EVALUATING MACROPHYTE SELECTION AND GERMINATION PROTOCOLS TO ENHANCE NUTRIENT SEQUESTRATION IN ENGINEERED WETLAND MODELS

by

Francesca Mary Fernandes

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Abstract

Evaluating Macrophyte Selection and Germination Protocols to Enhance Nutrient Sequestration in Engineered Wetland Models

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Francesca Mary Fernandes

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Ryerson University

Research examining contaminant sequestration using engineered wetlands has been conducted for many years but the implementation of sustainable, biodiverse strategies is still in its infancy. A major gap in knowledge still exists regarding the kinds of macrophytes to be selected, especially the inclusion of non-invasive native flora. There is a lack of information about macrophyte selection criteria and germination protocols. Thus, this study attempted to redress this dearth in knowledge. The first part of this thesis critically assessed a list of macrophytes provided by Environment Canada (1996) and created "selection criteria" for choosing specific macrophytes. Germination protocols were then compiled to determine and outline optimized germination protocols for these aquatic macrophytes.

In the second part of this study, two different constructed wetlands models were designed for laboratory purposes (a "floating" constructed wetland model and a "stationary" constructed wetland model). Water samples were assed for biological impact and phosphorus concentration.

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Dedication

I would like to dedicate my thesis in loving memory of my dearest uncle, **Derek Noronah**, for believing in me against all odds and even when no one, including myself, believed in me. Thank you for showing me that falling did not always mean failing, and that sometimes it was simply a lesson on how to graciously stand up again. He taught me not to be fearless, but to be courageous because, “Fools are fearless, the brave are courageous.”

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1.0 INTRODUCTION

Overview

A major problem besetting the Lower Great Lakes of North America, particularly Lake Erie, is the non- point source (NPS) runoff of contaminants originating from agricultural biomes into adjacent bodies of water (Neilson *et al*., 1995; International Joint Commission (IJC), 2014). These pollutants often include excess nutrients from fertilizers such as phosphorous and nitrogen, crop pesticides, and pharmaceuticals such as steroids and antibiotics from animal manure (Frank and Sirons, 1979; Bau *et al*., 2006; Allinger and Reavie, 2013). Additionally, in the past several decades, treated municipal waste, commonly referred to as biosolids, has also been land-applied in agricultural systems as supplements to costly commercial fertilizers (Canadian Council of Ministers of the Environment (CCME), 2009; Puddephatt, 2013). Subsequent runoff during heavy rainstorms can occur carrying both traditional agricultural and biosolids constituents. The contaminants that enter the waterbody may have deleterious impacts on the aquatic food-web, with one extensively-documented example being excessive concentrations of phosphorous and subsequent algal growth (Schindler *et al*., 1973; Schindler *et al*., 2008). With massive nutrient input and perfect abiotic conditions (sunlight, warmth), enormous algal blooms lead to eutrophication and subsequent fouled water systems.

Naturally-occurring wetlands that straddle the zone between the land and the water have helped to ameliorate some of these issues in the past by capturing and sequestering the phosphorous-laden runoff, thereby decreasing the amount of nutrients reaching aquatic systems (Vymazal, 2011). Other nutrients such as nitrogen running off land, may also be sequestered by wetlands. For the purpose of this study, the major focus will remain phosphorus. The removal over the past century of many of these wetlands (72%) has led to an increase in NPS pollution and there is much research being conducted to determine whether implementing engineered or constructed wetlands can assist in providing remediation strategies. The broad mandate of this thesis is to attempt to continue to build upon the "engineered wetlands" knowledge that is currently available. By deeply mining the existing literature and selecting appropriate native macrophytes, lab protocols will be developed to germinate and grow these macrophytes and build a constructed wetland that can be used to capture and sequester agricultural runoff

constituents, particularly phosphorus as it is a limiting nutrient and directly influences algae growth in receiving waters.

Literature Review

1.1 Background on Wetlands

Wetlands are dynamic ecosystems with characteristics of both water and land (Wetzel, 1982). Each wetland is unique; having traits that have developed over time based on geographic location and specific flora and fauna (Figure 1). The term 'wetland' is used to describe a land that is either saturated or submerged with water seasonally or annually, and was first formally defined prior to the 1900s by an unknown source (van der Valk, 2012).

Figure 1: Algonquin Natural Wetland, Ontario, Canada (Hendry, 2008)

There are different types of wetlands based on their water source, depth, and microclimates and the Cowardian classification system has five different kinds of wetlands: palustrine, marine, estuarine, riverine, and lacustrine (Cowardin *et al.*, 1979). This classification system relies on the location of the wetland i.e. inland, ocean, river, or lake adjacent.

Wetlands may also be categorized by their nutrient concentration and include oligotrophic, mesotrophic, eutrophic, and hypereutrophic conditions (Wetzel, 1983). These wetlands may be found in a wide range of places including coastal marshes in the Everglades, water-soaked peat in the Rocky Mountains, sphagnum-dominated bogs in the Arctic, and forested tropical floodplains in the Amazon Basin (Davis, 1991; Finlayson and Moser, 1991; Halsey *et al.*, 2000). These naturally-occurring ecosystems serve different purposes including the preservation of ecological diversity, habitat for organisms, flood prevention in certain areas, recreation, and even wastewater treatment in some communities (Boyer and Polasky, 2004; IJC, 2014).

1.1.1 Wetlands within Canada

Fourteen percent of Canada's total land mass is comprised of wetlands, with most being concentrated in the northern regions. Since 1906, the wetland concentration around the coasts of the lower Great Lakes has been approximately 5%, with many naturally-occurring wetlands being destroyed for agricultural needs ((Environment Canada and Climate Change (ECCC), 2016; ECCCb, 2016). On the shores of these Laurentian Great Lakes, there has been a continual loss of natural wetlands, wetlands that would have provided a buffer for terrestrial runoff and helped to ameliorate lake contamination. Research has estimated that the total southern Ontario wetland area before European settlement (prior to 1600s) to have been 2,026,591 hectares. As of 2002, this area has diminished to 560,844 hectares of pre-settlement wetlands. This is approximately a 72% loss of natural wetlands in southern Ontario. Between 1982 and 2002, 70, 854 ha of the original pre-settlement wetlands were drained to create space for built-up lands and agriculture (Ducks Unlimited Canada, 2010).

To compensate for the loss of wetlands and the functions they would have naturally carried out, engineered wetlands have been constructed for use in stormwater retention, flood reduction, runoff treatment, to reduce eutrophication effects, and decrease possible freshwater contamination (Jones and Lee, 1982; Oberts and Osgood, 1991; Konyha *et al*., 1993). Hereafter the term 'constructed wetland' will be used synonymously with the term 'engineered wetland.'

1.1.2 The Role of Wetlands in Treating Wastewaters

While the functions of wetlands are many and varied, for the purposes of the current thesis, their role as contaminant retention systems of non-point source runoff will be the major focus. Wetlands are effective systems in sequestering excess nutrients and capturing carbon (Wetzel and van der Valk, 1996). The ability of wetland macrophytes to capture nutrients and contaminants from terrestrial runoff enable them to be useful in treating both point source and non-point source pollution (wastewater) and wetlands have been used for centuries. However, Vymazal (1998) suggested that early wetlands were used, out of convenience, to dispose of wastewater which contained organics, mainly nutrients, heavy metals, and pathogens.

The usefulness of wetlands as a method for filtering out excess nutrients was observed in the 1970s in the 'Village of Bellaire', Michigan. Eighteen hectares of wetland outside the town was used to treat sewage from their lagoons at a rate of 30 million gallons per year (Kadlec, 1983). Parameters such as hydrology, water quality, total phosphorus, total nitrogen, suspended solids, vegetation, and soils were collected and analyzed. These wetlands proved useful for retaining nutrients such as nitrogen and phosphorus and suspended solids. Wetlands use mechanisms such as sedimentation, microbial denitrification, matric adsorption, filtration and plant uptake to remove nutrients and pollutants (See section 1.6 for more details). However, the wetlands could not remove enough pathogens, resulting in significant damage to vegetation near the wastewater inlet that was the result of viral and bacterial infections to the vegetation. Thus, this practice of using the wetland to treat their wastewater was stopped in the 1980s (Kadlec, 1983). While many places around the world used wetlands as treatment strategies, wetlands have now been abandoned in favour of using wastewater treatment facilities that are more efficient in killing pathogens, in point source contaminant strategies.

Nichols (1983) reviewed the long-term barriers that had been faced using wetlands to sequester excess nutrients from wastewater. It was observed that while short-term strategies using wetlands demonstrated high efficiency in nutrient removal, they eventually lost their ability to absorb nutrients. One reason for this may have been over enrichment of the vegetation as proposed by Verhoeven (1986). Over enrichment occurs when the amount of nutrients in the environment is higher than the nutrient uptake capacity of the plant and nutrient uptake reaches oversaturation resulting in temporary halt in nutrient uptake. Since then, these issues have been

addressed as wetland long-term efficiencies have been successfully implemented with the harvesting of wetland vegetation and subsequent use for various purposes (van Wirdum, 1993).

Along with natural wetland use and evaluation in wastewater treatment, constructed wetlands became a developing concept in Europe. The earliest, formally-documented constructed wetland patent belonged to Cleophas Monjeau (Wallace and Knight, 2006). Monjeau, born in Quebec in 1839, traveled to the United States and Germany for further education and research. After settling in Middleton, Ohio, he assembled the first documented constructed wetland to filter water 1901 (Hallock *et al.*, 1881; Wallace and Knight, 2006). His earliest design comprised of a vertical flow system, where wastewater traveled through layers of substrate to get filtered, with aerated fluctuating water levels passing through vegetation (Monjeau, 1901). For over a century, facilities have been centred around physical, chemical, and biological processes to treat wastewater; however, macrophytes had not yet been considered in wastewater treatment (Figure 2). The earliest study of the mechanisms by which vegetation in constructed wetlands filter wastewater was studied by Dr. Kathe Seidel in 1953 at the Max Planck Institute in Krefeld, Germany (Vymazal, 2011). Her initial designs were composed of a series of vertical flow and horizontal flow system with *Phragmites australis*.

Figure 2: Evolution of wastewater disposal to treatment using natural and constructed wetlands (Vymazal, 2011)

1.1.3 Biosolids Nutrient Runoff from Terrestrial Biomes

The nutrients used to test the macrophyte nutrient-removal efficiency in this study and consequently, constructed wetland ability to remove pollutants, was biosolids. Biosolids are the product of treated wastewater (McCarthy *et al.*, 2004) that may be produced from sewage

effluent or pulp and paper effluent through aerobic or anaerobic digestion (Puddephatt, 2013). Biosolids are the solid components left behind after dewatering and digesting the influent at wastewater treatment facilities. This source of nutrients is environmentally-relevant due to its application on agricultural land as an alternative, inexpensive source of commercial fertilizers and contaminants found in biosolids may continue to persist in the top soil, or be transported in different ways. Thus, in 2004, McCarthy *et al*. tested the long-term effects of these land applications and their impact on organisms in terrestrial and receiving water bodies. Based on the lack of significant impact to organisms in their study's results, they concluded that carefully regulated biosolids land-application was a viable and environmentally-sound alternative to biosolids disposal by landfilling and incineration.

During heavy thunderstorms, including those observed in Ontario during spring and fall, along with an increased frequency in extreme weather events, contaminants in the soil could enter aquatic ecosystems through runoff (Environment Canada, 2009). Though the biosolids did not show any significant negative impact on biota, the nutrients in biosolids that enter receiving water bodies may induce algae growth that could result in eutrophication (Hanief *et al.,* 2015).

1.1.4 Eutrophication

Eutrophication is the direct impact observed in conditions of excess nutrients, and is often the result of non-point source phosphorus runoff from agricultural systems. The high nutrients in aquatic environments provide more food and optimal growing conditions for autotrophs. The result is a bloom in algae, cyanobacteria, and phytoplankton species (Janus and Vollenweider, 1981). Eventually, these organisms die and as they decompose, this process carried out by microbial enzymes in the water column use up oxygen in various layers of the ecosystem. The decomposition of organic matter requires oxygen in a 1:1 molar ratio and the products from this reaction are CO_2, H_2O, and inorganic nutrients (Robarts, 1986). This depletion of dissolved oxygen (DO) reduces the available O_2 needed for respiration. Consequently, many organisms die due to these anaerobic conditions, especially, in the lower layers of aquatic ecosystems and these bottom layers subsequently become hypoxic.

An increase in dead zones, reduces the possibility of other aerobic species from coming back to populate these habitats (Schindler, 1974). At 20°C, O_2 takes 10, 000 times longer to diffuse in water than in air i.e. taking O_2 approximately 7 hours to move a distance of 1 cm through molecular diffusion in water (van der Valk, 2012). The low diffusion rate of O_2 in water, along with the consumption of O_2 for respiration, quickly exhausts DO in the water column and sediments. Thus, an increase in eutrophication, uses up an already depleting source of O_2, producing anoxic conditions (Rose and Crumpton, 1996). These impacts of eutrophication have been observed in Lake Erie repeatedly.

1.1.5 Eutrophication in Lake Erie

As the International Joint Commission has rightfully named them, Harmful Algal Blooms (HABs), caused aquatic havoc in the 1960s (IJC, 2014). The most common species observed were cyanobacteria. These organisms were single-celled prokaryotes that photosynthesized. Approximately 2% of cyanobacteria produce harmful toxins (Landsberg, 2002). Even though this makes up for a very low fraction of toxin-producing organisms, high concentrations of organisms produce more toxins. The increase in non-point source runoff from agricultural lands around Lake Erie have provided optimal growing conditions for phytoplankton in general. Due to invasive species, such as zebra mussels that filter fed the waters of the non-harmful organisms, the toxin producers were left with no competitors (IJC, 2014). Over the last fifty years, the most common species in these cyanobacterial blooms have included these species: *Microcystis aeruginosa, Planktothrix, Anabaena, Cladophora*, and *Lyngbya*. Species such as *Microcystis aeruginosa* are of grave concern due to the neurotoxins and hepatotoxins that they release, targeting neurons and liver cells respectively (Mayer and Clifford, 2011). These organisms can severely contaminate drinking water sources.

1.1.6 Impacts of Eutrophication

There are number of environmental, social, economical, and medical impacts caused by eutrophication. The excess nutrients from runoff cause a shift in the habitat characteristics due to changes in aquatic plants (IJC, 2014). The toxins produced by HABs create odour, and along

with affecting the taste of drinking water supplies, increase the operating costs of treatment facilities. Excess aquatic weed growth tends to clog irrigation canals and disrupts navigation. Aquatic ecosystems used for recreational purposes are abandoned due to slime, weed infestation, and noxious odours. Due to a loss of fish species and tourism, eutrophication causes major economic losses (IJC, 2014).

OBJECTIVE

The overall long-term benefit of this study was to assess the efficiency of using an engineered wetland to reduce eutrophication effects from excessive nutrient runoff, and mitigate possible contaminants found in aquatic ecosystems from biosolids runoff. In attempting to achieve this long-term goal, this study had two major objectives:

A. Evaluate selected macrophyte germination and growth for the development of optimal engineered wetland systems.

B. Use these macrophytes in a small constructed laboratory wetland to sequester excess nutrient and remove other pollutants.

PART I – Evaluate Selected Macrophytes, Germination and Growth

1.2 Macrophytes: Aquatic Vegetation

An essential component of wetlands, natural or engineered, is its vegetation. Aquatic plants that have a tolerance for saturated or water-logged soils, with high root adaptability to fluctuating water levels, are called 'macrophytes' (Vymazal, 2007). Studies carried out in the last few decades have demonstrated that while macrophytes play a crucial role in habitat formation, they are also important for nutrient sequestration (Whigham and Simpson, 1978; Tanner, 1996). Macrophytes sequester excess nutrients in wetlands for their own growth requirements and their roots, often fibrous in structure, act as a filter by providing an adhesive surface for contaminants to adsorb onto in a wetland (Ulrich and Burton, 1984). Moreover, macrophytes provide an increase in surface area for microbes to grow, attach, and use reduced carbon and oxygen in the rhizosphere (Brisson and Chazarenc, 2009). This interaction between microbes such as *nitrosomonas* and *nitrobacter* with the macrophyte roots is symbiotic because the plants receive bioavailable nitrogen forms such as nitrates for their assimilation while the microbes find a place to attach and grow.

1.2.1 Native Macrophytes and Invasive Macrophytes

The current debate about macrophytes in wetlands examines species-specific efficiency. Macrophyte comparison studies for nutrient sequestration and specific pollutant removal have increased current interest in future phytoremediation possibilities (Brisson and Chazarenc, 2009). For example, a study carried out in Denmark in 2009 examined three macrophytes for their ability to reduce triclosan in a constructed wetland receiving treated sludge. Triclosan is an antibacterial agent found in hand sanitizers, soaps, detergents etc. This study used *Typha latifolia, Phragmites australis,* and *Phalaris arundinacea* (Chen *et al*., 2009). They found a 70% reduction in triclosan at the end of their twelve-month study. However, this reduction was attributed to degradation and volatilization because all three macrophytes and the non-vegetated treatment produced the same results (Chen *et al*., 2009).

'Native' species are those that are found in a geographical location without human introduction. This is the globally-accepted definition, also referred to as 'indigenous' species

(Lavergne and Molofsky, 2004). 'Invasive' species are organisms whose reproduction rate is so high that it out competes other organisms and in doing so, reduces biodiversity in a location (Vitousek and Funk, 2007). It is interesting to note that invasive species could be native to the general landscape but, when relocated to a pristine subsection of the original landscape, they might become invasive. There are multiple hypotheses for this behaviour; i) relocation allows for previous resource allocation towards anti-herbivore defence to be allocated towards reproduction (Keane and Crawley, 2002; Power and Mitchell, 2003); ii) invasives might react to an increase in positive and mutualistic soil microbial interactions (Klironomos, 2002; Callaway and Ridenour, 2004), iii) native species may be more vulnerable to alleopathic responses from invaders due to novel competitive strategies such as propagation through rhizomes (Callaway, 2002; Lavergne and Molofsky, 2004). Regardless of reason for aggressive propagation, while invasive macrophytes reduce biodiversity, they also dramatically alter ecosystems (D'Antonio and Vitousek, 1992; Blossey *et al*., 2001) which result in million-dollar economic impacts annually (Pimentel *et al*., 2000).

1.2.2 Typha latifolia and Phragmites australis

Growing systems for treating wastewater, stormwater, and biosolids and traditional agricultural runoff are known as, "phytotechnologies." Mench *et al*. (2009) suggested that this term refers to applying plants and their associated microbes to scientific and engineering solutions. Phytoremediation then becomes the investment into long-term projects to remediate contaminated soils and waters (Mench *et al*., 2008; Mench *et al*., 2009; Vangronsveld *et al.,* 2009). Species chosen for such treatment depend on the objective of treatment. Plants help assimilate contaminants by absorption and provide a surface for microorganisms to grow (Mench *et al.,* 2010). The two most commonly used wetland species in Europe during this time were *Phragmites australis* and *Iris pseudacorus*. These two species are native to the European continent and to some parts of Asia as well as Africa (Manceau *et al*., 2008). Other commonly used species to treat pollutants include: *Typha spp., Scirpus spp., Phalaris arundinacea, Eichhlornia, Azolla* and *Lemna* (Vymazal, 2009).

In North America, the most common macrophyte species used is *Typha sp* (Tiley, 2013; Rozema *et al*., 2016). These species have been efficient in surface flow, horizontal subsurface

flow, and vertical subsurface flow constructed wetlands for removal of excess nutrients, primarily phosphorus and nitrogen. These wetlands have been used to treat mainly dairy wastewater (Smith *et al*., 2006; Munoz *et al*., 2009; Wood *et al*., 2008). Research carried out by Tiley (2013) in our laboratory examined the uses of *Typha latifolia* in nutrient sequestration as part of constructed wetlands. In his research, with the growing above-ground biomass and rhizome propagation, it soon became overwhelmingly abundant that *Typha* was an invasive species. Species such as *Phragmites* that exhibit very similar aggressive propagation, with high biomass production resulting in invasive behaviour are continuously used in constructed wetlands regardless of their status as invasive species. The Centre for Alternative Wastewater Treatment (CAWT) at Fleming College in Lindsay, Ontario, continues to use *Phragmites* out of convenience (Figure 3), simply for their high nutrient sequestration abilities (pers. comm. G. Balch, CAWT).

Figure 3: Constructed Wetland at CAWT depicting *Phragmites* Biomass Production (photo by Dr. Gordon Balch)

Over the last decade, many researches have conducted comparison studies to determine which macrophyte species are the most efficient in nutrient removal. A study conducted by Yu *et al*. (2012) looked at the efficiency of *Schoenoplectus lacustris, Vetiveria zizanioides, Acorus calamus, Cana india, Zizania latifolia, Phragmites communis,* and *Iris pseudacorus* for nitrogen

and phosphorus removal. These species are native to southern China (Gude *et al*., 2013) and their efficiencies were evaluated by examining their above-ground biomass production. The average biomass produced was 67%. Additionally, through phytoextraction, plant can take up heavy metals that are essential to their own growth, commonly heavy metals such as Fe, Mn, Cu, Zn, Mg, Mo and Ni along with other metals like Pb, Cr, and Hg. Many others are taken up but these have unknown biological functions (Cho-Ruk *et al*., 2006; Tangahu *et al*., 2011).

Similar macrophyte comparison studies have been carried out to test the efficiency of heavy metals removal. Qian *et al*. (2012) compared *Betula populifolia, Rhus copallinum, Polygonum cuspidatum,* and *Artemisia vulgaris* and their removal of As, Zn, Cu and Cr. The uptake rate was different for each of these species for each of the heavy metals and this demonstrated the need for future study regarding specific macrophyte selection. Other research with *Phragmites spp.* among other macrophytes (*Cyperus alternifolius, Cannaindica* and *Acorus calamus*) used a constructed wetland to treat wastewater produced from cleaning and maintaining livestock farms (Zhu *et al*., 2012). In this study in China, it was found that the presence of vegetation had little effect in reducing the chemical oxygen demand, suspended solids, ammonia nitrogen, total phosphorus and total nitrogen (Zhu *et al.*, 2012).

While more studies have compared macrophyte nutrient uptake efficiencies, this current research strategy examines native plants for macrophyte selection. Unfortunately, certain species can grow rapidly even if they are native and these macrophytes have been known to out-compete other native species (Rodriguez and Brisson, 2015). Thus, it is important to consider multiple species to create a healthy biodiversity and a macrophyte's potential to become invasive must be considered even when choosing native species.

1.3 Macrophyte Selection

For more than half a century, wetlands have been known to critical habitats for many organisms including amphibians, reptiles, fish, birds, and mammals (Bardecki, 1984; USEPA, 2016). Concerns regarding habitat restoration have helped initiate wetland restoration and in 1996 Environment Canada provided a list of macrophytes that were suitable for use in engineered wetlands and wetland restoration (Environment Canada, 1996; Miller, 2008; Erwin, 2009). The report by Environment Canada was written with the purpose of informing the general

public of the importance of wetlands, the difference between native and exotic species, seed collection, germination and macrophytes to use in their own backyard wetland restoration projects (Environment Canada, 1996). However, this list was produced 21 years ago, and needs revision with the new knowledge that has been gained in the intervening years. For example, species such as *Typha latifolia* have been identified as having invasive characteristics (Tiley, 2013) and *Sparganium chlorocarpum* is now considered the same species as *Sparganium eurycarpum,* making *S. chlorocarpum* now an invalid species name (USDA, nd). Additionally, many species on this list had not been previously used in engineered wetlands or are lacking data on nutrient-removal efficiency, while other species are either slow to germinate, have very little biomass to sequester sufficient quantities of nutrients, or are of "special concern" and are part of an endangered species class. Thus, this current study was initiated to assess the species in this 1996 list as suitable macrophytes for constructed wetland strategies. Additionally, due to the length of the list and out-dated information, revision of the selection criteria became a primary mandate.

1.3.1 Environment Canada Macrophyte Species List (Environment Canada, 1996)

For the purpose of this study, only the emergent macrophytes were assessed from the Environment Canada list. All available information on their ecology, anatomy, and presence in past nutrient removal studies are outlined below (Table 1). Macrophyte taxonomic keys can be found in Appendix I

Table 1: Common Marsh Plants for use in Wetland Restoration Provided by Environment Canada in 1996

EMERGENT	
Water Plantain	*Alisma plantago-aquatica*
Swamp Milkweed	*Asclepias incarnata*
Sedges	*Carex spp.*
Turtlehead	*Chelone glabra*
Spike Rushes	*Eleocharis spp.*
Water Horsetail	*Equisetum fluviatile*
Wild Blue Flag	*Iris versicolor*
Rushes	*Juncus spp.*
Pickerelweed	*Pontederia cordata*
Arrowhead	*Sagittaria latifolia*
Hard-Stemmed Bulrush	*Scirpus acutus*
Black Bulrush	*Scirpus atrovirens*
Soft-Stem Bulrush	*Scirpus validus*
Green Fruited Bur-Reed	*Sparganium chlorocarpum*
Giant Bur-Reed	*Sparganium eurycarpum*
Cattails	*Typha spp.*
American Brooklime	*Veronic americana*
SUBMERGENT	
Coontail	*Ceratophyllum demersum*
Waterweed	*Elodea canadensis*
Watermilfoil	*Myriophyllum exalbescens*
Sago Pondweed	*Potamogeton pectinatus*
Pondweed	*Potamogeton richardsonii*
Bladderworts	*Utricularia vulgaris*
Tape Grass	*Vallisneria americana*
FLOATING-LEAVED	
Yellow Water Lily	*Nuphar variegata*
White Water Lily	*Nymphaea odorata*
Water Smartweed	*Polygonum amphibium*
Variable-Leaved Pondweed	*Pontamogeton gramineus*
Floating Pondweed	*Pontamogeton natans*
FREE-FLOATING	
Common Duckweed	*Lemna minor*
Star Duckweed	*Lemna trisulca*
Greater Duckweed	*Spirodela polyrhiza*

1.3.2 Ecology and anatomy of Alisma plantago-aquatica (Water plantain)

Alisma plantago-aquatica commonly known as European water-plantain, common water-plantain or mad-dog weed is found on water saturated soil or in fresh water ecosystems. This perennial macrophyte is a monocot that produces flowers (USDA, 2006). This general information on macrophyte growth and reproductive organs is useful in identifying macrophyte life stage. This macrophyte grows to height of 1.0 m in shallow water. The plant has fibrous roots and long stemmed broad leaves. The leaf length varies from 15 cm to 30 cm long. The stem is triangular shaped with the plant producing inflorescence emitting small flowers of three petals each (Figure 4). This macrophyte has three sepals and six stamens per flower. The carpel of this plant is arranged as a single flat whorl (Fernald, 1946).

Figure 4: *Alisma plantago-aquatica* (© 2011 Dr. Amadej Trnkoozy)

This plant is native to Europe, Asia, and northern and central Africa. Water plantain has been introduced to certain parts of south Africa, Alaska, British Columbia, Washington State and Connecticut (Kew, nd). In Ireland, there have been a few studies that have used water plantain with other species to determine the constructed wetland efficiency. However, these experiments do not include any information on single macrophyte nutrient removal efficiencies. One such study conducted to treat dairy farmyard wastewater used water plantain, cattails and reed canary grass to reduce the total phosphorus. The constructed wetland retained 5-84% of phosphorus yearly, with the least retention capacity in winter (Dunne *et al.*, 2005). Another study conducted in the United States with constructed wetlands and this macrophyte (water plantain) along with four other species (*Carex stricta, Iris versicolor, Juncus effuse,* and *Ponterderia cordata*) found that a combination of macrophytes produced a biomass of 2.6 kilograms. These floating wetlands were able to capture 3.1 kilograms of sediments while filtering out 0.2 kilograms of nitrogen over a 137-day period (McAndrew *et al.*, 2016). The second macrophyte that was examined in this study was *Asclepias incarnata*.

1.3.3 Ecology and anatomy of Asclepias incarnata (Swamp milkweed)

Asclepias incarnata is an herbaceous perennial dicot macrophyte that is commonly known as swamp milkweed, rose milkweed, rose milkflower, swamp silk weed, and white Indian hemp. The swamp milkweed grows to a height ranging from 1.0 m to 1.5 m. This species has thick white roots, branched stems, with leaves that range from 7.5 to 15 cm long and 1.0 to 4.0 cm wide. The plant flowers have pink petals on an elevated central crown that grows in a compound umbel (Figure 5). Flat red seeds are grown attached to silk hairs in pods that split open when ripe (Dickinson *et al.*, 2004).

Figure 5: *Asclepias incarnata* (© 2003 Jeff Abbas)

This aquatic plant is native to North America and it grows in wet soils. The pink flowers of this plant often attract pollinator, such as the monarch butterfly that falls under the species of "special concern" by Species at Risk - Ontario. These butterflies lay their eggs on the leaves of the *Ascclepias incarnata*. The eggs develop into larvae (caterpillars) that feed on these leaves. These larvae eventually enter the pupae stage by forming a chrysalis connected to the plant stem. Monarch butterfly larvae only consume milkweed, making this macrophyte an important wetland species (USDA, 2011; MNRF 2015).

Literature does not exist on this macrophyte's nutrient removal efficiency individually. Only when found at constructed wetland locations, it may be used among various higher biomass producing macrophytes. Planted in a series of small constructed wetlands to treat municipal effluent, the ornamental macrophyte uses its extensive root system to filter suspended solids. Swamp milkweed, along with other wetland plants, was able to reduce the ammonia by 56% and the phosphorus by 80% (Steer *et al.,* 2002).

The third macrophyte that was examined in this study was *Carex sp.* Note that this is the genus name and that there are almost 2000 species (Hipp, 2007). For the purpose of germination and nutrient removal efficiency tests, *Carex vulpinoidea* was used due its native seed availability in Ontario.

1.3.4 Ecology and anatomy of Carex vulpinoidea (Fox sedge)

Carex vulpinoidea is a perennial monocot sedge species. The mature height for sedges range from 0.3 m to 1 m. The root depth can range from 0.4 to 0.5 m. These species are propagated by seed or sprigs (Frye and Lea, 2001). The inflorescence of this plant is a dense flower cluster of spikes that grow 10 cm long and 1.5 cm wide. The sedge width grows 2 mm wide. This macrophyte produces numerous small yellow seeds (Michaux, 1803).

