ABSTRACT

Title of Document: INTERACTIVE EFFECTS OF PLANT SPECIES AND ORGANIC CARBON ON NITRATE REMOVAL IN CHESAPEAKE BAY TREATMENT WETLANDS

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Nitrate from agricultural runoff are a significant cause of algal blooms in estuarine ecosystems such as the Chesapeake Bay. These blooms block sunlight vital to submerged aquatic vegetation, leading to hypoxic areas. Natural and constructed wetlands have been shown to reduce the amount of nitrate flowing into adjacent bodies of water. We tested three wetland plant species native to Maryland, *Typha latifolia* (cattail), *Panicum virgatum* (switchgrass), and *Schoenoplectus validus* (soft-stem bulrush), in wetland microcosms to determine the effect of species combination and organic amendment on nitrate removal. In the first phase of our study, we found that microcosms containing sawdust exhibited significantly greater nitrate removal than microcosms amended with glucose or hay at a low nitrate loading rate. In the second phase of our study, we confirmed that combining these plants removed nitrate, although no one combination was significantly better. Furthermore, the above-ground biomass of microcosms containing switchgrass had a significantly greater percentage of carbon than microcosms without switchgrass, which can be studied for potential biofuel use. Based on our data, future environmental groups can make a more informed decision when choosing biofuel-capable plant species for artificial wetlands native to the Chesapeake Bay Watershed.

INTERACTIVE EFFECTS OF PLANT SPECIES AND ORGANIC CARBON ON NITRATE REMOVAL IN CHESAPEAKE BAY TREATMENT WETLANDS

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Chapter 1: Introduction

Pollution in the Chesapeake Bay is a longstanding problem that is the result of years of industrial and agricultural contamination throughout the Bay Watershed. In particular, agricultural runoff into the Chesapeake Bay adversely affects surrounding aquatic, terrestrial, and industrial life, as well as residents around the Chesapeake Bay Watershed. Elevated levels of some nutrients result in a poor quality of life for plants and animals alike, and leave many residents who depend on the Chesapeake Bay without the resources needed to sustain their businesses and families.

1.1 The Problem: Effects of Pollutants from Agricultural Runoff

Throughout the last half of the 20th century, agricultural pollution has degraded the water quality in the Chesapeake Bay. About 28% of the Chesapeake Bay Watershed is used for agricultural purposes ("Chesapeake Bay Watershed Initiative," 2011). Nitrate and phosphate are two nutrients commonly found in many agricultural fertilizers and enhancers. Nitrate and phosphate from fertilizers used in these agricultural areas are running off into the Chesapeake Bay Watershed at increasing rates. Sediments from the mid-90s contain two to three times as much organic carbon and nitrogen as sediments from the early 20th century. These chemicals cause harmful algal blooms that can lead to massive dead zones as oxygen vital to aquatic life are depleted (Carpenter et al., 1998).

Algae are a necessary component of the Chesapeake Bay and serve many functions, such as supporting the food web that includes food sources like fish and shellfish. However, in an unnaturally high abundance, algae can become toxic ("Harmful Algal Blooms in Maryland, n.d."). Algal blooms deplete oxygen from the surrounding waters and result in areas that have little to no aquatic life or nutrients

necessary for organism growth. Algal blooms also decrease water clarity and quality; moreover, they inhibit aquatic life from thriving, leading to the loss of various aquatic species (D. M. Anderson, Glibert, & Burkholder, 2002). In fact, throughout the last half of the 20th century, the Bay lost over 90 percent of submerged aquatic plants (Arnold, Cornwell, Dennison, & Stevenson, 2000; Cornwell, Conley, Owens, & Stevenson, 1996).

In addition, reducing runoff into the Bay is vital to the success of the fishing industry, the health of seafood consumers, and the biodiversity of the Chesapeake Bay Watershed. In 1997, all seafood industry segments had a combined 10% decrease, corresponding to a lost sales volume of $43 million ("Harmful Algal Blooms in Maryland, n.d."). Algal blooms throughout the 2000s caused fish kills and beach closings in the Chesapeake Bay, leading to large losses of sales and tourism.

Our project aims to mitigate the effects of nitrate by identifying plant species that are both efficient at absorbing nitrate from agricultural runoff pollution and show potential as biofuel crops. By utilizing water-purifying plants that can also act as biofuels, we hope to select a combination of plants that can both maximize nitrate removal in a wetland environment located in the Chesapeake Bay Watershed and be utilized as an alternative energy source. Alternative energy sources are necessary because fossil fuels are unsustainable and (Naik, Goud, Rout, & Dalai, 2010). For the Chesapeake Bay in particular, biofuels are a good option. Biofuels release less CO2 into the atmosphere because they accumulate as much carbon as they release (Naik et al., 2010). Planting them in wetlands also reduces the stress on agricultural land and helps maintain a healthy wetland system. In addition, the potential for biofuels provides a financial incentive to build these wetlands along the Bay.

1.2 The Research Question

Our experiments were based on the question: "What combination of native plants with the potential to be used as biofuels most efficiently removes nitrate, the result of agricultural runoff, from the Chesapeake Bay Watershed in a wetland environment?" Efficiency was defined as the percent of nitrate removed in the system over a specified period of time. Nitrate was the focus of this study, as phosphate removal in a wetland environment has been shown to require extensive resources that extend beyond our scope (Vymazal, 2010). Nitrate is the more limiting nutrient near the mouth of the Bay, and removing nitrate would have a greater effect on reducing anoxic regions (Cerco, 1995). In addition, despite increases in phosphorus loading, there has been little increase in phosphorus concentration within the Bay throughout most of the century (Cornwell et al., 1996).

Since the Chesapeake Bay is a large body of water, our team chose to focus on a smaller, more accessible river that is part of the watershed. After reviewing scientific literature, we chose to emulate the conditions of the Choptank River, a major tributary of the Chesapeake Bay that has been adversely affected by agricultural runoff (U.S. Geological Survey Virginia Water Science Center, 2005). Sixty percent of the land surrounding the Choptank River is used for agricultural purposes, so the majority of runoff into that river is composed of nitrate and other agricultural pollutants (Figure 1-1). For the sake of accessibility and convenience while collecting water samples, we used the Tuckahoe Creek, a representative branch of the Choptank River (Whitall et al., 2010).

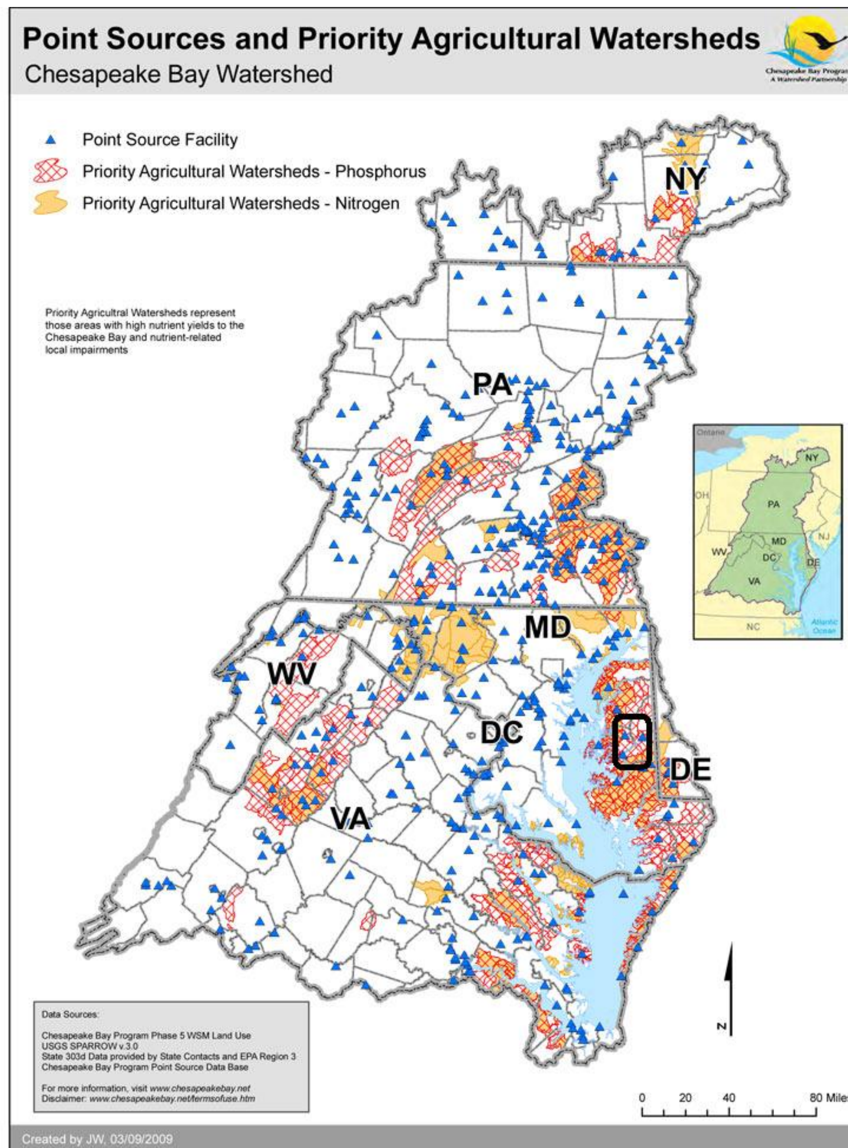

Figure 1-1: The Chesapeake Bay Watershed and Nutrient Sources.
The area enclosed by the box in the main map is the Tuckahoe Creek region (*Chesapeake Bay Program*, 2009).

1.3 Research Hypotheses

Our study was guided by several statistical hypotheses for each of the two separate phases of our research design. In the first phase, we tested the effectiveness of three organic amendments: sawdust, hay, and glucose. We examined which combination is most efficient at magnifying the difference in nitrate uptake across microcosms. The null hypothesis was: there is no difference in the nitrate uptake of plants when the organic factors are added to the system. The alternative hypothesis

was: there is a difference in the nitrate uptake of plants when sawdust, hay, or glucose is added to the system.

The second phase of the study tested different combinations of plants with the organic amendment determined from the results of Phase I to find an optimal combination for efficient nitrate removal. The null hypothesis for this phase was: there is no difference in nitrate uptake among different plant combinations. The alternative hypothesis was: there is a difference in nitrate uptake among different plant combinations.

We begin by discussing the basis of our research through the context of a literature review. We then outline our research methodology, starting with a general overview of our experimental design followed by our experimental setup and detailed protocol. A thorough analysis of our data and results follow. We conclude by discussing the significance of our results and questions that could be investigated in the future.

Chapter 2: Literature Review

2.1 Algal Blooms

Algae are naturally occurring photosynthetic organisms that support fish and shellfish. Found both as floating blooms (microscopic phytoplankton) or large algal mats on bottom sediments, algae can grow in a range of sizes ("Harmful Algal Blooms in Maryland," n.d.). The size of algal communities is dependent on environmental growing conditions. Nutrient levels as well as carbon dioxide concentration, light levels, temperature, and pH affect the ability of algae to proliferate (Kratz & Myers, 1955).

Nutrient sources that stimulate and support the growth of algal blooms include sewage, atmospheric deposition, groundwater flow, and agricultural runoff (D. M. Anderson et al., 2002). Past studies have demonstrated a strong correlation between total nitrogen and phosphorus inputs and microscopic algal bloom production (D. M. Anderson et al., 2002). Nitrogen input in the Bay's watershed originates from a number of sources (Figure 2-1) but historically has been linked to agricultural fields and other human-related land use (D. M. Anderson et al., 2002).

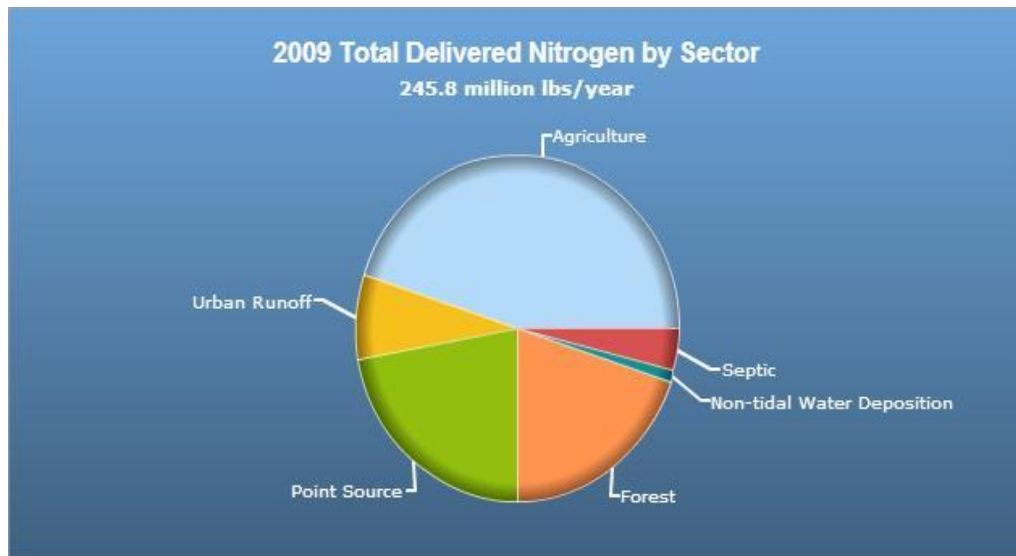

Figure 2-1: 2009 Total Delivered Nitrogen by Sector in the Chesapeake Bay. (Committee on the Evaluation of Chesapeake Bay Program Implementation for Nutrient Reduction to Improve Water Quality, 2011)

Since the beginning of industrialization, human activity has significantly increased the amount of reactive nitrogen in the Earth's environment. In the three centuries since humans transitioned from a purely agricultural society to an industrialized global society, the creation of reactive, biologically available nitrogen has roughly doubled, with the most contribution from synthetic nitrogen fertilizers (Howarth, 2008). Before the industrial revolution, it is believed nitrogen fixation on land was 90-195 Tg N per year and fixation by cyanobacteria in the sea was 200-300 Tg N per year with natural creation of biologically reactive nitrogen estimated at 300-500 Tg N per year. In 2000, manufactured biologically reactive nitrogen was approximately an additional 165 Tg N per year, globally (Howarth, 2008). From these figures, it can be observed that at one time, a stable process of biologically reactive nitrogen creation and fixation existed, with an average of 400 Tg of reactive N being introduced and fixed each year. The addition of 165 Tg N extra to the balanced system in the year 2000 increases the nitrogen production to over 140% of its natural value. This excess of nitrogen finds its way into runoff and ultimately into

streams and rivers where elevated nutrient loads stimulate abnormally fast rates of algae growth.

Harmful algal blooms (HABs), defined as ones which negatively affect other organisms, can be damaging in two ways: 1) the size of the "mat" of HABs displaces cohabitant organisms and alters the ecosystem's equilibrium in a process called eutrophication, and 2) they produce harmful toxins (D. M. Anderson et al., 2002). Effects of HABs include significant alteration of the ecosystem's natural equilibrium and environment; depletion of fish and shellfish populations due to loss of habitat and disease; human illness or death from exposure to harmful toxins through ingestion (toxic seafood), inhalation, or water contact; and death of other ecosystem organisms including marine mammals and seabirds (D. M. Anderson et al., 2002).

Eutrophication is the process of nutrient loading in bodies of water that leads to the formation of large algal blooms, and eventually, hypoxic dead zones. An overabundance of nutrients such as nitrogen and phosphorous stimulates rapid rates of algae growth, which blocks sunlight from reaching submerged aquatic vegetation (SAV) and robs the water of oxygen ("Chesapeake Bay Program," n.d.). When the SAV no longer receives sunlight, it cannot generate energy or produce oxygen via photosynthesis. Without light for photosynthesis, the choked plants will die. The decomposition process of the plants (both the algae and the SAV) consumes still more dissolved oxygen. With large algal communities, the biomass decay is so large that the rate of dissolved oxygen diffusing into the water does not match the consumption rate of dissolved oxygen (Minnesota Pollution Control Agency, 2009). The overall net effect is a low dissolved oxygen level, which can become hazardous to the local aquatic environment. When the level of oxygen is so low that it cannot sustain aquatic life, the area is considered a dead zone. This process is a major problem in

the United States – 50 percent of impaired lakes and 60 percent of impaired rivers are classified as such due to problems caused by eutrophication (Carpenter et al., 1998).

HABs also cause damage by releasing varying types and amounts of toxins. Within the Chesapeake Bay, cyanobacteria, haptophytes, dinoflagellates, green algae, raphidophytes, euglenophytes, diatoms, and cryptophytes all can produce toxins, but cyanobacteria and dinoflagellates have been known to produce severe toxins that can have hazardous effects on humans. Toxins are produced within the organism's cells and are released when the cell breaks open (Sellner, Doucette, & Kirkpatrick, 2003; "What are Harmful Algal Blooms?," n.d.). Some evidence suggests that the presence of algal predators stimulates toxin production, and that these toxins decrease competition by inhibiting the growth and survival of competing species (Jonsson, Pavia, Toth, & Karl, 2009). In some cases the toxins can be extremely potent, resulting in human fatalities even at low concentrations (D. M. Anderson et al., 2002). Internationally, different varieties of algal toxins may be the cause of nearly 60,000 human intoxication incidents each year (Van Dolah, Roelke, & Greene, 2001). Although the algal blooms produce the toxins, they can be passed from organism to organism through the food chain, disrupting the ecosystem's structure and survival at multiple levels (Hoagland, Anderson, Kaoru, & White, 2002).

In addition to impacting their immediate ecosystems, HABs and their toxins can also result in economic and health repercussions on the surrounding communities. One study found that HABs were responsible for "the loss of millions of dollars to coastal communities through costs associated with beach cleanup, closing of commercially important fisheries, and decreased tourism" (Hoagland et al., 2002; Van Dolah et al., 2001). One particular toxin from the species *Pfiesteria*, directly aggravated by excess phosphates, was estimated to have caused $43 million dollars in

lost sales volume during 1997 ("Harmful Algal Blooms in Maryland," n.d.). Furthermore, HAB toxins have been known to have negative impacts on health. For instance, HAB toxins are possible carcinogens that can contaminate drinking water. Further research is also being conducted to examine the relationship between certain HAB toxins and neurological diseases ("What are Harmful Algal Blooms?," n.d.). Saxitoxins, brevetoxins, and other toxins found in the Chesapeake Bay are all associated with seafood poisoning syndromes (Van Dolah, 2000). Additionally, HAB toxins are known to be fatal to waterfowl and livestock, and in some cases, dogs and humans ("What are Harmful Algal Blooms?," n.d.).

A comprehensive study concluded that estuaries of the mid-Atlantic are most severely affected by HABs, and that this region is the most affected by eutrophication in the nation (D. M. Anderson et al., 2008). In the Chesapeake Bay area alone, 34 different algal species within harmful algal blooms have been identified. *Prorocentrum minimum, Karlodinium veneficum Pfiesteria piscicida,* and *Pfiesteria shumwayae* are a few of the species that have been linked to harmful effects on the Bay, such as fish kills (D. M. Anderson et al., 2008).

2.2 Agricultural Runoff

Agricultural land accounts for a significant part of land use in the Chesapeake Bay Watershed. Between 25-30 percent of the over 165,759 square-km basin is devoted to agriculture ("Chesapeake Bay Watershed Initiative," 2011). Agricultural runoff is one of the most significant sources of pollution to the Chesapeake Bay Watershed (Fig 2-1). Pollution to bodies of water can be classified as either point or nonpoint sources. Point sources of pollution, such as sewage treatment plants or runoff from waste disposal sites, are single input locations. Agricultural runoff,

however, is classified as a nonpoint source of pollution, meaning that the pollution it contributes to bodies of water cannot be attributed to a single location. Nonpoint sources are derived from wide spans of land and are carried over land, underground, or through the atmosphere before reaching their destination in receiving waters. The constant fluctuation in the amount of pollution added to a body of water at a given time makes it difficult to monitor and control the issue in affected areas; the measures needed to do so can also be disruptive to daily life (Carpenter et al., 1998).

A main cause of the high concentrations of nitrogen and phosphorous in agricultural runoff are the fertilizer and manure used in the agricultural industry. On average, crops in produce farms absorb only 18 percent of nitrogen from fertilizer, leaving behind a surplus of about 174 kilograms per hectare per year of surplus nitrogen in the soil in the farmland (Isermann, 1990). This excess nitrogen is left to be stored in the environment in various ways – it can accumulate in soils, volatilize into the atmosphere, or leach into surrounding surface and ground waters (Carpenter et al., 1998).

Farms in the United States are responsible for about 500 million tons of manure per year as a byproduct of animal feeding operations, and poorly managed facilities allow nutrients to seep into bodies of water ("Protecting Water Quality from Agricultural Runoff," 2005). This nitrate- and phosphate-rich agricultural runoff can cause a steep increase in the nutrient concentration of the neighboring bodies of water, leading to eutrophication.

Additional causes of agricultural runoff include livestock grazing, irrigation, and pesticides. As animals deplete grasses, erosion becomes more prevalent and the sediment problem worsens. Excess water and inefficient irrigation used on crop fields pose runoff problems by eroding soil, “transporting nutrients, pesticides, and heavy

metals, or decreasing the amount of water that flows naturally in streams and rivers" ("Protecting Water Quality from Agricultural Runoff," 2005).

Various studies have shown that nonpoint agricultural runoff is a major concern that warrants mitigation. The National Water Quality Inventory reported that in 2000, agricultural nonpoint source pollution was the greatest contributor to poor water quality and contamination of the tested bodies of water. In another study that surveyed 86 different rivers, the researchers found that nonpoint sources of nitrogen were the cause of over 90 percent of nitrogen inputs to more than half of those rivers (Newman, 1995, as cited in Carpenter et al., 1998). Regarding the coastline of the North Atlantic Ocean, nitrogen pollution from nonpoint sources of runoff are approximately nine times greater than that contributed by wastewater treatment plants (Howarth et al., 1996, as cited in Carpenter et al., 1998). A study specific to the Chesapeake Bay found that point sources such as wastewater treatment plants contribute only approximately 25 percent of nitrogen and phosphorous pollution to the Bay, and the rest were therefore from nonpoint sources (Boynton et al., 1995, as cited in D. M. Anderson et al., 2002).

2.3 River Selection

As the largest estuary in the United States, the Chesapeake Bay stretches for 332 kilometers from Virginia to Maryland ("The Chesapeake Bay: Geologic Product of Rising Sea Level," 1998). The Bay's watershed and drainage basin encompass portions of six states and the District of Columbia, covering an area of more than 165,759 km^2. The Bay watershed is home to more than 17 million people, many of whom depend on the Bay for their livelihood and resources. The Bay is a major attraction for tourism, recreation and seafood. The blue crab market alone generated

$61 million dollars in 2004 ("Chesapeake Bay Program," n.d.). Furthermore, the Bay has over 100,000 smaller creeks, rivers, and streams, each of which has its own sub-watershed.

In addition to its seafood and tourism reputation, the Chesapeake Bay is also arguably one of the most polluted bodies of water in the United States (Whitall et al., 2010). Agricultural runoff and chemical pollutants from industrial and population growth contribute to a grave disequilibrium in the Bay watershed and ecosystem. A 2009 Executive Order directed federal agencies to apply "their expertise and resources to … improving the health of the Chesapeake Bay" (Whitall et al., 2010). In 2011, the Chesapeake Bay received a rating of 38 out of 100 on the Bay Health Index, its second lowest score since assessments began in 1986. Declines from 2010 were seen in both water quality and biotic indicators, and there were significant deficits in chlorophyll, water clarity, aquatic grasses and benthic community ("Chesapeake Bay Report Card," 2011). The difficulty in analyzing the entire Chesapeake Bay and determining how to best address the pollution within our time constraints forced us to adjust the scope of our project. We narrowed our search by turning to a major tributary of the Bay, the Choptank River.

The Choptank River Basin is exposed to many of the same issues as the Chesapeake Bay Watershed, but on a smaller scale. The Choptank River Basin was included in a national study of agricultural best management practices called the Conservation Effects Assessment Project (CEAP) (McCarty & McConnell, 2008). Geared at improving conservation and management practices for agricultural land, the CEAP, in part, targets watersheds that include sizeable agricultural regions with high pollutant runoff into nearby bodies of water ("Conservation Effects Assessment Project," n.d.). Land use within the Choptank Basin is approximately 60%

agricultural, and agricultural runoff is a primary source of nitrate within the Choptank River Basin. As nitrate removal is the focus of this research project, the Choptank River is an ideal environment to emulate in lab conditions.

Certain portions of the river have also been classified as “impaired waters” since 1998 under the Federal Clean Water Act (Whitall et al., 2010). This is due to a number of problems within the waters, including low dissolved oxygen in the river’s depths, phytoplankton overpopulation, and high nutrient concentrations (MDE, 2004 as cited in Whitall et al., 2010). Since 1997, the mouth of the Choptank River has suffered an 85 percent decrease in area that supports submerged aquatic vegetation (Whitall et al., 2010, Figure 2-2). Furthermore, it received a rating of 26 out of 100 on the Bay Health Index, showing that the river conditions are more severe than the Chesapeake Bay average ("Choptank River - Chesapeake Bay Report Card," 2011). Though it represents only 1.5% of the Chesapeake Bay watershed area, the Choptank River Basin emulates many of the overall conditions of the watershed.

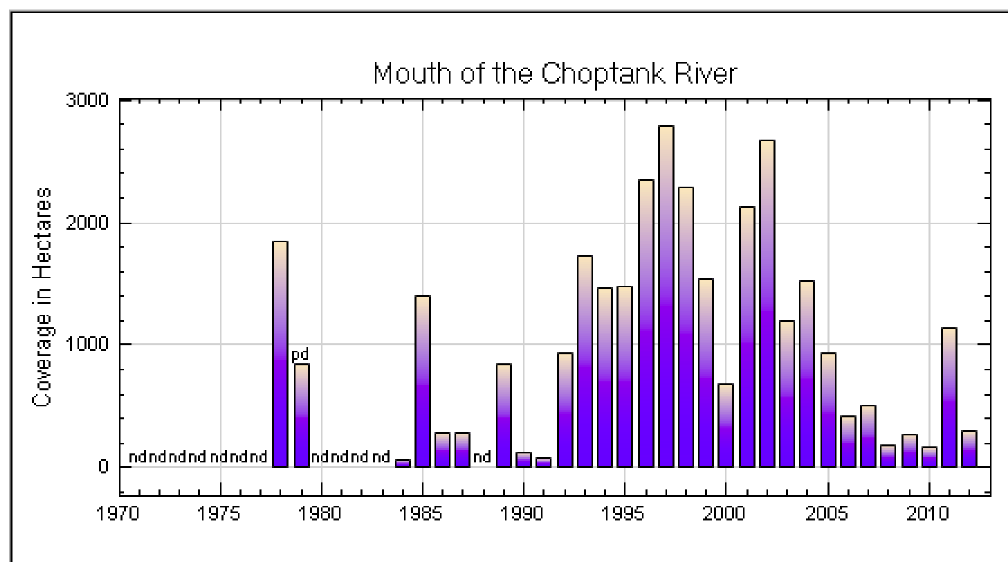

Figure 2-2: Submerged Aquatic Vegetation Coverage at the Mouth of the Choptank River.
Labels “nd” indicates SAV area was not mapped, “pd” indicates only partial mapping (Orth et al., 2012).

