.42 ± 0.03 yr^{-1} > *T. latifolia* 0.31 ± 0.03 yr^{-1}> *P. australis* 0.19 ± 0.01 yr^{-1} > *S. cyperinus* 0.13 ± 0.01 yr^{-1} (Tables 3.2 and S3.2) and was comparable with other litterbag studies from the literature (Table S3.3). This order of *k* was consistent in almost all the individual freshwater marshes in ABR, BC and OHM, indicating a very strong initial litter quality control on *k* (Figure 3.2a-e and 3.3a-e). We determined that litter *k* values were significantly positively correlated to initial N concentration in five out of eight wetlands (r=0.61-0.98) while less consistently positively correlated to initial P concentration (significant for two wetlands, r=0.41-0.97) and had no clear relationship with initial N:P quotient (r=(-0.68)-(0.09); Table S4).

Wetland characteristics and its interactions with litter species also significantly affected the decomposition rate (Table 3.2). In ABR, litters in seasonally inundated restored wetlands (ABR-R10, ABR-R17 and ABR-R18) had significantly faster decomposition rates (by 22%, 35% and 65%) relative to the adjacent intact wetland ABR-Intact that was permanently inundated (Figure 3.2f). This significant difference suggests a strong positive influence of the dry-wet dynamics on *k*, which was particularly evident in readily decomposable litters (*P. arundinacea* and *T. latifolia)* relative to more recalcitrant litters (*P. australis* and *S. cyperinus*). We did not find consistent relationships between litter *k* values and restoration age for four litters in ABR (r=(-0.09)-(0.79)), although strong positive relationships between litter *k* values and restoration age was observed for *P. arundinacea* ($y = 0.02x + 0.17$, R^2=0.76; $p<0.05$; n=9) and *P. australis* ($y = 0.01x + 0.05$, R^2=0.57; $p<0.05$; n=9; data not shown).

Wetlands in Manitoba varied greatly in their surrounding land uses. BC-IG was surrounded by a grassland while BC-IC and BC-R10 were both surrounded by conventional cropland in a standard canola-wheat rotation (Figure 3.1; Agriculture and Agri-Food Canada 2020, 2021). OH was a constructed marsh that received the sewage from the adjacent interpretive center. Results showed that the average k at the four wetlands in Manitoba were in the order of BC-IC (0.35 ± 0.06 yr^{-1}) > OH (0.22 ± 0.02 yr^{-1}) > BC-IG (0.20 ± 0.03 yr^{-1}) > BC-R10 (0.15 ± 0.02 yr^{-1}) and litters decomposed significantly faster in BC-IC ($p<0.05$) than the other wetlands (Figure 3.3f). In particular, the k value of *T. latifolia* (0.59 ± 0.06 yr^{-1}) was twice that at other wetlands (Figure 3.3a-e).

Overall, litters decomposed faster in wetlands of ABR in Ontario, which has a warmer summer and shorter frozen period in winter than BC and OHM in Manitoba (Figure 3.2e and 3.3e), especially for *P. arundinacea* (0.51 ± 0.03 yr^{-1} in ABR) which was 1.5 times the average rate in BC and OHM (0.34 ± 0.03 yr^{-1}) ($F=18.5, p<0.001$; One-way ANOVA). *P. australis* and *S. cyperinus* also decomposed significantly faster in ABR than BC and OHM with average rates of 0.21 ± 0.02 yr^{-1} *versus* 0.17 ± 0.02 yr^{-1} ($F=4.15, p=0.05$; One-way ANOVA), and 0.15 ± 0.01 *versus* 0.11 ± 0.01 yr^{-1} ($F=9.65, p<0.01$; One-way ANOVA) respectively. Nevertheless, the *T. latifolia* in wetlands of BC and OHM, especially in BC-IC, decomposed at a similar rate (0.30 ± 0.05 yr^{-1}) as those in wetlands in ABR (0.31 ± 0.03 yr^{-1}) (Figure 3.2e and 3.3e), largely due to the exceptionally fast decomposition rate at BC-IC (Figure 3.3a).

3.4.4 N and P dynamics

Over a 2.1-year decomposition period, the loss, retention or gain of N and P varied greatly among different litter species in both ABR and BC and OHM (Figure

3.4; Table 3.2; Table S3.5). *P. arundinacea* lost both N and P across all 8 wetlands with the average loss of 45 % N and 65 % P, respectively and showed an increase in the litter N:P quotient from 16 ± 1 to 28 ± 2 (Figure 3.4; Table S3.2, S3.5). In contrast, *T. latifolia* and *S. cyperinus* showed a net gain in both N and P in most wetlands with average retention of 110 % and 121 % N and 133 % and 120 % P, respectively (Figure 4). The more P retention than N in *T. latifolia* resulted in slight decline in the N:P quotient from 30 ± 1 to 26 ± 2 over the 2.1-year decomposition while the N:P quotient in remaining *S. cyperinus* litters (23 ± 1) remained very similar to the initial N:P quotient as 22 ± 1 (Table S3.2). *P. australis* lost 15% N while retained 115 % P on average, leading to the most decrease in the N:P quotient from 36 ± 4 to 28 ± 2 among all litters. The fractions of initial N and P remaining were both negatively associated with the initial litter N and P concentration (r ranging from -0.51 to -0.99 and from -0.61 to -0.99, respectively, across eight wetlands) (Table S3.4). Nevertheless, the remaining N:P quotients of litters had no clear relationship with the initial N:P quotients (r ranging from -0.62 to 0.90, $p>0.05$) nor the initial N or P concentration across wetlands (r ranging from -0.13 to 0.87 and from -0.56 to 0.82, respectively, $p>0.05$; Table S4). Overall, the N:P quotient of all litters converged between 21-25 for wetlands in ABR and 25-31 for wetlands in BC and OHM at the end of the study period (Figure 3.4).

Surrounding land uses (e.g., with/without sewage inputs) significantly affected the fractions of initial P remaining and the N:P quotient in litters (Table 3.2). OH which receives sewage inputs significantly elevated the P remaining of all the litter species ($p<0.05$; Figure 3.4; Table S3.5). The N remaining in OH, however, did not differ significantly from other wetlands, resulting in significant lower N:P ratios of remaining litters in OH compared to other marshes in BC ($p<0.05$; Figure 3.4). The remaining N and P of litters in BC-IC and BC-R10 which were affected by the surrounding agricultural activities did not differ significantly from those in BC-IG (intact surrounding by grasslands), except that noticeable net gains in N and P were observed for *T. latifolia* in BC-R10. Among all the litters, surrounding land uses most significantly affected *T. latifolia* which had the highest SE (Figure 3.4). Although *T. latifolia* showed on average net gain in N and P in both ABR and BC wetlands, significant losses of litter N in ABR-R17, ABR-R18 and BC-IC as well as loss of litter P in ABR-R18 and BC-IC were observed (Figure 3.4; Table S3.5). Apart from *T. latifolia*, the other three litters generally showed no significant difference in the remaining N and P across wetlands in ABR with varying hydrological conditions or in BC with varying surrounding land uses. The overall gains of N in litters were more significant in BC than ABR while in contrast, P gains were more significant in ABR than BC (Figure 3.4). Consistently lower N:P ratios in the remaining litters of ABR than those of BC and OHM were observed (F=18.75, p<0.001; One-way ANOVA).

3.5 Discussion

3.5.1 Litter decomposition rates in wetlands

We find a consistent order of the exponential decay constant k (yr^{-1}) of four macrophytes *P. arundinacea* > *T. latifolia* > *P. australis* > *S. cyperinus* across different freshwater marshes which is in line with other studies in temperate regions (e.g., Gingerich and Anderson 2011; Dolinar et al. 2016). This suggests a predominant litter species control on decomposition rates in wetlands under different land managements. Among all the litter quality parameters measured in this study, we observed the initial litter N concentration was most significantly positively related to the litter k. This significant litter N or N/lignin control on litter decomposition rate has long been recognized in both forest and wetland ecosystems (Hobbie 2005; Xie et al. 2019) although sometimes litter P concentration can be an equally important predictor (Rejmánková and Houdková 2006). The relative importance of multiple litter quality parameters can vary with the natural ranges of the indices of the selected litters (Taylor et al. 1987). In this study, we have very similar litter P concentrations for three out of four macrophytes which may obscure the relationship between litter P concentration and *k*. The slowest decomposition observed for *S. cyperinus* may be due to its lowest initial N concentration as well as its water-resistant waxy cuticle which acts as a barrier slowing litter leaching and the colonization and depolymerization of decomposers (Dolinar et al. 2016; Zukswert and Prescott 2017).

Litters decomposed significantly faster in the restored wetlands of ABR compared to their adjacent intact wetlands. The faster litter decomposition in ABR-R10, R17 and R18 than ABR-Intact is likely due to the seasonal inundation of the restored wetlands which dried in summer and rewetted in autumn. Alternating between dry and wet conditions favored decomposition, mineralization and CO_2 emissions in many ephemeral-flooded forests and wetlands (Battle and Golladay 2001; Jarvis et al. 2007; Capps et al. 2014). Exposing litter to an aerobic environment during summer when water levels drop below the soil surface likely enhances the microbial activities and mineralization rates relative to those experienced in wetlands that are more permanently inundated where exposure to oxygen is limited (Battle and Golladay 2001). Moreover, the subsequent wetting in autumn can boost the fragmentation of litters and the leaching of soluble carbon and nutrients which both stimulate litter mass loss (Dolinar et al. 2016). The soil moisture during the dry period is also an important parameter determining litter decomposition rates since drought (e.g., moisture < 20%) can greatly inhibit microbial growth and enzymatic activities which slows down litter decomposition (Schimel 2018). During the dry period in this study, we noticed a greater coverage (>80%) of vegetation in our wetlands which suggests a still moist condition for plant growth and litter decomposition. Thus, a mild drying of litters in summer could potentially elevate the average annual litter decomposition rate of restored marshes.

At BC, we determined significantly faster litter decay the intact wetland BC-IC surrounded by croplands than the other two wetlands. Given the permanently wet conditions of all marshes at BC, we owe this variance to the different soil substrate quality, especially soil N concentration of wetlands. The soil total N concentration (%) of the top 10 cm core around the litterbags was twice and three times in BC-IC of those in BC-R10 and BC-IG, respectively, while soil total P concentrations (%) were very similar between the three wetlands. According to the previous studies in the prairie pothole regions, wetlands surrounded by croplands can potentially receive large amounts of potassium (K), phosphate (PO_4^{3-}) and ammonium nitrate (NH_4NO_3) from the surrounding fertilization activities (Badiou et al. 2018), which is very likely to elevate the soil nutrient levels, especially soil N in our finding. Although the impact of soil N enrichment on litter decomposition rate (accelerate, decelerate or not significantly impact) can vary with site nutrient quality, litter type and the extent of N additions (Hobbie et al. 2005; Corstanje et al. 2006), an average acceleration of 11% in litter decomposition rate under N fertilization in wetland ecosystems was reported by a recent meta-study comprising ~10 field fertilization experiments (Su et al. 2021).

3.5.2 Nutrient dynamics in wetlands

The nutrient dynamic pattern (loss, retention or gain) of litters is not affected by inundation periods, or wetland class (intact *versus* restored) in this study but is largely dependent on the litter species and the site and surrounding anthropogenic activities where litters decomposed (ABR *versus* BC *versus* OHM). Our findings partially confirmed that when initial litter nutrient concentration is above a certain value (e.g., 1 % of litter N) or initial litter C-to-nutrient ratio is below the critical ratio, nutrient mineralization prevails immobilization and otherwise immobilization prevails mineralization and nutrient retention/gains usually occurred (Parton et al. 2007; Manzoni et al. 2010; Moore et al. 2011). *P. arundinacea* with the richest N (1.0 %) and P (0.06 %) content showed net loss of both nutrients at all wetlands and *S. cyperinus* with the poorest N (0.44 %) and relatively poor P (0.02 %) retained N and P in almost all wetlands. Nevertheless, the other two litters with similarly limited N and P did not show consistent retention as expected. We found *P. australis* with relatively low initial N concentration (0.62 %) lost N while retained P at many wetlands probably because its initial P was more limited (P concentration <0.02 %).

The net loss of N and net gain of P of litters or *vice versa* were also noticed in other litterbag studies (e.g., Moore et al. 2011) which could result from an imbalanced N and P supply in the initial litters for the microbial nutrient demands (Manzoni et al. 2010; Mooshammer et al. 2014). *T. latifolia* also lost N or P significantly in many wetlands but gained N and P in others. However, despite the inconsistency in its N and P dynamic patterns amongst wetlands, the N:P quotients of the remaining *T. latifolia* litters at the end of decomposition were highly constrained. The CIDET dataset (Moore et al. 2011) compiling over 100 observations in the N and P dynamics through 12-year litter decomposition experiment and many other individual litterbag dataset such as Morris and Lajtha (1986) and Xu and Hirata (2005) also revealed a strong convergence of litter N:P stoichiometry through time regardless of the wide range of initial litter C:N and C:P ratios. In this study, litter remaining N:P quotient all converged to ~25 in ABR, ~30 in BC and ~20 in OHM. Morris and Lajtha (1986) reported the remaining N:P quotient of 13-20 for *Carex* spp. and *Typha* spp.

decomposed in a tidal marsh of Massachusetts for one year. Xu and Hirata (2005) studied 7 litter leaf tissues of subtropical forest with a range of 29-57 in their initial N:P quotients and found the remaining N:P quotient approached 30 to 40 after a two-year decomposition. These findings all suggested a stoichiometric balance is achieved in the remaining litter N:P and this balance can vary with sites and ecosystems.

The magnitude of N immobilization is also strongly regulated by initial litter N availability as microbes confront severe N starvation in N-poor litters (Manzoni et al. 2012; Bonanomi et al. 2017). Mooshammer et al. (2012) measured the mineralization and immobilization rates and reported negative relationship between initial litter N and N immobilization rates. In this present study, with similar initial litter P concentrations, we did not find a significantly higher percent of P retention for P-limited litters but greatest N retention in *S. cyperinus* occurred in all wetlands was indeed noticed. On average, the percent of N retention in litters is significantly negatively related to the initial litter N concentration.

When litter nutrients are limited, decomposers can effectively absorb the nutrients from the surrounding soil substrate which makes site nutrient availability and surrounding anthropogenic activities another important regulator on the magnitude of litter N and P gained as well as the stoichiometric balance in remaining litters. For instance, Rejmánková and Houdková (2006) compared P retention in litters among NH_4NO_3 enriched, PO_4^{3-} enriched, $NH_4NO_3 + PO_4^{3-}$ enriched and reference wetlands and reported highest P retention and lowest litter remaining C:P quotient at the PO_4^{3-} enriched and $NH_4NO_3 + PO_4^{3-}$ enriched site. In agreement with their findings, the constructed wetland in this study which received sewage from human activities also showed the highest percent of P gain in all litters. Also, the lowest N:P quotients in remaining litters at OH were observed. However, we did not observe equally significant N gain nor increased N:P quotient in remaining litters in BC-IC which has a two-and -three- fold N in its surrounding soil substrate than other wetlands. This insignificant response to N enrichment can be ascribed to the still not enough N addition or a more profound limitation on P than N for microbes in wetlands which makes N addition insensitive. When N and P are both rich, a consistently higher percent of P loss than N loss in *P. australis* also suggests this suspension.

3.6 Acknowledgements

We thank the Contributing Editor and two anonymous reviewers for their constructive comments improving this manuscript. We thank Brendan Brooks, Janice Neumann, Calder Jones, Mike Dalva and Paula Kestelman for their great assistance in the field and laboratory, and Dr. Frieda Beauregard for plant identification. This study was funded by Natural Sciences and Engineering Research Council of Canada (NSERC STPGP 506809).

3.7 Tables and figures

Table 3.1 Descriptions of the eight wetlands in Atocas Bay Reserve (ABR), Broughton's Creek (BC) and Oak Hammock Marsh (OHM) from this study. 'MAT' and 'MAP' refers to mean annual temperature and precipitation, respectively. 'Water Level' refers to the average water level above soil surface at the open water section close to litterbag emplacement of eight wetlands during growing seasons (June to October) of year 2019, 2020 and 2021 for wetlands ABR-Intact, ABR-R10, ABR-R17 and ABR-R18 and year 2019 and 2020 for wetlands BC-IC, BC-IG, BC-R10 and OH.

Wetland	Location	MAT(°C) /MAP(mm)	Land Management	Surrounding Land Uses	Inundation Periods	Water Level (cm)	Soil Texture
ABR-Intact	ABR, Ontario	6.7 / 895	Intact	Grasslands	Permanently	35.4	Clay
ABR-R10	ABR, Ontario		Restored since 2010	Grasslands	Semi-permanently	10.8	Clay
ABR-R17	ABR, Ontario		Restored since 2003	Grasslands	Semi-permanently	28.9	Clay
ABR-R18	ABR, Ontario		Restored since 2002	Grasslands	Semi-permanently	19.4	Clay
BC-IC	BC, Manitoba	3.2 / 400	Intact	Croplands	Permanently	64.2	Clay Loam
BC-IG	BC, Manitoba		Intact	Grasslands	Permanently	66.7	Clay Loam
BC-R10	BC, Manitoba		Restored since 2010	Croplands	Permanently	33.1	Clay Loam
OH	OHM, Manitoba		Constructed	Sewage treatments	Permanently	35.9	Clay Loam

Table 3.2 Results of Two-way ANOVA for the effects of litter species, wetland characteristics and their interactions on litter exponential decay (k, yr^{-1}) and remaining N and P (%) and remaining N:P quotient (mass based), in two wetland groups (Atocas Bay Reserve, Broughton's Creek and Oak Hammock Marsh).

Site	Treatment	df	k (yr^{-1})		Remaining N		Remaining P		Remaining N:P	
			F value	Sig.	F value	Sig.	F value	Sig.	F value	Sig.
ABR	Litter species	3	193.14	**<0.001**	127.21	**<0.001**	117.77	**<0.001**	6.68	**<0.001**
	Wetland characteristics	3	32.91	**<0.001**	22.91	**<0.001**	2.94	**<0.05**	9.23	**<0.001**
	Litter species × Wetland characteristics	9	8.72	**<0.001**	3.73	**<0.01**	5.82	**<0.001**	6.28	**<0.001**
BC and OHM	Litter species	3	55.50	**<0.001**	41.56	**<0.001**	118.88	**<0.001**	3.69	**<0.05**
	Wetland characteristics	3	33.04	**<0.001**	2.67	0.06	98.76	**<0.001**	30.59	**<0.001**
	Litter species × Wetland characteristics	9	7.40	**<0.001**	2.54	**<0.05**	10.98	**<0.001**	1.88	0.09

Figure 3.1 (A) Locations of four wetlands in Atocas Bay Reserve; (B)-(E) Locations of four wetlands in Broughton's Creek and Oak Hammock Marsh across different surrounding land managements.

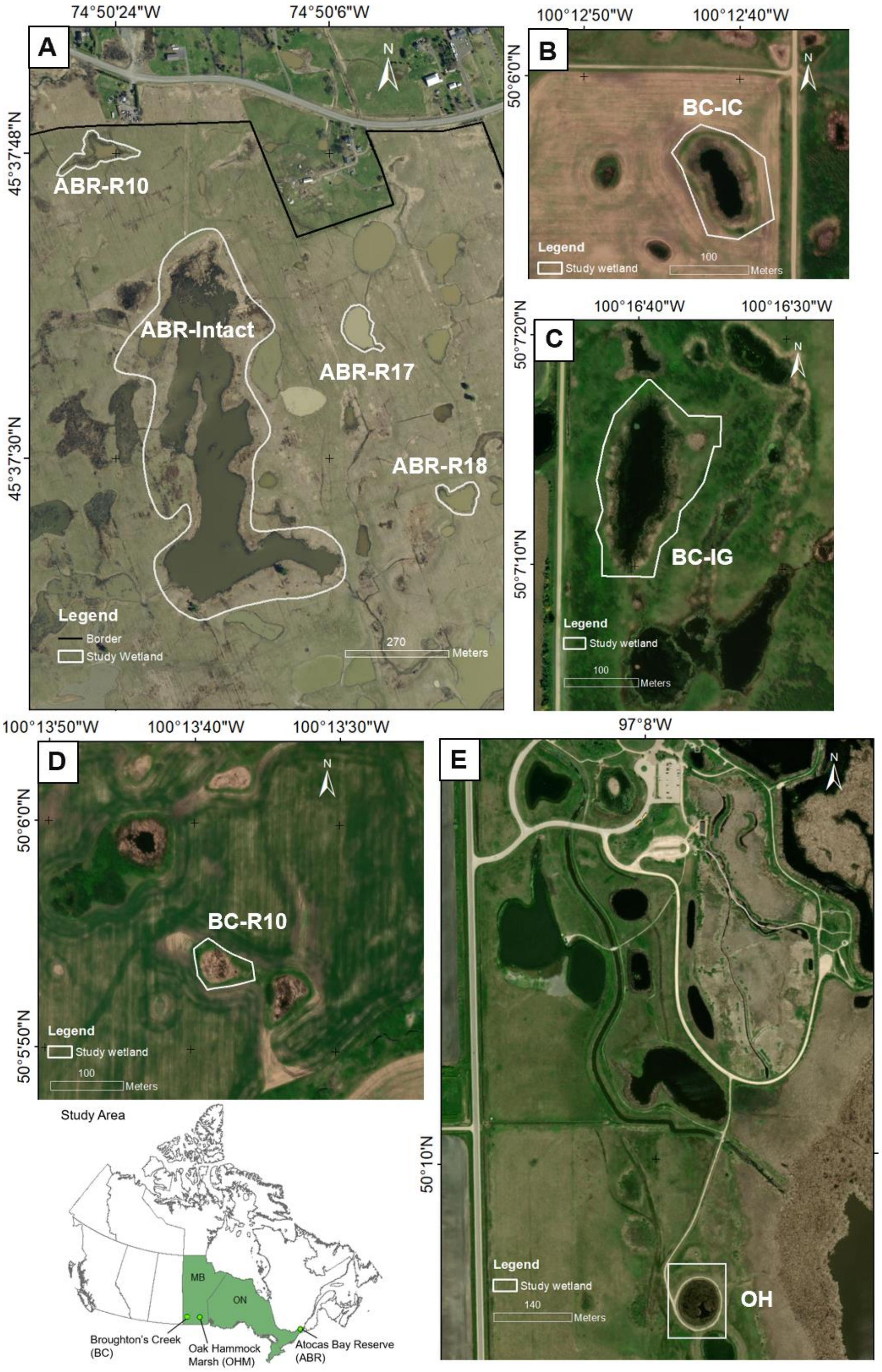


Figure 3.2 (a)-(d) Fraction of initial mass remaining (± SE) of four macrophytes at 0.34-, 1.16-, 1.44- and 2.10-years, harvested in 4 centered wetlands in Atocas Bay Reserve (ABR), respectively. Mean *k* ± standard deviation was estimated from the dynamics of litter remaining mass through 2.1-year decomposition. 'P', 'R', 'S' and 'T' refers to litter *P. australis, P. arundinacea, S. cyperinus* and *T. latifolia*, respectively; (e)-(f) Comparisons of the mean (± SE) *k* of four different macrophytes and across four centered wetlands of ABR during a 2.1-year decomposition, respectively.

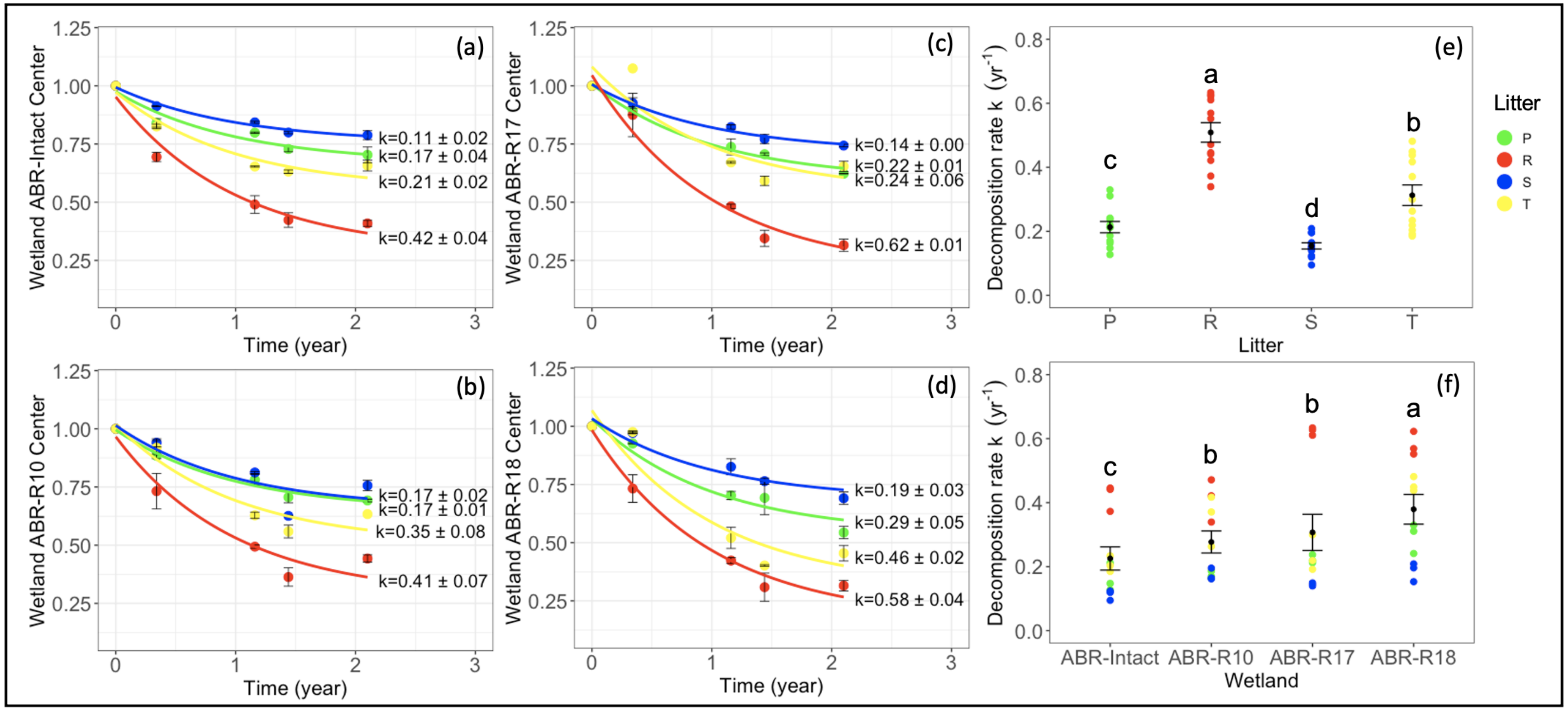


Figure 3.3 (a)-(d) Fraction of initial mass remaining (± SE) of four macrophytes at 0.33-, 1.20-, 1.46 and 2.10-years, harvested in 4 centered wetlands in

Broughton's Creek (BC) and Oak Hammock Marsh. Mean *k* ± standard deviation was estimated from the dynamics of litter remaining mass through 2.1-year decomposition. 'P', 'R', 'S' and 'T' refers to litter *P. australis, P. arundinacea, S. cyperinus* and *T. latifolia*, respectively; (e)-(f) comparisons of the mean (± SE) *k* of four different macrophytes and across four centered wetlands (BC-IC, BC-IG, BC-R10 and OH) during a 2.1-year decomposition, respectively.

