MICROBIAL COMMUNITY DYNAMICS IN DENITRIFYING BIOFILTERS RECEIVING AGRICULTURAL DRAINAGE

BY

MATTHEW DAVID PORTER

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, 2011

Urbana, Illinois

Advisers:

Research Assistant Professor Julie L. Zilles
Associate Professor Angela D. Kent
Abstract

Subsurface drains (tile drains) used to augment drainage in agricultural fields serve as a major pathway for agricultural nitrate pollution to enter surface waters. Used primarily in the Midwestern United States, nitrates from tile drainage systems contribute to eutrophication within the Gulf of Mexico, ultimately leading to the formation of the Gulf of Mexico hypoxic zone. One cost-effective solution for reducing the quantity of nitrate entering surface waters is the denitrifying biofilter. A typical denitrifying biofilter consists of a woodchip-filled trench inline with the drainage tile; woodchips provide a carbon substrate to the microorganisms that convert nitrate to nitrogen gas through the denitrification pathway.

Research to date has focused on applying traditional engineering approaches to improve biofilter performance and reliability. Although previous work has produced valuable results related to the selection of appropriate biofilter media, and optimization of operational parameters, denitrifying biofilters still perform somewhat unpredictably. Therefore, in this work we sought to understand how environmental and management factors affect the microbial communities responsible for biofilter functional.

To do so, we employed two different approaches. First, in our spatial study we sampled one biofilter over the course of an afternoon in 2007 to determine how total and denitrifying bacterial communities varied by depth, transect, and position along a transect. Second, in our temporal study we sampled three biofilters over two years, January 2009 – December 2010, to determine how total bacterial, denitrifying bacterial, and fungal communities correlated with environmental and management variables over time. Total bacterial community structure was analyzed by Automated Ribosomal Intergenic Spacer Analysis (ARISA), denitrifying bacteria community structure was determined by Terminal Restriction Fragment Length Polymorphism.
(T-RFLP) of \textit{nosZ} (one of the nitrous oxide reductase genes), and fungal ARISA (FARISA) was used to determine fungal community structure.

Spatial and temporal results from the biofilter studies have provided valuable insight into how microbial communities, essential to the functionality of the denitrifying biofilter, vary over space and time. Results from our spatial study indicate that the composition of the total bacterial community varied by depth and sampling transect, but not by sampling position along a transect. Denitrifying bacteria community composition, unlike total bacteria, showed little variance by depth, transect, or sampling position.

Results from our temporal study indicate that depth and season were two of the most important factors influencing the structure of total bacterial, fungal, and denitrifying bacterial communities within all three biofilters. Correspondence analysis results suggest that microbial community structuring by depth may have been driven by moisture and temperature gradients. In addition to depth, microbial community composition was influenced by seasonal factors within all three denitrifying biofilters. For 2009 and 2010 bi-annual seasonal variation was observed for samples collected in January – June or July – December. Results from correspondence analysis suggest that seasonality was likely driven by moisture, water flow, and temperature. In addition to observing patterns in community composition related to depth and season, we were able to identify small subsets of the total bacterial, denitrifying, and fungal populations that were either influential in shaping the overall community structure, were correlated to strong biofilter performance, or both.

The application of denitrifying biofilters in tile drain networks shows the promise of significantly reducing anthropogenic inputs of nitrogen into aquatic ecosystems. By developing an understanding of how microbial population dynamics, environmental parameters, and
management factors relate to biofilter performance, reliability, stability and resilience, the effectiveness and viability of the denitrifying biofilter as a treatment technology will ultimately be enhanced.
To My Beautiful Wife Jessica
Acknowledgements

I would like to acknowledge and thank my two advisers, Dr. Julie Zilles and Dr. Angela Kent. I particularly would like to express my profound gratitude and appreciation to Dr. Zilles for providing me with the opportunity to participate in this work, for her unwavering support, and her incredible ability to understand the realities of life outside the lab. Also, I would like to express my sincere appreciation to Dr. Kent for spending countless hours instructing me one-on-one on how to perform and interpret multivariate statistics.

The other members of the denitrifying biofilter project, and our collaborators deserve special recognition. In particular I would like to thank Dr. Luis Rodriguez for his support and helpfulness in coordinating with our collaborators. Dr. J. Malia Andrus for beginning this project, and for providing valuable lab and field instruction. Nick Bartolerio for his help with field sampling and sample processing. Ariane Peralta and Sara Paver for their insights into the world of microbial ecology. And finally, Dr. Richard Cooke for allowing access to our three study sites.

I would also like to thank friends and family for being supportive throughout this process. Finally, in the tradition of saving the best for last, special recognition is due to my wife Jessica, for her incredible patience and never ending support.

This work was funded by the National Science Foundation Environmental Sustainability program.
# Table of Contents

Chapter 1: Introduction .................................................................................................................. 1

Chapter 2: Literature Review ......................................................................................................... 6
  2.1 Introduction ........................................................................................................................... 6
  2.2 Current and Parallel Denitrifying Biofilter Research ............................................................. 6
  2.3 Bacterial Denitrification ....................................................................................................... 7
  2.4 Role of Fungi ....................................................................................................................... 8
  2.5 Spatial Studies of Microbial Communities ........................................................................... 9
  2.6 Time Series Studies of Microbial Communities .................................................................. 10

Chapter 3: Methodology .................................................................................................................. 12
  3.1 Study Sites ......................................................................................................................... 12
  3.2 Determination of Environmental Variables ...................................................................... 17
  3.3 Sample Collection ............................................................................................................. 18
  3.4 DNA Extraction and Purification ....................................................................................... 19
  3.5 Microbial Community Structure Determination ............................................................... 19
  3.6 Fragment Analysis ............................................................................................................ 21
  3.7 Statistical Analysis ............................................................................................................ 22

Chapter 4: Temporal Study Results ............................................................................................. 26
  4.1 Introduction ....................................................................................................................... 26
  4.2 FP07 .................................................................................................................................. 28
  4.3 FP03 .................................................................................................................................. 38
  4.4 DE01 .................................................................................................................................. 47
  4.5 Influential Microbial Communities .................................................................................... 54
  4.6 Populations Associated with Strong Nitrate Removal ....................................................... 56
  4.7 Summary ............................................................................................................................ 58

Chapter 5: Spatial Study ................................................................................................................ 61
  5.1 Introduction ....................................................................................................................... 61
  5.2 Methodology ...................................................................................................................... 61
  5.3 Results ............................................................................................................................... 63
  5.4 Discussion .......................................................................................................................... 68

Chapter 6: Discussion .................................................................................................................... 70
  6.1 Introduction ....................................................................................................................... 70
  6.2 Microbial Community Composition Differences by Depth .............................................. 70
  6.3 Microbial Community Composition Differences By Season ......................................... 73
  6.4 Influential Populations and Populations Associated with Strong Nitrate Removal ........ 76
  6.5 Conclusions and Future Work .......................................................................................... 77

References ....................................................................................................................................... 81

Appendix ......................................................................................................................................... 88
  A.1 Sample Processing, DNA Extraction, and DNA Purification Procedures ....................... 88
  A.2 Sample Locations and Miscellaneous Information ............................................................ 93
Chapter 1: Introduction

The agricultural usage of nitrogen-based fertilizers in the United States has increased approximately 446% over the past 49 years, from 4.5 million tons in 1960 to 24.8 million tons in 2009 (62), and with considerable consequences to environmental quality. Possibly the most well known environmental consequence of agricultural application of nitrogen fertilizers has been the establishment and expansion of large coastal hypoxic dead zones, such as that found in the northern Gulf of Mexico (49, 50, 59, 61). This annually recurring hypoxic zone has averaged 13,500 km$^2$ between 1985 and 2009, with a maximum area of 22,000 km$^2$ (49).

Coastal hypoxia in the Gulf of Mexico occurs during the summer when warm water combines with large inputs of nutrients, such as nitrogen, from the Mississippi River. This combination results in favorable growth conditions for phytoplankton. When the large plumes of phytoplankton die they are decomposed by aerobic bacteria, depleting oxygen from coastal waters in the Gulf of Mexico (50). Once oxygen levels have been depleted to a concentration of 2 mg/L or less (49, 53) the water is defined as hypoxic. Hypoxic water conditions have a detrimental effects on aquatic wildlife, including mortality and the forced migration of fishery resources (50).

Contributing to the excess of nutrient inputs to the Gulf of Mexico, and ultimately the formation of hypoxic zones, are agricultural activities in the Mississippi River drainage basin. Fertilizer runoff from fields in the Midwestern United States eventually makes its way into the Mississippi River (31) and ends up in the Gulf of Mexico. Further contributing to the export of nitrate from agricultural fields to the Gulf of Mexico, is the utilization of agricultural subsurface drainage systems.
Subsurface drainage networks, otherwise known as tile drains, are commonly installed in agricultural fields in the Midwestern United States to drain lands that would otherwise be too saturated for agriculture. Tile drains in the Midwestern United States are widely utilized, covering an estimated 20 million ha of agricultural land (4). In recent years, however, these systems have come under scrutiny for exacerbating the export of nitrate from agricultural fields to surface waters (4, 16). Research on these subsurface drainage systems suggests that tile drains serve as a direct conduit for nitrate transport between agricultural fields and receiving surface waters, bypassing wetlands and riparian zones that serve as natural regions of denitrification (4).

With subsurface drainage required for agriculture in much of the Midwestern United States, and nitrogen fertilizers essential to continued high yields, it is necessary to find innovative and cost-effective solutions to treat tile drain effluent. One promising solution currently under investigation is the denitrifying biofilter (Figure 1) (13, 17, 63). Inexpensive to install and maintain, denitrifying biofilters are placed inline with drainage tile, and consist of a woodchip-filled trench (13, 17, 32, 43, 57, 63, 64). Native microorganisms colonize the woodchips and convert nitrate to nitrogen gas through the process of denitrification (68). Woodchips within the biofilter function as a carbon source and electron donor for microorganisms, while nitrate functions as the electron acceptor.
Field-scale denitrifying biofilters have proven effective in reducing nitrate loads from entering surface waters. In a recent two-year study, Woli et al. demonstrated that field-scale biofilters were effective in removing up to 33% of annual nitrate loads from subsurface drainage effluent (64). Previous denitrifying biofilter research at the University of Illinois has focused on traditional engineering approaches, specifically determining cost-effective carbon substrates and appropriate hydraulic retention times (13, 17). The results of these studies have provided valuable conclusions regarding the effectiveness of woodchips as a carbon substrate, as well as demonstrating the correlation between biofilter performance and hydraulic retention time (HRT) (13, 17). Unfortunately, however, there remain instances where denitrifying biofilters have performed inconsistently.
To augment traditional engineering approaches, we sought to address denitrifying biofilter performance and reliability issues by achieving a greater understanding of the microbial communities (total bacteria, fungi, and denitrifying bacteria) responsible for biofilter functionality. Specifically, the goals of my project were twofold. First, to characterize relationships between microbial populations, measured environmental variables, and biofilter performance over time. Second, to develop an understanding of the spatial structure of microbial communities within one biofilter.

The research presented in this thesis is comprised of two related denitrifying biofilter studies. The majority of this work involves a two-year temporal study of microbial community composition in three denitrifying biofilters. This research took place from January 2009 – December 2010. Microbial community “fingerprinting” techniques and multi-variate statistical analyses were utilized to study the effect of environmental and management factors on the total bacterial (ARISA), denitrifying bacterial (nosZ T-RFLP), and fungal communities (FARISA) within each biofilter. A smaller component of this research was a spatial study of the total (ARISA) and denitrifying bacterial communities (nosZ T-RFLP) within one biofilter. Samples for this spatial study were collected at a single time point (March 16, 2007) prior to the start of the two-year temporal study. Spatial study results and discussion are located in Chapter 5.

While taking a microbial ecology approach to improving the functionality of an engineered system may seem indirect, we believed it to be the best course of action given the current state of biofilter development. With over ten years of research related to different media types, retention times, flow characteristics, and shapes, the next logical step in the development of this technology is to understand the microorganisms responsible for biofilter function. Therefore, we sought to better understand how environmental and management conditions
affected biofilter microbial communities and to ultimately be able to more accurately predict biofilter performance and reliability, stability, and resilience.
Chapter 2: Literature Review

2.1 Introduction

The purpose of this literature review is to provide background information pertinent to our two-year temporal biofilter study, one-day spatial study, and current research on denitrifying biofilters. The information provided here is organized into the following sections: current and parallel denitrifying biofilter research; bacterial denitrification; wood degrading fungi; spatial studies of microbial communities; time series studies of microbial communities. From each of these sections it is my goal to introduce the reader to the design and function of denitrifying biofilters, and factors that may possibly affect microbial populations within a biofilter.

2.2 Current and Parallel Denitrifying Biofilter Research

Recently there have been a number of studies that have investigated a broad range of topics related to denitrifying biofilter performance and longevity. In one study the authors demonstrated a 55% nitrate mass reduction in a 5-year study of a woodchip-filled denitrification wall (32), and a 2-year denitrifying biofilter study showed an average nitrate load reduction of 33% (64). Long-term research into denitrifying biofilter performance has demonstrated sustained potential for denitrification for over 9 years (43), and other work has shown that denitrification can be supported for up to 15 years without replacing woodchips (57).

Current denitrifying biofilter research is contributing valuable information related to the long-term viability and potential of the denitrifying biofilter as a treatment option for nitrate runoff. Lacking from this research, however, is insight into the microbial communities responsible for biofilter functionality. By better understanding microbial communities within the denitrifying biofilter we will be able to better predict denitrifying biofilter performance, reliability, stability, and resilience.
2.3 Bacterial Denitrification

Bacterial denitrification is the basic function that makes the denitrifying biofilter possible as a treatment technology. Therefore, it is essential to have a basic understanding of the principles of bacterial denitrification before studying microbial communities within a denitrifying biofilter. Bacterial denitrification, or the conversion of nitrate (NO$_3^-$) to dinitrogen (N$_2$) is a necessary component of the global nitrogen cycle (68). Denitrification is the stepwise reduction from nitrate (+5) to dinitrogen (0) with three intermediate compounds: nitrite (NO$_2^-$), nitric oxide (NO), and nitrous oxide (N$_2$O) (22, 41, 68). The enzymes necessary for denitrification are coded by: the respiratory nitrate reductase genes (nar), respiratory nitrite reductase genes (nir), nitric oxide reductase genes (nor), and the nitrous oxide reductase genes (nos) (Figure 2) (68).

