MICROBIAL COMMUNITY ASSEMBLY AND HYDROLOGIC VARIABILITY AS CONTROLS ON POTENTIAL DENITRIFICATION RATES IN RESTORED FRESHWATER WETLANDS

BY

DORA BOYD COHEN

DISSERTATION

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Doctoral Committee:

Professor Angela D. Kent, Chair and Director of Research
Assistant Professor Jeffrey W. Matthews
Associate Professor Anthony C. Yannarell
Research Assistant Professor Julie L. Zilles

ABSTRACT

Denitrification is a microbial process in wetlands that transforms nitrate pollutants into nitrogen gas under anaerobic conditions. Restored wetlands do not always reach equivalent denitrification rates as those they were intended to replace, and this is sometimes linked to differences in microbial composition. Further, increased drought and variability in flood regimes represents an additional threat because the stress of fluctuation between extreme dry or saturated conditions could alter the microbial community itself and the ability of remaining microbial taxa to transform nitrate via denitrification.

One of the goals of this dissertation was to identify either microbial or hydrologic factors that could limit the ability of restored wetland communities to reach high potential denitrification rates. Based on a survey of 30 restored and 15 reference wetlands, restored wetlands surprisingly reached equivalent rates as observed from reference sites. This survey was conducted twice: Once during a drought in 2012, and again in 2013 following unusually intense floods. Similar results were found both years, but average rates were an order of magnitude greater in 2013 than in 2012. These potential denitrification assays were performed under identical saturated anaerobic conditions in the lab each year, so the differences observed must be due to inherent differences in the active microbial community. The magnitude increase in rates between the two years could be explained by soil variables such as pH and moisture, as well as to the abundance of *nirS*-harboring denitrifiers in the community.

Soil moisture and oxygen availability strongly influence microbial community composition, so regions of a single floodplain wetland may contain vastly different communities depending on the hydrology. Another goal of this dissertation was to determine if past

hydrologic variability filters microbial taxa such that these communities exhibit high resistance or resilience and high functional stability in the face of altered hydrology. I established an experiment where I manipulated the hydrology of soil collected from upland, lowland and the transition region of a single wetland. The transition region contained a unique composition compared to the upland and lowland regions, and composition remained relatively stable compared to the other regions, indicating either high resistance or resilience. This community also exhibited stable potential rates compared to the other regions.

Since denitrification can also be influenced by soil factors, such as pH and texture, which differ among wetlands and even differ among regions of the same wetland, my final study included an experimental design that removed any differences between soil types. Sterilized soil mesocosms were inoculated by live wetland soil collected from wetlands that exhibited different historical flood regimes: Flashy floods, long extended floods, and high variability from year to year. These wetlands exhibited distinct microbial composition initially. Following inoculation of the sterile mesocosms, I manipulated the hydrology as I did in the previous study, but this time I measured potential denitrification at multiple time points, rather than only at the beginning and end. The manipulated hydrology was the strongest driver of rates, but the mesocosms inoculated with communities from the wetlands with high variability from year to year consistently showed lower rates than those inoculated by communities from the other wetlands.

Increased drought followed by intense floods due to climate change will influence denitrification function in wetlands. Restored wetlands can reach equivalent denitrification rates as those that they were intended to replicate, but both restored and reference wetlands are now threatened by increased drought and hydrologic variability.

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Dedicated to my parents and my family,
and to the teachers who would not give up on me
who continue to inspire me to love learning and to share that love through teaching others

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CHAPTER 1: INTRODUCTION

Risks to wetlands and wetland ecosystem function

Wetlands were once common in the Midwestern United States but a vast majority of these wetlands were drained for agricultural expansion. Continued destruction of wetlands led to a federal policy that aims for "no-net-loss" of wetland acreage and function. In accordance with this policy, the US Army Corps of Engineers provides permits for wetland drainage and development as long as additional wetlands are restored (Erwin 2008, Bendor 2009, Hossler et al. 2011). Wetland processes carry an important role in the large scale management of nitrate pollutants (Rabalais et al. 2002, Ardón et al. 2010), so the effectiveness of restoration to truly replace the N cycling services in damaged or destroyed wetlands must be investigated (Rey Benayas et al. 2009, Moreno-Mateos and Comin 2010).

In addition to drainage and development, wetland ecosystems face increasing disturbance from altered precipitation regimes, which will lead to increased drought intensity. Droughts are expected to become more frequent and last a longer duration while the precipitation events in between droughts will be larger, especially in the Midwestern United States (IPCC 2014). Increased drought would affect a range of ecosystem services, like the provisioning of seasonal habitat for animals (Elphick et al. 2010, Engle 2011) and facilitation of high productivity and C storage (Odum et al. 1995, Gitlin et al. 2006, Mitsch and Gosselink 2007). In particular, microbe-mediated nitrogen cycling processes that require saturated anaerobic conditions will be disrupted by drought (Friedl and Wüest 2002, Pinay et al. 2002).

Not all microbial taxa are equally capable of handling hydrologic stress (Potts 1994). An important microbe-mediated ecosystem service is the cycling of nitrate into nitrogen gas by

denitrification (Groffman et al. 2006). Denitrification is performed by particularly diverse microbial taxa (Zumft 1997, Schimel et al. 2007), and thus denitrifiers display high variability in their physiological tolerance to environmental stress (Doi et al. 2009, Green et al. 2012, Bissett et al. 2013, Griffiths and Philippot 2013). A less desired consequence of denitrification is that it can also be a source of nitrous oxide (N_2O), a harmful greenhouse gas, if the final step of N_2O reduction to N_2 does not occur (Weier et al. 1993). Some denitrifiers lack the enzymes necessary to reduce N_2O to N_2, so denitrifier composition can also influence greenhouse gas emissions that occur during denitrification (Philippot et al. 2011). Studies on how drought in wetlands will affect denitrifier community composition and their function are essential in order to manage excess nutrient pollutants and minimize greenhouse gas production in the face of a rapidly changing climate.

Restoration of microbial services in wetlands

In order to determine if a restored wetland is capable of similar function as it was before conversion, it would be ideal to compare the restored site to historical information about the ecology and processes that occurred in the original wetland. However, wetland drainage in Illinois began as far back as the 1600s when European settlers first arrived, and the majority of recorded wetland loss occurred between 1890 and 1930 (Barringer and Balding 1996, Dahl and Allord 1996). Instead, ecologists use hydrologic and vegetative data to identify reference wetlands that might represent a typical wetland for that region.

Denitrification has proven particularly difficult to restore since restored freshwater wetlands fail to exhibit equivalent denitrification rates when compared to reference wetlands (Hossler et al. 2011, Marton et al. 2014). Preliminary studies have demonstrated a correlation

between differences in function in restored and reference wetlands and a difference in denitrifier composition (Flanagan 2009, Peralta et al. 2010). Since denitrifiers encompass a wide range of phylogenetic diversity and vary in metabolic rates and strategies, the presence of specific microbial taxa may influence the denitrification capacity of a wetland community (Salles et al. 2012). On the other hand, denitrifiers might so diverse that they exhibit a high degree of functional redundancy (Torsvik and Øvreås 2002, Allison and Martiny 2008, Shade et al. 2012), which would suggest that rates in restored wetlands are influenced more by abiotic controls.

Differences in denitrification function between restored and reference wetland may persist simply because the microbial communities in the restored wetlands are slow to develop. If restoration trajectories of the belowground communities are as stochastic as trajectories of the aboveground community (Trowbridge 2007, Matthews et al. 2009, Matthews and Spyreas 2010), then there is a need to understand which environmental factors influence the outcome of these trajectories. If microbial community composition directly controls denitrification capacity, then research on denitrifier compositional trajectories may help restoration practitioners identify abiotic properties that can be managed to influence the long-term outcome of restoration.

Work remains to determine (a) which microbial taxa are functionally important for denitrification in wetlands, (b) what drives differences in microbial composition to persist between restored and reference wetlands even after many years following restoration, and (c) whether such differences in taxonomic composition are relatively important drivers of function when compared to abiotic controls on denitrification function.

Using potential activity assays to study microbial ecology

Denitrification can be measured either *in situ* in a wetland or by collecting soil to use later in activity assays in a laboratory. The laboratory-generated activity rates are considered to represent potential rates rather than the actual activity rates. Potential denitrification assays were used in this dissertation to quantify functional rates for three reasons:

(1) Denitrification activity is limited by the presence of oxygen and by low substrate concentration (Tiedje et al. 1989, Hanson et al. 1994, Ballantine et al. 2014). The work presented in this dissertation is focused on identifying the biotic controls of denitrification with reduced confounding effects from the environment. During potential denitrification assays, oxygen is purged to create anaerobic conditions, and most of the assays in this dissertation add excess nitrate and a carbon source. This allowed me to measure the potential level of activity that can be performed by the denitrifier population in the soil, regardless of the abiotic controls on activity that would have been present *in situ*.

(2) Potential denitrification assays can be conducted with added acetylene to measure overall denitrification ($N_2O + N_2$), rather than only N_2O emissions that arise from incomplete denitrification. A gas chromatograph with an electron capture detector (GC-ECD) is a popular choice environmental scientists use to quantify N gas, but this type of detector can only measure N_2O, not N_2 (Sitaula et al. 1992, Grob and Barry 2004). During the assays, I added acetylene gas to block the reduction of N_2O to N_2, which allowed me to use a GC-ECD to measure the sum of both N_2O and N_2 produced (Balderston et al. 1976, Yoshinari et al. 1977). In Chapter 5, I adapted this method to include a second treatment without acetylene, and I was able to calculate potential rates of both complete and incomplete denitrification.

(3) Finally, this approach allowed me to include a large number of replicate wetlands in my studies, and I was able to quickly visit wetlands that were geographically distant. Measuring denitrification rates *in situ* requires the establishment of semi permanent collars to be placed in the soil of the wetlands. The researcher must remain at a single site for a few hours to collect hourly gas samples from the collars (Groffman et al. 2006). Chapters 2, 3, and 5 each describe studies where wetlands were located at least an hour drive away from one another, and many that were more than 3-4 hours away from our laboratory. The relatively quick process of collecting soil allowed fieldwork to proceed twice as quickly as I would have been able to otherwise.

Certainly, there are drawbacks associated with the potential activity assays described in this dissertation. Since these assays were intended to measure activity from only the current denitrifiers, the antibiotic chloramphenicol wads added to inhibit cell division and prevent growth of new denitrifier biomass. However, chloramphenicol has been documented to reduce denitrification rates from environmental communities (Pell et al. 1996), and to directly inhibit denitrification enzyme activity in the model denitrifier *Paracoccus denitrificans* (Brooks et al. 1992). All denitrification rates reported in this dissertation were generated using the same concentration of chloramphenicol (10 mg/L) and this chloramphenicol concentration matches published assay conditions (Peralta et al. 2016). I can compare rates reported in each of the following chapters, and I can compare the results in this dissertation to potential denitrification rates published in the literature. However, I caution against interpreting potential rates to represent the maximum possible denitrification.

Denitrification activity in soils is often characterized by high variability, where one soil core may display remarkably high rates while a neighboring soil core collected from a meter away may not show any activity (Groffman et al. 2006). It is important to point out that the

methods used to quantify denitrification in this dissertation were intended for the comparison of potential rates between wetlands (with the exception of rates generated during the manipulative soil experiments in Chapters 4 and 5). Because of this, I did not attempt to describe fine-scale variation between individual soil cores. Instead, I collected field replicates from random locations within each wetland in order to characterize the variability across the site, and each field replicate included multiple homogenized soil cores. Collecting field replicates allowed me to use formal statistical tests to compare potential rates between wetlands. I observed variability in potential denitrification within the wetlands, but this variability was probably lower than would have been observed between individual soil cores.

Molecular techniques to study wetland microbial ecology

In conjunction with assays that quantify potential functional rates, there are also molecular techniques that we can use to identify important microbial taxa, and detect changes in abundance over time or between wetlands. First, taxa can be identified via 16S rRNA gene sequences (Caporaso et al. 2011). This technique involves a broad survey of the entire belowground microbial community. This would identify other taxa besides the denitrifiers, and could allow us to detect complex ecological interactions. Differences in 16S rRNA gene sequences have been correlated with differences in potential denitrification rates in Great Lakes sediment communities (Small et al. 2016), so this method can serve to illuminate broad patterns in microbial composition-function relationships. A major disadvantage of this technique is that it relies on the assumption that we know the 16S rRNA gene sequence of all the ecologically important denitrifiers. Unfortunately, many 16S rRNA gene sequences are still unknown, and may be labeled with only a broad phylogenetic classification, such as “unidentified

alphaproteobacterium #234", which yields limited insight to predict the function performed by these taxa.

Second, we can use quantitative PCR (qPCR) to quantify functional genes known to encode for an enzyme responsible for individual steps of the denitrification process. Denitrifier taxa may harbor genes encoding an enzyme for any one of the four steps: NO_3^- reductase, NO_2^- reductase, NO reductase, and finally N_2O reductase (Philippot 2002). Microbial ecologists tend to focus on either NO_2^- reductase (*nirK* or *nirS*) or N_2O reductase (Clade I or Clade II *nosZ*) genes as diagnostic genes for the ability to do denitrification. The process of NO_3^- reduction is also part of DNRA or anammox in addition to denitrification (Kartal et al. 2007, Yang et al. 2017), and at least three different gene complexes encode for different types of NO_3^- reductase: The *nas* complex encodes a non-respiratory reductase, while *nar* and *nap* are part of a respiratory pathway (Richardson et al. 2001, Philippot 2002). Denitrifiers that use a *nir* gene to reduce NO_2^- to NO must also be able to reduce NO to N_2O immediately due to its toxicity (Zumft 1993, Ye et al. 1994). Thus, the denitrifiers that harbor a *nir* gene are considered N_2O-producing denitrifiers, while the denitrifiers that harbor a *nos* gene are considered N_2O-consuming denitrifiers (Philippot et al. 2011).

Many denitrifier genomes contain both a *nir* gene and a *nos* gene, but some taxa possess either one or the other (Jones et al. 2013, Jones et al. 2014). A benefit of directly quantifying either *nir* or *nos* genes is that it immediately allows us to compare the relative abundance of taxa that are capable of doing a known function in the denitrification process. The ratio of these genes has been correlated to N_2O emissions (Philippot et al. 2011), and *nir* or *nos* gene abundances sometimes correlate to functional rates (Salles et al. 2017). However, a number of studies demonstrate a lack of a consistent correlation between functional gene abundance and functional

rates (Chroňáková et al. 2009, Song et al. 2010, Philippot et al. 2013, Brower et al. 2017). There may be important functional differences between microbial taxa that share the same functional genes, so a disadvantage using qPCR is that we have no way to know if these are all the same species, or many different species that possess a similar gene. Gene quantification by itself cannot reveal whether composition differs between sites if the sites contain similar abundances of these genes.

Third, taxa belonging to specific functional groups can be identified via sequencing of the diagnostic functional genes, rather than simply quantifying these genes. A benefit of this technique is that it allows us to know whether denitrifiers in one wetland are different from the denitrifiers in another wetland. However, a disadvantage is that we will not be able to determine relative abundance of functional groups in the overall community, and we often still will not know the taxonomic assignment of these gene sequences. Many of these sequences would be labeled as "unidentified denitrifier #305" for example. This information would still help us to understand whether the presence of specific microbial taxa influence denitrification capacity of a wetland community.

Use of a combination of these techniques should allow us maximum insight into the ecological dynamics of microbial functional groups involved in denitrification in wetlands. Each of the following chapters uses at least one of these PCR-based methods, if not all three, to describe the soil microbial communities. A complete list of the names and sequences of the PCR primers used in this dissertation is shown in Table 1.1.

Overview:

The following chapters aim to address these specific objectives: (a) Identify correlations between potential rates of denitrification and the abundance of specific microbial OTUs or groups of microbes, (b) determine which abiotic characteristics in wetlands drive differences in microbial composition, especially between restored and reference wetlands, and (c) determine if those differences in composition correlate to differences in potential function. Chapters 2 and 3 use an observational approach to observe how restored and reference wetland communities differ in composition and function, and whether these differences persist from year to year. Extreme hydrologic events (drought followed by floods) allowed me to observe whether restored and reference wetland communities are affected similarly by hydrologic disturbances. Chapters 4 and 5 use an experimental approach to evaluate how hydrologic variability, including periods of drought, might structure denitrifier composition and influence their ability to function in increasingly variable environmental conditions.

In Ch. 2: *Extended drought limits potential denitrifier activity in restored and reference freshwater wetlands equally*, I conducted a large comparative survey that included 30 restored wetlands and 15 reference wetlands. Soil samples were collected once during a drought in 2012 and again in 2013, which followed a spring season with particularly intense flooding by contrast. Potential rates were affected strongly by drought conditions despite the denitrification function being quantified under the exact same conditions in the lab with adequate moisture and nutrients, suggesting some sort of limitation by the microbial community. I also found that denitrifier gene abundances were similar between restored and reference wetlands, but these genes were in greater abundance during 2012 under a drought. Since these findings contradict a preliminary study conducted in Illinois (Flanagan 2009), it is possible that restored wetlands simply require

more time for the denitrifier communities to develop functional capacity and become similar to the reference wetland communities.

In saltmarshes, restored communities converge toward a composition that is similar to communities in unimpacted saltmarshes (Bernhard et al. 2012) and saltmarsh denitrifiers develop along a smooth restoration trajectory (Bernhard et al. 2015). Further, a chronosequence of marsh communities demonstrated that the development of the denitrifier community after restoration is correlated with an increase in potential denitrification rates (Salles et al. 2017). However, when I attempted to coerce the 30 restored freshwater wetlands from Ch. 2 into a chronosequence, I found the denitrifier communities and their potential functional rates were so diverse that no discernible patterns could be detected. Thus, I identified a need to directly observe changes in composition between paired wetlands over time, where each wetland could serve as its own control for comparison. In Ch. 3: *Tracking temporal patterns in denitrification and microbial community composition following wetland restoration in Illinois*, I studied microbial communities in soil collected in 2007, 2012, 2013, 2014, and 2015 from four paired restored and reference wetlands. Surprisingly, the four restored wetlands actually diverged in microbial composition over time, and not toward their own respective paired reference wetlands. Potential function was strongly influenced by external hydrologic conditions, where a peak was observed in 2013 following the intense flooding across the entire state of Illinois, despite identical laboratory assay conditions throughout the study. However, two of the reference wetlands showed a larger peak in potential denitrification in 2013 than any of the other wetlands, and these two wetlands showed distinct composition from the others throughout the eight-year study. The microbial taxa at these two sites may have been better able to respond to favorable

hydrologic conditions in the environment, and then were able to utilize conditions in the laboratory assays that were even more ideal for promoting denitrification activity.

Hydrology is a major factor that influences the soil conditions in which microbial taxa have to be able to survive (Schimel et al. 2007). In dry soils, nutrients become unavailable for microbial uptake and microbes must be able to divert resources toward physiological protection from desiccation. In saturated soils, oxygen is used up quickly, and microbial taxa must be able to switch to less efficient anaerobic respiration. There is often a transition region in floodplain wetlands were soil conditions alternate between dry and saturated. Past research has already demonstrated that this transition region contains a unique community compared to the relatively constant upland and lowland regions (Peralta et al. 2014, Peralta et al. 2016). In Ch. 4: *Environmental variability shapes microbial community response to altered hydrology*, I experimentally tested the functional stability of the unique community in the transition region of a wetland. Compared to communities from the relatively constant upland and lowland regions, this community was both compositionally resistant to altered hydrology in a two-month mesocosm experiment and remained more functionally stable than the communities from the other two regions. The historical flood regime of a wetland seems to be a powerful structuring force on the composition and function of soil denitrifier communities, and this may limit the future restoration of denitrification services in wetland.

However, potential denitrification is also influenced by abiotic factors like soil texture and nutrient profile (Groffman and Tiedje 1989, Burgin et al. 2010), and these factors also influence denitrifier community composition (Foulquier et al. 2013, Morrissey and Franklin 2015). It is difficult to truly isolate the effects the abiotic and biotic components of an ecosystem have on its function. Many have used statistical modeling techniques to quantify the relative

contribution of abiotic and biotic factors to process rates (Foulquier et al. 2013, Jones et al. 2014, Morrissey and Franklin 2015, Graham et al. 2016), and some of these attempts have served as inspiration for the statistical modeling techniques utilized in Ch. 2 and Ch. 3. Statistical modeling can have limited power, though, when it comes to identifying causal relationships rather than correlative relationships. In order to determine whether differences in composition directly influence potential denitrification rates, there is a need to conduct experiments that are designed to control for differences in soil texture and chemistry. In Ch. 5: *Historical flood patterns drive microbial community composition in restored wetlands but not the functional response to altered hydrology*, I established an experiment that evaluated the functional response to different hydrologic treatments of microbial communities from wetlands that possess contrasting historical flood patterns. These communities were all placed in a similar soil environment with similar texture and chemistry to eliminate the effect of the local soil environment on denitrification function. I combined an observational field study investigating wetland microbial composition with an experimental approach inspired by plant-soil feedback experiments (Bever et al. 1997), where sterilized soil was inoculated with live wetland soil. The two-month experiment included a range of hydrologic treatments from full saturation to fluctuating to fully dry conditions. The most surprising result was that hydrologic treatment was a far stronger influence on potential rates than differences in the starting community composition. However, the microbial communities from wetlands with high variability in flood intensity from year to year consistently showed lower rates than communities from wetlands with more consistent flood patterns from year to year. These results mean that differences microbial community composition that arose from past hydrologic filtering can influence the

maximum rate of potential denitrification, but potential denitrification activity is far more strongly influenced by recent fluctuations in water availability.

Significance:

Restored wetlands do not always produce the rates that would be expected based on manipulation of abiotic factors alone (Orr et al. 2007, Hossler et al. 2011, Marton et al. 2014). Existing research on microbial controls of denitrification has yielded mostly correlative information, and these studies do not always agree with each other (Flanagan 2009, Manis et al. 2014). My dissertation has demonstrated that potential denitrification rates in wetlands are strongly influenced by recent hydrologic events (either drought or intense flooding in the environment, as well as experimental hydrologic manipulation), and I have provided experimental evidence demonstrating that differences in microbial composition do influence potential denitrification rates to some degree. This experimental evidence can be used to confirm the correlative modeling approaches that have been attempted on large datasets, which show that microbial denitrification activity in wetlands is controlled by both biotic and abiotic factors (Foulquier et al. 2013, Graham et al. 2016).

Table

Table 1.1. Complete list of the names and sequences of the PCR primers used in this dissertation.

Primer name	Sequence	Target gene	Reference	Primer was used for sequencing	Primer was used for qPCR
515F	5'-GTGYCAGCMGCCGCGGTAA-3'	16S rRNA	Caporaso et al. (2011)	Ch. 3, 4, 5	
806R	5'-GGACTACVSGGGTATCTAAT-3'	16S rRNA	Caporaso et al. (2011)	Ch. 3, 4, 5	
Arch349F	5'-GYGCASCAGKCGMGAAW-3'	Archaeal 16S rRNA	Takai and Horikoshi (2000)	Ch. 4	
Arch806R	5'-GGACTACVSGGGTATCTAAT-3'	Archaeal 16S rRNA	Takai and Horikoshi (2000)	Ch. 4	
Arch-amoAF	5'-STAATGGTCTGGCTTAGACG-3'	Archaeal *amoA*	Francis et al. (2005)		Ch. 4
Arch-amoAR	5'-GCGGCCATCCATCTGTATGT-3'	Archaeal *amoA*	Francis et al. (2005)		Ch. 4
amoA-1F	5'-GGGGTTTCTACTGGTGGT-3'	Bacterial *amoA*	Rotthauwe et al. (1997)	Ch. 4	Ch. 4
amoA-2R	5'-CCCCTCKGSAAAGCCTTCTTC-3'	Bacterial *amoA*	Rotthauwe et al. (1997)	Ch. 4	Ch. 4
nirK876	5'-ATYGGCGGVCAYGGCGA-3'	*nirK*	Henry et al. (2004)	Ch. 3, 4, 5	Ch. 2, 3, 4
nirK1040	5'-GCCTCGATCAGRTTRTGGTT-3'	*nirK*	Henry et al. (2004)	Ch. 3, 4, 5	Ch. 2, 3, 4
nirSCd3aF	5'-AACGYSAAGGARACSGG-3'	*nirS*	Kandeler et al. (2006)	Ch. 3, 4, 5	Ch. 2, 3, 4
nirSR3cd	5'-GASTTCGGRTGSGTCTTSAYGAA-3'	*nirS*	Kandeler et al. (2006)	Ch. 3, 4, 5	Ch. 2, 3, 4
nosZ1F	5'-WCSYTGTTCMTCGACAGCCAG-3'	Clade I *nosZ*	Henry et al. (2006)	Ch. 3, 5	Ch. 2, 3
nosZ1R	5'-ATGTCGATCARCTGVKCRTTYTC-3'	Clade I *nosZ*	Henry et al. (2006)	Ch. 3, 5	Ch. 2, 3
nosZ-II-F	5'-CTIGGICCIYTKCAYAC-3'	Clade II *nosZ*	Jones et al. (2014)		Ch. 2, 3
nosZ-II-R	5'-GCIGARCARAAITCBGTRC-3'	Clade II *nosZ*	Jones et al. (2014)		Ch. 2, 3

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CHAPTER 2:

EXTENDED DROUGHT LIMITS POTENTIAL DENITRIFIER ACTIVITY IN RESTORED AND REFERENCE FRESHWATER WETLANDS EQUALLY

Abstract

A valuable function of wetlands is to remove nitrate pollutants via the anaerobic microbial process of denitrification. Previous research has determined that current restoration practices fail to reproduce denitrifier communities with potential function equivalent to that of reference wetlands. Further, increased drought as a consequence of climate change is likely to interrupt anaerobic processes like denitrification, and little is known about the long-term effects on wetland denitrifier communities, which may possess a range of physiological tolerance to drought stress. Restoration practitioners need to understand which factors promote the greatest response of denitrification rates once anaerobic conditions favorable to denitrification return after post-drought floods. In this study, we seek to understand if restoration practices have been effective in promoting denitrification function that is comparable to the wetlands they were intended to mimic, especially following an extreme drought. We measured potential denitrification rates in 30 restored and 15 reference wetlands in 2012 during a drought, and again in 2013 after the drought. Denitrification rates were similar between restored and reference wetlands both years, but potential rates were an order of magnitude greater during the following year after unusually intense spring floods. We conclude that restoration practices seem to reproduce wetlands of equivalent functional potential after 8 to 20 years post restoration, but not all restored wetlands were able to reach equivalently high rates once saturated conditions returned after a drought year. Statistical modeling demonstrated that potential denitrification

rates during the year following the drought were strongly influenced by both wetland soil and microbial characteristics, namely soil pH and abundance of *nirS*-containing denitrifiers. Future research efforts should focus on examining the potentially causal relationships and interactions between pH, denitrifier composition, and denitrification capacity in a wetland following drought.

Introduction

Restoration of denitrification function in wetlands is contingent on restoring both the physical and biological components of an ecosystem. Drought events can alter abiotic properties, such as oxygen availability and nutrient flux, and changes in abiotic properties would impose physical stress on the microbial organisms responsible for denitrification. Flood regimes are expected to change due to altered precipitation regimes, and this will lead to extended periods of drought, especially in the Midwestern United States (Hey and Philippi 1995, IPCC 2014). This is only one example for how climate change is quickly altering the playing field, and restoration practitioners now must aim to reach moving a target to achieve desired ecosystem characteristics (Suding 2011).

Restoration practitioners often aim to restore hydrology to create the saturated conditions that facilitate anaerobic microbial denitrification activity (Zumft 1997, Wallenstein et al. 2006). Simply restoring the flood pulse has not predictably returned function to a desired level at the scale of an entire wetland (Orr et al. 2007, Peralta et al. 2010). Direct comparisons between denitrification rates in restored and reference wetlands have revealed that restoration does not result in similar rates as would be desired (Hossler et al. 2011). Most concerning, even potential rates of denitrification under identical laboratory conditions fail to show similar functional capacity between restored and reference wetlands (Flanagan 2009, Marton et al. 2014).

Microbial function and the delivery of ecosystem services appear to remain limited by intrinsic factors, as well as by extrinsic environmental factors related to the hydrology and flood pulse.

Differences in potential function may be partially explained by differences in microbial community composition. Denitrifiers encompass a wide diversity of organisms with a range of metabolic capabilities (Zumft 1997, Schimel and Gulledge 1998). Denitrifier composition and richness are often correlated with differences in denitrification rates (Philippot et al. 2013, Morrissey and Franklin 2015, Powell et al. 2015, Graham et al. 2016). Further, denitrifier communities in wetlands are quite different from one another in general (Peralta et al. 2012), and differences in potential denitrification in restored and reference wetlands has been directly linked to denitrifier composition (Flanagan 2009). The denitrifier community composition of a site intended for future wetland restoration may remain constrained by priority effects, which prevent these communities from producing similar rates to reference wetlands even once soils are treated to identical potential microbial activity rate assays.

Wetlands are likely to experience major disturbances from altered precipitation patterns, especially from increased drought intensity. Community assembly processes are altered by drought conditions in wetlands (Chaparro et al. 2016), which would complicate any efforts to restore belowground communities in wetlands. Current restoration practices often already overlook microbial community characteristics (Harris 2009, Bodelier 2011, Griffith 2012), despite how important these communities are for a wide range of ecosystem services (Knelman and Nemergut 2014, Delgado-Baquerizo et al. 2016, Graham et al. 2016, Laforest-Lapointe et al. 2017).

Extended drought presents an additional challenge for microbial populations in soil because these taxa must possess physical or molecular machinery to protect themselves from

desiccation in order to persist and become active again once the flood returns (Fierer and Schimel 2003, Schimel et al. 2007, Evans and Wallenstein 2012). Low activity in general is expected to occur under drought conditions (Gleeson et al. 2008, Zeglin et al. 2010, Estop-Aragonés and Blodau 2012, Buelow et al. 2016, Goordial et al. 2016). Drying of soils can limit microbial activity by physically inducing dormancy (Jones and Lennon 2010, Angel and Conrad 2013) or through immobilization of nutrients (Davidson 1993, Stark and Firestone 1995, Buelow et al. 2016). Most importantly, desiccation has long-lasting effects on the microbial community, where greater intensity of drought conditions affect the response of the community to altered conditions (Fierer and Schimel 2002, Alster et al. 2013, Meisner et al. 2017) and may change the mechanisms by which microbial taxa utilize available resources (Zeglin et al. 2013). Drying and subsequent re-wetting can alter the active portion of the community (Aanderud et al. 2015) and eliminate specific taxa that do not possess physiological tolerance to a rapid transition from oxic to anoxic. This type of disturbance alters the community over a longer time period (Evans and Wallenstein 2012, Placella et al. 2012, Brower et al. 2017). Droughts are expected to become more frequent and persist for a greater duration as global precipitation regimes change (IPCC 2014). In general, microbial communities tend to be sensitive to disturbances in terms of both altered composition and function (Allison and Martiny 2008, Shade et al. 2012a). Thus, it important to understand whether denitrifier communities in restored wetlands can achieve denitrification capacity following a drought that is equivalent to those in reference wetlands in order to determine whether drought has long-term effects that limit the recovery of function during the years following the drought. This will become even more important to understand as restoration practitioners attempt to recreate wetlands in the greater context of a changing climate and properly replace the ecological function of wetlands that have been destroyed.

In 2012, the Midwestern U.S. experienced one of the most intense droughts on record (Mallya et al. 2013, Hoerling et al. 2014) with negative consequences for U.S. agriculture and the economy in the region (Al-Kaisi et al. 2013). In the summer of 2012, a large survey was conducted comparing dozens of restored wetlands located across the state of Illinois with reference wetlands (Jessop et al. 2015). This drought offered an opportunity to study the recovery of the belowground wetland community and denitrification capacity following drought, and to compare that recovery in both restored and reference wetlands, so soil sampling was conducted again from the same wetlands again the next year, immediately following record-breaking spring floods. Using this dataset, we asked whether denitrifier communities in restored wetlands differed in their recovery from extended drought compared to reference wetlands. We hypothesize that current restoration practices fail to replicate a belowground community whose denitrification function is equally as resilient or resistant to intense drought as those in reference wetlands. A suite of environmental factors were also quantified for each wetland, including published information about the vegetation (Jessop et al. 2015) and additional information about soil chemistry and denitrifier abundance in the soil. Using these factors, we further asked whether the denitrification capacity that was achieved following the drought could be correlated to characteristics of the wetland itself.

Methods

Study sites and sampling design

Wetlands in this study were located in 23 different Illinois counties and represent a range of environmental variability present across the entire state. In total, 45 wetlands were included, which consisted of 30 restored wetlands and 15 reference wetlands that were each geographically

adjacent to a restored site. In 2012, sampling transects were established as described previously (Jessop et al. 2015). Out of the ten plant community quadrats along each of the four transects, soil cores were collected from two randomly selected 1-m^2 quadrats along each transect, yielding eight technical replicates per wetland for microbial and soil analysis. The location of each sampling quadrat was recorded via GPS, and soil cores were collected from the same approximate locations in 2013. Following each sampling event, soil cores were placed in plastic bags and put on ice for transportation back to the laboratory for analysis.

Soil chemistry analysis

Soil pH was determined using a 1:1 dried soil:deionized water. Concentration of available ammonium (NH_4^+), combined nitrate (NO_3^-) and nitrite (NO_2^-), and inorganic phosphate (PO_4^-) in soil extracts was determined using a Lachat QuikChem (Lachat Instruments, Loveland, CO, USA) following the Berthelot method as described previously (Sims et al. 1995, Rhine et al. 1998). Total organic carbon and nitrogen were determined using combustion analysis (ECS 4010, COSTECH Analytical Instruments, Valencia, CA, USA). Gravimetric water content was also determined for every sample.

Potential denitrification enzyme assays

Potential denitrification was determined using a laboratory assay to measure the amount of nitrogen gas produced per hour under ideal conditions. The assay protocol was similar to the assay described by Peralta et al. (2016), except the media contained an added carbon source and nitrate. Soil slurries were made by combining 25 g soil with 25 mL media containing 45.83 mM dextrose, 14.28 mM nitrate, and 10 mg/L chloramphenicol to inhibit denitrifier cell division. The bottles were purged with helium gas to create anaerobic conditions. Acetylene gas was added to prevent the reduction of N_2O to N_2. Gas samples were collected at the beginning and after three

hours. Gas samples were collected at the beginning and after three hours. This denitrification assay protocol uses the initial gas sample as the assay control, which is analogous to the frozen control in the nitrification assay, and no technical replicates were established. The concentration of N_2O was determined using a gas chromatograph (GC-2014 with ECD, Shimadzu Corp., Kyoto, Japan). The concentration of N_2O was used as a proxy for N_2 concentration under the assumption that it would be reduced under natural conditions (Groffman et al. 2006). The same soil used for the assays was oven dried, and the final denitrification rate was corrected by the dry weight of the soil.

Soil DNA extraction and purification

Genomic DNA was extracted from 0.5 g freeze-dried soil using a FastDNA kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. Humic acids were removed using cetyl trimethyl ammonium bromide (CTAB) purification (Sambrook and Russell 2001). CTAB is a cationic surfactant that solubilizes complex carbohydrates and secondary metabolites that might interfere with PCR (Azmat et al. 2012). Following CTAB purification, the absorbance ratio at 260:230 nm was determined on a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), and purified DNA samples with a 260:230 nm ratio greater than 1.5 were used for qPCR. The purified DNA was adjusted to approximately 20 ng/µL and stored at -20˚C until further microbial community analysis.

Quantitative PCR of microbial denitrification genes

Quantitative PCR (qPCR) was used to determine the number of *nirS* and *nirK* genes present in each community as a measurement of N_2O producer abundance, and N_2O consumer abundance was measured by quantifying the number of Clade I *nosZ* genes and Clade II *nosZ* genes present in the community.

In order to quantify gene copy number in each of the qPCR assays, replicate serial dilutions of standard template were amplified simultaneously with the samples to produce a standard curve. The template DNA for these standards were generated by first amplifying the gene of interest from a mixed wetland soil sample using PCR without fluorescent dyes: Reactions were carried out in a 50μL volume and contained 50 mM Tris (pH 8.0), 25 μg/mL of T4 gene 32 protein, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 20 pmol of each primer (four separate reactions: *nirK*, *nirS*, Clade I *nosZ*, and Clade II *nosZ*), 2.5 U of Taq polymerase (Promega, Madison, WI, USA), and 100 ng of extracted soil DNA. PCR conditions included initial denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 53 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 15 min. Next, the amplicons were separated from primer dimers using a QIAquick gel extraction kit according to the manufacturers' protocol (Qiagen, Valencia, CA, USA). The concentration of purified amplicon was determined using a Qubit DNA fluorometer (ThermoFisher Scientific, Waltham, MA, USA), and the exact copy number of the gene of interest in each the standard could be calculated from the concentration. The final copy number of each gene in the unknown soil samples was normalized by the ng of template DNA used in the qPCR reaction. The MIQE guidelines (Minimum Information for qRT-PCR Experiments) explained by Bustin et al. (2009) were used to evaluate assay performance based on the standard curves for the *nirK, nirS*, Clade I *nosZ*, and Clade II *nosZ* qPCR assays, and the results of this analysis are summarized in Table A.1 and Fig A.1. All gene copy numbers calculated in unknown wetland soil DNA samples were within the range of the respective standard curve for each gene.

PCR amplification reactions were carried out in triplicate in a 5 μL volume containing 1X FastStart Universal SYBR Green master mix (Roche Applied Science, Germany) with 125

μg T4 gene 32 protein (Roche Applied Science, Germany), and 0.4 μM of each forward and reverse primers was added to their respective reactions. The *nirK* gene was amplified using the primers nirK876 and nirK1040 (Henry et al. 2004), and the *nirS* gene was amplified using the primers, nirSCd3aF and nirSR3cd (Kandeler et al. 2006). The following protocol was used to amplify both *nirK* and *nirS* genes: 5 min initial denaturation at 95˚ C and then 40 cycles of 95˚ C for 45 sec, 54˚ C for 1 min, and 72˚ C for 1 min. The Clade II *nosZ* gene was amplified using the primers nosZ-II-F and nosZ-II-R (Jones et al. 2014) according to the following protocol: 5 min initial denaturation at 95˚ C and then 40 cycles of 95˚ C for 45 sec, 54˚ C for 1 min, and 72˚ C for 1 min. The Clade I *nosZ* gene was amplified using the primers, nosZ1F and nosZ1R (Henry et al. 2006), and a touchdown protocol was used: 15 minutes initial denaturation at 95˚ C, 2 cycles of 95˚ C for 15 sec, 68˚ C for 1 min, and 72˚ C for 30 sec, 2 cycles of 95˚ C for 15 sec, 66˚ C for 1 min, and 72˚ C for 30 sec, 2 cycles of 95˚ C for 15 sec, 64˚ C for 1 min, and 72˚ C for 30 sec, and ending with 35 cycles of 95˚ C for 15 sec, 62˚ C for 1 min, and 72˚ C for 30 sec. The primer sequences are shown in Table 1.1 (Chapter 1). All reactions were carried out separately for each target sequence in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The quantitative cycle threshold (C_q) was determined using SDS software v 2.4 (Applied Biosystems, Foster City, CA). Gene copy number was quantified by comparing C_q of samples against those produced by a standard curve constructed from gel-purified environmental amplicons for each gene. For all reactions, 1 μL of template was used, which contained approximately 10-15 ng genomic DNA. Primer sequences are shown in Table 1.1 (Chapter 1).

Statistical analyses

To determine differences between variables described for both 2012 and 2013 (e.g. denitrification rates, gene copy numbers), a paired Student's t-test was used, while unpaired t-

tests were used to determine any differences between restored and reference wetlands due to the inclusion of some restored wetlands that were not paired to a reference wetlands. All tests were performed using the entire wetland as a single sampling unit (n_{ref} = 15 reference wetlands, n_{res} = 30 restored wetlands). Post-hoc power analyses were performed using the R package "pwr" (Champely 2009) to determine if there was adequate statistical power to detect differences in potential denitrification in restored and reference wetlands. Power was calculated for a two-tailed t-test, significance was set to 0.05, and the effect size was determined by dividing the difference in means by the pooled variance.

In order to visualize differences in denitrifier composition, a principle component analysis (PCA) ordination was constructed with the "rda" function in the R package "vegan" (Oksanen et al. 2013, R Core Team 2014) using the standardized copy number of each of the four denitrification genes quantified, as well as the standardized log ratio of *nirK*:*nirS*, the standardized log ratio of Clade I *nosZ*:Clade II *nosZ*, and the standardized log ratio of total *nir* genes (*nirK*+*nirS*) to total *nos* genes (Clade I *nosZ* + Clade II *nosZ*). The function "envfit" was used to evaluate whether this ordination displayed significant differences in denitrifier gene composition between restored and reference wetlands and between 2012 and 2013. All univariate linear regression and ANCOVA models were constructed using the "lm" function in R.

In order to determine which variables were associated with wetlands that are likely to achieve the highest denitrification function following a drought, we created four models where the response variable was the average potential denitrification rate from each wetland in 2013. Each of the four models was linear model with multiple predictor variables including (1) plant community characteristics, (2) surrounding land use, geography and hydrology, (3) soil chemistry, and (4) microbial community characteristics. Correlated predictor variables

(Pearson's $r > 0.8$) were removed before the analysis. We used the "dredge" function in the "MuMIn" package in R to generate all possible models from each data set, and we selected a subset of models with delta AICc values that were no more than four times the AICc of the model with the lowest AICc value (Burnham and Anderson 2004). The "model.avg" function in R was used to calculate average estimates for each variable from this subset, producing an average model for each data set. We report the adjusted R^2 for each average model to show explanatory power. Increasing the number of variables can artificially increase R^2, so we also report average AICc values for each model because AICc faces a penalty increase when more variables are added. The average AICc reported was the weighted average of each AICc for the subset of models used to generate the final model.

Results

Drought effects on denitrification function and denitrifier composition in restored wetlands

No difference in potential denitrification rates was observed between restored and reference wetlands under drought conditions or during the following year, but rates were an order of magnitude lower during the drought in 2012 (Fig. 2.1A). The statistical power was remarkably low (<10% in both 2012 and 2013), which can be expected because there was no difference between rates in restored and reference wetlands. Half of the 30 restored wetlands were paired with geographically adjacent reference wetlands, so a paired-test was also conducted only using rates generated from the 15 paired wetlands, but still no difference was detected. The paired t-tests exhibited greater statistical power than the unpaired t-tests with unequal sample size, but power was quite low overall. The paired t-test power was calculated to be 19% in 2012 and 16% in 2013. There was a significant log linear relationship between wetland soil moisture

(gravimetric water content) and potential denitrification (adjusted $R^2 = 0.18$, $p < 0.001$; Fig. 2.1B), despite the completely saturated assay conditions employed to generate these rates during both years.

Overall, the relative proportions of denitrifier gene copies were affected by year (envfit $R^2 = 0.09$, $p < 0.001$), but did not differ between restored and reference wetlands (Fig. 2.2). However, gene abundances were often lower in 2013 when greater activity was observed than in 2012, and the individual abundances of denitrification genes in restored wetlands generally did not differ across years (Fig. A.2). The only gene that differed between restored and reference wetlands was Clade I *nosZ* in 2013, but there was a far lower abundance of Clade I *nosZ* than any of the other genes quantified (Fig. A.2D).

Factors associated with denitrification potential during the year following the drought

Four separate multiple linear regression models were generated using different sets of predictor variables in order to identify factors that may predict the ability of wetland denitrifier communities to achieve high function following a severe drought. One model built from explanatory variables relating hydrology and geography found a positive relationship between potential denitrification rates and latitude (Table 2.1). Another model built on vegetative community metrics found a relationship between potential denitrification rates and invasion by reed canary grass (*Phalaris arundinacea*) (Table 2.2). The rates were significantly correlated with the percent cover of reed canary grass in the PCoA ordination, providing additional support for this relationship with reed canary grass invasion (Fig. A.3). The model using only soil variables found a significant positive relationship between potential denitrification rates and phosphate (Table 2.3). The two microbial variables that significantly correlated with potential denitrification was the *nirS* and Clade I *nosZ* gene copy numbers (Table 2.4).

Discussion

Many restoration ecologists have evaluated the performance of denitrifying services in restored wetlands and found them to be lacking when compared to reference wetlands (Peralta et al. 2010, Hossler et al. 2011, Marton et al. 2014). However, the current study was able to employ a greater number of replicate wetlands than were included many of those studies. Further, the drought in 2012 provided the unusual opportunity to study how drought affects the restoration of denitrification and denitrifier communities. Here, we report that denitrification function in 30 restored freshwater wetlands and 15 reference freshwater wetlands were equally limited by intense drought conditions. The ability to recover function appears to be associated with geography, as well as soil nutrients, like phosphate, and with the abundance of *nirS*- and Clade I *nosZ*-containing denitrifiers in the microbial community. Differences between restored and reference wetlands made little difference to the maximum rate achieved during the year following drought.

The restored wetlands in this study ranged from 8 to 20 years post-restoration by the time the drought occurred in 2012. Many restoration ecologists are interested in the question 'how long is enough?' when it comes to restoration (Matthews et al. 2009) or whether there are permanent barriers to achieving restoration goals (Suding 2011). Wetland plant communities exhibit stochastic trajectories following restoration (Trowbridge 2007, Matthews and Spyreas 2010), especially within the first 10 years. Marsh chronosequences have provided evidence to show that potential denitrification may increase continuously with age over 100 years (Salles et al. 2017) but not within a 14-year range (Smith and Ogram 2008). Contrary to saltwater coastal marshes, we found that the freshwater inland wetlands are too diverse to coerce into a

chronosequence framework. Potential denitrification showed no relationship with restoration age (data not shown). A previous study found that a subset of these paired restored and reference wetlands showed functional differences in potential denitrification rates 4 to 10 years post-restoration (Flanagan 2009). Contrary to preliminary findings, we conclude that 8 to 20 years post-restoration might be enough time to restore denitrifying services in freshwater wetlands.

There may be differences that persist within the microbial community despite functional similarity between restored and reference sites. Our results suggest that the abundance of some denitrifiers may respond to drought differently in restored than reference wetlands. For example, abundance of both Clade I and Clade II *nosZ* was greater in the reference wetlands during the drought than during the following year, while the restored wetlands showed little change between the two years. Other studies that more directly investigated differences in restored communities compared to a reference are often in agreement that restoration does not result in an identical community (Flanagan 2009, Peralta et al. 2010, Bernhard et al. 2012, Murphy and Foster 2014, Bernhard et al. 2015). A recent study shared strong evidence that microbial communities in restored Appalachian forests follow a trajectory of convergence toward an undisturbed metacommunity over 30 years post-restoration (Sun et al. 2017). Microbial community composition may follow some sort of successional trajectory following restoration, yet remain functionally redundant in the face of environmental stressors, such as drought. It is possible that the restored wetlands in our study were in the process of converging, but were strongly influenced by the effects of the drought.

Most interestingly, microbial assemblages in restored and reference wetlands were influenced by drought equally, where drought conditions experienced in the environment decreased the ability of soil communities to transform nitrate even under ideal saturated

conditions in a laboratory. Certainly, antecedent effects of recent oxygen availability are known to have a strong influence on denitrification rates even once anoxic conditions are returned (Scholefield et al. 1997, Attard et al. 2011, Bergstermann et al. 2011). This could be due to physical changes to the soil brought on by desiccation that trap nutrients in inaccessible forms (Stark and Firestone 1995, Buelow et al. 2016), short term changes to the microbial community (Shade et al. 2012b, Youngblut et al. 2013), or even drought-induced metabolic changes in the present community, thus altering activity in general (Zeglin et al. 2013). In our study, we actually found denitrifier populations tended to decrease in abundance during the year following the drought, possibly reflecting a lag response to drought stress. Even if microbial community composition is different between restored and reference wetlands, the microbial assemblages of restored wetlands are capable of equivalent denitrification capacity as those in the reference wetlands.

Potential denitrification rates can be influenced by different environmental factors, and historically these have been difficult to disentangle (Groffman et al. 2006). Here, we were interested in using these variables to explain the variability in potential denitrification rates in 2013 only. Dry conditions will limit denitrification rates, so a useful trait to focus on is the maximum denitrification rates that can be achieved during the year following drought. We established four separate models in order to evaluate the relative contribution of wetland characteristics that may be informative for restoration ecologists performing future research: (1) wetland hydrology and restoration history, (2) vegetative quality indicators, (3) soil characteristics, and (4) microbial community metrics.

(1) We did not find a difference between floodplain vs. depressional wetlands or between wetlands restored via intentional vegetation planting vs. excavation. Previous comparisons found

that restored floodplain (i.e. riparian) wetlands performed similarly to corresponding reference riparian wetlands, while restored depressional wetlands exhibited lower potential denitrification rates compared to their reference ecosystem (Marton et al. 2014). The Olentangy wetlands in Ohio offer an interesting case study for the comparison of wetlands restored via intentional planting or by 'self-design' (Mitsch and Wilson 1996, Mitsch et al. 1998), where two floodplain wetlands fed by the same river source were restored according to each method and monitored over more than 20 years. The naturally colonized wetland had lower vegetation richness but greater productivity, N and P retention, and C sequestration compared to the planted wetland, but denitrification was most strongly influenced by temperature and historic saturation levels (Mitsch et al. 2014, Song et al. 2014). In our study, we found neither distinction to have a significant effect on magnitude of potential denitrification. Geography (i.e. latitude) most strongly influenced rates, but variability among rates could not be explained by differences in surrounding land use type (data not shown), and thus 'geography' likely incorporates many interacting controlling variables.

(2) Plant community indicators were not strongly correlated with potential denitrification rates either, with the exception of percent cover of reed canary grass (*Phalaris arundinacea*). Previous work established that potential denitrification rates were inversely correlated with indicators of vegetation quality (e.g. floristic quality index - FQI), and the authors hypothesized that this represents an indirect effect of nutrient runoff (Jessop et al. 2015). Excess nutrients often favor the growth of a monoculture of *P. arundinacea* (Zedler and Kercher 2005, Martina et al. 2014), and excess soil nitrate can stimulate microbial denitrification even when the potential rate assay incubation includes a non-limiting amount nitrate (Hanson et al. 1994). It is possible

that the relationship observed between *P. arundinacea* and potential denitrification is simply because both variable would be elevated in wetlands that typically receive more nitrate runoff.

(3) The strongest influence of soil on the increase in potential denitrification rates was seen in phosphate, with pH as a strong but not significant factor. Phosphate is also commonly found in runoff containing nitrate (Hubbard et al. 1989, Shuman 2002), and this may represent a similar relationship as observed with *P. arundinacea* (though these were not correlated variables - data not shown). Soil pH can influence denitrification rates from soil organisms grown in culture (Saleh-Lakha et al. 2009, Dörsch et al. 2012), and has been observed to influence rates in the environment as well (Van den Heuvel et al. 2011). Both pH and soil nutrients are strong variables driving differences in microbial communities as well (Bárta et al. 2010, Griffiths et al. 2011, Peralta et al. 2012).

(4) The microbial model revealed that both *nirS* gene abundance and Clade I *nosZ* gene abundance in the microbial community were primary factors influencing potential denitrification rates. Previous literature has suggested that the specific hydrology of the environment influences denitrifier composition, where *nirS*-containing denitrifiers tend to dominate in constantly saturated regions of wetlands (Ligi et al. 2014), but greater abundance of *nirS* genes does not always correlate to greater potential denitrification rates (Song et al. 2010). Others have found that the diversity of *nirS* gene sequences influences rates (Salles et al. 2012, Morrissey and Franklin 2015). There has been some indication that greater abundance of denitrifier genes in general corresponds to higher denitrification rates (Chroňáková et al. 2009), but many have determined that denitrifier abundance is simply a secondary controlling factor when also considering hydrology or variables pertaining to the soil environment (Attard et al. 2011, Baxter et al. 2012, Manis et al. 2014). Using the modeling approach reported in this study, soil variables

and microbial abundance variables each explained 43% and 49% of the variability in potential denitrification rates, respectively. This adds to a growing body of evidence for the importance of considering both soil and microbial variables as controlling factors of microbially-mediated ecosystem processes (Graham et al. 2016).