At 71 miles long, the Choptank River is the largest river on the Delmarva Peninsula and a major tributary of the Chesapeake Bay. It has two main tributaries of its own: the Tuckahoe Creek and the Upper Choptank River. Because the Tuckahoe Creek is a significant tributary of the Choptank River, our team chose to incorporate the Tuckahoe Creek's natural environment and conditions into the project. The greater accessibility of the Creek made it more desirable as a collection and testing site over the Upper Choptank. In addition, the Tuckahoe is a suitable location for treatment wetlands, as there are treatment wetlands currently in operation there.

2.4 Nitrogen Cycle

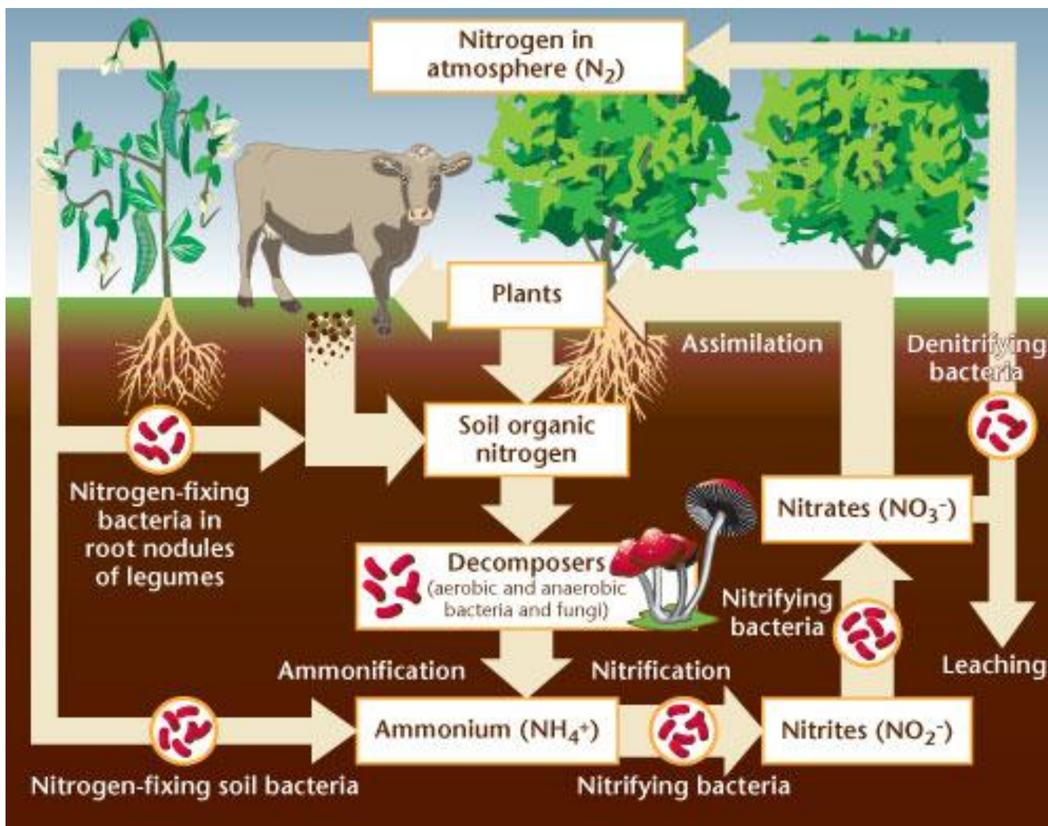

Figure 2-3: Nitrogen Cycle.
This experiment focused on assimilation and denitrification, processes related to nitrate removal (Webber, 2011).

The nitrogen cycle describes the transformation of nitrogen throughout an ecosystem. In wetlands, nitrogen can be found in a variety of oxidation states, mainly

N_2, NO_3^-, and NH_4^+. There are a number of physical processes that transfer nitrogen from one point to another without any molecular transformations (Kadlec & Wallace, 2009), such as uptake by plant roots or the subsurface water flow. In addition, there are several processes that actually transform nitrogen from one form to another: ammonification (mineralization), volatilization, nitrification, denitrification, and fixation (Mitsch & Gosselink, 2000). Microbial denitrification is a prominent process in wetland soils that releases nitrogen into the atmosphere. Because the microbes that carry out denitrification need an anaerobic environment to thrive, the anoxic conditions of wetlands create a suitable environment for the microbes to grow (Mitsch & Gosselink, 2000). These transformations will be discussed in the following sections, with a particular emphasis on denitrification as it is the most pertinent transformation to this research.

2.4.1 Transformations

Ammonification refers to a series of transformations that converts organically bound nitrogen to ammonium nitrogen as the organic matter is being decomposed, and can occur under both anaerobic and aerobic conditions. Different processes can occur after the ammonium ion (NH_4^+) is formed. Volatilization can take place under high pH conditions ($pH>8$), which is when ammonium is converted to NH_3 and released into the atmosphere. Ion exchange can also occur, where the ammonium ion is immobilized to negatively charged sites on soil particles. However, the presence of a thin oxidized layer at the surface of many wetland soils creates a gradient between the aerobic and anaerobic conditions in these soils. This causes ammonium to diffuse upwards in the soil into the aerobic environment. Nitrification can then take place by the aerobic bacteria *Nitrosomonas* and *Nitrobacter*, in which the ammonium ion is

oxidized to nitrite (NO_2^-) and eventually nitrate (NO_3^-). Nitrification can also occur in the oxidized rhizosphere of plants where there is adequate oxygen to convert ammonium nitrogen to nitrate nitrogen. Unlike ammonium, nitrate [NO_3^-] is not commonly immobilized by microorganisms. Generally, ammonium is preferred because not all microbes have nitrate reductase. If it is not assimilated immediately, it can be reduced via several pathways, of which reduction to ammonia and denitrification are the most common. Nitrogen fixation is a less prevalent pathway that results in the conversion of N_2 gas to organic nitrogen through the activity of certain organisms in the presence of the enzyme nitrogenase. In wetlands, fixation can occur in overlying waters, the aerobic soil layer, the anaerobic soil layer, the oxidized rhizosphere of the plants, and on the surfaces of plants (Mitsch & Gosselink, 2000).

2.4.2 Denitrification

Nitrate removal in wetlands can be attributed to several underlying mechanisms. Denitrification, the most important process for our research, is the process in which nitrate is converted into nitrogen gas via the intermediates nitrite, nitric oxide, and nitrous oxide. This process is carried out by facultative heterotrophs, which are organisms that can use either oxygen or nitrate as terminal electron acceptors. Nitrate is converted, in order, to nitric oxide (NO), nitrous oxide (N_2O), then nitrogen gas (N_2). Carbon drives the overall stoichiometric nitrate dissimilation reaction. Various carbon sources can be used. For example, in the presence of glucose, nitrate is converted into atmospheric nitrogen, carbon dioxide, and water ($NO_3^- + 0.208\ C_6H_{12}O_6 \rightarrow 0.5\ N_2 + 1.25\ CO_2 + 0.75\ H_2O + OH^-$). Because most

denitrification is accomplished by heterotrophic bacteria, the process is strongly dependent on carbon availability.

While denitrification theoretically does not occur in the presence of dissolved oxygen because the microbes that carry out denitrification are typically anaerobic, it has been observed in systems with considerable dissolved oxygen. This reaction is due to the spatial zonation in a wetland. Oxygen gradients allow both aerobic and anoxic reactions to proceed in close vertical proximity. Therefore, nitrate formed in surface waters can diffuse down into the top anoxic soil where it is denitrified. Other known mechanisms of nitrate loss include assimilation by plants and microbiota, and dissimilatory reduction to ammonium nitrogen. These have been documented to comprise 1-34% of total nitrate loss (Kadlec & Wallace, 2009).

The presence of vegetation also has many effects on nitrate loss. Aside from direct assimilation associated with growth, plant life can also affect other processes. The vegetation can act as a carbon source which drives the denitrification reaction, mainly through its decomposition products. In addition, it can harbor epiphytic (other organisms living on a plant) microbial biofilms (polysaccharide matrixes) on both living and dead plant material. The bacterial activity within these biofilms is regulated by diffusion of nutrients into the biofilm and by internal processes. However, living vegetation also produces oxygen via photosynthesis, which can inhibit the denitrification process to varying degrees (Kadlec & Wallace, 2009).

2.4.3 Human Contribution

Humans have drastically increased the amount of nitrogen in the global nitrogen cycle directly through the use of fertilizers and the combustion of fossil fuel. From 1950 to 1990 alone, industrial fixation of N fertilizer increased from <10

million metric tons of N per year (Tg yr^{-1}) to 80 Tg yr^{-1}. Due to this large increase of nitrogen entering the land-based nitrogen cycle, wetlands are being investigated for the ability to serve as nitrogen sinks (Vitousek, Mooney, Lubchenco, & Melillo, Jul. 25, 1997).

2.5 Constructed Wetlands

Constructed wetlands are manufactured systems that mirror processes found in natural wetlands in order to treat bodies of water affected by different types of pollutants. They typically consist of vegetation, soils, and associated microbes that aid in the treatment of these affected waters (Vymazal, 2010). Constructed wetlands are popular systems used to treat agricultural nutrients and nonpoint sources of pollution. Along with intercepting runoff, they are low-cost and can provide aesthetic and other ecological and remedial benefits (Díaz, O'Geen, & Dahlgren, 2012).

Wetlands are transitional areas between land and water and, therefore, do not have distinct boundaries. A common characteristic of all wetlands is that they have surface or near surface water levels. This creates a low-oxygen environment, which restricts plant life to only the plants that can grow in hypoxic conditions (i.e. the only plants that grow there are plants that are capable of growing in a low oxygen root zone) (Davis, 1995).

Constructed wetlands have been used to treat industrial, agricultural, and municipal wastewater; heavy metals in landfill leachate; and stormwater (Lenhart, Hunt, & Burchell, 2012; Vymazal, 2010). Their designs can vary to target specific pollutants. These designs are based on plant type (free floating, free floating leaved, emergent, or submerged), and the water flow (surface flow or subsurface flow). Subsurface flow can be categorized as vertical or horizontal (Stottmeister et al., 2003).

Since we kept our microcosms saturated and collected outflow from the bottom of our microcosms, we used emergent plants in a varied type of vertical, subsurface flow.

Microbial and vegetative anaerobic and aerobic processes taking place in the soil and root zones of constructed wetlands help reduce nitrate concentrations in runoff, as well as above-ground plant growth. Many studies have reported mean nitrate removal rates for constructed wetlands higher than 50% (Beutel, Newton, Brouillard, & Watts, 2009; Leverenz, Haunschild, Hopes, Tchobanoglous, & Darby, 2010; Vymazal, 2010). A review of 57 wetlands worldwide found that 80% of the wetlands studied decreased nitrogen loadings (Fisher & Acreman, 1999). Increases in N concentrations generally occurred in the soluble N form, not as total or particulate N (Fisher & Acreman, 1999).

Some limitations to constructed wetlands as nitrate removal systems are that they require large areas of land, are sensitive to toxins, and must be maintained at a specific water level (Davis, 1995). They have many advantages, however. Most wetlands cost little to build and maintain. Only limited periodic labor is required for wetland upkeep. They are aesthetically pleasing and they provide an ecosystem in which wildlife and aquatic organisms can thrive (Davis, 1995). Finally, there have been wetlands that have operated for more than 14 years that still have little or no loss in nitrate removal efficiency (Davis, 1995; Fisher & Acreman, 1999).

2.6 Plant Selection

In 1998, Bachand and Horne found that constructed macrocosms containing more than one wetland plant species removed, in some cases, more than three times more nitrate than macrocosms containing single plant species (Bachand and Horne, 1998). A mass balance showed that most of the nitrate removal occurred through

denitrification, with plant and soil uptake comprising a fraction of the nitrate removed. In this study, Bachand and Horne used wetland plant species that are both native to the U.S. and show high efficiency for nitrate removal. As our study centers on determining the effect of specific species combinations on nitrate removal, we selected plant species that are both native to the Chesapeake Bay Watershed and have been proven to be effective at removing nitrate. These plant species also must be capable of biofuel use to further add to the sustainability aspect of our project as well as a promising future direction. The biofuel capability of these plants will be discussed in Section 2.7. Two of the plant species chosen for our study, soft-stem bulrush (*Schoenoplectus validus)* and cattail (*Typha latifolia)*, were used in the aforementioned study by Bachand and Horne. The third plant species used in this study is switchgrass (*Panicum virgatum*). We decided to test multiple species because they differ in nitrate removal efficiency and perform well under different conditions (Brisson & Chazarenc, 2008).

2.6.1 Cattail

Cattail (*Typha latifolia)* is native to the Chesapeake Bay Watershed and has been researched extensively for its use in constructed wetlands (Lippson, 2006). Cattails are often used in wetland restoration and are considered an "obligate wetland indicator plant species," meaning they are always found in or near wetlands and water bodies (Slattery et al., 2003). Cattail participates in C3 carbon fixation, so they thrive in moderate sunlight and temperatures, and high carbon dioxide concentrations. Furthermore, cattails are tolerant of harsh water and weather conditions, including flood and drought, making them an ideal year-round wetland plant species (Slattery et al., 2003). The USDA recommends that cattails be used in combination with other

plants in a wetland environment to help restore and maintain wetlands while adding to a balanced ecosystem (Slattery et al., 2003). Various species of the genus *Typha* have also been shown to remove significantly more nitrate in mesocosms, when compared to similar plants. Gebremariam et al. (2008) found that cattail (*Typha* spp.) caused a significantly higher nitrate removal rate than bulrush (*Scirpus* spp.) in constructed mesocosms. Furthermore, the same study found that cattail degraded more easily and supported more microbiological activity than bulrush species, indicating that cattail species offer a more advantageous overall environment for denitrification (Gebremariam et al., 2008). Fraser et al. found that *Typha latifolia* is efficient at removing nitrate in low concentrations while similar plants are more efficient at removing nitrate in high concentrations, logically supporting the hypothesis that combinations of these plants improve the overall efficiency of nitrate removal in a microcosm environment (Fraser et al., 2004). Because cattail has been thoroughly studied in a wetland environment and has been proven to significantly remove nitrate pollution, *Typha latifolia* is the first plant species chosen for this project.

2.6.2 Switchgrass

Switchgrass (*Panicum virgatum*) is a widely used wetland plant in soil conservation. Because switchgrass is uses C4 carbon fixation, it can more efficiently fix carbon dioxide, allowing it survive in drought, high temperatures, with low CO_2 concentration. As a versatile perennial grass, it can survive in varied environments, including diverse soil types and water levels as well (USDA, 2012). Its peak growing period occurs during the spring and fall; it becomes dormant and unproductive during cold months (USDA, 2012). Switchgrass currently exists as a native Chesapeake Bay watershed plant species found in fresh and brackish water and tidal and nontidal

marshes; its versatility in different environments allows it to survive in varied conditions throughout the watershed (U.S. Fish & Wildlife Service, 2011). Previous studies have shown that switchgrass in a wetland environment will remove nitrate, however, at a lower rate than other species such as cattail (Wu, 2011). Although it is not widely used in constructed wetlands to remove nitrate, switchgrass is known for its high bioenergy yield and positive effects on soil quality and stability (McLaughlin et al., 1998). Because of its ability to survive in a wide range of environments with a high biofuel potential, switchgrass was chosen as one of the three plant species in this study. The addition of switchgrass to our species combination study will allow us to compare nitrate removal potential against bioenergy potential for experimental microcosms containing switchgrass.

2.6.3 Soft-Stem Bulrush

The third plant included in our study, soft-stem bulrush (*Schoenoplectus validus*), is also a common wetland plant and is native to Maryland and the general Chesapeake Bay watershed (U.S. Fish & Wildlife Service, 2011). This species partakes in C3 fixation, just as cattail, so the optimum growing conditions require moderate sunlight and temperature with a high carbon dioxide concentration (Fraser et al., 2004). Its growing season, however, is very similar to that of switchgrass, and its versatility in different growing environments ensures its survival even in harsh environments (U.S. Fish & Wildlife Service, 2011). Soft-stem bulrush is also known to be an efficient species for nitrate removal in microcosm environments. Fraser et al. (2004) conducted a study comparing nitrate removal from microcosms containing four different plant species individually and in combinations. At low nitrate treatment, *S. validus* alone and a four species mixture (*S. validus*, *C. lacustris*, *P. arundinacea*,

and *T. latifolia*) had significantly less nitrogen in soil leachate compared to other plant treatments (Fraser et al., 2004). Furthermore, another study found that amongst vegetated wetlands, *S. validus* was the most effective nitrate-removing plant when compared with *Phragmites communis* and *Typha latifolia* (Gersberg et al., 1986). Because the literature review suggests soft-stem bulrush is very versatile and one of the top wetland plant species in nitrate removal, *Schoenoplectus validus* is the third species that was chosen in this study.

2.7 Biofuel Capability of Plants

When selecting the plants to use in our study, we decided to choose plants that are biofuel-capable in order to potentially accommodate changing energy and environmental needs. The idea of harvesting biomass from constructed wetlands for energy production can be dated back to the 1970s, when it was discovered that ideal biofuel crops invaded wetlands (Jakubowski, Casler, & Jackson, 2010). This new opportunity for biofuel production alleviated the concerns of using vital agricultural lands for cellulosic biofuel production rather than food production. These wetland plants can be used as biomass, in this case cellulose, to then be converted to glucose through a series of treatments. The glucose then can be used to feed other organisms to produce ethanol, such as *E. coli* bacteria or other microorganisms. Using wetland plants as biomass could alleviate the use of food or wood resources as feedstocks and provide an effective alternative that could also decrease the agricultural runoff pollution (Sedjo & Sohngen, 2012).

Harvesting crops for biofuel production also helps to maintain a healthy wetland system. Over time, wetlands can become oversaturated with nutrients, decreasing their ability to filter nitrogen and phosphorous from the water. Harvesting

biomass from these wetlands "can act as the wringing out of the sponge (Jakubowski et al., 2010)," which ultimately helps decrease eutrophication downstream. After the plant is harvested, an increased amount of nitrogen and phosphorous will be absorbed by the plants from the environment as they begin to grow and form new biomass (Jakubowski et al., 2010). Selecting plants that are biofuel-capable gives our wetland an added component that will aid in its nitrate removal capacity as well as add to its economic value.

For our wetland, plants that were native to the Chesapeake Bay watershed, portrayed significant nitrate removal, and had high biofuel potential were chosen. The biofuel capability was assessed by the amount of biomass produced per unit area as well as energy content. From our literature review, we found three species of plants that fulfilled these requirements: cattail, switchgrass, and soft-stem bulrush.

2.7.1 Cattail

Many species of cattail, a common wetland plant, have been shown to have high biofuel potential. Cattail is a promising wetland plant species because it offers good growth potential and adaptation to harsh conditions, as well as rich concentrations of starch and sugar, which can be used for ethanol production. The cattail stalk can be used as quality fiber and pulp; new technological pulping techniques preserve the structure of the rind as biomass while separating the juice, which is an excellent sugar source (Korth, 2008).

One study analyzed a potential means for harvesting cattail as a source of ethanol using a hot-water pretreatment process with a Dionex accelerated solvent extractor. The research team varied the temperature and the duration of heating in order to obtain the maximum product of cellulose. A pretreatment at 190°C for 10

minutes effectively dissolved the xylanose. This harvested cellulose was then turned into glucose at a 77.6% yield (Zhang, Shahbazi, Wang, Diallo, & Whitmore, 2010). The glucose then could be used as a source of food for other microorganisms that produce ethanol or other biofuels. With an average conversion efficiency of 43.4% for pretreated cattail, a hectare can produce up to 4,012 L of ethanol. This compares very favorably to corn stover's 1,665 L ha^{-1} at a 60% conversion rate (Suda, Shahbazi, & Li, 2007). In addition, although initial trials achieved only 43.4% efficiency, more advanced conversion organisms would obtain better results. At the same 60% conversion rate of corn stover, cattail would produce 9,680 L ha^{-1}.

In addition to its above-ground mass, the roots of cattail can also be used in the production of biofuel. The rhizomes of cattail are high in starch content, at around 30% to 40%. The starch that is obtained can then be easily degraded to a sugar source for biofuel production. Cattail is also promising in terms of its potential for growth. Two years after germination, cattail can spread over an area of 58 m^2, and hybrid cattail can reach 37 t ha^{-1} (Beule, 1979). Therefore, cattail can yield a large mass that can be harvested. The promise of cattail as a biofuel source provided our team with the idea to include biofuel potential as a secondary element of the plant selection and screening process.

2.7.2 Switchgrass

After determining that cattails are a highly viable biofuel crop, we further researched biofuel-capable plants and cross-referenced with a list of Maryland-native, Chesapeake Bay area plants. Switchgrass is a common plant studied for its biofuel capabilities and its ability to filter agricultural runoff from Chesapeake Bay waters. Switchgrass is high in cellulose, which makes it a good candidate as a biomass crop

for cellulosic ethanol production. This perennial plant can grow up to seven feet in height and has an average life span of 10 to 20 years (Dale, 2010). Switchgrass needs to grow and become established in the first year of growth; however, by the second year, about 14.83 to 19.77 t ha^{-1} can be harvested, with yields approaching 24.71 to 29.65 t ha^{-1} yr^{-1} (Dale, 2010). Estimates from previous studies conclude the net energy output of switchgrass is almost 20 times better than corn, and each acre can produce 1892.71 to 3785.41 L of ethanol (Dale, 2010).

A Virginia Tech study of switchgrass and its biomass yields found that in 1989, a single hectare plot of switchgrass yielded 21.0 dry megagrams of biomass. The study compared switchgrass to other biofuel-capable plants, including sorghum-sudangrass (*Sorghum x drummondii*), birdsfoot trefoil (*Lotus corniculatus*), and flatpea (*Lathyrus sylvestris*). Out of all of the plants in the study, switchgrass consistently yielded the highest amount of dry biomass per hectare (Wright & Turhollow, 2010). Because of the large biomass production and high energy output of switchgrass, it proved to be an ideal candidate for our study. Since switchgrass can produce ethanol at a rate of 116.2 mg g^{-1}, a hectare of switchgrass yields 2440.2 kg, or 3092.8 L of ethanol (W. Anderson, Casler, & Baldwin, 2008).

2.7.3 Soft-Stem Bulrush

Finally, soft-stem bulrush was included in the experiment, as it is a high-performing plant that is both biofuel-capable and native to the Bay. Soft-stem bulrush was previously found to have the largest cellulose to lignin ratio due to its high cellulose content (Ruhland, 2011). This property makes bulrush a good candidate for cellulosic ethanol production as well.

Another study found that out of 20 wetland species, soft-stem bulrush ranked second in energy output per unit mass. The average energy content was 20.5 kilojoules per gram (kJ g^{-1}), only surpassed by cattail with an energy content of 21.5 kJ g^{-1}. In addition, soft-stem bulrush had a high biomass yield per unit area, ranging from 18 to 42 metric tons per hectare (Fedler, Hammond, Chennupati, & Ranjan, 2007). A standard hydrolysis, fermentation, and distillation method has been shown to produce an ethanol yield of 5.07% of the bulrush biomass (Sari, 2010). Therefore, a hectare of soft-stem bulrush yields 1521.1 kg, or 1927.9 L of ethanol. While this isn't as high as cattail or switchgrass, there has not been as much development on ethanol production from bulrush so there is room for improvement. Since creating and maintaining a wetland requires significant resources, periodically harvesting the plants for biofuels can help offset those costs. Due to the high cellulose concentration in bulrush and the high energy content, soft-stem bulrush was chosen as the final wetland species in this study.

2.8 Organic Amendments

Much like fertilizer enhances crop growth and vegetable gardens flourish with fresh compost, soil amendments have been shown to increase nitrogen uptake and improve biomass yield (Paustian, Parton, & Persson, 1992). Successful organic amendments provide nutrients which drive nitrogen fixation, mineralization, and immobilization within soils. An effective soil amendment originates from its respective lignin content and carbon-nitrogen ratio. Plants with high lignin content often have high concentrations of polysaccharides within the cell walls to diffuse water throughout the plant. Presence of increased numbers of polysaccharides was found to be synonymous with increased denitrification potential and an increased

biodegradable organic carbon concentration (Sirivedhin & Gray, 2006). When decomposing, lignin acts as a sink for atmospheric carbon, which may have an effect on plant and bacterial communities present within the microcosm soil. Similarly, the carbon-nitrogen ratio of the respective amendments further adds to the carbon available to the denitrifying bacteria and other mechanisms involved in nitrogen dissimilation (Warneke et al., 2011).

In the study "Influence of Macrophytes on Nitrate Removal in Wetlands," organic carbon forms, also called detritus, are listed as a stimulating mechanism for denitrification (Weisner, Eriksson, Granéli, & Leonardson, 1994). In a comparison of areas cleared of vegetation and areas with established wetlands, higher denitrification rates were observed in the planted areas. Furthermore, the authors claim that organic carbon availability is necessary for denitrifying bacteria. The organic carbon present in the study was the naturally occurring plant litter and debris from the plants' seasonal growth patterns. Independent of detritus location (suspended in water, on sediment surface, or mixed in sediment), positive effects were associated with the presence of organic carbon (Weisner et al., 1994).

Addition of carbon-based soil amendments to individual microcosms was deemed a plausible method in developing contrast in nitrogen removal data across different trials. We anticipated that regardless of how effective one combination of plants is against another at removing nitrate, the differences might be too small to establish a statistically significant conclusion. By instituting an organic amendment, the variability among the plant combinations is expected to be more significant and thus should lead to a more significant result. A great deal of research has been conducted across a large range of organic materials to identify effective soil amendments.

A number of organic amendments have been tested within many research projects across different disciplines and for different purposes. We chose organic amendments for our experimental design based on a thorough literature review. The team chose three carbon sources to add to the microcosms for the initial phase of testing to gauge the effectiveness of each soil amendment, allowing us to determine which organic amendment was best suited for the conditions of our experiment. The soil amendments used to supplement the denitrification process within both plant and non-plant testing conditions were glucose, sawdust, and hay. Further descriptions of each material and their individual properties are discussed below.

