ASSESSING RESTORATION POTENTIAL IN RELICT WETLAND SOILS: INVESTIGATING THE EFFECT OF WETLAND HYDROLOGY ON SOIL MICROBIAL COMMUNITY COMPOSITION AND DENITRIFICATION POTENTIAL

BY

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THESIS

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ABSTRACT

Microbial communities are known to strongly influence rates of biogeochemical cycling in wetland ecosystems; specifically, they are strong determinants of rates of denitrification typically observed. Human induced land use changes have significantly reduced the acreage of wetland habitats, with dam building in the eastern United States figuring prominently in these losses. Wetlands located behind these dams turned into ponds, and this ecosystem alteration significantly lowers denitrification potential. Due to these ecosystem land use changes, higher exports of anthropogenic nitrate continue to leach off the land, ultimately ending up in open bodies of water. The damming and subsequent ponding of these floodplain systems have caused radically altered environmental conditions for relict wetland soils. Microbial communities are resistant to change, and may undergo periods of dormancy when conditions are not conducive to activity, but it is uncertain how long they can withstand the effects of ecosystem alteration. By removing the dam and the depositional sediments, wetland restoration efforts attempt to stimulate higher rates of denitrification observed at the site. However, understanding if the microbial community inhabiting the relict hydric soil has a high restoration potential (e.g., capability of performing higher rates of denitrification when wetland hydrology is returned) is important when studying sites targeted for restoration. This thesis attempts to address wetland restoration potential with two studies. In the first part, the hydric soil from Big Spring Run (a site targeted for wetland restoration) was surveyed for both denitrification potential and microbial community composition. Big Spring Run (BSR) was the site of a floodplain wetland prior to European settlement; however, in the 1800s, a dam was erected downstream, filling the wetland to create a mill pond. The dam was removed in the mid-1900s; however the relict wetland soil remained buried under settled pond sediments. The relict wetland soil at BSR was surveyed to see if the microbial communities present in the relict hydric soil (which has been buried for over 200 years) are still functional. Results indicate that the current buried hydric soil performs denitrification at significantly lower rates than any of the surveyed reference wetlands (Nov 2010 $F_{[4,31]} = 13.75$, $p<0.0001$; March 2011 $F_{[4,42]} = 84.6$, $p<0.0001$; June 2011 $F_{[4,35]} = 71.89$, $p<0.0001$). Bacterial community composition was also distinct between all of the sites sampled (ANOSIM $R=0.693$, $p<0.001$) indicating that no specific community composition is needed to perform denitrification. The second part of this thesis attempts to determine if the microbial community contained within relict hydric soil will perform high rates of denitrification again

when dynamic wetland hydrology is restored. Soil from the altered BSR site was transplanted to nearby wetland ecosystems and denitrification rates and microbial community composition were assessed before and after transplanting. Results indicate that when stable wetland environmental parameters dominate, the microbial communities perform higher rates of denitrification in the transplanted sites than in their current altered conditions ($F_{[5,66]}$= 8.459, $p<0.0001$). However these denitrification rates, while improved over their current conditions, are still significantly lower than any of the surveyed reference wetland sites. Results were mixed when other environmental parameters (e.g., cold temperatures, persistent flooding, and high stream flow) affected the results. Further work is needed to test the long term implications of these findings; however the preliminary results indicate that while the hydric soil microbial communities do show slight improvements in denitrification potential after transplanting, the soil microbial community at BSR seems resistant to a full recovery of denitrifying potential.

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CHAPTER 1

Introduction:

Wetlands perform many important ecosystem functions, including providing habitat for a wide variety of wildlife, carbon sequestration, and water purification. Wetlands provide more ecosystem services than any other habitat (e.g., Craig *et al.* 2008, Richardson 2008) including hydrological flow regulation, flood control, pollution control, detoxification of hazards, and biogeochemical cycling (Millennium Ecosystem Assessment 2005). Microbial community processes are integral in performing many of these ecosystem services, particularly concerning biogeochemical transformations in wetland systems (e.g., Sylvia *et al.* 2005, Smith and Ogram 2008, Moseman *et al.* 2010). Much has been learned about how microbial communities function in natural wetlands systems, especially in regard to transformations occurring in the nitrogen cycle (denitrification, nitrification, ammonia oxidation, etc.) (Gutknecht *et al.* 2006).

Denitrification is of particular interest in wetland ecosystems. Denitrification is an anaerobic process that converts nitrate (NO_3^-) to nitrogen gas (N_2), and the denitrification pathway is one of the main nitrate removal mechanisms responsible for preventing nitrate loading in aquatic ecosystems (Soil Survey Staff 1999). Wetlands tend to support a wide range of denitrification rates, due to hydrological variability typically observed in these ecosystems. Under saturated conditions, atmospheric oxygen is prevented from cycling into the soil pore spaces, and as a result the soils become anoxic (oxygen deficient; e.g., Mid-Atlantic Hydric Soils Committee 2004). These conditions support high rates of anaerobic respiration, and high rates of denitrification can be observed. However, during dry periods, atmospheric oxygen can diffuse into the soil and return aerobic conditions (albeit briefly), resulting in depressed rates of denitrification (Orr *et al.* 2007). These variable environmental conditions support facultative anaerobes – bacterial communities capable of functioning in both aerobic and anaerobic conditions (e.g., Hartman *et al.* 2008). During saturated, anaerobic conditions, these organisms will perform denitrification, however during the drier periods; these bacterial communities will be capable of performing aerobic respiration. Rates of denitrification in wetlands can also be influenced by the microbial community composition (MCC) of the wetland being studied, particularly when the denitrifying community is surveyed (e.g., Dandie *et al.* 2011, Peralta *et al.* 2010).

This denitrification process in wetlands is highly important, as anthropogenic exports of nitrate have dramatically increased over the past century (e.g., Keeny 1973, Galloway *et al.* 2003). Left unchecked, high concentrations of nitrate in aquatic systems can lead to eutrophication, where the nutrients support high rates of phytoplankton growth and results in large algal blooms that cover the water surface (Mid-Atlantic Hydric Soils Committee 2004). When these organisms die, the subsequent decomposition pathway removes oxygen from the water, eventually resulting in "dead zones" – areas of hypoxia in large bodies of water. This phenomenon has been observed in various coastal environments, most noticeably in the Gulf of Mexico and Chesapeake Bay in the United States (Rabalais *et al.* 2002, Kemp *et al.* 2005).

Wetland microbial communities possess the ability to prevent excess nitrate from leaching into aquatic systems, however land use changes in the past few centuries have dramatically reduced wetland acreage (Vitousek *et al*. 1997, Dahl 1990). Wetlands were once considered useless features of the landscape, however their value is now recognized. The biogeochemical processes, especially in the nitrogen cycle, they control are now widely appreciated and desired. As a result of this perceived value, wetland restoration projects have grown in number and in popularity. However, what "defines" a successful wetland restoration is ambiguous with respect to ecosystem services, and places no value on the biogeochemical function of the site. The criteria used to identify wetlands only focus on: (1) wetland hydrology; (2) hydric soils (anoxic soils); and (3) hydrophytic plants (plants that can tolerate standing water) (Soil Survey Staff 1999, Mid-Atlantic Hydric Soils Committee 2004). Under these criteria, the biogeochemical function of the site might not be established (e.g., no denitrification, no pollutant removal) but the restoration could still be considered a success. Along with this, little effort has been placed on observing how the local microbial community adapts to the restoration. As a result, many restorations, while returning wetland hydrology, fail to restore microbially mediated ecosystem services – particularly denitrification (Orr *et al.* 2007, Bossio *et al.* 2006). Studying how denitrifying bacterial communities adjust to changing environmental conditions is therefore of high importance when trying to achieve a successful wetland restoration.

This following thesis consists of a literature review detailing how wetland restoration projects are conducted, with a particular emphasis on the importance of microbial community function in wetlands; an investigation of the microbial communities contained within various soil strata found in a Pennsylvania stream site targeted for restoration, with a focus on the

denitrifying potential of each of those communities; an investigation of how microbial communities in buried hydric sediment respond to wetland hydrology and conditions (observing both biogeochemical changes along with compositional changes); and a summary of how this work relates to the larger scope of wetlands restoration.

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CHAPTER 2

Literature Review: Wetland ecosystem services and the role of microbial community composition in a restoration context.

Wetland Function:

Wetland environments are highly important and productive habitats that provide an array of beneficial ecosystem services including carbon sequestration, habitat for plants and wildlife, groundwater recharge, pollutant degradation, flood control, and biogeochemical transformations (Millennium Ecosystem Assessment 2005). Wetlands provide more services than any other ecosystem (Craig *et al.* 2008, Richardson 2008). Of these services, water purification is one of the most important as wetlands have the ability to transform nutrients and breakdown toxins (Mid-Atlantic Hydric Soils Committee 2004, Taylor *et al.* 1990). The bulk of these ecosystem services are carried out by the plants and microbes that inhabit the distinctive hydric soils found in wetland habitats. These hydric soils develop in wetland habitats as a result of extensive periods of saturation periods contribute in forming anaerobic conditions (Soil Survey Staff 1999). This anoxic environment provides favorable conditions for anaerobic organisms to transform and remove nutrients that would otherwise negatively impact water quality. Because of these properties, wetlands are used to treat the effects of anthropogenic pollution, such as wastewater treatment and phosphorus and nitrogen based fertilizer removal (Keeny 1973, Lee *et al.* 1969, Nichols 1983).

One of the most important functions that wetlands perform is their role in the transformation of nitrogen – specifically in the denitrification pathway. Fertilizer application rates on agricultural fields have skyrocketed in the past half century (Ruhl 2000) and the subsequent runoff has generated high nitrate (NO_3^-) loads in surrounding aquatic environments (Kemp *et al.* 2005, Rabalais *et al.* 2002). Because of the anaerobic conditions typically found in wetlands, the microbial communities inhabiting the anoxic soils will use nitrate as the terminal electron acceptor in anaerobic respiration (denitrification) – resulting in a transformation of NO_3^- to N_2 (nitrogen gas) (e.g., Inglett *et al.* 2011, Song *et al.* 2010). However, denitrification is a strictly anaerobic process – thus if oxygen is present, denitrification will not occur (Mid-Atlantic Hydric Soils Committee 2004) and soluble nitrate will continue to leach into aquatic systems. Because wetland soils are predominantly saturated and their soil communities generally remain

in anaerobic conditions, high rates of denitrification are typically observed in these ecosystems (e.g., Millennium Ecosystem Assessment 2005, Nichols 1983).

The denitrification process prevents nitrate from entering aquatic ecosystems, as the initial substrate (NO_3^-) is transformed into a gaseous byproduct (N_2). This process is an especially important function carried out by wetland communities (e.g., Smith and Ogram 2008, Forshay and Stanley 2005, Craig *et al.* 2008) as high nitrate concentrations in the water can contribute to eutrophication. Left unchecked, eutrophication can lead to the production of algal blooms, hypoxia following decomposition of algal biomass, and an abrupt change in the structure of the overall ecosystem. Wetlands can help in preventing eutrophication by removing nitrate prior to its export into surface waters (Gilliam 1994, Comin *et al.* 1997), and the microbial communities contained within the hydric soils contribute significantly to the total reduction of nitrate.

Wetland Loss:

Unfortunately, cases of eutrophication and subsequent hypoxia in open bodies of water have increased significantly in the past century, specifically in the Chesapeake Bay and the Gulf of Mexico (e.g., Kemp *et al.* 2005, Rabalais *et al.* 2002, Zedler 1996). Land changes, mostly brought about by human interference, have significantly reduced the acreage of wetland habitats, as wetlands were once considered useless features of the landscape (Vitousek *et al.* 1997, Dahl 1990). The United States is estimated to have lost more than half of its original amount of wetland acreage – an estimated 89 million hectares in 1600 have been reduced to less than 42 million hectares today (Millennium Ecosystem Assessment 2005). Some states have fared even worse, with 10 states registering less than 10% of their original wetland acreage remaining (Environmental Protection Agency 2005).

Agricultural industries drained wetlands adjacent to streams (riparian zones) to increase farming area, and as a result, the nutrient runoff from the land is exported directly into the stream, and ultimately into open water (e.g., Ruhl 2000). The tillage of the land up to the banks of the stream keeps the soil aerated, preventing anaerobic processes (e.g., Ruhl 2000, Buckley and Schmidt 2003). Without riparian wetlands, denitrification is not encouraged – as no appropriate anaerobic conditions are provided for microbial communities to denitrify. Besides agricultural draining, damming of rivers and the subsequent flooding of wetland communities

also has significantly reduced wetland acreage (Walter and Merritts 2008). The dams cause upstream flooding, and as a result, wetlands are converted to ponds and lakes. As a result, sediments that normally would have flowed through the system settle and contribute to the burial of the relict wetland soil (Walter and Merrits 2008). Thousands of acres of wetlands have been lost in the eastern United States due to wetland burial, and as a result, denitrification does not continue to occur because the hydric soil communities are cut off from nutrient recharge (Forshay and Mayer 2009). Even through these soils remain anoxic in nature, anthropogenic nitrate does not seep into the former wetland soils and is instead exported downstream. Because riparian wetlands no longer provide a buffer between anthropogenic nitrate sources and aquatic ecosystems, higher loads of nitrate are exported to surface water – contributing to increasing eutrophication (Kemp *et al.* 2005, Rabalais *et al.* 2002).

In the past 100 years, thousands of mill dams have been removed (or breached) as mills were abandoned (Walter and Merritts 2008). The breaching of these dams resulted in the incision of streams into the mill pond deposited sediments as the stream attempted to return to their original elevation. Over the course of this process, buried relict hydric soils can be re-exposed. However, examining these incised systems has shown that meters of "legacy" (e.g., millpond depositional sediments) now lay on top of the former wetland soils (Walter and Merritts 2008), preventing wetland hydrology from occurring. Even though the dams have been removed and the stream has cut back to its original hydrological depth, the legacy sediments create steep banks that do not allow for wetland formation. During high flow periods, flood waters rush down the channel instead of spreading out over the floodplain – resulting in a high export of anthropogenic pollutants. The removal of these "legacy" could allow for a floodplain to be reconnected – hopefully encouraging a wetland to form in the relict soil (Walter and Merritts 2008, Forshay and Mayer 2009).

Wetland Restoration:

Over the past few decades however, the importance of wetland ecosystems has been realized. As evidence of this recent shift, the United States government instituted a "no net loss" policy in 1989 in the hopes of stopping continued wetland loss (Environmental Protection Agency 2005). Because of the "no net loss" policy, programs such as mitigation banks and site specific wetland restorations have become more common (Zedler 1996). However, while

wetland restoration projects have increased in number, the method for determining the "success" of a restoration can be ambiguous, and leave many important functions performed by a natural wetland (water purification and denitrification) absent or impaired in the restored ecosystem. The method for monitoring the success of a restoration includes three conditions that must be met: (1) recreation of wetland hydrology; (2) hydric (anoxic) soils; and (3) hydrophytic plants (e.g., plants that tolerate saturated soils) (Mid-Atlantic Soil Survey 2004). None of these criteria take into consideration biogeochemical cycling, so a "successful" restoration may not necessarily perform denitrification or help in water purification and detoxification (Orr *et al.* 2007, Bossio *et al.* 2006). Often times, the only performance standard used to test for wetlands restoration "success" is a high percent presence of native hydrophytic plants – which is often a very poor indicator of nutrient cycling and water purification potential (Spieles 2005). Occasionally, studies will measure soil type, hydrologic regime, and wildlife (Breaux and Serefiddin 1999), but in most cases, plant type is used as the sole indicator of restoration success.

Because of the limited guidelines used to evaluate restoration progress, the wetlands restored often fail to regain the full suite of ecosystem functions provided by natural wetlands (Zedler and Callaway 1999), and in the case of mitigation banks, the restoration does not take place at the site of the wetland loss. Mitigation banks, by definition, are large areas of restored wetlands where companies/industries can buy credits if they destroy a wetland in the process of developing the land. Mitigation banks aid in the "no net loss" policy in that if an acre of wetlands is destroyed by a company, they could buy an acre of wetlands in one of these banks to offset the damage to existing wetlands (Zedler and Callaway 1999). The problem is that the mitigation banks are often distant from the site of the damage, meaning that a unique community is potentially lost (Brinson and Rheinhardt 1996). Also, because of the distance from the original damaged wetland, the services restored in a mitigation bank are often not in the same watershed as the original ecosystem (Brown and Lant 1999). Thus while no net loss has been effectively accomplished, loss of ecosystem services *has* occurred. In addition, mitigation banks, while effective in preserving total wetland acreage, do very little to ensure that wetland diversity and wetland heterogeneity are conserved (Zedler 1996).