The study we describe here shows that drought can alter the ability of wetland soil microbes to respond to ideal conditions in a laboratory assay. One year later, the microbial community recovered this ability, but there may still be long-term consequences of the drought on the community because abundance of the denitrifier population was greatly decreased by then. Most surprisingly, we found that restored and reference wetland communities did not differ in potential function, contrary to previous studies (Peralta et al. 2010, Hossler et al. 2011, Marton et al. 2014). Potential denitrification was strongly influenced by both soil chemistry and microbial community, so we agree with previous studies that calling for careful selection of the soil environment when restoring wetlands (Peralta et al. 2012, Peralta et al. 2016), especially for mitigation purposes. We further report a relationship between *nirS*- and Clade I *nosZ*-containing denitrifiers, in particular, with the ability of a wetland microbial community to recover denitrification function following a drought, and this potentially causal relationship warrants further investigation.

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Tables

Table 2.1. Geographic and hydrologic variables that predict potential denitrification rates generated in 2013. Estimates generated from the average of 12 models selected based on having AICc no larger than four times the AICc of the best model. Significant predictors ($p < 0.05$) are indicated in bold text.

	Estimate	Importance	No. of models
(Intercept)	***** 747.52**		
Latitude	***** 576.49**	**1**	**12**
Floodplain (yes/no)	159.82	0.48	6
Excavated (yes/no)	-146.99	0.44	6
Gravimetric soil moisture	-66.15	0.21	4
Longitude	44.31	0.20	4
Adjusted R^2	0.46		
Mean AICc	718.54		

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 2.2. Plant community variables that predict potential denitrification rates generated in 2013. Estimates generated from the average of 15 models selected based on having AICc no larger than four times the AICc of the best model. Significant predictors ($p < 0.05$) are indicated in bold text.

	Estimate	Importance	No. of models
(Intercept)	***** 801.22**		
% Reed Canary Grass	***** 499.67**	**1.00**	**15**
% Native Perennial	-345.93	0.60	8
% Native	-164.08	0.34	6
% Perennial	217.33	0.31	6
Richness	-130.45	0.23	4
Floristic Quality Index	-73.76	0.14	3
Adjusted R^2	0.45		
Mean AICc	660.79		

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 2.3. Soil variables that predict potential denitrification rates generated in 2013. Estimates generated from the average of 13 models selected based on having AICc no larger than four times the AICc of the best model. Significant predictors ($p < 0.05$) are indicated in bold text.

	Estimate	Importance	No. of models
(Intercept)	***** 747.52**		
Phosphate	**** 409.54**	**0.95**	**12**
pH	§ 230.14	0.65	7
Ammonium	171.76	0.49	7
Nitrate	65.79	0.22	5
Total C	84.94	0.21	4
Adjusted R^2	0.43		
Mean AICc	720.24		

§ $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 2.4. Microbial variables (based on quantitative PCR of denitrification genes) that predict potential denitrification rates generated in 2013. Estimates generated from the average of 14 models selected based on having AICc no larger than four times the AICc of the best model. Significant predictors ($p < 0.05$) are indicated in bold text.

	Estimate	Importance	No. of models
(Intercept)	***** 747.52**		
nirS	***** 799.94**	**1**	**14**
Clade I *nosZ*	**** -387.37**	**0.97**	**13**
log(*nir*:*nos*)	168.84	0.39	6
log(Clade I:II *nosZ*)	108.43	0.28	5
Clade II *nosZ*	-19.19	0.15	3
nirK	1.23	0.14	3
log(*nirK*:*nirS*)	31.97	0.14	3
Adjusted R^2	0.49		
Mean AICc	715.95		

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figures

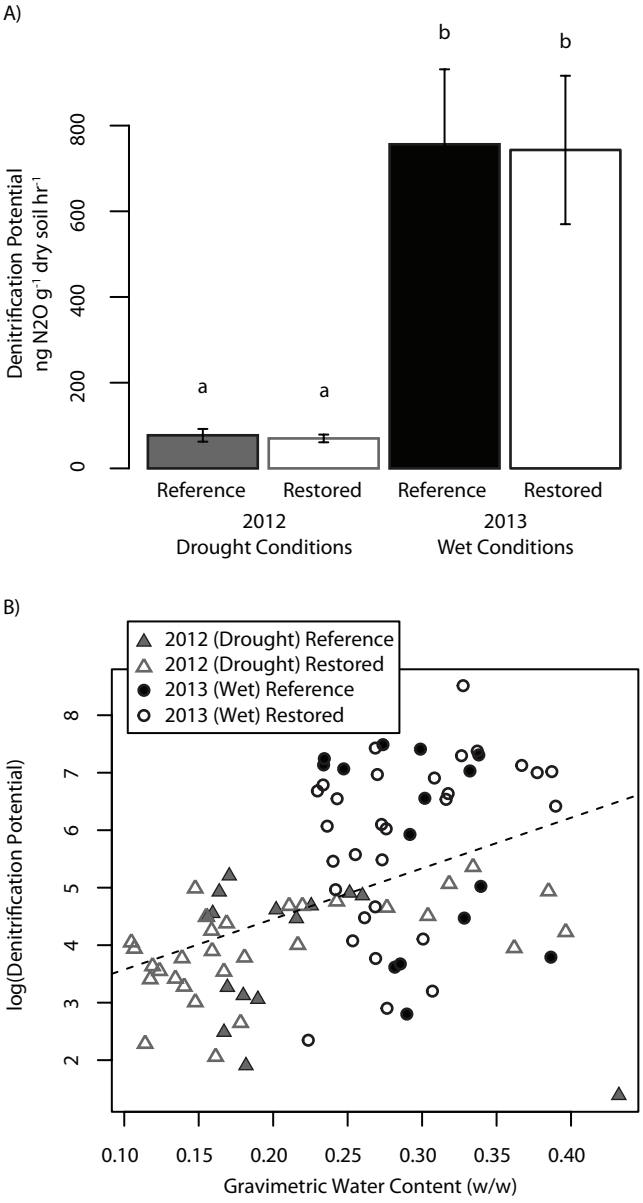

Figure 2.1. (A) Comparison of mean potential denitrification rates in reference wetlands (n = 15) and restored wetlands (n = 30) determined during a year with intense drought conditions, 2012, and during the year after when conditions returned to a more typical wet state. Error bars show standard error of the mean. Lower case letters indicate significant grouping. (B) Linear relationship between original soil moisture (gravimetric water content) and log denitrification rates ($R^2 = 0.18$, $p < 0.001$). Potential denitrification rates were determined under identical saturated conditions for all soil assays.

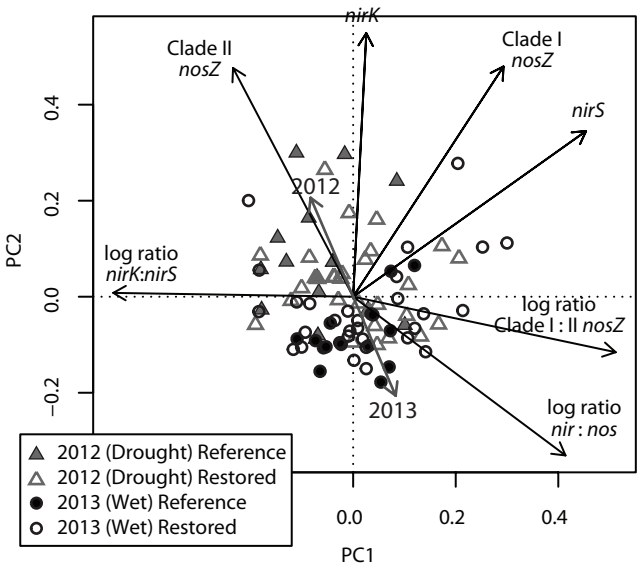

Figure 2.2. Principle component analysis (PCA) ordination based on standardized gene abundance values and standardized log ratios of total *nir* genes to total *nos* genes, *nirK* to *nirS*, and Clade I to Clade II *nosZ*. Axes for the seven variables are plotted as black arrows. "Year" was significantly correlated to the ordination ($p < 0.05$) using the 'envfit' function, and axes for both years are plotted as grey arrows. Wetland "type" (reference vs. restored) was not significantly correlated to this ordination.

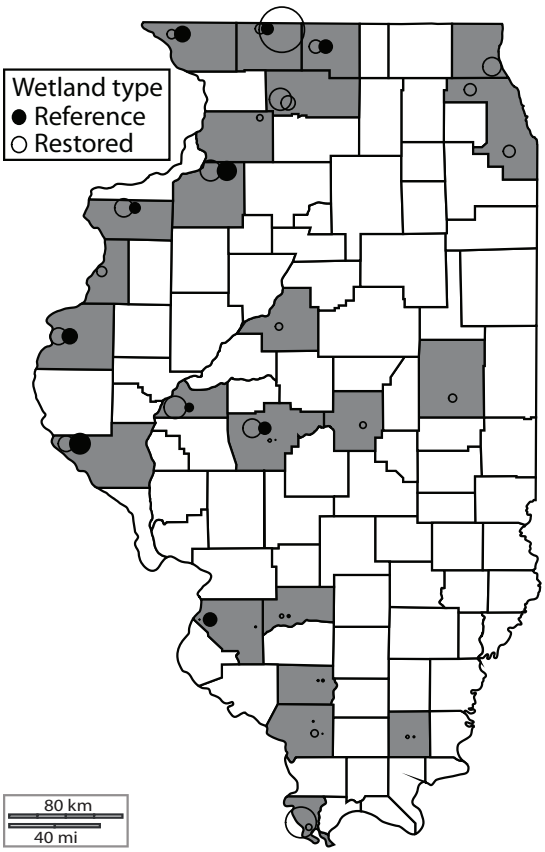

Figure 2.3. Location of 45 wetlands used in this study. Size of circle represents relative potential denitrification rates produced from soil collected in 2013. Filled circles represent reference sites, and open circles represent restored wetlands. Counties are shaded if they include at least one wetland used in this study.

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CHAPTER 3:

TRACKING TEMPORAL PATTERNS IN DENITRIFICATION AND MICROBIAL COMMUNITY COMPOSITION FOLLOWING WETLAND RESTORATION IN ILLINOIS

Abstract

During restoration, ecological concepts like succession and community assembly are utilized to alter a damaged ecosystem and restore it to a desired state. Some functions have proven difficult to control using current restoration practices, such as denitrification, which is an important ecosystem function performed by wetland microbial communities. Microbial communities are strongly influenced by abiotic soil properties like texture, pH, and organic matter, and soil properties develop quite slowly. It is possible that denitrifier communities in restored sites simply need time to develop to a fully functional state. To characterize the trajectory of wetland soil communities post restoration, soil samples were first collected in 2007 from four restored wetlands (7 to 10 years post restoration) that were paired with geographically adjacent reference wetlands, and annual sampling continued from 2012 to 2015. Potential denitrification assays were performed annually, and changes in the overall microbial and denitrifier communities were determined using DNA sequencing. The ratio of N_2O producing and consuming denitrifiers was also monitored annually by quantitative PCR. During this study, there was a drought in 2012, which was followed by unusually intense flooding in 2013. Potential denitrification was strongly influenced by the external conditions experienced before the assay, where the highest rates were observed in 2013. We also observed high variability in the maximum denitrification capacity of these communities, where two of the reference wetlands achieved greater rates in 2013 than the other wetlands. These two reference wetlands exhibited

unique soil community composition compared to the other wetlands in the study. Composition changed temporally in all of the restored and reference wetlands, but communities in the restored wetlands did not follow a trajectory toward the communities in their respective reference wetlands. The persistent differences between restored and reference wetland communities may have led to the observed variation in maximum denitrification rates. This suggests that the restoration practices employed did not restore the belowground ecology found in the damaged or destroyed wetlands these were meant to replace.

Introduction

Trajectories of community development and the delivery of ecosystem services can be stochastic following wetland restoration, causing problems for restoration ecologists who aim for a specific outcome. Wetland community development is likely influenced by a variety of external environmental factors, which can lead to alternative ecosystem states instead of the target ecosystem (Trowbridge 2007, Matthews et al. 2009, Hall and Zedler 2010). Belowground ecosystem processes that support the delivery of services, such as denitrification and nitrate removal, are not often monitored in practice (Matthews and Endress 2008). Studies have demonstrated that restored wetlands rarely exhibit denitrification rates that are comparable to rates observed in reference wetlands (Hossler et al. 2011, Marton et al. 2014), indicating that this service in particular is not truly replaced once a wetland is restored to mitigate wetland losses. Differences in denitrifier community composition are associated with differences in rates of denitrification function (Flanagan 2009, Peralta et al. 2010), and microbial community trajectories may influence ecosystem services over time (Salles et al. 2017). Studies that investigate the development of the microbial community over time following restoration, in

conjunction with the assessment of process rates, should be able to identify whether differences in function arise due to an ecological constraint associated with the microbial community.

A possible constraint on functional capacity in wetlands could arise from random trajectories of microbial community development following restoration. Matthews and Spyreas (2010) developed a framework in the context of plant community restoration that juxtaposes four different possible outcomes of community succession following restoration: (1) convergence of multiple restored communities toward a single desired state, (2) convergence of communities toward an undesired state, (3) divergence of communities toward multiple acceptable states, and (4) divergence of communities toward undesirable states. The authors found that wetland plant communities in restored wetlands exhibited convergence over the first four years, but remained quite distinct from the reference wetlands that were used as targets to identify what a desired community might look like. For microbial ecologists, characterization of a natural wetland community has only recently become a possibility, and restoration of the microbial community itself is a relatively new idea (Cockell and Jones 2009, Harris 2009, Bodelier 2011, Griffith 2012). Since microbial taxa vary in stress tolerance (Potts 1994, Schimel et al. 2007, Orwin et al. 2016) and denitrifier taxa vary in function due to physiological diversity (Bakken et al. 2012, Dörsch et al. 2012, Suenaga et al. 2018), it may be possible to identify specific taxa or specific combinations of taxa that result in desirable denitrification function. Communities that contain these taxa could be considered "target" communities for restoration. The characterization of such a reference community might allow restoration practitioners to manipulate abiotic drivers to favor colonization and establishment of these taxa following restoration.

Since the ability to participate in the process of denitrification is found in a wide phylogenetic range of microbial taxa (Schimel and Gulledge 1998), there may be multiple

combinations of taxa that are capable of performing similar rates of denitrification in restored wetlands. Experimental evidence has demonstrated that denitrification rates are at least partially controlled by denitrifier richness (Philippot et al. 2013) and composition (Morrissey and Franklin 2015), indicating that communities that contain different denitrifier taxa will exhibit different rates. Alternatively, other studies have observed that functional redundancy can occur between different denitrifier communities (Mustafa and Scholz 2011, Manis et al. 2014). The taxonomic diversity among denitrifiers as a functional group could explain discrepancies between the conclusions of these studies, where some communities exhibit low rates but multiple denitrifier communities are capable of high rates.

Microbial community composition is likely to change slowly regardless of whether restored wetland communities converge toward a target "reference" community or diverge toward multiple states that are functionally equivalent. Microbial community composition is strongly affected by the local soil environment (Griffiths et al. 2011, Peralta et al. 2012, Foulquier et al. 2013, Jones et al. 2014, Peralta et al. 2014) and soil development occurs slowly over time (Ballantine and Schneider 2009). The process of restoration can be seen as an event that initiates ecological secondary succession (Odum 1969, Zedler and Callaway 1999, Hall and Zedler 2010), where the ecosystem is altered by the removal of undesired vegetation and modified hydrology. Studies on successional processes that occur following glacial retreat have demonstrated that slow changes in soil communities mirror the slow building of soil over time (Kandeler et al. 2006, Schütte et al. 2009). In wetland plant communities, indicators of desired vegetation characteristics, like floristic quality index and native species richness, can increase after only a few years post restoration (Matthews et al. 2009). While changes in the above-ground plant community may influence belowground community composition to some degree

(Cline and Zak 2015, Reese et al. 2018), a long-term prairie restoration study demonstrated that the microbial community is more strongly influenced by the soil than by the development of the aboveground community (Murphy and Foster 2014).

A 2007 study involving geographically adjacent, paired restored and reference wetlands found that denitrifying services differed between the two types of wetlands, even up to 10 years post restoration (Flanagan 2009). However, a follow-up study conducted 5 years later found that rates were actually similar between restored and reference wetlands (Ch. 2 - *Extended drought limits potential denitrifier activity in restored and reference freshwater wetlands equally*). While the follow-up study involved a much larger dataset, it contained limited information about microbial community composition. It is possible that microbial communities in the restored wetlands are transitioning toward the communities in the reference wetlands over time, and denitrification services are also approaching similar rates.

The approach used here involves a small case study to determine if the microbial communities in the restored wetlands have begun to approach the compositional state of those in the reference wetlands, and if any differences in composition are linked to differences in function across a 4-year time series. During the time series, the Midwestern U.S. experienced one of the most intense droughts on record (Mallya et al. 2013, Hoerling et al. 2014), while the following year was characterized by usually intense floods. Belowground soil organisms can become stressed by desiccation during the drought or by anoxic conditions that arise during floods (Schimel et al. 2007). The flooding could also be expected to carry high nutrient loads (Jordan et al. 2010), which might favor fast-growing taxa (Gobler et al. 2016). Thus, we also aim to determine if trajectories of compositional change were altered by these extreme weather events.

Further, we seek to identify specific denitrifier taxa whose relative abundance is

correlated to denitrification capacity, and determine whether the abundance of these taxa change through time. The possibility that dissimilar microbial communities may still exhibit similar denitrification rates also leads to the question of whether a typical reference wetland community can be characterized, or if it is important to make such a distinction. The primary aim of our study is to lay the groundwork to establish whether microbial communities in restored wetlands are changing in composition over time and to relate changes in composition to changes in potential denitrification activity.

Methods

Sampling and field sites

Four geographically adjacent pairs of reference and restored wetlands were selected for this study, and the four pairs are located in three different Illinois counties (Fig. 3.1). Sites A, B, and D exhibit floodplain hydrology while the pair of wetlands at Site C are depressional wetlands, and the soil texture at these sites ranged from clay loam to silt loam (Table 3.1). Eight replicate soil samples were collected in the summer of 2007 from each wetland as described previously by Flanagan (2009) when the restored sites were 7 – 10 years old (Table 3.1). Eight replicate soil samples were collected from each wetland again in the summers of 2012, 2013, 2014, and 2015, when the restored sites were 15 – 18 years old. Soil from the paired wetlands at Site C was not collected in 2014 due to logistic complications. In 2012, the 8 replicate soil samples from each wetland were selected randomly using the method described in Ch. 2. In 2013, 2014, and 2015, a handheld GPS unit was used to collect samples from the same approximate locations as in 2012.

Potential denitrification activity assays

In 2007, potential denitrification rates were determined using soil collected from the field as described previously by Flanagan (2009). In 2012-2014, potential rates of denitrification were determined using a similar assay with minor changes. For these rates, 25 g of field moist soil was placed into 125-mL Wheaton jars with 25 mL of deionized water with chloramphenicol as described by Peralta et al. (2016). In 2012-2015, separate potential denitrification assays were performed with added 45.83 mM dextrose and 14.28 mM nitrate to generate the maximum possible rates under excess nutrients. In 2015, in order to quantify how much N_2O may be released by incomplete denitrification, a second assay was performed simultaneously with inert helium added instead of acetylene. Thus, potential rates of N_2O-producing incomplete denitrification activity could be determined as well. Gas samples were collected at the beginning and after three hours. The concentration of N_2O was determined using a gas chromatograph (GC-2014 with ECD, Shimadzu Corp., Kyoto, Japan). The final denitrification rate was corrected by the dry weight of the soil.

DNA extraction

Genomic DNA was extracted from 0.5 g freeze-dried soil using a FastDNA kit (MP Biomedicals, Santa Ana, California) according to the manufacturer's instructions. Humic acids were removed using cetyl trimethyl ammonium bromide (CTAB) purification (Sambrook and Russell 2001). CTAB is a cationic surfactant that solubilizes complex carbohydrates and secondary metabolites that might interfere with PCR (Azmat et al. 2012). Following CTAB purification, the absorbance ratio at 260:230 nm was determined on a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), and purified DNA samples

with a 260:230 nm ratio greater than 1.5 were used for qPCR. The purified DNA was adjusted to approximately 20 ng/μL and stored at -20˚C until further microbial community analysis.

Microbial community composition analysis

Illumina sequencing was used to target bacterial and archaeal rRNA genes, as well as *nirS*, *nirK*, and Clade I *nosZ* genes (Illumina, San Diego, CA). Sequencing amplicons were prepared by PCR using a Fluidigm Access Array IFC chip, which allowed simultaneous amplification of each target gene (Fluidigm, San Francisco, CA). Initial reactions were carried out according to a 2-step protocol using reagent concentrations specified by the manufacturer (FastStart High Fidelity PCR System, dNTPack (Roche, PN 04-738-292-001). The first PCR was performed in a 100-μL reaction volume using 1 ng DNA template and an annealing temperature of 58˚C, and this PCR amplified the target DNA region using both the gene-specific primers with Fluidigm-specific amplification primer pads CS1 (5'-ACACTGACGACATGGTTCTACA-3') and CS2 (5'-TACGGTAGCAGAGACTTGGTCT-3'), which produced amplicons comprised of (1) CS1 Fluidigm primer pad, (2) 5'-forward PCR primer, (3) amplicon containing the region of interest, (4) 3'-reverse PCR primer, and (5) CS2 Fluidigm primer pad. A secondary 30-μL PCR used 1 μL of 1:100 diluted product from the first PCR as template, and added Illumina-specific sequencing linkers P5 (5'-AATGATACGGCGACCACCGAGATCT-3') and P7 (5'-CAAGCAGAAGACGGCATACGAGAT-3'), along with a 10-bp sample-specific barcode sequence, so the final construct consisted of (1) Illumina linker P5, (2) CS1, (3) 5'-primer, (4) amplicon containing the region of interest, (5) 3'-primer, (6) CS2, (7) sample-specific 10-bp barcode, and (8) the Illumina linker P7. Final amplicons were gel-purified, quantified (Qubit; Invitrogen, Carlsbad CA, USA), combined to the same concentration, and then sequenced from both directions on an Illumina HiSeq 2500 2x250 bp Rapid Run. Fluidigm amplification and

Illumina sequencing was conducted at the Roy J. Carver Biotechnology Center (Urbana, IL, USA).

Overall community composition was assessed by sequencing the bacterial and archaeal 16S ribosomal rRNA gene V4 region using the forward primer 515F and reverse primer 806R (Caporaso et al. 2011). The NO_2^- reducing denitrifier composition was assessed by sequencing both *nirK*, with forward primer nirK876 and reverse primer nirK1040 (Henry et al. 2004), as well as *nirS*, with forward primer nirSCd3aF and reverse primer nirSR3cd (Kandeler et al. 2006). The N_2O reducing denitrifier composition was assessed by sequencing Clade I *nosZ* with forward primer nosZ1F and reverse primer nosZ1R (Henry et al. 2006). Primer sequences are listed in Table 1.1 (Chapter 1).

Paired-end 16S sequences were merged using Fast Length Adjustment of SHort reads (FLASH) software v. 1.2.11 (Magoč and Salzberg 2011). Only the first end of the sequences ("Read 1") was used for analysis of the functional genes. Quality filtering of fastq files was performed using software in the FASTX-Toolkit (Gordon and Hannon 2010). Sequences with fewer than 90% of bases showing at least 99.9% base accuracy were removed. The *nirK* sequences were shorter than 300-bp, so they were trimmed to the appropriate size using the FASTX-Toolkit. The fastq files were then converted to fasta format, and sequences were binned into discrete OTUs based on 97% similarity using USEARCH v. 8.1.1861 (Edgar 2010). Taxonomic assignments were made using QIIME software (Caporaso et al. 2011). For 16S gene assignments, the GreenGenes database v. 13.5 was used (McDonald et al. 2012) with the UCLUST algorithm (Edgar 2010). Functional gene databases were created by downloading sequences from the RDP FunGene website (Fish et al. 2013), and assignments were made in QIIME with the BLAST algorithm (Altschul et al. 1990).

Quantification of N_2O producing and consuming denitrifiers

Quantitative PCR (qPCR) was used to determine the number of *nirS* and *nirK* genes present in each community as a proxy for N_2O producer abundance, and N_2O consumer abundance was measured by quantifying the number of Clade I and II *nosZ* genes present in the community.

In order to quantify gene copy number in each of the qPCR assays, replicate serial dilutions of standard template were amplified simultaneously with the samples to produce a standard curve. The template DNA for these standards were generated by first amplifying the gene of interest from a mixed wetland soil sample using PCR without fluorescent dyes: Reactions were carried out in a 50μL volume and contained 50 mM Tris (pH 8.0), 25 μg/mL of T4 gene 32 protein, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 20 pmol of each primer (four separate reactions: *nirK*, *nirS*, Clade I *nosZ*, and Clade II *nosZ*), 2.5 U of Taq polymerase (Promega, Madison, WI, USA), and 100 ng of extracted soil DNA. PCR conditions included initial denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 53 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 15 min. Next, the amplicons were separated from primer dimers using a QIAquick gel extraction kit according to the manufacturers' protocol (Qiagen, Valencia, CA, USA). The concentration of purified amplicon was determined using a Qubit DNA fluorometer (ThermoFisher Scientific, Waltham, MA, USA), and the exact copy number of the gene of interest in each the standard could be calculated from the concentration. The final copy number of each gene in the unknown soil samples was normalized by the ng of template DNA used in the qPCR reaction. The MIQE guidelines (Minimum Information for qRT-PCR Experiments) explained by Bustin et al. (2009) were used to evaluate assay performance based on the standard curves for the *nirK, nirS*, Clade I *nosZ*, and

Clade II *nosZ* qPCR assays, and the results of this analysis are summarized in Table B.1 and Fig B.1. All gene copy numbers calculated in unknown wetland soil DNA samples were within the range of the respective standard curve for each gene.

PCR amplification reactions were carried out in triplicate in a 5 µL volume containing 1X FastStart Universal SYBR Green master mix (Roche Applied Science, Germany) with 125 µg T4 gene 32 protein (Roche Applied Science, Germany), and 0.4 µM of each forward and reverse primers was added to their respective reactions. The *nirK* gene was amplified using the same primers used for sequencing, nirK876 and nirK1040 (Henry et al. 2004), and the *nirS* gene was also amplified using the same primers as used for sequencing, nirSCd3aF and nirSR3cd (Kandeler et al. 2006). The following protocol was used to amplify both *nirK* and *nirS* genes: 5 min initial denaturation at 95˚ C and then 40 cycles of 95˚ C for 45 sec, 54˚ C for 1 min, and 72˚ C for 1 min. The Clade II *nosZ* gene was amplified using the primers nosZ-II-F and nosZ-II-R (Jones et al. 2014) according to the following protocol: 5 min initial denaturation at 95˚ C and then 40 cycles of 95˚ C for 45 sec, 54˚ C for 1 min, and 72˚ C for 1 min. The Clade I *nosZ* gene was amplified using the same primers as used for sequencing, nosZ1F and nosZ1R (Henry et al. 2006), and a touchdown protocol was used: 15 minutes initial denaturation at 95˚ C, 2 cycles of 95˚ C for 15 sec, 68˚ C for 1 min, and 72˚ C for 30 sec, 2 cycles of 95˚ C for 15 sec, 66˚ C for 1 min, and 72˚ C for 30 sec, 2 cycles of 95˚ C for 15 sec, 64˚ C for 1 min, and 72˚ C for 30 sec, and ending with 35 cycles of 95˚ C for 15 sec, 62˚ C for 1 min, and 72˚ C for 30 sec. All reactions were carried out separately for each target sequence in a 7900HT Fast Real-Time PCR System (Applied Biosysterms, Foster City, CA). The quantitative cycle threshold (C_q) was determined using SDS software v 2.4 (Applied Biosysterms, Foster City, CA). Gene copy number was quantified by comparing C_q of samples against those produced by a standard curve

constructed from gel-purified environmental amplicons for each gene. For all reactions, 1 μL of template was used, which contained approximately 10-15 ng genomic DNA.

Statistical analyses

The microbial community was analyzed separately in three different components: The total microbial community based on 16S rRNA gene sequences, the N_2O producing denitrifiers based on a concatenated OTU table of *nirK* and *nirS* sequences, and the N_2O consuming denitrifiers based on Clade I *nosZ* sequences. Principle coordinate analysis (PCoA) ordinations were used to visualize differences among communities using the 'cmdscale' function in the 'vegan' package in R (Oksanen et al. 2013, R Core Team 2014). In order to determine whether the microbial communities in the restored sites were converging toward the communities in the reference wetlands, the average Bray-Curtis distance between replicate samples collected in the two communities was calculated for each pair individually in each year, and Bray-Curtis distance was calculated using the 'vegdist' function also in the 'vegan' package. Bray-Curtis distance was also used to calculate how much the reference communities themselves changed over time by comparing the reference community year to the initial reference community observed in 2007. Permutational analysis of variance (PERMANOVA) models were built using the 'adonis' function in 'vegan' in order to determine the relative proportion of variability in composition explained by year of sampling, wetland site, and type of wetland (reference or restored). Analysis of variance (ANOVA) models were constructed using the 'aov' function in R to determine the differences among univariate data, such as Bray-Curtis distances, denitrification rates, or qPCR-based gene abundances. Specific differences between groups were identified using Tukey's honest significant difference test using the 'TukeyHSD' function. Simple linear regression models were built using the 'lm' function in R.

Separate analyses were used to identify two sets of "indicator denitrifier taxa" based on two different traits: (1) "High functioning" denitrifier OTUs whose relative abundances correlated to potential denitrification rates and (2) "Flood sensitive" denitrifier OTUs that changed in relative abundance (either by decrease or increase) in 2013 when unusually intense flooding occurred across central Illinois.

In order to identify denitrifier OTUs correlated with potential denitrification rates, a redundancy analysis (RDA) ordination was created with the 'rda' function in 'vegan' using a concatenated OTU table including *nirK*, *nirS*, and *nosZ* sequences and setting potential denitrification rates as the constrained RDA axis. The OTUs with RDA axis loadings within the highest 30% of all RDA values were selected as "indicator denitrifiers". This was done separately for rates generated without excess nutrients and for rates generated with excess nutrients.

In order to identify specific changes in the denitrifier community that arose in response to particularly large floods that occurred during the spring before sampling in 2013, a similarity percentage (SIMPER) analysis was conducted using the 'simper' function in 'vegan' on the concatenated OTU table based on *nirK*, *nirS*, and *nosZ* sequences. A new categorical variable was created where data collected in 2007 and 2012 were assigned to "Pre-Flood", data collected in 2013 were assigned to "Flood", and data collected in 2014 and 2015 were assigned to "Post-Flood". The top 12 denitrifier OTUs identified to drive compositional differences either between "Pre-Flood" and "Flood" or between "Flood" and "Post-Flood" were selected. Average relative abundance during "Pre-Flood" and "Post-Flood" was then calculated as the mean of the mean abundances detected during both years included in for each group, and relative abundance during "Flood" as the mean abundance detected during 2013 only. Heatmaps were created using the

'heatmap' function in R based on one large matrix of mean relative abundance values in order to keep the color scale consistent between all 12 OTUs.

Results

Change in microbial community composition over time

We detected 11,645 different microbial OTUs from 16S rRNA gene sequencing, and the total number of reads was over 9.5 million. For the N_2O producing denitrifiers, 7547 OTUs were observed from the *nirK* sequences with more than 5 million *nirK* reads in total, and 2613 OTUs were observed from the *nirS* sequences with more than 775,000 *nirS* reads in total. For the N_2O consuming denitrifiers, 2509 OTUs were observed from the Clade I *nosZ* sequences with more than 2 million *nosZ* reads in total.

Overall microbial composition and the composition of both N_2O producing and consuming denitrifiers significantly varied across years, though composition also remained distinct between wetlands sites and between restored and reference wetlands (Table 3.2). PCoA ordinations showed that the overall community and the N_2O producing denitrifiers in the restored wetland at Site B remained similar to the community in the paired reference wetland, while the communities in the paired wetlands at the remaining three sites remained distinct from one another throughout the study (Fig. 3.2A & 3.3A; Table 3.2). The ordination of the N_2O consuming denitrifier communities did not reveal strong site-to-site differences, and instead showed the restored wetland communities clustering distinctly from the reference wetland communities (Fig. 3.4A; Table 3.2). The reference communities changed through time (Fig. B.2), where simple linear regression showed a significant effect of time on the distance between the overall microbial community in each reference and the initial community ($R^2 = 0.53$, $p <$

0.001), as well as for the N_2O producing and consuming denitrifiers ($R^2 = 0.67$, $p < 0.001$; $R^2 = 0.81$, $p < 0.001$; respectively).

Using the reference community of each pair for comparison for each wetland community, we were able to show that communities in the restored wetlands are not converging toward the communities in the reference wetlands. The overall microbial community in the restored wetlands did not become any more similar to the community in the reference wetlands by the end of the time series than was observed at the beginning (Fig. 3.2B), and time was not a significant factor in the ANOVA model intended to explain these differences. For both the N_2O producing and consuming denitrifier communities, the communities in the restored wetlands were almost entirely dissimilar from the communities in the reference wetlands, where the Bray-Curtis distances were greater than 0.9 for most of the pairs at the beginning and remained equally as distant throughout the time series (Fig. 3.3B & 3.4B).

Potential denitrification activity over time

Individual wetland pairs at Sites A, C, and D showed differences between rates observed in the restored and reference wetland throughout the study (Table 3.3). There were no significant differences between mean rates in restored and reference wetlands, contrary to results observed in 2007 by Flanagan (2009). Potential denitrification peaked in 2013 for some wetlands but not all (Fig. 3.5), which shows that wetland soil communities varied in their maximum potential denitrification.

Indicator denitrifier OTUs correlated with potential denitrification

Six denitrifier OTUs were identified as "indicator taxa" correlated with potential denitrification rates based on their placement within the top 70th percentile of redundancy analysis axis loadings. Six OTUs were found to correlate with rates generated without excess

nutrients. Two OTUs were correlated with the rates generated with excess nutrients, and these were also included in the first six. Thus these six OTUs were selected as "indicator denitrifiers" for potential denitrification in general. Two *Azoarcus*-like OTUs, one *Azospira*-like OTU, and an OTU that was identified as *Arenimonas donghaensis* were identified from *nirS* sequences, one *Alcaligenes*-like OTU was identified from its *nirK* sequence, and a *nosZ* sequence-based OTU was identified as *Rhodopseudomonas palustris*. ANOVA models showed that time was a significant factor influencing the relative abundances of one of the *Azoarcus*-like OTUs, the OTU identified as *Arenimonas donghaensis*, and the *Alcaligenes*-like OTU. Tukey's HSD test revealed that abundance did not increase consistently over time and the relative abundances of indicator denitrifier taxa did not follow the same patterns (Fig. 3.6). Further, these taxa showed different patterns in different wetlands, which shows that there was a random pattern of community development over the duration of this study.

Denitrifier OTUs driving changes in composition before and after the flood in 2013

SIMPER analyses revealed that 12 denitrifier OTUs explained a small proportion of the compositional changes between both "Pre-Flood" (2007+2012) and "Flood" (2013) and between "Flood" and "Post-Flood" (2014+2015). These 12 OTUs explained 6.5% of the difference between "Pre-Flood" and "Flood" and 7.1% of the difference between "Flood" and "Post-Flood". Though it is clear that these OTUs were sensitive to flood conditions in some sites (e.g. OTU "LTTS_nirS_0007" in the reference wetland at Site C), the change in relative abundance did not follow a consistent pattern across all eight wetlands throughout the study (Fig. 3.7).

Change in ratio of N_2O producing and consuming denitrifiers and relationship with potential N_2O emissions

The log ratio of N_2O producers to consumers significantly increased through time (ANOVA: Year, $F = 13.4$, $df = 4$, $p < 0.001$). The ratio differed between wetland sites (ANOVA: Site, $F = 3.3$, $df = 3$, $p = 0.02$), such that Site B showed a lower ratio than Sites A and C. This ratio was not significantly different between wetland types ($p = 0.07$), but the reference wetlands tended to have a greater log ratio of N_2O producers to consumers, especially by the end of the study in 2014 and 2015 (Fig. 3.8). There was no significant correlation between the log ratio of N_2O producers to consumers and potential N_2O production during the denitrification assays performed in 2015, and N_2O production was not correlated to the ratio of *nirK* to *nirS*, ratio of Clade I *nosZ* to Clade II *nosZ,* or total *nir* gene copies or total *nos* gene copies. There was a significant negative linear relationship between the number of Clade I *nosZ* gene copies per ng total soil DNA and the proportion of N_2O produced by denitrification during the potential activity assays (linear regression: adjusted $R^2 = 0.66$, $p < 0.01$; Fig. B.3), but there was no relationship with Clade II *nosZ* gene copies or with *nirK* or *nirS* gene copies. Restored and reference wetlands did not significantly differ in total potential denitrification rates, incomplete denitrification rates, or in the proportion of N_2O produced during the potential activity assays.

Discussion

In our study, microbial community composition in restored wetlands appears to be dynamic, even up to 18 years after restoration, but the restored wetland communities remained dissimilar from the reference wetlands over time. Further, reference wetlands also changed in composition over time, and we observed high variation between reference wetland communities.

Thus, we were not able to identify a single "target" reference microbial community. In this system, we acknowledge that multiple community types may serve the same purpose, where denitrifiers could be equally as active in a restored wetland as those in a reference wetland despite differences remaining in the composition of the microbial community. However, potential denitrification rates were often greater in the reference wetlands by the end of the time series, which is consistent with published literature (Hossler et al. 2011, Marton et al. 2014).

The findings reported previously in Chapter 2 suggest that reference and restored communities would exhibit equivalent function, even if wetlands difference in community composition. The study described in Chapter 2 encompasses a greater number of replicate wetlands but lacks fine scale details about the microbial community. The study described in the current chapter serves as more of a case study to observe possible restoration outcomes for the microbial community. We conclude that these particular restored communities exhibited divergence toward undesirable states (Matthews and Spyreas 2010) since the microbial communities in the restored wetlands in this study followed unique trajectories and did not exhibit equivalent function compared to the reference wetlands.

Evidence from the literature suggests that microbial communities are strongly influenced by soil properties (Foulquier et al. 2013, Murphy and Foster 2014, Morrissey and Franklin 2015), and this likely leads to the slow development of the microbial community, which mirrors soil development (Kandeler et al. 2006, Schütte et al. 2009, Ferrenberg et al. 2013). It is possible that the communities in restored wetlands exhibited divergent behavior due to environmental differences that remained in the soil itself, despite similar hydrology and aboveground vegetation as in the reference wetlands. Soil texture was fairly homogenous across all of the study sites in our system, but the reference wetlands typically had greater total soil N and total soil C than the

restored wetlands (Fig. B.4). The restored wetlands may not have accumulated equivalent organic matter in the soil by the time our study was conducted, and this may have allowed differences in microbial community composition to persist in the restored wetlands.

Dynamic conditions, like seasonal flood intensity and corresponding redox conditions may affect both reference and restored communities equally but not enough to drive composition toward a similar state. We identified specific denitrifier taxa whose relative abundance changed during a year that experienced particularly large floods across the entire state. Taxa closely related to *Rhodopseudomonas palustris* and *Azoarcus* increased in abundance in that year. *R. palustris* is commonly found in saturated sediments (Harwood and Gibson 1988) and it was not unexpected to observe such a response to flood conditions. *Azoarcus* has the cellular machinery to participate in denitrification though it is typically known for being an associative N-fixer (Liu et al. 2006). This particular *Azoarcus* OTU may an opportunist that can switch from aerobic respiration to denitrification under flooded anaerobic conditions (Fernández et al. 2014). Other denitrifiers decreased in relative abundance in 2013 and then rebounded during the following years, such as taxa related to *Ensifer adhaerens* and *Pseudomonas*. *E. adhaerens* is another N-fixer that possesses the ability to carry out denitrification (Rudder et al. 2014), while the *Pseudomonas* genus includes well-known denitrifiers that can carry out denitrification under aerobic conditions (Davies et al. 1989, Su et al. 2001). The fact that this taxon was in greater abundance both before and after the particularly intense flood year, at least in Site A, may reflect some type of competitive advantage over other denitrifiers regardless of O_2 availability. Most notably, some of the unique denitrifier OTUs that were determined to drive compositional changes in response to flood conditions exhibited conflicting behavior in different wetlands, such as one of the denitrifier taxa related to *Paracoccus*. This demonstrates that hydrologic

disturbances have a highly stochastic effect on restoration trajectories, as has been suggested before for wetland plant communities (Richardson et al. 2007, Trowbridge 2007, Matthews and Spyreas 2010).

Only three of the four paired wetlands in this study showed a significant difference between denitrification potential in the restored and reference wetlands throughout the time series, and restored and reference wetlands did not significantly differ in rates overall, though this result may have been due to high variability in rates between each of the pairs (e.g. Site A pair vs. Site B pair) and high variability among years. Contrary to our results, we expected a clear difference between restored and reference wetlands because numerous studies have reported some sort of limitation of denitrification rates in restored wetlands (Orr et al. 2007, Flanagan 2009, Hossler et al. 2011, Marton et al. 2014). However, there was a striking difference in the potential denitrification rates observed in restored and reference wetlands in 2013, when data were collected following a spring season with intense flooding, and rates increased the most from the reference wetlands in Site A and Site C. These rates were generated in anaerobic laboratory assays, so oxygen availability in the environment should have no effect on the denitrification potential reported in this study. The seasonal flood conditions in 2013 must have either altered the community or induced dormant individuals into an active state so they were better able to take advantage of the ideal conditions provided in the laboratory than the soil communities collected during the other years of the study.

A previous study found that potential denitrification rates in restored wetlands decreased from spring to summer in a single year, even though denitrifier composition remained constant during that time (Peralta et al. 2016). Results of studies that investigate functional differences among restored and reference wetlands at only a single time point may change depending on

environmental conditions that year or even on the time of year in which sampling occurred, and the link between composition and function may also change. By identifying indicator denitrifier taxa, we were able to observe differences in relative abundance of important denitrifying taxa throughout the experiment, but these relative abundances did not increase or decrease linearly over time. Rather, abundance appeared to respond to changes in external weather conditions, such as the flooding the occurred in 2013. This could either mean that the actual abundance of these taxa is changing in response to external conditions, or the abundance of these taxa remained steady while the abundance of the rest of the community fluctuated. Thus, subtle differences in composition occurred, but the process of community development did not follow a smooth trajectory following restoration, and the reference wetland community was also changing over time. This is consistent with the broad differences observed earlier in the overall community and the denitrifier communities across the study, and further shows that the community response to environmental cues but may be transient.

We have shown that microbial communities in 7- to 18-year-old restored freshwater wetlands are not converging toward a desired reference community, and the soil community present in the restored sites will not always be able to achieve similar rates of denitrification as the reference communities even under identical conditions. The soil environment itself may be physically constraining the development of community composition toward a reference state. For example, the reference wetlands were observed to have greater organic matter, which could be a filter on community composition. Thus, we reiterate calls for careful management of the soil, and selection of sites that already exhibit soil properties similar to wetlands that have been damaged or destroyed, in order to rapidly replace these wetlands (Flanagan 2009, Peralta et al. 2012). We further caution that it may take decades to truly replace wetlands of equivalent function.

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Tables

Table 3.1. Site description for paired restored and reference wetlands that were sampled during the summers in 2007, 2012, 2013, 2014 (except for the Site B pair), and 2015. Soil texture data came from the USDA Web Soil Survey online tool (Soil Survey Staff).

Pair	Illinois County	Longitude	Latitude	Age in 2007 (years)
Site A	Sangamon	-89.67	39.88	7
Site B	Pike	-91.35	39.73	10
Site C	Stephenson	-89.65	42.46	7
Site D	Sangamon	-89.65	39.84	10
	Restored Site:		Reference Site:	
Pair	Hydrology	Soil Texture	Hydrology	Soil Texture
Site A	Floodplain	Silt Loam	Floodplain	Silty Clay Loam
Site B	Floodplain	Clay Loam	Floodplain	Silt Loam/Clay Loam
Site C	Depressional	Silt Loam	Depressional	Silt Loam
Site D	Floodplain	Silty Clay Loam	Floodplain	Silty Clay Loam

Table 3.2. PERMANOVA results from the 'adonis' function showing the proportion of variability explained (PERMANOVA R^2 statistic) by Site, Type (reference or restored), Year, and all possible interactions in the overall microbial community, N_2O producing denitrifier community, and N_2O consuming denitrifier community.

	Overall microbial community PERMANOVA R^2	N_2O producing denitrifier community PERMANOVA R^2	N_2O consuming denitrifier community PERMANOVA R^2
Site	*** **0.165**	*** **0.072**	*** **0.057**
Type	*** **0.088**	*** **0.035**	*** **0.036**
Year	*** **0.010**	*** **0.006**	** **0.006**
Site X Type	*** **0.111**	*** **0.045**	*** **0.042**
Site X Year	*** **0.014**	** **0.011**	* **0.011**
Type X Year	** **0.005**	** **0.004**	* **0.004**
Site X Type X Year	** **0.010**	** **0.011**	** **0.012**
Residuals	0.597	0.814	0.832
Total	1.000	1.000	1.000

Significance: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

Table 3.3**.** *F*-values from ANOVA models explaining denitrification rates for each wetland pair.

		Potential denitrification without excess nutrients				Potential denitrification with excess nutrients			
	df	Site A	Site B	Site C	Site D	Site A	Site B	Site C	Site D
Year	3	*** **461.3**	*** **10.8**	*** **14.1**	*** **7.4**	*** **56.3**	*** **226.2**	*** **684.4**	*** **29.3**
Type	1	*** **478.4**	0.3	* **5.6**	*** **30.2**	** **9.8**	1.2	*** **303.7**	*** **89.9**
Year X Type	3	*** **281.8**	2.2	** **4.7**	*** **13.8**	* **4.0**	0.5	*** **130.1**	*** **13.4**

Significance: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

Figures

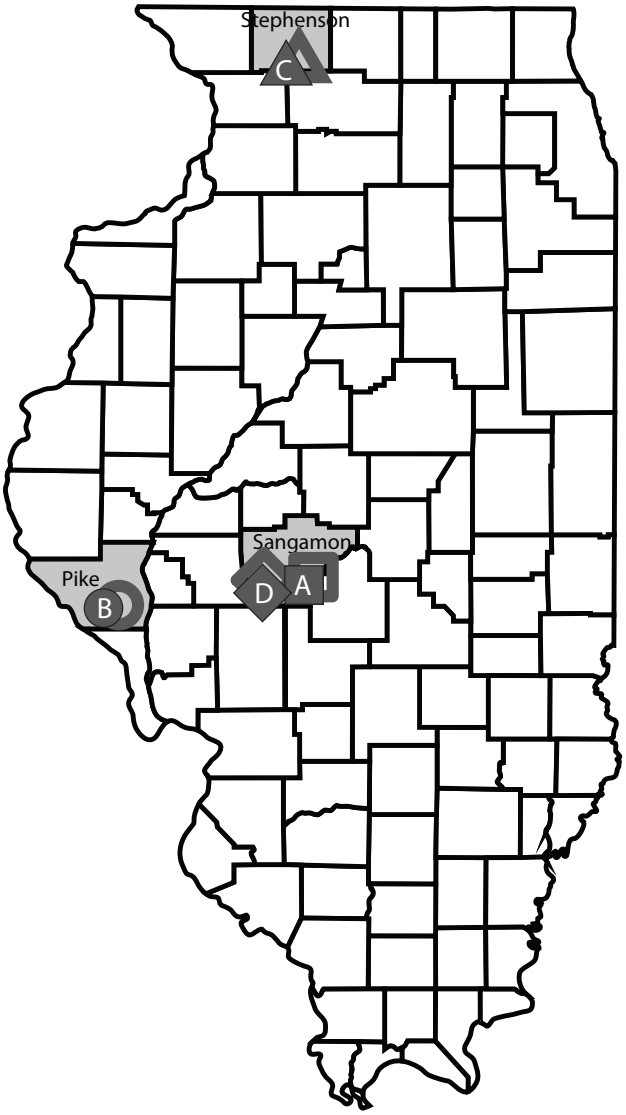

Figure 3.1. Map of Illinois with counties containing paired restored and reference wetlands shaded grey. Symbol shapes are overlaid on approximate location of corresponding wetlands.

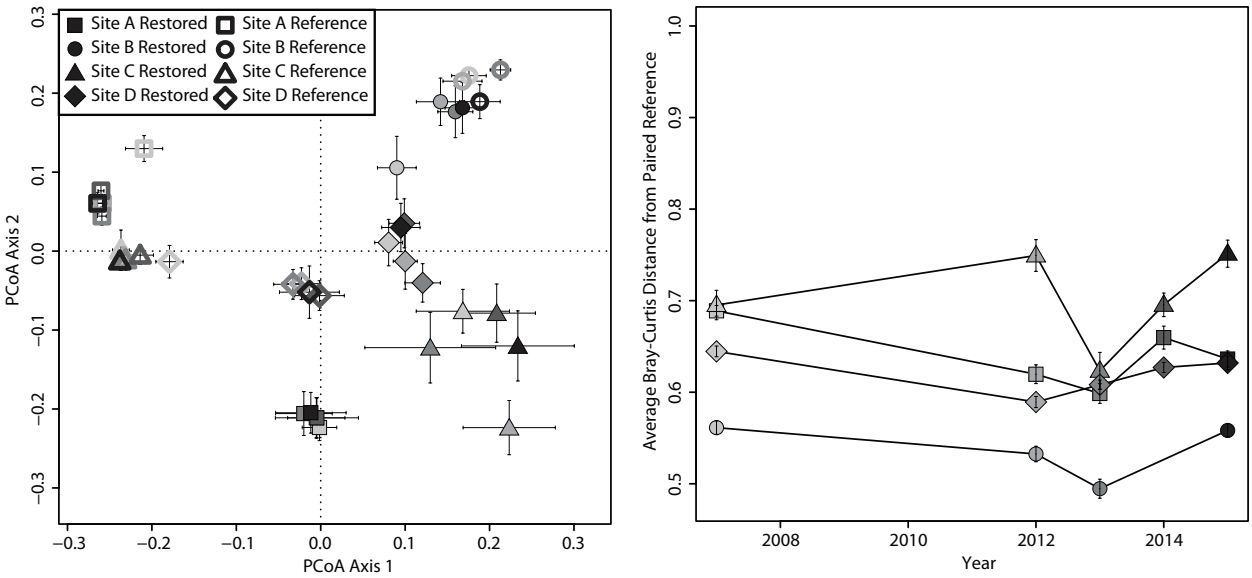

Figure 3.2. (A) Principle Coordinate Axis (PCoA) ordination of the microbial community generated from16S rRNA gene sequences. (B) Bray-Curtis distance between the restored and reference wetland in each pair over time. Symbols correspond to wetland pair, and shading indicates year, where the lightest color is from 2007 and the darkest represent data collected in 2015. Error bars show standard error of the mean.

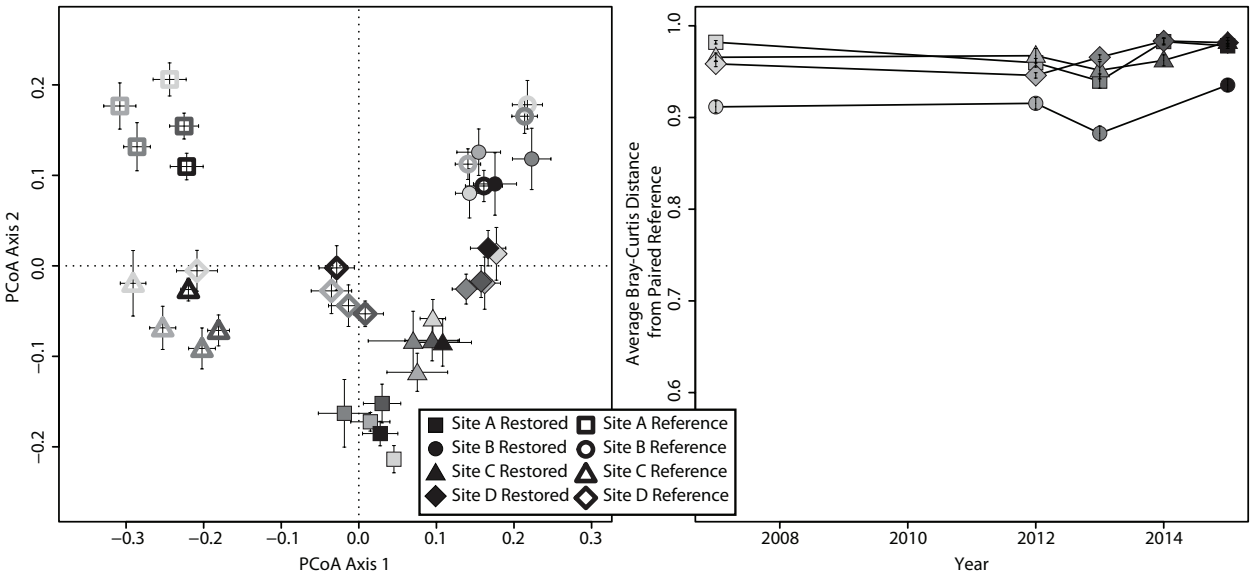

Figure 3.3. A) Principle Coordinate Axis (PCoA) ordination of the nitrite reductase-containing denitrifier community generated from *nirK* and *nirS* gene sequences. (B) Bray-Curtis distance between the restored and reference wetland in each pair over time. Symbols correspond to wetland pair, and shading indicates year, where the lightest color is from 2007 and the darkest represent data collected in 2015. Error bars show standard error of the mean.