![Figure 2](image_url)

Figure 2. Denitrification pathway indicating the sequence of enzymes involved in bacterial denitrification. Nar = respiratory nitrate reductase, Nir = respiratory nitrite reductase, Nor = nitric oxide reductase, Nos = nitrous oxide reductase.

Bacterial denitrification requires the following conditions (22):

1. Bacteria that have the metabolic ability to denitrify must be present.
2. Favorable electron donors such as organic carbon compounds must be present.
3. An anaerobic or low oxygen (O$_2$) environment must exist.
4. Nitrogen oxides, such as NO$_3^-$, must be present to serve as terminal electron acceptors.
Knowledge of the mechanisms of bacterial denitrification, and environmental conditions that influence the community composition and activity of bacteria involved in denitrification is essential to understanding patterns in denitrifying biofilter microbial community structure. Denitrifying bacteria are responsible for transformations that remove nitrate from the system. Therefore it is essential to not only understand how these bacteria are affected by environmental conditions, but to also understand how fungi and other bacterial populations relate to denitrifying bacteria.

2.4 Role of Fungi

Fungi comprise one of the three microbial communities studied in this work. The interest in studying fungal community structure was driven in part by previous research (2), which suggested that fungi might play an indirect role in the denitrification potential of a biofilter. While there are some fungi within the phyla Ascomycota that have the ability to denitrify (68), previous work by Appleford et al. suggested, based on denitrifying enzyme assays, that bacteria are the main source of denitrification within a biofilter (2). Appleford et al. did propose, however, that fungi might play an indirect role in biofilter performance – possibly by providing carbon substrate to denitrifying bacteria (2). In this role, fungi may be critical to biofilter functionality, as previous research has indicated that any increases in carbon availability in soil environments would likely lead to an increase in denitrification (22).

Fungi are known for their ability to degrade wood. Prominent wood-degrading fungi are those within the phyla Basidiomycota (3, 21, 30, 41). Within the Basidiomycetes there are two groups of fungi, brown-rot and white-rot, responsible for degrading wood (21, 41). Brown-rot fungi comprise the minority of known wood-rotting fungal species (21) and have been shown to preferentially degrade cellulose and hemicellulose over lignin in wood (21, 41). The other group
of wood degraders, white-rot fungi, are considered extremely important in nutrient cycling due to their ability to effectively and preferentially degrade lignin (21, 30, 41).

While more research is needed, it is possible that the presence of fungi within the denitrifying biofilter helps to improve biofilter functionality. As previous research has suggested (2), fungi may not directly denitrify but rather aid biofilter performance indirectly by cellulose and lignin in woodchips, and thus making organic carbon more accessible to denitrifying bacteria. Therefore, fungi may be a critical component to denitrifying biofilter performance.

2.5 Spatial Studies of Microbial Communities

To understand the microbial communities responsible for denitrifying biofilter functional, the spatial variation of these communities must be taken into consideration. Whether it is the result of preferential flow paths, depth, or water level differences, a multitude of potentially different environmental niches exist within a denitrifying biofilter. Understanding how this environmental heterogeneity affects biofilter microbial communities will help to guide biofilter design, and ultimately improve performance and reliability.

Research on natural systems has demonstrated that free-living microorganisms exhibit spatial patterns in regard to abundance, distribution, and diversity (42). In a study of salt marsh sediments along a creek bank, Franklin et al. (24) concluded that microbial community composition varied much more vertically along a creek bank than it did horizontally. In another study of an agricultural field, Franklin et al. (25) determined microbial community spatial structure at small spatial scales (>40 cm), and was able to link microbial community structure to total soil carbon and nitrogen content.

Microbial community spatial structure among denitrifying bacteria has been observed in constructed ecosystems. Kjellin et al. (36) demonstrated spatial patterns among denitrifiers using
throughout the paper. Enwall et al. (20) correlated the spatial locations of
denitrifying bacteria with environmental and farm management variables. Furthermore, Philippot et al. (48) associated the spatial distribution of denitrifying bacteria in three grassland fields to
the degree that they were impacted by cattle.

Previous research has demonstrated microbial community spatial structure in natural and
constructed systems (20, 24, 25, 36, 42, 48). This research has provided evidence that
environmental factors such as total soil carbon and nitrogen, as well as land management
practices, affect microbial community spatial structure. By better understanding the drivers of
microbial community spatial structure, it may be possible refine biofilter designs to provide
optimum environmental conditions for denitrifying microbial communities, and ultimately
improve performance and reliability.

2.6 Time Series Studies of Microbial Communities

In addition to understanding how and why microbial communities within a denitrifying
biofilter vary spatially, it is also important to understand how and why they vary over time. Just
as the environment within a denitrifying biofilter is not homogenous spatially, the environment
also varies temporally. As seasons change over the course of a year so do the environmental
conditions that affect microbial community structure within a denitrifying biofilter—most
notably temperature and water flow. The potential exists for changes in microbial community
composition to affect the functionality of a denitrifying biofilter. Therefore, by understanding
how these environmental changes affect microbial communities within a denitrifying biofilter
over time, we will be able to better predict system failures and improve biofilter design.
While there has been little research into the microbial dynamics of denitrifying biofilters, there have been temporal studies of bacterial communities in natural systems. Long-term research has shown predictable seasonal and annual patterns in bacterial community composition in fresh water lakes (34, 58, 65). Seasonal and annual community structure has also been observed in long-term studies of marine bacterioplankton (27, 45). Furthermore, correlations between microbial seasonal patterns and measured environmental variables (temperature, salinity, dissolved oxygen) have also been observed (27). In addition to research on total bacterial communities, there have been several temporal studies of denitrifying bacteria. Field studies of denitrifying bacteria have shown that denitrifying communities in soil are affected by changes in pH (15) and disturbances such as tillage (8).

Previous work in natural systems has shown that microbial community composition will vary in response to environmental and seasonal factors. Determining how microbial communities within a denitrifying biofilter respond to environmental factors over time will allow us to improve denitrifying biofilter design and performance.
Chapter 3: Methodology

3.1 Study Sites

Samples were collected from three denitrifying biofilters, FP07, FP03, and DE01, (Table 1) located in central Illinois from January 2009 through September 2010 (DE01 biofilter) or December 2010 (FP07 and FP03 biofilters). Woodchip samples were collected along the biofilter flow path from sampling ports installed on November 13, 2008. Sampling ports were constructed from PVC and consisted of a 10.16 cm diameter perforated outer casing extending from the bottom to the top of the biofilter, and a 7.62 cm diameter woodchip-containing, perforated, and removable inner port (Figure 3). Sampling ports were constructed at two different depths, 0.76 m and 1.52 m, corresponding to approximately the bottom and halfway from the bottom of the biofilter, respectively. Finally, sampling ports were installed at alternating depths and spaced at 6.1 m increments—spacing previously determined to provide optimal spatial resolution (1).

Figure 3. Sampling port side view from within a denitrifying biofilter. Sampling ports were constructed from perforated PVC and sized to either 0.76 m or 1.52 m.
Each biofilter was unique in shape (square, L-shaped, rectangular), drainage area, water flow characteristics, and management practices (Table 1). The denitrifying biofilters were installed and maintained by Dr. Richard Cooke and his laboratory (Dept. of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign).

Table 1. Study site location, design, size, and sampling information.

<table>
<thead>
<tr>
<th>Biofilter</th>
<th>Location</th>
<th>Shape</th>
<th>Drainage Area</th>
<th>Biofilter Dimensions</th>
<th>Dates Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Width</td>
<td>Length</td>
</tr>
<tr>
<td>FP07</td>
<td>Decatur, IL</td>
<td>Square</td>
<td>2.0 ha</td>
<td>6.1 m</td>
<td>6.1 m</td>
</tr>
<tr>
<td>FP03</td>
<td>Decatur, IL</td>
<td>L-Shape</td>
<td>5.0 ha</td>
<td>1.5 m</td>
<td>30.5 m</td>
</tr>
<tr>
<td>DE01</td>
<td>De Land, IL</td>
<td>Rectangle</td>
<td>6.1 ha</td>
<td>3.1 m</td>
<td>12.4 m</td>
</tr>
</tbody>
</table>

3.1.1 FP07

The FP07 biofilter was square in shape and contained five woodchip sampling ports: 3 shallow ports (0.76 m) and 2 deep ports (1.52 m) (Figure 4). Samples were collected from FP07 every two weeks from January 2009 – March 2010, and then every four weeks from April 2010 – December 2010. Preliminary temporal lag analysis indicated that microbial community variance within all three denitrifying biofilters was significant only after approximately 150 days (1). This result led to a change in sampling frequency in April 2010, with sampling beginning in April occurring every four weeks instead of every two.
Figure 4. Schematic of the FP07 biofilter with flow direction, sampling port locations, and biofilter dimensions indicated.

The FP07 biofilter was unique among all three biofilters in two ways. First, and perhaps most important, FP07 was the only biofilter unaltered over the duration of the two-year study. Therefore, the results obtained from this biofilter are the most comparable between years (2009 and 2010) of the three biofilters. Second, the FP07 biofilter experienced little seasonality in water flow.

3.1.2 FP03

The FP03 biofilter was L-shaped and contained seven woodchip sampling ports: five shallow ports (0.76 m) and two deep ports (1.52 m) (Figure 5). Sampling of the FP03 biofilter occurred every two weeks from January 2009 – March 2010, followed by every four weeks from April 2010 – December 2010, again based on preliminary analysis of the time scale of variation.

The FP03 biofilter was disturbed at an unknown time early in 2009 when the flow control boards, responsible for maintaining a pre-established water height, were removed from the biofilter flow control structure; water height and flow control were not restored until April 2010. Removing flow control had the effect of eliminating a predetermined minimum hydraulic
retention time within the biofilter during periods of flow. Therefore, the results obtained from FP03 must be analyzed with the caveat that during times of tile drain flow, water was not predictably retained within the biofilter.

Figure 5. Schematic of the FP03 biofilter with flow direction, sampling port locations, and biofilter dimensions indicated.

3.1.3 DE01

The final of the three study sites was the rectangular biofilter, DE01. Similar to FP07, DE01 contained three shallow (0.76 m) and two deep (1.52 m) sampling ports (Figure 6). The DE01 biofilter was fully operational for all of 2009 but was disturbed on March 9, 2010 during a renovation. The purpose of this renovation was to raise the biofilter floor from below to even with the tile drain level, to reduce the possibility of methylmercury production in stagnant water at the bottom of the biofilter. This renovation involved removing all of the woodchips from the biofilter, adding soil to raise the bottom of the biofilter, mixing new woodchips with those removed, and then replacing the new woodchip mixture and sampling ports. In addition, approximately 1 meter of topsoil and soybean crop was added to the top of the DE01 biofilter,
effectively creating a barrier between the top of the biofilter and the atmosphere; the PVC sampling ports were consequently buried as well.

Figure 6. Schematic of the DE01 biofilter with flow direction, sampling port locations, and biofilter dimensions indicated.

Perhaps the most important change made to the DE01 biofilter, however, was the accidental severing of the tile drainpipe connected to the DE01 biofilter during the renovations. There was no water flow through DE01 after March 9, 2010. Further affecting results from the DE01 in 2010 was the fact that following the biofilter renovation only three of the five sampling ports (two 0.76m and one 1.52 m) could be accessed after the biofilter had been buried with top soil.

Samples were collected from the DE01 biofilter every two weeks from January 2009 – March 2010. Unlike the other two biofilters, however, DE01 was only sampled from May – September 2010 every four weeks. Woodchip samples were not collected from DE01 in April 2010, as a result of the difficulty in locating buried sampling ports. Sample collection at the
DE01 biofilter ceased after September 2010, when we came to the conclusion that water flow would not be restored during the 2010 calendar year.

3.2 Determination of Environmental Variables

Environmental variables utilized in correspondence analysis were collected during field sampling. Temperature and dissolved oxygen data (in sampling ports containing water) were gathered utilizing a YSI Professional Plus handheld multiparameter meter with Quattro cable and probe 1003 (pH/ORP) (YSI, Yellow Springs, OH). Water samples for nitrate analysis were collected in acid-washed 100 mL Nalgene (Thermo Fisher Scientific, Rochester, NY) sampling bottles from all ports containing water, and transported to the lab on ice. Sulfuric acid (0.25 mL) was added as a preservative, and samples were stored at 4°C until analysis. Nitrate concentrations in water grab samples were determined by the Agricultural and Biological Engineering Water Quality Laboratory (University of Illinois, Urbana, IL) using EPA Method 353.1.

Woodchip moisture content was determined by weighing woodchip samples following collection, placing them overnight in a drying oven at 105°C to remove moisture, and weighing again. Meteorological data were collected for each sampling site from the online weather database WeatherUnderground (http://www.wunderground.com/). Weather data included temperature and precipitation values for the day of sampling and averaged values for the week leading up to and including the day of sampling.

3.2.1 Flow Data

Water flow data were collected using MULTI MINI-SAT™ field stations (Automata Inc. Nevada City, CA) with v-notch weirs, pressure transducers, and data loggers placed at both the inlet and outlet flow control structures of each biofilter. Water flow values were reported hourly.
and were accessible via an online database managed by the Agri Drain Corporation (Agri Drain Corporation, Adair, IA). Flow data was accessed at (http://www.agridrain.com/) by logging into the Data Center, located under the Smart Drainage Sys tab. Flow data from both the inlet and outlet flow sensors were reported to the Agri Drain website hourly. Our analyses used flow data only from the outlet flow sensor, as this was an appropriate measure of water flowing through the biofilter; readings from the inlet flow sensor included both water flowing into the biofilter and water by-passing the biofilter through the biofilter overflow.

To best utilize flow data for our analyses, instantaneous flow data were extrapolated for the hour reported, and then summed for a given calendar day, producing a daily flow value in L/day. Rolling averages were then used to calculate 5-day average flow, 7-day average flow, and 14-day average flow.

3.3 Sample Collection

Woodchip samples were collected from the bottom of each inner PVC sampling port and placed into tared and autoclaved 250 mL Nalgene bottles (Thermo Fisher Scientific, Rochester, NY). Following a sampling event, woodchips from the area immediately adjacent to the sampling ports were added to the top of the inner sampling column to replace those removed. All samples were stored on ice during transport back to the lab. Environmental data (pH, dissolved oxygen, temperature) were collected while the inner sampling port was removed by lowering the handheld sampling probe to the bottom of the sampling port.

