PERFORMANCE, DENITRIFICATION ACTIVITY, AND MICROBIAL COMMUNITY DYNAMICS OF DENITRIFYING BIOFILTERS UNDER FLUCTUATING WATER LEVEL

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Environmental Engineering in Civil Engineering
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

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Abstract

Agricultural runoff through tile drainage systems is a significant source of nitrogen to coastal water bodies, causing water quality degradation. Denitrifying biofilters have been identified as a technology that reduces the export of nitrate from tile-drained fields. A better understanding of the microbial communities that allow the biofilter to function may provide insight to improve performance.

Two studies were performed to achieve this aim. Laboratory-scale biofilters were operated for two years with variations in water level. Nitrate removal performance was measured. Denitrifying enzyme assays (DEAs) were performed regularly to measure the denitrification potential in different parts of the two reactors. A comparison of the microbial communities of agriculture, natural wetland, restored wetland, and biofilter habitats was also carried out to determine the relative effects of niche and legacy on the structure of the microbial communities. For both studies, total bacterial communities were analyzed using automated ribosomal intergenic spacer analysis (ARISA), and denitrifying bacterial communities were analyzed using terminal restriction fragment length polymorphism (tRFLP) of *nosZ*, the gene encoding the catalytic subunit of nitrous oxide reduction.

In the laboratory biofilter study, the performance was sensitive to changing water level, with 31% nitrate removed at high water level and 59% at low water level. The denitrification potential was the same under almost all conditions, ranging from 0.000015-0.004 mg N_2O-N/hour/dry g woodchip; the microbial communities were still able to carry out denitrification, unaffected by the water level, unless dried out for an extended period of time. The denitrifying bacterial communities were not significantly different from each other, regardless of water level. This indicates resistance of these bacteria, meaning that the bacterial communities did not change

in response to disturbance. The total bacterial communities became more distinct between the two reactors once the regular disturbance period began, with a stronger effect on the most severely disturbed port. The communities were not distinguishable based on high or low water level, though, in the same place during the regular disturbance period, indicating that disturbance communities were created, rather than high or low water level communities.

In the habitat comparison, the biofilter microbial communities were distinct from those of the other habitats. This was true for both the total and denitrifying bacterial communities. This suggests a stronger influence of environmental parameters on the microbial community structure than the legacy effects of the agricultural bacteria that were initially present and continued to enter with the influent water.

These results show the biofilters to be stable systems with resistant and functionally redundant bacterial communities. Water level and HRT should be considered in the design of the biofilters, as they influenced the nitrate removal. Overall, the biofilters show promise as a method to reduce the amount of nitrogen pollution from agricultural fields to surface water.

Acknowledgements

I would like to thank my advisors, Dr. Julie Zilles and Dr. Angela Kent. I would like to express my gratitude to Dr. Zilles for her excellent feedback on my writing and managing my time and for challenging me to think carefully about the questions I'm asking and how best to answer them. I am appreciative of Dr. Kent for introducing me to microbial ecology and the lab techniques and multivariate statistics that go with it, as well as for her never-ending enthusiasm.

Thanks must also go to the rest of the biofilter group: Dr. Luis Rodríguez, for his helpfulness and feedback in biofilter meetings. Graham Kent helped me in the field. I am grateful to Dora Boyd Cohen for good discussions about our work and also, for her friendship. Paul Choi put in frequent hard work to help me in lab. Nick Bartolerio helped me get on my feet in lab with the biofilters, and he and Matt Porter were always helpful and willing to share information with me after leaving Urbana-Champaign.

The members of the Kent and Zilles labs have been wonderful colleagues, and, in particular, Sara Paver and Jason Koval helped me as I learned new lab techniques. Corey Mitchell has also been wonderfully helpful when I was using the GC.

I would also like to express my gratitude to my wonderful friends and family for all their support. Thanks especially to Adrian, for his patience, encouragement, and willingness to bring dinner during late nights in lab.

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Chapter 1: Introduction

Human activity has caused radical alteration to the global nitrogen and phosphorus cycles. In particular, the widespread use of synthetic fertilizer in modern farming has increased nutrient loads to water bodies. The rate these nutrients are transported from land to ocean has more than doubled compared to the non-anthropogenically influenced rate (Howarth et al, 2000).

Excess nutrient loading in water bodies is a major cause of water quality degradation. Fertilization of surface water encourages the growth of excess algae. The eventual decomposition of the algae depletes dissolved oxygen in the water, creating hypoxic zones. This can lead to fish kills, which are particularly problematic in hypoxic areas with significant fishing industries, like the Chesapeake Bay and Gulf of Mexico. Some of the algal blooms are made up of toxic red tide organisms, which result in fish kills, and in some cases, human poisoning by shellfish and death of marine mammals. Algal blooms also alter the environment by changing the light patterns in the water. All these changes in the aquatic habitat favor different species than are naturally present and can result in biodiversity losses.

Although the chemistry of different water bodies varies, a review of research on nutrient limitation in coastal marine ecosystems suggests that many coastal ecosystems where hypoxia is a problem are nitrogen-limited (Howarth & Marino, 2006). Thus, it is important for efforts to reduce nutrient pollution to focus on preventing the release of excess nitrogen into coastal ecosystems.

One of the sources of nitrogen in these ecosystems is agricultural runoff. Tile drain usage is widespread in the agricultural Midwest, and subsurface runoff from fertilized agricultural

fields with tile drainage is a major source of nitrate in the Mississippi River Basin, which discharges to the Gulf of Mexico (David et al, 2010). In central Illinois, twelve year average nitrate losses from tile-drained agricultural fields were 23 to 33 kg N/ha, with average nitrate concentrations from 15 to 20 mg N/L in subsurface drains (Kalita et al, 2006), higher than the EPA maximum contaminant level for drinking water (10 mg/L).

Agricultural runoff poses treatment challenges, because it is a dispersed source of pollution and cannot easily be diverted to a centralized treatment plant. Treatment technologies must be sufficiently small-scale and low-maintenance to be employed next to cropped fields or runoff-receiving bodies. Methods to decrease nitrogen export in runoff include biological treatment systems like constructed wetlands (Carleton et al, 2001), decreased fertilizer use, and slowing the flow of runoff with buffer strips (Osborne & Kovacic, 1993, Gopalakrishnan et al, 2012) or drainage ditches.

Another such option is edge-of-field denitrifying biofilters. These biofilters consist of a trench of woodchips through which tile drainage is directed. The woodchips provide a filter media on which bacteria can grow as well as a carbon source for denitrification. The biofilters provide an environment in which denitrification can occur, reducing the nitrate load carried by the tile drainage water to surface water. Denitrifying biofilters have been implemented with success in Illinois (Wildman, 2002, Woli et al, 2010), Iowa (Jaynes et al, 2008), Ontario (Blowes et al, 1994, van Driel et al, 2006), and New Zealand (Schipper et al, 2010a).

Although the biofilters have been shown to remove nitrate from subsurface agricultural runoff, there is some inconsistency in performance. Anecdotally, problems have included variable performance for the first year of start-up, poor performance at peak flows, and a lag in performance after flow resumes following dry periods. Although the poor performance at peak

flows is likely related to residence time, the other issues could likely be related to the microbial communities in the biofilters. Microbial communities within biofilters have only been minimally investigated, and further study would provide insight into the function and structure of the microbial communities that enable the biofilter to remove nitrate. This knowledge would be helpful in improving biofilter design and start-up

Previous work has shown that the microbial communities of full-scale, field biofilters correlate with season and depth within the reactor, driven by temperature and moisture gradients (Porter, 2011). To study this further, the first part of this project examines the effect of changing water level on the performance of the biofilters, their microbial community composition, and denitrification potential. Two laboratory-scale reactors were used in this study for ease of sampling and control of operating conditions.

Engineered ecosystems are dependent on the presence and functioning of beneficial organisms. Denitrifying biofilters require microbes to reduce nitrate to dinitrogen gas, so the second part of this project checks the success of developing these microbial communities in biofilters. The total and denitrifying bacterial communities of field biofilters were compared with those of agricultural fields and natural and restored wetlands. Agricultural fields and wetlands were chosen, because they represent, to a degree, the starting microbial communities, in the case of the agricultural fields, and the target environment, in the case of the wetlands. This study looks at the relative importance of niche and legacy effects on the composition of bacterial communities and tests success in encouraging the growth of the desirable microbial communities in engineered ecosystems. Many other engineered ecosystems are also dependent on the establishment of the necessary microbial communities, so this study has broader implications regarding the ability of humans to engineer self-sustaining biological systems.

The next chapter of this thesis will review literature on the design and performance of denitrifying biofilters, the denitrification process and the factors that affect it, and the effect of legacy versus current environmental conditions on microbial communities. Then, chapter three will detail the methods used for both parts of this project. The results from the lab reactor study will be presented after this in chapter four, and then the results from the study comparing microbial communities from different habitats in chapter five. Chapter six will discuss the results, and finally, chapter seven will give conclusions and make recommendations for future work.

Chapter 2: Literature Review

2.1 Denitrification

Denitrification is the microbial conversion of nitrogen oxides to dinitrogen gas. Bacteria use nitrate, nitrite, nitric oxide, and nitrous oxide as terminal electron acceptors for their metabolic reactions. The denitrification pathway is mapped in Figure 1.

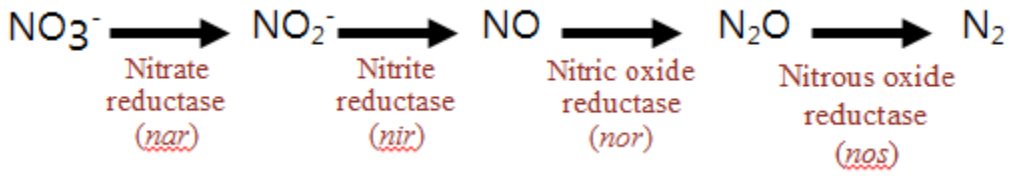


Figure 1. Pathway for denitrification from nitrate to dinitrogen gas, including the necessary enzymes for each step.

Enzymes have been identified that carry out each step of the process and are reviewed by Philippot (2002). The nitrate reductases are Nar, which is membrane-bound, and Nap, which is periplasm-bound. Nir has been identified as a nitrite reductase. The nitric oxide reductase complex has two subunits encoded by the *norB* and *norC* genes. The nitrous oxide reductase is encoded by the *nosZ*, with a necessary regulatory protein encoded by the *nosR* gene and other necessary proteins by the *nosDFYL* genes.

These functional genes are used in studies of microbial communities of denitrifiers, with *nirK* and *nirS* as the most commonly targeted genes for this (Wallenstein et al, 2006). Many bacteria that cannot carry out other steps of denitrification can reduce nitrate, such as for the dissimilatory reduction of nitrate to ammonia. Thus, the *narG* and *napA* genes are not widely used in studies of denitrifiers (Wallenstein et al, 2006). *NosZ* is also a widely used molecular

marker for investigating denitrifier communities, partly because nitrous oxide reductase catalyzes the final step of the denitrification pathway to inert N_2 (Wallenstein et al, 2006). However, several atypical *nosZ* genes have been found that are not accounted for with common *nosZ* primers, but are capable of nitrous oxide reduction (Sanford et al, 2012).

A phylogenetically diverse set of organisms have been observed to be able to carry out denitrification. Most denitrifiers are facultatively anaerobic heterotrophs, although a number of autotrophs have been observed to denitrify (Zumft, 1997). Several extremophiles have also been identified as denitrifiers. Among bacteria, denitrifying capability has been found in the Actinobacteria, Aquificae, Bacteroidetes, Firmicutes, and Proteobacteria phyla (Zumft, 1997). There are a few Archaea that are denitrifiers (Zumft, 1997). Fungi have also been observed to carry out denitrification, specifically yeasts, filamentous fungi, and many Fungi Imperfecti (Zumft, 1997).

Several studies have examined the environmental conditions that control denitrification. In their review, Wallenstein et al (2006) categorize these controls as either distal or proximal, where distal controls influence the composition and diversity of the denitrifier communities and proximal controls influence the instantaneous denitrification rates. Distal controls are determined by comparing environmental parameters to microbial communities, measured by either gene sequencing or DNA fingerprinting methods. Proximal controls are determined by comparing environmental parameters to denitrification rates measured by assays. Proximal controls on denitrification include oxygen availability, carbon availability, pH, temperature, and nitrate availability. Distal controls include carbon availability, temperature, moisture, oxygen availability, pH, predation, and disturbances. The averages and variability of these controls are

also important, in that a consistent temperature is different from a highly variable temperature, even if the averages are the same.

A consensus has not been reached regarding the relation between denitrifier community and denitrification enzyme activity. Several studies have found a link between community composition and denitrification activity. For example, a study comparing the denitrifier communities and denitrification rates of two geomorphically similar soils found the different communities to have different denitrification rates under identical incubation conditions as well as different sensitivity to pH and oxygen (Cavigelli and Roberston, 2000). Similarly, Holtan-Hartwig et al (2000) compared the denitrification rates of soils from three agricultural fields under identical laboratory conditions, and found different kinetics for each soil, also suggesting that the communities influence the denitrification rates. A study of denitrification in streams in an agricultural area found *nosZ* gene abundance and T-RF (terminal restriction fragment) number, determined by redundancy analysis, to be secondary controls on the denitrification rate in the streams (Baxter et al, 2012).

Other studies, though, have found denitrification rates to be independent of the denitrifying community composition. A study comparing denitrification activity and *nosZ* communities in agricultural soil, riparian soil, and creek sediment found that, although there were differences in both community composition and nitrous oxide reducing activity, they appeared uncoupled (Rich and Myrold, 2004). In a reciprocal transplant study, Boyle et al (2006) a change in denitrification rates after transplanting did not correspond to a change in microbial communities. Ma et al (2008) were unable to find a link between denitrifying (*nosZ*) communities from cultivated and uncultivated wetlands and nitrous oxide production in laboratory studies.

While some studies found a coupling between denitrifier communities and denitrifying activity, and some did not, it is not clear what determines the difference. Biofilters are attempting to replicate ecosystem services provided by wetlands, and study of wetlands is likely the most similar environment to the biofilters. This suggests that a change in community will not independently bring about a change in activity. Both Rich et al (2003) and Boyle et al (2006) compared denitrifier communities and activity in forest and meadow soils in Oregon's Western Cascade Mountains, though, with opposing conclusions in this regard. Therefore, it does not appear that the correlation or lack thereof between denitrifier community structure and denitrification activity in one environment will be a good predictor of the extent of the correlation in a similar habitat.

2.2 Denitrifying Biofilters

Several technologies have been proposed to biologically remove nitrate from water leaving agricultural fields. These technologies include vegetated buffer strips (Osborne & Kovacic, 1993, Gopalakrishnan et al, 2012), constructed wetlands (Carleton et al, 2001), denitrification walls (Robertson & Cherry, 1995, Schipper & Vojvodic-Vukovic, 1998), and denitrifying biofilters (Wildman, 2002, van Driel et al, 2006, Jaynes et al, 2008). In the literature, denitrifying biofilters are also referred to as denitrification beds, and the term denitrifying bioreactors includes both denitrifying biofilters and denitrification walls. Denitrification walls treat groundwater flowing through them, whereas biofilters have influent and effluent pipes that the water flows through.

This literature review will include studies on the effectiveness of field-scale biofilters, their design parameters, long-term performance, microbial communities, and greenhouse gas emissions. The results of many studies on denitrifying walls and biofilters, particularly laboratory studies, can give insight about the other type of system, so this biofilter-focused review includes relevant studies on denitrification walls.

2.2.1 Biofilter Effectiveness

Several studies have looked at the effectiveness of denitrifying biofilters under field conditions and found them to reduce the amount of nitrate exported from agricultural fields. As reviewed by Schipper et al (2010b), sustained nitrate removal rates under field conditions vary from 2-22 g $N/m^3/d$, with differences primarily attributed to temperature. A five year comparison between a denitrifying biofilter, a deep tile drain system, and a conventional drainage system showed the bioreactor treatments to have an average nitrate concentration of 8.8 mg N/L, whereas the conventional system had 22.1 mg N/L and the deep tile system had 20.5 mg

N/L (Jaynes et al, 2008). This is a 55% reduction in nitrate load from the biofilter system compared to the conventional one, without a difference in crop yields. Woli et al (2010) monitored a nitrogen budget over two to three years for a free drainage tile system and a controlled drainage tile system equipped with a denitrifying biofilter and observed an average nitrogen loss of 17 kg N/ha/year from the field with the biofilter, compared to 57.2 kg N/ha/year from the free drainage system. These results are encouraging and show that denitrifying biofilters bring about a substantial reduction in nitrate export from agricultural fields. Greater understanding of the factors influencing biofilter performance, though, could increase nitrate removal even further.

2.2.2 Biofilter Design

Much of the research on denitrifying biofilters to date has focused on engineering design parameters. Specifically, studies have attempted to determine the best shape and layout for the biofilters, the hydraulic residence time necessary for good performance, and the optimal filter media.

A comparison of rectangular, channel-shaped, and trapezoidal pilot-scale bioreactors suggests that the channel-shaped biofilter may be preferable (Christianson et al, 2010). Little difference in nitrate removal of rectangular, channel-shaped, and trapezoidal pilot-scale biofilters was observed, but the channel bioreactor seemed to have the most consistent performance. Based on his work with field-scale biofilters in central Illinois, Wildman (2002) recommends that for a biofilter managing a 30 hectare drainage area, pipes into and out of the biofilter extend at least six meters into the media, so that water volume entering the reactor is not limited.

Effective nitrate removal has been observed in studies with hydraulic residence time (HRT) ranging from a few hours to almost ten days. Doheny (2003) suggests a HRT of ten hours to reduce nitrate in tile drainage with 20-30 mg/L NO_3-N to below the EPA limit of 10 mg/L. Greenan et al (2009) also compared bioreactor performance at HRTs of 2.1, 2.8, 3.7, and 9.8 days, but used influent nitrate concentration of 50 mg NO^3-N/L, much higher than typically seen in Illinois tile drainage. At the lowest HRT tested, 2.1 days, a 14.7 mg/L decrease in nitrate-N concentration was observed, which would decrease the tile drainage concentrations observed in central Illinois to below the EPA limit. In a study of three pilot-scale biofilters, observed HRTs were between 1.5 and 2.2 times the theoretical HRTs (Christianson et al, 2011). The channel-shaped reactor behaved as a plug-flow reactor, and the rectangular and trapezoidal reactors had higher dispersion. 30-70% nitrate removal from tile drain water with HRTs from 4 to 8 hours was observed.