To avoid the problems associated with mitigation banks, recent work has focused on planning wetland restoration projects *at the site of first destruction* (Bossio *et al.* 2006). This allows for ecosystem services to be restored to the area that lost the wetland community in the

first place, meaning that ecosystem services have been recovered at the site (unlike mitigation banks where ecosystem services are lost at the destruction site). However, while many site restorations have been successful at producing a wetland, they have often focused mostly on restoring the floodplain and replicating wetland plant species rather than the microbial ecology necessary for biogeochemical cycling (Orr *et al.* 2007, Richardson 2008). Even wetlands that are classified as "successful" may fail to deliver microbially-mediated ecosystem services like denitrification (Orr *et al.* 2007). In a recent restoration project attempted in Wisconsin, a floodplain was reconnected to the Baraboo River system by removing a series of levees. The area was restored and it was expected that the reconnected floodplain would allow for higher rates of denitrification to be observed. Following restoration, however, it was found that while the potential for denitrification was present, the restored floodplain hydrology did not noticeably improve denitrification rates (Orr *et al.* 2007). The authors noted that the project was deemed a success because of the return of native wetland plants to the area; however that success was denitrification rates were not noticeably improved along the restored stream reach. Even though the wetland plant community was reproduced, the restoration effort did not achieve its overall goal of significantly enhancing denitrification rates (Orr *et al.* 2007).

Much of the blame lies on the failure of restoration efforts to recognize the importance of microbial communities in wetland function (Harris 2003). Most of the time and resources are spent ensuring that the plants are similar to those found in wetland communities, because many of the wetland assessment criteria focus on these characteristics (Mitsch *et al.* 2005). Unfortunately many restoration efforts spend no time ensuring that the microbial community present at the restoration is even capable of performing denitrification when given ideal wetland conditions (e.g., dynamic hydrological conditions, saturated conditions). Because microbial communities mediate many of the desirable functions of a wetland, there is a fundamental need to better understand how soil microbial communities respond to land use change (e.g., being buried under other sediments) and how the microorganisms' functions and composition are altered.

Microbially-mediated denitrification in restored wetlands:

Because of some of the biogeochemical shortcomings in wetland restoration criteria, recent wetland restoration work has stressed the importance in both the macro-ecological

approach to wetlands restoration (e.g., focusing on the plants and animals being reintroduced to the area) and the micro-ecological (biogeochemical) approach; where stress is placed on ecosystem services provided by the restored area (e.g., Inglett *et al.* 2011, Peralta *et al.* 2010). The expansion of wetland restoration goals was significantly influenced by the continued expansion of coastal eutrophic areas, particularly in the Gulf of Mexico and the Chesapeake Bay. With anthropogenic inputs continually increasing, generally from excess fertilizer applied to agricultural fields, the zones of hypoxia found in these bodies of water continued to increase in size (Kemp *et al.* 2005, Rabalais *et al.* 2002). Because natural wetlands are adept at preventing excess nutrients from entering stream reaches, stream and wetland restorations near agricultural areas have significantly increased, with stated goals of increasing denitrification rates (e.g., Kaushal *et al.* 2008, Klocker *et al.* 2009, Smith and Ogram 2008, Filoso and Palmer 2011). Studying microbial community composition became extremely important in wetland restorations (Peralta *et al.* 2010, Song *et al.* 2010), as the goals now were to provide high rates of denitrification – a microbially-mediated ecosystem service. Under these more specified goals, the effects of environmental variables, particularly the flooding regime, were studied in how they influenced the makeup and function of the targeted microbial community (Unger *et al.* 2009).

Because of the interest in biogeochemical cycling, and denitrification specifically; restoration projects started comparing natural wetland processes with the results generated from restoration zones (Bruland and Richardson 2006, Brinson and Rheinhardt 1996). Rates of denitrification generated at wetland restoration sites then had a point of comparison, with not only the stated goals of the project, but with the rates of denitrification generated at reference natural wetlands. Restorations could now be compared, both with biogeochemical cycling abilities and with overall microbial community structure, to their natural wetland counterparts (e.g., Bossio *et al.* 2006, Dandie *et al.* 2011, Peralta *et al.* 2010). When comparisons were made with reference wetland sites, it was often noted that while rates of denitrification were improved post-restoration over initial conditions, they were still far lower than any of the natural surveyed locations (e.g., Peralta *et al.* 2010, Song *et al.* 2010, Orr *et al.* 2007). These results led to the overall study of how denitrifying bacteria react to the restoration over a longer time period (2+ years) – especially as the overall ecosystem has time to stabilize after a period of intense restoration work; both in terms of hydrology and overall community makeup (Smith and Ogram 2008, Song *et al.* 2010). Results from this type of work seemed to show that as the

environmental conditions typical of a wetland developed and stabilized (e.g., dynamic hydrology, stabilized plant community, etc.) denitrifiers would become more active, and contribute to lower nitrate levels in the adjacent streams (Kaushal *et al.* 2008, Song *et al.* 2010). However, results were again inconclusive, with some studies showing restored denitrification rates similar to natural denitrification rates (e.g. Song *et al.* 2010, Dandie *et al.* 2011), and other showing either no improvement (e.g., Orr *et al.* 2007, Peralta *et al.* 2010) or only slight improvement (e.g., Kaushal *et al.* 2008).

Physical environmental factors influencing microbial community composition:

Environmental factors exert a large influence on the structure and function of wetland microbial communities (Gutknecht *et al.* 2006). One of the unique features of wetlands that may contribute to the low success of restoration efforts is that wetland ecosystems, by definition, have extremely variable environmental conditions. Wetland functions (e.g., denitrification, nutrient cycling) are strongly influenced by their hydrologic gradients, but it can be difficult to try and recreate this in a restoration effort (Zedler 1996). Hydrology (wetlands are usually saturated) generally determines the structure of the soil environment and the types of plant, animal, and microbial communities can inhabit the ecosystem. Because of the continual presence of water, conditions are created that support the growth of specially adapted plants and the formation of characteristic wetland soils – hydric soils (Mid-Atlantic Hydric Soils Committee 2004). Hydric soils in natural wetlands have been developed over many decades, and it can be difficult to try and restore wetlands if the original hydric soils have been destroyed.

Water availability plays a huge role in determining the processes that can be performed by a wetland. In general, more saturated environments (aquatic wetlands and flooded riparian wetlands) experience higher rates of anaerobic respiration – such as denitrification, methanogenesis, iron reduction, and sulfate reduction; and depressed rates of aerobic processes – like aerobic respiration and nitrification (Mid-Atlantic Hydric Soils Committee 2004). Constant saturation prevents atmospheric oxygen from diffusing into soil pore spaces, causing microorganisms to turn to other terminal electron acceptors for respiration (Balser, 2006). Many microorganisms (particularly facultative anaerobes) are adept at using other available terminal electron acceptors for respiration in anaerobic environments, and the lack of oxygen in wetland soils generally allows for high rates of denitrification to be performed (Trevors and Starodub

1987). Environments that experience wetting and drying cycles tend to be able to support both aerobic and anaerobic functions depending on the conditions experienced. During wet cycles, when sediments are depleted of oxygen, anaerobic pathways can be used for energy (dentrification, etc.) while in dry cycles, oxygen is present allowing for aerobic processes to occur. Wetting and drying cycles will strongly influence the magnitude of nitrate removal in wetland restorations (Filoso and Palmer 2011, Kaushal *et al.* 2008), so it is important to ensure that saturation is maintained (and with it, anaerobic conditions) if high rates of denitrification are to be returned.

Along with hydrology, the soil targeted for restoration should be studied in order to determine if the soil microbial communities can return to performing high levels of denitrification (Nannipieri *et al.* 2003). Often in restoration efforts, a floodplain is created, and a marsh rehabilitated, but the soil type is ignored (Orr *et al.* 2007, Bruland and Richardson 2006). In many cases, the soil that used to support a wetland has been altered significantly from its original environmental conditions and, as a result, the microbial community will be altered from the original wetland microbial community structure. Microbes are significantly influenced by soil type, depth, chemistry, and physical conditions (oxygen availability, light, temperature, etc.) (e.g., Blume *et al.* 2002, Gutknecht *et al.* 2006, Fierer *et al*. 2003, Song *et al.* 2010), so generating the appropriate soil conditions is often important to markedly improving wetland denitrification rates. Wetland soils (i.e. hydric soils) do form naturally under constant saturation (Mid-Atlantic Hydric Soils Committee 2004, Soil Survey Staff 1999), so even if the initial restoration is not able to immediately create a hydric soil, it will develop over time as long as the saturated hydrology is returned (Mid-Atlantic Hydric Soils Committee 2004). Restorations that can combine these beneficial environmental factors (soil type, dynamic wetland hydrology, and biogeochemical conditions) may show high rates of denitrification because the microbial organisms capable of performing this process have all the beneficial environmental variables already working for them (Moseman *et al.* 2010). Studies targeting these variables as the primary source of restoration potential have observed greater rates of denitrification in the years post restoration (Klocker *et al.* 2009, Inglett *et al.* 2011). Combining the physical parameters (e.g., hydrology, soil type) of wetlands restoration with a survey of the bacterial community performing denitrification is integral to future restoration projects.

Because of recent work done in the field, some studies have started to focus on the restoration of the microbial communities present in the wetland ecosystem (Song *et al.* 2010, Inglett *et al.* 2011). Instead of focusing on just the restoration of hydrophytic plants, efforts have been made to also ensure that the denitrification potential of the microbial communities is restored as well. Some studies have chosen to focus on microbial aspects of the system, as it is these communities that perform many of the biogeochemical processes to be restored (Harris 2003, Buckley and Schmidt 2003). Other studies have examined the role anthropogenic forces play in shaping microbial communities and how variable these communities can be in both structure and function (Hartman et al. 2008, Smith and Ogram 2008). What all of these have in common is the fundamental recognition that the microbial community cannot be ignored. Future studies must continue to focus on how the microbial community adapts through all phases of the restoration (Song *et al.* 2010). It is important, therefore, to determine whether or not a restoration can be successful. While many studies have observed how microbial communities react during and after a restoration has occurred (e.g., Peralta *et al.* 2010, Inglett *et al.* 2011, Song *et al.* 2010) few have attempted to gauge how a soil microbial community targeted for restoration will adapt *prior to* any field alterations. Determining if the targeted soil community could successfully adapt to restored wetland conditions (e.g., wetland hydrology) and return high rates of denitrification to the site would have significant implications for the field of restoration ecology. One way to study this is through a transplant experiment where soils from the attempted restoration site are placed in a natural wetland (Reed and Martiny 2007, Balser *et al.* 2002). If the microbial community is able to regain biogeochemical function (specifically in showing increased rates of denitrification) given all the correct environmental parameters, that could suggests that a whole site restoration effort could be effective at rebuilding proper wetland function.

Microbial resiliency, redundancy, and dormancy in wetlands:

Because of the extensive variability seen in wetland environmental conditions, diverse microbial populations will be able to populate the area and flourish. Certain microbes will be able to function in the dry, aerobic conditions while others will be able to function in the wet, anaerobic conditions (Gutknecht *et al.* 2006). Some organisms, namely facultative anaerobes, will be able to function during both sets of conditions; using aerobic respiration when the soils

are oxygenated, and aerobic respiration (denitrification) when the soils are anoxic. During periods of sub-optimal conditions for certain phylogenetic groups of microbes, they will be able to either switch to new terminal electron acceptors (switch from oxygen as a TEA to nitrate) or undergo a "hibernation" of sorts until favorable conditions return (Roszak and Colwell 1987, Jones and Lennon 2010). In this sense, microbial communities can become dormant for extensive periods of time and then return to full functionality when environmental conditions become favorable once again (Epstein 2009). Due to microbial resiliency and dormancy, it is possible that once remnant wetland soils are uncovered and restored, the microbes that have lain dormant for decades can return to normal function (Bossio et al. 2006). This idea follows the basic premise of "everything is everywhere, but the environment selects" (Baas-Becking 1934), meaning that microorganisms are not dispersal limited. So if wetland hydrology is restored, desired (e.g., denitrifying) bacterial communities should be available to regain denitrifying function in the soil.

The adaptability of microorganisms creates optimal conditions for functional redundancy – where multiple organisms inhabiting a given habitat are capable of performing the same tasks (Yannarell *et al.* 2007). When delving into a molecular level, even though the microbes themselves are phylogenetically different, they contain genes capable of performing the same function (Moseman *et al.* 2009). In some cases, ecosystem perturbations will inhibit certain populations of bacterial communities, however, due to functional redundancy, ecosystems processes (e.g., denitrification) will continue because other bacterial populations will not be affected by the perturbation (Bodelier 2011). In ecosystems exhibiting high levels of microbial diversity, the ecosystem function itself (e.g., carbon cycling, denitrification, etc.) is resistant to disturbances because a wide range of taxa can contribute to the process (Song *et al.* 2010). When systems were studied that exhibited low microbial diversity, community resistivity dramatically decreased – resulting in a loss of ecosystem function (Bodelier 2011, Gamfeldt *et al.* 2008).

This idea can be applied to microorganisms that carry out denitrification in wetlands. Many different bacterial taxa are capable of performing denitrification and that is because, even though phylogenetically different, they share the set of enzymes responsible for catalyzing nitrate reduction (Sylvia *et al.* 2005, Rich *et al.* 2003). Functional redundancy then becomes especially important in wetland ecosystems where the environmental conditions change so frequently. With multiple microbes capable of performing the same task, a more stable

community is created and biogeochemical transformations and ecosystem function will continue even if certain microbial communities are inhibited at a given time. Extensive microbial diversity allows for functional stability because a wide range of taxa can perform the same function. So if certain populations of denitrifiers are inhibited by a perturbation, others will remain resistant or even thrive, to that perturbation and denitrification will continue (Bodelier 2011). Both microbial resiliency and functional redundancy are important in ensuring the rehabilitation of high rates of denitrification in a wetland restoration project (Allison and Martiny 2008). Determining the denitrification potential of pre-restoration sediments in conjunction with microbial community composition present in the sediments, could be quite useful in aiding ongoing restoration efforts.

Summary

Microbial communities present in wetland ecosystems are extremely important in supporting high rates of denitrification. In order to achieve this, wetland restoration practices must shift from merely providing something that looks like a wetland, to developing an ecosystem that functions (biogeochemically, and hydraulically) like a wetland. In projects where remnant hydric soil will be utilized for a wetland restoration, it is important to determine how the microbial communities still contained within the soil layer will respond to restored dynamic hydrology and a returned hydrophytic plant community. Successful restoration of denitrification banks on the idea that the denitrifying community can be restored to full functionality when placed back in dynamic hydrological conditions. Taking a targeted approach to wetlands restoration, by studying how microbial communities shift in both structural composition and function, is extremely important in determining if the site targeted for rehabilitation will perform high rates of denitrification.

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CHAPTER 3

Assessing microbial community composition and denitrification potential in a stream targeted for restoration

Abstract

Microbial communities are responsible for nitrogen cycling in wetland ecosystems. Recent wetland restoration work has stressed the importance of returning high levels of denitrification to the restored ecosystems. High rates of denitrification can reduce nitrate loads entering aquatic ecosystems and improve water quality. To investigate the denitrification potential of a given wetlands restoration, microbial community composition (MCC) and denitrification (DeN) potentials were observed for soil strata found at a site targeted for wetland restoration and compared to 4 local natural and restored wetland ecosystems. At this former mill pond site, hydric soil has lain in an altered state (buried under legacy sediments) for the past 200 years. This study observed whether communities present in the altered hydric soils are capable of performing denitrification once the overlying legacy sediments are removed. The 5 locations exhibited a broad range of denitrification potential and total microbial community structure, but the relict hydric soils exhibited significantly lower rates of denitrification than any of the other wetland soils over the three sample periods ($F_{[4,31]}$ =13.75, $p<0.0001$; $F_{[4,42]}$ =84.6, $p<0.0001$; and $F_{[4,35]}$ =71.89, $p<0.0001$ respectively). The denitrifier community was surveyed, and significant differences were observed in the microbial communities inhabiting each soil strata (ANOSIM $R=0.306$, $p<0.001$). When the relict hydric soil was compared to reference wetland sites no differences were observed in the denitrifier communities ($R= 0.127$, $p=0.079$), few denitrifying bacterial populations were amplified in the relict soil contributing to a wide spread is similarity. Denitrifying bacteria still inhabit the relict hydric soil even after hundreds of years of altered conditions. However the denitrifying potential of these communities are significantly lower than any of the other surveyed wetland microbial communities.