Figure 6: *Carex vulpinoidea* (© 2010 Dean Wm. Taylor, Ph.D)

Carex vulpinoidea is commonly known as fox sedge and American fox-sedge. This macrophyte is native to North America and is commonly found in Canada and the United States (USDA, 2006b). Fox sedge has been reported in some places in the Dominican Republic, and Mexico and has been introduced in Europe and New Zealand. This macrophyte is found in damp soils with cyclic exposure to high and low water levels. This macrophyte is one of the most common organisms in the Northen hemisphere wetlands (Bernard, 1990). It is tolerant to slightly basic and anaerobic conditions (USDA, 2006b).

There have been numerous studies conducted with various species of Carex sp. A study done by Hunt *et al*. (1999) in the United States investigated the hydraulic flow of a constructed wetland with *Juncus effuses, Setaria glauca, Phalaris arundinacea, Verbena hastate, Carex vulpinoidea,* and *Juncus tenuis*. Due to the flat plain geographical location, insufficient inflow of water resulted in reduced biomass growth (Hunt *et al*., 1999). Another study in the United States used *Scirpus validus, Carex lacustris, Phalaris arundinacea,* and *Typha latifolia* to reduce the total nitrogen and total phosphorus from soil leachate in constructed wetlands (Fraser *et al*., 2004). This study observed *S. validus* as most effective in nutrient removal in the constructed wetland while *P. arundinacea* was the least effective.

The forth macrophyte that was examined in this study was *Chelone glabra.*

1.3.5 Ecology and Anatomy of Chelone glabra (Turtlehead)

Chelone glabra is a perennial dicotyledonous herbaceous macrophyte. The turtlehead grows to approximately 0.25 m and produces white flowers in the shape of turtleheads giving this macrophyte its common name. This plant is native to eastern North America and is commonly known as turtlehead. The closest relatives to this plant are the *Chionophile* and *Nothochelone* from western North America. The turtlehead macrophyte has not been reported to be seen elsewhere or introduced to other continents (Nelson and Elisens, 1999). There is little else known about this macrophyte nutrient removal studies is lacking.

Figure 7: *Chelone glabra* (Mohlenbrock and USDA SCS, 1989)

The fifth macrophyte that was examined in this study was *Eleocharis spp.*

1.3.6 Ecology and anatomy of Eleocharis spp. (Spike Rush)

Eleocharis is a perennial monocot macrophyte that belongs to the sedge family. Spike rushes grow to approximately 0.4 m long. and produce dark green leaves and brown seeds (Ogle, 2005). This species is said have a "cosmopolitan distribution" because it is available widely around the world in growth-optimal conditions such as wet soils. It has been reported in the Amazon rainforest, as well as in Australia, North America, South Africa, and Asia (Goyaerts and Simpson, 2007). There was no research available on the use of this macrophyte as part of engineered wetlands to reduce nutrients.

Figure 8: *Eleocharis spp.* (Hagwood, 2004)

The sixth macrophyte that was examined in this study was *Equisetum fluviatile.*

1.3.7 Ecology and anatomy of Equisetum fluviatile (Horsetail)

Figure 9: *Equisetum fluviatile* (Luc Viatour, 2006)

Equisetum fluviatile is a vascular perennial monocot macrophyte. The water horsetail can grow between 0.3 to 1 m in length with a stem diameter of 2 to 8 mm. It is commonly known as water horsetail and swamp horsetail. This macrophyte is native to North America but is also found in Spain, northern Italy, and parts of Asia. Historically, this macrophyte was used for sanding and filling because its stems contained high quantities of silica (USDA, nd). There is a lack of research conducted with this species, like many of the macrophytes above, in engineered wetlands.

The seventh macrophyte that was examined in this study was *Iris versicolor*.

1.3.8 Ecology and Anatomy of Iris versicolor (Wild blue flag)

Figure 10: *Iris versicolor* (© Jim Stasz)

Iris versicolor is a perennial monocot macrophyte commonly known as wild blue flag, harlequin blue flag, larger blue flag, and northern blue flag. This macrophyte grows 0.1 to 0.8 m high with leaves 1 cm wide. The roots form thick clumps of creeping rhizomes. Wild blue flag flowers form three petals and flat sepals (USDA, 2002a). The petals are often varied combinations of yellow, green, white, and blue. This species is native to North America, in eastern United States and Canada. Commonly found along shorelines, it is adapted to wet soil. There is no nutrient-removal efficiency research available on this ornamental macrophyte, likely due to its low biomass production capacity.

The eighth macrophyte that was examined in this study was *Juncus spp.*

1.3.9 Ecology and Anatomy Juncus spp. (Rush)

Juncus spp. are flowering perennial monocot macrophytes that are commonly known as rushes and this genus contains almost 300 species (Brooks and Clemants, 2000). It resembles sedges with its long dark leaf blades and is often incorrectly identified. Rushes can grow 2.2 m in length and produces a high above-ground biomass but grows slowly. This macrophyte produces dark green foliage with yellow leaves. It grows in wet soils and is native to some parts of Canada (Ontario, Quebec, British Columbia) and most of the US (USDA, 2002b). This macrophyte is commonly found in Lake Erie (Herdendorf, 1987). Although it has a slow growth rate, it is frequently used in engineered wetlands due to its biomass production.

The nutrient-removal efficiency of Juncus have been studied by many researchers noted above (Hunt *et al*., 1999; McAandrew *et al*., 2016). In 2013, Wiessner *et al*. conducted studies in Germany to examine the nitrogen removal capacity of this macrophyte in the rhizosphere. *Juncus spp* had a 45% efficiency in nitrogen removal. Regarding phosphorus removal, a study by Menon and Holland (2013) compared the phosphate removal efficiency of three species: *Juncus effuses, Carex lurida,* and *Dichanthelium acuminatum*. This study found no significant difference in nutrient consumption among the different species; however, they confirmed a difference in nutrient removal between vegetated and non-vegetated constructed wetlands. The mixed cultures had a phosphate removal efficiency of 77% (Menon and Holland, 2013).

Figure 11: *Juncus spp.* (© Bodner *et al.*, 2005)

The ninth macrophyte that was examined in this study was *Pontederia cordata.*

1.3.10 Ecology and Anatomy of Pontederia cordata (Pickerelweed)

Pontederia cordata is an aquatic perennial monocot ornamental plant. Pickerelweed reproduces by means of branching rhizomes. Flowers are tristylous with three different morphologies occurring within the same population and sometimes on the same individual. This macrophyte grows 1.0 m high with a fibrous root depth of 0.3 m. Leaf shape differs over population and on single individuals (USDA, 2002c). It is native to the American continent and commonly called pickerelweed. It grows successfully in wetlands and ponds and in flooded conditions. The pickerelweed is often grown on the shoreline for its aesthetics. As an ornamental crop, this species also has an extensive root system that allows for shore anchorage and nutrient absorption. This macrophyte provides food for birds such as water fowls. This macrophyte is commonly found in Lake Erie (Herdendorf, 1987).

Figure 12: *Pontederia cordata* (Mohlenbrock and USDA NRCS, 1995)

Pontederia cordata has a high above-ground biomass that acts as storage for nutrients. Studies in the United States and Taiwan assessed the biomass production and nutrient storage of *P. cordata.* They both, independently, concluded that this macrophyte must be harvested in summer to have the highest nutrient efficiency (Chen *et al.*, 2009). Unlike most macrophytes that translocate their nutrients to tubers below-ground at the end of fall, *P. cordata* transfers most of its nutrients to its below-ground storage organs at the beginning of fall (Want *et al*., 2014). Thus, to increase nutrient removal efficiency by harvesting the above-ground biomass, the aerial structures must be harvested in the summer.

The tenth macrophyte that was examined in this study was *Sagittaria latifolia.*

1.3.11 Ecology and Anatomy of Sagittaria latifolia (Arrowhead)

Sagittaria latifolia is a perennial monocot macrophyte. It is commonly called arrowhead, duck potato, katniss, swamp potato, tule potato, and wapato. This perennial macrophyte grows horizontal creeper rhizomes. It has large arrow-shaped leaves that make up most of its above ground biomass while it also has underwater tubers that store high quantities of biomass and are edible. The flowers form three petals. At maturity, this plant only grows to 0.3 m with an unknown root depth (Stevens, 2003). This species is native to the American continent, although there are some native species of *Sagittaria* in Europe, Africa, and Asia as well. This macrophyte is commonly found in Lake Erie (Herdendorf, 1987).

Figure 13: *Sagittaria latifolia* (Anderson, 2001)

Some researchers have theorized that this macrophyte's ability to remove nutrients relates to its high-water intake capacity. Thus, by making up for the plant's overall low biomass, it still allows for some nutrient removal (Chen *et al.,* 2009). Like some of the other species on this list, the macrophyte is not used alone in engineered wetlands, but collectively with many other species. Thus, the nutrient removal efficiency is unknown for this aquatic plant specifically.

The eleventh, twelfth, and thirteenth macrophytes that were examined in this study were *Scirpus acutus, Scirpus atrovirnes, and Scirpus validus.*

1.3.12 Ecology and Anatomy of Scirpus sp. (Bulrush)

Environment Canada provided lists for three species in the Scirpus genus: *S. acutus* (hard-stem bulrush), *S. atrovirens* (black bulrush), and *S. validus* (soft-stem bulrush). All three species are perennial monocot macrophytes and are native to the North, Central, and South American continent. As part of the giant sedge family, their growth ranges between 1.0 to 3.0 m. There are very few leaves found at the base of this macrophyte and the plant grows a terminal panicle at the top spike. Fruits and flowers are dark brown on the *S. acutus* and *S. validus*, while the *S. atrovirens* produces red flowers on the ends of its stems (Favorite, 2003a). These organisms propagate through seeds and rhizomes that require a root depth of 0.5 m. These macrophyte are commonly found in Lake Erie (Herdendorf, 1987).

Figure 14: *Scirpus acutus* (© Derek Tilley, USDA-NRCS)

Figure 15: *Scirpus atrovirens* (© USDA)

S. acutus is commonly called tule, hard-stem tule, tule rush, hard-stem bulrush, and viscid bulrush. This is because the stems grow 1.0-2.0 cm thick. In the early 1900s, it was often planted to create a buffer against strong wind and water forces on shorelines to reduce soil erosion. *S. acutus* is infrequently used in engineered wetland studies due to its rigid stems that impede hydraulic flow. Thus, there are no nutrient removal efficiencies available for this macrophyte individually (Favorite, 2003a).

S. atrovirens is present on every continent except Africa and Antarctica. It is commonly called deer-grass or grassweed. This macrophyte has grass-like leaves and is often found in freshwater ponds and wetlands. It is also observed to produce dense vegetation along

rivers, yet this species in this genus is infrequently used in engineered wetland studies. Thus, there are no nutrient removal efficiencies available for this macrophyte individually.

Figure 16: *Scirpus validus* (© James H. Miller)

S. validus is a macrophyte that is present all over the world. It is commonly known as soft-stem bulrush, grey blubrush, great bulrush, and giant bulrush. Note that this is not the same species as the giant rush. The giant rush (*Juncus ingens*) is an invasive species that grows to 5.0 m. Due to its high above-ground biomass production, the soft-stem bulrush is used in many constructed wetlands. Researchers studied the nutrient removal efficiency of this macrophyte among other high biomass species such as *Typha*. In the constructed wetlands, soft-stem bulrush had the highest nutrient removal efficiency (Finlayson and Chick, 1983; Fraser *et al.*, 2004). This macrophyte, though not yet labeled invasive, due to its fast-growing abilities and propagation through root and sprigs, may become invasive if grown as a monoculture.

Figure 17: *Sparganium eurycarpum* (© St. Mary's College of California)

The next species assessed in this study was *Sparganium eurycarpum.*

1.3.13 Ecology and Anatomy of Sparganium eurycarpum (Giant bur-reed)

Sparganium eurycarpum is a perennial monocot macrophyte native to the Americas. It is commonly called giant bur-reed or broad fruit bur-reed. At maturity, the giant bur-reed has a height of 1.5 m with a root depth of 0.3 m. It reproduces by producing creeping root rhizome systems. This perennial plant has a high above ground biomass, higher even than *Phragmites* and *Typha* at times. Studies conducted on the

nutrient capture in shoots, roots, and leaves of all three-species found that the bur reed had a higher nutrient removal rate than the common reed but a lower efficiency than the cattail (Liu *et al.*, 2012). Like *Phragmites* and *Typha*, this macrophyte has a significant above-ground biomass that gives it a competitive advantage over other wetland species. However, unlike *Phragmites* and *Typha* that grow rapidly, this macrophyte has a moderate growth rate and can be contained in a location if maintained regularly. This macrophyte is commonly found in Lake Erie (Herdendorf, 1987).

The last macrophyte species assessed was *Veronica americana.*

1.3.14 Ecology and Anatomy of Veronica americana (American brooklime)

Veronica Americana is a perennial dicot macrophyte native to the Americas. It is commonly called American brooklime and American speedwell. The American brooklime does not grow very tall with a height of 0.2 m at maturity. Its thin stems and roots do not produce sufficient biomass for nutrient removal. Thus, it is not used in engineered wetland studies.

Figure 18: *Veronica americana* *(*Hagwood, 2004)

1.4 Seed Germination

After carful macrophyte selection, the next mandate of protocol development was the examination of germination strategies. Under laboratory or greenhouse conditions, most seeds require one of two processes before germination. Firstly, germination is described as the breaking of the seed coat at which point, the radicle emerges to form the root and the hypocotyl grows to become the stem, see Figure 19 (Cavanagh, 1980).

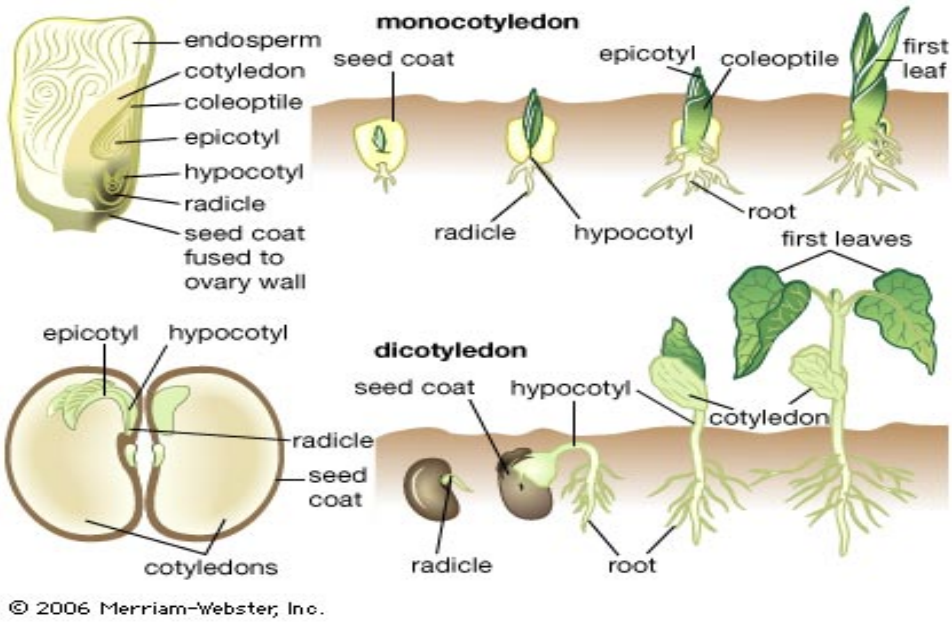


Figure 19: General germination phases in monocot and dicot seeds

Seed germination begins with a process termed "imbibition" during which the seed absorbs water which activates enzymes to increase the rate of metabolic activities. During the dormant phase, seeds continue to undergo metabolic activities; however, the rate of these processes is much slower in order to conserve energy. Dormancy can be increased by a physical hard, thick layer of seed coat or many thin layers of seed coat tightly packed together (Cavanagh, 1980).

In botany, "scarification" refers to a weakening of the seed coat in order to induce germination. To initiate germination artificially, seeds either require a superficial treatment to scarify the seed coat, or they need rigorous treatment to mimic changes in seasons, signaling optimal growing conditions (Fraser *et al*., 2014). In nature, seeds can break dormancy in many ways such as by the gastric acids in organisms that consume seeds, in being buried and re-exposed to light a few times, falling on hard surfaces, etc. Perennial plants, species that survive more than one growing season, are exposed to fall, winter, spring, and summer conditions (Cavanagh, 1980). Thus, these seeds may require rigorous treatment for scarification to occur. There are physical, mechanical, and chemical forms of seed coat scarification that result in the end of dormancy (Figure 20). The most commonly used laboratory and greenhouse methods of

seed scarification used by horticulturalists are on acid rinse, a base rinse, cold-wet storage, cold-dry storage, sandpaper treatment, or peeling the seed coat (seed nicking) to increase water absorption.

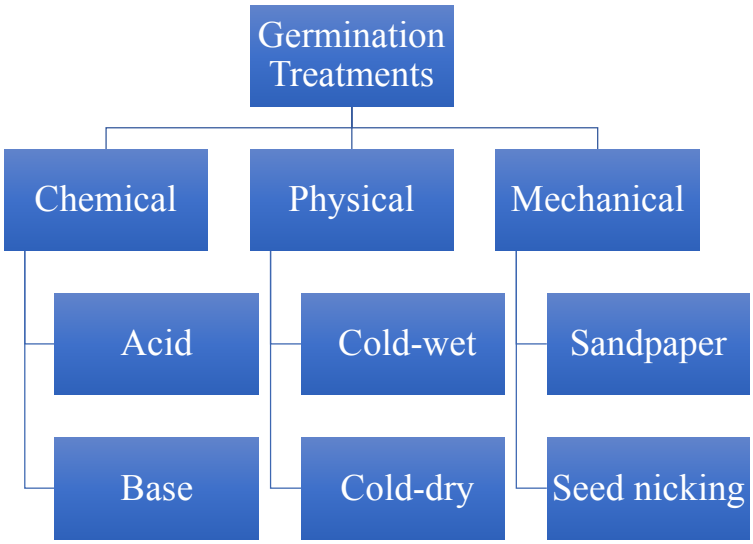


Figure 20: Germination treatments to induce scarification

1.4.1 Acid Treatment

Acid scarification is widely used as a treatment to induce imbibition and the most commonly-used acid is sulphuric acid (Can *et al.*, 2009). However, an alternative to sulphuric acid is hydrochloric acid (HCl) as HCl is present in stomach acids of organisms thus making it environmentally relevant. It is generally agreed that acid-rinsing seeds increases germination, yet the concentrations used differ among plant species and amongst research teams around the world (Pandrangi *et al.*, 2003). One reason for these differences is the length of time the seeds have spent in dormancy. Seeds that have spent longer in this state will need stronger treatment to induce water intake. Another reason is the difference in species in the genus tested; a lack of replication in germination tests with the same methods for the same species at the same dormancy age results in varied results globally.

1.4.2 Base Treatment

Base rinsing works in a similar manner to acid scarification. The most commonly-used base is sodium hypochlorite, also known as bleach. This solution acts as a seed disinfectant, rinsing away any possible fungus or infection that might decrease plant survival upon germination (Ervin and Wetzel, 2002; Butola and Badola, 2008). The longer a seed is in the dormant stage, the higher the likelihood of it becoming susceptible to pathogens. Not all seeds are stored dry and this is especially true for macrophyte seeds such as *Pontederia cordata* and *Zizania aquatica*. These seeds are not water-dependent for germination; however, they are temperature-dependent for germination. Rather than dry seeds in these species entering a dormant stage, loss of water results in termination of metabolic activities, instead of reduced metabolic rates.

Both acid rinsing and base rinsing work as effective disinfectants. Although bleaching is not environmentally-relevant, it is a laboratory measure used by horticulturalists to germinate many terrestrial seeds (Fieldhouse and Sasser, 1975; Thomas, 1981; Drew and Brocklehurst, 1984). Some botanists have observed bleach rinsing to yield a higher percentage of germination, although the biological mechanism and interaction between the base and seed coat is yet unknown.

1.4.3 Mechanical Treatment

Seeds that do not require rigorous chemical treatment may be processed using mechanical treatment to induce scarification. "General purpose" sandpaper, made from aluminium oxide, is sometimes used to lightly file down the seed coat (Baes *et al.*, 2002; Patane and Gresta, 2006; Can *et al.*, 2009). This type of sandpaper is available with different grit sizes for use on materials with different textures. Sandpaper with 60 to 150 grits is most often used by botanists in germination treatments. Another method

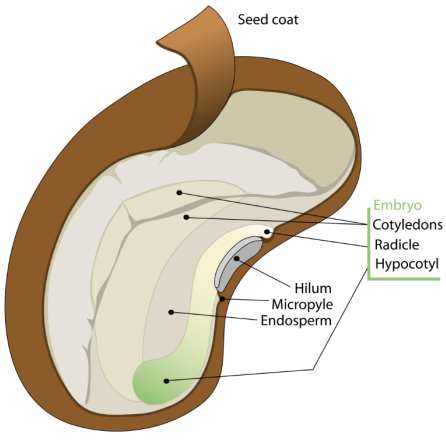


Figure 21: Seed structure (© Wikimedia Commons)

for mechanical scarification is by partial peeling of the seed coat (seed nicking) back from the hilum. The hilum is also known as the "eye" of a seed (Figure 21). It is a scar at the location where the seed detached from the plant ovary wall. Peeling the seed coat partially from this location exposes the endosperm to water, causing an expansion in endosperm that pushes the radicle out of the seed to initiate germination.

1.4.4 Cold-Wet Treatment

In the environment, the macrophyte seeds would have fallen into the water after the previous growth season. These seeds would overwinter at the bottom of the aquatic environment throughout the winter and would germinate when the water started to get warmer with rising spring temperatures (Donohue, 2005). Thus, by placing all the seeds in cold dark water we attempted to imitate winter.

1.4.5 Cold-Dry Treatment

A few types of seeds require cold and dry conditions to scarify the seed coat. The dry environment results in seed coat shrinking. Since there is no change in endosperm mass, this reduction in seed coat results in thinning and eventually cracking of the seed coat. This may be mimicked in a laboratory by mixing the seeds in vermiculite evenly. Vermiculite is a hydrous phyllosilicate mineral and is used in gardening for moisture removal and recommended for germination treatments by the USDA (2011).

PART II – Developing a Small Constructed Laboratory Wetland to Sequester Contaminants

1.5 Engineered Wetlands

Engineered wetlands are like natural wetlands except that they are constructed. They are built to mimic the biological, chemical, and physical processes that occur in a wetland (Vymazal, 2007). Constructed wetlands are built to serve specific objectives and thus components such as

vegetation, substrate, width, depth, hydraulic loading rate, and water retention time are chosen or calculated accordingly.

1.5.1 Use of Constructed Wetlands for Non-Point Source Pollution Removal

After decades of research, excess nutrients from terrestrial biomes running off into adjacent freshwater and marine ecosystems are still an issue. This concern has moved from focusing on point-source phosphorous input, particularly from detergents in municipal sewage treatment plants (Schindler, 1974) to excess nutrients from non-point source phosphorus and nitrogen in agricultural runoff. Whether commercial fertilizers, pesticides, or biosolids (See Section 1.1.3) are being added to farms, heavy thunderstorms can cause runoff of these substances into nearby aquatic ecosystems.

The use of constructed wetlands specifically to treat agricultural runoff was first documented decades ago. In 1987, a wetland was assembled using a plastic liner and some gravel on the shore of Lake Tahoe (Reuter *et al*., 1992). The objective of this research was to reduce nitrogen, phosphorus, iron, and suspended sediments and other runoff constituents from entering the lake. The data collected over the next two years showed an 85 to 90% reduction in nitrate, 47% removal of particulate phosphorus, and 84% reduction in total reactive iron. In this experiment, no vegetation was reportedly used. Due to the limitations of their design and gravel contamination during construction, the total Kjeldahl-N that passed through increased by 3% and the soluble phosphorus increased by 28% (Reuter *et al*., 1992).

Research was carried out by Berg (1998) on pollutant removal strategies proposed by the National Center for Environmental Assessment (NCEA) legislation in Baltimore. The project used a 30-m swale and berm system to collect surface water runoff. These created basins with their altered macrophytes allowed for longer water retention times during storms and resulted in exceeding the phosphorus removal specified by the Baltimore City Critical Area Program by 10% (Berg, 1998).

Studies examining constructed wetlands used to treat stormwater runoff analyzed components such as nitrate, ammonium, and phosphate (Johengen and LaRock, 1993). Macrophytes such as *Pontedaria sp.* and *Lemna sp.* were planted in some basins while other

basins were filled primarily with sediments and plankton in Florida, (United States). A concentration range of 0.5-1.5 mg/L of nutrients was added to the constructed wetlands and the concentration was monitored over time (Johengen and LaRock, 1993). The vegetated wetlands displayed the highest removal of nitrate (67%), ammonium (87%) and phosphate (62%) per day compared to the sediment mesocosms which removed 52, 59, and 49% per day respectively. The quantity of phosphorus being removed was greater than the phosphorus quantity required to support aquatic organisms and biomass (Johengen and LaRock, 1993). This research was an example of small-scale constructed wetlands. While these wetlands prove highly successful in nutrient removal, it is difficult to replicate wetland efficiency results due to the number of variables that interact with each other. Nevertheless, a literature review conducted by the University of Waterloo on the uses of constructed wetlands for removal of excess nutrients found small-scale constructed wetlands to have a higher efficiency of nutrient removal due to a larger water-to-soil surface area ratio (Cheng and Basu, 2017).

Large-scale constructed wetlands have been built for stormwater runoff nutrient removal in the Everglades, south of Lake Okeechobee (Figure 22).

Figure 22: South Florida everglades constructed wetland aerial view (By South Florida Water Management District)

Six constructed wetlands were assembled to cover an area of 16,400 hectors in 1994. One of the main major focuses was the reduction of phosphorus loading while defining a concentration threshold that would still be to support flora and fauna (Guardo *et al.*, 1995). Nutrients such as phosphorus, that are essential for the growth of many organisms, in reduced or excess amounts can disrupt biodiversity (Vymazal, 2011). Thus, the objective in the Everglades study was to reduce excess nutrients entering the Everglades. This project attained a phosphorus reduction median of 71% annually by 1999 and is on-going in this area (Guardo *et al*., 1999).

As the design guidelines for constructed wetlands improved, their use in removing pesticides from agricultural runoff increased. Along with pesticides, other agricultural contaminants were analyzed and their removal was compared to the water residence time (Rodgers and Dunn, 1992). The water residence time refers to the duration when the water remains in a constructed wetland before flowing out of it. The treatment of non-point source pollution from agricultural lands was advanced using constructed wetlands with vegetation such as cattails (*Typha sp*.), bulrush (*Scripus sp.*), and reeds (*Phragmites sp*.). At the time, these native species were considered to provide good ground cover during seasons of high nutrient runoff (Hammer, 1992).

The St. John Valley watershed of northern Maine observed huge algal blooms contaminating Long and Cross Lakes. Constructed wetland systems were assembled to treat the agricultural runoff. The design they used consisted of sedimentation basins, vegetated strips with wetland between them, and multiple detention ponds (Bouchard *et al*., 1995) as they ascertained that the structure and function of the constructed wetland are linked. Thus, the design parameters dictate the efficiency of the engineered wetland and in this particular study, the design helped reduce the total phosphorus by 85-88% and the suspended solids by 96-97% (Bouchard *et al*., 1995).

1.5.2 Global use of Engineered Wetlands

On other continents, constructed wetlands for nutrient removal in runoff have shown varying degrees of effectiveness using different parameters. Below are some of these studies.

1.5.2.1 Single Parameter Modification

In Northern Ireland, many lakes began to face eutrophication due to excess phosphorus from agricultural systems (Wood and McAtamney, 1996). The subsequent constructed wetlands were assembled by using laterite in the substrate, a mineral commonly found in this geographic location. It is a clay-like substance that is rich in iron and aluminium. Phosphorus entering the engineered wetland binds to these metal ions. These bound compounds settle out in the constructed wetland bed due to sedimentation. Thus, the resulting effluent from the constructed wetland contains significantly lower quantities of phosphorus entering the lakes. Pilot-scale wetlands that used laterite showed a 96% removal of phosphorus (Wood and McAtamney, 1996). This research demonstrates that a slight change in a constructed wetland parameter, in this case such as the addition of laterite to the substrate, can greatly alter the nutrient removal efficiency of an engineered wetland.

1.5.2.2 Multiple Parameter Modification

Two groups of researchers conducted experiments separately in the Ukraine and in the United Kingdom (UK) with constructed wetlands. In the Ukraine, they used a combination of vertical and horizontal (Figure 23) systems with a hydraulic retention time of days (Magmedov *et al.*, 1993).

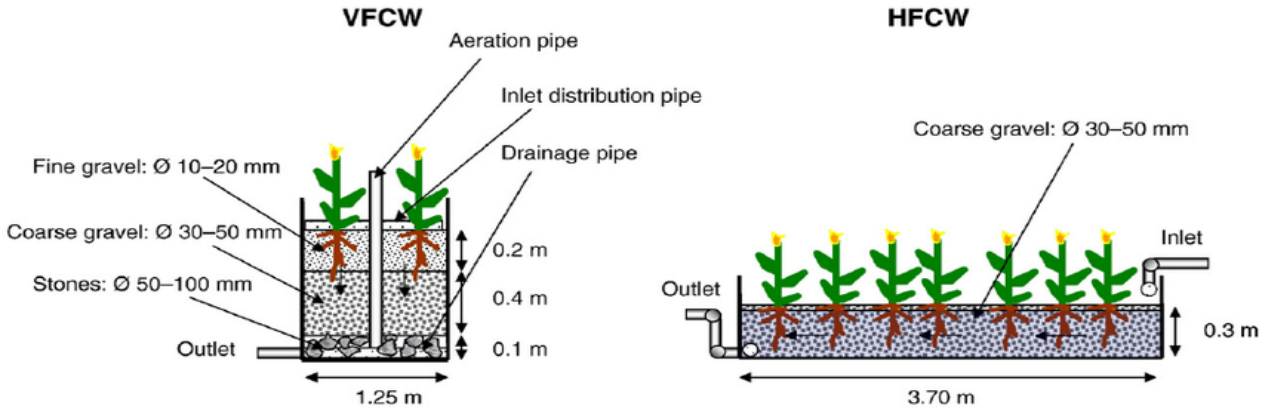


Figure 23: Vertical Flow Constructed Wetland (VFCW) and Horizontal Flow Constructed Wetland (HFCW)

Vertical systems rely on gravity and sedimentation of large particulates for water filtration whereas horizontal systems rely on the water movement rate as it moves over a certain distance. In the United Kingdom (UK), researchers changed the water retention times to minutes from days for a horizontal flow system. They built a second wetland in the UK which had a vertical flow and a water retention time in days (Cooper and Green, 1994). In the Ukraine, *Phragmites* and *Typha* were planted, while in the UK mainly *Phragmites* was planted (Magmedov *et al.*, 1996). Varied wetland widths, depths, loading rates, and hydraulic retention times still produced satisfactory results in these qualitative studies (Magmedov *et al.*, 1996) although the constructed wetland in the Ukraine demonstrated higher efficiency. That wetland attained a high purifying efficiency for biological oxygen demand species, suspended solids removal, a removal efficiency of 60-90% for ammonia, 80-95% for nitrate and 90-98% for coliform removal, while P removal was described as "mediocre." While phosphate removal was deemed "mediocre," the key point that this research emphasized is that the parameters of a constructed wetland play a significant role and can be manipulated with minimal changes that effect the constructed wetland efficiency results.