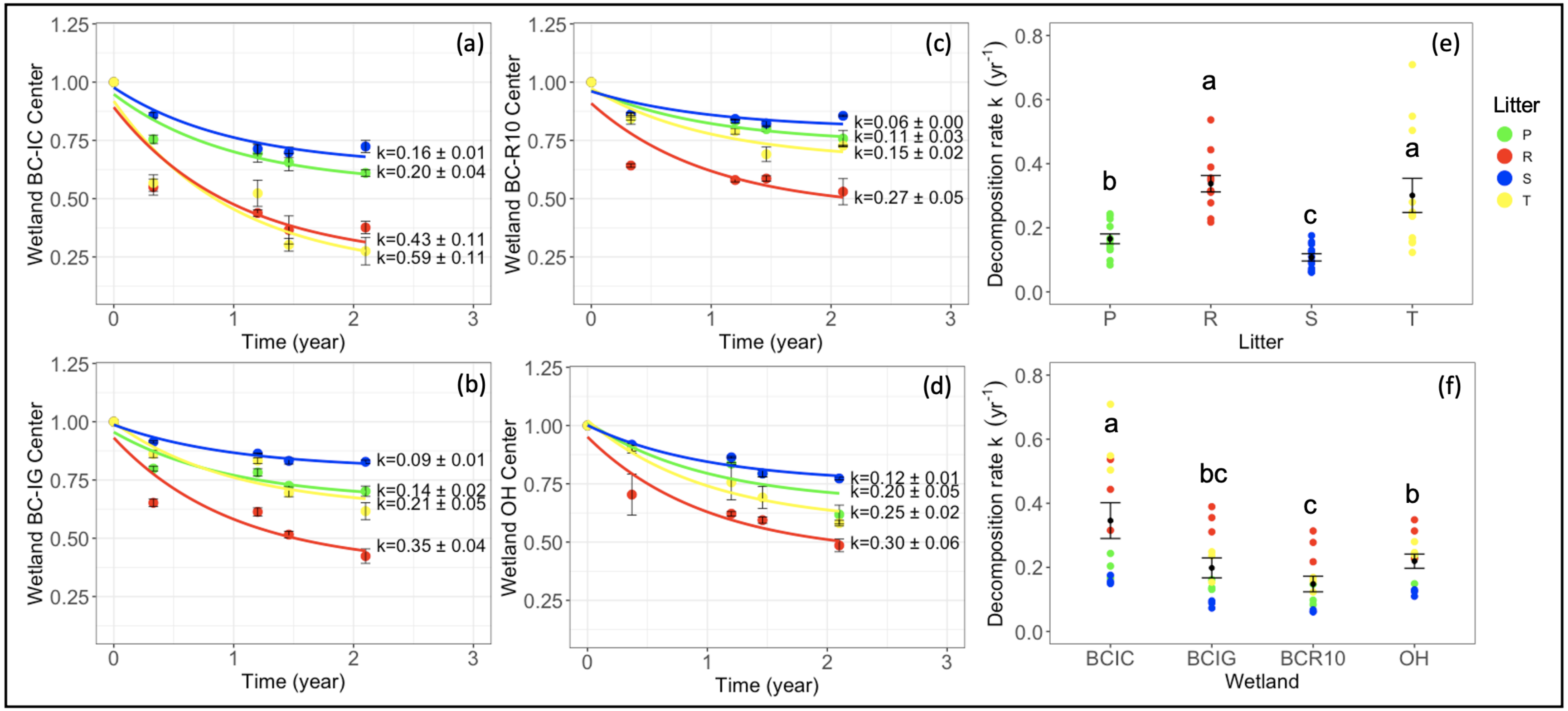
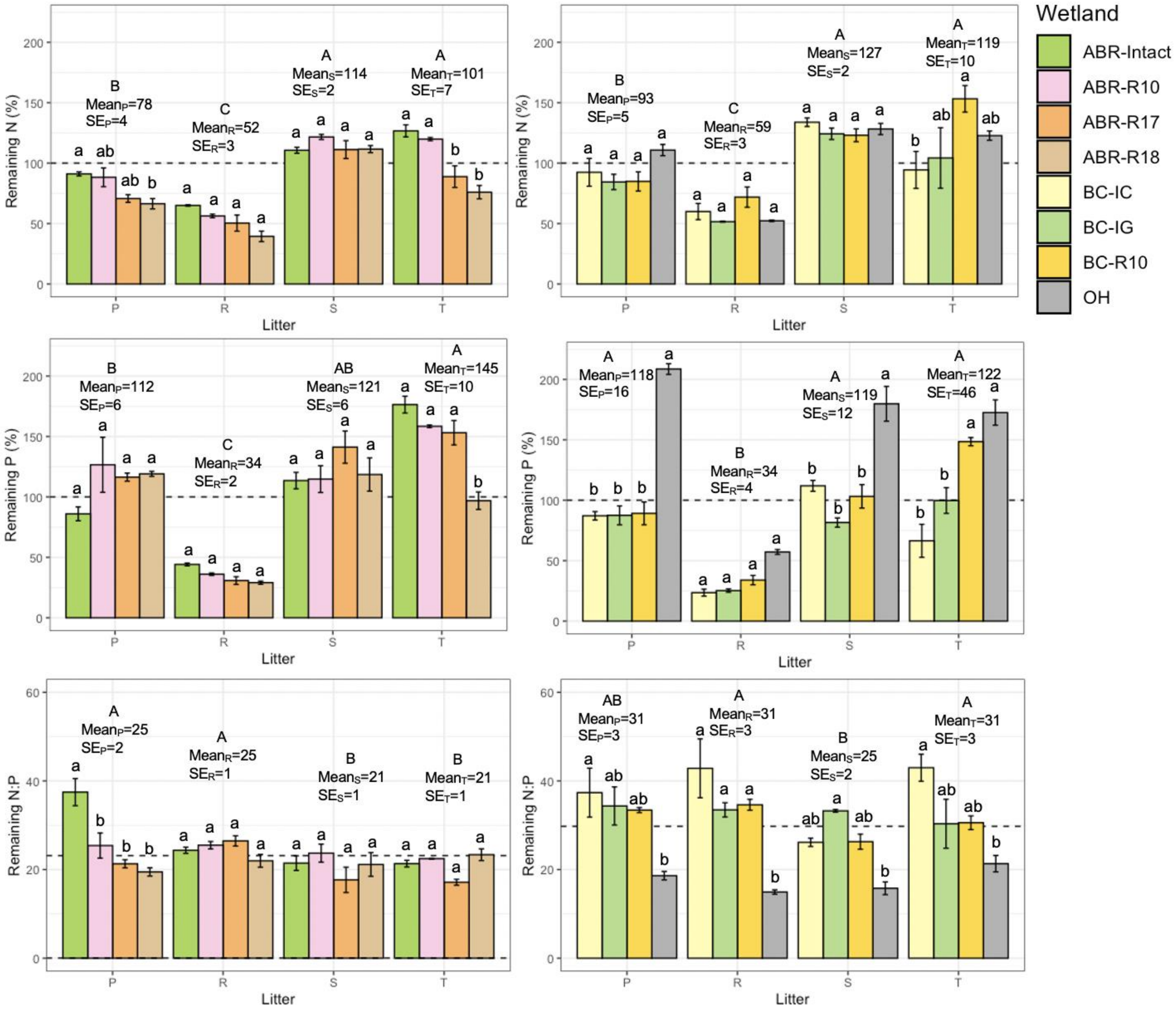


Figure 3.4 Mean (± SE) fractions of remaining N and P and the N:P quotients of litters in Atocas Bay Reserve (ABR) (left) and Broughton's Creek (BC) and Oak Hammock Marsh (OHM) (right), respectively, at the end of decomposition experiment. 'P', 'R', 'S' and 'T' refers to litter *P. australis, P. arundinacea, S. cyperinus* and *T. latifolia*, respectively. Dashed line of the remaining N:P plot indicates the average N:P quotient in the remaining litters in wetlands in ABR and in BC and OHM, respectively. Uppercase letters presented the significant difference in remaining N and P and the N:P quotient between four macrophytes and lowercase letters indicate significant difference in the remaining N and P and the N:P quotient of litters across different wetlands in ABR and BC and OHM, respectively.



3.8 Supplementary materials

Figure S3.1 Water level dynamics above soil surface of 4 freshwater marshes in Atocas Bay Reserve (ABR) from year 2019 to 2021. The records were not continuous due to the technical issues of the loggers. The precipitation data for wetlands ABR-Intact, ABR-R10, ABR-R17 and ABR-R18 were derived from station "OTTAWA INTL A". ABR-Intact: intact surrounded by grasslands; ABR-R10, ABR-R17, ABR-R18: restored since 2010, 2003 and 2002, respectively, all surrounded by grasslands.

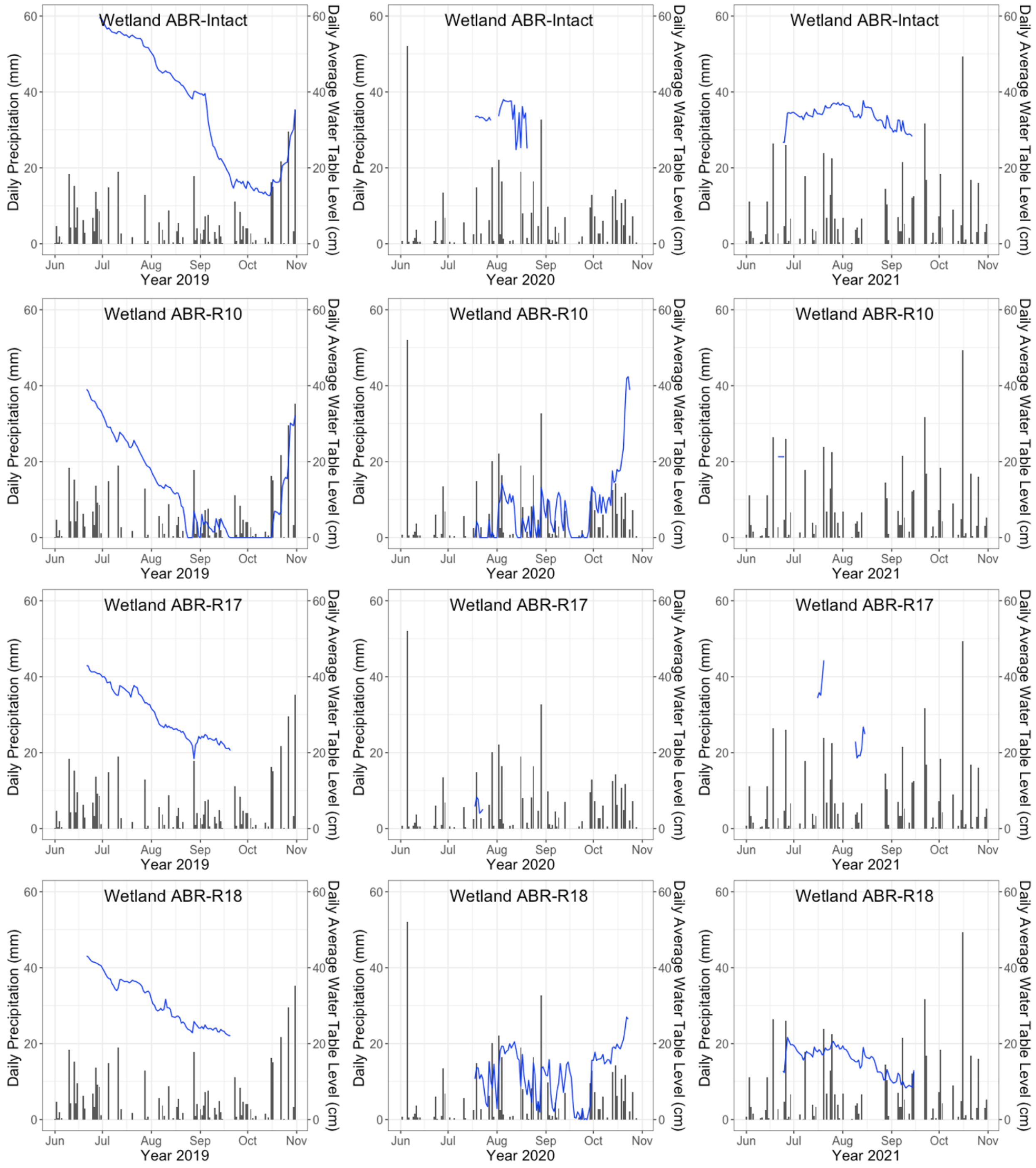


Figure S3.2 Water level dynamics above soil surface of 3 freshwater marshes in Broughton's Creek (BC) and 1 constructed marsh in Oak Hammock Marsh (OHM), respectively. Data recorded from year 2019 to 2020. The precipitation data for wetlands BC-IG, BC-IC, and BC-R10 in Broughton's Creek and OH in Oak Hammock Marsh were derived from station "RIVERS PETTAPIECE" and "WINNIPEG A CS", respectively. BC-IC: intact surrounded by croplands; BC-IG: intact surrounded by grasslands and BC-R10: restored since 2010 surrounded by croplands. OH: constructed sewage treatment wetland.

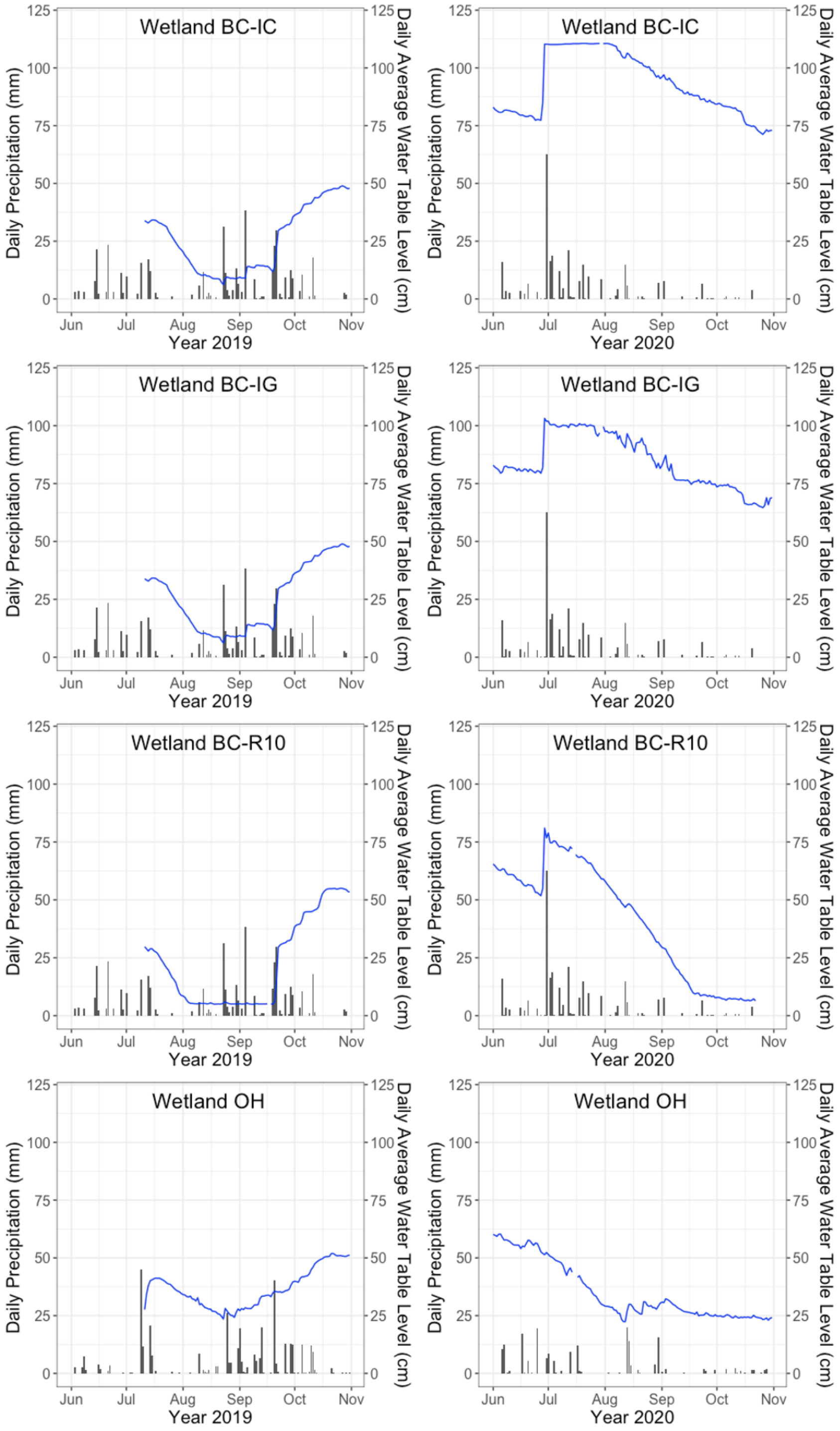


Table S3.1 Soil physiochemical properties at 0-10 cm depth within three wetlands in Broughton's Creek (BC) sampled in June of 2019, two wetlands in Atocas Bay Reserve (ABR) sampled in July of 2021. "NA" indicates not available. BC-IC: intact surrounded by croplands; BC-IG: intact surrounded by grasslands and BC-R10: restored since 2010 surrounded by croplands; ABR-Intact: intact surrounded by grasslands; ABR-R10:restored since 2010 surrounded by grasslands.

Wetland	C (%)	N (%)	C:N quotient	P (%)	C:P quotient	N:P quotient	pH	Bulk density (g cm^{-3})
BC-IC	13.2 ± 0.6	1.7 ± 0.1	8.0 ± 0.6	0.04 ± 0.01	346 ± 42	44 ± 7	7.22	0.22 ± 0.07
BC-R10	9.8 ± 1.4	0.7 ± 0.2	14.1 ± 0.9	0.05 ± 0.01	207 ± 15	15 ± 1	5.58	0.25 ± 0.02
BC-IG	7.1 ± 0.6	0.6 ± 0.2	13.2 ± 2.7	0.05 ± 0.00	145 ± 13	12 ± 3	7.56	0.50 ± 0.22
ABR-Intact	6.7 ± 0.7	0.5 ± 0.1	12.5 ± 0.3	0.06 ± 0.00	115 ± 10	9 ± 1	6.95	0.58 ± NA
ABR-R10	4.6 ± 0.6	0.4 ± 0.0	13.3 ± 0.5	0.07 ± 0.00	70 ± 7	5 ± 0	5.98	0.96 ± 0.45

Table S3.2 Initial carbon and nutrient concentrations and C:N, C:P and N:P quotients of four litters together with their average annual decay rates (k) and remaining N:P quotient through a 2.1-year decomposition. Data are expressed as the mean ± standard error. “C:N”, “C:P” and “N:P” are the mean (n=3) of the quotients of litter C to N concentration, C to P concentration and N to P concentration, respectively. Data for “k” and “Remaining N:P” are expressed as the average (n=24) ± standard error of eight wetlands. Lowercase letters indicate significant difference at 95% confidential interval (p<0.05).

Species	Type	C (%)	N (%)	P (%)	C:N	C:P	N:P	k (yr^{-1})	Remaining N:P
P. australis	stems	44.3 ± 0.1^{a}	0.62 ± 0.01^{b}	0.018 ± 0.002^{b}	71 ± 1^{b}	2556 ± 293^{a}	36 ± 4^{a}	0.19 ± 0.01^{c}	28 ± 2^{a}
P. arundinacea	leaves	45.2 ± 2.2^{a}	1.01 ± 0.03^{a}	0.062 ± 0.004^{a}	45 ± 1^{c}	741 ± 72^{b}	16 ± 1^{c}	0.42 ± 0.03^{a}	28 ± 2^{a}
S. cyperinus	stems	45.9 ± 0.0^{a}	0.44 ± 0.00^{c}	0.020 ± 0.001^{b}	103 ± 1^{a}	2263 ± 119^{a}	22 ± 1^{bc}	0.13 ± 0.01^{c}	23 ± 1^{a}
T. latifolia	leaves	48.4 ± 0.1^{a}	0.67 ± 0.01^{b}	0.023 ± 0.001^{b}	72 ± 1^{b}	2144 ± 79^{a}	30 ± 1^{ab}	0.31 ± 0.03^{b}	26 ± 2^{a}

Table S3.3 A summary of the exponential decay *k* value of *Typha* spp., *Phragmites australis, Phalaris arundinacea* and *Scirpus* spp. in intact and restored freshwater marshes from the literature. The average and range of *k* of the four macrophytes in this study are included.

Litter species	Site Location	Land management	*k* (yr^{-1})	Duration (yr)	Reference
Typha glauca	Delta Marsh, Manitoba, Canada	Natural and flooded	0.42-0.79	1.0-1.4	van der Valk et al. (1991)
Typha spp.	Ramsey, Hennepin and Dakota counties, Minnesota, USA	Minimal disturbed	0.29-0.52	0.8	Emery and Perry (1996)
Typha latifolia	Allegheny Mountain ecoregion, West Virginia, USA	Natural and restored	0.25-0.28	2.0	Gingerich and Anderson (2011)
Typha spp.	Ontario and Manitoba, Canada	Natural and restored	0.31 (0.15-0.59)	2.1	*this study*
Phragmites australis	Delta Marsh, Manitoba, Canada	Natural and flooded	0.11-0.26	1.0-1.4	van der Valk et al. (1991)
Phragmites australis	Gorenje Jezero, German	Flooding and drought	0.12-1.32	0.6	Dolinar et al. (2016)
Phragmites australis	Ontario and Manitoba, Canada	Natural and restored	0.19 (0.11-0.29)	2.1	*this study*
Phalaris arundinacea	Allegheny Mountain ecoregion, West Virginia, USA	Natural and restored	0.36-0.41	2.0	Gingerich and Anderson (2011)
Phalaris arundinacea	Ontario and Manitoba, Canada	Natural and restored	0.42 (0.27-0.62)	2.1	*this study*

Scirpus lacustris	Delta Marsh, Manitoba, Canada	Natural and flooded	0.44-0.48	1.0-1.4	van der Valk et al. (1991)
Scirpus cyperinus	Ontario and Manitoba, Canada	Natural and restored	0.13 (0.06-0.19)	2.1	*this study*

Table S3.4 Summary of Pearson correlation coefficients (n=4) of litter exponential decay (*k*), remaining N and P (%), remaining litter N:P quotient (mass based), and the initial litter nutrients concentrations and the N:P quotient (mass based) from eight individual wetlands. '*' $p<0.05$, '**' $p<0.01$. ABR-Intact: intact surrounded by grasslands; ABR-R10, ABR-R17, ABR-R18: restored since 2010, 2003 and 2002, respectively, all surrounded by grasslands. BC-IC: intact surrounded by croplands; BC-IG: intact surrounded by grasslands and BC-R10: restored since 2010 surrounded by croplands. OH: constructed sewage treatment wetland. ABR: Atocas Bay Reserve. BC: Broughton's Creek.

X_1	X_2	ABR-Intact	ABR-R10	ABR-R17	ABR-R18	BCIC	BCIG	BCR10	OH
k	Ln(Initial litter N)	**0.95***	0.85	0.93	**0.96***	0.61	**0.98****	**0.98****	**0.96***
k	Ln(Initial litter P)	**0.96***	0.82	**0.97***	0.82	0.41	0.92	0.93	0.71
k	Initial litter N:P	-0.63	-0.40	-0.68	-0.32	0.09	-0.50	-0.53	-0.15
Remaining N	Ln(Initial litter N)	-0.67	-0.84	-0.92	**-0.97***	**-0.99****	-0.94	-0.51	-0.89
Remaining N	Ln(Initial litter P)	-0.73	-0.78	-0.70	-0.72	-0.75	-0.79	-0.51	-0.94
Remaining N	Initial litter N:P	0.65	0.54	0.28	0.23	0.25	0.41	0.49	0.70
Remaining P	Ln(Initial litter N)	-0.65	-0.63	-0.82	-0.90	**-0.96***	-0.72	-0.58	-0.80
Remaining P	Ln(Initial litter P)	-0.61	-0.88	-0.89	**-0.99****	-0.89	-0.93	-0.75	**-0.99****
Remaining P	Initial litter N:P	0.67	0.90	0.74	0.72	0.44	0.88	0.77	0.83
Remaining N:P	Ln(Initial litter N)	0.05	0.44	0.82	0.30	0.86	-0.01	0.87	-0.13
Remaining N:P	Ln(Initial litter P)	-0.31	0.41	0.82	0.36	0.50	0.08	0.54	-0.56
Remaining N:P	Initial litter N:P	0.43	-0.40	-0.62	-0.13	0.12	-0.32	-0.06	0.90

Table S3.5 Initial and remaining litter nitrogen (N) and phosphorus (P) mass (mg) (mean ± SE, n=3) in eight wetlands over a 2.1-year decomposition. ABR-Intact: intact surrounded by grasslands; ABR-R10, ABR-R17, ABR-R18: restored since 2010, 2003 and 2002, respectively, all surrounded by grasslands. BC-IC: intact surrounded by croplands; BC-IG: intact surrounded by grasslands and BC-R10: restored since 2010 surrounded by croplands. OH: constructed sewage treatment wetland. ABR: Atocas Bay Reserve. BC: Broughton's Creek.

Wetland		Initial Litter Nitrogen and Phosphorus Mass (mg)					Remaining Litter Nitrogen and Phosphorus Mass (mg)			
		P. australis	*P. arundinacea*	*S. cyperinus*	*T. latifolia*		*P. australis*	*P. arundinacea*	*S. cyperinus*	*T. latifolia*
ABR-Intact	N:	29.45±0.53	38.31±0.19	20.20±0.40	25.69±0.15	N:	26.84±0.84	25.00±0.10	22.34±0.24	32.56±1.33
	P:	0.84±0.02	2.35±0.01	0.93±0.02	0.87±0.01	P:	0.72±0.06	1.04±0.02	1.05±0.08	1.53±0.07
ABR-R10	N:	28.64±1.11	38.25±0.10	20.59±0.68	25.74±0.06	N:	25.20±1.98	21.54±0.53	25.03±0.71	30.85±0.41
	P:	0.82±0.03	2.35±0.00	0.94±0.03	0.87±0.00	P:	1.03±0.19	0.85±0.02	1.08±0.11	1.37±0.01
ABR-R17	N:	31.01±0.39	38.41±0.08	20.48±0.19	26.05±0.16	N:	21.93±1.05	19.37±2.60	22.80±1.73	23.16±2.44
	P:	0.89±0.01	2.36±0.00	0.94±0.01	0.88±0.01	P:	1.03±0.04	0.73±0.08	1.32±0.12	1.35±0.10
ABR-R18	N:	31.58±0.83	38.37±0.43	20.80±0.52	25.59±0.02	N:	20.87±0.92	15.12±1.63	23.24±1.13	19.43±1.41
	P:	0.90±0.02	2.35±0.03	0.95±0.02	0.86±0.00	P:	1.07±0.01	0.68±0.03	1.12±0.10	0.84±0.06
BC-IC	N:	26.66±0.45	38.75±0.38	17.31±0.27	25.44±0.15	N:	24.54±2.71	23.25±2.72	23.18±0.78	24.05±4.00
	P:	0.76±0.01	2.38±0.02	0.79±0.01	0.86±0.01	P:	0.66±0.03	0.55±0.06	0.89±0.03	0.57±0.12
BC-IG	N:	26.72±0.78	39.85±0.41	17.16±0.15	25.31±0.18	N:	22.60±2.06	20.55±0.20	21.32±0.78	26.30±6.19
	P:	0.76±0.02	2.45±0.03	0.79±0.01	0.85±0.01	P:	0.66±0.04	0.62±0.03	0.64±0.03	0.85±0.09
BC-R10	N:	27.03±0.82	38.87±0.25	17.13±0.08	25.29±0.10	N:	23.07±2.83	27.99±3.44	21.07±0.81	38.74±2.67
	P:	0.77±0.02	2.39±0.02	0.79±0.00	0.85±0.00	P:	0.69±0.09	0.81±0.10	0.81±0.07	1.27±0.02
OH	N:	24.77±0.64	38.78±0.22	17.26±0.11	25.13±0.15	N:	27.42±1.09	20.28±0.20	22.14±0.84	31.07±0.79
	P:	0.71±0.02	2.38±0.01	0.79±0.00	0.85±0.00	P:	1.47±0.02	1.36±0.05	1.42±0.11	1.47±0.10

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Chapter 4: Hydrologic, plant and soil controls on microbial biomass and enzyme activities in a natural and a restored freshwater marsh of eastern Ontario, Canada

Bridging statement to Chapter 4

In chapter 3, we have seen that plant litter species and wetland hydrology both play key roles on the rates and efficiency of litter decomposition in restored marshes. Within its landscapes, the variance in hydrological condition and vegetation communities also exists. It is interesting to further detect the spatial variability in the decomposition and mineralization within the marsh landscapes. Rather than conducting another litterbag study, the extracellular enzymatic activities can effectively indicate the mineralization potential in soil. In this chapter, we examined the extracellular enzyme activities over nine vegetation plots distributed over the centre-wet meadow-upland topographic transect and related the enzyme activities with significant environmental factors including the microbial biomass, plant root density, plant substrate quality and the soil physiochemical properties. We found permanent inundation depressed enzymatic activities significantly in both intact and restored marshes. The degree of inundation and dominant plant species mainly determined the surface soil microbial biomass and extracellular enzyme activities, rather than land management or soil properties.

Note: The manuscript is ready for submission to *Freshwater Biology*.