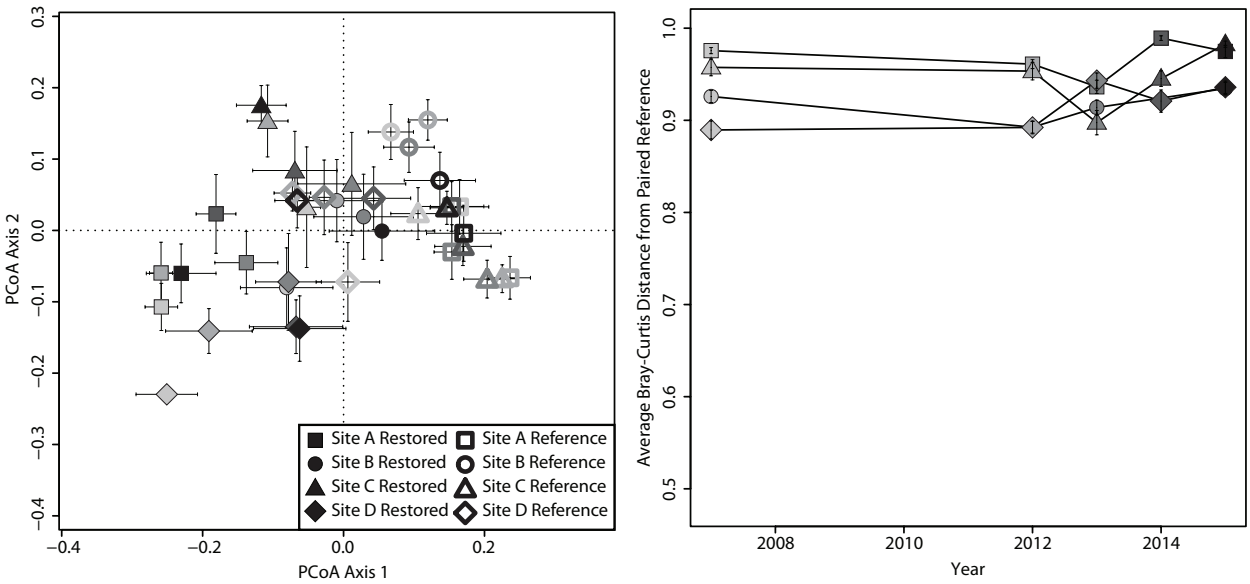

Figure 3.4. (A) Principle Coordinate Axis (PCoA) ordination of the nitrous oxide reductase-containing denitrifier community generated from Clade I *nosZ* gene sequences. (B) Bray-Curtis distance between the restored and reference wetland in each pair over time. Symbols correspond to wetland pair, and shading indicates year, where the lightest color is from 2007 and the darkest represent data collected in 2015. Error bars show standard error of the mean.

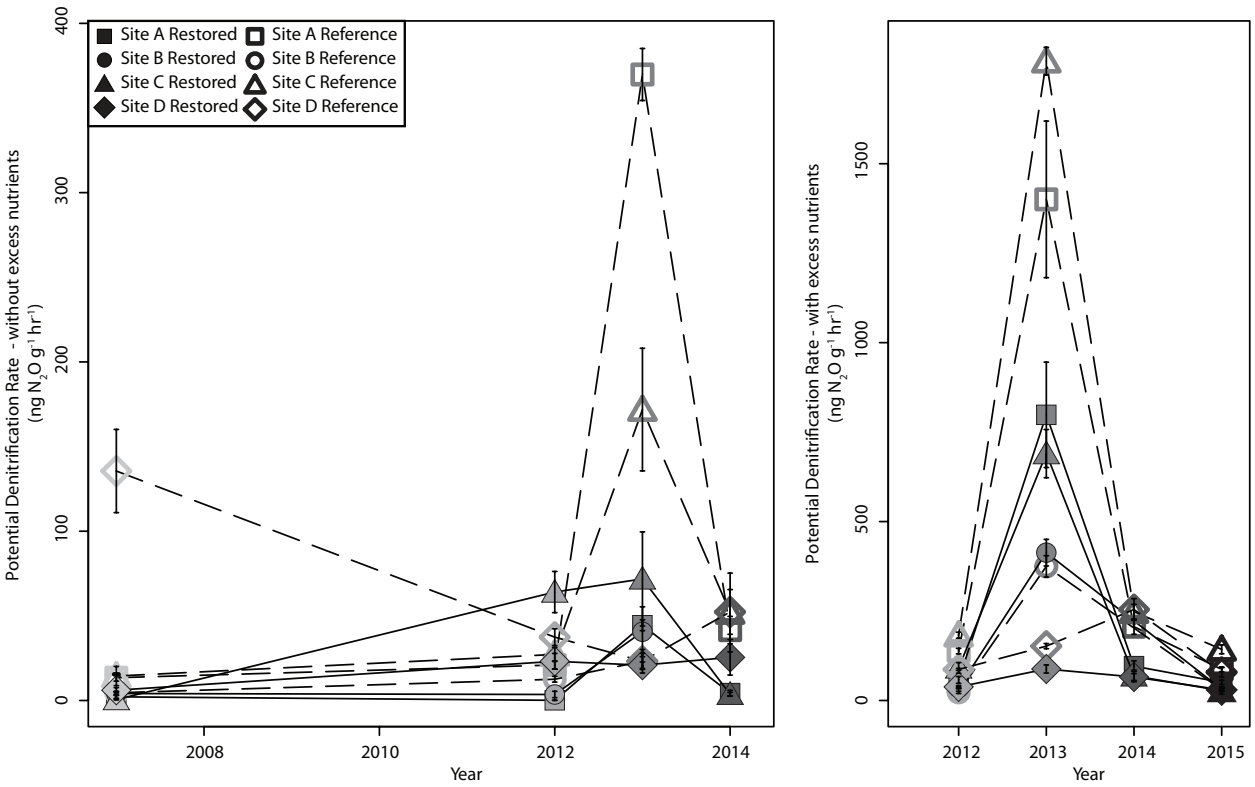

Figure 3.5. (A) Potential denitrification rates generated in a laboratory environment without any added nutrients. (B) Potential denitrification rates generated in a laboratory environment with excess potassium nitrate and dextrose added. Symbols correspond to wetland pair, and shading indicates year, where the lightest color is from 2007 and the darkest represent data collected in 2015. Error bars show standard error of the mean.

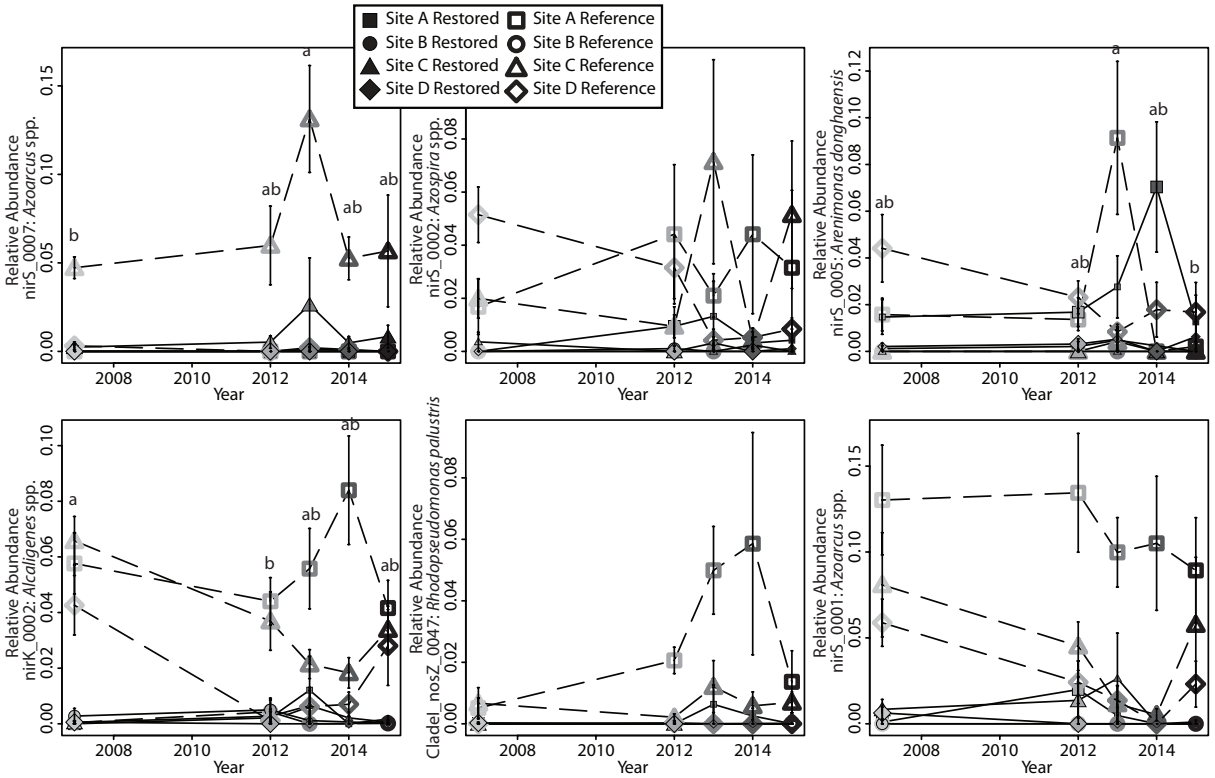

Figure 3.6. Relative abundance of the top six denitrifier OTUs found to correlate with potential denitrification rates using redundancy analysis (RDA) ordinations with rates as the constrained axis. Shading indicates year of sampling, symbols correspond to wetland pair, and error bars show standard error of the mean. Lower case letters indicate significant groupings between years determined by Tukey's HSD test.

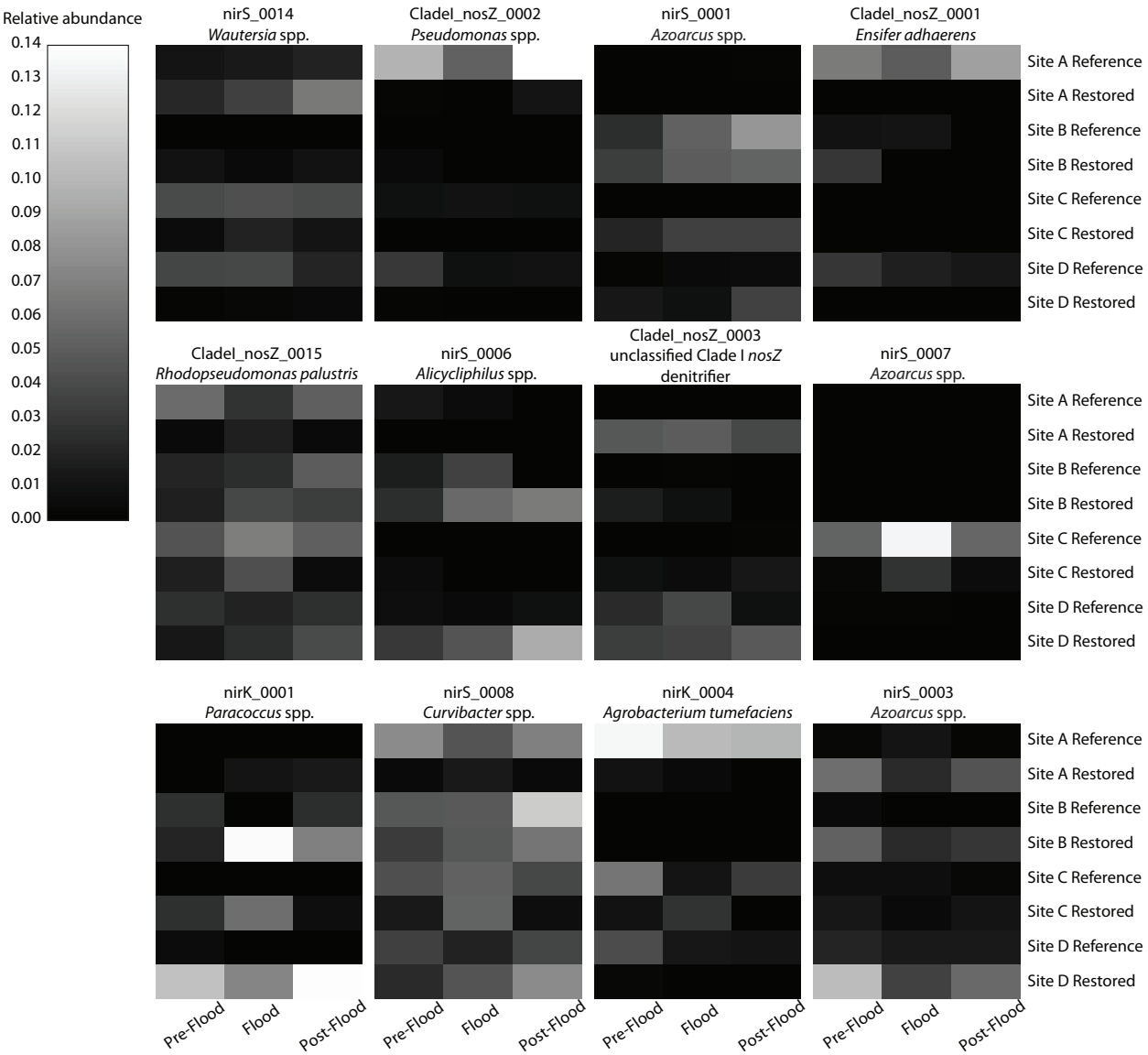

Figure 3.7. Heatmaps showing average relative abundance of the top 12 denitrifier OTUs found to drive changes in composition between "Pre-flood" (2007+2012) and "Flood" (2013) and between "Flood" and "Post-flood" (2014+2015) using a similarity percentage (SIMPER) analysis. Denitrifier OTUs that were undetected are shown as black, and those in higher abundance are indicated in white or lighter shades of grey. Sequence ID is shown at the top and the closest taxonomic identification found is directly below.

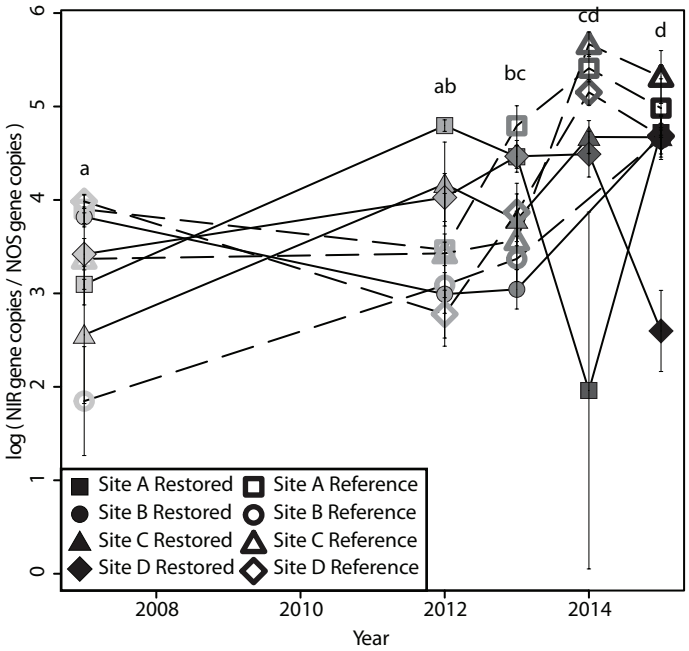

Figure 3.8. Change in the log ratio of genes present in N_2O producers ("NIR") and consumers ("NOS") over time. Shading indicates year of sampling, symbols correspond to wetland pair, and error bars show standard error of the mean. Lower case letters indicate significant groupings between years determined by Tukey's HSD test.

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CHAPTER 4:

ENVIRONMENTAL VARIABILITY SHAPES MICROBIAL COMMUNITY RESPONSE TO ALTERED HYDROLOGY IN A FLOODPLAIN WETLAND ECOSYSTEM

Abstract

Wetland soil communities that experience frequent flooding may also exhibit high functional stability. The dynamic environment itself might act as a filter that selects for microbial taxa with a unique physiological tolerance to both dry and saturated conditions. Soil communities in wetlands are responsible for nitrogen cycling functions, such as nitrification and denitrification. Most denitrifiers are facultative anaerobes and should exhibit high tolerance to both aerobic and anaerobic conditions, while nitrifiers are strict aerobes predicted to have limited tolerance for saturate anaerobic conditions. To test whether variability acts a filter and influences functional stability, soils were collected from upland, lowland, and hydrologically variable regions of a restored floodplain along the Illinois River, and each was subjected to dry, saturated, and variable hydrologic manipulation in a mesocosm experiment. Potential rates of nitrification and denitrification were generated, and the 16S ribosomal RNA gene and diagnostic functional genes *amoA*, *nirK*, and *nirS* were sequenced. Communities were distinct between the three regions with different hydrologic regimes. As predicted, the community present in the variable region exhibited the greatest functional stability and displayed the smallest degree of compositional change following the experiment compared to the other two communities, suggesting that this community included a high proportion of physiologically tolerant taxa. Most surprisingly, nitrification and denitrification activity showed similar responses to altered hydrology despite contrasting physiology of the functional groups. Historical variability filters

the community and influences microbial functional stability, and this might be able to further inform predictions about the effects of global change on the nitrogen cycle.

Introduction

Ecosystem stability is often attributed to species richness (Naeem and Li 1997) because high diversity means there is low probability that an entire functional group will be removed by a disturbance. Microbial communities are predicted to have high functional redundancy in general because the number of microbial taxa far outnumber the processes they perform (Torsvik and Øvreås 2002, Franklin and Mills 2006). However, if this were true, then compositional differences between communities would have little influence on the provisioning of microbially mediated ecosystem functions such as nutrient cycling. Since soil functional rates are often correlated to differences in the richness or composition between microbial communities (Nemergut et al. 2014, Graham et al. 2016), this idea more ore less represents a null hypothesis. The null hypothesis can be used to answer questions about *when* composition matters for function and *which* traits of the microbial community correspond to high functional stability. Richness certainly contributes to predictions of functional rates, and possibly functional stability (Griffiths et al. 2000, Bell 2010, Tardy et al. 2014). However, the taxonomic identity and physiology of individual taxa present in the community is likely to be important when richness alone fails to explain functional stability.

Most microbial communities are sensitive to a wide range of different types of disturbances, where composition is altered by the disturbance (Allison and Martiny 2008, Shade et al. 2012). This raises a question about why a few communities do exhibit compositional stability. Microbial taxa vary in physiological tolerance to environmental stress (Potts 1994,

Schimel et al. 2007, Placella et al. 2012). Because individual taxa vary in tolerance, communities should vary in stability, depending on the proportion of taxa that are particularly tolerant to the stress. In the face of a disturbance, communities that contain a high proportion of "tolerant taxa" will exhibit high compositional and functional stability, while functional stability might be lower for communities with a low proportion of tolerant taxa. This idea further predicts that two communities with different proportions of tolerant taxa will not be functionally redundant with one another, even if those communities exhibit similar taxonomic richness.

If high taxonomic richness provides a sort of "insurance" for functional stability through functional redundancy, then specific microbial functions should exhibit high stability because they are performed by diverse phylogenetic groups (Schimel and Gulledge 1998, Schimel et al. 2007). For example, microbial taxa involved with denitrification, the multistep reduction of nitrate to dinitrogen gas, are found among the Proteobacteria, as well as Verrucomicrobia, Gemmatimonadetes, Chloroflexi, Firmicutes, Bacteroidetes, and Spirochaetes (Jones et al. 2014). The ability to participate in the denitrification pathway has also been documented in archaea (Philippot 2002, Kraft et al. 2011) and eukaryotes (Seo and DeLaune 2010, Higgins et al. 2016). In contrast, nitrification, the conversion of ammonium to nitrate, is a process limited to a handful of bacterial genera in the β- and γ-Proteobacteria, as well as a small group of archaea, which perform the initial, rate-limiting step, ammonia oxidation (Prosser and Nicol 2008). If high diversity within a group confers high functional redundancy, then we would expect denitrification to exhibit greater functional stability than nitrification. This may also be reflected by a positive relationship between denitrifier richness and stability, while nitrifier richness and nitrification stability would not exhibit a relationship due to the limited diversity available to begin with.

Fluctuation between extreme states, such as oxic-anoxic fluctuations experienced by microbial communities in soils, is a particularly acute stressor (Pett-Ridge and Firestone 2005, Pett-Ridge et al. 2013). Either oxic or anoxic soil conditions alone present a strong environmental filter on community composition. The soils that regularly experience fluctuation between oxic and anoxic conditions may filter taxa that possess unique physiological tolerance to a range of redox conditions. Floodplain wetlands provide a study system to evaluate the effects of historical redox regimes on the functional stability of microbial processes due to the existence of the natural flood pulse (Odum et al. 1995, Hernàndez and Mitsch 2006, Altor and Mitsch 2008). Floodplains include a dry upland and flooded lowland, while the transition region between experiences regular intervals of oxic-anoxic fluctuation. This transition region sometimes harbors a unique community when compared to either the upland or lowland (Peralta et al. 2014, Peralta et al. 2016). The community present in the transition region may include an unusually large proportion of microbial taxa that are tolerant to both extremes, and thus uniquely tolerant to fluctuating oxic and anoxic conditions.

Both nitrification and denitrification are important wetland processes because they influence the pool of nitrate, a pollutant that leads to eutrophication in aquatic ecosystems, and they both produce N_2O, a potent greenhouse gas (Jordan et al. 2010, Batson et al. 2012). Previous work by Peralta et al. (2013) has established that nitrification and denitrification activity in wetland soils are both affected by changes in hydrologic conditions. We theorize that the distinct microbial assemblages observed across different regions of a floodplain reflect selective pressures from the hydrologic disturbance regime, and this should influence functional stability. In this study, we expect that communities from a naturally fluctuating environment will contain a high proportion of taxa that can tolerate hydrologic disturbances, which would result in

a more resistant or resilient response of the community compared to those that originate from a more constant environment. We also expect denitrifier activity to be more stable than nitrifier activity, as has been predicted for microbial functional groups with high and low diversity (Schimel and Gulledge 1998, Schimel et al. 2007). Denitrifiers should also exhibit greater stability than nitrifiers due to their flexible metabolic strategy (Wallenstein et al. 2006), while nitrifiers are aerobic organisms that would be highly stressed under anaerobic conditions (Francis et al. 2007). Experiments such as the one in this study are essential to investigate the effects of global change. Flood regimes in the Midwestern United States are expected to become more variable because climate change is expected to alter precipitation patterns in this region (IPCC 2014). The results of this study could provide valuable information for current predictive models of changing ecosystem processes.

Methods

Site description and sampling

Soil was collected in July 2012 from the La Grange wetland mitigation bank in Brown Co., IL, which is located adjacent to the Illinois River (39.97˚ N, 90.52˚ W). The La Grange site is a 1645-acre mitigation bank that was established by the Illinois Department of Transportation (IDOT) in 2004 to mitigate for impacts on other wetlands from road construction, and the majority of the site consists of former wetland area that has been restored (Brooks 2005, Plocher et al. 2009). The wetland is almost fully separated from the river by a levee, with the exception of a breach in the southeastern area that allows seasonal flooding. Triplicate samples were collected from three locations representing distinct hydrologic settings: “Upland Source 1” was collected from in an upland region that was flooded for an average of 0-1 days per year from

2003 to 2011. “Variable Source 2” was collected from a region located toward the middle of the wetland where seasonal floods occur (average annual flood duration was 90-111 days per year). “Lowland Source 3” was collected from a region located near the breach in the levee where the soil is typically flooded (flooded for >125 days per year on average; Fig. C.1). Sources 1 and 3 are considered to have relatively stable hydrologic conditions (dry and saturated, respectively), while Source 2 represents a hydrologically variable environment. Soil was collected from the top 10 cm with an ethanol-sterilized metal spade, and stored in a covered ethanol-sterilized 5-gal bucket. The triplicate samples collected from each source was collected from locations approximately 5 m away from each other. Soil was transferred to the laboratory on the same day of sampling, and stored in a cool, dark room before the soil experiment was set up on the day that immediately followed sampling.

Hydrologic disturbance experiment

In order to isolate the effect of altered flood regimes on microbial composition and function, a fully factorial mesocosm experiment was established in a greenhouse environment, where soil from each source location was subjected to every hydrologic treatment. All source soil was kept separate and was first processed using identical methods before being placed into identical mesocosms, which restricted any migration that may otherwise occur between different source soil communities had they been connected hydrologically. Soil was sieved to 2 mm and divided into experimental mesocosms constructed out of 6" plastic horticultural pots with drain holes at the bottom. Mesocosms that were subjected to saturation for any duration of the experiment were placed inside of larger plastic buckets that lacked drain holes to maintain the hydrological treatments. Autoclaved deionized water was used to manipulate water levels according to three treatments: (1) "Dry" mesocosms were not placed inside a larger bucket and

were kept dry for the full two months of the experiment. (2) "Variable" mesocosms were allowed to dry for one week, then placed inside a larger bucket and saturated up to 1 inch above the surface of the soil for one week. This was repeated three more times for a total of four two-week cycles. (3) "Saturated" mesocosms were placed inside of buckets and kept saturated up to 1 inch above the surface of the soil for the full two months of the experiment. In order to ensure that each hydrologic treatment fostered redox conditions that would be expected, one Indicator for Reduction in Soils (IRIS) tube (InMass Technologies, West Lafayette, IN, USA) was placed in a representative replicate mesocosm for each of the nine source soil × hydrologic treatment combinations for the two-month duration of the experiment. Daily monitoring was performed to ensure that evaporated water was replaced immediately. All mesocosms were located on the same bench in the same room in the greenhouse. Greenhouse temperature was maintained at 29˚C during the day and 25˚C during the night, and day length was set to 14 hours. Both immediately before and at the end of the experiment, a representative portion of soil was set aside for soil chemistry analyses and potential nitrification and denitrification assays, and a small portion of freeze-dried soil was stored at -20˚C for microbial analyses.

Soil chemical analyses

In order to determine whether some functional rates were strongly controlled by abiotic factors, a suite of soil chemistry data was collected before and after the experiment. Soil pH was determined using a 1:1 soil:deionized water slurry for each sample before and after treatment. Available ammonium (NH_4^+) and combined nitrate (NO_3^-) and nitrite (NO_2^-) were extracted from soil using 2M KCl and a ratio of 5 mg soil to 50 mL KCl. The concentration of extracted NH_4^+ and NO_3^-/NO_2^- was determined using a Lachat QuikChem (Lachat Instruments, Loveland, CO, USA) following the Berthelot method as described previously (Sims et al. 1995, Rhine et al.

1998). Total organic carbon and nitrogen were determined using combustion analysis (ECS 4010, COSTECH Analytical Instruments, Valencia, CA, USA). Gravimetric water content was also determined for every sample.

Soil N cycling enzyme activity assays

Potential rate assays were conducted in order to quantify the maximum rate that each community was capable of performing before and after the experiment. Since this study builds on work published by (Peralta et al. 2013, Peralta et al. 2016), the protocols for both potential nitrification and denitrification were similar to the protocols used in the previous studies.

The potential nitrification assay determined the rate of ammonium transformation into nitrite following a 5-hour incubation. First, assays were conducted where 5 g soil was made into a slurry with 20 mL 1M $(NH_4)_2SO_4$ and 0.1 mL 2M $NaClO_3$ to inhibit NO_2^- oxidation. Duplicate assays were conducted at room temperature under oxygenated conditions, while a third was kept frozen at -20˚C. Next, the concentration of NO_2^- was determined using colorimetric methods (Kandeler and Margesin 1996), and the rate reported is the average of the duplicate assays at room temperature with the concentration in the frozen control subtracted.

Potential denitrification was determined using a laboratory assay to measure the amount of nitrogen gas produced per hour under ideal conditions. The assay protocol was similar to the assay described by Peralta et al. (2016), except the media contained an added carbon source and nitrate. Soil slurries were made by combining 25 g soil with 25 mL media containing 45.83 mM dextrose, 14.28 mM nitrate, and 10 mg/L chloramphenicol to inhibit denitrifier cell division. The bottles were purged with helium gas to create anaerobic conditions. Acetylene gas was added to prevent the reduction of N_2O to N_2. Gas samples were collected at the beginning and after three hours. Gas samples were collected at the beginning and after three hours. The concentration of

N_2O was determined using a gas chromatograph (GC-2014 with ECD, Shimadzu Corp., Kyoto, Japan). The concentration of N_2O was used as a proxy for N_2 production under the assumption that it would be fully reduced in the absence of acetylene (Groffman et al. 2006). The same soil used for the assays was oven dried, and the final denitrification rate was corrected by the dry weight of the soil.

Soil DNA extraction

Genomic DNA was extracted from 0.5 g freeze-dried soil using a FastDNA kit (MP Biomedicals, Santa Ana, California) according to the manufacturer's instructions. Humic acids were removed using cetyl trimethyl ammonium bromide (CTAB) purification (Sambrook and Russell 2001). CTAB is a cationic surfactant that solubilizes complex carbohydrates and secondary metabolites that might interfere with PCR (Azmat et al. 2012). Following CTAB purification, the absorbance ratio at 260:230 nm was determined on a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), and purified DNA samples with a 260:230 nm ratio greater than 1.5 were used for sequencing and qPCR. The purified DNA was adjusted to 20 ng/μL and stored at -20˚C until further microbial community analysis.

Microbial community composition analysis

Illumina sequencing was used to target bacterial and archaeal rRNA genes, as well as *amoA*, *nirS*, and *nirK* genes (Illumina, San Diego, CA, USA). Sequencing amplicons were prepared by PCR using a Fluidigm Access Array IFC chip, which allowed simultaneous amplification of each target gene (Fluidigm, San Francisco, CA). Initial reactions were carried out according to a 2-step protocol using reagent concentrations according to Fluidigm recommended parameters, and an annealing temperature of 55°C was determined to be optimal for the combined set of primers. The first PCR was performed in a 100-μL reaction volume using

1 ng DNA template, and this PCR amplified each target DNA region using the gene-specific primers with Fluidigm-specific amplification primer pads CS1 (5'-ACACTGACGACATGGTTCTACA-3') and CS2 (5'-TACGGTAGCAGAGACTTGGTCT-3'), which produced amplicons including (1) CS1 Fluidigm primer pad, (2) 5'-forward PCR primer, (3) amplicon containing the region of interest, (4) 3'-reverse PCR primer, and (5) CS2 Fluidigm primer pad. A secondary 30-µL PCR used 1 µL of 1:100 diluted product from the first PCR as template, and PCR primers with CS1 and CS2 sequences and Illumina-specific sequencing linkers P5 (5'-AATGATACGGCGACCACCGAGATCT-3') and P7 (5'-CAAGCAGAAGACGGCATACGAGAT-3'), along with a 10-bp sample-specific barcode sequence, so the final construct consisted of (1) Illumina linker P5, (2) CS1, (3) 5'-primer, (4) amplicon containing the region of interest, (5) 3'-primer, (6) CS2, (7) sample-specific 10-bp barcode, and (8) the Illumina linker P7. Final amplicons were gel-purified, quantified (Qubit; ThermoFisher Scientific, Waltham, MA, USA), and then sequenced from both directions on an Illumina HiSeq 2500 2x250 bp Rapid Run. Fluidigm amplification and Illumina sequencing were conducted at the Roy J. Carver Biotechnology Center (Urbana, IL, USA).

Overall microbial community composition was characterized by sequencing the bacterial and archaeal 16S ribosomal RNA gene V4 region using the forward primer 515F and reverse primer 806R (Caporaso et al. 2011). Bacterial nitrifier composition was assessed by sequencing the bacterial *amoA* gene using the forward primer amoA-1F and reverse primer amoA-2R (Rotthauwe et al. 1997). Archaeal nitrifier composition was assessed by specifically targeting the archaeal 16S rRNA gene using the forward primer Arch349F and reverse primer Arch806R (Takai and Horikoshi 2000). Illumina sequencing of the archaeal *amoA* gene via the Fluidigm chip yielded an uneven distribution of reads from each sample in this study, where some of the

communities produced only 3 reads, and most of the Source 3 Lowland communities produced fewer than 100 reads (data not shown). A wealth of published sequence data has informed us that only a limited number of different archaeal species have the capacity for ammonia oxidation (Prosser and Nicol 2008, Alves et al. 2018). Over 60,000 *amoA* sequences have been published on GenBank as of 2018, and there are over 9,000 Thaumarchaea 16S rRNA sequences included in the Ribosomal Database Project (Cole et al. 2014). Based on this information, taxonomic identity determined by 16S rRNA gene sequences can be used to distinguish nitrifier archaea from non-nitrifier archaea. Denitrifier composition was assessed by sequencing the *nirK* gene using the forward primer nirK876 and reverse primer nirK1040 (Henry et al. 2004), and by sequencing the *nirS* gene using the forward primer nirSCd3aF and reverse primer nirSR3cd (Kandeler et al. 2006). Primer sequences are listed in Table 1.1 (Chapter 1).

Paired-end sequences were merged using Fast Length Adjustment of SHort reads (FLASH) software (v. 1.2.11) for both the bacterial and archaeal 16S rRNA gene sequences as the archaea-specific 16S rRNA gene sequences (Magoč and Salzberg 2011). Only Read 1 sequences were used for the functional gene sequences. Some of the amplicons would not have produced overlapping reads, and Read 1 was selected to be used for consistency. Quality filtering of fastq files was performed using software in the FASTX-Toolkit (Gordon and Hannon 2010). Sequences with fewer than 90% of bases showing at least 99.9% base accuracy were removed. The *nirK* primers encompass a 165-bp region, which is shorter than the 250-bp reads produced by Illumina HiSeq, so the *nirK* Read 1 sequences were trimmed to 165-bp also using FASTX-Toolkit software. The fastq files were then converted to fasta format. Sequences were binned into discrete OTUs based on 97% similarity using USEARCH v. 8.1.1861 (Edgar 2010). For the 16S rRNA gene sequences, taxonomic assignments were made using QIIME software (Caporaso et

al. 2011) with the UCLUST algorithm (Edgar 2010) and GreenGenes database v. 13.5 (McDonald et al. 2012). For functional genes, taxonomic identity was assigned by QIIME using with the BLAST algorithm (Altschul et al. 1990) and custom gene-specific databases compiled from the FunGene repository (Fish et al. 2013).

Quantification of N cycling functional group abundance

Broad differences in abundance of microbial functional groups may also determine potential functional rates so we quantified diagnostic genes for ammonia oxidation and nitrite reduction. Quantitative PCR (qPCR) was used to determine the number of both archaeal and bacterial *amoA* genes present in each community, and denitrifier abundance was determined by quantifying the number of *nirS* and *nirK* genes.

In order to quantify gene copy number in each of the four qPCR assays, replicate serial dilutions of standard template were amplified simultaneously with the samples to produce a standard curve. The template DNA for these standards were generated by first amplifying the gene of interest from a mixed wetland soil sample using PCR without fluorescent dyes: Reactions were carried out in a 50μL volume and contained 50 mM Tris (pH 8.0), 25 μg/mL of T4 gene 32 protein, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 20 pmol of each primer (four separate reactions: archaeal *amoA*: amoA-1F/ amoA-AR; bacterial *amoA*: amoA-1F/ amoA-2R'; *nirK*: nirK876/nirK1040; *nirS*: nirSCd3aF/nirSR3cd; Table 1.1), 2.5 U of Taq polymerase (Promega, Madison, WI, USA), and 100 ng of extracted soil DNA. PCR conditions included initial denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 53 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 15 min. Next, the amplicons were separated from primer dimers using a QIAquick gel extraction kit according to the manufacturers' protocol (Qiagen, Valencia, CA, USA). The concentration of purified amplicon

was determined using a Qubit DNA fluorometer (ThermoFisher Scientific, Waltham, MA, USA), and the exact copy number of the gene of interest in each the standard could be calculated from the concentration. The final copy number of each gene in the unknown soil samples was normalized by the ng of template DNA used in the qPCR reaction. The MIQE guidelines (Minimum Information for qRT-PCR Experiments) explained by Bustin et al. (2009) were used to evaluate assay performance based on the standard curves for the archaeal *amoA*, bacterial *amoA, nirK,* and *nirS* qPCR assays, and the results of this analysis are summarized in Table C.1 and Table C.2. All gene copy numbers calculated in unknown wetland soil DNA samples were within the range of the respective standard curve for each gene.

For qPCR amplification of archaeal and bacterial *amoA*, reactions were carried out in triplicate technical replicates in a 10 µL volume containing 1X FastStart Universal SYBR Green master mix (Roche Applied Science, Germany) with 250 µg T4 gene 32 protein (Roche Applied Science, Germany). The archaeal *amoA* gene was amplified using 0.4 µM of forward primer Arch-amoAF and 0.4 µM of reverse primer Arch-amoAR (Francis et al. 2005) using the following protocol: 5 min initial denaturation at 95˚ C and then 40 cycles of 95˚ C for 45 sec, 51˚ C for 1 min, and 72˚ C for 1 min. The bacterial *amoA* gene was amplified using 0.4 µM of the same forward primer used for sequencing, amoA-1F (Rotthauwe et al. 1997), and 0.4 µM of reverse primer amoA-2R' (Okano et al. 2004) using the following protocol: 5 min initial denaturation at 95˚ C and then 40 cycles of 95˚ C for 45 sec, 56˚ C for 1 min, and 72˚ C for 1 min. Both reactions were carried out in a 7900HT Fast Real-Time PCR System (Applied Biosysterms, Foster City, CA, USA). The quantitative threshold cycle (C_q) was determined using SDS software v 2.4 (Applied Biosysterms, Foster City, CA, USA). Gene copy number was quantified by comparing C_q of samples against those produced by a standard curve. For all

reactions, 2 µL of template was used, which contained approximately 20-30 ng genomic DNA, and triplicate reactions were carried out for each sample.

For qPCR amplification of *nirS* and *nirK*, a microfluidic Fluidigm Gene Expression chip was used to quantify both genes simultaneously, and 10 technical replicates per sample were used for this qPCR assay. Both genes were amplified using the same primer sets used for sequencing: nirK876 and nirK1040 (Henry et al. 2004) and nirSCd3aF and nirSR3cd (Kandeler et al. 2006). In order to increase the amount of template DNA, a specific target amplification (STA) reaction was performed similar to previously described (Ishii et al. 2014), except with 0.5 µM of each primer, and 1.25 µl of the DNA template in a 5 µL reaction volume. The following program was used: 95 ºC for 10 min followed by 14 cycles of 95 ºC for 15 sec and 58 ºC for 4 min. A 5-µL mixture was then prepared with a final concentration of 1X SsoFast EvaGreen Supermix with Low Rox (Bio-Rad Laboratories, Hercules, CA, USA), 1X DNA Binding Dye Sample Loading Reagent (Fluidigm, San Francisco, CA, USA), and 2.25 µl pre-amplified product. A separate master mix was prepared with a final concentration of 1X Assay Loading Reagent (Fluidigm, San Francisco, CA, USA), 0.5X DNA Suspension Buffer (Teknova, Hollister CA), and 50 µM of each forward and reverse primer. Each 5-µL mixture containing product was mixed with 5 µL of master mix and loaded onto a 96.96 Fluidigm Gene Expression chip. Amplification was performed according to the following program: 70 ºC for 40 min, 58 ºC for 30 sec, 95 ºC for 1 min followed by 30 cycles of 96 ºC for 5 sec, 58 ºC for 20 sec, and followed by dissociation curve. All the samples and standards were analyzed in 12 replicates. The C_q was determined using Fluidigm Real-Time PCR Analysis software version 4.1.3, and gene copy number was quantified by comparing C_q of samples against those produced by a

standard curve. Fluidigm qPCR was conducted at the Roy J. Carver Biotechnology Center (Urbana, IL, USA).

Statistical analysis

The overall microbial community was analyzed as a table of the relative abundances of 16S rRNA gene sequences. The nitrifier community was represented as a concatenated table of relative abundance of bacterial and archaeal ammonia oxidizers. The denitrifier community was represented as a concatenated table of relative abundance of *nirS* sequences and relative abundance of *nirK* sequences in each sample.

The goal of this study was to determine whether hydrologic variability itself favors a unique community that includes taxa that are physiologically tolerant to a range of hydrologic conditions, but first we needed to verify that the variable region of the wetland harbored a unique community. We used Venn diagrams to examine the number of microbial OTUs present only in this community and compared that to the number of OTUs that are present in all of the sites. Venn diagrams were created using the 'venneuler' function in the 'venneuler' package in R (Wilkinson and Urbanek 2011), and the number of OTUs reported in the Venn diagrams represent the total unique OTUs detected in at least one replicate within each group in the comparison (either soil source or treatment). Next, we used principle coordinate analysis (PCoA) ordinations calculated from Bray-Curtis distances to visualize community differences among sites, as well as changes in composition that may have occurred following experimental treatments. PCoA plots were created with the 'cmdscale' function in the 'vegan' package in R (Oksanen et al. 2013, R Core Team 2014). Relative effects of soil source and treatment on community composition were assessed by creating permutational analysis of variance (PERMANOVA) models with the 'adonis' function, which is also in the 'vegan' package. In

order to determine which specific OTUs differed between the three source communities, an indicator species analysis was performed using the 'multipatt' function in the 'indicspecies' package in R (De Cáceres and Legendre 2009). The top 20 most abundant "indicator OTUs" for each of the three sources were identified. Since richness can also influence functional stability if functional redundancy is high, the Chao1 index was calculated using the function 'estimateR' in 'vegan', and compared among source communities and treatments.

If the community from the variable region harbored an unusually large number of taxa that are physiologically tolerant to a range of hydrologic conditions, then we would expect this particular community to change the least when compared to communities from more stable regions, especially when compared across the range of hydrologic treatments to which these communities were exposed. To directly compare the magnitude of change from initial to final composition among these communities, the 'vegdist' function in 'vegan' was used to calculate Bray-Curtis dissimilarity values between the initial source soil community composition and the composition following each hydrologic manipulation.

Redundancy analysis (RDA) was carried out as a "follow up analysis" to identify whether changes in relative abundance of individual microbial OTUs could be correlated to changes in potential nitrification or denitrification rates. Two separate analyses were conducted using the 'rda' function in the 'vegan' package in R: (1) Concatenated table of bacterial and archaeal ammonia oxidizer OTUs using potential nitrification as the constrained axis, and (2) concatenated table of *nirK*- and *nirS*-containing denitrifier OTUs using potential denitrification as the constrained axis. The OTUs with RDA axis loadings within the highest 30% of all RDA values were selected as "indicator OTUs" for nitrification or denitrification.

For pairwise comparisons among univariate measurements (e.g. Chao1, gene copy numbers, or soil chemistry measurements), significant differences were determined by a two-tailed paired t-test at a significance level of 0.05. Error for all values reported was calculated as the standard error of the mean. Significant differences in soil factors and microbial functional gene abundances among multiple soil sources or treatments were determined by creating an ANOVA model using the 'aov' function, and specific differences were identified using the 'TukeyHSD' function, both of which are in the 'stats' package (R Core Team 2014).

Results

Environmental differences among sites and changes in physical properties

Initially, the three regions with distinct hydrologic history also differed in soil properties (Table 4.1). Significant differences were detected among sources in terms of gravimetric water content (GWC), pH, extractable NH_4^+, extractable combined NO_2^- and NO_3^-, total proportion of soil C (total C), total proportion of soil N (total N), and C:N ratio. Generally speaking, moisture, pH, and nutrients were greatest in the saturated lowland, while the upland was drier, more acidic, and had the lowest total C and lowest total N. However, both ammonium and nitrate were greatest in the upland compared to the other two, where both potential nitrification and denitrification rates were also the lowest.

Most of the soil variables that were tested remained unaffected by the hydrologic treatments in the two-month mesocosm experiment (Fig. C.2). However, pH in the Source 1 Upland soils increased (became more neutral) following the Variable and Saturated treatments (Tukey's HSD, $p < 0.05$), and the concentration of soil NH_4^+ and combined NO_2^- and NO_3^- differed considerably between treatments. The Saturated treatment led to a significant increase in

soil NH_4^+ across all three source soils when compared to what was extracted from the initial soil (Tukey's HSD, $p < 0.05$), where the biggest increase was seen following saturation of the Source 1 Upland soils. The combined concentration of NO_2^- and NO_3^- tended to decrease, especially following the Variable and Saturated treatments of the Source 1 Upland and Source 2 Variable soils (Tukey's HSD, $p < 0.05$). Combined NO_2^-/NO_3^- was low in the Source 3 Lowland soils initially, but significantly increased following the Variable treatment (Tukey's HSD, $p < 0.05$).

The IRIS tubes placed in the mesocosms that were subjected to the Dry treatment indicated that they had remained aerobic because none of the initial ferrous coating was reduced and lost. The IRIS tubes in mesocosms subjected to the Saturated treatment lost all of the ferrous coating, indicating that sustained anaerobic conditions were achieved with this treatment. The Variable treatment fostered an environment where some of the ferrous paint was reduced and flaked off, but approximately half of the paint remained by the end of the experiment.

Functional stability in the three source soil communities

Both potential nitrification and denitrification rates were influenced by the hydrological treatments (nitrification ANOVA, Treatment: $F = 12.82$, $df = 3$, $p < 0.001$; denitrification ANOVA, Treatment: $F = 23.51$, $df = 3$, $p < 0.001$). Nitrification and denitrification rates exhibited similar patterns as each other in response to hydrologic treatments (Fig. 4.7). Rates observed in the Upland Source 1 soils decreased following the Saturated treatment, while rates in the Lowland Source 3 soils decreased following the Dry treatment. Nitrification rates from the Variable Source 2 community decreased following the intense drying but denitrification rates from the same source did not decrease increased following any of the treatments, and both rates increased following the Variable treatment.

Microbial community differences among sites

We detected 1736 different microbial OTUs from 16S rRNA gene sequencing, and the total number of reads was over 290,000. For the nitrifier analysis, there were 6655 archaeal ammonia oxidizer reads pulled from the archaeal 16S rRNA gene sequences, and 6432 bacterial ammonia oxidizer reads generated by sequencing the bacterial *amoA* gene. For the denitrifier analysis, more than 113,000 *nirK* reads were generated, and more than 57,000 *nirS* reads were generated. 12-23% of the OTUs detected in each source community were unique to one of the three regions, and 18-30% of nitrifier OTUs and 27-33% of denitrifier OTUs were unique to one of the three regions (Fig. 4.1).

The source communities from the three hydrologically distinct regions differed in overall microbial richness calculated as the Chao1 index, but richness did not differ among sources in either the nitrifier or denitrifier communities (Table 4.1). Microbial community composition significantly differed among the three source soils as well, and the nitrifier and denitrifier communities differed (Table 4.2). Before the experiment, the overall microbial community from Variable Source 2 had the greatest number of OTUs overall (1214). The Lowland Source 3 showed the second highest number of OTUs (1007), while the Upland Source 1 had the lowest number of OTUs (817). The nitrifier and denitrifier communities from the Variable Source 2 also had the greatest number of OTUs (14 nitrifier OTUs and 450 denitrifier OTUs).

Community resistance or resilience following the hydrologic experiment

Bray-Curtis comparisons between initial and final composition revealed that the Variable Source 2 community changed the least over the 2-month experimental treatments compared to both the Upland Source 1 and Lowland Source 3 communities (Fig. 4.5A and 4.5B). For the denitrifier community, both the Variable Source 2 and Lowland Source 3 denitrifier communities

experienced a smaller degree of change than the Upland Source 1 community (Fig. 4.5C). A relatively small degree of change following the experiment as a whole (indicated by a small Bray-Curtis distances from the initial community) suggests that the community may be resistant or resilient to disturbances (Allison and Martiny 2008, Shade et al. 2012). Communities were strongly influenced by the original source location, and PERMANOVA also revealed a significant effect of hydrologic treatments on the composition of the overall community but not the nitrifiers or denitrifiers (Table 4.2). The PCoA ordination further shows that the experimental long-term saturation treatment led to a change in the Upland Source 1 community (Fig. 4.2). The nitrifier community and denitrifier community differed among the three source soils (Table 4.2, Fig. 4.3, and Fig. 4.4).

The Chao1 richness index of the overall microbial community (based on 16S rRNA sequences) differed among treatments (ANOVA, Treatment: $F = 3.768$, $df = 3$, $p = 0.024$; Fig. C.3), and this result was solely driven by a decrease in richness in the Upland Source 1 community following the Saturated treatment. The Chao1 richness of the denitrifiers also differed by treatment (ANOVA, Treatment: $F = 3.424$, $df = 3$, $p = 0.033$), but this was driven by greater richness in the Upland Source 1 denitrifiers following the Variable treatment compared to the Saturated treatment. There was no significant difference between initial denitrifier richness and denitrifier richness after any of the treatments. Neither source nor treatment affected the nitrifier Chao1 index.

Differences in functional gene copies belonging to AOA, AOB, *nirK* denitrifiers, and *nirS* denitrifiers were observed between sites, but gene copy number was relatively unaffected by hydrologic treatment. In general, there were fewest ammonia oxidizers in the lowland region and more upland, while denitrifiers showed the opposite pattern and there were more in the lowland

than in the upland region (Table 4.1). The *nirS* gene was the only gene where the abundance was significantly affected by treatment (ANOVA, Treatment: $F = 3.858$, $df = 3$, $p = 0.022$). This was driven by a decrease in *nirS* copy numbers present in the Upland Source 1 community following all three treatments (Fig. 4.6). No significant differences were observed among archaeal *amoA*, bacterial *amoA*, or *nirK* copy numbers.

Relationship between individual microbial OTUs and N cycling activity rates

Redundancy analysis (RDA) identified one nitrifier OTU whose relative abundance positively correlates to potential nitrification rates, a taxon in the Gammaproteobacteria genus *Nitrosococcus*, referred to as "Nitrifier OTU" below (accession number: KY802142). There were two denitrifier OTUs whose relative abundance correlated with potential denitrification rates, and both were identified from *nirK* sequences: A taxon in the Betaproteobacteria genus *Alcaligenes* ("Denitrifier OTU 1"; accession number: KY803903) and the Alphaproteobacteria *Starkeya novella* ("Denitrifier OTU 2"; accession number: KY803905). The relative abundance of the Nitrifier OTU differed among sources (ANOVA, Source: $F = 20.573$, $df = 2$, $p < 0.001$), but it did not differ among treatments. Both of the denitrifier OTUs also differed among sources (Denitrifier OTU 1 ANOVA, Source: $F = 6.404$, $df = 2$, $p < 0.01$; Denitrifier OTU 2 ANOVA, Source: $F = 7.435$, $df = 2$, $p < 0.01$) but not among treatments (Fig. C.7).

Discussion

The central question of this study was whether historical environmental variability selects for individuals that are physiologically tolerant to a range of conditions. Many microbial communities are sensitive to disturbances (Allison and Martiny 2008, Shade et al. 2012), which means disturbances can have consequences for important functions performed by the community

(Mendes et al. 2015, Delgado-Baquerizo et al. 2016, Gravuer and Eskelinen 2017). However, functional stability can also occur when there is a shift in composition but functional capacity of the new individuals is similar to those who were there before ("redundancy" between initial and final community composition), or composition itself might remain similar to what is was before. The latter scenario would suggest that the community is comprised of physiologically tolerant individuals. Our results showed that potential activity rates from the community unique to the transition region of a floodplain were the least likely to decrease following experimental hydrologic manipulation, which indicates a higher degree of functional stability compared to the upland and lowland communities. The microbial community in this region also changed in composition by the smallest degree compared to the other two. Together, this evidence supports our hypothesis that variability itself is an environmental filter that selects for individuals that are unusually tolerant of changing conditions.