2.8.1 Glucose

Many studies support the use of glucose as a soil amendment. Dissolved glucose solutions are commonly used to generate the polysaccharide interaction, described in section 2.8, during naturally occurring biomass degradation. Since glucose is often added as a dissolved solution, the denitrifying bacteria and other communities can quickly make use of the readily available polysaccharides distributed throughout the soil. Weisner et. al. (1994) maintains that addition of polysaccharides drastically increases nitrogen removal rates within artificial wetlands. Their experiment involved the addition of sucrose to a well-established (eighteen-year-old) stand of *Phragmites australis* in southern Sweden in late May. With standardized natural sunlight and temperatures, the authors observed that the sucrose-amended soil substrates were able to reduce the nitrate concentration faster than the neighboring, non-amended phragmites stand – removing approximately 2.9 g m^{-2} over seven days compared to 2.2 g m^{-2} in control treatments (Weisner et al., 1994).

Several other studies have reported similar results. In one study, exposure of seedlings and microbial colonies to nitrate and glucose solutions (C:N ratios varied from 0:1 through 100:1) increased the bacterial colony numbers up to six times the initial count in 15 days (Ritz & Griffiths, 1987). In a separate study, soils amended with glucose concentrations up to 10 mg g^{-1} soil were incubated at 22˚C for 14 days. Biomass and microbe activity increased in direct proportion to initial soil glucose concentrations (Sparling, Ord, & Vaughan, 1981). Elevated microbial activity is associated with improved denitrification rates and thus we anticipated glucose to be effective at stimulating high rates of denitrification.

Addition of a glucose solution provides bacteria with an immediate, highly available carbon source. However, due to its high availability, the stimulating effects of glucose may diminish after a comparatively short incubation period of about 7-14 days (Sparling et al., 1981). Thus, the effects of glucose may be most prevalent in the first two weeks of testing but may not have lasting, long-term effects on the denitrification process for trials lasting in excess of 14 days.

2.8.2 Sawdust

Sawdust was chosen to be the second organic amendment tested because of its potential to remove toxic substances from water and its relatively easy accessibility (Shukla, Zhang, Dubey, Margrave, & Shukla, 2002). Sawdust is a natural agricultural byproduct and can therefore be cheaply obtained in high quantities from lumber mills and other places where it can often pose problems of disposal. If sawdust could effectively be used as an organic amendment in treatment wetlands, not only would it provide an easy and low-cost alternative to increase nitrate removal, but it would also benefit the agricultural community by providing a market for sawdust and facilitating

its disposal. However, unlike glucose, the benefits of sawdust as an amendment are not instantaneous. The wood shavings must begin to degrade in order for simple sugars to become involved in the denitrification process.

While glucose was found to be more effective on the scale of a few days at increasing denitrification rates, sawdust amended tests reached comparable denitrification rates eight days into the trial (Hien, Park, Jo, Yun, & Minh, 2010). Long-term effectiveness of sawdust is also supported by a year-long trial conducted between 1996 and 1997. A mixture of soil and sawdust was inserted into a denitrification, made by digging a 1.5 m deep trench into the ground, mixing the soil and sawdust, and replacing it back in the trench. Over the course of 12 months, denitrification enzyme activity and microbial biomass were relatively stable. Water sample testing showed that the denitrification wall was effective at removing nitrate from intercepted, shallow groundwater before reaching the receiving waters (Schipper & Vojvodic-Vukovic, 1998).

In addition to providing a fuel source for the soil microbial community during decomposition, sawdust provides the ability for direct removal of materials in a wetland. Sawdust amendments are comparative to *Sphagnum* peat in their abilities to absorb materials. While this method is currently studied in heavy metal fixation from wastewater runoff, the basic principles apply to sawdust's effective removal of nitrate from water due to wood's natural adsorptive character, as previously discussed in section 2.8 (Shukla et al., 2002). In the same study, the lignin content of various woods was quantified and related to the denitrification process. Tropical woods had the highest lignin content, followed by soft (25-35% lignin), then hard (18-25% lignin) woods, making tropical woods the ideal sawdust amendment. Although a majority of commercial sawdust is generated from either soft or hard woods, it

nevertheless serves as an additional, long-term amendment source supporting denitrification mechanisms.

2.8.3 Hay

Our third chosen soil amendment, hay, is similar to wheat straw (Barrington, Choinière, Trigui, & Knight, 2002), which is considered by many soil and wetland researchers to enhance the denitrification process. Wheat straw is considered by many soil and wetlands researchers to have a constructive impact on the denitrification process. Research shows that wheat straw has the potential to increase denitrification rates for approximately one week. Beyond seven days, it was found that wheat straw's effect on denitrification rate gradually waned (Soares & Abeliovich, 1998). Wheat straw, however, is not hay. Hay was used because it was more readily available than wheat straw. This interchangeable relationship between hay and straw with respect to denitrification capabilities is supported by Table 2 within the article "Effect of carbon source on compost nitrogen and carbon losses." The study observed comparable material characteristics for wheat straw and hay (Barrington et al., 2002).

Wheat straw was observed to remove high amounts of nitrate in a long-term test at variable temperatures. Removal rates for the first ten months were reported as 18.7±4.2 and 22.7±1.7 g m^{-3} d^{-1} for 14˚C and 23.5˚C testing conditions, respectively. Between 10 and 23 months, wheat straw removed nitrate at a rate of 5.8±1.4 g m^{-3} d^{-1} (14˚C) and 7.8±1.6 g m^{-3} d^{-1} (23.5˚C). Between the first ten months and the second thirteen months, it was concluded that the carbon amendments' abilities to support denitrification had declined (Cameron & Schipper, 2010).

2.9 Carbon Cycle

The carbon cycle consists of all cycling of carbon in the Earth's crust. Carbon exists in a multitude of forms on Earth, ranging from fossil fuels, air-borne carbon dioxide, and organic material in the Earth's crust. The portion of the carbon cycle that has most to do with wetlands is the cycling of carbon through organic and non-organic material (Amundson, 2001).

The total biomass of non-plant organic matter (i.e. soil microbes) in soil can vary from 0.2 to 4% of the total mass. This living pool of organisms makes soil a dynamic reservoir of carbon rather than a static one. Even within the soil, the carbon does not cycle at the same rate and the levels of carbon are not homogenous throughout the soil. Low density carbon, such as glucose, cycles more quickly than high density carbon, such as graphite, while water-soluble carbon cycles more quickly than insoluble carbon. The rate of carbon cycling is also affected by precipitation and temperature (Amundson, 2001).

The amount of organic matter in soil reflects the long-term balance between rates of input and output. There are many factors that cause different rates of soil formation. These factors include climate, topography, parent material, biota, time, and human activity. Furthermore, the residence time of carbon in soil is affected by rainfall and temperature. The major source of lost carbon is through the release of carbon dioxide. Overall, the soil carbon content is constrained by temperature and environmental conditions such as eutrophication. However, under a natural state, the amount of carbon stored in soil tends to increase with time (Amundson, 2001).

The carbon cycle, such as it is, consists of photosynthesis in the plants and the decomposition of biomass, which adds carbon to the soil, as well as the release of carbon as CO_2. Photosynthesis participates in this cycle through the conversion of

CO_2 to oxygen and glucose in the above-ground portion of the plants (Barrington, 2002). However, in this study we focused on the below-ground carbon cycle, in which denitrification occurs. The amount of carbon in the soil is also directly correlated with nitrogen levels in the soil (Keller, 2011).

Decomposition in wetland environments depends on a number of separate reactions. This is much different from decomposition in dry environments, where the process can mostly be completed by a single organism. As in all decomposition, dead biomass is initially hydrolyzed into monomers. However, in wetland ecosystems, the anoxic conditions mean that the same microbes that cause this process cannot then decompose the products. Instead, these monomers go through a fermentation process that eventually produces fatty acids, alcohols, hydrogen and carbon dioxide gas. Methanogens then use these products to produce methane. These same products are also consumed by various microbes as well (Keller, 2011).

The biggest difference in decomposition caused by the anoxic wetland environment when compared with aerobic decomposition is time. The low dissolved oxygen levels of wetlands dramatically slow the decomposition rate. This slow decomposition rate allows the creation of peat and other forms of carbon storage (Keller, 2011).

Another interesting facet of decomposition in wetlands when compared with aerobic decomposition is the existence of fermentation. The resultant production of alcohols and other products slows down the reaction rates of the microbes. Furthermore, the small amount of oxygen production inhibits the largely anaerobic microbes in the environment.

The organic carbon in the soil also provides a source of nutrition for bacteria, more importantly, denitrifying bacteria. In a wetland environment, the soil possesses

favorable conditions for denitrification, the process of removing nitrate by conversion to nitrogen gas. The denitrifying bacteria require an anaerobic environment as well as a consistent carbon source for growth (Amundson, 2001). The high activity of these bacteria is a factor as to why constructed wetlands have been researched as a potential solution for nitrate pollution. In this study, organic carbon is manually added to the experimental microcosms to further improve the denitrification process, which will be discussed in a later section.

Chapter 3: Methodology

The project was divided into a preliminary phase and two main experimental phases, Phase I and Phase II. Phase I aimed to determine which organic amendment was most effective in increasing denitrification when used with cattail, while Phase II was conducted to determine the optimal plant combination with that organic amendment. Each phase began with an eight-week acclimation period for the plants to grow in the constructed microcosms. Organic amendments for both phases were added two weeks before data collection, within the acclimation period. A total of four one-week trials were conducted during each experimental phase, and the microcosms were flushed of nitrate once a day for four days in between each trial. After completing Phase II, the team recorded additional environmental parameters, including pH, dissolved oxygen, total carbon, and total nitrogen in the microcosm soil, and total carbon and total nitrogen of the microcosm plants.

3.1 System Descriptions

3.1.1 Emulating Tuckahoe Creek Conditions

Prior to the start of experimentation, water and soil samples were collected from the Tuckahoe Creek. Water samples collected from the Tuckahoe Creek during the spring and fall of 2011 were tested for their nitrate concentrations to serve as a basis for the microcosms' initial nitrate input concentration. Soil samples taken from the Tuckahoe Creek during the summer of 2011 were used as a basis for our microcosms' soil composition. Later, additional soil samples were taken from the same location during the winter and directly added to the microcosms in order to inoculate the soil with a native wetland bacterial ecosystem (Rice et al., 1998). A sampling site was chosen within Tuckahoe State Park, an area of flooded wetlands at

the border between Queen Anne's County and Caroline County, MD, which encompassed the inflow to the Tuckahoe Creek (Figure 3-1).

Figure 3-1: Sampling location at Tuckahoe State Park, Queen Anne, MD. Sampling location denoted by white arrow (USGS, 2013).

Approximately one liter of unfiltered stream water and 0.019 m^3 of submerged soil were collected for microcosm inoculation from the edge of a 60-acre lake leading into the Tuckahoe Creek. All samples were stored at -20°C after collection. Water samples were collected during the late spring season when nitrate levels are at a reported high due to agricultural activity in the surrounding area (Whitall et al., 2010). Prior to nitrate testing, collected water samples were frozen at -20°C to prevent nitrate degradation (EPA, 2012). Soil samples for inoculation were collected in late October 2011 and early February 2012, once at the start of each phase of testing, and added to all microcosms two weeks before data collection.

3.1.2 Microcosm Design: Preliminary Phase

The preliminary phase was conducted to test the microcosm experimental design and to optimize conditions for plant growth in the greenhouse environment. To construct the microcosms, one-half inch (1.27 cm) holes were drilled into the 10-gallon (37.8 L) plastic bins to allow for water drainage and collection. Each hole was fitted with one-half inch wide, one foot long (30.48 cm) plastic tubing, which was held in place by caulking the one-half inch hole and tubing. This plastic tubing was cinched with clothespins to prevent leakage during the trials. A fine mesh at the bin-tubing interface was installed to prevent the soil from clogging the tubing. This preliminary design had several problems. First, many of the plastic tubes became clogged by the end of the summer, making the collection of water samples difficult. When the water did pass through the tubes, it was often cloudy, making testing challenging. In addition, the tubing that did not get clogged leaked throughout the summer, leading to dry microcosms and lost water samples. Second, many of the microcosms became warped with time, as the heat and weight of the soil was too much for the plastic bins. Third, the shallow dimensions and weak structural integrity of the plastic bins made it difficult to drain all of the water from the microcosms, leaving residual water in the microcosms between trials.

3.1.3 Microcosm Design Improvements

To prevent a recurrence of the preliminary phase's microcosm structural problems, a new microcosm design was developed, emphasizing a sturdy structure and an effective filtering system. This new microcosm improved the collection method and quality of the samples.

To avoid problems with sturdiness, five gallon (18.9 L) buckets (Home Depot) were chosen for the microcosms' containers. The buckets were large and robust enough to hold the required amounts of gravel, soil, plants, and water. Additionally, the buckets were taller and narrower than the original buckets, allowing for more root growth and a more effective filtration system. Furthermore, narrowing the microcosms placed the plants in closer proximity, making it more comparable to a true wetland environment. Also, the buckets' rigidity made it easier to install a spout.

In order to avoid the filtration problems observed during the preliminary phase, two countermeasures were installed. A thin layer of mesh landscape fabric (Easy Gardener Weedblock) was added, encasing the soil and plants. Beneath the mesh, a thick layer of gravel was also added to the bottom of the microcosm to prevent soil from flowing down to the water collection region of the microcosm. To ensure that the filtration system would block the soil while still allowing water and nitrate to pass through, the mesh was tested by observing a continuous flow of water when added to a bag constructed from mesh. The layer of gravel was added at the bottom of the bucket to a depth reaching above the effluent pipe. This gravel filtered any soil that was able to pass through the mesh.

Finally, a better outflow system was designed. Instead of using plastic tubing, a spigot was installed. This spigot consisted of a PVC ball valve, bulkhead fittings, and a PVC pipe. The components were held together and kept watertight with PVC plumbers' glue (Home Depot).

3.1.4 Microcosm Construction: Phase I and Phase II

The microcosms were constructed in the following manner for Phase I and Phase II (Figure 3-2). To create the spigot assembly, a hole was first drilled into the

side of the microcosm five centimeters from the bottom of the bucket. Next, a one-half inch bulkhead fitting was added and bound to the hole using plumbers' glue. A segment of one-half inch PVC pipe with a ball valve was attached to this bulkhead fitting. Next the gravel was added to the bottom of the bucket. The gravel was first washed to remove all powder and residue. Then, this gravel was added to the microcosms until the spigot holes were covered with gravel, approximately 5 centimeters from the bottom of the bucket.

After the gravel was added, a sheet of mesh landscape fabric (Easy Gardener WeedBlock), approximately 1 meter x 1 meter, was placed on the gravel. The edges of the mesh were taped to the edges of upper edges of the microcosm, creating a mesh bowl along the edges of the microcosm. The total mass of the bucket, gravel, and mesh bowl averaged about 6 kg.

Subsequently, the sand and topsoil were mixed in an approximately 1:1 ratio and added to the microcosms. The ratio was determined by drying and sifting the Tuckahoe Creek soil samples, after which the approximate ratio of sand to soil was estimated. This combination of sand and soil served to both facilitate the growth of the plants and to support the types of microbial populations that exist in natural wetlands. This soil-sand mixture was manually mixed. A total of six, two-inch (5.08 cm) plant plugs (Environmental Concern Nursery, St. Michael's, MD) were planted in each microcosm excluding control groups, which contained no plants.

Figure 3-2: Final Microcosm Design

3.1.5 Amendment Acquisition

Amendments were obtained from different sources. Due to limited funds, amendment collection was based on ease of acquisition and cost-effectiveness. Hay was procured from a team member's farm in Susquehanna, Pennsylvania. Raw sawdust was collected from the University of Maryland Woodshop. Glucose (D-(+)-Glucose, $\geq$ 99.5% (GC)) was ordered from Sigma-Aldrich (St. Louis, MO).

3.1.6 Organic Amendment Addition and Soil Inoculation

The team postulated that different communities of bacteria thrive in different environments. Accordingly, the microcosms would have very different bacterial compositions when compared to a natural wetland. Therefore the wetland's bacterial communities were brought to the microcosms by taking wetland soil from the Tuckahoe Creek. These soil samples would contain the natural bacterial communities

of the wetland. In October of 2011 a portion of the team traveled to the Tuckahoe Creek and collected soil to be used as a bacterial inoculant. About one liter of inoculant was added to each microcosm on top of the soil. The inoculant was approximately 250 mL wetland substrate and 750 mL wetland water.

Organic amendments were added at 10 percent of the mass of the soil (Hien et al., 2010) to the top of each microcosm. Given the different compositions of the organic amendments, they were added into the microcosms in different ways. To add the glucose, it was diluted into a 0.1 M solution of glucose so that the glucose solution would be 10 percent of the mass of the soil, as shown in the calculations below. To add the sawdust, the team similarly made a suspension of sawdust and water. Due to the insolubility of sawdust, the sawdust needed to be well mixed into the water. To add the hay, the team first cut the hay into small pieces using scissors, before again making a 10 percent by mass mixture of hay and water.

3.1.7 Nitrate Solution Preparation and Addition

Each microcosm was watered with a 6.45 mg L^{-1} nitrate solution. To prepare the solution, a 12.911 g L^{-1} nitrate solution was made by adding 12.911 g of ammonium nitrate to 1 L of water. Then, this solution was diluted by 20 times (50 mL of high concentration solution into 1 L of water) to get 0.645 g L^{-1} (or 645 mg L^{-1}). Finally, 100 mL of this solution was used to create 10 L of a 6.45 mg L^{-1} solution. The resulting nitrate solution was then added to each microcosm to fully saturate the soil. The exact amount of nitrate solution needed per microcosm varied. Upon initial saturation at the start of the first trial, these amounts were noted on a label on each microcosm so the same amount of nitrate solution would be added at the start of each trial.

Nitrate solution was added at the beginning of each trial week. Microcosms were watered once during the week with tap water to maintain soil saturation, and these volumes were recorded. The recorded volumes were later used to calculate total nitrate concentration in the effluent.

3.1.8 Greenhouse Conditions

The microcosms were housed in a temperature-, light-, and humidity-controlled greenhouse room in the Research Greenhouse Complex at the University of Maryland, College Park. Temperatures were maintained at 27°C during the daytime and lowered to 21°C at night. Low natural sunlight was supplemented with high-intensity discharge (HID) lighting during the hours of 7 am to 10 pm, as needed. Microcosms were watered two times a week until they were visibly saturated. This was done in order to maintain an anoxic environment in the soil, similar to the conditions in a natural wetland. Experimental groups were arranged randomly in the greenhouse room in order to compensate for variations in sunlight intensity in different areas of the greenhouse.

3.1.9 Environmental Parameters

After completing Phase II, environmental parameters were tested to see how the microcosms compared to each other and the natural environment as denoted by previous literature.

3.1.9a Microcosm pH

The first parameter obtained from each microcosm was pH. This data was collected within a month after the end of Phase II, during which the plants were

maintained and watered as through the experimental trials. A Luster Leaf 1845 Rapitest Digital Soil pH meter (Luster Leaf Products, Inc., Woodstock, IL) was used to test the pH in each microcosm. To test pH, the meter was inserted approximately four centimeters below the surface of the soil. After allowing the pH meter to equilibrate, the meter was placed into the same microcosm in a different location and again inserted four centimeters into the soil. This was then repeated a third time, and these three data points were averaged.

3.1.9b Dissolved Oxygen Content

Dissolved oxygen (DO) was collected one month after the last effluent water sample was collected in Phase II. AYSI DO 200 meter (YSI Incorporated, Yellow Springs, OH) was placed in effluent collected from each microcosm. Sample water was stirred gently with the DO meter to mix the sample. DO was recorded after the meter equilibrated. After sampling a microcosm, the dissolved oxygen meter was rinsed with deionized water to reduce the chance for cross-contamination (YSI Incorporated, Yellow Springs, OH).

3.1.9c Percent Nitrogen and Percent Carbon

To identify the nitrogen that was taken up and fixed by the plants and the nitrogen that was retained in the soil of each microcosm, at the end of Phase II, plants and soil were analyzed for total percent N (nitrogen) and percent C (carbon). Total percent C was analyzed in order to provide biomass information to investigate the effectiveness of harvesting these plants for biofuel production. Before each plant and soil sample was analyzed, it had to be dried, weighed and ground (Appendix E, p.125).

All analyses were conducted by the Environmental Science and Technology Department's Analytic Lab at UMD.

3.2 Experimental Design

3.2.1 Experimental Design: Preliminary Phase

The purpose of the Preliminary Phase was to evaluate and refine our initial microcosm design and methodology in preparation for Phase I and Phase II. Each microcosm contained six narrow-leaved cattail plugs evenly spaced in a 1:1 top soil to sand mixture, to which soil from the Tuckahoe Creek was also added. Plants were watered once with 10% Hoagland solution (hydroponic nutrient solution) until the soil was saturated after they were planted, and watered with regular tap water for the remainder of the 6 week acclimation period. A total of three eight-day long trials were then conducted, with nitrate added on the first day and samples collected on the eighth day. Microcosms were flushed with tap water for four days in between trials. Because of the leaky tubing and poor experimental design, sample volumes varied and samples were often cloudy.

3.2.2 Experimental Design: Phase I

Organic amendments can provide a way to amplify differences in nitrate removal among experimental groups of different plant combinations by increasing denitrification rate, which may otherwise be relatively small given the small scale of the microcosms (Sirivedhin & Gray, 2006; Hien et al., 2010). Thus, the goal of Phase I of testing was to determine which organic amendment has the greatest positive impact on denitrification in combination with cattail, the most extensively researched wetland plant. Nitrate concentration reduction was compared between microcosms containing cattail and one of three organic amendments: hay, glucose, and sawdust.

Control microcosms with organic amendments and without plants, and with plants and without organic amendments provided a basis for comparison. Immediately after the microcosms were constructed, a total of six cattail plugs were planted in each microcosm. The plants were allowed an acclimation period of six weeks to give the plants an opportunity to adapt to new conditions and mature before testing began (Brisson & Chazarenc, 2008). However, because Phase I started in mid-fall and continued until the end of January, the cattail entered the dormant period of their natural growth cycle. During this period, all plants lost their color and waned, despite favorable growth conditions of the greenhouse.

Eight-day trial periods were determined based on review of literature (Hien et al., 2010; Zhu & Sikora, 1995). There was a four-day gap between trials during which microcosms were flushed with tap water and drained daily to remove any residual nitrate solution. Microcosms were saturated with tap water, and then drained and re-saturated the next day. At the last day of flushing before the start of the next trial, microcosms were drained and not watered. Microcosms were saturated with nitrate solution the following day, the fifth day after the end of the previous trial, to start the next trial.

Four trials were conducted in each phase to ensure that there would be enough data to statistically test our hypothesis as well as to account for school closures during the winter and summer when team members would not be available to carry out the project. From these trials, a large enough sample size can be used (28 data points) so that the sample can be considered approximately normal, as stated by the Central Limit Theorem (Devore, 2000).

The experimental microcosms used in Phase I were organized as follows:

Table 3-1: Phase I experimental microcosms

	Cattail	Glucose	Hay	Sawdust
Four Microcosms	✓			
Four Microcosms	✓	✓		
Four Microcosms	✓		✓	
Four Microcosms	✓			✓
Four Microcosms		✓		
Four Microcosms			✓	
Four Microcosms				✓
Four Microcosms				

3.2.3 Experimental Design: Phase II

Phase II tested the effect of the species of wetland plant (*Typha latifolia* (cattail), *Schoenoplectus validus* (soft-stem bulrush), *Panicum virgatum* (switchgrass) on nitrate removal in a microcosm. Sawdust, the organic amendment that resulted in the greatest nitrate concentration reduction in Phase I was added to each experimental group in Phase II. Control microcosms with sawdust and without plants, and without plants or sawdust provided a basis for comparison. The same trial length and methods as described in Phase I were used.

The experimental microcosms used in Phase II were organized as follows:

Table 3-2: Phase II experimental microcosms

	Sawdust	Cattail	Switchgrass	Bulrush
Four Microcosms	✓			
Four Microcosms	✓	✓		
Four Microcosms	✓		✓	
Four Microcosms	✓			✓
Four Microcosms	✓	✓	✓	
Four Microcosms	✓	✓		✓
Four Microcosms	✓		✓	✓
Four Microcosms	✓	✓	✓	✓
Four Microcosms				

3.3 Data Collection Methods

3.3.1 Water Sample Collection

At the end of each trial week, the water from every microcosm was collected independently. All water was drained from each microcosm through the spigot and then stirred in its own bucket. 40 mL were obtained from the stirred effluent and stored in 50 mL Falcon tubes (BD Biosciences; San Jose, CA). Before draining the next microcosm, the collection buckets were rinsed with faucet water.

3.3.2 Water Sample Testing: Preliminary Phase

Nitrate concentration in water samples was analyzed using a Hach spectrophotometer (DR 5000™ UV-Vis Spectrophotometer; Hach Company, Loveland, CO). This process entailed filtering the water (Whatman 1827-047 Glass Microfiber Filter, 4.7 cm diameter), adding a nitrate test packet (NitraVer® 5 Nitrate Reagent Powder Pillows, for 10 mL samples), waiting for the reaction to reach equilibrium, and then testing the change in absorbance using the spectrophotometer (Appendix E). Initial attempts at testing the nitrate concentration with this method proved very inconsistent, likely resulting from ammonium nitrate degradation in the samples as well as an insufficient testing range for the Hach spectrophotometer. The testing ranges of nitrate test packets (High Range 0.3-30 mg L^{-1} of nitrate-N; Mid Range 0.1-10 mg L^{-1} of nitrate-N) lacked the precision to ensure consistent, accurate measurement of the effluent nitrate concentration.

3.3.3 Water Sample Testing: Phase I and Phase II

After collection, samples were stored in a freezer until they were packed and shipped, to prevent degradation of nitrate (EPA, 2012). Frozen samples were shipped

to the University of Maryland Center for Environmental Studies (UMCES) Appalachian Laboratory (Frostburg, MD) to be tested for nitrate concentration. Nitrate, nitrite, and ammonium levels were tested in all samples using nitrate-nitrite by cadmium reduction colorimetry. In this procedure, a filtered sample passes through a granulated copper-cadmium column and nitrate is reduced to nitrite. Both the converted nitrite and the original nitrite in the sample undergo a reaction that forms an azo dye that can be measured with a spectrophotometer to determine concentration (NEMI, 1974).

3.3.4 Plant Sample Collection and Testing

Plant sampling was conducted after completion of Phase II only. Aboveground biomass was collected in each microcosm to test for plant-specific characteristics. Plants were cut from each microcosm at the soil level and placed into brown paper bags. The bagged plants were dried at 30-35°C for two weeks in an oven at the University of Maryland Research Greenhouse Complex. After the samples were dried (no loss of mass noted after three days), plant masses were measured and recorded (Table 5-1).