4.1 Abstract

The hydrologic conditions of freshwater marshes can impact soil microbial biomass and extracellular enzymes and thus the biogeochemistry and functioning of marshes. However, the high spatial variation in water inundation coincides with a change in dominant plant communities. This makes it a challenge to identify the factors that regulate microbial biomass and enzyme activities in marsh systems. To address this, we determined the microbial biomass carbon (MBC), nitrogen (MBN), and the activities of four hydrolytic extracellular enzymes involved in C, N, and phosphorus (P) decomposition, together with above and below ground plant and soil properties at nine vegetation plots across three hydrological conditions in two freshwater marshes of eastern Ontario, Canada, one intact and one restored. Relationships between microbial biomass, extracellular enzyme activities (EEAs) and wetland hydrologic, plant and soil properties were examined. We show that soil EEAs, MBN and the ratios of MBC to soil C and MBN to soil N were suppressed significantly under permanently inundated conditions in both marshes compared to intermittently and never inundated conditions. The lower EEAs under permanent inundation appeared to be a function of reduced microbial abundance or population rather than differences in soil or plant properties. Under permanent inundation, the MBC and MBC/MBN ratio were notably higher in the intact wetland where *Typha latifolia* dominated than the restored marsh dominated by *Potamogeton epihydrus* and *Sagittaria latifolia*, probably due to the oxygen release from *T. latifolia* roots which enhances microbial growth. Soil phosphatase activity was consistently the highest across all vegetation plots and had a positive linear relationship with plant root density ($R^2=0.32$; $p<0.01$) where *Carex* spp. and *Phalaris arundinacea* plots were the highest. Although soil C concentration of the restored wetland was only half of the intact one, their microbial biomass and EEAs were similar, probably due to the comparable aboveground

biomass. Our study revealed a strong negative impact of permanent inundation on microbial biomass and enzyme activities and a noticeable vegetation rhizosphere impact on the microbial properties in natural and restored freshwater marshes.

4.2 Introduction

Natural and restored freshwater marshes are widely regarded as important ecosystems for accumulating carbon (C) and retaining excessive nutrients such as nitrogen (N) and phosphorus (P) from agricultural runoff (Reddy et al. 1999; O'Geen et al., 2010). Within the marsh landscape from the center to upland, great variations exist in the hydrological conditions, soil properties and vegetation communities which often result in obvious zonation patterns in their primary productivity, decomposition rates, and organic C and nutrient accumulation potentials (Neckles and Neill, 1994; Bruland and Richardson, 2004; Xiao et al., 2012). These spatial differences within a freshwater marsh, are expected to impact microbial activity, especially of extracellular enzymes that influence decomposition and the supply of bioavailable C, N, and P, and thus the maintenance of wetland biogeochemical functions (Gutknecht et al., 2006). While variable soil moisture is a strong factor regulating microbial communities, it is unclear how the coinciding spatial variation in plant communities and soil C and nutrient status will moderate microbial responses to changing hydrology, particularly within natural and restored freshwater marshes (Wang et al., 2019; Wang et al., 2021).

The hydrological conditions affecting microbial community composition and EEAs vary across marsh transects (Mentzer et al., 2006; Unger et al., 2009). The center of marshes is typically permanently inundated (PI), transitioning to intermittently inundated (II) further from the center and never inundated (NI) at the upland zones. The most significant differences in microbial activity across these hydrological conditions typically occurs between PI condition and the other two conditions (Kang et al., 1998; Bruland and Richardson, 2004; Rinklebe and Langer, 2006). This is likely because under permanent inundation, anoxia restricts the presence of aerobic fungi and bacteria and their capacity for producing oxidases and potentially many hydrolytic enzymes (Freeman et al., 2001; Bossio et al., 2006; Burns et al., 2013). The increased concentrations of reduced metal ions (e.g., Fe^{2+}) under waterlogged conditions could also inhibit many extracellular enzymes such as phosphatase and cellulases (Pulford and Tabatabai, 1988; Freeman et al., 1996; Tejirian and Xu, 2010). Relative to NI conditions, intermittent inundation creates more variable soil redox and moisture conditions. This variability could potentially support more diverse microbial communities and increase microbial biomass concentrations, facilitating a more resilient and active microbial community compared to the PI and NI marsh areas (Wan et al., 2008; Gao et al., 2016). Occasional flooding also fosters mobilisation of elements into the porewater and enhances the release of labile C, N, and P which likely support higher microbial growth and activity (e.g., respiration, EEAs) (Schönbrunner et al., 2012; Peralta et al., 2014; Kim et al., 2014). Thus, the expected increased influx of nutrients and C and more metabolically diverse microbial community associated with the fluctuating moisture, suggest that II zones of freshwater marshes might be the most active area for microbial biomass production and EEAs. For instance, Peralta et al. (2014) show that areas under variable periods of inundation have more diverse bacterial communities compared to those that were permanently or never inundated in two restored marshes which suggests both aerobic and anaerobic microorganisms might be well-adapted to variable inundation.

Wetland plant species also change within a marsh hydrological gradient, further affecting microbial nutrient, C, and oxygen supply such that hydrology and plant species may interact to determine soil microbial biomass and EEAs (Gutknecht et al., 2006; Yarwood, 2018). Previous studies have found significantly enhanced microbial biomass and EEAs (e.g., β-glucosidase, β-D-xylosidase and phosphatase) with the presence of vegetation compared to non-vegetated or clipped controls (Gagnon et al., 2007; Zhang et al., 2010; Porter, 2011). Plants could affect the soil microbial biomass and EEAs through both litter- and root-related mechanisms. For instance, under similar soil conditions, Yang et al. (2018) reported lower C-acquiring enzymes (BG, phenol oxidase) when litter C inputs were more chemically labile. Rejmánková and Sirová (2007) found significant impacts of litter nutrients on microbial phospholipid fatty acid content and aminopeptidase and arylsulfatase activity in decomposing litters, although the soil nutrient concentrations likely play a more important role. In the rhizosphere, some emergent wetland plants (e.g., *Typha* spp.) release oxygen that greatly foster microbial growth (Wießner et al., 2002). Moreover, many plants release phosphatase which can be controlled by the aboveground biomass, P concentration of plant tissues, and soil nutrients conditions (Rejmánková et al., 2008; Robroek et al., 2009). Wetland plant species with different litter quality, root characteristics (e.g., density, surface area) and root exudation may thus lead to diverging microbial biomass and EEA patterns over the landscape but few field observations are available in natural and restored freshwater marshes.

To examine how hydrologic, plant and soil properties affect the microbial biomass and EEAs in freshwater marshes, we sampled nine vegetation plots under three hydrological conditions (PI, II, NI) within a natural and a restored marsh in eastern Ontario, Canada (Fig 4.1).

We hypothesize that: 1) soil MBC and MBN and EEAs vary among three hydrological conditions and likely increase from PI to NI and being the highest under II conditions and 2) variations in MBC, MBN and EEAs occur across different vegetation communities, associated with their litter and/or root characteristics.

4.3 Materials and methods

4.3.1 Study area

The study was conducted in two freshwater marshes in Atocas Bay Reserve (45°63’N, 74°84’W; 647 ha), a wetland restoration site in eastern Ontario, Canada. It has a mean annual temperature and precipitation of 6.7 °C and 895 mm, respectively (2012-2021 average for station OTTAWA INTL A, Environment Canada 2021). About 80% of the reserve was drained for agricultural uses and later restored by Ducks Unlimited Canada between 2000 and 2010. Old ditches were plugged with clay materials so that wetlands could be rewetted by precipitation and the spring snowmelt. Drained wetlands have gradually been rewetted and covered by vegetation after 12-to-22-year succession, though some intact wetlands remain, creating a mosaic of intact and restored wetlands. The two freshwater marshes we studied, one intact (INT) and one restored (RES) 12 years earlier, are 350 m apart and both depressional, isolated, permanently inundated in their center and underlain by clay-rich sediments (> 50 % clay). The centers of the marshes are permanently inundated and dominated by reeds (*Typha latifolia*) and emergent forbs (*Potamogeton epihydrus* and *Sagittaria latifolia*). Surrounding wet meadows are intermittently inundated and dominated by perennial grasses (e.g., *Phalaris arundinacea, Phleum pratense*) and sedges (e.g., *Carex* spp., *Scripus cyperinus*) with the presence of *Echinochloa crus-galli* and *Eleocharis obtusa*. The uplands are never inundated and dominated by perennial forbs (*Galium album*) and grasses (e.g., *Phragmites australis*) with sporadic occurrence of other forbs (e.g., *Fragaria virginiana*, *Lotus corniculatus* (legume), *Solidago rugosa* and *Trifolium pratense*).

Nine total plots (10 m × 10 m) along the PI- II- NI transects of the two marshes

(INT or RES) were selected and sampled based on their dominant vegetation. These plots were: PI conditions dominated with *Typha latifolia* (INT) or *Potamogeton epihydrus* and *Sagittaria latifolia* (RES); II conditions dominated with *Carex* spp. (INT), *Phalaris arundinacea* (INT), *Phalaris arundinacea* (RES), or *Scripus cyperinus* (RES); NI conditions dominated with *Phragmites australis* (INT), *Galium album* (INT), or *Galium album* (RES).

4.3.2 Wetland hydrological condition and vegetation sampling

We recorded continuous water levels from June to August, 2022 using a 2 m deep water logger placed at the center of the two marshes. The water levels of INT and RES center averaged 140 cm and 68 cm above sediment surface, respectively (Fig S4.1). At each vegetation plot, water table position, below or above the sediment surface, was determined in July, August and September, 2021 and May, June and July, 2022 (Table S4.1). In mid-July 2022, aboveground living biomass was collected from triplicate randomly selected quadrats (0.5×0.5 m^2) within each plot. In in the laboratory, we oven-dried the green biomass at 60 °C until it was a constant weight to calculate aboveground living biomass (g m^{-2}). From each quadrant, we also collected brown biomass of the dominant plant species that had fallen to the soil surface. Brown biomass was oven-dried at 60 °C until constant weight, and ground by a ball mill for determining the C, N and P concentrations of senesced litter entering the soil. At the center of RES where *P. epihydrus* and *S. latifolia* dominated, brown litters were degraded and could not be identified and thus 50% of the nutrient concentrations in living biomass was used to estimate their litter nutrient concentrations.

4.3.3 Soil and root sampling and processing

We collected triplicate soil cores (7.5 cm diameter, 10 cm deep) at each selected vegetation plot in mid-July 2022, stored in polyethylene bags, kept cool at 4 °C and visible plant tissues including roots and litter were removed. Extracellular enzyme and microbial biomass assays were completed on field-moist core samples within 3 and 14 days after sampling, respectively. Soil subsamples were used to determine the soil pH, soil gravimetric moisture (%), and soil C and nutrient concentrations. Adjacent to where soil cores were taken, we excavated 10 ×10 ×10 cm^3 volume of bulk soils to estimate the plant root density (g cm^{-3}). The excavated soil was washed with water, and the live roots were collected in a 1 mm mesh sieve and dried at 60 °C.

4.3.4 Soil physiochemical properties and litter quality determination

We estimated total C and total N concentrations (%) of soil (C_{soil}, N_{soil}) and brown plant litters (C_{plant}, N_{plant}) with a CHN analyzer (EA 1112, Carlo Erba, Milan, Italy). Soil and litter total P (P_{soil}, P_{plant}) concentration (%) were determined colorimetrically by the Malachite Green method (Ohno and Zibilske, 1991) after dilution in a H_2SO_4-H_2O_2 mixture following Akinremi et al. (2003). For the *P. epihydrus* and *S. latifolia*-RES plot, C_{plant}, N_{plant} and P_{plant} were the average of the two species. The stoichiometric ratios of soil (C/N_{soil}, C/P_{soil}, N/P_{soil}) and brown plant litter (C/N_{plant}, C/P_{plant}, N/P_{plant}) were calculated from total C, total N, and total P concentrations (%) of soil and litter, respectively.

Soil dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) concentrations (mg kg^{-1}) were determined following the procedure described in Jones and Willett (2006). Briefly, 2 g field moist soil was mixed in polypropylene bottles with 0.5 M K_2SO_4 at a 1:5 w/v ratio and shaken (250 rev min^{-1}) for 30 min at room temperature. The mixture was then centrifuged for 10 min (4000 rpm, 20 °C) to remove suspended solids. The filtrate was passed through 0.45 μm filter paper and diluted 10 times for DOC and TDN determination on a TOC/N analyzer (Shimadzu TOC-500). Standard solutions for DOC (glucose) and TDN (amino acid) were measured along with the samples. Soil gravimetric moisture was used to calibrate field moist soil to oven dry soil in DOC and TDN determination.

4.3.5 Soil microbial biomass and enzyme assays

Soil MBC and MBN were measured by the fumigation extraction method (Voroney et al., 1993). Briefly, MBC and MBN were estimated from the averaged values of the DOC and TDN concentrations in duplicate fumigated soil samples (10 g) minus paired duplicate non-fumigated samples and then divided by the extraction coefficients 0.45 (MBC) and 0.54 (MBN), respectively (Vance et al., 1987). Ratios of MBC/C_{soil} and MBN/N_{soil} were calculated from the MBC and MBN (mg kg^{-1}) divided by soil total C and total N concentrations (mg kg^{-1}), respectively.

Owing to the often-positive correlations between a suite of extracellular enzymes that target related substrates (Moorhead et al., 2013), only representative extracellular enzymes that are commonly used to indicate the overall EEAs on C-, N- and P- related compounds were determined. These included β-glucosidase (BG), β-N-acetyl-glucosaminidase (NAG), leucine aminopeptidase (LAP) and acid phosphatase (AP) for the degradation of cellulose, chitin and peptidoglycan, proteins and organic P substrates (e.g., phospholipids), respectively (Sinsabaugh and Follstad, 2011; Moorhead et al., 2013). Potential EEAs of BG, NAG, LAP, and AP were measured by the fluorescence method described in Saiya-Cork et al. (2002). Briefly, 1 g fresh soil was mixed with 70 mL 50 mM HCl-sodium-acetate buffer (pH=6.1) in a blender and homogenized for 1 min. Then 200 μL soil slurry together with 50 μL 200 μM substrate solution (4-Methylumbelliferyl-β-glucosidase, 4-Methylumbelliferyl-N-acetylglucosamine, L-Leucine-7-amio-4-Methylumbelliferyl, and 4-Methylumbelliferyl-phosphate) were pipetted to a 96-well microplate for incubation at 20 °C in the dark. Each soil sample per assay had 16 replicate wells on the microplate. Eight replicated blank controls (200 μL soil slurry + 50 μL buffer) and negative controls (200 μL buffer + 50 μL substrate) were included alongside the analytical replicates. Additional quench standard of 50 μL 10 μM 4-Methylumbelliferyl (MUB) for BG, NAG and AP and 7-amio-4-methylcoumarin (AMC) for LAP were pipetted to one column of 200 μL soil slurry wells with a reference standard (50 μL MUB or AMC + 200 μL buffer) processed alongside. After an incubation of 5 hrs, fluorescence assays were determined with 330 nm excitation and 450 nm emission filters by a fluorometer. We calculated final soil potential EEAs (nmol h^{-1} g dry $soil^{-1}$) after correction from negative controls and quenching.

4.3.6 Statistical analyses

We used one-way analysis of variance (ANOVA) followed by Bonferroni correction for multiple comparisons to determine the significant differences in soil EEAs and microbial biomass (MBC and MBN) across nine vegetation plots and three hydrological conditions, with residuals' normality and homoscedasticity confirmed by Shapiro-Wilk and Bartlett's tests, respectively. Because the normality or homoscedasticity assumptions were sometimes not met for ANOVA, we used non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons (Bonferroni-corrected) to examine the significant differences in microbial MBC/MBN, MBC/C_{soil}, MBN/N_{soil} ratios and plant and soil properties among nine vegetation plots and three hydrological conditions. When determining the variance of variables with vegetation as the main factor (across nine vegetation plots), we used triplicate samples within each vegetation plot (n=3) to conduct the one-way ANOVA or Kruskal-Wallis test. When determining the variance of variables with hydrology as the main factor (across three hydrological conditions), we pooled the triplicate plot samples and vegetation plots under one hydrological condition into one group and then compared the means and mean ranks among three hydrological groups using ANOVA and Kruskal-Wallis test, respectively: *n*=6 for PI (2 vegetation plots × 3 samples), *n*=12 for II (4 vegetation plots × 3 samples) and *n*=9 for NI (3 vegetation plots × 3 samples). Data homoscedasticity among nine vegetation groups and three hydrological groups were both assessed. When we conducted post-hoc comparisons for ANOVA and Kruskal-Wallis test among nine vegetation plots and three hydrological conditions, we used Bonferroni-corrected α as 0.0014 and 0.017 to avoid Type I errors. In all other cases, we regard significant difference at $\alpha = 0.05$.

Pearson correlation coefficients were calculated among EEAs, microbial biomass (MBC, MBN) and the environmental variables with two variables (pH, TDN) lg-transformed to improve the normality. The normality and homoscedasticity of the variable matrix were verified by quantile-quantile plot, Shapiro-Wilk and Breusch-Pagan test, respectively. The intercorrelations among soil EEAs, microbial biomass and plant root, litter, and soil parameters were conducted through a Redundancy Analysis (RDA) with four soil EEAs as dependent variables and other parameters as explanatory variables, displayed in a correlation biplot (Zurr et al., 2007) in CANOCO software version 5.0. For the covariates (e.g., N_{plant} and C/N_{plant}), only one parameter (e.g., N_{plant}) with better normality was used in the Pearson correlation and the RDA. We used a linear least squared regression model to determine the relationship between acid phosphatase activity and plant root density with data normality, homoscedasticity and absence of autocorrelation verified. Other than the RDA, all statistical analyses were completed in R (version 4.2.2).

4.4 Results

4.4.1 Plant litter nutrients and stoichiometry

Among all plant species, *P. epihydrus* and *S. latifolia*, *P. arundinacea* and *G. album* had the highest N and P concentrations (Appendix C). We found no significant variations in litter nutrient concentrations among the three hydrological conditions (Kruskal-Wallis test, p>0.05), nor between the intact and restored marsh within one plant species.

The average C_{plant}, N_{plant}, and P_{plant} concentrations of plant litters ranged between 39.3-47.7 %, 0.35-1.55 % and 0.009-0.044 %, respectively, and their C/N_{plant}, C/P_{plant} and N/P_{plant} ratios ranged between 25-135, 901-4978, and 15-57, respectively (Table S4.2). The estimated C_{plant}, N_{plant}, P_{plant} concentrations of *P. epihydrus* and *S. latifolia* were 33.0%, 1.42%, 0.043% and 43.0%, 2.14%, 0.044%, respectively with an average of 39.3%, 1.55%, 0.044%. Three forbs (*P. epihydrus*, *S. latifolia* and *G. album*) had the highest N and P concentrations, followed by *P. arundinacea*. The N concentration of *P. arundinacea* in the intact marsh were higher than that in the restored marsh while the P concentration remain very similar. *Carex* spp. and *P. australis* were especially low in P and had the highest C/P_{plant} and N/P_{plant} ratios (C/P_{plant} > 3500, N/P_{plant} >50). *T. latifolia* was low in P compared to N and had the C/N_{plant} and C/P_{plant} ratios as 73 and 2091, respectively. *S. cyperinus* was low in both N and P with a C/N_{plant} of 135 and C/P_{plant} of 2074, respectively. Nutrient-rich species occurred at all hydrological conditions: PI (e.g., *P. epihydrus* and *S. latifolia*), II (e.g., *P. arundinacea*) and NI (e.g., *G. album*).

4.4.2 Soil properties and plant root density

The C_{soil} and N_{soil} concentrations were between 4.6-6.7 % and 0.41-0.53% in the intact marsh, significantly higher than those in the restored marsh (2.4-4.6 % and

0.21-0.35 %) (Kruskal-Wallis test, $p<0.001$). For both marshes, C_{soil} and N_{soil} declined from PI to II and NI conditions (Table S4.3). However, P_{soil} (0.052-0.086 %) did not vary significantly among the three hydrological conditions nor between the two marshes and thus the variation in C/P_{soil} ratios was mainly due to the variations in C_{soil} rather than of P_{soil}. The C/N_{soil} was highly constrained to 11-13 across all sampled plots (Table S4.3).

Soil DOC (63-190 mg kg^{-1}) and TDN (1.9-10.7 mg kg^{-1}) had high variations across different vegetation plots and did not show significant variations among the three hydrological conditions. DOC was especially high (>100 mg kg^{-1}) in *Carex spp.*-INT, *P. arundinacea*-INT and RES, and *P. australis*-INT plots, where there was high root density (>0.01 g cm^{-3}). Overall, a significantly positive relationship between soil DOC and plant root density was found ($r=0.51$, $p<0.01$; Appendix E). However, DOC was also high in two centre PI plots despite their low root density (*T. latifolia*-INT, *P. epihydrus* and *S. latifolia*-RES), probably owing to the inflows from surrounding wet meadows. We did not find significant correlations between DOC and MBC nor TDN and MBN. Soil TDN was the highest at *S. cyperinus*-RES plot, which may be due to the low N plant uptake from soils with the least plant living biomass produced (Table S4.2, S4.3).

Plant root density varied greatly among vegetation communities and declined in the order of *Carex* spp.-INT > *P. arundinacea*-INT > *P. arundinacea*-RES and *P. australis*-INT > *S. cyperinus*-RES and *T. latifolia*-INT > *P. epihydrus* and *S. latifolia*-RES and *G. album*-RES > *G. album*-INT. Among the PI-II-NI plots, II plant species (e.g., *Carex* spp.-INT and *P. arundinacea*-INT and RES) showed the highest root density (Table S4.3).

4.4.3 Microbial biomass

At both marshes, plots under PI conditions (*P. epihydrus* and *S. latifolia*-RES and *T. latifolia*-INT) showed substantially lower MBN (52-84 mg kg^{-1}), MBC/C$_{soil}$ ratios (0.5-1.5 %) and MBN/N$_{soil}$ ratios (1.5-1.6 %) than plots under II and NI conditions with MBN, MBC/C$_{soil}$ and MBN/N$_{soil}$ ratios ranging between 116-185 mg kg^{-1}, 1.7-5.4 % and 3.4-9.0 %, respectively (Fig 4.2; Table S4.3). Soil MBC, MBN, MBC/C$_{soil}$ and MBN/N$_{soil}$ ratios between II and NI conditions did not vary significantly. The MBC/MBN ratios also showed a narrower range among II and NI plots (5.4-7.4) compared to PI plots (4.5-13.8) (Fig 4.2).

Under PI conditions, MBC appeared to be higher with the nutrient-poor plants, where MBC of *T. latifolia*-INT was around three times that at *P. epihydrus* and *S. latifolia*-RES. The *T. latifolia*-INT also had significantly higher MBC/MBN ratio than *P. epihydrus* and *S. latifolia*-RES (13.8 vs. 4.5) (Fig 4.2), suggesting different microbial community composition or abundance between the vegetative PI plots.

There were no significant differences in MBC and MBN among vegetation plots under II and NI conditions except that *P. arundinacea*-INT and RES showed slightly higher MBC and MBN than *Carex* spp.-INT and *S. cyperinus*-RES under the II conditions and *G. album*-INT and RES showed higher MBC and MBN than *P. australis*-INT under the NI conditions (Fig4.2). The MBC and MBN concentrations were similar between INT and RES for *P. arundinacea*, and for *G. album*, although the MBC/C$_{soil}$ and MBN/N$_{soil}$ were higher in the RES than INT due to the significantly lower C$_{soil}$ and N$_{soil}$ in restored wetland (Fig4 .2; Table S4.3). Overall, no significant correlations were found between MBC, MBN and litter and soil chemical parameters (e.g., N$_{plant}$, P$_{plant}$, C$_{soil}$, N$_{soil}$, P$_{soil}$, DOC, Lg(pH), Lg(TDN)) or plant root density (Table S4.4).

4.4.4 Soil enzyme activities

We saw the largest differences in soil EEAs when comparing across the hydrological conditions. The EEAs were generally lower in PI, between 48 and 73% of the activities under the other two hydrological conditions, similar to that observed for microbial biomass. BG, NAG and LAP activities did not vary between II and NI conditions, though AP was higher under the II condition compared to NI (Fig 4.3), which coincided with the high root density of *Carex* spp.-INT, *P. arundinacea*-INT and RES plots (Table S4.3). Overall, AP activity showed a significant positively linear relationship with root density ($R^2=0.32$, $p<0.01$; n=27; Fig 4.4).

Soil EEAs were often similar across different dominant vegetation within each hydrological zone with a few exceptions (Fig 4.3). The EEAs within one plant species were also similar despite of the soil differences between the two marshes (Fig 4.3; Table S4.3). Under the II conditions, *P. arundinacea*-RES generally had the highest activities of all C-, N- and P- acquiring enzymes and *S. cyperinus*-RES showed the lowest EEAs, particularly for the N-acquiring enzymes (NAG and LAP) (Fig 4.3). The EEA of AP was consistently the highest across all vegetation plots (371-603 nmol h^{-1} g dry $soil^{-1}$), followed by BG (156-271 nmol h^{-1} g dry $soil^{-1}$), NAG (79-190 nmol h^{-1} g^{-} dry $soil^{1}$) and LAP (29-109 nmol h^{-1} g dry $soil^{-1}$). We did not observe a strong influence of intact versus restored marsh on soil EEAs. Two plant species *P. arundinacea* and *G. album*, sampled from two marshes, showed little variation in their EEAs, despite some significant differences in C_{soil} and N_{soil}. This could be ascribed to the similar soil TDN, total P and aboveground living biomass produced between intact and restored marshes which compensate for the C degradation in the sediment due to the past drainage (Table S4.2, S4.3).

The Pearson correlation and RDA results both showed that microbial biomass (especially MBN) most significantly correlated with all four EEAs, followed by soil gravimetric moisture which significantly negatively correlated with four EEAs except for AP (Fig 4.5; Table S4.4). Similar to the linear regression results, AP activity was significantly positively correlated with the plant root density (r=0.57; p<0.01). Neither soil chemical parameters nor plant litter nutrients significantly correlated with the soil EEAs (Appendix E). The activities of all four EEAs were significantly positively correlated with each other and the correlations were stronger between C-acquiring (BG) and N- acquiring enzymes (NAG and LAP) than with the P-acquiring enzyme (AP) (Fig 4.5; Table S4.4).

4.5 Discussion

4.5.1 Wetland hydrologic impact on microbial biomass and enzyme activities

Testing hypothesis 1, we found the lowest microbial biomass (especially MBN), MBC/C_{soil} and MBN/N_{soil} ratios under the PI conditions for both wetlands, although the lowest MBC only occurred in the restored wetland (Fig. 2; Appendix D). These observations support many previous wetland studies (McLatchey and Reddy,1998; Bruland and Richardson, 2004; Rinklebe and Langer, 2006). Anoxia caused by permanent inundation is known to induce anaerobic respiration and fermentation which yield less energy for microbial biomass production per mineralized substrate than aerobic respiration (Bastviken et al., 2003). Moisture-saturated conditions are also less favorable for microbial growth and activities than soils below saturation (e.g., 30-70 % water holding capacity) (Rinklebe and Langer, 2006; Iovieno and Bååth, 2008).

Low MBC/C_{soil} and MBN/N_{soil} ratios can either result from the less degradable substrates (Anderson and Domsch, 1989) or unfavorable soil conditions for living microorganisms (Bauhus and Khanna, 1999). In line with our findings, McLatchey and Reddy (1998) reported significantly lower MBC/C_{soil} ratios under reduced, anaerobic conditions, ranging from 0.1- 0.8 % compared to 2.2 % under aerated conditions. The MBC/C_{soil} and MBN/N_{soil} ratios under II and NI conditions in this study were comparable to forest and agricultural soils with MBC-to-organic C and MBN-to-total N ratios ranging from 1-4 % and 3-5 %, respectively (Anderson and Domsch, 1989; Bauhus and Khanna, 1999; Joergensen et al., 1995). In our study, the lower MBC and MBN of the permanently inundated marsh areas coincided with more N- and P-rich dominant plant species and also had higher soil C and N concentrations compared to the II and NI areas. Thus, we suspect that the lower MBC and MBN that we observed, are less because of lower substrate bioavailability and more likely due to oxygen limitations.

