Biogeochemical cycles and microbial community dynamics in replicate woodchip bioreactors

by

Abby Elizabeth Schaefer

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Agricultural & Biosystems Engineering

Program of Study Committee:
Adina Howe, Co-major Professor
Michelle Soupir, Co-major Professor
Kaoru Ikuma

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2021

DEDICATION

To my grandparents: Carolyn & John Becker, Shirley & Franklin (Pete) Schaefer

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

ACKNOWLEDGMENTS

Iowa State University is located on the ancestral lands and territory of the Baxoje (bah-kho-dzhe), or Ioway Nation. The United States obtained the land from the Meskwaki and Sauk nations in the Treaty of 1842. I wish to recognize our obligations to this land and to the people who took care of it, as well as to the 17,000 Native people who live in Iowa today.

Thank you to my committee, Michelle Soupir, Adina Howe, and Kaoru Ikuma, for their guidance and support throughout the course of this research. Thank you to Tom Moorman, Beth Douglass, Gary Feyereisen, and Ulrike Tschirner for their collaboration and support of my work. Thank you to Leigh Ann Long, about whom there can never be enough kind words said. Thank you to Thanwalee (JiJy) Sooksa-Nguan for assisting me in the GERMS lab.

Thank you to my colleagues: Lindsey and Adam Hartfiel, Laura Alt, Alexis Slade, Tim Neher, Heather Tenboer, Brady Nahkala, Sam Phillips, Emily Waring, Kayla Walls, Alyssa Iverson, Jade Gerlitz, and Ji Yeow Law. All of you helped make graduate school feel more like a team sport.

Thank you to Isaac Rempe, Adam Buseman, Carolyn White, Kyle Werning, and Megan Lukas for their laboratory skills and expertise. Thank you to Kris Bell, our fearless leader. Thank you to the Sukup and Elings Hall custodial staff, especially Ahmed Cheklal. Thank you to the Katherine and Harold Guy family for their monetary support of my graduate education.

Thank you to my family: Mary Kay, Craig, Elizabeth, and Emily. Thank you to the Cook Family: Matthew, Anne, Dave, and Nathan. Thank you to the Kraus Family, especially Karla, Ralph, Nick, and Matt, for their thoughtful questions about my work and sincere interest in its impact.

It takes a village to complete a thesis; if you are reading this, you are part of my village.

ABSTRACT

Woodchip bioreactors have been extensively studied to understand factors that influence denitrification in order to achieve nutrient reduction goals from nonpoint sources of pollution. Further study will allow us to better understand the usable lifetime of these systems and the microbial community that drives denitrification, as well as the impact of hydraulic retention time (HRT) on these factors. We performed a battery of physical and chemical tests on woodchips excavated from replicate woodchip bioreactors following two years of continuous operation. Though we did not observe preferential woodchip consumption based on particle size, we observed spatial patterns of woodchip degradation that may be related to nitrate load. We also extracted DNA from woodchips sampled during two years for 16S rRNA amplicon sequencing and qPCR measurement of various denitrification genes. We observed patterns in the microbial community that may be related to the differences in substrate composition between HRTs. We did not observe statistical differences in qPCR results between HRTs, which may imply that detection of denitrifying genes may not be the most practical method of predicting system performance at the field or pilot scale. Replacing a portion of the bioreactor fill material may be an attractive way to minimize woodchip bioreactor management costs.

CHAPTER 1. GENERAL INTRODUCTION

The Nitrogen Cycle

Nitrogen easily changes its oxidation state through a variety of processes in what is known as the nitrogen cycle. There are three possible fates of fixed nitrogen once it has entered the nitrogen cycle: 1. Export via air or water, 2. Loss from the fixed N pool by conversion to molecular nitrogen (N_2), or 3. Storage and transformation within the watershed. Stored forms of nitrogen may later be exported or transformed into N_2. Exported nitrogen may impact the downstream ecosystem and stored nitrogen may accumulate to problematic levels, i.e., concentrations greater than 5 mg L-1 nitrate-as-nitrogen (NO_3^-- N) may cause excessive algae growth. Elevated amounts of fixed nitrogen in biogeochemical cycles lead to increased nitrogen export. The amount of fixed nitrogen (nitrogen in forms other than N_2) entering biogeochemical cycles has increased in since the 20th century due to anthropogenic activities, including agricultural intensification and urban sprawl as a result of population growth (Vitousek et al., 1997).

More specifically, nitrous oxide emissions from human activities make up about 9% of human-driven global warming and are the biggest cause of destruction of beneficial atmospheric ozone (Bakken et al., 2012). The Gulf of Mexico, the Great Lakes, and the Chesapeake Bay are three regions in the United States that have been impacted by the ecological effects of nitrogen excess. Specifically, deposited nitrogen triggers an algae bloom and successive algae die-off event. Aglae biomass is decomposed by other microorganisms, and these decomposers consume dissolved oxygen which leads to hypoxia. As an example, specifically within the Chesapeake Bay, submerged bay grasses, blue crab, oysters, and fish species have been harmed by nutrient-induced hypoxia.

Microbial Nitrogen Transformations

Microorganisms that are capable of performing denitrification are referred to as denitrifying microorganisms or, simply, denitrifiers. Plants and animals only remove nitrate temporarily whereas microbial denitrification is required for the permanent removal of nitrate from a system. Denitrification is a stepwise, enzymatic process by which nitrate (NO_3^-) is reduced to N_2, proceeding through the intermediates nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) (Averill & Tiedje, 1982; Hollocher, 1983).

Microorganisms are primarily responsible for the nitrogen transformations that occur within the nitrogen cycle. The key forms of bioavailable nitrogen are ammonium and nitrate, the former of which is an oxidizable cation while the latter is a reduceable anion. Nitrogen cycling within the environment depends on microorganisms that perform a variety of metabolic functions to change the oxidation state of nitrogen, or, the number of electrons association with the nitrogen atom (Jeannotte, 2014; Kraft et al., 2011; Petersen et al., 2012; Reisinger et al., 2016) In native, non-engineered environments, there is evidence that many factors influence the structure of denitrifying microbial communities which provides further grounds for the study of microorganisms inhabiting denitrifying bioreactors. Levels of bioavailable nitrogen are modulated by microbial activity that changes the oxidation state of molecular nitrogen. Microbes fix nitrogen using an assimilatory or a dissimilatory pathway. In assimilatory nitrate reduction, the key enzymes are in the cytoplasm and used to build biomass. In dissimilatory nitrate reduction, the key enzymes are membrane-bound and used for respiration.

Microbial denitrification favors anoxic conditions and can occur with either carbon, iron, or sulfur serving as the final electron acceptor (Collins et al., 2010). When organic carbon serves as the electron donor for denitrification, the process is known as heterotrophic denitrification. Thermodynamically, organic carbon tends to be oxidized preferentially by the e^- acceptor that

yields the most energy to bacteria (oxygen most favorablly, then nitrate). Nitrate reduction in carbon-limited aquifers favors denitrification over dissimilatory nitrate reduction to ammonia (DNRA). Denitrification can be identified as a nitrate removal process through isotope fractionation, specifically the measurement of $^{18}O/^{16}O$, which is preferred over the measurement of $^{15}N/^{14}N$. Carbon dioxide is produced through the oxidation of organic carbon and bicarbonate (HCO_3^-) may be produced from CO_2 or directly from heterotrophic denitrification (Korom, 1992).

Denitrification is a four-step process and each step is enzyme-catalyzed. These genes can be targets for identifying community members that are important in the denitrification process, but many of the genes are also present in non-denitrifiers. The most common genetic marker that is studied is nitrite reductase (*nir*) (Wallenstein et al., 2006). Denitrification is a phylogenetically diverse process, so rRNA genes are not typically targets for community analysis; therefore, functional genes are more commonly targeted.

There are several key genes involved in the metabolism and assimilation of nitrogen in microbes, including *nirS*, *nirK*, *nosZ*, and *nrfA*. Several experimental designs are described that use these nitrate respiration genes as markers for study. Microarrays and qPCR have been used to identify denitrifiers in a community. However, the diversity of species and genes that can be involved in denitrification creates a challenge for these types of studies in that it is impossible to generate primers for all possible denitrification genes (Kraft et al., 2011). Transcription of denitrification genes is dependent on transcription factors Anr, Dnr, NarXL, and NirQ; these factors are modulated by environmental levels of molecular oxygen, phosphate, and nitrate (Arat et al., 2015).

When denitrification is incomplete, nitrous oxide, a potent greenhouse gas, is produced. The enzyme nitrous oxide reductase (*nosZ*) is responsible for the conversion of nitrous oxide to molecular nitrogen, and it is thought that when microorganisms expressing this enzyme are less abundant, nitrous oxide emissions are higher. Environmental pH is also thought to affect the synthesis of nitrous oxide reductase and therefore would impact nitrous oxide emissions (Bakken et al., 2012).

Studying the Nitrogen Cycle

There are several other pathways within the nitrogen cycle, but denitrification has been implicated as the major fate of nitrate within denitrifying woodchip bioreactors. Heterotrophic denitrification is hypothesized to be the primary method of nitrate removal in bioreactors. This has been confirmed using label N species in laboratory settings. In a bioreactor, the rate of nitrate removal is comparable to that within the first 15 cm of an organic rich field soil. Denitrifiers prefer ^{14}N vs ^{15}N, and ^{15}N is often enriched in bioreactors. N immobilization does not prefer one isotope over the other (Schipper et al., 2010).

Community analysis methods that involve PCR can be biased; for example, there is inherent bias involved in primer design (Wallenstein et al., 2006). Denitrification genes can also be measured directly with gene probes, with the same biases as those in primer design for PCR. mRNA analysis allows us to determine if an organism is expressing denitrifying genes, as opposed to just examining whether an organism has the potential to perform denitrification when examining genomes. Population dynamics of denitrifier communities is not well understood. We also do not understand whether populations that differ in structure also differ in function. Another area that needs to be explored is the identification of new PCR primer sequences by finding previously unknown denitrification gene sequences (Wallenstein et al., 2006).

Denitrifying Bioreactors

Briefly, denitrifying bioreactors are an edge-of-field practice that promote nitrate-nitrogen removal from subsurface drainage by intercepting and impounding the water and providing a substrate for denitrifying microorganisms. These systems have been widely studied with respect to nitrate-nitrogen removal potential and multiple literature reviews have been synthesized (Addy et al., 2016). Questions that warrant further study involve the design lifetime of the cellulosic fill material and the synergistic interactions of the microbial community that performs both substrate degradation and denitrification.

Bioreactor Physical Parameters/Engineering Design

Denitrifying bioreactor performance is evaluated in two ways: on a mass removal basis (Warneke et al., 2011) or on a concentration reduction basis. Reporting denitrification performance on a mass basis per volume of bioreactor and unit time facilitates comparison between various bioreactor designs. However, it is necessary to compare final effluent concentrations to a standard such as the United States Environmental Protection Agency's drinking water standard of 10 mg L^{-1} NO_3^- -N (Office of Ground Water and Drinking Water, 2016). Current design standards require a 30% annual reduction in nitrate-nitrogen load from the effluent flow of the bioreactor and also specifies treatment of volumes between 10-20% of peak flow events (Ikenberry et al., 2014). Other reviews have addressed the engineering design of these systems (Addy et al., 2016; Christianson et al., 2012).

Briefly, the volume of the bioreactor can be determined by selecting a volume that allows for a hydraulic retention time (HRT) between 6 and 8 hours (USDA, 2011). However, during storm events, HRT will decrease. Although nitrate mass removal in bioreactors is decreased by as much as 50% during storms, various problems can arise as bioreactor volumes

increase (Christianson et al., 2011; Hassanpour et al., 2017). Additional factors influencing nitrate removal have been determined to be temperature, dissolved oxygen, age of the bioreactor, and influent nitrate concentration (Christianson et al., 2012), with HRT causing arguably the largest influence on nitrate removal.

Implications of Biological Design on Performance

Denitrification

Despite the standardized denitrifying bioreactor design, variable denitrification rates have been observed in denitrifying bioreactors. For example, on a percent basis, removals have ranged from as low as 7% up to 100% in lab-, pilot-, and field-scale bioreactors (Bell, 2013; Chun et al., 2010; Hoover et al., 2016; Hua et al., 2016; Jaynes et al., 2016; Martin et al., 2019; Woli et al., 2010) Some of the variability comes from different operating conditions such as temperature, dissolved oxygen, HRT, etc. In terms of a removal rate, reduction as little as 0.38 g NO_3-N m^{-3} d^{-1} in a field-scale bioreactor study up to a rate of 121 g NO_3-N m^{-3} d^{-1} in a lab-scale bioreactor operated at 15°C with a sodium acetate additive have been observed (Christianson et al., 2011; Jang et al., 2019). The highest removal rate observed from a traditional woodchip bioreactor is a single day removal rate of 116 g NO_3-N m^{-3} d^{-1} or a range of monthly averages of 23-44 g NO_3-N m^{-3} d^{-1} for a field-scale bioreactor during its first year of operation (Addy et al., 2016). In order to minimize variation and improve bioreactor consistency, it is important to understand the biological components involved in denitrifying bioreactors in order to create designs that stimulate the denitrifying activity of the microorganisms. Specifically, nitrate and carbon availability, the presence of oxygen, and pH are all abiotic factors involved in influencing biotic denitrification activity (Wallenstein et al., 2006).

Microorganisms that can carry out metabolic activity under both aerobic and anaerobic conditions are called facultative anaerobes. To be capable of such metabolic flexibility, these microorganisms must have a system in place to determine when oxygen is no longer available. In molecular genetics, a regulon is a group of genes that is under regulatory control by the same genetic element. This feedback system ensures optimal ATP synthesis rates by using energetically favorable terminal electron acceptors. In *Escherichia coli*, this system has been determined to be the fumarate nitrate reductase (FNR) regulon (Unden & Schirawski, 1997). The FNR regulon is activated in the absence of oxygen, initiating the transport of nitrate into the cell. Several transporters are involved in this process across bacteria and archaea, and the detection of their encoding genes can be used as evidence that denitrifying microorganisms might be present (Kraft et al., 2011; Kuypers et al., 2018).

It is hypothesized that communities of microorganisms work together to carry out the process of denitrification, especially because some microorganisms do not possess all the enzymes required to complete the entire process (Kuypers et al., 2018). Denitrifying communities can be thought of as possessing highly ordered divisions of labor that allow each member to have their metabolic needs met, a type of interaction known as syntrophy (de Roy et al., 2014). Like communities of macrofauna, microorganisms form complex and dynamic communities that respond to changes in their environment (Gonze et al., 2018).

Several genes of interests that are associated with nitrate reduction have been identified as indicators of denitrification, including membrane-bound nitrate reductase (*narG*), nitrite reductase (*nirS, nirK*), nitric oxide reductase (*nor*), and nitrous oxide reductase (*nosZ*); however, *nirS, nirK*, and *nosZ* are most often used in studies (Kraft et al., 2011). *nosZ* gene copy numbers

have been used as proxy for denitrification potential and *nirS* gene copy numbers have been positively correlated with denitrification rate (Fatehi-Pouladi et al., 2019; Ilhan et al., 2011).

Literature addressing the microbial community composition of denitrifying bioreactors is limited. A two-year study done in Illinois, USA sampled woodchip and water samples to track the microbial community composition of pilot-scale bioreactors and their response to environmental change. The study found that the community varied with respect to both season and bioreactor depth. Saturation levels could vary with bioreactor depth, therefore the community variation observed could be correlated to woodchip moisture content. Additionally, the study found that samples collected 125 days apart were less similar than samples collected 300 days apart, showing that this bioreactor community cycles on a roughly annual basis (Porter et al., 2015). Others have reported 125-day cycles for denitrifying bioreactors, with community structure correlated with temperature, inlet nitrate concentration, pH, moisture content, and depth (Andrus, 2011). Another pilot-scale study done by the same group investigated whether denitrifying bioreactor microbial communities were similar to those found in soil or wetland environments (Hathaway et al., 2015). The results showed that the bioreactor, soil, and both constructed and natural wetlands contained distinct microbial communities. This suggests that other factors dictate community structure besides the desired function of denitrification.

Other studies of bioreactor microbial communities have been done on the bench-scale. One study compared the effect of bioreactor water level on microbial community structure (Hathaway et al., 2017). This study showed that constantly changing water levels in a lab-scale reactor resulted in the formation of a distinct community compared to a reactor that had a constant water level. Although both reactors showed similar levels of nitrate removal, the disturbed reactor inadvertently had a longer HRT than the control reactor, which may have

confounded these results. A second study found that transcript levels of *nirK* were elevated in denitrifying microcosms compared to non-denitrifying microcosms and that Pseudomonas spp., Polaromonas spp., and Cellumonas spp. were identified as important bacteria for denitrification at low temperatures (Jang et al., 2019). The denitrification mechanisms present along the height of an up-flow bench reactor have also been investigated. The experiment showed that carbon-degrading, denitrifying, and fermentative microorganisms were important for bioreactor performance (Zhao et al., 2018). Additionally, the abundance of each type of microorganism was determined by resource availability, which varied along the height of the reactor. For example, organisms performing aerobic carbon degradation were found at the bottom of the reactor, where influent flow caused higher levels of dissolved oxygen. The carbon-degrading and fermentative microorganisms provide carbon to the denitrifiers to remove nitrate, demonstrating a division of labor among the community.

In addition to protein-encoding (functional) genes, other DNA regions that are of interest in studying microbial communities are rDNA (16S rRNA genes) and internal transcribed spacer (ITS) regions between rDNA units (Johnston-Monje & Lopez Mejia, 2020). Automated ribosomal intergenic spacer analysis (ARISA) and fungal automated ribosomal intergenic spacer analysis (FARISA) are techniques used in ITS gene amplification (Hathaway et al., 2017). Lastly, terminal restriction fragment length polymorphisms (TRFLPs) of functional genes are another community fingerprinting technique that has been used to study microbial community composition in denitrifying bioreactors (Porter et al., 2015). Selective primers are used for amplifying all target genes that are specific for bacterial or fungal species, minimizing contamination from chloroplast, mitochondrial, or plant rDNA (Thijs et al., 2017).

Substrate Availability

In bioreactors, denitrifying microbes compete for nitrate with organisms that perform other nitrogen-using processes such as the production of ammonium. One nitrogen-using process, the production of nitrous oxide gas, will be addressed later in this review. Although it is widely thought that anaerobic conditions are necessary for denitrification to occur within denitrifying bioreactors, it has also been shown that some organisms primarily responsible for the breakdown of cellulose and lignin require oxygen (Tavzes et al., 2001; Brown & Chang, 2014). Therefore, the ideal oxygen conditions for denitrification and substrate breakdown may be opposed, which has potential negative implications for the synergy of substrate availability and denitrification.

Most studies of denitrifying bioreactor substrate have focused on carbon:nitrogen ratio and carbon quality. C:N ratio has been shown to decrease in proportion to nitrate load (Ghane et al., 2018; Moorman et al., 2010). In addition, carbon quality decreases over time as sugars are preferentially consumed over lignin, which is more recalcitrant to microbial degradation based on its chemical structure (van der Lelie et al., 2012). Other work has shown that cellulose and hemicellulose can be converted to carbon dioxide and methane under anaerobic conditions, but anaerobic breakdown of lignin has not been demonstrated (Ko et al., 2009).

Recent studies of denitrifying bioreactors have investigated the effects of cyclical aerobic and anaerobic periods (Maxwell et al., 2019). It is thought that the aerobic periods stimulate the release of labile C from the woodchips. This could be because it is hypothesized that lignin degradation is performed by aerobic, heterotrophic white-rot fungi (Toljander et al., 2006). White-rot fungi are well-adapted to perform lignin degradation due to the extra-cellular enzymes they produce, which are necessary because lignin cannot be endocytosed (Dashtban et al., 2010). There is also evidence that bacterial and fungal species work together to breakdown

lignocellulosic materials, where bacteria consume the products of fungal wood degradation such that the lignin-degrading enzymes are not hindered by feedback inhibition (van der Lelie et al., 2012). However, work studying lignin and cellulose metabolism genes and microbial community members is extremely limited. There is a need to study both denitrifying gene detection and microbial community composition via 16S rRNA gene analysis. Further study is needed on denitrifying bioreactor communities in order to better engineer these systems for optimal nitrogen removal. Additionally, a better understanding of lignin degradation could lead to increased efficiency of substrate utilization or novel uses for spent bioreactor fill.

Bottle sedge, barley straw, and pine woodchips were used as the electron donor in lab-scale bioreactors operating for 270 days. 16S rRNA sequencing was used for microbial community profiling. There appears to be a relationship between nitrate removal rates and labile carbon content in the denitrification substrate. Substrate type affected the genetic potential for denitrification and also controlled the potential for the two nitrate reduction pathways differently as reflected in the nitrate removal rates (Hellman et al., 2020).

New Zealand soils are different from soils in the midwestern USA and have flashier drainage flows; therefore, there is a need to evaluate the effectiveness of woodchip bioreactors in this setting. Higher removal efficiencies at longer HRTs have been observed in New Zealand; and it is hypothesized that the longer HRTs provided more opportunity for microorganisms to perform denitrification. Greater dissolved organic carbon discharge during 2017 supported the idea that there was greater electron donor availability during the first year of operation. Hydrogen sulfide and methane production were observed during periods with very long HRTs. They concluded that nitrate removal efficiencies of greater than 50% were not achieved without generating hydrogen sulfide or methane production but hypothesized that removal efficiencies

greater than 50% could be achieved with an amendment of available carbon during periods when flow and N load peaks are concomitant (Rivas et al., 2020).

Woodchip bioreactors modified for wet detention ponds may provide nitrate removal in suburban/urban settings. They observed consistent nitrate and chlorophyll-a levels; with higher estimates of observed nitrate removal rate compared to those observed in agricultural settings. Wet detention ponds are a stormwater control method (SCM) that are intended to reduce peak flows and remove particulate pollutants. There were no statistical differences in nutrient concentrations when comparing samples based on distance from the bioreactor (Pluer et al., 2018).

As a result of this literature review, further study of both the mechanisms of substrate availability and the composition and function of the microbial community of woodchip bioreactors is warranted. The goals of the two studies presented here are to understand the impact of HRT on indicators of bioreactor aging, the microbial community as detected through 16S rRNA amplicon sequencing, and the detection of functional denitrification genes through qPCR. Our specific research goals include: 1) Understanding the impact of HRT on the chemical and physical properties of woodchips; 2) Determining the impact of HRT on the microbial community, measured through 16S rRNA amplicon sequencing; 3) Determining the impact of HRT on the detection of *nirS*, *nirK*, *nosZ*I, and *nosZ*II functional genes.