1.5.2.3 Hydrological Changes Affecting Engineered Wetland Efficiency

Research done during this same time in Victoria, Australia, examined the capacity of constructed wetlands to sequester excess nutrients from stormwater runoff (Raisin *et al.*, 1997). Due to the extreme hydrological events and seasonal conditions, a single large constructed wetland at shore level only retained 11% total nitrogen (TN) and 17% total phosphorus (TP) annually. Raisin *et al*. (1997) observed that a few, smaller-sized wetlands up stream had a better cumulative effect in reducing excess nutrients that would have collected lower in the downstream watershed.

In New Zealand, constructed wetlands were used to remove excess nitrogen in drainage coming from irrigated and rain-fed dairy pastures. Parameters such as organic nitrogen (Org-N) and total nitrogen (TN) were analyzed over a three-month period. The median volume of nitrogen entering the wetlands ranged from 6.5 to 10 g/m^3. Comparisons of nutrients in the influent and effluent showed a 99.8% removal for Org-N and 96% for TN (Tanner *et al.*, 2003). Further research on the same constructed wetlands over a few years demonstrated that the

increase in nitrogen entering the engineered wetlands was directly related to rainfall, soil-water status, and irrigation events. When more water was added to the pasture, naturally or manually, if soil absorption capacity was surpassed, there was an increase in nutrient flow (Tanner *et al.*, 2005).

Though the macrophytes used in both studies above were unidentified, the key point here to note was that the changes in hydrological activity influenced the wetland efficiency of nutrient removal. An increase in hydrological activities would result in low water retention times and higher water movement rates which, in a large wetland that had a low water-to-soil surface area ratio, result in less interaction time for nutrient removal.

1.5.2.4 Seasonal Effects on Constructed Wetland Efficiency

Jiang *et al*. (2005) looked at non-point source nutrient removal and the difference in nutrient sequestration in macrophytes. They studied the vertical and horizontal distribution of organic matter and TN in reeds (*Phragmites communis*) and wild rice (*Zizania latifolia*) and discovered that macrophyte uptake of nutrients in the above-ground biomass depends on the season. The below-ground sediments and macrophyte biomass had a higher nutrient and organic retention throughout the year. During the fall season, the plants were harvested to reduce the return of nutrients into the wetland. The harvested reeds removed 818 Kg/hm^2 of N and 103.6 Kg/hm^2 of P while the harvested wild rice removed 131 Kg/hm^2 of N and 28.9 Kg/hm^2 of P (Jiang *et al.*, 2005).

1.5.3 Types of Engineered Wetlands

Engineered wetlands are characterized based on their flora, water inflow, direction of water flow within the engineered wetland, and depth. Different types of engineered wetlands are used to meet diverse objectives. The four most common types of biomass used are: emergent plants (ex. *Carex sp.*), floating-leaved plants (ex. *Nuphar variegate*), free-floating plants (ex. *Lemna minor*), and submerged plants (ex. *Elodea canadensis*). The free-floating and floating leaved vegetation have their roots in the water column, whereas the emergent and submerged

macrophytes have their roots in the sediments (Figure 24). Emergent macrophytes can also be grown on a buoyant surface on the water to create floating constructed wetlands.

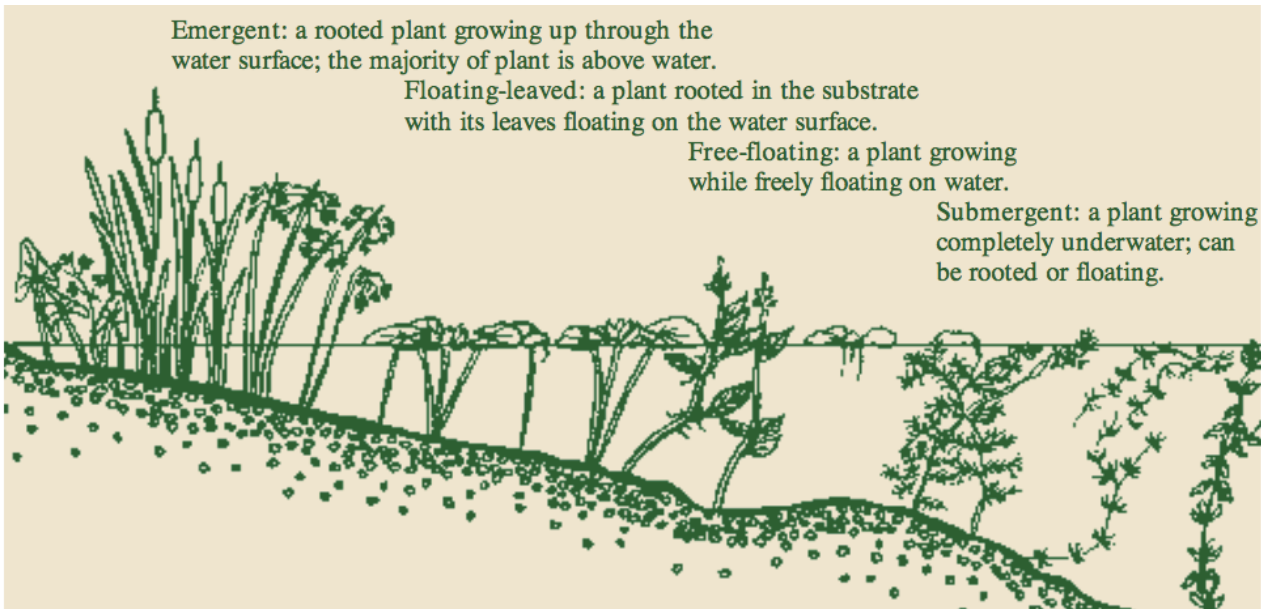


Figure 24: Wetland macrophytes (Environment Canada, 1996)

The water entering a constructed wetland could be from overland runoff and is referred to as 'Surface flow' or through a pipe leading the influent into the wetland, under the land and called 'Sub-surface flow' (Vymazal, 2007) (Figure 25). Free floating and floating-leaved macrophytes are often exposed to surface flow water. This design allows their roots, that may be closer to the surface, to have immediate and increased exposure to incoming nutrients. Due to emergent plants having part of their body in the water column and above the water surface, they can thrive in surface and sub-surface water flow. However, the submerged macrophytes require a subsurface flow of water for the nutrients to be more effectively absorbed by their roots, grounding the sediment (Figure 25) (Vymazal, 2007).

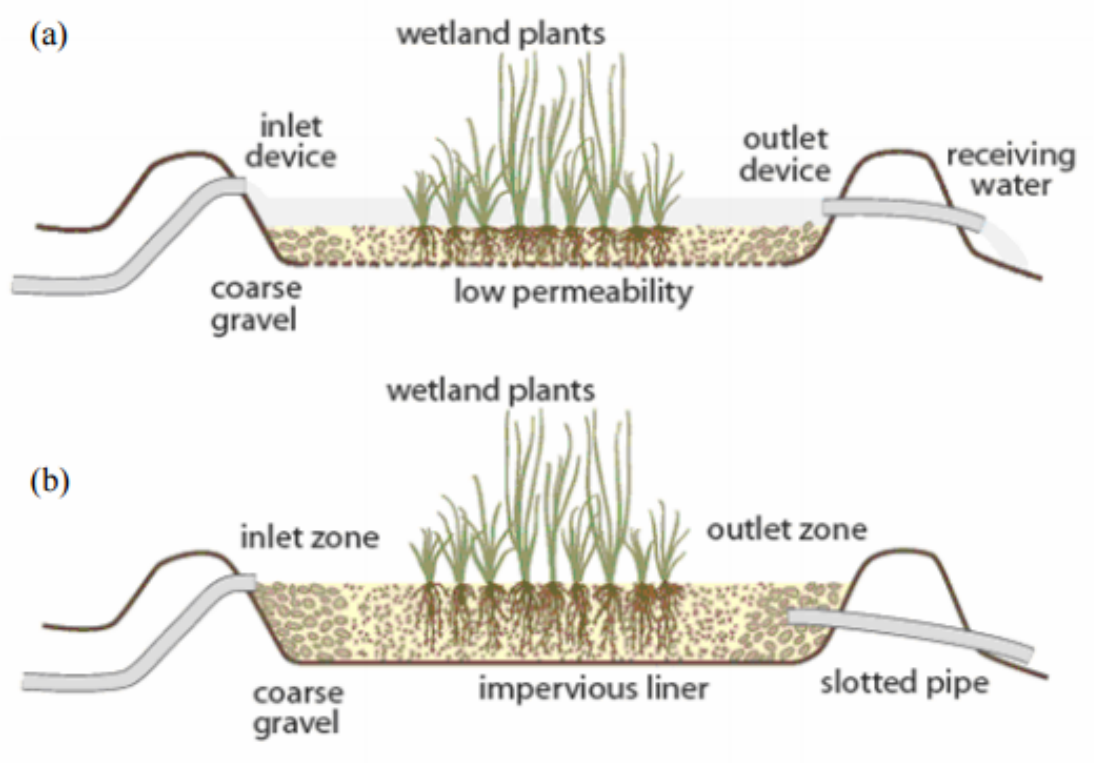


Figure 25: Types of constructed wetlands (a) Surface flow, (b) Subsurface flow (Gearheart, 2006)

The water in an engineered wetland can be treated by using a horizontal flow, vertical flow or a hybrid of the two flows. Treatment refers to the chemical, biological, and physical interactions the constituents of the water have with the wetland as it flows from inlet to outlet. The direction of water treatment will then dictate the depth of the water in an engineered wetland. In a horizontal flow constructed wetland, a larger surface area with shallow to moderate depth is required for nutrient removal. On the other hand, a vertical flow constructed wetland would need greater depth and layering of varied size sediments to aid in nutrient and contaminant filtration with a smaller surface area (Vymazal, 2007). Most engineered wetlands, to some degree, have a hybrid system of vertical and horizontal flow. Figure 26 outlines these differences clearly. For the purpose of this study, a hybrid system of an engineered wetland, with emergent plants is chosen.

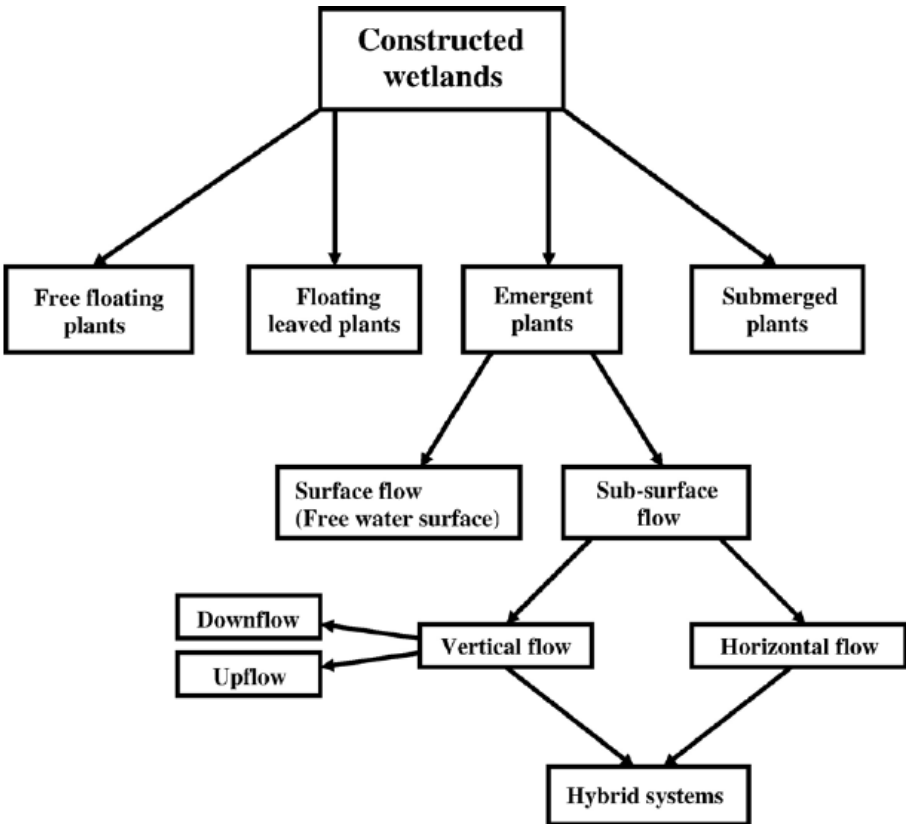


Figure 26: Breakdown of the Different Types of Constructed Wetlands (Source: Modified from Vymazal, 2001)

1.6 Pollutant Removal Mechanisms in Engineered Wetlands

Table 2 below briefly lists the diverse mechanisms. The main processes occurring are: sedimentation and accretion, filtration, matrix adsorption, plant uptake and organism absorption, and microbial denitrification. (Brown, 1985; Richardson, 1985; Gehrels and Mulamoottil, 1990; Mitsch and Reeder, 1991).

Table 2: Contaminant removal mechanisms in an engineered wetland (Cooper *et al.*, 1996)

Wastewater Constituents	Removal Mechanisms
Suspended Solids	- Sedimentation and accretion - Filtration
Soluble organics	- Aerobic microbial degradation - Anaerobic microbial degradation
Phosphorus	- Matrix sorption - Plant uptake
Nitrogen	- Ammonification followed by microbial nitrification - Denitrification - Plant uptake - Matrix adsorption - Ammonia volatilization (mostly in SF system)
Metals	- Adsorption and cation exchange - Complexation - Precipitation - Plant uptake - Microbial oxidation / reduction
Pathogens	- Sedimentation - Filtration - Natural die-off - Predation - UV radiation (SF system) - Excretion of antibiotics from roots of macrophytes

1.6.1 Sedimentation and Accretion

The water in an engineered wetland is often stagnant, depending on the hydraulic loading rate and retention time. This allows for gravity to attract floating particulates in the water to sink to the bottom sediments of the wetland. This process helps to filter out contaminants by separating the solids from the liquid (Puddephatt, 2013). To achieve the highest efficiency of this process, the volume of water inflow and residence time of the water within the wetland must be

taken into consideration. Depending on the purpose of the engineered wetland, compounds such as aluminium, iron, or calcium may be added to increase the adhesion of particulates to each other. The formation of such precipitates in an engineered wetland is known as accretion (Wood and McAtamney, 1996).

1.6.2 Filtration

Runoff from agricultural lands can carry with it many contaminants from the land that are insoluble. These suspended solids such as detritus and particulates are often filtered out of the water when moving horizontally brushing against and sticking to the macrophyte stems and/or leaves. In a vertical flow constructed wetland, these contaminants filter through the different gravel sizes, blocking larger particulates from moving further down the wetland (Wood and McAtamney, 1996).

1.6.3 Matrix Adsorption

Constructed wetland vegetation with fibrous root systems allow for high surface area. This increase in area provides a larger surface for particles in the water to bind and attach. The process of this adhesion, of one substance onto another, is called adsorption. Phosphates may adhere to macrophyte structures or larger suspended particulates (Cooper *et al.*, 1996).

1.6.4 Plant Uptake and Organism Absorption

Plants require certain quantities of nutrients for their growth and reproduction and phosphorus, being a limiting nutrient in freshwater ecosystems, is crucial to organism development (Schindler, 1974). The nutrients from the water in the wetland is taken up by the plants through root absorption. As new leaves, fruits, and seeds are formed, the nutrients are stored in these organs (Richardson, 1985).

1.6.5 Microbial denitrification

Plant roots are often in symbiotic relationships with micro-organisms. Heterotrophic bacteria such as *Pseudomonas* help convert nitrites and nitrates to nitrogen gas. This gas can then leave the water, entering the atmosphere in a gaseous state (Fennessy and Cronk, 1997). These bacteria use organic matter as an energy source. Within the constructed wetland, this source can come from decaying leaves. In the absence of decaying vegetation, bacteria such as *Planctomycetes* and *Nitrosomas eutropha* can oxidize ammonia to get energy to reduce nitrites to nitrogen gas (Kadlec and Wallace, 2009).

1.6.6 Phosphorus Removal

Since phosphorus acts as a growth-limiting nutrient in many aquatic ecosystems, mechanisms used specifically for phosphorus removal in wetlands are crucial to this study. Phosphorus is present in mainly two forms in wetland water: dissolved and particulate. The primarily dissolved form of phosphorus is formed as orthophosphate which is readily bioavailable to algae and plants. Phosphorus removal occurs through absorption and desorption, plant and microbial uptake, fragmentation and leaching, mineralization, and sedimentation and burial. This compound may go through various transformations during the life-time of a wetland. These processes are displayed in Figure 27 by Reddy and D' Angelo (1997).

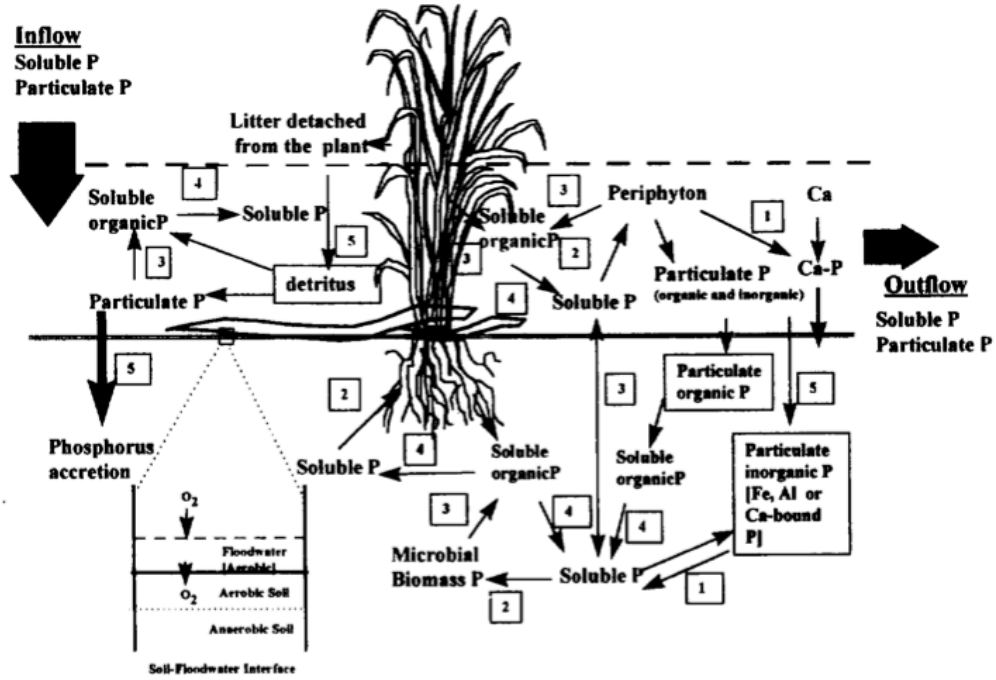


Figure 27: Phosphorus transformations in constructed wetlands (from Reddy and D'Angelo, 1997). (1) adsorption and desorption, (2) plant and microbial uptake, (3) fragmentation and leaching, (4) mineralization and (5) sedimentation and burial.

Adsorption is the process by which a molecule attaches to the surface of another substance. In wetlands, phosphorus removal, cycling, and storage may occur through coprecipitation with Al and Fe oxides and cations (Nichols, 1983; Reddy and D'Angelo, 1997). Acidic to neutral pH environments are required for such reactions and wetlands are usually in this range (Kadlec and Wallace, 2009). An increased amount of phosphorus is removed from wetlands through macrophyte absorption, rather than cation adsorption.

Phosphorus uptake by plants has been the most significant mechanism of phosphorus removal in wetlands due to their high biomass. Microbial populations also take up phosphorus; however, due to their low biomass and rapid turnover rate, they are unable to store phosphorus for long periods of time (Nichols, 1983; Vymazal, 2007). As microbes complete their life span, plant structures such as leaves break away, the movement of water may induce fragmentation of phosphorus particulates. These fragmented phosphorus particulates sometimes adsorb onto the wetland matrix and leach into the sediment. The accumulation of phosphorous in wetland

sediment or peat is known as the long-term method for phosphorous removal (Nichols, 1983; Reddy and D'Angelo, 1997; Vymazal, 2007; Mustafa *et al*., 2011). The sedimentation of phosphourus into the substrate is usualy the result of phosphorus-bound suspended solids that range from 1μm to > 100 μm (EPA, 2000). These particulates settle to the bottom of the wetland due to gravity (White *et al*., 2000; Mayer *et al*., 2006; Kadlec and Wallace, 2009).

Due to microbial and plant uptake of oxygen in the top layers of wetlands, anoxic conditions at the bottom of a wetland reduce microbial decomposition of organic matter due to lack of oxygen, resulting in permanent phosphorus burial (Smolders *et al*., 2006). This phosphorus can be mineralized into bioavailable phosphorus again through the addition of bicarbonate that is produced by macrophyte rhizosphere (Smolders *et al*., 2006).

1.6.7 Nutrient testing using bioassays

To test the efficiency of a constructed wetland for its reduction of excess nutrients, biological tests can be carried out. Phytoplankton bioassays are often used as a measure of nutrient availability for reproduction (Miller *et al*., 1978; Bostan, 2000). A common test organism used for the bioassay is *Pseudokirchneriella subcapitata.*

1.7 Test organisms - *Pseudokirchneriella subcapitata*

These organisms are a unicellular, chlorophyll-producing, and crescent-shaped algae (40-60 um^3). They are commonly present in North American fresh waters, which makes them a relevant test organism (ECCC, 2007). Under stressful conditions, these organisms reduce their chlorophyll production, loose their crescent shape, and reduce density (McCarthy, 1994). *P. subcapitata* (Figure 28) has been used extensively in biological monitoring and thus has been very well established in acute, and chronic toxicity studies. Cultures of *P. subcapitata* are easy to grow, maintain, and inexpensive to raise in a laboratory (EPA, 2002).

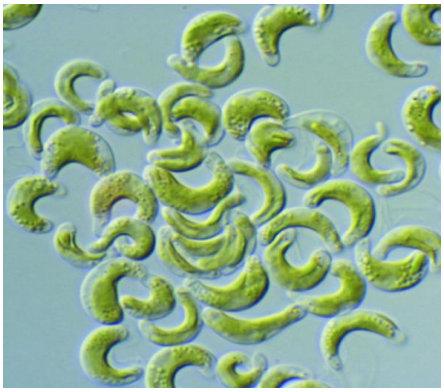


Figure 28: ***Pseudokirchneriella subcapitata*** **(40-60 µm^3)**

2.0 HYPOTHESES

The mandate of this study is to alleviate non-point sources of contaminants, leading to eutrophication, for example phosphorus, by using constructed wetlands. A key component of these engineered wetlands is the vegetation and thus macrophyte selection, germination, and use in a simple laboratory model will help advance this mandate. Lastly, in using bioassays and chemical analysis, a rudimentary experimental section assessed phosphorus sequestration by the macrophytes.

We propose the following hypotheses:

1. Varied germination treatments will yield different seed germination efficiencies across the different macrophytes.

2. Water samples from constructed wetland will vary in impact on water-column organisms in the phytoplankton bioassay over time.

3. The concentration of phosphorus in water sampled from the vegetated engineered wetland will change over time.

A summary of the experiments carried out to test these hypotheses can be found in Figure 29 on page 47 and Figure 39 on page 65

3.0 METHODOLOGY – PROTOCOL DEVELOPMENT

This research was conducted in an effort to determine the best macrophytes and emergent wetland models to be developed in combination with runoff treatment to adjacent bodies of water. This section describes all the testing used in this research to develop:

1. A selection method for appropriate constructed wetland macrophytes

2. An efficient engineered wetland laboratory model to sequester nutrients

The primary objective of this study was to develop a protocol and hence the methodology section was written in a format similar to that of government protocols. It must be noted that while most of the individual test methods described henceforth originated from research described in the literature, modifications allowed the development of a new protocol for small wetland development.

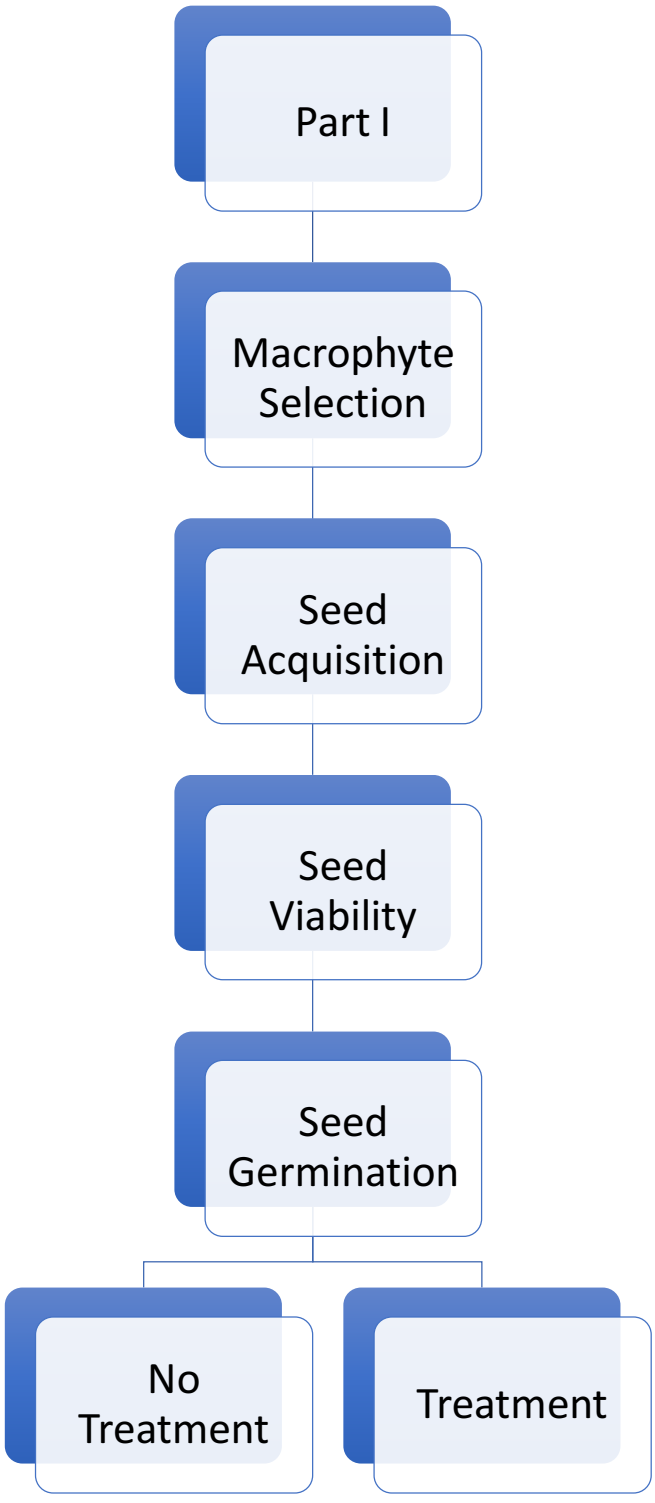


Figure 29: Summary of methodology for Part I from Macrophyte Selection to Germination Treatments Protocols used

3.1 McCarthy Laboratory Protocol for Cleaning Glassware and Other Objects used in Experiments (modified from Environment Canada; Puddephatt, 2013)

3.1.1 Materials and Equipment

Non-phosphate detergent, Extran (purchased from VWR Scientific)

Plastic Container with sufficient depth and width to completely submerge items being cleaned

Hydrochloric Acid (HCl) 10% (v/v)

Milli-Q water, from Millipore Corporation with 18.2 MΩ·cm at 25°C

Distilled water

3.1.2 Cleaning Protocol (Modified from Environment Canada; Puddephatt, 2013)

Note: Prior to use in experiments, all glassware and other items must be thoroughly cleansed.

1. In a large enough container to hold all the items to be cleaned, a soap solution of dechlorinated municipal drinking water (DMDW) with a sufficient quantity of Extran was prepared as per the manufacturer's guidelines.
2. Items were submerged in the solution and soaked for a minimum of 15 minutes.
3. Proceeding the wait period, the items were thoroughly finger-scrubbed and rinsed with DMDW to remove any residue.
4. These items were then soaked in a solution of (10% v/v) HCl for a minimum of 10 minutes to remove any remaining particulates, metals, or bases. This was followed by rinsing each item thrice with distilled water.
5. After the distilled water rinse, these items were rinsed three times with Milli-Q water and left to air dry.

3.2 Decision-Making for Macrophyte Selection

In comparing the list of emergent macrophyte species (Table 1) with those studied in the literature including botanical specimens grown both locally and globally, the macrophytes list (Environment Canada, 1996) was modified based on multiple characteristics such as origin, invasiveness, life span, growth rate, ability to absorb large quantities of nutrients, food web status, and root structures. These characterises were relevant for this study to understand the long-term effectiveness these macrophytes would have in constructed wetlands for the purpose of nutrient and contaminant removal. These characteristics might differ for other studies based on their study objectives. Built on these established objectives for our study, the following protocol was developed as a general procedure to be used for macrophyte selection in future laboratory constructed wetland studies.

3.2.1 Protocol development for Macrophyte Species Selection for use in Constructed Wetlands

1. Based on constructed wetland model chosen for assembly, determine which types of macrophytes will have the highest surface area-to-water ratio: emergent, floating-leaved, free-floating, submerged

2. Categorize the list of available native non-invasive macrophyte species into the four types of aquatic plants: see the comments above for emergent, floating-leaved, free-floating, submerged. Choose one or two group of plants to work with. Choose multiple species to create a sustainable biodiversity.

3. Using the list of species created in step 2, cross-check the list with the literature, botanical and horticultural societies, and government data bases to acquire information on the growth rate and life cycle of the chosen macrophytes. Macrophytes with a moderate to fast growth rate are preferable over slow growth rate. A slow growth rate indicated inefficient uptake and/or inefficient use of nutrients for macrophyte biochemical requirements.

4. Create new categories based on the study objective to test macrophytes. These new categories aid in sifting through the macrophyte list for trait- specific macrophytes.

5. Check for species that might be endangered, toxic to animal consumption, and eaten by birds and insects. Endangered species might be difficult to acquire compared to other macrophyte speeds. Aquatic plants that are toxic to animal consumption might be harmful and must thus be avoided. Macrophyte species consumable by birds and insects are acceptable and help increase ecosystem biodiversity when not choose ornamental plants for aesthetic appeal.