Limited oxygen could strongly restrict the presence of aerobic microorganisms especially fungi which have higher C concentrations and thus higher MBC/MBN ratio than bacteria (Drenovsky et al. 2004; Bossio et al., 2006). The notably lower MBC/MBN ratio at the *P. epihydrus* and *S. latifolia*-RES plot indicates a reduced proportion of fungi in the microbial community under PI condition compared to II and NI conditions. However, the MBC and MBC/MBN ratio at *T. latifolia*-INT plot were substantially higher compared to *P. epihydrus* and *S. latifolia*-RE*S* plot, suggesting greater microbial growth, especially fungi and a fungal-dominated microbial community, presumably due to the rhizosphere O_2 released from *T. latifolia* roots. *P. epihydrus* and *S. latifolia*, however, are floating plants and develops a limited quantity of fibrous roots and might not be able to release O_2 (Heisey and Damman, 1982; Brix, 2003). Confirmed O_2-released plants in marshes from this study include *P. australis*, *T. latifolia* and *Scirpus* spp. (Brix, 2003). The rich N of *P. epihydrus* and *S. latifolia* litters might also affect the microbial community and contribute to the low

MBC/MBN ratio at *P. epihydrus* and *S. latifolia*-RE*S* plot (Table S4.2; Fig 4.2).

As hypothesized, lowest soil EEAs also occurred under PI conditions (Fig 4.3). Depressed soil EEAs under waterlogged conditions have been widely reported in wetland systems including peatlands, salt marshes and constructed freshwater marshes (Freeman et al., 1996; Kang et al., 1998; Steinmuller et al., 2020), and several mechanisms have been identified to explain this phenomenon. An ‘enzyme latch’ mechanism was proposed in inundated peatlands which suggest that, hydrolases, like those we measured, are depressed when phenolic concentrations are high, typical of anoxic ecosystems (Freeman, 2001). This mechanism was further confirmed in several C-rich mangrove swamps and peatlands worldwide (Saraswati et al., 2016; Pinsonneault et al., 2016). Nevertheless, it might not be applicable for mineral marshes (including this study) where phenolic compounds are less likely to accumulate and inhibit hydrolase activity. First, mineral marshes, dominated by forbs and grasses, have lower concentrations of phenolic compounds in their tissues compared to moss peatlands, and thus fewer inputs of phenolics in mineral marshes (Mentzer et al., 2006; Bernal and Mitsch, 2008). Second, the high concentrations of reactive metals in the mineral matrix (e.g., Fe^{2+} and Mn^{2+}) can help support phenol oxidase activity under waterlogged conditions, further minimizing the accumulation of phenolics (Van Bodegom et al., 2005; Sinsabaugh, 2010). Thus, we do not suspect that the low EEAs we observed in the PI areas is due to phenolic inhibiting hydrolases activity.

Another explanation for our low enzyme activities might be the overall lower abundances of microbial populations. Similar to many other wetland studies, our results, revealed a substantial decline in microbial biomass in permanently inundated conditions compared to the never or intermittently inundated conditions (Bruland and

Richardson, 2004; Rinklebe and Langer, 2006). This suggests that the decreased microbial population might be limiting enzyme production and causing slow decomposition in permanently waterlogged areas (Frankenberger and Dick, 1983; McLatchey and Reddy,1998; Kang et al., 1998). Meanwhile, altered microbial composition (e.g., absence of some critical fungi) and/or shifted metabolic pathways from aerobic to anaerobic respiration/fermentation under waterlogged conditions could also reduce the hydrolytic enzyme activities (Romaní et al., 2006). To our knowledge, there is no discussion of the validity of these mechanisms in mineral freshwater marshes and our results mainly support the ‘decreased microbial biomass’ explanation, although other mechanisms might also contribute.

We also hypothesized that the intermittently inundated areas would exhibit the highest enzyme activities and microbial biomass. However, our results do not generally support this. The MBC, MBN and soil EEAs were often similar under II conditions compared to NI conditions, despite some variations caused by specific vegetation plots (Fig 4.2, 4.3). This could be because the two important premises to support this hypothesis were not observed in our marshes. First, we did not find significantly higher labile carbon and nutrients (DOC, TDN) nor higher total nutrients concentrations (N_{soil} and P_{soil}) under the fluctuating II conditions than the drier NI conditions (Table S4.3). Second, we expected that II conditions could possibly allow more diverse microbial community and higher microbial biomass but the MBC, MBN and MBC/MBN ratios between II and NI plots in this study were very similar, at least indicating no significant difference in their microbial population size and fungi-to-bacteria dominance. The similarities in microbial biomass between the II and NI may result from the clay-rich mineral matrix (> 50 %) in this study which diminishes the fluctuating impact on soil nutrients and the microbial biomass and EEAs. Clay-rich soils typically form smaller pores and have high water absorbance such that drying and wetting is more gradual which may minimize microbial biomass vulnerability to moisture fluctuations (Scow et al., 1997). Further, clay-rich soils often form stable aggregates, protecting organic compounds from physical fragmentation which prevents the release of labile carbon and nutrients upon flooding (Six et al., 2006). Nevertheless, the magnitudes of this protection mechanism on microbial biomass and enzyme activities in soils with varying clay content require further determination.

4.5.2 Wetland vegetation impact on microbial biomass and enzyme activities

Interactions between wetland plant and microbial communities are increasingly studied (Yarwood, 2018) but there are few field observations from natural and restored freshwater marshes. Our results support our second hypothesis that on top of hydrology, the dominant plant species further influences soil microbial biomass and EEAs. We found this vegetative impact was more likely exerted through root-related mechanisms, rather than litter-related mechanisms. There are at least two important pathways of marsh plant roots affecting soil microbial biomass and EEAs. First, some emergent plant species develop aerenchyma tissues in roots (e.g., *T. latifolia*, *P. arundinacea*) which release O_2 and change the microenvironments of surface soils (Wießner et al., 2002; Brix, 2003; Zhang et al., 2017). Fang et al. (2021) compared the soil microbial properties between the rhizosphere and non-rhizosphere soils of three wetland species in a constructed wetland and found different oxidation-reduction potential, microbial metabolic activity, abundance, and community structure in the rhizosphere soils largely due to the O_2 and organic compounds released from the roots of emergent plants. Second, wetland plant species could affect the soil EEAs through direct root-induced enzyme releases (Rejmánková et al., 2008). In most cases, microbial secreted enzymes are the major component for soil potential enzyme activities (Dick, 1994). But in some nutrient-poor conditions, root-induced enzymes can also be significant, especially phosphatase (Dakora and Phillips, 2002; Robroek et al., 2009). In this study, the consistently high AP across nine vegetation plots suggests a strong P-limitation for the microbial communities. Moreover, we found highest AP activity at the *Carex* spp.-INT, *P. arundinacea*-INT and RES plots which had the highest root density, suggesting possible direct rhizodeposition of phosphatases. High plant root surface areas, root biomass and root AP released from *P. arundinacea* were observed by Gagnon et al. (2007) and Salvato et al. (2012) in mesocosm experiments. *P. australis*, although it also had high root density, did not show high AP activity in this study, probably because its vigorous root systems can develop down to 70-90 cm the soil surface and our 0-10 cm sampling would have failed to capture this rhizodeposition effect (Moore et al., 2012).

4.6 CRediT authorship contribution statement

Dan Dong: Conceptualization, Methodology, Formal analysis, Visualization, Investigation, Data Curation, Writing - original draft, Validation. Cynthia M. Kallenbach: Methodology, Resources, Supervision, Writing - review and editing, Validation. Tim R. Moore: Methodology, Supervision, Writing - review and editing, Funding acquisition, Validation.

4.7 Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships involved in this study.

4.8 Data availability

Data will be available from the corresponding author upon request.

4.9 Acknowledgements

We thank Dr. Christian von Sperber, Calder Jones, Dr. Joann Whalen, Aidan De Sena, Lijun Hou, Rachael Harman-Denhoed, Hannah P. Lieberman, Paula Kestelman and Mike Dalva for their great assistance in the laboratory. We also thank Calder Jones for the help with plant and soil sampling in the field and Dr. Frieda Beauregard, Shir Gruber and Alexa Brunet for the identification of plant species. We also thank people from Ducks Unlimited including Rick Robb, Erling Armson, Joanne Barbazza, Kyle Borrowman, Mallory Carpenter and Tatjana Radulovic for their great efforts on land construction and management for Atocas Bay Reserve. This study was funded by Natural Sciences and Engineering Research Council of Canada (NSERC STPGP 506809).

4.10 Tables and figures

Figure 4.1 Locations of nine sampled vegetation plots. Plots were under permanently inundated (PI), intermittently inundated (II) and never inundated (NI) conditions of two freshwater marshes of Atocas Bay Reserve, Ontario, Canada. INT: Intact marsh; RES: restored marsh.

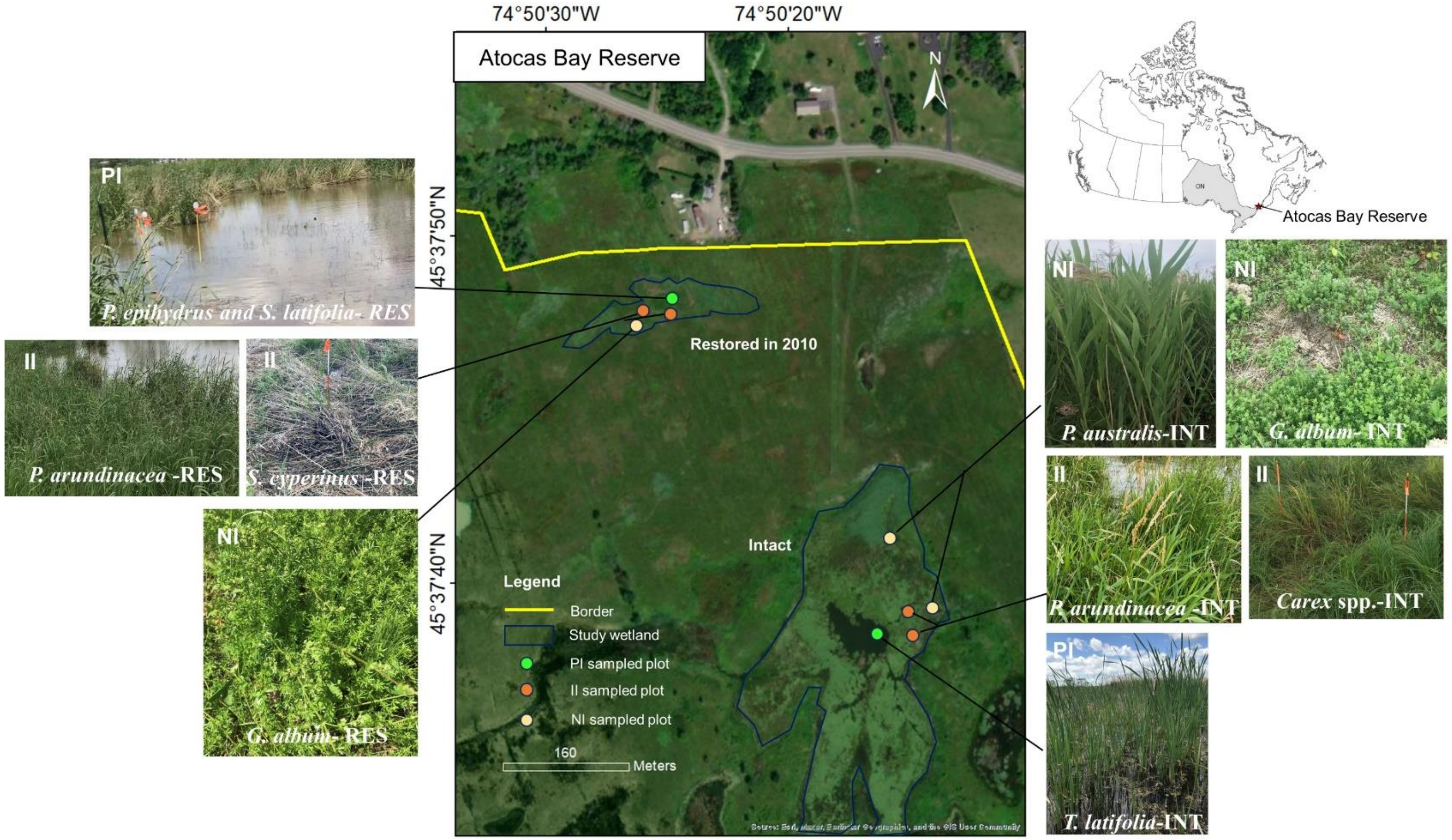


Figure 4.2 Microbial biomass properties of nine vegetation plots. Microbial biomass C (MBC; mg kg^{-1})), N (MBN; mg kg^{-1}), ratios of MBC/MBN and microbial biomass C/ (soil total C concentration) (MBC/C$_{soil}$; %) of nine vegetation plots were grouped by three hydrological conditions (PI for permanently inundated; II for intermittently inundated and NI for never inundated). Upper letters and lower letters indicate significant difference among different hydrological conditions (Bonferroni-corrected α=0.017) and among different vegetation plots (Bonferroni-corrected α=0.0014), respectively. Values presented in mean ± standard error (*n*=3). INT: intact marsh; RES: restored marsh.

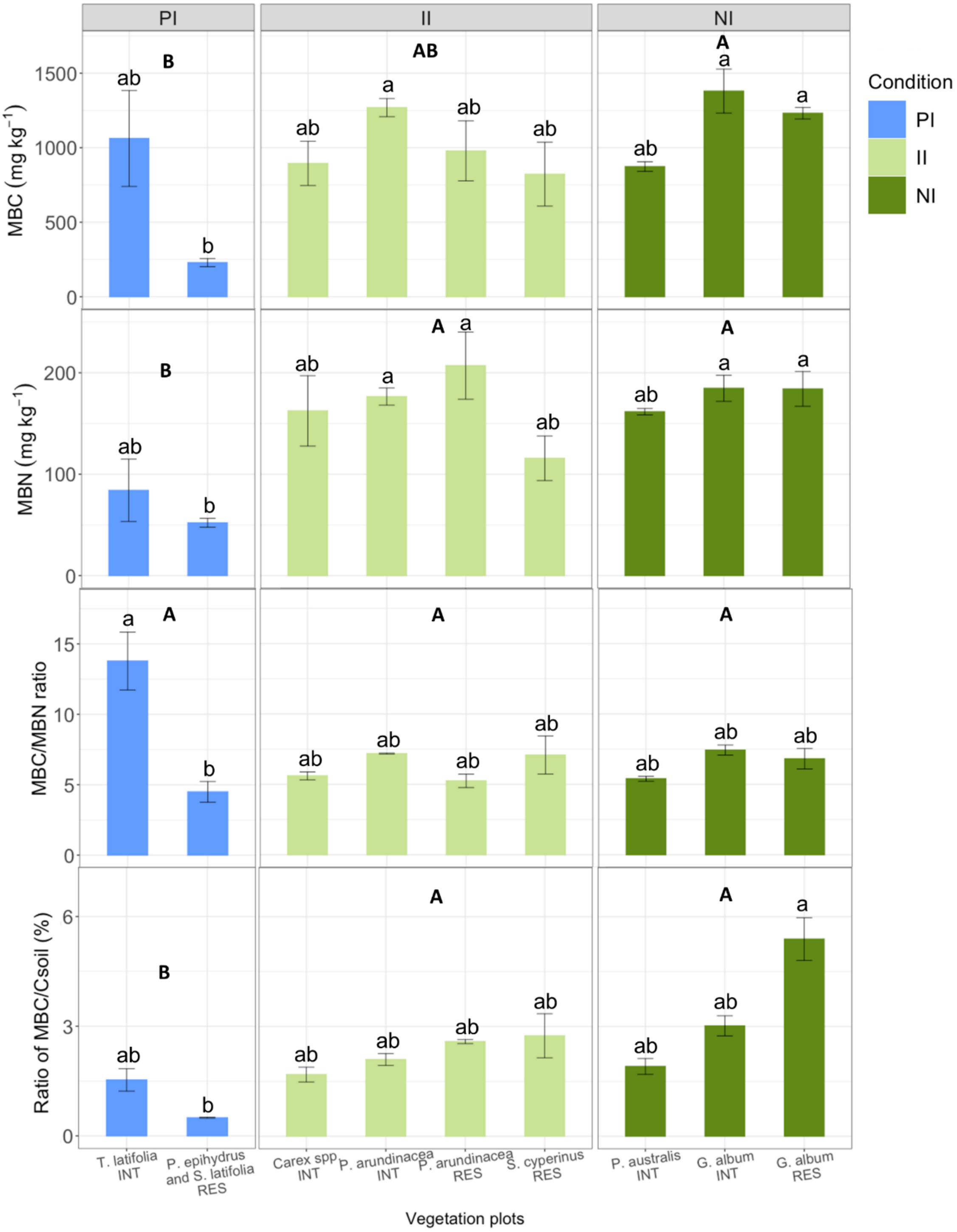


Figure 4.3 Extracellular enzyme activities of nine vegetation plots. Soil β-glucosidase (BG), β-N-acetyl-glucosaminidase (NAG), leucine aminopeptidase (LAP) and acid phosphatase (AP) (mean ± standard error; nmol h^{-1} g dry $soil^{-1}$) of nine vegetation plots were grouped by three hydrological conditions (PI for permanently inundated; II for intermittently inundated and NI for never inundated). Upper letters and lower letters indicate significant difference among different hydrological conditions (Bonferroni-corrected α=0.017) and among different vegetation plots (Bonferroni-corrected α=0.0014), respectively. INT: intact marsh; RES: restored marsh.

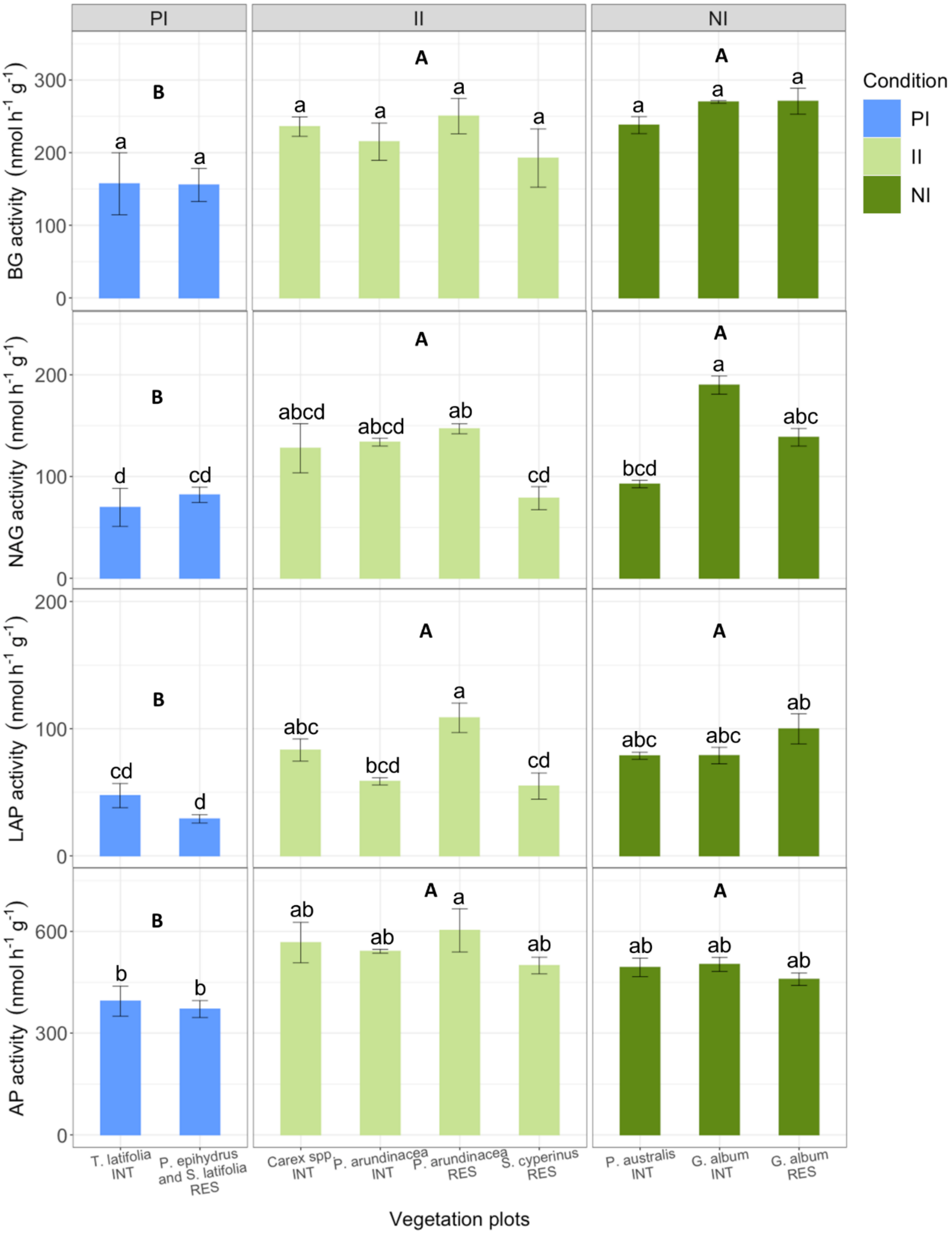


Figure 4.4 Linear regression between acid phosphatase activity and plant root density. (*n*=27, R^2=0.32, p<0.01). Samples were from permanently inundated (PI), intermittently inundated (II) and never inundated (NI) conditions, respectively.

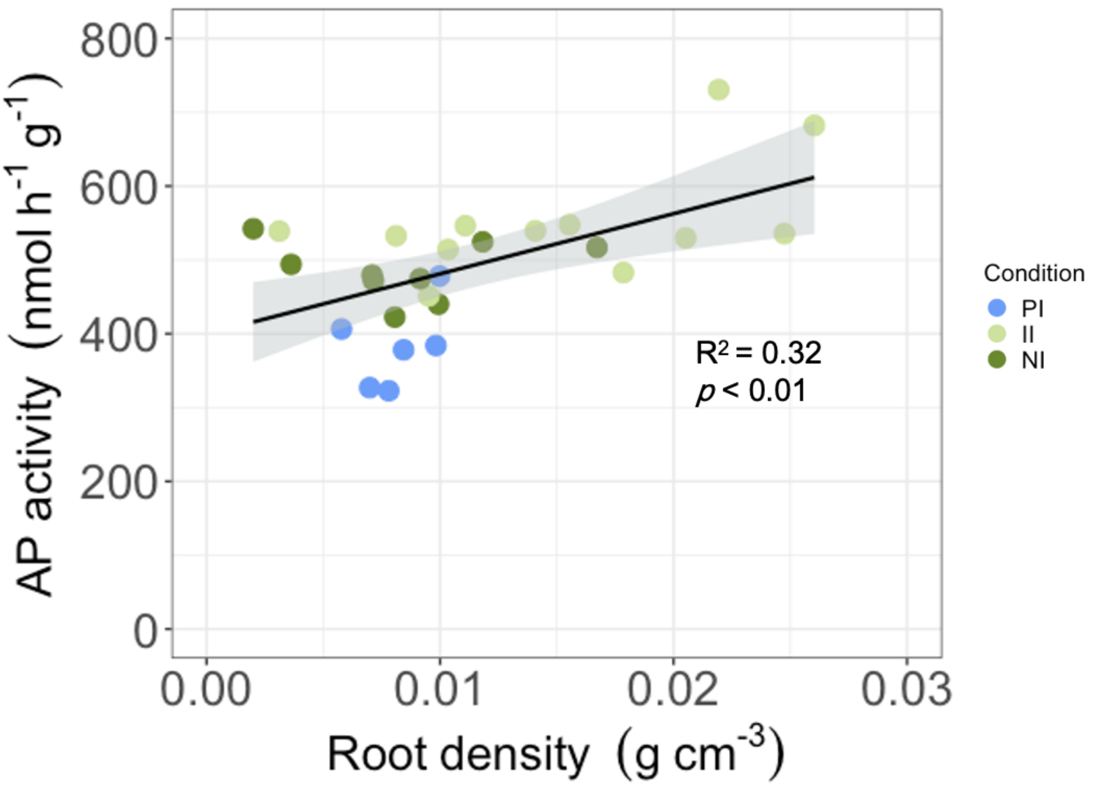
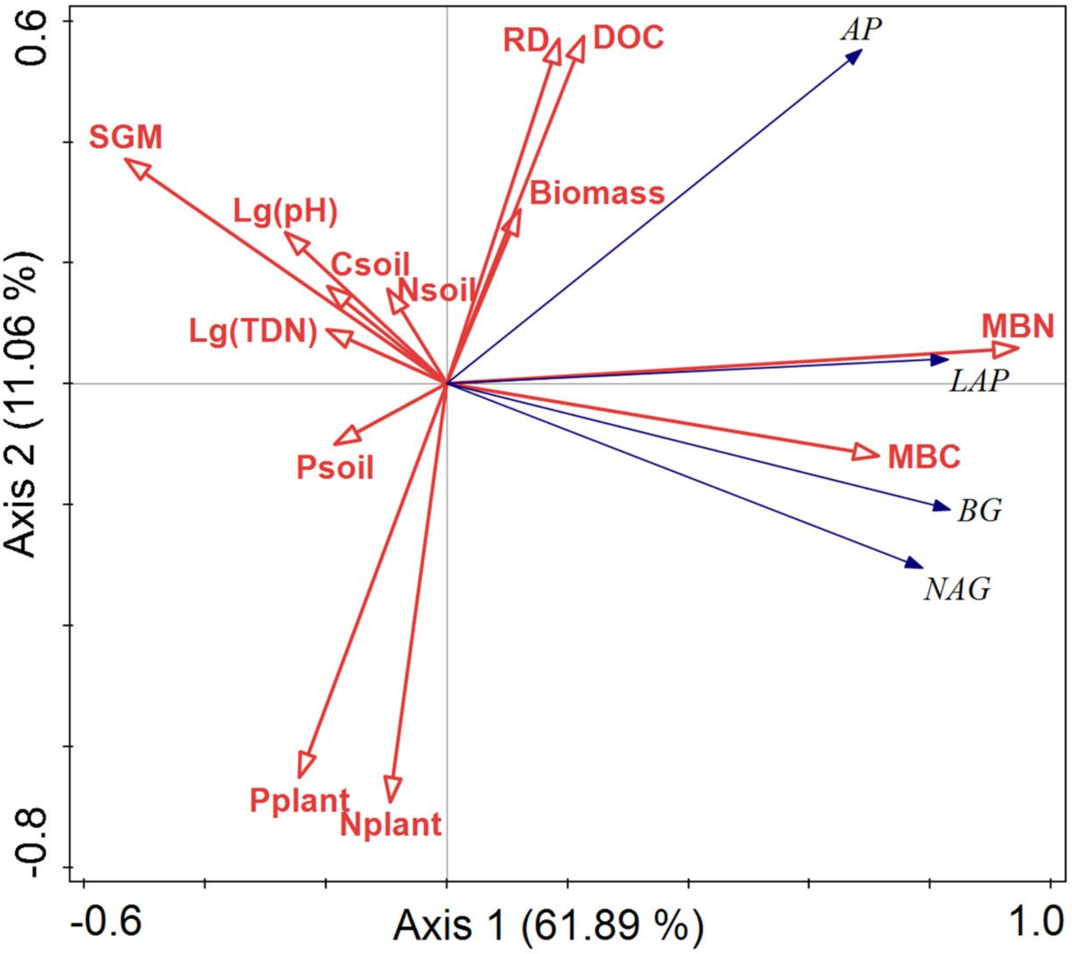


Figure 4.5 RDA correlation biplot of four EEAs and the explanatory variables. BG, β-glucosidase, NAG, β-N-acetyl-glucosaminidase, LAP, leucine aminopeptidase and AP, acid phosphatase. N_{plant} and P_{plant}, the N and P concentration of plant litter; RD, root density; Biomass, aboveground living biomass; MBC, MBN, microbial biomass C and N; C_{soil}, N_{soil}, P_{soil}, soil total C, N and P; DOC, soil dissolved organic carbon; Lg(TDN), soil total dissolved nitrogen (lg-transformed); Lg (pH), soil pH (lg-transformed); SGM, soil gravimetric moisture.



4.11 Supplementary materials

Figure S4.1 Daily average water level in the permanently inundated plots of the two wetlands (intact; INT and restored; RES) and the daily precipitation recorded from climatic station "OTTAWA INTL A" from June to August, 2022.