Several studies have examined different sources of organic carbon to determine the best filter media for the denitrification walls and denitrifying biofilters. Pine woodchips, eucalyptus woodchips, sawdust, corn cobs, wheat straw, and green waste were compared in a 23 month laboratory study, in which corn cobs were associated with the highest nitrate removal rate: 19.8 g N m^{-3} d^{-1} and 15.0 g N m^{-3} d^{-1} at 14^{o}C and 23.5^{o}C, respectively (Cameron and Schipper, 2010). Ammonium and total Kjeldahl nitrogen were leached from the bioreactors with corn cobs longer than from the bioreactors with other media. A follow-up study (Warneke et al, 2011) also found the corn cob treatments to release high concentrations of methane. They suggest that this could be because the corn cob treatments may be nitrate, rather than carbon limited, allowing methanogenesis to better compete against denitrification. Although Cameron and Schipper (2010) found corn cobs to be associated with the highest nitrate removal, Cooke et al (2001)

found woodchips to have superior nitrate removal than corn cobs, possibly due to the greater surface area of the woodchips. Batch tests comparing softwood, hardwood, coniferous, mulch, willow, compost, and leaves as carbon sources for denitrification found that softwood was associated with the most efficient denitrification without leaching nitrogen, with hardwood and coniferous sources also performing well (Gibert et al, 2008). Similarly, Greenan et al (2006) compared woodchips, woodchips saturated with soybean oil, dried cornstalks, and paper fibers from corrugated cardboard as a carbon source for denitrification in 180 day batch tests. They found cornstalks to support the highest degree of nitrate removal, followed by cardboard fibers, woodchips with oil, and then woodchips alone, but that the nitrate removal rates were steady with woodchips, and would likely continue longer than with cornstalks. The soybean oil increased the nitrate removed by 30-70% compared to the woodchips alone. Other media, including wheat straw (Soares and Abeliovich, 1998) and low quality cotton (Volokita et al, 1996) have also been found effective as a carbon source for denitrification of groundwater, but have been rapidly degraded.

Comparisons between media of different size shows no difference in nitrate removal rates for field (van Driel et al, 2006) or laboratory reactors (4-61 mm pine chips) (Cameron and Schipper, 2010).

2.2.3 Long-term Results

Some studies have observed bioreactors over several years to determine their long-term performance and the longevity of the media. Long-lasting media is important, because it results in lower maintenance on the biofilter, thus encouraging their successful implementation. Moorman et al (2010) observed sustained denitrification potential and nitrate removal from a

field biofilter operated for nine years. After that time period, one meter below the surface, 25% of woodchips remained, and deeper in the biofilter, at least 80% remained. A denitrification wall with soil-sawdust media was removing 90% of nitrate from the influent after 14 years, and approximately half of the carbon remained (Long et al, 2011). At this rate of sawdust degradation, the total carbon in the denitrification wall would not be fully depleted for 66 years, although that does not indicate when denitrification would become limited by insufficient accessible carbon. In a sawdust and sand denitrification wall treating a septic system plume with concentrations as high as 100 mg/L NO_3-N, denitrification rates measured during the 15^{th} year of operation were about 50% of those measured in the first year (Robertson et al, 2008). Together, these studies suggest that the longevity of denitrifying biofilters likely depends on local factors, such as influent nitrate loading, but they can reasonably be expected to remove nitrate for ten years or more.

2.2.4 Microbial Communities

Microbial communities in denitrifying biofilters have been studied in both field and lab biofilter environments, but many questions in this area remain unanswered. Porter (2011) performed a two year temporal study of total bacterial, total fungal, and denitrifying bacterial communities in three field biofilters. He found the communities to be strongly influenced by depth and season and that the depth differences were driven by temperature and moisture gradients. He observed bi-annual seasonal variation in samples, with the greatest distinction between samples from January-June or July-December. A spatial study of total and denitrifying bacteria in an L-shaped biofilter was also performed (Andrus, 2011, Porter, 2011). Total bacterial communities were found to vary by depth and along the flow path, but not orthogonal to flow-path, but denitrifying bacteria were not found to vary greatly throughout the biofilter.

Additionally, a third study looked at the abundance and relative proportions of total bacteria (16S), nitrite-reducing bacteria (*nirS* and *nirK*), and nitrous oxide-reducing bacteria (*nosZ*) after 2.5 years in continuously operated laboratory-scale bioreactors with several different filter media (Warneke et al, 2011). They found pine woodchips to have the lowest denitrification gene abundance and corn cobs to often have the highest, and that the nitrate removal rate increases exponentially with nitrite reductase gene abundance. The woodchips, though, had communities with a higher proportion of nitrite reductase genes making up the total bacterial communities. They also observed a higher ratio of nitrite reductase genes to nitrous oxide reductase genes at higher temperatures.

2.2.5 Greenhouse Gas Production

Nitrous oxide, an intermediate in the denitrification pathway, is a potent greenhouse gas (Forster et al, 2007). There is concern that attempts to remove nitrate using denitrification could trade a nutrient pollution problem for a climate change one (Groffman et al, 2000). Some studies have included nitrous oxide measurements in biofilter effluent to be aware of unintended consequences on air quality. In a study with several laboratory scale bioreactors, nitrous oxide production accounted for only 0.003-0.028% of the nitrate denitrified, and N_2 was the primary denitrification end product (Greenan et al, 2009). Moorman et al (2010) found lower overall indirect nitrous oxide emissions from the biofilters than from tile drain water not treated in a denitrifying biofilter. Denitrifying enzyme assays (DEAs) have been performed on biofilter samples inhibited and uninhibited by acetylene (Andrus, 2011). Nitrous oxide was below detection limit in the uninhibited DEAs, indicating that almost all the denitrification proceeded fully.

Research on denitrifying biofilters, to date, has shown them to be an effective technique for removing nitrate from agricultural drainage, although further improvements are anticipated. Channel-shaped reactors with woodchip media seem to be the best design for treating tile drainage. Although greenhouse gas production could be of concern, a few studies have shown that biofilters do not significantly increase nitrous oxide emissions (Greenan et al, 2009, Moorman et al, 2010, Andrus, 2011). Studies on the microbial communities of denitrifying biofilters have indicated that depth and season result in different communities and are correlated with temperature and moisture gradients (Porter, 2011).

2.3 Niche-Legacy Effects

Many different factors can have an effect shaping microbial community composition. These factors can generally be divided between "niche" or "legacy" effects. In ecology, a niche has a very specific definition, that "an n-dimensional hypervolume is defined, every point in which corresponds to a state of the environment which would permit the species to exist indefinitely" (Hutchinson, 1957). For this thesis, a more broad definition of niche will be used, that a niche is the set of conditions and resources that encourage or discourage the growth of a species. A community shaped by niche effects has species that have been selected for by the environmental conditions present at that time. Legacy effects have been defined as the "impacts of a species on abiotic or biotic features of ecosystems that persist for a long time after the species has been extirpated or ceased activity and which have an effect on other species" (Cuddington, 2011). A community shaped by legacy effects contains many species that reflect the historical conditions of the ecosystem.

In the case of the biofilters being studied, the influent coming into the reactors is coming from an agricultural field. If the biofilters were colonized and dominated by the microbes common in the agricultural fields, this would suggest that the legacy effects are central in shaping the community structure. The biofilters are designed to have wet, anoxic conditions that encourage denitrification, such as in a wetland. If the microbial communities are similar to those found in wetlands, it would indicate that the niche effects are governing the structure of the biofilter's microbial communities.

It is important to determine whether niche or legacy effects control the composition of microbial communities in engineered systems. The successful establishment and performance of

the biofilters is dependent on the ability of the filter's conditions to encourage the growth of denitrifying bacteria, and, to a degree, to approximate the nitrogen removal function of wetlands.

Although few papers specifically address the question of whether niche or legacy effects dominate in controlling microbial communities, there is a large body of literature that provides insight on this. This literature review attempts to provide a representative sample of study types and results that can shed light on this question. First, reciprocal transplant experiments and common garden experiments will be discussed together, starting with those that suggest legacy effects, followed by those that suggest niche effects. These will be followed by studies comparing the microbial communities of similar ecosystems with different land use histories. The last group of studies to be discussed will be comparisons between communities that have undergone land use change to those that have not been disturbed in this way. Table 1 includes all the papers included in this literature review, as well as a summary of relevant information and conclusions, to facilitate comparisons between papers. A discussion of these papers will conclude this section of the literature review.

Reciprocal transplant studies have been used by several researchers to test the functional redundancy of microbial communities. This type of experiment involves removing organisms from one habitat and growing them in another, where organisms from multiple habitats are all grown separately in each habitat. Similarly, common garden experiments involve growing organisms from multiple habitats under the same conditions. In some cases, the data collected on microbial community composition in these studies can also offer insight about the roles of legacy versus niche in shaping microbial community structure, because the transplanting represents a disturbance in the communities. If communities change from their original composition to become like those grown in the same habitat, this indicates a stronger niche

effect, whereas, if communities remain more like those from the same original habitat, this indicates a stronger legacy effect.

In one reciprocal transplant study supporting the importance of legacy effects, Langenheder et al. (2005) compared bacteria from four different lakes growing in media created from each lake's water. The bacterial communities clustered by medium and by inoculum, indicating that both their current and previous habitat influenced community composition. The new bacterial communities had between 250 and 300 hours to reach their new structure. If given more time, the communities might have converged to be more similar to other communities growing in the same medium. These experiments were closed to migration by outside bacteria.

As a follow up, Langenheder et al. (2006) performed a common garden experiment in which aquatic bacteria from eight sampling sites with different characteristics were incubated under identical conditions. The bacterial communities had low similarity between the inoculum and the community post-incubation. The similarity among the bacterial communities did not increase from the original inocula to the communities after incubation. These results show a stronger effect by the initial community than the growth conditions. Again, it may be relevant that no new bacteria could migrate into the communities, and 10 to 20 days may be insufficient time for the communities to converge.

Another reciprocal transplant experiment was performed by Strickland et al. (2009), in which three different types of leaf litter habitat and bacteria from the habitats were crossed with each other. While both habitat and inoculum showed an effect on the microbial community, the communities on the same litter habitat did not converge by the end of the 300 day experiment.

The communities on the more chemically complex litter habitats came closer to converging than those on the less complex habitats.

In contrast to the legacy effects observed in the previous studies, Ayarza & Erijman (2011) set up activated sludge sequencing batch reactors seeded with varying proportions of different inocula. The reactors were operated in the same manner and received the same influent. The bacterial communities were functionally stable and converged over time. These experiments lasted 40 days (10 solids retention times). These reactors were open to the atmosphere and could receive immigrant populations, so that may be part of why they converged when the other, closed systems did not.

In a reciprocal transplant study showing the importance of niche effects, Griffiths et al. (2008) inoculated two types of sterilized soil with bacteria from their own and each other's environment. After a three month incubation, the communities in the same types of soil converged to be more similar to each other than to the communities inoculated with the same microbial source. These communities were not open to migrant bacteria.

In another reciprocal transplant study, gut microbes from zebrafish and mice were used to inoculate the digestive tracts of other zebrafish and mice (Rawls et al., 2006). In this case, the microbial communities in the gut of a particular species are similar to each other, whether they had grown there since birth or were inoculated from the other species. This study suggests that the habitat is more important than the legacy in shaping the gut communities.

Studies comparing restored and natural habitats can also offer insight about the relative effects of legacy versus niche in the structuring of microbial communities. Peralta et al. (2010) compared bacterial and denitrifier communities in restored and reference wetlands. The bacterial

communities of the natural wetlands clustered together, separate from the restored wetlands, as did the denitrifiers in some cases. This suggests the importance of the legacy of different land use in shaping community composition.

A similar comparison can be made between primary and secondary communities, as in Fraterrigo et al (2006). The microbial communities of forests that had been logged, farmed, or undisturbed 50-75 years prior and since returned to forest were compared. The community structures were different among each type of site, particularly the farmed site. This was not explained by differences in the substrate pool or live biomass, suggesting the historic land use directly influenced the communities. The authors suggest that the farming practices may have altered the fungi community, which showed the strongest differences among site classifications.

In another comparison between primary and secondary communities, Kulmatiski and Beard (2008) decoupled the effects of land use legacy and the presence of native versus non-native plants on soil microbial communities. They sampled soils disturbed by agriculture and nearby undisturbed soils with native or non-native plants growing on them. There were distinct soil communities for each treatment, indicating both niche (native vs non-native plants) and legacy effects. The authors did not address any other potential differences between the disturbed and undisturbed sites which may be the direct causes of differences in community structure.

Another way to look at the relative effects of niche and legacy is to compare the microbial communities of formerly similar ecosystems, some of which have undergone land use changes. Waldrop et al (2000) compared microbial communities in Tahitian pineapple plantations of different ages in different cultivational stages between each other and nearby forest. The plantations were cleared and burned between six and fourteen years prior to

sampling. The microbial communities from the younger and older plantations were more similar to each other than they were to the nearby forests. This suggests a niche effect shaping the microbial communities, rather than the legacy of the previous forest ecosystem.

In a similar set-up, Jesus et al (2009) compared the bacterial communities of formerly forested soil communities with different land use. Cropped, pasture, agroforestry, young and old secondary forest, and primary forest sites were included. The microbial communities from each land use grouped together to varying degrees. They were correlated with different environmental parameters more than site classification, but the initial similarity of the soils present leads the authors to conclude that these differences are land use driven. The communities of the secondary forests were more similar to the primary forest than to the cropped or pasture sites. The communities from cropped sites were significantly different from the forest communities, even though they had been converted to cropland within the previous year. This study supports the idea that these soil communities are influenced more by niche effects than by legacy effects.

Table 1. Papers addressing niche vs legacy effects and their results.

Paper	Study type	Habitat	Open/closed to immigration	Time since change	Results: niche/legacy
Griffiths et al, 2008	reciprocal transplant	soil/leaf litter	closed	3 months	niche
Langenheder et al, 2005	reciprocal transplant	lake water	closed	250-300 hrs	legacy/both
Rawls et al, 2006	reciprocal transplant	zebrafish & mice guts		3-7 days	niche
Strickland et al, 2009	reciprocal transplant	soil/leaf litter	closed	300 days	legacy/both
Ayarza & Erijman, 2011	common garden	activated sludge	open	40 days	niche
Langenheder et al, 2006	common garden	lake water	closed	10-20 days	legacy
Peralta et al, 2010	natural/restored comparison	wetlands	open	2-3 years	legacy
Fraterrigo et al, 2006	primary/secondary comparison	forests	open	50-75 years	legacy
Kulmatiski & Beard, 2008	primary/secondary comparison	shrub-steppe soil, some formerly farmed	open	3-55 years	both
Jesus et al, 2009	disturbed/undisturbed comparison	forested/formerly forested soil	open	1-30 years	niche
Waldrop et al, 2000	disturbed/undisturbed comparison	forested/formerly forested agricultural soil	open	6-14 years	niche

A consensus has not been reached regarding the importance of niche versus legacy effects in shaping microbial communities. Certainly, they both matter to a degree. It is difficult to separate the effect land use has directly from the effects it has on environmental parameters that influence community structure. The tropical soils studied by Jesus et al (2009) and Waldrop et al (2000) may be able to recover more easily than the sites studied by Peralta et al (2010), Fraterrigo et al (2006) and Kulmatiski et al (2008). The shorter disturbance period caused by slash-and-burn agriculture may allow for microbial community recovery, whereas sites where agriculture has been carried out for much longer cannot recover easily.

There were also variable results for the reciprocal transplant experiments. Different bacteria grow at different rates, and the Langenheder et al (2005, 2006) and Strickland (2009) experiments might have converged if given more time. It makes sense that the activated sludge and gut bacteria in Ayarza & Erijman (2011) and Rawls et al (2006) might grow more quickly given their higher nutrient input. However, this does not explain why the microbial communities studied by Griffiths et al (2008) converged; perhaps the inocula were more similar to each other to begin with than in Langenheder et al (2005, 2006) and Strickland (2009).

The habitat in the biofilters is expected to be most like the wetland studied by Peralta et al (2010), as they both have high moisture content, low dissolved oxygen, and high nitrogen inputs. As this study found differences in microbial communities and their functioning between environmentally similar sites with different histories, it reinforces the importance of studying the relative importance of niche and legacy in the biofilter environment.

Chapter 3: Methods

3.1 Reactor Set-up

Two laboratory-scale biofilters were previously constructed by Nick Bartolerio, and a thorough description and schematic can be found in his thesis (Bartolerio, 2011). The biofilters are 13 cm wide by 46 cm long by 30.5 cm tall and are diagrammed in Figure 2.

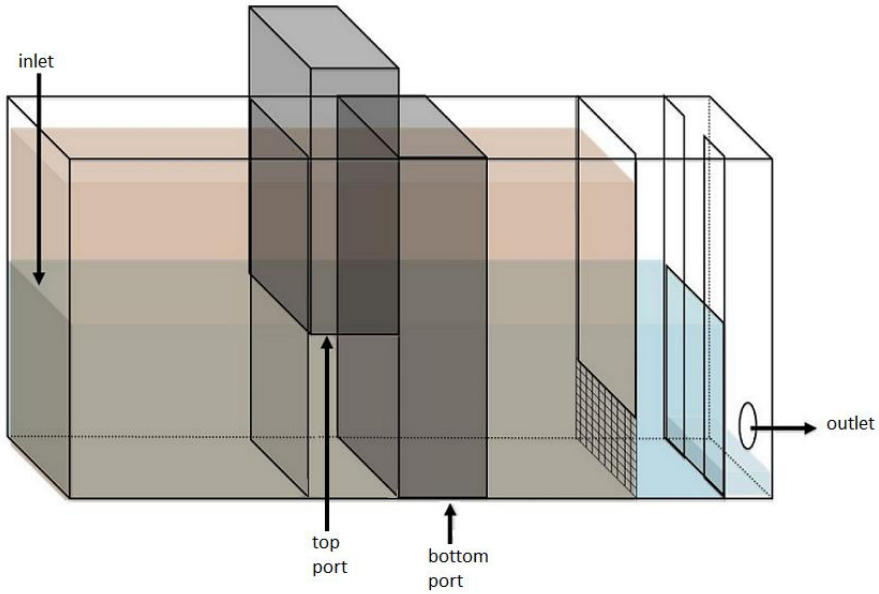


Figure 2. Diagram of laboratory-scale biofilter. Arrows indicate influent and effluent locations. The brown area shows the woodchip bed and the blue area shows the water level. The darker rectangular prisms show the location of the sampling ports.

Most of the reactor volume consists of a large woodchip bed. Within the bed, each reactor has two sampling ports. They are 13 cm wide by 9 cm long by 34 cm tall and are constructed from ¼" aluminum mesh. A removable mesh bottom allows for woodchip sampling from the depth of the port bottom. The woodchips in the port move down incrementally after each sampling event to fill that space. The top port was inserted at a height of 15.5 cm from the bottom, closer to the reactor influent. The bottom port was inserted at a height of 10 cm from the bottom, closer to the reactor effluent. The ports are adjacent to each other in the reactor, and ¼"

aluminum mesh separates the area they occupy from the rest of the reactor on either side, to prevent the woodchips from collapsing when the ports are removed.

At the end of the reactor is the overflow weir/drain compartment. An acrylic baffle wall extends from the top of the reactor to 5 cm above the bottom and separates this compartment from the woodchip bed. The area below the baffle wall has ¼" aluminum mesh that prevents woodchips from the rest of the reactor from getting into the overflow weir/drain compartment.

Water level in the biofilter is controlled by an adjustable overflow weir. Screws through the effluent side reactor wall hold the weir in place and allow it to be removed.

Tap water was dechlorinated by flowing through an activated carbon column and used as dilution water. It was mixed with concentrated synthetic tile drainage in a mixing chamber. The diluted synthetic tile drainage was pumped into the biofilters.

3.2 Reactor Operation

The laboratory-scale biofilters were initially filled with woodchips collected from a full-scale biofilter (FP07) in Decatur, IL. Approximately half of the woodchips came from a stockpile next to the biofilter and half from the bottom of the biofilter. As woodchips were depleted from sampling, they were re-stocked with woodchips collected from the stockpile.

The initial woodchip bed porosity at the time of inoculation was 0.60 in the manipulated reactor and 0.62 in the constant reactor (Bartolerio, 2011).