References

Addy, K., Gold, A. J., Christianson, L. E., David, M. B., Schipper, L. A., & Ratigan, N. A. (2016). Denitrifying Bioreactors for Nitrate Removal: A Meta-Analysis. Journal of Environment Quality, 45(3), 873. https://doi.org/10.2134/jeq2015.07.0399

Andrus, J. M. (2011). Microbial community analysis for denitrifying biofilters. Dissertation Abstracts International. Vol. 73, no. 01, suppl. B, 235 p. 2011. Retrieved from https://login.proxy.lib.duke.edu/login?url=https://search.proquest.com/docview/1093459776?accountid=10598%0Ahttp://pm6mt7vg3j.search.serialssolutions.com?ctx_ver=Z39.88-2004&ctx_enc=info:ofi/enc:UTF-8&rfr_id=info:sid/Aquatic+Science+%26+Fisheries+Abstract

Arat, S., Bullerjahn, G. S., & Laubenbacher, R. (2015). A network biology approach to denitrification in Pseudomonas aeruginosa. PLoS ONE. https://doi.org/10.1371/journal.pone.0118235

Averill, B. A., & Tiedje, J. M. (1982). The chemical mechanism of microbioal denitrification. FEBS Letters, 138(1), 8–12. https://doi.org/10.1016/0014-5793(82)80383-9

Bakken, L. R., Bergaust, L., Liu, B., & Frostegård, Å. (2012). Regulation of denitrification at the cellular level: A clue to the understanding of N2O emissions from soils. Philosophical Transactions of the Royal Society B: Biological Sciences. https://doi.org/10.1098/rstb.2011.0321

Bell, N. (2013). Characterizing Transient and Seasonal Responses of Bioreactors for Subsurface Drains, Master's Thesis.

Christianson, L., Bhandari, A., Helmers, M., Kult, K., Sutphin, T., & Wolf, R. (2012). Performance Evaluation of Four Field-Scale Agricultural Drainage Denitrification Bioreactors in Iowa. Transactions of the ASABE, 55(6). Retrieved from https://lib.dr.iastate.edu/abe_eng_pubs/307

Christianson, L. E., Bhandari, A., & Helmers, M. J. (2012). A practice-oriented review of woodchip bioreactors for subsurface agricultural drainage. Applied Engineering in Agriculture.

Christianson, L. E., Collick, A. S., Bryant, R. B., Rosen, T., Bock, E. M., Allen, A. L., … Easton, Z. M. (2017). Enhanced Denitrification Bioreactors Hold Promise for Mid-Atlantic Ditch Drainage. Agricultural & Environmental Letters, 2(1), 170032. https://doi.org/10.2134/ael2017.09.0032

Christianson, Laura E, Bhandari, A., Asce, M., & Helmers, M. J. (2011). Pilot-Scale Evaluation of Denitrification Drainage Bioreactors: Reactor Geometry and Performance. https://doi.org/10.1061/(ASCE)EE.1943-7870.0000316

Chun, J. A., Cooke, R. A., Eheart, J. W., & Cho, J. (2010). Estimation of flow and transport parameters for woodchip-based bioreactors: II. field-scale bioreactor. Biosystems Engineering. https://doi.org/10.1016/j.biosystemseng.2009.09.018

Collins, K. A., Lawrence, T. J., Stander, E. K., Jontos, R. J., Kaushal, S. S., Newcomer, T. A., Cole Ekberg, M. L. (2010). Opportunities and challenges for managing nitrogen in urban stormwater: A review and synthesis. Ecological Engineering, 36(11), 1507–1519. https://doi.org/10.1016/j.ecoleng.2010.03.015

Dashtban, M., Schraft, H., Syed, T. A., & Qin, W. (2010). Fungal biodegradation and enzymatic modification of lignin. International Journal of Biochemistry and Molecular Biology. e-Century Publishing Corporation.

Davis, M. P., Martin, E. A., Moorman, T. B., Isenhart, T. M., & Soupir, M. L. (2019). Nitrous oxide and methane production from denitrifying woodchip bioreactors at three hydraulic residence times. Journal of Environmental Management, 242, 290–297. https://doi.org/10.1016/j.jenvman.2019.04.055

De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T., & Boon, N. (2014). Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. Environmental Microbiology. https://doi.org/10.1111/1462-2920.12343

Easton, Z. M., Bock, E., & Stephenson, K. (2019). Feasibility of Using Woodchip Bioreactors to Treat Legacy Nitrogen to Meet Chesapeake Bay Water Quality Goals. Environmental Science and Technology, 53(21), 12291–12299. https://doi.org/10.1021/acs.est.9b04919

Fatehi-Pouladi, S., Anderson, B. C., Wootton, B., Button, M., Bissegger, S., Rozema, L., & Weber, K. P. (2019). Interstitial water microbial communities as an indicator of microbial denitrifying capacity in wood-chip bioreactors. Science of the Total Environment, 655, 720–729. https://doi.org/10.1016/j.scitotenv.2018.11.278

Feyereisen, G. W., Moorman, T. B., Christianson, L. E., Venterea, R. T., Coulter, J. A., & Tschirner, U. W. (2016). Performance of Agricultural Residue Media in Laboratory Denitrifying Bioreactors at Low Temperatures. Journal of Environmental Quality, 45(3), 779–787. https://doi.org/10.2134/jeq2015.07.0407

Ghane, E., Feyereisen, G. W., Rosen, C. J., & Tschirner, U. W. (2018). Carbon quality of four-year-old woodchips in a denitrification bed treating agricultural drainage water. Transactions of the ASABE, 61(3), 995–1000. https://doi.org/10.13031/trans.12642

Gonze, D., Coyte, K. Z., Lahti, L., & Faust, K. (2018). Microbial communities as dynamical systems. Current Opinion in Microbiology. https://doi.org/10.1016/j.mib.2018.07.004

Graf, D. R. H., Jones, C. M., & Hallin, S. (2014). Intergenomic Comparisons Highlight Modularity of the Denitrification Pathway and Underpin the Importance of Community Structure for N2O Emissions. PLoS ONE, 9(12), e114118. https://doi.org/10.1371/journal.pone.0114118

Grießmeier, V., Bremges, A., McHardy, A. C., & Gescher, J. (2017). Investigation of different nitrogen reduction routes and their key microbial players in wood chip-driven denitrification beds. Scientific Reports, 7(1). https://doi.org/10.1038/s41598-017-17312-2

Hassanpour, B., Giri, S., Pluer, W. T., Steenhuis, T. S., & Geohring, L. D. (2017). Seasonal performance of denitrifying bioreactors in the Northeastern United States: Field trials. Journal of Environmental Management. https://doi.org/10.1016/j.jenvman.2017.06.054

Hathaway, S. K., Bartolerio, N. A., Rodríguez, L. F., Kent, A. D., & Zilles, J. L. (2017). Denitrifying Bioreactors Resist Disturbance from Fluctuating Water Levels. Frontiers in Environmental Science, 5. https://doi.org/10.3389/fenvs.2017.00035

Hathaway, S. K., Porter, M. D., Rodríguez, L. F., Kent, A. D., & Zilles, J. L. (2015). Impact of the contemporary environment on denitrifying bacterial communities. Ecological Engineering. https://doi.org/10.1016/j.ecoleng.2015.05.005

Healy, M. G., Ibrahim, T. G., Lanigan, G. J., Serrenho, A. J., & Fenton, O. (2012). Nitrate removal rate, efficiency and pollution swapping potential of different organic carbon media in laboratory denitrification bioreactors. Ecological Engineering, 40, 198–209. https://doi.org/10.1016/j.ecoleng.2011.12.010

Hellman, M., Hubalek, V., Juhanson, J., Almstrand, R., Peura, S., & Hallin, S. (2020). Substrate type determines microbial activity and community composition in bioreactors for nitrate removal by denitrification at low temperature. Science of the Total Environment, 143023. https://doi.org/10.1016/j.scitotenv.2020.143023

Hollocher, T. C. (1983). The pathway of nitrogen and reductive enzymes of denitrification. Antonie van Leeuwenhoek, 48(6), 531–544. https://doi.org/10.1007/BF00399539

Hoover, N. L., Soupir, M. L., VanDePol, R. D., Goode, T. R., & Law, J. Y. (2017). Pilot-scale denitrification bioreactors for replicated field research. Applied Engineering in Agriculture. https://doi.org/10.13031/aea.11736

Hoover, Natasha L., Bhandari, A., Soupir, M. L., & Moorman, T. B. (2016). Woodchip Denitrification Bioreactors: Impact of Temperature and Hydraulic Retention Time on Nitrate Removal. Journal of Environment Quality. https://doi.org/10.2134/jeq2015.03.0161

Hua, G., Salo, M. W., Schmit, C. G., & Hay, C. H. (2016). Nitrate and phosphate removal from agricultural subsurface drainage using laboratory woodchip bioreactors and recycled steel byproduct filters. https://doi.org/10.1016/j.watres.2016.06.022

Ikenberry, C. D., Soupir, M. L., Schilling, K. E., Jones, C. S., & Seeman, A. (2014). Nitrate-Nitrogen Export: Magnitude and Patterns from Drainage Districts to Downstream River Basins. Journal of Environment Quality. https://doi.org/10.2134/jeq2014.05.0242

Ilhan, Z. E., Ong, S. K., & Moorman, T. B. (2011). Dissipation of Atrazine, Enrofloxacin, and Sulfamethazine in Wood Chip Bioreactors and Impact on Denitrification. Journal of Environmental Quality, 40(6), 1816–1823. https://doi.org/10.2134/jeq2011.0082

Jang, J., Anderson, E. L., Venterea, R. T., Sadowsky, M. J., Rosen, C. J., Feyereisen, G. W., & Ishii, S. (2019). Denitrifying bacteria active in woodchip bioreactors at low-temperature conditions. Frontiers in Microbiology, 10(APR), 635. https://doi.org/10.3389/fmicb.2019.00635

Jaynes, D. B., Moorman, T. B., Parkin, T. B., & Kaspar, T. C. (2016). Simulating Woodchip Bioreactor Performance Using a Dual-Porosity Model. Journal of Environmental Quality, 45(3), 830–838. https://doi.org/10.2134/jeq2015.07.0342

Jeannotte, R. (2014). METABOLIC PATHWAYS | Nitrogen Metabolism. Encyclopedia of Food Microbiology, 544–560. https://doi.org/10.1016/B978-0-12-384730-0.00199-3

Johnston-Monje, D., & Lopez Mejia, J. (2020). Botanical microbiomes on the cheap: Inexpensive molecular fingerprinting methods to study plant-associated communities of bacteria and fungi. Applications in Plant Sciences, 8(4). https://doi.org/10.1002/aps3.11334

Ko, J. J., Shimizu, Y., Ikeda, K., Kim, S. K., Park, C. H., & Matsui, S. (2009). Biodegradation of high molecular weight lignin under sulfate reducing conditions: Lignin degradability and degradation by-products. Bioresource Technology, 100(4), 1622–1627. https://doi.org/10.1016/j.biortech.2008.09.029

Korom, S. F. (1992). Natural denitrification in the saturated zone: A review. Water Resources Research, 28(6), 1657–1668. https://doi.org/10.1029/92WR00252

Kraft, B., Strous, M., & Tegetmeyer, H. E. (2011). Microbial nitrate respiration - Genes, enzymes and environmental distribution. Journal of Biotechnology. https://doi.org/10.1016/j.jbiotec.2010.12.025

Kuypers, M. M. M., Marchant, H. K., & Kartal, B. (2018). The microbial nitrogen-cycling network. Nature Reviews Microbiology, 16(5), 263–276. https://doi.org/10.1038/nrmicro.2018.9

Martin, E. A., Davis, M. P., Moorman, T. B., Isenhart, T. M., & Soupir, M. L. (2019). Impact of hydraulic residence time on nitrate removal in pilot-scale woodchip bioreactors. Journal of Environmental Management, 237(3), 424–432. https://doi.org/10.1016/j.jenvman.2019.01.025

Maxwell, B. M., Birgand, F., Schipper, L. A., Christianson, L. E., Tian, S., Helmers, M. J., Youssef, M. A. (2019). Drying-Rewetting Cycles Affect Nitrate Removal Rates in Woodchip Bioreactors. Journal of Environmental Quality, 48(1), 93–101. https://doi.org/10.2134/jeq2018.05.0199

Moorman, T. B., Parkin, T. B., Kaspar, T. C., & Jaynes, D. B. (2010). Denitrification activity, wood loss, and N2O emissions over 9 years from a wood chip bioreactor. Ecological Engineering, 36(11), 1567–1574. https://doi.org/10.1016/j.ecoleng.2010.03.012

Office of Ground Water and Drinking Water. (2016). National Primary Drinking Water Regulations. Retrieved from https://www.epa.gov/sites/production/files/2016-06/documents/npwdr_complete_table.pdf

Petersen, D. G., Blazewicz, S. J., Firestone, M., Herman, D. J., Turetsky, M., & Waldrop, M. (2012). Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates across a vegetation gradient in Alaska. Environmental Microbiology, 14(4), 993–1008. https://doi.org/10.1111/j.1462-2920.2011.02679.x

Pluer, W. T., Hoffman, R., & Walter, M. T. (2018). Reducing Stormwater Nitrogen with Denitrifying Bioreactors: Florida Case Study. Journal of Sustainable Water in the Built Environment, 4(4), 06018002. https://doi.org/10.1061/jswbay.0000867

Porter, M. D., Andrus, J. M., Bartolerio, N. A., Rodriguez, L. F., Zhang, Y., Zilles, J. L., & Kent, A. D. (2015). Seasonal Patterns in Microbial Community Composition in Denitrifying Bioreactors Treating Subsurface Agricultural Drainage. Microbial Ecology, 70(3), 710–723. https://doi.org/10.1007/s00248-015-0605-8

Reisinger, A. J., Groffman, P. M., & Rosi-Marshall, E. J. (2016). Nitrogen-cycling process rates across urban ecosystems. FEMS Microbiology Ecology, 92(12), fiw198. https://doi.org/10.1093/femsec/fiw198

Rivas, A., Barkle, G., Stenger, R., Moorhead, B., & Clague, J. (2020). Nitrate removal and secondary effects of a woodchip bioreactor for the treatment of subsurface drainage with dynamic flows under pastoral agriculture. Ecological Engineering, 148. https://doi.org/10.1016/j.ecoleng.2020.105786

Robertson, W. D. (2010). Nitrate removal rates in woodchip media of varying age. Ecological Engineering, 36(11), 1581–1587. https://doi.org/10.1016/j.ecoleng.2010.01.008

Schipper, L. A., Robertson, W. D., Gold, A. J., Jaynes, D. B., & Cameron, S. C. (2010). Denitrifying bioreactors—An approach for reducing nitrate loads to receiving waters. Ecological Engineering, 36(11), 1532–1543. https://doi.org/10.1016/J.ECOLENG.2010.04.008

Thijs, S., De Beeck, M. O., Beckers, B., Truyens, S., Stevens, V., Van Hamme, J. D., … Vangronsveld, J. (2017). Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys. Frontiers in Microbiology, 8(MAR). https://doi.org/10.3389/fmicb.2017.00494

Toljander, Y. K., Lindahl, B. D., Holmer, L., & Högberg, N. O. S. (2006). Environmental fluctuations facilitate species co-existence and increase decomposition in communities of wood decay fungi. Oecologia, 148(4), 625–631. https://doi.org/10.1007/s00442-006-0406-3

Unden, G., & Schirawski, J. (1997). The oxygen-responsive transcriptional regulator FNR of Escherichia coli: the search for signals and reactions. Molecular Microbiology, 25(2), 205–210. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9282732

USDA. (2011). Natural Resources Conservation Service Conservation Practice Standard. Natural Resources Conservation Service Conservation Practice Standard, (May), 1–3.

van der Lelie, D., Taghavi, S., McCorkle, S. M., Li, L. L., Malfatti, S. A., Monteleone, D., Tringe, S. G. (2012). The metagenome of an anaerobic microbial community decomposing poplar wood chips. PLoS ONE, 7(5). https://doi.org/10.1371/journal.pone.0036740

Verbaendert, I., Hoefman, S., Boeckx, P., Boon, N., & De Vos, P. (2014). Primers for overlooked nirK, qnorB, and nosZ genes of thermophilic Gram-positive denitrifiers. FEMS Microbiology Ecology, 89(1), 162–180. https://doi.org/10.1111/1574-6941.12346

Vitousek, P. M., Mooney, H. A., Lubchenco, J., & Melillo, J. M. (1997). Human domination of Earth's ecosystems. Science, 277(5325), 494–499. https://doi.org/10.1126/science.277.5325.494

Wallenstein, M. D., Myrold, D. D., Firestone, M., & Voytek, M. (2006). Environmental controls on denitrifying communities and denitrification rates: Insights from molecular methods. Ecological Applications. https://doi.org/10.1890/1051-0761(2006)016[2143:ECODCA]2.0.CO;2

Warneke, S., Schipper, L. A., Bruesewitz, D. A., McDonald, I., & Cameron, S. (2011). Rates, controls and potential adverse effects of nitrate removal in a denitrification bed. Ecological Engineering. https://doi.org/10.1016/j.ecoleng.2010.12.006

Warneke, S., Schipper, L. A., Matiasek, M. G., Scow, K. M., Cameron, S., Bruesewitz, D. A., & McDonald, I. R. (2011). Nitrate removal, communities of denitrifiers and adverse effects in different carbon substrates for use in denitrification beds. Water Research, 45(17), 5463–5475. https://doi.org/10.1016/j.watres.2011.08.007

Woli, K. P., David, M. B., Cooke, R. A., McIsaac, G. F., & Mitchell, C. A. (2010). Nitrogen balance in and export from agricultural fields associated with controlled drainage systems and denitrifying bioreactors. Ecological Engineering. https://doi.org/10.1016/j.ecoleng.2010.04.024

Zhao, J., Feng, C., Tong, S., Chen, N., Dong, S., Peng, T., & Jin, S. (2018). Denitrification behavior and microbial community spatial distribution inside woodchip-based solid-phase denitrification (W-SPD) bioreactor for nitrate-contaminated water treatment. Bioresource Technology. https://doi.org/10.1016/j.biortech.2017.11.011

CHAPTER 2. IMPACT OF FLOW ON WOODCHIP PROPERTIES AND SUBSIDENCE IN DENITRIFYING BIOREACTORS

Accepted by Agrosystems, Geosciences, & Environment, October 2020

Available online 15 March 2021

A. Schaefer[a], K. Werning[a], Natasha Hoover[a], Ulrike Tschirner[b], Gary Feyereisen[c], Thomas B. Moorman[d] Adina C. Howe[e], Michelle L. Soupir[f]

[a]Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, IA 50011, USA

[b]University of Minnesota, Department of Bioproducts and Biosystems Engineering 204 Kaufert Lab 2004 Folwell Avenue St. Paul, MN, USA 55108

[c]USDA-ARS, Soil and Water Management Research Unit 1991 Upper Buford Circle 439 Borlaug Hall St. Paul, MN, USA 55108

[d]USDA-ARS National Laboratory for Agriculture and the Environment, 2110 University Boulevard, Ames, IA 50011, USA

[e]Iowa State University, Genomics and Environmental Research in Microbial Systems Lab, Dept. of Agricultural and Biosystems Engineering, 3346 Elings Hall, USA

[f]Iowa State University, Water Quality Research Lab, Dept. of Agricultural and Biosystems Engineering, 3358 Elings Hall, USA

Abbreviations: DO, dissolved oxygen; DOC, dissolved organic carbon; HRT, hydraulic residence time; LCI, lignocellulose index; MDI, Morrill Dispersion Index; S, short circuiting

Abstract

Woodchip bioreactors are edge-of-field practices that remove nutrients from agricultural drainage water, with an effective lifespan estimated between 10 and 30 years. Subsidence, or bioreactor settling and subsequent depression formation, is a concern of producers and stakeholders and little is known regarding its effect on bioreactor performance. Six woodchip bioreactors set at 3 different hydraulic residence times (HRTs 2, 8, 16 h) were excavated after 2 years of operation, with wood samples collected from multiple depths and distances from the bioreactor inlet. Subsidence was observed in all 6 bioreactors and was greater near the inlet.

Particle size distribution did not change over the study period, indicating that smaller woodchips were not degrading preferentially or washing out of the bioreactor while the macropore space was simultaneously decreasing. Flow path analysis showed an increase in Morrill Dispersion Indices and short-circuiting as well as decreases in drainable porosity and hydraulic efficiency; these changes were uniform across all three HRTs, suggesting that the decline in hydraulic properties was independent of flow. Further, despite increased woodchip decomposition as measured by C:N ratio in the 2-h HRT bioreactors (mean ± standard deviation = 64.9 ± 13.7) compared to the 8-h and 16-h HRT systems (90.3 ± 19.0, 95.6 ± 27.2, respectively), denitrification was still supported at all HRTs based on the results from a batch denitrification test. To offset wood aging, bioreactor fill material nearest the inlet could be replenished without excavation of the entire bioreactor.

Core Ideas

HRT is optimized to maximize nitrate removal in denitrifying bioreactors.

Three HRTs were controlled in triplicate pilot-scale bioreactors.

Hydraulic properties did not differ after 3 years at different HRTs.

Woodchip degradation is impacted by HRT due to differences in influent nitrate load.

Introduction

Excessive nutrient loading resulting in rapid production of biomass and subsequent oxygen depletion has caused the hypoxic zone that has plagued the northern Gulf of Mexico since the 1970s. The dead zone has been linked to human population growth in coastal areas, increasing agricultural activity in upland watersheds, and growing demand for food and energy (Rabalais et al., 2002). Subsurface agricultural drainage networks in the Upper Mississippi River Basin contribute to eutrophication by serving as efficient conduits from row-crop fields

(Ikenberry et al., 2014; Mitsch et al., 2001; Schilling & Helmers, 2008). Further, bioavailable forms of nitrogen such as nitrate have been specifically linked to the Gulf of Mexico hypoxic zone due to increased mobility in the dissolved form. In 2008, the Gulf of Mexico Hypoxia Action Plan called on Midwest states to reduce their contributions to nutrient loading within the Mississippi River Watershed and many conservation practices have been developed to achieve these water quality goals (Iowa Department of Agriculture and Land Stewardship, 2017; Mississippi River/Gulf of Mexico Nutrient Task Force, 2008).