6. Combine the aforementioned categories studied into a table of results. Assign the species a binary system of 'Yes' for meeting criteria and 'No' for not meeting the criteria or lack of information.

7. Assign each 'Yes' a single point and each 'No' a zero. Add up all the points and select the macrophytes that make the third quartile. The third quartile in statistics includes the entities that are in the highest scoring range, i.e. meeting 75% or more of the criteria.

3.3 Seed Acquisition

The development of a decision-making protocol to determine macrophyte selection (section 3.2), was followed by acquiring these macrophyte seeds. The seeds for seven different species were purchased from two different companies. *Alisma plantaga-aquatica (*Water plantain*), Carex vulpinoidea (*Sedge*), Pontederia cordata (*Pickerelweed*), Sagittaria latifolia* (Arrowhead)*, Scirpus validus* (Soft-stem bulrush*), and Sparganium chlorocarpum (*Giant bur reed*)* were bought from Speare Seeds, a Canadian distributor in Harriston, Ontario. Seeds for *Asclepias incarnata* (swamp milkweed) were purchased from Wildflower Farm, located in Coldwater, Ontario.

Speare Seeds sold seeds "by the pound". Thus, 453.6 grams (one pound) of each of the six macrophyte seeds were purchased. These seeds were harvested from wild fields by horticulturists and supplied to the company. The company receives the seeds in one of two stored forms: dried or wet (pers. comm. Krista Hale, Office manager, Speare Seeds). Once the seeds are collected from the field, most suppliers increase preservation by drying them and subsequently storing in a freezer. Some suppliers store seeds in water in a dark space. The company packaged the seeds in plastic zip-lock bags and mailed them to us. Five out of the six different macrophyte

seeds ordered from this company arrived in dry-sealed polythene bags. The seeds for *Pontederia cordata* arrived in a sealed polythene bag filled with water. This bag was placed in a larger airtight plastic covering to prevent leakage during transportation.

The second company used to acquire seeds was Wildflower Farm. This company has been in business since 1988. They have their own farm and greenhouse in Coldwater, Ontario. They harvest the seeds from the native plants that they themselves grow and use the same seeds to grow new crops depending on demand (pers. comm. with Paul Jenkins, co-founder, Wildflower Farm). Five grams of seeds were ordered from this company. Seeds were transported dry in a paper envelope sealed within a larger mailing envelope.

3.4 Seed Viability

Angiosperms are flowering plants and their seeds go through a process called embryogenesis (Evert and Eichhorn, 2013). In this process, the plant forms embryos after fertilization to produce seeds. During this phase, the cells differentiate to form the endosperm, plumule, radicle, hypocotyl, and cotyledon. At the end of embryogenesis, the maturation phase begins and the seed undergoes desiccation losing approximately 90% of water. At this stage, the seed coat forms around the embryo as protective covering seen in Figure 30 (Evert and Eichhorn, 2013).

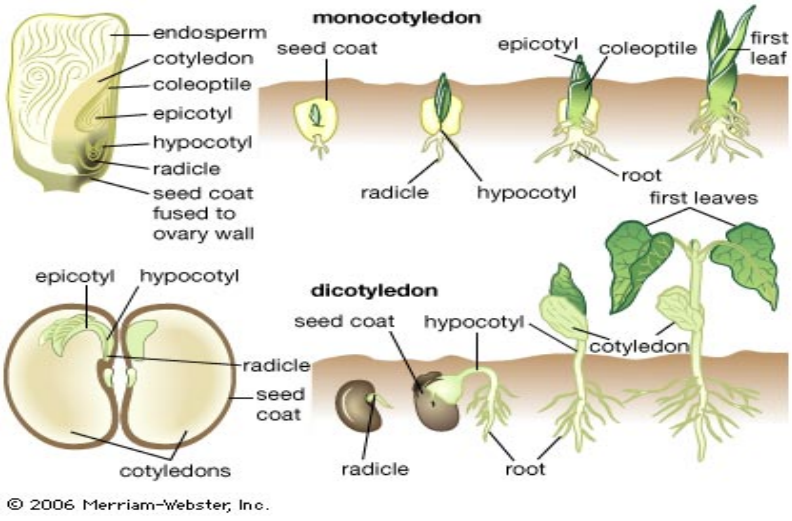


Figure 30: General germination phases in monocot and dicot seeds

This results in reduced metabolism of the seed. The seeds may then enter a dormant stage until favourable germination conditions are met. Dormant seeds carry out all the same metabolic processes as non-dormant seeds, only at a lower rate (Bewley, 1997). Since dormant seeds and dead seeds appear alike even under a 10x magnifying glass, a viability test would increase the chances of germination by aiding us in distinguishing the two types of seeds. Protocols for terrestrial seed viability exist in Canada, but only for agricultural crops by the Canadian Food Inspection Agency (CFIA) (CFIA, 2012). For many terrestrial seeds, a tetrazolium chloride test is sometimes carried out to detect viability. This test is not yet officially recognized in Canada, except for western wheatgrass (CFIA, 2012), but is accepted for agricultural crop seeds in the United States (Wickert *et al*., 2017). The protocol for carrying out a tetrazolium chloride test can be found in the Handbook on Tetrazolium Testing by the International Seed Testing Association (Muschick, 2010). By carrying out a simple aquatic seed viability test, we can estimate the percent success rate of germination. A common viability test is conducted by checking for seed respiration. Below is the protocol for such a test.

3.4.1 Protocol for Aquatic Seed Viability Testing

3.4.1.1 Materials and Equipment

Glass Beakers, 50 mL to 150 mL (depending on seed size)

Macrophyte seeds

Hot plate

Thermometer

Distilled water

3.4.1.2 Seed Viability Test Protocol

1. Fill beakers halfway with distilled water. Test the temperature of the water using a thermometer. If the water is below 15.0°C, heat beakers on a hot plate to raise

temperature. If water is above 15.0°C, cool water by placing in a refrigerator until temperature is 15.0°C.

2. Place seeds in 15.0°C water in the beaker and wait for eight hours.

3. Regularly monitor seeds for carbon dioxide production through bubble formation.

Note: This temperature was chosen because it causes a stratification response in seeds by mimicking early spring water temperatures (Roberts, 1988).

3.5 Germination Containers

Upon determining seed viability, germination strategies are developed. These first tests involved simple germination with no prior "seed treatment" such as scarification. Initial seed germination for *Alisma plantaga-aquatica* (water plantain*), Asclepias incarnata* (swamp milkweed) *Carex vulpinoidea* (sedges*), Pontederia cordata (*pickerelweed*), Sagittaria latifolia (*arrowhead*), Scirpus validus (*soft-stem bulrush*), and Sparganium chlorocarpum* (giant bur reed*)* was attempted in plastic germination trays as suggested by the CFIA (2012) (Figure 31). Each tray had dimensions of 0.53 m by 0.26 m by 0.06 m. Within this tray, there were 72 cells and each hexagonal cell had dimensions of 0.03 m by 0.03 m by 0.06 m. These trays were purchased from Pro-Hex. To the germination trays was added sieved reference soil using a sieve with 0.2 mm spacing. Reference soil was obtained from Stratford, Ontario from an Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) research agricultural site.

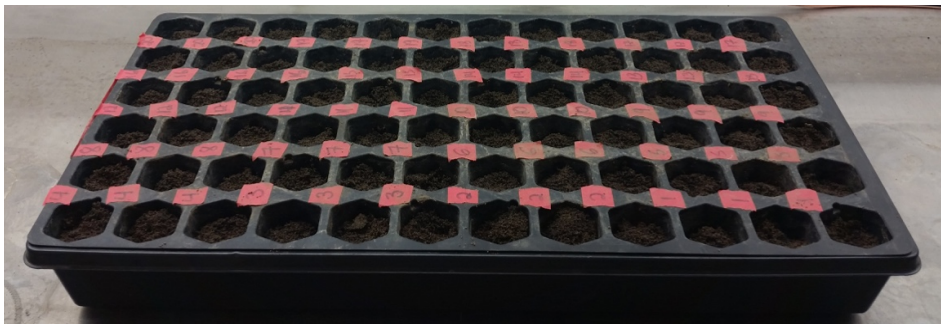


Figure 31: Germination tray with 72 cells

Three seeds were placed 1 mm deep in each moist compartment under a 12-hour light and 12-hour dark cycle. Observations on macrophyte growth were made every 24 hours. Large seeds (seeds from *P. cordata* and *S. chlorocarpum)* were placed 2 mm deep in their respective compartments. Since these seeds were heavier than the other seeds, under natural conditions, they are more likely to fall deeper into the soil in the environment and placing them more deeply into the soil attempts to mimic conditions. The germination trays were examined every two days and replenished with 10 mm of distilled water. Using a 10 mL beaker, water was added to the bottom of each tray. Moisture was also measured using a moisture meter (For more information on germination conditions please refer to Section 3.7).

After an initial lack of germination in these trays, these germination trays were replaced with growth chambers (Figure 32) that included a plastic dome to reduce evaporation. The difference between germination trays and growth chambers is that growth chambers have a plastic dome that helps retain moisture. Growth chambers came in two dimensions: 0.27 m by 0.27 m by 0.05 m and 0.25 m by 0.09 m by 0.05 m and both were used based on commercial availability. These chambers were purchased from Jiffy Greenhouses and consisted of pods filled with sphagnum moss. Each pod had dimensions of 0.03 m by 0.03 m (length x width).

The initial “germination without treatment" did not yield any germination due to dormancy and thus required treatment to induce scarification.

Figure 32: Growth chambers by Jiffy Greenhouses

3.6 Germination Treatment

All seeds were put through the viability test (section 3.4) before being sown for germination tests. Figure 33 outlines the different treatments used to trigger germination.

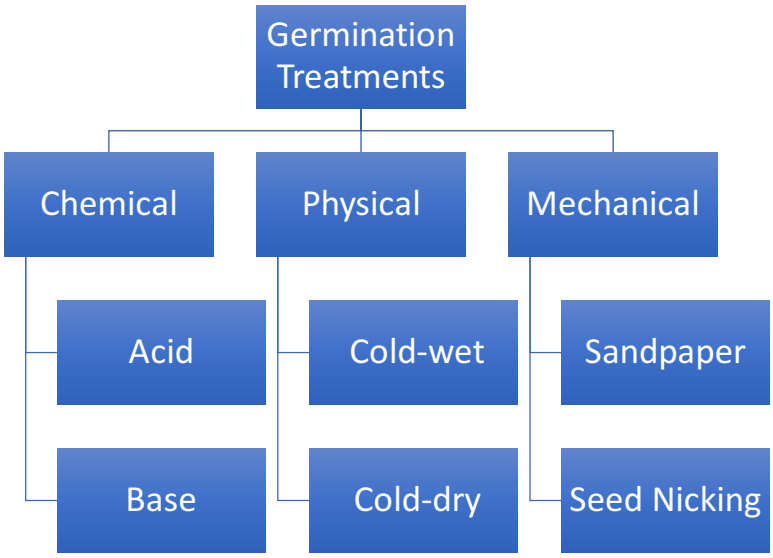


Figure 33: Seed treatment methods used to induce successful seed germination for the selected macrophytes

Chemical germination treatments that included acid and base rinses were carried out after the viability tests (i.e. post-viability). This was done to treat only the alive seeds to induce germination. Physical (cold-wet and cold-dry strategy) germination treatments were carried out before the viability test (i.e. pre-viability). By carrying out the treatment prior to the viability test, the seeds were moved from a cold to a warm environment. If the reverse was carried out, i.e. viability test first followed by cold-wet treatment, this would induce dormancy by unintentionally mimicking seasonal change from summer to fall. The mechanical (sandpaper scarification and seed-nicking) germination treatments were also carried out before the viability test (i.e. pre-viability). This was not a temperature dependent string of events, however in carrying out the mechanical treatment first, I avoided a higher chance of obtaining false-negative results. Since passing the viability test relies on water uptake and consequently bubble production due to gas release, if the seed coat was brittle, as it was for the seeds that underwent

the mechanical treatment, there was a higher chance of alive seeds not being able to take up water similar to dead seeds due to their thick seed coats. These differences, when the viability test was carried out, were to account for the purpose of conducting a viability test that was threefold: (1) distinguish between alive yet dormant seeds compared to dead seeds, (2) induce water absorption, and (3) to act as a reference i.e. no treatment. For the purpose of acting as a reference point of comparison between treated and non-treated germination results, all these seeds were directly sown after the viability test without any treatment before or after the viability test. The non-reference seeds received their treatment according to Table 3.

The highest water absorption is theoretically achieved when the seed coat is at its most vulnerable and these germination treatments aid in increasing this vulnerability. Selection of treatments was based on seed size and seed coat texture. This selection was necessary for environmental relevance and to reduce seed coat damage (Table 3). For example, small thin seeds would not require mechanical treatment versus large seeds (Roberts, 1988).

Table 3: Germination treatments assigned for selected macrophyte seed based on seed coat and texture of seed coat

	10% Acid	25% Acid	10% Base	25% Base	Sandpaper	Seed Nicking	Cold Wet	Cold Dry
A. plantaga-aquatica	✓	✓	✓	✓	✘	✘	✓	✘
A. incarnata	✓	✓	✓	✓	✓	✘	✓	✓
C. vulpinoidea	✓	✓	✓	✓	✘	✘	✓	✘
P. cordata	✓	✓	✓	✓	✘	✘	✓	✘
S. latifolia	✓	✓	✓	✓	✘	✘	✓	✘
S. validus	✓	✓	✓	✓	✓	✘	✓	✘
S. eurycarpum	✓	✓	✓	✓	✓	✓	✓	✘

3.6.1 Protocol Development for Aquatic Seed Germination Treatment

3.6.1.1 Materials and Equipment

Hydrochloric Acid (HCl) 10% v/v and 25% v/v

Sodium Hypochlorite (NaClO) 10% v/v and 25% v/v

Glass Beakers, 50 mL to 150 mL (depending on seed size)

Graduated Cylinder, 100 mL

Sandpaper, 150 grits (can be purchased from Mastercraft)

Plastic brush, 2 cm long bristles

Utility Knife, 18 mm (can be purchased from Canadian Tire)

10x Magnifying glass

Parafilm Tape

Refrigerator

Vermiculite

Distilled water

3.6.1.2 Acid Treatment (Pandrangi et al., 2003; Can et al., 2009)

As mentioned earlier in the literature review, acid treatment helps to thin the seed coat by mimicking the gastric acid environment of herbivores. There are two concentrations used due to a lack of standardization for acid treatment germination of aquatic seeds. If the seeds did not germinate in two different concentrations, then it could be concluded that acid rinsing these seeds is inadequate and would not be carried out in further testing of these seeds.

Protocol

1. Make a 10% v/v and 25% v/v acid solution by adding 10 mL and 25 mL HCl to 90 mL and 75 mL distilled water in a large glass.

2. Place twenty seeds in two separate glass beakers and pour each prepared solution in the beaker sufficient to submerge seeds.

3. Let seeds stand in the solutions for thirty minutes. Rinse seeds with distilled water. Dry seeds using paper towels.

3.6.1.3 Base Treatment (Fieldhouse and Sasser, 1975; Thomas, 1981)

Similar, to acid treatment, base treatment helps to thin the seed coat. Though this treatment is not environmentally relevant, it is a verified horticulturalist laboratory technique used in seed germination. Two separate concentrations were once again used to test if this treatment had any effect on these particular seeds.

Protocol

1. Make a 10% v/v and 25% v/v base solution by adding 10 mL and 25 mL NaClO to 90 mL and 75 mL distilled water in a large glass.

2. Place twenty seeds in two separate glass beakers and pour each prepared solution into the beaker sufficient to submerge seeds.

3. Let seeds stand in the solutions for thirty minutes. Rinse seeds with distilled water. Dry seeds using paper towels.

3.6.1.4 Sandpaper Treatment (modified from Fathahi et al., 2011)

This is a mechanical treatment that results in seed coat thinning. In nature, seeds are often exposed to rough surfaces like stones and rocks that they rub against and this treatment mimics this environmental situation that could induce water intake and thus result in germination.

Protocol

1. Cut the aluminum oxide sandpaper in squares of 0.05 m length. If seeds are larger than 0.03 m, cut larger squares of sandpaper sufficient to cover seeds once folded.

2. Place a single seed at a time in a single square of 150 grits sandpaper. Fold the sandpaper in half to cover the seed on both sides. Gently file down the seed coat for 30 seconds.

3. Dust off seed coat particles on the seed and sandpaper using small plastic brush.

4. Reuse the same sandpaper square until the grits are worn down more than 50%. This may be assessed by counting the number of aluminum oxide protrusions remain on the square after use.

3.6.1.5 Seed Nicking Treatment (modified from Tadros et al., 2011)

This treatment is only carried out for large seeds with thick skins that might require slight incisions (Figure 34) in the seed coat before water absorption to expand the endosperm and germinate.

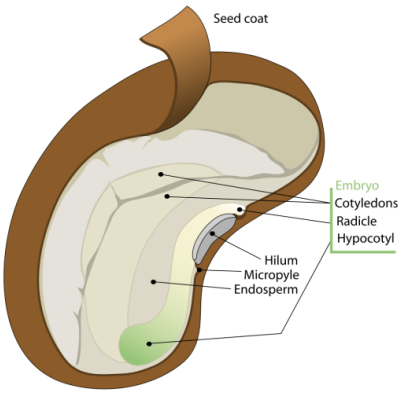


Figure 34: Seed structure (© Wikimedia Commons)

Protocol

1. Using a 10x magnifying glass, observe the seed and find the hilum.

2. From the hilum (Figure 34), with the help of a utility knife, gently peel back 50% of the seed coat to expose the plumule and part of the endosperm.

3.6.1.6 Cold-Wet Treatment (Donohue, 2005)

This treatment helps to mimic the environment's winter frost period. On exposing the seeds to warm temperatures, the seasonal change mimicked activates the enzymes to promote an increase in metabolic rate and induce water absorption.

Protocol

1. Place twenty seeds in a beaker. Fill this beaker with distilled water sufficient to submerge the seeds.

2. Use one beaker for each macrophyte seed. Cover the top of the beaker with parafilm tape.

3. Place the beakers in a dark refrigerator at 1.0°C for one month.

3.6.1.7 Cold-Dry Treatment (USDA, 2011)

Seed coat drying is a commonly used method for the swamp milkweed seed to break open the scarified seed coat. Drying helps to shrink the seed coat just enough to make it vulnerable to water absorption.

Protocol

1. Place a layer of vermiculite at the bottom of a plastic bag. Transfer a layer of twenty seeds on top of the vermiculite. Place a second layer of vermiculite on top of the seeds.

2. Force out any excess air in the bag and seal the bag with tape.

3. Place the bag in a dark refrigerator at 1.0°C for one month.

3.7 Environmental Conditions for Seed Germination

3.7.1 Light

The growth chambers were placed under a custom-built light-bank that was 1.2 m wide by 2.4 m long. The light bank consisted of five 1.2 m T8 VitaLux light bulbs. The light was measured using a digital light meter that provided a light reading in Lux. This unit was converted to uE/m^2/s and thus the lightbank emitted 100 μE/m^2/s for 12 hours a day. Light intensity is a measure of brightness at the surface of measurement. Light irradiance is the flux of radiant energy per unit area (Quaschning, 2003). One Einstein is equal to one mole of photons. This

measure is named in honour of Albert Einstein because of his work on light quanta and explanation of photoelectric effects in 1905 (Cerny 2000; Puddephatt, 2013). Though $\mu E/m^2/s$ is not a standard SI unit, it is more appropriate for measuring the irradiance compared to Lux (an SI unit) that measures the intensity of light. Germination chambers were randomly arranged under the light bank and rearranged every two days.

3.7.2 Moisture, pH, and temperature

Soil moisture was measured using a soil moisture meter. This instrument displayed a reading between 0 (dry) to 10 (saturated). Although most plant water needs are species-dependent, a reading between 6 to 8 was acceptable for most plants (Soil Moisture Meter, nd). Dry soil was kept moist by replenishing the bottom of the germination chambers with 5 mm additions of water as needed. The reference soil was tested for ideal pH values between 6 and 7 using a pH meter. The temperature under the light bank was 23°C ± 1°C (Tian *et al*., 2015).

Figure 35: Germination chamber setup for various treatments under light bank

3.8 Peat Mix

Once the cotyledons emerged, the macrophytes were transplanted into larger plastic containers. These containers were partially filled with a peat mix. This mix consisted of sphagnum moss mixed with water in a composition of 8.5 L of raw peat with 2 L of H_2O. This is done to treat the peat because dry peat is hydrophobic and would have dried out the soil. The peat is important for the plants because it provides the plants with organic matter (Jurgen *et al.,* 2017) and mimics the substrates found in many natural wetlands. Note that the germination pods in the germination growth chambers also consisted of sphagnum peat moss allowing consistency in the growing environment.

3.9 Transplantation

When the plants began to display their cotyledons, seedlings were transplanted to larger containers, to allow for the roots to have sufficient depth, adequate nutrients, and enough soil for anchorage. These young plants were transplanted into different containers to identify the best environment for them to grow.

3.9.1 Square containers

Initially, the plants that germinated in the growing chamber were relocated into plastic planters with dimensions of 10.5 cm by 10.5 cm by 12 cm. These containers had four holes at the bottom to allow for water uptake from the water tray while also allowing for roots to grow through (Figure 36). These were 5 cm in diameter. The square containers were filled with a 1:1 peat-to-reference soil mix.

Figure 36: Plastic single plant growth containers

3.9.2 Rectangular Containers

In order to allow for increased horizontal space, the macrophytes were subsequently transplanted into a rectangular container with dimensions of 58.5 cm by 17.5 cm by 13 cm. The rectangular containers were filled with a 1:1 peat- to- reference soil mix. Since these containers were longer, they permitted multiple plants to be relocated into one container (Figure 37). This helped to increase the biomass in a single container, compared to having lower biomass in multiple containers. The seedlings were hence, moved from the germination chambers to the rectangular containers to create models with higher biomass.

Figure 37: Rectangular multiple plant growth containers

3.9.3 Polyvinyl Chloride (PVC) Tubes

For the purpose of growing the seedlings during the first two months, the afore mentioned (Figure 36 and 37) containers sufficed. However, as these macrophytes began to grow, the lack of soil depth arrested the above-soil biomass production. Thus, new macrophyte seedlings were transplanted into 3 L open ended tubes (Figure 38). Each end of the tubes was covered with 0.5 mm polyester mesh sheets to keep the soil in place yet allowed the roots to grow through. Each tube contained mixtures of peat and soil at a ratio of 1:1 to provide nutrients and rooting media for initial macrophyte growth. These 0.36 m tubes allowed for increased root depth and root-soil anchorage.

Figure 38: Vertical growth tubes for macrophyte biomass elongation

3.10 Plant Biomass Bioassay

In order to determine the effects of biosolids (detailed in subsequent sections) on biomass production, parameters such as stem length, stem width, leaf length, and leaf width were measured periodically (every week) using a digital micrometer.

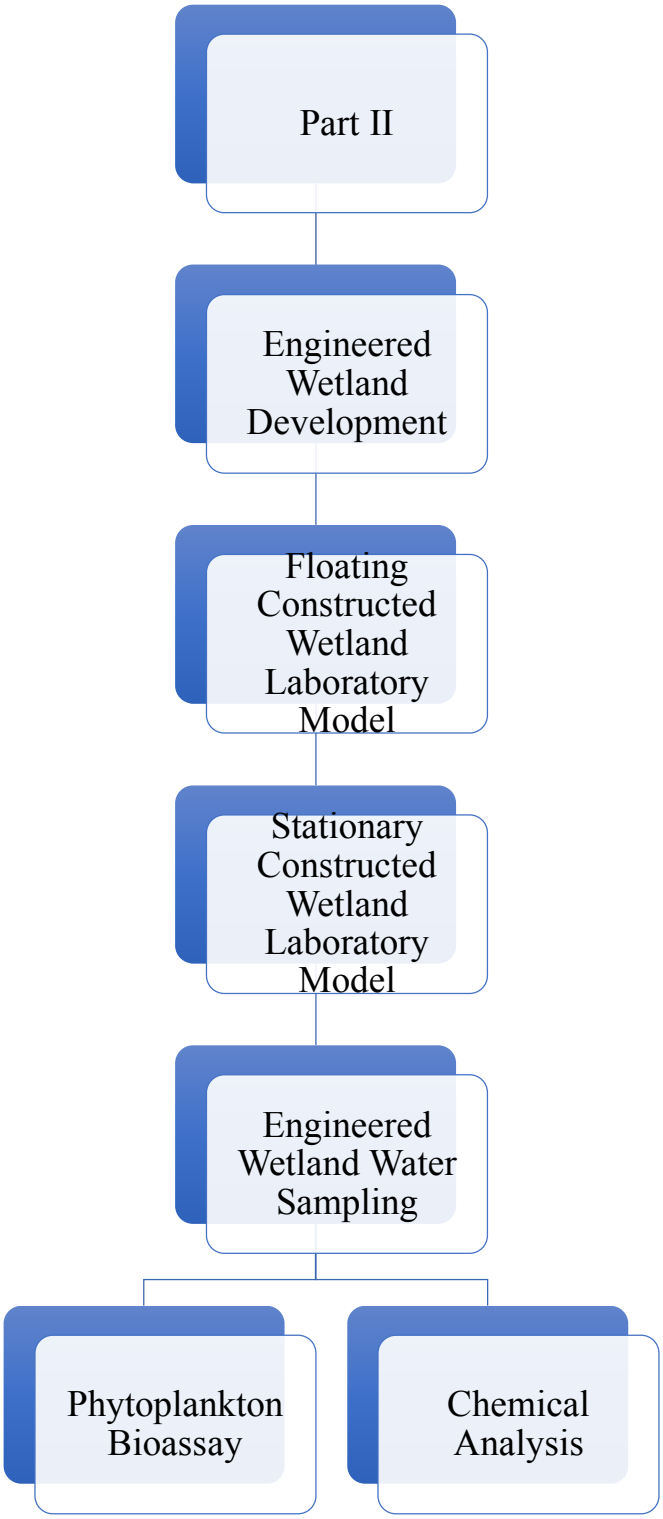
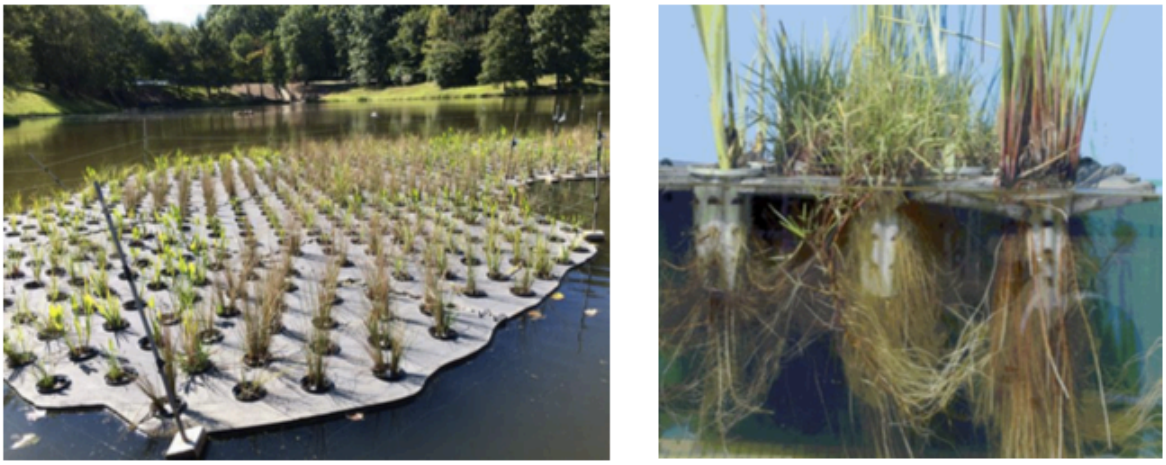


Figure 39: Summary of methodology for Part II: Developing an Engineered Laboratory Model and Test for Efficiency of that Model

3.11 Developing a "Floating Constructed Wetland" Model

The floating constructed wetland (Figure 40) is a rapidly- increasing model type that is being studied in the United States, especially at George Mason University in Virginia, United States (McAndrew *et al*., 2016).



(a) (b)

Figure 40: An example of a floating constructed wetland on Mason Pond, United States (©Beermats LLC 2016)

An advantage that this model has over the other two models (horizontal and vertical constructed wetland models) is that the wet "land" itself is floating on an aquatic water body. This model maybe constructed from different materials such as rubber tarpaulin or a buoyant sheet. Macrophytes are then transplanted into small openings in the tarpaulin (Figure 40). The floating wetland may be used seasonally and is easily harvested at the end of summer or fall to avoid the return of nutrients into the water. Floating wetland systems follow a hybrid system of hydraulic flow.

3.11.1 Materials and Equipment

Trough, large enough to grow macrophytes

Ethylene propylene diene monomer (EPDM) pond liner, without algaecide (used to prevent leaching into the soil from building materials) (Puddephatt, 2013)

Substrate, reference soil

Peat mix

Plastic planter

Plastic mesh, 1mm spacing (can be purchased from Home Depot)

Adhesive

Pontoons, made of foam

Macrophyte

Light bank

Distilled Water

3.11.2 Protocol for Developing a "Floating Constructed Wetland" Laboratory Model

1. Line troughs with a double layer of pond liner. Test the lined trough for leaks over 24 hours by filling the trough with distilled water to 25% of the trough volume.

2. Use the adhesive to attach the plastic mesh to the bottom of the planter to reduce substrate loss yet allow for root elongation.

3. Attach one pontoon to each side of the planter. Pontoons are buoyant floating devices that help keep attached containers afloat, if evenly distributed to balance the weight of the container.

4. Fill the planter with peat mix and test its buoyancy and balance by lowering it into the trough with water.

5. If the planter tips over, the pontoons must be adjusted on each side by resizing the pontoons.

6. Once the planter is stable, transplant a macrophyte seedling into the planter. Arrange the trough under a light bank and fill the leak proof trough to 70% of its volume.

7. Add 0.1 m depth of substrate to the bottom of the trough. Let the substrate sink and settle at the bottom of the trough.

8. Add the floating units into the trough and top the trough with sufficient water to fill it 0.05 m below its brim. Maintain water levels in troughs by regularly adding distilled water to the trough.