Dried plant matter was ground at the Environmental Science and Technology (ENST) Analytical Lab (University of Maryland) following their standard protocol for solid sample testing. After grinding, approximately 0.2 g of each sample was packed into 6x15mm tin capsules, to be analyzed by the Analytical Lab (Appendix E).

3.3.5 Soil Sample Collection and Testing

Soil sampling was conducted after completion of Phase II only. Since only a portion of the soil from each microcosm could be tested –and the tests would provide

percent N and percent C data-- the total mass of soil from each microcosm had to be measured prior to the collection of samples so that total nitrogen and carbon per microcosm could be calculated. To eliminate all water weight from the soil, the microcosms were dismantled. Soil from each microcosm was allowed to air dry in the greenhouse for one week. The air-dried soils were homogenized for each microcosm and representative samples were collected in special soil bags, paper bags lined with plastic on the inside. To collect samples, the soil was spread out in its original mesh and mixed using a pick. The soil was then mixed by hand to break up large clumps. Large roots were removed from the soil samples, to eliminate confounding data. Non-uniform dispersion of roots in the soil samples would add variability between samples (and affect accuracy and precision of percent nitrogen and percent carbon data). However, because smaller roots were present throughout the soil, samples were collected as a combination of soil and these smaller roots. Therefore, total percent N and percent C below ground would be accounted for. A sample of the same brand of top soil and sand was also mixed and collected to account for the starting percent N and percent C in the soil, before experimentation.

After the soil was dried at 50-60°C for three days, samples were ground using the Dynacrush soil crusher, model: DC-2 (Custom Laboratory Equipment, Inc., Orange City, FL) and passed through a 2mm sieve. The soil was then packed in 6x15 mm tin capsules following the Analytical Lab's protocol (Appendix E).

3.4 Data Analysis Methods

All statistical tests were analyzed using SAS 9.2 software. Data was deemed significantly different for p-values less than 0.05.

3.4.1 Preliminary Phase

The preliminary phase consisted of three data points from four separate microcosm groups. Three data points, marked with an asterisk in Table 4.1, were not included in statistical tests due to inadequate sampling. The remaining nine data points were analyzed using a Student's t-test to determine the effect of microcosms on nitrate removal, as well as the effect of new trial weeks on sample nitrate concentration.

3.4.2 Phase I

After the nitrate concentration reduction was determined for each microcosm, the significance of the differences between conditions was analyzed. An ANOVA F-test was used to test for significant differences among treatments. Specifically, a two factor ANOVA with one repeat measure was used, with microcosm treatment and week of the trial as the two factors analyzed. Organic amendments were analyzed across microcosms containing plants, and then across all microcosms (plants and no plants). Nitrate removal was also tested between microcosms with plants and microcosms with no plants. After identifying significant differences ($p<0.05$), the Student Newman Keuls (SNK) method was used to compare which group had a significantly greater nitrate removal. While the ANOVA test examined various organic carbon treatments, the SNK method tested the presence versus the absence of an amendment.

Nitrate removal was defined as the difference between the expected output nitrate concentration and the measured output concentration.

3.4.3 Phase II

The difference in nitrate concentration between the input and output solutions for each trial week was averaged to calculate nitrate removal. Data from Week 3 was collected but not included in the final data analysis because of significantly higher input nitrate concentrations ($p<0.001$). This higher input would have skewed the data, leading to artificially higher results. By removing Week 3, outliers did not have an effect on the determined results.

As in Phase I, the significance of the nitrate removal was analyzed. A single factor ANOVA test was used to analyze differences between nitrate concentration reductions for different plant combinations. After this, the SNK method was used to analyze the significance of individual factors in the results, again with a cutoff of $p=0.05$. While the ANOVA test examined various combinations, the SNK method could test the presence versus the absence of a single species.

3.4.4 Nitrogen Balance

To complete the nitrogen mass balance and determine removal of nitrate by denitrification, the total masses of nitrogen input and output was determined for each microcosm. Total mass of nitrogen input into a microcosm across the four-week trial period includes nitrogen load from the nitrate solution administered at the start of each trial week and existing soil nitrogen at the start of the four-week trial period. Nitrogen loads from nitrate solutions were calculated by multiplying input nitrate, nitrite, and ammonia concentrations for each trial week by the volume of solution administered. The amount of water required to saturate the soil determined this volume. Therefore, the nitrogen load can vary across microcosms, even within the

same experimental treatment, but stays constant across trial weeks. In all the equations, N = (NO_3^- + NO_2^- + NH_3). In equation E-1, $N_{soil(start)}$ was measured as the mass of nitrogen in the soil at the beginning of the trial (percent N multiplied by soil mass). $N_{influent}$ was calculated as the initial nitrate concentration multiplied by the initial nitrate solution volume added.

$$\Sigma N_{in} = N_{influent} + N_{soil(start)} \qquad \text{(E-1)}$$

Correspondingly, total mass of nitrogen effluent ($N_{effluent}$) was calculated by multiplying the final output nitrate concentrations by the total input volume (i.e. the initial nitrate solution and water added midweek to maintain saturation). In equation E-2, $N_{biomass}$ was calculated as the mass of nitrogen in the above-ground portion of the plants, $N_{soil(end)}$ as the mass of nitrogen in the soil at the end of the trial, and $N_{denitrification}$ as the mass denitrified.

$$\Sigma N_{out} = N_{effluent} + N_{biomass} + N_{soil(end)} + N_{denitrifcation} \qquad \text{(E-2)}$$

As plants and substrate were not replaced for new trials, nitrogen concentration reduction data collected at the end of the fourth trial week represented accumulation of nitrogen over the length of all four trial periods. Nitrogen mass removed by denitrification was calculated by combining the above equations and rearranging terms to yield:

$$\Sigma N_{in} = \Sigma N_{out} \qquad \text{(E-3)}$$

$$N_{influent} + N_{soil(start)} = N_{effluent} + N_{biomass} + N_{soil(end)} + N_{denitrifcation} \qquad \text{(E-4)}$$

$$N_{denitrifcation} = [N_{influent} + N_{soil(start)}] - [N_{effluent} + N_{biomass} + N_{soil(end)}] \qquad \text{(E-5)}$$

Using these above equations, a nitrogen balance was created for the analysis of Phase II.

Chapter 4: Phase I – Results and Discussion

4.1 Preliminary Phase

Water samples collected from the Tuckahoe Creek in the spring and fall of 2011 had nitrate levels of 2.67 mg-N L^{-1} and 2.65 mg-N L^{-1}, respectively. While this concentration was not used in the trials for the initial solution of nitrate water that the microcosms were treated with, the consistent nitrate loading across both seasons demonstrated that the same concentration of nitrate solution could be used throughout the phases and would be representative of the natural conditions. For the preliminary trials, as well as Phases I and II, the initial nitrate concentration was 5 mg L^{-1}.

Sample nitrate concentration of effluent was significantly lower than input nitrate concentration for all trials; there was no significant difference in sample nitrate concentration among trial weeks for each microcosm ($p=0.5079$). The data demonstrated that nitrate concentration was reduced in the microcosms and that the experimental setup worked.

Several issues with the microcosm procedures were identified from the preliminary phase. First, the microcosm containers did not effectively filter the effluent that was collected, so the samples were often cloudy and contained a significant amount of suspended soil particles. Second, while nitrate removal was apparent for each sample tested, the amount of nitrate removed varied greatly among samples (Table 4-1). To obtain more consistent results for the next phase, protocols were updated and clearly defined in order to minimize any errors. The new microcosm design also ensured that a cleaner sample would be collected.

Table 4-1: Nitrate outflow concentrations, preliminary phase.
Units of outflow for NO^{3-} are mg-N L^{-1}. Targeted inflow was 5 mg-N L^{-1}.

	Microcosm			
Week	P1	P2	P3	P4
Week 1	2.7	2.8	1.4	3.5
Week 2	5.0*	4.4*	1.7	1.7
Week 3	2.5	12.8*	1.5	2.6

*samples with significantly less than 10 mL collected

4.2 Phase I Results

4.2.1 Nitrate concentration reduction in planted and unplanted microcosms

Nitrate concentration in microcosms with plants was compared to microcosms without plants. The team found that across all weeks, the microcosms with plants removed significantly more nitrate than those without plants ($p<0.05$, Figure 4-1, Table 4-2). These results are promising because they support the fundamental assumption of this project that plants play a significant role in the reduction of nitrate concentration.

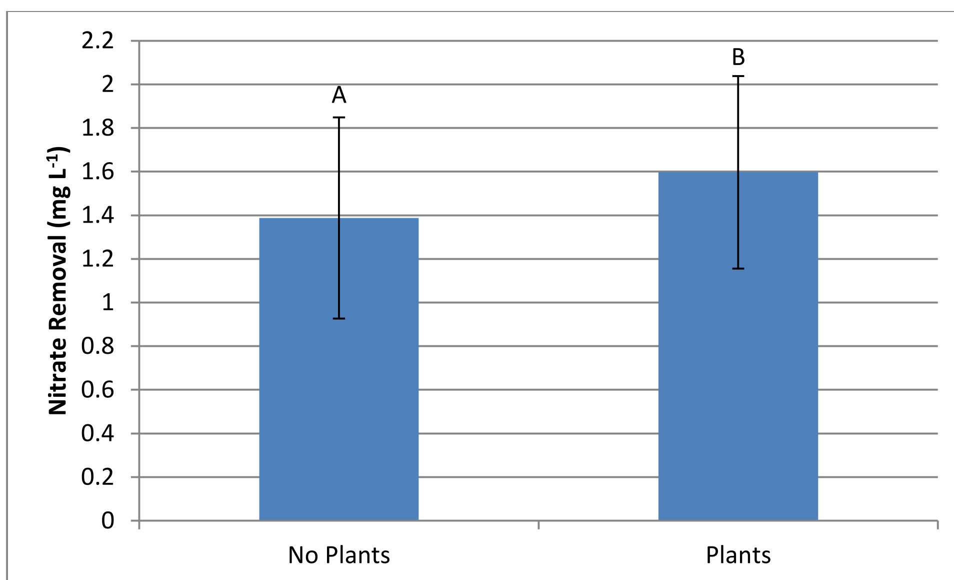

Figure 4-1: Nitrate Removal in Plant and No Plant Microcosms, Phase I.
Data collected over all four weeks with plants (n=48) and without plants (n=64). Error bars are standard deviation based on four trials. Plants have greater nitrate removal ($p<0.05$). Groups with different letters are statistically different.

Table 4-2: Decrease in Nitrate Concentration by Week, Comparing Plants and No Plants, Phase I.
Microcosms with plants (n=12) and without plants (n=16) are presented.

NO_3^- Decrease (mg L^{-1})	Week 1	Week 2	Week 3	Week 4
Plants	1.69	1.58	1.59	1.54
No Plants	1.24	1.56	1.44	1.31

4.2.2 Sawdust-amended nitrate removal in planted and unplanted microcosms

Sawdust-amended microcosms reduced the concentration of nitrate by a significantly higher amount than the control amendment conditions regardless of whether microcosms had plants or no plants, ($p<0.05$, Figure 4-2, Figure 4-3).

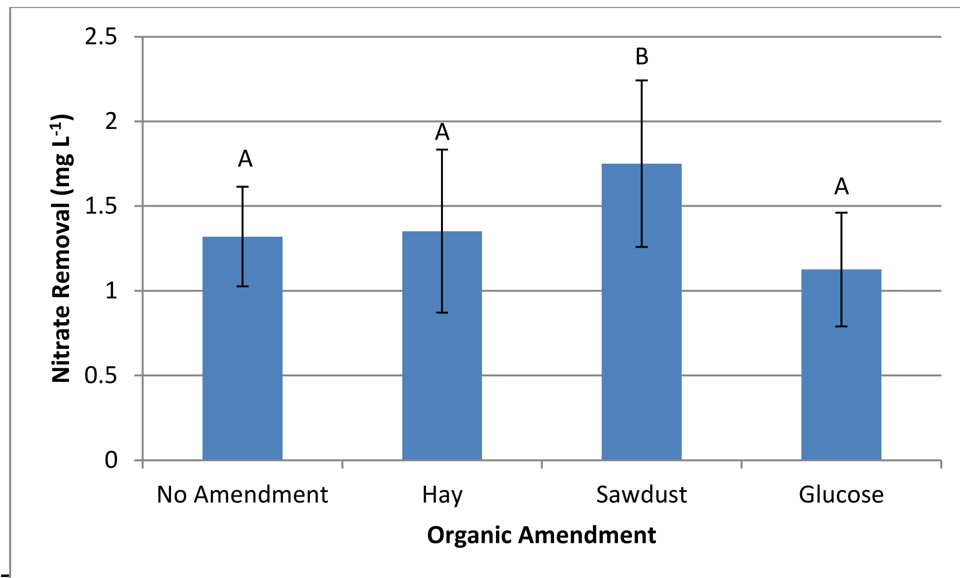

Figure 4-2: Nitrate Removal in No Plants by Organic Amendment, Phase I.
Error bars are standard deviation based on four trials. Sawdust has greater nitrate removal than other organic amendments ($p<0.05$). Groups with different letters are statistically different.

Among microcosms with plants, only microcosms with sawdust removed more nitrate than the control microcosms, which contained plants and no amendments ($p<0.05$, Figure 4-3); the glucose and hay microcosms did not remove more nitrate than the control group. Based upon these results, we decided that sawdust was the best amendment to add to our microcosms during Phase II of the experiment.

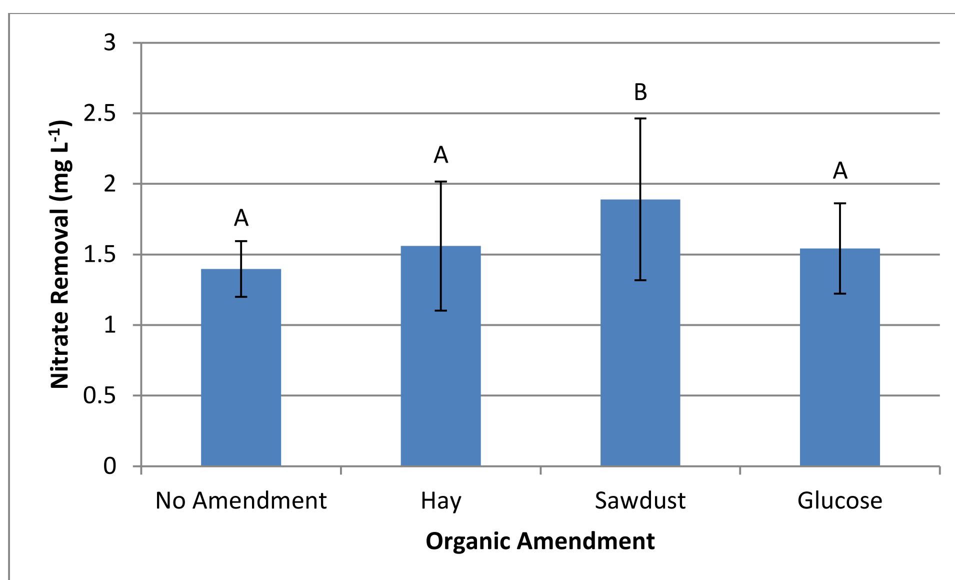

Figure 4-3: Nitrate Removal in Plants Only by Organic Amendment, Phase I. Error bars are standard deviation based on four trials. Sawdust has greater reduction in nitrate concentration than other organic amendments ($p<0.05$). Groups with different letters are significantly different.

Table 4-3: Decrease in Nitrate Concentration by Week Comparing Organic Amendments in Plants Only, Phase I
For all conditions, there were four replicates.

NO_3^- Decrease (mg L^{-1})	Week 1	Week 2	Week 3	Week 4
Plants+Hay	1.53	1.58	1.41	1.72
Plants+Sawdust	1.91	1.83	1.79	2.03
Plants+Glucose	1.88	1.48	1.65	1.16
Plants Only	1.42	1.42	1.51	1.24

There was no significant difference in nitrate removal among different trial weeks ($p=0.779$, Table 4-3).

The often cited time-dependent relationship between organic amendments and nitrate removal in microcosm experiments (Hien et al., 2010; Soares & Abeliovich, 1998; Weisner et al., 1994) was not observed in this four-week study, presumably because samples were only collected at the end of the trials. It is possible that more sampling would be required throughout the week to detect such an effect.

4.3 Discussion of Phase I

The team's findings that microcosms containing plants reduced a significantly greater concentration of nitrate than microcosms without plants is consistent with the results of previous studies as discussed in our literature review (Beutel et al., 2009; Leverenz et al., 2010; Vymazal, 2010). Other studies have shown that plants are important for fostering the growth of bacterial communities that facilitate the reduction of nitrate (Mitsch & Gosselink, 2000). Additionally, plants have been shown to increase nitrate removal through uptake (Fisher & Acreman, 1999). Confirming that plants play a critical role in the removal of nitrate in wetlands was critical to the justification of our project. Because plants are essential to reducing the concentration of nitrate, it was important to conduct Phase II of the study to determine which plants or combinations of plants would best facilitate the removal of nitrate from the Chesapeake Bay Watershed.

It is also important to note that the microcosms with plants and sawdust removed a greater amount of nitrate than those microcosms with only plants ($p<0.05$, Figure 4-3Table 4-3). While this result encouraged us to choose sawdust as the organic amendment to add to our microcosms to increase the difference in nitrate removal across test groups, the team did not consider that sawdust might be so effective at removing nitrate from the system as to mask the effect that different plants would contribute to nitrate concentration reduction. Among all organically amended microcosms, sawdust was observed to remove a significantly higher amount of nitrate than glucose or hay ($p<0.05$, Figure 4-3).

In planted microcosms, the presence of sawdust led to higher nitrate removal compared to planted microcosms with other or no organic amendments, indicating that the performance of cattail is improved with the addition of sawdust ($p<0.05$,

Figure 4-3). Sawdust's ability to enhance the nitrate removal in plant microcosms, coupled with the failure of glucose and hay to do so, indicated that sawdust should be used as the organic amendment in Phase II. The finding that glucose and hay did not increase nitrate removal disagrees with the literature (Cameron & Schipper, 2010; Soares & Abeliovich, 1998; Sparling et al., 1981; Weisner et al., 1994).

Comparison of the nitrate removal results of different organic carbon sources was inconsistent with results in literature. Glucose is one of the most easily biodegradable carbon sources and is readily consumed by denitrifying bacteria (Weisner, Eriksson, Granéli, & Leonardson, 1994). Many studies showed that glucose significantly increases microbial activity, thus demonstrating a significantly improved denitrification rate (Sparling, Ord, & Vaughan, 1981). In contrast, the experimental data does not support the literature in that microcosms with glucose did not perform significantly better than ones without any additional carbon source. This may be caused by the high availability of glucose to the bacteria. Since glucose is easy to metabolize, it should degrade quickly in biologically active soils—especially during the short trial period (8 d) of the experiment—which should lead to a reduced stimulatory effect on denitrification (Sparling et al., 1981). Thus, increased denitrification rate from glucose might disappear after two weeks. In order to acclimate the microbial communities to the organic carbon, the microcosms were incubated for two weeks after the addition of the amendments. Within the acclimation period, the glucose may have already been completely consumed by the bacterial communities. Since the glucose may have already been completely depleted during the data collection period, the microcosms that had additions of glucose may have had little to no external organic carbon available. This would explain why there

was no significant difference found between microcosms with glucose and microcosms without any organic carbon source.

Similarly, there was no significant difference found in the nitrate removal between microcosms with hay and microcosms without hay. In one study, the addition of wheat straw (similar properties to hay as an organic carbon source) was observed to remove a significantly higher amount of nitrate over a period of ten months with a decline in denitrification afterwards (Cameron & Schipper, 2010). However, in another study, denitrification efficiency declined within one week (Soares & Abeliovich, 1998). A study comparing bulking agents for composting found that chopped hay removed nitrates more than chopped wheat straw (Adhikari, Barrington, Martinez, & King, 2009). Because the acclimation period and data collection period was less than two months, the effectiveness of hay should have continued until the end of Phase I. A possible explanation for this difference may be the contrasting properties of wheat straw and hay. High quality hay is green and includes plant heads, leaves, and stems. Poor quality hay is dry, bleached, and coarse-stemmed. Both are edible by farm animals and can be used for nutritional value, but the compositions are different. The hay used in this study was dry and bleached, which may have contributed to different results. Because a less effective hay source was used, the hay may have degraded less than expected in the two-week acclimation period. The hay might have been an ineffective organic carbon source during the data collection period; it did not accelerate nitrate removal. An alternative consideration is that although the hay was cut into small pieces before it was added to the soil, it was not necessarily fully mixed within the soil. The localization of the hay amendment at the surface of the microcosm may have inhibited interactions between denitrifying bacteria and the carbon amendment. Without bacterial-amendment interaction,

regardless of hay quality or stage of degradation, the traditional organic amendment effect from the hay will remain non-existent.

Sawdust, however, was shown as the only organic amendment to provide significant nitrate removal as compared to no-amendment microcosms. Previous studies have come to the consensus that sawdust is a long lasting amendment, having a stable effect on denitrification enzyme activity and microbial biomass for over 12 months (Schipper & Vojvodic-Vukovic, 1998). Sawdust was also found to directly reduce the concentration of nitrate in solutions by adsorbing the materials via ion exchange and hydrogen bonding (Shukla et al., 2002). Most materials in the cell walls of sawdust are ion exchange compounds, such as cellulose, lignin, and hydroxyl groups. The sawdust components bond to nitrate and heavy metals, removing them from the solution (Shukla et al., 2002). Because the acclimation and data collection occurred within a two-month period, sawdust's effectiveness should have been consistent throughout the process.

The ability of sawdust to significantly increase nitrate removal over soil without amendments may have been due to adsorption. Initially, sawdust will absorb nitrate, thus providing a higher nitrate removal than other microcosms (Shukla et al., 2002). However, for durations longer than a year, the sawdust will begin degrading and releasing available nitrate. Sawdust applied to a biofuel-capable wetland would work properly in the one-year period before harvesting, so the full nitrogen removal effect would be realized. Higher nitrate levels from the sawdust will aid in the growth of plants. Overall, the results from Phase I support previous studies suggesting sawdust's effectiveness as an organic amendment.

Within each experimental group, there was no significant difference in nitrate removal between consecutive trials ($p=0.5451$, Table 4-3). Nitrate solution was added

to each microcosm at the beginning of each week and collected the effluent at the end of the week. This was repeated four times, producing results for four separate eight-day trials. Comparing nitrate concentration reduction between each week showed that the performance of the microcosms did not change during that month-long period. Each type of microcosm removed approximately the same amount of nitrate each week, indicating that plant activity and effects of organic amendment sources did not vary significantly from week to week. The flushing period, during which water was added and drained for four days to remove any excess nitrate, helped maintain consistent microcosm conditions.

Data from Phase I demonstrated several important results. First, the use of plants to reduce nitrate concentrations in wetlands is more effective than using no plants, reiterating the benefits and ecological importance of constructed wetlands. Second, sawdust is a valuable organic amendment in wetlands. Microcosms with plants and sawdust performed better than microcosms with plants but no sawdust, indicating the nitrate removal potential of this organic amendment. Among microcosms with plants, the sawdust treatment removed more nitrate than the control, glucose, and hay treatments. Lastly, more nitrate was removed in microcosms with sawdust and plants than in those with sawdust and no plants, further demonstrating the benefits of combining plants with organic amendments.

Chapter 5: Phase II – Results and Discussion

5.1 Phase II Results

5.1.1 Effect of Wetland Plants and Sawdust on Inflow Nitrate Removal

In all sampled microcosms, effluent nitrate concentrations were lower than inflow nitrate concentrations ($p<0.05$, Table 5-3).

5.1.2 Plants vs. No Plants

The average nitrate removal for microcosms containing plants (1.365 mg L^{-1}; n=28) was not greater than in microcosms without plants (1.353 mg L^{-1}; n=8, p=0.880, Figure 5-1).

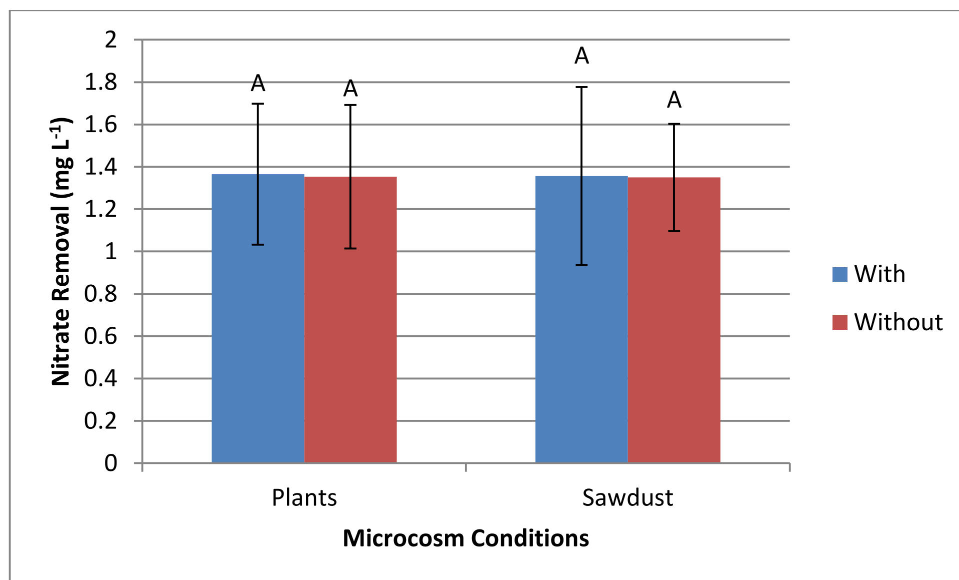

Figure 5-1: Nitrate Removal Comparing Presence of Plants and Sawdust, Phase II. Error bars are standard deviation based on three trials. No difference between microcosms with plants and without plants (p=0.880). No difference between microcosms with organic amendments and without organic amendment (p=0.964). Inflow concentration was 5 mg L^{-1}. Groups with different letters are statistically different, only within the same microcosm conditions.

5.1.3 Sawdust vs. No Sawdust

In microcosms containing no plants, the average nitrate removal for microcosms containing sawdust (1.356 mg L^{-1}; n=4) was not greater than for microcosms without sawdust (1.350 mg L^{-1}; n=4, p=0.964, Figure 5-1).

5.1.4 Nitrate Removal by Individual Plant Type

In microcosms containing plants (n=28), nitrate removal was compared across the absence or presence of an individual plant species using a T-test. Microcosms with bulrush did not have significantly higher nitrate removal than microcosms without (p=0.819). Microcosms with cattail did not have significantly higher nitrate removal than microcosms without cattail present (p=0.924). Microcosms with switchgrass did not have significantly higher nitrate removal than microcosms without switchgrass (p=0.589). Reduction in nitrate concentration in microcosms with or without a given plant species was not significantly different (Figure 5-2).

