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The Effect of Edge-of-Field Nutrient Management Practices on Microbial Concentrations in Subsurface Drainage Water and the Associated Risk of Antibiotic Resistance Dissemination

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THE EFFECT OF EDGE-OF-FIELD NUTRIENT MANAGEMENT PRACTICES ON MICROBIAL CONCENTRATIONS IN SUBSURFACE DRAINAGE WATER AND THE ASSOCIATED RISK OF ANTIBIOTIC RESISTANCE DISSEMINATION

BY

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DISSERTATION ACCEPTANCE PAGE

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This dissertation is dedicated to the memory of my beloved father, Hojatolah, a big-hearted man whom I miss every moment, and to my wonderful mother, Malektaj, a strong and smart woman with love and eternal appreciation.

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ABBREVIATIONS

AMR:	Antimicrobial resistance
ARGs:	Antibiotic resistant genes
BOD:	Biological oxygen demand
C:	Carbon
$CaCl_2$:	Calcium chloride
CFU:	Colony forming unit
CH_4:	Methane
CH_3Hg^+:	Methyl mercury
CO_2:	Carbon dioxide
$CuSO_4$:	Copper (II) sulfate
DO:	Dissolved oxygen
EC:	Electrical conductivity
E. coli:	*Escherichia coli*
EPA:	Environmental protection agency
Fe:	Iron
FIB:	Fecal indicator bacteria
H^+:	Hydrogen ions
H_3BO_3:	Boric acid
HCO_3^-:	Bicarbonate
HGT:	Horizontal gene transfer
HRT:	Hydraulic retention time
H_2O:	Water

H_2S: Hydrogen sulfide gas

K^+: Potassium

KH_2PO_4: Monopotassium phosphate

KNO_3: Potassium nitrate

K_2SO_4: Potassium sulfate

L: Liter

MCL: Maximum concentration level

$MgSO_4$: Magnesium sulfate

Mn: Manganese

$MnSO_4$: Manganese (II) sulfate

MPN: Most probable number

N_2: Nitrogen gas

Na_2MoO_4: Sodium molybdate

Nar: Nitrate reductase

NH_4^+: Ammonium

Nir: Nitrite reductase

NO: Nitric oxide

NO_3^-: Nitrate

N_2O: Nitrous oxide

NO_2^-: Nitrite

Nor: Nitric oxide reductase

Nos: Nitrous oxide reductase

OD: Optical density

OH^-: Hydroxyl ions

PBS: Phosphate buffer saline

PRB: Permeable reactive barrier

RPM: Revolutions per minute

SD: South Dakota

SDSs: Subsurface tile drainage systems

SO_4^{2-}: Sulfate

TKN: Total Kjeldahl nitrate

TOC: Total organic carbon

WBs: Woodchip bioreactors

$ZnSO_4$: Zinc sulfate

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ABSTRACT

THE EFFECT OF EDGE-OF-FIELD NUTRIENT MANAGEMENT PRACTICES ON MICROBIAL CONCENTRATIONS IN SUBSURFACE DRAINAGE WATER AND THE ASSOCIATED RISK OF ANTIBIOTIC RESISTANCE DISSEMINATION

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Many in-field and edge-of-field management practices have been used to reduce nutrient loads from agricultural fields. The denitrification woodchip bioreactor (WB) is one edge-of-field management practice that has proven to be effective in removing nitrate from subsurface drainage water. The success in nitrate removal achieved with WBs has raised interest in expanding their capabilities for removing other agricultural pollutants, including phosphorus, by using other types of media like phosphorus-sorbing filters or combining these filters with woodchips to remove both nitrate and phosphorus as dual-nutrient removal systems. Despite the extensive research done on WBs and nutrient filter materials, little consideration has been given to the potential effect of these removal systems on other contaminants, including microbes. Therefore, the main goal of this study was to quantify the potential effect of WBs and nutrient filter materials on altering drainage water microbes, including the potential for these systems to decrease unwanted microbes (e.g.,

E. coli), change the general microbial population, and alter antimicrobial resistance (AMR) microbe concentrations in subsurface drainage waters. To achieve this goal, two laboratory-studies and one field-scale study were conducted. The results of this research demonstrated the potential for WBs to alter microbial concentrations in subsurface drainage waters. The results of the laboratory study revealed that WBs are capable of significantly reducing *E. coli* concentrations (49% - 77%) and increasing culturable microbial concentrations (250% - 573%) from synthetic tile drainage water. Additionally, the recovered isolates from the general microbial populations from the influents and effluents had similar ratios of AMR. The similar ratio of AMR combined with the increased culturable microbial population detected in the effluents of laboratory WBs indicates the potential for increased concentrations of AMR microbes in tile drainage water when these waters pass through WBs. However, the results from monitoring an in-situ WB varied. Thirteen out of 19 samples resulted in an increase in *E. coli* concentrations (2% - 1700%) and the majority of sample pairs processed for culturable microbes (five out of six) had an increase in general microbial concentrations (53% - 902%); however, neither increases were significant. In addition, the estimated AMR concentrations did not significantly increase in the tile drainage water from the inlet to the outlet due to the lack of significant change in AMR ratios as well as culturable microbial population; however, the sample size was limited ($n = 6$) and the p-value was at the edge of significance ($p = 0.063$). In addition,