Figure 41: Floating Constructed Wetland Model

3.11.3 Stationary Constructed Wetland Model

The "stationary constructed wetland" has a surface-flow system which is a hybrid system of the horizontal and vertical flow systems and it provides a two-way hydraulic flow. Unlike vertical flow Wetlands that require at least 0.5 m depth, the surface flow wetland can compromise on depth if there is surface water movement as in a horizontal flow wetland. Figure 42 demonstrates the stationary constructed wetland containers used to assemble this model for this study. The procedure for assembling this model is below.

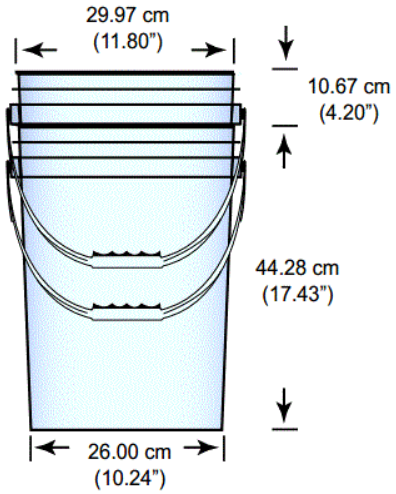


Figure 42: Constructed Wetland container measurements (© Home Depot)

3.11.4 Developing a Stationary Constructed Wetland Laboratory Model

3.11.5 Materials and Equipment

Trough or bucket, large enough to grow macrophytes

Peat mix

Polyvinyl chloride (PVC) tubes

Polyester mesh sheet, 0.5 mm (can be purchased from Home Depot)

Adhesive

Macrophyte

Fan

Light bank

Distilled Water

3.11.6 Protocol for Developing a Stationary Constructed Wetland Laboratory Model

1. Test the buckets for leaks over 24 hours by filling the buckets with distilled water to 25% of the bucket volume.

2. Using adhesives, attach the fiberglass sheet to the bottom of the PVC tube to reduce substrate loss, yet allow for root elongation (as described previously in 3.9.3).

3. Fill the tube with peat mix and test its standing stability by lowering it into the bucket with water.

4. If the tube tips over, the polyester mesh must be adjusted on each side by evening it out at the bottom.

5. Once the tube is stable, transplant macrophyte seedling into standing tube. Arrange the bucket under a light bank and fill the leak- proof bucket to 70% of its volume.

6. Add multiple tubes into the bucket and top the bucket up with sufficient water to fill it 0.03 m below its brim. Maintain water levels in troughs by regularly adding distilled water to the bucket.

7. Place an electric fan facing the buckets to aid in horizontal movement of the water.

These containers were placed on small caster-bound platforms that allowed for ease of spatial randomization (Figure 43).

Figure 43: Experimental setup of stationary constructed wetland mesocosm

In the following bioassays, only influent from the "stationary" model was used. Although the floating constructed wetland model is theoretically efficient, its success depends on the biomass production as observed in Figure 40 above. With a low biomass combined with the high surface-water evaporation due to the large trough, the data collected from these constructed wetlands would not be an accurate representation of the macrophyte's ability to sequester nutrients. Thus, the floating constructed wetland was dismissed in further analysis and only the stationary constructed wetland was used. Future studies will take a closer look at ways to reduce this potential error to evaluate floating constructed wetlands as nutrient removal system.

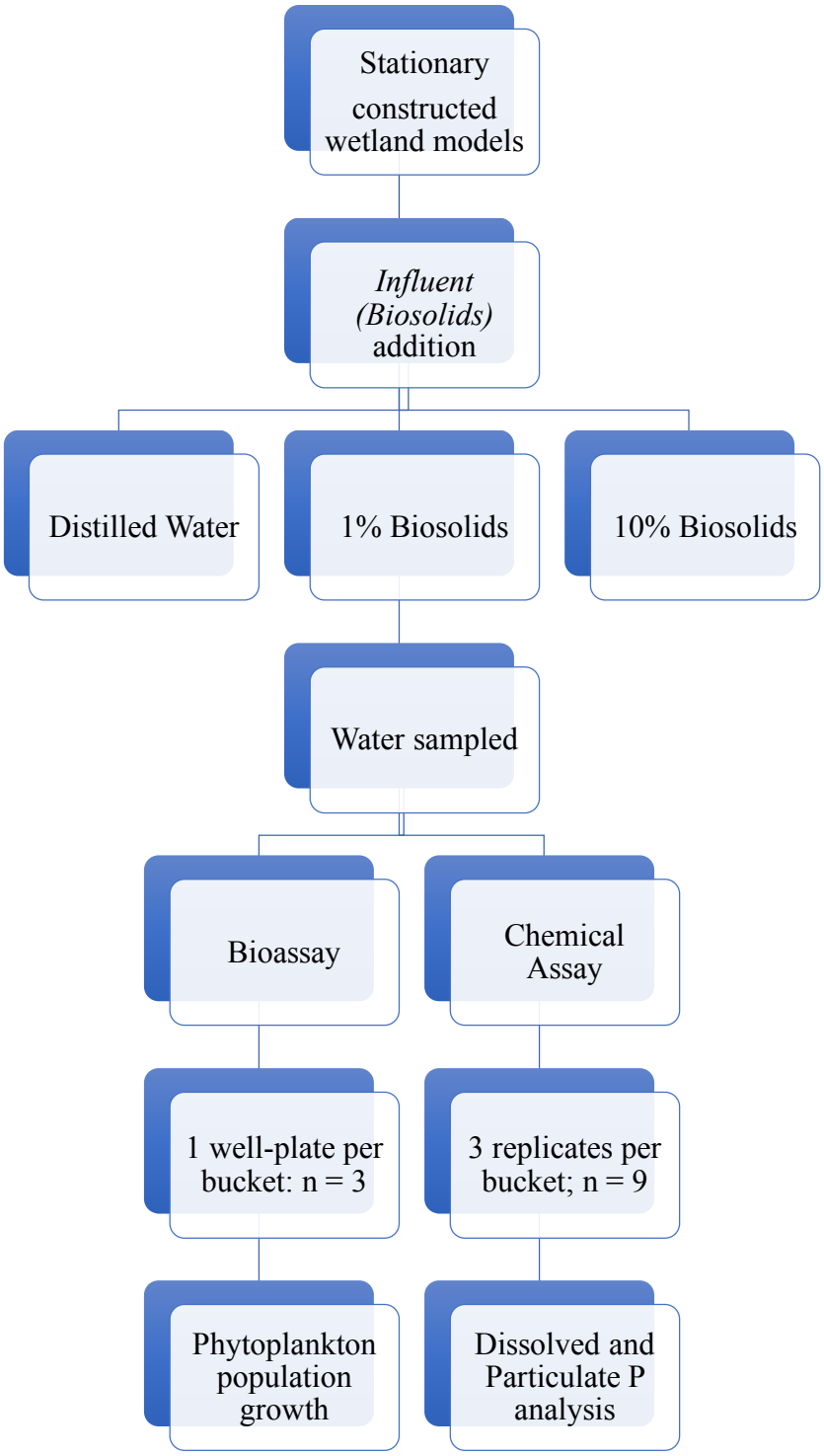


Figure 44: Diagrammatic Representation of Methodology Used to Test the Efficiency of the Constructed Wetland

3.12 Assessing Phosphorus Capture from Biosolids Runoff in a Stationary constructed wetland model

The influent used in the study to test the constructed wetland efficiency of nutrient removal was a biosolids solution. The biosolids used were obtained from Ashbridges Bay Wastewater Treatment Plant. Previous studies on biosolids runoff in this laboratory concluded that the upper limit concentration to runoff into nearby water bodies was 1% biosolids (Gebert, 2010). Two concentrations of influents were tested: 1% biosolids treatment and 10% biosolids treatment. The 10% biosolids treatment was tested to evaluate the constructed wetland efficiency in an extreme weather event that resulted in a larger than usual quantity of biosolids runoff. The biosolids sludge was mixed with 99% and 90% distilled water respectively to attain the two treatment concentrations. In the Reference containers, equal quantities of only distilled water were added. The two Treatments and the Reference each had a sample size of three from each constructed wetland group (3 groups with 3 stationary constructed wetland models per group; n = 9).

3.13 Water Sampling

3.13.1 Bioassay Water Sampling

In 50 mL beakers, 20 mL water samples were collected from 0.2 m depth in each bucket prior to influent addition. This provided a "background reference" condition. These samples were tested in well plates (Figure 45) to observe the initial nutrient conditions of the buckets. 'Well plates' also known as 'microtiter plates' are flat plates with multiple depressions that act as small test-tubes and are used in analytical tests. At 48 hours after adding the influents to the buckets, 20 mL water samples were taken and plated again. This sample collection was taken to observe immediate macrophyte nutrient removal behaviour when exposed to the nutrients. The samples were not taken at a shorter time period to allow time for spatial randomization of the buckets at 24 hours. This randomization was achieved through moving the buckets randomly under the light bank. After randomization, nutrients would turnover due to movement of the buckets, thus suspending any nutrients that were removed from the water column due to sedimentation. After these two water sample collections, the next collection was taken i) after one month, ii) after two months, and iii) after three months. This extended time frame was

chosen to allow for macrophyte nutrient uptake and biomass growth. The objective was to evaluate nutrient removal efficiency over time.

3.13.2 Chemical Analysis Water Sampling

Concomitant to biological assessment of nutrient (phosphorous) sequestering, water samples were collected for chemical analysis of reactive phosphorus. Literature has suggested that sample filtration helps to separate the dissolved and particulate forms of phosphorus in a water sample. A 0.22 um pore filter is increasingly being used within academic research compared to the previously used 0.45 um pore filter. The 0.22 um pore helps to separate a much smaller particle size, thus reducing error in sample analysis (Smith *et al.,* 1993).

Two types of water samples were collected from each bucket. Using a 50 mL syringe, 40 mL of water were collected and stored in 50 mL falcon tubes. These samples will be called the unfiltered samples. Using similar syringes, 40 mL of samples were once again collected from each bucket. These samples were passed through a 0.22 um pore filter that drained into new 50 mL falcon tubes. These samples will be known as the filtered samples. Both unfiltered and filtered samples were frozen and retrieved only when used for colorimetric analysis. Samples were stored in conditions below -10° C. Freezing the samples allowed for preservation and reduced absorption of phosphorus to the walls of the plastic container.

3.13.3 Pseudokirchneriella subcapitata culturing (US-EPA, 2002)

The green algae were obtained from the Carolina Biological Supply Company. The algae culture was grown on enriched broth (Bristol's media). The stock concentration for the nutrients can be found in Appendix IV. Algae culture was grown at 25°C ± 1°C in a 125 mL Erlenmeyer flask. Due to the dimensions of this algae, it has a tendency to settle out of the water column and end up at the bottom of the flask if not regularly re-suspended. Flasks were shaken by hand twice daily to re-suspend the algae culture. The algae cultures were placed under a light bank with 8.6 $\mu E/m^2/s$. A new culture was prepared every week by transferring 2 mL from the previous stock to a new 125 mL flask containing 100 mL of medium. This was done to maintain a supply of

"healthy" cells. Algae cell counts were carried out using a haemocytometer before each set of trials. The cell count used in the phytoplankton bioassay was approximately 1.0×10^5 cells per mL.

3.13.4 Nutrient Analysis using Phytoplankton Bioassay

This phytoplankton bioassay method is adapted from Bostan (2000) and is originally based on the US-EPA standard algal assay bottle test (Miller *et al.,* 1978). Samples collected from the buckets were tested using sterile well plates (Figure 45). Each sample was divided into 5 aliquots of 2 mL each. The plate was used for easy comparison between potential toxicity and effects of limiting nutrients on the algae. The plates were placed on a 120 rpm shaker under a 12-hour light and 12-hour dark cycle light bank for two light cycles. At the end of each light cycle, the cells in each plate were counted using a hemocytometer. Each column was assigned a letter for clarification.

The first aliquot of the original sample was unaltered (A). The second aliquot was mixed with 10^{-4} mg of P (B). The third aliquot was mixed with 2×10^{-3} mg of N (C). These last two aliquots (B and C) were used to text potential toxicity and thus were mixed with sufficient nutrients as per the growth media. The fourth aliquot was kept undiluted (D), while the fifth aliquot was diluted to 50% (E). The last column (F) was filled with growth media and used as a reference to compare the growth in the absence and presence of wetland effluent samples.

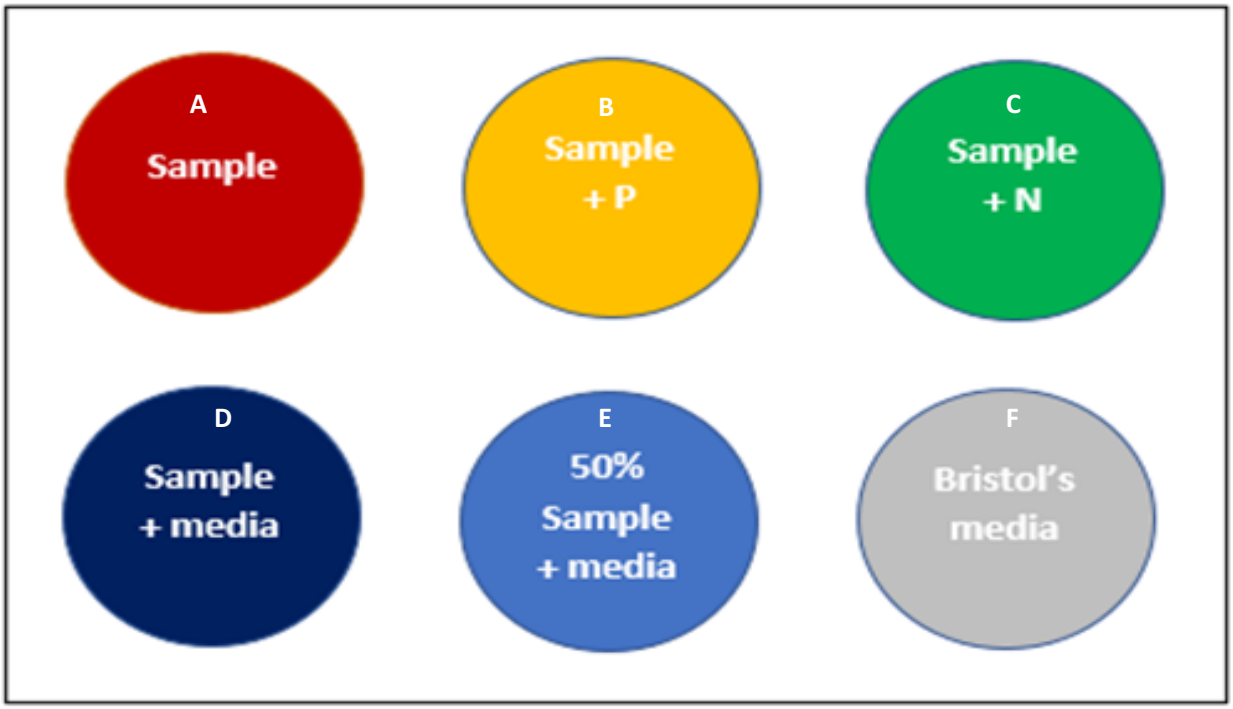


Figure 45: Well plate setup for Phytoplankton Bioassay.

3.13.5 Chemical Analysis

Reactive phosphorus (P^R), from aquatic environments, is taken up by macrophytes and is mainly composed of orthophosphate. It is termed "reactive phosphorus" because it easily responds to colorimetric tests without requiring primary sample digestion. P^R can be found in dissolved and suspended forms (Rice *et al.,* 2012). Testing the reactive phosphorus quantity in the bucket mesocosms over time helps to analyse any changes in this nutrient concentration within the engineered wetland. This allows for chemical testing of a macrophyte is effect in nutrient/pollutant and potential contaminant removal.

Since we are testing for P^R, there was no need for primary sample digestion. The samples were processed for colorimetric analysis under the ascorbic acid method. This method is suitable for detecting a phosphorus range of 0.01 to 6 mg of P/L

3.13.6 Ascorbic Acid Method

The stocks used to prepare the combined reagent used for colorimetric analysis can be found in Appendix VI. Ammonium molybdate and potassium antimonyl tartrate react in acid with the reactive phosphorus in our samples that is mainly composed of orthophosphate. This reaction forms a heteropoly acid called phosphomolybdic acid. When exposed to ascorbic acid, phosphomolybdic acid is reduced and consequently produces a strongly coloured molybdenum blue (Hanief *et al.,* 2015; Rice *et al.,* 2012). By using a spectrophotometer, the intensity of the 880nm wavelength of light emitted can be recorded. The higher the concentration of reactive phosphorus within a sample, the stronger the intensity of light that will be emitted by the sample and detected by the machine. A cuvette with a light path of 1 cm was used to detect the P concentration range from 0.15 mg/L to 1.30 mg/L.

The combined reagent is formed by mixing the above stock solution for a total volume of 100 mL reagent. 50 mL of stock I, 5 mL of stock II, 15 mL of stock III, and 30 mL of stock IV are combined in that order. Solutions are added to a flask one at a time. Before the next solution is added, the flask is swirled until any precipitate that might develop is dissolved. Solutions are mixed at room temperature and if the temperature of the combined reagent is out of this range during this process, the mixture is set aside to return to room temperature before the next stock solution is added (Rice *et al.,* 2012). The combined reagent is stable for only three to four hours after preparation (Figure 46). The standard phosphate solution is prepared by diluting 50 mL of stock V in 1000 mL of d.H_2O to attain 2.50 μg P in 1.00 mL solution.

Figure 46: Sample preparation for UV-spectrophotometer analysis

3.14 Statistical Analysis

The statistical tests carried out were based on the data collected with consideration given to the objectives of the experiment. There were four tests conducted: i) seed germination; ii) plant biomass bioassay; iii) phytoplankton bioassay, and iv) chemical assays.

The independent variable for the seed germination test was "Treatment" (Reference - warm water vs warm water with enhancers) while the dependent variable was "percent germination". The independent variable for the plant biomass bioassay, phytoplankton bioassay, and chemical assay experiments was "treatment" (Reference vs. 1% biosolids; reference vs. 10% biosolids). The dependent variables for the plant biomass bioassay were data collected on stem length, stem width, leaf length and leaf width. The dependent variable for the phytoplankton bioassay was reproduction of cells. For the chemical assay, the dependent variable was quantity of dissolved, particulate, and total phosphorus over time.

All the dependent variables were quantitative in nature (numerical) where actual measurements could be obtained, as opposed to qualitative data based on subjective observations. The germination results could be categorical or quantitative based on the objective of the study (Puddephatt, 2013). In this case, the germination efficiency was of importance and thus, quantitative data was more appropriate to collect.

The percent germination data were discrete in nature and thus no assumption was made regarding their distribution. These data were analyzed using non-parametric Friedman test (Whitlock and Schluter, 2014). The number of replicates (n) was three for the plant biomass bioassay and phytoplankton bioassay due to spatial restrictions, whereas the replicates for the chemical assay were nine. These replicate values were small which makes it difficult to determine if the data followed a normal distribution and thus met the assumptions of a parametric test (Zar, 1984; Rodger, 2004; Puddephatt, 2013). However, the sampling was random, independent, and had homogeneities of variance. Thus, by meeting the other assumption of a parametric test, the data could be analyzed using a one-way ANOVA to compare treatment means (Whitlock and Schluter, 2014). This is an appropriate analysis method since three groups were analysed; Reference, 1% biosolids and 10% biosolids. SPSS Statistics software was used to carry out the statistical analysis in this study.

4.0 RESULTS AND DISCUSSION

Overview

The use of vegetation in engineered wetlands has long been understood to result in higher pollutant removal compared to stormwater ponds (Whigham and Simpson, 1978; Ulrich and Burton, 1984; Mench *et al.*, 2009). Research on the different kinds of constructed wetlands has demonstrated the effects of changing parameters on pollution removal efficiencies. Vymazal *et al*. (2007) effectively described the different types of constructed wetlands and their purposes for types of wastewater treatment. However, research on macrophyte selection for engineered wetlands and aquatic plant germination strategies continues to be sparse and thus, this thesis attempted to address this dearth. Additionally, and critically, mitigating the effects of non-point source pollution, particularly nutrient runoff, is critical.

The following results have been acquired through using the above-mentioned methodologies (Chapter 3). The Results and Discussion section has been divided into two parts:

Part I: Macrophyte protocol development that examined i) macrophyte selection, ii) germination, iii) plant biomass bioassay
Part II: Assessing pollution sequestering efficiencies of macrophytes in the engineered wetland design through the use of phytoplankton bioassays and contaminant chemical analysis.

An outline of the results is presented in Figure 47 on the next page and Figure 55 on page 100.

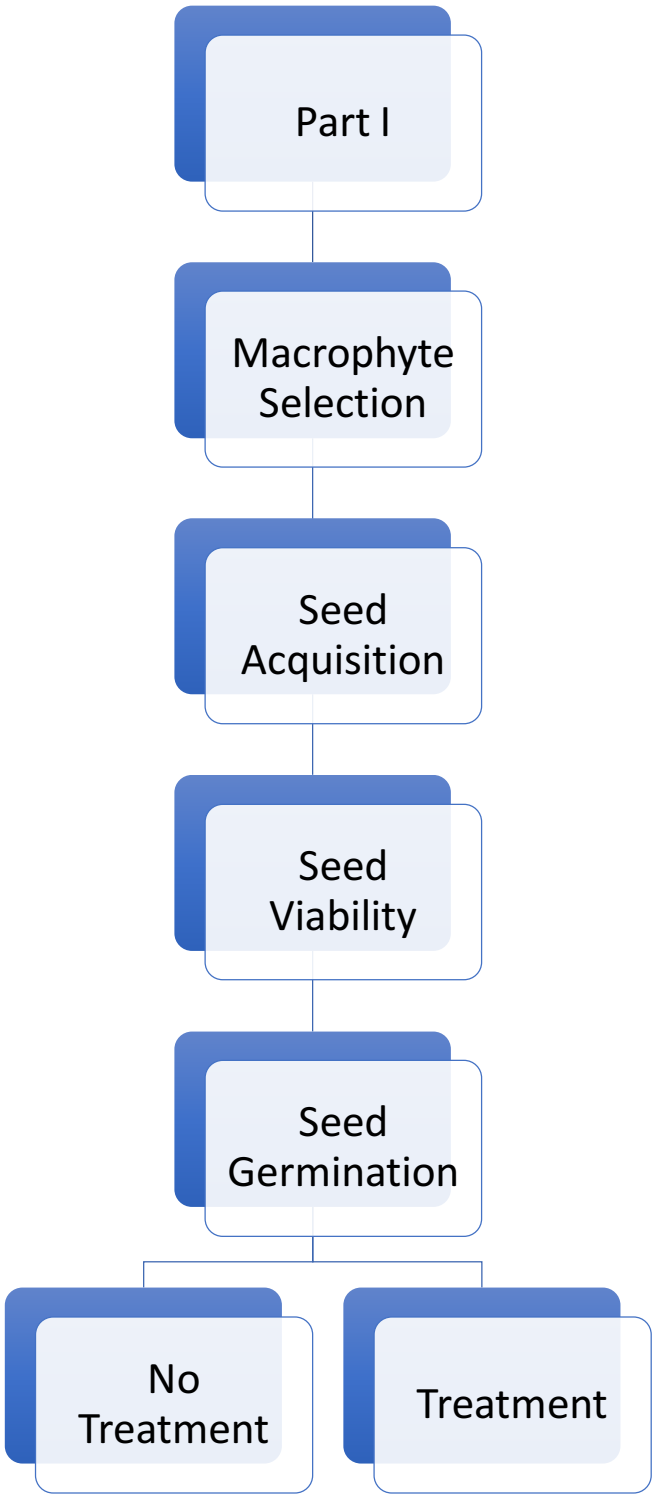


Figure 47: Summary of methodology for Part I from Macrophyte Selection to Germination Treatments Protocols used

PART I

4.1 Macrophyte Selection

Using the information available from the Literature Review, Environmental Canada (1996), Canadensys, United States Department of Agriculture (2002a, 2002b, 2002c, 2003, 2005, 2005b, 2006, 2011), Table 3, on page 93, was completed. The information in the Literature Review revealed the macrophytes most commonly used in engineered wetlands for nutrient-removal. Some studies provided information why certain species were chosen often mainly due to local and geographical availability. In assessing the nutrient-removal efficiency of different models of engineered wetlands, the macrophytes were often used as mixed cultures, thus measuring only the efficiency or inefficiency of the constructed wetland design used and not the nutrient efficiency of the macrophyte species individually. This format presents an interesting twofold issue during comparison and cross-checking information among various studies. Firstly, as mentioned in Chapter 1, section 1.5.1, there are different combinations of categories, with multiple variables, that go into engineering a constructed wetland such as macrophytes, substrates, hydraulic flow, water retention time and, size of constructed wetland. The probability of finding two or more studies that followed the same combination of variables from each category is greatly reduced.

Table 4: Macrophyte Selection Criteria for the Sixteen Different Emergent Species on the Environment Canada (1996) List

1	2	3	4	5	6	7	8
Plant Name *Scientific Name* Common Name	Emergent Aquatic	Native	Growth Rate S/M/F	Perennial	Removal (P, N, HM)	Insect/bird Food	Fibrous or Extensive Root System
Alisma plantago-aquatica Water plantain	✔	X	M	✔	✔	✔	-
Asclepias incarnata Swamp milkweed	✔	✔	M	✔	✔	✔	✔
Carex spp. Sedge	✔	✔	F	✔	✔	-	✔
Chelone glabra Turtlehead	✔	✔	-	✔	-	-	-
Eleocharis spp. Spike rushes	✔	✔	M	✔	-	✔	✔
Equisetum fluviatile Water horsetail	✔	✔	F	✔	-	-	-
Iris versicolor Wild blue flag	✔	✔	-	✔	-	-	✔
Juncus spp. Rushes	✔	✔	M	✔	✔	✔	✔
Pontederia cordata Pickerelweed	✔	✔	M	✔	✔	✔	✔
Sagittaria latifolia Arrowhead	✔	✔	M	✔	-	✔	-
Scirpus acutus Hard-stem bulrush	✔	✔	M	✔	-	✔	-
Scirpus atrovirens Black bulrush	✔	✔	M	✔	-	-	-
Scirpus validus Soft-stem bulrush	✔	✔	F	✔	✔	✔	-
Sparganium eurycarpum Giant bur-reed	✔	✔	M	✔	✔	-	-
Typha spp. Cattails							
Veronica americana American brooklime	✔	✔	F	✔	-	-	-

Secondly, in scenarios where monocultures were used for macrophyte comparisons, due to the present lack of standardized methods to denote macrophyte efficiency in nutrient removal, their measurements were often incomparable. For example, some studies used above ground and below ground biomass growth over a period of time to compare efficiency of same-aged macrophytes. Other studies used a decline in nutrient concentrations within constructed wetlands over time; and even these results were presented in multiple ways such as concentration of nutrient or percent of nutrient removed. Thus, the second issue in comparing results once two studies have used the same combination of variables from each category, is the measurements used to convey nutrient-removal efficiency. Hence Table 3 (column 6) presents only information about whether the macrophyte was used more than once or twice (outliers) for nutrient removal in engineered constructed wetlands.

Environment Canada (1996) provided the possible list of species that may be used in wetland restoration and constructed wetland. Plant fact sheets, plant guides, and plant profiles from the USDA were used to fill in the rest of the information. There are three different symbols used in this table. The ' ✓ ' represents categories that were met successfully. The 'x' symbolizes plants that did not meet the needs of that category. The '-' represents areas that do not have sufficient or any conclusive research and documentation. In column four, 'Growth Rate,' the variation in plant development is represented by 'S', 'M', and 'F' for slow, medium and fast respectively.

The categories chosen in the development of a macrophyte selection protocol are focused on developing an environmentally relevant, sustainable and low-maintenance population that is effective in nutrient uptake. In selecting species that are native, the introduction of foreign, potentially invasive species is negated. By choosing species that have a moderate-to-fast growth rate and are perennial, the need to replant species annually is avoided. There are a number of species on the list that are noticeably missing information, especially regarding nutrient-removal efficiency. One of the major reasons for this is the overuse of well-known nutrient-removal efficient macrophytes such as *Phragmites* and *Typha* (Sasaki *et al.,* 2003; Gray and Sedlak, 2005; Conkle *et al.*, 2008; Vymazal, 2009).

Macrophytes such a *Phragmites* and *Typha* include native and introduced strains across all of Canada and the United States. Although a trained horticulturist would be capable of

distinguishing native from introduced species, the concern with using these two macrophytes runs deeper than strain type. The roots of these macrophytes, literally, run deeper than most species (approximately 0.5 m) regardless of strain, propagating through creeping rhizomes that return to the surface in different locations. Though these macrophytes are capable of high nutrient removal, their use as a monoculture is only justified in a closed system to avoid an outbreak that will exterminate local biodiversity. This proves to be impossible because wetlands, and constructed wetlands alike, are open systems that require the input and output of material such as influent and effluent.

In using *Phragmites* and *Typha* in mixed cultures of macrophytes, the immediate threat of local biodiversity extermination is temporarily delayed but not negated. *Phragmites* is capable of growing 4.5 m high and thus a growing concern is the fire hazard this dry plant matter can pose due to the combustible nature of its dry stalks (Ontario Ministry of Natural Resources (OMNR), 2011). In states such as Michigan (United States) one way landowners are controlling the growth of this macrophyte is through herbicide application followed by controlled burning with the aid of the fire department and permits from local government agencies (Michigan Department of Environmental Quality (MDEQ), nd). The macrophyte selection criteria were also a tool to raise awareness about the other species available and previously recommended for use by a government agency such as Environment Canada.

Macrophytes such as *Alisma plantago-aquatica* (water plantain) is an introduced species found in Washington, Alaska, and British Columbia. Even though this macrophyte is not native, its low biomass, moderate growth rate and presence in the third quartile showed that it met most of the other categories in the selection process. Moreover, unlike macrophytes such as *Phragmites* and *Typha, Alisma* can only propagate through seed and sprigs. Sprigs are small stems with leaves that are cut and immediately sown. This macrophyte is a vascular plant that has xylem (Figure 48). If air bubbles enter this plant structure through the cut side of the stem, water uptake is blocked, resulting in plant death. Thus, invasion through sprigs is unlikely.