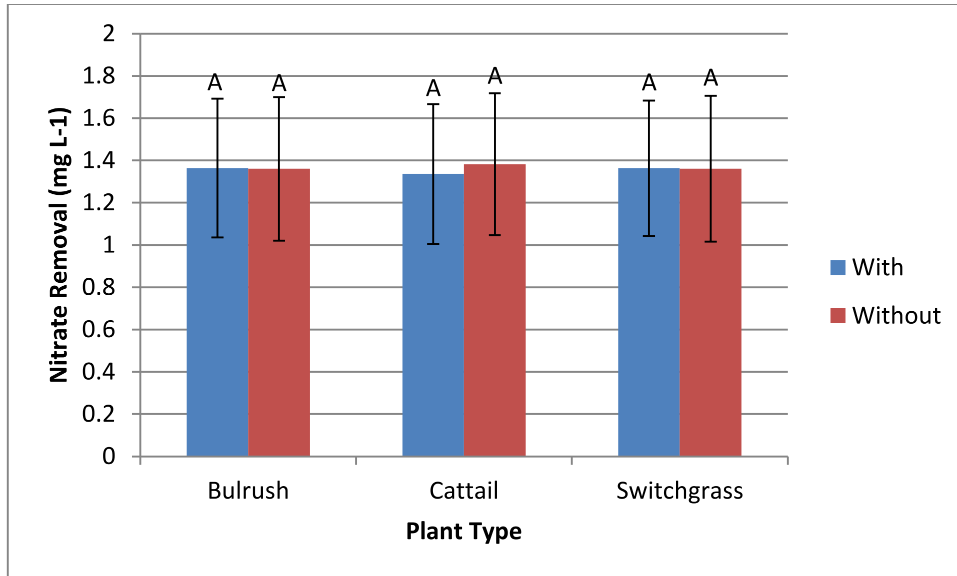

Figure 5-2: Nitrate Removal Comparing Presence of Plant Type, Phase II.
Error bars are standard deviation based on three trials. The presence of any individual species had no effect on nitrate removal. With versus without: bulrush ($p=0.819$), cattail ($p=0.924$), switchgrass ($p=0.589$). Inflow concentration was 5 mg L^{-1}. Groups with different letters are statistically different, only within the same species.

5.1.5 Nitrate Removal by Plant Combinations

In a comparison of the different plant species combinations, no single combination had significantly higher nitrate removal than the others ($p=0.612$, Figure 5-3). Switchgrass alone had an average removal of 1.339 mg L^{-1}. Just bulrush had an average removal of 1.416 mg L^{-1}. Just cattail had an average removal of 1.420 mg L^{-1}. The average removal of switchgrass plus bulrush was 1.400 mg L^{-1}. The average removal of switchgrass plus cattail was 1.287 mg L^{-1}. The average removal of bulrush plus cattail was 1.282 mg L^{-1}. The removal of all three species in a single microcosm was 1.358 mg L^{-1}.

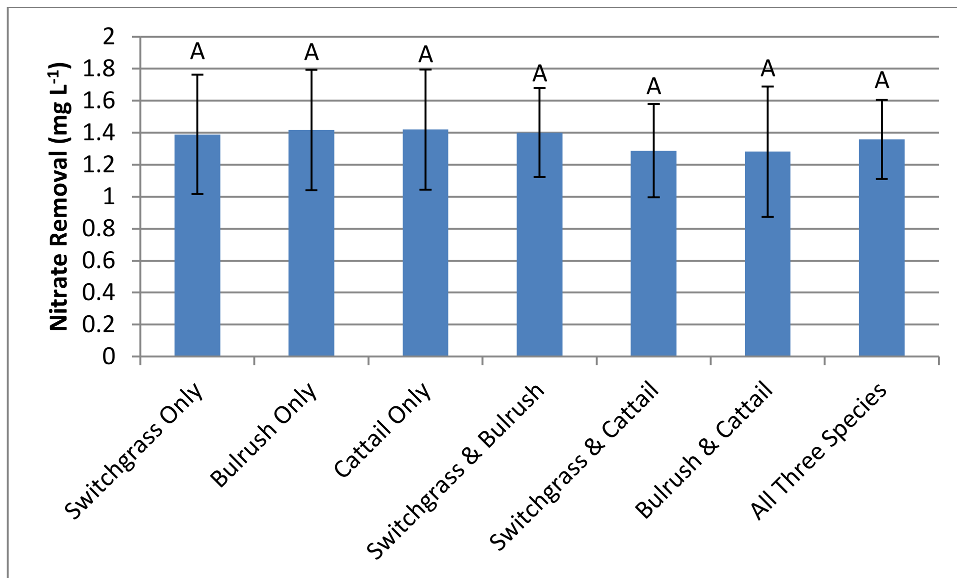

Figure 5-3: Nitrate Removal by Microcosm Combination, Phase II.
Error bars are standard deviation based on three trials. No plant combination had a significant advantage over the others at reducing nitrate concentration ($p=0.612$). Groups with different letters are statistically different.

5.1.6 Nitrogen and Carbon Budget within Microcosms

A variety of plant and soil samples were collected from the microcosms to understand where the input nitrate was stored. Above ground biomass and subsurface core samples were collected for each microcosm (Table 5-1, Table 5-2).

5.1.7 Aboveground Biomass: Nitrogen Levels

Comparing single plant species microcosms, switchgrass contained lower nitrogen mass compared to bulrush and cattail ($p<0.05$). However, the presence of switchgrass in plant combinations did not result in lower nitrate removal compared to microcosms without switchgrass ($p=0.138$, Table 5-1).

5.1.8 Aboveground Biomass: Carbon Content

Testing for carbon provides biofuel capability data. Carbon content for each microcosm's harvestable biomass was calculated by multiplying percent C by the

dried above ground plant mass recorded for each microcosm and dividing by the surface area of the microcosm (Figure 5-7). The averages of the data from four replicates of each condition were calculated (Table 5-1).

Among single plant species microcosms, the presence of bulrush exhibited an increased carbon mass per unit area ($p<0.05$). The presence of cattail also had a positive impact on carbon mass per unit area ($p<0.05$). The presence of switchgrass did not influence the carbon mass per unit area ($p=0.127$, Table 5-1, Figure 5-4).

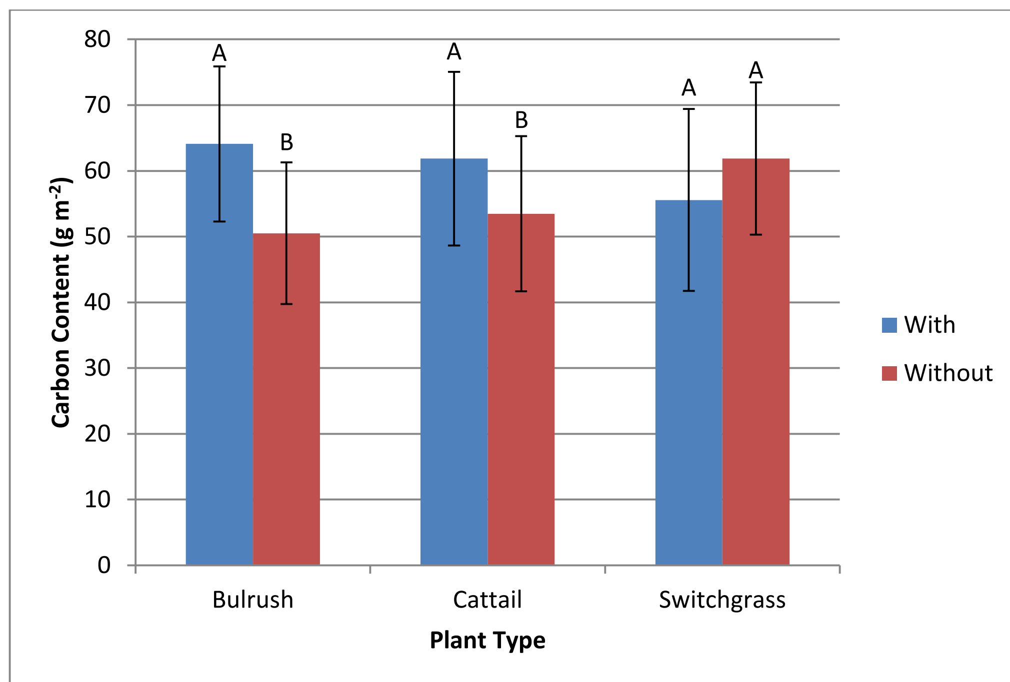

Figure 5-4: Carbon Content in Grams per Unit Area by Plant Species, Phase II. Error bars are standard deviation based on four replicates. Bulrush microcosms (64.09 g-C m^{-2}) had greater carbon mass than non-bulrush microcosms (50.51 g-C m^{-2}). Cattail (61.87 g-C m^{-2}) also had greater mass than non-cattail microcosms (53.48 g-C m^{-2}). Switchgrass did not have a significant difference mass-wise (55.57 g-C m^{-2} versus 61.87 g-C m^{-2}). Groups with different letters are statistically different, only within the same plant species.

5.1.9 Aboveground Biomass: Percent Carbon Content

When analyzing the effect of a single plant species on percent carbon, it was found that microcosms with bulrush contained a lower percentage of carbon than microcosms without bulrush ($p<0.05$). The presence of cattail did not significantly

affect percent carbon (p=0.679). The presence of switchgrass increased overall percent carbon in microcosms ($p<0.05$, Table 5-1, Figure 5-5).

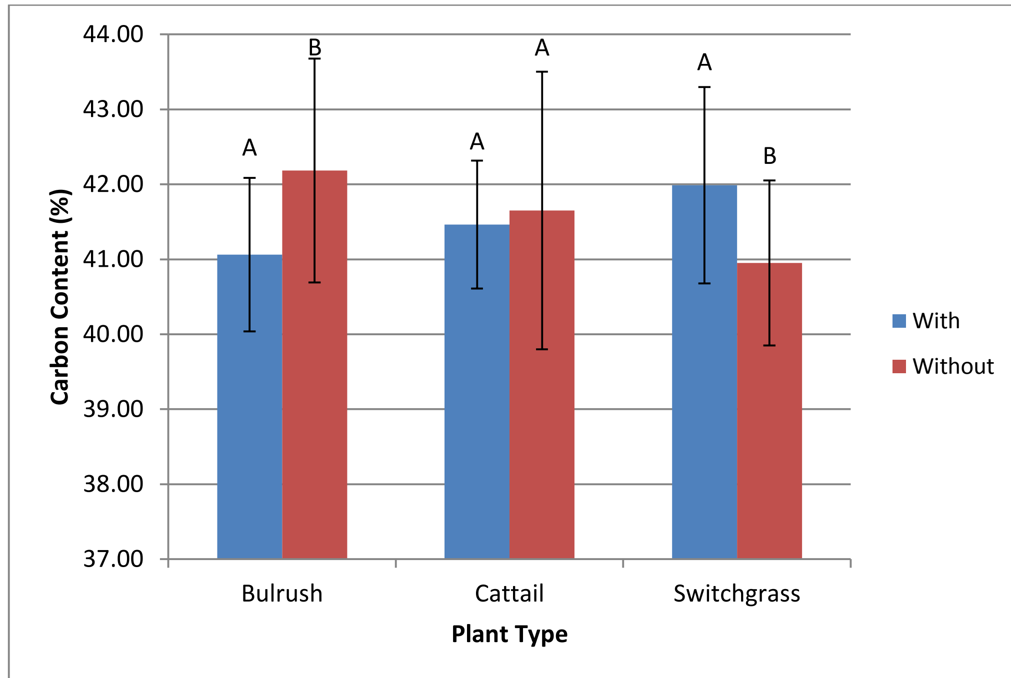

Figure 5-5: Percent Carbon Content by Plant Species, Phase II.
Error bars are standard deviation based on four replicates. Non-bulrush microcosms (41.29%) had greater carbon percent than bulrush microcosms (41.06%). Cattail (41.46%) did not have a significant difference from than non-cattail microcosms (41.65%). Switchgrass microcosms (41.99%) had greater carbon percent than non-switchgrass microcosms (40.95%). Groups with different letters are statistically different, only within the same plant species.

When comparing all plant combinations, switchgrass by itself had the highest percent carbon ($p<0.05$, Figure 5-6). In terms of microcosm weight, switchgrass had the lowest plant mass ($p<0.05$, Figure 5-7).

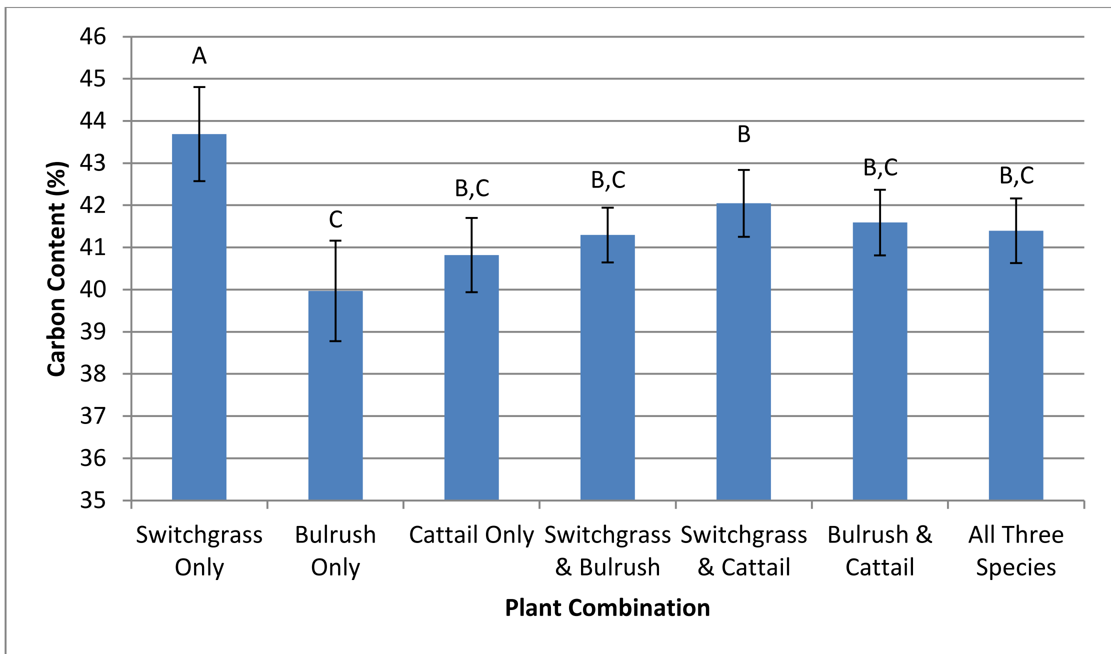

Figure 5-6: Percent Carbon Content by Microcosm Combination, Phase II.
Error bars are standard deviation based on four replicates. Switchgrass only (43.69%) has significantly greater percent content than other combinations. Groups with different letters are statistically different.

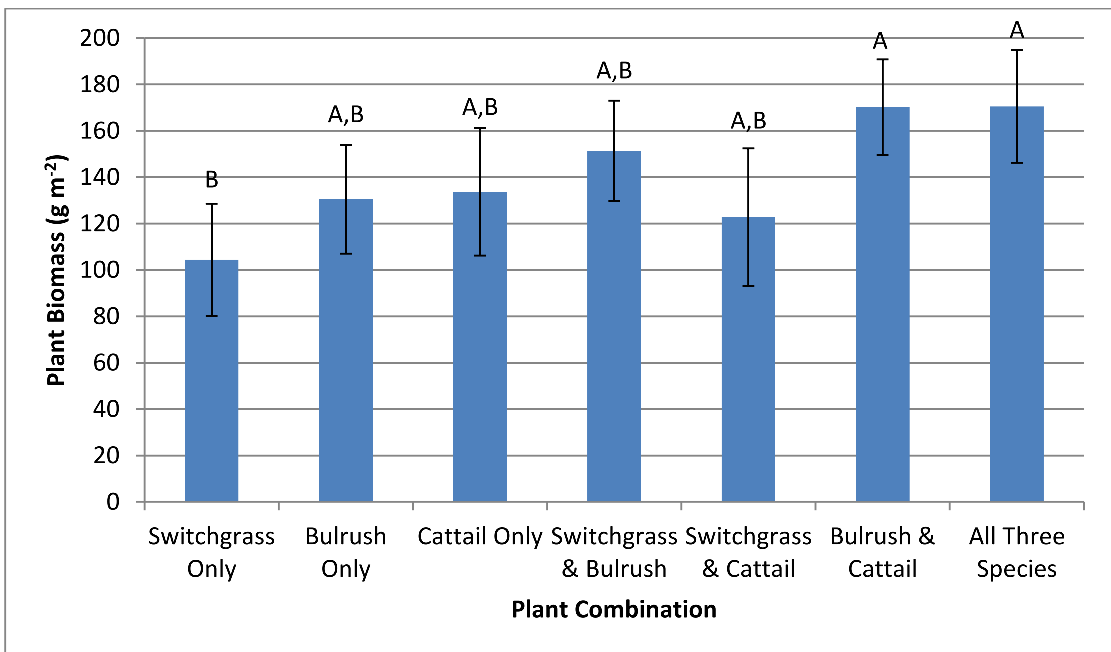

Figure 5-7: Plant Biomass Dry Weight in Grams per Unit Area by Microcosm Combination, Phase II.
Error bars are standard deviation based on four replicates. Switchgrass only microcosms (104.35 g m^{-2}) had the lowest above ground biomass of all combinations. Groups with different letters are statistically different.

Table 5-1: Nitrogen and Carbon Content in Above Ground Biomass, Phase II.
Averages are reported based on four replicates over four trials. Nitrogen content of plants was calculated by multiplying percent N analysis results by the total dry above ground plant mass for each microcosm. Carbon content was calculated by multiplying percent C analysis by total above ground plant mass divided by area of the microcosm. ('OA' stands for sawdust organic amendment)

Above Ground Biomass	Switchgrass + OA	Bulrush + OA	Cattail + OA	Switchgrass + Bulrush + OA	Bulrush + Cattail + OA	Switchgrass + Cattail + OA	Switchgrass + Bulrush + Cattail + OA
%N	0.372	0.565	0.609	0.575	0.571	0.535	0.548
Total N (g)	0.029	0.053	0.060	0.063	0.071	0.048	0.068
%C	43.69	39.97	40.82	41.30	41.59	42.05	41.40
Total C ($g\ m^{-2}$)	45.50	52.36	54.37	62.58	70.68	51.67	70.75

Table 5-2: Nitrogen and Carbon Content of Subsurface Cores, Phase II.
Averages are reported based on four replicates. Subsurface core nitrogen and carbon content was calculated identically to above ground biomass content. ('OA' stands for sawdust organic amendment)

Subsurface Core	Original Soil	No Plants, No OA	No Plants + OA	Switchgrass + OA	Bulrush + OA	Cattail + OA	Switchgrass + Bulrush + OA	Bulrush + Cattail + OA	Switchgrass + Cattail + OA	Switchgrass + Bulrush + Cattail + OA
%N	0.046	0.0340	0.038	0.042	0.045	0.051	0.040	0.041	0.035	0.039
Total N (g)	3.516	2.868	2.835	3.259	3.568	3.952	3.058	3.080	2.623	2.960
%C	4.127	2.262	2.345	2.400	2.451	2.956	2.211	2.659	1.931	2.106
Total C ($kg\ m^{-2}$)	4.295	2.231	2.389	2.577	2.651	3.142	2.297	2.705	1.997	2.197

5.1.10 Subsurface Cores: Nitrogen and Carbon Levels

To supplement the above ground biomass measurements and complete a nitrogen balance for each testing condition, the microcosms' root and soil were tested for percent nitrogen and carbon. The nitrogen content of mixed root and soil was calculated by multiplying percent N results by the total dry mass for each microcosm. There was no difference in percent N of subsurface biomass between all experimental groups ($p=0.683$, Table 5-2). The presence of sawdust did not result in significantly greater nitrogen removal compared to absence of sawdust ($p=0.456$, Table 5-2).

The carbon content of soil and roots was calculated by multiplying percent C by the total dry mass of each microcosm core. The averages of the data from four replicates of each condition are shown in Table 5-2. There was no difference in carbon content between the experimental groups ($p=0.635$, Table 5-2).

5.1.11 Denitrification

Over the four week trial period, $N_{denitrification}$ was significantly less than $N_{soil(end)}$ for all treatments ($p<0.05$, Table 5-3). Small and/or negative values for $N_{denitrification}$ indicate low denitrification activity.

Mass retention was calculated as the difference between $N_{influent}$ and $N_{effluent}$ as used in equations E-1 and E-2. Total N in the input and output is expressed as a mass loading rate per unit area, using the area of the microcosm substrate surface (Table 5-4).

Table 5-3: Nitrogen Balance for Different Plant Combinations, Phase II.
Numbers represent average value of four microcosms per experimental group in grams N. *= no above ground biomass from lack of plants

	No Plants, No OA	No Plants + OA	Switchgrass + OA	Bulrush + OA	Cattail + OA	Switchgrass + Bulrush + OA	Bulrush + Cattail + OA	Switchgrass + Cattail + OA	Switchgrass + Bulrush + Cattail + OA
$N_{influent}$	0.0253	0.0255	0.0265	0.0259	0.0259	0.0277	0.0268	0.0259	0.0267
$N_{soil(start)}$	3.3358	3.4516	3.6601	3.6601	3.5906	3.4979	3.4284	3.4979	3.5211
$N_{effluent}$	0.0006	0.0005	0.0006	0.0005	0.0006	0.0005	0.0017	0.0006	0.0005
$N_{biomass}$	0*	0*	0.0286	0.0532	0.0598	0.0625	0.0712	0.0479	0.0676
$N_{soil(end)}$	2.8675	2.8350	3.2591	3.5678	3.9523	3.0583	3.0803	2.6230	2.9600
$N_{denitrifcation}$	0.4929	0.6416	0.3983	0.0645	-0.3963	0.4042	0.3019	0.8523	0.5202

Table 5-4: Mass Retention Calculation for Different Plant Combinations, Phase II.
Numbers represent average value of four microcosms per experimental group in units of g m^{-2} yr^{-1}.

	No Plants, No OA	No Plants + OA	Switchgrass + OA	Bulrush + OA	Cattail + OA	Switchgrass + Bulrush + OA	Bulrush + Cattail + OA	Switchgrass + Cattail + OA	Switchgrass + Bulrush + Cattail + OA
Input	4.5260	4.5656	4.7409	4.6262	4.6262	4.9425	4.7869	4.6262	4.7869
Output	0.1157	0.0916	0.1017	0.0903	0.1008	0.0844	0.3029	0.1061	0.0952
Mass Retention	4.4103	4.4740	4.6392	4.5359	4.5254	4.8582	4.4840	4.5200	4.6917
Retention %	97.43	98.00	97.86	98.02	97.83	98.30	93.67	97.73	98.01

Table 5-5: Carbon Nitrogen Ratio of Soil by Microcosm Combination, Phase II.

	No Plants, No OA	No Plants + OA	Switchgrass + OA	Bulrush + OA	Cattail + OA	Switchgrass + Bulrush + OA	Bulrush + Cattail + OA	Switchgrass + Cattail + OA	Switchgrass + Bulrush + Cattail + OA
C:N	56.341	60.928	57.987	54.843	54.843	55.193	66.944	55.135	53.732

5.1.12 Soil Carbon Effects and Carbon Nitrogen Ratio

Soil carbon content did not affect nitrate removal (Figure 5-8).

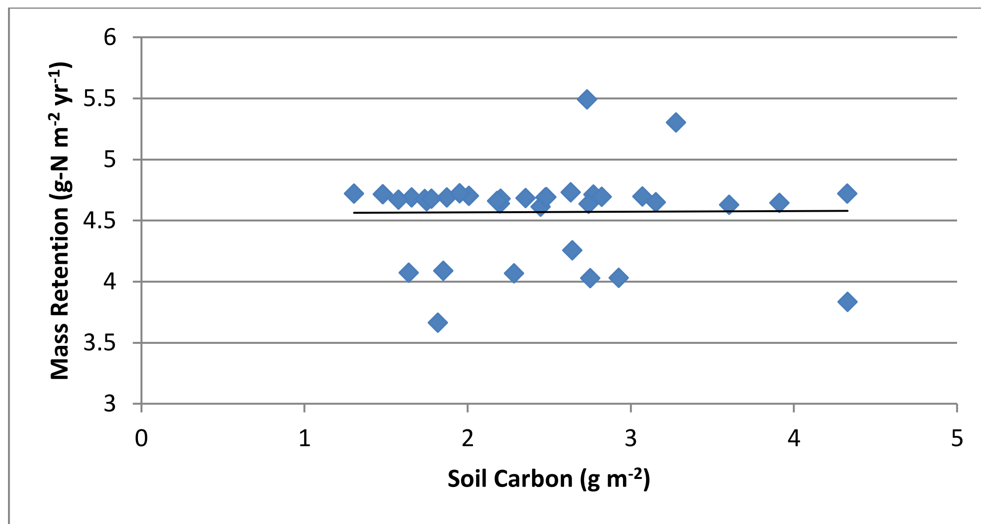

Figure 5-8: Scatterplot of Nitrate Removal and Soil Carbon Content, Phase II. Trendline is line of best fit.

The average carbon-to-nitrogen ratio for all microcosm soil was 57.748. No microcosm environment had a significantly higher carbon-nitrogen ratio than the others (p=0.516, Table 5-5).

5.2 Phase II Discussion

5.2.1 Explanation of Statistically Insignificant Nitrate Concentration Difference Between Experimental Groups

On average, microcosms containing plants did not show significantly greater nitrate removal than microcosms containing no plants. In addition, among microcosms containing plants, no single plant species or species combination showed significantly greater nitrate removal over other groups. Phase I data showed that the addition of sawdust to microcosms containing plants resulted in significantly greater nitrate removal compared to plant microcosms without sawdust. In contrast, Phase II data showed no significant difference in nitrate removal between experimental

treatments with and without sawdust. It is therefore possible that adding the sawdust to each microcosm allowed for a high level of nitrate concentration reduction in all of our microcosms, masking the differences in nitrate removal caused by the presence of various plant species. This result from Phase II suggests that nitrate removal by sawdust was not fully responsible for statistical insignificance observed across different plant combinations; instead, nitrogen may have been removed by soil uptake or even biomass uptake.