the results showed the capability of systems with steel turnings, woodchips, woodchips followed by steel turnings, and woodchips combined with biochar to remove *E. coli* (43% - 97%) from water passing through these systems. Higher concentrations of *E. coli* in the influent decreased the efficiency of nutrient removal systems to remove the *E. coli*. Additional laboratory and future in-field studies are warranted to support the development of an effective design for microbial contaminant removal from waters passing through these nutrient removal systems.

CHAPTER 1: GENERAL INTRODUCTION

1. General Introduction

Over the past few decades, nutrient loading has become one of the primary water quality concerns in the U.S. (USEPA, 1990), largely because of its substantial impacts on the eutrophication of surface waters and the large hypoxic zone in the Gulf of Mexico (David et al., 2010; Rabalais et al., 1996; USGS, 2000). Agricultural sources are recognized as a major source for nutrients that enter the Mississippi River which are then delivered to the Gulf of Mexico (Alexander, 2008; Goolsby et al., 2001; USGS, 2014).

The application of fertilizers, including manure, to agricultural land is a major source of a variety of pollutants into the environment (Goolsby and Battaglin, 2000; Heuer et al., 2011; Reddy et al., 1981). The pollutants, including nutrients, antibiotics, microbes, and antimicrobial resistance (AMR) via antibiotic resistant genes (ARGs), can be transported into the soil and tile drainage water mainly by infiltration via soil macropores (Hruby et al., 2016; Kay et al., 2004, 2005; Kladivko et al., 1999). Subsurface drainage systems then provide a direct pathway for the pollutants to surface waters (Hoang et al., 2013; Hunter et al., 2000; Jaynes et al., 1999; Kay et al., 2005; Luby et al., 2016; Washington et al., 2018), where they can adversely impact water quality (Pinheiro et al., 2013; Skaggs et al., 1994).

Many in-field and edge-of-field management practices have been developed to decrease nutrient loads from agricultural fields. Denitrification woodchip bioreactors (WBs) are one such practice that has proven to be effective in reducing nitrate in tile drainage water (Christianson et al., 2012; Schipper et al., 2010). Denitrification bioreactor systems traditionally consist of a lined trench at the edge of an agricultural field that is

filled with carbonaceous media, typically woodchips (Schipper et al., 2010). The tile drainage water is diverted through the bioreactor, treating between 23% and 98% of the annual nitrate load passing through tile drainage systems (Verma et al., 2010; Woli et al., 2010). There has been recent interest in examining similar ideas for other agricultural pollutants such as phosphorus-sorbing, in-line filters (Penn et al., 2007; Thapa, 2017) as well as assessing the effectiveness of other types or combinations of media on removing nitrate, phosphorus, organic contaminants, and pesticides (Bock et al., 2016; Goodwin et al., 2015; Inyang and Dickenson, 2015; King et al., 2010; Pluer et al., 2016). For example, biochar has been added to denitrifying bioreactors to act as dual-nutrient removal systems, promote denitrification (Bock et al., 2016; Pluer et al., 2016), and increase phosphorus removal (Bock et al., 2015). In other cases, industrial waste, such as steel by-products (Christianson et al., 2017; Goodwin et al., 2015; Hua et al., 2016), or phosphorous-immobilizing materials, such as water treatment residuals (Zoski et al., 2013), were combined or paired with woodchip media to remove both nitrate and phosphorus from nutrient-laden waters, including subsurface drainage water.

Despite the extensive research done on WBs and nutrient filter materials, little consideration has been given to the potential effect of these removal systems on other contaminants, including microbes. Biological processes play a crucial role in treating nitrate through denitrification bioreactors. However, the application of carbonaceous materials, like woodchips and biochar, in these systems may also provide opportunities for altering drainage water microbes and their characteristics through physical and biological mechanisms, alone or in combination. For example, fecal indicator bacteria (FIB), including *E. coli*, have the capability of growing in environments external to a host if given

adequate conditions which include temperature, pH, availability of water, nutrients, and energy sources (Van Elsas et al., 2011). On the other hand, filtration, competition, or predation may result in reduced concentrations of these undesirable microbial populations (Alufasi et al., 2017; Haig et al., 2015; Liao et al., 2015; Stevik et al., 2004). While there are a few studies demonstrating the efficiency of WBs in removing *E. coli* from wastewaters through pilot- and full-scale studies (Rambags et al., 2016, 2019; Robertson et al., 2005; Tanner et al., 2012), few studies have examined the potential effect of WBs on the *E. coli* populations within tile drainage water, particularly at the field-scale (Soupir et al., 2018; Zoski et al., 2013). In addition to altering the concentration of *E. coli* in tile drainage water, bioreactors can alter the concentration of other microbes (e.g., total coliform (Zoski et al., 2013)), possibly through high carbon and nutrient concentrations found within the bioreactors which provide favorable conditions for microbes to grow (Madigan et al., 2010). Furthermore, as denitrification bioreactors promote nutrient-rich environments, they, in turn, may promote cell reproduction and lead to an overall increase in the copies of unwanted genes, such as ARGs, leaving the bioreactor systems in tile drainage water. Biofilm formation, such as occurs on bioreactor woodchips (Chun et al., 2009; Damaraju et al., 2015), may also provide “hotspots” for horizontal gene transfer (Nesse and Simm, 2018), leading to a potential increase in AMR released into the environment.