High diversity in a community may either mean there is a high chance that particularly resistant or resilient taxa will be present and can remain in the community following a disturbance (Chaer et al. 2009, Royer-Tardif et al. 2010), or that multiple microbial taxa perform the same level of function as one another (Yuste et al. 2014). Experimental reduction of diversity has been shown to decrease functional stability, but the functional response greatly depends on the type of disturbance (e.g. high richness is more likely to rescue function from heat stress than from inorganic metal contamination) (Griffiths et al. 2000, Tardy et al. 2014). We found that the overall microbial community (based on 16S) in the Source 2 Variable region exhibited significantly greater richness than those in the Source 1 Upland and Source 3 Lowland regions. In combination with the high functional stability of potential nitrification and denitrification from this region, this implies that overall richness may contribute to functional stability. However, we

did not find differences in the richness of functional groups between each of the three regions. Richness is only one dimension that can be used to characterize a microbial community, where taxonomic composition is another. Biodiversity may be important for the stability of overall community function (Isbell et al. 2011, Bradford et al. 2014), while differences in composition can determine the stability of individual processes. Microbial composition contributes to differences between functional rates even when richness is determined to be most important (Bell et al. 2005, Graham et al. 2016, Orwin et al. 2016). Since the microbial functional groups in this study differed in composition between the three regions, but not in richness, this can be interpreted as evidence that the effects of microbial richness on functional stability are moderated by the specific composition of taxa present.

We also observed a similar functional response pattern between nitrification, a narrow process performed by few taxa, and denitrification, a broad process performed by many taxa. High diversity within a functional group may mean that functional redundancy is more likely to rescue processes like denitrification than processes like nitrification (Schimel and Gulledge 1998, Schimel et al. 2007), but we did not find evidence to support that idea in this particular study. Instead, since both functional groups exhibited unique composition in the Source 2 Variable region, and the Source 2 communities remained relatively similar to initial composition following the experiment, it is more likely that the specific nitrifier and denitrifier OTUs observed in that region were physiologically tolerant to the types of disturbances they experienced in this study, regardless of functional group richness. Some experimental results suggest that the presence of stress first alters the microbial community, and the resulting community exhibits greater functional stability than the one before (Philippot et al. 2008, Sjöstedt et al. 2018). This phenomenon may reflect the same filtering effect we propose because

the fluctuation between oxic and anoxic conditions also represents a "stress". We further found that the community in the Source 2 Variable region was functionally stable in the face of both dry and saturated conditions, not just the treatment that presented fluctuating conditions. This means these taxa are remarkably tolerant to a range of hydrologic stresses, not just the stress under which they were conditioned.

Given the difference in metabolic strategies between aerobic nitrifiers and facultatively anaerobic denitrifiers, we were quite surprised to observe similar patterns between these two processes in response to the same altered hydrology, especially from saturated soils. Nitrification would not be a favorable process under the anaerobic conditions present at both the Source 3 Lowland region and in the mesocosms that were kept saturated throughout the experiment, yet these soils yielded high potential nitrification rates. The phenomenon of unexpectedly high potential nitrification from saturated soils has been observed previously, where lowland wetland soils produced greater potential nitrification rates than dry upland soils (Peralta et al. 2013, Peralta et al. 2016). Nitrification and denitrification can become coupled in wetlands (Seitzinger 1988, Vila-Costa et al. 2016, Racchetti et al. 2017), meaning that aerobic nitrification processes physically occur close enough to anaerobic soils to provide the nitrate for denitrification. However, coupled processes would not be expected to influence potential rates obtained separately during laboratory assays. Also, while high nitrification *in situ* can stimulate denitrification, the high nitrifier activity observed from saturated soils remains unexplained.

One possibility is that the potential nitrification assay used in this study may have inadvertently measured NO_2^- accumulation from processes other than nitrification, though we argue this is also unlikely. There are anaerobic N cycling processes other than the ones addressed by this study (e.g. anammox and DNRA), but these processes would not have contributed to the

NO_2^- accumulation that occurred during the nitrification assays. Anaerobic ammonia oxidation (anammox) could not have produced NO_2^- as a byproduct. Instead, NH_4^+ is oxidized by an anaerobic alternative to oxygen, sometimes including NO_2^- itself, to produce a hydroxylamine intermediate and eventually nitrogen gas (van de Graaf et al. 1995, van de Graaf et al. 1997, Schmidt et al. 2002, Zhang et al. 2011). On the other hand, NO_2^- accumulation could arise from the dissimilatory reduction of nitrate to ammonium (DNRA). DNRA competes with denitrification to reduce NO_3^-, and NO_2^- occurs as a byproduct (Kraft et al. 2014, van den Berg et al. 2017). The only ATP-generating step of DNRA is the reduction of NO_3^- to NO_2^- (Tiedje 1988, Megonigal et al. 2014) and DNRA processes are just as common in soils as nitrification and denitrification processes (Yang et al. 2015). It is entirely possible that there were organisms present that were capable of this step. Facultatively anaerobic organisms capable of DNRA could have become enriched and remained active in anaerobic microsites in the assay slurries. However, the addition of $NaClO_3$ to inhibit nitrite oxidation to nitrate during the nitrification assays would have also inhibited the Nap enzyme that performs this initial reduction step of DNRA (Rusmana and Nedwell 2004). Thus, we are fairly confident that the accumulation of NO_2^- we measured during the nitrification assays was due to the activity of aerobic ammonia oxidizers, even in soils that had been saturated and anaerobic before the assay.

Another possible explanation for observing unexpectedly high potential nitrifier activity is that the few nitrifiers present in the Source 3 Lowland community (based on low *amoA* gene copy numbers) may have been particularly active nitrifiers. This would be consistent with findings reported in a recent review, where it was reported that the ammonia oxidizer activity observed during studies that utilize stable isotope probing techniques are often attributed to only a small handful of archaeal OTUs (Alves et al. 2018). Further, the “rare biosphere” has been

suggested to be ecologically important for the overall microbial community (Sogin et al. 2006), not just for nitrifiers, and this rare biosphere is possibly even more important than the most abundant organisms because the total community often consists of dormant individuals (Jones and Lennon 2010, Aanderud et al. 2015). This would suggest that the presence of specific, rare taxa within functional groups might be more important for predicting functional rates than differences in either taxonomic richness or abundance organisms that possess the corresponding diagnostic gene.

Indicator OTU analyses of the overall microbial community (based on 16S) revealed that completely different microbial OTUs were enriched in the communities found at each of the three regions, which further reflects environmental filtering. Interestingly, the top 20 most abundant indicator OTUs from the Source 2 Variable region were characterized by OTUs that belong to *Chloroflexi* and *Cyanobacteria*, both of which are primary producers. It is not uncommon to detect microbial taxa that belong to either of these phototrophic phyla in wetland sediments (Wang et al. 2012, Jin et al. 2017), and we did detect phototrophic OTUs across all three regions in this study. The specific OTUs identified as Source 2 indicator taxa may be responding to something unique about the Source 2 environment. Microbial primary producers will often "bloom" in response to excess nutrients (Gobler et al. 2016, Berry et al. 2017), but the Source 2 Variable region did not exhibit high soil nitrate compared to the other two regions. One possibility is that each drying or saturation event leads to a process similar to succession, but on a very brief time scale. During succession, fast growing *r*-selected individuals quickly colonize open niches in the soil (Odum 1969, Torsvik and Øvreås 2002, Ciccazzo et al. 2016). Microbial phototrophs are thought to play a key role in the initial accumulation of soil organic matter following glacial retreat (Frey et al. 2013). The phototrophic OTUs identified here may play a

similar ecological role following flood withdrawal from a wetland, when dissolved nutrients can be exported downstream and away from the wetland.

Contrary to the indicator taxa identified for Source 2, there were no primary producers among the top 20 indicator OTUs for Source 1 Upland and only one phototrophic OTU identified for the Source 3 Lowland communities. Instead these groups of indicator taxa were characterized by taxa that might be well adapted to constant conditions. The top 20 most abundant lowland indicator OTUs were characterized by the presence of sulfate-reducers. Sulfate metabolism would be expected to occur in sediments that have been anaerobic for an extended period of time, rather than nitrate metabolism, because the more favorable anaerobic electron acceptors like nitrate have become unavailable (Kojima and Fukui 2011, Watanabe et al. 2017). The top 20 most abundant upland indicator OTUs were characterized by OTUs belonging to the acidophilic, desiccation-tolerant *Koribacter* genus (Ward et al. 2009) and OTUs in the aerobic *Gaiellaceae* family in the *Actinomycetes* (Albuquerque et al. 2011). *Actinomycetes* can possess high tolerance to drought conditions (LeBlanc et al. 2008), and we speculate that these upland *Gaiellaceae* OTUs also possess high tolerance to drought.

Microbial community composition is influenced by both random historical dispersal events and by many abiotic characteristics of the soil that filter particular taxa with adaptions suited to the specific conditions (Webb et al. 2002). Environmental filtering can influence the functional stability of the soil community (Sjöstedt et al. 2018). Strong abiotic filters of soil communities include soil pH (Lauber et al. 2009, Griffiths et al. 2011) and redox conditions (Pett-Ridge and Firestone 2005, Peralta et al. 2014). Here, we suggest that the variability of an abiotic filter is also a filter itself. We demonstrated that the microbial community in the Source 2 Variable region is quite different in composition and exhibited greater compositional and

functional stability compared the other two communities. Thus we conclude that microbial community composition influenced functional stability in this system, and composition was more important that taxonomic richness for predicting function. We further speculate that the historical pattern of fluctuation between saturated and dry conditions filtered out microbial taxa that were not tolerant to either saturated or dry conditions, leaving behind primarily taxa that possess physiological adaptations to both extremes and can switch between them. Historical variability should be taken into account in order to predict how microbial functions may respond to a rapidly changing environment following global change.

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Tables

Table 4.1**.** Initial chemical properties, activity rates, and microbial properties for each of the three source soils.

	Source 1 – Upland	Source 2 – Variable	Source 3 – Lowland	ANOVA F ($df = 2$)	p-value
GWC (w/w %)	2.18 ± 0.38^{a}	14.57 ± 0.22^{b}	48.80 ± 1.68^{c}	578.60	< 0.001
pH (1 soil: 1 water)	5.60 ± 0.08^{a}	7.84 ± 0.02^{b}	7.78 ± 0.05^{b}	478.30	< 0.001
Total C (w/w %)	1.42 ± 0.03^{a}	2.21 ± 0.16^{b}	2.38 ± 0.01^{b}	29.86	< 0.001
Total N (w/w %)	0.13 ± 0.003^{a}	0.19 ± 0.01^{b}	0.23 ± 0.003^{c}	76.00	< 0.001
C:N	10.65 ± 0.04^{ab}	11.40 ± 0.30^{a}	10.20 ± 0.17^{b}	9.31	0.015
NH_4^+ ($\mu g\ g^{-1}$)	4.29 ± 0.43^{a}	0.47 ± 0.007^{b}	3.32 ± 0.45^{a}	30.58	< 0.001
Combined NO_2^-+NO_3^- ($\mu g\ g^{-1}$)	7.97 ± 2.33^{a}	4.25 ± 0.66^{ab}	0.15 ± 0.003^{b}	7.83	0.021
Potential nitrification (mg $NO_3^-\ g^{-1}\ hr^{-1}$)	8.32 ± 1.09^{a}	50.08 ± 1.29^{b}	395.36 ± 48.30^{c}	57.96	< 0.001
Potential denitrification ($\mu g\ N_2O\ g^{-1}\ hr^{-1}$)	13.59 ± 0.48^{a}	56.20 ± 0.71^{b}	268.39 ± 19.44^{c}	119.10	< 0.001
16S Chao1 Index	756.51 ± 7.29^{a}	1100.37 ± 16.09^{c}	909.10 ± 24.64^{b}	96.86	< 0.001
AOA (gene copy # per ng DNA)	949.07 ± 316.17^{a}	212.48 ± 30.00^{a}	22.14 ± 2.79^{b}	7.13	0.026
AOB (gene copy # per ng DNA)	4.59 ± 1.40^{a}	32.16 ± 5.85^{b}	3.54 ± 1.25^{a}	20.91	< 0.01
Nitrifier Chao1 Index	9.33 ± 0.88	9.44 ± 1.56	7.33 ± 1.45	n.s.	n.s.
nirK (gene copy # per ng DNA)	80.69 ± 40.27^{a}	278.71 ± 30.93^{b}	97.87 ± 4.12^{a}	13.91	< 0.01
nirS (gene copy # per ng DNA)	3.94 ± 0.08^{a}	169.98 ± 29.19^{a}	616.93 ± 140.09^{b}	14.73	< 0.01
Denitrifier Chao1 Index	168.25 ± 15.63	234.86 ± 31.55	220.00 ± 3.39	n.s.	n.s.

Error was calculated as standard error of the mean. Lower case superscript letters indicate significant grouping. Non-significant differences are indicated by an 'n.s.' instead of reporting an F statistic. Nitrifier Chao1 was calculated from a composite OTU table formed from the archaeal and bacterial ammonia oxidizers. Denitrifier Chao1 was calculated from a composite OTU table formed from *nirS*- and *nirK*-containing denitrifiers. Abbreviations: 'GWC', gravimetric water content; 'Total C', total combustible carbon content; 'Total N', total combustible nitrogen content; 'C:N', C to N ratio; 'AOA', archaeal *amoA* gene copy number; 'AOB', bacterial *amoA* gene copy number.

Table 4.2. PERMANOVA results from the 'adonis' function showing the proportion of variability explained (PERMANOVA R^2 statistic) by source soil and treatment in the overall microbial community, nitrifier community, and denitrifier community.

	Overall microbial community PERMANOVA R^2	Nitrifier community PERMANOVA R^2	Denitrifier community PERMANOVA R^2
Source	*** **0.74**	*** **0.54**	*** **0.27**
Treatment	*** **0.06**	0.05	0.07
Source X Treatment	*** **0.10**	0.10	0.14
Residuals	0.10	0.31	0.52
Total	1.00	1.00	1.00

Significance: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

Figures

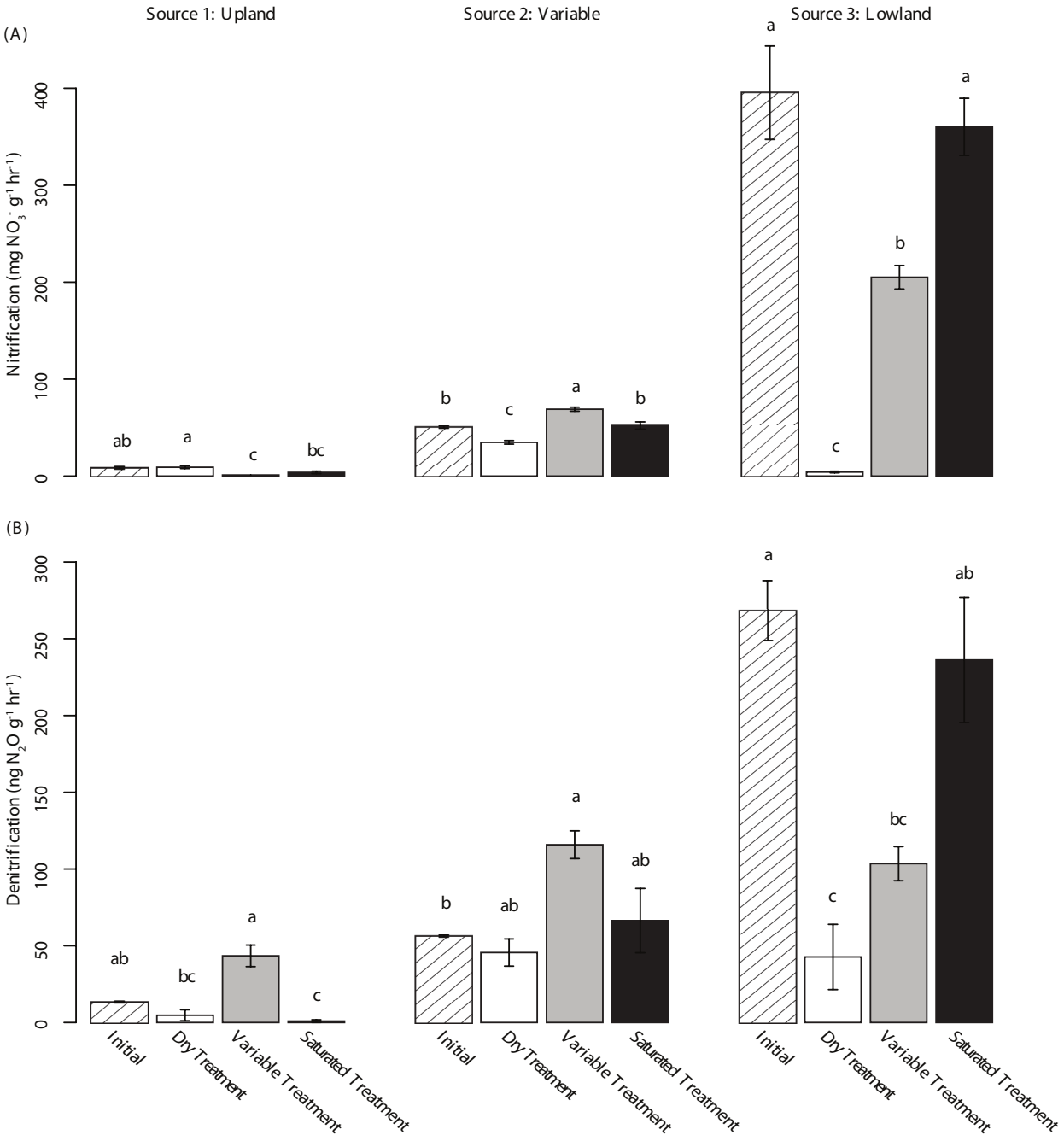

Figure 4.1. Activity rates from each source before and after the experiment. Lower case letters indicate significant groupings ($p < 0.05$), and error bars represent the standard error of the mean.

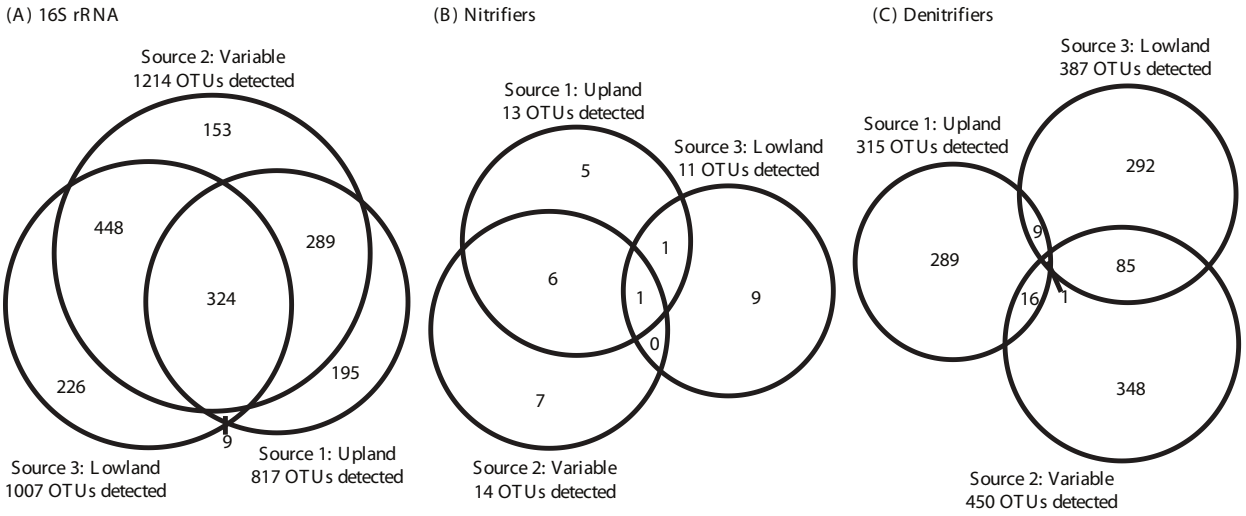

Figure 4.2. Venn diagrams showing microbial OTUs observed in communities in the three source soils before the experiment in (A) the overall community based on 16S rRNA gene sequences, (B) the nitrifier community based on archaeal ammonia oxidizer 16S rRNA sequences and bacterial *amoA* sequences, and (C) the denitrifier community based on *nirS* and *nirK* sequences. The one nitrifier OTU that was detected in all three source soils belongs to the archaeal genus *Nitrososphaera*. The one denitrifier OTU detected in all three soils was identified as a member of the *nirS*-containing taxon *Prosthecomicrobium hirschii* in the Alphaproteobacteria.

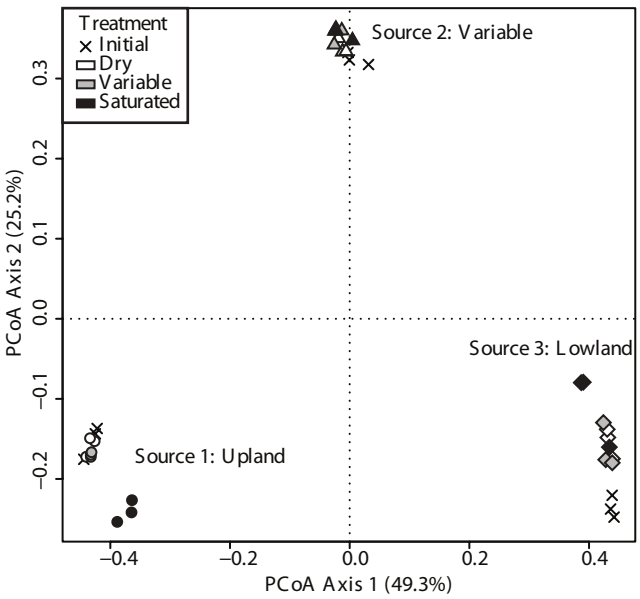

Figure 4.3. Principle coordinate analysis (PCoA) ordination of the overall microbial community based on 16S rRNA gene sequences.

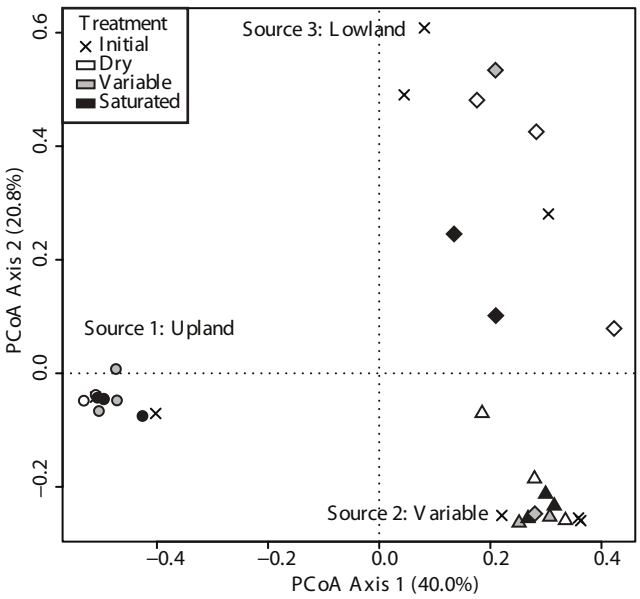

Figure 4.4. Principle coordinate analysis (PCoA) ordination of the nitrifier community created from a concatenated OTU table of archaeal 16S rRNA sequences that were identified as ammonia oxidizers and bacterial *amoA* sequences.

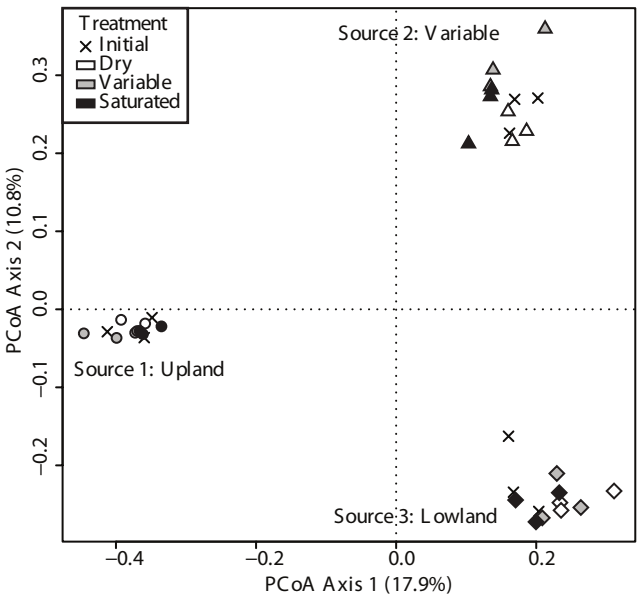

Figure 4.5. Principle coordinate analysis (PCoA) ordination of the denitrifier community created from a concatenated OTU table from *nirS* and *nirK* sequences.

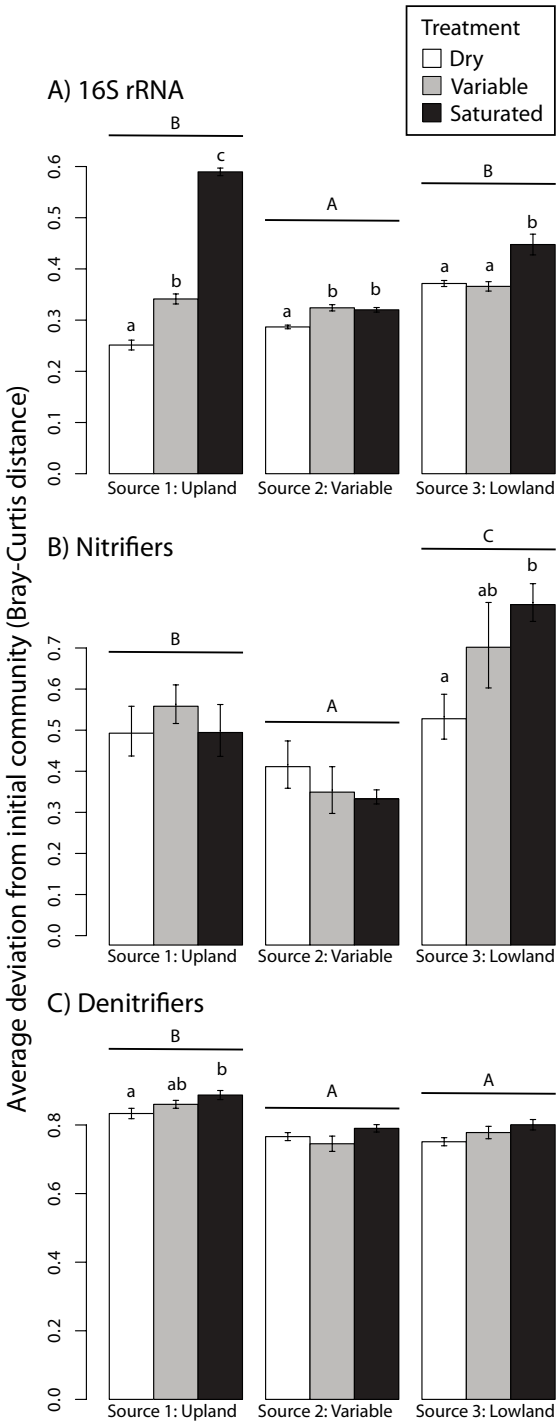

Figure 4.6. Change in microbial community composition following hydrologic alteration shown as Bray Curtis distance from the initial community. Bray Curtis ranges from 0 (no difference) to 1 (complete difference). Significant differences were observed in the degree of change in (A) the overall microbial community composition, and observed among (B) nitrifier and (C) denitrifier communities in the three source soils. Lower case letters indicates significant differences between treatments for each source, and significant differences between entire source communities are indicated by upper case letters ($p < 0.05$). Error bars show standard error of the mean.

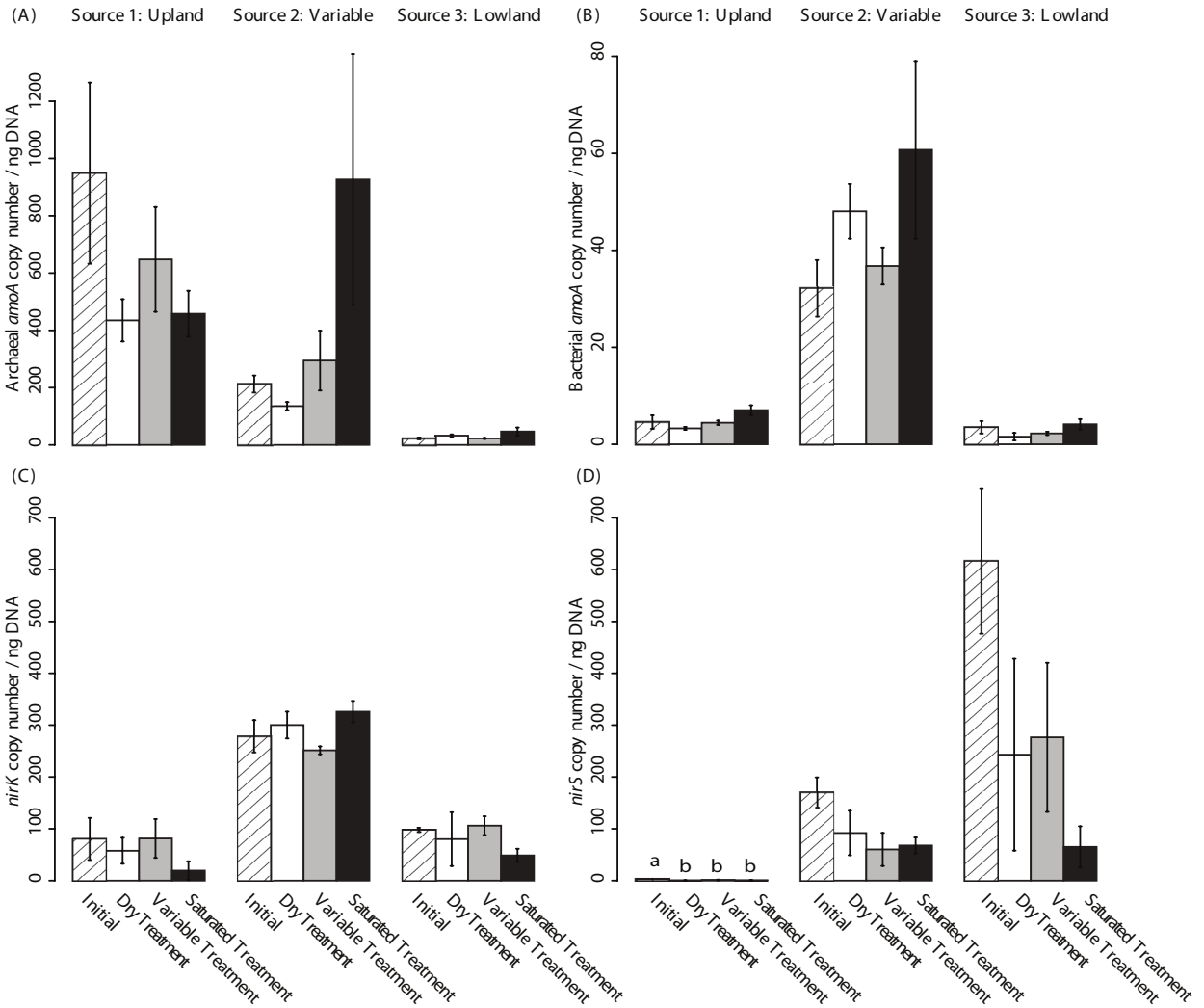

Figure 4.7: Gene copy number of (A) archaeal *amoA*, (B) bacterial *amoA*, (C) *nirK*, and (D) *nirS*. Copy number was standardized by the total ng of DNA from which the gene was amplified. Only the *nirS* abundance in the Source 1 soil community was affected by treatment, and significant groups ($p < 0.05$) are indicated by lower case letters. Error bars represent the standard error of the mean.

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CHAPTER 5:

HISTORICAL FLOOD PATTERNS DRIVE MICROBIAL COMMUNITY COMPOSITION IN RESTORED WETLANDS BUT NOT THE FUNCTIONAL RESPONSE TO ALTERED HYDROLOGY

Abstract

Historical flood patterns impose an environmental filter that structures soil microbial community composition, and composition can affect ecosystem function. An important microbial function in wetlands is the removal of nitrate via denitrification, but restored freshwater wetlands sometimes fail to achieve an equivalent rate of denitrification when compared to reference sites, even once hydrology of the site has been altered during restoration. It is possible that the historical hydrology of a wetland has already selected microbial taxa that differ in denitrification capacity, and dispersal limitation between wetlands prevents recruitment of new denitrifier taxa. In order to address this, we first surveyed denitrifier community composition in wetlands that were categorized to have contrasting historical flood patterns. Next, we established a controlled experiment where sterile soil mesocosms were inoculated with denitrifier communities from wetlands with contrasting historical flood patterns, and measured the response of potential denitrification activity rates to different hydrologic treatments over two months. Historical hydrology influenced wetland denitrifier composition at least to some degree but there were still strong site-specific differences. The wetland communities that were used to inoculate experimental mesocosms exhibited similar functional responses to the hydrologic treatments, regardless of contrasting historical flood patterns. In particular, mesocosms subjected to dry periods displayed reduced potential rates for the remainder of the experiment. Experimentally

removing the dispersal limitation between wetland denitrifier communities did not result in a different response to the hydrologic treatments, implying that differences in functional capacity at least are not due to dispersal limitation of denitrifier communities in newly restored wetlands. Thus, past flood patterns drive microbial composition in restored wetlands, but past flood patterns do not necessarily drive the response of denitrifier activity to experimental changes in hydrology.

Introduction

Historical flood patterns drive microbial community assembly by altering oxygen availability in the belowground environment, and composition in turn determines the functional capacity of these communities. Denitrification, an important microbial-mediated anaerobic process in wetlands, is strongly influenced by soil factors like pH and total C, but a need remains to determine the extent to which functional potential is further influenced by historical microbial assembly processes. The relationship between communities and their associated ecosystem process rates has been an ongoing discussion in ecology (Naeem and Li 1997, Tilman et al. 1997, Tilman et al. 2001). There is mounting correlative evidence to suggest that microbial community function is influenced by both the surrounding environment as well as by specific components of the microbial community (Bell et al. 2005, Knelman and Nemergut 2014, Graham et al. 2016).

In wetland restoration, denitrifying services have proven difficult to restore (Peralta et al. 2010, Hossler et al. 2011, Marton et al. 2014), so there is a practical need to identify microbial controls on desired ecosystem service rates. Restored wetland denitrifier communities possess different composition than those in reference wetlands, and this has been linked to differences in

function (Wallenstein et al. 2006a, Flanagan 2009). Microbial community differences in general have been correlated to functional rates in a variety of observational studies (Salles et al. 2012, Morrissey and Franklin 2015), as well as in controlled experiments (Philippot et al. 2013, Aanderud et al. 2015). Denitrifiers are a subset of the overall community that encompasses a phylogenetically diverse range of bacterial species (Zumft 1997). These taxa are capable of performing at least one step of the denitrification pathway because they harbor either nitrite reductase (*nir*) genes, nitrous oxide reductase (*nosZ*) genes, or both (Jones et al. 2013, Jones et al. 2014). In addition to the observed link in composition and denitrification, studies have also found a correlation between potential denitrification rates and the abundance of at least one of these denitrification genes (Iribar et al. 2015, Salles et al. 2017).

Denitrification as an process is also moderated by complex abiotic factors, including stress from water availability. Hydrologic stress arises from both the removal of oxygen and change in redox conditions that occurs following complete saturation, as well as from drought conditions (Zeglin et al. 2009, Song et al. 2010, Wilson et al. 2011). More importantly, the community composition can be altered by hydrologic stress because not all microbial taxa possess the physiology required to tolerate a range of hydrologic conditions (Schimel et al. 2007) and this can indirectly affect rates of activity performed by the community. For example, experimental rewetting can increase the abundance of rare taxa that are already present, thus altering the composition (Aanderud et al. 2015). The same experiment demonstrated that the rise in abundance of rare taxa was concurrent with an increase in respiration, indicating that such changes in composition have consequences on function (Aanderud et al. 2015). Historical flood regimes, and thus historical patterns in redox conditions, impose an environmental filter on

wetland denitrifier community assembly, and could have important implications for the functional response of these communities to a changing environment.

Further, dispersal limitation between sites can affect community assembly patterns (Leibold et al. 2004, Vellend 2010), and dispersal limited community assembly patterns have been demonstrated to influence microbial activity (Adams et al. 2014). In aquatic systems, dispersal is less restricted than in terrestrial systems because sediment communities can disperse between sites through attachment to small particles that float from upstream to downstream systems (Crump and Baross 2000, Crump et al. 2012). On the other hand, since wetlands are terrestrial during periods with low precipitation, it is still possible that denitrifiers in restored floodplain wetlands remain dispersal limited between sites (Whitaker et al. 2003, Van der Gucht et al. 2007, Kembel 2009, Cline and Zak 2014, Székely and Langenheder 2014). Limitation of function in newly restored wetlands may be due to compositional differences that persist in the microbial community following restoration, first by previous hydrologic filtering of the soil community, and second by dispersal limitation among existing wetlands.

Composition determines function at least to some degree, as has been demonstrated in the literature (Graham et al. 2016), and supported by correlative results in previous chapters of this dissertation. In order to further understand abiotic and microbial controls that limit the restoration of denitrification function in wetlands, we ask if historical hydrology limits functional potential by filtering the community, and whether future functional potential remains limited by dispersal limitation to new wetlands. We combined an observational field study approach, and an additional experiment to address three hypotheses: (1) If historical hydrology is one of the strongest structuring forces on below-ground ecology, and if these wetlands are also hydrologically connected, then wetlands with similar historical hydrologic patterns will exhibit

similar below-ground communities as one another. (2) If historical hydrology limits the future functional potential of belowground communities, then wetland communities with different historical hydrologic patterns will exhibit different functional responses to experimentally altered hydrology. (3) If microbial dispersal to newly restored wetlands is in fact limited, then removal of the dispersal limitation will also alter the functional response of the community to experimentally altered hydrology, where function will likely approach an intermediate rate compared to rates observed from communities in separate wetlands.

Methods

Wetland site selection

In order to categorize wetlands into groups with distinct historical hydrologic regimes, a principal component analysis (PCA) ordination was created using flood history data provided by the Illinois State Geological Survey. The ISGS records flood frequency, depth, duration, and timing for restored compensatory wetlands. There were 23 restored wetlands with flood data collected for at least two years, and these 23 wetlands were geographically distributed across the state of Illinois. The flood history variables included to construct the PCA were average annual flood frequency, average flood depth, average annual maximum flood depth, and average flood duration, as well as a coefficient of variation that was calculated for each variable. One outlier wetland was removed due to infrequent flooding, and a second wetland was removed because it was a floodplain wetland inaccurately grouped due to a levee separating it from the adjacent river, which altered the recorded flood data. The resulting PCA is shown in the supplementary material (Fig. D.1). The 21 remaining sites fell into three broad categories of flood histories. Sites with floods that typically achieve a greater depth were separated on the right side of the

ordination, and these sites were also lower in the watershed than the other wetlands. The bottom left quadrant of the ordination held sites that displayed the greatest variability in terms of both flood depth and duration from year to year. Wetlands located along the top half of the ordination experienced the most frequent flooding and the smallest duration, indicating a distinctive frequent pulsing flood regime.

In order to select wetlands that clearly belong within each of the three groups, a *k*-means clustering algorithm was employed. This technique uses an iterative process to assign multivariate data to groups based on similarity. The algorithm was set to assign wetlands to three groups, and 15 independent trials were run in succession. Sites that grouped together most often were selected to represent each of the three groups. Sites labeled as F1, F2, and F3 in the PCA ordination belong to the group with frequent, short-duration flood patterns, and these were assigned to the same group during all 15 trials of the *k*-means algorithm. Sites labeled as V1, V2, and V3 belong to the group that aligned with the coefficients of variability, and thus were determined to have high interannual variability, and these three wetlands were also assigned to the same group in all 15 trials. Only two sites clearly aligned with long-duration flood periods in the PCA ordination, L1 and L2. These two sites were grouped together in 13 out of the 15 trials, but other sites were rarely assigned to the same group as these two. Average values of the hydrologic PCA axes for each group are shown in Table 5.1. The eight wetlands selected for this study were also distributed across the state of Illinois in order to reduce potential confounding effects between geographic region and hydrologic history (Fig. 5.1).

Field sampling

For the initial evaluation of wetland community composition and soil characterization, six soil samples were collected from the top 10 cm of soil at the eight selected wetlands in March

of 2015. In order to capture within-site variability, two samples were collected from a relatively dry region, two samples from a transition region, and two samples were collected from a lowland region. Eight soil cores were collected from the top 10 cm at each sampling location and homogenized to produce a total of six samples from each wetland. The soil was transported on ice to the laboratory. At least 10 g of soil was removed immediately under sterile conditions, frozen at -20˚C, and freeze-dried for DNA extraction later. A portion of wet soil was set aside for analysis of extractable nutrients, while another portion was air dried for pH analysis, as well as analysis of total C and total N via combustion analysis. Sampling was repeated in May 2015, August 2015, and October 2015 to evaluate whether temporal changes occurred that might influence the planned experiment in terms of the original soil characteristics and microbial activity of the source soil.

Soil for the hydrologic manipulation experiment was collected in June 2015. Approximately 3.5 kg of soil was collected from the top 10 cm from each of the two lowland locations at each wetland, and transported back to the laboratory on ice. The two 3.5 kg samples from each wetland were homogenized before they used to inoculate the sterile experimental mesocosms, producing a total of eight separate inocula.

Experimental mesocosm set-up

Denitrification is strongly influenced by both soil properties (Groffman and Tiedje 1989, Strong and Fillery 2002, Schaller et al. 2004) and microbial properties (Wallenstein et al. 2006b, Foulquier et al. 2013, Iribar et al. 2015, Morrissey and Franklin 2015). In order to set up mesocosms with different communities but comparable soil properties, mesocosms were constructed from 90% autoclave-sterilized soil and inoculated with 10% live wetland soil. For the sterile soil base, a sandy loam mixture (2 parts sand : 1 part potting soil) was pasteurized

at 170˚C and further autoclaved at 121˚C. Since some soil enzymes remain active following autoclave sterilization (Carter et al. 2007), a simple preliminary experiment was conducted to ensure the effectiveness of the pasteurization+autoclave sterilization technique for reducing microbial activity. Since soil texture is a strong driver of denitrification activity, this experiment was also used to verify the ability of the inoculation technique to produce discernible activity rates (Foulquier et al. 2013). It was determined that pasteurization by itself reduced potential denitrification activity to 0, but the additional autoclave step would still be included as a precaution. The inoculated soils produced 10% of the original denitrification activity observed in fresh wetland soil collected from the same source.

All mesocosms were constructed one at a time by initially wetting the sterile sandy loam mixture with autoclaved deionized water, and then mixing in 10% inoculum by volume. Following one week of equilibration under moist conditions, the experiment was conducted for an additional two months with 9 additional weekly sampling events. There were four hydrologic treatments: (1) Stable saturated to mimic wetlands that are not connected directly to a flood pulse, (2) saturated for 6 weeks, then allowed to drain for the last two weeks to mimic a long early summer flood, (3) short alternating cycles of saturated for a week and dried for a week to mimic frequent flood conditions, and (4) a dry control. Destructive sampling of replicate mesocosms was conducted the day after establishing the mesocosms, the day after the beginning the hydrologic treatments (following one week of equilibration under moist conditions), and then on each day following a change in saturated or dry conditions for the third treatment (Fig. 5.2). While the long and short flood treatments mimic ‘home’ conditions that might be experienced by two of the hydrologic groups (“long flood group” and “frequent flood group”, respectively), a two-month experiment could not provide a ‘home’ treatment for the source communities from

wetlands that experience high variability from year to year. We anticipate these four treatments together to encompass the range of conditions that might be experienced by the "high interannual variability" group.

In order to simulate the removal of dispersal limitation among wetland communities, a ninth inoculum was created by homogenizing all eight wetland inocula in equal parts by volume, hereafter termed the MIX inocula. There were three replicate mesocosms per treatment per sampling event for each of the eight individual wetland inocula. Since we anticipated that mixing source communities could lead to particularly high variability among experimental replicates, ten replicate mesocosms per treatment per sampling event for the one MIX inoculum in order to capture this excess variability. A single uninoculated negative control was sampled for each treatment on each sampling event.

A total of 1400 soil mesocosms were assembled and destructively sampled for this experiment. The mesocosms themselves were created from 6" clear plastic tubes with rubber caps on the bottoms (VisiPak, Fenton, MO). Saturation was always performed with autoclaved deionized water, and the water level was maintained daily to combat evaporation or potential leaks in the rubber seal. The mesocosms were allowed to drain by replacing the rubber caps on the bottom with a piece of double layer cheesecloth secured with a rubber band. All mesocosms were placed in the same room in a greenhouse, and were randomly arranged in a grid.

DNA extraction and molecular methods

Total genomic DNA was extracted from freeze-dried wetland soil samples collected in March 2015 and May 2015 using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH). Genomic DNA was further purified using a cetyl trimethyl ammonium bromide (CTAB)

extraction to remove contaminating humic acids (Sambrook and Russell 2001). DNA concentration was adjusted to a standard concentration of 10 ng/µl in each sample.

Illumina sequencing was used to target bacterial and archaeal rRNA genes, as well as *nirS*, *nirK*, and Clade I *nosZ* genes (Illumina, San Diego, CA). Sequencing amplicons were prepared by PCR using a Fluidigm Access Array IFC chip, which allowed simultaneous amplification of each target gene (Fluidigm, San Francisco, CA). Initial reactions were carried out according to a 2-step protocol using reagent concentrations according to Fluidigm parameters. The first PCR was performed in a 100-µL reaction volume using 1 ng DNA template, and this PCR amplified the target DNA region using both the gene-specific primers with Fluidigm-specific amplification primer pads CS1 (5'-ACACTGACGACATGGTTCTACA-3') and CS2 (5'-TACGGTAGCAGAGACTTGGTCT-3'), which produced amplicons including (1) CS1 Fluidigm primer pad, (2) 5'-forward PCR primer, (3) amplicon containing the region of interest, (4) 3'-reverse PCR primer, and (5) CS2 Fluidigm primer pad. A secondary 30-µL PCR used 1 µL of 1:100 diluted product from the first PCR as template, and added Illumina-specific sequencing linkers P5 (5'-AATGATACGGCGACCACCGAGATCT-3') and P7 (5'-CAAGCAGAAGACGGCATACGAGAT-3'), along with a 10-bp sample-specific barcode sequence, so the final construct consisted of (1) Illumina linker P5, (2) CS1, (3) 5'-primer, (4) amplicon containing the region of interest, (5) 3'-primer, (6) CS2, (7) sample-specific 10-bp barcode, and (8) the Illumina linker P7. Final amplicons were gel-purified, quantified (Qubit; Invitrogen, Carlsbad CA, USA), combined to the same concentration, and then sequenced from both directions on an Illumina HiSeq 2500 2x250 bp Rapid Run. Fluidigm amplification and Illumina sequencing was conducted at the Roy J. Carver Biotechnology Center (Urbana, IL, USA).

Overall community composition was assessed by sequencing the bacterial and archaeal 16S ribosomal rRNA gene V4 region using the forward primer 515F and reverse primer 806R (Caporaso et al. 2011). The NO_2^- reducing denitrifier composition was assessed by sequencing both *nirK,* with forward primer nirK876 and reverse primer nirK1040 (Henry et al. 2004), as well as *nirS,* with forward primer nirSCd3aF and reverse primer nirSR3cd (Kandeler et al. 2006). The N_2O reducing denitrifier composition was assessed by sequencing Clade I *nosZ* with forward primer nosZ1F and reverse primer nosZ1R (Henry et al. 2006). Primer sequences are listed in Table 1.1 (Chapter 1).

Paired-end 16S sequences were merged using Fast Length Adjustment of SHort reads (FLASH) software v. 1.2.11 (Magoč and Salzberg 2011). Only the first end of the sequences ("Read 1") were used for analysis of the functional genes. Quality filtering of fastq files was performed using software in the FASTX-Toolkit (Gordon and Hannon 2010). Sequences with fewer than 90% of bases showing at least 99.9% base accuracy were removed. The *nirK* sequences were shorter than 300-bp, so they were trimmed to the appropriate size using the FASTX-Toolkit. The fastq files were then converted to fasta format, and sequences were binned into discrete OTUs based on 97% similarity using USEARCH v. 8.1.1861 (Edgar 2010). Taxonomic assignments were made using QIIME software (Caporaso et al. 2011). For 16S gene assignments, the GreenGenes database v. 13.5 was used (McDonald et al. 2012) with the UCLUST algorithm (Edgar 2010). Custom functional gene databases were created by downloading sequences from the RDP FunGene website (Fish et al. 2013), and assignments were made in QIIME with the BLAST algorithm (Altschul et al. 1990).

Soil chemical analyses

Soil pH was determined using a 1:1 soil:deionized water slurry for each sample before and after treatment. Concentration of available ammonium (NH_4^+), combined nitrate (NO_3^-) and nitrite (NO_2^-), and inorganic phosphate (PO_4^-) extracted from soil in 2M KCl was determined using colorimetric methods in a spectrophotometric microplate reader (Epoch Microplate Spectrophotometer, BioTek, Winooski, VT, USA). Available NH_4^+ was measured at 650 nm using a Berthelot method (Weatherburn 1967), NO_3^-/NO_2^- was measured at 540 nm by a vanadium method (Doane and Horwath 2003), and PO_4^- was measured at 630 nm using a Malachite green method (Lajtha et al. 1999). Total organic carbon and nitrogen were determined using combustion analysis (ECS 4010, COSTECH Analytical Instruments, Valencia, CA, USA). Gravimetric water content was also determined for every sample. All of these analyses were performed for the wetland soils. Almost all of these analyses were performed for the 1400 mesocosm samples as well, with the exception of total N and total C, which were only determined for the initial and final mesocosm soil samples to verify that overall C and N remained similar throughout the experiment.

Potential denitrification assay

Potential rates of denitrification were determined under ideal conditions using 25 g of either wetland soil or experimental mesocosm soil. Soil was placed into 125-mL Wheaton jars with 25 mL of deionized water with chloramphenicol as described by Peralta et al. (2016), except with added 45.83 mM dextrose and 14.28 mM nitrate to generate nutrient unlimited rates. All jars were purged with He gas to create anoxic conditions. In order to quantify complete denitrification, acetylene gas was added to the jar headspace to block the conversion of N_2O to N_2 and allow for detection on a GC. In order to quantify how much N_2O might be released via

incomplete denitrification, a second assay was performed simultaneously with He gas added instead of acetylene. Thus, relative potential rates of N_2O-producing incomplete denitrification and total denitrification activity could be determined. Gas samples were collected at the beginning and after three hours. The concentration of N_2O was determined using a gas chromatograph (GC-2014 with ECD, Shimadzu Corp., Kyoto, Japan). The final denitrification rate was corrected by the dry weight of the soil.

Statistical analyses

For the wetland site selection, the principal component analysis (PCA) ordination was constructed using the 'rda' function in the 'vegan' package in R (Oksanen et al. 2013), and the *k*-means clustering was performed using the 'kmeans' function in the 'stats' package in R (R Core Team 2014). Principal coordinate analysis (PCoA) ordinations were created using the 'cmdscale' function, and significant differences in composition were evaluated using the 'adonis' function in the 'vegan' package to run permutational analysis of variance (PERMANOVA) tests. The "core microbiome" for each of the source communities were defined as the microbial taxa detected in at least three of the six soil samples collected from each wetland. The "shared core microbiome" for each of the three hydrologic groups were defined as the core microbiome taxa that were determined for all source communities within each group (i.e. in all three "frequent flood" wetland source communities, all three "variable" wetland source communities, or in both of the "long flood" wetland source communities). To identify whether there exists particular representative taxa for the different flood groups, the 'multipatt' function in the 'IndicSpecies' package (De Cáceres and Legendre 2009) was used to run indicator species on only the most abundant taxa, defined as taxa whose total abundance summed to at least 0.05. To determine if hydrologic history selected for microbial taxa that belong to particular taxonomic groups, the

'DESeq' function in the 'DESeq2' package was used on the taxa identified from 16S rRNA gene sequences (Anders and Huber 2010, Love et al. 2014). To determine whether differences in microbial composition and potential denitrification rates were correlated in the source wetlands, Mantel correlation tests were run using the 'mantel' function in the 'vegan' package.

To determine relative effects of experimental hydrologic treatment and source community on univariate variables, the 'aov' function was used to run two-way ANOVA tests. To determine the effects of time, a repeated measures ANOVA was run, where 'time' was a third categorical factor that represented the sampling event. Specific differences were determined using the 'TukeyHSD' function to run Tukey's HSD test. To test whether denitrification rates were influenced by chemical or physical differences that persisted between inoculated experimental mesocosms, classification and regression tree (CART) analyses were run using the 'rpart' function in the 'rpart' package (Therneau et al. 2015) using all factors that had been tracked throughout the two-month experiment: Time, source community, moisture, pH, ammonium, nitrate, and phosphate. To detect changes in total C or total N in the mesocosms from the beginning of the experiment to the end, a paired t-test was run using the 't.test' function in R. To check whether either C or N influenced rates, simple linear regression models were constructed using the 'lm' function.