Conservation practices that have demonstrated nitrate reduction practices can be classified as land conversion, in-field, or edge-of-field; examples of land conversion and in-field practices include retiring land, converting to perennial land cover, or changing the timing and rate of nitrogen application in corn production (Christianson et al., 2013). However, most in-field management practices only have the capacity to decrease nitrate loads by 4 – 10% (Randall et al., 2003). Cover crops are an in-field practice that have higher nitrate removal potential (24 – 60% reduction) but require extensive management on the part of the producer (Kaspar et al., 2007; Kaspar et al., 2012). Additionally, the economic feasibility of broad land conversion is questionable (Qi et al., 2011; Tomer et al., 2010). Edge-of-field practices have the advantage that they minimally impact crop yield and are low-maintenance, which can otherwise be a barrier to practice implementation (Liu et al., 2018). Therefore, edge-of-field practices with high nitrate load reduction potential are attractive options for meeting nutrient reduction goals.

Since the early 1990s, edge-of-field denitrifying bioreactors have been widely studied due to their potential to significantly reduce nitrate loading, with observed average seasonal load reductions ranging between 40-60%, but with the potential to be as high as 80-95% (Addy et al., 2016; Blowes et al., 1994; Christianson et al., 2012; Greenan et al., 2009; Schipper et al.,

2010a). Bioreactors are a habitat for a complex consortium of microbial species (Jang et al., 2019; Porter et al., 2015; Yao et al., 2020) which enable denitrification processes (Dandie et al., 2008; Kraft et al., 2011; Kuypers et al., 2018; Melillo et al., 1984). Denitrifying bioreactors utilize a lignocellulosic substrate (typically woodchips) that a population of microorganisms, including denitrifying species, metabolizes via reduction of nitrate to nitrogen gas (Christianson, et al., 2011; Schipper et al., 2010b).

Two main processes strike a balance within denitrifying bioreactors. On one hand, anaerobic conditions have been shown to promote the denitrification process because oxygen is preferred as an electron acceptor over nitrate (Averill & Tiedje, 1982; Tesoriero et al., 2000; Warneke et al., 2011). However, the breakdown of cellulose is a process that is thought to be mainly mediated by various obligate-aerobic fungal species due to their ability to secrete hydrolytic enzymes and physically compromise the woodchip cell walls with their hyphae (Eriksson et al., 1990); anaerobic conditions have been shown to inhibit this catabolic process (Mattila et al., 2020; Tavzes et al., 2001). As the organic fill material in an aging bioreactor is consumed through denitrification, redox potential increases and nitrate removal efficiency decreases in the long term (Easton et al., 2015; Elgood et al., 2010). Further, differences in media redox potential and oxygen levels within denitrifying bioreactors can stratify microorganism populations spatially (Jansen et al., 2019; Porter et al., 2015). The organisms and mechanism of denitrification as well as the species performing lignin and cellulose degradation are targets for further study (Brown & Chang, 2014; Janusz et al., 2017).

Extensive engineering optimization of denitrifying bioreactor systems has occurred regarding media, geometry, managing bypass flow, and HRT (Cameron & Schipper, 2010; Christianson et al., 2011; Hoover, et al., 2016; Martin et al., 2019). HRT has been emphasized to

maximize the volume treated while still constraining these systems to a reasonable size. Typically, HRT is selected to between 4 – 8 h to allow for substantial mass removal of nitrate while minimizing by-pass flow, with HRTs less than 6 -h exhibiting decreased cumulative nitrate removal and longer HRTs required to reach the same nitrate removal efficiency at lower temperatures (Addy et al., 2016; Christianson et al., 2011; Hassanpour et al., 2017; Warneke et al., 2011).

Denitrifying bioreactor fill material and longevity has not previously been studied as a function of HRT. Recent studies evaluated the lifespan of the bioreactor fill material as these systems were initially designed to require little intervention by producers (Christianson et al., 2020; Ghane et al., 2018). Initial estimations of the timeframe for replenishing the fill material were ten-year intervals based on the rate of lignocellulosic substrate depletion, though decades-long intervals have since been estimated (Moorman et al., 2010; Robertson et al., 2000; Robertson et al., 2008). Since woodchip bioreactors are a relatively new conservation practice, there are limited field studies of their performance over longer time scales to validate this assumption. Additionally, few studies have examined the long-term performance and substrate characteristics of a woodchip bioreactor, but differences in woodchip particle size, C:N ratio, and carbon quality as measured by the lignocellulosic index (LCI) have been demonstrated with degradation resulting in mass loss, lower C:N ratios, and higher LCIs (Ghane et al., 2018; Moorman et al., 2010). Further, woodchip degradation has not yet been evaluated as a function of operational flowrate or HRT. Flowrate is known to be associated with dissolved oxygen (DO) levels within bioreactors and oxygen is associated with rapid woodchip degradation (Ghane et al., 2018). Specifically, the changes in the properties of the woodchips themselves contribute to the changes in overall hydraulic properties of the bioreactors. There have been few long-term

monitoring studies of denitrifying bioreactors due to the newness of the technology. Therefore, there is a need to study the effects of HRT on the decomposition of woodchip fill material and thus the implications for woodchip bioreactor lifespan. Determining the effect of HRT on woodchip decomposition will allow better estimation of the design lifetime of denitrifying bioreactors. The objective of this study is to determine the impact of varying HRT on indicators of bioreactor aging.

Materials and Methods

Site Description

The replicate woodchip bioreactors at the Iowa State University (ISU) Agronomy and Agricultural Engineering Research Farm (42.019861, −93.776872) provide a unique opportunity to evaluate the effect of HRT on the aged characteristics of this conservation practice (Hoover et al., 2017). The experimental set-up at ISU is part of a long-term monitoring study of denitrifying bioreactors, and in 2018 excavation was done to exchange portions of the woodchip media for corn cobs.

The site is described extensively in Hoover et al., 2017. Briefly, nine woodchip bioreactors were installed in parallel in September 2014. Each bioreactor consists of a concrete trench filled with a mixture of hardwood chips obtained from Golden Valley Hardscapes, Story City, Iowa, USA. The trenches were filled to cover the trench sides with woodchips (See Appendix A). A 3,000-gallon storage tank intercepts a nearby 12-inch diameter county tile line and an underground storage cistern supplies water to the bioreactors. Each bioreactor is fitted with its own influent control structure and dosing port. The outlet structures were fitted with stoplog drainage control structures which can be adjusted to change the active volume of the

bioreactors. Three HRTs (2, 8, and 16 h) are controlled in triplicate at this site. The bioreactors were saturated throughout the year, with the flow through the bioreactors occurring during the sampling season. The bioreactors remained saturated during winter months to inhibit rodents from bedding into the woodchips.

Woodchip Preservation, Transport, and Analysis

Six woodchip bioreactors were installed in fall 2014 and operating continuously during summer months beginning in 2016 through 2018. Excavation was initiated to change the experimental conditions by replacing the ¼ or ¾ proportion of the woodchips closet to the inlet with corncobs. Woodchips were sampled from three locations within replicate denitrifying bioreactors for analysis and a tracer study was conducted to evaluate hydraulic properties.

Three bioreactors were left undisturbed and served as the source of woodchips for a denitrification assay in 2019. The soil caps of the excavated bioreactors were removed to partially expose the tops of the concrete trenches. For all excavated bioreactors, woodchips were sampled from 0.30 –, 0.61 –, 0.91 –, and 1.22 – m vertical depths at the inlet and at a location 1/3 the length of the bioreactor (Figure 2.1A). To examine impact of distance from bioreactor inlet length on woodchip properties, additional samples from a distance of 2/3 the bioreactor length were taken from an additional 3 bioreactors, for a total of 8 or 12 sample locations from each bioreactor, depending on the excavation (Figure 2.1B). The overburden was removed using an excavator and remaining soil material was removed with a shovel. Representative samples from 0 – 0.3 m were collected using a shovel. Woodchips from the 0.30 – 0.61 m, 0.61 – 0.91 m, and 0.91 – 1.22 m depths were collected by shovel or post hole digger. All samples were mixed in a bucket or plastic tote before storage in a labeled 1-gallon (4 L) resealable plastic bag. All samples filled at least 75% of the gallon bag. Woodchip samples were stored in a cooler and

transported to ISU, where they were stored at 4°C. Subsidence (or settling) of the woodchips below the depth of the original fill on the ISU bioreactors was observed and measured with a tape measure at the inlet location and location B, 4.3 m from the inlet, during the 2018 excavation (See Appendix A). Observing subsidence as early as 3 years into bioreactor operation is of note and could be concerning to producers and stakeholders.

Tracer Study to Determine Hydraulic Characteristics

KBr tracer tests were performed in May 2015 prior to system operation and in May 2018 to study changes in flow characteristics over two years of operation. The bioreactor tracer study water samples were analyzed for Br^- using a Dionex ICS-2100 Ion Chromatography System (Thermo Fisher Scientific, Waltham, MA, USA) using method EPA 300.1 (Pfaff, Hautman, & Munch, 1997). The results from the flow path analysis provide insight into the Morrill Dispersion Index (MDI), short – circuiting (S), and hydraulic efficiency (λ) for each bioreactor. Briefly, MDI is a dimensionless measure of the amount of fluid recirculation or deviation from plug flow. An MDI of 1.0 indicates ideal plug flow, and values of 22 or greater indicate complete-mix reactor conditions. Hydraulic efficiency is a different metric for evaluating plug flow conditions, with values between 0.5 and 0.75 being considered satisfactory (Hoover et al., 2017). Short-circuiting is indicated with a value near 0, and an S value of 1.0 indicates ideal conditions (no stagnation) (Ta & Birgnal, 1998).

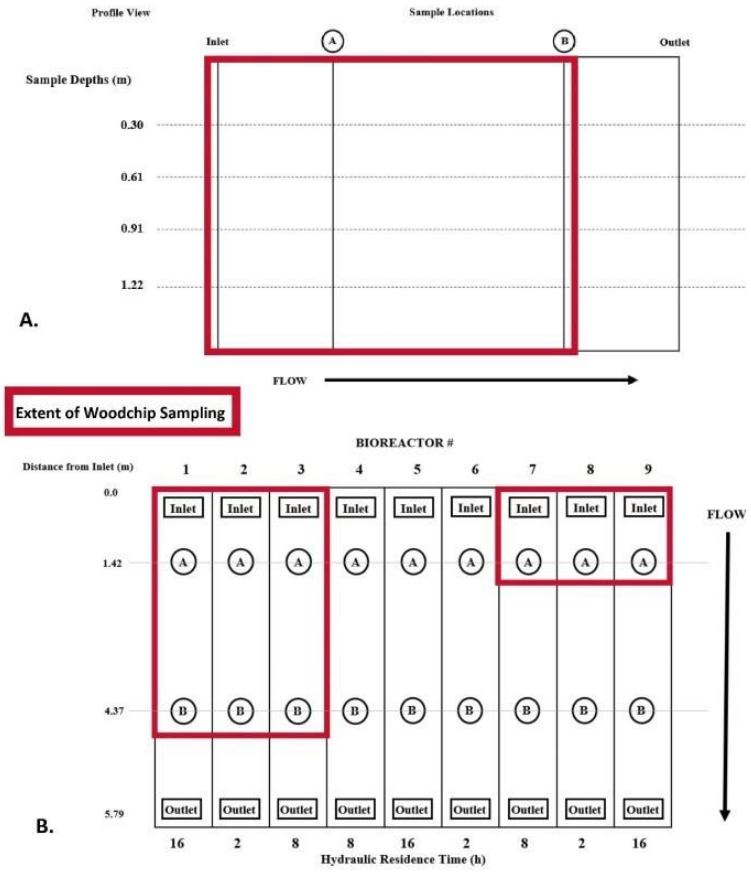


Figure 2.1 Woodchips were collected from three separate locations and four depths: 0 – 0.30 m, 0.30 – 0.61 m, 0.61 – 0.91 m, and 0.91 – 1.22 m. A) Profile view of woodchip sampling plan in 2018. B) Top view of woodchip sampling plan in 2018.

Flow-path analysis was performed as outlined in Hoover et al., 2017. Flow rates were adjusted at the beginning of the study to achieve retention times of approximately 4 hours. A 1 L dose of 36.5 g KBr L^{-1} was introduced into each dosing port and outflow samples were collected for 1,180 minutes in 20-minute increments. The Br^- concentrations at 20-minute sample intervals were used for MDI calculations to maintain even sampling intervals. Short circuiting, S, was calculated using the Ta & Birgnal 1998 method and hydraulic efficiency was calculated

using the Persson et al., 1999 method, as described in Hoover et al., 2017. Drainable porosity was determined by the following: the saturated depth in each bioreactor well was measured and the average saturated depth for each bioreactor was calculated. The bioreactors were gradually drained by gravity so that all the flow volume was measured by the individual flow meters at the outlet of each bioreactor. The final flow volume was recorded after 2 days. Drainable porosity was calculated by dividing the flow volume by the saturated volume of the bioreactor.

Woodchip Particle Size, Particle Density, and Chemical Composition Analysis

Approximately 750 g of woodchips were weighed and placed in a 23 cm by 28 cm aluminum pan and oven dried at 60°C for 48 hours or until no change in mass was observed. The mass at which further drying produced no change was used to determine gravimetric moisture content. To determine the C and N content, the woodchips were ground in a coarse Wiley mill (2.0 mm) followed by a fine Wiley mill (1.0 mm) and placed in labeled bags before shipment to the USDA ARS Laboratory in Saint Paul, MN. Prior (> 24hr) to samples being weighed for C:N analysis, paper bags containing the samples were again oven dried at 60°C to ensure all moisture had been removed. Each sample was analyzed in triplicate for percentages of ash, C, and N. The C:N ratio was calculated. Ash content was determined by combustion at 550°C for 4 hours in a muffle furnace. C and N analysis was performed with the Dumas combustion method using an element analyzer (vario MAX cube, Elementar, Hanau, Germany). Dried samples were analyzed for cellulose, lignin, and hemicellulose composition, also called “carbon quality” as in Ghane et al., 2018. Briefly, Klason lignin, glucose, mannose, xylose, galactose, and arabinose concentrations after acid hydrolysis were determined twice using a high-performance liquid chromatograph (model 1525 binary pump, Waters Corp., Milford, MA, USA) and pre-packed carbohydrate analytical column (Aminex HPX-87P, Bio-Rad, Hercules, CA, USA). An in-line

de-ashing cartridge (No. 125-0118, Bio-Rad) was used with the carbohydrate analytical column and a refractive index detector (model 2414, Waters Corp.) at a flow rate 0f 0.3 mL min^{-1} and 80ºC column temperature (Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, 2012).

Approximately 100 g oven-dried batches of each sample were sieved with a vibratory shaker for 10 – 15 minutes through a nested sieve set with the sieve sizes indicated in the axis range of Figure 2.3 and a catch pan. The mass of woodchip material in each sieve was recorded to determine the particle size distribution curve and median particle size (D50). To determine woodchip particle density, woodchip samples in the previously described resealable gallon bags were saturated with deionized water for 24 hours. Additionally, a sample of fresh woodchips collected from Golden Valley Hardscapes was also saturated in the same way. A corner was cut from each bag and the bags were drained by gravity for 24 hours. A 100-mL aliquot of deionized water was added to a 250-mL graduated cylinder and the volume was recorded. The volume change was recorded when 50 g of woodchips were added to the graduated cylinder and fully submerged to determine the particle volume and this volume was used to calculate particle density.

Batch Kinetic Study

To assess the NO_3—N-removal capability of the aged woodchips, a batch kinetic study was conducted. Four types of woodchips were used in the study: those collected from each of the undisturbed bioreactors at the ISU Agronomy and Agricultural Engineering Farm (2, 8, and 16-h HRT samples) and fresh hardwood chips that had not been used as a denitrifying substrate. Woodchips were excavated from the top 10 cm of bioreactors 4, 5, and 6 (8, 16, and 2 h HRTs,

respectively) at a location 1.1 m from the inlet at the ISU Agronomy and Agricultural Engineering Farm in October 2019 (Figure 2.1). One-gallon resealable bags were filled approximately 75% full of woodchips and transported in a cooler to ISU, where they were stored at 4°C until the batch study was performed. Nutrient solution (25 L of 30.0 mg L^{-1}) was prepared according to Hoover et al., 2016. Triplicate 10.0 g samples of woodchips from each bioreactor were aliquoted into clean, quart-sized, glass Mason jars for each time point for a total of 30 jars per bioreactor. The fresh woodchips were saturated with 190 mL of DI water for 48 hours at 4°C prior to the test to prevent the woodchips from floating during the batch test. To bring the final concentration of NO_3—N to 30.0 mg L^{-1} in each jar, 200 mL of nutrient solution was added to each jar of aged woodchips, and a spike of 10.0 mL of concentrated nutrient solution was added to the jars of fresh woodchips. The jars were gently swirled, sealed, and kept at 21°C during the test. At timepoints of 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 12, 24, 36, and 48 hours, three jars from each were destructively sampled. The jars were un-sealed and liquid was decanted into 125 mL Nalgene bottles. The samples were stored on ice before vacuum filtering with a 45-µm filter for NO_3—N analysis on the AQ2 (Seal Analytical, Mequon, WI, USA) with method EPA-114-A, Rev. 7 (equivalent to US EPA method 353.2 ver.2 (1993)).

Data Analysis

Data manipulation, statistical analyses, and figure generation were performed using the RStudio software package with R version 4.0.0 (R Core Team, 2020). R packages “tidyverse,” “Hmisc,” “olsrr,” and “rstatix” were used for data analysis (Wickham et al., 2020; Harrell Jr. et al., 2020; Hebbali 2020; Kassambara 2020). The distribution of observations for woodchip C:N ratio, woodchip chemical composition, D50 value, and particle density were tested for normality with the Shapiro-Wilk test ($p < 0.05$). A factorial ANOVA was conducted to compare the main

effects of sampling location, depth, and HRT and the interaction effect of location, depth, and HRT on woodchip C:N ratio, woodchip chemical composition, D50 value, and particle density. Location included three levels (Inlet, A, B), depth included four levels (0.30 m, 0.61 m, 0.91 m, 1.22 m) and HRT included three levels (2 h, 8 h, 16 h). This ANOVA technique was also used to assess statistical differences in the mass retained in each sieve in order to compare particle size distributions. A separate factorial ANOVA was performed to compare the main effects of location and HRT and the interaction effect of location and HRT on bioreactor subsidence. Location included two levels (Inlet, B) and HRT included three levels (2 h, 8 h, 16 h). We used a Tukey *post hoc* analysis between factors in RStudio for both ANOVA analyses to determine differences between main effect means and interactions.

The following analyses were also carried out in R. Two-sided student's t-tests were used to compare means of the flow characteristics (MDI, S, drainable porosity, and hydraulic conductivity) between years 2016 and 2018. Carbon and nitrogen concentrations were compared to literature values with one-sided student's t-tests. Linear regression was used to fit a zero-order model to the nitrate nitrogen concentration over time in the batch removal test. Time and three indicator variables representing woodchip type were used as explanatory variables in a multiple linear regression to test for significant differences in the regression slopes.

Results

Flow-Path Analysis

The MDIs determined in 2018 (2.9-3.9) (Appendix A) were higher than the 2016 MDIs (2.8 ± 0.3) for all bioreactors except for bioreactor 9 (MDI = 2.7, HRT = 16 h) (Table 1.1). The highest MDI observed was 3.9 in bioreactor 5 (HRT = 16 h). This suggests a deterioration of ideal plug flow towards flow with greater dispersion (Metcalf & Eddy, 2003). However, the

calculated MDIs in this study are similar to other reported MDI values for field woodchip bioreactors of 3.5 and 4.2 (Christianson et al., 2011). Similarly, the calculated short-circuiting values (S) are all lower than the mean 2016 value and range from 0.61 in bioreactor 5 to 0.69 in bioreactor 7. An S value of 1.0 indicates ideal flow, while an S value of 0.0 indicates short-circuiting (Ta & Brignal, 1998). Short-circuit-like conditions increased in all reactors in 2018 when compared to 2016. All drainable porosity values determined in 2018 are lower than the 2016 average, indicating a decrease in pore volume. The lowest drainable porosity was observed in bioreactor 2 (0.41 m^3 m^{-3}, HRT = 2 h), and the highest drainable porosity was observed in bioreactors 1 and 6 (0.47, HRT = 16 h and 2 h, respectively). All the calculated hydraulic efficiency values in 2018 were greater than 0.50, which is considered satisfactory (Hoover et al., 2016). The lowest hydraulic efficiency was observed in bioreactor 5 (0.60) and the highest hydraulic efficiency was observed in bioreactor 9 (0.80). The mean of each flow characteristic determined in 2018 was statistically different than the mean determined in 2016 ($p < 0.01$, Student's t test, unequal variance).

Table 2.1 Hydraulic properties of nine denitrifying bioreactors before and after two years of operation.

		2018 Avg ± SD	**2016 Avg ± SD**	**Percent Change**
Tracer Residence Time	h	5.3 ± 0.9	2.3 ± 0.3	N/A (Test Parameter)
MDI		3.3 ± 0.4	2.8 ± 0.3	+17.9%
Short Circuiting (S)		0.66 ± 0.02	0.73 ± 0.03	-9.6%
Drainable Porosity	m^3 m^{-3}	0.45 ± 0.021	0.51 ± 0.02	-11.7%
Hydraulic Efficiency (λ)		0.70 ± 0.06	0.78 ± 0.03	-10.3%

Bioreactor Subsidence

The subsidence for six woodchip bioreactors ranged between 10.8 and 25.4 cm, with a mean of 17.3 ± 4.6 cm (Figure 2.2A). Subsidence generally increased at the inlet location when compared to location B, 4.3 m from the inlet. The ANOVA main effect for location yielded an F ratio of $F(1, 30) = 17.067$, $p < 0.05$, indicating a significant difference between the inlet location and location B. Additionally, we observed that subsidence increased for the 2 h HRT compared to the 8 h and 16 h HRTs (Figure 2.2A).

Woodchip Properties

Particle density was not a parameter that was determined in 2016, therefore comparisons between years are not discussed here. The particle densities were similar at all three locations for all bioreactors. Particle density was not a factor that was affected by hydraulic retention time, location, or depth (Figure 2.2B). The woodchip particle size distribution for all HRTs was similar to the distribution reported in 2016 (Figure 2.3). There was only one analysis conducted for the particle size distribution in 2016 (n=1), and as a result there is little statistical power to compare to the 2018 woodchips. There was a statistically significant difference in the mass retained in the 19mm sieve from the 8 h HRT bioreactor compared to both the 2 h and 16 h woodchips ($p < 0.05$) but no other statistical differences in percent mass retained within sieves among woodchip type were observed. Results of the 3-way ANOVA showed D50 was not a parameter that appeared to be influenced by HRT, location, depth, or the interaction between these effects $F(12, 24) = 0.548$, $p > 0.05$).