It is important to understand the potential impacts of nutrient filter removal systems on undesirable microbes, such as *E. coli*, and undesirable traits, such as antimicrobial resistance, to improve the prediction of pathogen removal and AMR changes in tile drainage water and support the development of an effective design for microbial

contaminant removal from waters passing through these filter materials.

1.1 Goal and Objectives

The goal of this study was to detect and quantify the potential effect of WBs and nutrient filter materials on microbial concentrations and AMR microbe concentrations in tile drainage water to understand the potential for the nutrient removal systems to reduce unwanted microbes (e.g., *E. coli*), change the general microbial community, and their potential to alter AMR microbe concentrations in subsurface drainage waters. This main goal was fulfilled through the following objectives:

i. Quantifying and comparing the change in microbes and antibiotic susceptibility of microbes from the influent to the effluent of WBs under different microbial communities and flow conditions.
ii. Quantifying and comparing the change in microbes from the influent to the effluent of beds filled with different nutrient filter materials and arrangements under different influent *E. coli* concentrations.
iii. Quantifying and comparing the change in microbes and antibiotic susceptibility of microbes from the influent to the effluent of an in-situ WB.

1.2 Hypothesis

The main hypotheses of this research were as follows:

i. Denitrification bioreactors will promote cell reproduction, which will lead to an overall increase in microbial communities and the copies of ARGs leaving the bioreactor systems in tile drainage water.

ii. Microbial populations and diversity at the influent of nutrient removal systems will have a significant impact on the microbial community through the nutrient removal systems.

iii. Nutrient filter materials will reduce *E. coli* concentrations, but will vary in their efficiency depending on the types of filters.

1.3 Dissertation Organization

This dissertation contains seven chapters. The first chapter provides a general overview of the issues, and the goal and objectives of the research presented in later chapters. The second chapter contains a literature review providing information on understanding the potential transport of microbial contaminations from agricultural lands receiving manure to subsurface drainage systems and the performance of denitrification bioreactors. Chapters three, four, five, and six follow a peer-reviewed publication format and report the results of this research. Chapter seven provides the main conclusions of this research and implications for future research.

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CHAPTER 2: LITERATURE REVIEW

2.1 Water Quality and Agricultural Drainage Systems

The application of subsurface tile drainage systems (SDSs) in the Midwestern U.S. started in the late 1800s (Dinnes et al., 2002), and SDSs are now implemented on over 15 million hectares in this region (Christianson, 2011). It is believed that the capability of SDSs to drain the "prairie pothole" region of the Midwest combined with the increased utilization of low-cost nitrogen fertilizers and manure caused the enormous increase in agriculture in this region, and subsequently had a substantial positive effect on the U.S. economy (Dinnes et al., 2002). In spite of the benefits of SDSs, these systems have been identified as a significant pathway for many pollutants to enter surface waters (Hoang et al., 2013; Jaynes et al., 1999; Kay et al., 2005; Washington et al., 2018), including nutrients, antibiotics, a variety of microbes (e.g., pathogenic and non-pathogenic microbes), and antimicrobial resistance (AMR) via antibiotic resistant genes (ARGs) (Hoang et al., 2013; Hunter et al., 2000; Jaynes et al., 1999; Kay et al., 2005; Luby et al., 2016; Washington et al., 2018). The application of fertilizers, including manure, to agricultural land is a major source of these pollutants (Goolsby and Battaglin, 2000; Heuer et al., 2011; Reddy et al., 1981). The pollutants can be transported into the soil and tile drainage water mainly by infiltration via soil macropores (Hruby et al., 2016; Kay et al., 2004, 2005; Kladivko et al., 1999). Subsurface drainage systems then provide a direct pathway for the pollutants to enter surface waters where they adversely impact water quality (Skaggs et al., 1994). Small amounts of each pollutant can accumulate and reach concentrations above allowable maximum concentration levels (MCL), resulting in water quality impairments and jeopardizing aquatic and terrestrial health (Brooks et al., 2016).

2.2 Agricultural Nutrients and their Transport to the Waterbodies

Nutrient loads have become a critical water quality concern in the U.S. (USEPA, 1990) due, in part, to their impacts on the eutrophication of surface waters and the large hypoxic zone in the Gulf of Mexico (David et al., 2010; Rabalais et al., 1996; USGS, 2000). Agricultural sources are recognized as a major contributor of nutrients to the Mississippi River, that delivers the nutrients to the Gulf of Mexico (Alexander, 2008; USGS, 2014).