Introduction

Processes carried out by microbial communities are fundamental determinants of nutrient cycling in wetland ecosystems (e.g., Song *et al.* 2010, Peralta *et al.* 2010, Gutknecht *et al.* 2006). Wetland ecosystems are noted for their abilities to support various transformations in the nitrogen cycle, and denitrifying bacteria inhabiting the hydric soils of these wetlands exclusively drive the high rates of denitrification typically observed at natural wetland sites (Hartman *et al.* 2008). Anthropogenic land use changes have significantly reduced the acreage of wetland ecosystems over the past 200 years (Vitousek *et al.* 1997, Dahl 1990), as people sought to drain or bury these purported "useless" ecosystems (Ruhl 2000). Locations that previously supported functional wetland habitats were subsequently destroyed, either becoming agricultural plots through burial or drainage, or becoming ponds and lakes behind dammed rivers (Dahl 1990, Walter and Merritts 2008). These land use changes significantly altered biogeochemical cycles, particularly in the nitrogen cycle. The coupling of wetland function loss with increased anthropogenic exports of nitrogen has resulted in higher nitrate loads entering aquatic ecosystems (Dahl 1990, Kemp *et al.* 2005, Rabalais *et al.* 2002).

Higher nitrate loads contribute significantly to degraded water quality (e.g. Kemp *et al.* 2005, Kaushal *et al.* 2008, Rabalias *et al.* 2002), as increased nutrient loads can lead to eutrophication and subsequent hypoxia of coastal ecosystems (Ruhl 2000, Rabalais *et al.* 2002). Hypoxic conditions strongly alter marine ecosystems, resulting in extensive fish kills and reduced water quality, which ultimately can contribute to economic losses (Ruhl 2000, Kemp *et al.* 2005, Vitousek *et al.* 1997). Acknowledging this problem, efforts have been made to try and reduce nitrogen loads into aquatic ecosystems. As a result, wetland restoration projects attempting to increase rates of denitrification in riparian zones have increased (Smith *et al.* 2008, Kaushal *et al.* 2008, Filoso and Palmer 2011). These restoration projects seek to rehabilitate denitrification potential by recreating environmental conditions typical of wetland ecosystems (e.g., dynamic hydrology, hydric soils, high organic matter, and hydrophytic plants) (Bruland and Richardson 2006, Hartman *et al.* 2008, Craig *et al.* 2008). Even with the same stated goals, results have been mixed among sites (Bernhardt and Palmer 2011). Certain restoration projects have reported significantly increased rates of denitrification, and a well-established denitrifying community (Song *et al.* 2010, Smith and Ogram 2008) while others have reported no net change in either the microbial community composition or the rates of denitrification (Orr *et al.* 2007,

Bossio *et al.* 2006). The lack of improvement could result from a variety of environmental factors (e.g., soil structure, hydrology, pH, water chemistry, etc.) which all will have significant effects on the structure and function of the denitrifier community (Blume *et al.* 2002, Fierer *et al.* 2003, Gutknecht *et al.* 2006). Most restorations use reference natural wetlands as a standard for the type of environmental conditions necessary to support active denitrifying bacterial communities (Brinson and Rheinhardt 1996, Spieles 2005, Peralta *et al.* 2010).

Some restoration projects are unique because the original hydric soil that previously supported a wetland can still be found intact underneath the anthropogenic land alteration (Walter and Merritts 2008). In these cases, dams were erected near wetlands, and the reservoir that formed behind the dam turned the wetland into an open body of stationary water (Walter and Merritts 2008). As a result, sediment that normally would have flowed through the wetland was deposited on top of the relict hydric sediments, effectively burying the former wetland. In the restoration, the dam and deposited sediments are removed, re-exposing the original remnant wetland soil (Forshay and Mayer 2009). This original hydric soil (and thus the communities inhabiting it) is then used for the targeted wetland restoration.

Work has been performed in a variety of ecosystems showing the resiliency of microbial communities when faced with a significant disturbance (e.g., Yannarell *et al.* 2007, Jones and Lennon 2010, Moseman *et al.* 2009); however these disturbances are generally only for a short period of time – ranging from a couple of weeks to a couple of years. Due to the relatively short time frame, the microbial communities are resilient enough to survive the disturbance, and then regain their initial function after the disturbance period. In cases of ecosystem alteration (such as burial), some microbial communities can enter a period of dormancy (Jones and Lennon 2010, Allison and Martiny 2008) and essentially hibernate in a state of low metabolic function until favorable conditions occur. This theory is applicable to wetland soils impacted by sedimentation and altered hydrology, as it is important to determine if the microbial communities extant in these altered soils can regain functionality (particularly denitrification) once wetland environmental conditions return (e.g., dynamic hydrology, nutrient recharge, anaerobic conditions). However the hydric soils found at such former mill pond sites targeted for restoration, and the microbial communities that inhabit them, have often been in an altered state for a much longer period of time (up to hundreds of years). In order to gauge the potential for successful restoration of denitrification, it is important to determine if the microbial communities

found at these proposed restoration sites can perform denitrification when favorable environmental conditions are returned.

To address this issue, the microbial community compositions and denitrification potentials of a buried remnant hydric soil were examined at a site of a future wetland restoration. The Big Spring Run (BSR) restoration project located in Lancaster, PA is attempting to reconstruct a wetland in the location of a former marsh ecosystem (Forshay and Mayer 2009; Fig. 3.1). The site was originally a wetland prior to European settlement, but the main stream exiting the wetland was dammed in the 1700s when a grain mill was constructed, causing the wetland to flood and become a permanent pond (Walter and Merritts 2008, Forshay and Mayer 2009). The dam was removed in the early 1900s, but the ponding of the system resulted in depositional sediments settling out on top of the former wetland soil (Forshay and Mayer 2009; Fig. 3.2). While the water table and stream of the site has returned to its original elevation, cutting though the layers of the deposited "legacy" sediments, the microbial communities in the hydric soils remain buried and disconnected from typical wetland hydrology. The restoration of this site will unfold in 2 steps: the removal of all legacy (deposited) sediments to restore wetland hydrology, and the reseeding of hydrophytic plants typically observed in wetlands across eastern Pennsylvania. The restoration aims to achieve higher rates of denitrification for the system that will result in a reduced nitrate load into the surrounding streams (Fig. 3.3).

The objectives of this study are as follows: to (1) survey the microbial community composition of all the soil strata found at BSR (surface, legacy, hydric, and stream) and determine the denitrifying potential of each stratum; (2) determine the difference in both denitrifying potential and microbial community composition between stream-exposed hydric sediments and sediments that have been completely buried; (3) survey the denitrifying bacterial community found in all soil layers present and the BSR restoration site; and (4) determine how the denitrifier bacterial community and the rates of denitrification compare to reference wetland sites located near BSR. Assessing these four objectives will provide insight into the potential for a wetland restoration at BSR to restore both denitrifier communities and the overall denitrification process.

Materials and Methods

Study Sites:

Soil bacterial communities were collected from various stream and wetland environments located in Lancaster County, Pennsylvania. The sites sampled represented a variety of habitats ranging from nearby (or adjacent) natural, undisturbed wetlands (Low Marsh and Wet Meadow), to restored wetlands (Banta wetland and Millport wetland), to current fluvial systems targeted for wetland restoration (Big Spring Run) (Table 3.1). The sites targeted for restoration (BSR) and the sites post-restoration (Banta and Millport) represent the same original environmental conditions – all were wetlands prior to the 1700s that were flooded during the 1800s and early 1900s when mill dams were constructed downstream. Subsequent deposition of sediments buried the wetland hydric soils, so when the dams were removed, several feet of deposited sediments rested on top of the former wetlands soils. The study sites enable comparisons by: (1) providing insight to microbial community structure and function in the current altered conditions compared to natural and restored wetland settings and (2) allowing for a comparison of pre-restoration conditions to post-restoration conditions of the soil microbial communities.

Sample Collection:

Initial samples collected at Big Spring Run consisted of 0-20 cm horizontal cores into an incised stream bank where surface, deposition, and hydric sediments could be found. Four horizontal cores were collected for each strata (surface, legacy, hydric, and stream) at 3 locations along the stream reach – one along each channel before the confluence point and one after the confluence with a 4 cm diameter plastic soil coring tube. Initial soil strata samples at BSR were collected in November 2009. Samples were stored briefly at 4^{o}C until denitrification assay could be completed, and subsamples were stored at -80^{o}C awaiting DNA extraction for microbial analyses.

To compare BSR hydric soils to surrounding natural and restored wetlands, additional exposed hydric sediment samples, along with natural and restored wetland soils, were collected in November 2010, March and June 2011. Eight vertical cores (0-20 cm in depth) were collected at each wetland location to compare to the relict wetland soil. To observe if stream exposure affected microbial community composition and function, horizontal cores were collected at 0-20 cm from the stream cut, and also at 60-80 cm from the exposed stream bank (distance away from

stream cut) during the November 2010, March 2011, and June 2011 sample periods. BSR strata cores were collected using a 4 cm diameter plastic soil coring tube (horizontal cores – into the bank), while reference wetland sites were collected with a 3 cm diameter metal soil sampler. Cores in the reference sites were collected for the 0-20 cm vertical depth. Cores were stored briefly following collection at 4^{o}C in the field until denitrification potentials could be assessed, and subsamples were stored at -80^{o}C until further DNA processing could be completed.

Soil Analysis:

Denitrification assays were completed using a modified version of the acetylene block method as used by the USEPA (Forshay and McElmurry, 2009). Briefly, in the modified version of this method, 25 g of soil from each site was measured into a 125 ml Wheaton jar complete with sealed cap and septa. Nutrient media containing dextrose and nitrate was added to each jar to provide conditions capable of measuring the maximum denitrification potential of the sampled soil. Jars were incubated at *in situ* observed temperatures for hours, with initial (t=0) and final (t=3) concentration of N_2O recorded. The protocol was followed as outlined by the EPA certified method (RSKSOP #310; Forshay and McElmurry 2009), and upon completion of the process a denitrification rate was assessed to each soil sample collected. N_2O concentrations were recorded at both the beginning and end of the incubation period to determine the rate of nitrous oxide evolved during the assay. Values were multiplied by 10^9 to give standardized values (recorded as nmoles N_2O hr^- g dry $soil^-$). Remnant soils from the assay were then dried over 5 days at 50^{o}C to provide a soil dry weight. Soils were then muffled at 500^{o}C for 4 hours to provide an estimate of organic matter (OM) found at each soil strata or location.

Soil DNA extraction and purification:

Rocks and roots were removed from collected soil, and samples were freeze dried to remove the water. After freeze drying, 500 mg of each soil sample was weighed out for DNA extraction using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH). DNA extraction was achieved following the instructions provided by the manufacturer. Following DNA extraction, purification with cetyl trimethyl ammonium bromide (CTAB) was carried out to remove the humic acids (Sambrook *et al.* 2001). Following purification, samples were stored at -20^{o}C until further processing could be completed.

Community composition analysis:

Bacterial community composition was determined using the automated ribosomal intergenic spacer analysis (ARISA) as described by Fisher and Triplett (1999). The intergenic spacer region between the 16s and 23s rRNA genes was amplified using the polymerase chain reaction (PCR) method with 6-FAM labeled, universal 16S rRNA primer 1406F (5'-TGYACACACCGCCCGT-3') and 23SR (5'-GGGTTBCCCCATTCRG-3') targeting the bacterial 23S rRNA gene. Thermal cycling conditions for this PCR protocol consist of an initial denaturation at 94^{o}C for 2 min, followed by 30 cycles of 94^{o}C for 35 sec, 55^{o}C for 45 sec, and 72^{o}C for 2 min. After completion of the 30 cycles, a final extension period of 72^{o}C for 2 min was carried out. This process was carried out in an Eppendorf MasterCycler Gradient (Eppendorf AG, Hamburg, Germany). The length of fragments was determined using denaturing capillary electrophoresis using an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Electrophoresis conditions were 63^{o}C and 15 kV with a run time of 120 min. A custom 100- to 1,250-bp rhodamine X-labeled (ROX) size standard (Bioventures) was used as the internal size standard for ARISA fragments.

Denitrifier community composition was assessed in the various locations using terminal restriction fragment length polymorphism (T-RFLP). For this project, the denitrifier populations refer to the set of microorganisms than can carry out the last step of denitrification, the conversion of $N_2O \rightarrow N_2$. The PCR reactions to amplify *nosZ*, which encodes the catalytic subunit of nitrous oxide reductase, allow comparison of soil denitrifier populations among BSR soils and reference wetland soils. The *nosZ* gene was amplified using the *nosZ-F-1181* (5'-CGCTGTTCITCGACAGYCAG-3') and the *nosZ-R-1880* (5'ATGTGCAKIGCRTGGCAGAA-3') to yield a 700 bp PCR product (Rich *et al.* 2003). The *nosZ* reverse primer was labeled with the phosphoramidite dye 6-FAM. Thermal cycling conditions for this PCR protocol consisted of an initial denaturation of 94^{o}C for 3 min, followed by 25 cycles of 94°C for 45 sec, 56°C for 1 min, and 72°C for 2 min, with a final extension carried out at 72°C for 7 min. The Qiagen MinElute PCR purification kit (Qiagen, Valencia, CA) was then used to combine and concentrate *nosZ* PCR products from two 50 µl reactions. PCR products amplified from each sample were then digested in single-enzyme restriction digests containing *Alu*I, *Hha*I, or *Mbo*I. The length of fragments was determined using denaturing capillary electrophoresis using an ABI

3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Electrophoresis conditions were 63^{o}C and 15 kV with a run time of 120 min. An ABI GeneScan ROX 1000 size standard was used as the internal size standard for *nosZ* generated fragments.

Size-calling was carried out using Genemarker version 1.85 (SoftGenetics, LLC, State College, PA, USA) by aligning peaks using the ROX-1250 standard for ARISA fragments and the ABI ROX-1000 standard for *nosZ* fragments. Each fragment represents a microbial population, and the combination of all the fragments from a given sample (termed "community fingerprint") was considered to represent the total assemblage of microbial populations present in a given soil sample. For bacterial ARISA, the fragments between 300-1000 base pairs in length were used for constructing community fingerprinting. For *nosZ* T-RFLP analysis, the fragments between 100-650 base pairs in length were used for constructing the denitrifier community fingerprint. The *nosZ* fragment analysis results from the 3 digests were concatenated and analyzed together to give a representation of the denitrifier community.

Statistical Analysis:

To compare total bacterial community composition (through ARISA) and denitrifier community composition (through *nosZ* T-RFLP) among the soils collected, the software package PRIMER 6 for Windows was used (PRIMER-E, Plymouth, United Kingdom). A Bray-Curtis similarity coefficient was generated among all pairs of samples which was used to create non-metric multidimensional scaling (NMDS) plots. These plots allow for the (dis)similarity of particular strata of soil to be plotted in ordinal space. Dissimilar microbial soil communities plot far away from one another while similar soil microbial communities plot close to each other. Comparisons in microbial community structure were able to be made between the various soils collected.

Differences in microbial community composition were tested using the analysis of similarity (ANOSIM) procedure on pairwise Bray-Curtis similarity values (Clarke and Warwick 2001). The ANOSIM procedure produces a test statistic, R, that is calculated based off of the following formula: $R = (r_b - r_w)/[1/4n(n-1)]$, where n is the total number of samples, r_b is the average of rank similarities from all groups of replicates between different sample sites, and r_w is the average of all rank similarities among sample replicates. The R test statistic calculated by the ANOSIM function provides a degree of similarity value between the various soil strata and

locations tested. The values range from 0 (the groups are identical) to 1 (the groups are completely dissimilar). Calculations of similarity coefficients and ANOSIM were carried out using PRIMER 6 for Windows (PRIMER-E, Plymouth, United Kingdom). Difference in denitrification (DeN) rates were analyzed using the analysis of variance (ANOVA) test with a post-hoc Tukey's test to determine differences in DeN rates between individual layers. Significances were determined at a $p<0.05$ level.

Results:

BSR Strata comparison

In assessing strata denitrification in November 2009, there was a significant difference in denitrification potential between the surface sediments and the legacy, hydric, and stream sediments collected ($F_{[3,20]} = 10.1$, $p = 0.0002$) (Fig.3.4, Table 3.2). The microbial community composition analysis of these strata showed a significant difference between the 4 exposed soil strata at BSR (surface, legacy, hydric, and stream) (ANOSIM global $R=0.514$, $p=0.001$; Table 3.3). Hydric soils had a high variability in MCC, exhibiting a high level of dissimilarity within group in relation to community composition in comparison to the more similar arrangement of the surface soils (Fig 3.5a). Comparisons in denitrifier community reflected these results, with significant differences shown between the surface, legacy, and hydric soils at BSR (Fig. 3.5b). Analysis of the *nosZ* data showed that more denitrifier populations were found in surface soils than either the relict hydric soil or the legacy sediments ($F_{[2,75]} = 25.43$, $p < 0.0001$; Fig 3.5c).