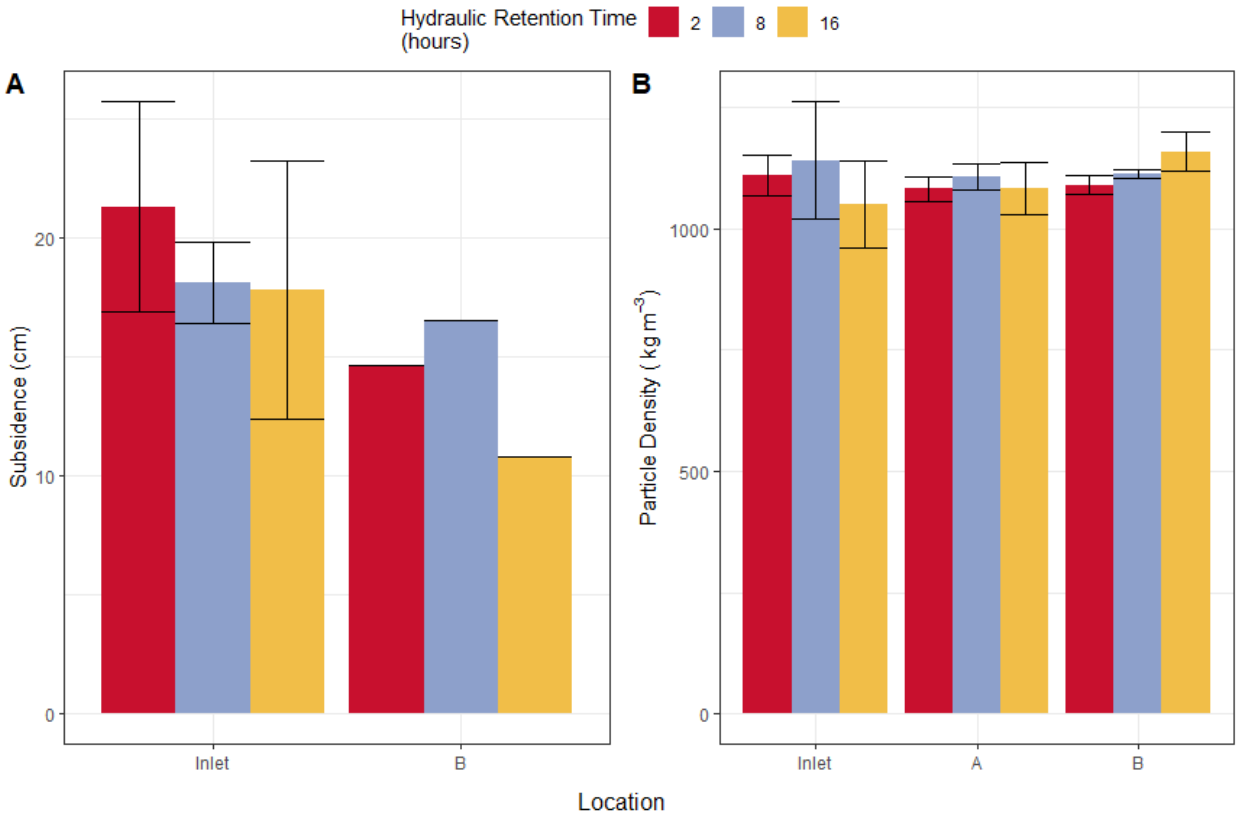


Figure 2.2 Subsidence and particle density of aged woodchips A) Mean subsidence in centimeters at the Inlet and B locations for denitrifying bioreactors operating at three HRTs. Error bars represent one standard deviation. Only one measurement was taken at location B due to the sampling design. B) Mean Particle density of woodchips sampled from three locations for denitrifying bioreactors operating at three HRTs. Error bars represent one standard deviation.

The reported C:N ratio for fresh mixed hardwood chips from the same supplier as those used in this study is 247 (Christianson et al., 2010). Others have reported C:N ratios ranging between 224 and 496 for hardwood, softwood, or mixed hardwood woodchip varieties (Ghane et al., 2018). The C:N ratios of the aged woodchips were below this range for all HRTs locations, and depths (Figure 2.4). Additionally, the highest C:N ratios were observed in the 16 h HRT

bioreactors at location B, while the lowest C:N ratios were observed in the 2 h HRT bioreactors at the inlet location.

There were statistically significant interaction effects between HRT and depth $F(6, 24) = 5.569$, $p < 0.05$), HRT and location $F(4, 24) = 4.955$, $p < 0.05$), and depth and location $F(6, 24) = 4.739$, $p < 0.05$) on C:N ratio. These differences are further displayed in Table 2.2. The Tukey *post hoc* comparisons showed statistically significant differences ($p < 0.05$) in C:N ratios between all three locations (Inlet, A, B).

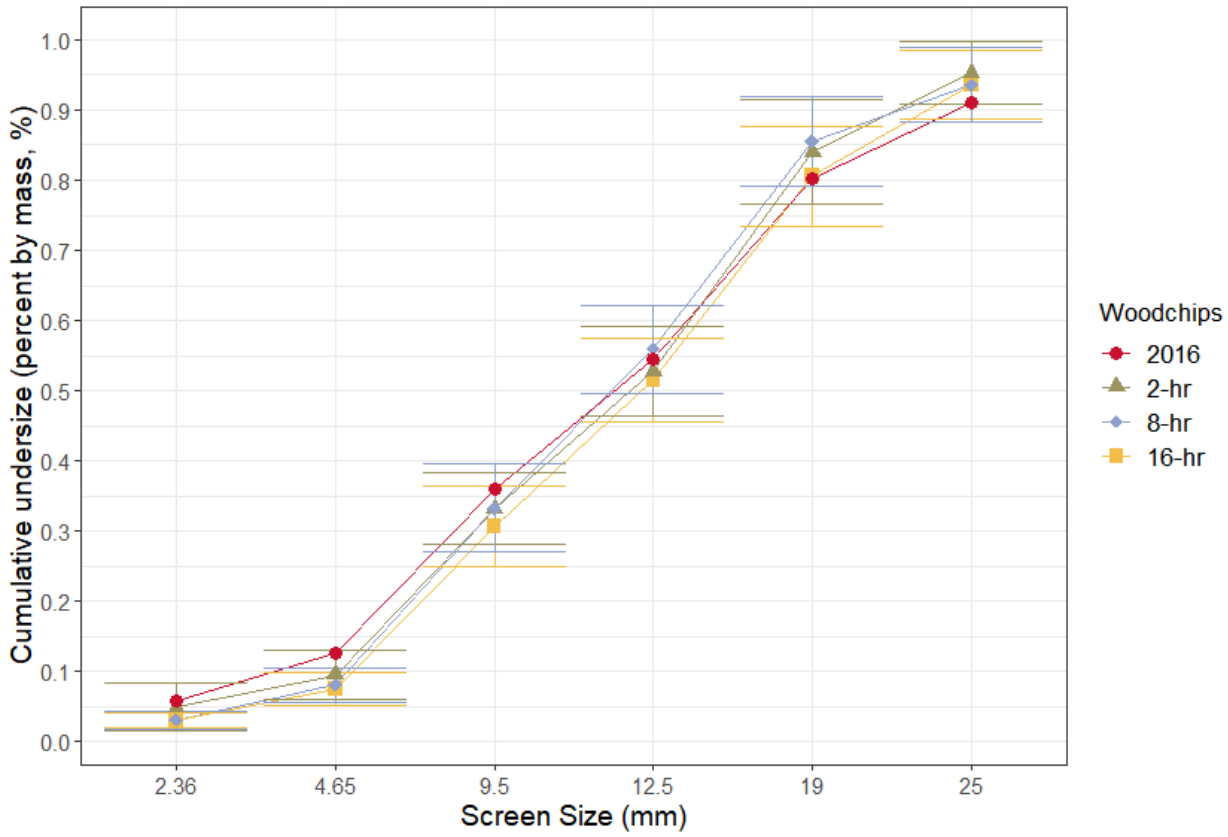


Figure 2.3 Mean particle size distributions of the aged woodchips compared to the distribution reported in 2016 (Hoover et al., 2017). Error bars represent one standard deviation.

There were statistically significant differences in C:N ratios in the 2 h HRT and both the 8 h and 16 h HRTs. There were statistically significant C:N ratios between the 0.30 m depth and

0.61 m depth, the 0.31 m depth and the 0.91 m depth, and the 0.91 m depth and the 1.22 m depth. When comparing the combined effect of HRT and depth, there were statistically significant differences between the 2 h HRT bioreactor and the 16 h HRT bioreactor at all depths except 0.30 m. The 0.30 m depth of the 16 h HRT was statistically different than all other depths in that bioreactor. None of the depths at any of the locations within the 2 h HRT bioreactor were significantly different from one another.

There were statistically significant differences between the 2 h HRT inlet location and all other locations in the 8 h and 16 h HRT bioreactors (Table 2.2). The 2 h inlet location was statistically different than the 2 h B location. The 2 h A and B locations were significantly different than both the 16 h A and B locations. The 8 h inlet location was statistically different than the 8 h A and B locations and the 16 h inlet location was significantly different than the 16 h A and B locations. Typical carbon concentrations (C%) for woodchips used in denitrifying bioreactors range between 47.0 – 51.0% and typical nitrogen concentrations (N%) range between 0.1 – 0.21% (Ghane et al., 2018). C% was lower in all bioreactor HRTs in 2018 compared to the mean literature value (one-sided t-test, $p < 0.05$) (data not shown). Additionally, the main effects of location and HRT on C% were significant ($F(2, 24) = 5.018$, $p < 0.05$ and $F(2, 24) = 7.692$, $p < 0.05$). Similarly, N% was higher in wood from all bioreactor HRTs compared to the mean literature value ($p < 0.05$) (data not shown). The main effects of location and HRT were significant on N% ($F(2, 42) = 7.421$, $p < 0.05$; $F(2,24) = 11.741$, $p < 0.05$).

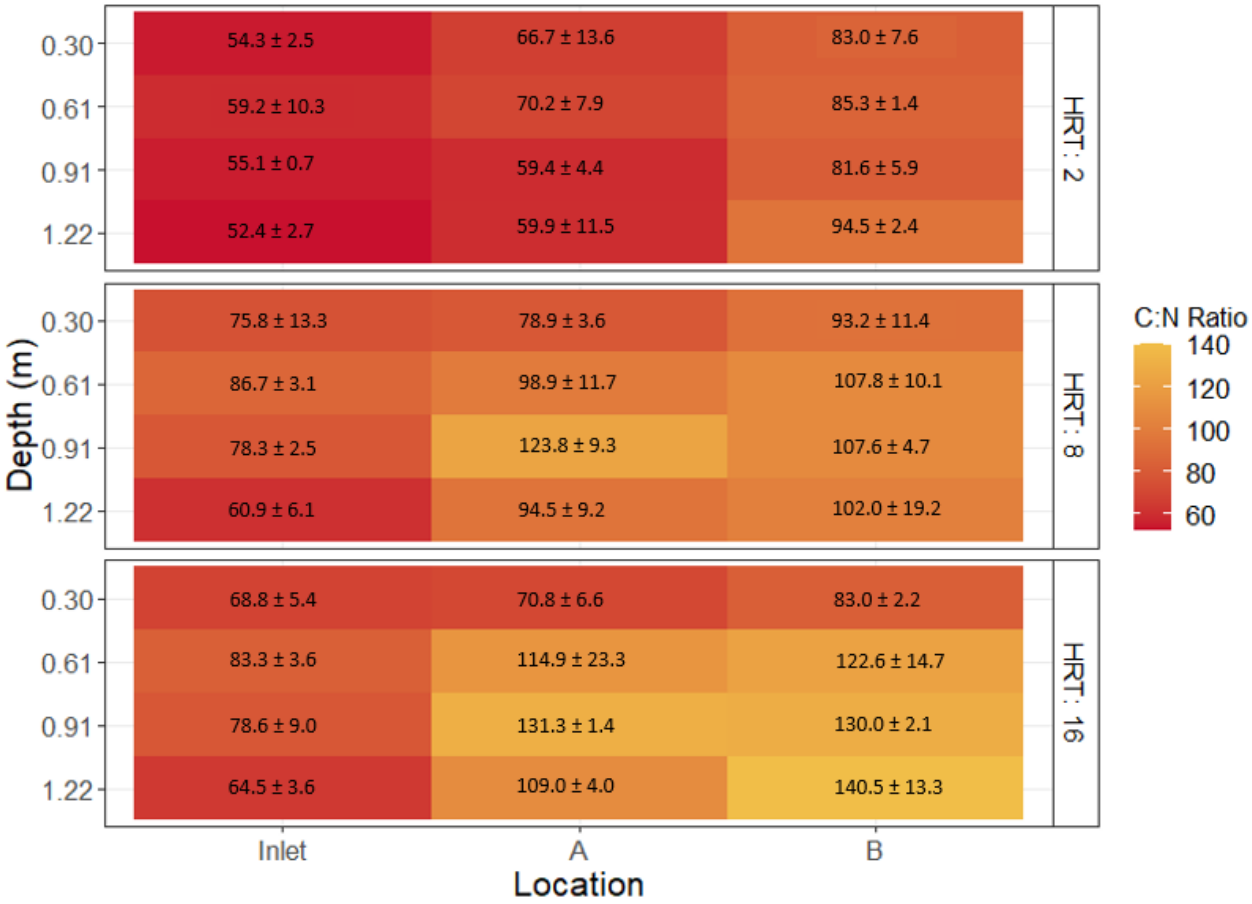


Figure 2.4 Heatmap comparing C:N ratios by HRT, location, and depth. Figure text displays mean C:N ratio ± standard deviation.

The effect of HRT on lignin content, glucose content, and ash content were all significant ($F(3, 24) = 18.769$, $p < 0.05$; $F(3,24)=5.665$, $p < 0.05$; $F(3, 24) = 7.750$, $p < 0.05$). The mean LCI for all aged woodchips was statistically different than that observed in the fresh woodchips ($p < 0.05$). The glucose content in the 2 h HRT woodchips was statistically different than the 16 h HRT and fresh woodchips ($p < 0.05$). The mean glucose content of the fresh woodchips was higher by 2.1% than that measured in the aged woodchips ($p < 0.05$). Additionally, mean ash and mean lignin contents of the fresh woodchips were lower than those measured in the aged woodchips by an average of 10.6% and 6.5%, respectively ($p < 0.05$).

Table 2.2 Mean C: N Ratios for combinations of HRT and Depth and HRT and Location. Within a row, means followed by the same lowercase letter are not significantly different ($p < 0.05$). Within a column, means followed by the same uppercase letters are not significantly different ($p < 0.05$).

Depth (m)	**Hydraulic Retention Time (h)**		
	2	8	16
	CN: Ratio (mean ± standard deviation)		
0.30	65.0 ± 13.2 aA	80.5 ± 10.5 bA	72.4 ± 8.2 abA
0.61	68.8 ± 12.6 aA	95.8 ± 11.8 bB	103.4 ± 22.1 bB
0.91	62.1 ± 11.0 aA	102.4 ± 22.0 bB	109.9 ± 27.1 bB
1.22	63.8 ± 17.6 aA	82.6 ± 20.6 bC	97.5 ± 31.0 cB
Location			
Inlet	55.3 ± 5.9aA	75.4 ± 11.4bA	73.8 ± 9.4bA
A	64.0 ± 9.5aB	99.0 ± 18.2bB	106.5 ± 25.0bB
B	86.1 ± 6.8aC	102.7 ± 12.3bC	119.0 ± 24.3cC

Table 2.3 Constituents of woodchips sampled from bioreactors operating at 3 HRTs compared to fresh woodchips. Means within columns followed by the same letters are not significantly different ($p > 0.05$).

Woodchips	Lignin (%)	Glucose Equivalents (%)	Ash (%)	LCI
2 h HRT	39.8 ± 3.6 a	29.8 ± 2.4 a	22.4 ± 13.3 a	0.61 ± 0.09 a
8 h HRT	36.6 ± 1.4 b	32.4 ± 2.1 b	12.2 ± 6.9 b	0.57 ± 0.06 b
16 h HRT	35.8 ± 3.6 b	33.5 ± 3.6 b	9.9 ± 6.3 b	0.56 ± 0.04 bc
Fresh	30.9 ± 0.03 c	34.0 ± 0.8 b	4.25 ± 0.13 b	0.51 ± 0.01 c

The 2 h HRT woodchips showed the largest percent decrease compared to the fresh woodchips in glucose (4.2%) and largest percent increases in lignin (8.9%) and ash (18.2%) content. Statistical differences in ash content between the 2 h HRT woodchips and the 8 h HRT, 16 h HRT, and fresh woodchips were observed ($p < 0.05$). These results are consistent with the increased LCIs observed in all aged woodchips.

Batch Kinetic Study

The 2 h HRT woodchip NO3—N concentrations observed after 48 hours were significantly different (p-adjusted < 0.05) than the 8 h and 16 h HRT woodchips (Figure 2.5). All aged woodchips showed greater concentration reductions over 48 hours compared to the fresh woodchips. The woodchips sampled from the bioreactor operating at the 2 h HRT showed the greatest concentration reduction (6.9 ± 0.57 mg L^{-1} in 48 h) while the fresh woodchips produced the smallest concentration reduction (1.1 ± 0.58 mg L^{-1}). The 8 h and 16 h woodchips showed similar concentration reductions over 48 h (4.4 ± 0.25 and 4.3 ± 0.73 mg L^{-1} in 48 h). A yellow color was observed in the liquid of the jars containing the fresh woodchips, which is indicative of dissolved carbon leached from the woodchips, however dissolved organic carbon (DOC) concentrations were not measured.

Multiple linear regression was used to predict nitrate – nitrogen concentration based on time and woodchip type. A significant regression equation was found ($F(7, 112) = 102.1$, $p < 0.05$), with an R^2 of 0.865. Significant regression coefficients for the interaction of time and indicator variables for 2 h, 8 h, and 16 h HRT woodchips ($p < 0.05$) indicated that the zero-order kinetic constants for all aged woodchips differed from the kinetic constant calculated for the fresh woodchips. A second multiple linear regression excluding the concentration results was performed to determine whether any of the kinetic constants for the aged woodchips were

significantly different. There was a significant regression equation ($F(5, 84) = 155.7$, $p < 0.05$) with an R^2 of 0.903.

A significant regression coefficient for the interaction of time and the indicator variable for 2 h HRT woodchips ($p < 0.05$) indicated that the zero-order kinetic constant for the 2 h HRT woodchips was statistically different from both the 8 h and 16 h HRT woodchip constants. A non-significant regression coefficient for the interaction of time and the indicator variable for 8 h HRT woodchips ($p > 0.05$) indicated that the zero-order kinetic constants for the 8 h and 16 h HRT woodchips were not statistically different.

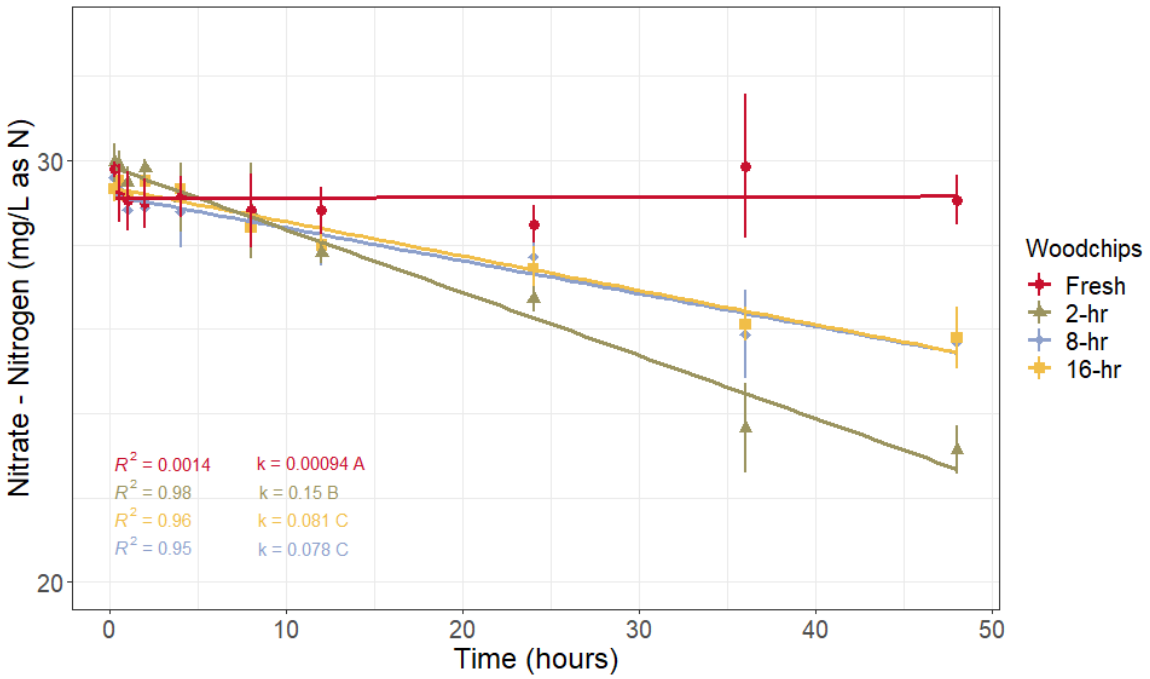


Figure 2.5 Linear regression was used to fit zero-order models to [NO3—N]. Figure label displays zero-order kinetic constants (mg L^{-1} h^{-1}) for linear models fit to the average concentration at each time. The constant for the fresh woodchips is not significantly different from zero ($p > 0.05$). Capital letters indicate significant differences between kinetic constants ($p < 0.05$).

Discussion

Our experiment allowed us to observe changes in woodchip properties and hydraulic characteristics in denitrifying bioreactors operating for two years to better understand the implication of hydraulic retention time as a design criterion. We observed decreases in C:N ratio and increased subsidence over time. There are several potential reasons for observed of subsidence, including damage due to field equipment, woodchips being washed out of the bioreactors, or mass loss from woodchips due to degradation. Subsidence in denitrifying bioreactors is a concern of producers due to aesthetics, but our results indicate that subsidence varied with HRT while changes in hydraulic properties did not, due to the differences observed in subsidence by HRT (Figure 2.1A) and lack of statistical differences in hydraulic properties between HRTs (Table 2.1). Our experiments do suggest that the cause of the subsidence is likely settling due to gravity and independent of the changes in hydraulic properties (MDI, S, λ, drainable porosity) that we observed.