Nitrate loadings are often high in the Midwest and can reach over 80 kg/ha in Iowa (Kaspar et al., 2007; Lawlor et al., 2008) with typical nitrate loads estimated to be between 25 kg/ha and 35 kg/ha, and typical flow weighted concentrations ranging from 10 mg/L to over 25 mg/L (Christianson, 2011). These high loads make it common to detect higher nitrate concentrations in tile drainage than the MCL of 10 mg/L set by USEPA for drinking water (Jaynes et al., 1999; Schilling, 2005; USEPA, 2011).

Phosphorus losses in tile drains can also be high. The concentration of phosphorus in tile drainage water varies from less than 0.01 to over 9.80 mg/L for total phosphorus according to a review study by Moore (2016). This is often above the concentrations necessary to stimulate eutrophication, which ranges from 0.02 – 0.03 mg/L (King et al., 2015).

The high nitrate and phosphorus losses through SDSs are concerning, due to their effect on eutrophication and hypoxic conditions (Dale et al., 2007; Rabalais et al., 1996) as well as the potential danger to human health by contamination of raw water supplies or potable waters (Anderson et al., 2002; Codd et al., 1999; Comly, 1945). Nutrients can promote and support the growth of algal blooms in the waterbodies, which can be toxic (Anderson et

al., 2002). Some of toxic algal blooms can cause skin or liver damage and even result in cancer (Davidson et al., 2014). Drinking water containing nitrate can also cause methaemoglobinemia or blue baby syndrome in infants (Brunato et al., 2003) which causes coma and death if it is not recognized and treated appropriately (Knobeloch et al., 2000).

Nutrient losses can be significantly different between U.S. tile drainage systems due to a variety of factors such as soil types, cropping systems, drainage designs, tillage systems, and weather patterns (King et al., 2015; Kladivko et al., 2004; Kleinman et al., 2015; Patni et al., 1996). Precipitation is cited as the most significant factor contributing to drainage flow volume as well as nutrient losses (King et al., 2015; Randall and Goss, 2008). Nutrient losses in tile drainage are a function of flow, which are typically at their peak during large precipitation events (King et al., 2015; Randall and Goss, 2008). Preferential flow paths are one cause of large nutrient losses measured in tile drainage, which is supported through experimental responses in tile discharge at the beginning of a precipitation event (Kung et al., 2000; Paasonen-Kivekäs and Koivusalo, 2006). Agricultural fields receiving nutrient applications above the recommended levels are more prone to nutrient losses via tile drains (Ball Coelho et al., 2007; Bolton et al., 1970; Hooda et al., 1999; Randall and Goss, 2008). Furthermore, agricultural land with shallow tile drains have a higher concentration of phosphorus (Culley et al., 1983; Poole et al., 2005) and lower nitrate export than deeper drains do (Poole et al., 2005). Cropping systems can also influence the amount of nitrate losses in subsurface drainage systems (Randall and Goss, 2008). As an example, much greater volumes of water and nitrate can enter tile drainage water with row crops (e.g., corn, soybean, etc.) than those with perennial crops (e.g., alfalfa and legume mix) (Baker and Melvin, 1994; Drury et al., 1993; Letey et al., 1977). The nitrate losses via row crops

are estimated to be up to 50 times higher when compared with those from fields with perennial crops (Randall and Goss, 2008). However, the literature suggests that phosphorus losses are more dependent on the rate of phosphorus application, phosphorus source and placement, and timing of phosphorus application rather than the cropping system (King et al., 2015).

2.3 Agricultural Microbial Contamination and Transport to Waterbodies

The microbial contamination of water largely comes from fecal matter associated with humans, domesticated animals, or wildlife (Jung et al., 2014) which can contaminate waterbodies through point sources (e.g., aquaculture effluents and wastewater treatment plants) and non-point sources (e.g., agricultural tile drainage waters and stormwater run-off) (Carpenter et al., 1998; Holt, 2000; Novotny, 1994). When manure is applied as fertilizer on agricultural land, it introduces large scale microbial contamination into the environment (Manyi-Loh et al., 2016) (Figure 2.1). These microbial pollutants can leach through the soil and move into groundwater and tile drainage water (Hruby et al., 2016; Kay et al., 2004, 2005; Kladivko et al., 1999) and negatively impact water quality (Garder et al., 2012; Skaggs et al., 1994). The SDSs are a major pathway for pathogen transport in tile-drained agricultural land amended with manure (Jamieson et al., 2002). The primary concern of pathogen transport is the likelihood of either human contact or ingestion of water contaminated with pathogens which can lead to severe sickness or even death (Pandey et al., 2014). For example, there is an increased risk of illness when swimming in waters contaminated with pathogenic microbes (Prüss, 1998).