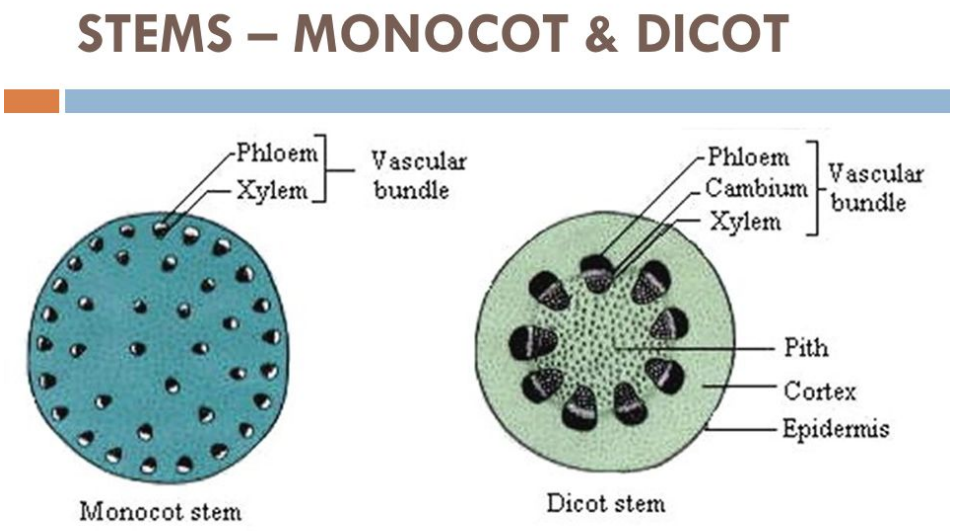


Figure 48: Vascular plant stem structures (© Scarlet Fox 2016)

Among the other macrophytes suggested by Environment Canada (1996), the criteria (Table 3) show letters for growth rate. These growth rates are based on the information found in the characteristics database on the Canadensys and USDA websites. The USDA database is well-known compared to Canadensys, a less popular database. The word "Canadensys" is a homonym of Canadensis, meaning of Canadian origin in Latin. Both of these websites contain biological, ecological, and geographical data collected by horticulturists, botanists, ecologists, hobbyists, and birdwatchers, then dedicated to the public domain. The data received by the organization are cross-referenced by a 'science and technology advisory board' made up of academic and government officials with qualifications in science and technology. Due to the vast amounts of information that needed processing, information such as growth rate is measured qualitatively and not quantitatively. Thus, Table 18 (column four) depicts letters such as 'S', 'M' and 'F' instead of length or biomass per unit of time.

As mentioned earlier (section 1.3.1), the Environment Canada (1996) list provided four groups of macrophytes: emergent plants, floating-leaved plants, free-floating plants, and submergent plants. In this thesis, only emergent plants from this list were assessed. Reasons included the following: i) biomass production is high in emergent versus other groups, ii) emergent species use three out of three parts of the constructed wetland ecosystem matrix (substrate, water, and above water atmosphere) giving them a higher competitive advantage, and

iii) emergent plants may also be used in a "floating constructed wetland model" (the first constructed wetland protocol model developed). Using additional information such as life cycle (perennial versus annual), food web status (toxic, food source, or neither) and root structures, the selection process accounted for the sustainability of these macrophytes with longevity based on ecosystem interactions. After this selection process was completed, a total of ten species met the criteria: *Alisma plantago-aquatica* (water plantain), *Asclepias incarnata* (swamp milkweed), *Carex spp.* (sedge), *Eleocharis spp.* (spike rushes), *Juncus spp.* (rushes), *Pontederia cordata* (pickerelweed), *Saggitaria latifolia* (arrowhead), *Scirpus acutus* (hard-stem bulrush), *Scirpus validus* (soft-stem bulrush), *Sparganium eurycarpum* (giant bur-reed).

4.2 Seed Acquisition

Among sixteen aquatic plant species listed in Environment Canada's 1996 list, nine species met the criteria to be used in our constructed wetlands. From these ten species, only seven had seeds available for purchase. These species were *Alisma plantago-aquatica (water plantain), Asclepias incarnata (swamp milkweed), Carex vulpinoidea (Carex spp. – sedge genus, fox sedge), Pontederia cordata (pickerelweed), Sagittaria latifolia (arrowhead), Scirpus validus (soft-tem bulrush), and Sparganium eurycarpum (giant bur-reed)*. These macrophytes were selected based on their ability to meet five out of the seven categories used to assess their potential success in our engineered wetland system.

4.3 Seed Viability

Among the seven species that underwent the viability test, only five of the macrophytes produced bubbles while sinking to the bottom of the beaker. These five species were: *Alisma plantago-aquatica* (water plantain), *Asclepias incarnata* (swamp milkweed), *Carex vulpinoidea* (fox sedge), *Saggitaria latifolia* (arrowhead), and *Scirpus validus* (soft-stem bulrush). The water pressure against the walls helped the seeds release trapped gases, breaking their dormant states. In the water, two situations occurred. Firstly, placing the seeds in water made it easier to check for respiration by observing the production of bubbles of carbon dioxide by the seeds (Cavanagh, 1980). Secondly, when the seeds were soaked in water, imbibition was initiated. Imbibition is a special kind of water diffusion that occurs in solids such as wood and seeds. The seeds absorb

water, causing an increase in seed volume (McDonald *et al.*, 1988). The seeds that produced bubbles and absorbed water sank to the bottom of the beakers. The sunken seeds were identified as viable and used for germination (Bewley *et al.,* 2013). Not all seeds passed this viability test.

The two macrophyte seeds that did not sink to the bottom of the beaker or produce bubbles were *Pontederia cordata* (pickerelweed) and *Sparganium eurycarpum* (giant bur-reed). Both these species had the largest seed size among all the other seeds (Figure 49 and 50). The *P. cordata* seeds were heavier than the *S. eurycarpum* seeds. These seeds likely did not sink for two reasons: i) the simplest explanation is that these seeds were likely dead ii) Seed buoyancy in water may have had another reason. Research carried out on wetland seed buoyancy in the Netherlands in 2005 noted that macrophytes native to geographical locations that were frequently flooded had higher buoyancy. This characteristic allowed the seeds to survive longer but there was a trade-off with seed persistence. Seeds were able to travel and disperse further during floods by not sinking. Seeds with higher buoyancy (i.e. lower capacity for water absorbance) rarely went through imbibition, and thus dried out (van den Broek *et al.,* 2005).

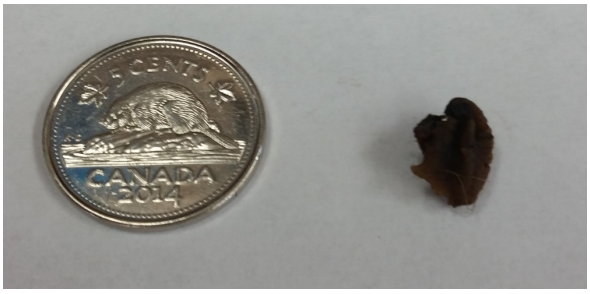


Figure 49: Pickerelweed seed

Figure 50: Giant bur-reed seed

Other macrophyte species that propagate through seeds, with the aid of dispersal from water, had similar characteristics to *Pontederia cordata* (pickerelweed) and *Sparganium eurycarpum* (giant bur-reed), where the seeds were large, contained larger volumes but lower seed-specific weights that resulted in high buoyancy. This evolved characteristic aids in greater seed dispersal (Lopez, 2001).

The seeds acquired from Speare Seeds and Wildflower Farm had a low overall percent viability. Less than 50% of any of the seeds in any given macrophyte seed tested were viable. Since most seeds are harvested from the wild, by the time they are put in cold storage, many of them may already be dead. Since wholesale seed companies such as Speare Seeds and

Wildflower Farm sell seeds by weight, (i.e. per pound, or per gram) they do not concern themselves with separating the dead seeds from the Alive seeds. Speare Seeds is only a seed distributor and thus, they do not carry information on germination techniques. Although Wildflower Farm is a producer and a distributor, minimal information on germination techniques was provided to protect trade secrets. A recommendation for distributors would be to sell seeds that have not been in storage for more than five years. Additionally, they should conduct their own viability tests on seed samples bi-annually. Although it is not common practice for distributors themselves to provide clients with seed guides, it is recommended that they supply the customer with at least common germination techniques to grow these macrophytes.

4.4 Germination Treatment

Although some seeds sank during the viability tests while others did not, the seeds that did sink themselves appeared undamaged from the outside. However, we could not conclude if they were dead or dormant. Thus, all seven macrophytes seeds were used in the germination efficiency tests. With the macrophytes that passed the viability tests, only viable seeds were chosen for germination. For macrophytes such as pickerelweed and giant bur reed, seeds that had been soaked in the warm water were chosen regardless of their descent in the vessel. For each treatment described below, it must be noted that the number of seeds used for each macrophyte was 10 and the number of replicates (n) for each entire experiment was 4, with germination tests carried out quarterly over a one-year period (n = 4 x 4). Figure 50 below provides a review of the treatments used to trigger seed germination.

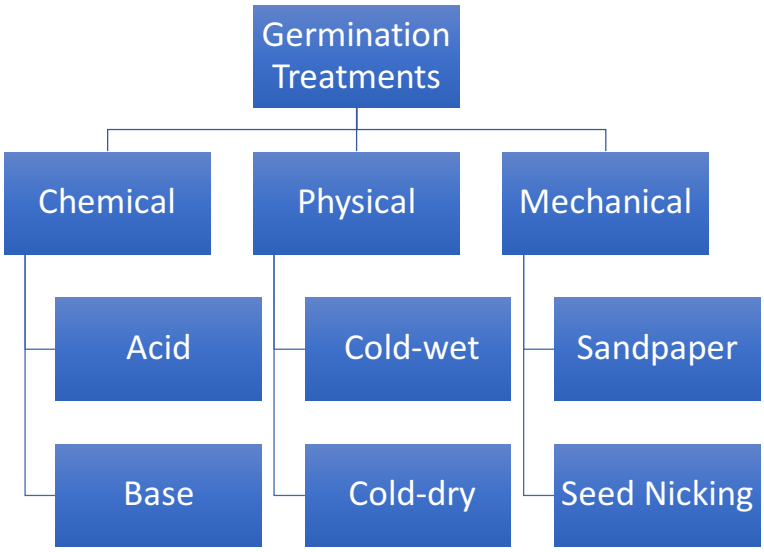


Figure 51: Seed treatment methods used to induce successful seed germination for the selected macrophytes

As mentioned in section 3.6, seed selection for germination treatment was carried out based on seed size and texture. Seeds that had fragile seed coats and could have been damaged through mechanical treatment were only processed using chemical treatment. Physical and chemical treatment were used for all macrophyte seeds (Table 5).

Table 5: Germination treatments assigned for selected macrophyte seeds based on seed coat and texture of seed coat (n=10)

	10% Acid	25% Acid	10% Base	25% Base	Sandpaper	Seed Nicking	Cold Wet	Cold Dry
A. plantaga-aquatica	✓	✓	✓	✓	✘	✘	✓	✘
A. incarnata	✓	✓	✓	✓	✓	✘	✓	✓
C. vulpinoidea	✓	✓	✓	✓	✘	✘	✓	✘
P. cordata	✓	✓	✓	✓	✘	✘	✓	✘
S. latifolia	✓	✓	✓	✓	✘	✘	✓	✘
S. validus	✓	✓	✓	✓	✓	✘	✓	✘
S. eurycarpum	✓	✓	✓	✓	✓	✓	✓	✘

Among the seven macrophytes that were sown and treated with various germination treatment processes, five out of seven species germinated: *Alisma plantago-aquatica* (water plantain), *Asclepias incarnata* (swamp milkweed), *Carex vulpinoidea* (fox sedge), *Saggitaria latifolia (*arrowhead), and *Scirpus validus* (soft-stem bulrush). These were the same five species that had passed the viability test. This might increase the credibility of the viability test and suggests its use to distinguish between dormant and dead seeds for future experiments.

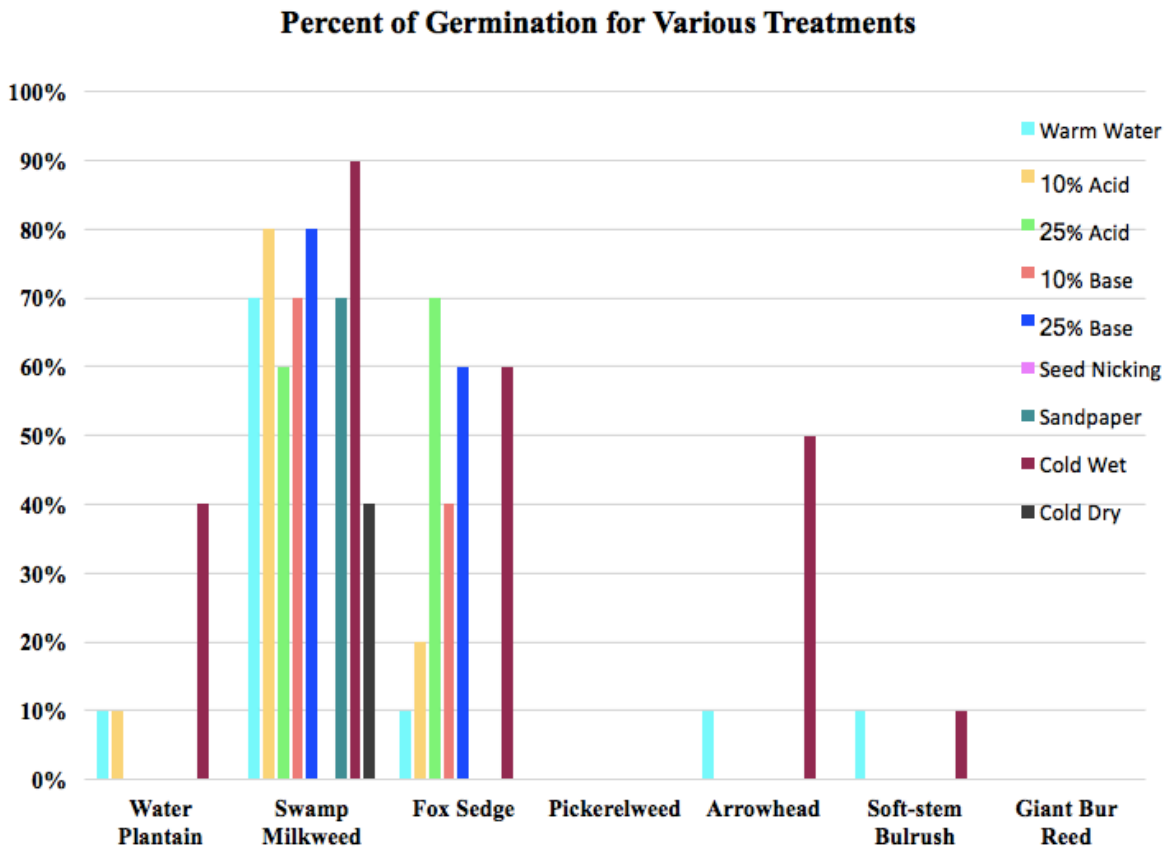


Figure 52: Macrophyte germination success rate for various treatments

4.4.1 Acid Treatment

The seeds were soaked in acid and then dried on paper towels. Two concentrations (10% v/v and 25% v/v) were used to scarify the seed coat. Two concentrations were used to verify if this treatment is suitable for any macrophyte seeds on the Environment Canada (1996) list. These concentrations were chosen to represent the upper and lower limit of the range of concentrations reported in the literature, since there is no standard for these treatments.

10% v/v of the acid treatment germinated 10% water plantain seeds, 80% swamp milkweed, and 20% fox sedge (Figure 52). The 25% v/v acid most likely damaged some of the seeds. Although this concentration of acid reduced seed germination of swamp milkweed to 60%, it increased germination of fox sedge to 70%. One reason for this could be that having a smaller seed size than the milkweed, the sedge seeds are more likely to get stuck on leaves and

fruits eaten by many organisms. Thus, it is exposed to high concentration of acids such as those present in the stomach.

As for the other seeds that did not germinate, they were analysed for any signs of damage such as cracks or scrapes on the seed coat. Under 10x magnification, the seed coats for the milkweed in 25% v/v acid and the arrowhead in 10% v/v and 25% v/v acid appeared wrinkled. This seed coat wrinkling caused by the acid wash may have damaged the embryo inside. Although there are very few germination treatment studies on wetland macrophytes, research on seed germination with grass seeds using acid treatment demonstrated a similar increase in percent germination for species such as *Panicum coloratum, Eragrostis curvula,* and *Erogrotis superba* (Voigt and Tischler, 1996). This study noted a difference in germination (seed coat break) and emergence (hypocotyl emergence from soil). They observed that although acid treatment increased the germination percentage, it did not increase emergence of seedlings (Voigt and Tischler, 1996). Other studies conducted using different chemicals such as acids, alcohols, ketones, amines, and alkaline solutions noted that acid treatment resulted in the highest seed germination in the numerous medicinal plant species tested (Bhardwaj *et al.,* 2016).

These findings contradict the results observed in this thesis because, while acid treatment did increase germination of seeds compared to the reference (warm water) in some species, this treatment did not yield the highest germination percent in most species. Acid treatment resulted in the highest germination percentage only for *Carex vulpinoidea* (fox sedge) at 70% This difference in results could be attributed to the difference in species used.

4.4.2 Base Treatment

Most seeds collected after the base rinse had lost their pigmentation. Like the acid rinse, the base rinse helped in thinning and disinfecting the seed coat. The base rinse had no effect on some seeds such as *Alisma plantago-aquatica* (water plantain)*, Pontederia cordata* (pickerelweed)*, Sagittaria latifolia* (arrowhead)*, Scripus validus* (soft-stem bulrush)*, and Sparganium eurycarpum* (giant bur-reed)*.* Conversely, seeds of the *Asclepias incarnata* (swamp milkweed) and *Carex vulpinoidea* (fox sedge) seem to germinate better with higher base concentrations. An increase in base concentration from 10 v/v to 25% v/v resulted in a 10% germination increase for swamp milkweed and 20% germination increase for fox sedge.

The yellow-brown colour of the fox sedge seeds turned white in the bleach. As noted earlier, the bleach acts as a disinfectant and thus (in doing so) helps clean the seeds by removing any contaminants that might be preventing it from breaking dormancy. At the highest base concentration, fox sedge and swamp milkweed had germinations of 60% and 80 % respectively. Some studies conducted on the effects of base treatment on seed germination have noted that certain species have higher percent germination when treated with sodium hypochlorite (bleach) versus acids. The biological mechanisms for these results are still unknown, however (Fieldhouse and Sasser, 1975; Thomas, 1981; Drew and Brocklehurst, 1984).

4.4.3 Mechanical Scarification Germination

Sandpaper scarification was used on the small yet brittle seeds of the soft-stem bulrush; however, it yielded no germination. The two types of mechanical scarification were used mainly for the bigger seeds such as pickerelweed and giant bur reed. These seeds did not appear to be damaged yet they resisted germination. Note that these were also the seeds that did not pass the viability tests. Thus, their lack of germination might not be the result of treatment but might be because they were dead to begin with (see section 4.3).

Some studies that have used sandpaper treatment with acid and base treatment for the germination of *Dracocephalum kotschyl* have found this treatment to yield the higest percent germination compared to base, which resulted in the lowest percent germination (Fathahi *et al.,* 2011). In other studies, seed nicking, also known as 'blade' treatment, was used to break dormancy for agricultural seeds such as *Leucaena leucucephala* and *Acacia famesiana*. These researchers found that this mechanical scarification increased germination by 56% for the seeds of *A. famesiana* while a simple hot water treatment at 70°C for 20 minutes yield a 97% germination for *Leucaena leucucephala* (Tadros *et al.,* 2011). This difference in seed treatment preferences in the process of breaking dormancy in various plant seeds is common, and results (more or less) vary among studies based on seed type, age, and treatment.

4.4.4 Cold-Water Germination

After most seeds had been transferred from the cold-water storage (in beakers) to the warm water beakers, germination began promptly. Following the first 12-hour light cycle, some seeds had begun to break open their seed coat. With macrophytes such as water plantain, fox sedge, and arrowhead having small seeds with thin seed coats, it was reasonable for the change to occur quickly within the water beakers themselves.

With the winter frost ending and warm spring waters washing over the seeds in the environment, the seeds are likely to germinate quickly to make the most of the short season, a small fraction of the year that allows for ideal growing conditions (Donohue, 2005). The larger seeds and seeds with a more brittle seed coat such as the swamp milkweed and the soft-stem bulrush germinated after the damp seeds were placed in the germination pods. The soft-stem bulrush only germinated with this treatment and yet it only had a germination of 10%.

The most observable change in the swamp milkweed seed was its change in colour from orange to red. The seed pigment lost was noticeable (physical change that displayed change) in the seed coat through treatment. The swamp milkweed seeds had the highest germination of 90% with this treatment. Seeds that were refrigerated were densely stored. Some studies on aquatic macrophytes have observed some density-dependent germination (Adler *et al.,* 1993; Linhart, 1976; Murray 1998). Germinating seeds often release chemical signals that might enhance the germination of other seeds (Linhart, 1976; Murray, 1998). Researchers found that cold-wet treatment used to germinate the macrophyte *Ottelia alismoides* resulted in similar high-density clusters after refrigeration and higher germination efficiencies (Yin *et al.,* 2009). These results are similar to the results observed for *Alisma plantago-aquatica* (water plantain), *Asclepias incarnata* (swamp milkweed), and *Sagittaria latifolia* (arrowhead) with highest percent germination of 40%, 90%, and 50% respectively.

4.4.5 Cold Dry Germination

The only macrophyte in this treatment was the swamp milkweed seeds. When these seeds were transferred into the warm water beaker, they did not lose as much colour as the seeds in the cold-water treatment had lost. However, they did take in water faster, sank faster and germinated

more quickly in their pods. Some of these seeds were observed to break their seed coat immediately following one 12-hour light and 12-hour dark cycle. This macrophyte attained an overall germination of 40% in this treatment over the course of one week. Tests conducted by the USDA (2011) found this treatment to yield twice the germination percentage for swamp milkweed seeds compared to no treatment germination.

4.4.6 Challenges with germination

During the initial germination trials, the only macrophytes that grew were swamp milkweed and fox sedge. The swamp milkweed, although successfully germinating at the time (30%), did not often survive post germination for more than a few months (Figure 53)

Figure 53: *Asclepias incarnata* (swamp milkweed)

Only one of three seeds that originally germinated grew 1.5 m and flowered after a year. The fox sedge, conversely was very resilient and was consistently successful in growing regardless of slight variations in temperature. These macrophytes germinated in water, on soil, on sand, and on cotton mesh. The sedges grew quickly and developed extensive fibrous root systems.

Due to varying laboratory conditions including inconsistent temperature, the germinating seeds were moved in the laboratory to find ideal and consistent temperature conditions. After the

laboratory temperature become consistent at 23°C ± 1°C, under a light bank, a higher germination rate was observed for swamp milkweed and fox sedge. The combined germination percent efficiencies for all the macrophytes tested in all the different treatments can be found above in Figure 52.

4.5 Statistical Results for Germination Treatments

The independent variable for the seed germination test was treatment (reference - warm water vs warm water with treatment). The dependent variable for the seed germination test was percent germination. The percent germination data were discrete in nature and no assumption was made regarding their distribution. Thus, a non-parametric Friedman test was used to analyze the germination treatments. This test was used to verify if there was a significant difference in germination percent for different treatments. This information is especially relevant when choosing one germination test over another to develop macrophytes.

Table 6: Friedman test result for germination scarification treatment

N	7
Chi-Square	24.109
df	8
Asymp. Sig.	.002

The critical value for the X^2 distribution with degrees of freedom (df) = 8 and significance level of $\alpha = 0.05$ is 15.51. The overserved value ($X^2 = 24.109$) is further out in the tail of the distribution and greater that the critical value of 15.51. Thus, there is a significant difference between germination scarification treatments used. The statistical analysis for these tests does not inform us of which test worked best because each macrophyte responded differently. Due to the number of variables (treatments) per macrophyte (also a statistical variable, in this case seven macrophytes, thus multiplying by seven more variables), these tests cannot conclude which treatment worked best for all species. Additional statistical tests conducted on each macrophyte may provide us with the ideal germination treatment for that

macrophyte. Yet, this information cannot be used to generalize that a single treatment works for all macrophyte seeds.

4.6 Plant Biomass Bioassays

In this thesis, the above-ground biomass of the macrophytes transplanted into the constructed wetlands was used to assess the difference in growth within Reference versus Treatment strategies. Biosolids were used as the source of excess nutrient runoff in this study. In order to assess for potential difference in biomass among the different concentrations of treatment (0% - reference, 1% biosolids and 10% biosolids), the macrophyte leaf length, leaf width, stem length and stem width data were collected for *Carex vulpinoidea* over the course of the experiment. The macrophytes used were the same age and received every other growth condition identically. The descriptive data used for this test can be found in Appendix III. The mean and standard deviation data are present in Table 6. The one-way ANOVA was carried out only for the final data collected.

Table 7: Mean and standard deviation data for plant biomass of *Carex vulpinoidea* in each treatment after 3 months

Category (mean, standard deviation)	**Reference**	**Biosolids 1%**	**Biosolids 10%**
Leaf Length	45.5 ± 2.66	43.8 ± 6.42	37.0 ± 3.06
Leaf Width	0.33 ± 0.05	0.30 ± 0.00	0.31 ± 0.03
Stem Length	3.93 ± 0.75	4.16 ± 0.60	3.70 ± 0.36
Stem Width	0.20 ± 0.00	0.23 ± 0.05	0.23 ± 0.05

The statistical analysis results for the plant bioassay show the different p-values for each variable (Appendix III). The ANOVA helps to determine whether the difference in sample means is significant or expected by chance (Whitlock and Schluter, 2015). If there is a real difference in means, the p-value < 0.05. This is not observed for any of the variables. Thus, this

result demonstrates that the different concentrations of nutrients did not affected the biomass production of this macrophyte (Figure 54). One reason for this may have been over-enrichment of the vegetation where the macrophyte roots temporarily pauses nutrient uptake as proposed by Verhoeven (1986).

A)  B) 

Figure 54: Macrophyte biomass different over a 3-month period for all three conditions (reference, biosolids 1% and biosolids 10%. A) Start date and B) End date

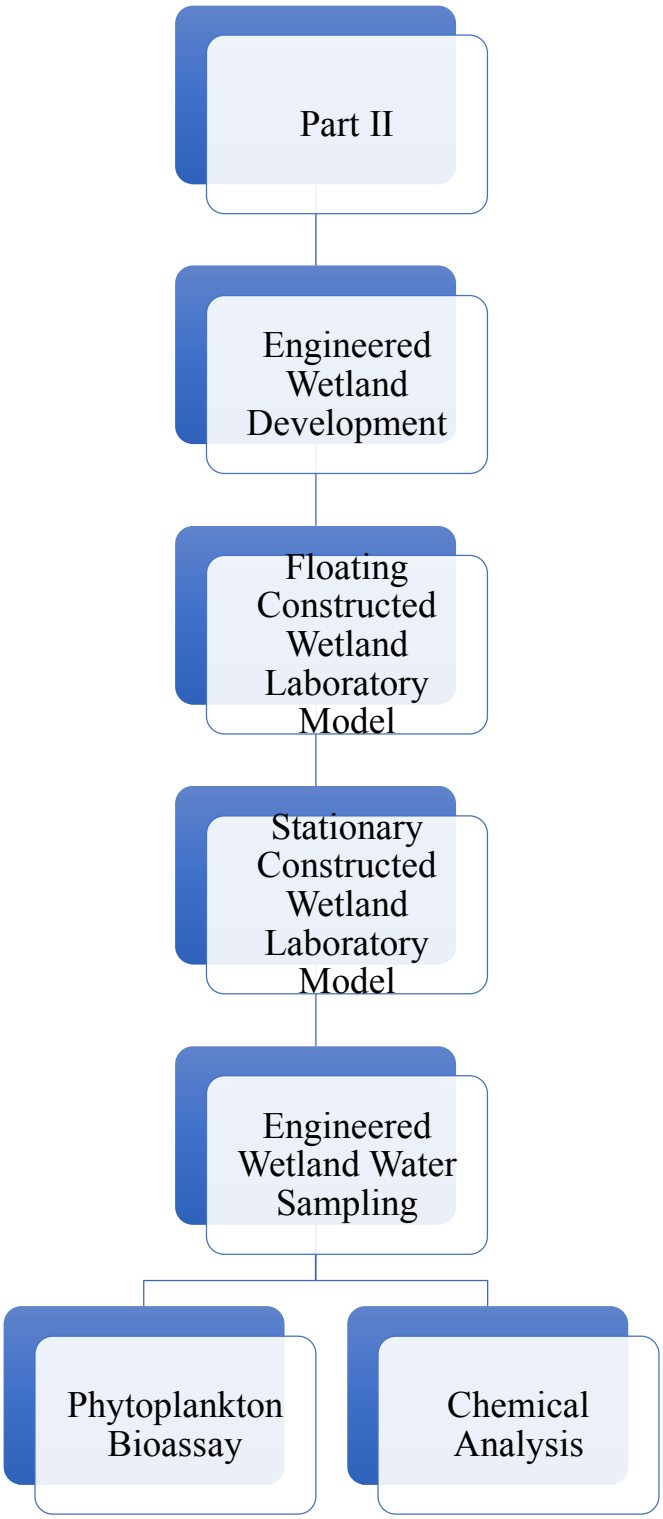


Figure 55: Summary of methodology for Part II from Developing an Engineered Laboratory Model to Testing for Efficiency of that Model

4.7 Engineered Wetland Model

The selection for the engineered wetland model evolved primarily based on water-to-biomass ratio. The water-to-biomass ratio represents the amount of available water for a given plant. If there is more water than plant, less nutrients will be sequestered from the water over time. In using a rectangular trough, the initial lack in macrophyte germination due to temperature fluctuations resulted in a high water-to-biomass ratio, (i.e. more water than plants.) Moreover, since the vessel used was a rectangular trough, a higher surface area of the water was exposed to light resulting in significantly higher evaporation rates. Thus, the concentration of nutrients in the troughs increased over a single day, skewing the results. In moving from the rectangular troughs to the bucket mesocosms (Figure 56), the water-to-biomass ratio greatly decreased. Previous studies have demonstrated a linear correlation between plant biomass and wetland water volume (Tanner, 1996). Therefore, constructed wetland design changes were necessary to collect unbiased data for constructed wetland efficiency. Multiple replicates for multiple treatments were possible for testing the pollutant and potential contaminant removal efficiency of *Carex vulpinoidea.*

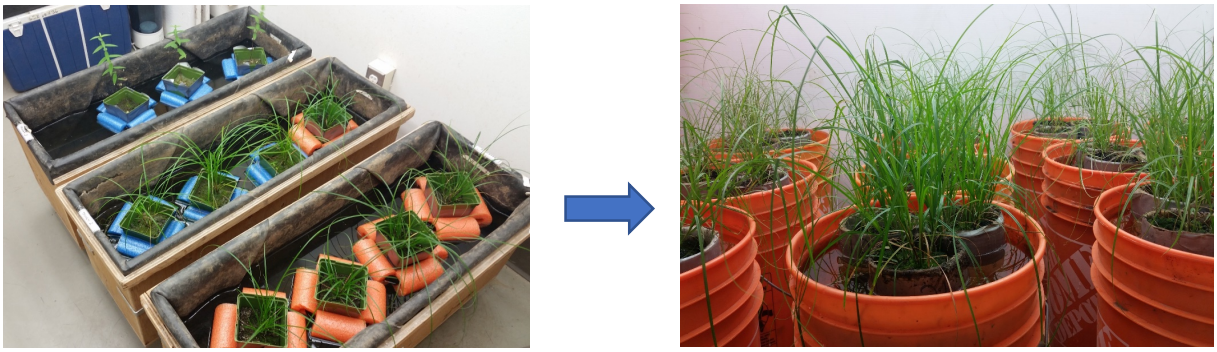


Figure 56: Regular trough (high surface area evaporation) to buckets (low surface area evaporation)

4.8 Phytoplankton Bioassay results used to Assess Constructed Wetland Efficiency

4.8.1 Developing "initial conditions"

In order to determine if the macrophytes could sequester contaminant constituents, particularly nutrients, in the constructed wetlands, an 'initial conditions' test was carried out with the water from the mesocosms. Water samples taken from each unit were tested in a bioassay with the test organism, *Pseudokirchneriella subcapitata*. From section 3.13.4 (A) was the original unaltered sample (B) was mixed with 10^{-4} mg of P (C) was mixed with 2 x 10^{-3} mg of N. The last two aliquots were used to text potential toxicity and thus were mixed with sufficient nutrients as per the growth media. (D) was kept undiluted, while (E) was diluted to 50% and (F) was filled with growth media and used as a control to compare the growth in the absence and presence of wetland water samples.