Our results suggest that across all six bioreactors pore space has decreased and flow characteristics have shifted towards unideal conditions during 2 years of flow. We hypothesize that the decrease in drainable porosity can be attributed to media compaction, or subsidence, due to gravity effects. Due to the controlled conditions at the Iowa State Research Farm, the observed subsidence is not likely attributable to damage by field equipment. Sedimentation is also unlikely in this system because of the use of a reservoir tank prior to the inlet into the bioreactors, although others have hypothesized sedimentation was occurring based on an increase in the proportion of media particles falling under 1.18 mm and an increase in observed ash content (Christianson et al., 2020; Feyereisen & Christianson, 2015; Ghane et al., 2018; Robertson et al., 2008).

Woodchip subsidence has been mentioned in the literature previously within the context that an unsaturated woodchip layer might be advantageous to replenish the bioreactor media after settling or consumption (Christianson & Schipper, 2016). Installation photos from 2014 (Appendix A) show that the woodchips were filled to at least the level of the concrete trench. Overfilling the bioreactor with media may be able to compensate for subsidence over time but with a cost trade-off.

The particle size distributions for all three HRTs from woodchips sampled in 2018 were identical to the distribution reported for the woodchips in 2016 (N. L. Hoover et al., 2017). This means that the proportion of woodchips of each size has remained relatively stable, as opposed to preferential degradation of particles of a given size. Ghane et al. (2018) reported a decrease in the proportion of larger-sized woodchips closest to the inlet location after 4 years, but did not report a D50 value or particle size distribution for the original woodchips (Ghane et al., 2018). Additionally, Christianson et al. (2020) reported a decrease in D50 over 9 years. Both these studies also indicated a decrease in porosity, likely from sedimentation as subsidence was not reported. In our study it is more likely that the decrease in porosity was from gravity effects due to the low likelihood of sedimentation and given that we did not observe a change in particle size distribution. These results combined show that the initial 2-year period of bioreactor operation is not when changes in particle size distribution or D50 occur.

Low C:N ratios are indicative of a depletion of carbon relative to nitrogen or accumulation of nitrogen relative to carbon (Moorman et al., 2010). Though aerobic respiration and other anaerobic processes such as dissimilatory nitrate reduction to ammonia (DNRA) may occur within denitrifying bioreactors, we expect that substrate depletion within these systems is proportional to influent nitrate load because denitrification has been shown to be the dominant

fate of nitrogen (Gibert, Pomierny, Rowe, & Kalin, 2008; Greenan, Moorman, Kaspar, Parkin, & Jaynes, 2006). During the period of operation between 2016 and 2017, the nitrate load to the 2 h HRT bioreactors was significantly higher than the 8 h and 16 h HRT bioreactors (Martin et al., 2019), explaining why the lowest C:N ratios were observed at all locations and depths in the bioreactors set at 2 h HRT.

An additional potential mechanism of substrate degradation in denitrifying bioreactors is aerobic respiration. Results from an earlier study of these bioreactors between 2016 and 2018 indicated anaerobic conditions for the A (1/3 bioreactor length) and B (2/3 bioreactor length) locations as well as the outlet in all bioreactors. This suggests that aerobic respiration had reduced the oxygen concentration sufficiently between the inlet and location A for all HRTs. The presence of anaerobic conditions in the bioreactor at location A suggests that woodchip degradation due to aerobic respiration primarily occurs between the inlet and location A. No statistical differences in DO levels were reported between HRTs for any of the locations, providing evidence that differences in nitrate load better explain the observed changes in C:N ratios between bioreactor HRTs (Martin et al., 2019). These results are supported by the conclusions of a different study where greater woodchip decomposition occurred nearest the inlet of denitrification beds (Ghane et al., 2018).

After two years, the observed LCIs had not yet decreased sufficiently to be indicative of decomposition stabilization (~0.70 – 0.80, DeBusk & Reddy, 1998; Melillo et al. 1989). The LCI for the fresh woodchips presented here (0.51 ± 0.01) is the highest reported for fresh fill material in the literature, with values typically between 0.2 – 0.25; the highest previously-reported value reported for fresh woodchip media is 0.45 (Christianson & Schipper, 2016; Feyereisen et al., 2016).

The spatial sampling design in our study provides potential insight into the chemical and biological stratification within denitrifying bioreactors. If the C:N ratios are taken as spatial indicators of denitrification activity, the results from our study indicate that substrate degradation primarily occurs within the first 75% lengthwise of the denitrifying bioreactor. These results, taken with the subsidence measurements, suggest that recharging the woodchips near the inlet location while leaving the outlet location undisturbed might be an effective management strategy to prolong the usable lifetime of these systems.

The components of the woodchips described as ash are not capable of being consumed by microbial metabolism and therefore the absolute amount of ash in a sample of woodchips should remain constant. With this in mind, the relative amount of ash should increase as other components are metabolized. This can mean that components that fail to increase in relative abundance at the same rate as ash are being degraded or consumed. Therefore, the smaller increase in percent lignin compared to the percent increase in ash can be taken to mean that the absolute amount of lignin decreased overall. These results are consistent with cellulose being the preferred energy source for denitrification.

Another study has reported zero-order reaction constants ranging between 0.38 ± 0.06 mg N L^{-1} h^{-1} for 7-yr old woodchips and 0.50 ± 0.01 mg N L^{-1} h^{-1} for 2-yr old woodchips (Robertson, 2010). The C:N ratio of these woodchips was not reported, so we cannot compare whether the higher rates observed here result from differences in substrate composition. Despite lower C:N ratios, the aged woodchips still contain sufficient labile C to support denitrification and the results from the chemical analysis of the aged woodchips showed that roughly 1/3 glucose content remained after two years. Based on the observed denitrification potential of the aged woodchips, we hypothesize that despite the elevated oxygen levels observed near the inlet of the

2-h HRT bioreactors, facultative anaerobic microorganisms are present that can perform denitrification. Further work could characterize the effect of oxygen load on microbial community composition in contrast to the selective pressure of nitrate load.

Our study suggests that the change in flow characteristics (MDI, S, λ) as well as decrease in drainable porosity is independent of flow rate. This is promising because it implies that designing for a target flow rate will not negatively impact the hydraulic properties of the bioreactor. Our results suggest that spatial changes in C:N ratio could be indicators of denitrification and that the woodchip degradation observed nearest the inlet suggests that partial excavation and replenishment of woodchips might serve to prolong the usable lifetime of denitrifying bioreactors. Additionally, our results suggest that woodchips are not selectively degraded based on particle size during the first two years of operation. Further research on the microbial characteristics of denitrifying bioreactors is warranted to explain the differences in decomposition and denitrification performance in denitrifying bioreactors operating at different HRTs.

Acknowledgments

The authors would like to thank Leigh Ann Long for assistance during bioreactor excavation, woodchip sample preservation, and batch study nutrient analysis. We would also like to thank Alexis Slade, Andrew Craig, Ana Barana, Elizabeth Reike, and Andrew Hillman for assistance during bioreactor excavation.

Conflict of interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: M. Soupir, T. Moorman, G. Feyereisen

Data curation: A. Schaefer, K. Werning, N. Hoover

Formal analysis: A. Schaefer, K. Werning, N. Hoover

Funding acquisition: M. Soupir, A. Howe

Investigation: A. Schaefer, K.Werning, N. Hoover, U. Tschirner

Methodology: M. Soupir, T. Moorman, G. Feyereisen, U. Tschirner

Project administration: M. Soupir

Resources: M. Soupir, T. Moorman, G. Feyereisen, U. Tschirner

Software: A. Schaefer

Supervision: M. Soupir, A. Howe

Validation: A. Schaefer

Visualization: A. Schaefer

Writing–original draft: A. Schaefer

Writing–review & editing: M. Soupir, A. Howe, N. Hoover, T. Moorman, G. Feyereisen, U. Tschirner

References

Addy, K., A.J. Gold, L.E. Christianson, M.B. David, L.A. Schipper, et al. 2016. Denitrifying Bioreactors for Nitrate Removal: A Meta-Analysis. J. Environ. Qual. 45(3): 873. doi: 10.2134/jeq2015.07.0399.

Averill, B.A., and J.M. Tiedje. 1982. The chemical mechanism of microbial denitrification. FEBS Lett. 138(1): 8–12. doi: 10.1016/0014-5793(82)80383-9.

Blowes, D.W., W.D. Robertson, C.J. Ptacek, and C. Merkley. 1994. Removal of agricultural nitrate from tile-drainage effluent water using in-line bioreactors.

Brown, M.E., and M.C.Y. Chang. 2014. Exploring bacterial lignin degradation. Curr. Opin. Chem. Biol. 19(1): 1–7. doi: 10.1016/j.cbpa.2013.11.015.

Christianson, L.E., A. Bhandari, M. Asce, and M.J. Helmers. 2011. Pilot-Scale Evaluation of Denitrification Drainage Bioreactors: Reactor Geometry and Performance. doi: 10.1061/(ASCE)EE.1943-7870.0000316.

Christianson, L.E., A. Bhandari, and M.J. Helmers. 2012. A practice-oriented review of woodchip bioreactors for subsurface agricultural drainage. Appl. Eng. Agric.

Christianson, L., A. Castelló, R. Christianson, M. Helmers, and A. Bhandari. 2010. Technical Note: Hydraulic Property Determination of Denitrifying Bioreactor Fill Media. Appl. Eng. Agric. 26(5). https://lib.dr.iastate.edu/abe_eng_pubs/305 (accessed 26 May 2020).

Christianson, L., G. Feyereisen, C. Hay, U. Tschirner, K. Kult, et al. 2020. Denitrifying bioreactor woodchip recharge: Media properties after nine years. Trans. ASABE 63(2): 407–416. doi: 10.13031/trans.13709.

Christianson, L., J. Tyndall, and M. Helmers. 2013. Financial comparison of seven nitrate reduction strategies for Midwestern agricultural drainage. Water Resour. Econ. 2–3: 30–56. doi: 10.1016/j.wre.2013.09.001.

Christianson, L.E., and L.A. Schipper. 2016. Moving Denitrifying Bioreactors beyond Proof of Concept: Introduction to the Special Section. J. Environ. Qual. 45(3): 757–761. doi: 10.2134/jeq2016.01.0013.

Dandie, C.E., D.L. Burton, B.J. Zebarth, S.L. Henderson, J.T. Trevors, et al. 2008. Changes in bacterial denitrifier community abundance over time in an agricultural field and their relationship with denitrification activity. Appl. Environ. Microbiol. 74(19): 5997–6005. doi: 10.1128/AEM.00441-08.

Easton, Z.M., M. Rogers, M. Davis, J. Wade, M. Eick, et al. 2015. Mitigation of sulfate reduction and nitrous oxide emission in denitrifying environments with amorphous iron oxide and biochar. Ecol. Eng. 82: 605–613. doi: 10.1016/j.ecoleng.2015.05.008.

Elgood, Z., W.D. Robertson, S.L. Schiff, and R. Elgood. 2010. Nitrate removal and greenhouse gas production in a stream-bed denitrifying bioreactor. Ecol. Eng. 36(11): 1575–1580. doi: 10.1016/j.ecoleng.2010.03.011.

Feyereisen, G.W., and L.E. Christianson. 2015. Hydraulic flow characteristics of agricultural residues for denitrifying bioreactor media. Appl. Eng. Agric. doi: 10.13031/aea.31.10552.

Feyereisen, G.W., T.B. Moorman, L.E. Christianson, R.T. Venterea, J.A. Coulter, et al. 2016. Performance of Agricultural Residue Media in Laboratory Denitrifying Bioreactors at Low Temperatures. J. Environ. Qual. 45(3): 779–787. doi: 10.2134/jeq2015.07.0407.

Ghane, E., N.R. Fausey, and L.C. Brown. 2015. Modeling nitrate removal in a denitrification bed. Water Res. 71: 294–305. doi: 10.1016/j.watres.2014.10.039.

Ghane, E., G.W. Feyereisen, C.J. Rosen, and U.W. Tschirner. 2018. Carbon quality of four-year-old woodchips in a denitrification bed treating agricultural drainage water. Trans. ASABE 61(3): 995–1000. doi: 10.13031/trans.12642.

Greenan, C.M., T.B. Moorman, T.B. Parkin, T.C. Kaspar, and D.B. Jaynes. 2009. Denitrification in Wood Chip Bioreactors at Different Water Flows. J. Environ. Qual. 38(4): 1664–1671. doi: 10.2134/jeq2008.0413.

Harrell Jr, F.E., with contributions from Charles Dupont and many others. 2020. Hmisc: Harrell Miscellaneous. R package version 4.4-0. https://CRAN.R-project.org/package=Hmisc

Hebbali, A. 2020. olsrr: Tools for Building OLS Regression Models. R package version 0.5.3. https://CRAN.R-project.org/package=olsrr

Hoover, N.L., A. Bhandari, M.L. Soupir, and T.B. Moorman. 2016. Woodchip Denitrification Bioreactors: Impact of Temperature and Hydraulic Retention Time on Nitrate Removal. J. Environ. Qual. doi: 10.2134/jeq2015.03.0161.

Hoover, N.L., M.L. Soupir, R.D. VanDePol, T.R. Goode, and J.Y. Law. 2017. Pilot-scale denitrification bioreactors for replicated field research. Appl. Eng. Agric. doi: 10.13031/aea.11736.

Ikenberry, C.D., M.L. Soupir, K.E. Schilling, C.S. Jones, and A. Seeman. 2014. Nitrate-Nitrogen Export: Magnitude and Patterns from Drainage Districts to Downstream River Basins. J. Environ. Qual. doi: 10.2134/jeq2014.05.0242.

Iowa Department of Agriculture and Land Stewardship, Iowa Department of Natural Resources, and Iowa State University College of Agriculture and Life Sciences. 2017. Iowa Nutrient Reduction Strategy: A Science and Technology-Based Framework to Assess and Reduce Nutrients to Iowa Waters and the Gulf of Mexico.

Jang, J., E.L. Anderson, R.T. Venterea, M.J. Sadowsky, C.J. Rosen, et al. 2019. Denitrifying bacteria active in woodchip bioreactors at low-temperature conditions. Front. Microbiol. 10(APR): 635. doi: 10.3389/fmicb.2019.00635.

Jansen, S., R. Stuurman, W. Chardon, S. Ball, J. Rozemeijer, et al. 2019. Passive Dosing of Organic Substrates for Nitrate-Removing Bioreactors Applied in Field Margins. J. Environ. Qual. 48(2): 394–402. doi: 10.2134/jeq2018.04.0165.

Janusz, G., A. Pawlik, J. Sulej, U. Świderska-Burek, A. Jarosz-Wilkolazka, et al. 2017. Lignin degradation: Microorganisms, enzymes involved, genomes analysis and evolution. FEMS Microbiol. Rev. 41(6): 941–962. doi: 10.1093/femsre/fux049.

Kassambara, A. 2020. rstatix: Pipe-Friendly Framework for Basic Statistical Tests. R package version 0.5.0. https://CRAN.R-project.org/package=rstatix

Kaspar, T.C., D.B. Jaynes, T.B. Parkin, and T.B. Moorman. 2007. Rye Cover Crop and Gamagrass Strip Effects on NO3 Concentration and Load in Tile Drainage. J. Environ. Qual. 36(5): 1503–1511. doi: 10.2134/jeq2006.0468.

Kaspar, T.C., D.B. Jaynes, T.B. Parkin, T.B. Moorman, and J.W. Singer. 2012. Effectiveness of oat and rye cover crops in reducing nitrate losses in drainage water. Agric. Water Manag. 110: 25–33. doi: 10.1016/j.agwat.2012.03.010.

Kraft, B., M. Strous, and H.E. Tegetmeyer. 2011. Microbial nitrate respiration – Genes, enzymes and environmental distribution. J. Biotechnol. 155(1): 104–117. doi: 10.1016/J.JBIOTEC.2010.12.025.

Kuypers, M.M.M., H.K. Marchant, and B. Kartal. 2018. The microbial nitrogen-cycling network. Nat. Rev. Microbiol. 16(5): 263–276. doi: 10.1038/nrmicro.2018.9.

Liu, T., R.J.F. Bruins, and M.T. Heberling. 2018. Factors influencing farmers' adoption of best management practices: A review and synthesis. Sustain. 10(2): 432. doi: 10.3390/su10020432.

O'Dell, J.W., 1993. Method 353.2 Determination of nitrate-nitrite nitrogen by automated colorimetry, EPA Doc. #. USEPA Environmental Monitoring Systems Lab, Cincinnati, OH.

Martin, E.A., M.P. Davis, T.B. Moorman, T.M. Isenhart, and M.L. Soupir. 2019. Impact of hydraulic residence time on nitrate removal in pilot-scale woodchip bioreactors. J. Environ. Manage. 237(3): 424–432. doi: 10.1016/j.jenvman.2019.01.025.

Mattila, H.K., M. Mäkinen, and T. Lundell. 2020. Hypoxia is regulating enzymatic wood decomposition and intracellular carbohydrate metabolism in filamentous white rot fungus. Biotechnol. Biofuels 13(1). doi: 10.1186/s13068-020-01677-0.

Melillo, J.M., R.J. Naiman, J.D. Aber, and A.E. Linkins. 1984. Factors controlling mass loss and nitrogen dynamics of plant litter decaying in northern streams. Bull. Mar. Sci. 35(3): 341–356.

Metcalf, W., and C. Eddy. 2003. Metcalf and Eddy Wastewater Engineering: Treatment and Reuse. Wastewater Eng. Treat. Reuse McGraw Hill. New York, NY.

Mississippi River/Gulf of Mexico Watershed Nutrient Task Force. 2008. Gulf Hypoxia Action Plan 2008 for Reducing, Mitigating, and Controlling Hypoxia in the Northern Gulf of Mexico and Improving Water Quality in the Mississippi River Basin. Washington, D.C.

Mitsch, W.J., J.W. Day, J. Wendell Gilliam, P.M. Groffman, D.L. Hey, et al. 2001. Reducing Nitrogen Loading to the Gulf of Mexico from the Mississippi River Basin: Strategies to Counter a Persistent Ecological Problem. Bioscience 51(5): 373. doi: 10.1641/0006-3568(2001)051[0373:rnlttg]2.0.co;2.

Moorman, T.B., T.B. Parkin, T.C. Kaspar, and D.B. Jaynes. 2010. Denitrification activity, wood loss, and N2O emissions over 9 years from a wood chip bioreactor. Ecol. Eng. 36(11): 1567–1574. doi: 10.1016/j.ecoleng.2010.03.012.

Oviatt, C., P. Doering, B. Nowicki, L. Reed, J. Cole, et al. 1995. An ecosystem level experiment on nutrient limitation in temperate coastal marine environments. Mar. Ecol. Prog. Ser. 116(1–3): 171–180. doi: 10.3354/meps116171.

Pfaff, J., Hautman, D., Munch, D., 1997. Method 300.1 Determination of Inorganic Anions by Ion Chromatography, EPA Doc. #. USEPA Evnironmental Monitoring Systems Lab, Cincinnati, OH.

Porter, M.D., J.M. Andrus, N.A. Bartolerio, L.F. Rodriguez, Y. Zhang, et al. 2015. Seasonal Patterns in Microbial Community Composition in Denitrifying Bioreactors Treating Subsurface Agricultural Drainage. Microb. Ecol. 70(3): 710–723. doi: 10.1007/s00248-015-0605-8.

Qi, Z., M.J. Helmers, R.D. Christianson, and C.H. Pederson. 2011. Nitrate-Nitrogen Losses through Subsurface Drainage under Various Agricultural Land Covers. J. Environ. Qual. 40(5): 1578–1585. doi: 10.2134/jeq2011.0151.

Rabalais, N.N., R.E. Turner, and W.J. Wiseman. 2002. GULF OF MEXICO HYPOXIA, THE DEAD ZONE; Annu. Rev. Ecol. Syst 33: 235–63. doi: 10.1146/annurev.ecolsys.33.010802.150513.

Randall, G.W., J.A. Vetsch, and J.R. Huffman. 2003. Nitrate Losses in Subsurface Drainage from a Corn-Soybean Rotation as Affected by Time of Nitrogen Application and Use of Nitrapyrin. J. Environ. Qual. 32(5): 1764–1772. doi: 10.2134/jeq2003.1764.

R Core Team. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Robertson, W.D. 2010. Nitrate removal rates in woodchip media of varying age. Ecol. Eng. 36(11): 1581–1587. doi: 10.1016/j.ecoleng.2010.01.008.

Robertson, W.D., D.W. Blowes, C.J. Ptacek, and J.A. Cherry. 2000. Long-term performance of in situ reactive barriers for nitrate remediation. Ground Water 38(5): 689–695. doi: 10.1111/j.1745-6584.2000.tb02704.x.

Robertson, W.D., J.L. Vogan, and P.S. Lombardo. 2008. Nitrate removal rates in a 15-year-old permeable reactive barrier treating septic system nitrate. Gr. Water Monit. Remediat. 28(3): 65–72. doi: 10.1111/j.1745-6592.2008.00205.x.

Schilling, K.E., and M. Helmers. 2008. Effects of subsurface drainage tiles on streamflow in Iowa agricultural watersheds: Exploratory hydrograph analysis. Hydrol. Process. 22(23): 4497–4506. doi: 10.1002/hyp.7052.

Schipper, L.A., W.D. Robertson, A.J. Gold, D.B. Jaynes, and S.C. Cameron. 2010a. Denitrifying bioreactors—An approach for reducing nitrate loads to receiving waters. Ecol. Eng. 36(11): 1532–1543. doi: 10.1016/J.ECOLENG.2010.04.008.

Schipper, L.A., W.D. Robertson, A.J. Gold, D.B. Jaynes, and S.C. Cameron. 2010b. Denitrifying bioreactors—An approach for reducing nitrate loads to receiving waters. Ecol. Eng. 36(11): 1532–1543. doi: 10.1016/J.ECOLENG.2010.04.008.

Ta, C.T., and W.J. Brignal. 1998. Application of computational fluid dynamics technique to storage reservoir studies. Water Sci. Technol. doi: 10.2166/wst.1998.0143.

Tavzes, C., F. Pohleven, and R.J. Koestler. 2001. Effect of anoxic conditions on wood-decay fungi treated with argon or nitrogen. Int. Biodeterior. Biodegrad. 47(4): 225–231. doi: 10.1016/S0964-8305(01)00096-8.

Tesoriero, A.J., H. Liebscher, and S.E. Cox. 2000. Mechanism and rate of denitrification in an agricultural watershed: Electron and mass balance along groundwater flow paths. Water Resour. Res. 36(6): 1545–1559. doi: 10.1029/2000WR900035.