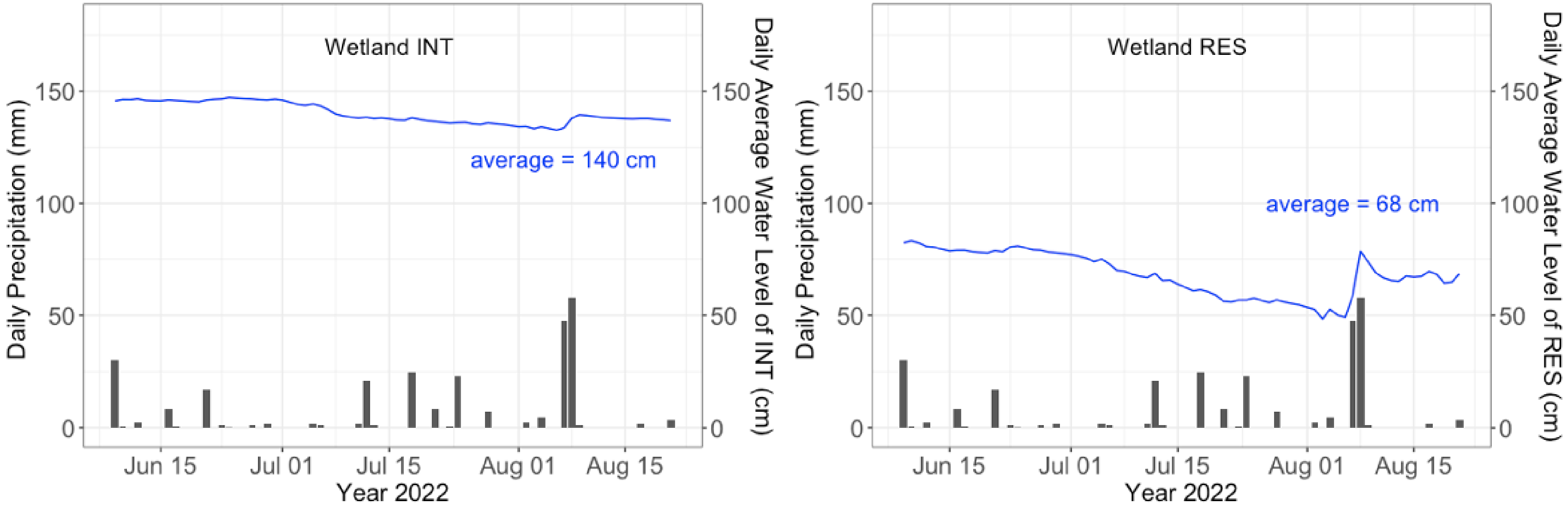


Table S4.1 Hydrological conditions (PI for permanently inundated; II for intermittently inundated and NI for never inundated) of the nine sampled freshwater marsh (intact; INT and restored; RES) plots and their dominant plant species between July 2021 and July 2022. 'I' and 'NI' inundated and not inundated by water, respectively.

Vegetation plot	Hydrological condition	Jul. 2021	Aug. 2021	Sep. 2021	May 2022	Jun. 2022	Jul. 2022
T. latifolia-INT	PI	I	I	I	I	I	I
P. epihydrus and *S. latifolia*-RES	PI	I	I	I	I	I	I
Carex spp.-INT	II	NI	NI	I	I	I	NI
P. arundinacea-INT	II	NI	NI	I	I	I	NI
P. arundinacea -RES	II	NI	NI	NI	I	I	NI
S. cyperinus-RES	II	NI	NI	NI	I	I	NI
P. australis-INT	NI	NI	NI	NI	NI	NI	NI
G. album-INT	NI	NI	NI	NI	NI	NI	NI
G. album-RES	NI	NI	NI	NI	NI	NI	NI

Table S4.2 Descriptions of the nine sampled vegetation plots: dominant plant species, degree of inundation, aboveground litter quality characterized by C $_{plant}$ (total carbon, %), N $_{plant}$ (total nitrogen, %), P $_{plant}$ (total phosphorus, %) and C/N$_{plant}$, C/P$_{plant}$ and N/P$_{plant}$ and aboveground living biomass (g m^{-2}) determined in mid-July 2022. Values are means of three replicates ± SE. Different letters in the same column indicate significant difference among vegetation plots at Bonferroni-corrected alpha level (α=0.0014). Significant difference in aboveground litter quality and living biomass across three hydrological conditions (PI, II, NI) were compared through Kruskal-Wallis test (α=0.05). INT: Intact marsh; RES: restored marsh.

Vegetation plot	Hydrological condition	Aboveground litter quality						Aboveground living biomass (g m^{-2})
		C$_{plant}$ (%)	N$_{plant}$ (%)	P$_{plant}$ (%)	C/N$_{plant}$	C/P$_{plant}$	N/P$_{plant}$	
T. latifolia-INT	PI	46.4±0.0^{ab}	0.64±0.01^{ab}	0.022±0.001^{ab}	73±1^{ab}	2091±48^{ab}	29±1^{ab}	474±137^{a}
P. epihydrus and *S. latifolia*-RES	PI	39.3±0.1^{b}	1.55±0.01^{a}	0.044±0.000^{a}	25±0^{b}	901±6^{b}	35±0^{ab}	355±46^{a}
Carex spp.-INT	II	44.1±0.1^{ab}	0.64±0.00^{ab}	0.013±0.000^{b}	69±0^{ab}	3503±51^{a}	51±1^{a}	476±123^{a}
P. arundinacea-INT	II	42.6±0.0^{b}	1.23±0.01^{ab}	0.031±0.001^{ab}	34±0^{ab}	1380±43^{ab}	40±1^{ab}	550±130^{a}
P. arundinacea-RES	II	44.3±0.1^{ab}	0.77±0.01^{ab}	0.028±0.000^{ab}	58±1^{ab}	1609±32^{ab}	28±0^{ab}	639±66^{a}
S. cyperinus-RES	II	47.7±0.0^{a}	0.35±0.00^{b}	0.023±0.000^{ab}	135±1^{a}	2074±23^{ab}	15±0^{b}	211±66^{a}
P. australis-INT	NI	46.4±0.0^{ab}	0.53±0.00^{b}	0.009±0.000^{b}	87±0^{a}	4978±140^{a}	57±2^{a}	1058±34^{a}
G. album-INT	NI	43.3±0.1^{ab}	1.28±0.01^{ab}	0.034±0.001^{ab}	34±0^{ab}	1284±33^{ab}	38±1^{ab}	331±6^{a}
G. album-RES	NI	43.8±0.1^{ab}	1.13±0.01^{ab}	0.033±0.001^{ab}	39±0^{ab}	1314±30^{ab}	34±1^{ab}	345±39^{a}
P-value Sig. (PI vs. II vs. NI)		0.70	0.29	0.35	0.41	0.33	0.10	0.06

Table S4.3 Soil properties, root density and microbial biomass features of the nine sampled vegetation plots. C_{soil}, N_{soil}, and P_{soil}, soil total C, N and P concentration (%); DOC, dissolved organic carbon (mg kg^{-1}); TDN, total dissolved nitrogen (mg kg^{-1}) including ammonium, nitrate and dissolved organic N; RD, root density (g cm^{-3}); MBC/C_{soil} and MBN/N_{soil} (%), the percent of MBC and MBN in soil total C and total N pool, respectively. Lowercase letters in the same column indicate significant difference among nine plots at Bonferroni-corrected alpha level (α=0.0014). Uppercase letters in the same column indicate significant difference among three hydrological conditions at Bonferroni-corrected alpha level (α=0.017). INT: Intact marsh; RES: restored marsh.

Vegetation plot	Condition	C_{soil} %	N_{soil} %	P_{soil} %	C/N_{soil}	C/P_{soil}	N/P_{soil}	DOC mg kg^{-1}	TDN mg kg^{-1}	RD g cm^{-3}	pH	MBC/C_{soil} %	MBN/N_{soil} %
T. latifolia-INT	PI	6.7^{a}	0.53^{a}	0.058^{a}	13^{a}	115^{a}	9.2^{a}	135^{a}	2.4^{a}	0.009^{ab}	6.95^{a}	1.5^{ab}	1.5^{b}
P. epihydrus and *S. latifolia*-RES	PI	4.6^{ab}	0.35^{a}	0.065^{a}	13^{a}	70^{a}	5.3^{a}	107^{a}	5.3^{a}	0.008^{ab}	5.98^{ab}	0.5^{b}	1.6^{b}
PI (n=6)		5.6^{A}	0.44^{A}	0.062^{A}	13^{A}	93^{A}	7.2^{A}	121^{A}	3.9^{A}	0.008^{B}	6.47^{A}	1.0^{B}	1.5^{B}
Carex spp.-INT	II	5.3^{ab}	0.44^{a}	0.052^{a}	12^{a}	101^{a}	8.4^{a}	190^{a}	1.9^{a}	0.023^{a}	6.43^{ab}	1.7^{ab}	3.6^{ab}
P. arundinacea-INT	II	6.2^{ab}	0.52^{a}	0.070^{a}	11^{a}	69^{a}	8.2^{a}	101^{a}	2.1^{a}	0.016^{ab}	6.32^{ab}	2.1^{ab}	3.4^{ab}
P. arundinacea-RES	II	3.0^{ab}	0.26^{a}	0.064^{a}	12^{a}	53^{a}	4.5^{a}	140^{a}	3.9^{a}	0.013^{ab}	6.07^{ab}	2.6^{ab}	6.1^{ab}
S. cyperinus-RES	II	2.9^{ab}	0.25^{a}	0.054^{a}	12^{a}	55^{a}	4.7^{a}	88^{a}	10.7^{a}	0.009^{ab}	5.85^{b}	2.7^{ab}	4.6^{ab}
II (n=12)		4.5^{A}	0.38^{A}	0.059^{A}	12^{AB}	77^{A}	6.5^{A}	130^{A}	4.6^{A}	0.015^{A}	6.17^{A}	2.3^{A}	4.1^{A}
P. australis-INT	NI	4.7^{ab}	0.41^{a}	0.086^{a}	11^{a}	68^{a}	5.9^{a}	139^{a}	2.6^{a}	0.013^{ab}	6.13^{ab}	1.9^{ab}	4.1^{ab}
G. album-INT	NI	4.6^{ab}	0.41^{a}	0.060^{a}	11^{a}	77^{a}	6.8^{a}	63^{a}	3.7^{a}	0.004^{b}	5.84^{b}	3.0^{ab}	4.5^{ab}
G. album-RES	NI	2.4^{b}	0.21^{a}	0.053^{a}	11^{a}	44^{a}	3.9^{a}	78^{a}	2.2^{a}	0.008^{ab}	6.01^{ab}	5.4^{a}	9.0^{a}
NI (n=9)		3.9^{A}	0.34^{A}	0.066^{A}	11^{B}	62^{A}	5.5^{A}	93^{A}	2.9^{A}	0.008^{B}	6.00^{A}	3.4^{A}	5.9^{A}
P-value Sig. (PI vs. II vs. NI)		0.12	0.43	0.59	**<0.001**	0.08	0.26	0.25	0.68	**<0.05**	0.13	**<0.01**	**<0.001**

Table S4.4 Pearson correlation coefficient matrix of four hydrolase enzyme activities β-glucosidase (BG), β-N-acetyl-glucosaminidase (NAG), leucine aminopeptidase (LAP) and acid phosphatase (AP) and environmental parameters characterizing plant litter nutrients, root density, soil microbial biomass, and soil physiochemical features (*n*=27). N_{plant} and P_{plant}, the N and P concentration of litters; RD, root density; MBC, MBN, microbial biomass C, N; $C_{soil,}$ $N_{soil,}$ P_{soil}, soil total C, N, P; DOC, dissolved organic carbon; Lg(TDN), Lg(soil total dissolved nitrogen); Lg(pH), Lg(soil pH); Biomass, aboveground living biomass; SGM, soil gravimetric moisture. * *P*-values < 0.05, ** *P*-values < 0.01, *** *P*-values <0.001.

	BG	NAG	LAP	AP	N_{plant}	P_{plant}	RD	MBC	MBN	C_{soil}	N_{soil}	P_{soil}	DOC	Lg (TDN)	Lg (pH)	Biomass	SGM
BG	1																
NAG	**0.74*****	1															
LAP	**0.80*****	**0.62*****	1														
AP	**0.62*****	**0.60****	**0.64*****	1													
N_{plant}	0.02	0.36	-0.19	-0.25	1												
P_{plant}	-0.11	0.22	-0.28	-0.31	**0.84*****	1											
RD	0.20	0.08	0.20	**0.57****	-0.28	**-0.46***	1										
MBC	**0.64*****	**0.64*****	**0.53****	**0.48***	-0.04	-0.11	0.09	1									
MBN	**0.81*****	**0.75*****	**0.81*****	**0.74*****	-0.05	-0.21	0.31	**0.79*****	1								
C_{soil}	-0.14	-0.07	-0.32	0.02	0.06	-0.17	0.26	0.23	-0.03	1							
N_{soil}	-0.07	-0.00	-0.24	0.09	0.02	-0.24	0.26	0.31	0.09	**0.98*****	1						
P_{soil}	0.08	-0.10	0.02	-0.02	0.01	-0.16	0.00	0.00	0.14	0.32	0.36	1					
DOC	-0.03	-0.13	0.16	0.31	-0.32	**-0.47***	**0.51****	-0.16	0.04	0.31	0.29	0.07	1				
Lg(TDN)	-0.29	-0.24	-0.28	-0.22	-0.09	0.25	**-0.45***	-0.37	-0.35	-0.38	**-0.41***	-0.10	-0.13	1			
Lg(pH)	-0.34	-0.36	-0.19	-0.16	-0.25	-0.36	0.25	0.07	-0.24	**0.64*****	**0.60****	-0.02	0.36	**-0.52****	1		
Biomass	0.15	-0.04	0.25	0.26	-0.29	**-0.51***	0.28	0.11	0.34	0.25	0.27	**0.49***	**0.53****	-0.31	0.20	1	
SGM	**-0.39***	**-0.40***	**-0.46***	0.00	-0.07	-0.07	0.33	-0.05	-0.30	**0.81*****	**0.72*****	0.18	0.31	-0.18	**0.65*****	0.20	1

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Chapter 5: Sediment C, N, P concentration and stoichiometry of intact, drained and restored freshwater marshes in the Prairie Pothole Region of Canada

Bridging statement to Chapter 5

In chapter 3 and 4, we have examined litter decomposition rates and microbial biomass and enzyme activities in the surface soil of restored marshes. This leaves unclear the C, N, P dynamics in subsurface and deep sediments which are related to physiochemical processes such as leaching, rock weathering, P adsorption and desorptio. In this chapter, we determined the vertical distribution of C, N, P concentration and stoichiometric characteristics in soils of intact, drained and restored marshes sampled to 30-150 cm depth. One set of three wetlands occurs in Broughton's Creek Watershed, Manitoba, where replicated core samples were taken from the edge of the wetland to the centre, to a depth of 50 cm. The other set of wetlands is from Camrose County, Alberta, with a larger number of wetlands, only the samples from the centre were taken. Combining the wetlands, we see land cultivation and surrounding agricultural activities profoundly affect C, N, P concentrations and distribution in sediment profiles. Rewetting on formerly drained and cultivated soils could potentially translocate dissolved C, N, P deeper in the sediment profile.

Note: This chapter involves two datasets (one acquired through chemical analysis by Dan Dong, one received from the project leader Dr. Irena F. Creed). The two datasets served similar research purposes but were not consistent in sampling design, chemical analysis and will be split into two manuscripts for publication.

5.1 Abstract

Wetlands undergo changes through drainage, cultivation and restoration, with interest in the fate of sediment C, N, P and stoichiometry. Here, we first examined three Manitoba wetlands (2 intact, 1 restored) which were sampled in centre, midslope and upland locations to a depth of 50 cm and analyzed for C, N and P concentrations. The study was extended to thirty wetlands in Alberta (3 intact, 3 drained, 24 restored) with more varied land uses, restored ages (2-23 years) and deeper sampling (30-150 cm) in the centre only. The stoichiometric relationships of C, N and P were derived from their concentrations. We found that cultivation and restoration both strongly affected sediment C, N, P, with C and N always tightly coupled, but not with P. In Manitoba, the croplands-bordered intact wetland contained 1.5-2 times C and N in profiles (both in concentration and density) of those in the grasslands-bordered intact and restored wetlands, despite similar P concentration among three marshes. In Alberta, the C, N, P concentrations of drained wetlands were overall 60-90 % of those in the intact reference at surface 0-30 cm. Restored wetlands however contained higher P concentration than the intact reference but only half the C and N concentration of intact states at both surface and subsoils. Compared to P, C and N seemed to lose to greater degrees from the intact benchmark when experiencing land conversions. Sediment C, N concentration, and C:P and N:P ratios in restored wetlands were undistinguished across varied restoration periods and consistently lower than the intact and drained references, suggesting potentially a more P-rich condition after rewetting. The vertical distribution of C and N in cultivated and restored soils also differed substantially from the intact reference. At 20-30 cm depth, there existed an exceptionally rich C and N layer in drained wetlands, likely resulting from N fertilizer input or N-fixation crops. In restored wetlands which were transformed from drained wetlands, similar thin layers of sharp increases of C, N, and P were seen, but occurred

in subsoils as well, possibly formed by the agricultural legacies (e.g., residual roots) that leached downward through the prevailing vertical fluxes in these pothole wetlands. Our findings suggest C and N always change correspondingly in cultivated and restored prairie pothole marshes while P likely decouples with C and N in changes over land conversions.

5.2 Introduction

The Prairie Pothole Region (PPR) is among the largest wetland complexes in North America, containing millions of isolated freshwater marshes, embedded within an agricultural dominated landscape (Ross and McKenna, 2023). These marshes under intact states form a thick layer of organic horizons through long-term inundation (up to 1 m; Richardson et al., 1994), burying considerable C, N, P in sediment. In addition, they trap allochthonous nutrients (e.g., nitrate and phosphate) and organic materials from the agricultural exports and land erosion, which mitigates the risks of eutrophication in downstream rivers and the significant loss of C to the atmosphere (Reddy, 2010; Loder and Finkelstein, 2020). Since the European settlement, extensive anthropogenic interventions have been undertaken in these marshes, for example, drainage, consolidation, cultivation, fertilization, and more recently restoration to compensate the historical wetland losses (Dal, 2014; Jones, 2021). Along with the extensive land conversions, there are growing interests in the changes in sediment C, N, P status under various land managements and surrounding human activities, and their vertical profiles (Bartzen et al., 2010; Anderson, 2017). While adequate assessments have been conducted on C storage and profile characteristics in response to drainage and restoration (e.g., by Euliss et al., 2006;Tangen and Bansal, 2020; Creed et al., 2022; Chizen et al., 2024), our knowledge is highly limited on the C, N, P distribution and stoichiometry in profiles among different land use history (e.g., intact *versus* drained *versus* restored) and under different surrounding land uses (e.g., grasslands *versus* croplands).

In most undisturbed ecosystems, C, N, P were tightly coupled and established some stoichiometric constraint in microbial biomass, mineral and organic soil organic matter (SOM), regional topsoil within 0-20 cm depth, and some consistent stoichiometric patterns over soil profiles (Tian et al., 2010; Kirkby et al., 2011; Wang et al., 2015; Tipping et al., 2016; Qiao et al., 2020; Schneider et al., 2021). For instance, two end-extremes of C:N:P ratios as 919:36:1 and 61:7:1 (mass-based) were modelled in a continuum of global organic- to- mineral SOM containing 0.1- 60 % C from thousands of soil samples throughout O, A, B and C horizons (Tipping et al., 2016). In their study, the C:N:P ratios of global SOM generally fell within the two extremes which are in between 7-36 and 61-919 for C:N and C:P quotients, respectively. Previous studies also reported comparatively constrained global microbial biomass molar C:N:P ratios as 72:6:1 (Cleveland and Liptzin, 2007) and 42:6:1 (Xu et al., 2013), and global wetland soil molar C:N:P ratio as 1347:72:1, equivalent to mass ratio as 521:16:1 (Xu et al., 2013). There are also regional studies which reported constrained C:N:P ratios in surface 0-10 cm of wetland soils (Zhang et al., 2012; Liu et al., 2017). Throughout wetland soil profiles, the C:N and C:P ratios often decrease when transitioning from O to A horizon because of the greater assimilation and retention of nutrients than C by microorganisms, then often continue to decline when transitioning from A to B and C horizons because of a greater depletion of C than N and P as SOM: mineral ratio decreases continuously. The relative abundance of C and N in subsoils depends on the inputs of C and N, quality of leached organic compounds, microbial activities such as N fixation and the specific environmental condition and soil texture of certain ecosystems. For instance, the abundant labile organic compounds leached to subsoils may trigger greater extent of C consumption than that in surface layer through microbial decomposition and make C:N ratio lower than surface soils (Qiao et al., 2020). In mineral soils, the N:P ratio mostly declined with depth when mineral matter increases because of the significant reduction of N while a less significant decline in total P (Ma et al., 2020).

N deposition and extensive fertilizer inputs are new N and P sources for disturbed wetland ecosystems and drained and cultivated wetland soils have shown strong deviations in C, N, P concentration and the stoichiometric balances from the intact states, although existing studies most concentrated on the surface organic soil layers (often less than 30 cm depth) where soils were most directly affected (Brown et al., 2017). After drainage and cultivation, significant loss of total and organic C and organic matter concentration was widely observed (Ewing et al., 2012; Werkmeister et al., 2018; Kaur et al., 2023). Changes in total N and P concentration in cultivated wetland can vary among studies, with increased total N (Lou et al., 2017), decreased total N (Wang et al., 2018); increased total P (Wang et al., 2014) and slightly decreased total P concentration (Badiou et al., 2018) compared to the intact reference all observed, largely owing to the different forms and amounts of fertilizer applied to specific study areas (Ross and and McKenna, 2023). In general, excessive N fertilizer inputs can increase the soil N:P ratios in short-term cultivation while after decades of cultivation, most studies have reported decreased N:P ratio compared to the adjacent intact wetlands, likely resulted from the greater retention of P fertilizers than N (Lou et al., 2017; Wang et al., 2014).

Restoration and rewetting of formerly cultivated wetlands can further influence their sediment C, N, P. In the short term, frequent wetting of dried and nutrient-rich soils could trigger instant pulses of C and N emissions to the atmosphere known as the ‘Birch effect’ (Birch, 1958; Jarvis et al., 2007). Meanwhile, these rich labile nutrients inherited from cultivated stage in the surface soils can be leached to subsoils and stabilized or adsorbed there owing to the slowed decomposition and elements turnover rates (Schneider et al., 2021), alternatively being flushed to surrounding lands through lateral water movements (e.g., spills in potholes) (Brunet and Westbrook, 2011). In the longer term, when wetlands become revegetated and constantly saturated, anoxia fosters the soil organic matter accumulation and ideally the net accretion of C, N and P can be re-achieved (Spohn et al., 2020; Creed et al., 2022). However, it’s been extrapolated from recent evidence that the concentration and storage of C in restored wetlands might be inconsistent among individuals and largely depends on the post-restoration environmental conditions such as the wetland sizes, surrounding environments, leaching intensity, soil texture and penetration, the periods of restoration and the speed of vegetation readaptation (Craft, 2022; Loder et al., 2023).

To date, few studies have assessed the C, N, P concentration and stoichiometric features of restored wetlands, at both surface and deep sediment. In this study, we determined wetland soil C, N, P concentration and their stoichiometries of 3 inland mineral freshwater marshes to depths of 50 cm in Manitoba and 30 of more varied depth ranges from 30 to 150 cm in Alberta, Canada. There are two objectives of this study. First, to examine the vertical and horizonal variability of C, N and P concentrations and stoichiometries in three representative Manitoba marshes, with limited variations in their surrounding land management and land uses. Second, we sought patterns in C, N and P vertical distribution and stoichiometric features in more sampled marshes of Alberta, with greater variability in land management classes (intact *versus* drained *versus* restored) and restored ages. We also looked for potential temporal trends in sediment C, N, P and stoichiometry over the chronosequence of 24 restored marshes in Alberta.

5.3 Method

5.3.1 Study areas

Our study locations involved the Broughton Creek Watershed (BCW, 50°06’N, 100°13’W; 26,034 ha) in the southeastern Manitoba and the Camrose County (CC, 52°03-36’ N, 111°24’-113°12’ W), ~3,000 km^{-2} in the central Alberta, both part of the Canadian Prairie Pothole Region (Table 5.1). This region contained large numbers of small and shallow pothole ponds (water depth <2 m, sizes ranged from 0.2-8 ha) formed from the Pleistocene glaciers retreat around 12,000 years ago (Shjeflo et al., 1962; Jones et al., 2021; Schlaffer et al., 2022). The climate is subhumid in the grassland ecoregion with mean annual temperature and precipitation ranged from 2.9-3.2 °C and 400-426 mm, respectively (data derived from station ‘Branden’ and ‘Camrose’ between 2010 and 2020, Environment Canada 2021). Due to the clay-rich glacial tills (>20 % in mass), these marshes often have low penetration in deep sediments and thus limited water exchange with groundwater (Hayashi et al., 2016). Instead, precipitation and surface inflows are two most dominant sources of water supply (Carroll et al., 2004). Potholes are not connected in hydrological network except in flooding periods (e.g., snow melts in early springs and heavy rains) when water spilled and filled the neighboring potholes (Montgomery et al., 2021). The predominant hydrodynamics in these potholes is vertical driven by evapotranspiration and rainfall with hydraulic conductivity > 1 m yr^{-1} in surface sediments (Hayashi et al., 2016; Schlaffer et al., 2022). Water levels of these marshes fluctuate intra-annually and inter-annually depending on the relative strengths of evapotranspiration and precipitation (Hayashi et al., 2016).

Soil series in both study areas are dominated by Chernozemic including Orthic Black Chernozem (the provincial soil of Manitoba), Gleyed Eluviated Black Chernozem, and Gleyed Rego Black Chernozem, depending on specific environmental conditions (e.g., topography, degree of drainage) (Pennock et al., 2011; Agricultural and agri-food Canada, 2015). Soil horizons of Chernozems typically involved 15-30 cm A horizon, a thin B horizon followed by C and R horizon. The rich mineral matter, shallow organic layer in Chernozemic soils (often less than 40 cm depth; Pennock et al., 2011) and occasional dried conditions prevent peat formation (Montgomery et al., 2021).

Soils in the centre of wetlands are Gleysols or Gleysol-Chernozemic combined, which are permanently or semi-permanently waterlogged, more eluviated and often develop thicker A and B horizon than the soil series from the surrounding drier land (Manning et al., 2001; Chizen et al., 2024). The organic A horizon can be up to 1 m in the centre of deep pothole marshes (Richardson et al., 1994). The parent materials in BCW are of limestone, granite and shale origins and are of sandstone, mudstone, siltstone and coal origins in CC. Vegetation of pothole marshes are dominated by floating and emergent plants in centre and sedges, grasses and forbs in the surrounding wet meadows (Jones, 2021). The average sedimentation rate in ~20 prairie pothole marshes in PPR ranged from 0.2 to 0.6 cm year^{-1}, synthesized by Ross and McKenna (2023). Soils are neutral to moderately alkaline: pH ranged from 6-8 in BCW and 5.5-8 in CC, with Ca^{2+} as the most dominant exchangeable cation, followed by Mg^{2+} and Na^{+} (Bowser, 1962; Pennock et al., 2011; Pascal et al., 2018). The main soil textures of BCW and CC are clay loam and loam, respectively (Bowser, 1962; Badiou et al., 2018).

Both study locations are under predominant agricultural uses, with coverages of

71 % and 76 % croplands in BCW (perennial wheat and barley as dominant crops) and CC (canola, wheat, barley, field pea as dominant crops), respectively (Badiou et al., 2018; Economic Development Camrose, 2024). Fertilizer containing N, P and potassium were applied annually to wheat and barley croplands while no fertilizer was applied to grasslands, remaining intact wetlands or restored wetlands, although restored wetlands were fertilized formerly (Euliss et al., 2006; Badiou et al., 2018). The recommended application rates of N and P fertilizer on barley and winter wheat fields by Government of Manitoba were 80-175 and 40-50 lb acre^{-1} yr^{-1}, respectively, with a fertilizer N:P ratio as 1.3-3.5:1 (Government of Manitoba, 2024).

5.3.2 Soil sampling and analyses for Manitoba wetlands

At the Broughton's Creek Watershed (BCW), we sampled 27 soil cores from 3 wetlands in July 2019: two intact surrounded by cropland and grassland, respectively, and one 10-year restored surrounded by grassland at 3 topographic positions (centre, midslope and upland) along 3 randomly established radial transects within each wetland. The orientations of transects evenly split 0-360^{o} for each wetland. Soil cores were sampled to 50 cm depth consistently using a peat auger (3.5 cm internal diameter) except for the surface 15 cm soils from the centre positions which were too loose and sampled by knife instead. Soil cores were stored in poly-ethylene bags for every 5 cm depth increment and transferred to laboratory in coolers for r analysis.