Figure 56 shows the cell production per volume for wells 'A' to 'E.' The red arrow displays where the expected cell concentration should be after two generation. The initial cells added to each well were 1 x 10^5 cells per mL (black arrow). After each 12-hour light cycle, this value is expected to double (US-EPA, 2002). Thus, after two light cycles, when the cell count was carried out for this assay, there should have been at least 4 x 10^5 cells (as depicted by the red arrow).

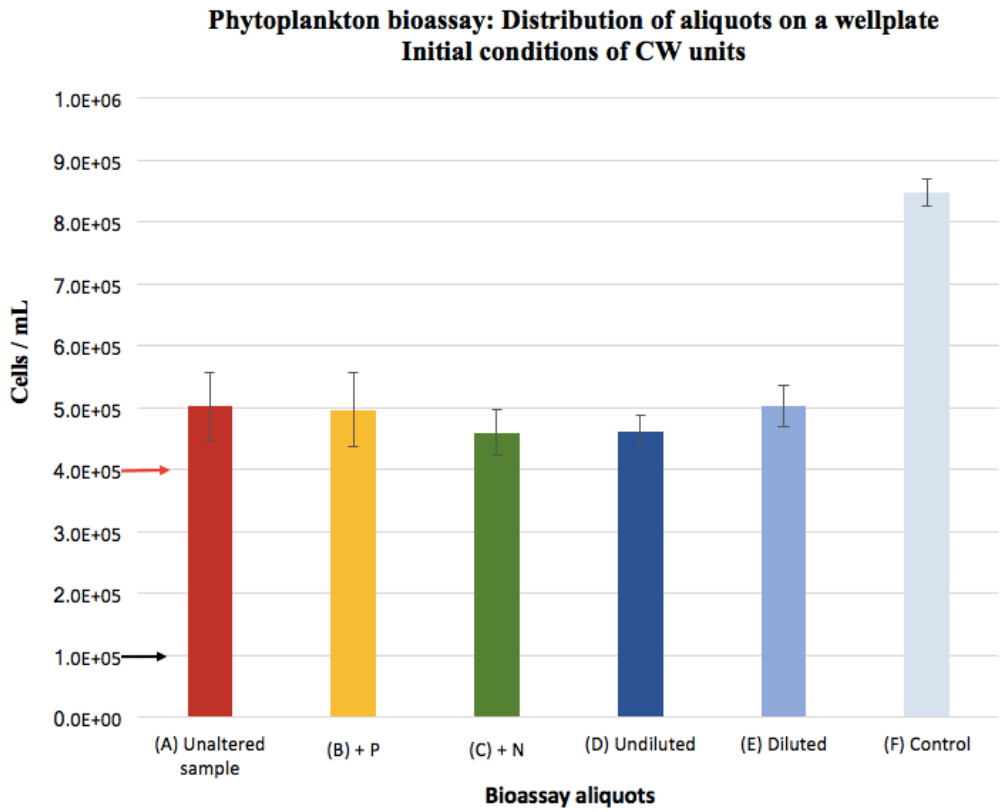


Figure 57: Phytoplankton bioassay to test initial conditions of constructed wetland units

In Well 'F,' (the control for this test), all necessary nutrients for algae growth were present. Thus, the cells in this Well were able to grow higher than the expected number. The rest of the Wells tested the water samples for different substances. Since Well 'A' tested the unaltered samples, when 'A' to 'F' were compared, a significant difference in cell growth was observed. This could be attributed to a lack of nutrients in the mesocosms. This conclusion is reasonable because the biosolids had not yet been added to the wetlands. Nutrients that were previously present in the buckets should have been taken up by the plants to grow. This 'initial conditions' test demonstrated that all the buckets being used to test had approximately the same concentration of nutrients (or lack of nutrients) in their water to deprive algae growth in well 'A'.

The reason we are calling this a preliminary test is because the next few Wells did not follow an expected trend. Well 'B' contained the sample and additional P. In theory, if the samples were lacking nutrients, as seen in Well 'A', adding nutrients should have resulted in an

increase in algae population. However, Well 'A' and 'B' have approximately the same population (Figure 57). The same situation is seen for Well 'C' that had the sample with added N. So perhaps in this case, the water samples were missing more than one nutrient and it was not sufficient to add just one nutrient to the wells, phosphorus and nitrogen. The first three wells 'A', 'B', and 'C' were used to analyze nutrient excess and deficiency.

The next two wells 'D' and 'E' were used to analyze for potential toxicity. By providing both water samples with nutrients at 100% and 50% respectively, we wanted to note any differences in cell concentrations. The results in the 'initial conditions' test showed no difference for both these wells. Once again, the cells concentrations were the same as wells 'A' to 'C' yet different from the control. Well 'F' is the only well on the plates that does not have the water sample added to it. The factors constant in wells 'A' to 'E' are the water samples and the cell concentrations (Figure 57).

One explanation for this cell concentration decrease is the presence of copper from the water used or perhaps the peat was contaminated with copper and it leached into the water. Copper has been found to be toxic to *Pseudokirchneriella subcapitata* in low phosphorous environments (Kamaya *et al.,* 2004). This reasoning would make sense for well 'A' and well 'C'. However, well 'B' that had added P and wells 'D' and 'E' that had all the nutrients should not display any toxicity because they contained adequate P concentrations. Well 'B', 'D', and 'E' concentration rates should have been higher than wells 'A' and 'C'. Further experimentation is required to assess the initial condition differences in these wetlands. Additionally, chemical analysis is necessary to illuminate this conundrum.

4.8.2 Phytoplankton Bioassay test after addition of biosolids (48 hours)

After the initial addition of biosolids (1% and 10%) to bucket mesocosms each respectively, water samples were collected to conduct a bioassay test. The cell counts were carried out after two light cycles and the results are displayed in Figure 56. The most noteworthy result in this figure is observed for well 'D' for all three treatments. The cell concentration was significantly lower even compared to the reference units that did not receive any biosolids. Under a light microscope, the cells from this well were found in clusters compared to the other

wells (Figure 59). Disruption of clusters was attempted with pipetting samples back and forth to even distribution. However, this process did not result in much differences. For future studies, sonication of samples is recommended if this occurs. In this well, only water sample and modified Bristol's media was added in a 1:1 ratio.

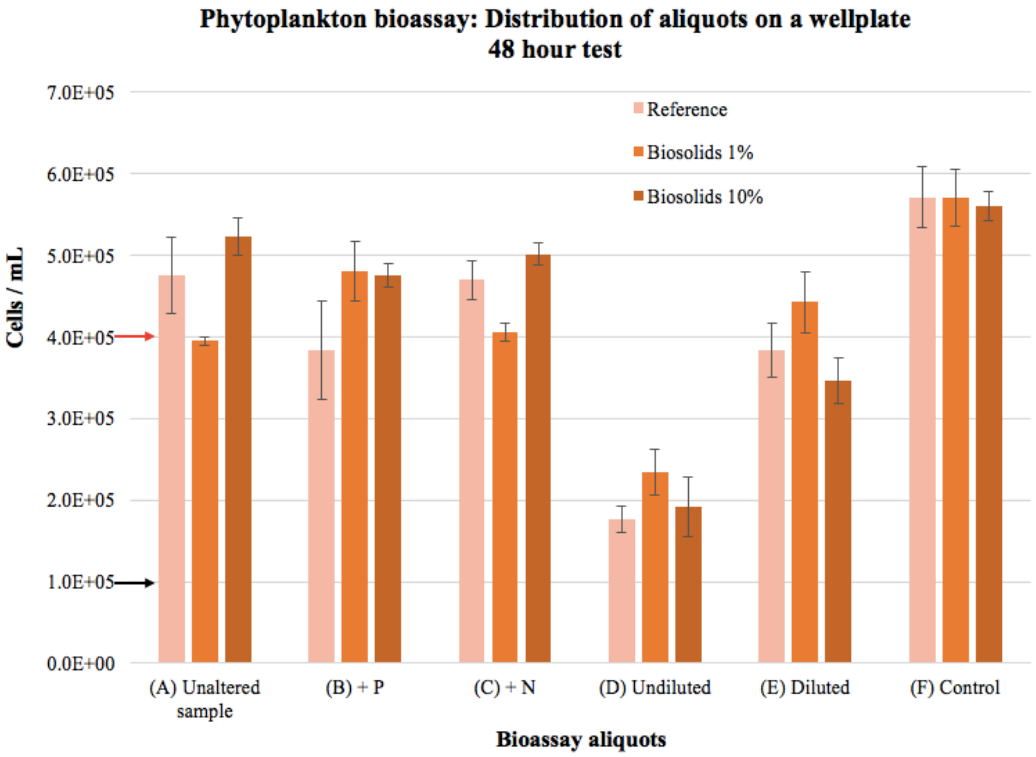


Figure 58: Phytoplankton bioassay carried out after first 48 hours of biosolids addition

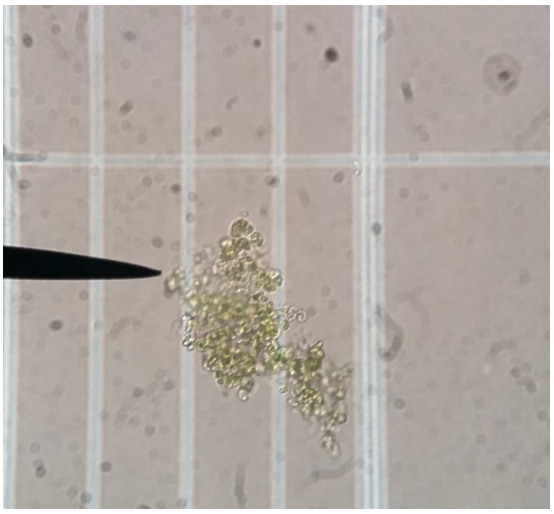
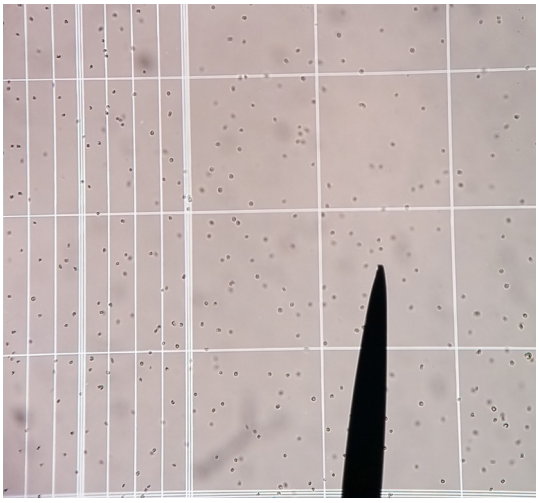
a)  b) 

Figure 59: a) Algal cells from well 'D' at 400x magnification; Cells from well 'F' at 100x magnification

The comparison of 'initial conditions' and 'Biosolids 1%' and 'Biosolids 10%' in Figure 56 displays a number of differences. When phosphorus is added to the sample of water in well 'B' and nitrogen is added in well 'C', there is an increase in cell concentration. However, the algal cell concentration declined in well 'D' for the Biosolids 1% and 10% treatment. However, due to the Reference in Figure 56 showing a similar decline in cell concentration, no conclusion regarding toxicity related to biosolids can be drawn. In well 'E' that contained 50% of the sample and all the nutrients needed for cell reproduction, the cell concentration shows a >20% decline than the control, yet it is higher than 'E' in the initial conditions. These observations were preliminary due to the decline in reference buckets as well as treatment buckets.

4.8.3 Phytoplankton Bioassay test after 2 months

Figure 60 displays the phytoplankton bioassay results for water samples taken two months after the initial biosolids runoff input. These data were collected two months later to allow the macrophytes time to adjust to the change in nutrient concentrations, develop biomass, and assess the difference nutrient concentrations in the water samples would have on the algal reproduction. The immediate difference in these results compared to those from the 48 hour

bioassay test (Figure 58) was the reduction in algal cells for all the wells, including the control well 'F'. After receiving two light cycles under the light-bank, the initial cell input of 1 x 10^5 cells/mL should have reached 4 x 10^5 cells per mL. However, this value was not reached even by the controls in these plates.

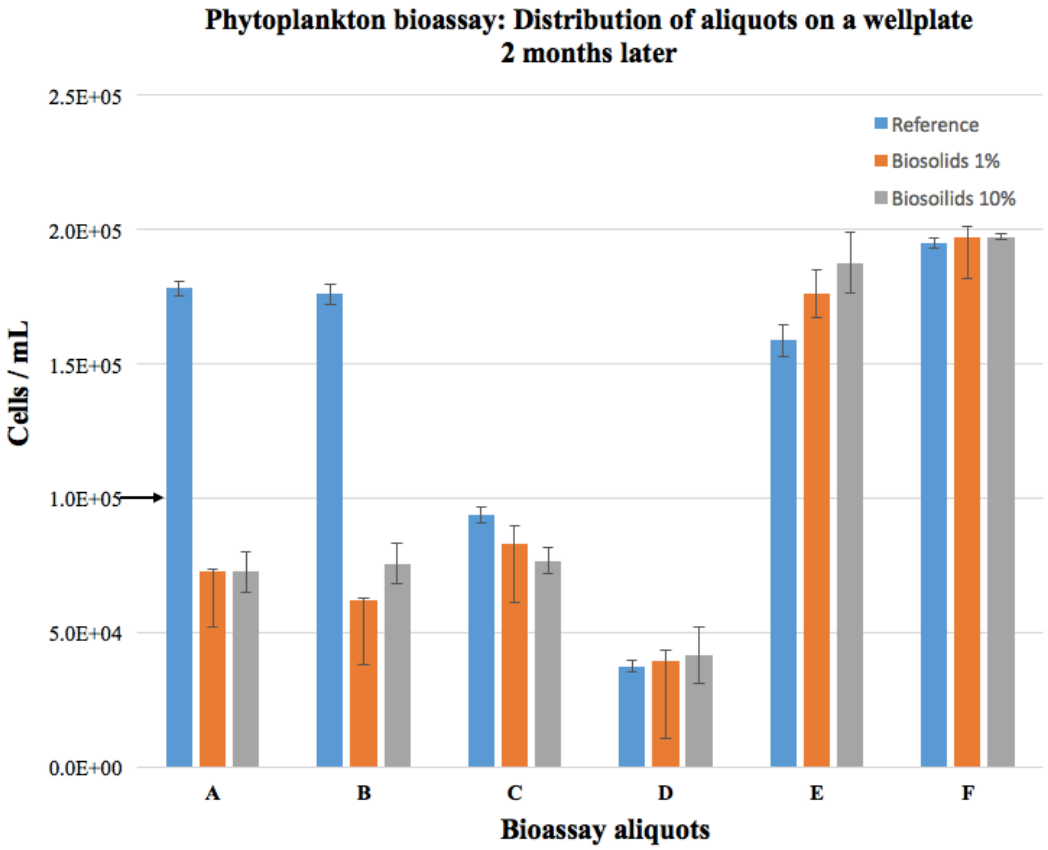


Figure 60: Phytoplankton bioassay carried out after first 2 months of biosolids addition

These outcomes may be the result of contamination in the algae cell stock culture. The consistency in these results compared to the results observed in the 48 hours bioassay can be seen for well 'D'. The cell counts for this well are still significantly lower compared to the other wells for each treatment. The statistical results for the phytoplankton bioassay can be found in Appendix V. These results confirm the significance in variance observed only for wells 'A' and 'B' between the treatments with $p < 0.05$. Based on these results, it could not be ascertained that the wetlands were efficient in nutrient removal. Further experimentation with a fresh culture is

necessary to use the phytoplankton bioassay to assess constructed wetland efficiency. If *P. subcapitata* is overly sensitive to test wetland water samples, the use of other test organisms such as *Euglena gracilis* may be warranted. A noteworthy observation relating the plant biomass to the nutrients is that biomass statistical analysis did show a difference in standard deviation for leaf length between the reference and the two treatments with a decrease in biomass for buckets treated with biosolids 10%.

4.9 Chemical Analysis

Orthophosphate is called "reactive" phosphorus and is the stable form that is bioavailable to macrophytes. One of the objectives of this thesis was to measure the efficiency of macrophyte nutrient (phosphorus) removal. Thus, reactive phosphorus was assessed in the water samples. Reactive phosphorus is present in the water in two forms, particulate and dissolved. The water samples used to collect the concentration of reactive phosphorus data were taken three weeks after the initial input of biosolids. The reference units have the lowest amount of dissolved phosphorus compared to the other two treatments. However, the reference treatment also had the highest amount of particulate phosphorus (Figure 61). Note that macrophytes use dissolved phosphorus for their growth (Vymazal, 2007). The concentration of particulate phosphorus is considerably lower than dissolved phosphorus in these constructed wetland mesocosms. As the concentration of biosolids increases, the concentration of dissolved phosphorus increases and the concentration of particulate phosphorus decreases within these constructed wetland units.

A statistical analysis test using a one-way ANOVA on these dissolved, particulate and total phosphorus concentrations revealed $p < 0.05$. These results suggested that the treatment wetlands had significantly different concentrations for dissolved, particulate and total phosphorus. The descriptive data used for this test can be found in Appendix VII. Note that Figure 60 shows no particulate P for biosolids 10% treatment. This is because the detection method had a range between 0.01 and 1 mg/L and was thus unable to detect any lower concentrations.

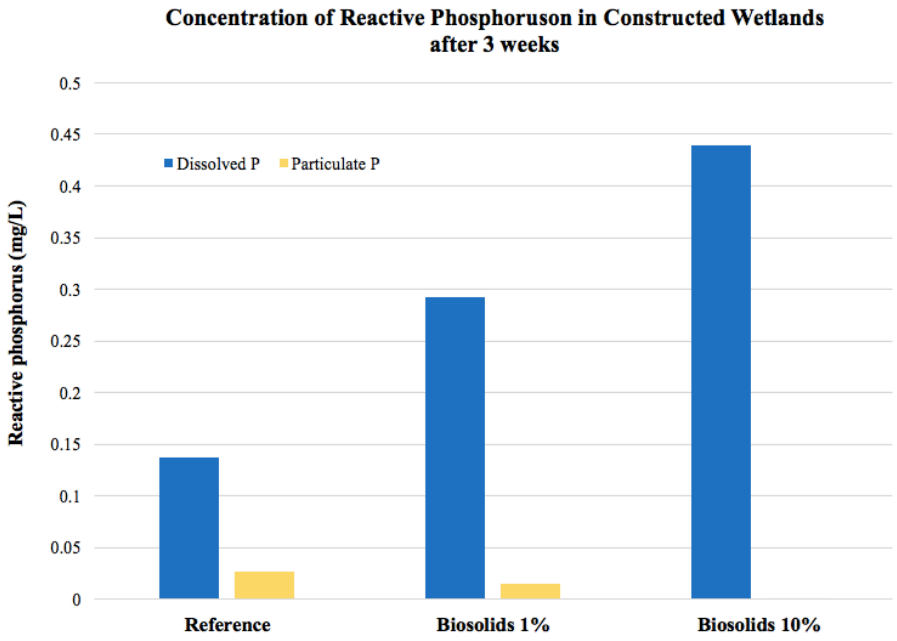


Figure 61: Concentration of dissolved and suspended phosphorus distribution among the three different treatments analyzed at 3 weeks

Water samples collected six weeks later displayed a decrease in dissolved phosphorus for the 10% treatment of biosolids. The descriptive data and one-way ANOVA results for this can be found in Appendix VII. The F-value for each treatment was higher than one, showing a significant difference in variance of treatments. Similar trends were observed for the water samples tested at six weeks after initial biosolids runoff input. Only the particulate phosphorus F-value was lower than one for the six-week test.

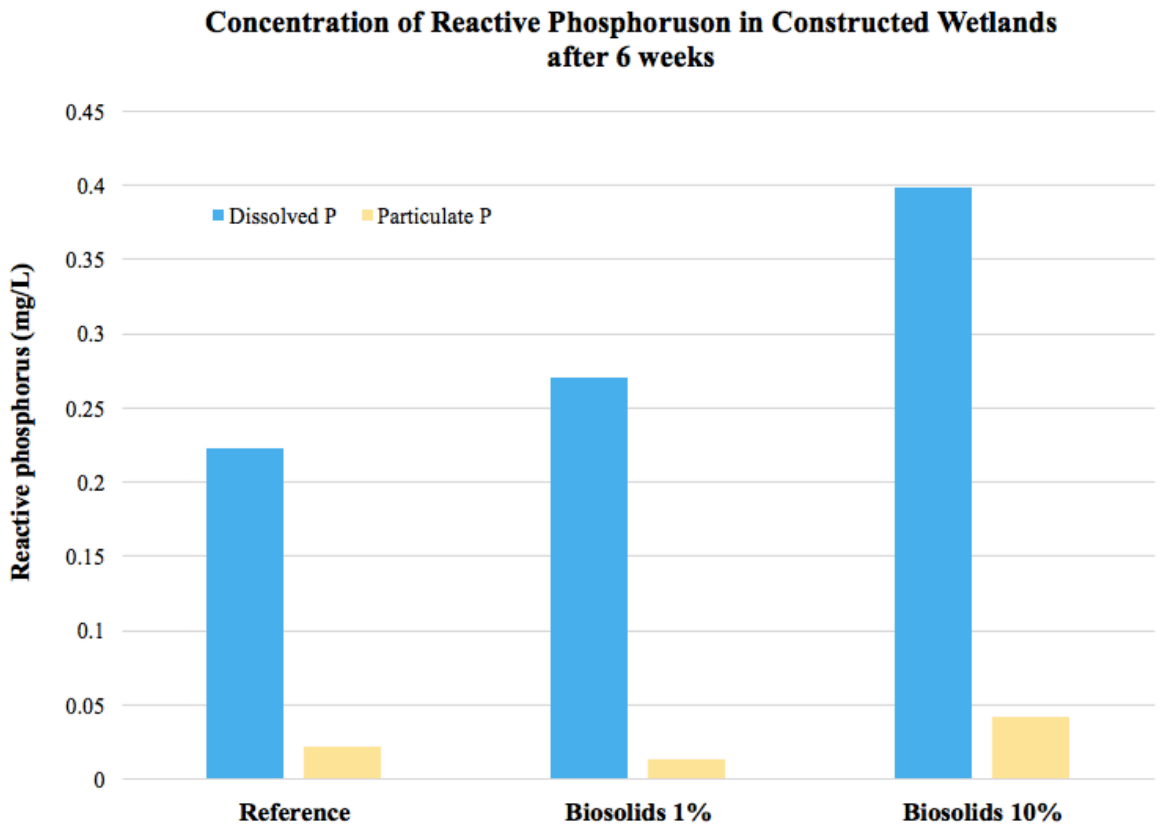


Figure 62: Concentration of dissolved and suspended phosphorus distribution among the three different treatments analyzed at 6 weeks

The particulate phosphorous concentrations were less than 0.1 mg/L in all treatments after three weeks and thus no significant difference was found in variance (Figure 62 and 63). This reduction in particulate phosphorus could be due to sedimentation to the bottom of the wetland or due to adsorption to the wetland matrix (Cooper *et al.,* 1996; Reddy and D' Angelo, 1997).

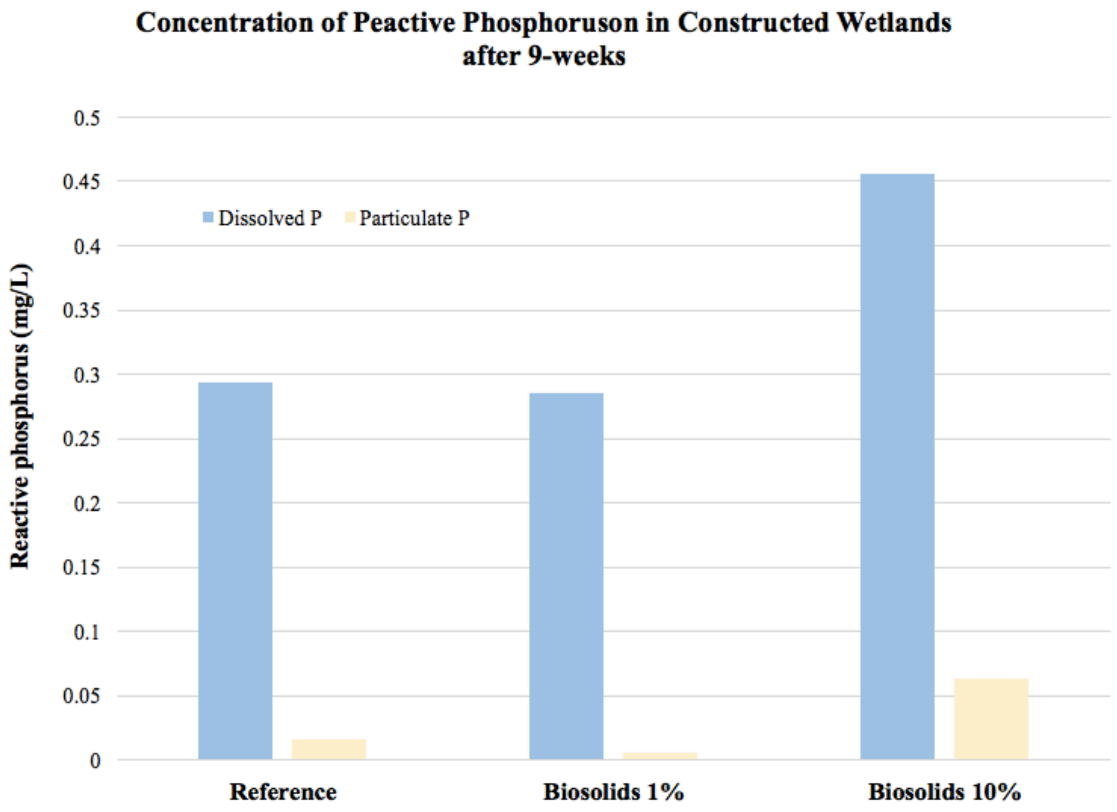


Figure 63: Concentration of dissolved and suspended phosphorus distribution among the three different treatments analyzed at 9 weeks

4.9.1 Total Phosphorus Analysis

The statistical analysis carried out on the total phosphorus concentration change in the three treatments over the nine-week period showed a significant difference in variance with F-values all greater than one. Although the total concentration of phosphorous differed between the three differences, it was not significantly reduced after nine weeks. This might suggest the need for longer water retention time, and higher below-ground biomass i.e. root structures. The descriptive data used for the total phosphorus one-way ANOVA test can be found in Appendix VII.

5.0 CONCLUSION

The Environment Canada (1996) list was useful in providing a starting point for macrophyte selection used for an engineered wetland. However, studies must develop criteria specific to their objectives and present those criteria in their work to demonstrate the effectiveness of selection in their research. These criteria must be standardized in some way to allow for comparison among macrophytes. Similarly, the selection of constructed wetland models and the efficiency of nutrient removal using constructed wetlands must be consistent throughout the research field.

Upon revisiting the hypotheses in Chapter 2, the first hypothesis on seed germination efficiencies based on various seed germination treatments can be accepted. Different germination treatments increased seed germination percentage in different seeds. Only two species did not germinate: *Pontederia cordata* (pickerelweed) and *Sparganium eurycarpum* (giant bur-reed). The following two hypotheses however, on phytoplankton growth in the biological test and nutrient concentrations in the nutrient analysis must be rejected. These tests did not show a variation in algae growth over time nor did it display a significant variation in nutrient concentration over time.

With regards to the "stationary" constructed wetlands used in this study (Figure 64a), a future recommendation to enhance nutrient removal would be to evolve the design. In the current design, the PVC tubes were directly in contact with the bottom surface of the buckets with a thick mesh in-between to reduce substrate loss from tubes. Several variables in this design must be altered and tested for efficiency. A change in mesh size that allows for increased root growth is imperative to increase direct contact between water and macrophyte roots i.e. increased surface area for absorption of nutrients. The PVC tubes should be placed at least 5 cm away from the bottom surface to reduce root damage over time. The last design change would be to incorporate a series of constructed wetlands to test the nutrient removal efficiency in future studies (Figure 64b).

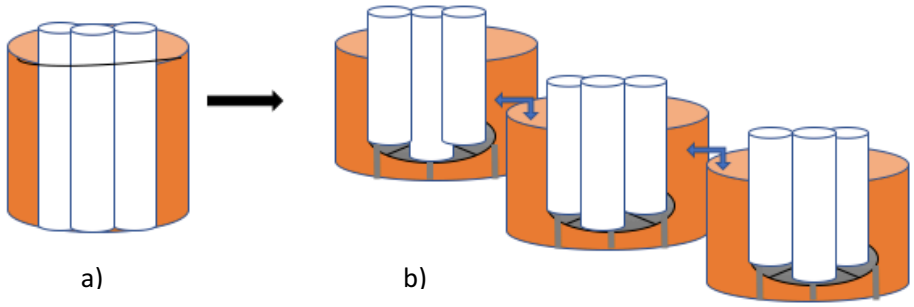


Figure 64: a) Current stationary constructed wetland model with b) future recommended changes to stationary constructed wetland model design

This thesis initiated with the intention of finding suitable macrophytes for constructed wetlands to treat excess nutrients. While this still holds true, the macrophytes play a much bigger role than we had originally anticipated. In moving forward, perhaps this journey the thesis has taken this team on along with the knowledge it has bestowed on us from past generations through the elders, James Whetung, and Jeff Beavers will raise awareness of the socio-cultural, economic and ecological importance of working to restore a sustainable environment.