Tomer, M.D., K.E. Schilling, C.A. Cambardella, P. Jacobson, and P. Drobney. 2010. Groundwater nutrient concentrations during prairie reconstruction on an Iowa landscape. Ecosyst. Environ. 139: 206–213. doi: 10.1016/j.agee.2010.08.003.

Warneke, S., L.A. Schipper, D.A. Bruesewitz, I. McDonald, and S. Cameron. 2011. Rates, controls and potential adverse effects of nitrate removal in a denitrification bed. Ecol. Eng. doi: 10.1016/j.ecoleng.2010.12.006.

Wickham, H., Averick, M., Bryan, J., et al., 2019. Welcome to the tidyverse. Journal of Open Source Software, 4(43), 1686, https://doi.org/10.21105/joss.01686

Yao, Z., L. Yang, F. Wang, L. Tian, N. Song, et al. 2020. Journal Pre-proofs Enhanced nitrate removal from surface water in a denitrifying woodchip bio-reactor with a heterotrophic nitrifying and aerobic denitrifying fungus. doi: 10.1016/j.biortech.2020.122948.

APPENDIX A. SUPPLEMENTAL MATERIAL

Figure 2.6 The concrete-lined trenches were filled to the top with woodchips during installation in 2014.

Table 2.4 Hydraulic properties of nine denitrifying bioreactors before and after two years of operation.

Bioreactor No. (HRT, h)	1 (16)	2 (2)	3 (8)	4 (8)	5 (16)	6 (2)	7 (8)	8 (2)	9 (16)
Tracer Residence Time (h)	5.1	5.6	5.1	6.1	6.7	5.7	4.0	4.0	5.4
MDI	3.0	2.9	3.4	3.3	3.9	3.3	3.8	3.8	2.7
Short Circuiting	0.69	0.66	0.66	0.65	0.61	0.68	0.69	0.65	0.67
Drainable Porosity	0.47	0.41	0.45	0.43	0.46	0.47	0.45	-	0.44
Hydraulic Efficiency (λ)	0.72	0.77	0.65	0.65	0.60	0.70	0.75	0.67	0.80

CHAPTER 3. MICROBIAL COMMUNITY AND DENITRIFYING GENES IN REPLICATED WOODCHIP BIOREACTORS

Abby Schaefer[a], Tom Moorman[b], Michelle Soupir[c], Adina Howe[d]

[a]Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, IA 50011, USA

[b]USDA-ARS National Laboratory for Agriculture and the Environment, 2110 University Boulevard, Ames, IA 50011, USA

[c]Iowa State University, Water Quality Research Lab, Dept. of Agricultural and Biosystems Engineering, 3358 Elings Hall, USA

[d]Iowa State University, Genomics and Environmental Research in Microbial Systems Lab, Dept. of Agricultural and Biosystems Engineering, 3346 Elings Hall, USA

Abstract

The microbial community inhabiting woodchip bioreactors is under-investigated at the pilot scale. In this study our goals were to understand the factors that influence woodchip bioreactor microbial community variation, investigate the factors that drive microbial community composition, and understand the link between the microbial community and denitrification. We used a combination of qPCR of *nirS*, *nirK*, *nosZ*I, and *nosZ*II and 16S amplicon sequencing to characterize the microbial communities of nine, pilot-scale woodchip bioreactors located at Iowa State University. Our results showed dynamic microbial communities but with persistent taxa between sampling years and hydraulic retention times (HRTs). Similarities between functional gene copy numbers across sampling year and HRT indicate that the function of denitrification is conserved despite differences in the microbial communities.

Introduction

Denitrifying bioreactors (also called woodchip bioreactors) are an edge-of-field practice that have been proven effective to reduce non-point source (NPS) nitrogen (N) loads to receiving surface waterbodies in the Upper Mississippi River Basin, Chesapeake Bay area, and Florida (Christianson et al., 2017; Easton et al., 2019; Pluer et al., 2018). Since the early 2010s, literature describing the performance of these systems has increased exponentially, with the primary focus being engineering design and optimizing nitrate removal characteristics. Denitrifying bioreactors have been shown to consistently and efficiently reduce NPS N loads by 60-80% (mass-basis) (Christianson et al., 2012).

Research has focused on the optimal influent nitrate concentration, bioreactor temperature, HRT, and media type, and the influence of these characteristics on performance is well-characterized. Briefly, increased influent nitrate concentration can improve nitrate removal rates; higher water temperatures also improve nitrate removal (Addy et al., 2016; Hoover et al., 2016). Longer HRTs lead to higher concentration reductions, with the tradeoff of excess production of harmful by-products such as methane, nitrous oxide, and methylmercury (Davis et al., 2019; Healy et al., 2012). While some forms of carbonaceous media provide higher initial substrate availability, woodchips are relatively recalcitrant and thus can provide stable nitrate removal rate due to their recalcitrance (Feyereisen et al., 2016; Robertson, 2010). Although engineering considerations have led to improved denitrifying bioreactor performance, bioreactor performance is limited in our understanding the microbial communities that directly drive denitrification and cellulose and lignin degradation. Particularly, it is unclear if representative denitrifying communities in bioreactors exist under varying bioreactor management, different times, and different installations exist. The rationale for identifying representative microbial

communities in bioreactors is that these microbes could be considered as keystone species and as central to optimizing both bioreactor performance for denitrification and management.

Generally, studies of bioreactor microbiomes focus on characterizing the community membership by identifying phylogenetic markers (e.g., 16S rRNA genes) or denitrification genes and linking these to observed denitrification rates and bioreactor performance. Previous studies have identified potential relationships between bioreactor performance and its microbiome. Temperature and substrate influence the abundance of functional denitrifying genes, with high temperatures selecting against *nirK* or selecting for *nirS* and *nosZ* (Warneke et al., 2011). The selection of bioreactor substrates influences denitrification, with woodchips having the highest proportion of indicators of nitrite reductase relative to maize cobs, wheat straw, green waste, or sawdust (Warneke et al., 2011). Influent nutrient concentrations and temperature also influence the denitrifying bioreactor microbial community. At low nitrate concentrations, microorganisms from the order Pseudomonadales appear to be important for denitrification, while members of Rhodocyclales and Rhizobiales appear important at higher nitrate concentrations (Grießmeier et al., 2017). *Pseudomonas* spp., *Cellulomonas* spp., and *Polaramonas* spp. have been identified as important denitrifying bacteria within woodchip bioreactors operating at low temperatures, with certain strains of *Cellulomonas* able to perform cellulose degradation (Jang et al., 2019). Other key factors that can play a role in structuring the denitrifying bioreactor microbial community are substrate moisture content and water saturation levels. Unsaturated and saturated portions of bioreactor were observed to form distinct microbial communities without an impact on denitrification performance (Hathaway et al., 2017; Porter et al., 2015). Overall, these observations indicate synergy between carbon-degrading microorganisms, denitrifying microorganisms, and bioreactor performance.

Comparing bioreactors and their associated microbiomes between studies is challenging due to differences in environmental or field conditions associated with varying installations. To overcome these challenges, we have installed replicated denitrifying woodchip bioreactors at the same site. These bioreactors were all installed in parallel in 2016 and receive similar inputs from a single county tile line. To evaluate the impact of bioreactor design on bioreactor microbiomes, three hydraulic retention times (HRTs, 2, 8, and 16 h) were tested in triplicate at this site. Additional samples were also taken two years after operation for comparison of microbiomes. This study is expanding our previous findings that these denitrifying bioreactors have shown significant differences in oxygen level, substrate C:N ratio, nitrate concentration, and greenhouse gas production between HRTs (Schaefer et al., 2021; Martin et al., 2019; Davis et al., 2019). These previous evaluations were focused mainly on operational characteristics of the bioreactor, in this study, we characterize these bioreactors for their microbial communities, assessing both the community membership and the presence of genes encoding denitrification activity. The specific goal of this study is to characterize the persistent membership in bioreactor microbiomes and understand the factors that influence this microbial membership.

Methods

Bioreactors

The replicate woodchip bioreactors at the Iowa State University (ISU) Agronomy and Agricultural Engineering Research Farm (42.019861, −93.776872) are part of a long-term monitoring study of denitrifying bioreactors. The site is described extensively in Hoover et al., 2017. Briefly, nine woodchip bioreactors were installed in parallel in September 2014. Each bioreactor consists of a concrete trench filled with a mixture of hardwood chips obtained from

Golden Valley Hardscapes, Story City, Iowa, USA. A 3,000-gallon storage tank intercepts a nearby 12-inch diameter county tile line and an underground storage cistern supplies water to the bioreactors. Each bioreactor is fitted with its own influent control structure and dosing port. The outlet structures were fitted with stoplog drainage control structures which can be adjusted to change the active volume of the bioreactors. Three HRTs (2, 8, and 16 h) are controlled in triplicate at this site. The bioreactors were saturated throughout the year, with the flow through the bioreactors occurring during the sampling season. The bioreactors remained saturated during winter months to inhibit rodents from bedding into the woodchips.

Woodchips of the same type as those used for the original bioreactor fill material were used to fill hand-sewn mesh bags with 30 g woodchips. The bags were weighted with marbles and submerged in the monitoring wells of the bioreactors. These woodchip bags were destructively sampled in September 2016 and June 2018 for DNA extraction and microbial community analysis. Woodchips were kept on ice during transportation to the lab and stored in the cooler prior to analysis. The wet weight and moisture content of the woodchips was determined so that all gene copy numbers could be reported on a per gram of dry woodchips basis.

DNA Extraction and Quantification

DNA was extracted from the woodchips with Powersoil 96 Well DNA Isolation kits (Qiagen) using 0.25 g of woodchips. DNA extracted from known strains were used as positive controls. *Pseudomonas stutzeri* (ATCC 14405) was used as a standard for targeted nitrogen cycling genes. A culture of *P. stutzeri* was grown overnight in marine broth and DNA was extracted (Qiagen's QIAamp DNA Mini Kit). Then, qPCR was performed with extracted DNA and *nosZ*1 primers with and without SYBR-Green. The PCR products without SYBR-Green

(used Qiagen HotStart Taq Plus* and increased the MgCl2 concentration by 1.0 mM in the reaction) were cloned into pCR-4TOPO vector followed by transformation of One Shot TOP10 chemically competent *Escherichia coli* using the TOPO TA cloning kit (Invitrogen Cat No K4575-01). The resulting clones were tested by extracting plasmid DNA (5 PRIME FastPlasmid Mini-Prep Kit) and then performing qPCR and gel electrophoresis to determine plasmid size.

A similar procedure was used to construct plasmids containing the PCR product amplified by *nosZ*II primers. In this case, the *nosZ* DNA was amplified from *Geobacillus thermodenitrificans* (ATCC 29492). These plasmids containing their respective *nosZ* gene fragments were utilized as standards. By knowing the size of the ratio of the inserted gene fragment to the total size of the plasmid, DNA concentrations can be converted into gene copy numbers.

Each qPCR run included positive standards (dilutions of plasmid DNA), and negative standards: water as template (no DNA) or *E. coli* (ATCC 43651) genomic DNA. Each dilution of standard, and each negative control were prepared in triplicate wells of the 96 well PCR plate. Before dilution of the standards, the DNA concentration was determined using an Eppendorf Biophotometer. Primer sequences and thermal cycling conditions are listed in Appendix B.

16S rRNA Amplicon Sequencing

Extracted DNA was quantified using the Qubit Fluorometer with the Qubit dsDNA assay (Thermo Science Scientific, Waltham, MA, USA). The 16S V4 amplification primers were used to amplify the V4 region of the 16S small subunit rRNA. 16S rRNA genes and sequenced

on the Illumina MiSeq with 150 bp paired-end libraries at Argonne National Laboratory (Argonne, IL, USA). Taxa were identified as amplicon sequence variants using the default dada2 pipeline. Taxonomic assignment of OTUs was completed by alignment to the most similar representative gene in the Silva 16S ribosomal database (version 123).

Data Analysis

Gene abundances were estimated as copy numbers per gram of substrate and were normalized to observed total 16S rRNA gene copies. The ratio of total *nosZ* copy numbers to total nitrite reductase gene copy numbers (both normalized to 16S rRNA gene copy numbers) was calculated to determine the genetic potential for complete nitrate reduction. Statistical comparisons were performed with 3-way ANOVA and with year, HRT, and sampling location as factors.

Dissimilarities in the composition of sample microbial communities were visualized by Principal Coordinates Analysis (PCoA) ordination of the Bray-Curtis distance between samples. PCoA ordinations were performed using the Phyloseq package in R. The adonis() function from the R package “vegan” was used to perform a non-parametric multivariate analysis of variance test of the null hypothesis that there is no difference in the composition of microbial communities between samples.

Results

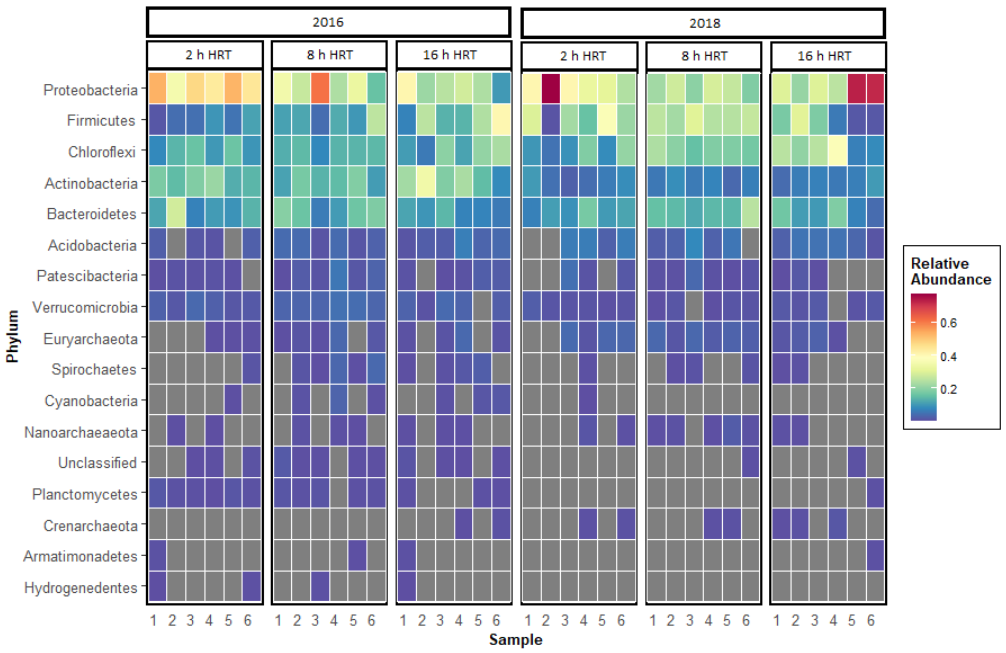
All nine bioreactors in this study received drainage water from a homogenized source (Hoover et al., 2017) and as a result, the main uncontrolled variables were nitrate loading rate and flow rate. To evaluate the bioreactor microbiomes under these operating conditions, samples were obtained for three HRTs and two sampling years, and the bioreactor microbiome was characterized through sequencing of 16S rRNA genes, a highly conserved

phylogenetic marker among bacteria. Generally, the most abundant taxa identified in bioreactor microbiomes were associated with 5 phyla and included Proteobacteria (36%), Firmicutes (14%), Chloroflexi (14%), Bacteroidetes (14%), and Actinobacteria (10%) (percentages represent proportions of the total abundances from all 36 samples). The most abundant taxa identified by HRT and by year were compared (Figure 3.1). These results indicate the presence of similar phylum between bioreactors with different HRTs and also from different years. Specifically, abundant phyla (> 10%), including Proteobacteria, Firmicutes, and Chloroflexi tend to be shared between both operating conditions and sampling years. Less abundant phyla (5% - 10%) are present in multiple samples but not as consistently identified across all samples.

The bioreactor communities observed in each sample were compared through a distance-based ordination method of the Bray-Curtis dissimilarity of observed taxa in each sample (Figure 3.2). These results show that the microbial community response in replicate denitrifying bioreactors is dynamic, with distinct communities forming between years and HRTs (NPMANOVA, $p < 0.05$; Table 3.1). The separation of samples by woodchip bioreactor management (i.e., HRT) is indicative of its influence on the microbial community (NPMANOVA, $p < 0.05$; Table 3.1). The year of sampling and HRT explained 15% and 9% of the variance observed, respectively.

Our observations of similar abundant phylum in our samples indicates a group of persistent taxa between years and HRTs (Figure 3.1). The significant differences observed in bioreactor microbiomes between HRTs and years indicates that while abundant phyla may persist between bioreactors, the presence of less abundant taxa or the abundance distribution of taxa can distinguish microbiomes from different HRTs and years.

A.



B.

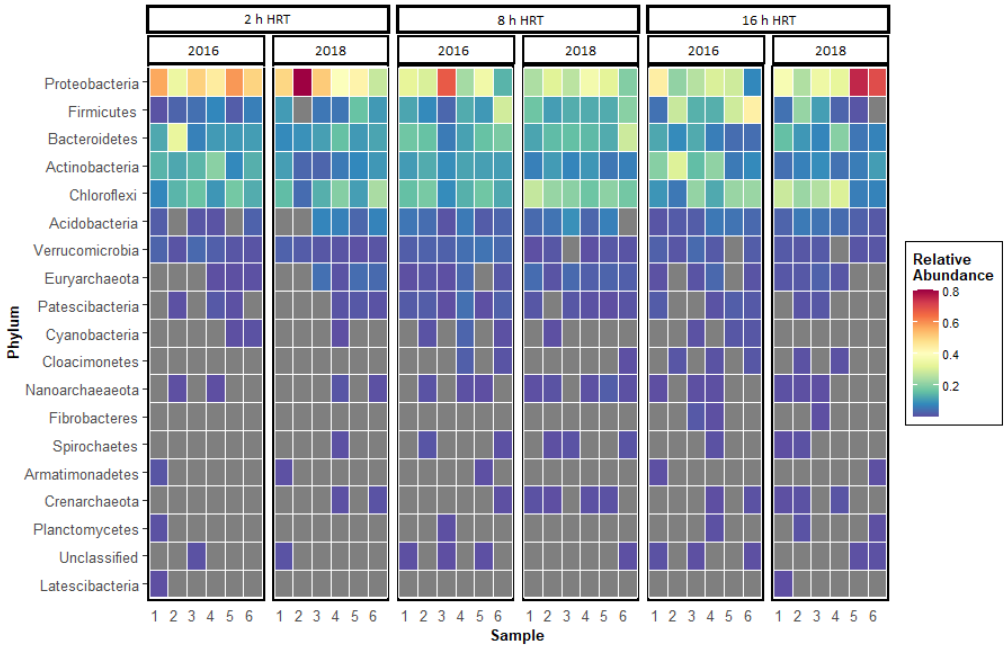


Figure 3.1 Relative abundances of taxa A) in both years grouped by HRT and B) in all HRTs grouped by years. For both A and B, dominant phyla included Proteobacteria, Firmicutes, and Chloroflexi.

Table 3.1 NPMANOVA results from the Bray-Curtis dissimilarities. Non-parametric analysis of variance of the effect of sampling year, location, and HRT on community dissimilarities.

	Df	SumofSqs	MeanSqs	F.Model	R^2	Pr(>F)
Year	1	1.5322	1.53217	6.2956	0.1468	0.001
Location	1	0.3962	0.39619	1.6279	0.03796	0.058
HRT	2	0.9640	0.48199	1.9805	0.09236	0.002
Residuals	31	7.5445	0.24337		0.72287	
Total	35	10.4368			1.00000	

To evaluate the impacts of abundance and persistence, we evaluated the average relative abundance of each taxa and its persistence across all samples (fraction of samples in which each taxon was observed). For samples from both 2016 and 2018 taxa identified in both groups of samples were found at both high occupancy and high abundance and low occupancy and lower abundance (Figure 3.3). Similar patterns were observed in samples from 2, 8, and 16 h HRTs. Overall, we observed that the average abundance and occupancy of common taxa was 87 ± 206 and 4 ± 4 between years and 77 ± 155 and 3 ± 2 between HRTs, respectively.

Our previous work in this system has shown that the chemical composition of the woodchip substrate varies among HRTs and across years (Schaefer et al. 2021). Therefore, our observed differences in the microbial community between HRT groups (and differences based on the degree of woodchip decomposition) are parallel to other studies that have shown differences in microbial community composition based on substrate type (Hellman et al., 2020).