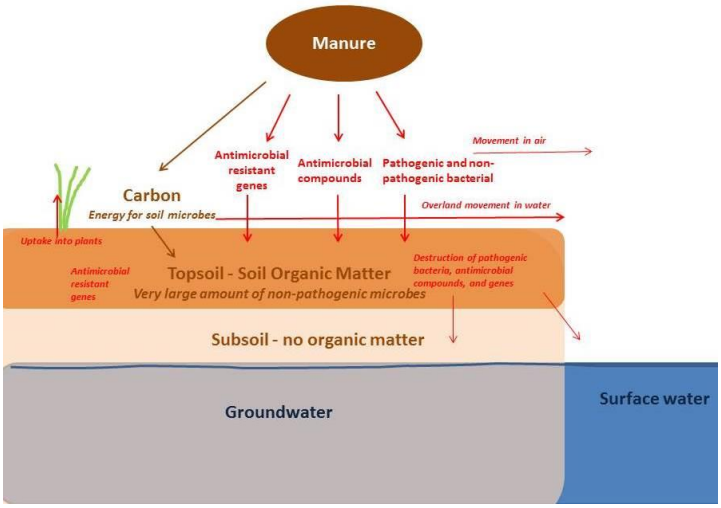

Figure 2.1 Pathways of microbial contamination from animal manure into the environment (WMABC, 2015).

Pathogenic contamination of waterbodies and their associated waterborne diseases, such as gastrointestinal illnesses, are a major water quality concern worldwide (Pandey et al., 2014) and is the number one cause of water quality impairments for rivers and streams in the U.S. (USEPA, 2014). Pathogens are the major cause of impairments for 2 million hectares of lakes as well as over 480,000 km of rivers and shorelines in the U.S. (USEPA, 2008).

Fecal indicator bacteria (FIB) serve as an indicator of fecal contamination and the associated health risk. The FIB include total coliforms, fecal coliforms, *Escherichia coli* (*E. coli*), and enterococci (USEPA, 2012). Microbial water quality is assessed through measuring the concentration of these FIB in waterbodies (Malakoff, 2002; Pandey and Soupir, 2012; Pandey and Soupir, 2013).

2.3.1 Survival of Microbes in Manure-Amended Soils

The survival rate of microorganisms introduced into the environment greatly impacts their potential to contaminate waterbodies (Reddy et al., 1981). Some enteric microbes (e.g., *E. coli*) are able to survive for an extended period of time in the environment (Fenlon et al., 2000) even in conditions that are not favorable, like on plastics or fabrics (Neely, 2000; Robine et al., 2000). In fact, many pathogenic microbes can persist in the environment for long periods of time under various environmental conditions (Table 2.1) (Chee-Sanford et al., 2009). In a review study conducted by Gerba et al. (1975), the survival rates of enteric microbes in the soil and groundwater were between two and four months. Filip et al. (1988) studied the survival capability of several microbes under saturated soil conditions and determined that most microbes examined, including *E. coli*, survived for more than 100 days at a low soil temperature (10 °C). Sjogren (1994) used exponential regression to approximate the survival rates of *E. coli* in soil and showed that the potential survival rate for *E. coli* ranged between 20.7 to 23.3 months. The land application of manure may change the soil conditions in a way that is more hospitable for pathogenic organisms (Dazzo et al., 1973) to survive and even regrow (Crane et al., 1980; Howell et al., 1996; Van Donsel et al., 1967). The survival rate of FIB and pathogens after applying manure in soils was assessed by Cook et al. (2014) and Rogers et al. (2011), and ranged from weeks to months. The long survival rate of microbes and their potential for regrowth in the soil system provides more opportunities for transport during storm events or irrigation through leaching or runoff.

Table 2.1 Persistence of pathogenic microbes in the environment based on a literature review by Chee-Sanford et al. (2009).

Estimate of survival time					
Environment	Temperature (ºC)	*Salmonella*	*Campylobacter*	*Yersinia enterocolitica*	*E. coli* O157:H7
Water	< 0	~ 6 mo*	≤ 8 wk*	> 1 yr*	> 300 d*
	~ 5	~ 6 mo	1wk - 4 mo	> 1 yr	> 300 d
	~ 30	~ 6 mo	~ 4 d	~ 10 d	> 84 d
Soil	< 0	> 6 mo	≤ 28 wk	> 1 yr	> 300 d
	~ 5	≤ 28 wk	~ 2 wk	> 1 yr	> 100 d
	~ 30	~ 4 wk	~ 1 wk	~ 10 d	~ 2 d
Slurry	-	≤ 75 d	< 112 d	≥ 28 d	≤ 100 d
Dry surfaces	-	≤ 7 d	~ 1 d	~ 1 d	~ 1 d

*yr, mo, wk, d are abbreviations for year, month, week, and day, respectively.