Denitrification potential in BSR hydric soils vs. reference wetland sites

When the targeted restoration soil (BSR hydric) was compared with the other wetland sites in 3 seasons (Nov. 2010, March and June 2011), denitrification potential was significantly lower in the hydric soils of BSR than in any of the other surveyed wetlands (Nov 2010 $F_{[4,31]} = 13.75$, $p<0.0001$; March 2011 $F_{[4,42]} = 84.6$, $p<0.0001$; June 2011 $F_{[4,35]} = 71.89$, $p<0.0001$) (Fig. 3.6, Table 3.4). No significant difference was observed in denitrifying potential between the buried hydric soils (60-80 cm horizontal cores) and the exposed hydric sediments (0-20 cm horizontal cores) when the two were examined over the three sampling periods ($t=1.982$, $p=0.16$). When comparing among sampling dates, all reference wetland and BSR hydric soils showed a significantly higher denitrification potential during the June sampling date compared to

samples collected in cooler months with the exception of the WM soils ($p<0.01$ for all strata except WM, Fig. 3.6).

Total Microbial Community Composition (MCC) for site locations

When all BSR strata microbial community fingerprints were combined to form a total BSR microbial community, the site was had a significantly different community makeup from the Banta (ANOSIM R=0.264, p=0.003) and Millport (R=0.388, p=0.001) wetland restoration sites. Smaller differences were observed between the BSR and Wet Meadow (ANOSIM R=0.085, p=0.149) and BSR and Low Marsh (R=0.239, p=0.002) communities. However each of the natural and restored wetlands surveyed had distinct assemblages of bacterial communities from each other with the exception of Low Marsh and Banta (Fig. 3.7a, Table 3.5). Exposed (0-20 cm core) and buried (60-80 cm core) relict hydric sediments were then separately compared with the natural and restored wetlands, as the surface and legacy sediments will be removed. A significant difference was observed between the two hydric locations chosen for sampling at BSR (ANOSIM R=0.92, $p<0.001$); however no significant difference was observed between the exposed and buried hydric sediments from their respective sites (ANOSIM R=0.144, p=0.84; R=0.376, p=0.012 for two locations surveyed). The community composition of BSR hydric soils was significantly different from any of the surveyed wetland sites (ANOSIM R=0.693, $p<0.001$) (Fig. 3.7b, Table 3.5). Denitrifiers were observed in the relict hydric soil of BSR, however a high level of variability was observed (Fig. 3.8). No significant difference was observed in denitrifier population when the restored and disturbed wetlands were compared with the BSR exposed and buried hydric soils (ANOSIM R=0.127, p=0.079; Fig 3.8).

Discussion:

Anthropogenic land use changes can have a profound impact on microbial community structure and function (e.g., Jones and Lennon 2010, Allison and Martiny 2008, Moseman *et al.* 2010), with altered environmental conditions strongly driving microbial ecosystem response (Gutknecht *et al.* 2006, Moseman *et al.* 2009, Smith and Ogram 2008, Hartman *et al.* 2008). Loss of dynamic wetland hydrology and linkage with hydrophytic plants can strongly depress rates of hydric soil denitrification, leaving nitrate available to flow into aquatic ecosystems (Orr *et al.* 2007, Kemp *et al.* 2005, Rabalais *et al.* 2002, Hartman *et al.* 2008). As wetland restoration

projects have sought to restore high rates of denitrification, interest in the denitrification potential of the microbial community targeted in the restoration has increased (Inglett *et al.* 2011, Song *et al.* 2010, Kaushal *et al.* 2008). However, when anthropogenic land use changes have fundamentally altered an ecosystem for hundreds of years (as in the case of BSR), it is often unknown what effect the extended ecosystem alteration will have on microbial structure and function when a restoration is attempted. Work has repeatedly shown the fundamental resilience of microbial communities (e.g., Allison and Martiny 2008, Yannarell *et al.* 2007, Jones and Lennon 2010), and the high inertia of soil microbial communities – basically their resistivity to change when met with an environmental alterations (e.g. Balser *et al.* 2002). That work, however, has mostly dealt with disturbances lasting weeks to months. What this study sought to observe was how microbial community structure and function of a long-term altered hydric soil (BSR) related to surrounding natural and restored wetland community structure and function.

In hydric soils that have been significantly altered from their natural wetland state, depressed rates of denitrification were observed. The rates of denitrification observed in the BSR hydric soil were significantly lower than their references in natural and restored wetland ecosystems. Rates of denitrification did improve in hydric soils during warmer months, but the range of improvement was still significantly below any of the reference sites (see Table 3.4). While the surface soils at BSR exhibited rates of denitrification similar to those found in wetland systems (see Table 3.2 and 3.4), these soils will be removed during the restoration, taking with them any denitrification potential they could bring to the restored system. These results underscore the complex nature of wetland restoration projects. The relict soils targeted for restoration currently do not perform high rates of denitrification, and if this problem persists, could lead to a failed biogeochemical restoration, as described by Orr and colleagues (2007). However, there are a few factors that suggest that a hydrological restoration should improve the low denitrification rates observed in the buried hydric sediments.

A major difference between all of the strata observed at BSR is that the surface sediments are currently the only soils in contact with plant communities. Previous work has stressed the importance of plant-microbe interactions (e.g. Spieles 2005, Reynolds *et al.* 2003) in enhancing wetland denitrification, and this lack of any plant-microbe interactions could significantly constrain high rates of denitrification in BSR hydric soil under current conditions (Speiles 2005). Wetlands in local restored watersheds utilizing the same restoration technique as BSR (e.g.,

Millport and Banta found near BSR) have shown significantly higher rates of denitrification than their counterparts in the current buried conditions. This is important because it suggests that the hydric soils can be rehabilitated to perform high rates of denitrification if wetland hydrology is restored. These two wetland sites in restored watershed have denitrification potentials orders of magnitude greater than the relict buried soils (see Fig. 3.6) so if this process can be replicated during the restoration of the BSR hydric soils, similar rates of denitrification should be observed.

Hydrology and soil physical issues also contributed to the depressed rates of denitrification. Under current buried conditions, the BSR hydric soil is compressed to a higher level of compaction than any of the surrounding natural wetlands in the area, as shown by Walter and Merritts (2008). The burial of these soils led to a removal from wetland hydrology – which allows water and nutrients to readily move through the hydric soils. In natural wetlands, wetting and drying cycles are frequent (e.g. Unger *et al.* 2009), which support denitrifier communities. This cycling allows for nutrients (specifically nitrate) to re-enter the system, so during saturated conditions which support anaerobic activities, denitrification can occur at rapid rates (Inglett *et al.* 2011, Unger *et al.* 2009). The BSR hydric soils have remained detached from any semblance of this cycle for over 200 years. The ponding and subsequent burial of these hydric sediments has prevented runoff nutrients from entering the anaerobic relict soils, and likely contributes to the depressed rates of denitrification observed.

The current microbial communities inhabiting the surface sediments have the ability to perform high rates of denitrification while the other strata contain communities that do not, even when provided with ideal conditions (e.g., anaerobic conditions, high levels of labile carbon and nitrogen). Also, the summer hydric comparison to nearby wetlands (Fig. 3.7b) shows that the buried hydric communities at BSR are significantly different from all other wetland microbial populations. It is important to note that while all of the wetland microbial communities surveyed have distinct total microbial assemblages from one another (Table 3.5), their denitrifying bacterial communities are not especially distinct (Fig. 3.8), even from the relict BSR soil – which is currently not functioning.

While denitrifiers are present in the BSR soil, group similarity (e.g., similarity between relict hydric soil samples) was low. While T-RFLP data does not give specific information on abundance, from the data analyzed it was clear that fewer distinct populations inhabited the hydric soils of BSR than any of the other wetlands ($t=10.21$, $p<0.0001$). So overall, a greater

diversity of denitrifiers was observed in the subsamples of the natural and restored wetlands than in the relict soil (e.g., more distinct peaks in T-RFLP = more distinct denitrifier communities). A higher level of diversity can foster a greater level of microbial (and functional) redundancy (Allison and Martiny 2008) – where many phylogenetically distinct organisms can perform the same desired function (e.g., denitrification). Because the diversity of denitrifiers is significantly lower in the relict soil than in any of the surveyed reference wetlands, alterations can completely inhibit function (denitrification) in the relict soil, while in the reference wetlands some microbial communities will resist the alteration and still perform high rates of denitrification. This high level of denitrifier diversity in the reference wetlands creates functional stability – allowing the ecosystem to retain function even when faced with a significant disturbance.

Denitrifiers are present in the buried hydric soil, yet are not currently performing significant rates of denitrification. Research, as performed by Song and colleagues (2010) and Smith and Ogram (2008), showed how denitrifying communities responded to a restoration gradient – eventually returning to full denitrification functionality as time progressed. Banta and Millport back this conclusion up – as both are wetland restoration sites that have had restored hydrology for multiple years, and currently show significantly higher rates of denitrification than the BSR relict soil. Because the BSR restoration will follow the same plan as these sites, an extended return of wetland hydrology should promote higher rates of denitrification performed by denitrifying organisms in the relict soil. Soil microbial communities are noted for being slow to respond to environmental change (Balser *et al.* 2002, Reed and Martiny 2007), so it is not surprising that when presented with the correct denitrifying conditions, the BSR hydric communities did not immediately respond. The findings of this study suggest that microbial communities, and specifically some denitrifying organisms, are still present in the relict wetland soil of BSR. Even though denitrifying microorganisms are present, the denitrification potential of the soil is still significantly below any of the surveyed reference sites. Because of the altered environmental conditions of the BSR hydric soil, it may take a longer period of time (e.g., Song *et al.* 2010, Balser *et al.* 2002, Reed and Martiny 2007) for these organisms to full functionality.

Conclusion:

Environmental disturbances are strong drivers in microbial community function and structure. Anthropogenic land use changes have significantly altered the environmental

conditions in many wetland ecosystems, disturbing the microbial community, and changing many of the biogeochemical cycles controlled by these habitats. By surveying a site targeted for wetland restoration, this study showed that the denitrifying potential of the relict hydric soil is significantly lower than any surveyed reference sites. Microbial community composition was also significantly different between all 5 of the wetland soils surveyed. However, even though the microbial community structure was significantly different between each of the 4 reference wetland sites, denitrification still occurred at high rates at these sites. These results suggest that no specific microbial community is needed to perform denitrification – microbial redundancy allows for a wide range of different microbial populations to contribute to the same process. While the *nosZ* T-RFLP results do not show differences between the five sites, the high variability among denitrifier communities also support functional redundancy – the distinction between soil microbial communities suggests that many different organisms are capable of performing denitrification. Even though denitrifiers are currently present in the relict soil, environmental constraints prevent the community from actively promoting high rates of denitrification. However, the T-RFLP data gives no information on abundance of denitrifying organisms inhabiting the tested soil, so it is possible that the relict soil, while containing denitrifiers, simply has a far lower abundance number of communities than any of the other surveyed sites. The lack of wetland hydrology likely contributes to the depressed rates of denitrification observed. Two wetland sites in the BSR watershed (Wet Meadow and Low Marsh) have already shown improved rates of denitrification once wetland hydrology was returned, and two nearby locations (Millport and Banta) have shown high rates of denitrification after a similar style of restoration occurred. If this process can replicated for the site restoration, higher rates of denitrification should be returned to Big Spring Run.

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Figures:

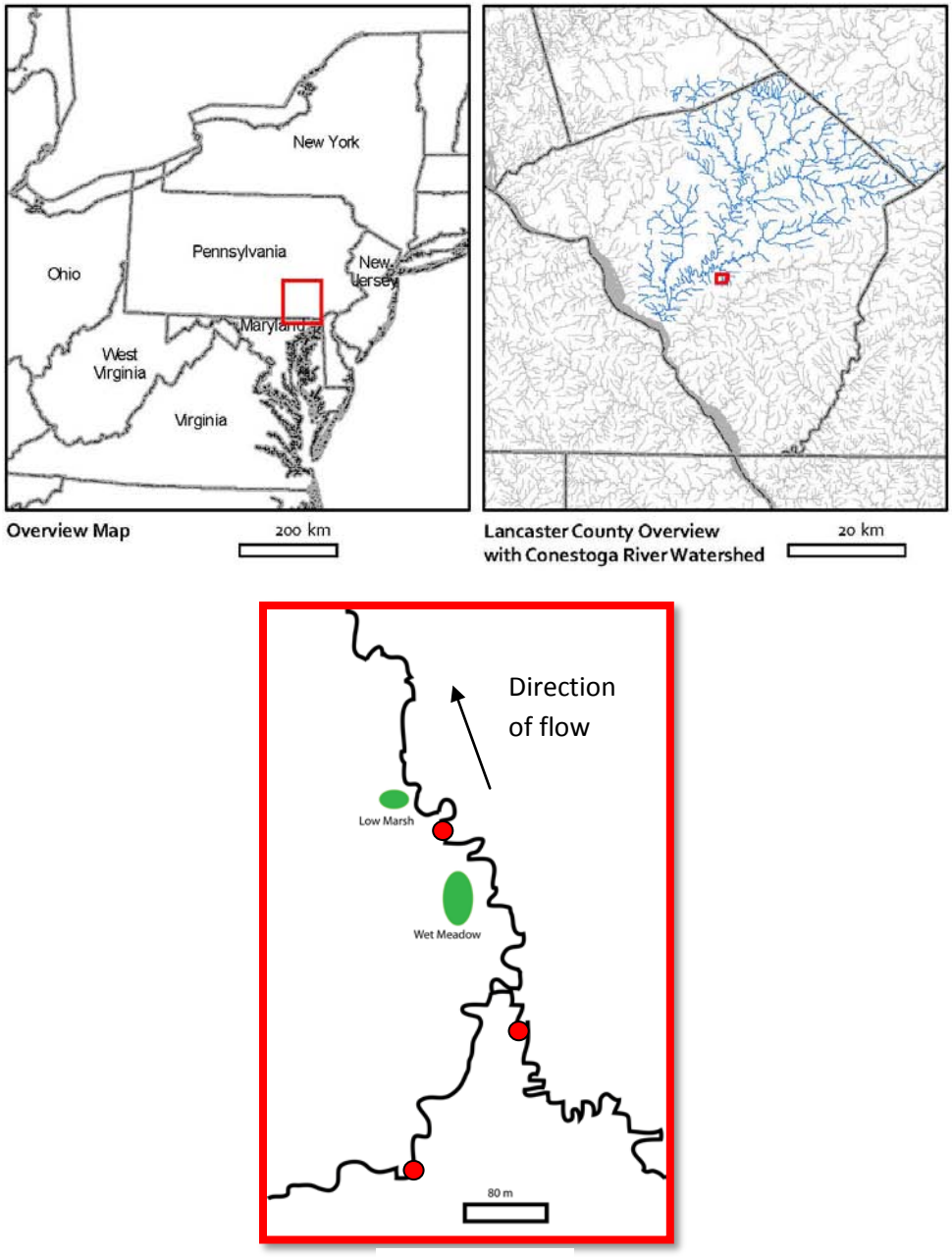


Figure 3.1. Representation of the Big Spring Run system located in Lancaster County, Pennsylvania. Wet Meadow and Low Marsh sites included on map as representation of natural wetlands found along Big Spring Run. Dots along stream reach indicate sample locations (2 locations prior to confluence, 1 after)

Figure 3.2. Picture of current altered environmental conditions at BSR. Bottom dark layer represents relict hydric sediments from a former wetland. Lighter top sediments are depositional legacy soils from ponding. Soils are labeled to represent where the 4 strata were collected. The surface and legacy sediments will be removed in the restoration.