6.0 EPILOGUE

A few months ago, it was determined that the inclusion of wild rice (Figure 64), along with the traditional knowledge associated with this species, would be a valuable addition to the macrophyte species being studied for nutrient removal in our constructed wetland models. Through research on native macrophytes, two experts on *Zizania aquatica* and *Zizania palustris* (two strains of wild rice) were found, James Whetung and Jeff Beaver (Courtesy of Drew Hayden Taylor, Curve Lake Community Centre and Alderville Community Centre). A brief background on James Whetung and on Jeff Beaver can be found in Appendix VIII. In June 2017, our team (Dr. Lynda McCarthy, Dr. Vadim Bostan and myself) received a chance to spend an entire day at Curve Lake and Alderville, near Peterborough, with these two experts to learn about wild rice. The objective of this meeting, was to explore the use of native macrophytes such as wild rice in constructed wetlands for nutrient removal. The message we came back to Toronto with, was the importance of macrophytes in a socio-cultural, economic and ecological perspective.

At Curve Lake (Figure 65, 66, 67), James talked about the differences between farming practices between the First Nations and European settlers beginning in the 1600s. One of the major differences he mentioned was the use of pesticides and herbicides. He related the effects of these chemicals on native macrophytes such as wild rice (*Zizania aquatica and Zizania palustris*). A decrease in this staple food availability resulted in numerous health problems within the community, a prominent heath concern being the rise in diabetes' cases. Jeff, at Alderville, had spent the last decade restoring Manomin beds and documenting Emily Creek, Pigeon Lake, and Rice Lake and continues to do so. As Jeff explained to us, Manomin is an Ojibwa term for wild rice that means "good berry." Wild rice is one of the four sacred foods of the Ojibwa peoples. It is considered scared because it is believed to be a gift from the creator. The other three foods are corn, fish and deer. Wild rice plays more than a nutritious role in the community, it has cultural significance.

With the farming practices changing due to land availability, the importance of growing wild rice heightened. This crop is grown in the water that is approximately six feet deep. However, with the increase in tourism and colonial arrogance, the land, lake and water were destroyed. The construction of the Trent canals between 1833 and 1920 made it possible for

steamships to navigate through these waters. The movement of these large vessels however damaged this aquatic crop.

Prior to the 1600s the Indigenous peoples had nationhood. As James said, “The water was the road.” The water in which the wild rice grew was also a nursery for many small fish and amphibians. The beaver, muskrats and eagles were all part of this biodiverse ecosystem. “The clothes of the muskrat were used to make clothes for the First Nation peoples,” said James. The biodiversity of Rice Lake included the American eel, trout, ducks, geese and waterfowl. Crops, fish and deer were consumed based on community needs. As Jeff said, “[Our ancestors] tried to plan ahead for seven generations.” Rice Lake gets its name from having, at some point, over 10, 000 acres of wild rice.

At the end of August, all the different First Nation bands would come to Rice Lake and help in hand harvesting this crop. This occasion was celebrated as a feast during which communities got an opportunity to meet relatives, trade and bond. Wild rice was harvested with hand and canoes by gently dropping the ripe seeds into the canoes. The seeds that were still growing were left on the grass to fall into the water and seed the following year’s crop. Once the grains were collected, they were slowly roasted for preservation and treated with wood ash. This helped preserve the rice to make it last through winter until the next harvest. Since dairy was not part of the cultural foods, the added wood ash increased the calcium in this staple food that was needed by the Indigenous peoples.

The First Nations consider some wetlands as spiritual points. There are numerous reasons for this belief. According to Jeff, wetlands are spiritual points because they provide nutritional food originally grown by ancestors of the tribes. As Jeff and James continually work to preserve their culture through educating the youth in their community on canoeing and harvesting practices, they also encouraged us to see the macrophytes as more than ‘biotech,’ but as a significant part of their community and the larger ecosystem.

Figure 65: Wild rice in Curve Lake in the floating leaf stage

Figure 66: James (on the right) with his associate Michelle (on the left) near the wild rice in the aerial stage

There are plenty of species that we considered to be native or foreign because of their origin. Some species such as *Phragmites* that were introduced by European settlers in the 1600s can grow and out compete many native species. While on Curve Lake, we observed hundreds of Water Lily pads covering the water (Figure 66), this is an introduced species as well. When we inquired with James and Jeff regarding the invasiveness of this species, it was interesting to observe both people not labeling this macrophyte as a danger to their wild rice. Instead, we were informed that each species has a life span to which they grow, fulfil their purpose and then decompose. Unlike our perspective of foreign versus innate, their paradigm showed us a much less compartmental and more inclusive view.

Figure 67: Water Lily pads on Curve Lake

7.0 PUBLICATION IN INFLUENTS, SUMMER 2017

Constructed wetlands as a eutrophication mitigation strategy for the Great Lakes

Francesca M. Fernandes, Ryerson University

The Laurentian Great Lakes consists of five major bodies of water, including Lakes Superior, Michigan, Huron, Erie, and Ontario. According to the United States Environmental Protection Agency, these lakes hold approximately 21% of the world's surface freshwater. From concerns regarding invasive species to pollutants, a recurrent issue has been excess nutrients entering the Great Lakes. Over the last half a century, Lake Erie has experienced the highest amounts of eutrophication. While it was first noted in the Great Lakes Water Quality Agreement of 1972, this effect is the direct result of excess nutrients that give rise to algae blooms. The 2014 Lake Erie Ecosystem Priority Report by the International Joint Commission, noted that phosphorus, the limiting nutrient for many phytoplankton and algae, was the cause of the current observed eutrophication. Consequently, this community has witnessed diminishing water quality and impacts on fisheries, tourism, and property values.

One of the many solutions that have been proposed to reduce excess nutrient runoff into the Great Lakes has been wetlands. Natural wetlands are dynamic ecosystems that share characteristics of both water and land. These naturally-occurring ecosystems serve different purposes such as the preservation of ecological diversity, habitat for organisms, flood prevention in certain areas, and recreation. Wetlands have also been used as a secondary or tertiary wastewater treatment system for almost a century by European settlers in North America.

In the past few decades, engineered wetlands have been constructed to compensate for the loss of natural wetlands, due to farming and urbanization. Previous research has demonstrated that vegetated wetlands have a higher capacity for sequestering nutrients such as phosphorus and nitrogen from wastewater, in comparison to non-vegetated stormwater ponds. Wastewater or runoff entering aquatic ecosystems mainly contains organics, nutrients, heavy metals, and pathogens. The macrophytes in an engineered wetland helps to remove nutrients while contaminants can be taken out through sedimentation and adhesion to the wetland matrix. Depending on the retention time, hydraulic flow, width, and depth of the wetland, the efficiency of contaminant removal varies.

The most common vegetation used in wetlands have been *Phragmites australis* (common reed) and *Typha latifolia* (cattail). These species have a high nutrient removal rate. However, these macrophytes are also able to out-compete many native species in North America, decreasing biodiversity and becoming invasive. Thus, macrophyte selection is a crucial step in constructing sustainable wetlands. The species may then be harvested and used for various purposes at the end of each growing season. Prior to European settlement, *Zizania aquatica* (wild rice) was a common lakeshore species planted as part of the aboriginal staple food strategy.

After decades of research, excess nutrients in freshwater ecosystems are still a concern. The source has moved from point-source phosphorous to non-point source phosphorus. Current extreme weather events such as heavy thunderstorms in short time frames can cause higher runoff. Over the last twenty years, wetlands have been used in some places as a buffer system to reduce contaminants from entering aquatic ecosystems. However, germinating indigenous macrophytes for this purpose, comparing polyculture efficiency in nutrient uptake, and maintaining viability are areas that bear further study.

At Ryerson University, through the Environmental Applied Science and Management program in collaboration with Ryerson Urban Water, studies are currently being carried out to evaluate the prior- and post-efficiency of using an engineered wetland as a method to treat agricultural runoff containing excess nutrients, before it enters an aquatic ecosystem such as Lake Erie. Researchers such as Dr. Lynda McCarthy and Dr. Vadim Bostan, along with myself, have been working with the Curve Lake First Nation community to reuse indigenous species such as wild rice and to plant them with other species such as *Carex sp.* (fox sedge) in constructed surface flow wetlands (Figure 1 (68)).

The Laurentian Great Lakes are more than just a geological creation. They form a unique ecosystem and provide important resources to the community. Protecting the source of our water and reducing the effects of eutrophication is an on-going process. Using a nature-mimicking system such as constructed wetlands, with non-invasive and diverse macrophyte species, could be the key to a promising eutrophication-free future for areas such as Lake Erie.

Figure 1(68): *Carex vulpinoidea* in stationary constructed wetland model

8.0 APPENDICES

APPENDIX I - Taxonomic Keys for Macrophyte List (Environment Canada, 1996)

Key to Alisma plantago-aquatica (USDA Alisma plantago-aquatica)

Kingdom:	Plantae
Subkingdom:	Tracheobinota – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta (Angiosperm) – Flowering plants
Class:	Liliopsida - Monocotyledons
Subclass:	Alismatidae
Order:	Alismatales
Family:	Alismataceae
Genus:	Alisma L.
Species	A. plantago-aquatica L.

Key to Asclepias incarnata (USDA Asclepias incarnata)

Kingdom:	Plantae
Subkingdom:	Tracheobinota – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta (Angiosperm) – Flowering plants
Class:	Magnoliopsida – Dicotyledons
Subclass:	Asteridae
Order:	Gentianales
Family:	Asclepiadaceae
Genus:	Asclepias L.
Species:	A. incarnata L.

Key to Carex vulpinoidea (USDA Carex vulpinoidea)

Kingdom:	Plantae
Subkingdom:	Tracheobinota – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta (Angiosperm) – Flowering plants
Class:	Liliopsida – Monocotyledons
Subclass:	Commenlinidae
Order:	Cyperales
Family:	Cyperaceae
Genus:	Carex L.

Key to Chelone glabra (USDA Chelone glabra)

Kingdom:	Plantae
Subkingdom:	Tracheobinota – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta (Angiosperm) – Flowering plants
Class:	Magnoliophyta (Dicotyledons)
Subclass:	Asteridae
Order:	Scrophulariales
Family:	Scrophulariaceae
Genus:	Chelone L.
Species	C. glabra L.

Key to Eleocharis spp. (USDA Eleocharis spp.)

Kingdom:	Plantae
Subkingdom:	Tracheobinota – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta (Angiosperm) – Flowering plants
Class:	Liliopsida – Monocotyledons
Subclass:	Commelinidae
Order:	Cyperales
Family:	Cyperaceae
Genus:	Eleocharis

Key to Equisetum fluviatile (USDA Equisetum fluviatile)

Kingdom:	Plantae
Subkingdom:	Tracheobinota – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta (Angiosperm) – Flowering plants
Class:	Equisetopsida
Order:	Equisetales
Family:	Equisetaceae
Genus:	Equisetum L.
Species	E. fluviatile L.

Key to Iris versicolor (USDA Iris versicolor)

Kingdom:	Plantae
Subkingdom:	Tracheobinota – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta (Angiosperm) – Flowering plants
Class:	Liliopsida -Monocotyledons
Subclass:	Liliidae
Order:	Liliales
Family:	Iridaceae
Genus:	Iris L.
Species	I. versicolor L.

Key to Juncus spp (USDA Juncus spp)

Kingdom:	Plantae
Subkingdom:	Tracheobinota – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta (Angiosperm) – Flowering plants
Class:	Liliopsida - Monocotyledons
Subclass:	Commelinidae
Order:	Juncales
Family:	Juncaceae
Genus:	Juncus L.

Key to Pontederia cordata (USDA Pontederia cordata)

Kingdom:	Plantae
Subkingdom:	Tracheobionta – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta – Flowering plants
Class:	Liliopsida - Monocotyledons
Subclass:	Liliidae
Order:	Liliales
Family:	Pontederiaceae
Genus:	Pontederia L.
Species	P. cordata L.

Key to Scirpus atrovirens (USDA Scirpus atrovirens)

Kingdom:	Plantae
Subkingdom:	Tracheobionta – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta – Flowering plants
Class:	Liliopsida - Monocotyledons
Subclass:	Commelinidae
Order:	Cyperales
Family:	Cyperaceae
Genus:	Scirpus L.
Species	S. atrovirens Willd.

Key to Scirpus validus (USDA Scirpus validus)

Kingdom:	Plantae
Subkingdom:	Tracheobinta – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta – Flowering plants
Class:	Liliopsida - Monocotyledons
Subclass:	Commelinidae
Order:	Cyperales
Family:	Cyperaceae
Genus:	Schoenoplectus
Species	S. tabernaemontani

Key to Sparganium eurycarpum (USDA Sparganium eurycarpum)

Kingdom:	Plantae
Subkingdom:	Tracheobionta – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Manoliophyta – Flowering plants
Class:	Liliopsida - Monocotyledons
Subclass:	Commelinidae
Order:	Typhales
Family:	Sparganiaceae
Genus:	Sparganium L.
Species	S. eurycarpum Engelm.

Key to Typha spp. (USDA Typha spp.)

Kingdom:	Plantae - Plants
Subkingdom:	Tracheobionta – Vascular plants
Superdivision:	Spermatophyta - Seed plants
Division:	Magnoliophyta – Flowering plants
Class:	Liliopsida - Monocotyledons
Subclass:	Commelinidae
Order:	Typhales
Family:	Typhaceae
Genus:	Typha L.

Key to Veronica Americana (USDA Veronica americana)

Kingdom:	Plantae - Plants
Subkingdom:	Tracheobionta – Vascular plants
Superdivision:	Spermatophyta – Seed plants
Division:	Magnoliophyta – Flowering plants
Class:	Magnoliopsida - Dicotyledons
Subclass:	Asteridae
Order:	Scrophulariales
Family:	Scrophulariaceae
Genus:	Veronica L.
Species	V. americana Schwein.

APPENDIX II – Discrete and ANOVA Data for Germination Treatment

Discrete data used to assess for significance in germination scarification treatments

	N	Mean	Std. Deviation	Minimum	Maximum
Rank	7	1.1429	2.60951	.00	7.00
Rank	7	1.5714	2.93582	.00	8.00
Rank	7	1.8571	3.18479	.00	7.00
Rank	7	1.5714	2.82000	.00	7.00
Rank	7	2.0000	3.46410	.00	8.00
Rank	7	.0000	.00000	.00	.00
Rank	7	1.0000	2.64575	.00	7.00
Rank	7	3.5714	3.40867	.00	9.00
Rank	7	.5714	1.51186	.00	4.00

APPENDIX III – Discrete and ANOVA Data for plant biomass

Descriptive data used in a one-way ANOVA to assess significance in plant biomass

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Max	Min
LeafLength	1	3	45.5000	2.66646	1.53948	38.8762	52.1238	43.40	48.50
	2	3	43.8333	6.42599	3.71005	27.8703	59.7964	38.90	51.10
	3	3	37.0667	3.06649	1.77044	29.4491	44.6842	35.10	40.60
	Total	9	42.1333	5.42333	1.80778	37.9646	46.3021	35.10	51.10
LeafWidth	1	3	.3333	.05774	.03333	.1899	.4768	.30	.40
	2	3	.3000	.00000	.00000	.3000	.3000	.30	.30
	3	3	.3000	.00000	.00000	.3000	.3000	.30	.30
	Total	9	.3111	.03333	.01111	.2855	.3367	.30	.40
StemLength	1	3	3.9333	.75056	.43333	2.0689	5.7978	3.20	4.70
	2	3	4.1667	.60277	.34801	2.6693	5.6640	3.60	4.80
	3	3	3.7000	.36056	.20817	2.8043	4.5957	3.40	4.10
	Total	9	3.9333	.55227	.18409	3.5088	4.3578	3.20	4.80
StemWidth	1	3	.2000	.00000	.00000	.2000	.2000	.20	.20
	2	3	.2333	.05774	.03333	.0899	.3768	.20	.30
	3	3	.2333	.05774	.03333	.0899	.3768	.20	.30
	Total	9	.2222	.04410	.01470	.1883	.2561	.20	.30

One-way ANOVA test result for plant biomass of Carex vulpinoidea

Variable		Sum of Squares	df	Mean Square	F	Sig.
Leaf Length	Between Groups	119.687	2	59.843	3.106	.119
	Within Groups	115.613	6	19.269		
	Total	235.300	8			
Leaf Width	Between Groups	.002	2	.001	1.000	.422
	Within Groups	.007	6	.001		
	Total	.009	8			
Stem Length	Between Groups	.327	2	.163	.464	.650
	Within Groups	2.113	6	.352		
	Total	2.440	8			
Stem Width	Between Groups	.002	2	.001	.500	.630
	Within Groups	.013	6	.002		
	Total	.016	8			

APPENDIX IV – Composition of Bristol's Media (Nichols, 1973)

Macronutrients	**Concentration (mg/L)**
$NaNO_3$	250
$CaCL_2 \cdot 2H_2O$	25
$MgSO_4 \cdot 7H_2O$	75
K_2HPO_4	75
KH_2PO_4	175
NaCl	25
EDTA	50
KOH	31
$FeSO_4 \cdot 7H_2O$	4.98
Conc. H_2SO_4	0.001 mL
H_3BO_3	11.42

Micronutrients	**Concentration (mg/L)**
$ZnSO_4 \cdot 7H_2O$	8.82
$MnCl_2 \cdot 4H_2O$	1.44
MoO_3	0.71
$CuSO_4 \cdot 5H_2O$	1.57
$Co(NO_3)_2 \cdot 6H_2O$	0.49

APPENDIX V – Statistical Results for Phytoplankton Bioassay

One-way ANOVA results for the phytoplankton bioassay tested 2 months after biosolids addition

Wells		Sum of Squares	df	Mean Square	F	Sig.
A	Between Groups	22302720000.000	2	11151360000.000	171.947	.000
	Within Groups	389120000.000	6	64853333.330		
	Total	22691840000.000	8			
B	Between Groups	23272106670.000	2	11636053330.000	164.952	.000
	Within Groups	423253333.300	6	70542222.220		
	Total	23695360000.000	8			
C	Between Groups	446008888.900	2	223004444.400	3.063	.121
	Within Groups	436906666.700	6	72817777.780		
	Total	882915555.600	8			
D	Between Groups	27306666.670	2	13653333.330	.109	.898
	Within Groups	750933333.300	6	125155555.600		
	Total	778240000.000	8			
E	Between Groups	1258382222.000	2	629191111.100	2.537	.159
	Within Groups	1488213333.000	6	248035555.600		
	Total	2746595556.000	8			
F	Between Groups	9102222.222	2	4551111.111	.235	.797
	Within Groups	116053333.300	6	19342222.220		
	Total	125155555.600	8			

APPENDIX VI: Ascorbic Acid Stock Solutions

Stock solution preparation for phosphorus analysis using ascorbic acid method

Number	Stock Solutions	Preparation
I	Sulphuric acid (2.5 M)	70 mL H_2SO_4 in 500 mL d.H_2O*
II	Potassium antimonyl tartrate solution	1.3715 g $K(SbO)C_4H_4O_6 \cdot {}^1/_2H_2O$ in 400 mL d.H_2O
III	Ammonium molybdate solution	20 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 500 mL d.H_2O
IV**	Ascorbic acid (0.1 M)	1.76 g $C_6H_8O_6$ in 100 mL d.H_2O
V	Phosphate solution	0.2195 g anhydrous KH_2PO_4 in 1000 mL d.H_2O

*d.H20 – distilled water

** Stock IV is stored at 4°C and is only stable for one week after preparation.

APPENDIX VII – Discrete and ANOVA Data for Chemical Assay

Descriptive data used for One-way ANOVA of Chemical Assay Water Samples three weeks after biosolids input into constructed wetlands

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Max	Min
Dissolved	1.00	9	.1367	.05362	.01787	.0955	.1779	.06	.20
	2.00	9	.2789	.08192	.02731	.2159	.3419	.16	.34
	3.00	9	.4411	.20582	.06861	.2829	.5993	.25	.72
	Total	27	.2856	.17902	.03445	.2147	.3564	.06	.72
Particulate	1.00	9	.0311	.03060	.01020	.0076	.0546	.00	.08
	2.00	9	.1200	.06982	.02327	.0663	.1737	.02	.22
	3.00	9	.2411	.16922	.05641	.1110	.3712	.00	.37
	Total	27	.1307	.13525	.02603	.0772	.1842	.00	.37
Total	1.00	9	.1122	.04438	.01479	.0781	.1463	.05	.16
	2.00	9	.2767	.07533	.02511	.2188	.3346	.18	.38
	3.00	9	.4378	.19961	.06654	.2843	.5912	.26	.72
	Total	27	.2756	.18154	.03494	.2037	.3474	.05	.72

One-way ANOVA for Chemical Assay Water Samples three weeks after biosolids input into constructed wetlands

Variable		**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Dissolved Phosphorus	Between Groups	0.418	2	0.209	12.061	0.000
	Within Groups	0.416	24	0.017		
	Total	0.833	26			
Particulate Phosphorus	Between Groups	0.200	2	0.100	8.709	0.001
	Within Groups	0.276	24	0.011		
	Total	0.476	26			
Total Phosphorus	Between Groups	0.477	2	0.238	15.065	0.000
	Within Groups	0.380	24	0.016		
	Total	0.857	26			

Descriptive data used for One-way ANOVA of Chemical Assay Water Samples six weeks after biosolids input into constructed wetlands

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Max	Min
Dissolved	1.00	9	.2244	.03283	.01094	.1992	.2497	.20	.31
	2.00	9	.2700	.01658	.00553	.2573	.2827	.25	.29
	3.00	9	.3978	.05995	.01998	.3517	.4439	.31	.47
	Total	27	.2974	.08433	.01623	.2640	.3308	.20	.47
Particulate	1.00	9	.0244	.03127	.01042	.0004	.0485	.00	.10
	2.00	9	.0156	.01667	.00556	.0027	.0284	.00	.04
	3.00	9	.0578	.02438	.00813	.0390	.0765	.03	.11
	Total	27	.0326	.03020	.00581	.0206	.0445	.00	.11
Total	1.00	9	.2022	.01563	.00521	.1902	.2142	.18	.22
	2.00	9	.2567	.01118	.00373	.2481	.2653	.24	.27
	3.00	9	.3578	.04024	.01341	.3268	.3887	.29	.39
	Total	27	.2722	.07018	.01351	.2445	.3000	.18	.39

One-way ANOVA for Chemical Assay Water Samples six weeks after biosolids input into constructed wetlands

		Sum of Squares	df	Mean Square	F	Sig.
Dissolved	Between Groups	.145	2	.073	44.067	.000
	Within Groups	.040	24	.002		
	Total	.185	26			
Particulate	Between Groups	.009	2	.004	7.231	.003
	Within Groups	.015	24	.001		
	Total	.024	26			
Total	Between Groups	.112	2	.056	84.587	.000
	Within Groups	.016	24	.001		
	Total	.128	26			

Descriptive data used for One-way ANOVA of Chemical Assay Water Samples nine weeks after biosolids input into constructed wetlands

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Max	Min
Dissolved	1.00	9	.2956	.03127	.01042	.2715	.3196	.25	.33
	2.00	9	.2844	.03206	.01069	.2598	.3091	.23	.31
	3.00	9	.4556	.04003	.01334	.4248	.4863	.40	.52
	Total	27	.3452	.08635	.01662	.3110	.3793	.23	.52
Particulate	1.00	9	.0400	.03279	.01093	.0148	.0652	.01	.09
	2.00	9	.0422	.02819	.00940	.0206	.0639	.00	.07
	3.00	9	.0811	.14887	.04962	-.0333	.1955	.00	.47
	Total	27	.0544	.08811	.01696	.0196	.0893	.00	.47
Total	1.00	9	.2778	.03492	.01164	.2509	.3046	.24	.32
	2.00	9	.2800	.02958	.00986	.2573	.3027	.23	.31
	3.00	9	.5189	.18072	.06024	.3800	.6578	.41	.99
	Total	27	.3589	.15488	.02981	.2976	.4202	.23	.99

One-way ANOVA for Chemical Assay Water Samples nine weeks after biosolids input into constructed wetlands

		Sum of Squares	df	Mean Square	F	Sig.
Dissolved	Between Groups	.165	2	.083	68.594	.000
	Within Groups	.029	24	.001		
	Total	.194	26			
Particulate	Between Groups	.010	2	.005	.601	.557
	Within Groups	.192	24	.008		
	Total	.202	26			
Total	Between Groups	.346	2	.173	14.917	.000
	Within Groups	.278	24	.012		
	Total	.624	26			

Descriptive data used for One-way ANOVA of Dissolved Phosphorus Chemical Assay Water Samples nine weeks after biosolids input into constructed wetlands

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Max	Min
Dissolved1	1.00	9	.1367	.05362	.01787	.0955	.1779	.06	.20
	2.00	9	.2789	.08192	.02731	.2159	.3419	.16	.34
	3.00	9	.4411	.20582	.06861	.2829	.5993	.25	.72
	Total	27	.2856	.17902	.03445	.2147	.3564	.06	.72
Dissolved2	1.00	9	.2244	.03283	.01094	.1992	.2497	.20	.31
	2.00	9	.2700	.01658	.00553	.2573	.2827	.25	.29
	3.00	9	.3978	.05995	.01998	.3517	.4439	.31	.47
	Total	27	.2974	.08433	.01623	.2640	.3308	.20	.47
Dissolved3	1.00	9	.2956	.03127	.01042	.2715	.3196	.25	.33
	2.00	9	.2844	.03206	.01069	.2598	.3091	.23	.31
	3.00	9	.4556	.04003	.01334	.4248	.4863	.40	.52
	Total	27	.3452	.08635	.01662	.3110	.3793	.23	.52

One-way ANOVA for Dissolved Phosphorus Chemical Assay Water Samples nine weeks after biosolids input into constructed wetlands

		Sum of Squares	df	Mean Square	F	Sig.
Dissolved1	Between Groups	.418	2	.209	12.061	.000
	Within Groups	.416	24	.017		
	Total	.833	26			
Dissolved2	Between Groups	.145	2	.073	44.067	.000
	Within Groups	.040	24	.002		
	Total	.185	26			
Dissolved3	Between Groups	.165	2	.083	68.594	.000
	Within Groups	.029	24	.001		
	Total	.194	26			

Descriptive data used for One-way ANOVA of Particulate Phosphorus Chemical Assay Water Samples nine weeks after biosolids input into constructed wetlands

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Max	Min
Particulate1	1.00	9	.0311	.03060	.01020	.0076	.0546	.00	.08
	2.00	9	.1200	.06982	.02327	.0663	.1737	.02	.22
	3.00	9	.2411	.16922	.05641	.1110	.3712	.00	.37
	Total	27	.1307	.13525	.02603	.0772	.1842	.00	.37
Particulate2	1.00	9	.0244	.03127	.01042	.0004	.0485	.00	.10
	2.00	9	.0156	.01667	.00556	.0027	.0284	.00	.04
	3.00	9	.0578	.02438	.00813	.0390	.0765	.03	.11
	Total	27	.0326	.03020	.00581	.0206	.0445	.00	.11
Particulate3	1.00	9	.0400	.03279	.01093	.0148	.0652	.01	.09
	2.00	9	.0422	.02819	.00940	.0206	.0639	.00	.07
	3.00	9	.0811	.14887	.04962	-.0333	.1955	.00	.47
	Total	27	.0544	.08811	.01696	.0196	.0893	.00	.47

One-way ANOVA for Particulate Phosphorus Chemical Assay Water Samples nine weeks after biosolids input into constructed wetlands

		Sum of Squares	df	Mean Square	F	Sig.
Particulate1	Between Groups	.200	2	.100	8.709	.001
	Within Groups	.276	24	.011		
	Total	.476	26			
Particulate2	Between Groups	.009	2	.004	7.231	.003
	Within Groups	.015	24	.001		
	Total	.024	26			
Particulate3	Between Groups	.010	2	.005	.601	.557
	Within Groups	.192	24	.008		
	Total	.202	26			

Descriptive data used for One-way ANOVA of Total Phosphorus Chemical Assay Water Samples nine weeks after biosolids input into constructed wetlands

						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Max	Min
Total1	1.00	9	.1122	.04438	.01479	.0781	.1463	.05	.16
	2.00	9	.2767	.07533	.02511	.2188	.3346	.18	.38
	3.00	9	.4378	.19961	.06654	.2843	.5912	.26	.72
	Total	27	.2756	.18154	.03494	.2037	.3474	.05	.72
Total2	1.00	9	.2022	.01563	.00521	.1902	.2142	.18	.22
	2.00	9	.2567	.01118	.00373	.2481	.2653	.24	.27
	3.00	9	.3578	.04024	.01341	.3268	.3887	.29	.39
	Total	27	.2722	.07018	.01351	.2445	.3000	.18	.39
Total3	1.00	9	.2778	.03492	.01164	.2509	.3046	.24	.32
	2.00	9	.2800	.02958	.00986	.2573	.3027	.23	.31
	3.00	9	.5189	.18072	.06024	.3800	.6578	.41	.99
	Total	27	.3589	.15488	.02981	.2976	.4202	.23	.99

One-way ANOVA for Total Phosphorus Chemical Assay Water Samples nine weeks after biosolids input into constructed wetlands

Variable		**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
TP 3 Weeks	Between Groups	0.477	2	0.238	15.065	0.000
	Within Groups	0.380	24	0.016		
	Total	0.857	26			
TP 6 Weeks	Between Groups	0.112	2	0.056	84.587	0.000
	Within Groups	0.016	24	0.001		
	Total	0.128	26			
TP 9 Weeks	Between Groups	0.346	2	0.173	14.917	0.000
	Within Groups	0.278	24	0.012		

APPENDIX VIII – James Whetung and Jeff Beaver

James Whetung (from to the Curve Lake First Nations) is the owner of Black Duck Wild Rice and has over 25 years of experience in growing, collecting and processing wild rice. His understanding of this macrophyte has been rooted in traditional knowledge and over the decades he has made this the foundation of his company. The other expert was Jeff Beaver (from the Alderville First Nations) who is a public speaker on Alderville traditions and culture and an ecologist. He has spent the last decade restoring wild rice beds and documenting them throughout Treaty 20, Williams Treaty territory.

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