Many factors influence the survival of enteric microbes in soil (Gerba et al., 1975), including soil type, soil moisture, temperature, nutrient availability, precipitation, temperature, pH, the microbial concentration on or in the soil profile, manure application rate and characteristics, and timing of tillage application (Evans and Owens, 1972; Jamieson et al., 2002; Mackie et al., 2006a; Onan and LaPara, 2003; Samarajeewa et al., 2012). For example, the survival of *E. coli* in soils is affected by high and low temperatures (Berry et al., 1991; Terzieva and McFeters, 1991), limited moisture (Byappanahalli and Fujioka, 2004), variation in soil texture (Desmarais et al., 2002), low organic matter content (Tate, 1978), high salinity (Tassoula, 1997), and predation (Byappanahalli and Fujioka, 2004; Chao and Feng, 1990).

The principal role of moisture on the survival of enteric microbes in the soil has been shown in numerous studies (Entry et al., 2000; Faust, 1982; Gerba et al., 1975; Mubiru et al., 2000; Reddy et al., 1981). For example, Hagedorn et al. (1978) monitored the degree of movement and subsequent groundwater contamination by fecal bacteria, and found that *E. coli* numbers were the highest after a rise in water table from extensive precipitation

events. Similarly, Tate (1978) determined that *E. coli* survival was greatest in organic soils under flooded circumstances. However, excessive moisture may impact the availability of organic carbon through dilution, and thereby result in a reduction of *E.coli* survival rates (Chandler and Craven, 1980). Low moisture conditions can also adversely affect microbial survival. Kibbey et al. (1978) reported a quick die-off of *Streptococcus faecalis* under low moisture conditions in the soil, while Entry et al. (2000) determined there was a strong positive correlation between the survival rate of fecal coliforms and the soil moisture in buffer strips which received swine waste.

Soil properties impact microbial survival through their effect on moisture retention. Soils with high water-holding capability (matric potential) and nutrient capacities are likely to have a longer survival period for microbes (Gerba and Bitton, 1984; Huysman and Verstraete, 1993). Soils with smaller particles (silt and clay) provide higher water-holding capacity through their larger surface area as compared to those with larger particles and smaller surface areas (sand) (Ball, 2011). Chau et al. (2011) showed the highest and lowest microbial concentrations were with the smaller particles (silt and clay) and the large soil particles (sand), respectively.

Soils with high organic matter also have higher moisture retention and, in turn, have greater microbial survival (Tate, 1978). Organic matter present in the soil can promote the survival and growth of enteric microbes (Gerba et al., 1975; NandaKafle et al., 2018). Increased microbial survival rates in organic soils were previously reported by Tate (1978), where the *E. coli* survival rate was three times greater in an organic soil (histosol) than a sandy soil.

Microbial survival is also different in different soil layers. Topsoil usually provides the greatest survival rates of fecal microbes (Zhai et al., 1995) due to more favorable conditions, specifically the high nutrient availability, that exist in the surficial soil layers (Chandler et al., 1981) as compared to the subsoil.

Temperature is another variable that affects microbial survival, as evidenced by being inversely correlated with microbial mortality (Gerba et al., 1975; Reddy et al., 1981) meaning lower temperatures result in an increase in microbial survival. Van Donsel et al. (1967) reported that 90% of fecal coliform bacteria died within approximately three days after manure application in the summer, but the survival time increased to around 13 days in the winter. Similarly, Reddy et al. (1981) detected that die-off almost doubled with a 10 °C rise in temperature. In addition to the inverse relationship between temperature and FIB survival, studies have determined that cool temperatures are favorable for the survival of fecal microbes. For example, *E. coli* survived for more than 100 days in water-soil combinations held at 10 °C (Filip et al., 1988). Similar trends were observed by Cools et al. (2001), where both *Enterococcus* and *E. coli* showed an increased persistence under increased soil moisture and lower temperatures.

Soil pH has an adverse effect on microbial mortality, with lower pH increasing microbial mortality (Gerba et al., 1975). The optimum pH for FIB survival is between 6 to 7 (Reddy et al., 1981). Soils with neutral to alkaline pH can lead to longer *E. coli* survival times than those with an acidic pH of similar texture (Sjogren, 1994).

Additionally, the type of manure (e.g., swine, poultry, cattle, etc.), as well as manure application characteristics (liquid or solid) are important factors affecting the

concentrations of pathogenic microbes introduced into the natural environment as well as the survival rate of microorganisms (Kudva et al., 1998; Manyi-Loh et al., 2016; Unc and Goss, 2004). For example, poultry and swine manure are more likely to introduce a greater concentration of pathogenic microbes into the soil than cattle manure (Unc and Goss, 2004). In terms of survivability, *E. coli* can survive up to 21 months in sheep manure which was not aerated, but only up to 47 days in bovine manure (Kudva et al., 1998).

Manure characteristics and application also impact the mobility and survivability of microorganisms (Manyi-Loh et al., 2016; Unc and Goss, 2004). The injection of manure into the soil decreases the mortality of microbes from ultraviolet solar radiation as compared to microbes introduced to the environment through manure application on the soil surface (Unc and Goss, 2004). Injection also increases the likelihood of microbial adsorption onto soil particles (Unc and Goss, 2004).