The reactors were continuously fed with synthetic tile drainage. A basic bacterial and fungal growth media (Tanner, 1997) was modified to resemble tile drainage for important constituents. Table 2, Table 3, and Table 4 give the recipe for the feed. A comparison of the

composition of the synthetic tile drainage to that of actual tile drainage can be found in Bartolerio (2011). Influent nitrate concentration was 15 mg NO_3^--N/L, which is similar to what would be found in tile drainage in central Illinois (Kalita et al, 2006).

Table 2. 10X concentrated synthetic tile drainage medium recipe derived from a basic bacterial and fungal media (Tanner, 1997) that was scaled to be similar in chemical composition with respect to major mineral components as actual tile drainage (Stone & Krishnappan, 1997, Blowes et al, 1994).

10X Synthetic Tile Drainage Medium		
KNO_3	1.08	g/L
Mineral Solution	2.5	mL/L
Trace Metal Solution	0.625	mL/L
Yeast Extract	0.025	g/L

Table 3. Trace metal solution recipe for use in synthetic tile drainage derived from a basic bacterial and fungal media (Tanner, 1997).

Trace Metal Solution		
Nitrilotriacetic acid	2	g/L
Adjust pH to 6 with KOH		
$MnSO_4 \cdot H_2O$	1	g/L
$Fe(NH_4)2(SO_4)2 \cdot 6H_2O$	0.8	g/L
$CoCl_2 \cdot 6H_2O$	0.2	g/L
$ZnSO_4 \cdot 7H_2O$	0.2	g/L
$CuCl_2 \cdot 2H_2O$	0.02	g/L
$NiCl_2 \cdot 6H_2O$	0.02	g/L
$Na_2MoO_4 \cdot 2H_2O$	0.02	g/L
Na_2SeO_4	0.02	g/L
Na_2WO_4	0.02	g/L

Table 4. Mineral solution recipe for use in synthetic tile drainage derived from a basic bacterial and fungal media (Tanner, 1997).

Mineral solution (1 L)		
NaCl	80	g/L
KCl	10	g/L
KH_2PO_4	10	g/L
$MgSO_4 \cdot 7H_2O$	20	g/L
$CaCl_2 \cdot 2H_2O$	4	g/L

Following start-up, the theoretical retention time for both reactors was maintained at twelve hours for both reactors for the duration of operation. The water level in one reactor, called the constant reactor, was maintained at 19 cm, which corresponds to a 5.76 L woodchip bed void volume. The water level in the other reactor, called the manipulated reactor, was 20.5 cm initially, which also corresponds to a 5.76 L woodchip bed void volume.

The high water level in the manipulated reactor was maintained for eight months. Then, the weir was removed, halving the water level; this water level was maintained for six months. After this, a period of regular disturbance was begun, in which the water level alternated between high and low every three weeks. This disturbance period lasted for eight months. The water level was then kept low for two months.

Two weeks before reactor shut-down, the influent to both reactors was modified. For most of the operational period, the tubing was inserted into the woodchips close to the high water line. When it was modified, a cylinder of 1/8" mesh was inserted all the way to the bottom of the reactor at the inlet. The influent tubing could then be inserted into the cylinder, and influent flowed into the reactor at the bottom. Table 5 gives the dates for all operational changes to the reactors.

Table 5. Dates of all changes to reactor operation

Date	Reactor	Action
8/27/2011	Manipulated	weir removed
1/18/2012	Both	pump clogging, reduced influent NO3- (5.6 mg N/L)
3/4/2012	Both	pump clogging, reduced influent NO3- (1.3 mg N/L)
3/8/2012	Both	pump clogging, reduced influent NO3- (7 mg N/L)
3/10/2012	Both	pump clogging, increased effluent NO3- (44.3 mg N/L)
4/9/2012	Both	pump clogging, increased effluent NO3- (35.7 mg N/L)
4/12/2012	Manipulated	weir replaced
5/1/2012	Manipulated	weir removed
5/22/2012	Manipulated	weir replaced
6/12/2012	Manipulated	weir removed
7/2/2012	Manipulated	weir replaced
7/24/2012	Manipulated	weir removed
8/14/2012	Manipulated	weir replaced
9/6/2012	Manipulated	weir removed
9/27/2012	Manipulated	weir replaced
10/18/2012	Manipulated	weir removed
12/4/2012	Both	inlet changed
12/18/2012	Both	reactor feed changed to water

3.3 Nitrate Concentrations

5 ml samples of the combined influent and each reactor's effluent were collected every other day, passed through 0.22 μm nylon syringe filters, and stored in 10 ml polyvinyl vials (Dionex, Sunnyvale, CA) at 4^{o}C until analysis.

3.4 Microbial Community Sample Collection

Woodchips were sampled from the bottom of each port in the lab reactor weekly. 15 g of woodchips were placed in autoclaved 250 ml Nalgene bottles. They were continuously shaken with 110 ml of Ringer's solution overnight in a 30° C room. The next day, the contents were shaken and transferred to 50 ml centrifuge tubes and centrifuged for three minutes at 5000 *x*g, and the supernatant was poured off. 2.5 ml of PBS and 5 sterile 5 mm glass beads were added, and the tubes were vortexed for two minutes and centrifuged for five minutes at 750 *x*g. The supernatant was removed to sterile 1.5 ml tubes and stored at -20^{o}C until extraction.

For the niche-legacy comparison, samples were taken from four different habitats: field biofilters, restored wetlands, natural wetlands, and agricultural fields. Biofilter samples were taken from three different reactors, FP03 and FP07 in Decatur, IL and DE01 in De Land, IL in summer 2010 (Porter, 2011). For the wetlands, sediment samples were taken from six pairs of natural and restored wetlands in Illinois in June 2007 (Flanagan, 2009). Each sample is the composite of eight soil cores (1.9 cm diameter) taken from within a 0.25 m^2 quadrat at a depth to 12 cm. A thorough description of the sampling sites and methods can be found in Flanagan, 2009, and more detailed information on the samples used is included in the appendix. For this niche-legacy study, two samples from each wetland were used. Three samples were also taken from a wetland at the discharge location of the FP03 biofilter in summer 2010. Each of these samples is a composite of three soil cores taken from within a 1 m^2 quadrat at depth to 6 cm. Agricultural soil samples were taken from a conventionally farmed field near one of the wetland pairs with similar sampling methods. Samples were also taken from the Morrow Plots on the University of Illinois, Urbana-Champaign campus in summer 2009. These samples are a composite of five soil cores to a depth of 12 cm. Three samples were taken from the field which drains into the FP03 biofilter in summer 2010 with similar sampling methods as the nearby wetland. One sample each was taken from a field planted with switchgrass (*Panicum virgatum*) at the Northern Illinois Agronomy Research Center in Dekalb, IL, a field planted with *Miscanthus* and one with switchgrass at Dixon Springs Agricultural Center in Simpson, IL, and a conventionally cropped field at UIUC SoyFACE in Champaign, IL in winter 2009.

3.5 DNA Extraction

Biofilter DNA was extracted from the woodchip washes using the FastDNA Spin Kit (MP Biomedicals, Solon, OH) according to the manufacturer's directions. DNA from soil and

sediment samples was extracted using the FastDNA Soil Spin Kit (MP Biomedicals, Solon, OH) according to the manufacturer's directions. The extracted DNA was purified using cetyl trimethyl ammonium bromide (CTAB) cleanup (Sambrook & Russell, 2001) to remove humic acid contamination. During the cleanup, the DNA was incubated overnight at -20°C in 100% ethanol to increase recovery. DNA concentration was determined using a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE), and each sample was standardized to 10 ng/ul. Samples were stored at -20°C.

3.6 Microbial Community Analysis

The community structure for all bacteria was determined using automated ribosomal intergenic spacer analysis (ARISA). The universal 16s rRNA gene 1406f forward primer (5'-TGYACACACCGCCCGT-3'), labeled with 6-carboxyfluorescein, and bacteria-specific 23S rRNA gene 23Sr reverse primer (5'-GGGTTBCCCCATTCRG-3') were used (Fisher & Triplett, 1999). Polymerase chain reactions (PCR) contained 2.5 µl 10X buffer (30 mM MgCl, 50 mM Tris (pH 8.0), 250 µg/mL bovine serum albumin,) (Idaho Technology, Inc, Salt Lake City, UT), 1.25 µl dNTPs (5 mM) (New England Biolabs, Ipswich, MA), 1 µl of each primer (10 µM), 0.25 µl Taq polymerase (5U/µl) (Promega, Madison, WI), 17 µl autoclaved nanopure water, and 2 µl DNA template (20 ng). All PCR reactions were carried out in an Eppendorf MasterCycler (Eppendorf, Hauppauge, NY). The PCR cycle started with an initial denaturation step at 94°C for 2 minutes, followed by thirty repetitions of 94°C for 35 seconds, 55°C for 45 seconds, and 72°C for 120 seconds, and ended with a final extension step at 72°C for 2 minutes. The PCR products were then held at 4°C.

The denitrifier community structure was assessed using terminal restriction fragment analysis (tRFLP) of the nitrous oxide reductase gene *nosZ*. The *nosZ* F-1181 forward primer (5'-

CGCTGTTCITCGACAGYCAG-3') and *nosZ* R-1880 reverse primer (5'-ATGTGCAKIGCRTGGAGAA-3'), labeled with 6-carboxyfluorescein, were used (Rich et al, 2003). Each reaction contained 5 µl 10X Tris buffer (pH 8.3), 2.0 µl dNTPS (5 mM), 4 µl 25 mM MgCl2 (Promega, Madison, WI), 1.25 µl 10 mg/ml bovine serum albumin (BSA) (New England Biolabs, Ipswich, MA), 1 µl of each primer at 10 µM, 0.5 µl Taq polymerase (5U/µl), 25.25 µl autoclaved nanopure water, and 10 µl DNA template (100 ng). The PCR cycle started with 3 minutes at 94°C, followed by thirty repetitions of 94°C for 45 seconds, 56°C for 60 seconds, and 72°C for 120 seconds, and then ended with 72°C for seven minutes. The PCR products were then held at 4°C. Three reactions per sample were carried out and then combined and concentrated using a Qiagen MinElute PCR Purification kit (Qiagen, Valencia, CA).

The DNA was digested with *AluI* and *HhaI* (New England BioLabs, Ipswich, MA). Each digestion reaction contained 1 µl 10X Buffer 4 (New England BioLabs, Ipswich, MA), 0.2 µl BSA, 0.5 µl restriction enzyme, 8.3 µl autoclaved nanopure water and 10 µl PCR product. Digests were carried out overnight at 37°C.

All ARISA and tRFLP products were analyzed using denaturing capillary electrophoresis with an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA) at the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois, Urbana, IL). The ABI GeneScan ROX 1000 size standard was used for all *nosZ* tRFLP reactions and ARISA reactions from the lab reactor. A custom 100 to 2000 bp Rhodamine X-labeled size standard (Bioventures, Murfreesboro, TN) was used for all the other ARISA reactions.

Size calling and peak definition of the chromatographs was carried out using GeneMarker 2.2.0 (SoftGenetics, LLC, State College, PA). For ARISA chromatographs, peaks between 300

and 1000 base pairs were included, and a minimum fluorescence threshold of 200 was used. For *nosZ* tRFLP chromatographs, peaks between 100 and 700 base pairs were included, and a minimum fluorescence threshold of 80 was used. The minimum and maximum size for each peak was defined using the allele calling features in GeneMarker. Each peak is considered to represent a distinct operational taxonomic unit (OTU).

3.7 Microbial Community Statistical Analysis

Lab reactor microbial communities were analyzed using PRIMER 6, version 6.1.10 (Primer-E, Ltd., Plymouth, UK). Non-metric multi-dimensional scaling (MDS) plots were generated to visually analyze the patterns in microbial community structure. The ANOSIM function (Clarke and Green, 1988) was used to generate similarity values among different groups of samples. The DIVERSE function was used to determine species richness.

Partial correspondence analysis was used for the niche-legacy comparison using Canoco for Windows 4.5.1 (Plant Research International, Wageningen, Netherlands) (ter Braak et al, 2002). The environmental variables used were moisture and habitat categorization as biofilter, natural wetland, restored wetland, or agriculture. The influence of location was removed from the analysis with latitude and longitude as covariables. The program parameters used were species, environment, and covariable data available; indirect gradient; unimodal (CA) response; inter-sample distances; biplot scaling; and no transformation of species data.

3.8 Denitrification Potential

Denitrification potential was measured through denitrification enzyme assays (DEA) with the acetylene block method (Tiedje et al, 1989). These assays were performed in duplicate for each sampling port. Ten grams of woodchips were incubated in a Wheaton bottle with 75 ml of solution containing 15 mg/L NO_3^- and 0.1 g chloramphenicol. The chloramphenicol prevents the

bacteria from synthesizing new protein, so only enzymes that are already present contribute to the denitrification potential. In selected assays, 1 mM glucose was included. The headspace was flushed with helium gas, and acetylene gas added to achieve a headspace concentration of about 20%. Acetylene prevents the final denitrification step from nitrous oxide to dinitrogen gas. Samples were taken at times zero, two, four, and six hours. Gas samples were taken and stored in 10 ml Vacutainers (BD, Franklin Lakes, NJ). Liquid samples were filtered with 0.22 μl syringe filters and stored in 10 ml polyvinyl vials (Dionex, Sunnyvale, CA). After each sampling point, the extracted volume was replaced with 10:1 helium: acetylene gas mix. The Vacutainers had silicon sealant spread on the top to prevent contamination. The liquid samples were stored at 4°C until analysis. After the assay, the headspace volume was measured, and the bottles were placed in a drying oven at 105°C to determine the dry weight of woodchips.

The nitrate concentrations were converted to total nitrate removed from the bottle. A linear regression of this was used to calculate the rate of nitrate removal, where the slope of the regression line is the mass of nitrate removed per hour. The t0 sample was not included in the regression, because it was usually not linear with the other time points. The nitrate removal rates were normalized per dry gram of woodchips.

The nitrous oxide concentrations were converted to total N_2O produced in the bottle. Similarly to nitrate removal, a linear regression was used to calculate the rate of nitrous oxide produced per hour. This rate was also normalized per dry gram of woodchips.

3.9 Statistical Analysis of Denitrification Potential

The denitrification potential values for multiple categories of disturbance and water level were averaged together. Significance of values was evaluated using paired t-tests for unequal variance in Microsoft Excel (Microsoft Corporation, Redmond, WA).

3.10 Tracer Studies

Tracer studies were performed on both reactors, twice with the changed inlet and twice with the original inlet. The manipulated reactor had a low water level during all the tracer studies. The first set of tracer studies took place in early December 2012, shortly before reactor shut down. The second set of tracer studies were performed because of problems with analysis of the first set of tracer studies. They took place six months after reactor shut down; dechlorinated water had been flowing through the reactors, but no nutrient feed. They are compared to tracer studies performed on the reactors by Nick Bartolerio (2011) in November 2011, when the manipulated reactor was being subjected to its extended low water period and the inlet had not been modified.

A step input of 100 ppm bromide was used as the tracer. Effluent samples were taken every hour for the first five hours, every half hour for the next thirteen hours, and every hour for the remaining eighteen hours.

The cumulative residence time distribution, $F(t)$, given in equation 1below, was determined from the effluent bromide concentrations.

$$F(t) = \frac{c_{eff}(t)}{c_0} \qquad (1)$$

where $c_{eff}(t)$ = effluent bromide concentration at time t
c_o = influent bromide concentration

The derivitive of $F(t)$ is the residence time density function, $f(t)$. The mean residence time, $\bar{t}$, is computed from $f(t)$ according to equation 2.

$$\bar{t} = \int_0^\infty f(t)t\ dt \qquad (2)$$

$f(t)$ at the sampling time j was approximated using equation 3.

$$f(t_j) \approx \left(\frac{\Delta F}{\Delta t}\right)_j = \frac{F(t_j) - F(t_{j-1})}{t_j - t_{j-1}} \quad (3)$$

The mean residence time could then be approximated according to equation 4.

$$\bar{t} = \frac{\sum_{j=1}^{jmax} f(t_j)\left(\frac{t_j + t_{j+1}}{2}\right)(t_{j+1} - t_j)}{\sum_{j=1}^{jmax} f(t_j)\,(t_{j+1} - t_j)} \quad (4)$$

3.11 Dissolved Oxygen Measurements

Dissolved oxygen was measured with a YSI Professional Plus handheld multiparameter meter with Quattro cable and probe 1003 (pH/ORP) (YSI, Yellow Springs, OH). Measurements were taken twice in each port of the reactor, once at low water level on September 28, 2012 and once at high water level on October 2, 2012. Because the top port of the manipulated reactor was not submerged at low water level, dissolved oxygen could not be measured in that port then. To allow the probe into the reactor, the sampling ports were removed when taking the measurements. Readings were taken as quickly as possible after removing the sampling port, but removing the port may have introduced some oxygen.

3.12 Analytical Measurements

Gas samples taken during the DEAs were analyzed using a Shimadzu GC-2014AFsc gas chromatography system with packed columns and GCSolution software (Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). Preconditioned, custom packed HayeSep N 80-100 columns were included in the set-up (Supelco, Bellefonte, PA). A standard curve was created for each run using standards with 0.11 ppm, 0.73 ppm, 1.09 ppm, 1.80 ppm, 3.67 ppm, and 7.33 ppm N_2O.

Liquid samples taken during the DEAs and for reactor performance were analyzed for nitrate concentration using an ICS-2000 ion chromatography system with Chromeleon Chromatography Management Software (Dionex, Sunnyvale, CA). An IonPac AS18 4x250 mm hydroxide-selective anion-exchange column was used in the set-up. A standard curve was created for each run using standards with 3.125 ppm, 6.25 ppm, 12.5 ppm, 25 ppm, 50 ppm, and 100 ppm NO_3^-. Samples taken during the tracer studies were analyzed for bromide this way, also. The same concentrations were used for the bromide standards as for the nitrate standards.

Chapter 4: Results of Laboratory Biofilter Study

4.1 Reactor Performance

Both reactors showed successful nitrate removal throughout the study period. The two reactors removed nitrate similarly when they both had the weir up for high water level (Figure 3). At a high water level, the constant reactor had an average of 31±7% nitrate removal, and the manipulated reactor had an average of 31±8% nitrate removal. The performance of the manipulated reactor was better when the water level was lower, even though the HRT remained constant. At the lower water level, the manipulated reactor had an average of 59±11% nitrate removal.