In the laboratory, fresh soils were sieved through 2 mm with plant materials and animals removed by hand. The clods in samples were gently hand crushed or by mortar and pestle when necessary. Gravel (>2 mm) was not found in the surface soils (0-30 cm), but occasionally existed in soils at 40-45 and 45-50 cm depths and was discarded before any soil analysis. Soils were oven-dried at 105 °C till constant weight to determine the soil gravimetric moisture, bulk density (at only the surface 0-

30 cm depth) and the soil organic carbon (OC) concentration. Due to the often-small variations in soil OC below 20 cm depth, we homogenized the samples from 20-50 depth into two composite depth increments at 20-30 and 30-50 cm depths, respectively, for further determination of soil total carbon (TC), total nitrogen (TN) and total phosphorus (TP) concentration.

Soil OC was estimated as 50 % of loss on ignition weight loss (550 °C, 4 hours, 1 g soil; Loder et al., 2023). Soil TC and TN concentration (%) was determined by the dry combustion method (ignited at 1000 °C) on a CHN-analyzer (EA 1112, Carlo Erba, Milan, Italy). Soil TP concentration (%) was determined colorimetrically using the Malachite Green method (Ohno and Zibilske 1991) after hot acid digestion at 360 °C in a H_2SO_4-H_2O_2 mixture described in Akinremi et al. (2003).

Our samples in BCW (n=162) had an average OC/TC ratio ~1, indicating no significant presence of inorganic carbon (e.g., $CaCO_3$). OC was used for data analysis to maintain the consistency with our Alberta data where OC concentration was determined.

5.3.3 Dataset of Alberta wetlands

From the University of Toronto, a data set containing the OC, TN, TP concentration of 30 prairie pothole wetland in the centre area sampled in June 2016 in Camrose County (see detailed wetland GPS in Supplementary Data S5.1) was obtained. In the dataset, 3 wetlands were intact (no land conversion history), 3 drained (wetland converted to agricultural fields) and 24 restored wetlands in a 2-to-21-year chronosequence (in 2016). Each wetland contained triplicate soil cores. Some background information including soil texture and parent materials was available.

The depth of the sampled cores ranged between 30-150 cm with 57 % between

30-50 cm depth, 23 % between 50-100 cm depth and 20 % between 100-150 cm depth (Fig S5.1). Soil cores were segmented into 1 cm, 2 cm, or 5 cm intervals for chemical analysis based on their final depth. Specifically, soil cores > 100 cm deep were segmented into every 5 cm increments, 30-100 cm deep into every 2 cm increments and 30 cm deep into every 1 cm increment. These raw data were converted to 5 cm increments to facilitate comparisons. Samples in 2 cm increments were combined following 0-2 and 2-4 cm into 0-5 cm and 4-6, 6-8, and 8-10 combined to 5-10 cm. The final depth used for mean and standard error calculation for each wetland correspond with the shortest depth of triplicate cores. Extra depth increments with only 1 observation were removed to avoid abrupt increase or decrease. Soil analysis was completed through 2016 to 2018 at the University of Manitoba.

Soil profiles were sectioned into three layers for comparisons following the practical thickness of soil horizons of Gleysols and Chernozems: 0-30 cm (mainly O and A horizon containing rich organic matter, plant roots and nutrients, and are directly affected by cultivation), 30-100 cm (mainly B and C horizons that are partially weathered and indirectly affected by cultivation), and 100-150 cm (mainly C and R horizon that were composed of parent materials).

5.3.4 Data analysis

In the Manitoba dataset (n=162), we used one-way analysis of variance (ANOVA) followed by Tukey HSD comparisons to determine the significant variance in soil C, N, P concentration and C:N, C:P, N:P ratios across different wetlands (1 intact cropland- surrounded, 1 intact grassland-surrounded, and 1 restored) and different topographic positions (center, midslope, and upland) and depth increments within each wetland. In each ANOVA test, residuals' normality and homoscedasticity were confirmed by quantile-quantile plot, Shapiro's and Levene's tests, respectively.

The coefficient of variance (CV, %) of C, N, P concentration and stoichiometric ratios at each depth increment was calculated by the ratio of standard deviation and mean.

In the Alberta dataset (n=1150), due to the inconsistent distributions of variables (negatively skewed, positively skewed and normally distributed) in the total dataset and multiple subsets divided by wetland classes and depth segments, we used the 5th, 50th (median) and 95th percentiles and arithmetic mean (mean) and standard error (S.E.) to describe variable distributions. All comparison analyses (among wetland classes, restoration age groups, and depth segments) were conducted through nonparametric Kruskal-Wallis rank sum test following Dunn's multiple comparisons tests (Bonferroni-corrected). We maintained the raw values of C, N, P concentration and stoichiometric ratios through analysis rather than logarithmically transformed them for better comparing our numbers with other studies.

The percentiles of variables were determined by the quantile function in 'stats' package. Residuals' skewness, normality and homoscedasticity were tested by histogram plots, Shapiro's and Levene's tests, respectively. Possible relationships between C, N, P concentration (logarithmically transformed) and restoration age in 0-30 cm soils were examined through least-squared linear regression models with residuals' normality, homoscedasticity, and no autocorrelation validated. We regard significance at 95% confidence level consistently.

5.4 Results

5.4.1 Sediment C, N, P concentration of Manitoba wetlands

There was substantial difference in sediment C and N concentration between the two intact wetlands (*p*<0.05, ANOVA) (Fig 5.1). The cropland-surrounded intact wetland (BCW-IC) contained 1.5-3 times C and N (both in concentration (%) and in density (kg m^{-3})) in the centre and midslope zones of that in the grassland-surrounded intact wetland (BCW-IG). Soil C, N, P concentration of the restored wetland BCW-R10G was more similar to the BCW-IG than BCW-IC (Fig.1), likely due to their same surrounding land uses.

On average, C and N concentration was consistently higher in the centre and midslope zones than upland zones within all three wetlands, while P concentration showed no significant zonal differences along the center-midslope-upland transect and were highly constrained to 0.04-0.05 % at all locations (Fig 5.1; Table S5.1).

Vertical distribution of C, N, P differed across landscape positions for all three wetlands. In midslope profiles, C, N and P were consistently concentrated in top 0-10 cm, whereas in centre profiles, highest C, N and P concentration appeared at both surface 0-10 and at deeper layers (20-50 cm) (Fig 5.1), achieving generally thicker C and N rich layers at the waterlogged centre than the drier midslope and upland positions.

The vertical distributions of N, P in restored wetland was distinct from two intact wetlands (Fig 5.1). In two intact wetlands, C. N, P were more concentrated in the top 0-20 cm than in 20-50 cm ($p<0.01$-0.1, ANOVA). Restored wetland, however, often showed higher N and P concentration in 20-50 cm than in 0-20 cm (Fig 5.1), although its C distribution followed similar pattern as two intact wetlands. Moreover, in 20-30 cm, the bulk density of restored wetland at midslope and upland increased and thus N and P density also increased (Fig. S5.2).

5.4.2 Sediment C, N, P stoichiometry of Manitoba wetlands

Two intact wetlands differed significantly in their C:N, C:P and N:P ratios ($p<0.05$, ANOVA) (Fig 5.2). BCW-IC had markedly lower C:N ratio (averaged 10) and higher C:P, N:P ratios (averaged 214 and 26) than BCW-IG where C:N, C:P and N:P ratios averaged 16, 138, 10, respectively. The C:N, C:P and N:P ratios of BCW-R10G was intermediate between the two intact ones, averaged 14, 159 and 12, respectively (Table S5.2).

The C:N ratio was generally more constrained than the C:P and N:P ratios over depth in three wetlands (Fig 5.2), particularly in the 0-20 cm. However, below 20 cm depth where mineral matter increased (Pennock et al., 2011), C:N ratio changed inconsistently among wetlands and across landscape positions (Fig 5.2). A general decrease of C:N was observed in BCW-R10 because of both the decrease of C and the increase of N. Most profiles of two intact wetlands showed an increase in C:N below 20 cm, indicating a greater loss of C than N in deeper sediments.

The C:P and N:P ratio of three wetlands mostly declined with depth in profiles, suggesting a prevailingly greater reduction in C and N over depth than P (Fig.2), except for the N:P ratios of BCR-R10G which consistently increased in 20-50 cm among three landscape positions, suggesting a greater abundance of N over P in the deeper layers, despite that N and P concentration both increased in 20-50 cm (Fig 5.1, 5.2).

The C:N, C:P and N:P ratios and vertical profile characteristics mostly remain similar across centre, midslope and upland positions in two out of three wetlands (BCW-IG and BCW-R10G). However, in BCW-IC, centre and midslope positions showed significantly higher C:P and N:P ratios than upland (Fig 5.2; Table S5.2), largely due to the exceptionally rich C and N.

5.4.3 Sediment C, N, P concentration of Alberta wetlands

Sediment C, N, P concentration of centre sediment profiles differed significantly among intact, drained and restored marshes in Alberta ($p<0.001$; Kruskal-Wallis test; Table S5.3) but their orders were inconsistent across depth increments, except that intact wetlands contained consistently the highest C and N concentration throughout the 0-150 cm profile (Fig 5.3; median of 15.2, 5.9 and 2.8 % C and 1.3, 0.4, 0.2 % N at 0-30, 30-100, 100-150 cm depth increments, respectively). In 0-5 cm, restored marshes showed overall higher C and N concentration than drained wetlands (Fig 5.3). However, drained wetlands had significantly higher C and N concentration than restored wetlands in deeper depths of the surface soils (10-30 cm; $p<0.05$). In subsoils (30-100 cm), restored wetlands contained overall higher C and N concentration than drained wetlands (Fig 5.3; Table S5.4; $p<0.05$). There was no significant difference in C and N concentration below 100 cm between drained and restored wetlands. On average, drained and restored wetlands contained 40-46 % and 44-46 % of the C and N concentration of the intact benchmarks, respectively, throughout the 1 m profile (calculated from averaged percentages of both median and mean in two depth segments (0-30 cm and 30-100 cm)).

Restored wetlands contained the highest P concentration in the uppermost 0-5 cm and most depths within 0-150 cm (median of 0.086, 0.049 and 0.065% P at 0-30, 30-100, 100-150 cm depth increments, respectively), followed by intact wetlands (median of 0.086, 0.040 and 0.041 % P at 0-30, 30-100, 100-150 cm, respectively), and then drained wetlands (median of 0.073, 0.039 and 0.110 % P at 0-30, 30-100, 100-110 cm, respectively). On average, P concentration of drained and restored wetlands was 92 and 112 % of the intact benchmarks in 1 m profile.

We see mostly no significant differences in sediment C, N, P concentration among restored wetlands under varying restoration periods (Fig 5.3; Table S5.3), except for the significantly lower C, N and P concentration in 0-30 cm (3.7 % C, 0.3 % N and 0.061 % P; medians) than other restored groups (5.8-6.5 % C and 0.5-0.6 % N, 0.086 to 0.098 % P; medians; $p<0.001-0.05$) and significantly higher P concentration in 6-10 yr restored wetlands than older restored wetlands (11-15 and 16-23 yr) in 30-100 cm depth (Fig. 5.3, Fig S5.3, Table S5.4). There was no significant relationship between soil C (or N, P) concentration (logarithmically transformed) and restoration age at 0-5 cm or 0-10 cm soils where new sediments in most recent 30 years were assumed to deposit (sedimentation rates of 0.2-0.6 cm/yr in Prairie Pothole Region (Ross and McKenna, 2023)) ($p=0.1-0.7$, $R^2<0.02$). These findings suggest the restoration period was not a key determinant of soil C, N, P concentration in surface restored soils.

We found distinct C and N vertical distribution between intact, drained and restored wetlands while less variable P vertical distribution among three wetland classes (Fig 5.3, Fig S5.3). In intact wetlands, soil C and N were most concentrated in the surface 0-30 cm, although this rich-C and N layer (>10 % C and >1 % N) expanded to varied depths (10-50 cm) among individual intact marshes (Fig S5.3). In drained wetlands, C and N were most concentrated in the middle layer and showed strong pulses at the depths of 20-30 cm and/or 40-50 cm (Fig S5.3) with no simultaneous increases of P. C and N profiles of restored wetlands shared some similarities with drained wetlands, most obvious for the 1-5 yr restored wetlands which also showed pulses of C and N at 20-30 cm depth. These abrupt increases of C and N also occurred in older restored marshes (6-23 years), but to less magnitude than drained wetlands and more randomly distributed throughout the 20-70 cm profiles (Fig S5.3). In the 0-20 cm depth, however, C and N profiles of restored wetlands deviated from the drained pattern and decreased sharply with depth (e.g., from 9 % to 1 % C in a 14-yr restored wetland, Wetland ID: FER1), rather than remained unchanged in the drained wetlands.

Regardless of the different land managements, soil P concentration was always the highest at the uppermost layer (0-5 cm) and declined with depth, although some random rich-P layers occurred in restored wetlands throughout the 10-70 cm depth (Fig 5.3, Fig S5.3). In deeper mineral layers (80-150 cm), P concentration of all wetlands increased continuously with depth, indicating a rather rich P source originated from the deposits of the glacial tills.

5.4.4 Sediment C, N, P stoichiometry of Alberta wetlands

The C:N ratio remained highly constrained across depth and across wetland classes (intact, drained and restored), ranged from 10-20 consistently (5th and 95th percentiles) (Fig 5.4; Table S5.4). However, C:P and N:P ratios were markedly lower in restored and drained wetlands than intact wetlands of which C:P ranged from 29 to 261 and N:P ranged from 1 to 20 (5th to 95th percentiles), mainly ascribed to the low C and N concentration (Fig 5.4, Fig S5.4). On average, the C:P and N:P ratios of drained and restored wetlands were 51 and 35 %, 51 and 36 % of the intact benchmarks, respectively, throughout the 1 m profile (Table S5.4).

There was no significant difference in any soil stoichiometric features among restored marshes under differed restoration ages, except that C:P ratio was substantially lower in 30-100 cm of the 6-10 yr restored wetlands than others due to its high P concentration (Fig 5.4, Fig S5.4, Table S5.4).

The C:P and N:P profiles showed distinct patterns between intact, drained and restored classes (Fig 5.4). In intact wetlands, C:P and N:P either increased with depth within 0-30 cm below which continuously declined or consistently declined throughout the 0-50 cm profile (Fig S5.4). The increase of C:P and N:P ratios in intact marshes were ascribed to the strong retention of C and N over P in the organic-rich surface layer (Fig S5.3). Drained and restored wetlands generally showed decreased C:P and N:P ratios with depth because P decreased to less magnitude than C and N, opposite to the intact wetlands (Fig 5.4, Fig S5.4). Abrupt increases of C:P and N:P ratios occurred throughout the 20-70 cm depth of drained and restored wetlands, mainly resulted from the elevated C and N concentration, rather than reduction of P. For instance, the C:P and N:P of drained wetlands was notably higher at 20-30 cm because of the remarkably high C and N concentration (Fig S5.3).

5.5 Discussion

5.5.1 Impact of cultivation on wetland soil C, N, P concentration and stoichiometry

Drainage and cultivation fundamentally alter wetland soil properties (e.g., bulk density, the concentration and forms of C, N, P, moisture, pH, aggregates content and sizes) and often reduce the total C and N storage, although they can elevate the labile fractions of C, N, P which facilitates plant accessibility (Cui et al., 2014; Brown et al., 2017; Badiou, et al., 2018). In agreement with the previous studies, our findings proved substantially lowered soil C and N concentration in drained and cultivated soils compared to intact soils while only slightly lowered P concentration. Badiou et al. (2018) also reported slightly reduced total P concentrations in drained marshes (0.076%) than the adjacent intact marshes (0.085 %) in the Canadian PPR, and the extractable phosphate concentration increased, but the P sorption capacity decreased. The continued supply of phosphate fertilizer could maintain the relatively rich P in surface soils and often decreased with depth within the 0-50 cm profile (Fig 5.3, Fig S5.3), in accordance with the declined P in the Chernozem profiles of German croplands (Gocke et al., 2021).

We noticed sharp increases of C and N in 20-30 and 40-50 cm depths of drained wetlands. This disproportionately rich C and N at the rooting depth likely resulted from the legume crops (e.g., field pea) fixing atmospheric N into the rhizomes (Guo et al., 2023) or the excessive supply of N fertilizers (e.g., nitrate) being leached downward through strong vertical water fluxes. It's been measured that the vertical hydraulic conductivity of pothole marshes can exceeds 1 m depth yr^{-1} within the 4-6 m depth (Hayashi et al., 2016; Schlaffer et al., 2022). The major forms of C, N and P in the leachate in wetlands often involves dissolved organic C, and organic and inorganic N and P, derived from the soluble fertilizers (e.g., nitrate and phosphate), plant roots, dissolved organic compounds that desorbed from the degraded organic matter (Kalbitz et al., 2000; Qualls and Richardson, 2008; Reddy et al., 2010).

Land cultivation and long-term fertilizer application could also deviate the C, N and P balances in wetland ecosystems (Noe et al., 2001, 2005, 2007). Intensified N fertilization and N deposition could decrease the soil C:N ratio, although still within the 5-25 range (Sardans et al., 2012; Deng et al., 2020; Schneider et al., 2020). The C:P ratio always declined after drainage due to the great loss of C in wetland soils (Badiou et al., 2018). Both decrease and increase in soil N:P ratios after drainage were noticed but most studies revealed a decline in soil N:P ratios (Wang et al., 2014, Lou et al., 2017). A global synthesis also concluded, on average, a reduction in soil N:P ratio when converting forests to croplands (Kim et al., 2023). The N:P ratio of croplands can be governed by the type of fertilizer (organic vs. inorganic), the application rates of N and P, the nitrification potential and the soil types (organic vs. mineral) (Deng et al., 2020; Gocke et al., 2021). Our study areas in PPR have mineral soils and strong nitrification potentials and showed on average ~50 % the soil N:P ratio of the intact benchmark. The low N:P ratio of restored marshes suggests a P-rich condition (N:P ratio <10) after restoration.

5.5.2 Impact of restoration on wetland soil C, N, P concentration and stoichiometry

When the drained croplands are rewetted and restored back to wetlands, enhanced water volume, fluctuations, leaching, and altered vegetation composition, biogeochemical processes (e.g., nitrification, P desorption and sorption) are likely happening (Reddy et al., 2010). To date, the C, N, P transformations over the restoration period in mineral marshes and their developing trajectories have not been clearly identified. Our study used a space-replacing-time approach to detect the potential temporal patterns in the C, N, P concentration and C:N, C:P and N:P stoichiometric features over two decades of restoration. We further involved the adjacent drained wetlands as reference of the original state where restored marshes evolved from and the intact reference where these restored marshes were expected to approach. Ideally, restored wetland characteristics should fall in between the two reference states and approach to the intact conditions with increasing ages, as observed for the successful C recovery in many studies (Yu et al., 2017; Creed et al., 2022; Yang et al., 2020).

In this study, we found on average higher C, N, P concentrations in restored marshes than drained wetlands in the uppermost 0-5 cm where the major C, N, P components were the plant residues and microbial biomass, suggesting some successful adaptation of aquatic plants and microbes (Reddy et al., 2010). However, below the upper 0-5 cm depth, restored marshes mostly showed lower C and N than drained and intact wetlands while higher P and then lower N:P and C:P ratios (Fig 5.3). This suggests that rewetting and restoration might further diminish soil C and N in the middle and bottom layer of surface soils through gaseous emissions or leaching, but successfully retained P, likely through chemical sorption (Reddy et al., 2010). This indicates a P recovery trajectory decoupling with C and N. In several marshes, we found the C, N, P could be leached downwards and showed random increases in the subsoils (Fig S5.3).

In mineral freshwater marshes, the P dynamics can be fundamentally distinct from C and N due to the lack of gaseous phase as well as the rich mineral matter and cations which involves strong sorption and desorption processes on top of the biotic microbial behaviors (e.g., nitrification, phosphatase activity). The deviated P patterns with C and N in two intact wetlands seems to further validate this (Fig S5.3). In waterlogged soils, the amorphous forms of Al and Fe provide more surface area for P adsorption than their crystalline forms, which will increase P sorption capacity (Navaratnam, 2013), despite that noticeable P release from Fe-P into soluble phosphate could happen as a result of rewetting (Aldous et al. 2007). Moreover, P could be adsorbed to calcium cation forming calcium-phosphates (Ca-P), which is the predominant exchangeable cation in our study region (Pennock et al., 2011; Badiou et al., 2018).

5.5.3 Restored soil transformations over time

The restored marshes regardless of restoration period were generally undistinguished in their C, N, P concentration and stoichiometric features (Table S5.4). In the new sediments (at 0-5 cm, 0-10 and 0-20 cm samples), we did not see any relationship between C, N, P with the restoration ages. Moreover, the occurrence, depth, extent and number of the sharp increases of C, N, P in the restored profiles seems to be highly random and unique for individual marshes, at least not following temporal trends (e.g., more increases in longer restored marshes). Our findings agreed with the view of Loder and Finkelstein (2020) and Loder et al. (2023) that the C status of restored marshes could be highly unique for individual marshes which can be determined by inherent characteristics of ponds such as sizes, slopes, water levels, hydroperiod, and the speed of revegetation (Loder et al., 2023).

5.5.4 Consistency in C, N, P concentration and stoichiometry among wetland topographic locations

Surrounding wet meadows and upland might also benefit from restoration through the end of harvesting and re-vegetation of grasses and forbs (Reddy et al., 2010). In this study, we found high spatial heterogeneity in C and N concentration and storage but not P upon restoration in BCW-R10G, largely due to the differed hydrological conditions and associated oxygen availability, leaching intensity among center, midslope and upland positions. Mideslope and upland soils are drier than centre, having greater biomass production, but faster decomposition and higher nitrification rates (Tangen et al., 2015). The strong nitrification and mineralization potential in upland soils may favor the C and N gaseous emissions when receiving allochthonous dissolved organic and inorganic N, rather than being retained in the system.

5.6 Acknowledgements

We thank Zeinab, Dr. David Lobb, and Anthony for completing the laboratory work and sharing the data for the Camrose County samples. We are also grateful for Dr. Irena Creed, Eric and Jackie for project organization, and Hicham Benslim, Paula Kestelman, Mike Dalva and Calder Jones for help in laboratory work. This study was funded by Natural Sciences and Engineering Research Council of Canada (NSERC STPGP 506809).

5.7 Tables and figures

Table 5.1 Descriptions of two wetland study sites within the agricultural-intensive Canadian Prairie Pothole Region.

Site	Province	MAT (°C) MAP (mm)[1]	Soil type[2]	Bedrock[2]	Soil texture	pH[3]	Wetland type	Dominant crops[4]
Broughton's Creek Watershed	Manitoba	3.2 °C, 400 mm	Black chernozemis, Gleysols	Limestone, granite, shale	Clay loam	7-8	Depressional and prairie	Barley and wheat
Camrose County	Alberta	2.9 °C, 426 mm	Black chernozemis, Gleysols	Sandstone, mudstone, siltstone, coal	Loam	5.5-8	Depressional and prairie	Canola, wheat, barley, field pea

[1] MAT and MAP refer to mean annual temperature and precipitation, respectively; data derived from 1981-2010 Environment Canada, weather station: Brandon and Camrose.

[2] soil type and bedrock sources: Pennock et al. (2011). Agricultural and agri-food Canada (2015).

[3] pH sources: Badiou et al. (2018); Bowser et al. (1962); Clare et al. (2021).

[4] dominant crops sources: Badiou et al. (2018); Economic Development Camrose (2024).

Figure 5.1 Organic carbon (OC), total nitrogen (TN) and phosphorus (TP) concentration of three Manitoba wetlands in 0-50 cm depth at the centre, midslope, and upland positions. Asterisk (*) denotes significant difference in C, N, P concentration at the corresponding depth among three wetlands (*, *p*<0.05; **, *p*<0.01; 'ns' denotes no significant differences). BCW-IC, BCW-IG, and BCW-R10G refer to intact wetland (cropland-surrounded), intact wetland (grassland-surrounded), and 10-year restored wetland (grassland-surrounded), respectively, in Broughton's Creek Watershed (BCW), Manitoba. Error bars at depth denote standard error.

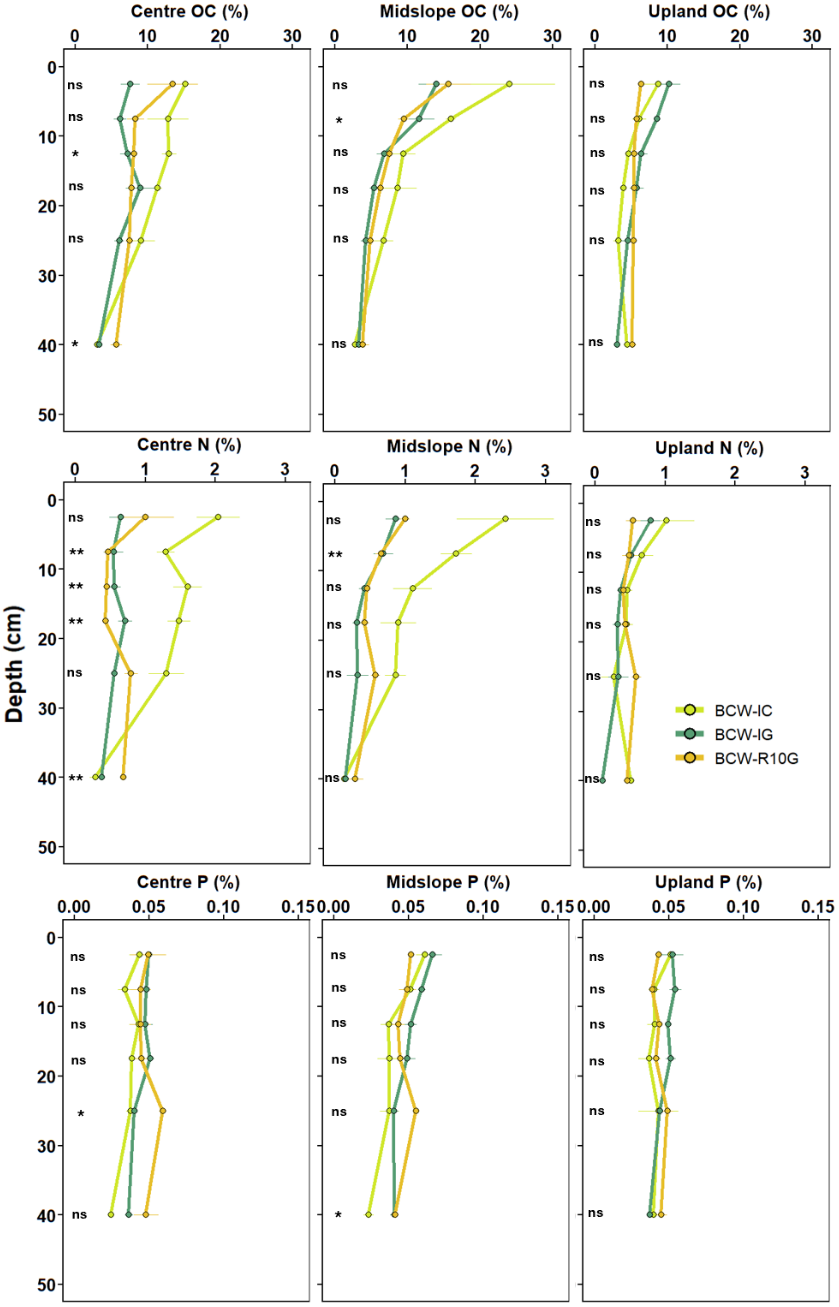


Figure 5.2 Stoichiometric ratios (mass-based) of three Manitoba wetlands in 0-50 cm depth at the centre, midslope, and upland positions. Asterisk (*) denotes significant difference in C:N, C:P and N:P ratios at the corresponding depth among three wetlands (*, $p<0.05$; **, $p<0.01$; 'ns' denotes no significant differences). BCW-IC, BCW-IG, and BCW-R10G refer to intact wetland (cropland-surrounded), intact wetland (grassland-surrounded), and 10-year restored wetland (grassland-surrounded), respectively, in Broughton's Creek Watershed (BCW), Manitoba. Error bars at depth denote standard error.