Upon returning from the field, 110 mL of Ringer’s solution (Oxoid, Cambridge, UK) was added to 30 g of woodchip sample. Samples were shaken overnight in a 30°C temperature controlled room. The day following sampling, the woodchips were removed and the woodchip wash for each sample was centrifuged at 5,000 g for 3 min to concentrate microorganisms. 2.5
mL of 1X PBS (Fisher Bioreagents #BP665-1) and five autoclaved 5 mm glass beads were added to each pellet. Each sample was then vortexed at full speed for 2 min and centrifuged with the glass beads for 5 min at 750 g. The supernatant was collected and stored at -20°C.

3.4 DNA Extraction and Purification

DNA was extracted from woodchip sample washes using a FastDNA Spin Kit (MP Biomedicals, Solon, OH) following the manufacturer’s instructions. Due to humic acid contamination of the DNA, extracted DNA was further purified using cetyl trimethyl ammonium bromide (CTAB) cleanup (56). During CTAB cleanup samples were left overnight in 100% ethanol at -20°C to increase DNA precipitation. Extracted and cleaned DNA was standardized to a concentration of 10 ng/µl using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) and stored at -20°C.

3.5 Microbial Community Structure Determination

3.5.1 ARISA: Total Bacteria

Automated ribosomal intergenic spacer analysis (ARISA) was used to compare total bacterial community structure among samples (23, 34, 35). Primers used for ARISA were the 1406f forward primer (universal 16S rRNA gene) and 23Sr reverse primer (bacteria-specific, 23S rRNA gene) (Table 2) (23). Polymerase chain reactions (PCR) contained 1X Tris buffer, 250 µM of each dNTP, 2.5 mM MgCl₂, 250 µM bovine serum albumin, 400 nM of each primer, 1.25 U of GoTaq Flexi DNA Polymerase (Promega, Madison, WI), and 20 ng of extracted woodchip DNA in a final volume of 25 µL. PCR reactions were carried out with an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 94°C for 35 s, 55°C for 45 s, and 72°C for 2 min. A final extension step was carried out at 72°C for 2 min. 2 µL of autoclaved nanopure water was
used as a negative control. PCR reactions were conducted using an Eppendorf MasterCycler Gradient (Eppendorf, Hauppauge, NY).

Table 2. Primers used for ARISA, FARISA, and nosZ T-RFLP. Fluorescent labels and primer references are noted.

<table>
<thead>
<tr>
<th>Method</th>
<th>Name</th>
<th>Sequence</th>
<th>Fluorescent Label</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARISA</td>
<td>1406f</td>
<td>5’- TGYACACACCGCCCGT-3’</td>
<td>6-FAMa</td>
<td>(23)</td>
</tr>
<tr>
<td></td>
<td>23Sr</td>
<td>5’-GGGTTBCCCATCRG-3’</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>FARISA</td>
<td>2234C</td>
<td>5’-GTTTCCGTAAGTGAACCTGC-3’</td>
<td>None</td>
<td>(51)</td>
</tr>
<tr>
<td></td>
<td>3126T</td>
<td>5’-ATATGCTTAAGTTCAGCGGTT-3’</td>
<td>HEXb</td>
<td></td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td>nosZ F-1181</td>
<td>5’-CGCTGGTTCITCGACAGYCAG-3’</td>
<td>None</td>
<td>(54)</td>
</tr>
<tr>
<td></td>
<td>nosZ R-1880</td>
<td>5’-ATGTGCAKICRTGGAGAA-3’</td>
<td>6-FAMa</td>
<td></td>
</tr>
</tbody>
</table>

a) 6-FAM = 6-carboxyfluorescein.
b) HEX = 6-carboxyhexafluorescein.

3.5.2 Fungal ARISA: Fungi

Fungal community structure was assessed using fungal automated ribosomal intergenic spacer analysis (FARISA) (51). The 2234C and 3126T primers were used for fungal ARISA (Table 2) (51). Each PCR reaction mixture contained 20 ng of extracted woodchip DNA, 500 nM of each primer, 1X of PCR buffer (Idaho Technology Inc. Part # 1770, Salt Lake City, UT), 200 μM of dNTPs, and 1.25 U of GoTaq Flexi DNA Polymerase (Promega, Madison, WI) in a total reaction volume of 25 μl. PCR reactions were carried out with an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 60 s, 55°C for 30 s, and 72°C for 2 min, with a final extension step of 72°C for 5 min. PCR controls consisted of consisted of a 2 μL of autoclaved nanopure water negative control and a positive control of 2 μL of extracted yeast DNA. All fungal ARISA PCR reactions were carried out using an Eppendorf MasterCycler Gradient (Eppendorf, Hauppauge, NY).
3.5.3 **nosZ T-RFLP: Denitrifying Bacteria**

Terminal restriction fragment length polymorphism (T-RFLP) of the nitrous oxide reductase gene (*nosZ*) was employed to assess the denitrifying community structure within each biofilter (47, 54, 68). The *nosZ* F-1181 forward primer and the *nosZ* R-1880 reverse primer were used for *nosZ* T-RFLP (Table 2), generating an amplicon of 700 bp (54). It should be noted that substantially more DNA template was needed for *nosZ* T-RFLP than for either ARISA or fungal ARISA due to the fact that *nosZ* T-RFLP targets a subset of the overall bacterial community.

PCR reactions consisted of 200 ng of extracted DNA, 1X Tris buffer, 2 mM MgCl$_2$, 250 µM bovine serum albumin, 200 µM of each dNTP, 200 nM of both the forward and reverse primer, and 2.5 U of GoTaq Flexi DNA Polymerase (Promega, Madison, WI) in a final volume of 50 µL. PCR reactions were carried out with an initial denaturation step at 94°C for 3 min, followed by 25 cycles of 94°C for 45 s, 56°C for 1 min, and 72°C for 3 min, with a final extension step of 72°C for 7 min.

PCR products from three 50 µL reactions were combined and concentrated using a Qiagen MinElute PCR purification kit (Qiagen, Valencia, CA). Following purification separate restriction enzyme digests using *Alu*I and *Hha*I (New England Biolabs, Ipswich, MA) were performed on each sample. Each digest contained 10 µL of purified PCR product, 1 µL 10X Buffer 4 (New England Biolabs, Ipswich, MA), 0.2 µL 100X BSA, and 0.5 µL restriction enzyme in a final volume of 20 µL. Digests were carried out at 37°C overnight.

3.6 **Fragment Analysis**

DNA fragments from ARISA, fungal ARISA, and *nosZ* T-RFLP were analyzed using denaturing capillary electrophoresis on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA) at The Keck Center for Functional Genomics (University of Illinois, Urbana,
Electrophoresis was conducted with a run time of 120 min at 63ºC and 15 kV, using the POP-7 polymer. A custom 100 to 2000 bp Rhodamine X-labeled size standard (Bioventures, Murfreesboro, TN) was utilized as the internal size standard for ARISA and fungal ARISA. For nosZ T-RFLP an ABI GeneScan ROX 1000 size standard was used (Applied Biosystems, Foster City, CA).

Fragment length data for total bacteria, fungi, and denitrifying community analyses were processed using GeneMarker software, version 1.95 (SoftGenetics, State College, PA). Samples with poor size calling or overall low fluorescence were excluded from all analyses. For ARISA and fungal ARISA fragment lengths between 300 – 1000 bp were analyzed. Likewise nosZ T-RFLP fragments between 100 - 700 bp (the full-length, uncut nosZ fragment) were analyzed (54). To standardize variation in ARISA and fungal ARISA created by different capillary electrophoresis runs, the relative fluorescence was determined by dividing the peak area of each fragment by the sum of fluorescence for each sample (35, 52, 66). Relative fluorescence of nosZ terminal restriction fragments (TRFs) was determined separately for each restriction digest and then concatenated, with the total relative fluorescence for the two combined digests summing to 2.

### 3.7 Statistical Analysis

#### 3.7.1 Correspondence and Partial Correspondence Analyses

Correspondence and partial correspondence analyses were conducted to determine community composition patterns in ARISA, fungal ARISA, and nosZ T-RFLP. All analyses were conducted using Canoco for Windows version 4.5.1 (Plant Research International, Wageningen, Netherlands) (5). Environmental variables available for all correspondence analyses included: daily water flow through the biofilter, 7-day average flow, day of sampling
average temperature, day of sampling precipitation, weekly precipitation, sampling port
temperature, weekly average temperature, woodchip moisture content, and daily biofilter water
flow. For all correspondence analyses the following program parameters were used: species and
environment data available, indirect gradient analysis; unimodal (CA) response model; inter-
sample distances; biplot scaling; no transformation of species data.

Partial correspondence analysis was utilized specifically to remove the influence of year,
allowing samples from 2009 and 2010 to be combined. This allowed us to determine if microbial
community patterns were present when analyzing all samples together. For partial
correspondence analyses the following program parameters were used: species, environment and
covariable data available; indirect gradient analysis; unimodal (CA) response model; inter-
sample distances; biplot scaling; no transformation of species data.

3.7.2 Redundancy Analysis

Redundancy analysis (RDA) was conducted using Canoco for Windows version 4.5.1 and
utilized to identify microbial populations that were strongly correlated with high rates of
nitrate removal. Nitrate removal data was only available for January 1, 2009 – September 11,
2009, therefore only samples from these dates were included in RDA analyses. Nitrate removal
data were determined by calculating the difference between inlet and outlet nitrate load values.
Nitrate load values were determined from water grab samples collected at the inlet and outlet
flow control structures; load reduction data were calculated only on days when water flowed
through the biofilter. 7-day moving averages of daily nitrate reduction were calculated and
utilized in all RDA analyses. For RDA analyses the following program parameters were used:
Species and environment data available; direct gradient analysis; linear (RDA) response model;
inter-species correlations; divide by standard deviation; do not transform; center and standardize
by species; do not forward select. The goal of this analysis was to determine the top 5-20 populations correlated with strong nitrate removal. RDA analysis forces nitrate removal to be the only variable on the x-axis. Therefore populations associated with strong nitrate removal are determined by filtering populations based on their minimum lower axis fit in CanoDraw.

### 3.7.3 Mean Centroid Distance

The mean centroid distance was calculated to quantify variability among groups classified in correspondence analysis. The mean centroid distance for a particular classification, such as depth or season, was calculated by taking the average of the Euclidean distance (Eqn. 1) of each sample within that classification.

\[
\sqrt{(x_n - \bar{x})^2 + (y_n - \bar{y})^2}
\]

Eqn 1.

Axis 1 and axis 2 scores—defining the Cartesian location of each sample within the correspondence analysis ordination (x and y)—were retrieved from the solution file for each correspondence analysis and utilized to calculate the mean centroid distance. The results from these calculations provide insight into the variability microbial communities within different classifications utilized in correspondence analysis. Mean centroid distances were used to compare variability within each microbial community due to depth and seasonal factors.

### 3.7.4 Analysis of Similarity

Analysis of similarity (ANOSIM) \((9, 10)\) was utilized to assess patterns in microbial community structure within each biofilter. Specifically, ANOSIM is used to compare the similarity of microbial communities within and among groups of samples. For our analyses, ANOSIM was used to evaluate the degree of microbial community variation within depth and
seasonal classifications. The analysis of similarity uses normalized relative fluorescence data to generate a Bray-Curtis similarity matrix for every possible sample pairing (6, 39) and generates a test statistic, R. An ANOSIM R value of 0 indicates complete similarity among samples tested, while an ANOSIM R value of 1 denotes complete differentiation. ANOSIM tests were conducted using the software package Primer 6, version 6.1.10 (Primer-E Ltd., Plymouth, United Kingdom).

3.7.5 Influential Populations: BVSTEP Procedure

The BVSTEP procedure (12) was used to identify population subsets within each microbial community that were most responsible for defining the microbial community structure; These population subsets are referred to as influential populations. The BVSTEP procedure utilizes a stepwise algorithm, similar to a stepwise multiple regression, to compare subsets of each microbial community to the whole (11). The result of this stepwise comparison was the identification of the smallest subset of populations that best explain the overall microbial community structure to a predetermined Spearman rank correlation coefficient (ρ). For our purposes we sought a Spearman rank correlation coefficient level that would provide us with a maximum of 10-15 of the most influential populations. Empirically, ρ = 0.85 was found to be the most appropriate Spearman rank correlation coefficient.

For all BVSTEP calculations a Spearman rank correlation of ρ = 0.85 was employed with Δρ < 0.001, and random selection with 6 restarts. All BVSTEP calculations were carried out in Primer 6, version 6.1.10 (Primer-E Ltd., Plymouth, United Kingdom).
Chapter 4: Temporal Study Results

4.1 Introduction

To determine how microbial populations (total bacteria, denitrifying bacteria, and fungi) within a denitrifying biofilter changed over time and responded to environmental variables, we studied three denitrifying biofilters (FP07, FP03, DE01) over the course of two years. Each biofilter was unique in its shape, volume, and the amount of agricultural land that it serviced. Furthermore, each biofilter experienced different operational and management characteristics for the duration of the study. The FP07 biofilter was operated in the same manner throughout 2009 and 2010, FP03 had its flow control boards removed at an unknown time in 2009 and they were not restored until April 2010, and the DE01 biofilter had the line connecting it to the tile drain network broken during a renovation in March 2010.

Due to the fact that microbial communities within each individual biofilter were likely influenced by differences in design and operation during the temporal study, each biofilter was considered a unique system. Therefore, all three biofilters were analyzed independently from one another. Furthermore, due to the system management and environmental differences between 2009 and 2010, results from each year are presented separately.

Although each biofilter was unique in design and operation for the course of the two-year study, microbial community composition in all three biofilters varied strongly by depth and season. In general, the depth that samples were collected at was important in shaping microbial community structure in all three biofilters. For each biofilter we were able to identify environmental and operational variables likely responsible for driving this depth distinction.

In addition to depth, microbial communities were structured by season. From our analysis we concluded that strong seasonal variation was observed for all three microbial communities in
each biofilter when comparing samples collected in January – June to those collected in July – December. From correspondence analysis we were able to determine common environmental variables that were likely responsible for driving seasonal patterns in microbial community structure within each denitrifying biofilter.

For all biofilters we found 435 unique ARISA fragments, 309 unique FARISA fragments, and a combined 270 nosZ TRFs from the AluI (156 TRFs) and HhaI (114 TRFs) digests. Due to the complexity of the microbial communities within each biofilter we sought to determine population subsets that were either influential, associated with good biofilter performance, or both. Influential populations responsible for defining approximately 85% of the overall structure within each microbial community were determined using the BVSTEP procedure. Although nitrate removal data was only available for January – September 2009, populations associated with strong nitrate removal, for this time period, were determined using RDA analysis. Influential populations in 2009 that were also associated with good nitrate removal are noted.
4.2 FP07

4.2.1 FP07 Hydrology

The FP07 biofilter was unique in that it did not experience strong water flow seasonality in 2009 or 2010 (Figure 7). In 2009 and 2010 the largest dry spells in FP07 were only 22 and 11 days in length, respectively. The average volume of water flowing through FP07 was more consistent throughout the year, for both 2009 and 2010, than in the other two biofilters.