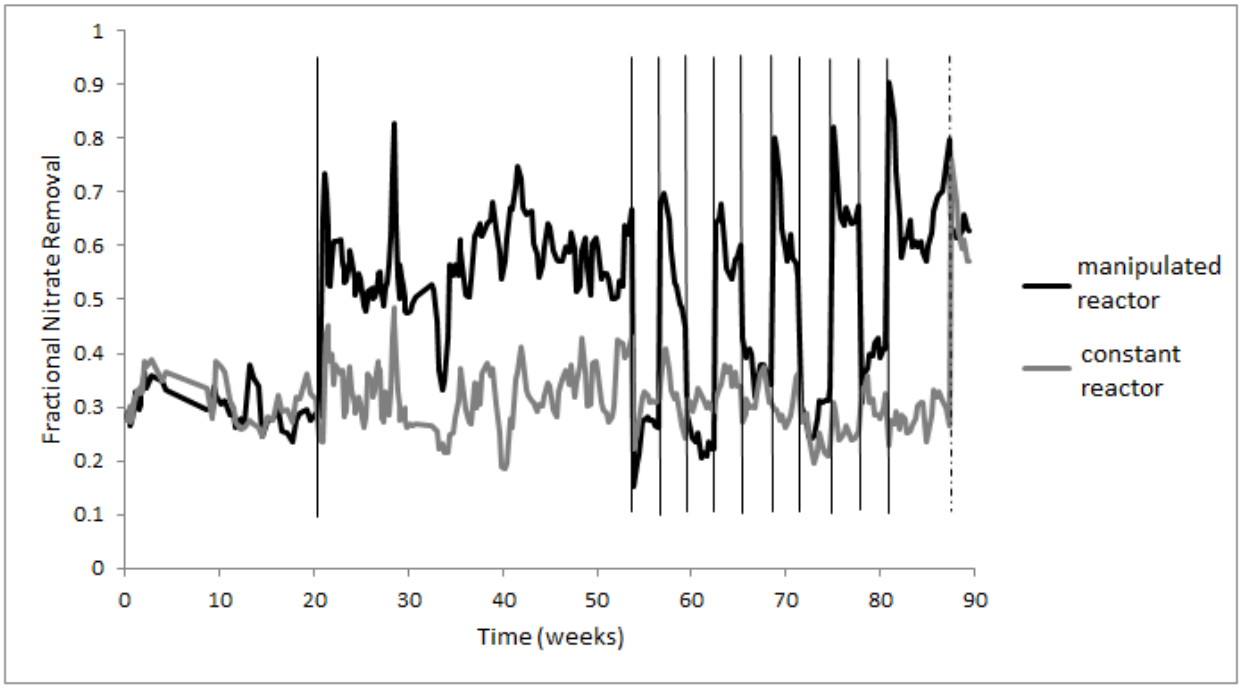


Figure 3. 5-day average of nitrate removal by each reactor from April 3, 2011 to December 18, 2012. The solid vertical lines indicate a weir change. The first line is the weir removal on August 23, 2011 at the beginning of the period of low water level. The second line is the weir raising on April 12, 2012 at the beginning of the disturbance period. The dashed line indicates the inlet change on December 4, 2012. The dates with severe influent concentration changes (listed in Table 5.) have been excluded. All the dates for operation changes are listed in Table 5.

To test whether differences in flow were affecting nitrate removal, the inlet was modified in December 2012. After this change, the performance of the constant reactor increased and became similar to the manipulated reactor. With the modified inlet, the constant reactor had an average of 64±11% nitrate removal, and the manipulated reactor had an average of 63±4% nitrate removal.

During the period of regular disturbance, different performance was observed in the manipulated reactor immediately following weir removal and the rest of the time during low water level periods. After weir removal, the performance spiked to nitrate removal of between 70 and 90%. Within a few days to a week at low water level, the performance decreased to low water level average nitrate removal levels. When the weir was not replaced after three weeks, the performance remained at this lower, average level for the next four weeks.

4.2 Denitrification Potential

For the DEAs, nitrate removal was measured over six hours to determine the denitrification potential. In most cases the nitrate removal rates were not significantly different between the reactors, ports, or water level (Table 6). The exception to this is the top port of the manipulated reactor during the period of extended low water level, as compared to itself during the period of regular disturbance or to any other ports during this time. The average nitrate removal rate in the top port of the manipulated reactor during the low water level was 0.004 ±0.001 mg NO_3-N/hour/dry g woodchips, lower than the nitrate removal during other periods or in other ports.

Table 6. Average nitrate removal rates in DEAs of woodchips sampled from the reactors under different disturbance classifications.

	Bottom Port		Top Port	
	Removal Rate (mg NO_3-N/hour/dry g woodchips)	Standard Deviation	Removal Rate (mg NO_3-N/hour/dry g woodchips)	Standard Deviation
Before disturbance[a]	0.007	0.001	0.004	0.001
After disturbance[b]	0.008	0.002	0.007	0.003
After disturbance, low water level[b]	0.007	0.002	0.008	0.003
After disturbance, high water level[b]	0.008	0.002	0.006	0.002
Less than one week after lowering weir[b]	0.007	0.003	0.007	0.003
More than two weeks after lowering weir[b]	0.007	0.002	0.009	0.004
Less than one week after raising weir[b]	0.007	0.002	0.0065	0.0009
More than two weeks after raising weir[b]	0.0091	0.0009	0.006	0.002
Manipulated reactor overall[b]	0.007	0.002	0.006	0.003
Constant reactor overall[b]	0.007	0.003	0.008	0.002

[a]The values reported here were measured between January 5 and April 5, 2012, when the manipulated reactor had sustained low water level.
[b]The values here were measured between April 13 and December 3, 2012, when the water level in the manipulated reactor changed every three weeks.

The denitrification potential was also measured in the DEAs as the nitrous oxide production rate, as this represents all but the last step of denitrification. Table 7 gives the average nitrous oxide production rates from DEAs of woodchips sampled under different operating conditions. A high level of uncertainty in these measurements makes it difficult to compare the nitrous oxide production rates from different reactor conditions.

Table 7. Average nitrous oxide production rates in DEAs of woodchips sampled from the reactors under different disturbance classifications.

	Bottom Port		Top Port	
	Production Rate (mg N2O-N/hour/dry g woodchips)	Standard Deviation	Production Rate (mg N2O-N/hour/dry g woodchips)	Standard Deviation
Before disturbance[a]	0.00027	0.00008	0.00003	0.00001
After disturbance[b]	0.0011	0.0009	0.0007	0.0008
After disturbance, low water level[b]	0.0011	0.0008	0.0011	0.0004
After disturbance, high water level[b]	0.001	0.001	0.0005	0.001
Less than one week after lowering weir[b]	0.001	0.001	0.0007	0.0004
More than two weeks after lowering weir[b]	0.0008	0.0003	0.0004	0.0004
Less than one week after raising weir[b]	0.001	0.002	0.001	0.001
More than two weeks after raising weir[b]	0.0010	0.0003	0.0009	0.0006
Manipulated reactor overall[b]	0.0009	0.0009	0.000568	0.0008
Constant reactor overall[b]	0.0009	0.0007	0.000794	0.0006

[a]The values reported here were measured between January 5 and April 5, 2012, when the manipulated reactor has sustained low water level.
[b]The values here were measured between April 13 and December 3, 2012, when the water level in the manipulated reactor changed every three weeks.

There was a discrepancy between the nitrate removal rates and the nitrous oxide production rates. The nitrous oxide production rates were about one tenth of the nitrate removal rates (Figure 4).

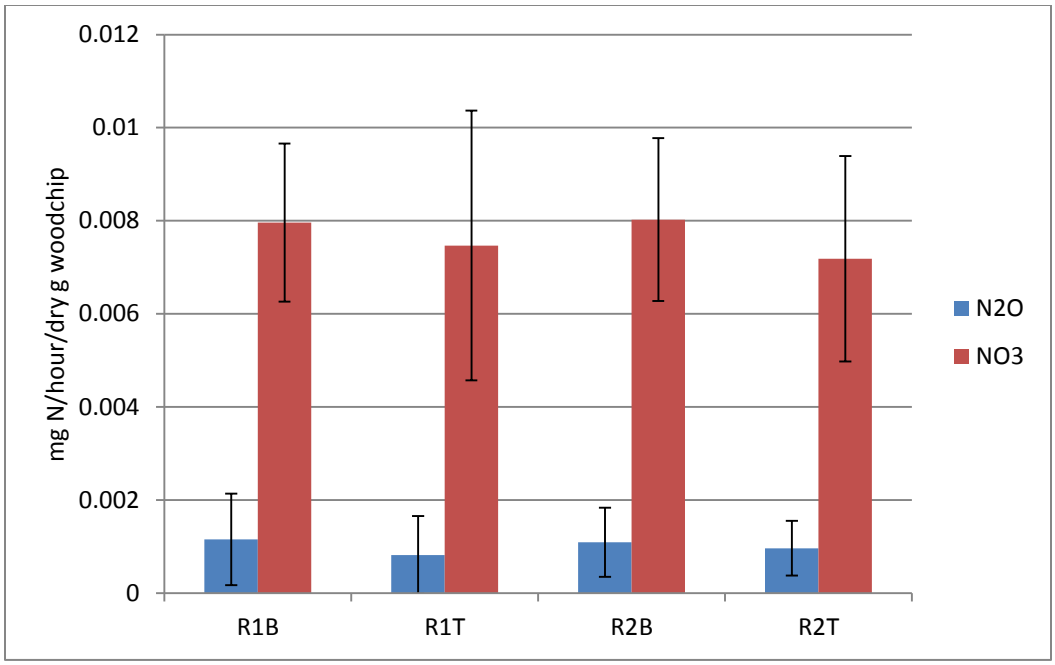


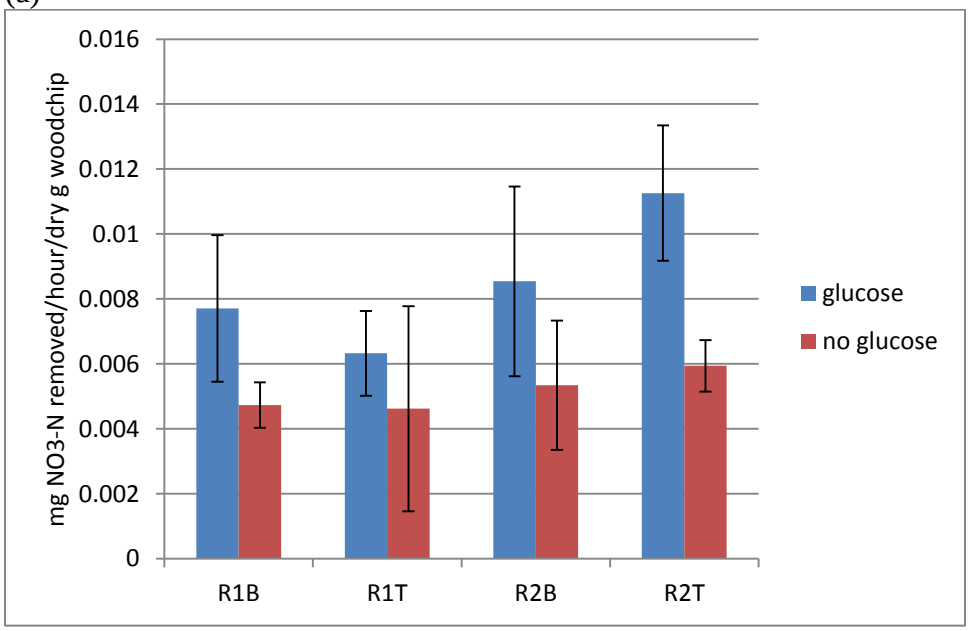
Figure 4. Average nitrate removal rates and nitrous oxide production rates. The error bars are one standard deviation. The DEAs represented here were performed between January 5, 2012 and December 3, 2012. R1 is the manipulated reactor and R2 is the constant reactor. B and T are the bottom and top ports respectively.

Three DEAs were also completed with and without glucose amendments. This was done to compare the effect of increased carbon on denitrification potential. If the removal of the weir on the manipulated reactor was causing the improved performance by increasing available carbon, a greater difference in denitrification potential with and without glucose would be observed in the constant reactor than the manipulated one.

The average nitrate removal rates are higher when measured with amended glucose than without for both ports of both reactors (Figure 5a). This general trend was also true for all of the individual DEAs, except for the bottom port of the constant reactor on July 7, 2012, when the unamended DEAs have higher removal rates, and the top port of the manipulated reactor on June 14, 2012 and the bottom port of the manipulated reactor on July 5, 2012, when the unamended and amended DEAs resulted in the same removal rate (Table 8). The average nitrous oxide production rates were also higher with amended glucose than without for both ports of both

reactors (Figure 5b). This was true for all individual DEAs, again except for the bottom port of constant reactor on July 5, 2012 (Table 9).

(a)



(b)

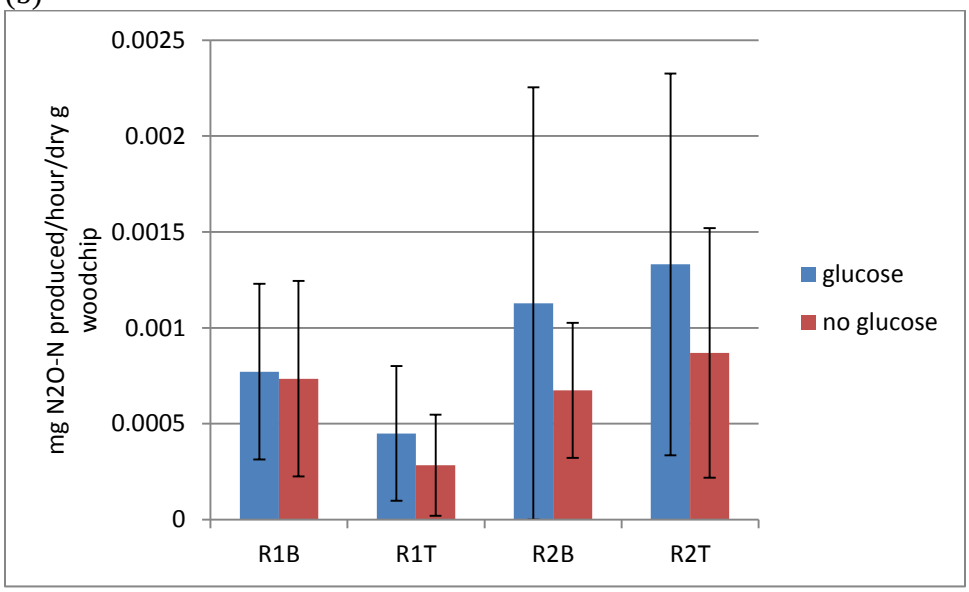


Figure 5. Average nitrate removal rates (a) and nitrous oxide production rates (b) comparing the nitrate removal rate in each port of each reactor measured with and without amended glucose. Error bars are one standard deviation. R1 is the manipulated reactor and R2 is the constant reactor. B and T are the bottom and top ports respectively.

Table 8**Error! Reference source not found.**. Average nitrate removal rates for each port of each reactor with and without glucose amendments.

Date	NO_3 removal rates (mg NO_3-N/hour/dry g woodchip)							
	Manipulated reactor, bottom port		Manipulated reactor, top port		Constant reactor, bottom port		Constant reactor, top port	
	glucose	no glucose	glucose	no glucose	glucose	no glucose	glucose	no glucose
6/14/2012[a]	0.010	0.0051	0.008	0.008	0.0112	0.0052	0.0136	0.0050
6/28/2012[a]	0.008	0.004	0.005	0.002	0.009	0.0035	0.010	0.00626
7/5/2012[b]	0.005	0.005	0.006	0.004	0.005	0.007	0.010	0.007

[a]Water level was low on this date.
[b]Water level was high on this date.

Table 9. Average nitrous oxide production rates for each port of each reactor with and without glucose amendments.

Date	N_2O production rates (mg N_2O-N/hour/dry g woodchip)							
	Manipulated reactor, bottom port		Manipulated reactor, top port		Constant reactor, bottom port		Constant reactor, top port	
	glucose	no glucose	glucose	no glucose	glucose	no glucose	glucose	no glucose
6/14/2012[a]	0.00028	0.000213	0.00046	0.00023	0.00028	0.00029	0.0004	0.00025
6/28/2012[a]	0.0012	0.0012	0.00009	0.00005	0.002406	0.00010	0.002	0.00155
7/5/2012[b]	0.0008	0.0008	0.0008	0.0006	0.0007	0.0008	0.00117	0.0008

[a]Water level was low on this date.
[b]Water level was high on this date.

Similarly to the unamended DEAs, the nitrate removal rates in the glucose-amended DEAs were about ten times higher than the nitrous oxide production rates.

4.3 Microbial Community Composition

My study on microbial community composition in the lab reactors focused on the effect of changing water level on the bacterial communities. How do the communities respond to regular disturbance: does it result in different OTUs? How is overall diversity affected? How do different parts of the reactor respond to the changing water level? Ideally, a comparison between the microbial communities and the performance of the reactors will also be possible.

4.3.1 Total Bacterial Communities

The analysis of total bacterial communities included 317 unique OTUs. Each sample contained between four and 123 OTUs, with a median of 70. The median number of OTUs was similar for both reactors.

The total bacterial communities in the manipulated reactor were similar to those in the same port, regardless of water level (Figure 6). ANOSIM R values are used to evaluate the similarity of different classifications of samples (eg. manipulated vs. constant reactor). A value close to 0 means that the samples are similar to each other, and a value close to 1 indicates that they are dissimilar. An insignificant ANOSIM R value indicates that the groups of communities being compared are indistinct from each other. The ANOSIM R value comparing the top port communities at low and high water levels was not significant, nor was the value comparing the bottom port communities at low and high water levels, meaning that the communities are not

significantly different from each other. This can also be seen in the MDS plot of these samples (Figure 6).

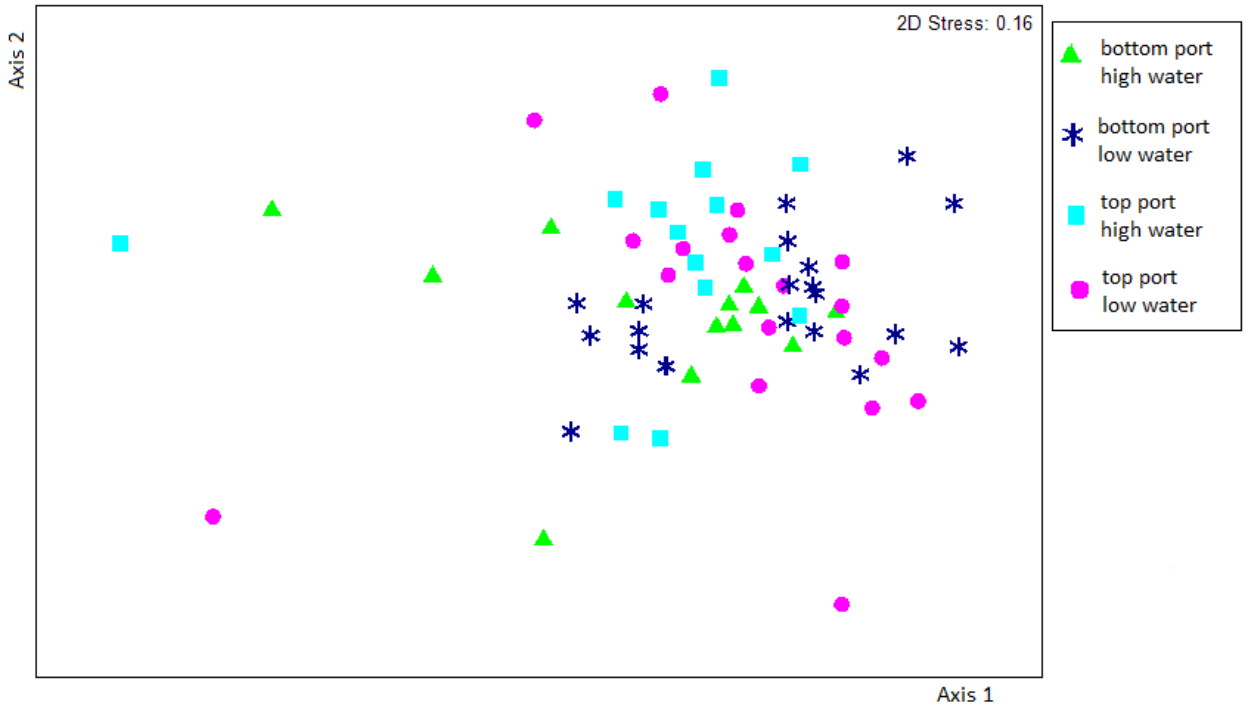


Figure 6. MDS plot of ARISA communities in the manipulated reactor during the disturbance period. Each point represents a community sample with multiple OTUs present. These samples were taken from April 16, 2012 through December 10, 2012. The communities are classified according to the port they were collected from and the water level at the time they were collected.