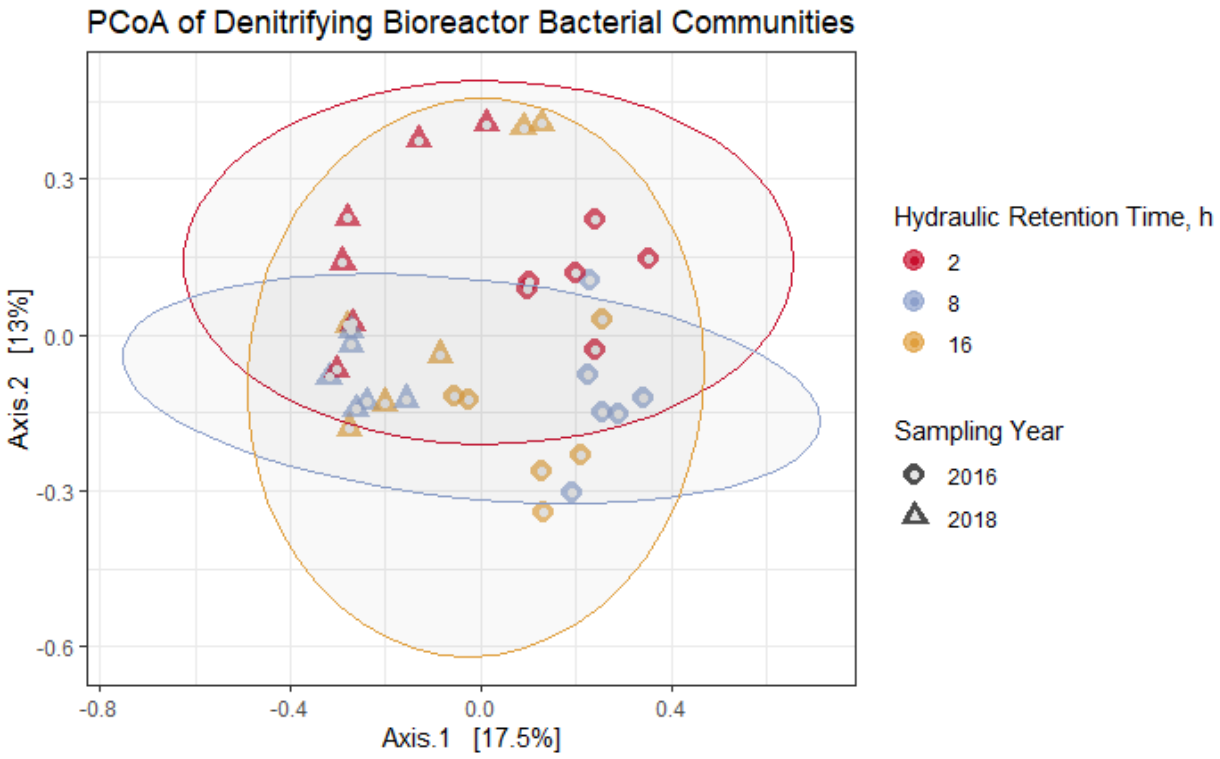
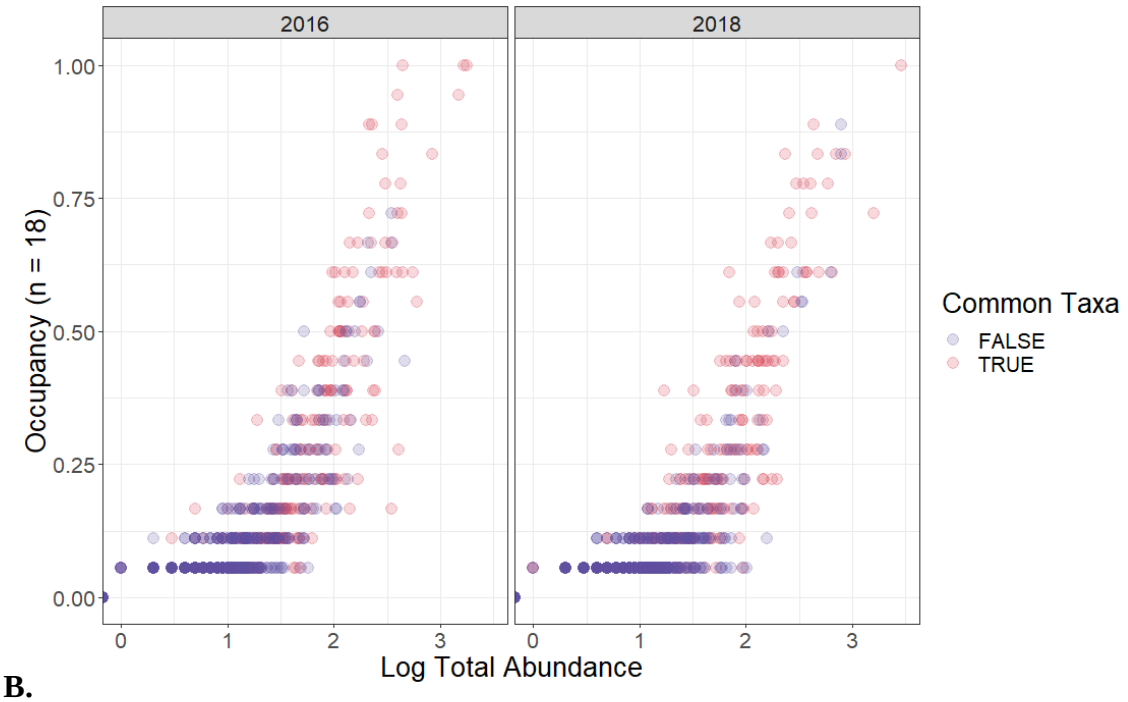


Figure 3.2 PCoA of Bray-Curtis dissimilarity of bioreactor microbiome samples, with color indicating HRT and shape indicating sampling year. Figure shows that the community within the 8 h HRT bioreactor is less variable between years, which suggests the NRCS design standard of 4-8 h HRT provides optimal conditions for the microorganisms.

Previous work on the nitrate mass removal rate (MRR) over the same study period also showed statistical differences between MRRs at different HRTs (Martin et al., 2019). Our observations are consistent with these results because of the observed differences in bioreactor microbiomes between HRTs (Figure 3.2). Additionally, we also quantified copies of nitrogen cycling genes, specifically, *nirS* and *nirK* genes, in our samples. The concentrations of these genes were not statistically different between years or HRTs (3-way ANOVA, $p > 0.05$; Figure 3.4).

A.



B.

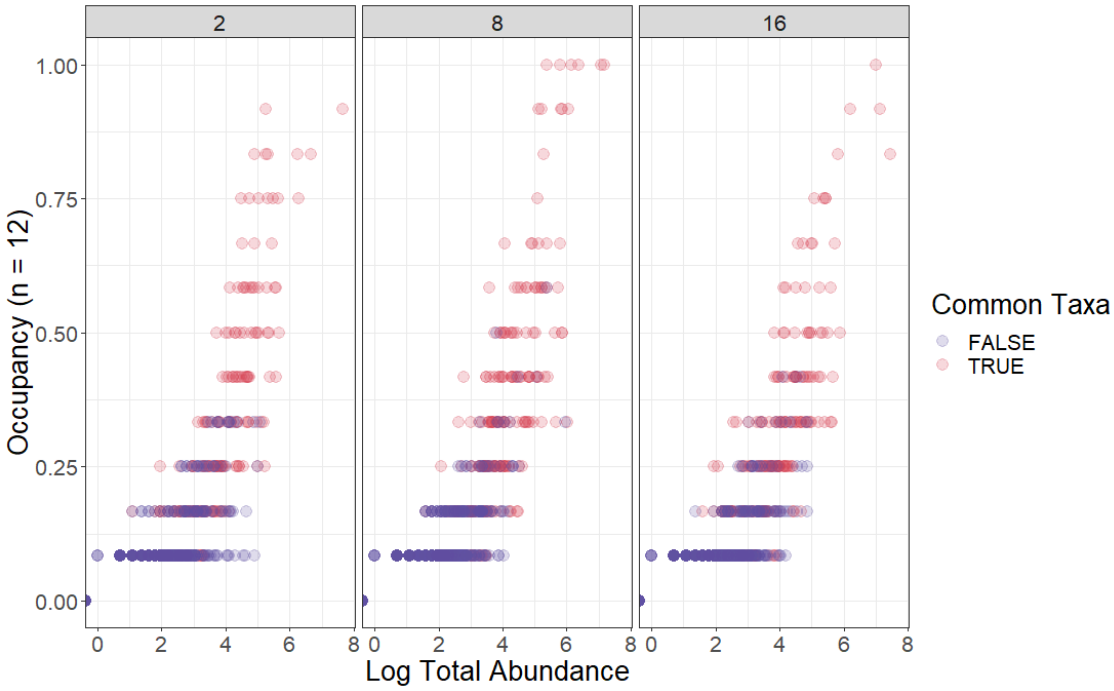


Figure 3.3 Abundance-occupancy plots A) grouped by year. B) grouped by HRT. Occupancy corresponds to the number of samples a taxon was detected in. Abundance corresponds to the total number of times a taxon was detected in any sample.

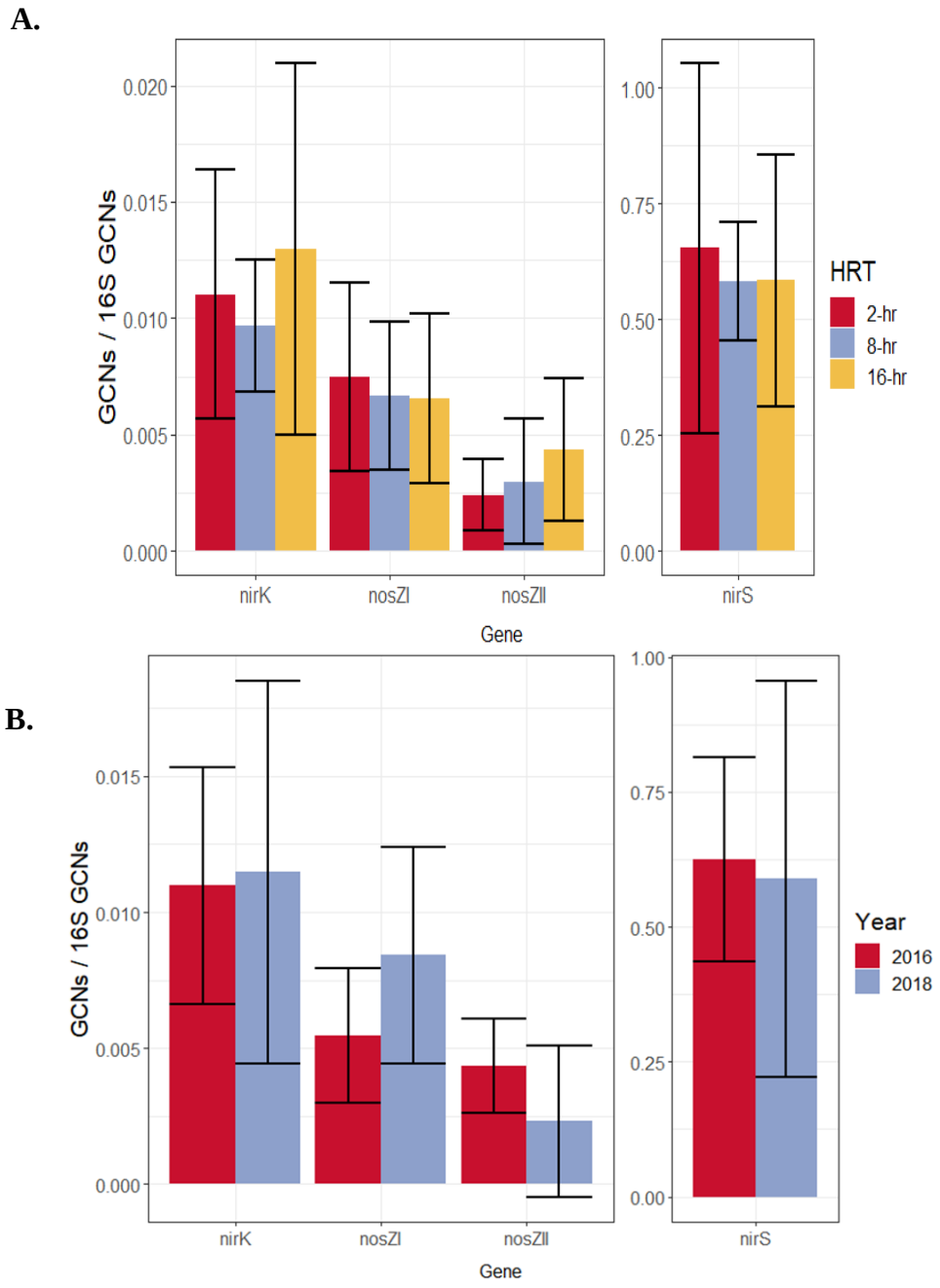


Figure 3.4 Ratio of functional gene copy numbers to 16S rRNA gene copy numbers for each HRT and B) for each year.

Therefore, we do not find evidence that differences in the targeted *nirS* and *nirK* genes influence the observed differences in MRR at the pilot scale or the differences observed in the total microbial communities. Similarly, we estimated the abundances

of *nosZ* genes (clade I and clade II) in both years (3-way ANOVA, $p < 0.05$). Our results also did not show significant differences in the ratio of nitrous oxide reduction potential to nitrite reduction potential across year and HRT (Figure 3.5).

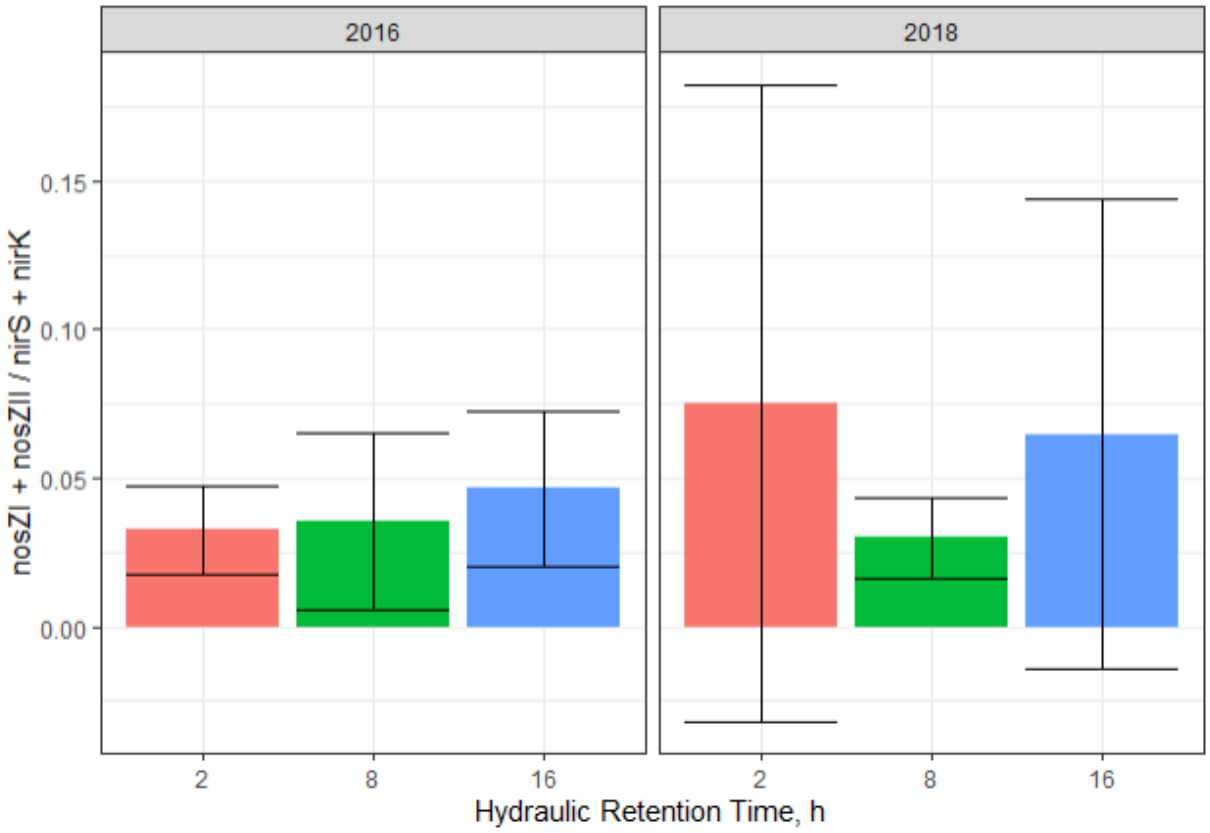


Figure 3.5 Ratio of total *nosZ* copy numbers to the sum of *nirS* and *nirK* copy numbers.

Discussion

There were three primary goals of this study. The first goal was to understand the variation among microbial communities in nine replicated denitrifying bioreactors. We observed that there was a higher relative abundance of Proteobacteria in the 2 h HRT bioreactors compared to other HRTs for both years (data not shown). Proteobacteria contain the phylum Pseudomonas, which has been implicated as a major driver of denitrification in terrestrial

systems. The higher relative abundance of Proteobacteria in the 2 h HRT bioreactors may be linked to the higher MRR of denitrification observed in this system over the same two-year study period (Martin et al., 2019). However, the 16 h had the highest concentration reduction of nitrate-nitrogen, suggesting that measurement of the proportion of potentially-denitrifying bacteria alone might not be the best predictor of denitrifying bioreactor performance.

The high occupancies of both Pseudomonas and Christensenellaceae support the idea that the microbial communities within denitrifying woodchip bioreactors strike a balance between denitrification and substrate decomposition. *Christensenella* is a saccharolytic genus that has been shown to be able to produce acetic acid as the end product of glucose fermentation. *Christensenella* could be potentially important for providing the electron donor for the denitrification process; acetic acid has been used as an additive to enhance denitrification in biological nutrient removal at drinking water plants. Acetic acid may also be used as the terminal electron acceptor in methanogenesis; between 2016 and 2018 these denitrifying bioreactors produced 0.5 – 1.7 g CH_4 m^{-3} d^{-1} (Davis, 2019).

Cellulomonas are a member of the Actinobacteria that possess cellulose-degrading enzymes. *Cellulomonas* were detected in 89% of samples in 2016 but only 44% of samples in 2018. This is potentially the result of changes in the woodchip substrate over time; during the study period substrate C:N ratio declined and LCIs increased indicating an increase in substrate recalcitrance.

We also observed similarities in functional gene copy numbers for 2016 and 2018. The lack of statistical differences between *nirS* and *nirK* copy numbers normalized to 16S gene copy numbers indicates that despite differences in the overall microbial communities between years, the function of denitrification is conserved. The increase in proportion of *nosZ* clade I gene copy

numbers from 2016 to 2018 and simultaneous decrease in proportion of *nosZ* clade II gene copy numbers is of note; however, work spanning a portion of this study period showed that sampling year was not a significant factor for nitrous oxide production (Davis et al., 2019). Further, difficulties with *nosZ* primer design have been reported and differences in detection with *nosZ* primers used might under-estimate gene copy count in situ (Verbaendert et al., 2014). Therefore, the differences in *nosZ* copy numbers we observed may not reflect the true phenotypic potential for nitrous oxide reduction.

Previous work has shown that *nosZ*II is dominant in non-denitrifying nitrous oxide reducers, and that *nosZ* in general has high co-occurrence with the *nirS* nitrite-reducing pathway compared to the nirK pathway (Graf et al., 2014). Our results are parallel to these findings because we detected higher gene copy numbers g^{-1} substrate for *nirS* than *nirK* and previous work with these systems has shown that nitrous oxide emissions account for ~5% or less of the total mass of nitrate-nitrogen removed (Davis et al., 2019). Typically, it is thought that non-detection of *nosZ* genes is correlated with higher nitrous oxide emissions, however there is evidence that shows the majority of *nosZ* genes present in the environment are atypical and would not be detected with conventional primers. The potential for nitrous oxide reduction compared to the potential for denitrification remained consistent across years, HRTs, and location within the bioreactor. This suggests that the HRT design criterion does not significantly impact either the selection for nitrous-oxide reducers over denitrifiers or the detection of these genes.

The second goal of this study was to understand the factors that influence microbial community composition. Other studies have shown that the main factors controlling denitrification within denitrifying bioreactors are substrate availability and

temperature (Warneke et al., 2011). Within the experimental set up described here, we had the opportunity to examine the impact of HRT on the denitrifying microbial community. Based on our results, it appears that a major contributing factor to the microbial community structure is sampling year. The principal coordinates analysis appears to show that time best explains the variation in our samples, compared to HRT or location, or the combination of both. Others have also shown this and hypothesized that unknown succession mechanisms play a role in the microbial community structure during bioreactor start up and the subsequent operating period. There also appears to be a strong link between substrate availability and denitrification rate, based on high-frequency monitoring work (Maxwell et al., 2019).

Previous work with the system at the ISU research farm has shown that in addition to the flow conditions, the influent nitrate loads are statistically different between bioreactors operating at different HRTs. Consequently, differential substrate degradation occurs, with the bioreactors receiving a higher influent nitrate load being more conducive to rapid substrate degradation (Schaefer et al., 2021). It is therefore possible that we would see differences in the bioreactor microbial communities based on the stage of woodchip decomposition.

As the woodchips are consumed through denitrification and other processes (aerobic respiration, etc.), we might expect to see a shift from microorganisms that readily degrade cellulose to those that degrade more recalcitrant woodchip components including lignin. However, samples from the same year were more comparable than those from different years but the same HRT. As a caveat to this point, the woodchips sampled for DNA extraction and subsequent analysis were finely ground and located within the sampling wells, and thus distinct from the woodchips previously analyzed for C:N ratio and LCI. The rate of degradation of these finer woodchips is unknown, and though we did not report preferential woodchip degradation

based on size, others have over longer monitoring periods (Ghane et al., 2018). If the woodchip degradation within the bags is more uniform across HRTs than that measured from the excavated woodchips, that may explain the similarities between samples from different HRTs but the same year.

The third goal of this study was to understand the link between the microbial community and denitrification. In our study, the impact of microbial community differences on woodchip bioreactor performance is unclear. Previous work on the nitrate MRR over the same study period showed statistical differences between MRRs at different HRTs (Martin et al., 2019). Taken with the lack of statistical differences in *nirS* and *nirK* between years or HRTs, we do not find evidence that differences in *nirS* and *nirK* gene proportions drive the differences in MRR. These results imply that the function of denitrification is conserved despite differences in microbial community composition.

Our results imply that an environmental factor other than nitrate availability drives microbial community selection. A possible driving factor influencing denitrifying microbial community structure is substrate electron donor availability.

Conclusions

Replicate woodchip bioreactors developed distinct microbial communities over two years. This is notable due to the homogenous drainage water source for all nine woodchip bioreactors and controlled initial condition of the woodchip media. Further study of the relationship between microbial community, electron donor availability, and nitrate removal is warranted. Further investigation to the persistent microbial community between woodchip bioreactors with different management is warranted to understand factors that drive stability within these systems.

References

Addy, K., Gold, A. J., Christianson, L. E., David, M. B., Schipper, L. A., & Ratigan, N. A. (2016). Denitrifying Bioreactors for Nitrate Removal: A Meta-Analysis. *Journal of Environment Quality*, *45*(3), 873. https://doi.org/10.2134/jeq2015.07.0399

Andrus, J. M. (2011). *Microbial community analysis for denitrifying biofilters. Dissertation Abstracts International. Vol. 73, no. 01, suppl. B, 235 p. 2011.* Retrieved from https://login.proxy.lib.duke.edu/login?url=https://search.proquest.com/docview/1093459776?accountid=10598%0Ahttp://pm6mt7vg3j.search.serialssolutions.com?ctx_ver=Z39.88-2004&ctx_enc=info:ofi/enc:UTF-8&rfr_id=info:sid/Aquatic+Science+%26+Fisheries+Abstract

Arat, S., Bullerjahn, G. S., & Laubenbacher, R. (2015). A network biology approach to denitrification in Pseudomonas aeruginosa. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0118235

Averill, B. A., & Tiedje, J. M. (1982). The chemical mechanism of microbioal denitrification. *FEBS Letters*, *138*(1), 8–12. https://doi.org/10.1016/0014-5793(82)80383-9

Bakken, L. R., Bergaust, L., Liu, B., & Frostegård, Å. (2012). Regulation of denitrification at the cellular level: A clue to the understanding of N2O emissions from soils. *Philosophical Transactions of the Royal Society B: Biological Sciences*. https://doi.org/10.1098/rstb.2011.0321

Bell, N. (2013). *Characterizing Transient and Seasonal Responses of Bioreactors for Subsurface Drains, Master's Thesis*.