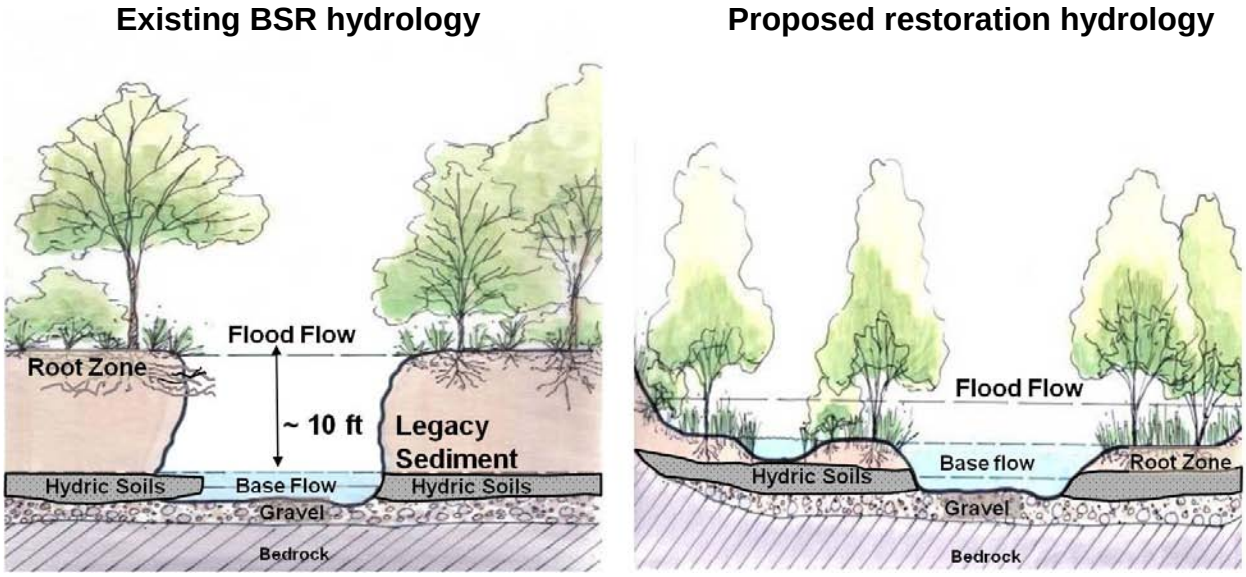


Figure 3.3. Representation of current environmental conditions at BSR and proposed restoration landscape. Legacy sediments will be removed to expose remnant hydric soils and improve hydrology of the site. Under current conditions, floodwaters do not spread out over the floodplain, but rather saturate the channel. The proposed restoration will reconnect the floodplain to the channel.

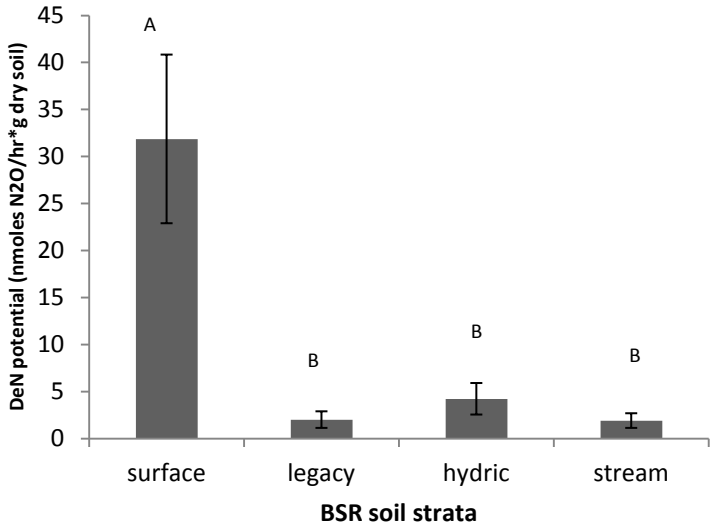
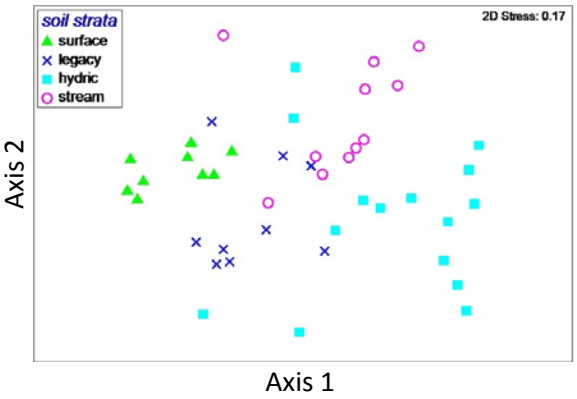
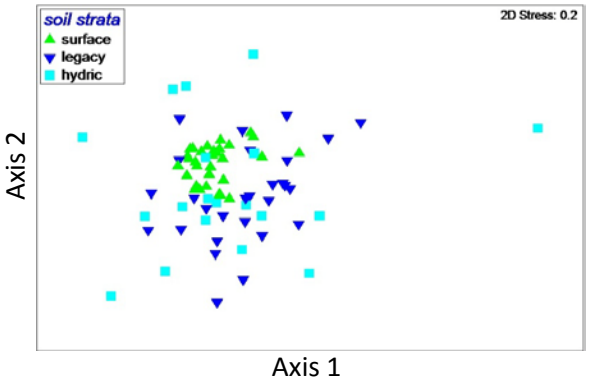


Figure 3.4. Comparison of BSR strata denitrification potential for the Nov. 2010 sampling. ANOVA of strata yielded $F_{(3,20)} = 10.1$, $p=0.0002$. Letters indicate post hoc significance at the $p=0.05$ level.

(A) BSR ARISA (total microbial community)



(B) BSR *nosZ* (denitrifier community)



(C) Denitrifier populations per BSR strata

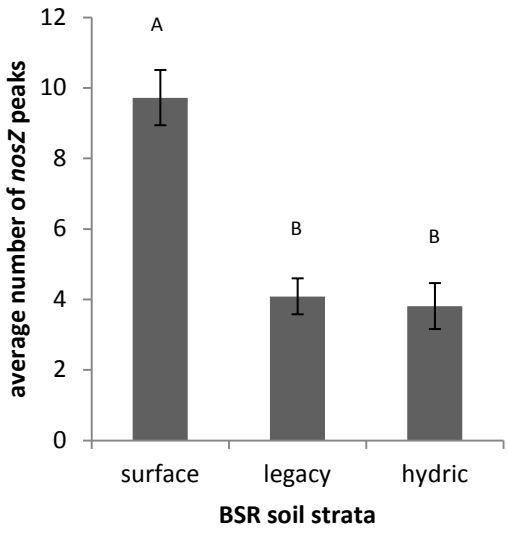


Figure 3.5 Non-metric multidimensional scaling (NMDS) plots for ARISA community composition analysis of BSR soil strata (A) and for *nosZ* denitrifier community composition of BSR soil strata (B). Each point on the plot represents the microbial community composition of a soil sample taken from BSR. Also, an estimate of the average number of unique denitrifier populations per strata is given (C). Each *nosZ* T-RF represents a unique denitrifying population, so number of fragments collected for each sample was averaged and then divided by 3 (to account for the 3 digests run) to give a simple representation of the diversity of denitrifying communities for each BSR strata.

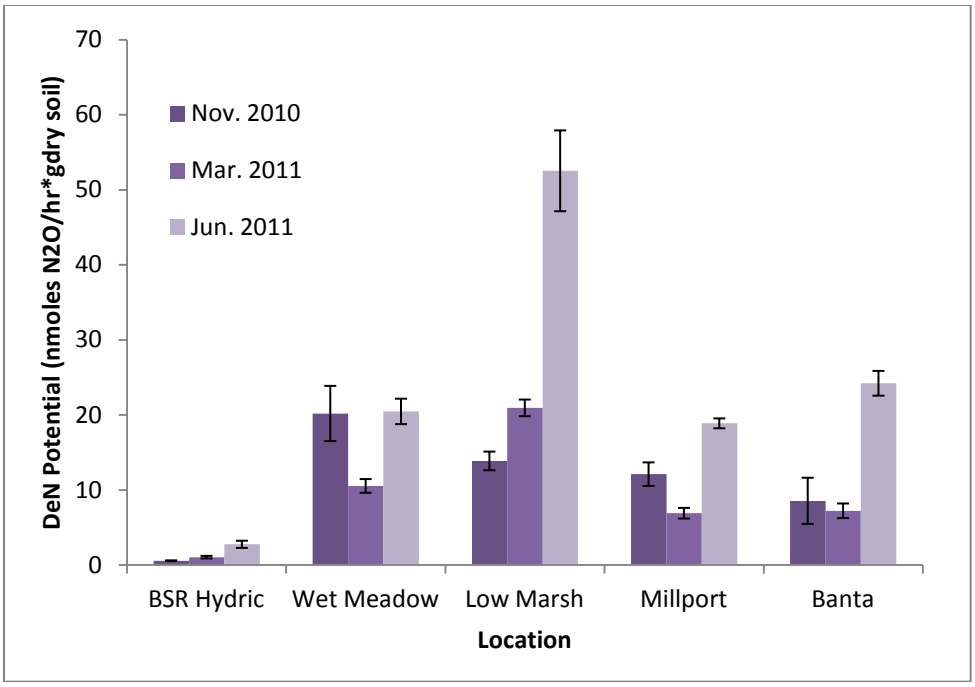


Figure 3.6. Comparison of denitrification potential of the various disturbed (BSR) natural (Wet Meadow, Low Marsh) and restored (Millport, Banta) wetland locations. Error bars expressed as one standard error about the mean.

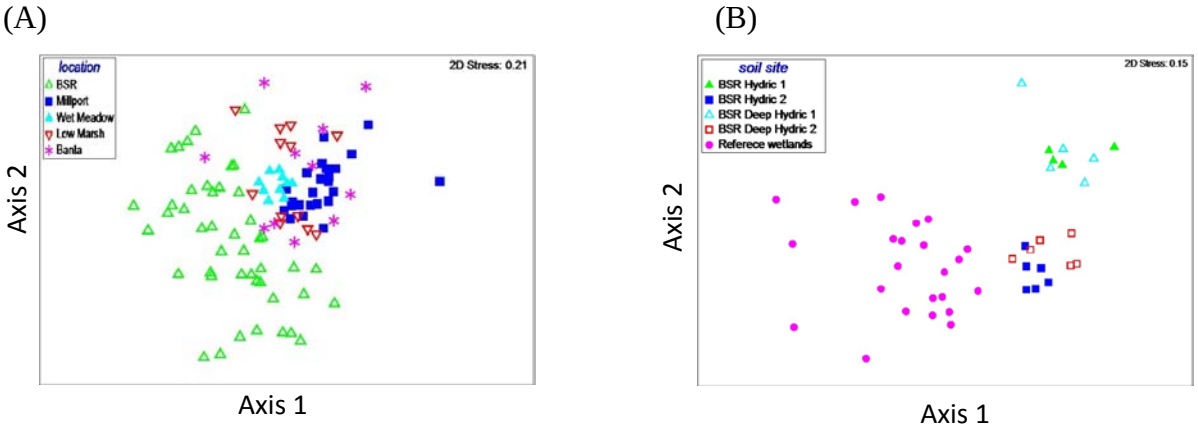


Figure 3.7. NMDS plots for ARISA comparison between all BSR soils (surface, legacy, hydric, and stream) and reference wetland sites (A) and between BSR hydric soils and reference wetland sites (B) taken during the summer sampling. Each point on the plot represents the microbial community surveyed for each core of soil collected at each of the sites. Hydric sites 1 and 2 in (B) represent the two stream locations where hydric soil was collected. Thus hydric 1 and deep hydric 1 represent a single location where a 0-20 cm horizontal core (hydric) was taken and a 60-80 cm horizontal core (deep hydric) was collected.

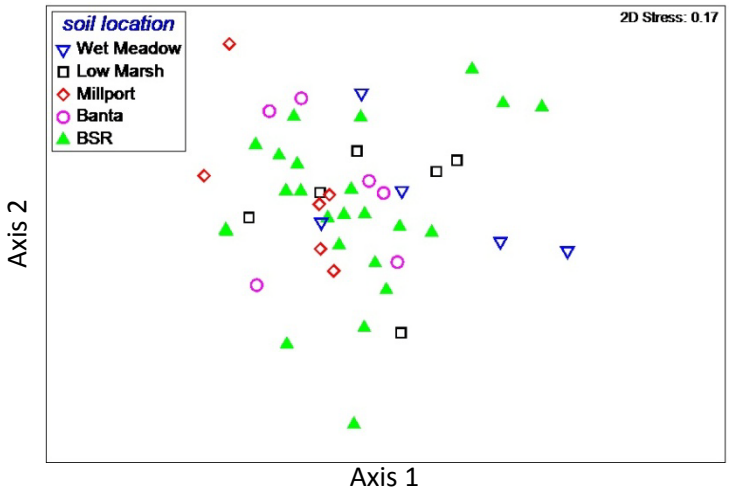


Figure 3.8. NMDS plots for *nosZ* denitrifier communities found in eastern PA hydric soils. Extensive overlap in denitrifier community composition was observed between all 5 tested locations. The four reference wetland sites did exhibit community level differences between each other, however the differences were not as distinct as the ARISA total community differences.

Tables:

Table 3.1 Wetlands Characteristics

Wetland	Latitude	Longitude	Wetland Status
Big Spring Run (BSR)	39° 59'36.9"N	76° 15'42.7"W	unrestored
Wet Meadow (WM)	39° 59'39.3"N	76° 15'50.1"W	natural
Low Marsh (LM)	39° 59'45.1"N	76° 15'51.5"W	natural
Millport (M)	40° 08'18.1"N	76° 15'28.2"W	restored
Banta (B)	40° 08'52.4"N	76° 16'24.6"W	restored

Table 3.2 Denitrification Rates for Nov. 2009 BSR soil strata

BSR Soil Type	Denitrification potential (nm N_2O/gdry soil*hr) Mean +/- SEM
Surface	31.853 +/- 8.971
Legacy	1.9951 +/- 0.882
Hydric	4.2211 +/- 1.677
Stream	1.8919 +/- 0.779

Denitrification potentials observed for the 4 soil strata at Big Spring Run. Significant different marked in Fig. 3.3

Table 3.3 ANOSIM comparison of BSR strata microbial communities

BSR soil strata	surface	legacy	hydric	stream
surface	-			
legacy	0.727*	-		
hydric	0.678*	0.385	-	
stream	0.846*	0.563*	0.273	-

Significant differences in microbial community composition ($p<0.05$) denoted with "*"

Table 3.4: Denitrification potential by sample date

	Sample Date					
	Nov-10		Mar-11		Jun-11	
Wetland Location	DeN potential	SEM	DeN potential	SEM	DeN potential	SEM
BSR Hydric	0.587	0.0222	1.0556	0.1721	2.7664	0.4815
Wet Meadow	20.1995	3.6796	10.5487	0.9141	20.4762	1.6966
Low Marsh	13.8807	1.2433	20.9463	1.1101	52.536	5.3833
Millport	12.1127	1.5746	6.9119	0.7082	18.8857	0.6593
Banta	8.5583	3.0804	7.2332	0.9744	24.223	1.6502

Denitrification (DeN) potentials and standard error about the mean (SEM) for 5 sites surveyed over the 3 sample dates.

Table 3.5 ANOSIM comparisons between relict hydric soils and reference wetland locations (R-values presented, only non significant difference is between Low Marsh and Banta communities)

Soil Location	BSR Hydric 1	BSR Hydric 2	Wet Meadow	Low Marsh	Millport	Banta
BSR Hydric 1	-					
BSR Hydric 2	0.92	-				
Wet Meadow	0.971	1	-			
Low Marsh	0.981	0.964	0.776	-		
Millport	0.962	0.96	0.616	0.626	-	
Banta	0.871	0.798	0.459	0.129	0.513	-

CHAPTER 4

Uncovering restoration potential in relict wetland soils: Using soil transplant experiments to assess how microbial community composition and denitrification potential respond to wetland hydrological conditions

Abstract

Anthropogenic land use changes are strong drivers of microbial community structure and function. Human alterations have significantly disturbed many wetland ecosystems – and thus the biogeochemical cycles their microbial communities mediate. Wetland restorations attempt to recreated and restimulate their processes (particularly concerning denitrification), and understanding how the microbial community targeted for restoration responds and adapts to changing parameters is important. In this experiment, hydric soils targeted for restoration were transplanted into 4 geographically similar wetlands to assess changing denitrification potentials and microbial community compositions. Results were mixed, with a spring transplant showing only a slight improvement in denitrification potential ($F=2.373$, $p=0.049$) from controlled conditions and a summer transplant showing significant improvement in overall denitrification ($F= 8.496$, $p<0.0001$) from controlled conditions. Seasonal variations could have contributed to these results as the spring transplant conditions were significantly colder than the summer conditions. A longer term experiment was also implemented and no significant change in denitrification rates was observed over the 3 month transplant experiment. Significantly, in all three transplant experiments, no deviation was recorded in microbial community composition between controls and transplant treatments, meaning that changes in denitrification potential observed were from the microbial communities living in the altered Big Spring Run hydric soil. The improvements noted in some of the experiments are likely due to increased activity of the relict soil microbial community. However, all increases in denitrification potential observed in transplanted relict hydric soil were significantly lower than the reference wetland locations. Restoration of wetland hydrology may not be enough to immediately promote high rates of denitrification in the relict soil microbial communities. A longer period of returned wetland hydrology may be needed to observe higher rates of denitrification.