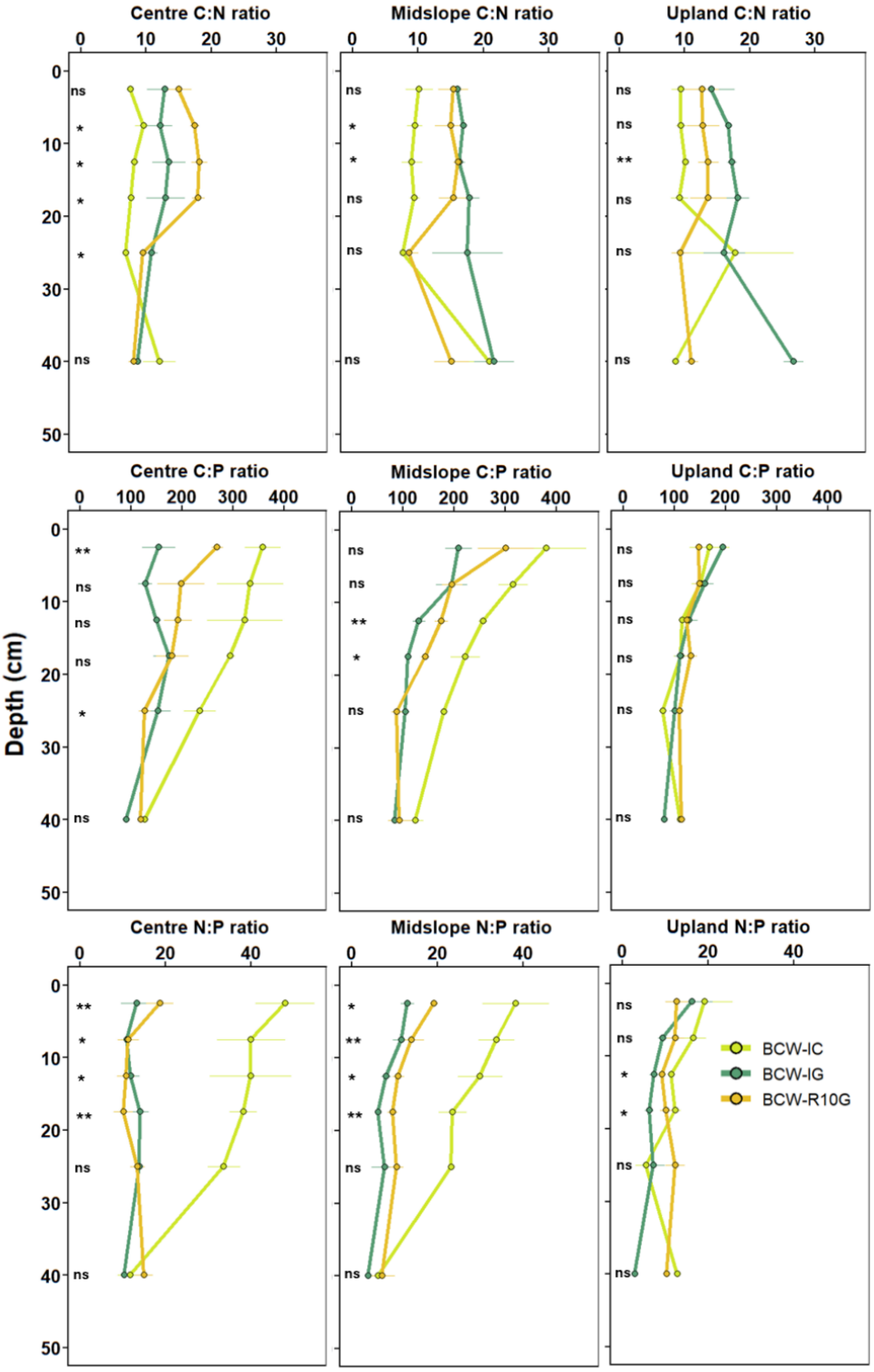


Figure 5.3 Average soil organic carbon (OC), total nitrogen (TN) and phosphorus (TP) concentration (%) in centre location of thirty Alberta wetlands in 0-150 cm depth grouped by different land uses and restored periods. Grey lines are raw C, N, P concentration profiles of independent replicates from each wetland. 'Mean' indicates the average ± standard error of the variables within each depth segment. Error bars denote standard error. *Note:* The sampling depth of individual wetlands varied with intact wetlands (n=3) sampled to 45, 145, 150 cm; drained wetlands (n=3) sampled to 60, 100, 115 cm; Restored 1-5 yr (n=6) sampled to 35, 40, 45, 45, 45, 150 cm; Restored 6-10 yr (n=7) sampled to 30, 35, 40, 50, 60, 70, 130 cm; Restored 11-15 yr (n=5) sampled to 30, 35, 40, 75, 90cm; Restored 16-23 yr (n=6) sampled to 30, 30, 35, 45, 65, 150 cm, respectively.

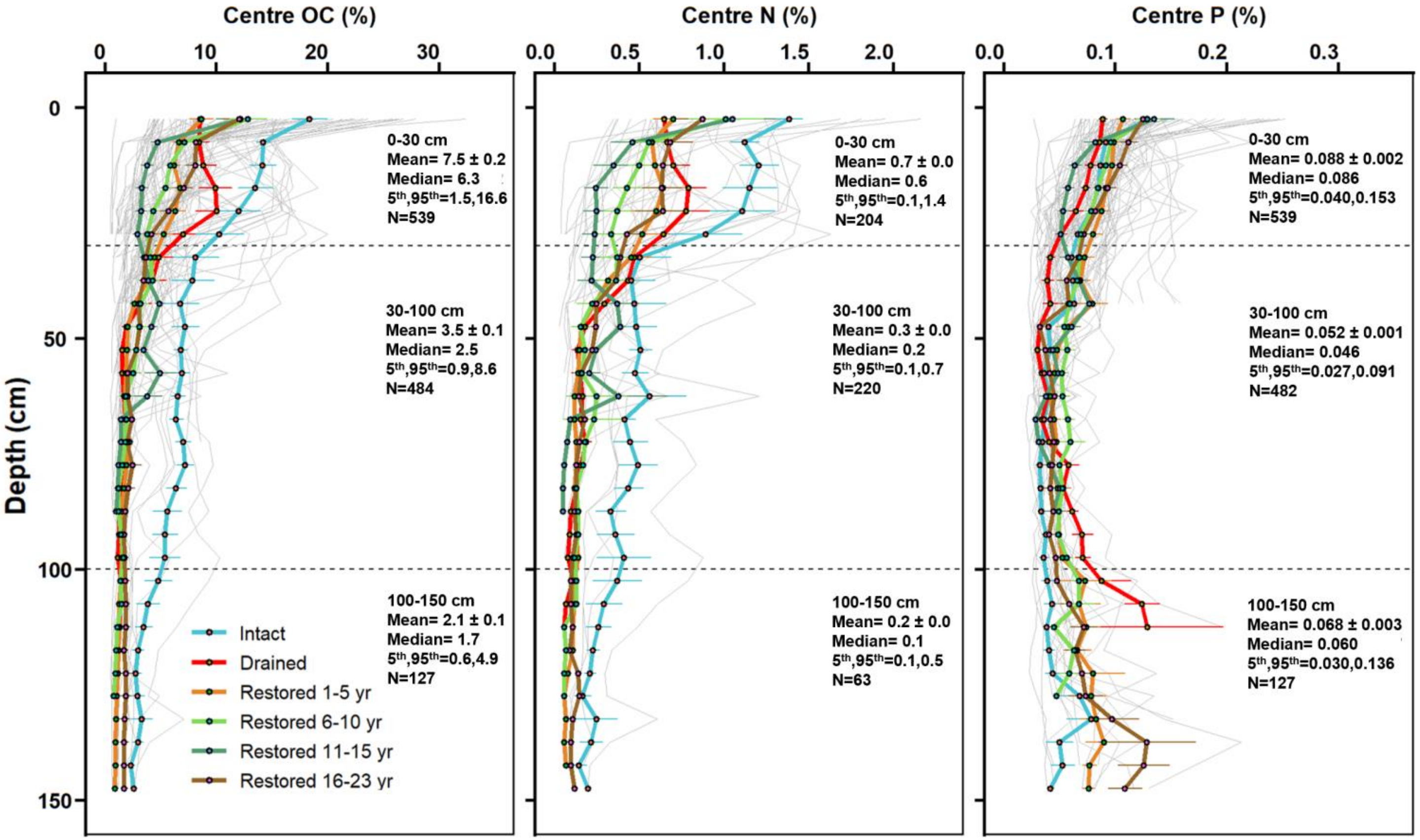
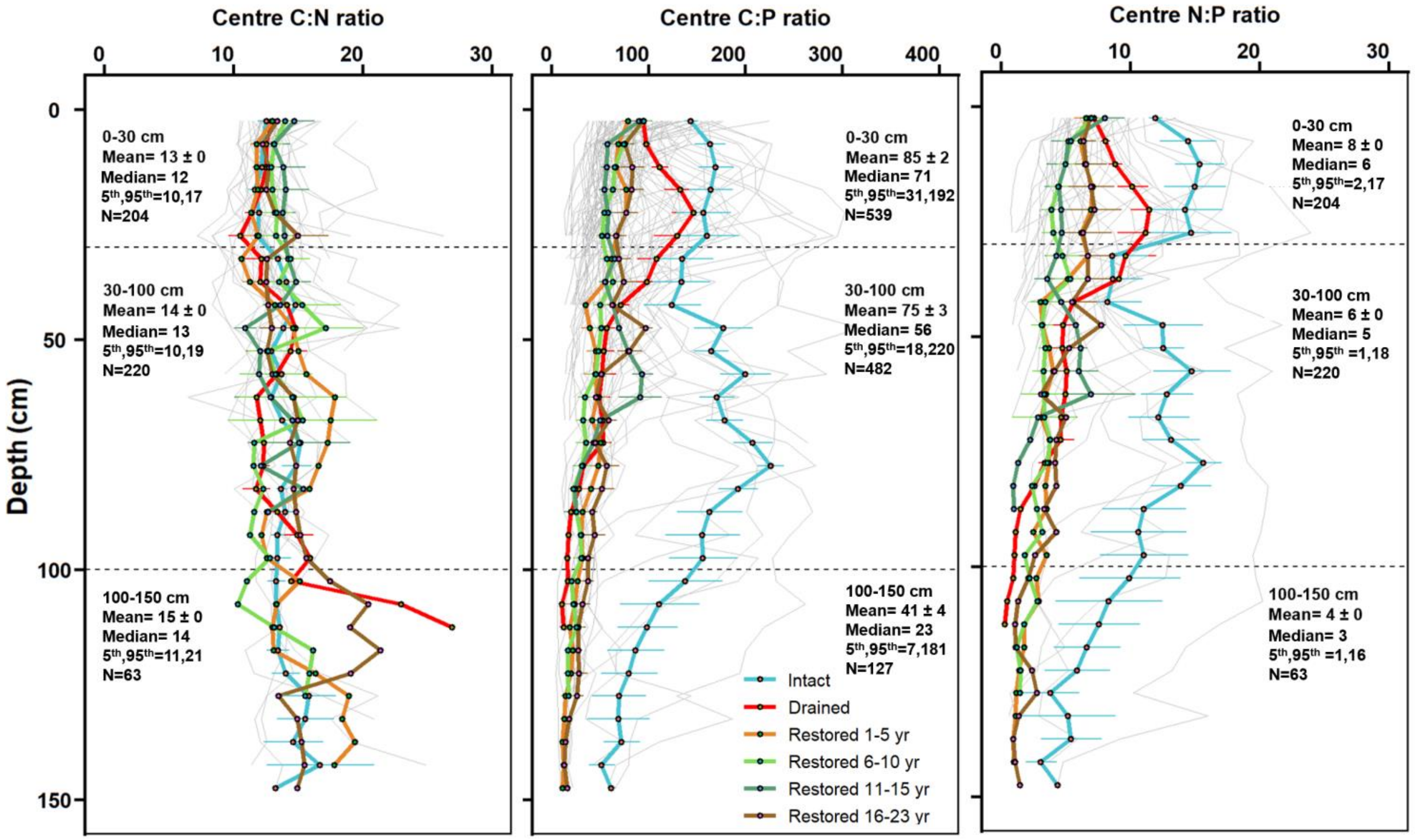


Figure 5.4 Average soil stoichiometric ratios (mass-based) in centre location of thirty Alberta wetlands in 0-150 cm depth grouped by different land uses and restored periods. C:N, C:P and N:P ratios were derived from organic carbon, total N and P concentration. Grey lines are raw C:N, C:P, and N:P profiles of independent replicates from each wetland. 'Mean' indicates the average ± standard error of the variables within each depth segment. Error bars denote standard error. *Note:* The sampling depth of individual wetlands varied with intact wetlands (n=3) sampled to 45, 145, 150 cm; drained wetlands (n=3) sampled to 60, 100, 115 cm; Restored 1-5 yr (n=6) sampled to 35, 40, 45, 45, 45, 150 cm; Restored 6-10 yr (n=7) sampled to 30, 35, 40, 50, 60, 70, 130 cm; Restored 11-15 yr (n=5) sampled to 30, 35, 40, 75, 90cm; Restored 16-23 yr (n=6) sampled to 30, 30, 35, 45, 65, 150 cm, respectively.



5.8 Supplementary materials

Data S5.1 Detailed sampling information on thirty wetlands in Alberta.

Figure S5.1 Frequency histogram of the sampling depth of Alberta wetlands (n=30).

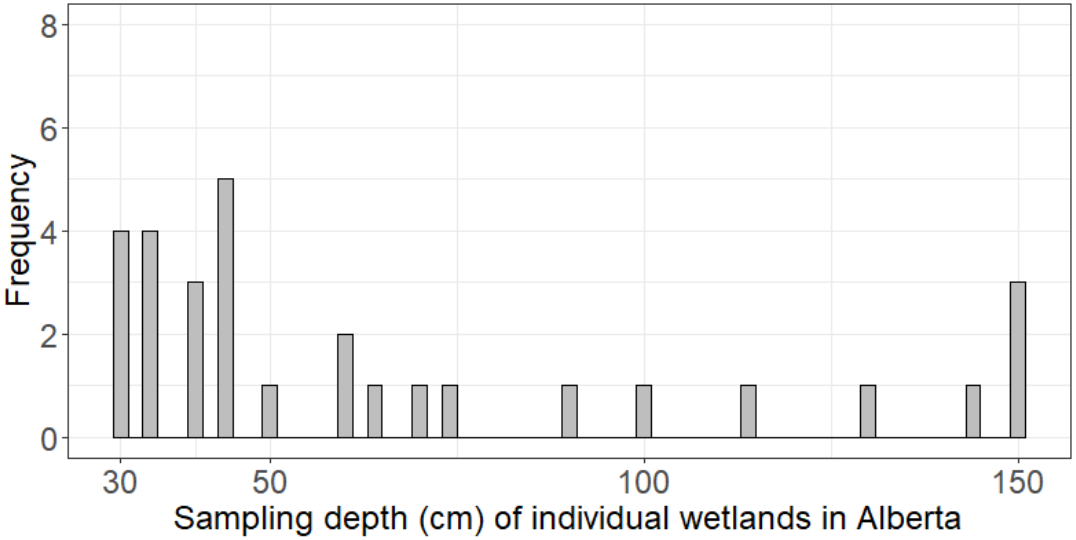


Figure S5.2 Soil bulk density (BD, g cm^{-3}) and the density of organic carbon (OC), total nitrogen (TN), and total phosphorus (TP) (kg m^{-3}) in 0-30 cm profiles at midslope and upland of three Manitoba wetlands. BCW-IC, BCW-IG, BCW-R10G refer to intact (cropland-surrounded), intact (grassland-surrounded) and 10-year restored (grassland-surrounded) wetlands in Broughton's Creek Watershed (BCW). Error bars represent standard errors. 'N' and 'Mean' indicate the number of observations and the average ± standard error of variables in 0-30 cm at each landscape position.

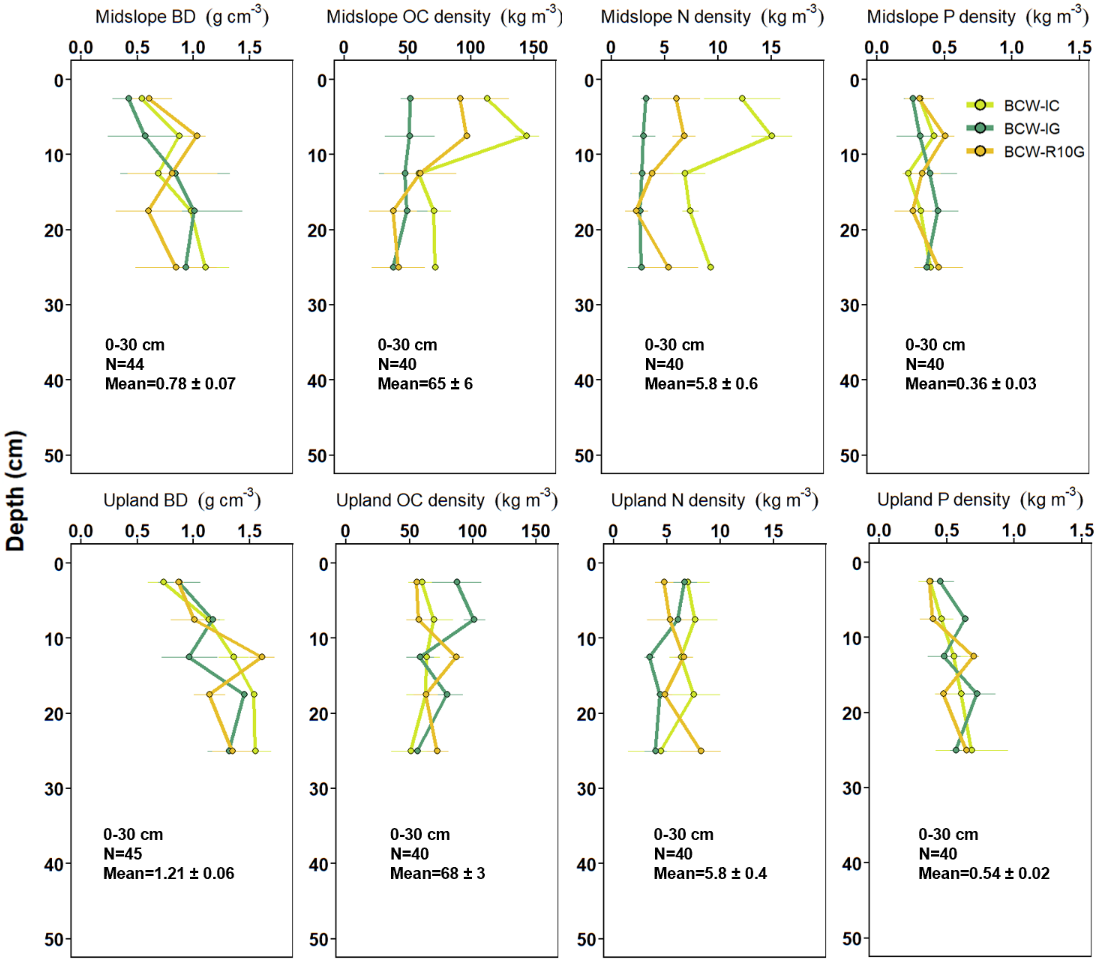


Figure S5.3 Soil organic carbon (OC), total nitrogen (TN), and total phosphorus (TP) concentration of thirty individual wetlands over depth in Alberta across different land managements (drained *versus* restored *versus* intact) and restoration periods (1-5, 6-10, 11-15 and 16-23 years). Values in each profile were averaged from 2 or 3 independent replicates from one wetland.

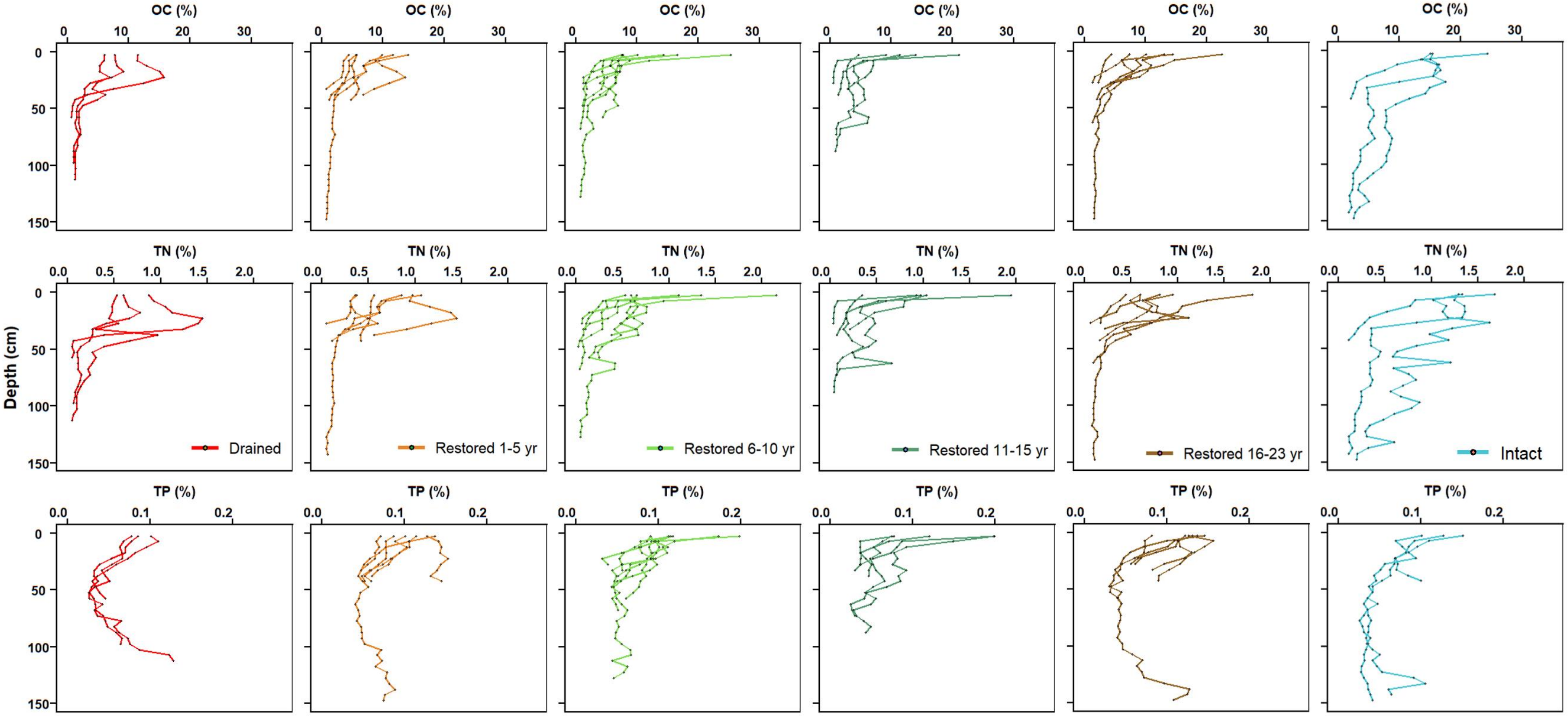


Figure S5.4 Soil stoichiometric C:N, C:P, N:P ratios (mass-based) of thirty individual wetlands over depth in Alberta across different land managements (drained *versus* restored *versus* intact) and restoration periods (1-5, 6-10, 11-15 and 16-23 years). Values in each profile were averaged from 2 or 3 independent replicates.

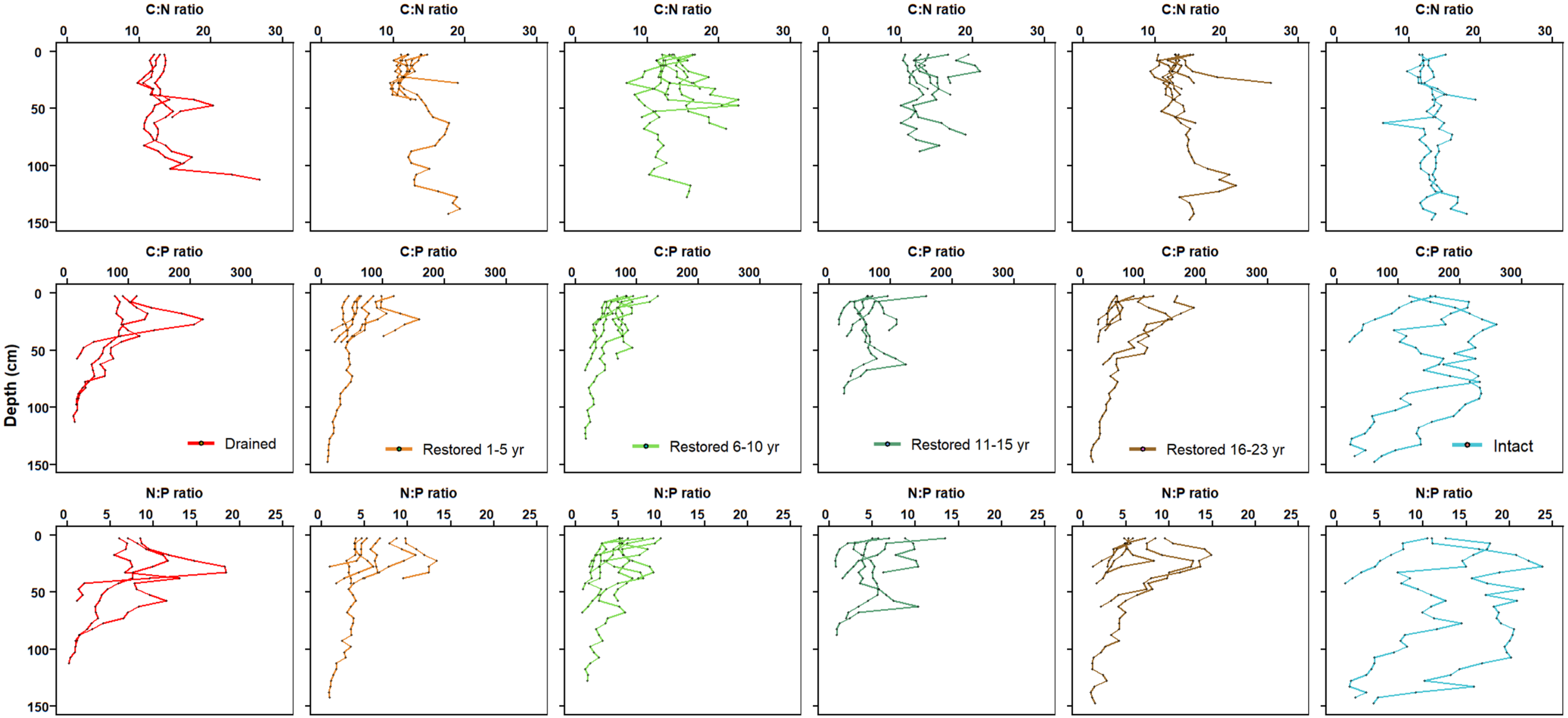


Table S5.1 One-way analysis of variance (ANOVA) results for soil organic carbon (OC), total nitrogen (TN), total phosphorus (TP) concentration and their stoichiometric ratios among wetlands, topographic positions, and depth increments in three Manitoba wetlands (significant at $\alpha = 0.05$). Significant *p* values are in bold.

Province	Factor	df	OC (%)		TN (%)		TP (%)		C:N		C:P		N:P	
			F	p	F	p	F	p	F	p	F	p	F	p
Manitoa	Wetland	2	2.4	0.09	22.0	**<0.001**	9.2	**<0.001**	22.9	**<0.001**	12.8	**<0.001**	39.9	**<0.001**
	Topography	2	8.8	**<0.001**	6.6	**<0.001**	1.7	0.2	3.7	**<0.05**	11.7	**<0.001**	8.9	**<0.001**
	Depth	5	19.5	**<0.001**	7.8	**<0.001**	7.2	**<0.001**	0.9	0.5	13.0	**<0.001**	4.1	**<0.01**

df: degree of freedom.

Table S5.2 Sediment organic carbon (OC), total nitrogen (TN), total phosphorus (TP) concentration and stoichiometric ratios, bulk density and OC, TN, TP density (mean ± standard error) at three topographic positions and across all positions of wetlands in Broughton's Creek Watershed (BCW), Manitoba. BCW-IC, BCW-IG, BCW-R10G refer to intact (surrounded by cropland), intact (surrounded by grassland), and 10-year restored (surrounded by grassland) wetlands.