Christianson, L., Bhandari, A., Helmers, M., Kult, K., Sutphin, T., & Wolf, R. (2012). Performance Evaluation of Four Field-Scale Agricultural Drainage Denitrification Bioreactors in Iowa. *Transactions of the ASABE*, *55*(6). Retrieved from https://lib.dr.iastate.edu/abe_eng_pubs/307

Christianson, L. E., Bhandari, A., & Helmers, M. J. (2012). A practice-oriented review of woodchip bioreactors for subsurface agricultural drainage. *Applied Engineering in Agriculture*.

Christianson, L. E., Collick, A. S., Bryant, R. B., Rosen, T., Bock, E. M., Allen, A. L., … Easton, Z. M. (2017). Enhanced Denitrification Bioreactors Hold Promise for Mid-Atlantic Ditch Drainage. *Agricultural & Environmental Letters*, *2*(1), 170032. https://doi.org/10.2134/ael2017.09.0032

Christianson, Laura E, Bhandari, A., Asce, M., & Helmers, M. J. (2011). Pilot-Scale Evaluation of Denitrification Drainage Bioreactors: Reactor Geometry and Performance. https://doi.org/10.1061/(ASCE)EE.1943-7870.0000316

Chun, J. A., Cooke, R. A., Eheart, J. W., & Cho, J. (2010). Estimation of flow and transport parameters for woodchip-based bioreactors: II. field-scale bioreactor. *Biosystems Engineering*. https://doi.org/10.1016/j.biosystemseng.2009.09.018

Collins, K. A., Lawrence, T. J., Stander, E. K., Jontos, R. J., Kaushal, S. S., Newcomer, T. A., … Cole Ekberg, M. L. (2010). Opportunities and challenges for managing nitrogen in urban stormwater: A review and synthesis. *Ecological Engineering*, *36*(11), 1507–1519. https://doi.org/10.1016/j.ecoleng.2010.03.015

Dashtban, M., Schraft, H., Syed, T. A., & Qin, W. (2010). Fungal biodegradation and enzymatic modification of lignin. *International Journal of Biochemistry and Molecular Biology*. e-Century Publishing Corporation.

Davis, M. P., Martin, E. A., Moorman, T. B., Isenhart, T. M., & Soupir, M. L. (2019). Nitrous oxide and methane production from denitrifying woodchip bioreactors at three hydraulic residence times. *Journal of Environmental Management*, *242*, 290–297. https://doi.org/10.1016/j.jenvman.2019.04.055

De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T., & Boon, N. (2014). Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environmental Microbiology*. https://doi.org/10.1111/1462-2920.12343

Easton, Z. M., Bock, E., & Stephenson, K. (2019). Feasibility of Using Woodchip Bioreactors to Treat Legacy Nitrogen to Meet Chesapeake Bay Water Quality Goals. *Environmental Science and Technology*, *53*(21), 12291–12299. https://doi.org/10.1021/acs.est.9b04919

Fatehi-Pouladi, S., Anderson, B. C., Wootton, B., Button, M., Bissegger, S., Rozema, L., & Weber, K. P. (2019). Interstitial water microbial communities as an indicator of microbial denitrifying capacity in wood-chip bioreactors. *Science of the Total Environment*, *655*, 720–729. https://doi.org/10.1016/j.scitotenv.2018.11.278

Feyereisen, G. W., Moorman, T. B., Christianson, L. E., Venterea, R. T., Coulter, J. A., & Tschirner, U. W. (2016). Performance of Agricultural Residue Media in Laboratory Denitrifying Bioreactors at Low Temperatures. *Journal of Environmental Quality*, *45*(3), 779–787. https://doi.org/10.2134/jeq2015.07.0407

Ghane, E., Feyereisen, G. W., Rosen, C. J., & Tschirner, U. W. (2018). Carbon quality of four-year-old woodchips in a denitrification bed treating agricultural drainage water. *Transactions of the ASABE*, *61*(3), 995–1000. https://doi.org/10.13031/trans.12642

Gonze, D., Coyte, K. Z., Lahti, L., & Faust, K. (2018). Microbial communities as dynamical systems. *Current Opinion in Microbiology*. https://doi.org/10.1016/j.mib.2018.07.004

Graf, D. R. H., Jones, C. M., & Hallin, S. (2014). Intergenomic Comparisons Highlight Modularity of the Denitrification Pathway and Underpin the Importance of Community Structure for N2O Emissions. *PLoS ONE*, *9*(12), e114118. https://doi.org/10.1371/journal.pone.0114118

Grießmeier, V., Bremges, A., McHardy, A. C., & Gescher, J. (2017). Investigation of different nitrogen reduction routes and their key microbial players in wood chip-driven denitrification beds. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-17312-2

Hassanpour, B., Giri, S., Pluer, W. T., Steenhuis, T. S., & Geohring, L. D. (2017). Seasonal performance of denitrifying bioreactors in the Northeastern United States: Field trials. *Journal of Environmental Management*. https://doi.org/10.1016/j.jenvman.2017.06.054

Hathaway, S. K., Bartolerio, N. A., Rodríguez, L. F., Kent, A. D., & Zilles, J. L. (2017). Denitrifying Bioreactors Resist Disturbance from Fluctuating Water Levels. *Frontiers in Environmental Science*, *5*. https://doi.org/10.3389/fenvs.2017.00035

Hathaway, S. K., Porter, M. D., Rodríguez, L. F., Kent, A. D., & Zilles, J. L. (2015). Impact of the contemporary environment on denitrifying bacterial communities. *Ecological Engineering*. https://doi.org/10.1016/j.ecoleng.2015.05.005

Healy, M. G., Ibrahim, T. G., Lanigan, G. J., Serrenho, A. J., & Fenton, O. (2012). Nitrate removal rate, efficiency and pollution swapping potential of different organic carbon media in laboratory denitrification bioreactors. *Ecological Engineering*, *40*, 198–209. https://doi.org/10.1016/j.ecoleng.2011.12.010

Hellman, M., Hubalek, V., Juhanson, J., Almstrand, R., Peura, S., & Hallin, S. (2020). Substrate type determines microbial activity and community composition in bioreactors for nitrate removal by denitrification at low temperature. *Science of the Total Environment*, 143023. https://doi.org/10.1016/j.scitotenv.2020.143023

Hollocher, T. C. (1983). The pathway of nitrogen and reductive enzymes of denitrification. *Antonie van Leeuwenhoek*, *48*(6), 531–544. https://doi.org/10.1007/BF00399539

Hoover, N. L., Soupir, M. L., VanDePol, R. D., Goode, T. R., & Law, J. Y. (2017). Pilot-scale denitrification bioreactors for replicated field research. *Applied Engineering in Agriculture*. https://doi.org/10.13031/aea.11736

Hoover, Natasha L., Bhandari, A., Soupir, M. L., & Moorman, T. B. (2016). Woodchip Denitrification Bioreactors: Impact of Temperature and Hydraulic Retention Time on Nitrate Removal. *Journal of Environment Quality*. https://doi.org/10.2134/jeq2015.03.0161

Hua, G., Salo, M. W., Schmit, C. G., & Hay, C. H. (2016). Nitrate and phosphate removal from agricultural subsurface drainage using laboratory woodchip bioreactors and recycled steel byproduct filters. https://doi.org/10.1016/j.watres.2016.06.022

Ikenberry, C. D., Soupir, M. L., Schilling, K. E., Jones, C. S., & Seeman, A. (2014). Nitrate-Nitrogen Export: Magnitude and Patterns from Drainage Districts to Downstream River Basins. *Journal of Environment Quality*. https://doi.org/10.2134/jeq2014.05.0242

Ilhan, Z. E., Ong, S. K., & Moorman, T. B. (2011). Dissipation of Atrazine, Enrofloxacin, and Sulfamethazine in Wood Chip Bioreactors and Impact on Denitrification. *Journal of Environmental Quality*, *40*(6), 1816–1823. https://doi.org/10.2134/jeq2011.0082

Jang, J., Anderson, E. L., Venterea, R. T., Sadowsky, M. J., Rosen, C. J., Feyereisen, G. W., & Ishii, S. (2019). Denitrifying bacteria active in woodchip bioreactors at low-temperature conditions. *Frontiers in Microbiology*, *10*(APR), 635. https://doi.org/10.3389/fmicb.2019.00635

Jaynes, D. B., Moorman, T. B., Parkin, T. B., & Kaspar, T. C. (2016). Simulating Woodchip Bioreactor Performance Using a Dual-Porosity Model. *Journal of Environmental Quality*, *45*(3), 830–838. https://doi.org/10.2134/jeq2015.07.0342

Jeannotte, R. (2014). METABOLIC PATHWAYS | Nitrogen Metabolism. *Encyclopedia of Food Microbiology*, 544–560. https://doi.org/10.1016/B978-0-12-384730-0.00199-3

Johnston-Monje, D., & Lopez Mejia, J. (2020). Botanical microbiomes on the cheap: Inexpensive molecular fingerprinting methods to study plant-associated communities of bacteria and fungi. *Applications in Plant Sciences*, *8*(4). https://doi.org/10.1002/aps3.11334

Ko, J. J., Shimizu, Y., Ikeda, K., Kim, S. K., Park, C. H., & Matsui, S. (2009). Biodegradation of high molecular weight lignin under sulfate reducing conditions: Lignin degradability and degradation by-products. *Bioresource Technology*, *100*(4), 1622–1627. https://doi.org/10.1016/j.biortech.2008.09.029

Korom, S. F. (1992). Natural denitrification in the saturated zone: A review. *Water Resources Research*, *28*(6), 1657–1668. https://doi.org/10.1029/92WR00252

Kraft, B., Strous, M., & Tegetmeyer, H. E. (2011). Microbial nitrate respiration - Genes, enzymes and environmental distribution. *Journal of Biotechnology*. https://doi.org/10.1016/j.jbiotec.2010.12.025

Kuypers, M. M. M., Marchant, H. K., & Kartal, B. (2018). The microbial nitrogen-cycling network. *Nature Reviews Microbiology*, *16*(5), 263–276. https://doi.org/10.1038/nrmicro.2018.9

Martin, E. A., Davis, M. P., Moorman, T. B., Isenhart, T. M., & Soupir, M. L. (2019). Impact of hydraulic residence time on nitrate removal in pilot-scale woodchip bioreactors. *Journal of Environmental Management*, *237*(3), 424–432. https://doi.org/10.1016/j.jenvman.2019.01.025

Maxwell, B. M., Birgand, F., Schipper, L. A., Christianson, L. E., Tian, S., Helmers, M. J., Youssef, M. A. (2019). Drying-Rewetting Cycles Affect Nitrate Removal Rates in Woodchip Bioreactors. *Journal of Environmental Quality*, *48*(1), 93–101. https://doi.org/10.2134/jeq2018.05.0199

Moorman, T. B., Parkin, T. B., Kaspar, T. C., & Jaynes, D. B. (2010). Denitrification activity, wood loss, and N2O emissions over 9 years from a wood chip bioreactor. *Ecological Engineering*, *36*(11), 1567–1574. https://doi.org/10.1016/j.ecoleng.2010.03.012

Office of Ground Water and Drinking Water. (2016). *National Primary Drinking Water Regulations*. Retrieved from https://www.epa.gov/sites/production/files/2016-06/documents/npwdr_complete_table.pdf

Petersen, D. G., Blazewicz, S. J., Firestone, M., Herman, D. J., Turetsky, M., & Waldrop, M. (2012). Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates across a vegetation gradient in Alaska. *Environmental Microbiology*, *14*(4), 993–1008. https://doi.org/10.1111/j.1462-2920.2011.02679.x

Pluer, W. T., Hoffman, R., & Walter, M. T. (2018). Reducing Stormwater Nitrogen with Denitrifying Bioreactors: Florida Case Study. *Journal of Sustainable Water in the Built Environment*, *4*(4), 06018002. https://doi.org/10.1061/jswbay.0000867

Porter, M. D., Andrus, J. M., Bartolerio, N. A., Rodriguez, L. F., Zhang, Y., Zilles, J. L., & Kent, A. D. (2015). Seasonal Patterns in Microbial Community Composition in Denitrifying Bioreactors Treating Subsurface Agricultural Drainage. *Microbial Ecology*, *70*(3), 710–723. https://doi.org/10.1007/s00248-015-0605-8

Reisinger, A. J., Groffman, P. M., & Rosi-Marshall, E. J. (2016). Nitrogen-cycling process rates across urban ecosystems. *FEMS Microbiology Ecology*, *92*(12), fiw198. https://doi.org/10.1093/femsec/fiw198

Rivas, A., Barkle, G., Stenger, R., Moorhead, B., & Clague, J. (2020). Nitrate removal and secondary effects of a woodchip bioreactor for the treatment of subsurface drainage with dynamic flows under pastoral agriculture. *Ecological Engineering*, *148*. https://doi.org/10.1016/j.ecoleng.2020.105786

Robertson, W. D. (2010). Nitrate removal rates in woodchip media of varying age. *Ecological Engineering*, *36*(11), 1581–1587. https://doi.org/10.1016/j.ecoleng.2010.01.008

Schaefer, A., Werning, K., Hoover, N., Tschirner, U., Feyereisen, G., Moorman, T., Howe, A., Soupir, M. (2021). Impact of flow on woodchip properties and subsidence in denitrifying bioreactors. *Agrosystems, Geosciences, & Environment*, 4(1). https://doi.org/10.1002/agg2.20149

Schipper, L. A., Robertson, W. D., Gold, A. J., Jaynes, D. B., & Cameron, S. C. (2010). Denitrifying bioreactors—An approach for reducing nitrate loads to receiving waters. *Ecological Engineering*, *36*(11), 1532–1543. https://doi.org/10.1016/J.ECOLENG.2010.04.008

Thijs, S., De Beeck, M. O., Beckers, B., Truyens, S., Stevens, V., Van Hamme, J. D., … Vangronsveld, J. (2017). Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys. *Frontiers in Microbiology*, *8*(MAR). https://doi.org/10.3389/fmicb.2017.00494

Toljander, Y. K., Lindahl, B. D., Holmer, L., & Högberg, N. O. S. (2006). Environmental fluctuations facilitate species co-existence and increase decomposition in communities of wood decay fungi. *Oecologia*, *148*(4), 625–631. https://doi.org/10.1007/s00442-006-0406-3

Unden, G., & Schirawski, J. (1997). The oxygen-responsive transcriptional regulator FNR of Escherichia coli: the search for signals and reactions. *Molecular Microbiology*, *25*(2), 205–210. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9282732

USDA. (2011). Natural Resources Conservation Service Conservation Practice Standard. *Natural Resources Conservation Service Conservation Practice Standard*, (May), 1–3.

van der Lelie, D., Taghavi, S., McCorkle, S. M., Li, L. L., Malfatti, S. A., Monteleone, D., Tringe, S. G. (2012). The metagenome of an anaerobic microbial community decomposing poplar wood chips. *PLoS ONE*, *7*(5). https://doi.org/10.1371/journal.pone.0036740

Verbaendert, I., Hoefman, S., Boeckx, P., Boon, N., & De Vos, P. (2014). Primers for overlooked *nirK*, *qnorB,* and *nosZ* genes of thermophilic Gram-positive denitrifiers. *FEMS Microbiology Ecology*, *89*(1), 162–180. https://doi.org/10.1111/1574-6941.12346

Vitousek, P. M., Mooney, H. A., Lubchenco, J., & Melillo, J. M. (1997). Human domination of Earth's ecosystems. *Science*, *277*(5325), 494–499. https://doi.org/10.1126/science.277.5325.494

Wallenstein, M. D., Myrold, D. D., Firestone, M., & Voytek, M. (2006). Environmental controls on denitrifying communities and denitrification rates: Insights from molecular methods. *Ecological Applications*. https://doi.org/10.1890/1051-0761(2006)016[2143:ECODCA]2.0.CO;2

Warneke, S., Schipper, L. A., Bruesewitz, D. A., McDonald, I., & Cameron, S. (2011). Rates, controls and potential adverse effects of nitrate removal in a denitrification bed. *Ecological Engineering*. https://doi.org/10.1016/j.ecoleng.2010.12.006

Warneke, S., Schipper, L. A., Matiasek, M. G., Scow, K. M., Cameron, S., Bruesewitz, D. A., & McDonald, I. R. (2011). Nitrate removal, communities of denitrifiers and adverse effects in different carbon substrates for use in denitrification beds. *Water Research*, *45*(17), 5463–5475. https://doi.org/10.1016/j.watres.2011.08.007

Woli, K. P., David, M. B., Cooke, R. A., McIsaac, G. F., & Mitchell, C. A. (2010). Nitrogen balance in and export from agricultural fields associated with controlled drainage systems and denitrifying bioreactors. *Ecological Engineering*. https://doi.org/10.1016/j.ecoleng.2010.04.024

Zhao, J., Feng, C., Tong, S., Chen, N., Dong, S., Peng, T., & Jin, S. (2018). Denitrification behavior and microbial community spatial distribution inside woodchip-based solid-phase denitrification (W-SPD) bioreactor for nitrate-contaminated water treatment. *Bioresource Technology*. https://doi.org/10.1016/j.biortech.2017.11.011

APPENDIX B. SUPPLEMENTAL MATERIAL

Table 3.2 Denitrifying Gene primers used for Field Bioreactor Woodchip Study 2016-2018. Forward and Reverse primers are paired for each gene listed.

Forward	**5'**							**3'**
EUB341f	CCT		ACG	GGA	GGC	AGC	AG	
nirK876	ATY		GGC	GGV	CAY	GGC	GA	
mod. nirSCd3aF	AAC		GYS	AAG	GAR	ACS	GG	
nosZ1F	WCS		YTG	TTC	MTC	GAC	AGC	CAG
nosZIIF$_{bd4}$	TCG		AAG	TTG	AAG	CCG	GGT	G
Reverse	**5'**							**3'**
EUB518r	ATT	ACC	GCG	GCT	GCT	GG		
nirK1040	GCC	TCG	ATC	AGR	TTR	TGG	TT	
mod. nirSR3cd	GAS	TTC	GGR	TGS	GTC	TTS	AYG	AA
nosZ1R	ATG	TCG	ATC	ARC	TGV	KCR	TTY	TC
nosZIIR$_{bd4}$	GAA	CGT	TCA	TCG	TCG	ACA	CCG	

Table 3.3 Thermal cycling conditions for denitrifying primers.

EUB	(95^{o}C, 3 min) X 1
	(95^{o}C, 10 s; 55.3^{o}C, 30 s) X 42
	(95^{o}C, 10 s; (65^{o}C to 95^{o}C, 5 s, increment 0.5^{o}C)), X 1
nirK	(95^{o}C, 3 min) X 1
	(95^{o}C, 10 s; 59^{o}C, 20 s) X 39
	(95^{o}C, 10 s; (60^{o}C to 95^{o}C, 5 s, increment 0.5^{o}C)), X 1
nirS	(95^{o}C, 3 min) X 1
	(95^{o}C, 10 s; 58^{o}C, 20 s) X 39
	(95^{o}C, 10 s; (65^{o}C to 95^{o}C, 5 s, increment 0.5^{o}C)), X 1
nosZI	(95^{o}C, 3 min) X 1
	(95^{o}C, 10 s; 60^{o}C, 30s) X 39
	(95^{o}C, 10 s; (65^{o}C to 95^{o}C, 5 s, increment 0.5^{o}C)), X 1
nosZII	(95^{o}C, 3 min) X 1
	(95^{o}C, 10 s; 60^{o}C, 30s) X 39
	(95^{o}C, 10 s; (65^{o}C to 95^{o}C, 5 s, increment 0.5^{o}C)), X 1

CHAPTER 4. OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

In Chapter 2, we observed spatial differences in woodchip substrate consumption that appeared to be related to the influent nitrate loading rate. In addition to denitrification, other processes such as aerobic respiration may serve as the mechanism of substrate consumption. Future work to confirm whether the spatial patterns we observed can be taken as spatial indicators of denitrification would clarify the dominant processes within woodchip bioreactors.

We hypothesized that replacing a portion of the bioreactor material as opposed to replacing the entirety of the fill material would prolong the usable lifetime while minimizing operational costs. However, if the dominant mechanism of substrate consumption at the inlet section was aerobic respiration, replacing the woodchips might not be warranted. The degree to which aerobic cycles are beneficial to the function of woodchip bioreactors as they pertain to electron donor availability is another avenue of further investigation.

As researchers, it may be beneficial to consider the bioreactor fill material lifespan as function of the nitrate load treated. Differences in influent nitrate load may explain the discrepancies in woodchip bioreactor lifespan detailed in the literature. However, it is useful to consider the lifetime as a number of years when communicating with producers and other stakeholders, our work shows differences in the degree of substrate decomposition based on differences in nitrate load under the same number of years.

In Chapter 3, we observed that the microbial communities within woodchip bioreactors are dynamic but with patterns of similarities. The differences in the microbial communities we observed are potentially related to the differences we observed in the carbon constituents of the woodchips. We also observed the least variation in the microbial community inhabiting the 8 h

HRT woodchip bioreactors. If we assume that stability in microbial community will lead to consistency in denitrification performance, our results reflect well on the current design standard HRT of 4-8 h. Based on the work presented here, we hypothesize that understanding the factors that lead to consistency in electron donor availability will lead to consistency in denitrification in these systems.

We were not able to detect a significant impact of the effect of HRT on key denitrification genes. These results may imply that measurement of functional genes is not the most practical way to predict system performance at the pilot scale. It also is possible that the lack of statistical differences in detection where due to the sparse sampling scheme presented here. Higher temporal resolution in sampling would allow us to determine if the patterns we observed here are part of larger, consistent trends. We would also be able to further understand the resilience in these systems and the response of the microbial community to environmental stimuli.

It would be beneficial to standardize woodchip bioreactor monitoring measurements. For example, obtaining an initial woodchip C:N measurement would have allowed us to quantify the rate and degree of woodchip decomposition after 4 years. Further, another measurement that may help us to understand the woodchip bioreactor microbial community is the saturated hydraulic conductivity. Preliminary evidence suggests that the bioreactor biofilm may be washed out during high-flow events based on temporary increases in saturated hydraulic conductivity (Johnson, Cooke, Christianson, & Christianson, 2020). Measurements of saturated hydraulic conductivity coupled with 16S rRNA assays of the microbial community could lead to an understanding of succession patterns following disturbance events.

References

Johnson, G. M., Cooke, R. A., Christianson, R. D., & Christianson, L. E. (2020). Subsurface drainage flow fluctuations impact denitrifying bioreactor hydraulic conductivity. In ASABE 2020 Annual International Meeting (pp. 1-). St. Joseph, MI: American Society of Agricultural and Biological Engineers. https://doi.org/10.13031/aim.202000157