Tillage can also impact on the conditions that microorganisms from manure experience after application (Unc and Goss, 2004). For example, microbial activity is greater close to the soil surface in the agricultural lands under no-tillage than those under conventional tillage (Levanon et al., 1994). Once manure is applied on agricultural fields, accessibility of mineral nutrients as well as carbon substrates is improved, resulting in increased soil microbial activities (Unc and Goss, 2004). Predatory populations can also increase as a result of the nutrients introduced by the addition of manure (Unc and Goss, 2004). Microbial competition in the soil can be a key factor in reducing the microbial communities introduced into the soils (Acea et al., 1988; Soda et al., 1998). The survival of pathogens in the soil can also be limited through a competition between soil microbes to obtain their essential nutrients (Jamieson et al., 2002). For example, Klein and Casida Jr (1967)

demonstrated that *E. coli* cells can be parasitized by some bacteriophages and free-living soil organisms which limited *E. coli* survival.

2.3.2 Transport of Microbes from Manure-Amended Soils to Tile Drainage System

Microbes enter and contaminate water resources (Goss et al., 2001; Reddy et al., 1981) from agricultural lands receiving manure application through processes such as surface runoff and infiltration (Bach et al., 2002; Gagliardi and Karns, 2002; Jamieson et al., 2002; Joy, 1998; Reddy et al., 1981; Tyrrel and Quinton, 2003). The transport of microbes is mostly dominated through the occurrence of rapid water fluxes (Unc and Goss, 2004); thus, the direction of microbial transport from manure can be identified via the water pathway, infiltration, or surface runoff (Unc and Goss, 2004). Fecal microbial transport under ideal matrix flow conditions is inversely related to particle size (Gerba and Bitton, 1984), as fine particles containing clay and silt particles strongly influence the physical filtration of microbial cells under ideal matrix flow conditions (Canter and Knox, 1985; Reddy et al., 1981). However, there are several column and field studies indicating that macropores (or non-matrix flow) are the main transport pathway for fecal microbes (Abu-Ashour et al., 1998; Fontes et al., 1991; McMurry et al., 1998; Shrestha et al., 1997). Macropores can facilitate preferential flow and increase the transport of fecal microbes from the topsoil to the groundwater or SDSs.

Field studies have demonstrated that tile drainage systems can receive significant concentrations of enteric microbes, depending on manure applications and environmental conditions (Jamieson et al., 2002). This suggests that SDSs can accelerate the transport of pathogens and their indicators from manure-amended soils to surface waters (Garder et al., 2012; Haack et al., 2016; Joy et al., 1998; Pappas et al., 2008; Soupir et al., 2006). The

SDSs have been identified as a major transport pathway for pathogenic microorganisms, especially during extreme precipitation events (Jamieson et al., 2002).

Soil moisture content at the time manure is applied as well as the amount of precipitation in the two to three weeks following manure application are the environmental factors with the highest effect on microbial transport to SDSs (Joy et al., 1998; Samarajeewa et al., 2012). The peak concentrations of FIB are often detected at peak flows caused by storm events immediately following manure application (Dean and Foran, 1992; Hunter et al., 2000; Joy et al., 1998; Pappas et al., 2008).

Another factor affecting microbial transport to SDSs is tillage application. Tillage can reduce microbial transport from manure-applied fields to SDSs by disrupting preferential flow paths (Hoang et al., 2013; Pappas et al., 2008; Samarajeewa et al., 2012; Stratton et al., 2005). Soils under tillage practices retain more microbes than soils without any tillage practices (Hruby et al., 2016; Stratton et al., 2005; Thiagarajan et al., 2007; Wang et al., 1998). Decreased macro-porosity and micro-porosity in soils having tillage practices are cited as the main factors contributing to the decreased presence of FIB (including *E. coli*) in tile-water (Abu-Ashour et al., 1998; Pappas et al., 2008; Samarajeewa et al., 2012; Stratton et al., 2005; Thiagarajan et al., 2007; Wang et al., 1998). The application of tillage prior to the application of liquid manure is recognized as an effective method to significantly reduce the concentration of manure-borne pathogens transported to tile drainage (Samarajeewa et al., 2012). Thiagarajan et al. (2007) showed that SDSs in no-till agricultural lands contributed further to *E. coli* loads in comparison with any other tillage-drainage combination. Similarly, Wang et al. (1998) demonstrated that disturbed soil columns retained more microbes compared to undisturbed soil columns. This signifies that

the soil under tillage practices can retard the microbial movement in the soil profile because of matrix flow, while preferential flow facilitated by the establishment of macropores in no-till fields transports more microbes and increases their loads to SDSs (Abu-Ashour et al., 1998; Dean and Foran, 1992; Thiagarajan et al., 2007).