To compute the nitrogen balance, equation E-5 was used. Two assumptions were made to limit the nitrogen balance to the variables in equation E-5. During each trial period, ammonium from input ammonium nitrate solution could have been lost to vaporization or converted to nitrate. However, it is assumed that volatilization of nitrogen within the microcosm was insignificant due to a moderately acidic soil pH measurement in all microcosms; the average soil pH was measured at 6.24 ± 0.45. Volatilization, nitrogen conversion to free ammonia (NH_3), is directly affected by soil pH (Rehm, 2010). Loss due to volatilization is higher when soil pH is greater than 7.4 (calcareous soils). The potential for loss of nitrate due to volatilization is much less in soils with an acidic or neutral pH due to the reduced availability of hydrogen ions (H^+). Similarly, NH_3 conversion to NO_3 was expected to be limited due to the anaerobic conditions present within the saturated microcosms. The average dissolved oxygen (DO) levels recorded in the microcosms were 1.94 ± 0.75 ppm at room temperature (23.13^{o}C ± 9.3%). Outliers in recorded values shifted the mean dissolved oxygen to a greater value, and contributed to a larger standard deviation. The two largest and smallest extrema within the data set were removed and the average dissolved oxygen levels were recorded as 1.84 ± 0.40 ppm at room temperature

(21.81°C ± 4.9%). These dissolved oxygen levels were low enough to limit the oxidation reaction which generates nitrate from ammonia (Princic, et al. 1988).

Phase II data did not show statistical significant differences in nitrogen mass removed by denitrification ($N_{denitrification}$) or nitrogen removal between plant combinations, but it did confirm nitrate removal trends noted within literature. Low nitrogen loading rate (<10 g-N m^{-2} yr^{-1}) corresponds with greater than 80 percent mass retention (Mitsch, et al. 2005). Observations from Louisiana's Caernarvon River division wetland off the Mississippi River demonstrated how low nitrogen loading rate leads to large mass retention percentages. In Mitsch's surveys from 1992 and 1993, nitrogen loading rate was measured at 5.60 and 7.30 g-N m^{-2} yr^{-1} respectively. Nitrogen outflow for those years was recorded as 0.17 and 1.54 g-N m^{-2} yr^{-1}, for a mass retention of 97 and 79 percent, respectively (Mitsch, et al. 2005). Nitrate loading rates and mass retention percentages from Phase II microcosms exhibited similar nitrogen dynamics to those noted in the Caernarvon wetlands. Average nitrate loading rate from Phase II was 4.6919 g-N m^{-2} yr^{-1} and average outflow rates ranged from 0.0844 to 0.3029 g-N m^{-2} yr^{-1}, resulting in mass retention percentages between 93.67 and 98.30 percent. In future studies, a higher nitrate loading rate can be used to yield lower nitrate mass retention percentages, thereby allowing detection of differences in nitrate removal between plant species combinations.

Mitsch's work along the Mississippi River yielded a relationship between nitrogen inflow and percent removal, demonstrating that at elevated loading rates, not all nitrogen can be retained within the wetland. In both the Caernarvon wetlands and the Olentangy River experimental wetlands, Mitsch documented that at 60 g-N m^{-2} yr^{-1} or more, nitrogen percent removal begins to level off. The same data suggests that

more than 80 percent of nitrogen is retained when inflow levels fall below 10 g-N m^{-2} yr^{-1}, which is the range our microcosms were. A microcosm study with a broader range of nitrate-nitrogen inflow levels than were used in this experiment would be necessary to generate similar results. This wider range of nitrogen inflow levels would likely result in a broader range of nitrogen retention. With a greater range in nitrogen retention, more significant differences between experimental treatments may be noted.

5.2.2 Effect of Nutrient Loading on Nitrogen Retention

Results for nitrogen uptake by plants were consistent with studies showing superior nitrate removal potential for both bulrush and cattail. In mesocosm experiments at Washington State University, cattail and bulrush were subjected to 13 L of 19 mg-N L^{-1} water at the beginning of a fourteen-day period. By the fourteenth day, approximately 1 mg-N $L^{-1}$was measured in the bulrush mesocosm, and less than 0.5 mg-N L^{-1} in the cattail treatment (Gebremariam & Beutel, 2008). These results show that at these levels of nitrogen, both cattail and bulrush can retain most of the nitrogen. In another study, nitrogen uptake by a variety of plants grown in quartz sandstone gravel, to eliminate confounding sequestration of nitrogen in the soil, was documented. One experiment introduced 16.4 L of 48 mg L^{-1} NO_3-N for a total nitrogen load of 70.3 mg-N (approximately 18.3 g-N m^{-2} yr^{-1}. After 120 hours, NO_3-N concentrations were measured at nearly zero mg L^{-1} for the bulrush and cattail (*Typha latifolia*). Similarly designed experiments demonstrated NH_4-N and NO_3-N concentrations were eliminated in 200 and 100 hours, respectively (Zhu & Sikora, 1995).

There is a lack of previous studies showing high nitrate removal potential of switchgrass (*Panicum virgatum*). Switchgrass is known for its high bioenergy potential, which was reflected in our results for percent carbon content, but not for carbon content in mass per unit area (Figure 5-5, Figure 5-6).

Mass retention results, supported by Gebremariam and Bechtel (2008) and Zhu and Sikora (1995), for all experimental groups suggested that a majority of the nitrogen was removed within our microcosms (Table 5-4). The eight-day trial periods were shorter than the fourteen-day Gebremariam and Bechtel (2008) experiment and equal to the eight-day Zhu and Sikora (1995) experiment. Additionally, a smaller amount of total nitrogen was supplied as compared to the other studies. However, all three experiments (Gebremariam and Bechtel, 2008; Zhu & Sikora, 1995) noted minimal residual total nitrogen in the outflow water. Even after the four consecutive one-week trials involving nitrogen loading and microcosm flushing between trials, the retention rates did not seem to plateau at some maximum nitrogen saturation threshold. By the end of each trial, the mass of output nitrogen was often no more than 10% the input mass across all treatments, indicating that over 90% of the nitrogen was retained or removed as a gas in or from the microcosms. In addition, there was no evidence of nitrogen saturation effects, in which the microcosms would not be able to retain any more nitrogen. The experimental design did not allow the group to identify during trials how the nitrogen was removed from the flow or where it was stored in the microcosms until above ground biomass and subsurface samples were tested for nitrogen content.

5.2.3 Aboveground Biomass Nutrient Accumulation

Analysis of the aboveground biomass in single species microcosms showed that switchgrass plant tissue contained lower nitrogen levels than the cattail or bulrush ($p<0.05$, Table 5-1). However, plant combinations with switchgrass contained similar plant tissue nitrogen levels compared to combinations without switchgrass (Table 5-1). Assuming nitrogen content in the original plants started out equal across all three species, it seems switchgrass is not as effective at taking up nitrogen in its above ground tissues as cattail or bulrush. Alternatively, it could mean that switchgrass' use of nitrogen was more efficient, perhaps due to its use of C4 Photosynthesis rather than the C3 photosynthesis used by cattail and soft-stem bulrush. However, without knowing the plant tissues' N concentration before the trial began, there is no guarantee that all three species began at a similar percent nitrogen initial value. Thus, since switchgrass took up significantly less nitrogen than bulrush and cattail, it may have facilitated the removal of the greatest percentage of nitrogen despite starting with very small percent nitrogen.

The total mass of nitrogen retained in aboveground bulrush and cattail tissue was nearly twice the total mass of nitrogen in switchgrass, partially because the plants were larger. Despite this result, microcosms with multiple plant species which included switchgrass did not reduce nitrate concentration less than those which did not include switchgrass. Nitrogen not taken up by switchgrass was still retained by cattail and bulrush within the same microcosm. Switchgrass may have taken up more nitrogen if a higher mass of nitrogen was put into the microcosms; the cattail and bulrush plants likely out-competed the switchgrass for available nitrogen.

Percent carbon testing provided a means of quantifying the viability of the plants for biofuels. Plants with high carbon mass content could be quality biofuel

crops. Switchgrass, cattail, and bulrush were selected for use in this investigation because they were wetland plants shown to perform well in treatment wetlands and as biofuel crops (Zhang et al., 2010; Dale, 2010; Ruhland, 2011). In quantifying the above ground harvestable biomass for each test condition, no single plant type had significantly greater carbon content than another (Figure 5-6). However, single plant microcosms had lower carbon content than microcosms with combinations of plant species ($p<0.05$) (Table 5-1), suggesting that species diversity may support greater growth. However, while switchgrass had the greatest aboveground percent carbon, its aboveground biomass was less than either bulrush or cattail.

5.2.4 Subsurface Nutrient Accumulation

Soil core sample analysis revealed that total subsurface nitrogen did not differ across the treatments, suggesting that plants did not have an effect on soil nitrogen. Contrary to Phase I results, the presence of the sawdust did not result in significantly greater nitrogen removal when compared to treatments without sawdust.

No relationship was found between mass retention of nitrogen and soil carbon content, evidenced by a near zero slope (Figure 5-8). The insignificant differences between nitrogen removal in microcosms with and without sawdust and the lack of noticeable nitrogen uptake by the soil demonstrated that nitrogen removal by belowground biomass and the soil did not play a role in nitrate removal of the system.

Subsurface carbon values were used to estimate denitrification at the microbial level, as denitrification rate is affected by available carbon. While not all carbon sources are equally consumed by denitrifying microbes, elevated amounts of carbon are associated with elevated denitrification rates (deCatanzaro & Beauchamp, 1985). Although other processes can consume carbon, the decrease in soil carbon content

after the experiment suggests that denitrification might have occurred. Between one half and one quarter of the total carbon measured in the original soil was unaccounted for in the subsurface samples after testing (Table 5-2). Since the rate of carbon consumption is related to denitrification, loss of carbon between the original soil and final experimental soil suggests microbial denitrification occurred at a high rate to consume between 100 and 200 grams of carbon (Table 5-2). Realistically, the carbon initially in the soil may not have been entirely consumed by microbes during denitrification, as carbon could have also leached out of the soil into the effluent water (Davis, Childers, & Noe, 2006). Unfortunately, the team did not measure the carbon levels in the effluent to confirm this. Statistically insignificant differences in carbon content between all experimental groups in the post-trial sampling suggest equivalent fates of the initial soil carbon – whether consumed by denitrifiers, leeched into the effluent, or some combination thereof.

Chapter 6: Conclusion and Future Direction

6.1 Conclusion

6.1.1 Summary of experiment

- Purpose: To test wetland plants and organic amendments for their combined ability to remove nitrate from water in a wetland microcosm.
- Phase I of this experiment determined whether the organic amendment (glucose, hay, or sawdust) affected nitrate removal.
- Phase II of this experiment investigated whether combinations of wetland plant species affected nitrate removal.
- The microcosms were designed to be similar to the conditions of a natural wetland. To achieve this end, the team used a soil-sand mixture similar to that found in a wetland on the Tuckahoe Creek on the Eastern Shore of Maryland, and inoculated the microcosms with a wetland soil sample from the Tuckahoe.

6.1.2 Results

- In Phase I, it was found that adding sawdust to the microcosms removed more nitrate than adding either glucose or hay.
- In Phase II, it was found that our microcosms could remove up to 98% of the nitrogen, but there were no differences in nitrate concentration reduction due to plant species.
- The high nitrogen mass retention was most likely affected by the low nitrate loading (5 mg L^{-1} at 4.6919 g-N m^{-2} yr^{-1}). The low nitrate concentration reflected values found in the Tuckahoe Creek in the spring

and fall. This signifies that natural wetlands along the Tuckahoe Creek are likely important sinks for nitrogen. However, the low concentration reduced the ability to detect differences among plant species. Treatment wetlands are known to remove nitrate at rates much higher than we tested. Future studies should use a wider range of nitrate input concentration to help determine whether plant species have an effect on removal rates.

6.1.3 Results for Society

- This project confirmed that wetlands do indeed remove nitrate from water. This supports a considerable amount of research on artificial wetlands as nitrate pollution treatment.
- Furthermore, the findings support that at low nitrate loading rates, the percentage of mass retained is very high (up to 98%).
- Microcosms with a diverse mix of plant species yielded higher carbon content than single species microcosms, suggesting that species diversity increases carbon storage of wetlands. In addition, this suggests that more diverse wetlands have increased potential as sources of cellulosic ethanol production.

6.2 Future Directions

There are a few aspects that could be improved upon in this project that will increase the depth, reliability, and relevance of the results. The following list describes 12 methods that can be used as future directions for our study.

6.2.1 Total Added Nitrate Volume versus Concentration

A potential future direction would be to perform a corrected version of the original experiment. One of the most important corrections to make is to account for

the continual flow of nitrate water instead of just adding an initial concentration. When the project was originally conducted, an amount of nitrate was added to create an initial nitrate concentration equal to that in the Tuckahoe Creek. However, it was determined that the nitrate should have been added on a g-N $m^{-2}yr^{-1}$ basis instead of a single concentrated volume in g-N L^{-1}, which was insufficient to replicate conditions in the Tuckahoe. This mistake was not caught during Phase I because the phases took place at different times of year. Phase I took place during the fall and part of winter while Phase II took place during the spring. The plants from Phase I were dormant for the majority of the phase, which likely lead to lower nitrate removal by the plants. Results from Phase I showed significant differences in nitrate uptake between various amendments. These significant results led the team to believe that this amount of nitrate would also be sufficient for Phase II. During Phase II, the plants reduced a higher concentration of nitrate, which may have in part been due to an increased amount of plant growth as compared to hibernation in Phase I. The insufficient nitrogen load was not realized until the end of all of the trials in Phase II because the samples from all trials were sent for testing at the end of the fourth trial.

6.2.2 Nitrate added at one time versus added over time

If the correct amount of nitrate were to be added, an experimenter could use two different methods for adding them. One could add a portion of the nitrate daily, or one could add all of the nitrate on the first day. In adding a portion of the nitrate daily, the experimenter would replicate a constant flow of nitrate from groundwater. In adding all of the nitrate in one day, the experimenter would be replicating a flood of surface water flowing from nearby farms. By testing both of these scenarios, an experimenter could discover how well wetlands perform when experiencing shocks of

nitrate addition versus a constant stream of nitrate. This, in turn, would help to elucidate how artificial wetlands are affected by increased water flow caused by more impervious surfaces, such as roofs, sidewalks, and roads. By comparing the two methods, the experimenter would be able to aid the design process of artificial wetlands by estimating the optimal water flow rate through the artificial wetland.

6.2.3 Variation of water levels

Another possible future direction for our project would be to vary the water levels in different microcosms. This would test the impact of the anoxic environment provided by the water cover. By conducting this experiment, one could also test the capability of different plants to remove nitrate at different water levels. This would also aid in the design of an artificial wetland. Potential problems with this technique would include observing how to keep the amount of nitrate constant even as the water level changes.

6.2.4 Macrocosm containing many specimens of plants

In the initial experimental design, the team had planned to do a third phase, which would incorporate all that we had learned from Phase I and II on a larger scale. This future direction would allow the experimenters to better understand how an artificial wetland planted in Maryland would remove nitrate. This would remove several innate problems with the microcosm design. First, it would mediate the prevalence of hard edges to the microcosm. The sides of the buckets may act as a foundation that bacteria can use for stability and growth. Second, the size of a macrocosm would minimize deviations in the data caused by sunlight and soil differences. Third, our large macrocosm was planned to have water running through

it to simulate a constant water flow environment. Having running nitrate water would allow us to better emulate conditions in the field.

6.2.5 Model of nitrate removal, given geographical data from region

This future direction, in conjunction with creating a larger microcosm, would allow a team of experimenters to predict the impact of a specifically located treatment wetland on the agricultural runoff entering the Chesapeake Bay. Given data about algal bloom responsiveness to different nitrate concentrations, the experimenters could even predict how much a system of such wetlands could reduce algal blooms. This data would allow us to make predictions about areas outside of the section of Tuckahoe that we had been researching. We would want to expand our scope to other areas since the Chesapeake Bay Watershed is so diverse. Without this, our data is only pertinent to a small section of the Chesapeake Bay Watershed.

6.2.6 Phosphate Experiments

A potential future direction, pending future research, would be to conduct the same experiment, but adding differing amounts of phosphates instead of nitrate. Phosphates are also a large part of agricultural fertilizer, and it would be interesting to see how much they would change in conjunction with nitrate in a treatment wetland.

6.2.7 Varying Soil Acidity

Another future direction would be to change the acidity of soil, either by adding lime to increase pH or by adding sulfur to decrease pH. By testing nitrate concentration reduction in different acidities, an experimenter would expose the effect that soil pH has on the removal of nitrate. Since bacteria play a critical role in

nitrogen uptake, the tests would focus on finding the optimum pH that would allow the bacteria to thrive. This would potentially introduce ways for an artificial wetland to be treated so that it is even more effective at removing nitrate from wastewater.

6.2.8 Testing with different plants

This future direction would allow one to test other plants for their ability to assist denitrification processes. The Chesapeake Bay is a very large and diverse environment, which includes over 2,700 different species of plants (Chesapeake Bay Program, 2012). Tests could be performed on plants that have been introduced into the Chesapeake Bay, such as phragmites. Phragmites is an invasive species, meaning that it will crowd out and occupy land previously covered by other species. However, since phragmites has become very common in the Chesapeake Bay, one could study to see if the species has any benefits, such as helping with the uptake of nitrogen. This study would have the potential for finding a wetland plant that is even more effective than cattail, soft-stem bulrush, or switchgrass.

6.2.9 Field Testing

A future direction would be to actually install a testing wetland on the shores of Tuckahoe Creek. Although much can be learned through microcosm testing, an actual wetland would allow the experimenter to observe what happens when the wetland is installed and then compare the actual results to the model results. This would identify problems with the testing protocol, while providing data to support the implementation of treatment wetlands. To increase the applicability of our results, we would have liked to install field tests in different areas of the Tuckahoe.

6.2.10 Variation of Sand/Soil Proportion

This future direction would allow the experimenters to investigate how different soil compositions affect nitrate removal. For Phases I and II, the team tried to emulate soil conditions seen at the Tuckahoe; however, there is a range of soil to sand ratios throughout the Creek. Because the population and growth rates of denitrifying bacteria are determined in large part by the composition of the soil surrounding them, altering this composition will allow us to determine the optimal ratio of soil to sand for bacteria growth. Also, by having different compositions of soil and sand, we would be able to increase the range of applicability to different sections of the river.

6.2.11 Variation of soil levels

Research and experimental results showed that soil by itself has the ability to retain nitrate. Our research shows that although there was a significant difference in nitrate uptake between microcosms with plants and without plants, nitrate concentration still decreased in microcosms with only soil. This future direction would explore how varying the amounts of soil affects nitrogen uptake. By varying the levels of soil, one could see how the plants being researched would react to different gradients in the soil. More soil could allow more bacteria to live in the microcosms, leading to more nitrate being denitrified.

Appendices for

INTERACTIVE EFFECTS OF PLANT SPECIES AND ORGANIC CARBON ON NITRATE REMOVAL IN CHESAPEAKE BAY TREATMENT WETLANDS

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Gemstone Senior Thesis

March 2013

This document includes:

Appendix A: Budgets

Appendix B: Supplemental Figures

Appendix C: Phase I Data/Charts

Appendix D: Phase II Data/Charts

Appendix E: Protocols

Appendix A – Budget

Spring 2011 – Sea Grant

Potting soil - $200
Seedling cattails - ($1+/plant * 60) - $100
Seedling soft-stem bulrush - ($1+/plant * 60) - $100
Seedling switchgrass - ($1+/plant * 60) - $100
Glucose (5 kg) - $80
Nitrate - $50
Fertilizer- $100
Pipettes (200 count 9 inch eye droppers) - $25
15mL conical tubes (1000 count) - $250
Water sample analysis (purchase of Cardy Twin Nitrate Meter) - $800
Miscellaneous Expenses: $60
Transportation to river - $60
Spring 2011 Total: $1925

Fall 2011 – Sea Grant

Potting soil mix of sand and soil (bags purchased individually and mixed by team members) - $325
Cattail plugs – ($1+/plant * 60) - $100
Microcosm supplies
- 5 gallon buckets (40 buckets * $2.50/bucket) - $100
- Gravel (8 bags, 0.5 cubic feet * $3.50/bag) - $28
- Spigots (40 * $5.50/spigot) - $220

Glucose (5 kg) - $80
Nitrate - $200
Travel expenses (to Tuckahoe Creek) - $250
15 mL conical tubes (500 count) - $140
Water sample analysis (300 nitrate replacement pillow packets) - $100
Fall 2011 Total: $1543

Spring 2012 – Sea Grant

Potting soil mix of sand and soil (bags purchased individually and mixed by team members) - $70
Cattail plugs ($0.70/plant * 75) - $52.50
Switchgrass plugs ($0.65/plug x 75) - $48.75
Soft-stem bulrush plugs ($.0.70/plug x 75) - $52.50
Microcosm supplies
- 5 gallon buckets (4 buckets * $2.50/bucket)) - $10
- Spigots (4 * $5.50/spigot) - $22

Glucose (5 kg) - $80
Travel expenses (to Tuckahoe Creek) - $250
15 mL conical tubes (500 count) - $140
Water sample analysis (135 samples * $7/sample + $50 shipping) - $995
Phase II water sample analysis - $995
Spring 2012 Total: $2715.75

Fall 2012 – Sea Grant

Plant tissue analysis (28 samples * $3/sample + sample analysis capsules) - $91
Soil sample analysis (37 samples * $3/sample + sample analysis capsules) - $120.25
Water sample analysis (146 samples * $5/sample) - $730
<u>Fall 2012 Total: $941.25</u>

Keeping Maryland Beautiful Grant

INTECOL International Wetlands Conference Registration ($450/person * 4 presenters) - $1800
Data analysis expenses
    15 mL conical tubes (500 count) - $140
    Water sample analysis (135 samples * $7/sample + $50 shipping) - $995
<u>Keeping Maryland Beautiful Total: $2935</u>

Appendix B – Supplemental Figures

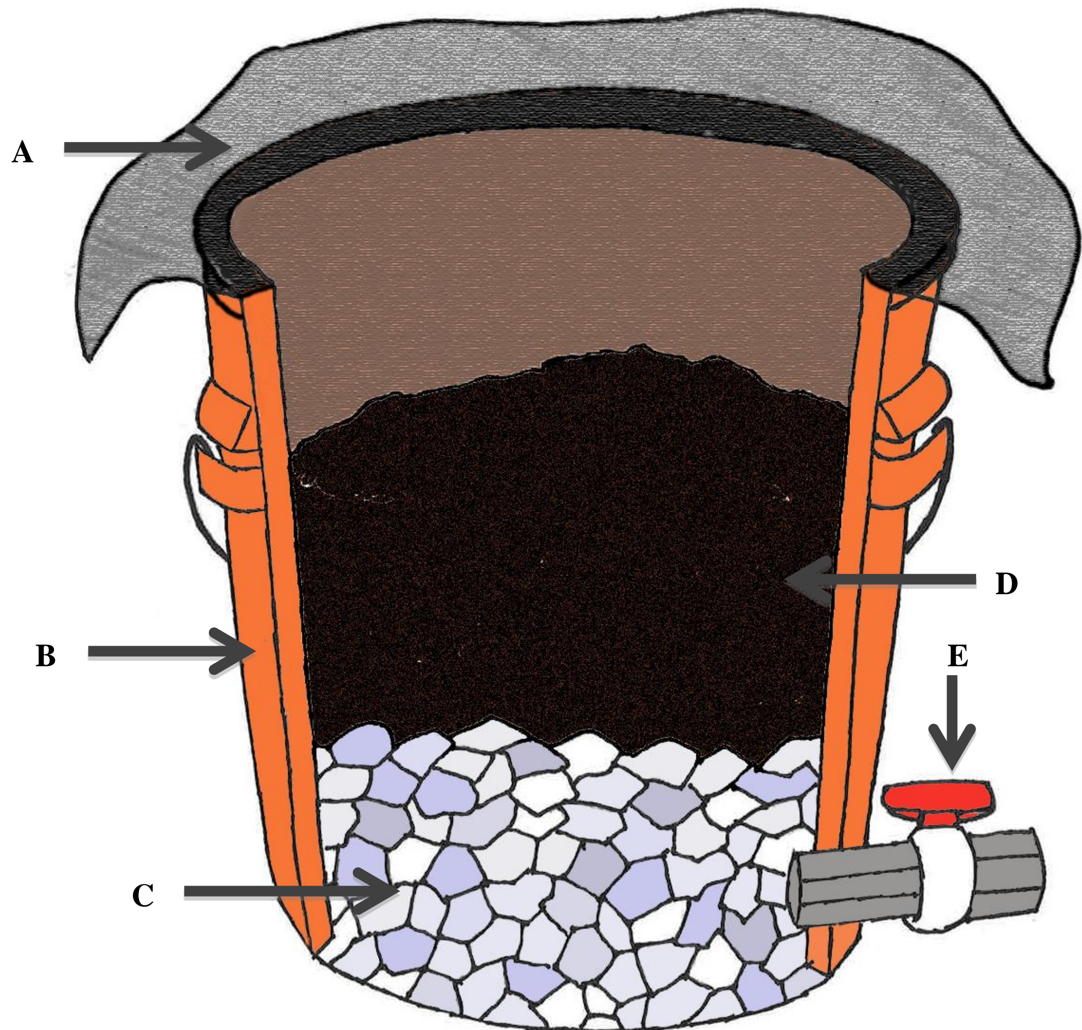
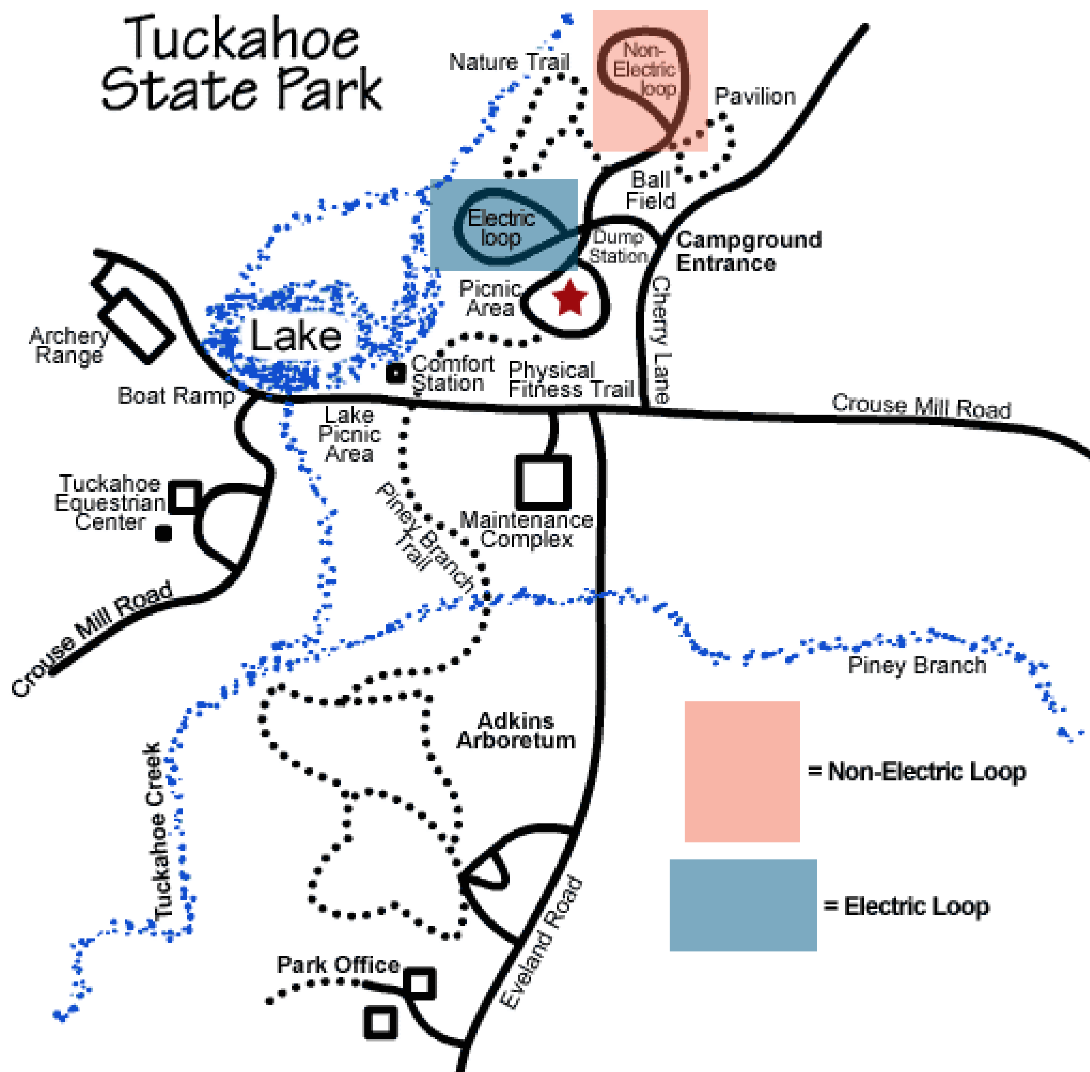


Figure AC1: Final microcosm experimental set-up. The final experimental design used for Phase I and Phase II included (A) mesh landscape fabric (B) microcosm bucket (C) gravel (D) inoculated soil and sand mixture (E) bulkhead fitting.