After the disturbance period, the bacterial communities of the reactors became more distinct from each other. The greater similarity between communities from each reactor prior to the disturbance period compared to during that period is visible in the MDS plots comparing the reactors during the time periods (Figure 7). The ANOSIM R value for the communities of both reactors before the disturbance period started was 0.146 (p=0.001). This included communities from the entire period before disturbance, when the weir on the manipulated reactor was up for an extended period of time and then down for an extended time. The ANOSIM R value for the communities of both reactors during the period of extended high water level was 0.114 (p=0.13),

which was insignificant, whereas it was 0.222 (p=0.001) for the period of extended low water level. The ANOSIM R value comparing both reactors after the disturbance period started was 0.314 (p=0.001). The ANOSIM R value during the disturbance period was higher than the values for the period prior to the disturbance, indicating an increase in dissimilarity between the communities of each reactor once the disturbance period began.

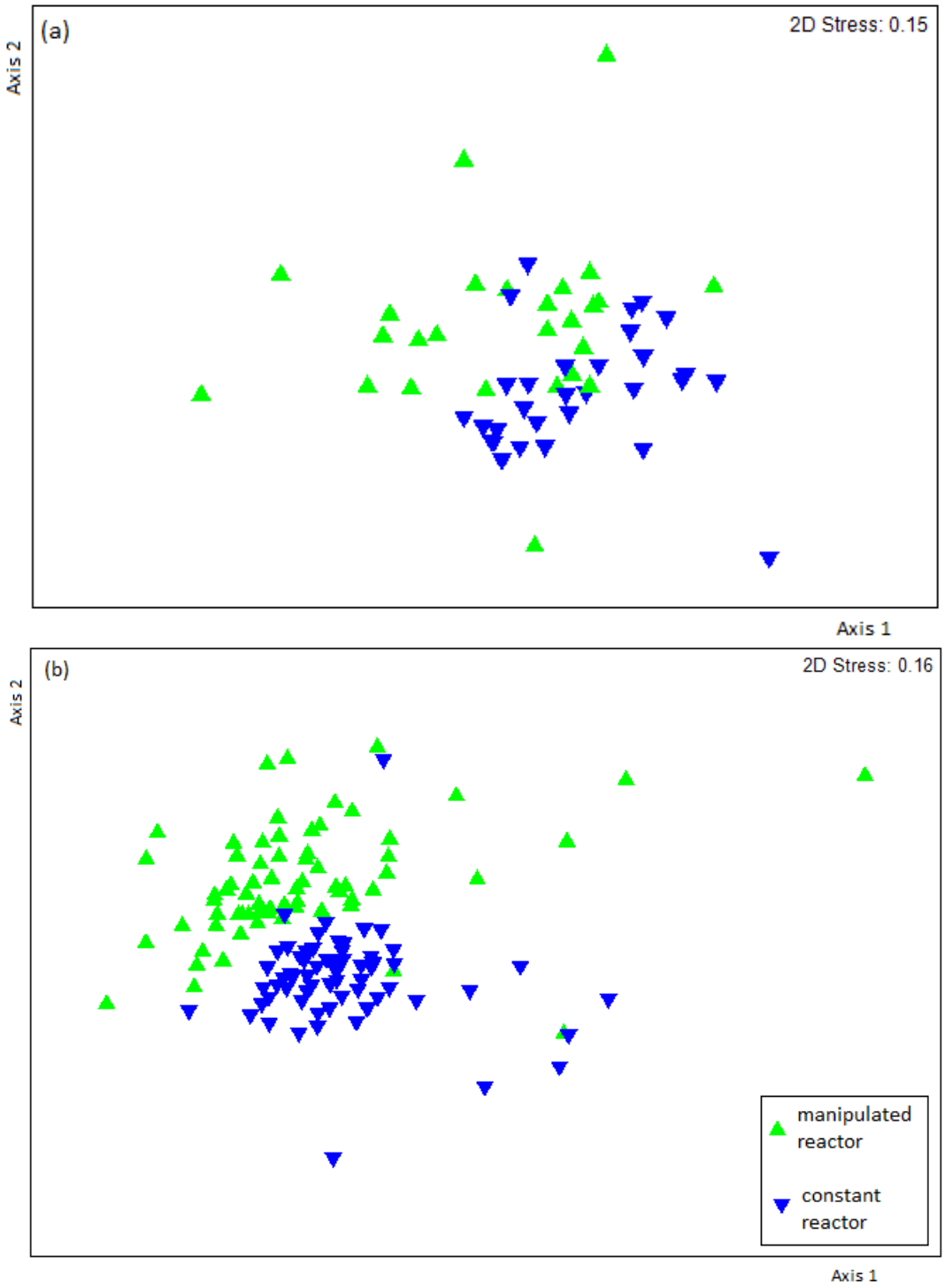


Figure 7. MDS plots showing a comparison of the ARISA communities of the manipulated and constant reactors before (a) and after (b) the beginning of the disturbance period. Each point represents a community sample with multiple OTUs present. The samples in (a) were collected between November 5, 2010 and April 9, 2012, and the samples in (b) were collected between April 16, 2012 and December 10, 2012. Both figures include communities experiencing low water level and high water level. The samples are categorized according to the reactor of origin.

The communities of each port were also compared to each other in all possible combinations. Table 10 shows the ANOSIM R values for these comparisons in order from most to least similar, and Figure 8 shows the MDS plot of these communities. The communities were most similar to the other communities in the same reactor, even from different ports. The communities of the manipulated reactor's top and bottom ports were more similar to each other than those of the constant reactor. Within the manipulated reactor, the communities of the bottom port were more similar to those of the constant reactor than the communities of the top port were to those of the constant reactor. This means that the communities of the top port in the manipulated reactor were the most distinct of those studied. The communities of the bottom port of the manipulated reactor were more similar to the top than bottom port of the constant reactor. The communities of the top port of the manipulated reactor were more similar to the top port than the bottom port of the constant reactor.

Table 10. ANOSIM R values and their significance comparing all combinations of the ARISA communities of each port of each reactor during the disturbance period.

	ANOSIM R	p
Manipulated top & bottom	0.057	0.005
Constant top & bottom	0.075	0.002
Constant top & manipulated bottom	0.272	0.001
Constant & manipulated bottom	0.298	0.001
Constant & manipulated top	0.381	0.001
Constant bottom & manipulated top	0.422	0.001

An ANOSIM R value close to zero indicates that the groups of samples being compared are similar, whereas a value close to one indicates that they are different from each other.

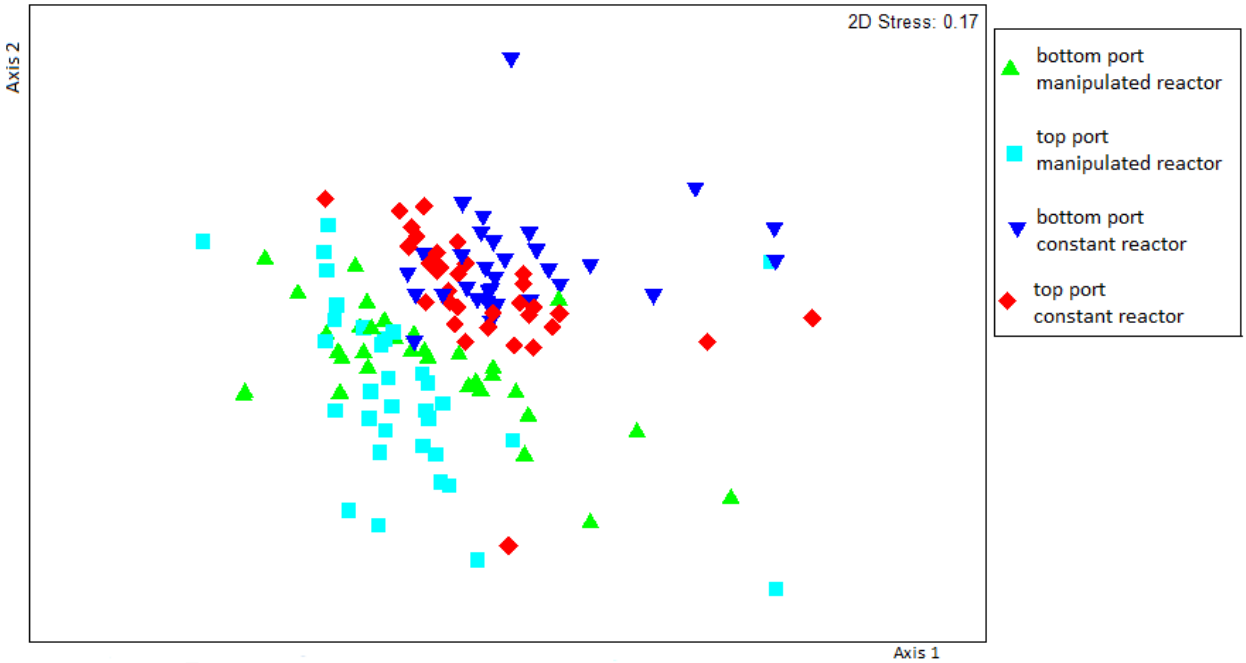


Figure 8. MDS plot of ARISA communities from both ports of both reactors during the disturbance period. Each point represents a sample with multiple OTUs present. These samples were collected from April 16, 2012 through December 10, 2012. The samples are classified according to the reactor and port of origin. This plot includes communities from periods of high and low water levels.

4.3.2 Denitrifying microbial communities

The results of the denitrifying communities data included 800 unique restriction fragments from digests with both restriction enzymes. Each sample contained between five and 468 OTUs, with a median of 36 fragments per sample, including both digests. There was a median of 17 fragments from the reactions digested with AluI and for those digested with HhaI. There was a maximum of 227 fragments per AluI digest and 241 fragments per HhaI digest. The median number of OTUs was 34 for the manipulated reactor and 38 for the constant reactor.

Unlike with the total bacterial communities, the similarity of the denitrifying communities between the two reactors did not change before and after the regular disturbance

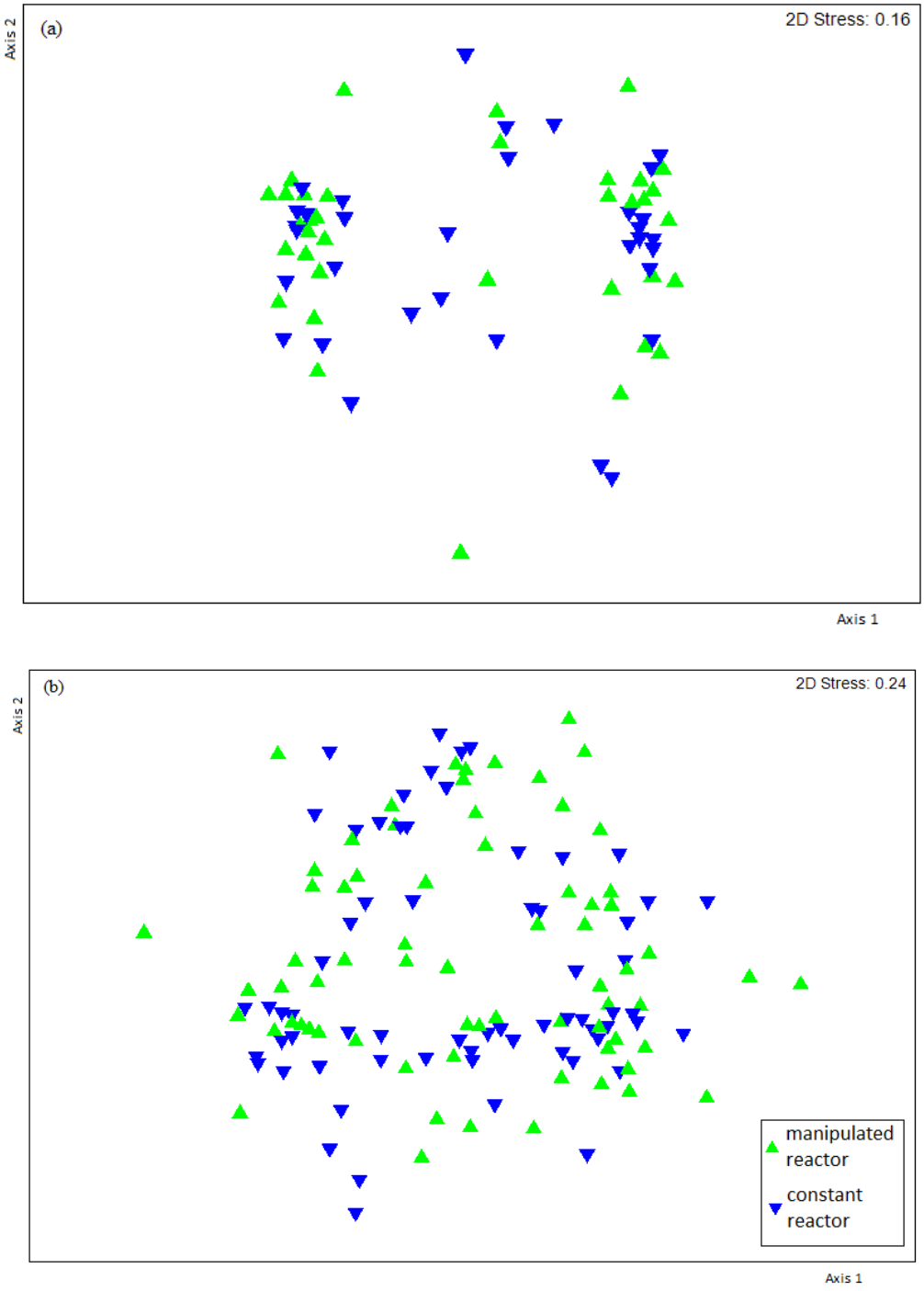


Figure 9. MDS plots showing a comparison of the *nosZ* communities of the manipulated and constant reactors before (a) and after (b) the beginning of the disturbance period. Each point represents a community sample with multiple OTUs present. The samples in (a) were collected between November 5, 2010 and April 9, 2012, and the samples in (b) were collected between April 16, 2012 and December 10, 2012. Both figures include communities experiencing low water level and high water level. The samples are categorized according to the reactor of origin.

period (Figure 9). The ANOSIM R values comparing the reactors during these operational periods were insignificant, indicating indistinctness from each other.

There were no discernible patterns in the structure of the denitrifying communities; the groups of samples were not distinct from each other. The overlap of the communities from each port can be seen in the MDS plot of them (Figure 10). The ANOSIM R values comparing the four different ports to each other were insignificant, indicating that the communities were indistinct from each other. There was also no distinction between the communities of the same ports of the manipulated reactor to each other from high and low water conditions during the period of regular disturbance (Figure 11), and the ANOSIM R values comparing the communities were insignificant.

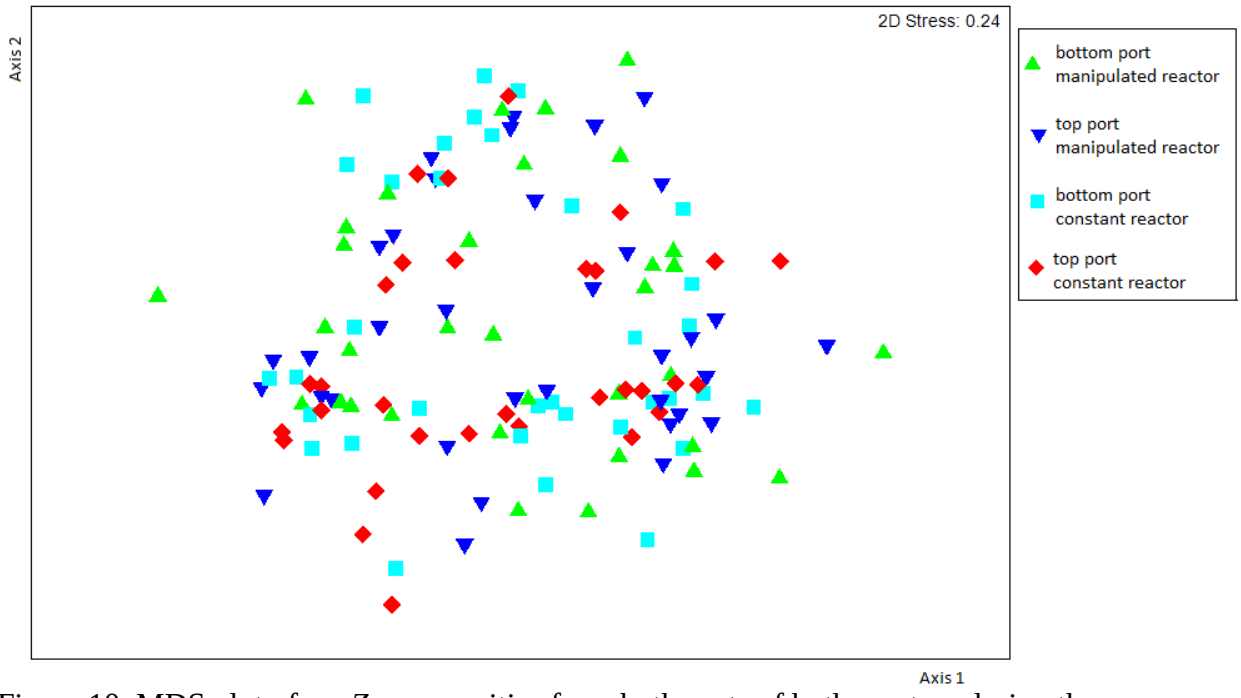


Figure 10. MDS plot of *nosZ* communities from both ports of both reactors during the disturbance period. Each point represents a community sample with multiple OTUs present. These samples were taken from April 16, 2012 through December 10, 2012. The samples are classified according to the reactor and port they were taken from. This plot includes communities from periods of high and low water levels.