Table 3. FP07 hydrology data for 2009 and 2010. Hydrology information includes the largest number of consecutive calendar days without flow in each year, the percentage of days with flow, average daily flow, and the average HRT for each seasonal classification (January – June or July – December).
Figure 7. Hydrographs of the FP07 biofilter for (A) 2009 and (B) 2010. These plots show the relative consistency of water flow between the January – June and July – December seasonal classifications in both 2009 and 2010.
4.2.2 FP07 Microbial Community Composition Structured by Depth

Depth was an important factor influencing microbial community structure within the FP07 biofilter. As shown visually by the clustering of symbols in (Figure 8), and confirmed by ANOSIM (Table 4), the two depths sampled show differences in their microbial community structure. ANOSIM provides a measure of the degree of similarity between two different groups, and typically ranges from 0 (samples are completely similar) to 1 (samples are completely dissimilar). ANOSIM results (Table 4) suggest that for both 2009 and 2010 denitrifying bacteria demonstrated the greatest differences in community structure between depths, followed by fungi and total bacteria.

By overlaying measured environmental parameters onto correspondence analysis plots (Figure 8), we can assess the environmental parameters that correlate most strongly with microbial communities observed at each depth. The strongest environmental variables correlated with microbial community structuring by depth were woodchip moisture content and sampling port temperature. Woodchip moisture content was positively associated with samples collected at the 1.52 m depth (Figure 8). Sampling port temperature was positively correlated with samples collected at 0.76 m (Figure 8).
Figure 8. Correspondence analysis biplots showing depth differences in FP07 microbial community composition. Points represent microbial community samples collected at either 0.76 m or 1.52 m. This plot illustrates differences in microbial communities between depths in 2009 and 2010, and that microbial communities collected at 0.76 m were positively correlated with port and weekly average temperature, while those from 1.52 m were positively correlated with % moisture. Axes represent theoretical environmental gradients. The percentage of microbial community variance explained by each axis is indicated in parenthesis. Vectors indicate measured environmental variables, specifically: % moisture, daily flow, 7-day average flow, weekly precipitation, weekly average temperature, and sampling port temperature. The value of each environmental variable increases in the direction of the vector, and the direction of the vector indicates the level of correlation to each axis.
Table 4. FP07 microbial community differences by depth (0.76 m or 1.52 m) represented by ANOSIM and Mean Centroid Distance values. ANOSIM is based on Bray-Curtis Similarity, while correspondence analysis values are utilized to determine Mean Centroid Distances.

<table>
<thead>
<tr>
<th>Community</th>
<th>ANOSIMa</th>
<th>Mean Centroid Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>P</td>
</tr>
<tr>
<td>ARISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.255</td>
<td>0.001</td>
</tr>
<tr>
<td>2010</td>
<td>0.255</td>
<td>0.001</td>
</tr>
<tr>
<td>FARISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.283</td>
<td>0.001</td>
</tr>
<tr>
<td>2010</td>
<td>0.391</td>
<td>0.001</td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.422</td>
<td>0.001</td>
</tr>
<tr>
<td>2010</td>
<td>0.436</td>
<td>0.001</td>
</tr>
</tbody>
</table>

a) ANOSIM values based on samples classified as 0.76 m or 1.52 m.
b) Bolded values are statistically significant ($P = 0.05$).

To evaluate the variability of microbial communities at 0.76 m or 1.52 m, the mean centroid distance, or the average distance between all samples within a group plotted in correspondence analysis and the group centroid, was calculated. The mean centroid distance provides a quantitative measure of the variability of sample groupings in correspondence analysis (Figure 8). Therefore, as shown in samples collected at the 1.52 m depth show higher variability than those collected at 0.76 m for all three microbial communities, except denitrifiers in 2009.

To examine microbial community differences among sample depths for the entire dataset, we utilized partial correspondence analysis. This analysis factors out the influence of year, allowing samples from 2009 and 2010 to be analyzed together (Figure 9). Partial correspondence analysis confirmed that woodchip % moisture content was strongly correlated with samples collected at 1.52 m, and sampling port temperature with samples collected at 0.76 m.
Figure 9. Partial correspondence analysis biplots showing depth differences in FP07 microbial community composition. Partial correspondence analysis allows samples from 2009 and 2010 to combined and analyzed together. Points represent microbial community samples collected at either 0.76 m or 1.52 m. This plot indicates that microbial communities varied by depth in 2009 and 2010, and that microbial communities collected at 0.76 m were positively correlated with port and weekly average temperature, while those from 1.52 m were positively correlated with % moisture. Axes represent theoretical environmental gradients. The percentage of microbial community variance explained by each axis is indicated in parenthesis. Vectors indicate measured environmental variables, specifically: % moisture, daily flow, 7-day average flow, weekly precipitation, weekly average temperature, and sampling port temperature. The value of each environmental variable increases in the direction of the vector, and the direction of the vector indicates the level of correlation to each axis.

4.2.3 FP07 Microbial Community Composition Structured by Season

Seasonal variation, in addition to depth, was another strong explanatory variable of microbial community structuring within this denitrifying biofilter. Correspondence analysis (Figure 10) and analysis of similarity results (Table 5) indicate strong patterns (or differences) in microbial community structure based on seasonal (January – June and July – December) classifications. Most notability, samples collected in 2009 showed the strongest seasonal trends. 2010 samples show slight seasonal grouping in total bacteria and fungi, but little among
Correspondence analysis plots suggest that high values for woodchip % moisture was correlated with samples collected in the January – June seasonal classification, while higher values for sampling port temperature and weekly average temperature were correlated with samples collected during July – December.

Table 5. FP07 microbial community differences by season (Jan – Jun or Jul – Dec) represented by ANOSIM and Mean Centroid Distance values. ANOSIM is based on Bray-Curtis Similarity, while correspondence analysis values are utilized to determine Mean Centroid Distances.

<table>
<thead>
<tr>
<th>Community</th>
<th>ANOSIM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean Centroid Distance</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P</td>
<td>Jan-Jun</td>
<td>Jul-Dec</td>
</tr>
<tr>
<td>ARISA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.304</td>
<td>0.001</td>
<td>0.16</td>
<td>0.20</td>
</tr>
<tr>
<td>2010</td>
<td>0.209</td>
<td>0.001</td>
<td>0.64</td>
<td>0.79</td>
</tr>
<tr>
<td>FARISA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.289</td>
<td>0.001</td>
<td>0.52</td>
<td>1.33</td>
</tr>
<tr>
<td>2010</td>
<td>0.173</td>
<td>0.001</td>
<td>1.05</td>
<td>0.99</td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.258</td>
<td>0.001</td>
<td>0.81</td>
<td>1.25</td>
</tr>
<tr>
<td>2010</td>
<td>0.041</td>
<td>0.074</td>
<td>0.74</td>
<td>0.93</td>
</tr>
</tbody>
</table>

<sup>a</sup> ANOSIM values based on samples classified as collected in either Jan-Jun or Jul-Dec.
<sup>b</sup> Bolded values are statistically significant ($P = 0.05$).
Figure 10. Correspondence analysis biplots showing season differences in FP07 microbial community composition. Points represent microbial community samples collected during either Jan – Jun or Jul – Dec. This plot indicates that microbial communities exhibited seasonal variation in 2009 and 2010, and that microbial communities collected in Jan - Jun were positively correlated with % moisture, while those collected in Jul - Dec were positively correlated with sampling port and weekly average temperature. Axes represent theoretical environmental gradients. The percentage of microbial community variance explained by each axis is indicated in parenthesis. Vectors indicate measured environmental variables, specifically: % moisture, daily flow, 7-day average flow, weekly precipitation, weekly average temperature, and sampling port temperature. The value of each environmental variable increases in the direction of the vector, and the direction of the vector indicates the level of correlation to each axis.
Examination of the variability of samples collected in January – June and July – December revealed that in general all three microbial communities were less variable during the first halves of 2009 and 2010 (Table 5). Results obtained from partial correspondence analysis confirm the seasonal groupings obtained from 2009 and 2010, and indicate that woodchip % moisture content, sampling port temperature, and weekly average temperature are likely important in driving this seasonal trend.

Figure 11. Partial correspondence analysis biplots showing season differences in FP07 microbial community composition. Points represent microbial community samples collected during either Jan – Jun or Jul – Dec. Partial correspondence analysis allows samples from 2009 and 2010 to combined and analyzed together. This plot indicates that microbial communities exhibited seasonal variation in 2009 and 2010, and that microbial communities collected in Jan - Jun were positively correlated with % moisture, while those collected in Jul - Dec were positively correlated with sampling port and weekly average temperature. Axes represent theoretical environmental gradients. The percentage of microbial community variance explained by each axis is indicated in parenthesis. Vectors indicate measured environmental variables, specifically: % moisture, daily flow, 7-day average flow, weekly precipitation, weekly average temperature, and sampling port temperature. The value of each environmental variable increases in the direction of the vector, and the direction of the vector indicates the level of correlation to each axis.
Water flow through the biofilter was not strongly correlated with either seasonal classification in correspondence (Figure 10) or partial correspondence analysis (Figure 11). This result was likely due to the fact that relatively similar water flow through the FP07 biofilter was observed during both seasons.
4.3 FP03

4.3.1 FP03 Hydrology

The FP03 biofilter experienced seasonality in water flow in 2009 and 2010 (Table 6). Although the percentage of days with flow in January – June and July – December of 2009 was similar, the July – December period was marked by an extended dry period of 109 days from July 6, 2009 – October 23, 2009 (Table 6). For 2010, the seasonality of flow through FP03 was even greater as FP03 experienced an extended period of no flow from July 6, 2010 to the end of 2010 (Table 6). Hydrographs (Figure 12) of the FP03 biofilter illustrate the seasonality of flow for 2009 and 2010. Furthermore, the magnitude of the average flow values for 2009 and 2010 illustrate the differences in flow through FP03 before, and after flow control was restored.

Table 6. FP03 hydrology data for 2009 and 2010. Hydrology information includes the largest number of consecutive calendar days without flow in each year, the percentage of days with flow, average daily flow, and the average HRT for each seasonal classification (January – June or July – December).

<table>
<thead>
<tr>
<th>Year</th>
<th>Most Consecutive Days no flow</th>
<th>% Days with flow</th>
<th>Average Flow (L/day)</th>
<th>Average HRT (Day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>109</td>
<td>43.1</td>
<td>38.0</td>
<td>141,370</td>
</tr>
<tr>
<td>2010</td>
<td>178</td>
<td>87.8</td>
<td>3.26</td>
<td>35,989</td>
</tr>
</tbody>
</table>
Figure 12. Hydrographs of the FP03 biofilter for (A) 2009 and (B) 2010. These plots show the distinction in water flow between the January – June and July – December seasonal classifications in both 2009 and 2010.
### 4.3.2 FP03 Microbial Community Composition Structured by Depth

Analysis of similarity (Table 7) and correspondence analysis (Figure 13) results indicate that while there was some microbial community variation by depth, overall depth was not a strong factor in microbial community structure in the FP03 biofilter. Correspondence analysis plots (Figure 13) indicate that sampling port temperature and woodchip % moisture content were strongly correlated with differences in microbial communities among samples collected at 0.76 m and 1.52 m, respectively. It is should also be noted that all three microbial communities demonstrated variation by depth in 2010 than in 2009 perhaps because in 2009 there was no flow control on the FP03 biofilter.

Table 7. FP03 microbial community differences by depth (0.76 m or 1.52 m) represented by ANOSIM and Mean Centroid Distance values. ANOSIM is based on Bray-Curtis Similarity, while correspondence analysis values are utilized to determine Mean Centroid Distances.

<table>
<thead>
<tr>
<th>Community</th>
<th>ANOSIM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean Centroid Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.76 m</td>
</tr>
<tr>
<td>ARISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.099</td>
<td>0.59</td>
</tr>
<tr>
<td>2010</td>
<td>0.146</td>
<td>0.55</td>
</tr>
<tr>
<td>FARISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.116</td>
<td>0.78</td>
</tr>
<tr>
<td>2010</td>
<td>0.241</td>
<td>0.84</td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.192</td>
<td>0.69</td>
</tr>
<tr>
<td>2010</td>
<td>0.205</td>
<td>0.74</td>
</tr>
</tbody>
</table>

a) ANOSIM depth values based on samples classified as 0.76 m or 1.52 m.
b) Bolded values are statistically significant ($P = 0.05$).
Figure 13. Correspondence analysis biplots showing depth differences in FP03 microbial community composition. Points represent microbial community samples collected at either 0.76 m or 1.52 m. This plot illustrates differences in microbial communities between depths in 2009 and 2010, and that microbial communities collected at 0.76 m were positively correlated with port and weekly average temperature, while those from 1.52 m were positively correlated with % moisture. Axes represent theoretical environmental gradients. The percentage of microbial community variance explained by each axis is indicated in parenthesis. Vectors indicate measured environmental variables, specifically: % moisture, daily flow, 7-day average flow, weekly precipitation, weekly average temperature, and sampling port temperature. The value of each environmental variable increases in the direction of the vector, and the direction of the vector indicates the level of correlation to each axis.
The variability of microbial communities at 0.76 m and 1.52 m was determined by calculating the mean centroid distance, or the spread of samples in correspondence analysis. Results from mean centroid distance calculations (Table 7) indicate that microbial communities observed at 0.76 m were less variable in community composition than samples from 1.52 m. Partial correspondence analysis was utilized to combine samples collected in 2009 and 2010. Results from partial correspondence analysis confirm that depth was not a strong factor in structuring microbial community composition in the FP03 biofilter (Figure 14).

Figure 14. Partial correspondence analysis biplots showing depth differences in FP03 microbial community composition. Partial correspondence analysis allows samples from 2009 and 2010 to combined and analyzed together. Points represent microbial community samples collected at either 0.76 m or 1.52 m. This plot indicates that microbial communities varied by depth in 2009 and 2010, and that microbial communities collected at 0.76 m were positively correlated with port and weekly average temperature, while those from 1.52 m were positively correlated with % moisture. Axes represent theoretical environmental gradients. The percentage of microbial community variance explained by each axis is indicated in parenthesis. Vectors indicate measured environmental variables, specifically: % moisture, daily flow, 7-day average flow, weekly precipitation, weekly average temperature, and sampling port temperature. The value of each environmental variable increases in the direction of the vector, and the direction of the vector indicates the level of correlation to each axis.
4.3.3 FP03 Microbial Community Composition Structured by Season

Analysis of similarity (Table 8) and correspondence analysis (Figure 15) results indicate that FP03 microbial communities varied by season. 2009 microbial communities showed slightly stronger seasonal variation than did samples collected in 2010 (Table 8). Correspondence analysis plots suggest that woodchip % moisture was positively correlated with microbial communities collected in January – June, while sampling port temperature and weekly average temperature were associated with samples collected in July – December (Figure 15).