Results

We detected 5423 different microbial OTUs from 16S rRNA gene sequencing, and the total number of reads was almost 2 million. For the nitrite reducing denitrifiers, 279 OTUs were observed from the *nirK* sequences with more than 1.6 million *nirK* reads in total, and 1706 OTUs were observed from the *nirS* sequences with more than 213,000 *nirS* reads in total. For the

nitrous oxide reducing denitrifiers, 1692 OTUs were observed from the Clade I *nosZ* sequences with more than 835,000 *nosZ* reads in total. Testing with a PERMANOVA model demonstrated that community composition did not change between March and May, so the community composition data reported in this chapter was generated from soil collected in March.

The microbial communities differed between the eight source wetlands included in this study (Fig. 5.3). The three hydrologic groups of wetlands significantly differed in overall microbial composition ($PERMANOVA_{group}$ $R^2 = 0.085$, $p < 0.01$), nitrite reducing denitrifier composition ($PERMANOVA_{group}$ $R^2 = 0.075$, $p < 0.001$), and nitrous oxide reducing denitrifier composition ($PERMANOVA_{group}$ $R^2 = 0.069$, $p < 0.001$). The eight source communities also differed in overall microbial composition ($PERMANOVA_{source}$ $R^2 = 0.090$, $p < 0.001$), nitrite reducing denitrifier composition ($PERMANOVA_{source}$ $R^2 = 0.050$, $p < 0.001$), and nitrous oxide reducing denitrifier composition ($PERMANOVA_{source}$ $R^2 = 0.042$, $p < 0.001$). Potential denitrification rates also differed among the source communities ($ANOVA_{group}$ $F = 5.3$, $df = 2$, $p < 0.001$), where the frequent flood wetlands exhibited greater rates than the long flood wetlands (Tukey's HSD, $p < 0.001$). The rates did not significantly differ between source communities within the frequent flood group or the long flood group, but they differed between communities in the variable flood group ($ANOVA_{source}$ $F = 45.6$, $df = 2$, $p < 0.001$), where all three source wetlands significantly differed in rates (Fig. 5.4). There was a small but significant correlation between overall microbial community composition and potential denitrification rates (Mantel $R = 0.1578$, $p < 0.01$). The same was seen for nitrite reducing denitrifiers (Mantel $R = 0.1425$, $p < 0.01$) and for nitrous oxide reducing denitrifiers (Mantel $R = 0.109$, $p < 0.05$).

A shared core microbiome was determined for each hydrologic group for the overall microbial community, the nitrite reducing denitrifiers, and for the nitrous oxide reducing

denitrifiers, though the number of shared denitrifier taxa was much lower than taxa shared in the overall community (Fig. 5.5). Of the total core taxa identified from the overall microbial community, 32.4% were assigned to only the frequent flood group, 2.6% were assigned to only the high interannual variability group, and 10.7% were assigned to only the long flood group. Of the nitrite reducing denitrifiers, 14.7% were assigned to only the frequent flood group, 2.9% were assigned to only the high interannual variability group, and 64.7% were assigned to only the long flood group. Of the nitrous oxide reducing denitrifiers, 12.5% were assigned to only the frequent flood group, none were assigned to only the high interannual variability group, and 66.7% were assigned to only the long flood group. Out of the total core taxa identified for each of the eight source communities, 14-59% of the core overall microbial taxa, 1-14% of the core nitrite reducing denitrifier taxa, and 0.7-22% of the core nitrous oxide reducing taxa were shared by all source communities within each group (Fig. D.2).

When "indicator taxa" were identified for each group using the "multipatt" function, these taxa only displayed high abundance in one or two source communities, and none were in high abundance in all source communities within a particular group (Fig. D.3, D.4, and D.5). Using the "DESeq2" function to identify and plot some of these taxa by rarified abundance, we found a similar result: No taxonomic groups were found to be representative of microbial communities in wetlands with similar hydrologic history (Fig. D.6) and taxa abundance in individual wetlands drives the differences detected between hydrologic groups. .

Using these source communities to inoculate sterile experimental mesocosms, the experimental hydrologic treatments most strongly influenced potential denitrification rates, while the original hydrologic group of the source community did not strongly influence rates. The time of sampling source communities to inoculate the mesocosms would have had little effect on the

potential denitrification rates observed during the experiment because there were no temporal trends detected in potential activity rates from the original wetlands between late spring and summer (Fig. D.7). The MIX treatment showed no differences in rates compared to the single source inocula (Fig. 5.6). A two-way repeated measures ANOVA revealed that treatment, hydrologic group, and time were all significant factors that influenced denitrification rates. Treatment showed the strongest effect ($ANOVA_{treatment}$, $F = 53.4$, $df = 3$, $p < 0.001$), where the Saturated treatment resulted in greater rates than the other treatments (Tukey's HSD, $p < 0.001$, for all three comparisons), and all treatments resulted in greater rates than the Dry treatment (Tukey's HSD, $p < 0.001$, for all three comparisons). This ANOVA model was constructed to compare the effect of source communities from wetlands belonging to the three hydrologic groups to each other, while simultaneously comparing the effect of mixing all source communities together in order to effectively remove dispersal limitation. The "hydrologic group" factor had four levels: Frequent flood, high interannual variability, long flood, and MIX. Hydrologic groups differed significantly as well ($ANOVA_{group}$, $F = 4.0$, $df = 3$, $p < 0.01$), but the only significant pairwise differences revealed were that the high interannual variability group showed lower rates than the frequent flood group (Tukey's HSD, $p < 0.05$) and also lower rates than the long flood group (Tukey's HSD, $p < 0.05$). The MIX inocula did not significantly differ from mesocosms inoculated with only a single source community. Time was significant ($ANOVA_{time}$, $F = 66.2$, $df = 9$, $p < 0.001$); Tukey's HSD showed that rates decreased between the fourth and fifth sampling events.

The ANOVA models were analyzed using "hydrologic group" as a factor instead of individual "site" because none of the individual source communities exhibited unique responses to hydrologic treatments when compared to the average response of the hydrologic groups (Fig.

D.8). Total potential denitrification rates were selected as the response variable, rather than potential N_2O production, because the rate of N_2O released by the experimental mesocosms was always much lower than total potential denitrification rates (Fig. D.9).

It was determined that the source inocula did not impart chemical or physical differences to the experimental mesocosms that might have influenced rates. Mesocosms inoculated by different sources may have exhibited differences at the beginning of the experiment, but these were not correlated with differences in potential rates throughout the experiment. CART analyses showed that both total potential denitrification rates and incomplete denitrification (potential N_2O production) throughout the experiment were influenced by soil moisture and time, such that rates were limited by low moisture and decreased over time, but not by soil nutrients or initial source community (Fig. D.10). The soil pH did not differ among mesocosms (Fig. D.11) and moisture followed patterns that reflected the treatments (Fig. D.12). Soil ammonium, nitrate, and phosphate did change over time (Fig. D.13, D.14, and D.15), but they did not change in a way that was correlated with denitrification rates. Total C and N were not included in the CART analyses because they were only evaluated at the beginning and end of the experiment, but neither total C nor total N influenced denitrification rates in the mesocosms at either sampling date. The total soil C in the experimental mesocosms was significantly lower by the end of the experiment, but these values were not correlated to rates, and total soil N did not change throughout the experiment (Fig. D.16).

Discussion

Hydrologic history effects on denitrifier composition and potential function

We have demonstrated that historical flood patterns exhibit at least some influence on overall microbial community composition, as well as denitrifier composition. There remains a large proportion of compositional variability attributed to site-to-site differences. These site-to-site differences seem to swamp out the influence of hydrologic history, which is likely due to additional community assembly processes and additional environmental filters that may not have been detected in the current study design. The structuring influence of hydrology has been observed before (Peralta et al. 2016), but here we added statistical power by including replicate wetlands with similar historical regimes. If flood regimes imposed a predictable filtering effect on composition, then we would expect to see particular microbial taxa selected in wetlands with similar histories. However, instead we found taxa enriched in individual wetland communities rather than across all of the wetlands that share a similar hydrologic history.

The significant historical hydrologic effect on microbial composition may indicate that multiple alternative states can arise from similar environmental drivers. For example, the rapid drying and rewetting typical of wetlands with a "frequent" flood pattern may filter out organisms that cannot adapt to rapidly changing conditions (Schimel et al. 2007, Buelow et al. 2016), but the organisms left would only include taxa that could colonize that site to begin with. There is no immediately obvious reason to assume that the same taxa were already present at all the sites within each group (Kembel 2009, Székely and Langenheder 2014). An example of this might include the taxon belonging to the *Flavisolibacter* genus that was identified as an indicator of species turnover between the three hydrologic groups, which was in high abundance in sites F1 and F3 but not F2 (Fig. D.6A). Another example includes the taxon in the alphaproteobacterial

genus *Rhodoplanes* identified as an indicator of hydrologic group, though it was only in high abundance in the site V3, and not V1 or V2 (Fig. D.6B). Even without dispersal limitation between sites with similar historical conditions, there may still be environmental drivers that differ between these sites, and those differences could represent a secondary filtering step. Soil texture, moisture, and soil C are strong drivers of community composition (Drenovsky et al. 2004, Cleveland et al. 2007, De Graaff et al. 2010, Foulquier et al. 2013). The soil at site F2 showed higher % silt, higher moisture, and lower total C than the soil at sites F1 and F3, and the soil at V3 showed lower % clay, higher % silt, lower moisture, and lower total C and N than the soil at sites V1 and V2 (Table 5.2 and Table 5.3).

Potential denitrification rates differed among hydrologic groups, and Mantel analyses revealed a weak correlation between potential rates and belowground community composition. It is difficult to untangle whether differences in rates and composition are simply due to differences in soil chemistry and texture or if the community has a directly causal effect on potential function. For example, soil C drives potential denitrification rates (Stanford et al. 1975) as well as microbial composition, and the wetlands with the greatest potential denitrification rates also displayed the greatest total soil C (Table 5.3 and Table 5.4). Other researchers have used statistical modeling to isolate the importance of both the environment and the microbial community to drive denitrification rates in environmental systems that were equally as complex as the wetlands in this study (Foulquier et al. 2013, Morrissey and Franklin 2015, Graham et al. 2016). We chose to use an additional experimental approach to remove the effect of the environment, which is addressed in the following section. Put together, our observational findings already suggest that historical flood patterns influence community assembly to some degree, and this leads to differences in potential denitrification rates from the soil.

Hydrologic history effects on denitrifier response to altered hydrology

We were able to isolate the microbial community from the confounding effects of soil chemistry and texture by inoculating experimental mesocosms with live wetland soil, and we found there was a small difference in the functional response to hydrologic manipulation between groups. However, the effect of different soil inocula was greatly overshadowed by the effect of the imposed hydrologic treatments. It is certainly possible that the sterile soil base used to construct the mesocosms acted as an environmental filter itself, thus altering mesocosm community composition to a similar state regardless of starting composition. However, reciprocal transplant experiments have demonstrated that the starting community strongly influences the final community composition even up to a year after being placed in a new environment (Waldrop and Firestone 2006, Reed and Martiny 2013). Instead, we may have observed a functionally redundant response, where functional redundancy refers to the situation where microbial communities are so diverse that two different communities are still capable of performing the same function at the same rate (Torsvik and Øvreås 2002, Allison and Martiny 2008). It has been speculated that denitrification will exhibit a particularly high degree of functional redundancy due to the high diversity or organisms that can participate in the denitrification process (Schimel and Gulledge 1998, Schimel et al. 2007).

Our methods were similar to an approach commonly utilized in plant-soil feedback experiments (Mills and Bever 1998, Bever 2002, Kardol et al. 2007, Mangan et al. 2010), where individual plant species are grown in controlled systems in order to filter unique soil communities for each species, and then those soils are used to inoculate sterilized soils. This allows researchers to compare plant growth in “home” and “away” soil communities. In our novel experimental approach, we wanted to be able to measure potential denitrification from

inoculated soils, but this approach would have led to rates that are sometimes too low to detect. Thus, we used a higher percentage by volume (10%) than is typical of plant-soil feedback studies (5%). The soil chemistry of experimental units was monitored at each sampling event to ensure that different wetland inocula did not impart physical differences that might further confound results. We found some chemical differences among mesocosms inoculated with particular wetland communities, but none of the chemical differences influenced rates, and thus differential responses must be due to differences in the starting community.

The importance of historical regimes on community composition may be irrelevant if different soil communities are equally able to respond to altered hydrologic conditions. Historical hydrologic patterns have been demonstrated to influence both composition and function in soil communities to some extent (Evans and Wallenstein 2012, Brower et al. 2017). Experiments in grassland ecosystems have demonstrated that increased precipitation can lead to the development of a soil community that is less resilient to future environmental changes (Gravuer and Eskelinen 2017), while extended drought altered the carbon use efficiency (CUE) of soil communities (Zeglin et al. 2013). On the other hand, fluctuations in water level and associated mobilization of nutrients can have strong effects on the activity of the microbial community across many cellular functions, including denitrification (Manis et al. 2014), as well as respiration and expression of stress response genes (Buelow et al. 2016). In our study, we found that the original hydrologic group of the source community had far less of an influence on potential functional rates compared to the experimental treatments, which indicates that wetland denitrifier communities in general may either exhibit high functional redundancy with each other, or their ability to function is equally susceptible to drought.

However, we detected a small difference in functional response from mesocosms inoculated by communities from wetlands with high interannual variability (source communities V1, V2, and V3). This group of inocula often displayed lower rates than those from the other two hydrologic groups, particularly under the constantly saturated treatment, which should have been most favorable treatment for taxa capable of anaerobic respiration, like denitrifiers (Zumft 1997). The defining characteristic of this group of wetlands is that they do not experience flooding every year, and the floods they experience are not always the same magnitude and at the same frequency from year to year. Flood events may be rare enough in these wetlands that they represent a disturbance, and thus these source communities may have simply not contained denitrifier taxa that were able to respond to the saturated treatment at the same rate as the other sources. Some of the denitrifiers that were enriched in this particular group possess the same taxonomic identity as those enriched in the other two groups (e.g. *Pseudomonas* sp., *Paracoccus* sp., and *Rhodopseudomonas palustris*) and this may indicate that there are physiological differences between closely related individuals. However, one OTU in particular, the *nirS*-containing denitrifier *Polymorphum gilvum*, was primarily enriched in the group of wetlands with high interannual variability (Fig. D.4C), and enriched in both the high interannual variability and long flood groups (Fig. D4B). This taxon has received attention from microbiologists because it can degrade a wide variety of hydrocarbons and is tolerant to high salinity, but sequencing its genome revealed that it also possesses an unusually high abundance of genes for motility proteins (Nie et al. 2012). *P. gilvum* may be remarkably well adapted to changing conditions because it would be able to move to neighboring regions as the surrounding soil conditions become less favorable.

Removal of dispersal limitation among restored wetland communities

While the initial community composition of these wetland source communities suggested that dispersal limitation was a historical factor driving composition, we did not find evidence that the removal of dispersal limitation influenced denitrification capacity in any way. Thus we cannot conclude that the limited denitrifier function reported in restored wetlands (Hossler et al. 2011, Marton et al. 2014) is simply due to dispersal limitation from existing wetlands to newly restored sites.

Dispersal limitation is still an important community assembly process to consider in wetland ecology, especially when faced with the challenge of restoring microbial communities that are functionally equivalent to those in wetlands that were destroyed or damaged. Dispersal can influence composition by rescuing populations of taxa that are particularly sensitive to stress (Székely and Langenheder 2017), and most microbial communities are sensitive to stress (Allison and Martiny 2008). In general, rare taxa are far more likely to be dispersal limited while the majority of the community remains subject to strong habitat filtering (Székely and Langenheder 2014), which could explain why dispersal had little effect in an experiment that examined the influence of dispersal on functional rates. The rare taxa may have limited importance for function on short time scales. However, these taxa would have long term consequences on function when opportunistic taxa increase in abundance once conditions are favorable (Aanderud et al. 2015, Székely and Langenheder 2017, Shen et al. 2018).

Experimentally manipulating dispersal rates between leaf litter decomposing communities, a dry system where the individual communities are not well connected, showed that even slow rates of dispersal alter the final composition (Albright and Martiny 2017). Dispersal limitation may not be as important during aquatic or semiaquatic microbial community

assembly though. Lake and large order stream microbial communities contain the same microbial taxa that are present in low order streams and even in the surrounding soil communities (Crump et al. 2012). Fully aquatic ecosystems can contain rare taxa that may not be well adapted to that particular region but are nevertheless still present, and if conditions are altered in such way that these rare taxa become favored, they increase in abundance and are no longer rare (Shen et al. 2018). It is possible that soil microbial community assembly in newly restored wetlands is not dispersal limited, and in fact, these communities face strong environmental filtering from soil properties that are slow to develop (Ballantine and Schneider 2009).

Conclusions

We were not able to provide evidence that restored wetlands are limited in potential function by dispersal limitation of the below-ground community, but our study was able to demonstrate that historical hydrology of a wetland certainly leaves a signature on microbial community composition. Denitrification in restored wetlands may be limited, to some degree, by differences in microbial community composition. Wetland denitrifier communities that historically face extended drought conditions may be limited in future functional capacity, even if hydrology is restored in a way that favors rapid denitrification.

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Tables

Table 5.1. Average hydrologic variables used to categorize wetlands into three distinct groups. The lower case letters represent significant groupings based on one-way ANOVA and Tukey's HSD ($p < 0.05$). The standard error of the mean for each variable is given in parentheses.

Hydrologic Group	Maximum Depth (m)		Mean Depth (m)		Duration (days)		Frequency (event/year)		Watershed Area (ha)
	Mean	CV	Mean	CV	Mean	CV	Mean	CV	
Frequent Flood (N=3)	1.00[a] (0.08)	0.67 (0.09)	0.54[a] (0.04)	0.75 (0.04)	3.85 (3.05)	1.05 (0.09)	9.50[a] (1.44)	0.55 (0.15)	1,297.97 (1,011.33)
High Interannual Variability (N=3)	0.89[a] (0.11)	1.01 (0.05)	0.43[a] (0.03)	0.80 (0.21)	12.75 (3.43)	1.30 (0.21)	1.88[b] (0.63)	0.54 (0.10)	12,098.80 (6,439.09)
Long Flood (N=2)	1.84[b] (0.17)	0.77 (0.12)	0.94[b] (0.04)	0.70 (0.08)	32.77 (13.27)	1.07 (0.28)	2.82[b] (0.52)	0.53 (0.07)	955,455.90 (890,458.90)

Table 5.2. Wetland location and soil type. Soil information was obtained from the USDA Web Soil Survey online tool (Soil Survey Staff).

Hydrologic Group	Wetland	County	Latitude	Longitude	%Clay	%Sand	%Silt	Texture	Parent Material
Frequent Flood	F1	Sangamon	39.76	-89.44	31.0	8.0	61.0	Silty clay loam	silty alluvium
	F2	Perry	38.04	-89.43	20.0	8.0	72.0	Silt loam	alluvium
	F3	Effingham	39.15	-88.57	11.5	32.3	56.2	Silt loam	sandy/ loamy alluvium
High Interannual Variability	V1	Henry	41.55	-90.18	31.0	5.0	64.0	Silty clay loam	silty alluvium
	V2	Sangamon	39.88	-89.67	31.0	8.0	61.0	Silty clay loam	silty alluvium
	V3	Jackson	37.79	-89.23	18.0	6.0	76.0	Silt loam	Loess/ silty alluvium
Long Flood	L1	Alexander	37.29	-89.51	31.0	10.0	59.0	Silty clay loam	alluvium
	L2	Brown	39.97	-90.52	43.0/ 22.5[§]	5.0/ 24.8[§]	52.0/ 52.7[§]	Silty clay/ Silt loam[§]	alluvium

[§]The upland region of wetland L2 displayed different values than the rest of the site, and those values are reported first, while the values from the lowland region are reported second. The source soil that was used to inoculate experimental mesocosms came from the lowland region.

Table 5.3. Average soil chemistry variables for each wetland at the time of sampling. The standard error of the mean for each variable is given in parentheses.

Hydrologic Group	Wetland	Moisture (% w/w)	Total C (% w/w)	Total N (% w/w)	C:N	NH_4^+ (mg/g soil)	NO_3^- (mg/g soil)	PO_4^- (mg/g soil)
Frequent Flood	F1	0.29 (0.02)	2.36 (0.16)	0.19 (0.01)	12.7 (0.45)	1.62 (0.26)	1.12 (0.02)	2.90 (0.66)
	F2	0.33 (0.01)	1.98 (0.13)	0.18 (0.01)	11.23 (0.31)	0.78 (0.13)	1.15 (0.01)	1.64 (0.28)
	F3	0.29 (0.004)	2.75 (0.18)	0.19 (0.02)	14.44 (0.12)	0.72 (0.92)	1.11 (0.02)	2.21 (0.66)
High Interannual Variability	V1	0.33 (0.02)	5.13 (0.1)	0.37 (0.01)	13.89 (0.11)	2.93 (0.24)	1.20 (0.03)	11.43 (0.30)
	V2	0.29 (0.01)	2.37 (0.12)	0.19 (0.01)	12.50 (0.28)	2.20 (0.47)	1.12 (0.02)	1.57 (0.33)
	V3	0.27 (0.02)	1.28 (0.24)	0.11 (0.02)	11.26 (0.17)	3.33 (0.19)	1.08 (0.05)	1.75 (0.21)
Long Flood	L1	0.25 (0.01)	1.92 (0.13)	0.13 (0.004)	14.69 (0.51)	1.30 (0.02)	1.05 (0.02)	3.35 (0.35)
	L2	0.31 (0.01)	2.30 (0.30)	0.21 (0.02)	11.09 (0.47)	1.04 (0.02)	1.18 (0.03)	2.06 (0.56)

Table 5.4. Average denitrification rates for each wetland at the time of sampling. The standard error of the mean for each variable is given in parentheses.

Hydrologic Group	Wetland	Potential Incomplete Denitrification (ng N_2O g^{-1} soil hr^{-1})	Potential Complete Denitrification (ng N_2O g^{-1} soil hr^{-1})	Proportion Incomplete Denitrification (N_2O / N_2O+N_2)
Frequent Flood	F1	13.72 (3.09)	94.84 (10.83)	0.16 (0.04)
	F2	29.53 (1.30)	95.34 (6.82)	0.31 (0.05)
	F3	7.24 (1.83)	80.75 (10.78)	0.15 (0.01)
High Interannual Variability	V1	7.17 (4.46)	129.63 (8.14)	0.05 (0.09)
	V2	17.19 (1.92)	56.40 (3.08)	0.35 (0.07)
	V3	12.60 (4.86)	24.18 (4.09)	0.53 (0.12)
Long Flood	L1	2.91 (3.48)	39.09 (8.85)	0.10 (0.03)
	L2	18.37 (2.23)	53.66 (18.08)	0.39 (0.06)

Figures

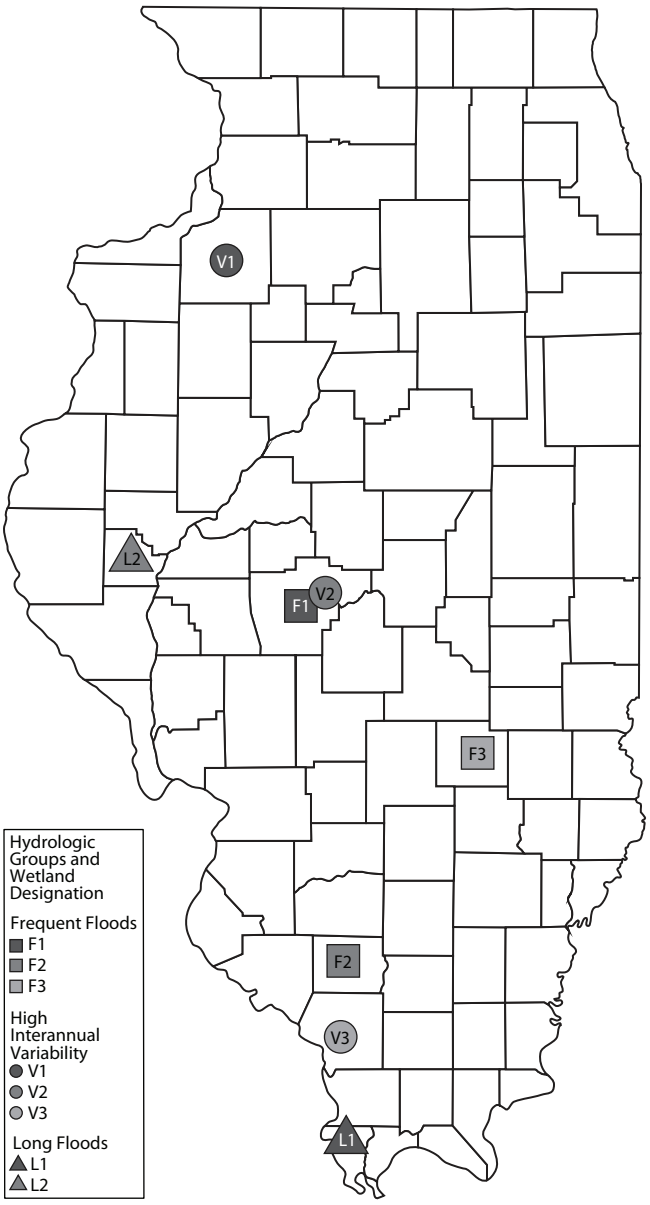

Figure 5.1. Map of Illinois showing the approximate location of the eight source wetland. The shape of the symbol represents the historical hydrologic group that the wetland was assigned to: Wetlands that belong to the "frequent" flood group are shown as squares, those that exhibit high variability from year to year ("high interannual variability") are shown as circles, and those in the "long" flood group are shown as triangles. The letter+number inside each shape is the unique wetland designation used to refer to each wetland throughout the study.

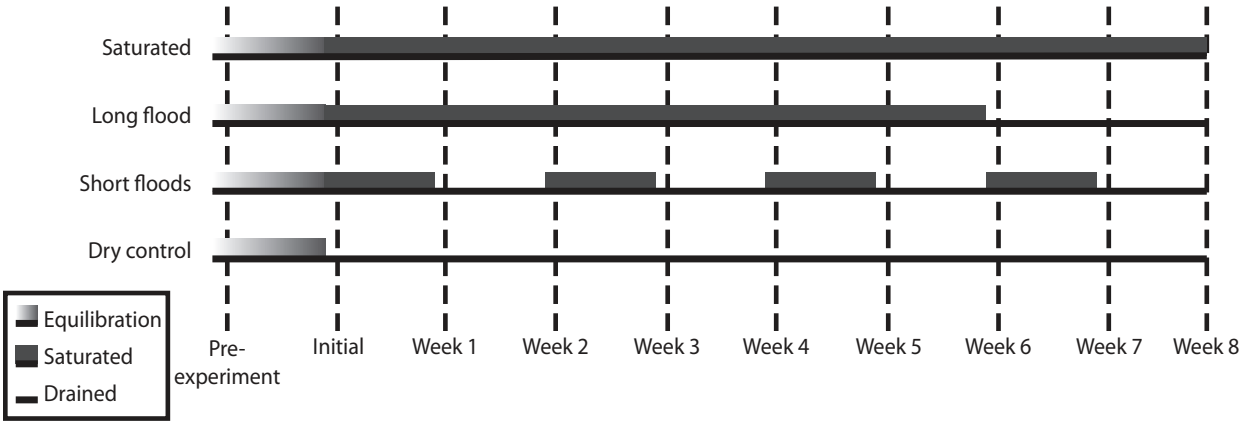

Figure 5.2. Hydrologic manipulation treatments in the experimental mesocosm study. The vertical dashed lines represent sampling events that occurred every 7 days, and each sampling event occurred one day after any change in hydrology throughout the experiment. The dark grey bars show the duration of time that each treatment involved complete saturation. The absence of a grey bar shows the duration of time when mesocosms were allowed to drain. All mesocosms were treated exactly the same during the first week of the experiment ("Pre-experiment" to "Initial"), where they were kept moist but not inundated to allow for equilibration.

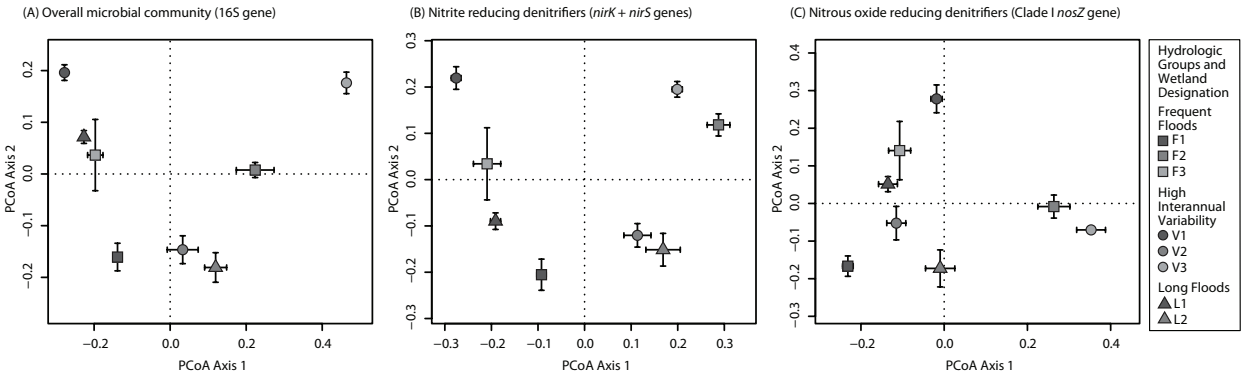

Figure 5.3. Principal coordinate analysis (PCoA) ordinations of different subsets of the microbial communities present at each source wetland: (a) the overall microbial community based on sequences of the 16S rRNA gene, (b) the nitrite reducing denitrifiers based on sequences of both *nirK* and *nirS* genes, and (c) the nitrous oxide reducing denitrifiers based on the Clade I *nosZ* gene. The shape of the symbol represents the historical hydrologic group that the wetland was assigned to: Wetlands that belong to the "frequent" flood group are shown as squares, those that exhibit high variability from year to year ("high interannual variability") are shown as circles, and those in the "long" flood group are shown as triangles. Points represent the mean centroid of six replicates collected from each wetland and error bars are the standard error of the mean.

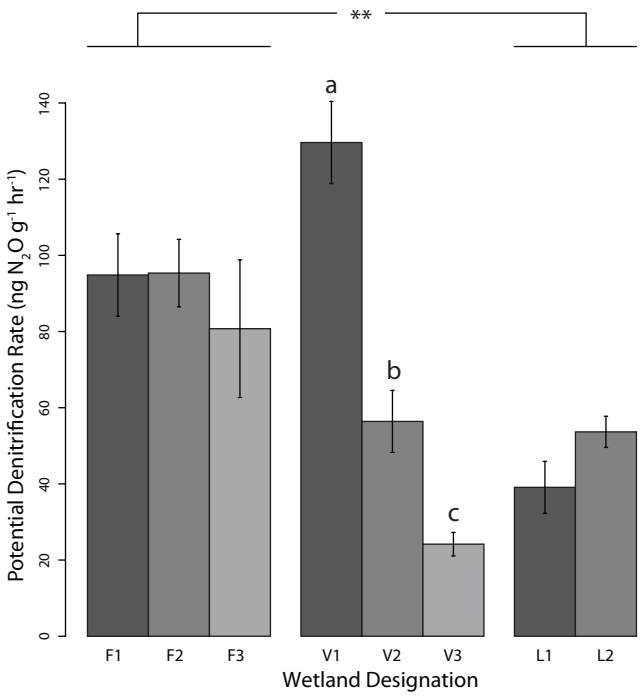

Figure 5.4. Potential denitrification rates from soil collected from each source wetland (N = 6 soil samples per wetland). The asterisks above show significant differences between hydrologic groups (** $p < 0.01$), and the lower case letters show significant differences between source wetlands within a group (based on Tukey's HSD with $p < 0.05$).

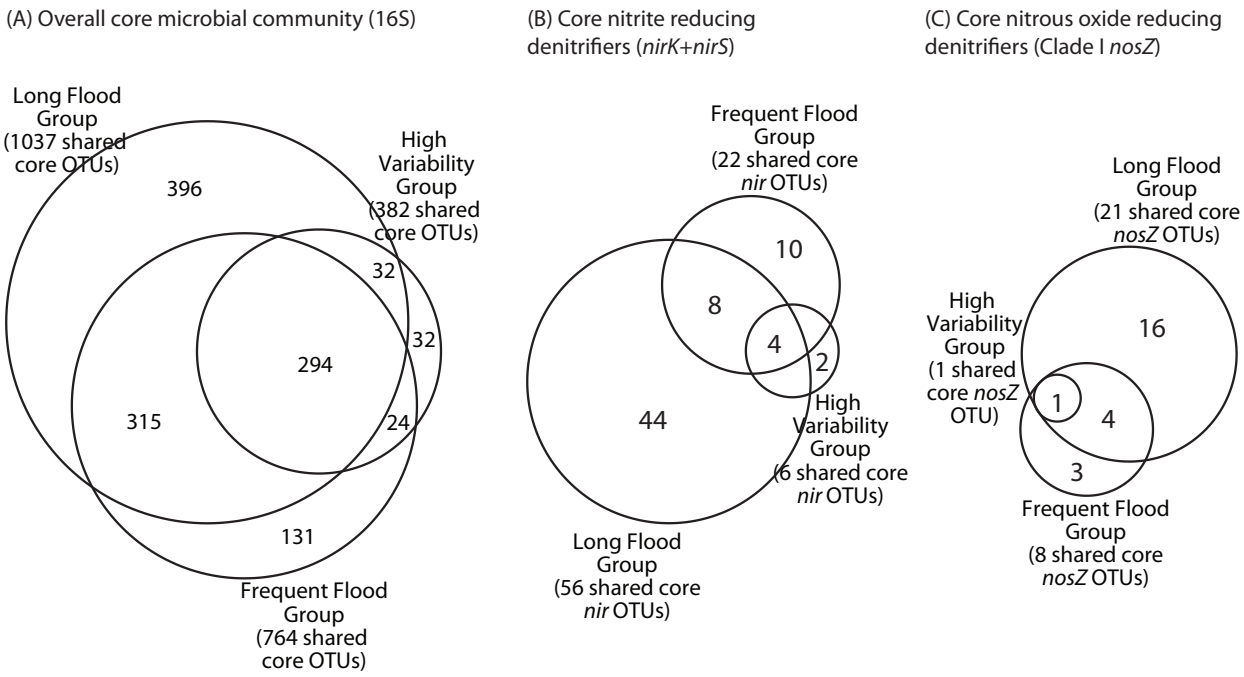

Figure 5.5. Number of core microbial OTUs that overlap between the three hydrologic groups.

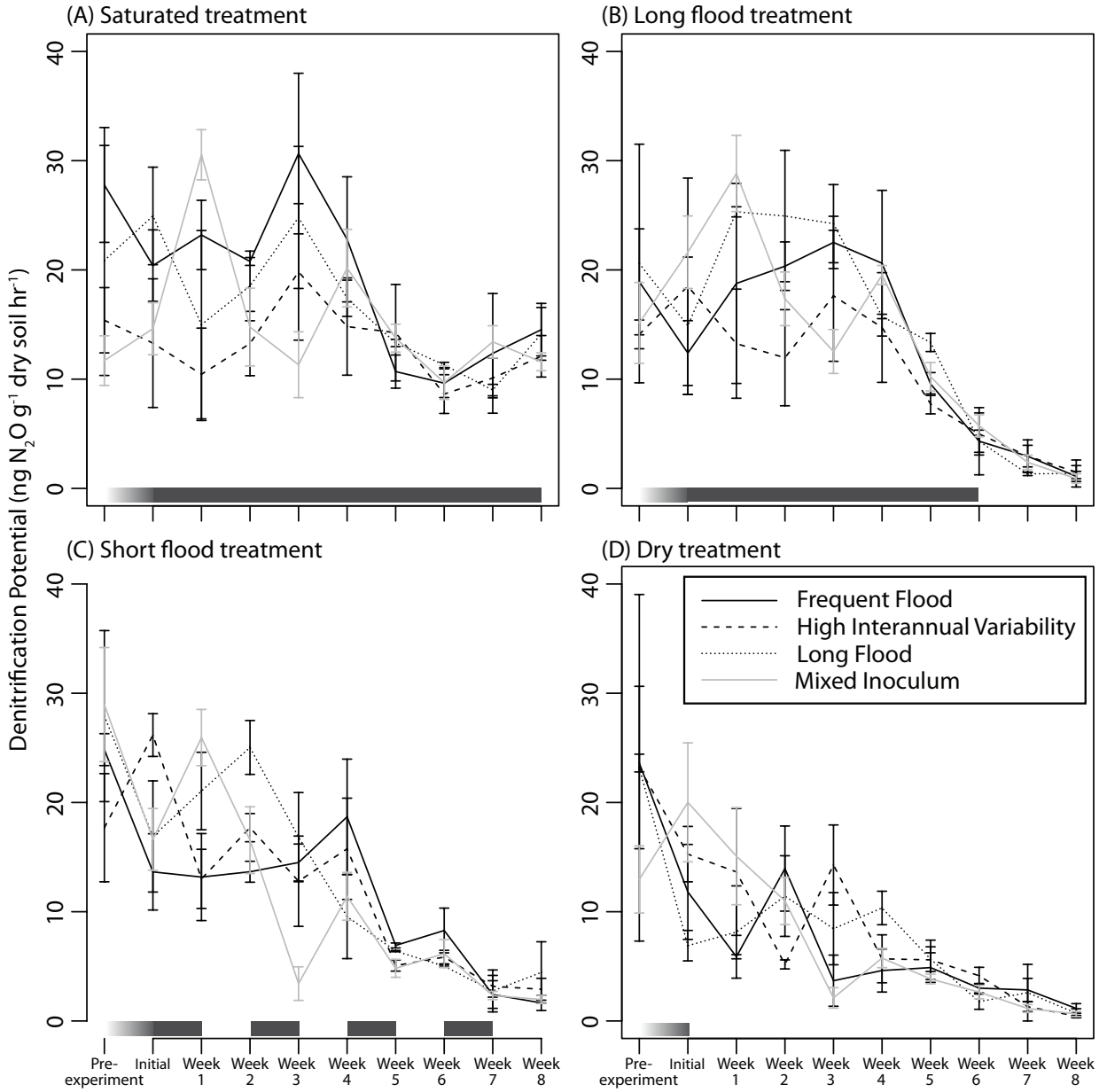

Figure 5.6. Potential denitrification rates from mesocosms. Each panel shows the results from different treatments: (a) Fully saturated, (b) long flood treatment — saturated for 6 weeks, drained for 2 weeks, (c) short flood treatment — alternating saturated for a week with drained for a week, and (d) dry control. The lines show the averages for mesocosms inoculated with wetland communities belonging to the distinct hydrologic groups: Solid black lines are mesocosms inoculated by the "frequent" flood wetland communities (N = 3 source wetlands), the dashed lines are mesocosms inoculated by communities from wetlands with "high interannual variability" (N = 3 source wetlands), and the dotted lines are mesocosms inoculated by communities from wetlands assigned to the "long" flood group (N = 2 source wetlands). The grey lines show the results from inoculating mesocosms with mixed soil from all 8 source wetlands (N = 10 replicate mesocosms). Error bars show standard error of the mean. The dark grey horizontal bars along the x-axis of each plot are a graphical representation of the hydrologic treatment (see Figure 5.2).

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CHAPTER 6: CONCLUSIONS

Wetland ecosystems are often complex and serve many ecological and public service functions (Rey Benayas et al. 2009, Moreno-Mateos and Comin 2010), and there is limited monitoring of the delivery of ecosystem services in restored wetlands that were intended to replace those that were damaged or destroyed (Matthews and Endress 2008). The studies that exist suggest that mitigation efforts do not effectively replace wetland ecosystem services when compared to those delivered by reference wetlands that represent a target ecosystem (Moreno-Mateos et al. 2012). Wetland ecosystems are also increasingly threatened by drought and hydrologic disturbances induced by climate change (Pinay et al. 2002). Thus, restoration efforts may be shooting for a moving target (Erwin 2008, Dunwiddie et al. 2009, Suding 2011).

This dissertation focused specifically on soil microbial communities in restored freshwater wetlands in Illinois, and on the potential for these communities to remove nitrate pollutants via denitrification. The existing literature suggests that soil communities in restored wetlands do not approach the same potential denitrification rates observed in reference wetlands (Hossler and Bouchard 2010, Marton et al. 2014) and sometimes this is coupled with a difference in the composition of the microbial community (Flanagan 2009, Peralta et al. 2010). Numerous studies have also documented a relationship between denitrifier activity and denitrifier composition, abundance, or richness (Morrissey and Franklin 2015, Graham et al. 2016, Salles et al. 2017) but these patterns are not always consistent (Chroňáková et al. 2009, Song et al. 2010, Philippot et al. 2013, Brower et al. 2017). My results point toward a complicated relationship between denitrifier composition and function in wetlands, where potential function is most strongly influenced by recent hydrologic events (drought, intense flood disturbance, or

experimentally altered hydrologic treatments). Statistical modeling in Chapters 2 and 3 showed that characteristics of the microbial community do influence potential rates to a degree. The experimental approach in Chapter 5 demonstrated that microbial communities from wetlands that experience high variability in flood regimes from year to year are not capable of achieving potential rates as high as communities from other wetlands, even once differences in soil texture and chemistry were removed.

The following three specific objectives were addressed:

(a) Identify correlations between potential rates of denitrification and the abundance of specific microbial OTUs or groups of microbes.

The first study described in Chapter 2 found that functional potential was at least partially correlated to the abundance of *nirS* genes present in a community (see Table 2.4). When I proceeded to characterize differences in wetland communities by using DNA sequences, I found they exhibit high variability from wetland to wetland (see Figures 3.2, 3.3, 3.4, and Figure 5.3). In general, wetlands that have different denitrification potential also have different microbial communities, but many wetlands exhibit similar functional potential but different communities. Thus, I was unable to identify specific microbial taxa that are consistent indicators of high functional capacity (see Figure 3.6, Figure C.7, and Figures D.3, D.4, D.5, and D.6).

In Chapter 3, I used a redundancy analysis (RDA) to identify taxa whose relative abundance correlated with potential denitrification rates over time, including taxa related to *Rhodopseudomonas palustris*, *Azoarcus* spp., and unidentified denitrifiers. Each of these taxa peaked in abundance during 2013 when all of the wetlands peaked in potential denitrification rates. However, none of the individual OTUs were in high abundance in more than one wetland,

even though two wetlands, Site A reference and Site C reference, both exhibited high potential rates. This suggests that functional redundancy is high among wetland denitrifiers. When experimentally tested in Chapter 4, however, functional redundancy between wetland denitrifiers did not automatically confer functional stability. Instead, the results of Chapter 4 suggest that functional stability is determined more by the historical context of the community.

(b) Determine which abiotic characteristics in wetlands drive differences in microbial composition, especially between restored and reference wetlands.

Recent hydrologic conditions at the time of sampling strongly influenced potential rates, but I did not find a straightforward effect on microbial composition. Using the study in Chapter 3 as a small case study, I found that microbial communities in restored wetlands are not exhibiting compositional convergence toward their respective reference wetlands, even up to 17 years post restoration. This might be related to differences in soil organic matter because these particular reference wetlands have greater soil C than the restored wetlands (see Figure B.4). In the same study, I found that recent hydrologic events *in situ* influenced potential rates generated in a laboratory, but these events did not result in a clear change in composition. Instead, a few specific denitrifier OTUs responded by either increasing or decreasing in relative abundance during a year that experienced intense flooding. In Chapter 4, contrasting historical flood regimes across a single wetland strongly influenced microbial community composition. In Chapter 5, I identified replicate wetlands with similar historical flood regimes, and I found that flood patterns do not necessarily yield a predictable influence on microbial composition. Composition differed between wetlands with contrasting flood regimes, but composition also differed between wetlands with similar flood regimes. Others have documented that contrasting

flood patterns or fluctuating redox states influence microbial composition (Pett-Ridge and Firestone 2005, Peralta et al. 2014). I found the hydrologic regime influenced composition to some degree, but other environmental filters might have structured composition as well. For example, soil texture can drive differences in microbial composition, and one of the "frequent flood" wetlands had soil that was 32% sand while the other two in the same group had 8% sand. Total C and total N was twice as high in one of the "high interannual variability" wetlands compared to the seven other wetlands.

(c) Determine if those differences in composition correlate to differences in potential function.

In Chapter 2, I observed that average denitrification rates across 45 wetlands were equivalent between restored and reference wetlands. Soil moisture at the time of sampling strongly influenced potential denitrification rates (see Figure 2.1 B). Other abiotic factors that were correlated with rates included phosphate and pH (see Table 2.3). However, soil moisture, phosphate, and pH were no different between restored and reference wetlands on average. Using a singly wetland in Chapter 4, I found that historical hydrologic variable structured a community that was more stable in composition, as well as in rates, compared to other community. In Chapter 5, I found that hydrologic manipulation was a far stronger influence on potential rates than either community composition or historical flood regimes. Interestingly, the mesocosms inoculated by source communities from wetlands with historically variable flood regimes from year to year exhibited consistently lower rates than mesocosms inoculated with source communities from wetlands with typically flashy or long extended flood patterns.

It is possible that the timeline along which hydrologic fluctuations occur in an environment is an important influence on the functional stability of a soil community. In Chapter

4, the transition region of a wetland experienced both dry and flooded conditions within a single season, and this structured a community that was relatively stable in function when compared to other communities in the same wetland. In Chapter 5, the wetlands with high variability from year to year may experience flooding some years, but also experience dry conditions during other years, even during the spring flood season. The taxa able to persist between extreme fluctuations on a longer timescale than a single season may simply be less active than taxa that are adapted to rapid fluctuations in flashy flood wetlands or adapted to extended flood conditions. The community in the transition region described in Chapter 4 did not exhibit the particularly high potential activity rates either, which is consistent with this hypothesis. This may indicate there are tradeoffs, where individual denitrifiers might be either "high functioning" or physiologically tolerant to conditions that fluctuate between extreme states (desiccation to saturation).

Final remarks

My work suggests that that increased drought followed by intense floods will influence denitrification function in wetlands. Climate change will likely exacerbate the existing struggle to restore wetlands of equivalent ecosystem services to those that were once delivered by wetlands. I did not observe a clear relationship between denitrifier community composition and functional stability, and this might be due to the high variability among wetland soil communities. Wetland communities varied in their ability to remain functional in the face of either prolonged drought or stress from fluctuating hydrology, but no single community or individual taxa stood out as the being the key to functional stability.

There are at least two possible explanations for the lack of a consistent relationship between the denitrifier community and denitrifier function. One possibility is that there was a mismatch between the scale at which the community was studied and the scale at which rates were quantified. Microbial organisms located even millimeters from one another may never interact with each other. When composition and function are examined at such a tiny scale, patterns do emerge (Bailey et al. 2013), but the information gained from such studies may not be practical for restoration ecology. Another possibility is that the DNA-based methods used in this dissertation captured a large proportion of dead and dormant individuals that would not contribute to the observed denitrifier rates (Jones and Lennon 2010). This also might explain why individual microbial OTUs can be correlated to denitrifier function, but the overall community rarely shows a straightforward correlation.

Future work should also investigate the presence of physiological traits that would allow some microbial communities to remain functional. For example, the presence or abundance of microbial taxa that produce extracellular polymeric substances (EPS) could extend protection from desiccation to the immediately surrounding community, regardless of whether the EPS producers are denitrifiers or non-denitrifiers. This type of research would not be easily conducted using molecular techniques. Like denitrification, the ability to produce EPS is found in phylogenetically diverse microbial taxa (Roberson and Firestone 1992, Dimopoulou et al. 2014, Pereira et al. 2015). However, the genetics that underlie EPS production are far more complex than those for denitrification, including multiple clusters of different polymorphic genes involved in a variety of biosynthetic pathways (Reeves et al. 1996, Schmid et al. 2015). There is no single diagnostic gene or set of diagnostic genes conserved across all of these taxa. Instead of using PCR assays to target an EPS "functional gene", this would likely require the use of chemical

assays to quantify EPS production across different soil and sediment communities, and correlate EPS production to functional stability. Experimental approaches could then be used to determine if EPS production by a non-denitrifier contributes to functional stability of the denitrifier community. These experiments might involve inoculation of soil communities with two different variants of the same bacterial strain, where one produces EPS and the other cannot (Tamaru et al. 2005).

The most surprising result was that some restored wetlands are able to reach equivalent denitrification rates as those that they were intended to replicate. This is better news for restoration ecologists than was expected. However, outcomes of restoration for the microbial community are still highly variable and unpredictable. Further manipulative experiments may be informative in order to identify why some restoration efforts lead to high functioning and functionally stable communities.

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APPENDIX A:

SUPPLEMENTAL MATERIAL FOR CHAPTER 2

Supplementary tables

Table A.1. Quantitative PCR assay performance values calculated for the standard curves. Standard curves included 12 technical replicates on each plate. Samples were analyzed across four separate reaction plates, so performance values were calculated separately for each reaction plate. Linear dynamic range and limit of detection were the same for each plate. Clade II *nosZ* was quantified using a single standard curve (with 12 technical replicates). Calculations are described in the MIQE guidelines (Bustin et al. 2009).

qPCR Assay	Efficiency	Linear Dynamic Range	Limit of Detection
nirK	0.81 ± 0.02	5.67×10^3 # copies to 5.67×10^9 # copies	5.67×10^3 # copies
nirS	0.94 ± 0.01	1.06×10^3 # copies to 1.06×10^9 # copies	1.06×10^3 # copies
Clade I *nosZ*	1.00 ± 0.03	1.40×10^3 # copies to 1.08×10^7 # copies	1.40×10^3 # copies
Clade II *nosZ*	0.63	7.85×10^2 # copies to 7.85×10^7 # copies	7.85×10^2 # copies

Table A.2. Wetland age, location, and characteristics.