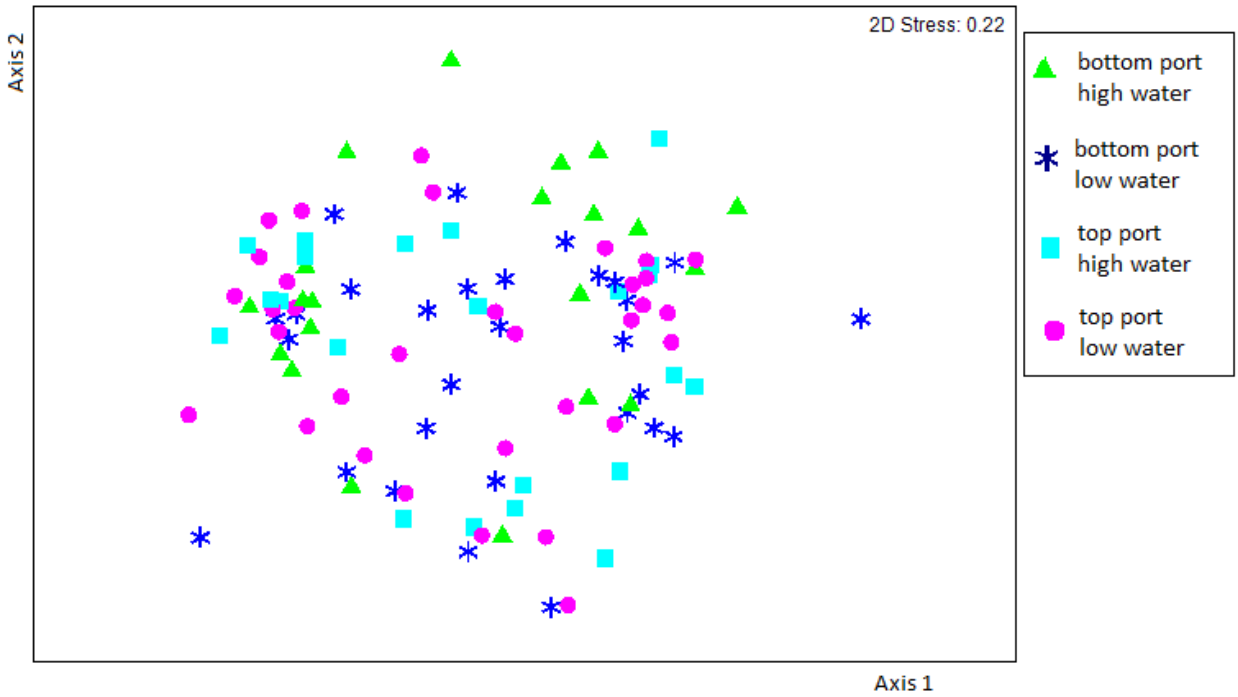


Figure 11. MDS plot of *nosZ* communities in the manipulated reactor during the disturbance period. Each point represents a community sample with multiple OTUs present. These samples were taken from April 16, 2012 through December 10, 2012. The communities are classified according to the port they were taken from and the water level at the time they were taken.

4.4 Tracer Studies

The tracer studies after the inlets were modified to flow in at the bottom of the reactors showed longer HRTs for both reactors than the studies before the inlet modification (Table 11). In both cases, the manipulated reactor had a longer HRT than the constant reactor, although the difference was much larger after the inlet was modified. A greater increase in HRT occurred in the manipulated reactor than the constant reactor.

Table 11. HRTs calculated from tracer studies

Date	Inlet Condition	HRT (hours)	
		Manipulated reactor	Constant reactor
Nov 5-6, 2011	original	8.8	7.5
Jun 24-25, 2013	modified	17.1	10.2

4.5 Dissolved Oxygen

Dissolved oxygen concentration in both ports of both reactors was measured twice, once during a high water period and once during a low water period (Table 12). The dissolved oxygen concentrations were less than 1 mg/L in the bottom port of the constant reactor. It was similarly low in the bottom port of the manipulated reactor when the water level was high. When the water level was low, the concentration in the bottom port was much closer to saturation (about 9 mg/L at room temperature). There was dissolved oxygen present in the top port of the constant reactor, a few mg/L less than saturation, as well as in the top port of the manipulated reactor at high water level.

Table 12. Dissolved oxygen concentration in each port. This was measured twice in fall 2012, once at low water level and once at high water level.

Date	Water level	Dissolved Oxygen (mg/L)			
		Constant reactor		Manipulated reactor	
		Top port	Bottom port	Top port	Bottom port
9/28/2012	low	7.8	0.95	a	7.55
10/2/2012	high	6.92	0.59	5.02	0.95

a The concentration could not be measured in the top port of the manipulated reactor at low water level, because it was not submerged.

Chapter 5: Results of Niche-Legacy Effects Study

5.1 Total Bacterial Communities

The comparison of agricultural soils, restored and natural wetlands sediments, and biofilters showed that each habitat had distinct communities of total bacteria (Figure 12). As measured by the distance between centroids (Table 13), the agricultural samples contained the most distinct communities, followed by the biofilters, the restored wetlands, and finally the wetlands. The biofilter ARISA communities were most similar to those from wetlands and least similar to those from agricultural fields.

Table 13. Centroid distances between each habitat's ARISA communities

Habitats	Centroid Distance
Wetland, restored wetland	0.2067
Biofilter, wetland	0.5261
Biofilter, restored wetland	0.5639
Agriculture, wetland	0.6852
Agriculture, restored wetland	0.7161
Biofilter, agriculture	1.2113

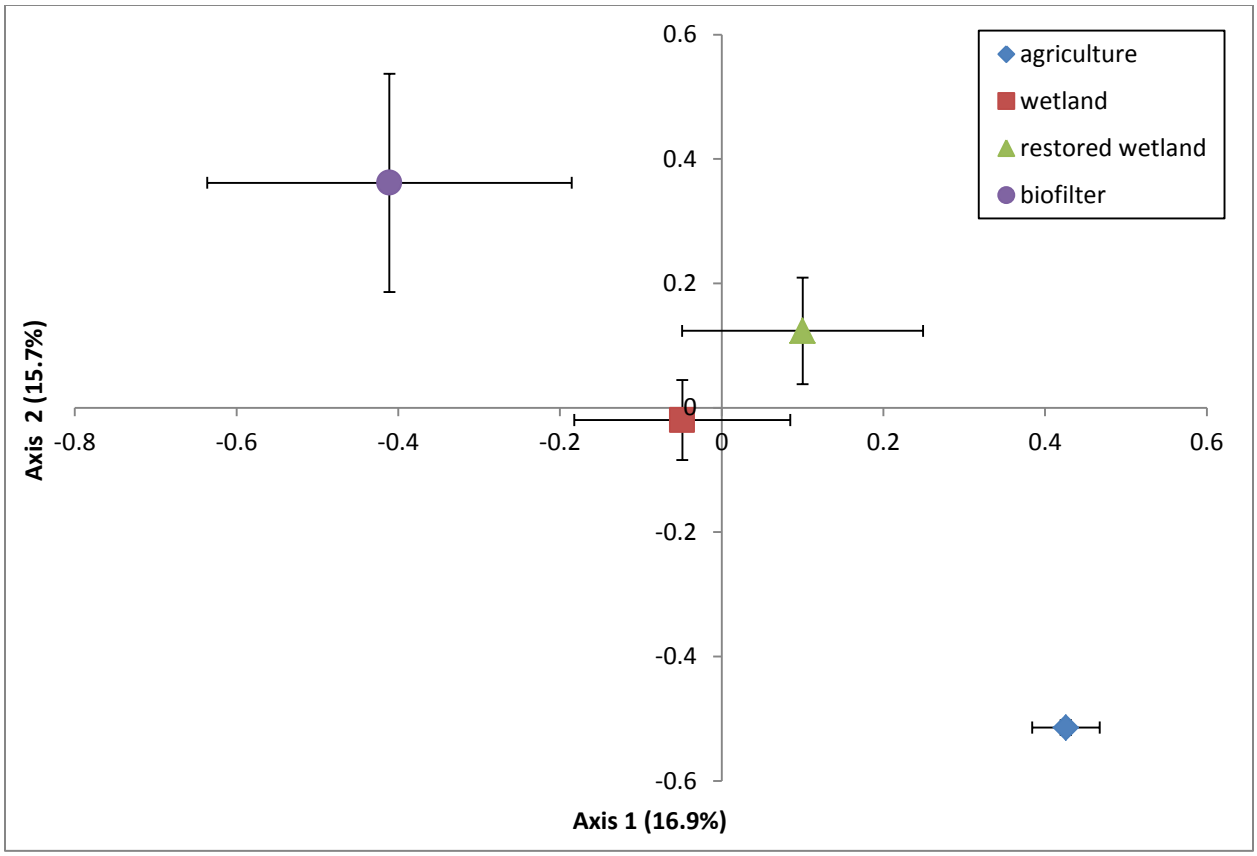


Figure 12. Centroids of partial correspondence analysis sample location of ARISA communities. Error bars show one standard deviation. Partial correspondence analysis removes the variance that can be attributed to location, allowing samples from multiple locations to be analyzed together. Each point is the average of all the sample scores of ARISA communities from that habitat. The sample score from each community collapses all the species abundance data into a point on the x and y axes. The axes represent theoretical environmental gradients. The percentage of microbial community variance explained by each axis is given in parentheses.

5.2 Denitrifying Bacterial Communities

The denitrifying bacterial communities from each habitat were also distinct from each other, although somewhat less so than the total bacterial communities (Figure 13). As measured by the distance between centroids (Table 14), the biofilter samples were the most distinct communities, followed by the agricultural samples, wetland samples, and finally the restored wetlands. The biofilter denitrifying communities were most similar to those from restored wetlands and least similar to those from agricultural fields.

Table 14. Centroid distances between each habitat's *nosZ* communities

Habitats	Centroid Distance
Wetland, restored wetland	0.6415
Agriculture, restored wetland	0.8062
Biofilter, restored wetland	0.9498
Agriculture, wetland	1.3019
Biofilter, wetland	1.2622
Biofilter, agriculture	1.5860

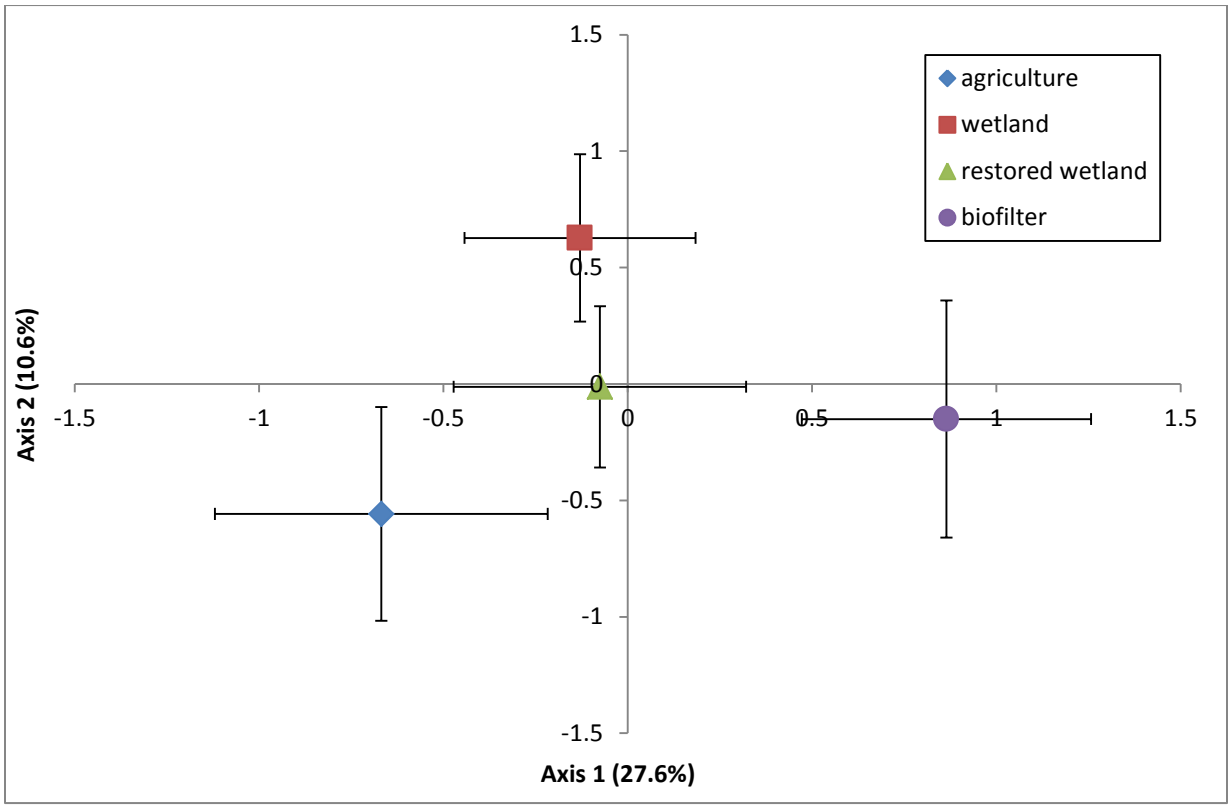


Figure 13. Centroids of partial correspondence analysis sample location of *nosZ* communities. Error bars show one standard deviation. Partial correspondence analysis removes the variance that can be attributed to location, allowing samples from multiple locations to be analyzed together. Each point is the average of all the sample scores of ARISA communities from that habitat. The sample score from each community collapses all the species abundance data into a point on the x and y axes. The axes represent theoretical environmental gradients. The percentage of microbial community variance explained by each axis is given in parentheses.

Chapter 6: Discussion

6.1 Laboratory Biofilters

The three main components of the laboratory biofilter study are the performance of the biofilters, the denitrification rates measured in the DEAs, and the microbial community structure, total bacteria and denitrifying bacteria. The response of these to the water level disturbances will be discussed, as will the relationships between performance, denitrification potential, and microbial community structure.

The laboratory biofilters successfully removed 31% of the nitrate at high water level and 59% of the nitrate at low water level. For comparison to other biofilters, the total nitrate removed per volume can be calculated given the influent nitrate loading and volume of the inundated woodchip bed. The removal rates were 5 g N/day/m^3 for both biofilters at high water level and 9 g N/day/m^3 for the manipulated biofilter at low water level. Therefore, the performance of the laboratory biofilters falls within the range of 2-22 g N/day/m^3 observed in the literature (Schipper et al, 2010b).

The laboratory biofilters had better performance when the water level was low than when it was high. This was different from the initial expectation that performance would be better at high water level, because a greater portion of the reactor would be anoxic. Hypotheses tested to explain this difference are that there was more dissolved oxygen at higher water level, short circuiting was occurring at high water level, or that more carbon was available at low water level. None of these were found to be conclusively true, although differences in carbon appear to be the most likely cause of the performance difference.

One potential explanation is that more carbon could be released when the water level is low. A greater portion of the reactor is aerobic, which could result in a faster carbon mineralization rate of the woodchips (Moorman et al, 2010). To determine whether differences in carbon availability were causing the difference in performance, DEAs with and without glucose amendments were performed. If there were little to no difference between the amended and unamended DEAs for the manipulated reactor but a difference between them for the constant reactor, this would indicate that carbon was limiting denitrification at the high water level, but not the low, causing the difference in performance. For both ports in both reactors, the nitrate removal rates and nitrous oxide production rates were higher when measured with glucose amendments than without, indicating that they are all carbon-limited. The difference in amended and unamended nitrate removal rates was larger for the constant reactor than the manipulated reactor. The smallest difference was between the DEAs for the top port of the manipulated reactor, which, being close to the water level, would likely feel the strongest effect of increased carbon. However, the differences in rates calculated from the two types of DEAs are not different enough between the two reactors to conclude that carbon availability was the main driver of performance differences.

Another possibility investigated is that differences in dissolved oxygen concentration in the reactors caused the change in performance at high and low water level. If dissolved oxygen were lower in the manipulated reactor at low water level than at high water level, this could explain the difference in performance. As expected, the dissolved oxygen was highest in the manipulated reactor when the water level was low, though, so dissolved oxygen concentrations do not seem to be causing the different nitrate removal.

A third possible explanation for the improved performance at low water level is that there are differences in actual HRT at different water levels, even though the theoretical HRT was the same. If the HRT was much higher at the low water level than the high, this would explain the differences in performance. The tracer studies performed by Bartolerio (2011) showed that the HRT was 8.84 hours in the manipulated reactor at low water level and 7.52 hours in the constant reactor. The HRT was longer at low water level than high, but only by 15%, whereas about twice as much nitrate was removed at low water level than high. Therefore, the difference in HRT does not fully explain the difference in performance. The similar construction of the two reactors and their similar performance at the same water level also suggested that the constant reactor and the manipulated reactor at high water level would have similar HRTs.

However, the change in performance after the inlet was modified suggests that differences in the way water flows through the reactors are causing the changes. The inlet had been modified so that the influent flowed in to the bottom of the reactor, rather than near the water line. Following the modification, the nitrate removal by the constant reactor doubled, whereas the removal in the manipulated reactor at low water level barely changed. The tracer study after the inlet was modified showed an increase in the difference between the two reactors' HRTs, which does not explain why the performance of the constant reactor became more similar to the manipulated reactor. Furthermore, if the increase in performance in the constant reactor were caused by a longer HRT, it seems likely that the even greater increase in the HRT of the manipulated reactor would result in better performance, which was not the case.

One possible explanation for the performance improvement in the constant reactor after modifying the inlet is that the bottom of the reactor, close to the inlet, could be an area of high denitrification activity that started receiving more flow in the constant reactor once the inlet was

changed, thus increasing the overall nitrate removal. The flow would have been passing through this part of the manipulated reactor at low water level anyway, which could be why the inlet modification did not have a large effect on the manipulated reactor's performance. The denitrification potential was not measured at the bottom of the reactor, close to the inlet.

Another unexpected result in nitrate removal was the difference in performance in the manipulated reactor right after weir removal and a week after weir removal. A potential explanation is that it could be caused by differences in carbon degradation. Possibly, the optimal conditions for carbon release from the woodchips occur in the portion of the reactor that was previously inundated just after weir removal. Exposed to the air, oxygen was available for aerobic respiration. This is supported by the faster degradation of woodchips near the top of field biofilters, where there is more oxygen (Moorman et al, 2010). The woodchips were still moist from the high water period, though.

DEAs were performed on woodchip samples from both ports of both biofilters to compare the denitrification activity in different parts of the reactors and to determine which operating conditions resulted in the highest denitrification rates. To my knowledge, all the DEAs performed as part of other biofilter studies only measured nitrous oxide production, as is typical for the assay, so only the nitrous oxide values in this study can be compared to the values found in the literature. The range of values found for the laboratory biofilters in this study was 0.000015-0.004 mg N/hour/g woodchips for all DEAs and 0.00005-0.004 mg N/hour/g woodchips when excluding the top port of the manipulated reactor during the extended dry period, which had significantly lower denitrification potential than usual. In DEAs of woodchip samples from a field biofilter, the denitrification rate ranged from 0.000176-0.00143 mg N/hour/g woodchips (Moorman et al, 2010). Denitrification rates measured in DEAs of

woodchip samples from several lab reactors range from about 0.001-0.004 mg N/hour/g woodchips (Warneke et al, 2011). The ranges of denitrification rates found in these two studies are both within the range of the laboratory biofilters. This indicates that nitrous oxide production rates observed in this work are reasonable for denitrifying biofilters. This study included many more DEAs than either of the ones cited here, which may explain the wider range of values in my work.

The denitrifying activity was very similar across reactors and across ports. Looking at several different ways of classifying the disturbance conditions, this similarity persists. The manipulated reactor was being disturbed by the changing water level, and indirectly the environmental conditions that control denitrification were being changed. The microbes were nevertheless able to maintain the same denitrification potential. Even with the changing microbial communities, the denitrification potential was not that different, suggesting that these communities were functionally redundant.

The exception to this similarity of denitrification potential is the top part of the disturbed reactor, before regular disturbance. It was at low water level, and fairly dried out for an extended period of time during this period, with moisture levels as low as 14% (data not shown). The denitrification potential in this port was lower during this time than all the other groupings of values. The water level does influence denitrification potential, but it takes a long time to see the effect.

A surprising result observed from the DEAs was the discrepancy between nitrate removal rates and the nitrous oxide production rates. If the nitrate is being removed by complete denitrification and the rate is being accurately measured in the DEAs, these two rates should be

the same. However, the nitrate removal rates were ten times higher than the nitrous oxide production rates.