Introduction

Microbial processes mediate many of the biogeochemical cycles observed in wetland ecosystems (e.g., Song *et al.* 2010, Dandie *et al.* 2011, Moseman *et al.* 2009). Bacterial populations inhabiting hydric soils found in wetland systems are particularly important in the nitrogen cycle – most notably in their ability to perform denitrification (Song *et al.* 2010, Peralta *et al.* 2010, Bossio *et al.* 2006). In natural wetlands, high levels of anthropogenic nitrate (NO_3^-) inputs can be transformed and removed by this microbial-mediated process prior to entering surface waters, as nitrate is converted back to atmospheric nitrogen (N_2) through the denitrification pathway (Lee *et al.* 1969, Gutknecht *et al.* 2006). Denitrifying populations of bacteria that thrive in the anaerobic conditions typical of natural wetlands are able to perform high rates of denitrification, and thus can prevent eutrophication from occurring in open water.

Alterations in the environmental conditions of these ecosystems can strongly drive both community composition and function of these microbial populations (e.g., Unger *et al.* 2009, Peralta *et al.* 2010, Gutknecht *et al.* 2006, Fierer *et al.* 2003). As human development of the land progressed, wetland acreage significantly decreased as these ecosystems were seen as "useless" features of the land (Vitousek *et al.* 1997, Dahl 1990). As a result, the environmental conditions in these wetland areas were radically altered and disturbed to support human-managed systems (agricultural fields, subdivisions, recreational uses, etc.). These newer conditions are not favorable for denitrification, as the human-managed systems often promote aerobic processes. These radical environmental changes significantly depressed stream denitrification rates, causing nitrate to not be removed from the system (Comin *et al.* 1997, Kemp *et al.* 2005, Rabalais *et al.* 2002).

Today, wetland restoration projects seek to restore many of these ecosystem services, and denitrification is important among them (e.g. Inglett *et al.* 2011, Filoso and Palmer 2011, Kaushal *et al.* 2008). Because of the integral role microbial communities play in providing wetland services, some projects study the composition and function of bacterial populations after a wetland restoration has been completed (Inglett *et al.* 2011, Peralta *et al.* 2010, Song *et al.* 2010). However, little research has been performed in assessing how microbial communities contained within the site targeted for restoration will adapt and function as the restoration progresses. By observing how targeted microbial communities functionally adapt when placed in wetland conditions, a gauge of restoration potential can be achieved. If the soil communities

respond to wetland hydrology and begin to perform denitrification at rates higher their current unrestored conditions, then an ecosystem restoration of that site should be successful at restoring denitrification.

Previous work observing microbial community composition in disturbed ecosystems has shown that microorganisms are resilient to changing environmental settings, and in some cases may enter a period of dormancy until favorable conditions return (e.g. Yannarell *et al.* 2007, Jones and Lennon 2010, Moseman *et al.* 2009, Allison and Martiny 2008). However, that work has often spanned a short timeframe of disturbance, ranging from weeks to months (Smith and Ogram 2008). In this study, we seek to test how microbial communities respond to having favorable wetland conditions returned after remaining in altered conditions for over 200 years. In these restorations, it is important to ensure that high rates of denitrification are supported. If the soil microbial communities targeted for restoration do not improve in structure and function when ideal wetlands conditions are provided, then restoration goals related to water quality (e.g. nitrate removal, dynamic hydrology, etc.) may not be met when the restoration work is completed. We seek to assess the potential for recovery of denitrification function in a Pennsylvania wetland restoration project by transplanting cores of restoration-targeted soil into other natural and restored wetland ecosystems, and observe how rates of denitrification change under conditions that approximate the targeted wetland hydrology.

At the restoration site in Lancaster, Pennsylvania, legacy sediments resulting from pond deposition are being removed to re-expose the hydric soil from a former wetland community (Forshay and Mayer 2009). In previous work (see Chapter 3) performed at this site, it was determined that the microbial communities inhabiting the hydric soil currently perform low rates of denitrification. The surface soils at this site performed high rates of denitrification; however these soils will be removed during the restoration to restore floodplain hydrology, uncovering the relict hydric soil (former riparian wetlands) remaining at the site. To determine how these relict hydric soils will respond to restoration, soil transplant experiments were conducted where collected hydric soil from the restoration site was transplanted into various natural and restored wetlands. It is important to determine if: (1) the microbial communities in the relict hydric soil, once placed in wetland hydrological conditions, will show improvement in denitrification potential; and (2) if the remnant hydric soil will allow for immigration of new microbes (specifically denitrifiers) that will improve denitrification potential.

We hypothesize that due to microbial resiliency and redundancy (see Bodelier 2011, Roszak and Colwell 1987, Wittenbolle *et al.* 2009), the microbial community contained within the relict hydric soils will show higher rates of denitrification once wetland conditions are returned. Previous work at this site has shown that even under the heavily altered conditions present in the relict hydric soils at BSR (e.g., burial, high soil compaction, low soil porosity and water flow), denitrifiers still inhabit the hydric soils and denitrification still occurs, though at very low rates (Forshay and Mayer, 2009, Chapter 3). We hypothesize that returning these communities to wetland hydrology and conditions should stimulate the denitrifier communities and restore high rates of denitrification.

We also posit that new microorganisms should migrate into the soil once wetland hydrology is established, further contributing to improved denitrification rates. Native wetland microorganisms in all of the transplant locations currently exhibit high rates of denitrification and a markedly different total microbial community makeup (Chapter 3) from those observed in relict hydric soils. However, the denitrifying communities present at each site are not strongly distinct from one other, suggesting that a return to wetland hydrology should encourage higher rates of denitrification in the relict soil. Given a long enough planting period (3 months) new microorganisms should immigrate into the planted relict soil. We hypothesize that the immigration of new denitrifying organisms should stimulate the soil and contribute to higher denitrification potentials.

Materials and Methods

Study Sites

Hydric soil bacterial communities were collected from the BSR restoration site as described in Chapter 3 (see Table 3.1). The cores collected from the hydric soil layer were then transplanted in various wetland communities including natural wetlands (Low Marsh and Wet Meadow) and restored wetlands (Banta and Millport restoration wetlands) (See Chapter 3 Table 1). The sites targeted for restoration and the post-restoration sites all come from the same previous environmental conditions – all were originally wetlands pre 1700s that were flooded during the 1800s and early 1900s when mill dams were constructed downstream (Walter and Merritts 2008). Subsequent deposition of sediments buried the wetland hydric soils, so when the dams were removed, several feet of deposited sediments rested on top of the former wetlands soils.

Transplant Experiments:

Two soil transplant experiments were designed and implemented. The first transplant experiment was replicated twice (March 2011, June 2011), with soil cores being placed in 8kDa cutoff dialysis tubing (Spectrum Labs) (e.g., Reed and Martiny 2007) and transplanted in the treatment conditions (explained below) for 9 days. The dialysis tubing allowed nutrients and water to move freely across the membrane, but prevented the migration of microbial communities into or out of the bag. This experiment allowed for the testing of the response of the relict hydric soil microbial community to restored wetland hydrology. The second experiment was performed over a 3 month planting period (March-June 2011) using nylon mesh (average pore size of 1.5 mm) to keep the soil conglomerated during the planting period. Soils remained in transplanted treatments for 3 months, and the nylon mesh, while retaining the relict hydric soil, allowed for nutrients, water, and microbial populations to move freely across the membrane (e.g., Balser *et al.* 2002). This experiment allowed for the testing of microbial immigration into relict hydric soil. Denitrification potentials and MCC were assessed prior to transplanting and post-transplanting to observe changes associated with experiment.

Sample Collection and Experimental Design:

Hydric soils from BSR were collected in March and June of 2011. Because of the variability seen in hydric soil microbial community composition (Chapter 3), two locations were chosen along the stream reach for collection (Fig. 4.1). At each location, 0-20 cm (exposed hydric) and 60-80 cm (buried hydric) horizontal cores were taken, using a 2 cm metal soil corer, to test effect of stream exposure on denitrifying potential and MCC. 20 cores were collected for each horizontal depth at each location (2 locations x 2 depths = **4** different hydric soils) and homogenized for each soil type (4 total homogenizations). Each homogenized soil was then divided into 3 replicates. Each replicate was further divided into 6 parts representing each of the soil treatments. The treatments were as follows: (1) control – taken back to lab and analyzed; (2) disturbance control – soil placed in bag (dialysis or nylon depending on experiment) back in original BSR bank. This tested the effect of soil mixing and aeration on MCC and denitrification potential; (3) Wet Meadow transplant; (4) Low Marsh transplant; (5) Millport transplant; and (6) Banta transplant. With this protocol, each hydric soil (4 total) had 3 replicates for each transplant

treatment. Each transplant experiment type (dialysis bag or nylon bag) used this same experimental design. In the long term (nylon bag) experiment, bank controls (7) were also collected (in the same manner as the initial sample hydric soil collection) to assess microbial community differences in structure and function in the current altered stream (BSR) between the March and June sampling periods. Upon collection, cores were briefly stored at 4^{o}C in the field until denitrification potentials could be assessed, and subsamples were stored at -80^{o}C until further DNA processing could be completed.

Soil Analysis:

Denitrification assays were completed using a modified version of the acetylene block method as used by the USEPA (Forshay and McElmurry, 2009). Briefly, in the modified version of this method, 25 g of soil from each site was measured into a 125 ml Wheaton jar complete with sealed cap and septa. Nutrient media containing dextrose and nitrate was added to each jar to provide conditions capable of measuring the maximum denitrification potential of the sampled soil. Jars were incubated at *in situ* observed temperatures for hours, with initial (t=0) and final (t=3) concentration of N_2O recorded. The protocol was followed as outlined by the EPA certified method (RSKSOP #310; Forshay and McElmurry 2009), and upon completion of the process a denitrification rate was assessed to each soil sample collected. N_2O concentrations were recorded at both the beginning and end of the incubation period to determine the rate of nitrous oxide evolved during the assay. Values were multiplied by 10^9 to give standardized values (recorded as nmoles N_2O hr^- g dry $soil^-$). Remnant soils from the assay were then dried over 5 days at 50^{o}C to provide a soil dry weight. Soils were then muffled at 500^{o}C for 4 hours to provide an estimate of organic matter (OM) found at each soil strata or location.

Soil DNA extraction and purification:

Rocks and roots were removed from collected soil, and samples were freeze dried to remove the water. After freeze drying, 500 mg of each soil sample was weighed out for DNA extraction using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH). DNA extraction was achieved following the instructions provided by the manufacturer. Following DNA extraction, purification with cetyl trimethyl ammonium bromide (CTAB) was carried out to

remove the humic acids (Sambrook *et al.* 2001). Following purification, samples were stored at -20^{o}C until further processing could be completed.

Community composition analysis:

Bacterial community composition was determined using the automated ribosomal intergenic spacer analysis (ARISA) as described by Fisher and Triplett (1999). The intergenic spacer region between the 16s and 23s rRNA genes was amplified using the polymerase chain reaction (PCR) method with 6-FAM labeled, universal 16S rRNA primer 1406F (5'-TGYACACACCGCCCGT-3') and 23SR (5'-GGGTTBCCCCATTCRG-3') targeting the bacterial 23S rRNA gene. Thermal cycling conditions for this PCR protocol consist of an initial denaturation at 94^{o}C for 2 min, followed by 30 cycles of 94^{o}C for 35 sec, 55^{o}C for 45 sec, and 72^{o}C for 2 min. After completion of the 30 cycles, a final extension period of 72^{o}C for 2 min was carried out. This process was carried out in an Eppendorf MasterCycler Gradient (Eppendorf AG, Hamburg, Germany). The length of fragments was determined using denaturing capillary electrophoresis using an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Electrophoresis conditions were 63^{o}C and 15 kV with a run time of 120 min. A custom 100- to 1,250-bp rhodamine X-labeled (ROX) size standard (Bioventures) was used as the internal size standard for ARISA fragments.

Denitrifier community composition was assessed in the various locations using terminal restriction fragment length polymorphism (T-RFLP). For this project, the denitrifier populations refer to the set of microorganisms than can carry out the last step of denitrification, the conversion of $N_2O \rightarrow N_2$. The PCR reactions to amplify *nosZ*, which encodes the catalytic subunit of nitrous oxide reductase, allow comparison of soil denitrifier populations among BSR soils and reference wetland soils. The *nosZ* gene was amplified using the *nosZ-F-1181* (5'-CGCTGTTCITCGACAGYCAG-3') and the *nosZ-R-1880* (5'ATGTGCAKIGCRTGGCAGAA-3') to yield a 700 bp PCR product (Rich *et al.* 2003). The *nosZ* reverse primer was labeled with the phosphoramidite dye 6-FAM. Thermal cycling conditions for this PCR protocol consisted of an initial denaturation of 94^{o}C for 3 min, followed by 25 cycles of 94°C for 45 sec, 56°C for 1 min, and 72°C for 2 min, with a final extension carried out at 72°C for 7 min. The Qiagen MinElute PCR purification kit (Qiagen, Valencia, CA) was then used to combine and concentrate *nosZ* PCR products from two 50 µl reactions. PCR products amplified from each

sample were then digested in single-enzyme restriction digests containing *Alu*I, *Hha*I, or *Mbo*I. The length of fragments was determined using denaturing capillary electrophoresis using an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Electrophoresis conditions were 63^{o}C and 15 kV with a run time of 120 min. An ABI GeneScan ROX 1000 size standard was used as the internal size standard for *nosZ* generated fragments.

Size-calling was carried out using Genemarker version 1.85 (SoftGenetics, LLC, State College, PA, USA) by aligning peaks using the ROX-1250 standard for ARISA fragments and the ABI ROX-1000 standard for *nosZ* fragments. Each fragment represents a microbial population, and the combination of all the fragments from a given sample (termed "community fingerprint") was considered to represent the total assemblage of microbial populations present in a given soil sample. For bacterial ARISA, the fragments between 300-1000 base pairs in length were used for constructing community fingerprinting. For *nosZ* T-RFLP analysis, the fragments between 100-650 base pairs in length were used for constructing the denitrifier community fingerprint. The *nosZ* fragment analysis results from the 3 digests were concatenated and analyzed together to give a representation of the denitrifier community.

Statistical Analysis:

To compare total bacterial community composition (through ARISA) and denitrifier community composition (through *nosZ* T-RFLP) among the soils collected, the software package PRIMER 6 for Windows was used (PRIMER-E, Plymouth, United Kingdom). A Bray-Curtis similarity coefficient was generated among all pairs of samples which was used to create non-metric multidimensional scaling (NMDS) plots. These plots allow for the (dis)similarity of particular strata of soil to be plotted in ordinal space. Dissimilar microbial soil communities plot far away from one another while similar soil microbial communities plot close to each other. Comparisons in microbial community structure were able to be made between the various soils collected.

Differences in microbial community composition were tested using the analysis of similarity (ANOSIM) procedure on pairwise Bray-Curtis similarity values (Clarke and Warwick 2001). The ANOSIM procedure produces a test statistic, R, that is calculated based off of the following formula: $R = (r_b - r_w)/[1/4n(n-1)]$, where n is the total number of samples, r_b is the average of rank similarities from all groups of replicates between different sample sites, and r_w is

the average of all rank similarities among sample replicates. The R test statistic calculated by the ANOSIM function provides a degree of similarity value between the various soil strata and locations tested. The values range from 0 (no separation – the groups are identical) to 1 (complete separation – the groups are completely dissimilar). Calculations of similarity coefficients and ANOSIM were carried out using PRIMER 6 for Windows (PRIMER-E, Plymouth, United Kingdom). Measures of species diversity, richness, and evenness were carried out using the DIVERSE function in PRIMER 6 for Windows (PRIMER-E, Plymouth, United Kingdom). Differences in these values between hydric soil locations were carried out using and analysis of variance (ANOVA) test with a post-hoc Tukey's test to determine pair-wise differences. Species diversity is a measure biodiversity, with higher values signifying a higher level of denitrifier biodiversity. Species richness is a simple measure of all of the distinct species (for the purposes of this study, species = denitrifier communities) observed in a given ecosystem. Species evenness ranges from 0 (complete variation between microbial communities) to 1 (no variation between microbial communities). Difference in denitrification (DeN) rates were analyzed an ANOVA with a post-hoc Tukey's test to determine differences in DeN rates between individual layers. Significances were determined at a $p<0.05$ level.

Results:

Dialysis transplant experiment

Denitrification and OM results from dialysis transplant

During the spring dialysis transplanting experiment, hydric soil transplanted from BSR into the 4 wetland sites did not significantly differ from the controls after 9 days planted ($F_{[5,63]}=2.373$, $p=0.049$; Fig. 4.2a). While the ANOVA value was significant, post hoc analysis revealed that the only pair-wise significance was between the undisturbed control (collected directly from the stream bank), and the disturbance control planted back in the stream bank ($p<0.05$, marked in Fig. 4.2a). During the summer transplant experiment, the hydric soils transplanted from BSR into the wetlands all significantly improved over the undisturbed control, and the cores planted in Low Marsh and Banta improved significantly over the disturbance control ($F_{[5,66]}= 8.459$, $p<0.0001$; Fig. 4.2b). All rates of denitrification potential were higher in the summer sampling than in the spring sampling period. However, all rates of denitrification for the transplanted soil were significantly lower than any of the reference wetland sites (see Table

4.1). Soil organic matter was significantly higher in the planting and control BSR hydric soils than in any of the surveyed wetlands ($F_{[2,93]}$= 50.92, p<0.0001), but there was no significant difference between the planting and control soils in amount of SOM (t=0.21, p=0.83; see Fig 4.3; Table 4.1).