(DNR, 2013)

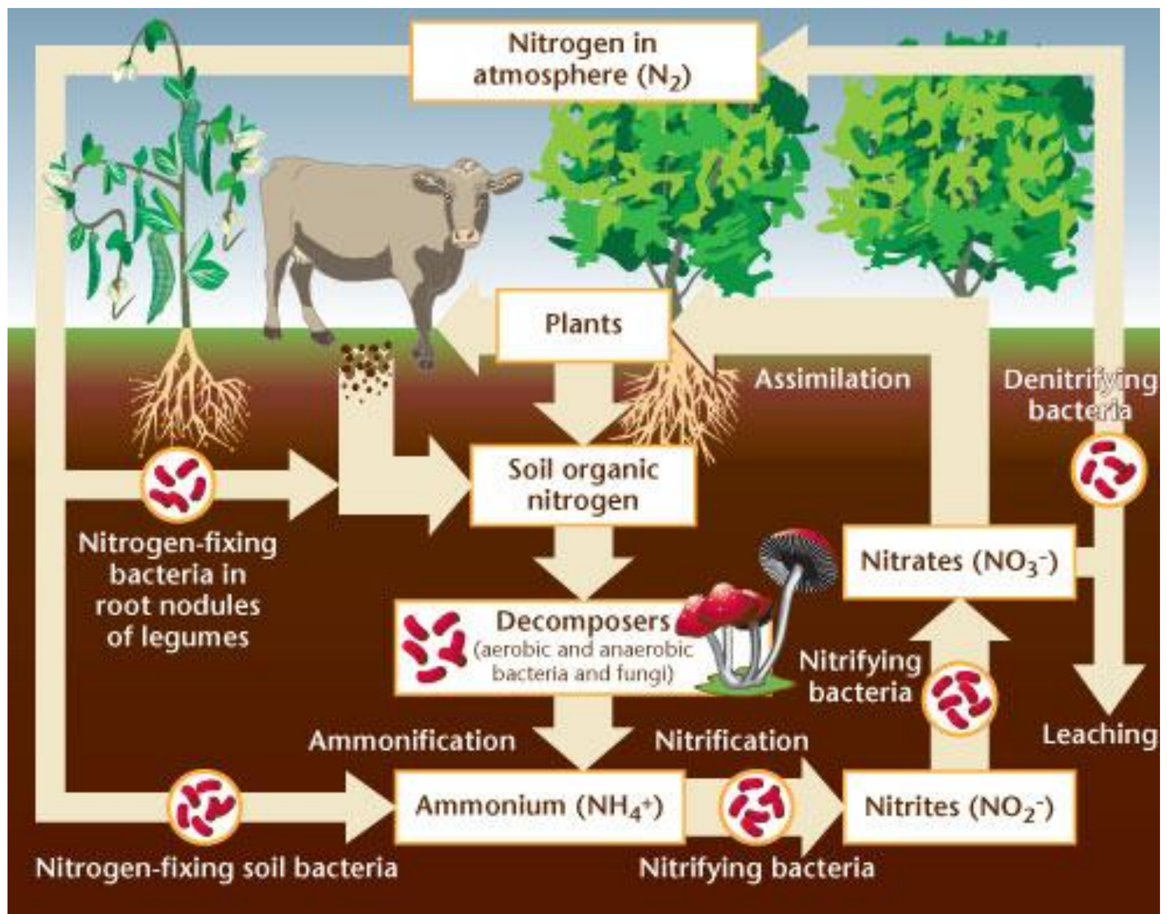
Figure AC2: Schematic map of the Tuckahoe Creek and its location within the Tuckahoe State Park. We chose the Tuckahoe Creek as the site of our water and soil sample collections because it is a major tributary of the Chesapeake Bay and is also relatively accessible. Water samples collected from along the Tuckahoe Creek were used to establish a baseline concentration for the microcosms' initial nitrate input. Soil samples were collected and used to determine the appropriate soil

composition to use in our microcosm experimental design and also to inoculate the soil.

Figure AC3: (A) Original microcosm experimental set-up. The original experimental design was abandoned because of the numerous problems encountered,

including ineffective water collection and drainage, and warping of the bucket. **(B) Arrangements of microcosms in the greenhouse.** Microcosms were lined against the walls of the greenhouse space.

Figure AC5: (A) Addition of hay as an organic amendment. (B) Addition of glucose as an organic amendment. (C) Addition of sawdust as an organic amendment. The cumulative nitrate filtration efficiencies of each organic amendment and cattail combination were tested during Phase I of testing. Sawdust yielded a significantly greater amount of nitrate filtration.



http://cahnrsalumni.wsu.edu/connections/2011/nspire/

Figure AC6: The nitrogen cycle. Our project was intended to test the nitrate filtration efficiencies of plants native to the Chesapeake Bay watershed when coupled with an organic amendment. The Chesapeake Bay receives a heavy input of nitrate pollution, primarily due to agricultural runoff and chemical pollutants. Fertilizers, animal waste, and the combustion of fossil fuel are just some of the sources that contribute directly to the soil organic nitrogen, leading to a nitrate buildup as depicted above.

Appendix C – Phase I

Phase I Amendment Calculations

14 kg (total weight of microcosm) – 6 kg (gravel + bucket) = 8 kg = 8000 g (1 g = 1 mL)

10% * 8000 mL = 800 mL of 0.1 M glucose solution

800 mL * 0.1 M = 0.08 mol glucose = 14.413 g

0.1 mol glucose = 18.016 g glucose

0.1 M glucose = 18.016 g (glucose)/L

0.1 M sawdust/hay = 180.16 g (sawdust/hay)/10L

Nitrate Water Added (in L)

Phase I	Trial 1	Trial 2	Trial 3	Trial 4
P + Hay 1	1	3	1	1
P + Hay 2	4	4	4	4
P + Hay 3	2.5	2.5	2.5	2.5
P + Hay 4	2.5	2.5	2.5	2.5
NP + Hay 1	1.5	2.5	1.5	1.5
NP + Hay 3	1	2	1	1
NP + Hay 2	1	2	1	1
NP + Hay 4	3	3	3	3
P + Sawdust 2	2	3	2	2
P + Sawdust 4	1	1	1	1
P + Sawdust 1	3	3	3	3

P + Sawdust 3	2.5	2.5	2.5	2.5
NP + Sawdust 2	3	3	3	3
NP + Sawdust 4	1	2	1	1
NP + Sawdust 3	1	2	1	1
NP + Sawdust 1	2.5	2.5	2.5	2.5
P + Glucose 3	2.5	2.5	2.5	2.5
P + Glucose 1	2.5	2.5	2.5	2.5
P + Glucose 4	2	3	2	2
P + Glucose 2	2	2	2	2
NP + Glucose 2	1.25	2	1.25	1.25
NP + Glucose 4	2.5	2.5	2.5	2.5
NP + Glucose 3	1.5	2.5	1.5	1.5
NP + Glucose 1	1	2	1	1
P + No Amendments 2	2	2	2	2
P + No Amendments 3	2	2	2	2
P + No Amendments 1	1.5	1.5	1.5	1.5
P + No Amendments 4	2	2	2	2
NP + No Amendments 2	2	3	2	2
NP + No Amendments 1	1.5	2.5	1.5	1.5
NP + No Amendments 4	2	2	2	2
NP + No Amendments 3	1.5	1.5	1.5	1.5

Phase I

Trial # 1	Initial mg Nitrate Added	Expected conc of Nitrate (mg L-1)	Actual conc of Nitrate (mg L-1)	Difference
1	2.66	0.591111111	0.0696	0.5215111

2	10.64	2.128	0.039	2.089
3	6.65	1.9	0.0232	1.8768
4	6.65	1.6625	0.0362	1.6263
5	3.99	1.14	0.0259	1.1141
6	2.66	0.76	0.0409	0.7191
7	2.66	0.76	0.0409	0.7191
8	7.98	1.995	0.0464	1.9486
9	5.32	2.128	0.0275	2.1005
10	2.66	0.76	0.0311	0.7289
11	7.98	2.66	0.0298	2.6302
12	6.65	2.216666667	0.0289	2.1877667
13	7.98	2.28	0.0297	2.2503
14	2.66	0.886666667	0.0198	0.8668667
15	2.66	0.886666667	0.0177	0.8689667
16	6.65	1.9	0.0443	1.8557
17	6.65	2.216666667	0.0238	2.1928667
18	6.65	1.9	0.0154	1.8846
19	5.32	1.773333333	0.038	1.7353333
20	5.32	1.773333333	0.0708	1.7025333
21	3.325	1.023076923	0.2031	0.8199769
22	6.65	1.9	0.2077	1.6923
23	3.99	1.33	0.8864	0.4436
24	2.66	1.064	0.1612	0.9028
25	5.32	1.33	0.0179	1.3121
26	5.32	1.773333333	0.0139	1.7594333

27	3.99	1.14	0.0208	1.1192
28	5.32	1.52	0.0129	1.5071
29	5.32	1.52	0.0211	1.4989
30	3.99	1.33	0.057	1.273
31	5.32	1.773333333	0.0172	1.7561333
32	3.99	1.14	0.0156	1.1244
Trial # 2	**Initial mg Nitrate Added**	**Expected conc of Nitrate (mg L-1)**	**Actual conc of Nitrate (mg L-1)**	**Difference**
1	6.912	1.8432	0.0724	1.7708
2	9.216	1.755428571	0.0503	1.7051286
3	5.76	1.536	0.1384	1.3976
4	5.76	1.536	0.0847	1.4513
5	5.76	1.536	0.0513	1.4847
6	4.608	1.417846154	0.0396	1.3782462
7	4.608	1.8432	0.0584	1.7848
8	6.912	1.974857143	0.0122	1.9626571
9	6.912	2.126769231	0.021	2.1057692
10	2.304	1.024	0.199	0.825
11	6.912	2.304	0.0087	2.2953
12	5.76	2.094545455	0.0117	2.0828455
13	6.912	2.304	0.0215	2.2825
14	4.608	1.536	0.0252	1.5108
15	4.608	2.048	0.092	1.956
16	5.76	1.92	0.0291	1.8909
17	5.76	1.92	0.6051	1.3149

18	5.76	1.92	0.1522	1.7678
19	6.912	1.728	0.345	1.383
20	4.608	1.675636364	0.2124	1.4632364
21	4.608	1.536	0.6663	0.8697
22	5.76	1.645714286	0.1642	1.4815143
23	5.76	1.772307692	0.4734	1.2989077
24	4.608	1.675636364	0.3912	1.2844364
25	4.608	1.536	0.2302	1.3058
26	4.608	1.675636364	0.093	1.5826364
27	3.456	1.3824	0.2674	1.115
28	4.608	1.8432	0.184	1.6592
29	6.912	1.728	0.0417	1.6863
30	5.76	1.772307692	0.2779	1.4944077
31	4.608	1.536	0.1321	1.4039
32	3.456	1.3824	0.2638	1.1186
Trial # 3	**Initial mg Nitrate Added**	**Expected conc of Nitrate (mg L-1)**	**Actual conc of Nitrate (mg L-1)**	**Difference**
1	1.97	0.985	0.0303	0.9547
2	7.88	1.97	0.0129	1.9571
3	4.925	1.407142857	0.0731	1.3340429
4	4.925	1.407142857	0.0165	1.3906429
5	2.955	1.182	0.0097	1.1723
6	1.97	0.985	0.0779	0.9071
7	1.97	0.985	0.0306	0.9544
8	5.91	1.97	0.019	1.951

9	3.94	1.97	0.0154	1.9546
10	1.97	1.313333333	0.0186	1.2947333
11	5.91	1.97	0.0063	1.9637
12	4.925	1.97	0.0282	1.9418
13	5.91	1.97	0.0382	1.9318
14	1.97	0.985	0.0216	0.9634
15	1.97	1.97	0.0399	1.9301
16	4.925	1.97	0.0049	1.9651
17	4.925	1.97	0.0099	1.9601
18	4.925	1.641666667	0.1024	1.5392667
19	3.94	1.576	0.0549	1.5211
20	3.94	1.576	0.0161	1.5599
21	2.4625	1.407142857	0.2617	1.1454429
22	4.925	1.641666667	0.0851	1.5565667
23	2.955	1.4775	0.0617	1.4158
24	1.97	1.313333333	0.0396	1.2737333
25	3.94	1.576	0.0093	1.5667
26	3.94	1.576	0.0757	1.5003
27	2.955	1.4775	0.0701	1.4074
28	3.94	1.576	0.0178	1.5582
29	3.94	1.576	0.0112	1.5648
30	2.955	1.4775	0.0398	1.4377
31	3.94	1.576	0.1614	1.4146
32	2.955	1.4775	0.0179	1.4596

Trial	**Initial mg**	**Expected conc of**	**Actual conc of**	**Difference**

# 4	Nitrate Added	Nitrate (mg L-1)	Nitrate (mg L-1)	
1	2.5175	1.007	0.057	0.95
2	10.07	2.237777778	0.0396	2.1981778
3	6.29375	2.097916667	0.1428	1.9551167
4	6.29375	1.798214286	0.0307	1.7675143
5	3.77625	1.5105	0.0299	1.4806
6	2.5175	1.007	0.0251	0.9819
7	2.5175	1.007	0.0637	0.9433
8	7.5525	2.157857143	0.0274	2.1304571
9	5.035	2.014	0.0492	1.9648
10	2.5175	1.25875	0.0465	1.21225
11	7.5525	2.5175	0.0269	2.4906
12	6.29375	2.5175	0.0582	2.4593
13	7.5525	2.5175	0.0382	2.4793
14	2.5175	1.678333333	0.0867	1.5916333
15	2.5175	1.678333333	0.0764	1.6019333
16	6.29375	2.097916667	0.0409	2.0570167
17	6.29375	1.25875	0.0208	1.23795
18	6.29375	1.25875	0.0428	1.21595
19	5.035	1.007	0.032	0.975
20	5.035	1.25875	0.0418	1.21695
21	3.146875	0.968269231	0.0969	0.8713692
22	6.29375	1.25875	0.0263	1.23245
23	3.77625	0.9440625	0.0286	0.9154625
24	2.5175	0.839166667	0.0344	0.8047667

25	5.035	1.25875	0.0284	1.23035
26	5.035	1.25875	0.0231	1.23565
27	3.77625	1.25875	0.0108	1.24795
28	5.035	1.25875	0.0254	1.23335
29	5.035	1.007	0.0183	0.9887
30	3.77625	0.9440625	0.3657	0.5783625
31	5.035	1.25875	0.0144	1.24435
32	3.77625	1.25875	0.1845	1.07425

Comparison of Week, Organic Amendment and Presence of Plants

The ANOVA Procedure

Class Level Information

Class	Levels	Values
PLANT	2	0 1
FACTOR	4	0 1 2 3
WEEK	4	1 2 3 4

Number of Observations Read	128
Number of Observations Used	128

Dependent Variable: DIFF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	6.63603972	0.94800567	5.56	<.0001
Error	120	20.44671015	0.17038925		
Corrected Total	127	27.08274987			

R-Square	Coeff Var	Root MSE	DIFF Mean
0.245028	27.66925	0.412782	1.491845

Source	DF	Anova SS	Mean Square	F Value	Pr > F
PLANT	1	1.40976139	1.40976139	8.27	0.0048

Source	DF	Anova SS	Mean Square	F Value	Pr > F
FACTOR	3	4.86096597	1.62032199	9.51	<.0001
WEEK	3	0.36531236	0.12177079	0.71	0.5451

Student-Newman-Keuls Test for DIFF

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	120
Error Mean Square	0.170389

Number of Means	**2**
Critical Range	0.1444761

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	PLANT
A	1.59679	64	1
B	1.38690	64	0

Student-Newman-Keuls Test for DIFF

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	120
Error Mean Square	0.170389

Number of Means	2	3	4
Critical Range	0.2043201	0.2448984	0.2688655

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	FACTOR
A	1.8200	32	2
B	1.4556	32	1
B			

Means with the same letter are not significantly different.			
SNK Grouping	**Mean**	**N**	**FACTOR**
B	1.3581	32	0
B			
B	1.3337	32	3

Student-Newman-Keuls Test for DIFF

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	120
Error Mean Square	0.170389

Number of Means	**2**	**3**	**4**
Critical Range	0.2043201	0.2448984	0.2688655

Means with the same letter are not significantly different.			
SNK Grouping	**Mean**	**N**	**WEEK**
A	1.5661	32	2
A			
A	1.5140	32	3
A			
A	1.4634	32	1
A			
A	1.4240	32	4

Appendix D – Phase II

Experimental Groups

Microcosm Number	Sawdust	Switchgrass	Cattail	Bulrush
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
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20				
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26				
27				
28				
29				
30				
31				
32				
33				
34				
35				
36				

Trial 1 Nitrate Removal Data

Trial 1	Nitrate Input (mg)	Input Nitrate Concentration (mg/L)	Output Nitrate Concentration (mg/L)	Nitrate Removal (mg/L)
1	4.6486	1.549533333	0.0148	1.534733333
2	4.6486	1.16215	0.0585	1.10365
3	4.6486	1.16215	0.0156	1.14655
4	4.6486	1.16215	0.0154	1.14675
5	4.6486	1.549533333	0.0148	1.534733333
6	4.6486	1.549533333	0.0115	1.538033333
7	4.6486	1.549533333	0.037	1.512533333
8	4.6486	1.549533333	0.025	1.524533333
9	4.6486	1.549533333	0.0256	1.523933333
10	4.6486	1.549533333	0.0097	1.539833333
11	4.6486	1.549533333	0.0551	1.494433333
12	4.6486	1.549533333	0.0052	1.544333333
13	2.3243	1.16215	0.0244	1.13775
14	2.3243	0.774766667	0.0019	0.772866667
15	4.6486	2.3243	0.0287	2.2956
16	2.3243	1.16215	0.014	1.14815
17	4.6486	2.3243	0.0162	2.3081
18	2.3243	1.16215	0.0099	1.15225
19	4.6486	2.3243	0.014	2.3103
20	2.3243	1.16215	0.0161	1.14605
21	4.6486	1.549533333	0.015	1.534533333
22	4.6486	1.549533333	0.0112	1.538333333
23	4.6486	1.549533333	0.0166	1.532933333
24	4.6486	1.549533333	0.0168	1.532733333
25	4.6486	1.549533333	0.0158	1.533733333
26	4.6486	2.3243	0.0273	2.297
27	4.6486	1.549533333	0.0109	1.538633333
28	2.3243	1.16215	0.0151	1.14705
29	4.6486	1.549533333	0.0062	1.543333333
30	2.3243	1.16215	0.0049	1.15725
31	2.3243	0.774766667	0.0134	0.761366667
32	4.6486	1.549533333	0.0109	1.538633333
33	2.3243	1.16215	0.0431	1.11905
34	4.6486	1.549533333	0.0092	1.540333333
35	4.6486	1.549533333	0.011	1.538533333
36	4.6486	1.549533333	0.0097	1.539833333

Trial 2 Nitrate Removal Data

Trial 2	Nitrate Input (mg)	Input Nitrate Concentration (mg/L)	Output Nitrate Concentration (mg/L)	Nitrate Removal (mg/L)
1	3.9972	1.59888	0.0342	1.56468
2	3.9972	1.59888	0.0566	1.54228
3	3.9972	1.59888	0.0681	1.53078
4	3.9972	1.59888	0.015	1.58388
5	3.9972	1.59888	0.0174	1.58148
6	3.9972	1.59888	0.0199	1.57898
7	3.9972	1.59888	0.008	1.59088
8	3.9972	1.59888	0.0082	1.59068
9	3.9972	1.59888	0.0422	1.55668
10	3.9972	1.59888	0.0352	1.56368
11	3.9972	1.59888	0.0346	1.56428
12	3.9972	1.59888	0.0206	1.57828
13	3.9972	1.59888	0.0091	1.58978
14	3.9972	1.59888	0.0106	1.58828
15	3.9972	1.59888	0.0182	1.58068
16	6.662	1.903428571	0.0128	1.890628571
17	3.9972	1.59888	0.0213	1.57758
18	3.9972	1.59888	0.0019	1.59698
19	3.9972	1.59888	0.0291	1.56978
20	3.9972	1.59888	0.0187	1.58018
21	3.9972	1.59888	0.0119	1.58698
22	3.9972	1.59888	0.0202	1.57868
23	3.9972	1.59888	0.0119	1.58698
24	3.9972	1.59888	0.0262	1.57268
25	3.9972	1.59888	0.0326	1.56628
26	3.9972	1.59888	0.0095	1.58938
27	3.9972	1.59888	0.0272	1.57168
28	3.9972	1.59888	0.0213	1.57758
29	3.9972	1.59888	0.0127	1.58618
30	3.9972	1.59888	0.0254	1.57348
31	3.9972	1.59888	0.0255	1.57338
32	3.9972	1.59888	0.0134	1.58548
33	3.9972	1.59888	0.012	1.58688
34	3.9972	1.59888	0.0027	1.59618
35	3.9972	1.59888	0.01	1.58888
36	3.9972	1.59888	0.0308	1.56808

Trial 3	Nitrate Input (mg)	Input Nitrate Concentration (mg/L)	Output Nitrate Concentration (mg/L)	Nitrate Removal (mg/L)
1	6.3204	2.1068	0.0531	2.0537
2	6.3204	1.5801	0.0399	1.5402
3	6.3204	1.5801	0.036	1.5441
4	6.3204	1.5801	0.0216	1.5585
5	6.3204	1.5801	0.0304	1.5497
6	6.3204	2.1068	0.0149	2.0919
7	6.3204	2.1068	0.0215	2.0853
8	6.3204	2.1068	0.0143	2.0925
9	6.3204	2.1068	0.0269	2.0799
10	6.3204	1.5801	0.0077	1.5724
11	4.7403	1.89612	0.0089	1.88722
12	6.3204	2.1068	0.0107	2.0961
13	6.3204	2.1068	0.0026	2.1042
14	4.7403	1.89612	0.0086	1.88752
15	6.3204	2.1068	0.0019	2.1049
16	6.3204	2.1068	0.0144	2.0924
17	6.3204	2.1068	0.0019	2.1049
18	6.3204	2.1068	0.0019	2.1049
19	6.3204	2.1068	0.0357	2.0711
20	6.3204	2.1068	0.0108	2.096
21	6.3204	2.1068	0.0187	2.0881
22	6.3204	2.1068	0.018	2.0888
23	6.3204	2.1068	0.0205	2.0863
24	6.3204	2.1068	0.0207	2.0861
25	6.3204	2.1068	0.0177	2.0891
26	6.3204	2.1068	0.0154	2.0914
27	6.3204	2.1068	0.0207	2.0861
28	6.3204	2.1068	0.0052	2.1016
29	6.3204	2.1068	0.0135	2.0933
30	6.3204	2.1068	0.0141	2.0927
31	6.3204	2.1068	0.0259	2.0809
32	6.3204	2.1068	0.0171	2.0897
33	6.3204	2.1068	0.0116	2.0952
34	9.4806	3.1602	0.0373	3.1229
35	6.3204	2.1068	0.0447	2.0621
36	6.3204	2.1068	0.0504	2.0564

Trial 3 Nitrate Removal Data

Trial 4 Nitrate Removal Data

Trial 4	Nitrate Input (mg)	Input Nitrate Concentration (mg/L)	Output Nitrate Concentration (mg/L)	Nitrate Removal (mg/L)
1	2.6976	1.07904	0.0142	1.06484
2	2.6976	1.07904	0.8435	0.23554
3	2.6976	1.07904	0.0217	1.05734
4	2.6976	1.07904	0.0064	1.07264
5	2.6976	1.07904	0.0055	1.07354
6	2.6976	1.07904	0.028	1.05104
7	2.6976	1.07904	0.0086	1.07044
8	2.6976	1.07904	0.0826	0.99644
9	2.6976	1.07904	0.0107	1.06834
10	2.6976	1.07904	0.0262	1.05284
11	2.6976	1.07904	0.0631	1.01594
12	2.6976	1.07904	0.0652	1.01384
13	2.6976	1.07904	0.0188	1.06024
14	2.6976	1.07904	0.0506	1.02844
15	2.6976	1.07904	0.0367	1.04234
16	4.496	1.284571429	0.0395	1.245071429
17	2.6976	1.07904	0.011	1.06804
18	2.6976	1.07904	0.0151	1.06394
19	2.6976	1.07904	0.0312	1.04784
20	2.6976	1.07904	0.0066	1.07244
21	2.6976	1.07904	0.0184	1.06064
22	2.6976	1.07904	0.0243	1.05474
23	2.6976	1.07904	0.0174	1.06164
24	2.6976	1.07904	0.0257	1.05334
25	2.6976	1.07904	0.0288	1.05024
26	2.6976	1.07904	0.061	1.01804
27	2.6976	1.07904	0.0275	1.05154
28	2.6976	1.07904	0.0174	1.06164
29	2.6976	1.07904	0.0114	1.06764
30	2.6976	1.07904	0.0469	1.03214
31	2.6976	1.07904	0.0148	1.06424
32	2.6976	1.07904	0.0741	1.00494
33	2.6976	1.07904	0.0458	1.03324
34	2.6976	1.07904	0.0506	1.02844
35	2.6976	1.07904	0.0046	1.07444
36	2.6976	1.07904	0.0051	1.07394

Plants vs. No Plants ANOVA

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
PLANT	2	0 1

Number of Observations Read	144
Number of Observations Used	144

Dependent Variable: DIFF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00516166	0.00516166	0.03	0.8679
Error	142	26.40055625	0.18591941		
Corrected Total	143	26.40571791			

R-Square	Coeff Var	Root MSE	DIFF Mean
0.000195	28.19503	0.431184	1.529290

Source	DF	Anova SS	Mean Square	F Value	Pr > F
PLANT	1	0.00516166	0.00516166	0.03	0.8679

Student-Newman-Keuls Test for DIFF

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	142
Error Mean Square	0.185919
Harmonic Mean of Cell Sizes	49.77778

Note: Cell sizes are not equal.