Table 8. FP03 microbial community differences by season (Jan – Jun or Jul – Dec) represented by ANOSIM and Mean Centroid Distance values. ANOSIM is based on Bray-Curtis Similarity, while correspondence analysis values are utilized to determine Mean Centroid Distances.

<table>
<thead>
<tr>
<th>Community</th>
<th>ANOSIM(^a)</th>
<th>Mean Centroid Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R(^b)</td>
<td>P</td>
</tr>
<tr>
<td>ARISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.328</td>
<td>0.001</td>
</tr>
<tr>
<td>2010</td>
<td>0.275</td>
<td>0.001</td>
</tr>
<tr>
<td>FARISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.358</td>
<td>0.001</td>
</tr>
<tr>
<td>2010</td>
<td>0.275</td>
<td>0.001</td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.679</td>
<td>0.001</td>
</tr>
<tr>
<td>2010</td>
<td>0.325</td>
<td>0.001</td>
</tr>
</tbody>
</table>

\(^a\) ANOSIM season values based on samples classified as collected in either Jan-Jun or Jul-Dec. \(^b\) Bolded values are statistically significant (P = 0.05).

Further examination of explanatory environmental factors reveals that both daily and 7-day average water flows correlate strongly with samples collected during the first half of 2010 (Figure 15). This result is interesting, because the FP03 biofilter experienced strong flow seasonality in 2009, not just in 2010. It is possible that flow was not strongly correlated with season in 2009 because the FP03 biofilter did not have its flow control boards installed.
Figure 15. Correspondence analysis biplots showing season differences in FP03 microbial community composition. Points represent microbial community samples collected during either Jan – Jun or Jul – Dec. This plot indicates that microbial communities exhibited seasonal variation in 2009 and 2010, and that microbial communities collected in Jan - Jun were positively correlated with % moisture, while those collected in Jul - Dec were positively correlated with sampling port and weekly average temperature. Axes represent theoretical environmental gradients. The percentage of microbial community variance explained by each axis is indicated in parenthesis. Vectors indicate measured environmental variables, specifically: % moisture, daily flow, 7-day average flow, weekly precipitation, weekly average temperature, and sampling port temperature. The value of each environmental variable increases in the direction of the vector, and the direction of the vector indicates the level of correlation to each axis.
Mean centroid distance analysis was utilized to examine the variability of samples collected within each seasonal classification. This analysis revealed greater variation for samples collected during July – December for all three microbial communities in 2009 and 2010 (Table 8). The seasonality of flow observed in the FP03 biofilter may provide an explanation for why samples collected during the second halves of 2009 and 2010 were more variable. In 2009 and 2010 water flow through FP03 stopped after July and did not resume until late October (2009), or not at all (2010).

Results from partial correspondence analysis, where the influence of year was removed and samples from 2009 and 2010 combined, demonstrate that all three microbial communities exhibited seasonal patterns (Figure 16). Furthermore, these results indicate that woodchip % moisture was highly correlated with samples collected in January – June, and that sampling port temperature and weekly average temperature were associated with samples collected in July – December. Partial correspondence analysis confirmed seasonal patterns observed in ANOSIM and correspondence analysis.
Figure 16. Partial correspondence analysis biplots showing season differences in FP03 microbial community composition. Points represent microbial community samples collected during either Jan – Jun or Jul – Dec. Partial correspondence analysis allows samples from 2009 and 2010 to combined and analyzed together. This plot indicates that microbial communities exhibited seasonal variation in 2009 and 2010, and that microbial communities collected in Jan - Jun were positively correlated with % moisture, while those collected in Jul - Dec were positively correlated with sampling port and weekly average temperature. Axes represent theoretical environmental gradients. The percentage of microbial community variance explained by each axis is indicated in parenthesis. Vectors indicate measured environmental variables, specifically: % moisture, daily flow, 7-day average flow, weekly precipitation, weekly average temperature, and sampling port temperature. The value of each environmental variable increases in the direction of the vector, and the direction of the vector indicates the level of correlation to each axis.
4.4 DE01

4.4.1 DE01 Hydrology

The DE01 biofilter experienced water flow seasonality in 2009, with an extended period of no flow for 110 days (June 21 – October 21, 2009) (Table 9). In 2010 water flow through DE01 ceased after the tile line connecting DE01 to the tile drain network was broken during a biofilter renovation. Hydrographs (Figure 17) of the DE01 biofilter illustrate the seasonality of flow for 2009, and the disruption in 2010.

Table 9. DE01 hydrology data for 2009 and 2010. Hydrology information includes the largest number of consecutive calendar days without flow in each year, the percentage of days with flow, average daily flow, and the average HRT for each seasonal classification (January – June or July – December).

<table>
<thead>
<tr>
<th>Year</th>
<th>Most Consecutive Days no flow</th>
<th>% Days with flow</th>
<th>Average Flow (L/day)</th>
<th>Average HRT (Day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>110</td>
<td>62.6</td>
<td>36.4</td>
<td>237,161</td>
</tr>
<tr>
<td>2010*</td>
<td>n/a</td>
<td>100</td>
<td>0</td>
<td>269,583</td>
</tr>
</tbody>
</table>

a) Flow was artificially stopped on March 9, 2010.
Figure 17. Hydrographs of the DE01 biofilter A) 2009 and (B) 2010. Plot A) shows the distinction in water flow between the January – June and July – December seasonal classifications, while plot B) in both 2009 demonstrates the result of the tile drain line being severed on March 9, 2010.
4.4.2 DE01 Microbial Community Composition Structured by Depth

Correspondence analysis (Figure 18) and ANOSIM (Table 10) results indicate that depth was an important factor in structuring microbial communities within the DE01 biofilter in 2009. Woodchip % moisture was strongly related to samples collected at 1.52 m, and sampling port temperature to samples collected at 0.76 m (Figure 18). Microbial community variation by depth was not apparent for total and denitrifying bacteria in 2010, while fungi demonstrated a large depth variance (Figure 18 and Table 10). This was likely the result of the biofilter renovation that occurred on March 9, 2010.

Table 10. DE01 microbial community differences by depth (0.76 m or 1.52 m) represented by ANOSIM and Mean Centroid Distance values. ANOSIM is based on Bray-Curtis Similarity, while correspondence analysis values are utilized to determine Mean Centroid Distances.

<table>
<thead>
<tr>
<th>Community</th>
<th>ANOSIM R</th>
<th>Mean Centroid Distance</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.76 m</td>
<td>1.52 m</td>
</tr>
<tr>
<td>ARISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.613</td>
<td>0.001</td>
<td>0.47</td>
</tr>
<tr>
<td>2010</td>
<td>0.077</td>
<td>0.101</td>
<td>0.84</td>
</tr>
<tr>
<td>FARISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.214</td>
<td>0.001</td>
<td>0.92</td>
</tr>
<tr>
<td>2010</td>
<td>0.459</td>
<td>0.001</td>
<td>0.46</td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.444</td>
<td>0.001</td>
<td>1.28</td>
</tr>
<tr>
<td>2010</td>
<td>0.052</td>
<td>0.192</td>
<td>0.96</td>
</tr>
</tbody>
</table>

a) ANOSIM depth values based on samples classified as 0.76 m or 1.52 m.  
b) Bolded values are statistically significant ($P = 0.05$).  

49
Figure 18. Correspondence analysis biplots showing depth differences in DE01 microbial community composition. Points represent microbial community samples collected at either 0.76 m or 1.52 m. This plot illustrates differences in microbial communities between depths in 2009 and 2010, and that microbial communities collected at 0.76 m were positively correlated with port and weekly average temperature, while those from 1.52 m were positively correlated with % moisture. Axes represent theoretical environmental gradients. The percentage of microbial community variance explained by each axis is indicated in parenthesis. Vectors indicate measured environmental variables, specifically: % moisture, daily flow, 7-day average flow, weekly precipitation, weekly average temperature, and sampling port temperature. The value of each environmental variable increases in the direction of the vector, and the direction of the vector indicates the level of correlation to each axis.
To compare the variability of samples from the 0.76 m and 1.52 m depths, mean centroid distances were calculated for each depth, both years, and all three microbial communities. With the exception of denitrifying bacteria, microbial communities collected at 1.52 m were more variable than samples from 0.76 m (Table 10).

Partial correspondence analysis of depth was not conducted for the DE01 biofilter. The biofilter renovation conducted on March 9, 2010 introduced additional variability that partial correspondence analysis could not effectively remove.

4.4.3 DE01 Microbial Community Composition Structured by Season

Correspondence analysis (Figure 19) and analysis of similarity results indicate (Table 11) seasonal variation in the DE01 biofilter during 2009. Samples collected in January – June showed a strong correlation to daily flow, 7-day flow, and woodchip % moisture, while samples collected in July – December correlate with weekly average temperature, sampling port temperature, and weekly precipitation. In 2009, the DE01 biofilter underwent a significant period of no flow from June 21 to October 9 (Figure 17). This period of drought may have contributed to the microbial community seasonal variation observed in DE01.

Due to the accidental breaking of the tile drain line in March 2010, seasonal analysis of this biofilter must be considered carefully. Further, it needs to be noted that sampling of the DE01 biofilter was completed in September 2010, not December. Therefore, the 2010 seasonal classifications used for DE01 were January – June and July – September. Correspondence analysis (Figure 19) and ANOSIM (Table 11) reveal some seasonal variation for total and denitrifying bacteria in 2010, but not for fungi.
Table 11. 2009 DE01 microbial community differences by season (Jan – Jun or Jul – Dec) represented by ANOSIM and Mean Centroid Distance values. ANOSIM is based on Bray-Curtis Similarity, while correspondence analysis values are utilized to determine Mean Centroid Distances.

<table>
<thead>
<tr>
<th>Community</th>
<th>ANOSIM(^a)</th>
<th>Mean Centroid Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(R)</td>
<td>(P)</td>
</tr>
<tr>
<td>ARISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.266</td>
<td>0.001</td>
</tr>
<tr>
<td>2010</td>
<td>0.157</td>
<td>0.076</td>
</tr>
<tr>
<td>FARISA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.339</td>
<td>0.001</td>
</tr>
<tr>
<td>2010</td>
<td>0.053</td>
<td>0.32</td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>0.209</td>
<td>0.001</td>
</tr>
<tr>
<td>2010</td>
<td>0.162</td>
<td>0.075</td>
</tr>
</tbody>
</table>

\(^a\) ANOSIM season values based on samples classified as collected in either Jan-Jun or Jul-Dec.  
\(^b\) Bolded values are statistically significant (\(P = 0.05\)).

Examination of seasonal variability by mean centroid distance analysis indicated that samples collected in the second six months of 2009 and 2010 were more variable than those collected during the first six months (Table 8). This variability may be related to water flow through the biofilter. In 2009 and 2010 there were significant periods of no water flow through DE01 (Figure 17) during the July – December/September seasonal classification.

Partial correspondence analysis of season was not completed for the DE01 biofilter. The biofilter renovation conducted on March 9, 2010 introduced additional variability that partial correspondence analysis could not effectively remove.
Figure 19. Correspondence analysis biplots showing season differences in DE01 microbial community composition. Points represent microbial community samples collected during either Jan – Jun or Jul – Dec. This plot indicates that microbial communities exhibited seasonal variation in 2009 and 2010, and that microbial communities collected in Jan - Jun were positively correlated with % moisture, while those collected in Jul - Dec were positively correlated with sampling port and weekly average temperature. Axes represent theoretical environmental gradients. The percentage of microbial community variance explained by each axis is indicated in parenthesis. Vectors indicate measured environmental variables, specifically: % moisture, daily flow, 7-day average flow, weekly precipitation, weekly average temperature, and sampling port temperature. The value of each environmental variable increases in the direction of the vector, and the direction of the vector indicates the level of correlation to each axis.
4.5 Influential Microbial Communities

Due to the complexity of the microbial communities within each biofilter, we sought to
determine population subsets from each community that were influential in driving the overall
community structure. To do this we utilized the BVSTEP procedure in Primer 6, which uses a
stepwise algorithm, similar to a stepwise multiple regression, test to compare subsets of each
microbial community to the entire community (11). The results from BVSTEP provide the
smallest subset of populations that best explain the overall microbial community structure to a
predetermined Spearman rank correlation coefficient ($\rho$). To achieve a higher Spearman rank
correlation coefficient, such as $\rho = 0.95$, the number of influential populations would range from
approximately 30 – 50 and would provide less clarity than the 10-15 populations achieved with
$\rho = 0.85$.

Influential populations for all three biofilters are presented in Table 12. Populations that
were influential in 2009 and 2010 within each biofilter are highlighted in bold. One interesting
result from this analysis is the difference between DE01 and the other two biofilters when
comparing populations that were influential in both 2009 and 2010. For DE01 there were only
three fungal populations that were influential in both years, whereas the FP07 and FP03 biofilters
had populations that were influential in both years in all three microbial communities (Table 12).
This difference is likely the result of the renovation done to the DE01 biofilter in March 2010.
Table 12. Influential microbial populations for the FP07, FP03, and DE01 biofilters. Numbers represent the specific size (number of base pairs) of unique ARISA and FARISA fragments, and nosZ terminal restriction fragments. nosZ TRFs include the restriction enzyme used to generate that specific TRF. Populations were determined using the BVSTEP function with $\rho = 0.85$.