The difference between nitrous oxide production and nitrate removal in DEAs is likely due to incomplete blocking by acetylene. Alternative explanations are nitrous oxide dissolved in the water, another method of nitrate removal besides denitrification, or incomplete denitrification. Nitrous oxide has a Henry's Law constant of 0.025 mol/kg/bar (NIST), which is 0.62 when converted to be dimensionless. The equilibrium concentration in water is less than in air, and the bottles in which the assays were performed were shaken prior to sampling to release dissolved gas. Nitrous oxide dissolved in the water would therefore not cause a ten-fold decrease in the measurement.

Another potential explanation for the discrepancy is that nitrate is being removed from the solution by methods other than denitrification. Nitrate can be used for cell growth. Given that the addition of chloramphenicol prevents new protein synthesis, it seems unlikely that this much nitrate would be removed via cell growth. Nitrate could also be removed via the Anammox process, in which nitrite is the electron acceptor for ammonia oxidation (Mulder et al, 1995). No ammonia is added to the solution in the DEAs, so it seems unlikely that sufficient ammonia is present for ten times more nitrate to be removed via Anammox than denitrification.

Incomplete denitrification is another potential explanation for the discrepancy between nitrous oxide production and nitrate removal measured in the DEAs. Possibly nitrate is being reduced, but is not being converted all the way to nitrous oxide. This would have potential implications for biofilter implementation, because if nitrate is not being transformed all the way to N_2, one environmental quality problem is being traded for another. No nitrite has been

detected in the water samples collected during DEAs, so denitrification is occurring at least past the reduction of nitrite. Nitric oxide has not been measured in the gas samples, so it is possible that this is the limiting step where denitrification is not going to completion. This leaves incomplete blocking of nitrous oxide reduction by acetylene and incomplete denitrification as the two likely causes of the discrepancy. If incomplete blocking is the cause, the nitrate removal rates are a better measurement of denitrification potential than the nitrous oxide production rates, but if incomplete denitrification is the cause, nitrous oxide production rates are the better measurement.

The total bacterial and denitrifying bacterial communities in the laboratory biofilters were investigated to determine the effects of the changing water level on them. Also, the differences in the diversity and community structure between the two reactors and between the top and bottom (constantly inundated) ports were compared.

The regular disturbance of the reactors resulted in the development of total bacterial communities distinct from the communities of the constant reactor. However, the communities of the manipulated reactor at low water level were not significantly different from those at high water level. This suggests that specific disturbance communities are being formed, rather than high water level communities and low water level communities.

Increased disturbance severity caused the greatest dissimilarity to other communities. This is evident from the ANOSIM R values comparing the top port of the disturbed reactor during regular disturbance to other ports at the same time. The top port was the most severely disturbed port in the reactor, because it was inundated at high water level, but just at the water level when the weir was removed. The bottom port, though, was inundated at all times, even

with the changing water level. Both ports of the constant reactor were more similar to the bottom port of the manipulated reactor than the more severely disturbed top port, indicating that the more severe disturbance was associated with a more distinct bacterial community.

The denitrifying bacterial communities, though, appear to be less affected by the changing water level than the total bacterial communities. No significant differences were observed between the communities of each reactor or between the communities of the manipulated reactor at high and low water level. This suggests that the denitrifying communities are resistant to disturbance by changing water level. This resistance may be because most denitrifying bacteria are facultative anaerobes (Zumft, 1997) and would be successful in a habitat with increased oxygen. Although moisture and carbon were also likely changing in the manipulated reactor with the varying water level, the denitrifying bacteria do not appear to have been sensitive to the change in these conditions.

6.2 Niche-legacy Study

In the comparison of microbial communities from different habitats, microbial communities in the biofilters were completely distinct from the communities found in agricultural soil and natural and restored wetland sediment. This suggests that the communities of the biofilters have developed primarily based on the influence of the environmental conditions there. The biofilter communities were even more dissimilar to those from agricultural soil than the wetland communities were from the agricultural soil. There was not an observable effect of the legacy of historical agricultural land use on the biofilter microbial communities. This was true for both the total bacterial communities and the denitrifying bacterial communities.

The literature review showed both niche and legacy having an effect on the microbial community structure. Of the papers included in the literature review of this thesis, both of the studies comparing habitats with different current land use primarily showed niche effects (Jesus et al, 2009, Waldrop et al, 2010). The habitat comparison in this thesis is most similar in concept to the experimental design of these disturbed/undisturbed habitat comparison studies, so it makes sense that the biofilter habitat comparison would have results similar to these studies. Like Peralta et al (2010), a difference in the microbial community structure of natural and restored wetlands was also observed in this habitat comparison. Looking at the larger scale, though, in which habitats are more environmentally different, shows a stronger niche effect on microbial community composition.

The results of this habitat comparison indicate that microbial communities can successfully be encouraged to develop through the implementation of the right environmental conditions. Many engineered ecosystems depend on microbes to degrade contaminants, as in the case of the denitrifying biofilters. This further validates the importance of studying microbial communities in engineered ecosystems and the ecological drivers that influence their composition.

Chapter 7: Conclusions

The laboratory biofilter study was conducted to observe the performance, denitrification activity, and microbial communities under constant conditions and under changing water level conditions. Active field biofilters experience variable flow and thus variable HRT, so the changing water level should give insight into the effects of this flow variability on denitrification within the reactors and the microbes responsible for carrying it out.

The performance of the laboratory biofilters was sensitive to the effects of changing water level, even with a constant HRT. About twice as much nitrate was removed at low water level than high. This difference is likely due to changes in flow patterns within the biofilters at different water levels. Based on the evidence of the DEAs with and without glucose, the lower water level also seems to increase the release of carbon from the woodchips, increasing the nitrate removal in the reactors. This suggests that the water depth of the biofilters should be considered in design to optimize nitrate removal.

The denitrification activity was similar in both reactors under almost all conditions. The only time water level appeared to influence the denitrification activity was in the top port during the period of long-term low water level, when these woodchips became dried out. This indicates that denitrification should not decrease due to short-term decreases in flow through the biofilter.

The bacterial communities from the two reactors became more divergent from each other after the period of regular disturbance began. Distinct disturbance communities appeared to develop, rather than high or low water level communities, with the most severely disturbed parts of the biofilter having the most different microbial communities. The changing bacterial communities did not affect denitrification.

The denitrifying bacterial communities were not different from each other between the different reactors, ports, or disturbance conditions. In spite of the changing water level, these communities exhibited a high level of resistance.

One of the original hypotheses that motivated the laboratory biofilter study was that the changing water level would encourage the development of microbial communities able to rapidly recover after disturbance. Functional redundancy was observed in the total bacterial communities with respect to denitrification and denitrifying bacterial communities were unaffected by the changing water level. Although the water level had a strong effect on the performance of the denitrifying biofilters, it seems to be primarily due to the environmental conditions in the reactors, rather than due to the microbial community composition.

The results of the niche-legacy comparison showed differentiation of the microbial communities of each habitat. Both the total and denitrifying bacterial communities in the field biofilters were independent of their source community, agricultural soil, and those from a somewhat similar environment, wetlands. This suggests that environmental conditions can be used to encourage the development of particular microbial communities in engineered ecosystems.

Some future work is recommended to further elucidate the effects of changing water level on the biofilters. To gain a better understanding of the effect of changing water level on performance, a shorter term study of the nitrate removal of the biofilters should be conducted. The inlet should be set up as it was after the modification, in which the water enters the biofilter at the bottom. This will test whether water level affects the performance of the reactor and how, when the inlet is not causing the differences. A study of the same length as the one already

performed is unnecessary, because a few months will be sufficient to observe the performance for several weeks at each water level. Although the results of the tracer studies did not show a shorter residence time in the reactors at high water level, the performance was the same in the two reactors at different water levels once the inlet was changed. This additional study will clarify the effect of water level, independent of changes it may have caused in flow through the reactor.

Second, to more completely study the effect of disturbance on the microbial communities, the biofilters should be pushed to failure. How long does it take for denitrification potential to return to its previous level? Does the microbial community structure return to its pre-disturbance state, and if so, how long does that rebound take? A more severe disturbance should be imposed that results in drastically less nitrate removal and a large change in microbial community composition. Some potential disturbances that are experienced by field biofilters include changes in influent flow rate, nitrate loading, or HRT. Injection of a pesticide is another potential way to realistically disturb the laboratory biofilters. This second proposed experiment would also be useful for reliability modeling of the denitrifying biofilters, continuing the work of Nick Bartolerio (2011).

Thirdly, the cause of the discrepancy between nitrate removal and nitrous oxide production should be further investigated. To do this, the gas samples collected during the DEAs should be analyzed for nitric oxide. If it accumulated over time during the assays, this indicates that nitric oxide reduction is the rate-limiting step of denitrification for the biofilter microbes. If this is the case, the nitrous oxide production rates are the better measure of denitrification potential. If the possibility of nitric oxide accumulation is eliminated, this leaves incomplete

blocking by acetylene of nitrous oxide reduction as the likely explanation. If this is the case, the nitrate removal rates are the better measure of denitrification potential in the biofilters.

Overall, the performance of the reactor is sensitive to the operating conditions, and the bacterial communities changed in response to the changing water level. However, the denitrification potential was not as sensitive, and the denitrifying bacterial communities within the reactors displayed resistance under the water level disturbance. The microbial communities of the biofilters were distinct from the communities in agricultural soil or natural or restored wetland sediments.

These results have positive implications for the removal of nitrate from agricultural runoff before it reaches sensitive water bodies through good performance by denitrifying biofilters. The functional redundancy of the total bacteria and resistance of the denitrifying bacteria under water level disturbance indicate that the microbes in the field biofilters should be able to successfully carry out denitrification, even when subjected to variable flow rate. The sensitivity of the biofilter performance to changing water level, though, suggests that nitrate removal in the reactors is highly dependent on either the HRT or other conditions. This can be addressed in the design of the biofilters to maximize nitrate removal. The niche-legacy habitat comparison also shows a strong effect of environmental conditions rather than legacy effects on the microbial community composition of the biofilters. This is encouraging in that environmental conditions can be used to develop microbial communities effective at nitrate removal.

References

Andrus, J.M. (2011) Microbial Community Analysis for denitrifying biofilters. PhD dissertation, University of Illinois at Urbana-Champaign, Urbana, IL.

Ayarza, J., Erijman, L. (2011) Balance of neutral and deterministic components in the dynamics of activated sludge floc assembly. *Environmental Microbiology*. **61**: 486-495

Bartolerio, N.A. (2011) Development and application of a microbial reliability model to analyze denitrifying biofilter stability. M.S. thesis, University of Illinois at Urbana-Champaign, Urbana, IL.

Baxter, A.M., Johnson, L., Edgerton, J., Royer, T., Leff, L.G. (2012) Structure and function of denitrifying bacterial assemblages in low-order Indiana streams. *Freshwater Science* **31**: 304-317.

Blowes, D.W., Robertson, W.D., Ptacek, C.J., Merkley, C. (1994) Removal of agricultural nitrate from tile-drainage effluent water using in-line bioreactors. *Journal of Contaminant Hydrology* **15**: 207-221.

Boyle, S.A., Rich, J.J., Bottomley, P.J., Cromack, J.K., Myrold, D.D. (2006) Reciprocal transfer effects on denitrifying community composition and activity at forest and meadow sites in the Cascade Mountains of Oregon. *Soil Biology and Biochemistry* **38**: 870-878.

Cameron, S.G., Schipper, L.A. (2010) Nitrate removal and hydraulic performance of carbon substrates for potential use in denitrification beds. *Ecological Engineering* **36**: 1588-1595.

Carleton, J.N., Grizzard, T.J., Godreh, A.N., Post, H.E. (2001) Factors affecting the performance of stormwater treatment wetlands. *Water Research* 35: 1552-1562.

Cavigelli, M.A., Robertson, G.P. (2000) The functional significance of denitrifier community composition in a terrestrial ecosystem. *Ecology* **81**: 1402-1414.

Christianson, L.E., Helmers, M.J., Bhandari, A. (2010) Bioreactor design geometry effects on nitrate removal. ASABE Paper #IDS-CSBE100073.

Christianson, L.E., Bhandari, A., Helmers, M.J. (2011) Pilot-scale evaluation of denitrification drainage bioreactors: reactor geometry and performance. *Journal of Environmental Engineering* **137**: 213-220.

Clarke, K.R. and Green, R.H. (1988) Statistical design and analysis for a" biological effects" study. *Marine Ecology Progress Series* **46**: 213-226.

Cooke, R.A., Doheny, A.M., Hirschi, M.C. (2001) Bio-reactors for edge-of-field treatment of tile outflow. ASABE Paper #0120181-17.

Cuddington, K. (2011) Legacy effects: The persistent impact of ecological interactions. *Biological Theory* **6**: 203-210.

David, M. B., Drinkwater, L. E,; McIsaac, G. F. (2010) Sources of nitrate yields in the Mississippi River Basin. *Journal of Environmental Quality* **39**: 1657-1667.

Doheny, A.M. (2003) Amelioration of tile nitrate-N and atrazine using inline biofilters. M.S. thesis, University of Illinois at Urbana-Champaign, Urbana, IL.

Fisher, M.M., and Triplett, E.W. (1999) Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Applied and Environmental Microbiology* **65**: 4630-4636.

Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., Fahey, D.W., Haywood, J., Lean, J., Lowe, D.C., Myhre, G., Nganga, J., Prinn, R., Raga, G., Schulz, M., Van Dorland, R. (2007) Changes in Atmospheric Constituents and in Radiative Forcing. In: Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M.Tignor and H.L. Miller (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Fraterrigo, J., Balser, T., Turner, M. (2006) Microbial community variation and its relationship with nitrogen mineralization in historically altered forests. *Ecology* **87**: 570-579.

Gopalakrishnan, G., Negri, M.C., Salas, W. (2012) Modeling biogeochemical impacts of bioenergy buffers with perennial grasses for a row-crop field in Illinois. *Global Change Biology Bioenergy* **4**: 739-750.

Griffiths, B.S., Hallett, P.D., Kuan, H.L., Gregory, A.S., Watt, C.W., Whitmore, A.P. (2008) Functional resilience of soil microbial communities depends on both soil structure and microbial community composition. *Biology and Fertility of Soils* **44**: 745–754.

Groffman, P.M., Gold, A.J., Addy, K. (2000) Nitrous oxide production in riparian zones and its importance to national emission inventories. *Chemosphere – Global Change Science* **2**: 291-299.

Gibert, O., Pomierny, S., Rowe, I., Kalin, R.M. (2008) Selection of organic substrates as potential reactive materials for use in a denitrification permeable reactive barrier wall (PRB). *Bioresource Technology* **99**: 7587-7596.

Greenan, C.M., Moorman, T.B., Kaspar, T.C., Parkin, T.B., Jaynes, D.B. (2006) Comparing carbon substrates for denitrification of subsurface drainage water. *Journal of Environmental Quality* **35**: 824-829.

Greenan, C.M., Moorman, T.B., Parkin, T.B., Kaspar, T.C., Jaynes, D.B. (2009) Denitrification in wood chip bioreactors at different water flows. *Journal of Environmental Quality* **38**: 1664-1671.

Holtan-Hartwig, L., Dorsch, P., Bakken, L.R. (2000) Comparison of denitrifying communities in organic soils: kinetics of NO_3^- and N_2O reduction. *Soil Biology and Biogeochemistry* **32**:833-843.

Howarth, R.W., Marino, R. (2006) Nitrogen as the limiting nutrient for coastal marine ecosystems: Evolving views over three decades. *Limnology and Oceanography* **51**: 364-376.

Hutchinson, G.E. (1957) Concluding remarks. *Cold Spring Harbor Symposium* **22**:415–427.

Jaynes, D.B., Kaspar, T.C., Moorman, T.B., Parkin, T.B. (2008) In situ bioreactors and deep-pipe drain-pipe installation to reduce nitrate losses in artificially drained fields. *Journal of Environmental Quality* **37**: 429–436.

Jesus, E., Marsh, T., Tiedje, J., Moreira, F. (2009) Changes in land use alter the structure of bacterial communities in Western Amazon soils. *The ISME Journal*. **3**:1004-1011.

Kalita, P.K., Algoazany, A.S., Mitchell, J.K., Cooke, R.A., and Hirschi, M.C. (2006) Subsurface water quality from a flat tile-drained watershed in Illinois, USA. *Agriculture, Ecosystems & Environment* **115**: 183-193.

Kulmatiski, A., Beard, K. (2008) Decoupling plant-growth from land use legacies in soil microbial communities. *Soil Biology and Biogeochemistry* **40**: 1059-1068.

Langenheder, S., Lindström, E. Tranvik, L. (2005) Weak coupling between community composition and functioning of aquatic bacteria. *Limnology and Oceonography*. **50**: 957-967.

Langenheder, S., Lindström, E. Tranvik L. (2006) Structure and function of bacterial communities emerging from different sources under identical conditions. *Applied and Environmental Microbiology* **72**: 212-220.

Long, L.M., Schipper, L.A., Bruesewitz, D.A. (2011) Long-term nitrate removal in a denitrification wall. *Agriculture, Ecosystems & Environment* **140**: 514-520.

Ma, W.K., Bedard-Haughn, A., Siciliano, S.D., Farrell, R.E. (2008) Relationship between nitrifier and denitrifier community composition and abundance in predicting nitrous oxide emissions from ephemeral wetland soils. *Soil Biology and Biogeochemistry* **40**:1114-1123.

Moorman, T.B., Parkin, T.B., Kaspar, T.C., Jaynes, D.B. (2010) Denitrification activity, wood loss, and N_2O emissions over 9 years from a wood chip bioreactor. *Ecological Engineering* **36**: 1567-1574.

Mulder, A., van de Graaf, A.A., Robertson, L.A., Kuenen, J.G. (1995) Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiology Ecology* **16**: 177-183.

Osborne, L.L. and Kovacic, D.A. (1993) Riparian vegetated buffer strips in water-quality restoration and stream management. *Freshwater Biology* **29**: 243-258.

Peralta, A., Matthews, J., Kent, A. (2010) Microbial community structure and denitrification in a wetland mitigation bank. *Applied and Environmental Microbiology* **76**: 4207-4215.

Philippot, L. (2002) Denitrifying genes in bacterial and Archaeal genomes. *Biochimica et Biophysica Acta* **1577**: 355-376.

Porter, M.D. (2011) Microbial community dynamics in denitrifying biofilters receiving agricultural drainage. M.S. thesis, University of Illinois at Urbana-Champaign, Urbana, IL.

Rawls, J.F., Mahowald, M.A., Ley, R.E., Gordon, J.I. (2006) Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell* **127**: 423-433.

Rich, J.J., Heichen, R.S., Bottomley, P.J., Cromack, J.K., Myrold, D.D. (2003) Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. *Applied and Environmental Microbiology* **69**: 5974-5982.

Rich, J.J., Myrold, D.D. (2004) Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA. *Soil Biology and Biochemistry* **36**: 1431-1441.

Robertson, W.D., Cherry, J.A. (1995) In situ denitrification of septic-system nitrate using reactive porous media barriers: field trials. *Ground Water* **33**: 99–111.

Robertson, W.D., Vogan, J.L., Lombardo, P.S. (2008) Nitrate removal rates in a 15-year-old permeable reactive barrier treating septic system nitrate. *Groundwater Monitoring & Remediation* **28**: 65-72.

Sambrook, J., and D. Russell (2001) *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanford, R.A., Wagner, D.D., Wu, Q., Chee-Sanford, J.C., Thomas, S.H., Cruz-García, C., Rodríguez, G., Massol-Deyáe, A., Krishnani, K.K., Ritalahti, K.M., Nissen, S., Konstantinidis, K.T., Löffler, F.E. (2012) Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proceedings of the National Academy of Science* **109**: 19709-19714.