Microbial community composition (MCC) of control and transplanted soils

When MCC was analyzed for the June 2011 planting experiment, there were significantly different microbial communities inhabiting the relict hydric soil in comparison to wetland planting sites (WM, LM, M, and B; Chapter 3, Fig. 3.6). Post transplanting ARISA analysis of these soils showed that there was no significant difference in community composition between the relict hydric soil controls and the communities of the relict soils transplanted to treatment wetlands (ANOSIM R=0.066, p=0.035; Fig. 4.4a). Both the controls and the transplants remained distinct from the reference wetland locations (ANOSIM R=0.917 and R=0.89 respectively). Denitrification rates were then overlaid onto MCC to give a representation of how effective each community was at performing denitrification. Even though the transplanted soils improved over their controls in the summer experiment (see Fig. 4.2b), all transplanted cores still had significantly lower denitrification potentials than their natural and restored wetland counterparts (Fig. 4.4b). Denitrifier community composition did not noticeably differ between any of the surveyed sites prior to (R=0.086, p=0.037) or post planting (R=0.073, p=0.02), meaning that time and season did not strongly influence denitrifier community makeup. The overall community composition for denitrifying bacteria showed a high degree of dissimilarity between all of the BSR hydric soils – with a high variability in denitrifier communities observed for all of the surveyed relict hydric soils (Fig. 4.5a). Reference wetland microbial communities showed a greater degree of similarity within location, but there were distinct differences in denitrifying community composition between some of the reference wetland sites (ANOSIM R = 0.494, p<0.001; Fig 4.5b)

Long Term nylon mesh experiment

Denitrification and SOM results from long term transplant experiment

For relict hydric soils planted in wetland conditions for 3 months (March-June 2011), no noticeable improvement in denitrification potential was noted in the transplanted cores compared

to the disturbance control (see Fig. 4.6). Also no improvement in denitrification potential was noted in any of the transplanted relict hydric soils compared to post control hydric sediments (cores taken in conjunction with transplant experiment collection). However, all of the transplanted soils showed significant improvement in denitrification potential over the March 2011 pre-controls ($F_{[6,76]}$=8.694, p<0.0001; Fig. 4.6; Table 4.1). Denitrification potential observed in the relict hydric controls and transplanted cores was still significantly lower than any of the denitrification potentials observed in reference wetland sites (Chapter 3, Fig. 3.4). Control BSR soils and planted BSR soils showed no significant difference in SOM percentage (t=0.68, p=0.499), however both soils had significantly higher SOM contents than any of the other surveyed wetlands ($F_{[2, 93]}$=119.74, p<0.0001; Fig. 4.8).

Microbial community composition of control and transplanted soils

After 3 months of being planted in restored and natural wetlands, microbial community composition of the planted soils did not noticeably differ from the control soils (see Fig. 4.7a), yet remained structurally distinct from all planting locations (all ANOSIM R>0.8, p<0.001 when comparing planting soil community structure to their wetland counterparts; Fig. 4.7a). When the community structure was coupled with denitrification potential, it was noted that while there was some improvement in soil DeN potential over the planting period, rates of DeN were still significantly below any of the other wetland sites (Fig. 4.7b, Table 4.1). When denitrifier communities were assessed, there was much variability but no patterns in community makeup between any of the disturbed hydric sites and the reference wetland locations (ANOSIM R=0.192, p=0.02). When the relict soil was removed from the statistical analysis some of the reference wetland sites were different from one another (WM vs. M; R=0.891, p<0.0001; see Fig. 4.5b) and showed a high level of similarity within the group.

Denitrifier Comparison:

Denitrifier communities were compared among the 5 hydric soils for denitrifier diversity indices and were compiled in Table 4.2. Overall the relict hydric soil had a lower overall species diversity (ANOVA F=4.32, p=0.012) and a lower species richness (ANOVA F=4.97, p=0.0011) than the reference wetland sites. Species evenness was not significantly different between any of the locations surveyed (Table 4.2).

Discussion:

Dialysis (short term) experiments

Relict hydric soil at BSR has repeatedly shown low rates of denitrification regardless of sampling date (Chapter 3); consistently lower than any hydric soils found in surrounding reference wetland ecosystems (Chapter 3, Fig 3.4). The dialysis transplant experiments sought to determine how the BSR hydric soil microbial communities would respond to being "restored", in a sense, to proper wetland hydrology for a short period of time. The overall results were interesting in that the spring transplant experiment yielded no significant results between planting treatments and the controls, and the summer experiment showed some significant results (Fig. 4.2a). Environmental factors, specifically temperature, are strong drivers of microbial metabolism (e.g., Hartman *et al.* 2008, Gutknecht *et al.* 2006, Bossio *et al.* 2003), and cold temperatures can significantly hinder rates of denitrification. During the spring sampling, temperatures below freezing were regularly recorded, likely inhibiting microbial activity. From Fig. 3.4 of Chapter 3, it was observed that rates of denitrification potential in early spring were all depressed in relation to summer rates. These conditions could have led to the results observed with the spring transplant experiment.

However, when temperatures were warmer, significant improvements were noted in denitrification rates of the transplanted soils (Fig. 4.2b). Although they were still significantly lower than the restored and natural wetland rates, denitrification potentials in the transplanted hydric soil were significantly higher than the relict hydric bank controls. As previous work as shown, no "correct" microbial community is needed to perform denitrification (e.g., Song *et al.* 2010, Inglett *et al.* 2011) and the fact that the microbial community remained unchanged over the planting period, yet improved in denitrification potential is indicative that a site restoration focusing on restoring dynamic wetland hydrology to BSR could stimulate the denitrifying community and encourage high rates of denitrification. The summer transplant results suggest that the soil microbial community in relict hydric soil – while inhabiting an altered state and currently not performing high rates of denitrification – can improve when wetland conditions are provided.

Nylon mesh (long term) experiment:

Because current denitrification rates are so low in BSR hydric soil, a longer-term study was performed exploring how the hydric soil reacts to new microbial communities. Soils were planted for 3 months in various wetland systems, and denitrification rates and MCC was assessed prior to, and post planting. Denitrification results from the study indicated that while all the soils improved significantly from the March 2011 undisturbed control (Fig. 4.6), none of the planted soil denitrification potentials varied from the disturbance control or the summer post control. These results suggest that the warming temperatures due to seasonal change likely drove any changes observed in the denitrification rate, rather than any restoration of wetland hydrology.

The results from this work showed that over the three month transplant period, the microbial community of the original soil (March 2011 pre controls) did not significantly change when planted in a structurally unique community. From Figure 4.7a, it was noticed that there was no shift in MCC of the planted soils towards the wetland locations they were planted in. Previous work in the field has stressed the high inertia (basically the resistivity) of the soil community to changing environmental conditions (e.g. Balser *et al.* 2002, Reed and Martiny 2007), so it could be that a longer planting treatment is needed to observe any noticeable shifts in MCC. Soil microbial communities are initially resistant to environmental perturbations (e.g., Yannarell *et al.* 2007) so it is likely that due to the length of anthropogenic land use changes found at BSR, the altered conditions have become the new "normal" and that the transplanting treatments simulated a perturbation. The legacy sediments resting on top of the relict hydric soil have altered the environmental conditions from the original wetland parameters. Because of this alteration, different communities of microorganisms could have been favored over the 200+ years of ecosystem alteration. Because of resistivity, the microbial communities might have resisted adapting to the new environmental parameters (e.g., wetland hydrology, soil density changes) when transplanted.

Along with this, denitrifying populations were not significantly different between any of the planting locations and the original BSR soil. These results again suggest that denitrifiers are present in the disturbed conditions, but need revitalized wetland hydrology to again function. Even though community composition was not distinct between the 5 surveyed sites, the reference wetlands did show higher levels of species richness and diversity among the denitrifiers than the relict hydric soil (Table 4.2). Denitrifiers are present in the relict soil, even under the altered

conditions; however the community is not nearly as diverse or rich as the reference communities. Work performed by Song and colleagues (2010) showed how over a restoration gradient, the denitrifier populations regained function. They found that in the first year post restoration, no significant improvement was noted in denitrifying potential, however, the second year showed marked improvements. This lends credence to the theory that a longer period of study is needed. The Banta and Millport wetland restorations support this theory as well – as both systems have shown high rates of denitrification multiple years after a restoration occurred. The BSR project will attempt to follow the same restoration plan as Banta and Millport, and the fact that these sites both function as desired years after restoration work was completed is encouraging. It is possible that the transplant timeline used in this study was too short for microbial communities to adapt to changing environmental conditions. Balser and colleagues (2002) surveyed forest soil microbial communities, and observed that changes in function sometimes took as long as 2 years to notice. Longer transplant experiments of hydric soil should be used to see if the same effect is noticed in wetland hydric soils targeted for restoration.

Soil Organic Matter

Previous work has stressed environmental variables as strong drivers of microbial community structure and function, and high levels of soil organic matter (SOM) can be a strongly correlated with denitrification rates (Hartman *et al.* 2008, Orr *et al.* 2007). In this study, high percentages of SOM were noticed in the buried hydric soils; significantly more so than any of the other wetland sites surveyed (Figs. 4.3 and 4.8). SOM can be used as a measure of the carbon and nitrogen content found in the soil community (Forshay and Mayer, 2009). While the values of SOM observed in the hydric soil were significantly higher than any of the reference wetland sites, it is possible that this represents a highly recalcitrant fraction of organic matter. Even though SOM is present, environmental conditions at the site along with recalcitrant material may have restricted decay and limited the amount of available carbon – thus resulting in the low levels of denitrification observed. The denitrifier population surveyed in BSR hydric soil is not noticeably different from any of its wetland counterparts; however this is likely due to the high level of community variability noted in the relict wetland soils. Even with denitrifying communities present, restored wetland hydrology, and high levels of SOM, the relict hydric soil did not show improved denitrification potentials over the transplant timeline. The relict hydric

soil community may be resistant to returning wetland hydrology, and may not provide high rates of denitrification once the site is restored.

Conclusion:

In summary, the hydric soil microbial communities did noticeably improve in denitrification potential when the planting occurred during more favorable environmental conditions (June 2011 dialysis experiment), however those rates were still far below any of the potentials measured in natural and restored reference wetlands. The spring transplant experiment occurred when low temperatures suppressed microbial activity, and the longer-term experiment failed to record any improvement in denitrification potential over a 3 month experimental timeframe, or a significant shift in MCC. Denitrifiers were present in the relict soil community, and they did not differ strongly from any of the surveyed wetland communities, however, fewer distinct denitrifier populations were present in the relict soil than any of the other surveyed locations (Table 4.2). This lack of diversity does not allow for high levels of microbial redundancy – meaning that under these altered conditions, the few denitrifiers that are still present in the relict soil are all inhibited and will not perform denitrification. When planted in reference wetland sites during temperate conditions, relict hydric soil community composition remained the same and showed improved rates of denitrification. However, the relict soil communities seem resistant to returning wetland hydrology, as even after three months of restored wetland hydrology; no improvement in denitrification potential was recorded for relict hydric soils. While returning wetland hydrology to the site may slightly improve rates of denitrification observed in the relict soils, a full restoration including high rates of denitrification may not be achieved immediately due to the resistivity of the soil microbial community.

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Figures:

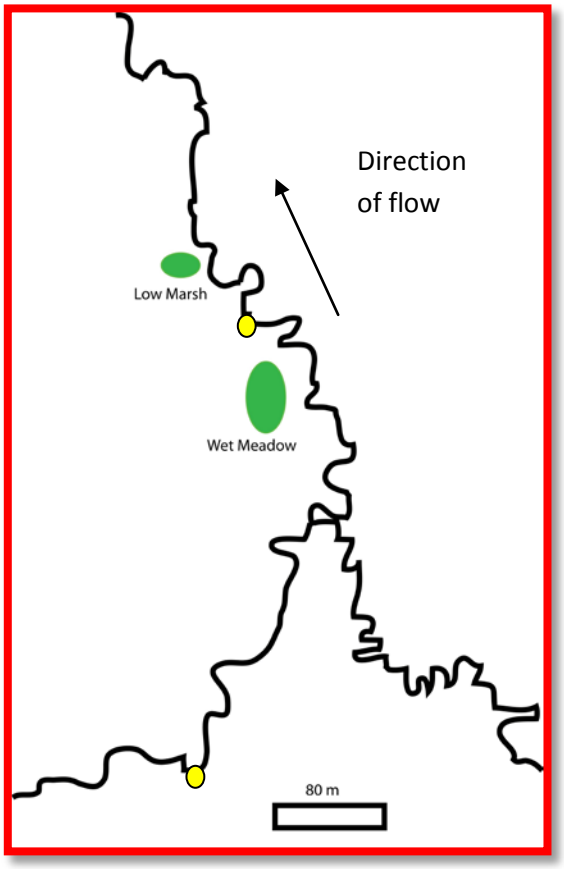

Figure 4.1. Representation of BSR restoration site. The large dots on the map represent locations from where hydric (0-20 cm horizontal cores) and deep hydric soil (60-80 cm horizontal cores) was collected Top dot along stream reach represents hydric site 2 (post confluence point) while bottom dot along stream reach represents hydric site 1 (pre confluence point). Wet Meadow and Low Marsh reference wetlands also labeled above.

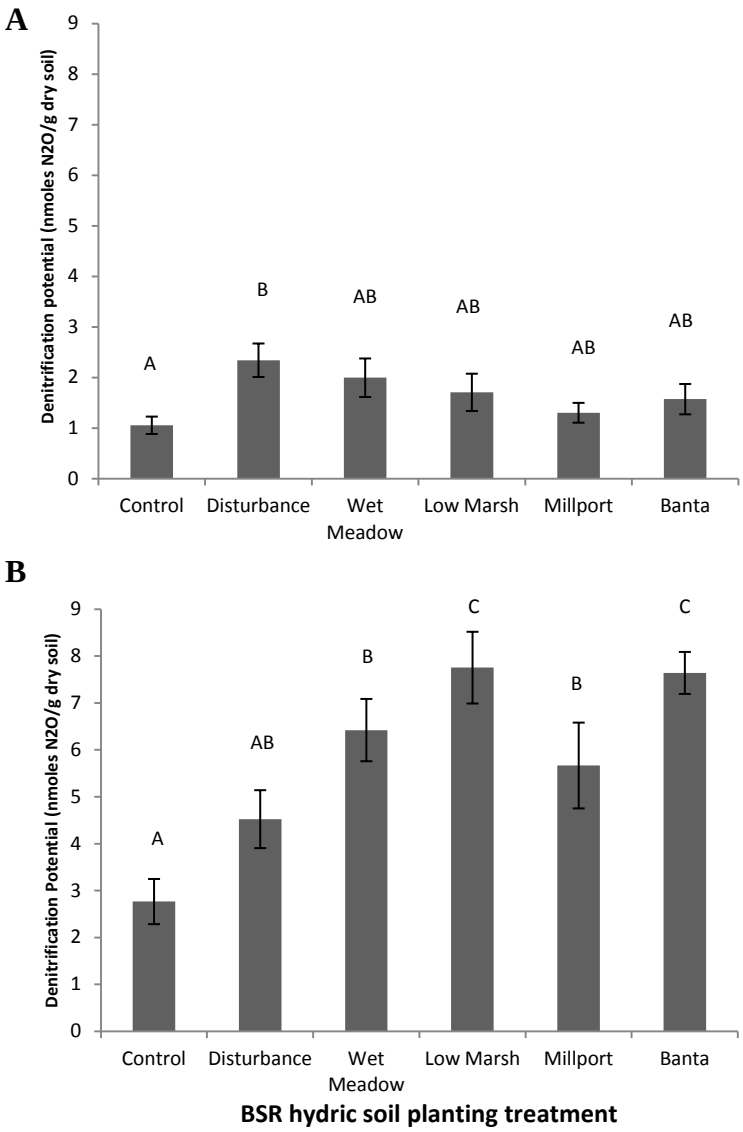

Figure 4.2. Denitrification potential for the spring (A) and summer (B) transplant experiments. Significant differences (at $p<0.05$) are represented by letters.