<table>
<thead>
<tr>
<th>Biofilter and Microbial Community</th>
<th>Influential Populations</th>
<th>$\rho$</th>
<th>% of 2009 Pops in 2010</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FP07</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARISA</td>
<td>488, 545, 602, 725, 749, 765, 782, 799, 869, 878, 889, 891, 899, 907, 968</td>
<td>0.855</td>
<td>40</td>
</tr>
<tr>
<td>2009</td>
<td>382, 384, 385, 425, 515, 545, 602, 763, 765, 770, 780, 782, 786, 795, 797, 805, 848, 878, 889, 921</td>
<td>0.851</td>
<td></td>
</tr>
<tr>
<td>FARISA</td>
<td>325, 430, 449, 493, 527, 533, 546, 555, 570, 613, 645</td>
<td>0.856</td>
<td>18</td>
</tr>
<tr>
<td>2009</td>
<td>325, 554, 559, 591, 613, 625, 631, 633, 758</td>
<td>0.862</td>
<td></td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td>Hha-105, Hha-208, Hha-224, Hha-225, Hha-226, Alu-435</td>
<td>0.872</td>
<td>50</td>
</tr>
<tr>
<td>2010</td>
<td><strong>Hha-224, Hha-225, Hha-226</strong>, Hha-247, Hha-320, Hha-353, Alu-270, Alu-404, Alu-413, Alu-463</td>
<td>0.854</td>
<td></td>
</tr>
<tr>
<td><strong>FP03</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARISA</td>
<td>646, 672, 720, 749, 760, 765, 770, 778, 793, 799, 805, 811, 914</td>
<td>0.851</td>
<td>46</td>
</tr>
<tr>
<td>2009</td>
<td>385, 720, 746, 755, 760, 765, 770, 805, 807, 841, 875, 914, 924</td>
<td>0.852</td>
<td></td>
</tr>
<tr>
<td>FARISA</td>
<td>430, 501, 521, 540, 544, 554, 555, 559, 566, 613, 790, 800</td>
<td>0.851</td>
<td>18</td>
</tr>
<tr>
<td>2010</td>
<td>389, 479, 482, 501, 557, 559, 625, 636, 645, 684, 725, 744, 758, 807</td>
<td>0.851</td>
<td></td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td><strong>Hha-105, Hha-225, Hha-321, Alu-203</strong></td>
<td>0.866</td>
<td>75</td>
</tr>
<tr>
<td>2009</td>
<td><strong>Hha-105, Hha-112, Hha-224, Hha-226, Hha-320, Hha-321, Alu-203</strong>, Alu-413, Alu-461, Alu-463, Alu-676</td>
<td>0.855</td>
<td></td>
</tr>
<tr>
<td><strong>DE01</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARISA</td>
<td>358, 364, 369, 880</td>
<td>0.516</td>
<td>0</td>
</tr>
<tr>
<td>2009</td>
<td>464, 517, 600, 602, 697, 777, 857, 935</td>
<td>0.853</td>
<td></td>
</tr>
<tr>
<td>FARISA</td>
<td>426, 527, 566, 684, 725, 758, 771</td>
<td>0.857</td>
<td>43</td>
</tr>
<tr>
<td>2009</td>
<td><strong>426, 566</strong>, 589, 631, 633, 638, 755, 758</td>
<td>0.858</td>
<td></td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td>Hha-266, Hha-351, Alu-231, Alu-597, Alu-678</td>
<td>0.613</td>
<td>0</td>
</tr>
<tr>
<td>2009</td>
<td><strong>Hha-105, Hha-191, Hha-226, Hha-246, Hha-320</strong></td>
<td>0.858</td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td><strong>Hha-105, Hha-191, Hha-226, Hha-246, Hha-320</strong></td>
<td>0.858</td>
<td></td>
</tr>
</tbody>
</table>

a) Bolded populations were influential to their respective biofilters in both 2009 and 2010.
4.6 Populations Associated with Strong Nitrate Removal

Similar to the rationale for determining populations influential in defining microbial community structure, we sought to determine a subset of microbial communities within each biofilter that were strongly correlated to strong nitrate removal. To determine populations associated with good nitrate removal, we selected the top 5 – 20 microbial populations correlated with high levels of nitrate removal, utilizing Redundancy Analysis (RDA). For this analysis we used nitrate removal data for January 1, 2009 – September 11, 2009. Therefore, for RDA analysis we only used samples collected during January 1, 2009 – September 11, 2009 (Figure 20).

Populations associated with strong nitrate removal are presented in Table 13. Comparisons made between populations associated with good performance and 2009 influential populations revealed the highest number of common populations in the FP07 biofilter, followed by FP03 and DE01 (Table 13). It is possible that because FP07 was the strongest performing biofilter (Figure 20) during this time period that microbial populations associated with good performance were more influential in determining microbial community structure in FP07 than in the other two biofilters. Further work is needed to determine the degree that microbial community structure is influenced by populations associated with good performance, particularly in both strong and weak performing systems.
Figure 20. Nitrate removal for January 1, 2009 – September 2011, calculated as nitrate removed from the inlet to the outlet flow control structure. Nitrate removal was consistently high in A) FP07 but not B) FP03. Nitrate removal was quite variable in C) DE01.
Table 13. Microbial populations associated with strong 7-day nitrate removal. Numbers represent the specific size (number of base pairs) of unique ARISA and FARISA fragments, and nosZ terminal restriction fragments. nosZ TRFs include the restriction enzyme used to generate that specific TRF. Populations were determined using the RDA analysis.

<table>
<thead>
<tr>
<th>Biofilter and Microbial Community</th>
<th>Populations Associated with Good Nitrate Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FP07</strong></td>
<td></td>
</tr>
<tr>
<td>ARISA</td>
<td>311, 312, 333, 353, 381, 382, 383, 428, 429, 430, 434, 548, 592, 594, 646, 712, 749, 933, 952</td>
</tr>
<tr>
<td>FARISA</td>
<td></td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td>Alu-174, Alu-197, Alu-204, Alu-205, Hha-191, Hha-207, <strong>Hha-208</strong>, Hha-209, <strong>Hha-224</strong>, <strong>Hha-225</strong>, Hha-667</td>
</tr>
<tr>
<td><strong>FP03</strong></td>
<td></td>
</tr>
<tr>
<td>ARISA</td>
<td>399, 401, 437, 452, 468, 495, 500, 519, 524, 556, 562, 579, 589, 611, 627, 675, 684, 807</td>
</tr>
<tr>
<td>FARISA</td>
<td></td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td>394, 404, 486, 487, 496, 533, 557, 585, 656, 716, 735, 746</td>
</tr>
<tr>
<td><strong>DE01</strong></td>
<td></td>
</tr>
<tr>
<td>ARISA</td>
<td>321, 364, 395, 398.5, 410, 418, 463, 506, 572, 578, 616, 626, 639, 644, 657</td>
</tr>
<tr>
<td>Fungal ARISA</td>
<td>357, 487, 548, 595, 611, 638, 647, 658, 769, 898</td>
</tr>
<tr>
<td>nosZ T-RFLP</td>
<td>Alu-195, Alu-196, Alu-202, Alu-204, Alu-284, Alu-342, Hha-112, Hha-114, 1Hha-137, Hha-184</td>
</tr>
</tbody>
</table>

a) Bolded populations signify those that were associated with good performance, and influential.

### 4.7 Summary

The overall goal for our two-year temporal study was to determine how denitrifying biofilter microbial communities changed over time, and how they responded to environmental and management variables. To accomplish this we studied three denitrifying biofilters (FP07, FP03, DE01) over the course of two years. We also determined small population subsets within each biofilter that were influential to determining microbial community structure, associated with good biofilter performance, or both.

Microbial communities responded to similar drivers in each biofilter over the duration of the two-year study. Two of the most important factors that influenced microbial community composition were depth and season. In general, total bacteria, fungi, and denitrifying bacteria all
exhibited different community structure at each depth. Correspondence analysis results indicated that microbial communities at 1.52 m were positively associated with moisture (1.52 m), while those at 0.76 m were positively associated with temperature. Furthermore, variability analysis revealed that samples collected at 0.76 m were in general less variable than those from 1.52 m.

In addition to depth, season was important in driving microbial community structure. Strong seasonal patterns in microbial community structure were observed by classifying samples based on sample collection date (January – June or July – December). Seasonal patterns were evident for all three microbial communities. Correspondence analysis results indicate that moisture/water flow was positively associated with microbial communities collected in January – June, while temperature was positively correlated with microbial communities collected in July – December. To varying degrees, all three biofilters exhibited more consistent water flow during January – June and higher temperatures during July – December. Variability analysis indicated that microbial communities in samples collected during January – June were less variable than those from July – December.

Finally, due to the complexity of denitrifying biofilter microbial communities, we determined small population subsets that were influential, associated with strong nitrate removal, or both. From our analysis we have determined that populations influential in 2009 were largely influential in 2010, with the exception of the DE01 biofilter, which was disturbed in 2010. Furthermore, we have also shown that while small, there is some overlap among populations that are influential in driving microbial community structure and those that are associated with strong biofilter performance. The value of knowing these populations will come when comparing the results from this two-year temporal study to future denitrifying biofilter studies. Further
investigation is needed to confirm if these populations are unique to FP07, FP03, and DE01 or if they are important to all denitrifying biofilters.
Chapter 5: Spatial Study

Acknowledgements: Biofilter sampling, sample processing, and initial analyses for the biofilter spatial study were conducted by J. Malia Andrus. I extracted and cleaned DNA, performed ARISA and nosZ T-RFLP, and conducted MDS and ANOSIM analyses on the samples collected for this study.

5.1 Introduction

A one-day study of the FP03 biofilter was undertaken to assess microbial community spatial structure. Sample collection for this spatial study was conducted on March 16, 2007, prior to the start of our two-year temporal study. ARISA and nosZ T-RFLP were used to assess variation in the total and denitrifying bacterial communities.

5.2 Methodology

The methods utilized for this spatial study were very similar to those employed for the two-year temporal study. There are, however, slight variations in sample collection, sample processing, molecular, and statistical methods. These differences are noted in this chapter; otherwise methods in Chapter 3 were used.

5.2.1 Study Sites

The FP03 biofilter was sampled for this study on March 16, 2007. On the day of sampling the biofilter was operating properly. Water flow through the biofilter on the day of sampling was approximately 44,928 L.

5.2.2 Biofilter Sampling

Woodchip samples were collected using a 10.2 cm diameter soil auger. Samples were collected at 2.74 m intervals along the length, 0.46 m intervals along the width, and from two
depths, 0.76 m and 1.52 m. Sample transect locations parallel to the flow path were labeled 1-12 (starting from biofilter inlet), and positions within each transect (orthogonal to the flow path) were labeled A-C (Figure 21).

![Figure 21. Schematic of the spatial study sampling regime. Samples were collected at three positions (A,B,C) along twelve transects (1-12). Image modified from Andrus (1).]

Due to fact that the biofilter was not of uniform width, several predetermined sample locations were along the edges of the biofilter. As a result, the auger removed soil instead of woodchips. At these locations no samples were retained for analysis.

### 5.2.3 Sample Processing

Woodchip samples were processed by adding 125 mL of Ringer’s solution (0.9% NaCl, 0.042% KCl, 0.024% NaHCO₃) to 20 g of woodchips (26). Bottles were shaken vigorously for 30 s to dislodge microbial cells from woodchips. Aliquots (1 mL) of the woodchip wash were concentrated 4-fold by centrifuging and re-suspending in 250 µl of sterile Ringer’s solution. Samples were stored at -80°C until further analysis.
5.2.4 ARISA and nosZ T-RFLP

nosZ T-RFLP was carried out using three restriction enzymes, *AluI, HhaI*, and *MboI*. Relative fluorescence was determined separately for each restriction digest and then concatenated, with the total relative fluorescence for the two combined digests summing to 3.

5.2.5 Statistical Analyses

Non-metric multidimensional scaling plots (MDS) were used to visualize community similarity between samples (52, 65). MDS plots were calculated from Bray-Curtis similarity matrices (6), and used specifically to visualize microbial community similarity by depth, transect, position, and sampling location. MDS analyses were conducted using the statistical software package Primer 6, version 6.1.10 (Primer-E Ltd., Plymouth, United Kingdom).

5.3 Results

5.3.1 Total Bacterial Community

Within the biofilter 394 distinct bacterial populations were detected by ARISA, with an average of 59 fragments per sample. The total bacterial community exhibited slight spatial structure by depth (Figure 22 and Table 14). Total bacterial spatial variation by sampling transect was evident for both depths combined, more so for samples collected at the 0.76 m depth, and slightly less for samples from the 1.52 m depth (Figure 23 and Table 14). While the total bacterial community appeared to be influenced in part by depth and transect, sampling position did not appear to be driving community structure (Figure 24 and Table 14).
Table 14. Microbial community differences for depth, position, and transect for total bacteria (ARISA) and denitrifying bacteria (nosZ T-RFLP). ANOSIM values are based on Bray-Curtis Similarity.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Total Bacteria (ARISA)</th>
<th>Denitrifying Bacteria (nosZ T-RFLP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANOSIM R</td>
<td>P-value</td>
</tr>
<tr>
<td>Depth</td>
<td>0.136</td>
<td>0.001</td>
</tr>
<tr>
<td>Transect (1-12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All Depths</td>
<td>0.261</td>
<td>0.001</td>
</tr>
<tr>
<td>0.76 m</td>
<td>0.333</td>
<td>0.001</td>
</tr>
<tr>
<td>1.52 m</td>
<td>0.197</td>
<td>0.005</td>
</tr>
<tr>
<td>Position (A,B,C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All Depths</td>
<td>0.033</td>
<td>0.054</td>
</tr>
<tr>
<td>0.76 m</td>
<td>0.033</td>
<td>0.166</td>
</tr>
<tr>
<td>1.52 m</td>
<td>0.072</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Figure 22. Non-metric multidimensional Scaling (MDS) plots of microbial community composition at each depth. A) total bacterial communities demonstrate structure by depth. B) Denitrifying bacteria do not demonstrate community structure by depth. Points represent microbial community samples collected at either 0.76 m or 1.52 m.
Figure 23. Non-metric multidimensional Scaling (MDS) plots of total bacterial community structure, classified by transect at A) all depths combined, B) 0.76 m depth only, C) 1.52 m depth only, and denitrifying community structure by transect at (D) all depths combined, E) 0.76 m depth only, F) 1.52 m only. Points represent microbial community samples collected at a specific transect (1-12).
5.3.2 Denitrifying Bacteria Structure

Among denitrifiers 206 total TRFs—comprised of 74 from *Alul*, 62 from *HhaI*, and 70 from *MboI*—were detected by *nosZ* T-RFLP. In contrast to the total bacterial community, denitrifying bacteria did not exhibit distinct community structure by depth (Figure 22 and Table 14), sampling transect (Figure 23 and Table 14), or sampling position (Figure 24 and Table 14).
Figure 24. Non-metric multidimensional Scaling (MDS) plots of total bacterial community structure by position at A) all depths combined, B) 0.76 m depth only, C) 1.52 m depth only, and denitrifying community structure by position at (D) all depths combined, E) 0.76 m depth only, F) 1.52 m only. Points represent microbial community samples collected at a specific position (A,B,C).
5.4 Discussion

Understanding the factors that determine microbial community structure and function in engineered ecosystems—particularly those reliant on microbial activity to perform a specific function—may be valuable in optimizing functional performance. Within the FP03 biofilter, the total bacterial community demonstrated spatial structure while the denitrifying bacterial community did not.