Schipper, L.A., Cameron, S.C., Warneke, S. (2010a) Nitrate removal from three different effluents using large-scale denitrification beds. *Ecological Engineering* **36**: 1552-1557.

Schipper, L.A., Robertson, W.D., Gold, A.J., Jaynes, D.B., Cameron, S.C. (2010b) Denitrifying bioreactors – An approach for reducing nitrate loads to receiving waters. *Ecological Engineering* **36**: 1532-1543.

Schipper, L., Vojvodic-Vukovic, M. (1998) Nitrate removal from ground water using a denitrification wall amended with sawdust: field trial. *Journal of Environmental Quality* **27**: 664–668.

Soares, M.I.M., Abehovich, A. (1998) Wheat straw as substrate for water denitrification. *Water Research* **32**: 3790-3794.

Stone, M. and Krishnappan, B. (1997) Transport characteristics of tile-drain sediments from an agricultural watershed. *Water Air & Soil Pollution* **99**: 89-103.

Strickland, M., Lauber, C., Fierer, N. & Bradford, M. (2009) Testing the functional significance of microbial community composition. *Ecology* **90**: 441-451.

Tanner, R.S. (1997) "Cultivation of bacteria and fungi" in *Manual of Environmental Microbiology*, C.J. Hurst, G.R. Knudsen, M.J. McInerney, L.D. Stetzenbach, and M.V. Walter, eds., American Society for Microbiology, Washington, D.C., 52-60.

ter Braak, C. J. F, and P. Smilauer (ed.). 2002. CANOCO reference manual and CanoDraw for Windows user's guide: software for canonical community ordination (version 4.5). Wageningen University and Research Center Publications, Wageningen, Netherlands.

Tiedje, J.M., Simkins, S., Groffman, P.M. (1989) Perspectives on measurement of denitrification in the field including recommended protocols for acetylene based methods. *Plant and Soil* **115**: 261-284.

van Driel, P.W., Robertson, W.D., Merkley, L.C. (2006) Denitrification of agricultural drainage using wood-based reactors. *Transactions of the ASABE* **49**: 565-573.

Volokita, M., Abehovich, A., Soares, M.I.M. (1996) Denitrification of groundwater using cotton as energy source. *Water Science and Technology* **34**: 379-385

Waldrop, M., Balser, T., Firestone, M. (2000) Linking microbial community composition to function in a tropical soil. *Soil Biology and Biogeochemistry* **32**: 1837-1846.

Wallenstein, M.D., Myrold, D.D., Firestone, M., Voytek, M. (2006) Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. *Ecological Applications* **16**: 2143-2152.

Warneke, S., Schipper, L.A., Matiasek, M.G., Scow, K.M., Cameron, S., Bruesewitz, D.A., McDonald, I.R. (2011) Nitrate removal, communities of denitrifiers and adverse effects in different carbon substrates for use in denitrification beds. *Water Research* **45**: 5463-5475.

Wildman, T.A. (2002) Design of field-scale bioreactors for bioremediation of nitrate-N in tile drainage effluent. M.S. thesis, University of Illinois at Urbana-Champaign, Urbana, IL.

Woli, K.P., David, M.B., Cooke, R.A., McIsaac, G.F., Mitchell, C.A. (2010) Nitrogen balance in and export from agricultural fields associated with controlled drainage systems and denitrifying bioreactors. *Ecological Engineering* **36**: 1558–1566.

Zumft, W.G. (1997) Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews* **61**: 533-616.

Appendix

A.1 Laboratory Procedures

A.1.1 Biofilter Woodchip Sampling Procedure for Microbial Community Analysis

Adapted from Matthew Porter's procedure for field biofilter sampling.

Prior to Sampling

1. Wash and autoclave one 250 mL Nalgene sampling bottle for each port.
2. Weigh each sampling bottle without the lid and write the weight on the bottle.
3. Prepare Ringer's solution by adding 250 mL of nanopure water to 2 Ringer's tablets (Oxoid Limited, UK) and autoclaving on a 30 liquid cycle.
4. Autoclave a 250 mL graduated cylinder, 5 mm glass beads, and 1.5 mL centrifuge tubes on a 30 gravity cycle.

Sample Collection

1. Sterilize tweezers by covering in 70% ethanol and flaming.
2. Add at least 15 g woodchips from the bottom of the port to the appropriate sampling bottle.

Sample Processing

1. Remove woodchips from each bottle until 15 g remain.
2. Add 110 mL of sterile Ringer's solution to each bottle using a sterile graduated cylinder.
3. Place the bottles on the shaker tray in the 30ºC room overnight.
4. Label 1.5 mL centrifuge tubes with the date, reactor, and port. Label three tubes for each port.
5. Remove the bottles from the warm room to work at the lab bench.
6. Vigorously shake the bottles with the woodchip wash (the Ringer's solution in which the woodchips have been shaken), and transfer as much of the liquid as will fit into sterile, appropriately marked 50 mL centrifuge tubes.
7. Spin down the woodchip wash for 3 minutes at 5000xg.
8. Pour off the supernatant into a waste beaker.
9. Repeat steps 6-8, adding the woodchip wash to the same centrifuge tubes as before, until all the woodchip wash has been used. This should take two to three spins.
10. Add 2.5 mL of 1X PBS and 5 sterile glass beads to each 50 mL centrifuge tube.
11. Vortex each 50 mL centrifuge tube for 2 minutes on the high setting.
12. After vortexing, centrifuge at 750xg for 5 minutes.
13. Pipet 1 mL of the supernatant into each of the sterile, labeled 1.5 mL centrifuge tubes using sterile large orifice pipette tips. There should be two to three 1.5 mL centrifuge tubes for each port.
14. Store the DNA sample tubes at -20ºC until extraction.

A.1.2 DNA Extraction Protocol

Modified from the manufacturers' instructions by J. Malia Andrus to increase DNA yields. This uses the FastDNA Spin Kit (MP Biomedicals, Solon, OH).

1. Before beginning, load five 2 mL mid-sci tubes with autoclaved nanopure H_2O. Place these tubes into the block heater. Set the heater on low and at the 65°C mark. The water will be needed in the washing step.
2. Use a 96-tube plate, and set up the following tubes in this order vertically:
    a. One lysis tube per sample (in soil spin kit)
    b. One 1.5 mL tube per sample (on bench top)
    c. One Spin Module per sample (in soil spin kit)
    d. One 1.5 mL tube per sample (on bench top)
3. Label the tops of each of the tubes with sample name and date.
4. Vortex each sample, and add 400 μL of the sample to its lysis tube.
5. Add 1 mL of CLS-TC to each lysis tube.
6. Load the lysis tubes in the Fast Prep Machine. Ensure that the machine is balanced and the spindle is securing each tube in place. Run for 40 seconds at 5 m/s.
7. Centrifuge the lysis tubes for 5 mins at max speed.
8. Pipette 700 μL of supernatant into each sample's respective 2 mL tube.
9. Add 700 μL (equivalent to the volume of DNA) of binding matrix, into each 2 mL tube.
10. Transfer all of the 1.5 mL tubes into a 96 well tray, place the lid on the tray and slowly turn the tray up and down for 5 mins.
11. After 5 mins are up, centrifuge the 1.5 mL tubes for 1 min at max speed.
12. Pipette about half of the liquid out (700 μL) into a waste liquid container, but make sure to leave some liquid near the top of the glass bead level.
13. Re-suspend the glass beads with the remaining liquid, and pipette into the basket tube.
14. Spin the basket tubes for 1 min at max speed, to separate the liquid from the beads.
15. Empty the liquid from the basket tube into the waste liquid container.
16. Pipette the remaining extraction liquid into the now empty 1.5 mL tube (should get about 500 μL).
17. Add an equivalent volume of binding matrix into each of the tubes.
18. Repeat steps 10 through 15.
19. Add 500 μL of SEWS to the basket tube and centrifuge for 1 min.
20. Empty the wash into the waste container.
21. Add another 500 μL of SEWS to the basket tube and centrifuge again for 1 min.
22. Empty the wash into the waste container.
23. Centrifuge for 1 min to remove any remaining ethanol.
24. Place the basket into the remaining 1.5 mL tube.
25. Add 100 μL of 65°C water to the glass beads in the basket.
26. Vortex the samples, and then centrifuge for 1 min (The tops will pop open, so place the tubes into the centrifuge so that the lid opens down.
27. Store at -20^{o}C until further analysis.

A.1.3 CTAB Cleanup Procedure

Established CTAB procedure of Angela Kent's laboratory.

working CTAB stock recipe (10% CTAB/0.7 M NaCl):

1. Dissolve 4.1 g NaCl in 80 mL H_2O.
2. Place on heater/stirrer with stirring bar.
3. Slowly add 10 g CTAB (cetyl trimethyl ammonium bromide)
4. Adjust final volume to 100 mL.
5. Autoclave.

Purification protocol

1. Pre-warm working CTAB stock to 65°C in a 1.5 mL tube in the heat block.
2. Transfer 100 mL of the extracted DNA into a 1.5 mL tube for each sample.
3. Label (long-term storage) one autoclaved 1.5 mL tube for each DNA extraction.
4. Add 16.25 mL of 5 M NaCl (autoclaved) to each DNA extract to adjust the NaCl concentration of each DNA extract to 0.7 M.
5. Add 12 mL of warm working CTAB stock (0.1 vol). Mix thoroughly and incubate at 65°C for 15 mins. *use filter tips
6. Add 128 mL (one volume) of chloroform:isoamyl alcohol (24:1). Mix carefully but thoroughly. Centrifuge at maximum speed (14,000 x g) for 5 mins.
   *Use filter tips; do not collect white interface layer (only transfer top layer for precipitation)
7. Carefully remove the top layer to a clean, well-labeled 1.5 mL tube (should get about 125 mL). Add 256 mL (two volumes) of cold 100% EtOH to precipitate the DNA. After adding/mixing 100% EtOH, increase precipitation of DNA by putting samples in the freezer (-20°C) overnight.
8. Mix thoroughly and centrifuge at maximum speed for 15 mins (increase time to increase expected yield).
   ***Make sure to orient the tubes in the same direction (e.g. hinge side point out) so you know the location of the DNA (if hinge side facing out, DNA will be along the side of the hinge).
9. Carefully avoiding the pellet, remove supernatant. Add 125 mL 70% EtOH (cold), flick to mix, and centrifuge at maximum speed for 7 mins.
10. Repeat step 9 once.
11. Remove supernatant. Allow pellet to air dry (approximately 15 mins – this may take longer and be careful not to dry down samples all the way). Place tubes upside-down, propped against a tube rack and over kim wipes.
12. Resuspend pellet in 50 mL dH_2O.
13. Wash back side of tube (where DNA is supposed to be) with dH_2O using the pipet. If you orient all your tubes hinge side out in the centrifuge, then the DNA should be along the hinge side, so make sure you run water down that side.
14. Store at -20°C until further analysis.

A.1.4 Denitrification Enzyme Assay Protocol

Adapted from Nicholas Bartolerio's DEA procedure, based on the acetylene block method described by Tiedje et al. (1989).

1. Mix a solution of 15 mg/L NO_3^- and 0.1 g/L chloramphenicol in nanopure water.
2. Measure solution into 75 mL aliquots for each DEA to be carried out. Aliquots should be stored in autoclaved 125 mL Nalgene sampling bottles.
3. Sample woodchips from each port using sterile tweezers, as for the microbial community sampling.
4. Add 10 g of woodchips into a labeled 150 mL Wheaton bottle. Duplicate assays should be performed for each port. Record the mass of the bottle empty and with woodchips.
5. Pour nitrate-chloramphenicol solution into each Wheaton bottle.
6. Close the Wheaton bottles with lids fitted with rubber septa.
7. Flush the bottle headspace with helium gas for 10 minutes. An extra needle should be in each septum, so the bottle doesn't become over-pressurized and explode. Feel the tops of the needles to ensure that gas is exiting. The bottles should be behind the shield, in case any break during flushing.
8. Extract 15 mL of headspace gas and replace it with acetylene gas to give an approximate acetylene concentration of 20% in the headspace.
9. Take a 12 mL gas sample from each bottle, and store them in labeled Vacutainers.
10. Take a 4 mL liquid sample from each bottle and filter through a 0.2 μm syringe filter. Store them in labeled Dionex IC vials.
11. After sampling, replace the removed volume with 16 mL of 90:10 He:acetylene gas mixture.
12. Repeat steps 9-11 at two, four, and six hours.
13. After all sampling, remove the caps and pour water from a graduated cylinder into the bottles until full to measure the headspace volumes.
14. Put bottles into the drying oven at 105^oC for a couple days.
15. Store the liquid samples at 4^oC until further analysis.
16. When the bottles are dry, measure and record the dry mass of the bottles with woodchips.
17. Analyze the liquid samples for NO_3^- using ion chromatography.
18. Analyze the gas samples for N_2O using gas chromatography.

A.1.5 Ion Chromatography Settings

Column: AS18
Sampling loop: 0.25 μm
Flow rate: 1.0 mL/min
Eluent concentration: 32.0 mM
Suppressor current: 80 mA

A.2 Sample Information

Samples used in niche-legacy comparison

Sample	Habitat	Location	Latitude	Longitude	Percent Moisture	Sampling Time	Original Sampler
B118	agriculture	Dekalb, NIARC	-87.7999	41.94533		Winter 2009	K. Woli
B137	agriculture	Simpson, Dixon Springs	-88.8625	41.80105		Winter 2009	K. Woli
B141	agriculture	Simpson, Dixon Springs	-88.8625	41.80105		Winter 2009	K. Woli
B171	agriculture	UIUC SoyFACE	-88.2398	40.18569		Winter 2009	K. Woli
MP1	agriculture	Morrow Plots	-88.2398	40.18569	20.4	August 2009	NRES 598
MP9	agriculture	Morrow Plots	-88.2398	40.18569	20.9	August 2009	NRES 598
MP11	agriculture	Morrow Plots	-88.2398	40.18569	20.8	August 2009	NRES 598
MP13	agriculture	Morrow Plots	-88.2398	40.18569	21.3	August 2009	NRES 598
MP15	agriculture	Morrow Plots	-88.2398	40.18569	23.5	August 2009	NRES 598
MP17	agriculture	Morrow Plots	-88.2398	40.18569	22.6	August 2009	NRES 598
MP19	agriculture	Morrow Plots	-88.2398	40.18569	24.0	August 2009	NRES 598
MP21	agriculture	Morrow Plots	-88.2398	40.18569	23.8	August 2009	NRES 598
MP23	agriculture	Morrow Plots	-88.2398	40.18569	25.2	August 2009	NRES 598
WL-404	restored wetland	JP	-88.78	42.03	58.81	June 2007	D. Flanagan
WL-405	restored wetland	JP	-88.78	42.03	34.17	June 2007	D. Flanagan
WL-409	natural wetland	JP	-88.78	42.03	62.56	June 2007	D. Flanagan
WL-410	natural wetland	JP	-88.78	42.03	51.11	June 2007	D. Flanagan
WL-421	restored wetland	32AP	-89.65	39.84	9.02	June 2007	D. Flanagan
WL-424	restored wetland	32AP	-89.65	39.84	15.92	June 2007	D. Flanagan
WL-427	natural wetland	32AP	-89.65	39.84	19.03	June 2007	D. Flanagan
WL-428	natural wetland	32AP	-89.65	39.84	16.61	June 2007	D. Flanagan
WL-434	restored wetland	13AB	-89.67	39.88	22.65	June 2007	D. Flanagan
WL-439	restored wetland	13AB	-89.67	39.88	10.51	June 2007	D. Flanagan
WL-443	natural wetland	13AB	-89.67	39.88	31.20	June 2007	D. Flanagan
WL-444	natural wetland	13AB	-89.67	39.88	26.23	June 2007	D. Flanagan

WL-453	restored wetland	35A	-91.35	39.73	13.34	June 2007	D. Flanagan
WL-455	restored wetland	35A	-91.35	39.73	15.85	June 2007	D. Flanagan
WL-459	natural wetland	35A	-91.35	39.73	34.19	June 2007	D. Flanagan
WL-461	natural wetland	35A	-91.35	39.73	14.83	June 2007	D. Flanagan
WL-467	restored wetland	11A	-89.65	40.44	9.99	June 2007	D. Flanagan
WL-468	restored wetland	11A	-89.65	40.44	11.55	June 2007	D. Flanagan
WL-475	natural wetland	11A	-89.65	40.44	15.70	June 2007	D. Flanagan
WL-476	natural wetland	11A	-89.65	40.44	16.66	June 2007	D. Flanagan
WL-483	restored wetland	1AB	-89.65	42.46	32.69	June 2007	D. Flanagan
WL-484	restored wetland	1AB	-89.65	42.46	26.79	June 2007	D. Flanagan
WL-490	natural wetland	1AB	-89.65	42.46	19.51	June 2007	D. Flanagan
WL-491	natural wetland	1AB	-89.65	42.46	22.59	June 2007	D. Flanagan
WL-514	agriculture	JP	-88.78	42.03	36.71	June 2007	D. Flanagan
WL-515	agriculture	JP	-88.78	42.03	25.42	June 2007	D. Flanagan
FP03-20100812-A2C	biofilter	FP03	-88.2261	40.10465	69	August 2010	M. Porter
FP03-20100812-B5C	biofilter	FP03	-88.2261	40.10465	72	August 2010	M. Porter
FP03-20100812-C2C	biofilter	FP03	-88.2261	40.10465	66	August 2010	M. Porter
FP03-20100812-D2C	biofilter	FP03	-88.2261	40.10465	69	August 2010	M. Porter
FP03-20100812-E2C	biofilter	FP03	-88.2261	40.10465	62	August 2010	M. Porter
FP03-20100812-F5C	biofilter	FP03	-88.2261	40.10465		August 2010	M. Porter
FP03-20100812-G2C	biofilter	FP03	-88.2261	40.10465		August 2010	M. Porter
F1	agriculture	FP03	-88.2261	40.10465		August 2010	M. Porter
F2	agriculture	FP03	-88.2261	40.10465		August 2010	M. Porter
F3	agriculture	FP03	-88.2261	40.10465		August 2010	M. Porter
W1	natural wetland	FP03	-88.2261	40.10465		August 2010	M. Porter
W2	natural wetland	FP03	-88.2261	40.10465		August 2010	M. Porter
W3	natural wetland	FP03	-88.2261	40.10465		August 2010	M. Porter
DE01-20100729-A2C	biofilter	DE01	-95.665	37.6	57	July 2010	M. Porter
DE01-20100729-B5C	biofilter	DE01	-95.665	37.6	63	July 2010	M. Porter
DE01-20100729-E2C	biofilter	DE01	-95.665	37.6	64	July 2010	M. Porter

FP07-20100812-A2C	biofilter	FP07	-88.2261	40.10465	66	August 2010	M. Porter
FP07-20100812-B2C	biofilter	FP07	-88.2261	40.10465	67	August 2010	M. Porter
FP07-20100812-C5C	biofilter	FP07	-88.2261	40.10465	71	August 2010	M. Porter
FP07-20100812-C5W	biofilter	FP07	-88.2261	40.10465		August 2010	M. Porter
FP07-20100812-D5C	biofilter	FP07	-88.2261	40.10465	63	August 2010	M. Porter
FP07-20100812-D5W	biofilter	FP07	-88.2261	40.10465		August 2010	M. Porter
FP07-20100812-E2C	biofilter	FP07	-88.2261	40.10465		August 2010	M. Porter