Number of Means	**2**

Number of Means	2
Critical Range	0.1708539

Means with the same letter are not significantly different.			
SNK Grouping	Mean	N	PLANT
A	1.54049	32	0
A			
A	1.52609	112	1

Amendment vs. No Amendment ANOVA

The ANOVA Procedure

Class Level Information		
Class	Levels	Values
AMEND	2	0 1

Number of Observations Read	32
Number of Observations Used	32

Dependent Variable: DIFF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.08632013	0.08632013	0.35	0.5581
Error	30	7.38220646	0.24607355		
Corrected Total	31	7.46852658			

R-Square	Coeff Var	Root MSE	DIFF Mean
0.011558	32.20130	0.496058	1.540491

Source	DF	Anova SS	Mean Square	F Value	Pr > F
AMEND	1	0.08632013	0.08632013	0.35	0.5581

Student-Newman-Keuls Test for DIFF

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	30
Error Mean Square	0.246074

Number of Means	2
Critical Range	0.3581765

Means with the same letter are not significantly different.			
SNK Grouping	Mean	N	AMEND
A	1.5924	16	1
A			
A	1.4886	16	0

Cattail vs. Switchgrass vs. Bulrush ANOVA

The ANOVA Procedure

Class Level Information		
Class	Levels	Values
COMBO	7	1 2 3 4 5 6 7
WEEK	4	1 2 3 4

Number of Observations Read	112
Number of Observations Used	112

Dependent Variable: DIFF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	13.91150849	1.54572317	31.40	<.0001
Error	102	5.02052118	0.04922080		
Corrected Total	111	18.93202967			

R-Square	Coeff Var	Root MSE	DIFF Mean
0.734813	14.53765	0.221858	1.526090

Source	DF	Anova SS	Mean Square	F Value	Pr > F
COMBO	6	0.31176598	0.05196100	1.06	0.3942
WEEK	3	13.59974251	4.53324750	92.10	<.0001

Student-Newman-Keuls Test for DIFF

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05

Error Degrees of Freedom	102
Error Mean Square	0.049221

Number of Means	2	3	4	5	6	7
Critical Range	0.1555824	0.1865589	0.204873	0.2178377	0.2278337	0.2359434

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	COMBO
A	1.58706	16	3
A			
A	1.57129	16	4
A			
A	1.56400	16	1
A			
A	1.55142	16	2
A			
A	1.50680	16	7
A			
A	1.45268	16	5
A			
A	1.44938	16	6

Student-Newman-Keuls Test for DIFF

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

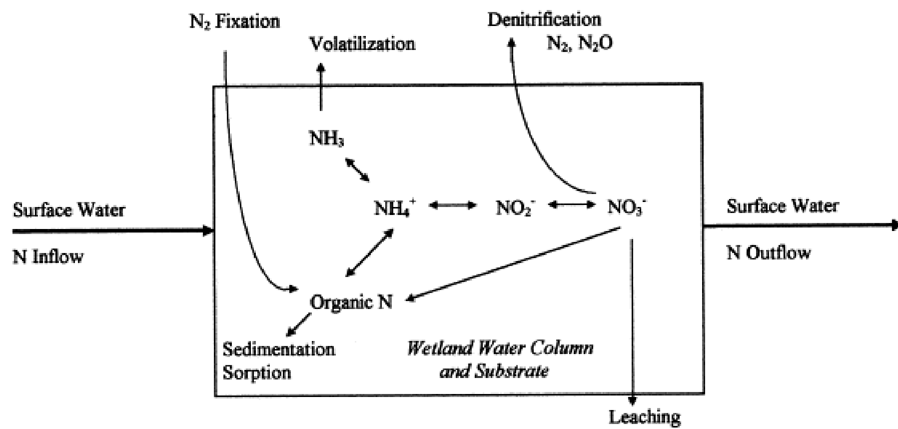
Alpha	0.05
Error Degrees of Freedom	102
Error Mean Square	0.049221

Number of Means	2	3	4
Critical Range	0.1176093	0.1410252	0.1548694

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	WEEK
A	2.01071	28	3
B	1.58644	28	2
B			
B	1.47577	28	1
C	1.03144	28	4

Nitrogen Balance



The above nitrogen balance schematic proposed by Mitsch (1999) was used to understand the movement of nitrogen within a wetland. After considering the Mitsch schematic, a nitrogen balance was developed to define the total nitrogen within the experimental microcosms.

Carbon Biomass by Plant

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
BUL	2	0 1
CAT	2	0 1
SWI	2	0 1

Number of Observations Read	28
Number of Observations Used	28

Dependent Variable: BIOMASS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	2019.134222	673.044741	6.19	0.0029
Error	24	2607.718580	108.654941		
Corrected Total	27	4626.852803			

R-Square	Coeff Var	Root MSE	BIOMASS Mean
0.436395	17.88777	10.42377	58.27314

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BUL	1	1264.307894	1264.307894	11.64	0.0023
CAT	1	482.669618	482.669618	4.44	0.0457
SWI	1	272.156711	272.156711	2.50	0.1266

Student-Newman-Keuls Test for BIOMASS

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	108.6549
Harmonic Mean of Cell Sizes	13.71429

Number of Means	2
Critical Range	8.2156323

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	BUL
A	64.093	16	1
B	50.514	12	0

Student-Newman-Keuls Test for BIOMASS

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	24

Error Mean Square	108.6549
Harmonic Mean of Cell Sizes	13.71429

Number of Means	**2**
Critical Range	8.2156323

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	CAT
A	61.869	16	1
B	53.479	12	0

Student-Newman-Keuls Test for BIOMASS

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	108.6549
Harmonic Mean of Cell Sizes	13.71429

Number of Means	**2**
Critical Range	8.2156323

Means with the same letter are not significantly different.

SNK Grouping	Mean	N	SWI
A	61.873	12	0
A			
A	55.573	16	1

Carbon Percentages by Plant

The ANOVA Procedure

Class Level Information

Class	Levels	Values
BUL	2	0 1
CAT	2	0 1

Class Level Information		
Class	**Levels**	**Values**
SWI	2	0 1

Number of Observations Read	28
Number of Observations Used	28

Dependent Variable: CARBONPER

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	16.25601815	5.41867272	4.00	0.0193
Error	24	32.55122470	1.35630103		
Corrected Total	27	48.80724286			

R-Square	Coeff Var	Root MSE	CARBONPER Mean
0.333066	2.803330	1.164603	41.54357

Source	DF	Anova SS	Mean Square	F Value	Pr > F
BUL	1	8.64004286	8.64004286	6.37	0.0186
CAT	1	0.23786786	0.23786786	0.18	0.6791
SWI	1	7.37810744	7.37810744	5.44	0.0284

Student-Newman-Keuls Test for CARBONPER

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	1.356301
Harmonic Mean of Cell Sizes	13.71429

Note: Cell sizes are not equal.

Number of Means	**2**
Critical Range	0.9178977

Means with the same letter are not significantly different.			
SNK Grouping	**Mean**	**N**	**BUL**
A	42.1850	12	0

Means with the same letter are not significantly different.			
SNK Grouping	**Mean**	**N**	**BUL**
B	41.0625	16	1

Student-Newman-Keuls Test for CARBONPER

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	1.356301
Harmonic Mean of Cell Sizes	13.71429

Note: Cell sizes are not equal.

Number of Means	**2**
Critical Range	0.9178977

Means with the same letter are not significantly different.			
SNK Grouping	**Mean**	**N**	**CAT**
A	41.6500	12	0
A			
A	41.4638	16	1

Student-Newman-Keuls Test for CARBONPER

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	1.356301
Harmonic Mean of Cell Sizes	13.71429

Note: Cell sizes are not equal.

Number of Means	**2**
Critical Range	0.9178977

Means with the same letter are not significantly different.			
SNK Grouping	**Mean**	**N**	**SWI**
A	41.9881	16	1
B	40.9508	12	0

Carbon Biomass by Combination

```
DATA PHASE2;
INPUT COMBO WEIGHT;
DATALINES;
1      132.1835623
1      76.375772
1      94.20135613
1      114.6322179
2      100.0975109
2      135.885799
2      157.0022602
2      128.8926852
3      129.166925
3      100.3717506
3      137.8054773
3      167.1491312
4      126.6987672
4      178.5300811
4      154.945462
4      145.3470706
5      156.7280205
5      166.3264119
5      157.2765
5      200.4692615
6      119.5685335
6      138.9024363
6      150.0091464
6      82.54616651
7      175.5134437
7      136.7085183
7      194.9844664
7      175.1020841
;
PROC ANOVA DATA=PHASE2;
CLASS COMBO;
MODEL WEIGHT = COMBO;
MEANS COMBO / SNK;
RUN;
```

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
COMBO	7	1 2 3 4 5 6 7

Number of Observations Read	28

Number of Observations Used	28

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	14699.13506	2449.85584	4.02	0.0077
Error	21	12784.46634	608.78411		
Corrected Total	27	27483.60140			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.534833	17.56383	24.67355	140.4793

Source	DF	Anova SS	Mean Square	F Value	Pr > F
COMBO	6	14699.13506	2449.85584	4.02	0.0077

Student-Newman-Keuls Test for WEIGHT

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	21
Error Mean Square	608.7841

Number of Means	2	3	4	5	6	7
Critical Range	36.282386	43.975977	48.629262	51.974775	54.582256	56.715882

Means with the same letter are not significantly different.

SNK Grouping		Mean	N	COMBO
	A	170.58	4	7
	A			
	A	170.20	4	5
	A			
B	A	151.38	4	4
B	A			
B	A	133.62	4	3
B	A			

Means with the same letter are not significantly different.				
SNK Grouping		**Mean**	**N**	**COMBO**
B	A	130.47	4	2
B	A			
B	A	122.76	4	6
B				
B		104.35	4	1

Carbon Percentages by Combination

```
DATA PHASEII;
INPUT COMBO CARBONPER;
DATALINES;
1       42.76
1       43.31
1       45.31
1       43.37
2       38.44
2       40.25
2       41.32
2       39.86
3       40.59
3       42.08
3       40.57
3       40.04
4       41.12
4       42.21
4       40.68
4       41.17
5       42.41
5       41.91
5       41.47
5       40.57
6       40.89
6       42.57
6       42.55
6       42.18
7       41.80
7       40.28
7       41.97
7       41.54
;
PROC ANOVA DATA=PHASEII;
CLASS COMBO;
MODEL CARBONPER = COMBO;
MEANS COMBO / SNK;
RUN;
```

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**

Class Level Information		
Class	**Levels**	**Values**
COMBO	7	1 2 3 4 5 6 7

Number of Observations Read	28
Number of Observations Used	28

Dependent Variable: CARBONPER

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	31.77284286	5.29547381	6.53	0.0005
Error	21	17.03440000	0.81116190		
Corrected Total	27	48.80724286			

R-Square	Coeff Var	Root MSE	CARBONPER Mean
0.650986	2.167953	0.900645	41.54357

Source	DF	Anova SS	Mean Square	F Value	Pr > F
COMBO	6	31.77284286	5.29547381	6.53	0.0005

Student-Newman-Keuls Test for CARBONPER

Note: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha	0.05
Error Degrees of Freedom	21
Error Mean Square	0.811162

Number of Means	**2**	**3**	**4**	**5**	**6**	**7**
Critical Range	1.3243963	1.6052313	1.7750876	1.8972071	1.9923866	2.0702691

Means with the same letter are not significantly different.			
SNK Grouping	**Mean**	**N**	**COMBO**
A	43.6875	4	1
B	42.0475	4	6

Means with the same letter are not significantly different.

SNK Grouping		Mean	N	COMBO
	B			
C	B	41.5900	4	5
C	B			
C	B	41.3975	4	7
C	B			
C	B	41.2950	4	4
C	B			
C	B	40.8200	4	3
C				
C		39.9675	4	2

1-SOA, 2- BOA, 3-COA, 4-SBOA, 5-BCOA, 6-SCOA, 7-SBCOA

Appendix E – Protocols

Using the Hach DR 5000™ UV-Vis Spectrophotometer

1. Select the correct program: Stored Programs →NI HR RP
2. Press "Start" (should be at a wavelength of 500nm)
3. Take the 10mL pipette, rubber bulb, vial and tube of sample water to the hood
4. Add 10mL of sample water to the vial (fill till the meniscus reaches 10mL)
5. Fill one vial with 10mL of distilled water
6. Measure the amount of nitrate:
    a. Press options → Timer button
    b. Empty the pillow packet into the vial
    c. Put a rubber stopper on the vial
    d. Start the shake timer and shake sample vigorously (1 min)
    e. Start the wait timer (5 min)
    f. Use a Kimwipe to wipe the outer surface of the vial properly
    g. Using the vial with distilled water, set a blank value (hit "zero")
    h. Put the sample in the machine (hit "read")
7. To dispose of materials, empty blank vial into sink, empty sample water into specified container
8. Wash all pipettes used

Pillow Packet Information

Hach Permachem reagents, Nitriver 2 Nitrile Reagent Powder, Pillows for 10mL sample, Cat. 21075-69 pk/100

Collecting and Drying Soil Samples

1. Take 5-7 cores from each microcosm using the soil probe. Make sure each core is to a consistent depth.
2. Mix the cores together while still moist. Add enough of the resulting mixture to fill up to indicated line on soil bag.
3. Label soil bag with microcosm number.
4. Dry soil samples at 60 C. Leave soil bag opened when they are in the dryer (soil bags have plastic lining that will retain moisture if the bag is closed).
5. After drying, squeeze out any air from the bag before closing it.

Soil Sample Crushing

From the ENST Analytical Lab Standard Operating Procedure prepared by Laboratory Supervisor Stanley Schlosnagle.

Air-dried soil is ground on a soil crusher. The crushed and mixed soil is passed through a U. S. standard 10 mesh sieve (2-mm opening). Soil that passes the sieve is collected for standard nutrient soil test analysis.

Two types of soil crushers are available. Air-dried soil is typically crushed on a Dynacrush soil crusher, model: DC-2 (Custom Laboratory Equipment, Inc., Orange City, Florida). The Dynacrush has a stainless steel flailer assembly inside a urethane body. However, if there is a concern of metal contamination from the stainless steel flailer assembly, then soil may be ground on a roller crusher that uses an 8-inch wide rubber belt that rotates beneath two 5-1/2 inch diameter metal rollers.

Dynacrush Soil Crusher

- Turn switch "on" to dust collector. Switch is located below the red and green caps of the wall-mounted box, left of soil grinders. Turn switch clockwise to "dust collector". Open door to the hall to prevent back pressure.
- Wear safety glasses or goggles
- Suggest wearing dust mask, especially if grinding many samples
- All operations that cause dust to become suspended must be conducted along the vents on the countertop of the east wall. Check that vents are not obstructed and that dust collector is turned "on".
- Turn switch "on" to Dynacrush soil crusher (standard method) or belt crusher, never walk away with soil grinder running.
- While running, open top lid to Dynacrush soil crusher and place about 1 cup of air-dried soil into crusher then close top lid. In 2 to 3 seconds, open lever on right side to allow soil to fall into sieve below (2-mm openings). Remove sieve and shake back-and-forth to advance soil through sieve. Collect soil that fell through sieve and discard remaining soil into a 5 gallon bucket.
- Clods may occasionally cause an obstruction in the grinder and can be cleared by tapping them gently with a small stick so they fall into the flailer assembly; otherwise, turn machine off and unplug from wall outlet before removing obstruction.
- Do not intentionally place soil into the grates of the dust collector but place excess soil in 5-gallon bucket located on top of the grates. The bucket should always be kept above the grates so that any dust created from pouring soil into the bucket will be sucked into the dust collector.

Shut Down

- Turn "off" switch to soil grinder when done or whenever leaving room.
- Sweep floor and work bench area
- Soil from bucket may be discarded in the outside dumpster in HH parking lot. Bring empty bucket back. Paper trash should be placed in plastic trash bag and taken to dumpster.
- Turn switch "off" to dust collector, switch in vertical position, when no one is using it and there are no plans of using it later that day. This dust collector provides service to the adjacent plant grinding room (room 0217).

Soil Roller Crusher

Use air cleaner as noted above. Keep fingers and clothes away from moving parts.

The roller crusher is used as an alternative to the dynacrush where the later may cause metal contamination from the use of the flailer assembly. Soil is crushed on an 8-inch wide belt that moves beneath two spring loaded metal rollers. Crushed soil falls from the belt into a vibrating U. S. standard 10 mesh sieve (2-mm opening) that is positioned directly above a plastic triangular shaped collection pan. Slowly pour soil on the moving belt so that the edge of belt remains free of soil and rock fragments.

Centering the 8" wide belt, conducted by lab supervisor:

- The eight-inch wide belt should be centered beneath the two metal rollers.
- If not properly centered, the edge of the moving belt will rub the metal frame, contaminate soil, and disintegrate the belt.

1) Turn motor switch "off" and remove plug from wall outlet.

2) Clean all surfaces that contain soil and rock fragments particularly areas along edges of belt.
3) Clean under belt (where soil should not contact)
4) Clean two metal rollers that belt goes around.
5) After cleaning, manually push belt to align edges of belt until they do not extend beyond the edge of the two metal rollers.

If above did not correct alignment of 8-inch wide belt then try to adjust belt's tension at front left roller:

1) Required tools: ¾ inch wrench or small channel locks, nail
2) While standing by motor and facing where soil is added to belt, loosen (clockwise) large ¾-inch nut located on bolt containing 3-$^{1}/_{2}$-inch length of threads (located by front left roller).
3) Insert nail in one of four wholes on this threaded bolt
4) With nail, turn threaded bolt
   a) Counterclockwise $^{1}/_{8}$ revolution if edge of soil belt rubbed frame at rear, (metal roller is moved toward sieve)
   b) Clockwise $^{1}/_{8}$ revolution if edge of belt rubbed frame at front, (metal roller is moved away from sieve)
5) Tighten ¾ inch nut (counterclockwise)
6) Manually push/pull to align edge of soil-belt to edge of metal rollers (do not force belt near its seam.)
7) Plug motor in and turn switch "on" to test alignment of soil belt.
8) Always keep soil away from the edges of belt.

Grinding Plant Samples

Equipment: Wiley Mill

1. Turn on the dust collector.

   Note: There is one switch that controls dust collection for two rooms. The switch is located in the room with the soil grinder; the plant grinder in the adjacent room is also controlled by this switch.

2. Move trash can next to the grinder for easy access. Put on safety goggles.

3. Open plant grinder door. Slide the silver sieve (curved plate with holes) into the appropriate notch located directly underneath the rotating blade assembly. Slide the collection tray into the appropriate notch underneath the sieve.

   Note: There are two sieves: silver-colored (2 mm holes) and gold-colored (1 mm holes). The grinder can not produce particles small enough to fall through the gold sieve.

4. Close door and tighten the latch by turning the screw. Turn the machine on.

5. Feed in plants from the top. Use the T-shaped wooden tool to push the plants down (this tool will not reach the blades). If you have a lot of plant material, you may need to break up the load into two parts.

6. After grinding, let the machine run for a while before turning it off.

   Note: This machine should be turned off in between samples.

7. Without opening the door, remove the collection tray (if you open the door, large plant particles may fall into the collection tray). Dump contents into paper bag.

8. Mix the grinded material by hand and put a small sample in a coffee grinder (fill to ½ the height of the metal tray). Place plastic guard above the blades

(this keeps the sample in contact with the blades). Grind until desired particle size. Dump contents in plastic plant sample bag. Clean coffee grinder.

Note: Coffee grinder will break if used too often.

9. Label plastic bag with a permanent marker. Squeeze out any air from the bag before closing it.
10. Repeat steps 3-9 for each plant sample. Be sure to clean the silver sieve, collection tray, grinding blades, and coffee grinder with a vacuum in between samples.
11. Walk back to soil grinding room and turn off the air cleaner.

Weighing and Packing

Grinded plants and soil need to be packed in tin capsules to be used in the Leco CHN-2000 Analyzer.

1. Use tweezers to transfer a capsule (Leco part# 502-040) to the balance. Close the side panes and tare the capsule (zero the weight of the capsule to only record the weight of the soil). Remove the capsule from the balance.
2. Use a spatula to fill up the tin capsules. The target weight is 0.2000 grams. For soil samples, fill the tin around halfway. Plant samples might fill up more of the capsule.
3. Squeeze air out of the top of the capsule. Exclude as much air as possible, because air contains nitrogen gas. This might be hard to do for plant capsules.
4. Use tweezers to transfer the capsule to the scale. Be careful not to transfer any soil/plant particles on the scale.
5. Close the panes and record weight to four decimal places.

6. Fold only the top millimeter of the capsule. Remove dust from outside of capsule.
7. Remove the capsule and place in labeled box (cells read from A1-10, then B1-10, etc.)
8. Record the cell number along with corresponding microcosm number and mass.
9. Repeat for each sample.

Determination of percent C and percent N

Analytical Method, from CHN-2000 Instruction Manual

Model: CHN-2000 Elemental Analyzer, manufactured by LECO® Corporation.

LECO® CHN-2000 Carbon, Hydrogen, and Nitrogen Analyzer.

Analysis occurs after combustion at 950 C. Carbon and hydrogen are quantified by infrared detection. Combustion with O_2 converts elemental C to CO_2 and elemental H to H_2O. Concentration of CO_2 and H_2O are determined by infrared (IR) radiation detection. The IR source is made of nichrome wire. Gases of CO_2 and H_2O absorb energy at specific wavelengths in the infrared spectrum . The increase in concentration of these gases result in an increase in IR energy absorbed and is indicated by a drop in voltage. During combustion, elemental N is converted to N_2 and NO_x. Next, NO_x is reacted with reagents to reduce it to N_2. Nitrogen is quantified by thermal conductivity. N_2 has a lower thermal conductivity than the carrier gas, helium, and an increase content of N_2 will result in an increase in the temperature of the measured filament, hence thermal conductivity. The resistors for thermal conductivity are made of tungsten wire.

Glossary

Algal bloom: A rapid increase in the numbers of algae in a body of water usually caused by a change in the flow, light, temperature or nutrient levels of the water in which it lives. Algal blooms deprive the water of oxygen and other nutrients necessary to aquatic life.

ANOVA: Analysis of variance (ANOVA) is a group of models and methods which associate variance in a single variable with different sources of variation.

Biofuels: A form of renewable fuel that's derived from biomass, which includes organic materials produced by plants, animals or microorganisms.

Carbon sink: A natural or artificial reservoir that accumulates and stores some carbon-containing chemical compound for an indefinite period.

Constructed wetland: Constructed wetland treatment systems are engineered systems that have been designed and constructed to utilize the natural processes involving wetland vegetation, soils, and their associated microbial assemblages to assist in treating wastewater. They are designed to take advantage of many of the processes that occur in natural wetlands, but do so within a more controlled environment.

Dead zones: Areas of low-oxygen water in the aquatic environment, often caused by decomposition of vast algal blooms.

Denitrification: The microbe-facilitated process by which nitrate is reduced which may eventually produce molecular nitrogen.

Denitrification Factors: A substance or substrate that aids the process of denitrification.

Effluent: Outflow of water or gas from a source.

Eutrophication: Overflow of nutrients into a body of water which can cause loss of oxygen and extreme population growth or loss.

Fossil fuels: Any fuel derived from hydrocarbon deposits such as coal, petroleum, natural gas and, to some extent, peat; these fuels are irreplaceable, and their burning generates the greenhouse gas carbon dioxide.

Greenhouse gases: A gas that traps heat into the atmosphere. The gas works in the same way as the glass in a greenhouse. Heat energy enters the atmosphere in a short wavelength form, but reflects off the earth in long wave form, so it gets trapped in the earth's atmosphere.

Hectare: A unit of area, 10,000 square meters, used in the measurement of land.

Hydrology: Movement, sources, amount, and properties of water in an environment.

Lignin: A complex chemical compound most commonly derived from wood, and an integral part of the secondary cell walls of plants and some algae.

Macrophyte: A large, multi-cellular, land based organism belonging to the plant kingdom.

Microcosm: Artificial ecosystems used to simulate natural conditions for the purpose of experimentation.

Microfauna: Small microscopic animals, but also including fungi and bacteria.

Nitrate: The nitrate ion is a polyatomic ion with the molecular formula NO^{-3}. It is the conjugate base of nitric acid, consisting of one central nitrogen atom surrounded by three identical oxygen atoms in a trigonal planar arrangement.

Nitrification: The conversion of ammonia to nitrate through oxygen addition.

Phosphates: Natural minerals containing phosphorus that are important to the maintenance of all life. They are used in laundry and dishwasher detergents and fertilizers. Their residues can cause growth of algal bloom in freshwater lakes and streams.

Polysaccharides: Defined as long carbohydrate molecules of repeating monomer units.

PVC film: Polyvinyl chloride (PVC) is a synthetically produced polymer plastic that is present in many different forms; PVC film is a clear malleable and waterproof plastic.

Runoff: Water flow from saturated soil that may contain man-made contaminants.

Salinity: The level of different salts in a body of water or soil usually reported in mg L^{-1} or parts per million.

Soil Inoculation: The process of mixing soil with a desired microbial community into a larger sample of soil in order to give the original microbial community to the larger sample.

Spectrophotometer: A spectrophotometer is a light intensity-measuring device that can measure intensity as a function of light source wavelength. It is useful in measuring absorption and therefore concentration differences because the spectrophotometer detects more light passing through the sample when more substance is absorbed.

Xylanase: A class of enzymes that degrade hemicellulose, a major component of plant cell walls.

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