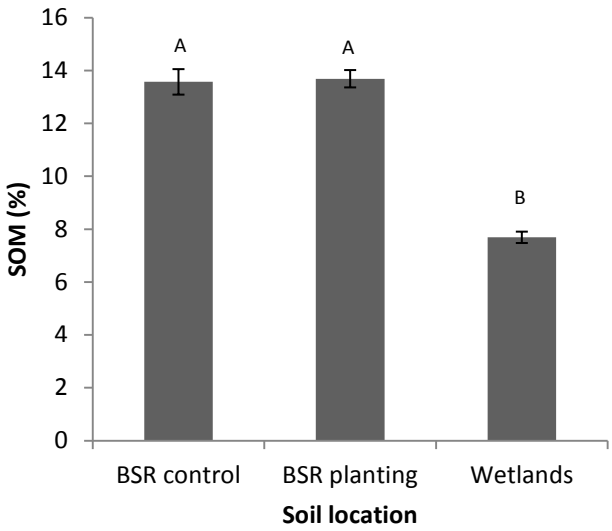

Figure 4.3. Soil Organic Matter (SOM) for each soil location during the June 2011 transplant experiment. Significant differences are shown with the small letters above each bar.

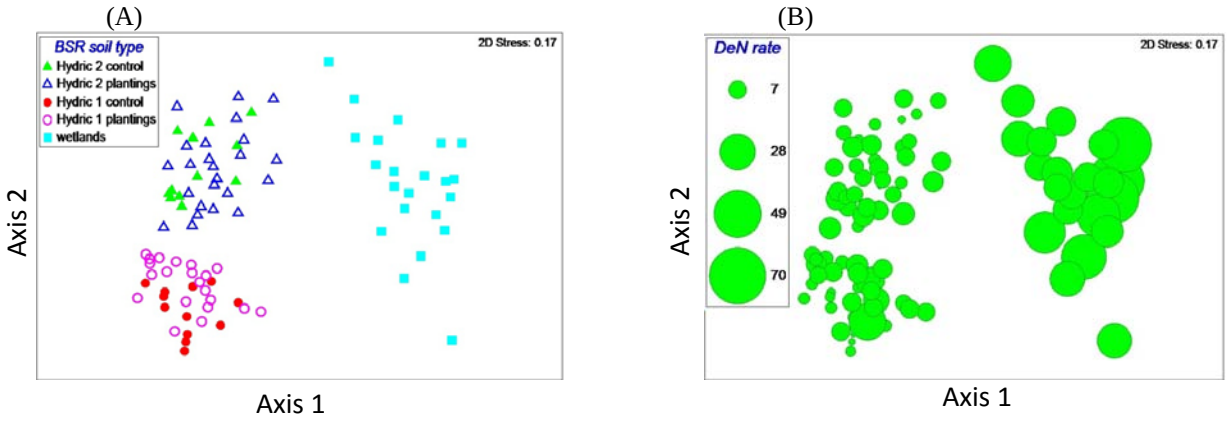

Figure 4.4. NMDS plots for summer transplant experiment total microbial community composition (ARISA) of relict soils and reference wetland sites (A). Community composition data was then overlaid with denitrification potentials (B) observed for June 2011 BSR dialysis transplant experiment. The hydric 2 and hydric 1 labels correlate to soils collected originally from BSR. The wetlands label is a conglomeration of the total MCC of the 4 remaining wetland sites.

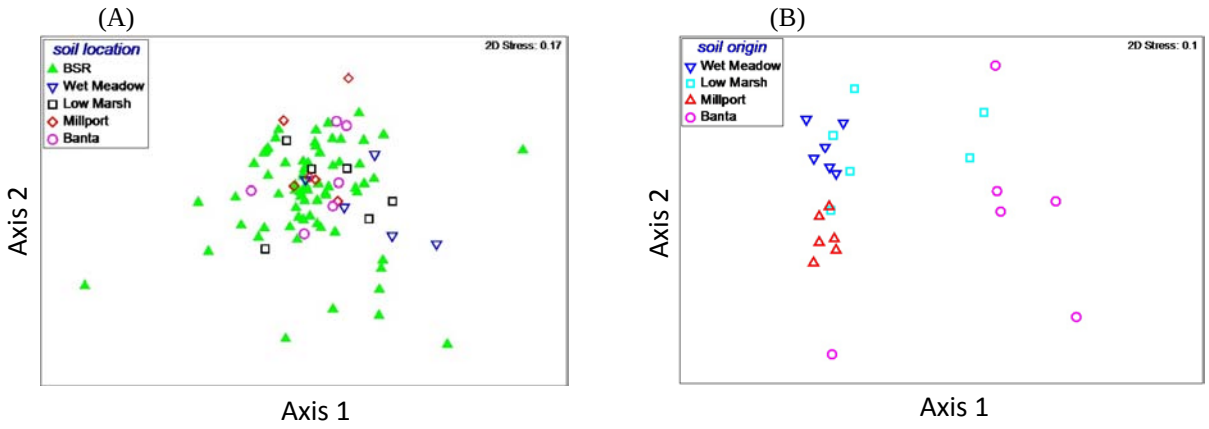

Figure 4.5. NMDS plot of denitrifier community composition (using *nosZ*) among various locations sampled (A). The BSR triangles represent all of the hydric soil collected (the controls), along with all of the transplanted cores (the plantings), as no distinct shift was noticed prior to, and post transplanting. An NMDS plot was also generated comparing the denitrifier populations between the reference wetland sites (B). Significant differences were observed between each of the reference wetland denitrifying communities.

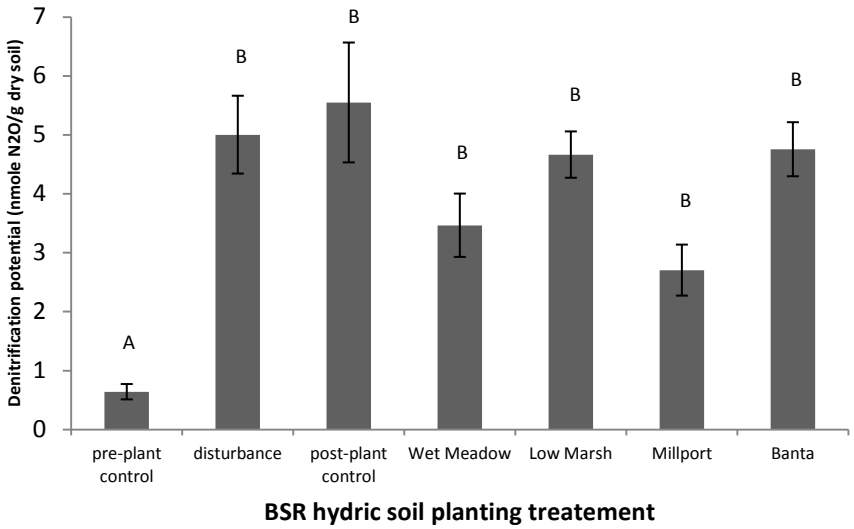

Figure 4.6. Denitrification potential of long term transplant experiment. Pairwise differences are shown with the letters above each of the bars

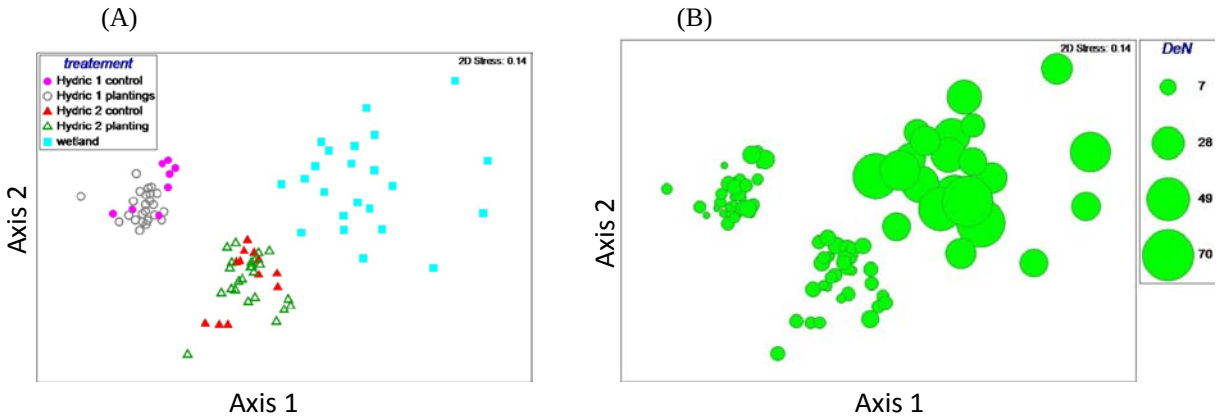

Figure 4.7. ARISA community composition for LT experiment (A), and then measured against rates of denitrification observed at each location (B). Both the pre (spring) and post (summer) bank controls are included in the "control" label. Natural and restored wetland systems had significantly higher rates of denitrification than any of the disturbed or planted soils from BSR.

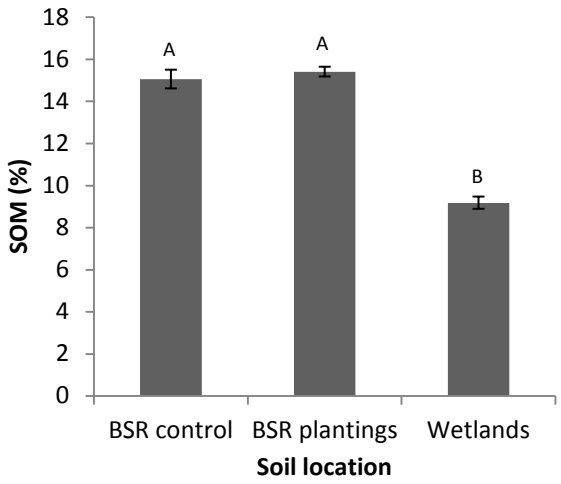

Figure 4.8. SOM levels for each combined treatments for the long term transplanting experiment. Significant pair-wise differences listed above the bars of the chart.

Tables:

Table 4.1 Soil chemistry data from transplant experiments

Experiment	Denitrification Potential (nmoles N_2O/g drywt*hr) Mean	(+/- SEM)	Soil Moisture Mean	(+/- SEM)	SOM (% by dry weight) Mean	(+/- SEM)
March 2011 Dialysis Experiment						
Relict Hydric Control	1.056	0.172	-	-	-	-
Disturbance Control	2.342	0.331	-	-	-	-
Wet Meadow transplant	1.996	0.381	-	-	-	-
Low Marsh transplant	1.707	0.369	-	-	-	-
Millport transplant	1.303	0.196	-	-	-	-
Banta transplant	1.572	0.300	-	-	-	-
Wet Meadow control	10.594	0.914	-	-	-	-
Low Marsh control	20.946	1.110	-	-	-	-
Millport control	6.912	0.708	-	-	-	-
Banta control	7.233	0.974	-	-	-	-
June 2011 Dialysis Experiment						
Relict Hydric Control	2.766	0.482	0.913	0.036	13.547	0.720
Disturbance Control	4.523	0.618	0.864	0.023	13.590	0.672
Wet Meadow transplant	6.423	0.664	0.886	0.027	13.645	0.731
Low Marsh transplant	7.754	0.765	0.909	0.021	13.636	0.613
Millport transplant	5.667	0.915	0.947	0.026	13.867	0.604
Banta transplant	7.641	0.448	0.944	0.023	13.602	0.666
Wet Meadow control	20.476	1.697	0.467	0.025	6.283	0.244
Low Marsh control	52.536	5.383	0.608	0.034	7.625	0.279
Millport control	18.886	0.659	0.657	0.018	8.472	0.138
Banta control	24.223	1.650	0.646	0.025	8.380	0.336
3 Month Long Term Experiment						
March 2011 hydric control	0.639	0.130				
Disturbance Control	5.001	0.661	0.953	0.023	15.468	0.481
June 2011 hydric control	5.547	1.017	0.821	0.030	14.738	0.694
Wet Meadow transplant	3.464	0.537	0.791	0.018	15.319	0.381
Low Marsh transplant	4.663	0.393	0.854	0.016	15.720	0.562
Millport transplant	2.702	0.433	0.919	0.018	15.043	0.283
Banta transplant	4.754	0.459	0.886	0.017	15.232	0.509
Wet Meadow control	27.647	2.918	0.491	0.018	7.454	0.161
Low Marsh control	52.498	5.769	0.645	0.021	8.852	0.180
Millport control	23.539	2.222	0.712	0.034	9.652	0.070
Banta control	34.363	5.745	0.718	0.023	10.691	0.511

Table 4.2. Diversity measures of hydric soil denitrifier communities

	Shannons Diversity Index		Species Richness		Species Evenness	
	Mean	SEM	Mean	SEM	Mean	SEM
Relict Soil	1.565	0.050	8.271	0.434	0.782	0.008
Wet Meadow	1.933	0.102	10.500	1.118	0.831	0.011
Low Marsh	1.585	0.111	7.833	0.792	0.778	0.019
Millport	1.922	0.101	13.667	2.030	0.755	0.010
Banta	1.968	0.111	13.333	2.951	0.795	0.021

Mean and Standard error about mean (SEM) for each diversity measure

CHAPTER 5

Summary

Microbial mediated biogeochemical cycling in wetland ecosystems are strongly driven by environmental parameters (e.g., Hartman *et al.* 2008, Song *et al.* 2010, Gutknecht *et al.* 2006). Anthropogenic alterations (through land use changes) disrupt the natural cycling that these systems perform, yet soil microbial communities can be resistant to the disturbance for short periods of time (e.g., Allison and Martiny 2008, Yannarell *et al.* 2007, Jones and Lennon 2010). During restoration of human-altered sites (specifically in wetland restoration projects), it is important to observe how the microbial community reacts to restored conditions, especially if the community has been removed from wetland conditions for hundreds of years. Based on previous work, wetland restoration projects have achieved mixed results, with some projects not stimulating active denitrifier communities (e.g., Orr *et al.* 2007, Bossio *et al.* 2006); while others have shown success and stimulating denitrifiers (e.g., Song *et al.* 2010, Smith and Ogram 2008). Based on these mixed observations, this thesis investigated microbial community composition and denitrification potential of a long-term altered hydric soil prior to a proposed restoration. We investigated the current altered conditions in relation to reference wetland sites (both in terms of microbial community composition and denitrifying potential), and then performed a soil transplant experiment that would determine if the relict soils would respond to favorable wetland hydrology (e.g., mimicking the site restoration).

To investigate the current altered relict soil conditions, we first examined the soil strata found at the site in terms of total community composition and denitrification potential. While the surface layer of the disturbed site showed high rates of denitrification ($F_{[3,20]} = 10.1$, $p = 0.0002$), the relict hydric sediments showed low denitrification potential. When compared to reference wetland ecosystems, the relict hydric soils were orders of magnitude lower (Ch. 3) than any of the other surveyed systems. Microbial communities, and more importantly, denitrifying communities still inhabited the hydric soils, however, they are not performing high rates of denitrification under the current altered conditions.

To then investigate if these communities could adapt and ultimately perform higher rates of denitrification, we then performed a transplant experiment, where cores of the relict hydric soil were planted in the reference wetland sites to observe how community composition and denitrification rates changed. Overall, community composition did not change over any of the 3

transplant experiments, but soil communities are notoriously resistive to change (e.g., Balser *et al.* 2002, Reed and Martiny 2007) and the time frame of transplant (ranging from 9 days to 3 months), may have been too short for any shift in microbial community to occur. However, denitrification rates did improve during the summer transplant experiment ((F= 8.496, $p<0.0001$) suggesting that the microbial community contained within the relict soil can be denitrify at higher rates when wetland parameters are restored (e.g., dynamic hydrology, hydrophytic plants). However, even these improved rates were still significantly lower than any of the surveyed reference wetlands (Table 4.1). When a longer transplant experiment was attempted, no observable improvement was recorded in the transplanted relict soils (Ch. 4) over their current conditions even after being planted in reference wetlands for 3 months. The microbial communities in the relict soils seem resistant to changing function and do not seem to support high rates of denitrification (like observed in the reference wetland sites).

Understanding how the microbial community adjusts to changing environmental conditions is important when studying wetland restoration projects. The goal of these projects is often to restore ecosystems services (e.g., biogeochemical cycling, nutrient removal, flood control) and many of these services are mediated by microbial communities. When an altered soil is targeted for restoration, the relict communities must be monitored to observe if they can regain beneficial function (e.g., higher rates of denitrification) when dynamic wetland conditions are returned. As shown by Song and colleagues (2010) and Balser and colleagues (2002), soil microbial communities are resistant to changing environmental parameters. Song noted that denitrification potentials remained low in the year following a wetland restoration, and only began to improve in the following years. This thesis studied the effects of short-term environmental changes on relict soil communities and found that improvements in relict soil denitrification potentials (if recorded at all) were slight. Because of microbial resistivity, a longer timeframe could be needed to see a noticeable improvement in Big Spring Run denitrification potentials.

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