Wetland	Position	OC (%)	TN (%)	TP (%)	C:N	C:P	N:P	Bulk density in 0-30 cm (g cm^{-3})	C density in 0-30 cm (kg m^{-3})	N density in 0-30 cm (kg m^{-3})	P density in 0-30 cm (kg m^{-3})
BCW-IC	centre	10.5±1.1	1.3±0.1	0.037±0.002	9±1	278±26	35±4	NA	NA	NA	NA
	midslope	11.0±2.0	1.3±0.2	0.043±0.003	11±1	235±28	27±3	0.9±0.1	85±10	9.9±1.2	0.36±0.03
	upland	4.9±0.6	0.6±0.1	0.041±0.003	11±1	124±11	14±2	1.3±0.2	62±5	6.6±0.8	0.53±0.06
	all	8.8 ±0.9a	1.1±0.1a	0.040±0.002b	10±1c	214±16a	26±2a	0.8±0.1a	59±5a	6.7±0.7a	0.33±0.04a
BCW-IG	centre	7.0±0.6	0.6±0.0	0.046±0.002	12±1	147±10	13±1	NA	NA	NA	NA
	midslope	7.5±1.1	0.5±0.1	0.051±0.003	18±1	138±13	9±1	0.8±0.1	48±6	2.9±0.4	0.36±0.06
	upland	6.3±0.6	0.4±0.1	0.048±0.002	18±1	129±10	9±1	1.1±0.1	74±6	4.8±0.5	0.56±0.05
	all	6.9±0.5a	0.5±0.0b	0.048±0.001a	16±1a	138±6b	10±1b	0.7±0.1a	48±5a	3.0±0.3b	0.36±0.04a
BCW-R10G	centre	8.5±0.8	0.6±0.1	0.048±0.003	14±1	181±15	13±1	NA	NA	NA	NA
	midslope	7.9±1.1	0.6±0.1	0.048±0.002	14±1	164±20	12±1	0.7±0.1	63±11	5.0±0.9	0.36±0.06
	upland	5.6±0.2	0.5±0.0	0.043±0.001	12±1	130±5	11±1	1.2±0.1	67±4	5.9±0.6	0.52±0.04
	all	7.3±0.5a	0.6±0.0b	0.047±0.001a	14±1b	159±9b	12±1b	0.8±0.1a	54±5a	4.5±0.4b	0.36±0.03a

'NA' denotes no available data. The average OC, TN, TP concentration and their stoichiometric ratios were determined across 0-50 cm depth while average bulk density and C, N, P density were only determined across 0-30 cm depth. Different letters in the same column indicate significant differences in average soil parameters among wetlands (ANOVA and Tukey's HSD comparisons, $p<0.05$).

Table S5.3 Kruskal-Wallis rank sum test results for soil organic carbon (OC), total nitrogen (TN), total phosphorus (TP) concentration and their stoichiometric ratios among different land managements (intact *versus* drained *versus* restored), restoration periods and depth segments in thirty Alberta wetlands (significant at $\alpha = 0.05$). Significant p values are in bold.

Province	Factor	Groups	df	OC (%)		TN (%)		TP (%)		C:N		C:P		N:P	
				H	p	H	p	H	p	H	p	H	p	H	p
Alberta	Land management	Intact vs. drained vs. restored	2	80.18	**<0.001**	20.13	**<0.001**	68.39	**<0.001**	5.34	0.07	198.51	**<0.001**	84.90	**<0.001**
	Restoration period	1-5 vs. 6-10 vs. 11-15 vs.16-23 yr	3	5.37	0.15	4.54	0.21	39.95	**<0.001**	7.21	0.07	8.74	**<0.05**	4.35	0.23
	Depth segment	0-30 vs. 30-100 vs. 100-150 cm	2	349.82	**<0.001**	164.31	**<0.001**	345.69	**<0.001**	43.74	**<0.001**	137.49	**<0.001**	52.89	**<0.001**

df: degree of freedom.

Table S5.4 Sediment organic carbon (OC), total nitrogen (TN), total phosphorus (TP) concentration (%) and stoichiometric ratios (mean ± standard error; 5th, 50th (median) and 95th percentiles in the bracket) and median within different depth ranges at the centre of drained, restored and intact wetlands in Alberta. 'X' refers to the sampling depth of individual wetlands. Upper and lower letters indicate significant difference among three wetland management classes (intact, drained and restored) and among four restored groups (1-5 yr, 6-10 yr, 11-15 yr and 16-23 yr), respectively (Kruskal-Wallis rank sum test, significant at $\alpha = 0.05$). For each wetland, average soil OC, TN, TP concentrations and C:N, C:P, N:P ratios were calculated to the deepest depth with at least 2 replicate cores sampled.

Variable	Depth ranges (cm)	Land management					
		Drained (n=3) X=60, 100, 115 cm	Restored 1-5yr (n=6) X=35, 40, 45, 45, 45, 150 cm	Restored 6-10yr (n=7) X=30, 35, 40, 50, 60, 70, 130 cm	Restored 11-15yr (n=5) X=30, 35, 40, 75, 90 cm	Restored 16-23yr (n=6) X=30, 30, 35, 45, 65, 150 cm	Intact (n=3) X=45, 145, 150 cm
OC (%)	X<30	8.8±0.6 B (2.7,8.0,16.3)	6.6±0.3 a (3.2, 5.8,13.9)	6.6±0.4 a (1.5, 6.0, 17.7)	5.0±0.5 b (0.7, 3.7, 13.7)	7.6±0.4 a (2.2, 6.5,15.3)	13.8±0.7 A (3.7, 15.2, 20.5)
			Restored (n=24): 6.5±0.2 C (1.2, 5.5, 15.0)				
	30<X<100	2.4±0.2 C (0.9,1.7,5.2)	2.9±0.3 a (0.9, 2.2,7.2)	2.9±0.2 a (0.8, 1.9, 7.3)	2.9±0.2 a (0.7, 3.0, 6.2)	2.8±0.1 a (1.4, 2.5, 4.4)	6.8±0.4 A (2.3, 5.9, 14.6)
			Restored (n=24): 3.0±0.1 B (0.9, 2.3, 7.2)				
	X>100	1.4±0.1 (1.0,1.4,1.6)	1.1±0.1 (0.6, 1.2, 1.8)	1.2±0.1 (0.6, 1.1, 1.8)	NA	1.8±0 (1.0, 1.9, 2.6)	3.3±0.3 (1.8, 2.8, 7.0)
			Restored (n=24): 1.4±0.1 (0.6, 1.3, 2.4)				
TN (%)	X<30	0.7±0.1 B (0.4,0.6,1.3)	0.6±0.1 ab (0.3, 0.5, 1.2)	0.5±0.1 ab (0.1, 0.5, 1.1)	0.4±0.1 b (0.0, 0.3, 1.0)	0.7±0.1 a (0.2, 0.6, 1.2)	1.1±0.1 A (0.4, 1.3, 1.6)
			Restored (n=24): 0.6±0.0 C (0.1, 0.5, 1.2)				
	30<X<100	0.2±0.0 B (0.1,0.1,0.6)	0.3±0.0 a (0.1, 0.2, 0.6)	0.2±0.0 a (0.1, 0.2, 0.7)	0.2±0.0 a (0.1, 0.2, 0.5)	0.2±0.0 a (0.1, 0.2, 0.5)	0.5±0.0 A (0.2, 0.4, 1.0)
			Restored (n=24): 0.2±0.0 B (0.1, 0.2, 0.6)				
	X>100	0.1±0.0 (0.1,0.1,0.1)	0.1±0.0 (0.1, 0.1, 0.1)	0.1±0.0 (0.1, 0.1, 0.1)	NA	0.1±0.0 (0.1, 0.1, 0.1)	0.2±0.0 (0.1, 0.2, 0.6)
			Restored (n=24): 0.1±0.0 (0.1, 0.1, 0.1)				
TP (%)	X<30	0.074±0.003 B (0.042, 0.073, 0.106)	0.094±0.003 a (0.056, 0.086,0.150)	0.091±0.003 a (0.043, 0.091,0.140)	0.074±0.005 b (0.030, 0.061, 0.185)	0.098±0.003 a (0.045, 0.098, 0.153)	0.089±0.003 A (0.054, 0.086, 0.142)
			Restored (n=24): 0.090±0.002 A (0.039, 0.086, 0.153)				
	30<X<100	0.044±0.002 B	0.062±0.004 ab	0.059±0.002 a	0.051±0.002 b	0.050±0.003 b	0.044±0.002 B

		(0.024, 0.039, 0.076)	(0.035, 0.052, 0.140)	(0.037, 0.054, 0.091)	(0.026, 0.046, 0.089)	(0.030, 0.043, 0.095)	(0.027,0.040,0.075)
			Restored (n=24): 0.056±0.001 A (0.030, 0.049, 0.096)				
	X>100	0.114±0.021 (0.060, 0.110, 0.180)	0.077±0.005 (0.045, 0.070,0.122)	0.059±0.004 (0.039, 0.060,0.084)	NA	0.085±0.007 (0.049, 0.072, 0.159)	0.050±0.004 (0.026,0.041,0.100)
			Restored (n=24): 0.076±0.004 (0.044, 0.065, 0.132)				
C:N	X<30	12±0 A (9, 12, 14)	12±0 a (10, 12, 14)	14±1 a (10, 13, 17)	14±1 a (11, 13, 20)	13±0 a (10, 13, 16)	12±0 A (11, 12, 13)
			Restored (n=24): 13±0 A (10, 12, 18)				
	30<X<100	13±0 A (10,13,18)	13±1 a (10, 13, 17)	14±1 a (9, 13, 22)	14±1 a (10, 13, 17)	13±0 a (12, 14, 16)	14±0 A (12, 14, 16)
			Restored (n=24): 14±0 A (10, 13, 19)				
	X>100	21±4 (15, 23, 26)	16±1 (13, 16, 19)	14±1 (11, 14, 16)	NA	17±1 (14, 16, 21)	14±1 (12, 13, 21)
			Restored (n=24): 16±1 (11, 16, 20)				
C:P	X<30	119±7 A (70, 108,223)	73±3 a (33, 66, 137)	68±3 a (32, 63, 123)	63±4 a (20, 56, 118)	80±4 a (36, 59, 163)	160±9 A (56, 162, 275)
			Restored (n=24): 71±2 B (28, 61, 145)				
	30<X<100	58±4 B (17, 49,115)	48±3 ab (18, 45,102)	47±3 b (15, 39, 91)	47±3 a (25, 62, 105)	63±4 a (26, 56, 114)	167±8 A (41, 182,263)
			Restored (n=24): 54±2 B (19, 50, 106)				
	X>100	13±2 (9, 12, 19)	18±2 (6, 15, 38)	20±2 (8, 20, 31)	NA	24±2 (11, 22, 46)	86±10 (19, 66, 215)
			Restored (n=24): 21±1 (6, 20, 42)				
N:P	X<30	9±1 (6, 9, 14) B	7±1 a (3, 6, 12)	5±0 a (2, 6, 9)	5±1 a (1, 5, 10)	7±1 a (3, 5, 14)	14±1 A (6, 15, 22)
			Restored (n=24): 6±0 C (2, 5, 13)				
	30<X<100	5±0 (1, 4, 11) B	4±0 a (3, 4, 9)	4±0 a (1, 3, 8)	4±0 a (1, 4, 7)	5±1 a (2, 4, 10)	12±1 A (3, 11, 20)
			Restored (n=24): 4±0 B (1, 4, 9)				
	X>100	1±0 (0, 1, 1)	2±0 (1, 1, 3)	2±0 (1, 2, 3)	NA	2±0 (11, 22, 46)	6±1 (1, 4, 18)
			Restored (n=24): 2±0 (1, 1, 3)				

‘NA’ denotes no available data due to short soil cores. Significant variance was only determined for 0-30 and 30-100 cm soil.

5.9 References

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Chapter 6: Synthesis, conclusions and insights to future research

Compared to northern peatlands and coastal marshes, freshwater marshes are less frequently studied in their C, N, P retention states, mechanisms and the recovery of C, N, P retention through restoration. The freshwater marsh system is distinct from coastal marsh and peatland systems in its pedogenesis, hydrology, water and soil chemistry, and plant communities, thereby requiring independent research to instruct specific restoration practice and land management. Recent studies revealed substantially varied efficacy of the C, N, P retention among restored freshwater marshes, which encourage more research to detect the controlling and limiting factors of marsh ecosystem recovery in terms of its structure, element cycling and biogeochemical functions. In chapter 3, 4 and 5, I have examined the C, N, P dynamics and retention status in the decomposing litter, microbial biomass and enzymatic activities in surface soil and C, N, P vertical profiles in deep sediment between the intact and restored freshwater marshes. In this chapter, I conclude my original findings by chapter and discuss how they collectively advance our knowledge on the C, N, P dynamics and retention in restored freshwater marshes and list what more research is needed in the future.

6.1 Chapter synthesis

In chapter 3, we found no consistent pattern in litter decomposition rate among wetland classes (intact versus restored) nor between different wetland restoration ages. Instead, we found litter quality and wetland characteristics, especially the inundation periods and surrounding land uses, significantly affected the decomposition rates. We further found the nutrient dynamic in litters was determined by both initial litter quality and surrounding human disturbances (e.g., sewage inputs to marshes), and achieved a steady stoichiometric balance for each site at the end of the decomposition experiment. These findings suggest the effectiveness of wetland restoration on slowing decomposition and enhancing nutrient accumulation largely depends on the quality of the input litters and wetland hydrological and nutritional characteristics.

In chapter 4, we observed a strong negative impact of permanent inundation on four hydrolytic enzyme activities and microbial biomass in both intact and restored freshwater marshes, although the lowest MBC only occurred in the permanently inundated plot from the restored wetland. The overall strong relationship between microbial biomass and enzyme activities suggests the reduced microbial biomass might be an important reason for the declined enzyme activities in mineral marshes. We further show that plant species and root characteristics in marshes can lead to variations in microbial biomass and enzyme activities, likely through the rhizosphere effects. In strongly P-limited marshes, plant root-released phosphatase might be an important mechanism for increasing P availability. Beyond hydrology and plant, soil differences (e.g., total C concentration) between the intact and restored marshes did not show a strong impact on microbial biomass and enzyme activities. Our results confirmed the strong hydrological and vegetation zonation effects on the microbial biomass and enzyme activities in natural and restored marshes.

In chapter 5, we showed cultivation and restoration of freshwater marshes not only affect surface soils but also subsoils (likely through vertical leaching), which is rarely noted before. Restored wetlands mostly failed to duplicate the C and N concentration in sediment to the intact references but often contained comparable P concentration to the intact reference. This suggest the restored marshes might be under a more P-rich condition than the intact reference. For both intact and restored marshes, the concentration and final fate of C, N, P in sediment was unique among individual marshes, despite of similar restoration ages and is likely determined by both anthropogenic impacts and wetland intrinsic characteristics (e.g., vegetation coverage, size, hydrodynamics, clay and mineral content).

6.2 Comprehensive discussion

As stated in Chapter 1, in this thesis, I aim to provide some insights to these questions:

1. Does rewetting of wetlands boost C and N and P retention or otherwise enhance the C and N and P losses from the inland marsh system?

My dissertation has assessed the C, N, P retention states in decomposing litters and surface and deep sediments between intact and restored freshwater marshes. Findings from Chapter 3 and 4 overall showed successfully slowed decomposition rates and depressed enzymatic activities in restored marshes when long-term inundation is established. This indicates rewetting drained wetlands is an effective approach to support C, N, P mass retention in the organic litter tissues. It also warns that irregular drying-and-wetting dynamics through restoration practices should be avoided as it could likely accelerate the litter decomposition and mineralization and cause greater C, N, P losses from the system than the permanently inundated conditions. Moreover, the changing climate and frequent extremes weather events also adds risk of occasional drought in wetlands (Eimers et al., 2008; Erwin, 2009) and should be considered when planning wetland restoration and construction programs.

In surface and deep sediments, my findings from Chapter 5 showed in most restored freshwater marshes, C and N concentration remained low (<10 % C and < 1 % N) and failed to resemble the adjacent intact profiles which stored rich C to a deeper depth (e.g., 30-60 cm) in sediment. This is despite the C, N concentration can be similar to the intact reference in the uppermost 0-5 cm where C and N should be mainly composed of decomposing litter tissues and microbial biomass.

These findings from three chapters collectively suggest that the recovery of wetland living organisms (e.g., vegetation biomass, microbial biomass) and biological-dominated processes (e.g., decomposition) in the uppermost soils might be easy and achievable within two decades of rewetting. However, long-term C, N, P burial and the achievement of thick SOM accumulation in sediments might not be achievable in only two decades of restoration. This result generally agrees with many C studies which reported still low soil C concentration and C accumulation rate in restored sediments despite decades of passive or active restoration (Moreno-Mateos et al., 2012; Loder et al., 2023). Long-term continuous accretion of C and N into the sediment further relies on the stabilization of these organic materials (e.g., decomposing litters, plant roots, microbial necromass and products) which involves more physiochemical processes such as particulate absorption, chemical binding, and soil aggregation to help resist the losses from leaching and biodegradation (Lehmann and Keeler, 2015; Paul, 2016). Historical cultivation and crop roots adaption may decrease the soil aggregate sizes and stability (characterized by mean-weight diameter) (Udom and Ogunwole, 2015), modify soil pore sizes and add challenge to the C, N, P stabilization after restoration. The differences in soil physical and chemical properties induced by land conversions such as bulk density, particle size, soil porosity, hydraulic conductivity, pH, cation concentrations, micro-and macro-aggregate contents from the intact conditions are often hard to recovery and less studied but can potentially be the important limiting factors of the SOM and C, N, P stabilization. For instance, in Chapter 5, we see a high potential of vertical losses of

C, N, P from the surface soils to deeper subsoils in restored marshes (Fig S5.3), possibly resulted from the failure of stabilization of the organic compounds.

2. Will carbon and nutrients be tightly coupled in the restoration process or decoupled and develop distinct C, N, P states from the intact conditions?

My findings from Chapters 3 and 5 showed often deviated litter and soil N-P stoichiometry of restored and constructed marshes from the intact reference, suggesting distinct C, N, P states and changing C, N and P balances between restored and intact conditions, although the validity of this conclusion largely depends on the stoichiometry of the sampled intact benchmarks.

In general, soil mass C:N, C:P and N:P ratio of intact marshes in this dissertation was 9-12, 147-278 and 13-35, comparable to the soil stoichiometry of the temperate and mineral freshwater marshes in Sanjiang Plain, China, with average C:N, C:P, and N:P ratio as 15, 142, and 10 (Zhang et al., 2012), but lower than the subtropical freshwater marsh-fen complex (peat formed in profiles) in the Everglades, Florida, with C:N, C:P and N:P ratio around 13, 700 and 60 (Craft and Richardson, 2008) (Table 6.1). In Chapter 5, soil P concentration in the restored freshwater marshes was comparable to and even exceeded the intact condition, although the C and N was lower than the intact reference. This abundance of total P in restored soils together with the low C and N concentration make average soil C:P and N:P ratio of restored marshes about half that of our intact references, as 71 and 6, respectively, more similar to forest and cropland soils than those from intact freshwater marshes and fens (Table 6.1).

The strongly decreased C:P and N:P ratios in restored marshes may have important ecological implications, although rarely studied. Previous studies have noted decreased C:P ratios due to the P enrichment. For instance, Reddy et al. (1993)

reported decreasing C:P and N:P ratios of Florida wetlands (N:P ratio from 56 to 21) when closer to agricultural inflows which suggested greater N restriction under P eutrophic conditions. The decline of soil C:P and N:P ratio was accompanied by decreased primary production together with decreased rates of C and N accumulation (Reddy et al., 1993). Ecosystem N deficiency will not only hamper the primary production but also diminish the vegetation diversity. For instance, studies have found noticeable vegetation succession from slow-growing species to fast-growing species resulting in reduced species diversity in response to P enrichment (Sterner and Elser et al., 2002; Wang et al. 2014).

This rich total P in the restored soils may also be the reason for the high biomass production of *Carex* spp. and *Phalaris arundinacea* in the restored marsh despite containing only half C concentration of the intact reference, as observed in Chapter 4. Plant species which can release phosphatases to acquire P and support growth through rhizomes may more readily dominate in the restored wetlands through succession than those who can not.

Table 6.1 Soil stoichiometric properties (mass-based) of international freshwater marshes and other typical ecosystems.

Ecosystem	Study region	Management	Sample number	Depth (cm)	Soil C:N	Soil C:P	Soil N:P	Reference
Freshwater marsh	Broughton's Creek, Canada	Intact, restored	3 wetlands	0-50	10-16	138-214	10-26	*this thesis*
	Camrose County, Canada	Intact, drained, restored	30 wetlands	0-30	12 (intact)	160 (intact)	14 (intact)	
					12 (drained)	119 (drained)	9 (drained)	
					13 (restored)	71 (restored)	6 (restored)	
	Sanjiang Plain, China	Intact	1 wetland (15 samples)	0-30	15	142	10	Zhang et al., 2012
	Everglade fen, US	Intact, P-eutrophic	8 stations	0-30	15-17	302-878	20-56	Reddy et al., 1993
	Everglade fen, US	Intact, P-eutrophic	9 stations	0-30	12-15 (median ~13)	372-5000 (median ~700)	26-410 (median ~60)	Craft and Richardson, 2008
Lake wetland	Southern Donting Lake	Intact, slightly reclaimed	2 wetlands	0-200	21.1	35.4	1.7	Wu et al., 2022
Northern peatland	Canadian bog, fen, swamps	Intact	400 samples	0-50	25-42	768-923	25-37	Wang et al., 2015
Estuarine marsh	Min River estuarine, China	Intact and adjacent cropland	153 samples	0-20	11.1-13.4 (intact)	44.4-57.5 (intact)	3.9-4 (intact)	Hu et al., 2024
					8.9 (cropland)	25.9 (cropland)	2.9 (cropland)	
Wetland (global)	Global	Mixed land uses	17 studies	Varied within 0-25 cm	15.9	521.4	28.4	Xu et al., 2013
Forest	Guangxi Province (subtropical)	Mixed land uses	115 sites	0-10	13.95	69.6	5.53	Lu et al., 2023
	Tiantong National Park (subtropical)	Intact	185 grids	0-20	15	190	12	Qiao et al., 2020
	Global	Mostly intact	47-55 samples	0-10 or 20	12.4	82	6.6	Cleveland and Liptzin, 2007
Grassland	Global	Mostly intact	72-75 samples	0-10 or 20	11.8	64.3	5.6	Cleveland and Liptzin, 2007

Cropland	Jiangxi Province	Cropland	16,547 samples	0-20 cm	11.73	38.31	3.38	Hu et al., 2022

Interestingly, my findings from Chapter 5 showed a highly stable and mostly unchanged C:N ratio throughout the sediment of mineral marsh, regardless of different soil depths, land managements, or surrounding land uses which potentially add additional N to soils (Table 6.1; Fig 5.4). Moreover, in Chapter 4, the C:N ratio (mass-based) in microbial biomass of two Ontario marshes (ranging from 5-15) was also constrained and close to the global average microbial C:N ratio as 6 and 10 (calibrated to mass ratio) reported from Cleveland & Liptzin (2007) and Xu et al. (2013), confirming a widely reported homeostasis of microbial biomass C, N (and potentially P) in most ecosystems.

In response to allochthonous N and/or P inputs (either from surrounding croplands, sewage inputs or potentially N and P deposition), this tight coupling of C and N seems being maintained as observed in this thesis and other wetland studies (e.g., Wang et al., 2014). The constraints of C:N ratio in marsh system may result from a suite of controlling mechanisms which connect the C and N cycling through litter decomposition, microbial activities and soil formation. For instance, higher N concentration of litter tissues always leads to higher C and N losses through decomposition (Parton et al., 2007; Berg and McClaugherty, 2008). When bioavailable C and N are not balanced, microorganisms can adjust their C or N use efficiencies (Zechmeister-Boltenstern et al., 2015) and higher N use efficiency was reported when providing litter with higher C:N ratios (Mooshammer et al., 2014). In response to drainage drying, microbial C and N emissions can be coupled by the oxygen consumption under aerobic conditions and in response to wetting, the losing pathways of C and N are both gaseous emissions and leaching.

The stabilization of C and N in SOM is also tightly coupled. During pedogenesis, N (derived from N fixation, deposition, and sometimes rock weathering) and C (derived from plant photosynthesis, plant biomass and sometimes limestone bedrocks) mostly accumulate in litter debris and microbial biomass which are both important precursors of SOM. Recent applications of isotope ^{13}C and ^{15}N allow an advanced understanding on the critical roles that N play in forming different fractions of SOM. For instance, N derived from non-structural compounds of litter and microbial residues greatly contributes to the formation of stable SOM whereas the remaining structural and recalcitrant components mainly form fairly labile SOM via physical compression (Cotrufo et al., 2013, 2015). In mineral SOM, the rich content of soil organic N and other minerals can form important proteinaceous layers which provide additional reactive spots for the attachment of the function groups for organic compounds (Kleber et al., 2007) and these mineral-organic interfaces foster the presence of stable SOM, likely meanwhile constrain the C:N ratios.

3. What mechanisms and factors may limit the accretion of carbon and nutrients retention in restored wetlands?

My findings showed overall large variance in sediment C, N, P concentration which is driven by some identified factors such as wetland sewage inputs and surrounding land uses in this dissertation, but mostly by the unknown factors which are not examined in this dissertation. Sewage inputs aided the P retention in decomposing litters (see Chapter 3). N enrichment might be a positive factor on C retention (see Chapter 5). However, the restoration age of marshes was not an efficient factor controlling sediment C, N, P concentration, decomposition rates (see Chapters 3 and 5). I suspect that soil texture, remained SOM content after drainage and wetland hydrodynamics and the leaching intensity might be closely related to the failure or success of the accretion of new SOM in restored marshes. For instance, Bruland and Richardson (2004, 2006) and Bruland et al. (2009) revealed SOM is always tightly associated with soil and microbial properties and topsoil additions to created wetlands can foster SOM accumulation, P adsorption, and microbial biomass.

My thesis findings further suggest that different wetland compartments may recover at different speeds and to different degrees over certain period of restoration. The recovery of hydrological regimes can be achieved shortly (less than 5 years) after ditch-blocking, although further depends on the local climate and wetland morphology. Vegetation and biomass recovery usually takes less than 10 years with some efforts to maintain and achieve (e.g., seedbank introduction, mowing), although the restored vegetation community might differ from the intact reference (Craft, 2022). Microbial community and soil C, N, P concentration recovery at the surface soils largely rely on the accumulation of SOM. However, wetland soil physical and chemical properties such as porosity, aggregate sizes, pH, cation concentrations are rarely successfully restored in field observations (Craft, 2022) and may require necessary human interventions.

6.3 Conclusions

In conclusion, this dissertation suggests the outcomes of freshwater marsh restoration can depend on wetting strategies, establishment of vegetation communities, historical cultivation (N or P inputs), as well as the ongoing anthropogenic impacts on the restored marshes such as fertilization on neighbouring croplands, sewage inputs. The accretion of C, N, P in thick layers mostly failed in our studied marshes and requires further studies to examine the limiting factors and mechanisms. To achieve full recovery of C, N, P retention to the intact states, necessary amendments on soils and wetland ecosystems might be required.

6.4 Suggested avenues of future research

I suggest four priority avenues for future research:

First, the controlling mechanism on the continuous stabilization of C and N in restored freshwater marshes is highly unclear. There could be more research on the soil amendment practices either on physical or chemical properties, such as topsoil addition experiments, pH adjustment experiment, cation addition experiment, and soil compiling experiment on freshwater marshes to detect their positive or negative impacts on sediment C, N, P retention.

Second, the sources and forms of P in the sediment of freshwater marshes surrounded by agricultural lands require further examination. Sampling could be conducted in restored marshes with similar age, area, hydrology and geology settings but under little and intense agricultural impacts, respectively, to compare the detailed P species in surface sediment. In particular, the potential flux of organic P to inorganic P over the drainage and rewetting processes merits further research.

Third, the decoupling of P with C and N after restoration in mineral marshes observed in this dissertation (although we did not measure the available P) may have further implications (e.g., risks of invasive plants intrusion), but are rarely assessed.

Fourth, I suggest when conducting restoration studies, more comprehensive information on wetland characteristics before and after restoration is needed for better assessment on wetland biogeochemical functioning recovery and mechanism testing. In many published literatures on restoration studies, the age of restoration is considered an important factor although many existing studies have shown no real relationships between measured parameters and the restoration ages. I suggest that other than ages, wetland morphology such as sizes, slopes, and surrounding land uses, soil cations, aggregate sizes, and vegetation information might be more useful for the assessment of wetland functioning recovery. Models that assess the restoration efficacy of wetland functions such as C, N, P accumulation rates, vegetation primary productivity, and enzymatic activities may also involve these factors.

6.5 Suggestions on efficient restoration practice to retain soil C, N, P

First, wetlands that readily dry (usually very small) are not preferred for restoration because the seasonal drying could elevate litter decomposition rate and reduce the C storage in soil. Wetlands that achieve permanent wet over years are a priority for restoration.

Second, the N inputs from surrounding agricultural lands might be a positive factor on C storage in restored freshwater marshes given the C:N ratio was highly constrained over multiple land use classes.

Third, the introduction of certain plant species to restored sites can be an efficient measure to determine the litter quality, decomposition rate and retention of C, N, P in surface soil.

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