Site	Type	Age in 2012	Latitude	Longitude	Hydrology	Excavated?
A	Restored	17	38.57	-90.13	Depressional	Yes
RefA	Reference	--	38.57	-90.13	Depressional	No
B	Restored	19	40.41	-90.95	Floodplain	No
RefB	Reference	--	40.41	-90.95	Floodplain	No
C	Restored	14	40.07	-90.30	Floodplain	No
RefC	Reference	--	40.07	-90.30	Floodplain	No
D	Restored	14	38.52	-89.63	Floodplain	No
RefD	Reference	--	38.52	-89.63	Floodplain	No
E	Restored	13	39.84	-89.65	Floodplain	Yes
RefE	Reference	--	39.84	-89.65	Floodplain	Yes
F	Restored	13	42.42	-90.40	Floodplain	No
RefF	Reference	--	42.42	-90.40	Floodplain	No
G	Restored	13	41.55	-90.18	Floodplain	No
RefG	Reference	--	41.55	-90.18	Floodplain	No
H	Restored	13	37.74	-88.69	Depressional	Yes
RefH	Reference	--	37.74	-88.69	Depressional	No
I	Restored	13	39.73	-91.35	Floodplain	No
RefI	Reference	--	39.73	-91.35	Floodplain	No
J	Restored	13	41.25	-90.59	Floodplain	No
RefJ	Reference	--	41.25	-90.59	Floodplain	No
K	Restored	12	42.46	-89.65	Depressional	Yes
RefK	Reference	--	42.46	-89.65	Depressional	No
L	Restored	11	39.88	-89.67	Floodplain	No
RefL	Reference	--	39.88	-89.67	Floodplain	No
M	Restored	10	38.01	-89.37	Floodplain	No
RefM	Reference	--	38.01	-89.37	Floodplain	No
N	Restored	10	37.79	-89.23	Floodplain	No
RefN	Reference	--	37.79	-89.23	Floodplain	No
O	Restored	7	42.33	-89.39	Floodplain	Yes
RefO	Reference	--	42.33	-89.39	Floodplain	No
P	Restored	19	42.01	-88.20	Depressional	Yes
Q	Restored	16	41.84	-89.70	Depressional	Yes
R	Restored	16	41.89	-89.41	Floodplain	Yes
S	Restored	16	41.89	-89.41	Floodplain	No
T	Restored	15	42.17	-87.97	Depressional	Yes
U	Restored	15	41.57	-87.89	Depressional	Yes
V	Restored	15	39.95	-88.27	Depressional	No
W	Restored	14	38.44	-89.84	Depressional	No
X	Restored	14	37.29	-89.51	Floodplain	No
Y	Restored	13	40.44	-89.65	Floodplain	No
Z	Restored	13	40.82	-91.07	Floodplain	Yes
AA	Restored	12	42.46	-89.65	Floodplain	No
AB	Restored	10	37.70	-89.22	Floodplain	Yes
AC	Restored	10	39.76	-88.98	Depressional	Yes
AD	Restored	8	37.26	-89.26	Floodplain	No

Table. A.3. Vegetation variables included in regression

Site	%Perennial	%Native Perennial	%Native	FQI	*Phalaris arundinacea* (% cover)	Plant Species Richness
A	67.24	45.61	31.58	14.20	4.75	13
RefA	87.50	81.25	75	11.88	0	5
B	87.50	81.25	75	11.88	0	4
RefB	84.21	73.21	67.86	19.34	0	15
C	84.21	73.21	67.86	19.34	0	10
RefC	66.15	63.08	63.08	18.46	0	4
D	77.31	62.71	56.78	27.72	20.50	20
RefD	89.29	81.82	78.18	23.10	0	21
E	70.42	57.14	52.86	17.99	0	15
RefE	85.71	74.29	60	15.04	0	13
F	84.48	70.18	61.40	17.40	52.38	7
RefF	79.31	63.79	60.34	18.24	47.50	17
G	78.41	65.52	59.77	25.25	0	23
RefG	75.41	67.21	60	19.97	0	7
H	73.68	63.16	52.63	17.25	4.75	10
RefH	91.07	80.36	71.43	21.78	0	9
I	84.54	68.75	56.25	24.83	10.63	22
RefI	81.67	76.67	70	21.57	2.63	15
J	66.15	63.08	63.08	18.46	0	1
RefJ	83.33	76.67	66.67	13.28	20.13	11
K	89.29	81.82	78.18	23.10	8.5	15
RefK	90	83.33	70	13.34	81.13	9
L	90.12	70	50.63	19.62	0	28
RefL	67.39	60	57.78	14.76	8.50	13
M	64.06	57.14	55.56	15.95	--	--
RefM	85.42	78.72	72.34	19.90	0	15
N	60.24	53.01	50.60	17.60	--	--
RefN	84.38	81.25	72.73	19.80	0	11
O	85.71	74.29	60	15.04	0	18
RefO	63.16	55.26	44.74	10.96	66.56	4
P	79.31	63.79	60.34	18.24	17.00	7
Q	75.41	67.21	60	19.97	70.00	3
R	91.07	80.36	71.43	21.78	0	10
S	81.67	76.67	70	21.57	1.88	19
T	81.48	71.25	71.25	25.83	0.38	18
U	83.33	76.67	66.67	13.28	20.13	4
V	90	83.33	70	13.34	12.50	17
W	77.27	59.09	53.03	17.00	56.88	9
X	67.39	60	57.78	14.76	0	17
Y	85.42	78.72	72.34	19.90	10.63	9
Z	85.86	71.13	65.63	26.04	--	--
AA	62.5	52.78	43.66	17.00	--	--
AB	84.38	81.25	72.73	19.80	0	23
AC	82.76	67.06	51.19	23.17	0	23
AD	81.63	73.47	63.27	17.44	57.81	2

Table A.4 Wetland potential denitrification rates and soil chemistry.

Site	Pot. denitrification (ng N_2O g^{-1} hr^{-1})		Water content (w/w %)		Soil pH (1:1 slurry)		Total C (w/w %)		Total N (w/w %)		Ammonium (mg NH_4^+ / g soil)		Nitrate (mg NO_3^- / g soil)		Phosphate (mg PO_4^- / g soil)	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
A	52.00 (9.55)	24.55 (5.46)	0.35 (0.01)	0.30 (0.01)	6.39 (0.13)	6.65 (0.13)	2.17 (0.42)	1.12 (0.35)	0.18 (0.03)	0.10 (0.03)	5.45 (1.75)	7.83 (2.40)	0.19 (0.10)	0.06 (0.02)	2.04 (0.35)	0.60 (0.10)
RefA	4.00 (1.45)	44.32 (10.60)	0.43 (0.02)	0.38 (0.01)	6.05 (0.10)	6.11 (0.07)	5.02 (0.60)	2.83 (0.21)	0.39 (0.05)	0.21 (0.02)	5.62 (0.52)	9.52 (1.84)	0.01 (0.004)	1.00 (0.18)	2.52 (0.12)	0.83 (0.22)
B	70.44 (6.71)	886.49 (76.44)	0.16 (0.01)	0.23 (0.02)	7.59 (0.03)	7.63 (0.02)	1.67 (0.13)	1.50 (0.20)	0.12 (0.01)	0.10 (0.01)	5.13 (0.43)	8.50 (1.50)	5.57 (2.20)	4.83 (1.22)	2.33 (0.16)	1.01 (0.10)
RefB	101.16 (17.13)	1255.87 (66.89)	0.20 (0.004)	0.23 (0.002)	7.31 (0.06)	7.42 (0.07)	2.02 (0.13)	1.94 (0.19)	0.17 (0.01)	0.13 (0.01)	6.81 (1.39)	5.69 (0.90)	7.71 (1.47)	8.19 (1.23)	3.45 (0.64)	1.19 (0.09)
C	26.61 (4.72)	445.98 (33.53)	0.14 (0.005)	0.26 (0.004)	7.86 (0.03)	7.95 (0.01)	1.71 (0.16)	1.84 (0.07)	0.11 (0.01)	0.10 (0.01)	3.84 (0.28)	7.47 (0.73)	1.49 (0.38)	0.66 (0.26)	2.46 (0.25)	1.02 (0.07)
RefC	94.91 (5.94)	702.76 (101.64)	0.16 (0.004)	0.30 (0.01)	7.69 (0.03)	7.80 (0.06)	2.63 (0.20)	2.40 (0.22)	0.18 (0.01)	0.15 (0.02)	6.69 (0.57)	10.65 (1.53)	4.19 (1.34)	2.31 (1.44)	1.85 (0.09)	1.06 (0.05)
D	20.29 (0.76)	60.75 (7.11)	0.15 (0.003)	0.29 (0.01)	5.88 (0.07)	6.15 (0.07)	1.95 (0.12)	1.78 (0.25)	0.17 (0.01)	0.15 (0.02)	25.62 (19.62)	8.37 (1.68)	3.26 (1.39)	0.61 (0.27)	1.55 (0.31)	0.36 (0.09)
RefD	26.24 (2.14)	87.44 (17.47)	0.17 (0.01)	0.32 (0.01)	5.86 (0.05)	5.86 (0.05)	2.70 (0.12)	2.99 (0.36)	0.22 (0.01)	0.23 (0.02)	9.99 (1.25)	11.39 (2.84)	4.36 (0.86)	0.43 (0.08)	1.62 (0.05)	0.21 (0.10)
E	57.41 (14.52)	798.28 (224.83)	0.10 (0.002)	0.23 (0.01)	8.10 (0.05)	8.08 (0.04)	2.78 (0.13)	2.66 (0.11)	0.13 (0.02)	0.10 (0.01)	2.70 (0.53)	4.52 (1.17)	0.22 (0.07)	0.79 (0.33)	1.70 (0.33)	0.74 (0.12)
RefE	137.99 (11.66)	1400.34 (334.29)	0.16 (0.001)	0.23 (0.01)	7.41 (0.04)	7.23 (0.04)	4.14 (0.16)	4.10 (0.16)	0.28 (0.01)	0.28 (0.01)	5.45 (0.58)	2.71 (0.97)	4.52 (0.93)	18.37 (1.84)	3.27 (0.41)	1.47 (0.13)
F	158.94 (32.92)	1098.04 (172.18)	0.32 (0.02)	0.38 (0.01)	7.49 (0.03)	7.67 (0.03)	3.13 (0.18)	2.75 (0.11)	0.23 (0.01)	0.20 (0.01)	6.02 (1.97)	11.23 (1.48)	20.57 (8.47)	0.70 (0.21)	2.11 (0.11)	0.94 (0.22)
RefF	89.05 (9.81)	1130.26 (85.18)	0.15 (0.01)	0.33 (0.01)	7.57 (0.03)	7.45 (0.02)	3.55 (0.17)	3.45 (0.16)	0.28 (0.02)	0.28 (0.02)	6.77 (0.80)	7.28 (2.24)	8.21 (2.56)	5.19 (2.04)	2.02 (0.19)	0.77 (0.24)
G	108.42 (5.35)	1683.23 (67.66)	0.22 (0.01)	0.26 (0.01)	7.89 (0.04)	7.72 (0.02)	4.53 (0.10)	4.74 (0.08)	0.30 (0.01)	0.31 (0.01)	7.82 (1.10)	9.48 (1.76)	16.82 (3.76)	36.24 (5.79)	11.53 (1.55)	3.76 (0.29)
RefG	108.44 (7.90)	1652.19 (141.61)	0.22 (0.004)	0.30 (0.01)	8.06 (0.01)	7.72 (0.03)	4.91 (0.16)	5.25 (0.40)	0.33 (0.01)	0.34 (0.02)	7.52 (0.63)	9.45 (1.87)	19.89 (3.48)	26.06 (3.84)	7.77 (0.43)	3.06 (0.42)
H	7.85 (3.30)	18.21 (4.50)	0.16 (0.01)	0.28 (0.01)	5.28 (0.09)	5.37 (0.04)	1.75 (0.19)	1.37 (0.14)	0.15 (0.01)	0.10 (0.01)	7.60 (1.09)	5.07 (0.95)	0.97 (0.55)	0.12 (0.05)	1.60 (0.05)	0.22 (0.08)
RefH	6.72 (1.05)	16.47 (4.50)	0.18 (0.003)	0.29 (0.01)	5.35 (0.05)	5.27 (0.08)	2.07 (0.19)	1.63 (0.19)	0.17 (0.01)	0.12 (0.01)	5.46 (0.41)	6.16 (1.38)	1.37 (0.50)	0.41 (0.14)	1.56 (0.04)	0.24 (0.07)
I	34.95 (3.62)	413.10 (56.71)	0.12 (0.01)	0.27 (0.01)	5.84 (0.29)	6.80 (0.16)	2.20 (0.15)	2.01 (0.50)	0.18 (0.01)	0.14 (0.03)	3.73 (0.68)	5.08 (1.17)	3.60 (0.77)	1.46 (0.42)	2.09 (0.18)	1.06 (0.16)
RefI	22.77 (4.82)	374.73 (45.66)	0.18 (0.01)	0.28 (0.01)	5.61 (0.16)	6.63 (0.12)	2.49 (0.33)	1.21 (0.16)	0.18 (0.02)	0.10 (0.01)	5.30 (0.56)	5.62 (0.44)	0.87 (0.49)	1.40 (0.32)	1.83 (0.11)	1.12 (0.17)
J	117.04 (7.80)	1597.29 (28.31)	0.24 (0.002)	0.33 (0.004)	7.30 (0.11)	7.33 (0.10)	3.97 (0.25)	3.21 (0.13)	0.30 (0.01)	0.25 (0.01)	6.97 (0.54)	13.70 (3.10)	4.77 (0.96)	0.75 (0.66)	2.34 (0.13)	1.01 (0.16)
RefJ	129.23 (15.53)	1172.05 (70.98)	0.27 (0.02)	0.24 (0.003)	7.15 (0.13)	7.10 (0.08)	3.34 (0.18)	2.85 (0.07)	0.26 (0.01)	0.24 (0.01)	8.52 (2.68)	5.12 (0.75)	11.82 (5.42)	10.39 (0.91)	4.87 (2.08)	0.85 (0.13)
K	91.03 (22.75)	689.85 (102.84)	0.30 (0.02)	0.32 (0.01)	7.38 (0.15)	7.47 (0.06)	2.82 (0.21)	1.73 (0.29)	0.23 (0.02)	0.13 (0.02)	3.96 (0.44)	6.92 (2.29)	34.86 (12.04)	1.11 (0.88)	2.96 (0.40)	1.10 (0.18)
RefK	182.46 (12.85)	1786.54 (59.12)	0.17 (0.003)	0.26 (0.01)	7.71 (0.10)	7.39 (0.05)	3.67 (0.23)	3.16 (0.20)	0.29 (0.02)	0.27 (0.02)	6.88 (0.32)	1.78 (0.50)	2.06 (0.53)	13.36 (2.24)	5.88 (0.34)	1.58 (0.08)

Table A.4 (continued)

Site	Pot. denitrification (ng N_2O g^{-1} hr^{-1})		Water content (w/w %)		Soil pH (1:1 slurry)		Total C (w/w %)		Total N (w/w %)		Ammonium (mg NH_4^+ / g soil)		Nitrate (mg NO_3^- / g soil)		Phosphate (mg PO_4^- / g soil)	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
L	37.95 (5.25)	87.98 (17.21)	0.12 (0.01)	0.26 (0.004)	7.30 (0.19)	7.13 (0.10)	1.92 (0.17)	1.77 (0.17)	0.15 (0.01)	0.14 (0.01)	4.02 (0.31)	5.89 (1.21)	2.88 (1.53)	6.55 (1.90)	1.52 (0.04)	1.08 (0.10)
RefL	86.73 (4.19)	151.89 (11.02)	0.21 (0.02)	0.34 (0.02)	7.02 (0.18)	6.99 (0.11)	3.82 (0.25)	3.72 (0.21)	0.29 (0.01)	0.29 (0.01)	5.02 (0.48)	13.71 (4.3)	14.87 (4.17)	6.00 (2.52)	2.34 (0.12)	0.87 (0.15)
M	34.54 (13.29)	43.32 (2.52)	0.16 (0.004)	0.26 (0.004)	5.79 (0.32)	6.28 (0.18)	2.08 (0.13)	1.99 (0.17)	0.18 (0.01)	0.18 (0.01)	15.66 (5.89)	6.66 (1)	11.59 (4.47)	0.34 (0.11)	1.72 (0.04)	0.26 (0.09)
RefM	12.02 (1.88)	37.25 (10.42)	0.16 (0.003)	0.28 (0.01)	4.39 (0.14)	5.56 (0.05)	1.94 (0.12)	2.26 (0.22)	0.17 (0.01)	0.21 (0.02)	7.61 (1.35)	0.96 (0.10)	1.38 (0.64)	2.18 (0.46)	1.86 (0.08)	0.66 (0.17)
N	9.85 (1.35)	10.45 (4.34)	0.10 (0.01)	0.22 (0.01)	4.77 (0.15)	5.62 (0.11)	1.46 (0.14)	0.83 (0.09)	0.11 (0.01)	0.08 (0.01)	3.77 (0.27)	4.59 (1.85)	1.32 (0.65)	0.02 (0.01)	1.41 (0.07)	0.27 (0.10)
RefN	21.32 (5.65)	39.40 (13.35)	0.19 (0.01)	0.29 (0.01)	4.74 (0.06)	5.48 (0.05)	2.85 (0.35)	2.35 (0.17)	0.22 (0.02)	0.22 (0.01)	5.65 (0.87)	1.46 (0.18)	3.41 (1.35)	3.47 (0.48)	1.59 (0.20)	0.23 (0.07)
O	108.38 (7.17)	1062.47 (85.69)	0.21 (0.004)	0.26 (0.01)	7.15 (0.20)	7.16 (0.11)	2.85 (0.30)	2.23 (0.13)	0.22 (0.01)	0.20 (0.01)	4.93 (0.59)	10.34 (5.51)	10 (2.38)	3.52 (1.77)	2.66 (0.29)	1.03 (0.10)
RefO	135.33 (14.68)	1493.62 (210.71)	0.25 (0.01)	0.34 (0.01)	7.27 (0.19)	7.04 (0.04)	5.02 (0.57)	3.92 (0.34)	0.40 (0.04)	0.35 (0.03)	6.22 (0.89)	7.46 (2.55)	12.61 (4.75)	11.28 (4.83)	3.92 (1.00)	0.87 (0.14)
P	68.75 (22.91)	612.85 (90.69)	0.36 (0.02)	0.39 (0.03)	7.12 (0.18)	7.42 (0.15)	7.66 (1.08)	6.68 (1.24)	0.42 (0.07)	0.36 (0.07)	10.45 (2.12)	17.54 (5.83)	1.45 (0.85)	1.11 (0.94)	1.81 (0.07)	0.92 (0.23)
Q	213.32 (51.94)	999.41 (118.19)	0.35 (0.05)	0.29 (0.02)	6.93 (0.09)	7.19 (0.09)	4.32 (0.27)	2.82 (0.31)	0.35 (0.02)	0.22 (0.03)	11.87 (1.96)	6.61 (1.81)	1.69 (0.71)	3.15 (1.90)	3.00 (0.58)	1.41 (0.40)
R	105.12 (18.46)	1117.67 (103.99)	0.29 (0.03)	0.38 (0.01)	7.64 (0.04)	7.89 (0.01)	4.50 (0.18)	4.36 (0.26)	0.29 (0.02)	0.25 (0.01)	5.83 (0.38)	28.21 (4.55)	20.19 (3.75)	0.11 (0.06)	5.00 (0.37)	1.71 (0.09)
S	89.44 (7.66)	1473.62 (267.56)	0.15 (0.01)	0.33 (0.03)	7.75 (0.03)	7.73 (0.02)	5.13 (0.29)	5.18 (0.50)	0.34 (0.03)	0.31 (0.04)	7.34 (0.81)	12.21 (1.94)	20.52 (3.05)	12.01 (2.24)	6.49 (0.67)	3.47 (0.26)
T	54.97 (16.23)	762.56 (190.99)	0.22 (0.03)	0.32 (0.02)	7.40 (0.07)	7.29 (0.12)	4.63 (0.21)	4.33 (0.33)	0.34 (0.03)	0.33 (0.03)	7.81 (1.65)	5.40 (1.51)	3.60 (0.81)	7.51 (3.82)	2.04 (0.62)	1.08 (0.10)
U	139.71 (49.42)	1245.04 (168.41)	0.39 (0.03)	0.36 (0.03)	7.43 (0.05)	7.48 (0.11)	4.04 (0.18)	3.77 (0.17)	0.27 (0.01)	0.22 (0.02)	25.03 (6.45)	13.39 (3.97)	0.51 (0.18)	1.11 (0.84)	1.71 (0.11)	0.83 (0.14)
V	44.16 (5.82)	263.85 (38.03)	0.18 (0.01)	0.25 (0.01)	6.64 (0.16)	6.96 (0.12)	5.21 (0.36)	5.22 (0.89)	0.30 (0.02)	0.29 (0.04)	9.87 (0.98)	9.06 (1.76)	2.46 (1.52)	2.54 (0.53)	1.46 (0.06)	0.66 (0.20)
W	51.49 (18.81)	58.88 (17.01)	0.10 (0.002)	0.24 (0.01)	6.69 (0.29)	6.78 (0.23)	1.67 (0.24)	1.94 (0.19)	0.13 (0.01)	0.16 (0.01)	5.05 (0.38)	5.23 (1.06)	0.58 (0.22)	0.65 (0.03)	1.92 (0.42)	0.90 (0.13)
X	30.24 (5.96)	697.25 (64.97)	0.11 (0.005)	0.24 (0.004)	7.98 (0.04)	7.74 (0.05)	1.39 (0.11)	1.65 (0.18)	0.08 (0.004)	0.12 (0.01)	2.62 (0.3)	10.27 (1.23)	2.16 (0.67)	4.97 (0.63)	1.78 (0.09)	1.11 (0.07)
Y	79.98 (16.55)	432.80 (129.31)	0.17 (0.02)	0.24 (0.01)	7.98 (0.02)	7.99 (0.04)	2.60 (0.20)	2.60 (0.31)	0.10 (0.02)	0.12 (0.03)	9.15 (4.81)	1.24 (0.34)	4.95 (1.05)	1.61 (0.28)	2.92 (0.30)	0.86 (0.05)
Z	14.16 (3.74)	106.37 (26.06)	0.18 (0.005)	0.25 (0.01)	5.70 (0.13)	6.31 (0.05)	1.64 (0.15)	0.99 (0.15)	0.12 (0.01)	0.07 (0.01)	1.85 (0.34)	1.83 (0.41)	0.06 (0.06)	1.82 (0.27)	1.82 (0.08)	1.13 (0.18)
AA	146.77 (27.22)	4983.78 (825.38)	0.15 (0.01)	0.33 (0.01)	8.05 (0.06)	7.76 (0.01)	3.58 (0.08)	3.67 (0.11)	0.27 (0.01)	0.25 (0.02)	7.07 (0.40)	16.71 (3.06)	5.29 (1.53)	4.34 (1.98)	9.20 (0.99)	2.17 (0.29)
AB	43.59 (4.81)	235.07 (34.37)	0.14 (0.01)	0.24 (0.01)	7.93 (0.09)	7.59 (0.07)	1.50 (0.13)	1.54 (0.16)	0.11 (0.01)	0.11 (0.01)	4.99 (0.48)	1.05 (0.15)	1.11 (0.62)	1.30 (0.3)	7.53 (6.00)	0.29 (0.06)
AC	49.43 (10.38)	240.88 (6.22)	0.16 (0.01)	0.26 (0.01)	8.09 (0.14)	7.78 (0.08)	3.38 (0.24)	2.54 (0.28)	0.20 (0.02)	0.17 (0.03)	3.25 (0.25)	3.36 (0.65)	5.97 (2.96)	0.46 (0.18)	1.94 (0.19)	0.78 (0.16)
AD	30.58 (2.58)	143.38 (42.3)	0.13 (0.01)	0.24 (0.02)	6.11 (0.54)	6.20 (0.35)	1.98 (0.14)	1.63 (0.24)	0.15 (0.01)	0.13 (0.01)	5.12 (0.63)	4.32 (1.58)	5.79 (2.56)	0.18 (0.16)	4.50 (2.97)	0.50 (0.10)

Table A.5. Abundance of microbial denitrifier functional groups in each wetland.

Site	10^3 *nirK* gene copies / ng DNA		10^3 *nirS* gene copies / ng DNA		10^3 Clade I *nosZ* gene copies / ng DNA		10^3 Clade II *nosZ* gene copies / ng DNA		log(*nirK*:*nirS*)		log(Clade I *nosZ*: Clade II *nosZ*)		log(total *nir*: total *nos*)	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
A	59.97 (6.29)	80.74 (8.95)	90.83 (9.70)	77.74 (19.13)	0.69 (0.09)	0.46 (0.08)	1.55 (0.53)	12.61 (3.61)	-0.41 (0.12)	0.10 (0.27)	-0.64 (0.24)	-3.17 (0.53)	4.31 (0.27)	2.63 (0.46)
RefA	37.44 (3.78)	35.24 (10.04)	37.13 (1.81)	32.04 (6.84)	0.51 (0.07)	0.59 (0.13)	4.36 (3.26)	1.80 (0.38)	-0.003 (0.15)	0.02 (0.22)	-1.40 (0.60)	-1.13 (0.26)	3.32 (0.58)	3.28 (0.23)
B	160.48 (24.94)	87.33 (4.50)	327.61 (39.63)	145.07 (16.78)	1.04 (0.04)	0.76 (0.05)	15.30 (11.82)	1.06 (0.14)	-0.73 (0.21)	-0.49 (0.16)	-1.87 (0.67)	-0.31 (0.08)	4.07 (0.61)	4.86 (0.15)
RefB	115.73 (18.39)	216.98 (43.14)	119.58 (33.27)	150.06 (37.21)	0.48 (0.06)	1.19 (0.26)	6.07 (1.80)	2.47 (0.74)	0.01 (0.23)	0.40 (0.11)	-2.28 (0.50)	-0.57 (0.22)	3.74 (0.32)	4.71 (0.17)
C	99.92 (32)	67.16 (13.93)	219.83 (31.83)	167.99 (28.10)	0.91 (0.21)	1.00 (0.19)	2.02 (1.57)	1.92 (0.77)	-0.88 (0.16)	-0.93 (0.14)	0.00 (0.51)	-0.31 (0.52)	5.09 (0.33)	4.51 (0.19)
RefC	76.30 (7.50)	76.92 (16.17)	200.81 (44.94)	131.05 (25.42)	0.71 (0.07)	0.82 (0.07)	2.21 (0.91)	1.81 (0.64)	-0.88 (0.31)	-0.54 (0.04)	-0.87 (0.46)	-0.63 (0.30)	4.65 (0.45)	4.41 (0.28)
D	120.50 (26.09)	107.83 (10.80)	81.57 (7.69)	85.96 (3.75)	1.08 (0.12)	0.81 (0.11)	3.36 (1.52)	3.13 (1.16)	0.34 (0.16)	0.21 (0.10)	-0.73 (0.49)	-1.20 (0.21)	4.02 (0.30)	4.02 (0.25)
RefD	190.85 (40.34)	100.36 (19.81)	99.96 (11.31)	54.40 (13.51)	0.96 (0.16)	0.67 (0.11)	6.96 (2.82)	2.59 (0.88)	0.58 (0.15)	0.65 (0.16)	-1.51 (0.60)	-1.19 (0.29)	3.90 (0.36)	3.94 (0.16)
E	82.94 (9.02)	77.64 (9.24)	146.13 (15.73)	178.35 (29.60)	1.00 (0.10)	1.34 (0.13)	0.89 (0.06)	1.55 (0.14)	-0.57 (0.07)	-0.82 (0.07)	0.11 (0.07)	-0.14 (0.14)	4.80 (0.06)	4.46 (0.12)
RefE	227.68 (26.54)	156.33 (18.56)	82.21 (3.85)	107.46 (7.41)	0.58 (0.03)	0.64 (0.12)	9.91 (2.62)	1.63 (0.42)	1.00 (0.13)	0.36 (0.07)	-2.74 (0.23)	-0.89 (0.31)	3.47 (0.17)	4.79 (0.21)
F	65.34 (7.71)	111.25 (26.98)	296.94 (81.97)	366.43 (68.07)	0.45 (0.04)	1.22 (0.31)	2.72 (0.53)	7.46 (2.27)	-1.42 (0.16)	-1.22 (0.14)	-1.76 (0.15)	-1.74 (0.19)	4.70 (0.13)	4.09 (0.14)
RefF	93.53 (28.89)	81.46 (16.36)	172.11 (56.67)	145.47 (31.42)	0.66 (0.10)	0.81 (0.07)	3.12 (0.99)	2.82 (0.44)	-0.53 (0.12)	-0.56 (0.15)	-1.48 (0.26)	-1.21 (0.16)	4.13 (0.31)	4.10 (0.13)
G	128.11 (40.55)	125.34 (22.14)	270.83 (72.20)	216.54 (20.66)	1.01 (0.17)	0.83 (0.10)	9.65 (4.83)	2.60 (0.40)	-0.72 (0.17)	-0.57 (0.09)	-1.82 (0.50)	-1.12 (0.08)	3.84 (0.35)	4.62 (0.18)
RefG	190 (36.58)	180.22 (15.86)	385.98 (72.98)	326.84 (49.46)	1.81 (0.24)	0.87 (0.11)	12.27 (3.22)	3.54 (1.01)	-0.70 (0.09)	-0.57 (0.13)	-1.81 (0.45)	-1.28 (0.21)	3.72 (0.44)	4.84 (0.21)
H	79.19 (18.45)	38.33 (26.45)	20.14 (10.98)	16.10 (5.16)	0.27 (0.07)	0.39 (0.06)	3.07 (0.72)	1.73 (0.44)	1.78 (0.35)	0.39 (0.34)	-2.44 (0.05)	-1.40 (0.27)	3.34 (0.29)	2.84 (0.74)
RefH	62.46 (7.35)	24.66 (7.92)	27.46 (7.59)	16.92 (6.53)	0.51 (0.05)	0.33 (0.06)	5.82 (1.56)	0.80 (0.20)	0.92 (0.18)	0.42 (0.11)	-2.34 (0.20)	-0.84 (0.10)	2.70 (0.10)	3.54 (0.13)
I	91.01 (30.81)	47.99 (19.56)	34.77 (8.27)	32.78 (8.48)	0.83 (0.14)	0.60 (0.13)	5.20 (1.47)	2.91 (0.86)	0.82 (0.25)	0.17 (0.41)	-1.72 (0.31)	-1.58 (0.28)	2.99 (0.56)	3.04 (0.21)
RefI	139.13 (22.99)	48.55 (9.85)	59.97 (12.25)	35.13 (5.00)	0.96 (0.22)	0.32 (0.04)	8.84 (2.14)	2.48 (0.29)	0.88 (0.17)	0.30 (0.11)	-2.18 (0.14)	-2.05 (0.22)	3.09 (0.13)	3.37 (0.24)
J	130.98 (40.10)	39.17 (11.61)	94.42 (33.82)	51.79 (10.41)	0.94 (0.11)	0.37 (0.09)	6.12 (1.89)	1.00 (0.43)	0.48 (0.16)	-0.46 (0.26)	-1.64 (0.36)	-0.69 (0.48)	3.35 (0.74)	4.33 (0.19)
RefJ	138.94 (25.43)	49.34 (12.86)	87.78 (22.52)	48.77 (16.12)	0.91 (0.05)	0.34 (0.09)	5.87 (1.26)	0.35 (0.09)	0.52 (0.17)	0.11 (0.22)	-1.79 (0.26)	-0.005 (0.28)	3.49 (0.09)	4.90 (0.20)
K	69.23 (33.21)	64.77 (23.82)	124.22 (26.74)	94.45 (30.08)	0.69 (0.19)	0.77 (0.07)	3.32 (2.01)	2.60 (0.78)	-0.79 (0.40)	-0.38 (0.22)	-1.14 (0.49)	-1.02 (0.47)	4.17 (0.45)	3.79 (0.28)
RefK	189.87 (10.26)	33.31 (9.98)	128.97 (15.15)	57.51 (13.70)	1.08 (0.27)	0.30 (0.04)	15.96 (9.31)	2.06 (0.26)	0.40 (0.17)	-0.60 (0.24)	-2.13 (0.79)	-1.92 (0.22)	3.43 (0.64)	3.57 (0.31)

Table A.5 (continued)

Site	10^3 *nirK* gene copies / ng DNA		10^3 *nirS* gene copies / ng DNA		10^3 Clade I *nosZ* gene copies / ng DNA		10^3 Clade II *nosZ* gene copies / ng DNA		log(*nirK*:*nirS*)		log(Clade I *nosZ*: Clade II *nosZ*)		log(total *nir*: total *nos*)	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
L	77.81 (8.34)	38.91 (12.13)	45.20 (7.56)	122.04 (22.92)	0.80 (0.12)	1.03 (0.28)	1.63 (0.76)	1.02 (0.33)	0.57 (0.20)	-1.20 (0.33)	-0.31 (0.72)	0.29 (0.46)	4.03 (0.26)	4.47 (0.17)
RefL	194.51 (52.08)	23.16 (8.79)	205.48 (54.10)	101.42 (48.96)	1.47 (0.35)	0.57 (0.14)	26.44 (9.11)	1.34 (0.62)	-0.03 (0.23)	-1.11 (0.31)	-2.69 (0.37)	-0.05 (0.77)	2.78 (0.26)	3.87 (0.31)
M	143.73 (30.89)	50.85 (14.60)	89.99 (28.77)	42.22 (14.03)	1.10 (0.14)	0.79 (0.16)	6.04 (2.35)	1.86 (0.74)	0.57 (0.13)	0.23 (0.11)	-1.26 (0.63)	-0.65 (0.40)	3.65 (0.22)	3.54 (0.35)
RefM	87.20 (7.42)	53.06 (12.32)	26.57 (6.94)	50.89 (10.21)	0.68 (0.09)	0.62 (0.09)	1.82 (0.53)	1.72 (0.29)	1.29 (0.26)	0.02 (0.26)	-0.88 (0.36)	-1.01 (0.15)	3.86 (0.17)	3.77 (0.09)
N	200.81 (17.82)	28.33 (5.61)	101.49 (16.91)	40.49 (13.59)	2.11 (0.17)	0.69 (0.02)	7.66 (1.48)	1.39 (0.52)	0.72 (0.26)	-0.28 (0.18)	-1.24 (0.17)	-0.29 (0.65)	3.47 (0.16)	3.52 (0.23)
RefN	154.27 (19.67)	56.64 (22.03)	48.98 (2.10)	58.23 (20.28)	0.49 (0.10)	0.29 (0.07)	8.00 (0.51)	2.65 (1.30)	1.13 (0.10)	-0.11 (0.11)	-2.85 (0.15)	-1.68 (0.49)	3.17 (0.14)	3.42 (0.65)
O	309.15 (20.60)	55.85 (16.43)	183.92 (12.80)	75.21 (24.39)	1.38 (0.16)	0.80 (0.15)	13.25 (1.53)	2.70 (1.31)	0.52 (0.09)	-0.25 (0.08)	-2.26 (0.21)	-0.51 (0.74)	3.53 (0.14)	3.82 (0.36)
RefO	310.97 (109.32)	73.37 (3.73)	216.82 (76.22)	114.07 (69.93)	1.75 (0.27)	0.65 (0.19)	13.42 (3.14)	2.25 (0.98)	0.25 (0.43)	0.66 (1.17)	-1.99 (0.10)	-0.20 (1.11)	3.42 (0.39)	4.51 (0.51)
P	56.35 (29.92)	12.11 (2.27)	150.97 (93.20)	28.55 (13.31)	0.80 (0.25)	0.59 (0.24)	2.04 (1.04)	9.22 (4.72)	-0.57 (0.55)	0.19 (1.33)	-0.30 (0.54)	-2.16 (0.58)	4.04 (0.20)	1.99 (0.67)
Q	154.51 (14.76)	61.94 (12.64)	171.72 (71.41)	74.21 (15.58)	0.77 (0.18)	0.63 (0.14)	4.94 (0.41)	1.36 (0.39)	0.22 (0.58)	-0.18 (0.25)	-1.94 (0.18)	-0.69 (0.46)	4.01 (0.13)	4.23 (0.22)
R	98.41 (10.09)	112.02 (29.27)	510.96 (77.91)	539.77 (82.82)	1.14 (0.07)	1.47 (0.19)	6.53 (1.40)	4.14 (1.36)	-1.62 (0.18)	-1.69 (0.24)	-1.67 (0.25)	-0.87 (0.26)	4.40 (0.13)	4.84 (0.26)
S	150.32 (24.26)	105.84 (31.07)	263.57 (58.35)	351.45 (134.47)	0.90 (0.16)	0.92 (0.21)	2.78 (0.77)	1.42 (0.72)	-0.50 (0.13)	-1.07 (0.26)	-1.04 (0.14)	-0.11 (0.34)	4.75 (0.11)	5.30 (0.07)
T	199.02 (70.77)	112.76 (8.94)	115.81 (38.27)	84.73 (27.15)	0.52 (0.20)	0.33 (0.04)	4.48 (1.72)	1.27 (0.20)	0.15 (0.39)	0.60 (0.55)	-1.92 (0.28)	-1.35 (0.19)	4.09 (0.11)	4.80 (0.10)
U	75.25 (36.58)	89.74 (52.76)	178.93 (90.03)	92.40 (32.80)	0.62 (0.29)	0.49 (0.11)	4.70 (3.54)	4.66 (0.48)	-0.80 (0.17)	-0.21 (0.57)	-1.51 (0.41)	-2.29 (0.17)	4.06 (0.35)	3.27 (0.45)
V	154.25 (14.62)	111.24 (23.20)	66.63 (10.12)	82.50 (46.54)	0.82 (0.14)	0.55 (0.15)	3.12 (1.05)	3.10 (1.32)	0.86 (0.12)	0.67 (0.38)	-1.14 (0.30)	-1.25 (0.60)	4.19 (0.29)	4.16 (0.26)
W	159.48 (11.25)	123.48 (34.06)	69.84 (12.24)	60.64 (27.84)	0.50 (0.04)	0.49 (0.08)	4.76 (1.06)	4.75 (2.01)	0.87 (0.19)	0.86 (0.23)	-2.18 (0.18)	-2.01 (0.48)	3.82 (0.12)	3.58 (0.33)
X	129.42 (33.11)	212.58 (30.35)	378.72 (112.29)	444.29 (62.79)	1.58 (0.50)	2.68 (0.25)	4.24 (2.16)	7.19 (1.10)	-1.07 (0.17)	-0.73 (0.15)	-0.49 (0.40)	-0.96 (0.19)	4.82 (0.40)	4.19 (0.09)
Y	99.82 (27.15)	161.83 (42.37)	194.66 (28.66)	206.98 (43.08)	0.64 (0.09)	1.14 (0.21)	3.62 (1.34)	2.89 (0.44)	-0.79 (0.24)	-0.30 (0.09)	-1.59 (0.28)	-0.95 (0.26)	4.31 (0.24)	4.44 (0.27)
Z	79.62 (11.89)	36.18 (9.25)	55.17 (30.59)	34.01 (12.41)	0.67 (0.24)	0.30 (0.08)	22.08 (15.23)	3.28 (1.94)	0.73 (0.37)	0.32 (0.32)	-2.17 (1.36)	-1.66 (0.85)	2.90 (0.89)	3.35 (0.41)
AA	170.24 (60.79)	115.74 (10.91)	117.11 (47.30)	602.33 (44.99)	0.50 (0.14)	1.63 (0.33)	5.85 (3.13)	3.27 (1.20)	0.51 (0.18)	-1.65 (0.05)	-2.18 (0.36)	-0.49 (0.50)	3.90 (0.21)	5.09 (0.25)
AB	136.79 (30.90)	71.76 (22.54)	143.33 (26.89)	106.47 (37.14)	0.65 (0.10)	0.48 (0.06)	11.36 (6.01)	2.20 (0.44)	-0.08 (0.12)	-0.46 (0.24)	-2.51 (0.59)	-1.49 (0.17)	3.44 (0.57)	3.92 (0.55)
AC	181.13 (25.84)	62.65 (26.01)	129.07 (49.04)	85.89 (28.24)	1.46 (0.40)	0.97 (0.22)	3.97 (0.93)	2.74 (0.79)	0.54 (0.26)	-0.41 (0.46)	-0.98 (0.34)	-1.00 (0.42)	4.06 (0.11)	3.62 (0.26)
AD	175.00 (44.94)	60.36 (15.59)	69.62 (14.89)	57.70 (14.71)	1.16 (0.21)	0.83 (0.16)	3.99 (1.49)	42.48 (40.70)	0.92 (0.23)	0.06 (0.07)	-0.95 (0.54)	-1.95 (1.39)	3.94 (0.16)	2.60 (1.36)

Supplementary figures

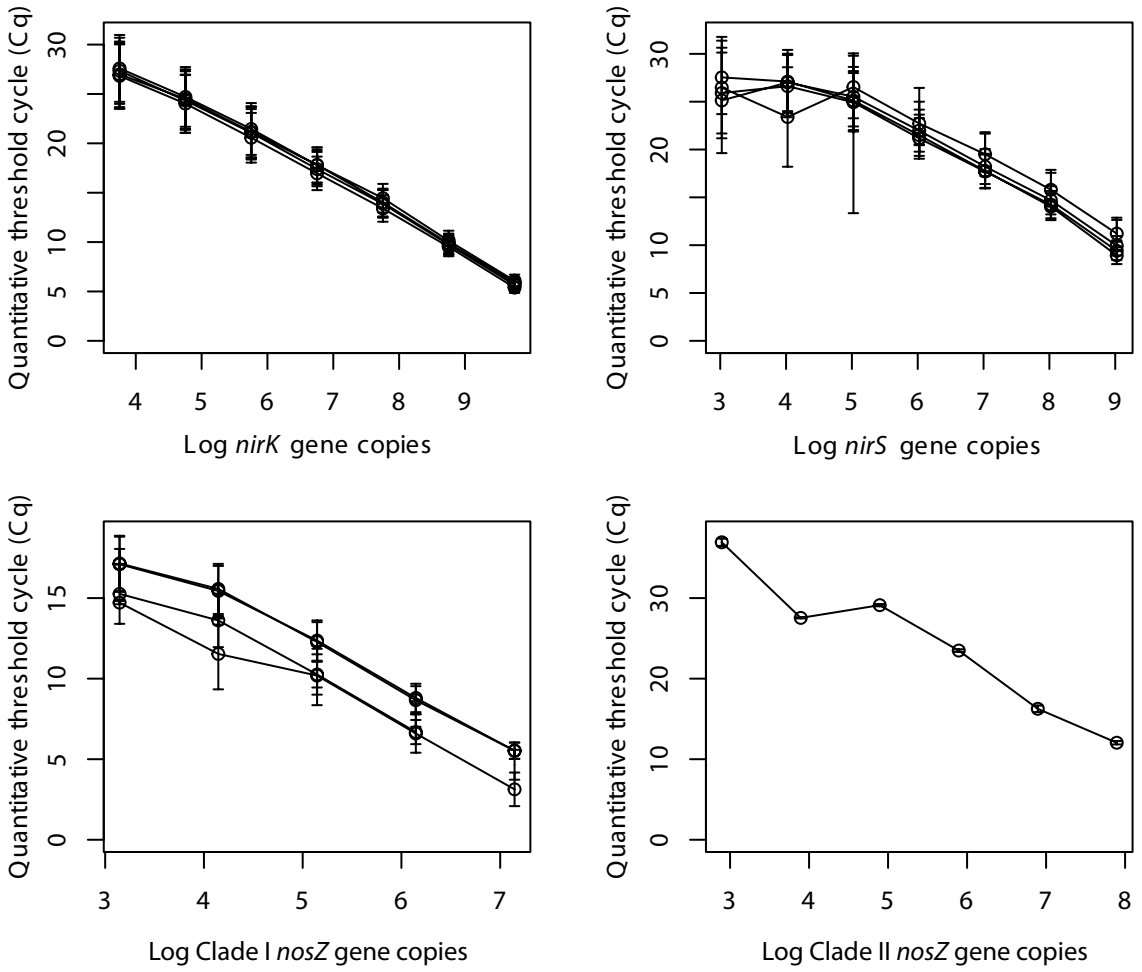

Figure A.1. Quantitative PCR assay precision shown as the standard error of the mean for each C_q value in the standard curve (between 12 technical replicates). Separate lines are shown for each reaction plate.

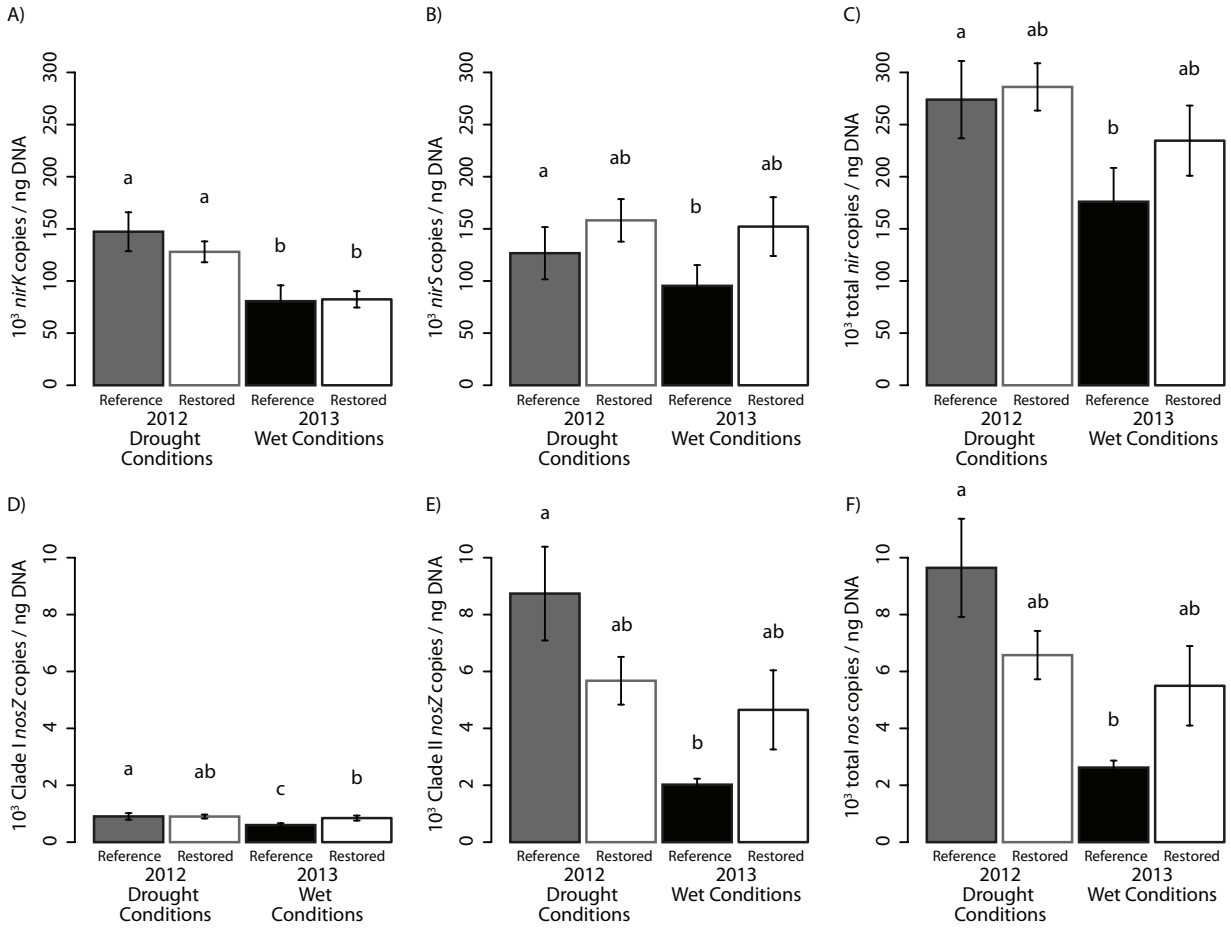

Figure A.2. Denitrification gene copy numbers. The bars represent overall means calculated from n = 15 reference wetlands and n = 30 restored wetlands. Errors bars show standard error of the mean. Lower case letters show significant groupings ($p < 0.05$).

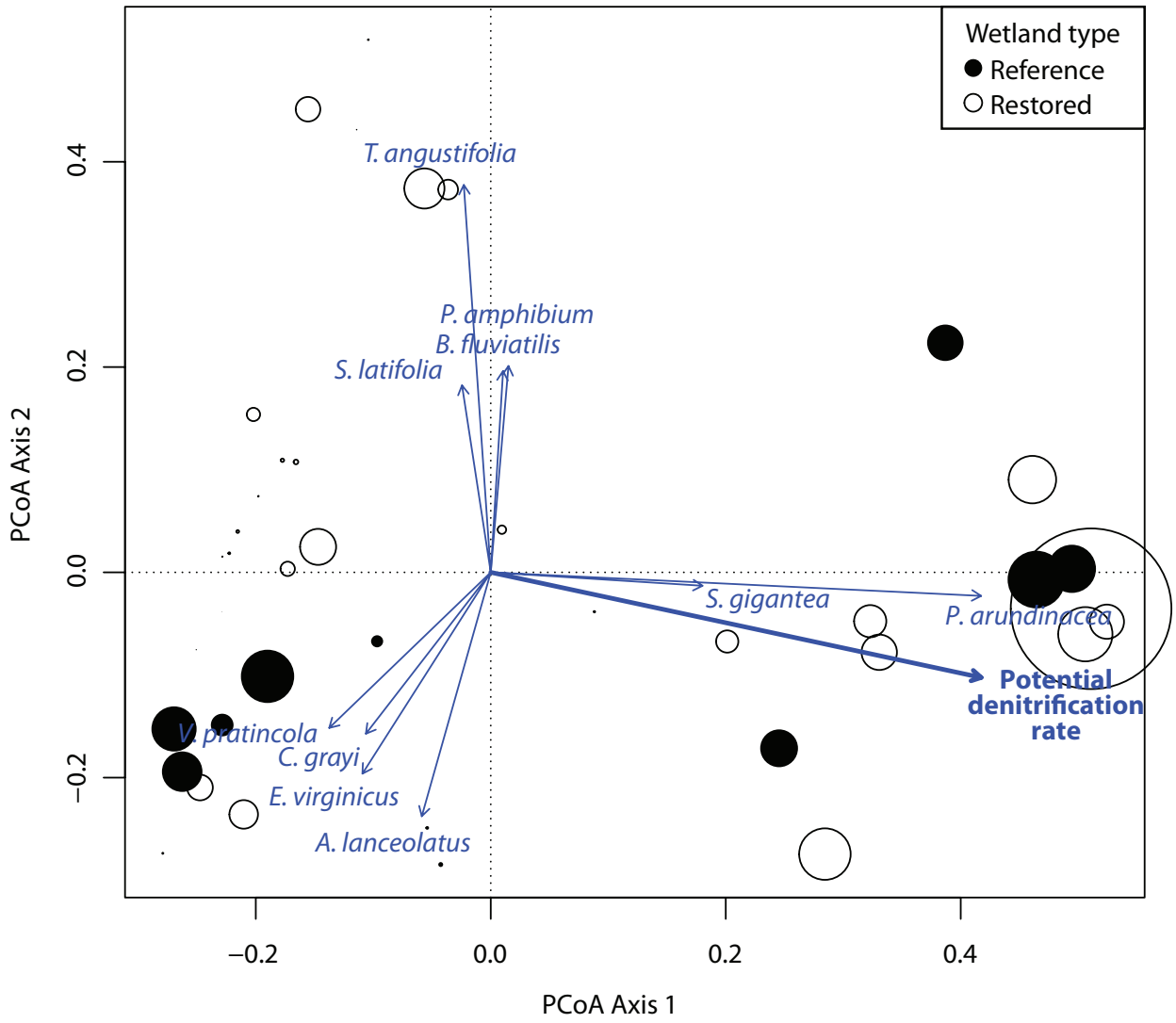

Figure A.3. Principle coordinate analysis (PCoA) ordination plot of the plant community in each wetland. Individual plant species were correlated to the ordination using the 'envfit' function and only plant species that significantly correlated to the ordination ($p < 0.05$) are shown in blue. A separate envfit model including the average denitrification potential of each wetland in 2013 revealed a significant correlation to the ordination ($p < 0.05$) and is also shown as a thick blue arrow.

APPENDIX B:

SUPPLEMENTAL MATERIAL FOR CHAPTER 3

Supplementary methods

Determination of soil C and N

Soil samples collected in 2007, 2012, 2013, and 2015 were air-dried and sieved to 2 mm. Total organic carbon and nitrogen were determined using combustion analysis (ECS 4010, COSTECH Analytical Instruments, Valencia, CA, USA).

Supplementary table

Table B.1. Quantitative PCR assay performance values calculated for the standard curves. Standard curves included 12 technical replicates on each plate. Samples were analyzed across five separate reaction plates, so performance values were calculated separately for each reaction plate. Linear dynamic range and limit of detection were the same for each plate. Clade II *nosZ* was quantified using a single standard curve (with 12 technical replicates). Calculations are described in the MIQE guidelines (Bustin et al. 2009).

qPCR Assay	Efficiency	Linear Dynamic Range	Limit of Detection
nirK	0.81 ± 0.02	5.67×10^3 # copies to 5.67×10^9 # copies	5.67×10^3 # copies
nirS	0.90 ± 0.04	1.06×10^4 # copies to 1.06×10^9 # copies	1.06×10^4 # copies
Clade I *nosZ*	1.00 ± 0.02	1.40×10^3 # copies to 1.08×10^7 # copies	1.40×10^3 # copies
Clade II *nosZ*	0.63	7.85×10^2 # copies to 7.85×10^7 # copies	7.85×10^2 # copies

Supplementary figures

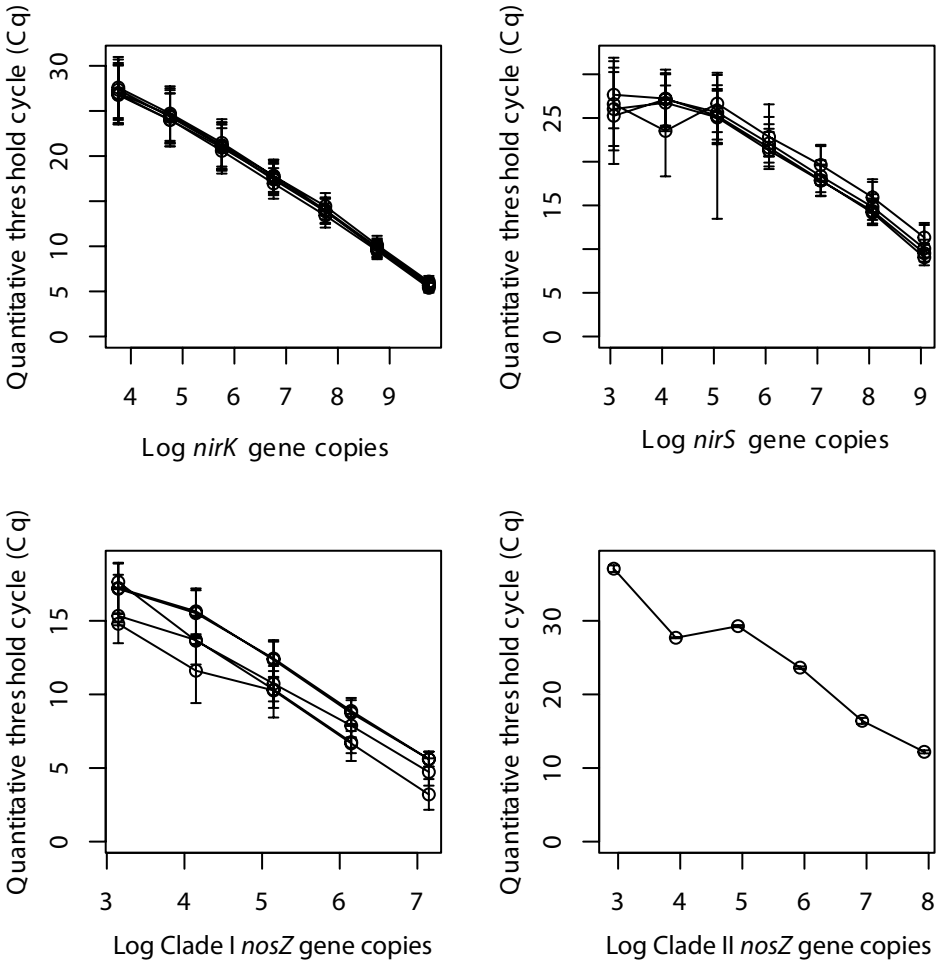

Figure B.1. Quantitative PCR assay precision shown as the standard error of the mean for each C_q value in the standard curve (between 12 technical replicates). Separate lines are shown for each reaction plate.