Although spatial structure was not evident among denitrifying bacteria within the biofilter, spatial structure among denitrifiers has been observed in other engineered ecosystems. Spatial structure in denitrifying bacterial communities has been observed in sediments collected from a wastewater treatment wetland (36), in soil samples from an active farm (20), and in soil samples collected from a grassland pasture (48). Explanations for why other studies observed denitrifying community spatial structure could be due to the fact that hydraulic retention time was not a factor in our study (36), that different microbial fingerprinting techniques were utilized (20, 36, 48), or that the study systems were different (20, 36, 48). T-RFLP also generally provides lower resolution than ARISA, which may have obscured some spatial structure in the denitrifying bacterial community (33, 46).

The shape of the denitrifying biofilter, and the fact that the biofilter was unlined may have influenced our spatial results. The biofilter studied was L-shaped, with a 90-degree turn immediately following the 8th transect. This shape, particularly at the 90-degree turn, likely influenced flow characteristics within the biofilter, and it is possible that this may have had an effect on microbial community structure (7, 28, 44). Specifically, it is possible that preferential flow paths through the woodchip medium contributed to gradient (dissolved oxygen, carbon, etc.) formation within the biofilter (28). Previous research has demonstrated that preferential
flow paths through soil, a porous medium, can create biological “hot spots” of increased microbial activity, by establishing favorable oxygen, nutrient, and moisture gradients (7, 44).

It is also important to consider that the biofilter was unlined, such that the biofilter woodchip medium was in direct contact with soil from the surrounding agricultural field on all borders. There are two consequences of the biofilter being unlined. First, microbial communities within the denitrifying biofilter were always in direct contact with soil microbial communities. Second, it is likely that there was groundwater seepage both into and out of the biofilter. Groundwater seepage would not only affect chemical gradients imposed by the biofilter flow regime, but could also introduce new microbial communities to the biofilter system.

From our one-day spatial study of the FP03 biofilter, we were able to demonstrate spatial structure among the total bacterial community, but not denitrifying bacteria. Results from this study were utilized to determine appropriate spacing for the PVC sampling ports utilized in our two-year study (1), ensuring that we had optimized our temporal biofilter sampling regime. In addition to providing valuable background information for our temporal study, this research also demonstrated for the first time that microbial communities are structured over space within a denitrifying biofilter. Further work is needed to determine if the spatial structure of microbial communities within the denitrifying biofilter is important to biofilter performance. Ultimately, by determining environmental factors that influence microbial community spatial variation and learning how spatial variation affects performance, denitrifying biofilter designs can be optimized to improve performance and reliability.
Chapter 6: Discussion

6.1 Introduction

By developing a greater understanding of the drivers and dynamics of microbial communities responsible for denitrifying biofilter functionality, we will be able to better predict system failures and improve performance. From this work we have determined that total bacterial, fungal and denitrifying bacterial communities demonstrated similar spatial and temporal patterns in microbial community composition over a two-year study of three unique biofilter systems. We have demonstrated distinct community structure between depth and season are two strong recurring patterns in biofilter microbial communities. Additionally, we have been able to identify populations within each microbial community that were either influential in shaping microbial community structure, correlated with strong nitrate removal, or both.

6.2 Microbial Community Composition Differences by Depth

Perhaps the strongest driver determining microbial community structure across all three denitrifying biofilters was depth. With the exception of DE01 in 2010, total bacteria, fungi, and denitrifying bacteria all exhibited distinct community structure by depth. Moreover, the degree of difference in microbial community structure between depths remained fairly consistent within each biofilter from 2009 to 2010. Overall the DE01 biofilter (2009) displayed the strongest difference in microbial communities between depths, followed by FP07 and FP03, respectively.

One explanation for why the FP03 biofilter had the smallest degree of microbial community structuring by depth is that its flow control boards were removed for an unknown length of time in 2009, and for approximately one-third of 2010. In addition to maintaining a minimum HRT, the flow control structures also provide a minimum water level during times of flow. In the absence of flow control boards, woodchip samples taken from deeper within the
biofilter were probably less frequently inundated with water, and therefore more similar to those collected from the 0.76 m depth than we would otherwise expect. While this hypothesis would explain the smaller degree of difference between depths within the FP03 biofilter, it is not consistent with the results from our 2007 spatial study. Sampling for the spatial study occurred at a time when the flow control boards were present in the FP03 biofilter, but neither the total nor denitrifying bacteria showed strong community structuring by depth. Therefore, it appears that the FP03 biofilter may not have the same degree of depth stratification as the other two biofilters for some other reason.

One possible explanation may be that FP03 lacks the same depth separation due to its unique flow regime. Water flows through the FP07 and DE01 biofilters linearly across all of the sampling ports. FP03, however, has a 90-degree turn located approximately half way between the inlet and outlet flow control structures. It may be that differences in flow dynamics were responsible for the lack of depth separation. By forcing tile drainage to make a 90-degree turn, within our system it is possible that the water followed a preferential flow path such that the majority of the flow largely bypassed the location of our sampling ports after the turn. If this hypothesis is correct the 0.76 m and 1.52 m ports after the turn would be more similar to each other than they are in other biofilters. Overall the cumulative effect of this increased similarity could result in less depth distinction among all 0.76 m and 1.52 m sampling ports in FP03, than in the other two biofilters. Future work should employ tracer tests to determine water flow characteristics within all three biofilters.

In all three biofilters, correspondence analysis results indicated that sampling port and weekly average temperature were positively correlated with samples collected at 0.76 m, and negatively correlated with samples from 1.52 m. This finding is not surprising as temperature
was more variable within the 0.76 m sampling ports. This variability was likely a result of the 0.76 m sampling ports being both irregularly inundated with water and closer to the ground surface. Studies of aquatic environments have noted the influence of temperature in determining microbial community structure (38, 58, 65, 67), and hypothesized that temperature may have a direct selective effect on bacterial community composition (67). Due to the higher variability of temperature at 0.76 m, it is probable that temperature was an important factor in driving biofilter microbial community depth structure.

In addition to temperature and moisture, the bioavailability of organic carbon likely played an important role in structuring biofilter microbial communities by depth. Correspondence analysis results indicate a positive correlation between woodchips at 1.52 m and woodchip % moisture. This result is not surprising, as woodchips at 1.52 m in each biofilter were almost always inundated with water, while those at 0.76 were variably inundated. One hypothesis is that by being regularly wet, organic carbon was more consistently bioavailable at 1.52 m. In contrast, the woodchips at 0.76 m often dried out, particularly in the summer during periods of little or no flow. Therefore, it may be possible that while organic carbon was consistently available at 1.52 m, its availability occurred in pulses at 0.76 m. This difference in the nature of organic carbon availability may be in part responsible for driving the depth differences observed in the three biofilters.

Studies of natural systems have correlated microbial community structure with organic carbon availability (18, 29, 37, 40), and organic carbon differences by depth (18, 29). Most relevant to our work was a study of soil microbial community composition, in which the authors determined that soil water content and the availability of organic carbon were influential in shaping community structure (18). If organic carbon bioavailability is correlated with moisture in
the biofilter, it may have consequences on performance. One hypothesis is that denitrifying bacteria may perform better at 1.52 m than at 0.76 m within the biofilter due to a ready supply of organic carbon, due to the fact that a favorable electron donor, such as organic carbon, must be present for bacterial denitrification to occur (22). Further research investigating organic carbon availability at depth, while taking into account moisture needs to be undertaken. If a link between moisture, organic carbon availability, and performance can be established, future biofilter designs can be altered to raise or lower water levels within the system.

One surprising finding comes from examination of microbial community variability at each of the two depths, using mean centroid distance analysis. We had hypothesized that samples collected at 1.52 m would be less variable than those from 0.76 m. This hypothesis was based on the fact that the 1.52 m sampling ports were typically inundated with water, which likely provided a more consistent environment than the variably wet 0.76 m sampling ports. This, however, proved not to be the case as microbial communities collected from 0.76 m were typically less variable than those from 1.52 m. From these results, I am unable at this time to determine a reasonable explanation for why samples collected at 1.52 m were more variable than those from 0.76 m. Future research should investigate whether there are differences in denitrification between the two depths. By determining if performance varies with depth we will be better positioned to assess whether depth variability is important to system performance, reliability, stability, and resilience, or if it is just an artifact of the system design.

### 6.3 Microbial Community Composition Differences By Season

One of the most interesting results from our two-year temporal study was the finding that biofilter microbial communities demonstrated distinct community structure by season. Consistent across all biofilters, the strongest patterns in community structure were observed when
classifying microbial communities bi-annually based on whether samples were collected in the months of January – June or July – December. With the exception of total and denitrifying bacteria in the DE01 biofilter in 2010, all three microbial communities within each biofilter exhibited seasonal patterns in community structure in 2009 and 2010.

The degree of seasonality was variable depending on the year and biofilter. In general all microbial communities experienced stronger seasonality in 2009. Among the three biofilters, microbial communities in FP03 demonstrated the highest seasonal variation followed by FP07 and DE01, respectively. Correspondence analysis results indicate that woodchip % moisture was positively correlated with samples collected in January – June, while higher sampling port temperature and weekly average temperature were associated with samples collected in July – December. The finding that woodchip % moisture was positively correlated with samples collected in January – June is not surprising, as water flow through all three biofilters was greater during the first six months of 2009 and 2010. The connection between sampling port, weekly average temperature, and samples collected in July – December is also logical, as both ambient temperature, and temperatures within the biofilter (sampling port temperature) were highest during the summer and fall months.

Due to the seasonality of water flow through the biofilter, we initially hypothesized that water flow was contributing to seasonal patterns in microbial community composition. This hypothesis was confirmed for the DE01 biofilter in 2009, the FP03 biofilter in 2010, and to a lesser degree the FP07 biofilter in 2010. In all three cases daily flow and 7-day average flow were positively correlated with samples collected in January – June. For the other three cases, where flow was not strongly associated with seasonal patterns in community structure there are plausible explanations. In 2010, flow to the DE01 biofilter was cut off after March 9th, artificially
altering DE01 flow dynamics. The FP03 biofilter had its flow control boards removed at an unknown time in 2009, drastically reducing HRT. Finally, the FP07 biofilter demonstrated little flow seasonality during 2009.

Analysis of microbial community variability by season indicated that microbial communities were generally less variable in January – June than in July – December. We hypothesized that samples collected in January – June would be less variable than those from July – December, due to the fact that water flow in each biofilter slowed or stopped completely around July.

To my knowledge, there has not been any other research that has demonstrated seasonal patterns in microbial community composition within a denitrifying biofilter. There have been, however, a handful of river, lake, and marine studies where seasonality in microbial community composition has been attributed to environmental factors (14, 27, 58, 65). In particular, the authors of a 2.5-year study of two temperate rivers concluded that bacterioplankton seasonal patterns were largely driven by temperature and variability in river water flow (14). The authors of a 6-year study of a freshwater lake attributed annual trends in bacterial community composition to temperature and lake water-column mixing (58). The results from these studies complement our finding of microbial community structure. Furthermore, they agree with our finding that predictable environmental factors, such as temperature or flow, play an important role in the seasonal variation of microbial community composition.

The results from our temporal biofilter study have indicated that the total bacterial, fungal, and denitrifying bacterial communities have remarkably similar seasonal dynamics. These results agree with previous findings of seasonal variation in natural systems (14, 27, 58, 65), and demonstrate that microbial community structure within the biofilter was driven by
environmental factors. More work is necessary to resolve whether seasonal variation has any bearing on performance, reliability, stability and resilience. By determining the interplay between microbial community seasonality and performance, it may be possible to alter biofilter designs to mitigate or exploit seasonal dynamics to improve system performance and reliability.

6.4 Influential Populations and Populations Associated with Strong Nitrate Removal

Due to the complexity of the microbial communities within each biofilter, we sought to determine subsets of each community that were either influential in shaping community structure, associated with strong nitrate removal, or both. Comparing the number of populations that identified as influential in both 2009 to 2010 indicated that while there was some turnover, a substantial portion were consistently important in explaining microbial community dynamics. It was from this analysis that the effects of disturbance on the DE01 biofilter can be assessed, as zero total and denitrifying bacteria populations from 2009 remained influential in 2010. Had the DE01 biofilter not been renovated in 2010, it is probable that the percentages of influential populations carrying over from 2009 to 2010 would have been similar to the results from FP07 and FP03.

In addition to examining influential populations, we also looked at populations correlated with a high level of 7-day average nitrate removal. Interestingly, there were no microbial communities correlated with strong nitrate removal common to all three biofilters. The greatest overlap occurred among denitrifying bacteria, as all three biofilters shared at least one common population. Comparisons between microbial populations associated with strong performance and influential populations from 2009, revealed that FP07 was the only biofilter to have at least one population that was classified as both influential and associated with strong performance for all three microbial communities.
The determination of population subsets, whether associated with strong nitrate removal or influential, is only an academic exercise unless this information can be used to improve biofilter design or functionality. The true value of this data will present itself when comparing these populations to those from other denitrifying biofilters. Specifically, if it can be established that specific populations regularly occur in strongly performing biofilters then the identity of those individual populations can be determined. Although the denitrifying biofilter is an engineered system it relies upon microorganisms to function. Determination of individual microorganisms that are influential or associated with good preforming systems may ultimately allow for the establishment of more reliable biofilters.

6.5 Conclusions and Future Work

The goal of this research was to determine how the microbial communities associated with denitrifying biofilters behaved over space and time, and how these communities were affected by environmental and management variables. From our one-day spatial study we concluded that the total bacteria, but not denitrifying bacteria varied significantly by depth and sampling transect along the flow path. Using the knowledge gained from this work we installed sampling ports in three denitrifying biofilters and collected samples over the course of two years.

Results from our two year temporal study demonstrated that total bacteria, fungi, and denitrifying bacteria showed consistent patterns in community structure by depth and season. Correspondence analysis suggested that microbial community differences between depths were likely the result of moisture and temperature differences between the two depths. Microbial composition was also distinct among seasons (January – June and July – December). Microbial community seasonality was likely the result of annually reoccurring environmental factors, specifically water flow, moisture, and temperature.
The results from our two-year biofilter study provide an excellent knowledge base for future studies to build from. Experiments investigating the strongest patterns (depth and season) and important populations (influential and populations associated with strong nitrate removal) should be undertaken. To do so we have constructed laboratory-scale denitrifying biofilters mimicking the design of our field systems. The following is an experimental plan detailing a schedule of experiments utilizing these laboratory reactors.