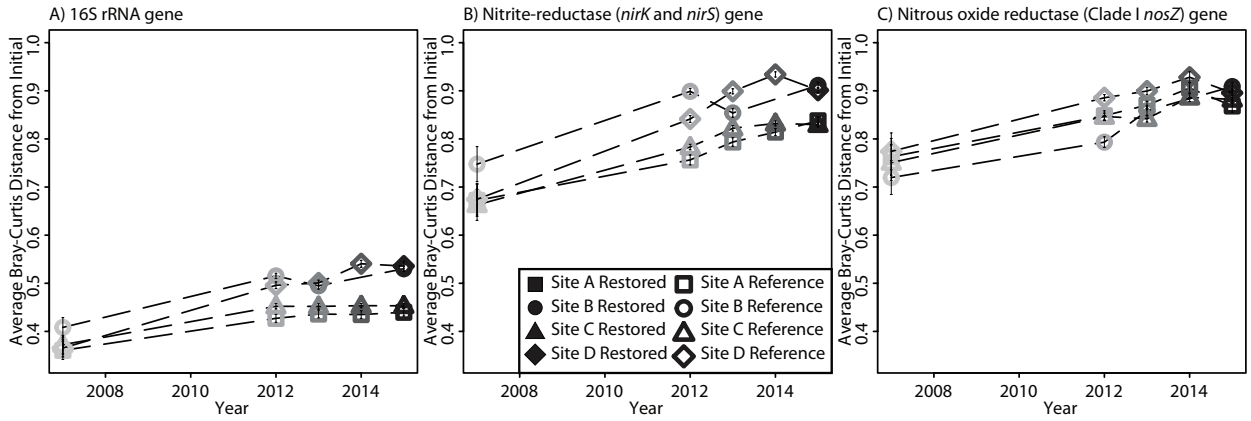

Figure B.2. Bray-Curtis distance between the reference wetland community over time and the initial reference wetland community observed in 2007 for (A) the overall community based on the 16S rRNA gene, (B) the N_2O producing denitrifiers based on *nirK* and *nirS* gene sequences, and (C) the N_2O consuming denitrifiers based on Clade I *nosZ* gene sequences. Symbols correspond to wetland pair, and shading indicates year, where the lightest color is from 2007 and the darkest represent data collected in 2015. Error bars show standard error of the mean.

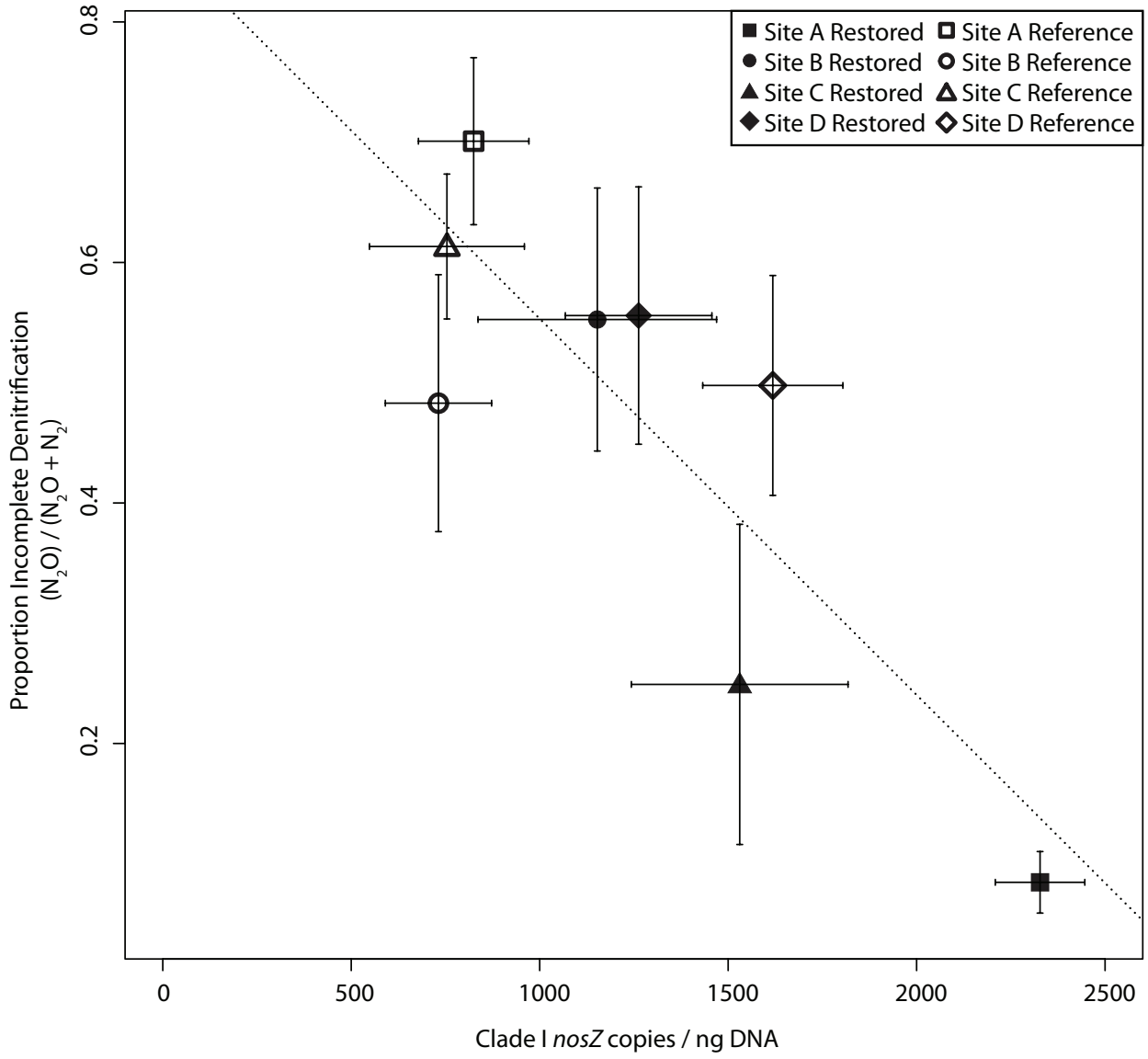

Figure B.3. Negative linear regression between the proportion of incomplete denitrification that occurred during potential rate assays and the Clade I *nosZ* gene copy number. This plot shows data generated in 2015. The error bars represent the standard error of the mean.

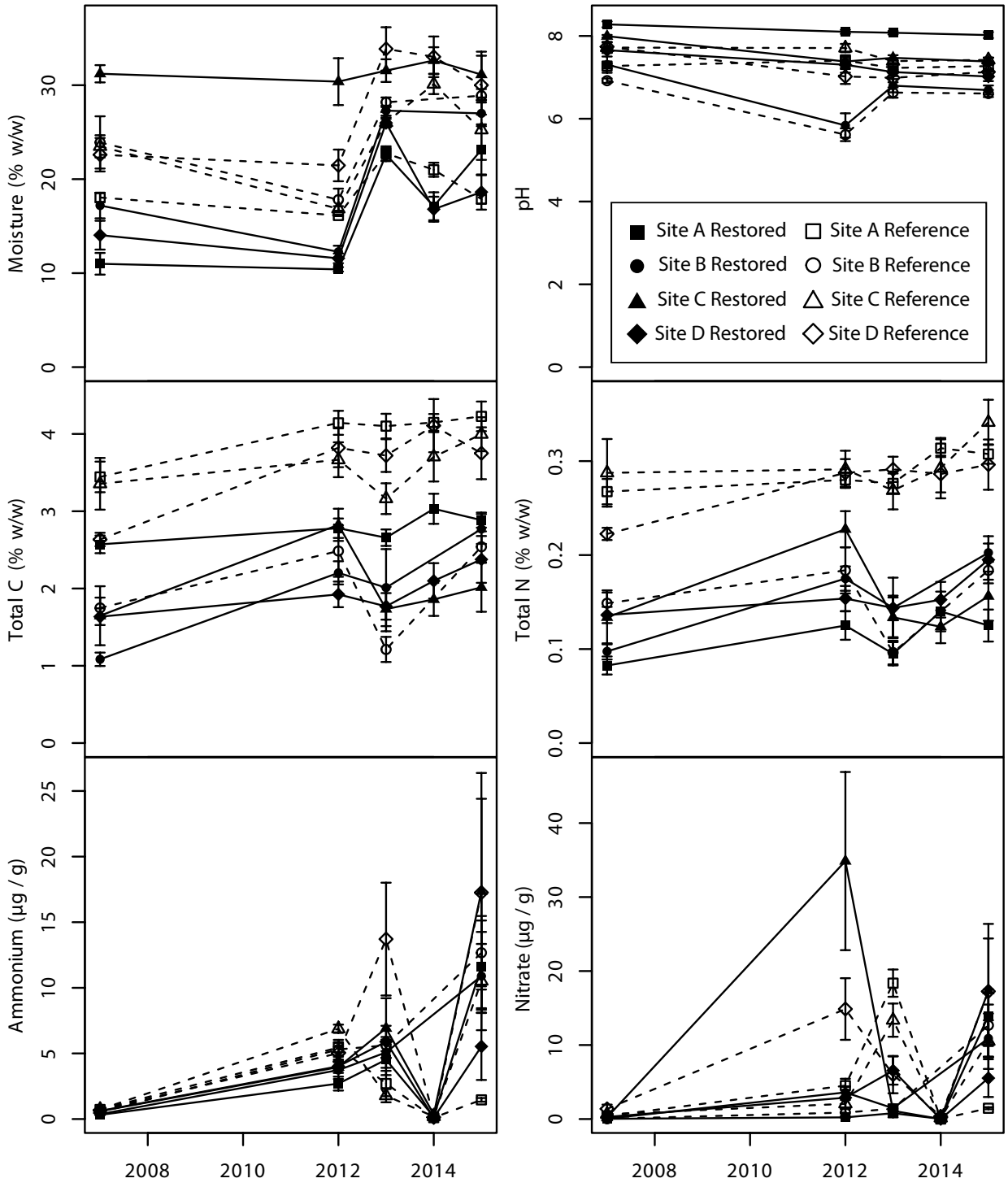

Figure B.4. Average soil chemistry variables in each wetland over time. Error bars show standard error of the mean.

APPENDIX C:

SUPPLEMENTAL MATERIAL FOR CHAPTER 4

Supplementary results

Changes in soil properties following hydrologic manipulation

Soil moisture was influenced strongly by the hydrologic treatments (ANOVA, Treatment: F = 886.9, df = 3, $p < 0.001$; Fig. C.2A). Regardless of initial moisture levels, the Dry treatment resulted in low moisture (<10% w/w), while the Variable and Saturated treatments resulted in high moisture levels (25% - 50% w/w). Extractable NH_4^+ was also significantly affected by treatment (ANOVA, Treatment: F = 209.83, df = 3, $p < 0.001$). Extractable NH_4^+ concentration increased in the Dry and Variable source soils following the Saturated treatment (Fig. C.2C). Extractable NO_x was affected by treatment (ANOVA, Treatment: F = 9.065, df = 3, $p < 0.001$). NOx typically decreased following both the Variable and Saturated treatments (Fig. C.2D). One exception is that the NOx in the soil from the Saturated site increased following the Variable treatment. Soil pH was significantly affected by treatment (ANOVA, Treatment: F = 39.71, df = 3, $p < 0.001$). There was an increase in pH observed in the soils collected from the Dry site following the Variable and Saturated Treatments (Fig. C.2B). Total C, total N, and the C:N ratio remained unaltered by the experiment (Fig. C.2E-C.2G), except for a non-significant decrease in total N from the Saturated source soils following the Saturated treatment, which caused a non-significant increase in the C:N ratio.

Supplementary tables

Table C.1. Quantitative PCR assay performance values calculated for the standard curves for each of the four qPCR assays reported in this study. Calculations are described in the MIQE guidelines (Bustin et al. 2009).

qPCR Assay	Efficiency	Linear Dynamic Range	Limit of Detection
Archaeal *amoA*	84.88%	6.86×10^{2} # copies to 6.86×10^{7} # copies	6.86×10^{2} # copies
Bacterial *amoA*	89.02%	7.45×10^{2} # copies to 7.45×10^{7} # copies	7.45×10^{2} # copies
nirK	85.47%	3.20×10^{1} # copies to 5.00×10^{5} # copies	3.20×10^{1} # copies
nirS	86.27%	8.00×10^{2} # copies to 1.00×10^{5} # copies	8.00×10^{2} # copies

Table C.2. Quantitative PCR assay precision calculated as the standard error of the mean for each standard curve C_q value for all four of the qPCR assays reported in this study. "Standard 1" had the lowest number of copies while "Standard 6" had the highest number of copies

qPCR Assay	Precision					
	Standard 1 mean C_q ± SEM	Standard 2 mean C_q ± SEM	Standard 3 mean C_q ± SEM	Standard 4 mean C_q ± SEM	Standard 5 mean C_q ± SEM	Standard 6 mean C_q ± SEM
Archaeal *amoA*	25.09 ± 0.19	22.03 ± 0.22	18.4 ± 0.08	14.81 ± 0.07	11.5 ± 0.12	6.82 ± 0.17
Bacterial *amoA*	26.76 ± 0.10	23.04 ± 0.04	19.56 ± 0.05	15.94 ± 0.06	12.65 ± 0.11	7.39 ± 0.07
nirK	17.83 ± 0.08	16.51 ± 0.04	13.47 ± 0.04	10.5 ± 0.04	7.59 ± 0.01	5.97 ± 0.01
nirS	16.44 ± 0.09	14.75 ± 0.04	11.93 ± 0.04	9.76 ± 0.02	6.25 ± 0.03	--

Supplementary figures

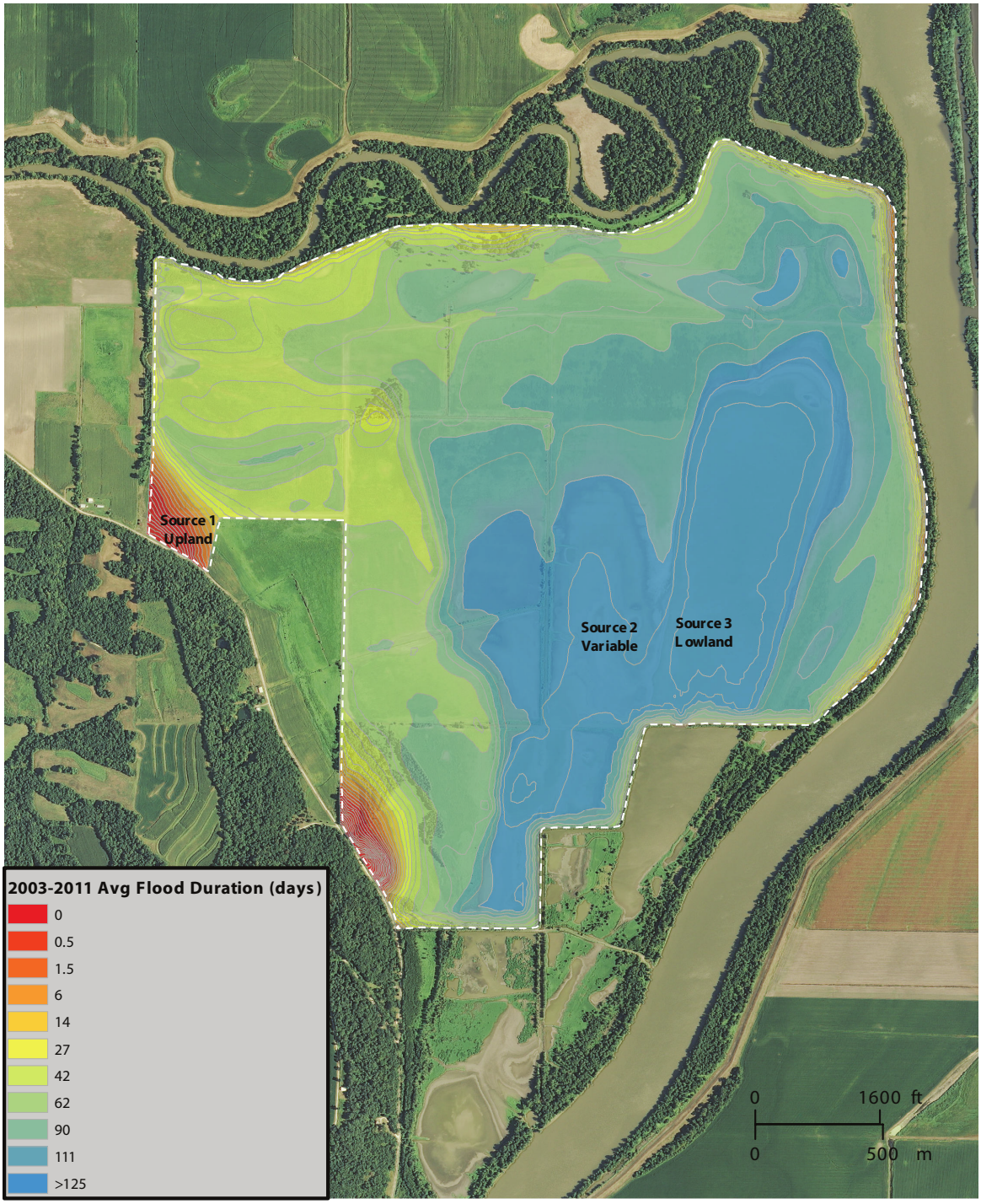

Figure C.1. Average flood patterns observed at the La Grange mitigation wetland. Approximate sampling locations for each of the three source communities are indicated in text. Figure was adapted from heatmap provided by G. Pociask, ISGS.

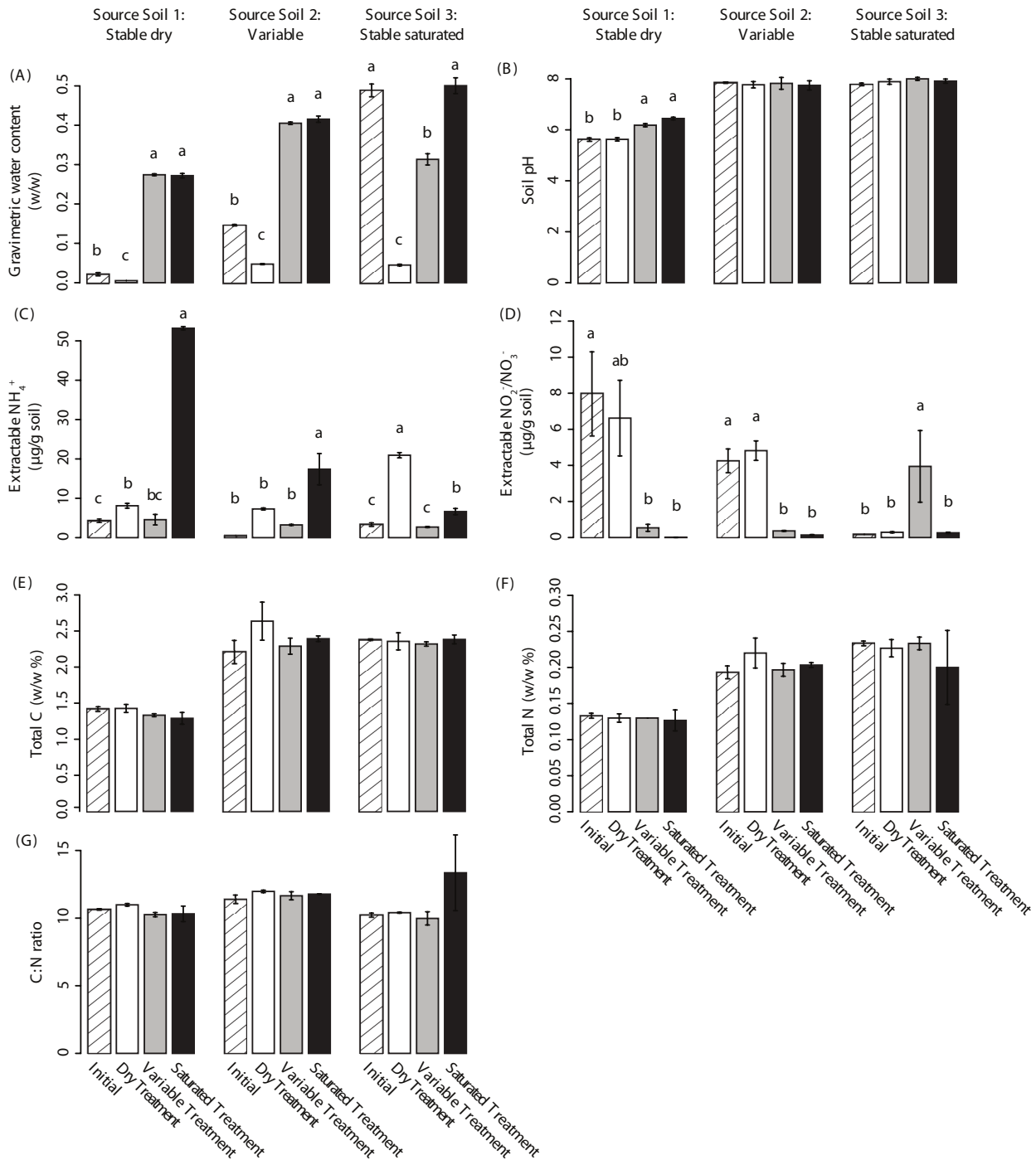

Figure C.2. Changes in soil properties before and after the experiment. Each panel shows a separate soil property, where each of the three source soils is shown on a separate graph. The individual sites are arranged by column within the panels, where the first column shows data from the Dry source soil, the middle column shows data from the Variable source soil, and the column on the right shows data from the Saturated source soil. Lower case letters indicate significant groupings ($p < 0.05$), except for instances where there were no significant differences detected between treatments. Error bars represent the standard error of the mean.

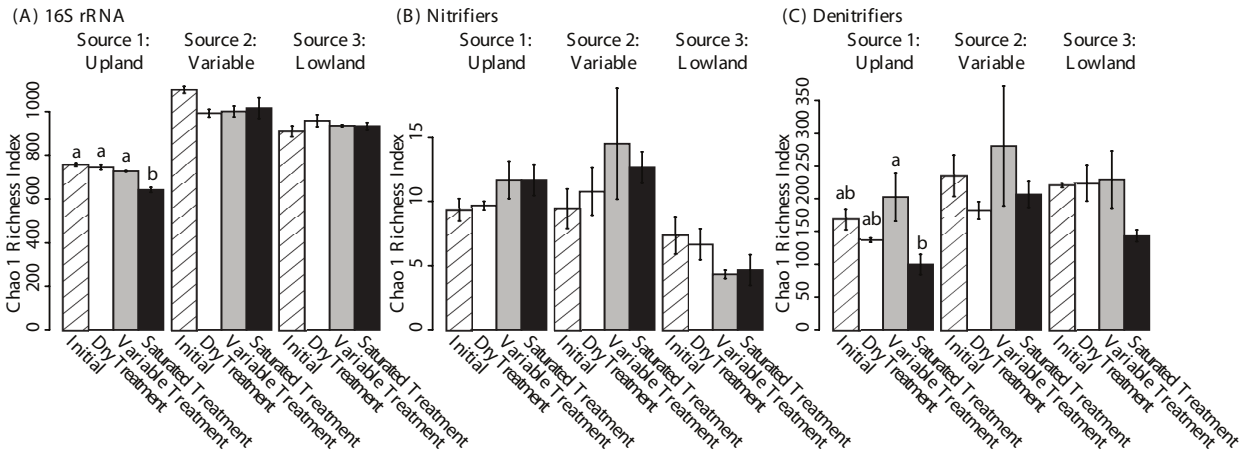

Figure C.3. Change in richness in (A) the overall microbial community based on 16S rRNA sequences, (B) the nitrifier community, and (C) the denitrifier community. Significant differences following treatments are indicated by lower case letters. Error bars show standard error of the mean.

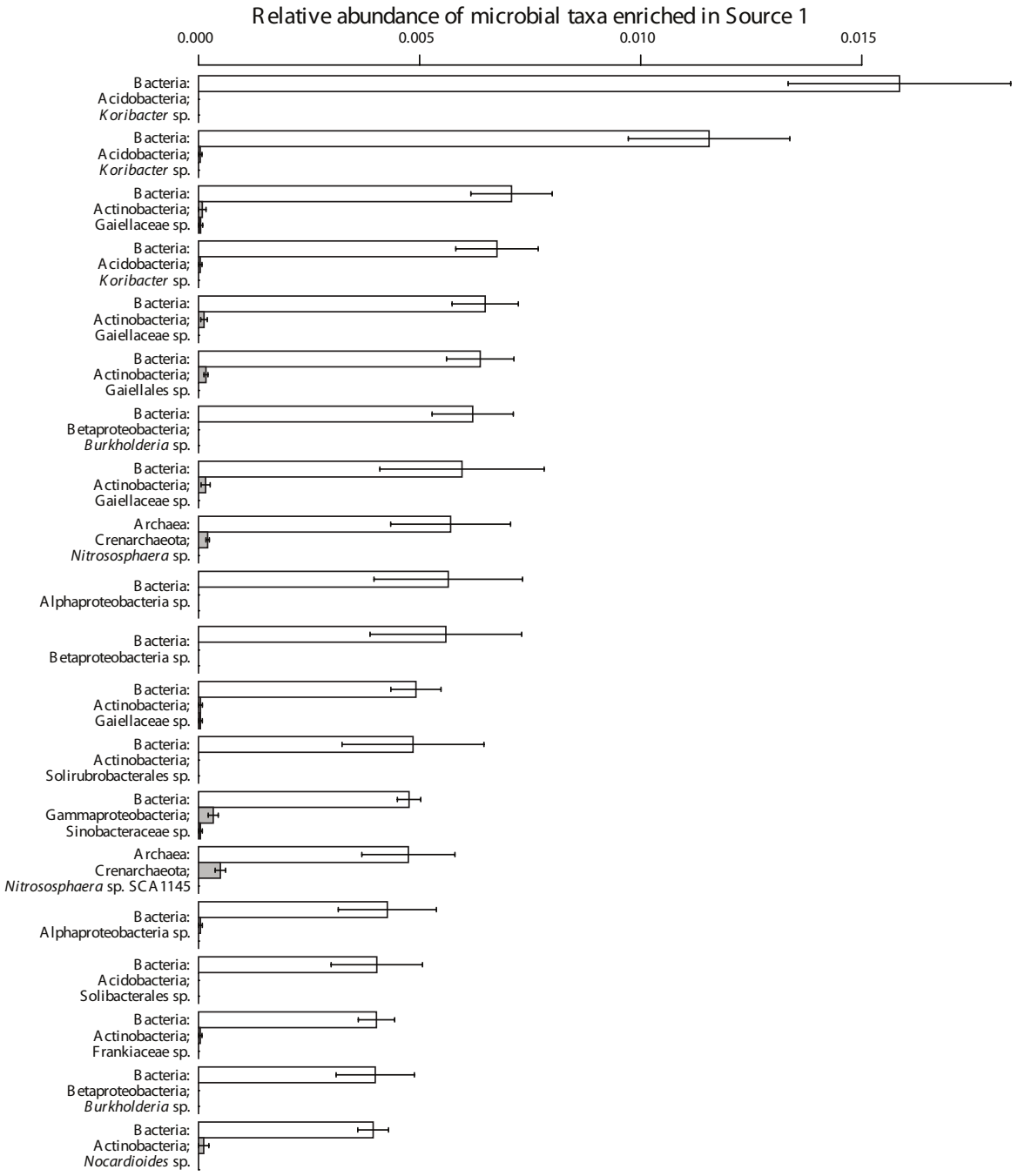

Figure C.4. Relative abundance of microbial taxa (based on 16S rRNA sequences) in the Source 1 soil community before the experiment (hatched bars) and following each treatment (white: dry, grey: variable, black: saturated). The top 20 OTUs shown were determined to explain 25.6% of the change in the community between the initial and saturated treatment using the 'simper' function in R.

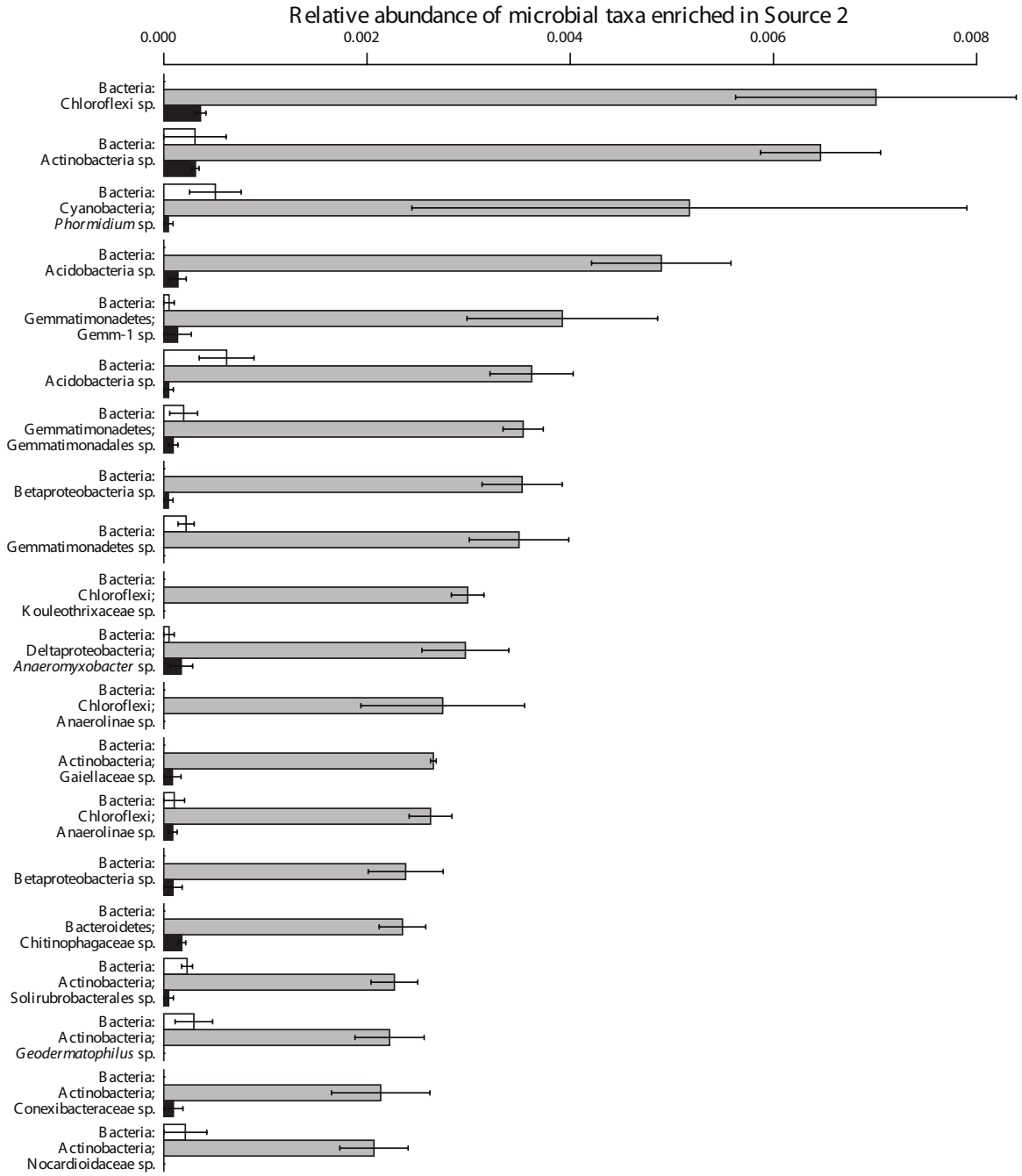

Figure C.5. Relative abundance of microbial taxa (based on 16S rRNA sequences) in the Source 3 soil community before the experiment (hatched bars) and following each treatment (white: dry, grey: variable, black: saturated). The top 20 OTUs shown were determined to explain 13% of the change in the community between the initial and following any of the treatments using the 'simper' function in R.

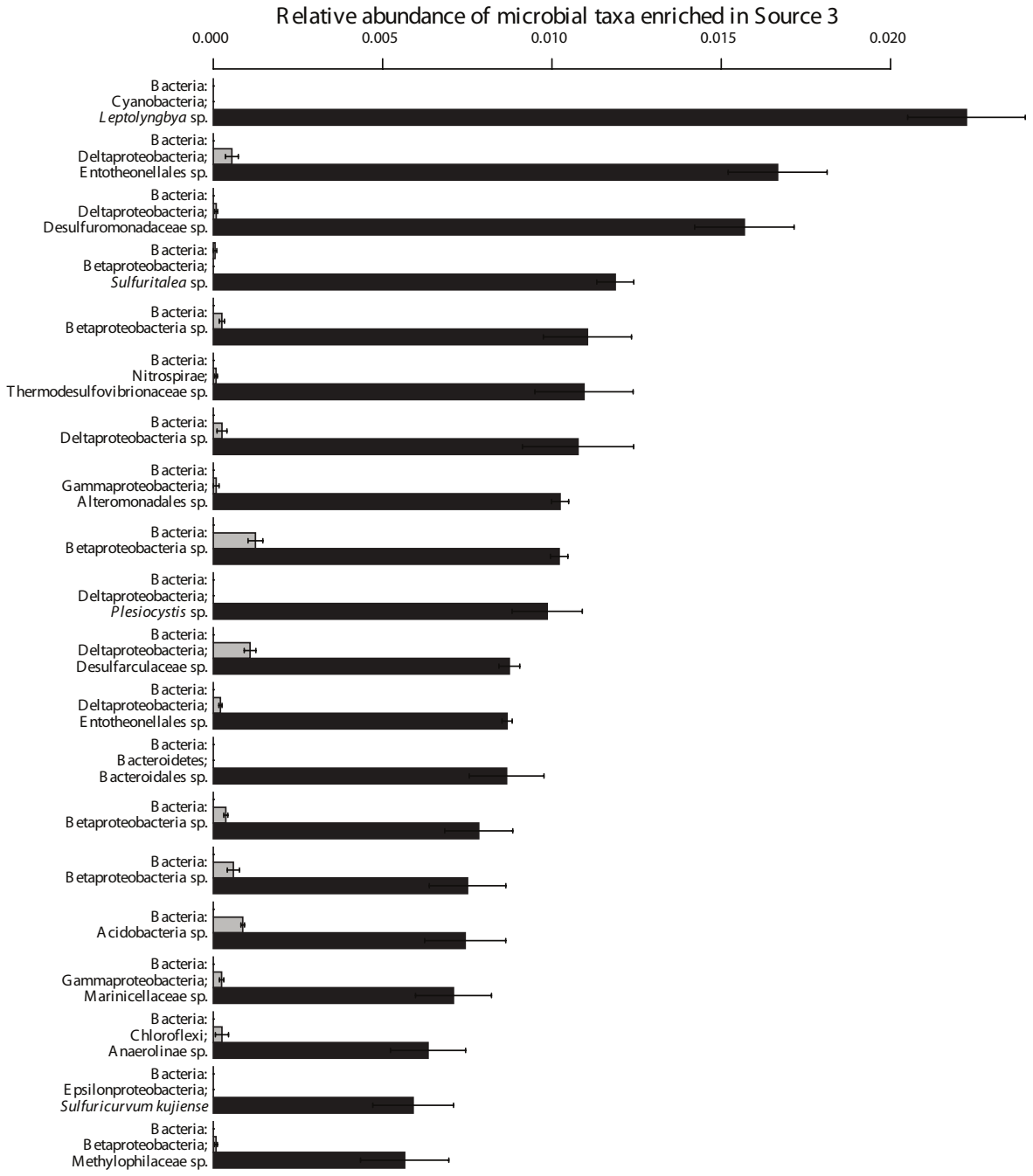

Figure C.6. Relative abundance of microbial taxa (based on 16S rRNA sequences) in the Source 2 soil community before the experiment (hatched bars) and following each treatment (white: dry, grey: variable, black: saturated). The top 20 OTUs shown were determined to explain 19.9% of the change in the community between the initial and following any of the treatments using the 'simper' function in R.

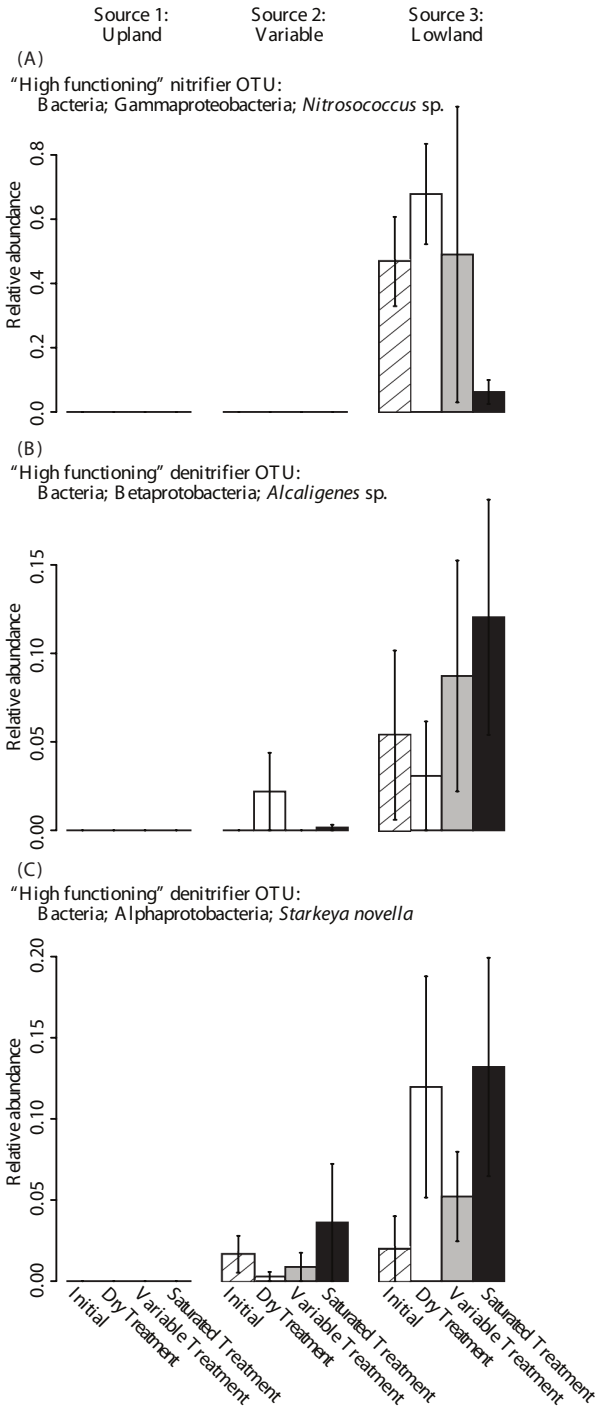

Figure C.7. Relative abundance of nitrifier and denitrifier OTUs identified to be "high functioning" by using the 'rda' function in R to calculate their loading along a constrained function axis in a redundancy analysis (RDA). These OTUs ranked in the top 10% of along their respective constrained axis (nitrification rate for the nitrifier OTU and denitrification rate for the denitrifier OTU). All three OTUs correlated positively with the respective functional rates when checked using linear regression. No significant differences in relative abundance were observed among treatments. Error bars show standard error of the mean.

APPENDIX D:

SUPPLEMENTAL MATERIAL FOR CHAPTER 5

Supplementary figures

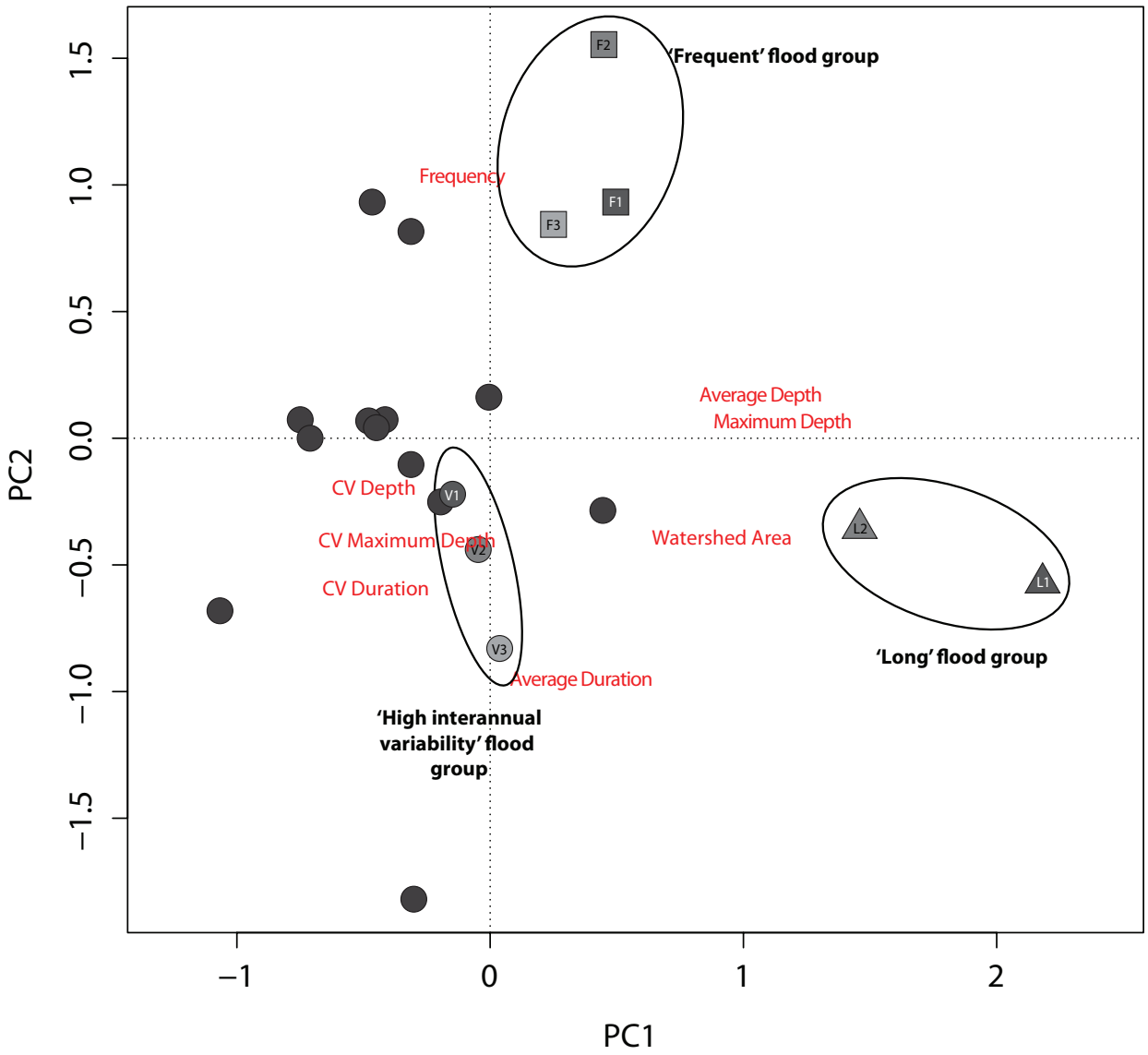

Figure D.1. Principal component analysis (PCA) ordination showing the historical hydrologic differences among 23 wetlands. The wetlands that were not selected for this study are shown as dark grey circles. The eight wetlands selected for this study are indicated with the same symbol shapes and letter-number combinations as used in the map in Fig. 1, and they are circled and labeled in bold text with the hydrologic group that they were assigned to. The flood variables used to build the PCA are shown in red. "CV" stands for coefficient of variability, and it was used as a means to measure variability from year to year.

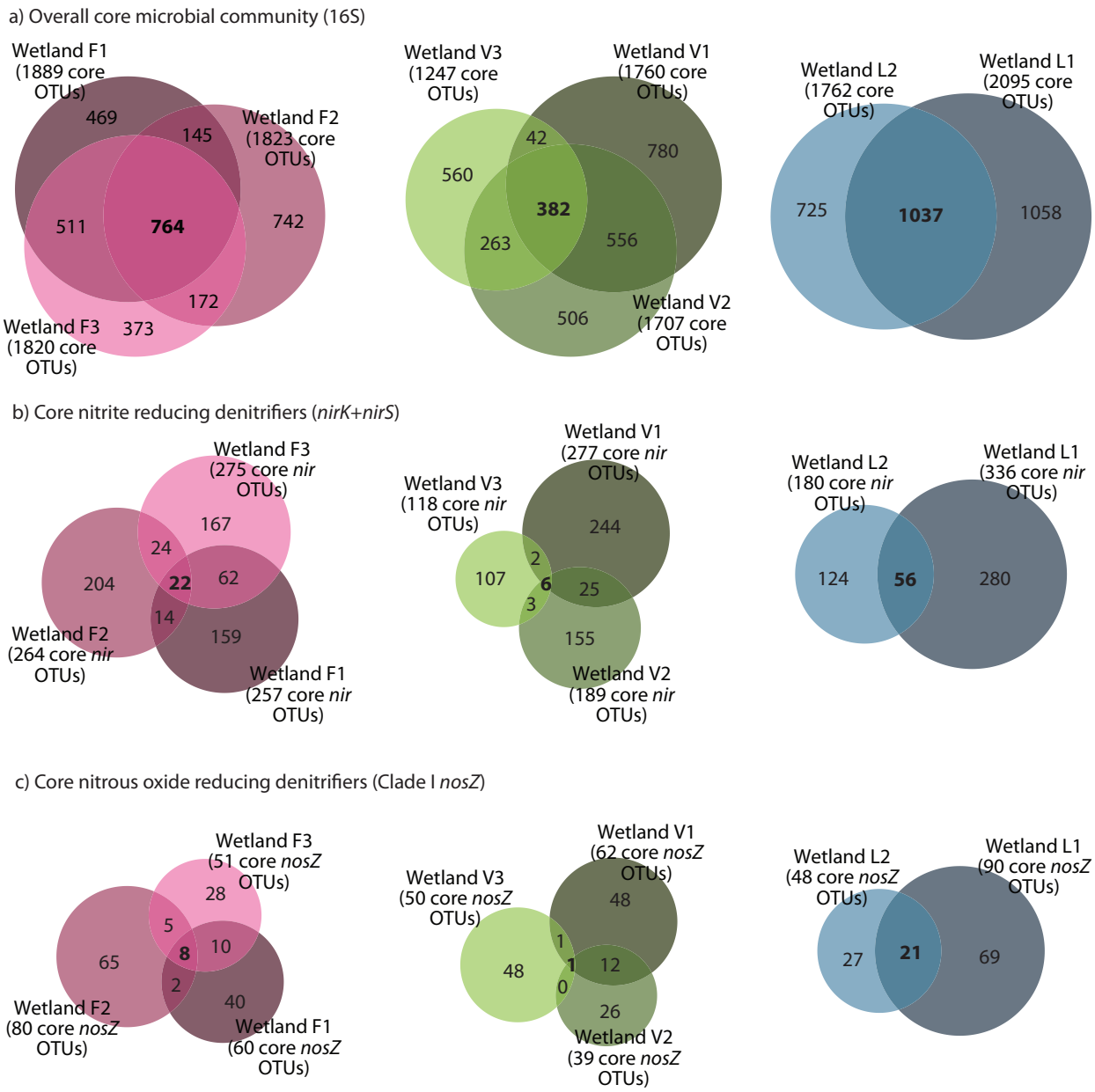

Figure D.2. Number of core taxa shared among each of the source wetland communities in each hydrologic group, including (a) the overall microbial community, (b) the nitrite reducing denitrifier community, and (c) the nitrous oxide reducing community. The overlapping region in the center of each diagram is defined as the "shared core microbiome" for each hydrologic group.

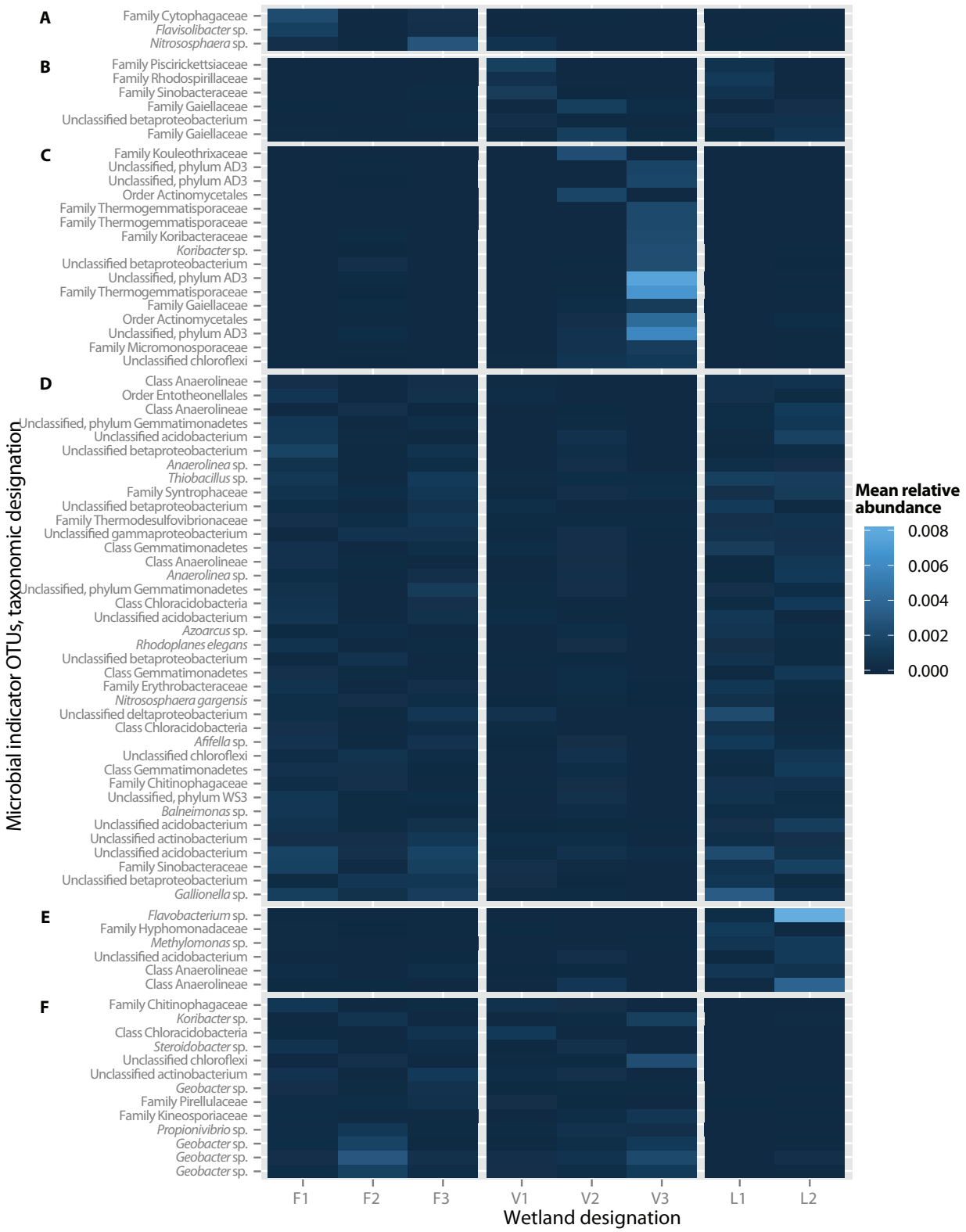

Figure D.3. Relative abundance of indicator microbial taxa identified for (A) frequent flood, (B) both frequent flood and high interannual variability, (C) high interannual variability, (D) high interannual variability and long flood (E) long flood, and (F) frequent flood and long flood wetland communities.

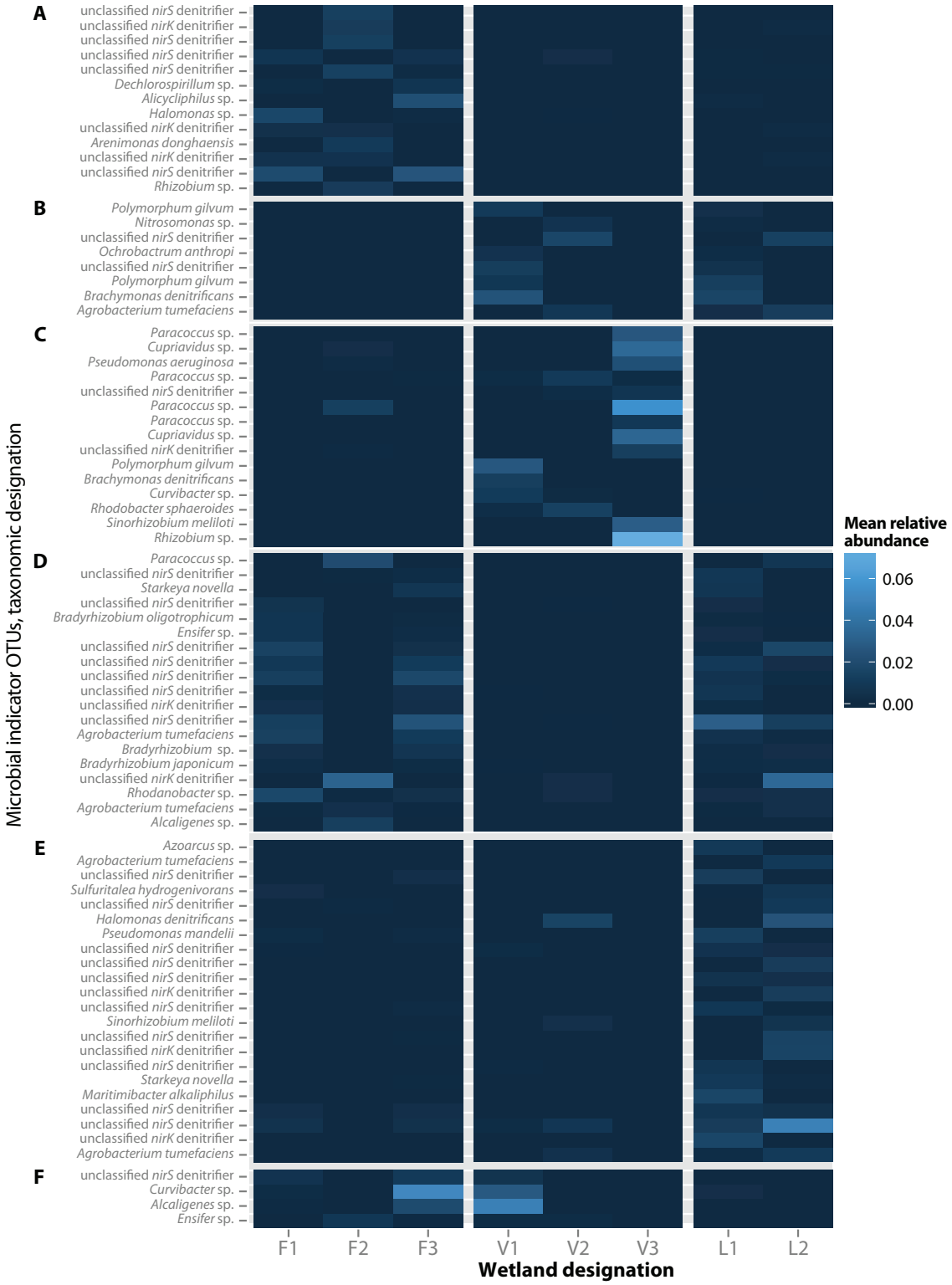

Figure D.4. Relative abundance of indicator nitrite reducing denitrifier taxa identified for (A) frequent flood, (B) both frequent flood and high interannual variability, (C) high interannual variability, (D) high interannual variability and long flood (E) long flood, and (F) frequent flood and long flood wetland communities.

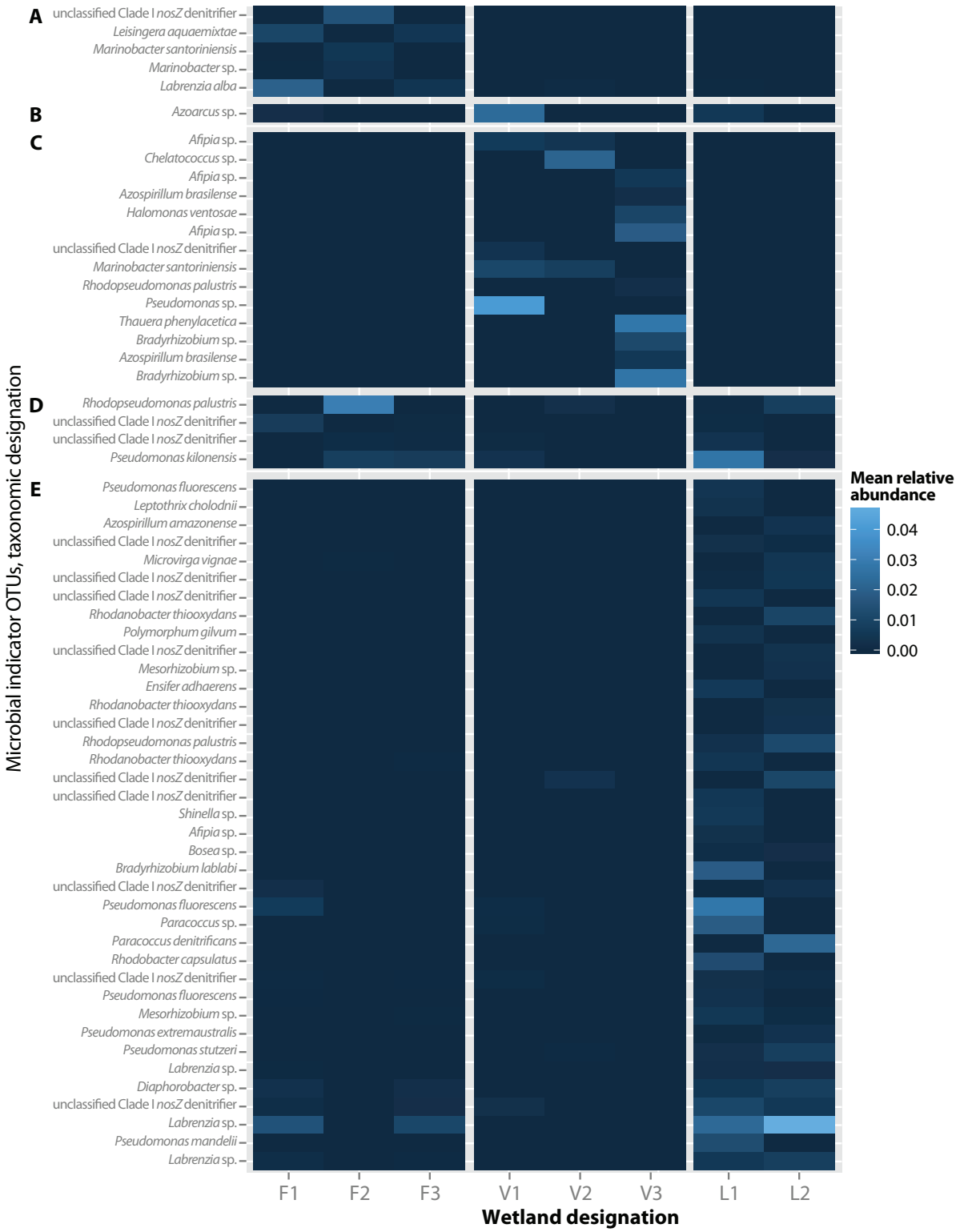

Figure D.5. Relative abundance of indicator nitrous oxide reducing denitrifier taxa identified for (A) frequent flood, (B) both frequent flood and high interannual variability, (C) high interannual variability, (D) high interannual variability and long flood and (E) long flood wetland communities. no indicator taxa were identified for both frequent flood and long flood wetland communities.

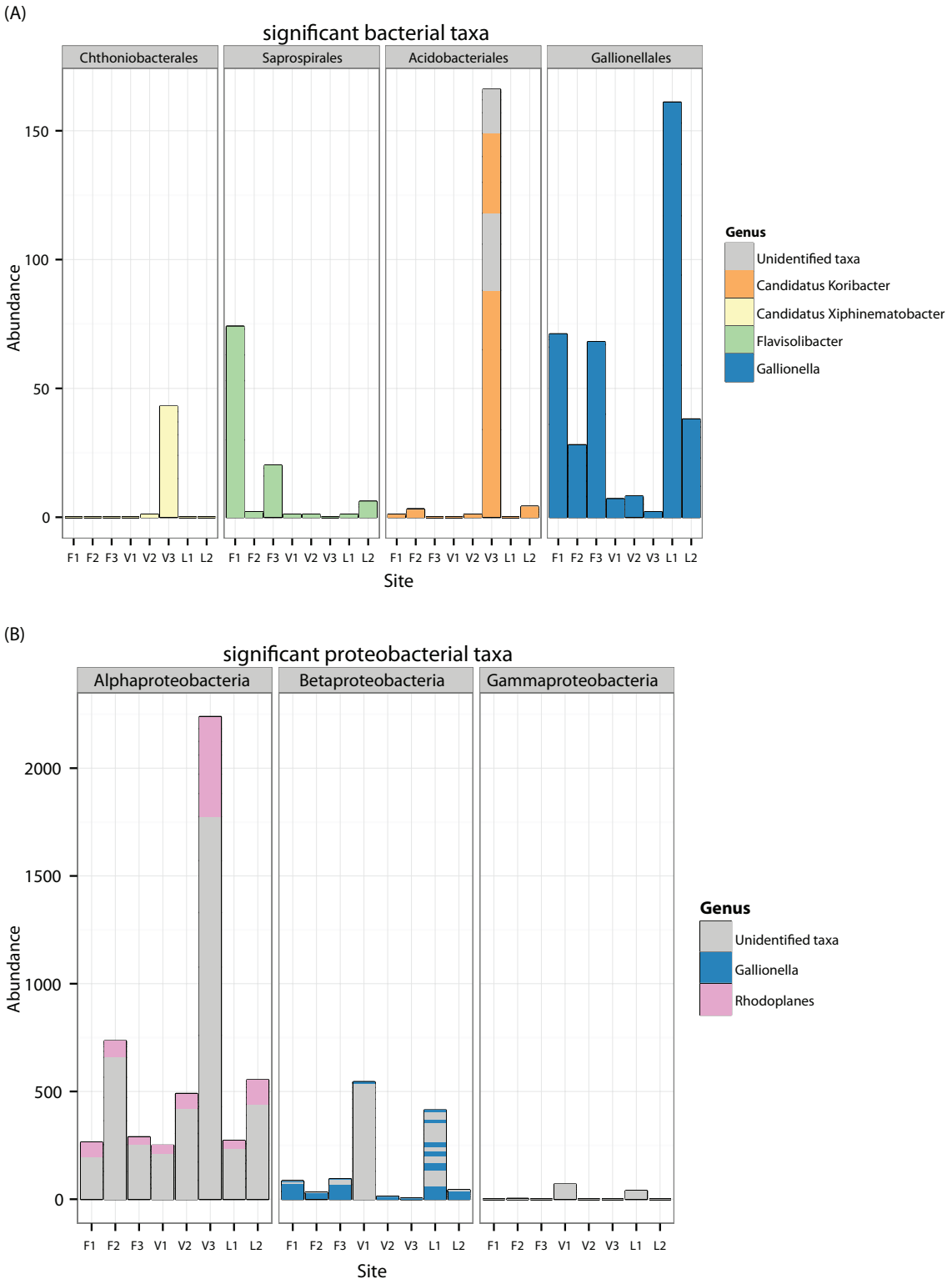

Figure D.6. Rarified abundance of bacterial reads that were identified to explain differences in composition between the three hydrologic groups (selected with significance level of alpha < 0.01 by the "DESeq2" function in "phyloseq" in R).

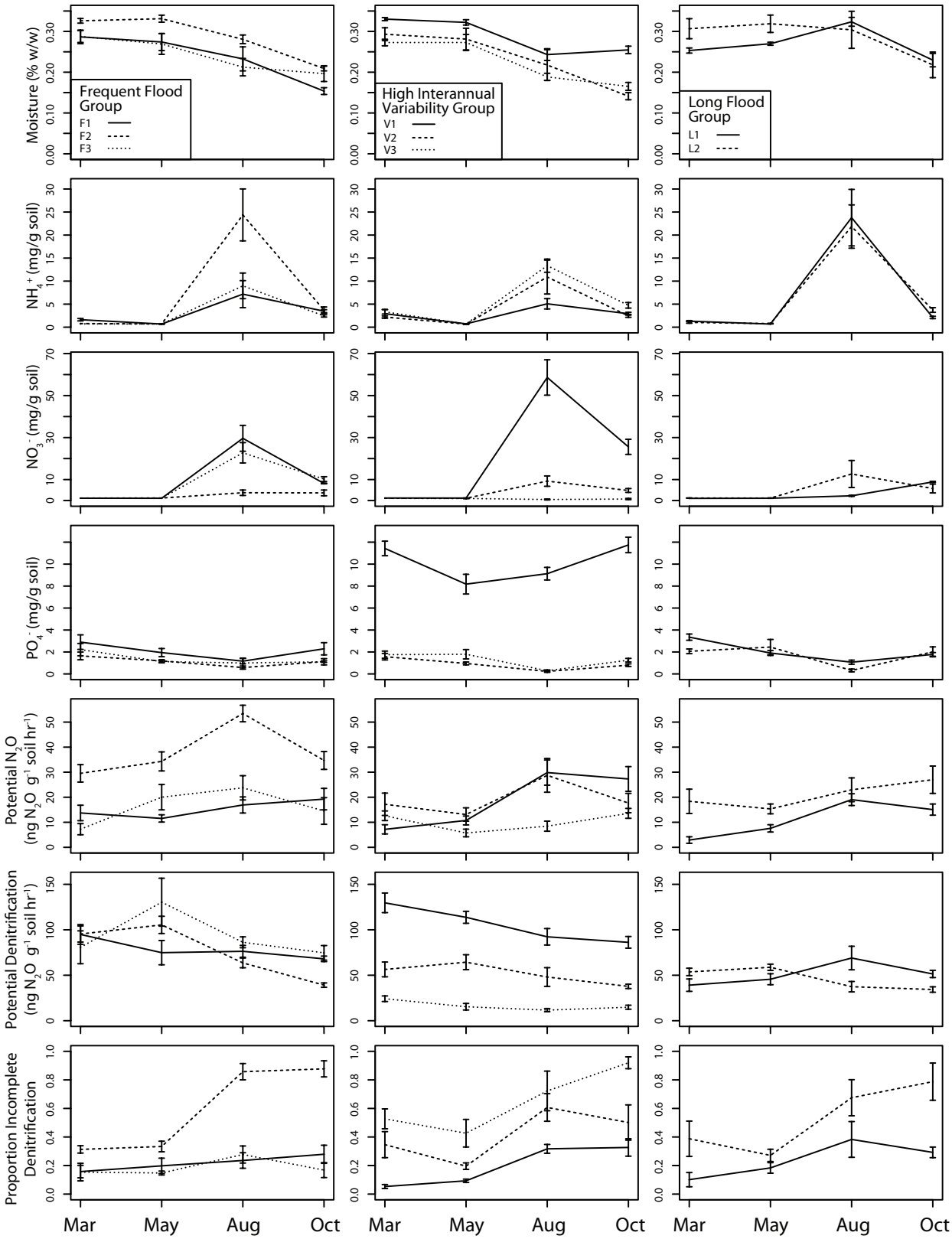

Figure D.7. Temporal trends in soil characteristics and potential functional rates in the source wetlands. Error bars show standard error of the mean.

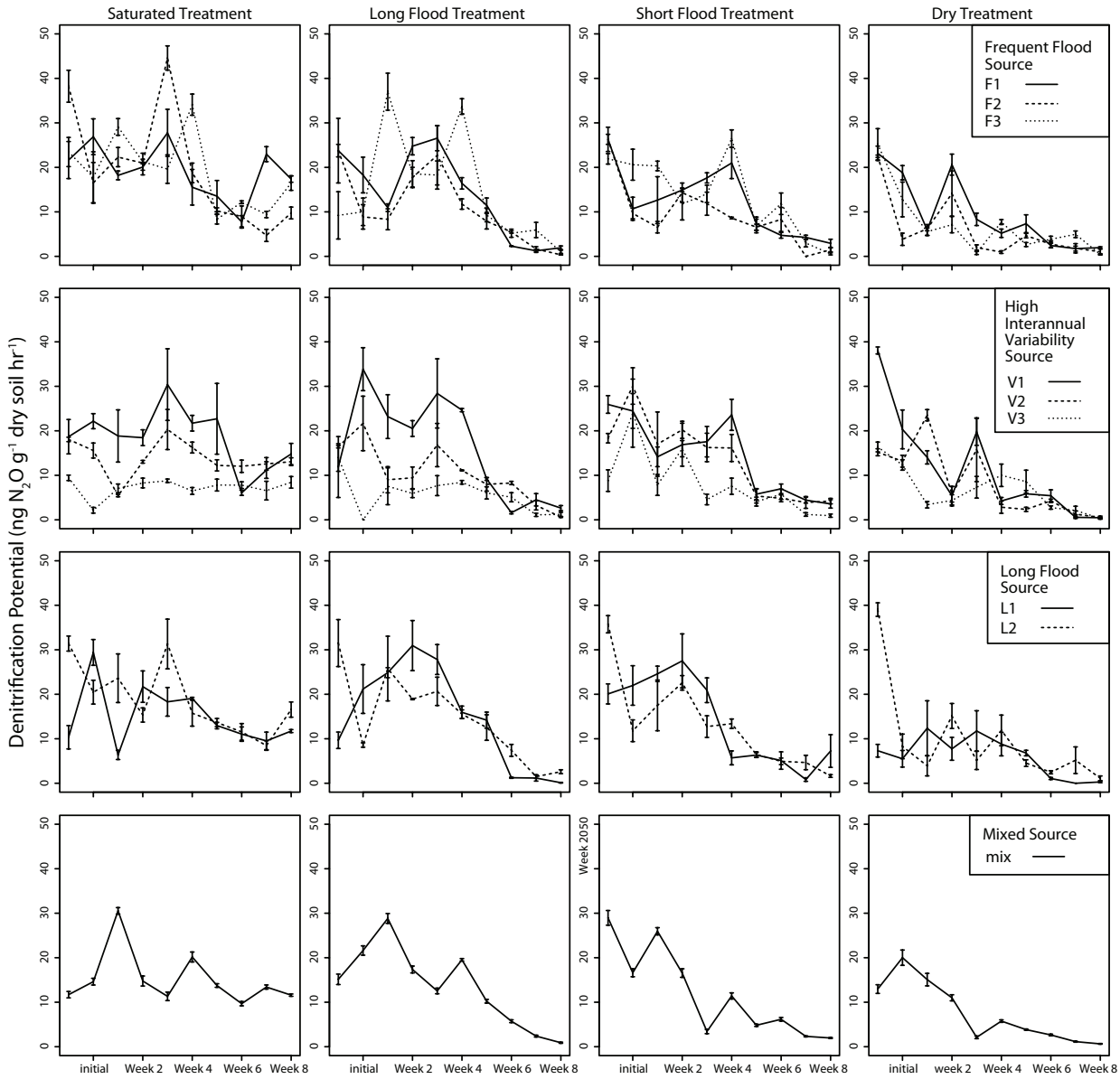

Figure D.8. Total potential denitrification rates in inoculated mesocosms over time. The means were calculated from replicate mesocosms each week (N = 3 for F1, F2, F3, V1, V2, V3, L1, and L2; N = 10 for mix). The error bars represent standard error of the mean.

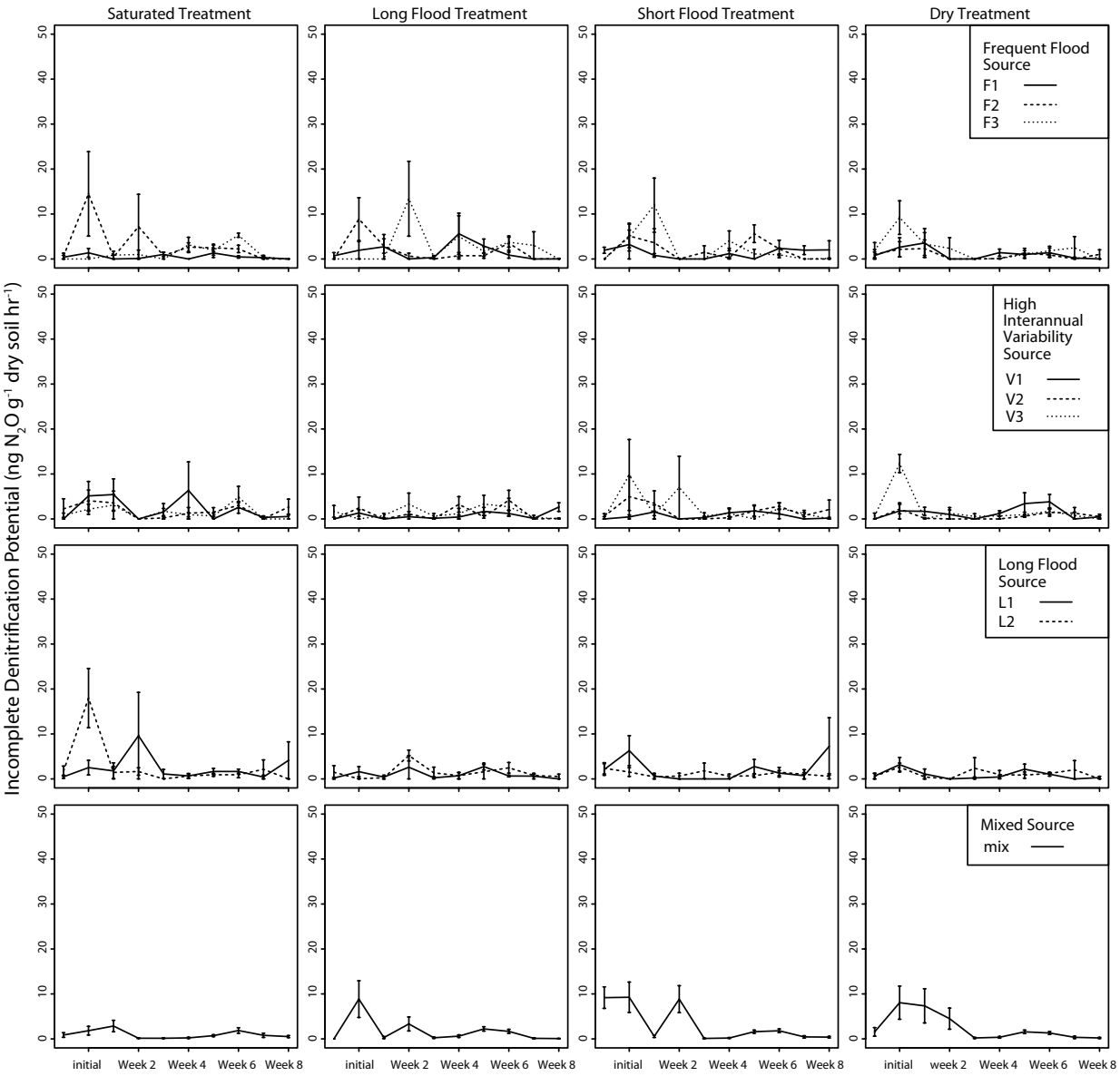

Figure D.9. Rates of N_2O released during potential denitrification assays (i.e. "incomplete" potential denitrification). The means were calculated from replicate mesocosms each week (N = 3 for F1, F2, F3, V1, V2, V3, L1, and L2; N = 10 for mix). The error bars represent standard error of the mean.

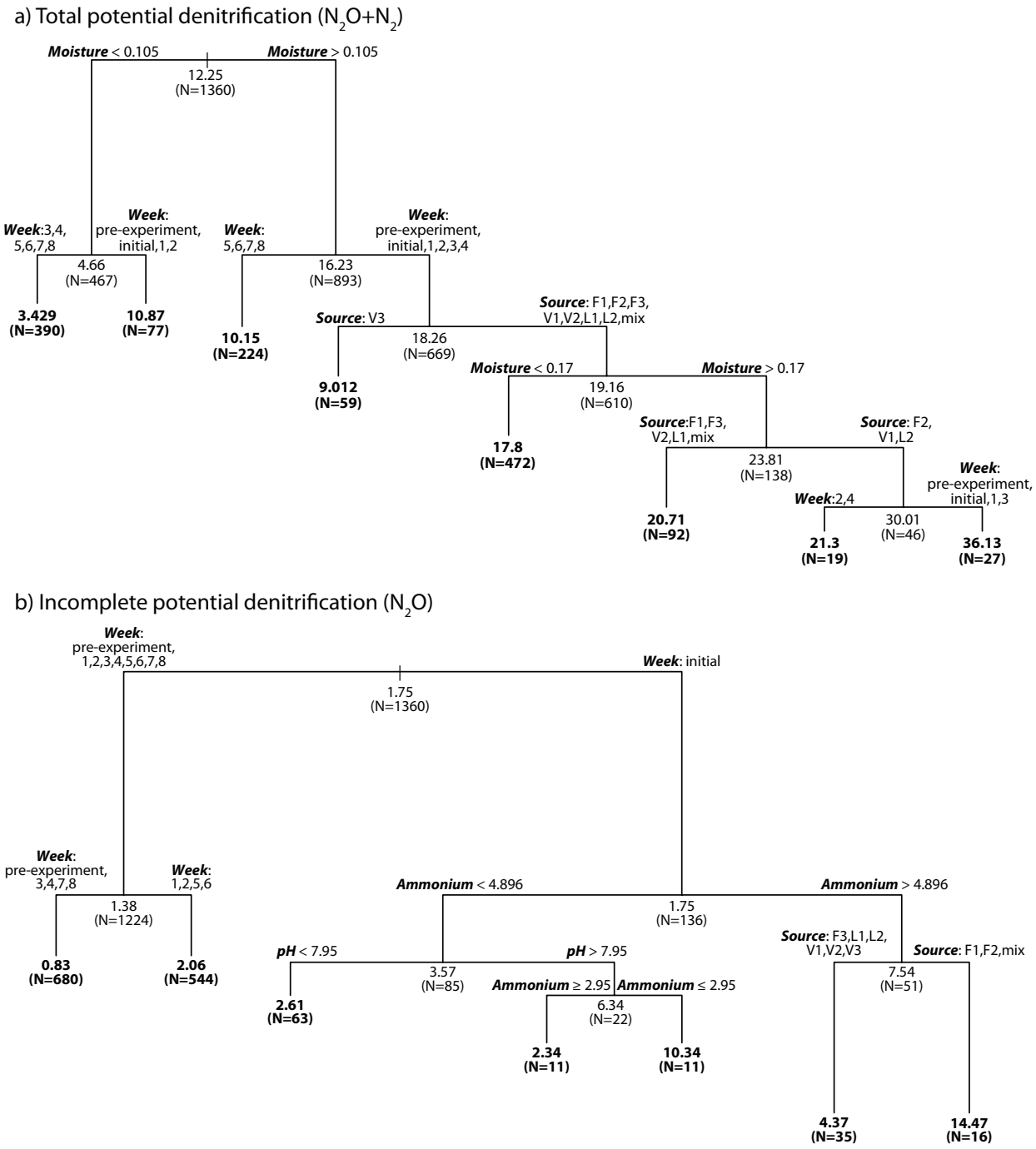

Figure D.10. Categorical and regression tree (CART) analyses of (a) total potential denitrification rates and (b) potential N2O emissions (incomplete denitrification).

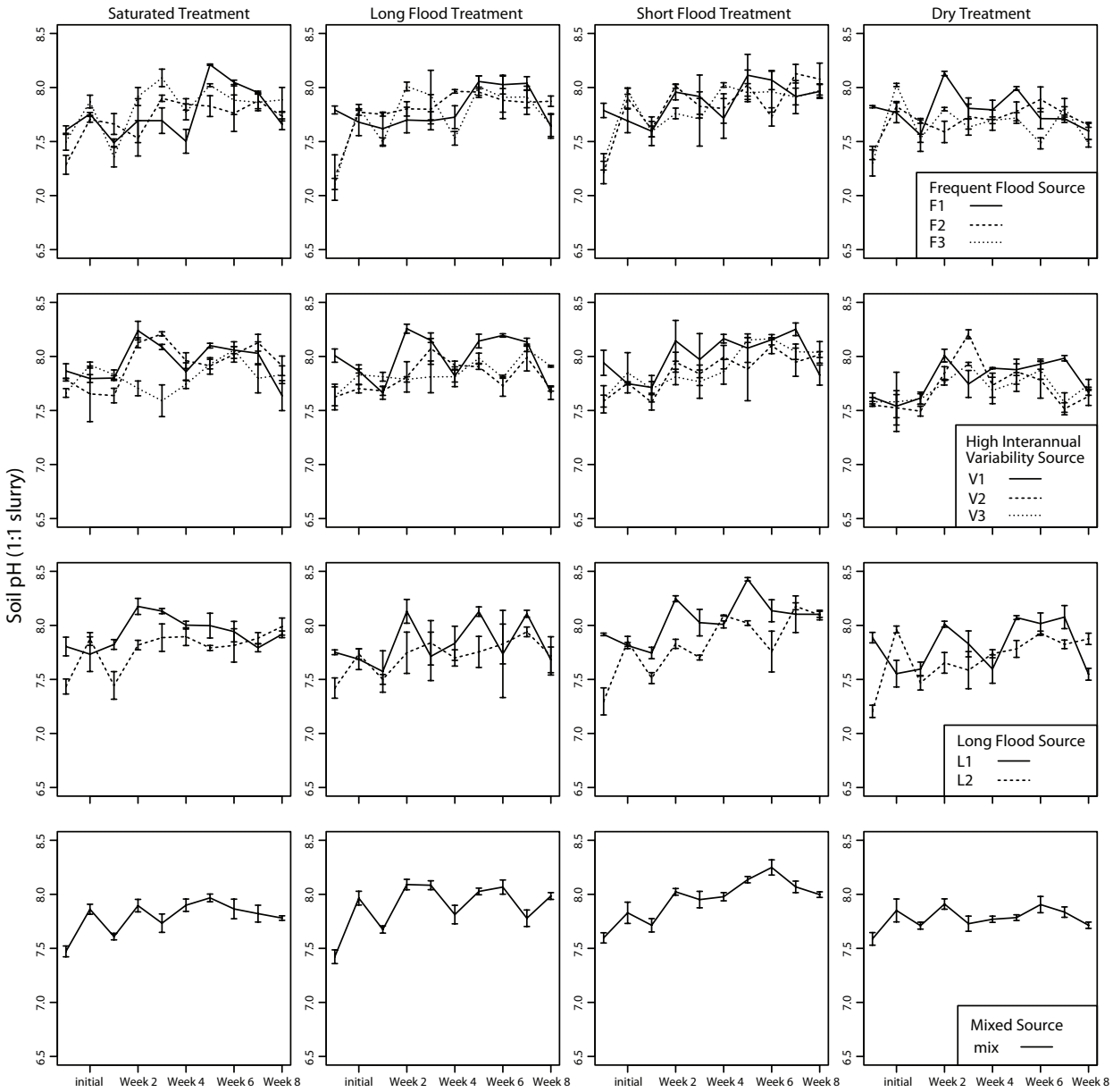

Figure D.11. Soil pH of experimental mesocosms over time. The means were calculated from replicate mesocosms each week (N = 3 for F1, F2, F3, V1, V2, V3, L1, and L2; N = 10 for mix). The error bars represent standard error of the mean.

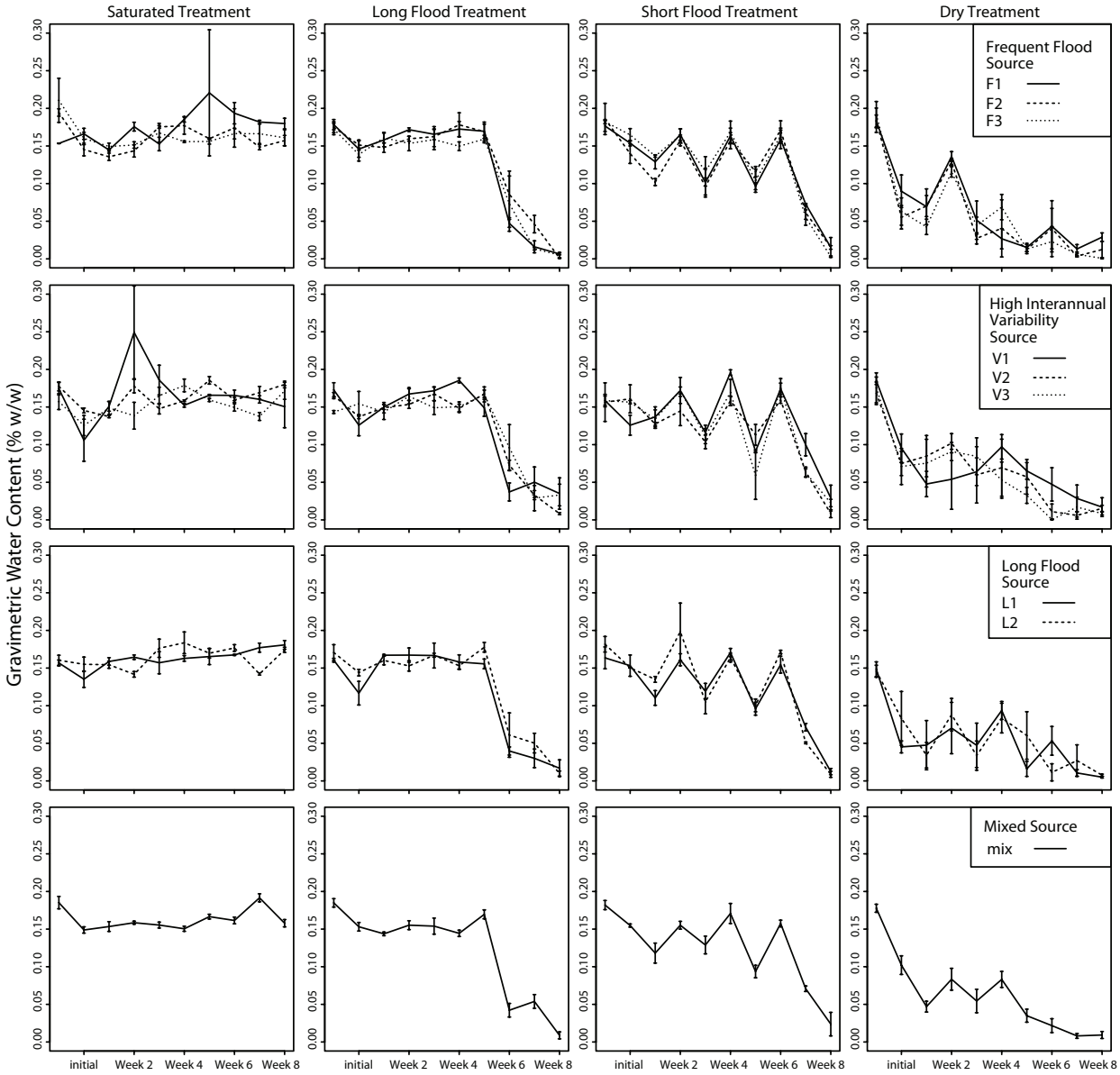

Figure D.12. Soil moisture of experimental mesocosms over time. The means were calculated from replicate mesocosms each week (N = 3 for F1, F2, F3, V1, V2, V3, L1, and L2; N = 10 for mix). The error bars represent standard error of the mean.

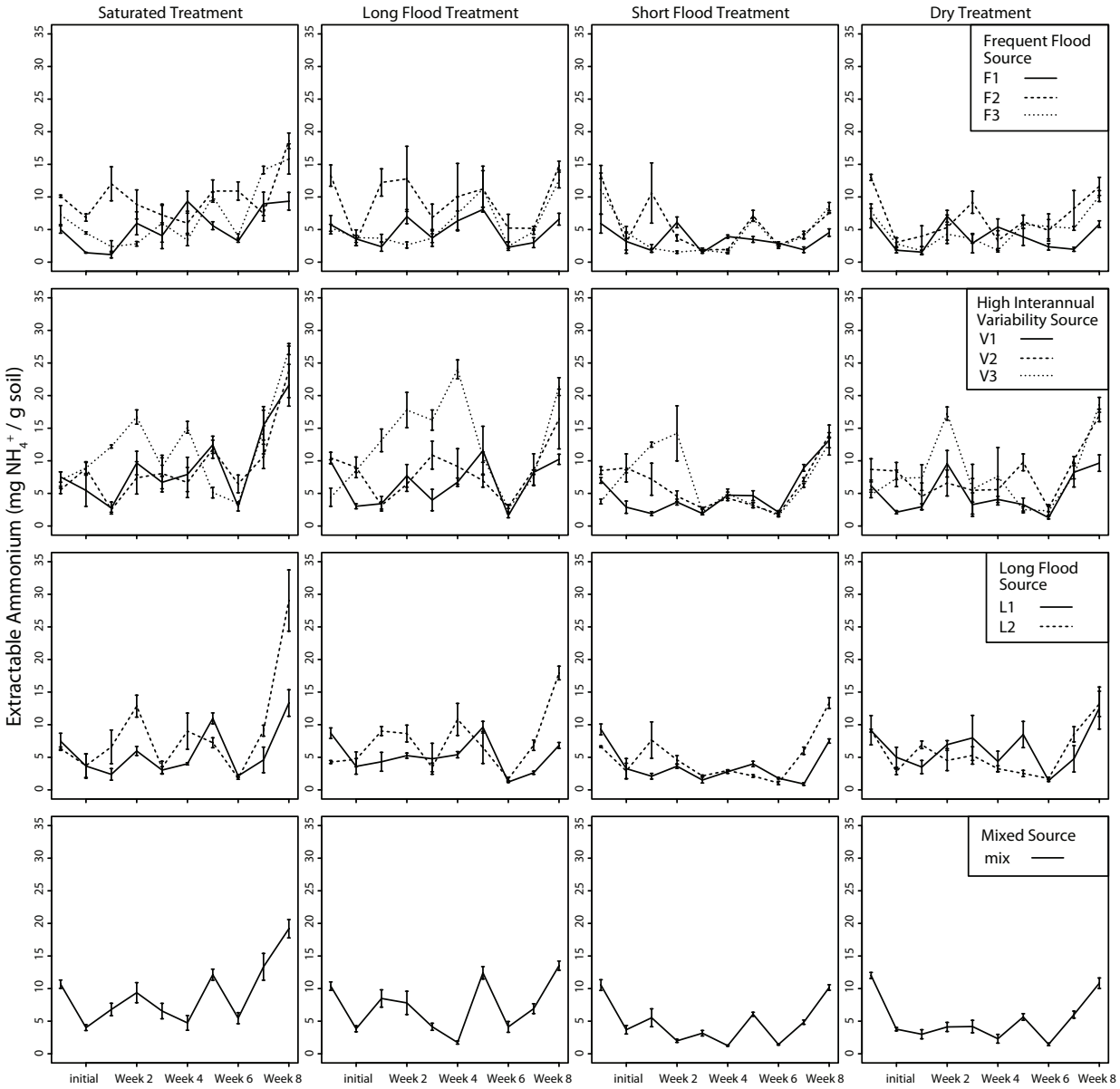

Figure D.13. Soil ammonium of experimental mesocosms over time. The means were calculated from replicate mesocosms each week (N = 3 for F1, F2, F3, V1, V2, V3, L1, and L2; N = 10 for mix). The error bars represent standard error of the mean.

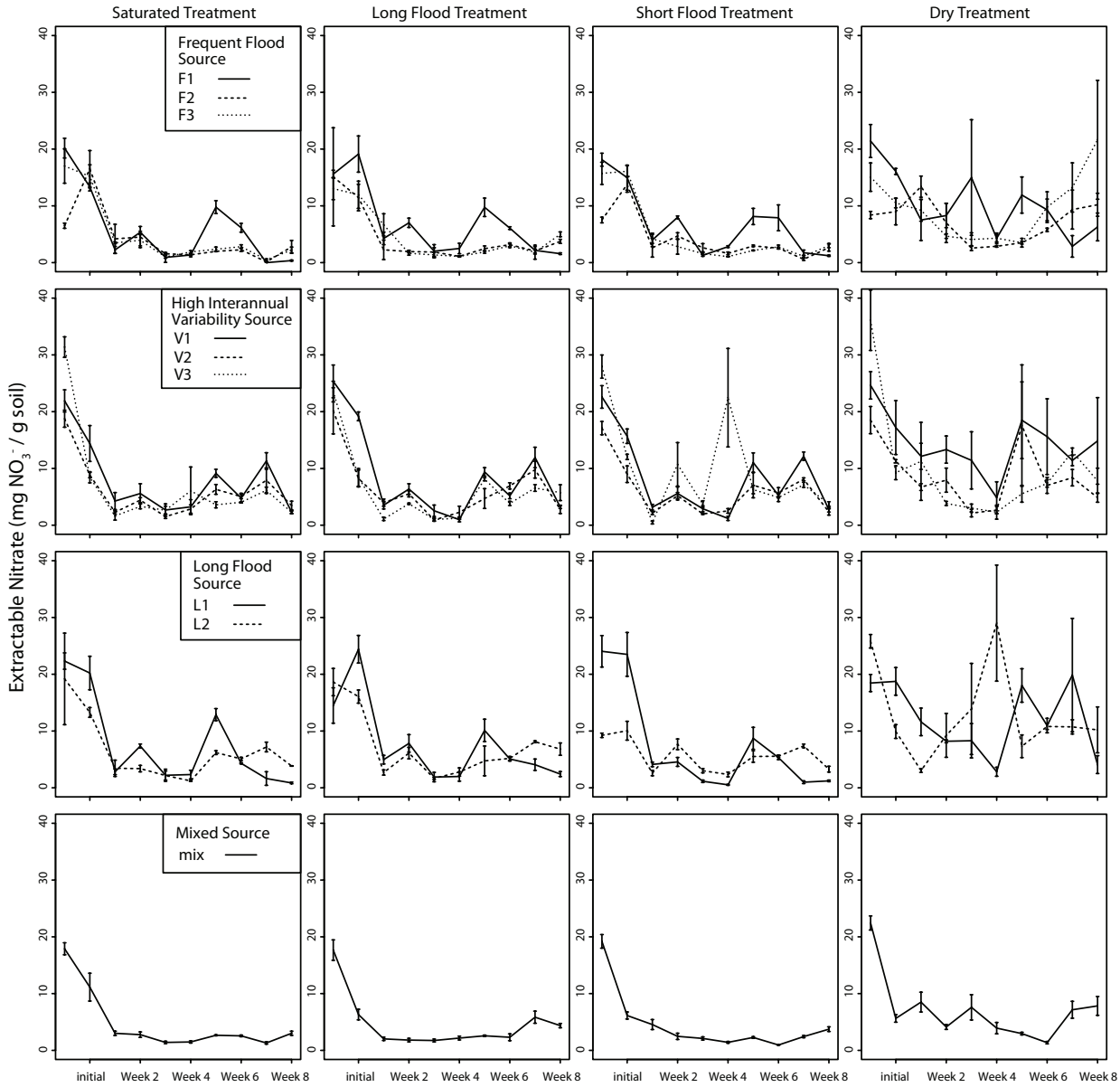

Figure D.14. Soil nitrate of experimental mesocosms over time. The means were calculated from replicate mesocosms each week (N = 3 for F1, F2, F3, V1, V2, V3, L1, and L2; N = 10 for mix). The error bars represent standard error of the mean.

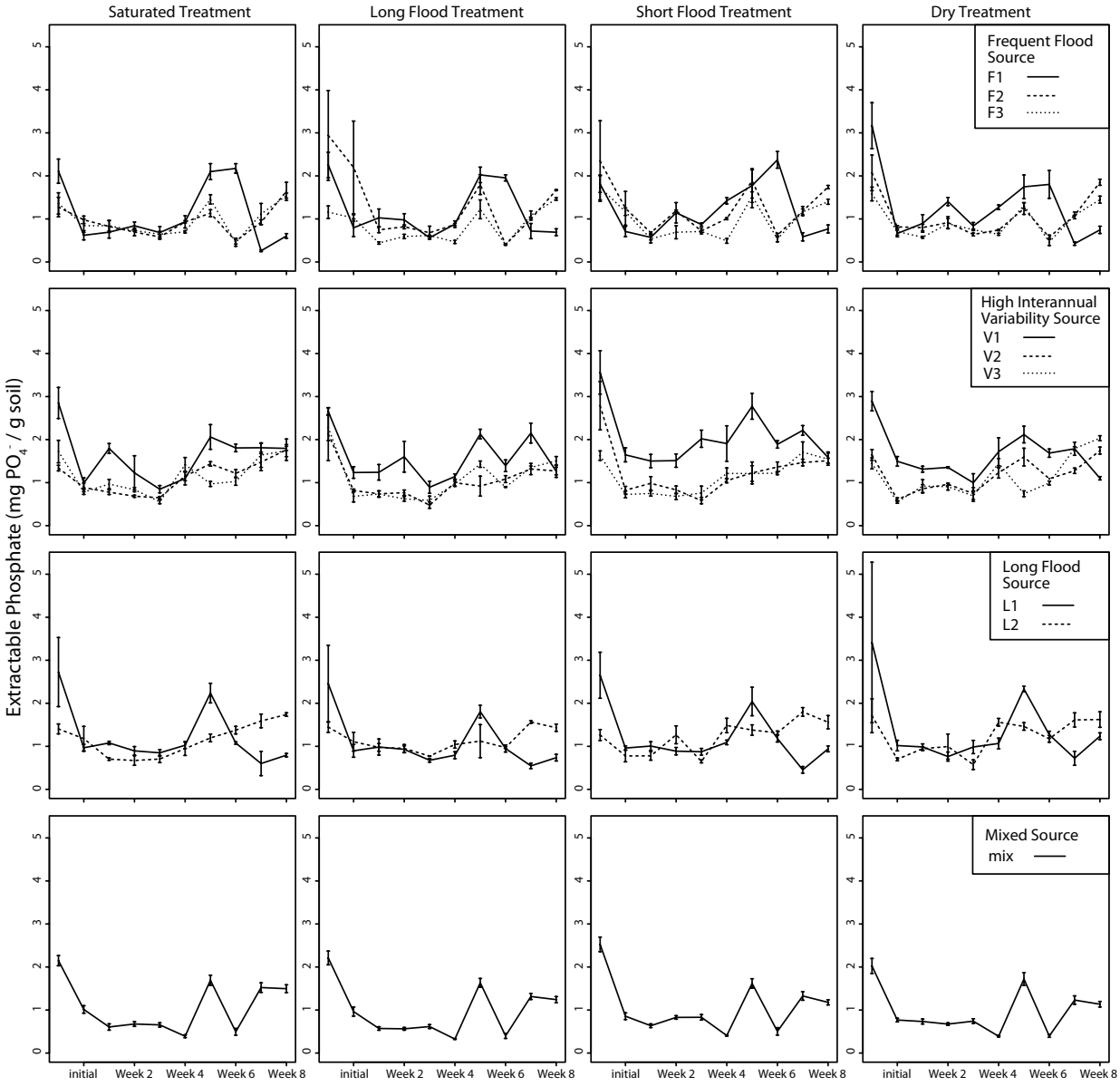

Figure D.15. Soil phosphate of experimental mesocosms over time. The means were calculated from replicate mesocosms each week (N = 3 for F1, F2, F3, V1, V2, V3, L1, and L2; N = 10 for mix). The error bars represent standard error of the mean.

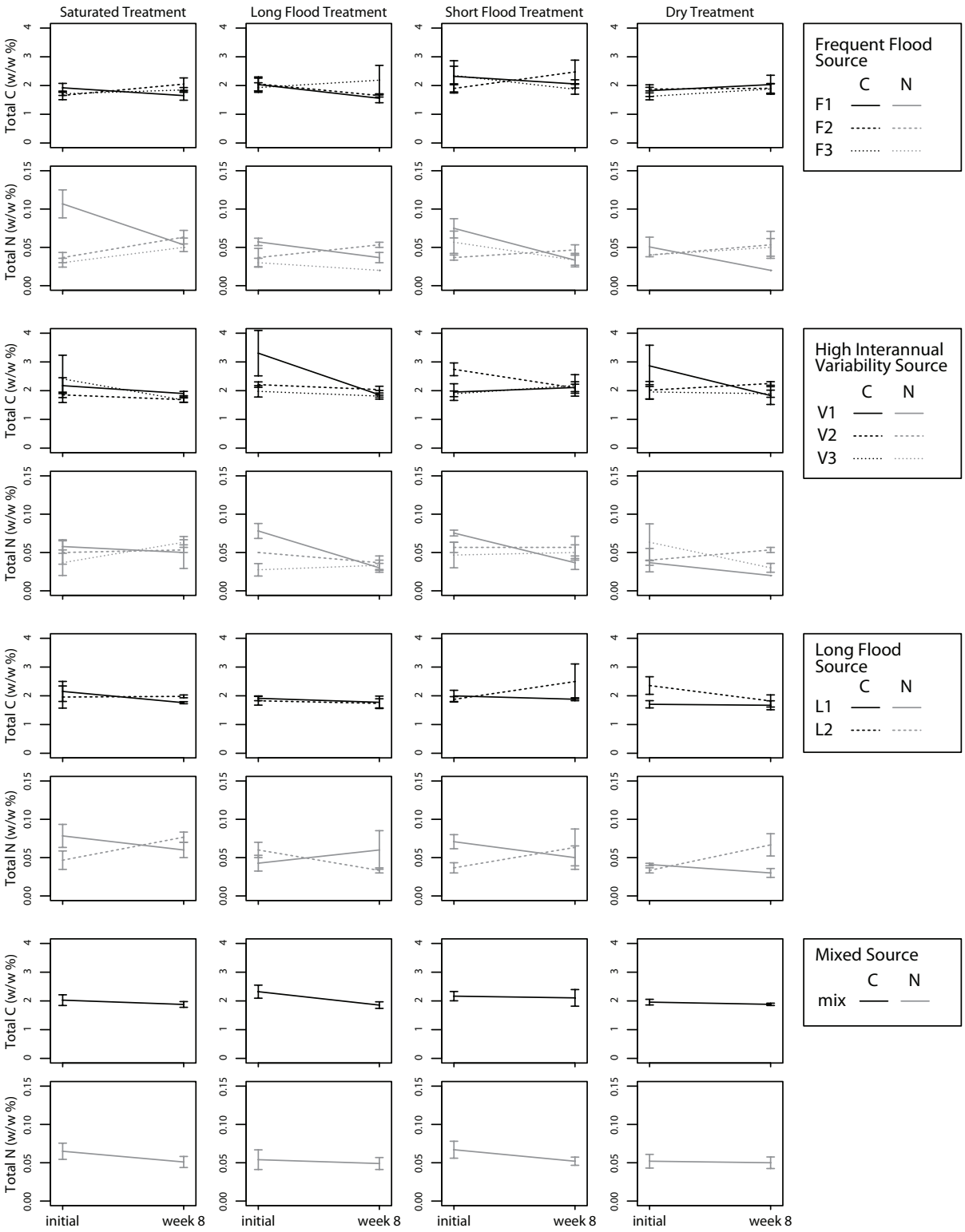

Figure D.16. Total soil carbon and nitrogen in the inoculated mesocosms at the beginning of the experiment (sampling event: "initial") and at the end (sampling event: "week 8"). The means were calculated from replicate mesocosms each week (N = 3 for F1, F2, F3, V1, V2, V3, L1, and L2; N = 10 for mix). The error bars represent standard error of the mean.