Correspondence analysis demonstrated that depth differences were correlated with moisture. From this result, I hypothesize that moisture differences between the two depths drive the bioavailability of organic carbon. To test this hypothesis, a set of experiments should be conducted utilizing adjustable weirs within the laboratory reactors. Specifically, I recommend running two reactors with different water levels. In one reactor the water level will be constant with woodchips continually inundated. In the second, woodchips will be typically dry but experience periodic water flow. Woodchips and water samples will be collected on a weekly basis to assess the microbial community composition and availability of organic carbon. Organic carbon will be determined using a Chemical Oxygen Demand (COD) analysis of reactor water (19). Finally, halfway through the experiment the flow conditions of the two reactors will be switched. After switching the flow conditions, I hypothesize that the typically dry reactor will see a spike of organic carbon before coming to a steady state, while the level of organic carbon in the normally wet reactor will slowly decrease with time. Results from this set of experiments will provide greater insight into whether or not moisture differences at the two depths are responsible for creating organic carbon gradients between the two depths and ultimately for structuring microbial communities.
The previous set of experiments will help to determine what is driving the depth distinction in microbial community structure; however, these experiments will not provide information pertaining to biofilter performance, reliability, stability, and resilience. Therefore, I propose conducting a series of batch experiments run in parallel with the reactor experiments using woodchips from each reactor. After flooding the typically dry reactor, woodchip and water samples will be collected from both reactors. Kinetic experiments utilizing denitrifying enzyme assays (2, 60) will assess the denitrification potential of woodchips collected from each reactor. These results will provide insight into the performance potential of woodchips at each depth.

In addition to depth, seasonal differences were observed in our temporal study. Correspondence analysis results suggest that moisture, flow, and temperature were likely responsible for driving microbial community seasonal patterns. Future experiments should seek to determine the performance, reliability, stability, and resilience of denitrifying biofilters experiencing seasonal changes. To do so, I propose running two reactors in parallel with the temperature and flow characteristics matching those observed in the field. Starting with conditions typical to January 1st date and running all the way to conditions consistent with December 31st, the reactors will be operated with flow and temperature characteristics consistent to those observed in the field. Water flow will maintain a constant and reasonable nitrate concentration. Woodchip and water samples will be collected regularly to assess microbial community structure and to determine biofilter performance. The results from these experiments will provide information that will help inform future designs to either dampen or accentuate seasonality within the biofilter.

Influential populations and populations associated with strong nitrate removal were determined for each biofilter over the course of our two-year study. The simplest way to
determine if these populations are important to biofilter performance and functionality is to assess what populations are influential and associated with strong nitrate removal during the laboratory reactor depth and season experiments. These results will provide information about how important microbial populations respond to varying environmental and operational characteristics.

Ultimately, it is my hope that these reactor studies will continue my work and will contribute information that will be used to improve denitrifying biofilter design. From our two-year study of three denitrifying biofilters I have determined that the structure of total bacterial, denitrifying bacterial, and fungal communities were structured by depth and seasonal factors, largely moisture and temperature. Furthermore, I have determined population subsets that were influential to shaping community structure, associated with strong nitrate removal, or both. By better understanding how microbial communities within the biofilter are affected by environmental and operational parameters, we will ultimately be able to improve biofilter design, performance, reliability, stability, and resilience. Determining innovative, cost-effective, and reliable solutions to reduce nitrate loads from exiting subsurface drainage networks will enable us to slow and possibly even reduce many of the deleterious environmental effects of nitrate—such as coastal eutrophication and hypoxia in the Gulf of Mexico.
References

1. **Andrus, J. M.** 2011. Microbial community analysis for denitrifying biofilters. University of Illinois at Urbana-Champaign, Urbana, IL.


25. **Franklin, R. B., and A. L. Mills.** 2009. Importance of spatially structured environmental heterogeneity in controlling microbial community composition at small spatial scales in an agricultural field. Soil Biology and Biochemistry **41:**1833-1840.


55. **Rodrigue, A. L.** 2006. Effects of sampling frequencies in the evaluation of nitrate-N transport from drainage-related BMP. University of Illinois at Urbana-Champaign, Urbana, IL.


Appendix

A.1. Sample Processing, DNA Extraction, and DNA Purification Procedures

A.1.1. Biofilter Woodchip Sampling Procedure

Prior to Sampling
1. Wash and autoclave one 250 mL and one 125 mL Nalgene sampling bottle and two 50 mL centrifuge tubes for each sample location.
2. Prepare Ringer's solution by adding 250 mL of nanopure water to 2 Ringer's tablets and autoclaving on a 30 liquid cycle.
3. Autoclave a 250 mL graduated cylinder, 5 mm glass beads, and 1.5 mL centrifuge tubes on a 30 gravity cycle.

Night of sampling
1. Remove woodchips from each bottle until 30 g remain. Place the removed woodchips in the appropriately marked weighing tin and record wet weight. Place the tins in the drying oven overnight at 105°C.
2. Add 110 mL of sterile Ringer's solution to each bottle using a sterile graduated cylinder. Place the bottles on the shaker tray in the 30°C room overnight.
3. Spin down each water sample in a sterile 50 mL centrifuge tube for 3 mins at 5000xg (this will require about three spins).
4. After the second spin, transfer 10 mL of the supernatant to a syringe with a 0.45 µm filter and filter into a 15 mL centrifuge tube (this will be used to measure pH).
5. Add 2.5 mL of 1X PBS and 5 sterile glass beads to each 50 mL centrifuge tube (use sterile tweezers to handle).
6. Store water samples overnight at 4°C.
7. Use the previously filtered water sample to measure the pH of water samples.

Day after sampling
1. Remove and weigh the tins from the drying oven and record the “dry weight.”
2. Spin down the woodchip wash (the Ringer's solution in which the woodchips have been shaken) in sterile, appropriately marked 50 mL centrifuge tubes for 3 mins at 5000xg (this should take two spins).
3. Add 2.5 mL of 1X PBS and 5 sterile glass beads to each 50 mL centrifuge tube.
4. Vortex each 50 mL centrifuge tube for 2 mins on the high setting.
5. After vortexing, centrifuge at 750xg for 5 mins.
   a. Label both the side and top of 2 sterile 1.5 mL tubes for each sample with the following:
      b. Sampling Date
      c. Sample Name
6. Pipet 1 mL of the supernatant into each of the sterile, labeled 1.5 mL centrifuge tubes using sterile large orifice pipette tips.
7. Store the DNA sample tubes at -20°C until extraction.
8. Clean up sampling bottles, put away all reagents.
A.1.2. Woodchip and Water DNA Extraction Procedure

The woodchip and water DNA extraction procedure was modified from the manufacturers instructions by J. Malia Andrus to increase DNA yields.

Step 1- Setup and Prep

1. Before beginning load five 2 mL mid-sci tubes with autoclaved nanopure H₂O. 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. Start by pulling out the FastDNA Soil Spin Kit.
3. 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 2 mL tube per sample (on bench top)
   c. One Spin Module per sample (in soil spin kit)
   d. One 1.7 mL tube without pop-top per sample (on bench top)
4. Label the tops of each of the tubes with sharpie (sample name and date)
5. Vortex each sample, and add 400 µL of DNA to its lysing tube, afterwards leave the tubes open.
6. Immediately add 1 mL of CLS TC to the top of each sample in the lysing tubes and put the tops back onto the tubes.
7. Walk over to the Fast Prep Machine and load the machine.
   a. Take care to load the samples in order, starting at the red dot.
   b. Make sure the Fast Prep Machine is balanced.
   c. Turn the spindle and secure each lysis tube in place.
   d. Set the Fast Prep Machine to 5 M/S for 40 seconds.
   e. **After each run, the Fast Prep Machine needs to rest for 5 mins!
8. Spin all of the lysing tubes for 5 mins at max speed.

Step 2 – Extraction

1. Pipette 700 µL of supernatant into each sample’s respective 2 mL tube.
2. Next pipette a volume equivalent to the volume of DNA, 700 µL of binding matrix, into each 2 mL tube, and shake well.
3. Transfer all of the 2 mL tubes into a 96 well tray, place the lid on the tray and slowly turn the tray up and down for 5 mins.
4. After 5 mins are up, centrifuge the 2 mL tubes for 1 min at max speed.
5. Pipette most 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.
6. Re-suspend the glass beads with the remaining liquid, and pipette into the 3rd tube with the basket.
7. Spin the basket tubes for 1 min at max speed, to separate the liquid from the beads.
8. Empty the liquid from the basket tube into the waste liquid container.
9. Now put the remaining extraction liquid into the now empty 2 mL tube.
10. Add 700 µL of binding matrix into each of the tubes.
11. Now mix the samples by placing the lid on the tray and slowly turn the tray up and down for 5 mins.
12. After 5 mins are up, centrifuge the 2 mL tubes for 1 min at max speed.
13. Pipette most of the liquid (700 µL) into a waste liquid container, but make sure to leave some liquid at the top of the glass bead level.
14. Re-suspend the glass beads with the remaining liquid, and pipette into the 3rd tube with the basket.
15. Spin the basket tubes for 1 min at max speed, to separate the liquid from the beads.
16. Empty the liquid from the basket tube into the waste liquid container.
17. Throw away the 2 mL tube.

Step 3 - Washing

1. Add 500 µL of SEWS to the basket tube and centrifuge for 1 min.
2. Empty the wash into the waste container.
3. Add another 500 µL of SEWS to the basket tube and centrifuge again for 1 min.
4. Empty the wash into the waste container.
5. Centrifuge for 1 min to remove any remaining ethanol.
6. Place the basket into the last tube, the 1.7 mL tube.
7. Add 100 µL of 65°C water quickly to the glass beads in the basket.
8. 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.

Step 4 – Finishing and Cleanup

1. Place the original DNA tubes back into the freezer.
2. Pull the basket from the 1.7 mL tube, throw it away, and place the extracted DNA into the box for extracted, but not clean DNA.
3. Turn off the heating block.
4. Rinse the 96 plate with water and leave to dry.
5. Use 409 to wipe down the inside of the centrifuge and rotor.
A.1.3. CTAB Purification of Extracted DNA

Due to humic acid contamination of the DNA, extracted DNA was further purified using cetyl trimethyl ammonium bromide (CTAB) cleanup (56). The following procedure was modified by the graduate students of the Kent lab, and was utilized in purify DNA used in all analyses.

CTAB solution recipe (10% CTAB/0.7 M NaCl):

1. Dissolve 4.1 g NaCl in 80 mL H₂O.
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.
6. This is the working CTAB stock.

Purification protocol

1. Pre-warm working CTAB stock to 65°C in a 1.5 mL tube in the heat block. Meanwhile, proceed to steps 2 and 3.
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. Adjust the NaCl concentration of each DNA extract to 0.7 M: add 16.25 mL of 5 M NaCl (autoclaved) to each DNA extract.
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) for at least 15 mins (increasing time will increase precipitation – overnight is best).
8. Mix thoroughly and centrifuge at maximum speed for 5 mins (increase time to increase expected yield). If low yield is expected, increase centrifugation time to ~15 mins. ***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 2 mins. If low yield is expected, increase centrifugation time to ~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 100 mL dH₂O. If low yield is expected, re-suspend DNA in 50 mL dH₂O.
13. Wash back side of tube (where DNA is suppose to be) with dH₂O 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.2. Sample Locations and Miscellaneous Information

A.2.1. Sample Locations

For the temporal biofilter study there remains some extracted, diluted, and purified DNA. The volume of DNA remaining varies largely, however. Table A1 contains the sampling dates and locations of the remaining clean DNA, as well as the locations of unextracted DNA. All clean DNA is located in 4105 Newmark, in a box labeled “Biofilter Project”. Backup unextracted samples are also located in the -20°C freezer in 4105 Newmark Civil Engineering Lab. Additionally, some unextracted and perhaps some extracted, cleaned, but not diluted DNA may be in the 3rd floor walk-in freezer in Turner Hall, next door to 319.

Table A1. Sampling Dates and locations of Temporal Biofilter Extracted and Unextracted Samples

<table>
<thead>
<tr>
<th>Sampling Date</th>
<th>Plate Name/Number</th>
<th>Extracted DNA Location</th>
<th>Unextracted Sample Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/14/2010 – 9/9/2010</td>
<td>Template #9</td>
<td>-20°C 4105 Newmark</td>
<td>-20°C 4105 Newmark</td>
</tr>
<tr>
<td>10/7/2010 – 12/7/2010</td>
<td>Template #10</td>
<td>-20°C 4105 Newmark</td>
<td>-20°C 4105 Newmark</td>
</tr>
</tbody>
</table>

No unextracted samples remain from the spatial biofilter study. Some extracted and purified samples do remain, and are located in the walk-in freezer on the 3rd floor of Turner Hall, next to C319. Additionally, for the spatial biofilter study there were some samples that do not exist, because soil was collected instead of woodchips. To the best of my knowledge these samples include: C5-b; C7-a; C7-b.
A.2.2. Sample Naming Scheme

Temporal biofilter samples were named in a manner that allowed them to be easily manipulated in Primer, Microsoft Excel, and Canoco. The following naming scheme incorporated information pertaining the biofilter sampled, sampling date, sampling port, depth of sampling port, and media type (woodchip or water).

<table>
<thead>
<tr>
<th>Biofilter Name</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DE01 = De Land, “rectangular biofilter”</td>
<td>1. Digits 1 – 4 = year</td>
</tr>
<tr>
<td>2. FP03 = Decatur East, “L-shaped biofilter”</td>
<td>2. Digits 5 &amp; 6 = month</td>
</tr>
<tr>
<td>3. FP07 = Decatur West, “square biofilter”</td>
<td>3. Digits 7 &amp; 8 = day</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sampling Port</th>
<th>Depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. First letter following date, (A-G)</td>
<td>1. Character following port</td>
</tr>
<tr>
<td>DE01: A-E</td>
<td>2: 0.76 m</td>
</tr>
<tr>
<td>FP03: A-G</td>
<td>5: 1.52 m</td>
</tr>
<tr>
<td>FP07: A-E</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Type of sample collected</td>
</tr>
<tr>
<td>W: Water</td>
</tr>
<tr>
<td>C: Woodchips</td>
</tr>
</tbody>
</table>

For Example the sample DE01-20090724-C2C was:

1. From the DE01 biofilter
2. Was collected on 7/24/2009
3. Was collected from sampling port C
4. Sampling port C is a 0.76 m port
5. This was a woodchip sample