Soil characteristics, including porosity, macropore structure, surface area, etc., play a large role in the movement of enteric microbes through their impact on gravitational movement and adsorption of microbes with water (Jamieson et al., 2002; Van Elsas et al., 2011). Many complicated physical and chemical phenomenon are involved in retaining, removing, or transporting microbial cells in the soil environment, as these processes are dependent on the interaction of the different properties of microbial cells, soils, as well as the suspending solutions (Unc and Goss, 2004). Microbial removal can occur through sedimentation and adsorption or at the soil surface by straining (Gerba et al., 1975); however, separating the filtration processes from adsorption processes is difficult (Reddy et al., 1981). Physical filtration is thought to be the primary process trapping microbes and limiting their movement (Gerba and Bitton, 1984). Microbes which are large, ranging from 0.2 to 5.0 µm, are subjected to more straining than microbes which are smaller in size (< 300 nm) (Jamieson et al., 2002), and Canter and Knox (1985) showed the effectiveness of smaller pore sizes on straining microbial cells. Suspended particles have the ability to attach at the soil surface and can also serve as a filter to trap additional microbes (Corapcioglu and Haridas, 1984). The physical filtration of microbes at the soil surface increases the likelihood of microbial losses during runoff (Crane et al., 1980). Adsorption is believed to be the main process limiting the transport of smaller microbes. Removing microbes via water filtration in the soil is inversely linked with the soil particle sizes (Gerba

and Bitton, 1984). The small soil particles (clay and silt) can absorb more microbes as compared to large soil particles (sand), especially under a high soil moisture content (Reddy et al., 1981).

Another important factor influencing the transport of microbes in the soil environment is the ability of microbial cells to aggregate and form flocs and clumps (Jamieson et al., 2002). This may cause microbes to be more susceptible to filtration (Abu-Ashour et al., 1998), and microbes are seldom present in suspension in the form of a single particle (McDowell-Boyer et al., 1986). Individual microbes may attach together, form bridges in soil pores, and inhibit further microbial movement in the direction of flow (Gerba and Bitton, 1984). Sakthivadivel and Irmay (1966) showed that bridging in soil pores occurred once the diameter of the soil particles was about 0.07 to 0.20 times lower than the diameter of the suspended particles moving through the soil.

2.4 Release of Antibiotics, Antimicrobial Resistance, and Antibiotic Resistant Genes from Manure-Amended Soils to the Environment

The considerable purposeful application of antibiotics for humans as well as animals leads to the constant release of antibiotics into the natural environment (Batt et al., 2006; Brown et al., 2006; Kümmerer, 2009). The primary concern for this release is the development of antimicrobial resistance (AMR) and antibiotic resistance genes (ARGs), which results in lowering the therapeutic impact of antibiotics on human and animal pathogens (Kemper, 2008; WHO, 2018; Zhang et al., 2009).

In the livestock industry, antibiotics are routinely used not only to treat and prevent disease, but also to promote animal production (Cromwell, 2002). Once they are applied,

only a small proportion of antibiotics are used by the digestive system of animals, while 70% to 90% is excreted in animal wastes (Chander et al., 2006; Dolliver and Gupta, 2008; Onan and LaPara, 2003). Along with the antibiotics, antibiotic resistant selection occurs in animal digestive tracts (Aminov et al., 2001) as well as the environment via excretion of the antibiotics in urine and feces. These animal wastes can serve as pools of resistance both from the resistant microbes in the excrement as well as the presence of agents, including antibiotics and metals, which can select for resistance (Seiler and Berendonk, 2012).

Land application of animal wastes on agricultural land is a regular method of animal waste disposal, which serves as a major nutrient source to plants. This practice, however, introduces a large amount of antibiotics as well as microbial communities rich with ARGs into the environment (Figure 2.1) (Binh et al., 2009; Chee-Sanford et al., 2009; Heuer et al., 2011; McKinney et al., 2018; Wang et al., 2012), and is a common pathway for these pollutants to enter the environment (Chee-Sanford et al., 2009). Soils receiving animal manure increase the environmental exposure to antibiotics and contribute to the development and distribution of antibiotic resistance (Heuer et al., 2011; Sarmah et al., 2006), possibly serving as sources of antibiotic resistance to nearby bodies of water (Luby et al., 2016; McKinney et al., 2018).

Manure-sourced antibiotic residues act as a tool for gene transfer between organisms via selective pressure and consequently increase the possibility of transmission of antibiotic resistance between microorganisms (Heuer et al., 2011). However, the selection can also occur without antibiotic selective pressure (Alonso et al., 2001), signifying that ARGs might be transferred or preserved in the environment with or without antibiotic selective pressure. There are some studies reporting an increase in AMR and ARGs in manure-

amended soils in the absence of significant concentrations of antibiotics in the manure (Heuer and Smalla, 2007; Zhou et al., 2010); however, more studies on this topic are required owing to inconsistent results as well as inadequate data (Franklin et al., 2016).

The transfer of ARGs from fecal organisms to native soil microbes can occur (Daane et al., 1996; Lorenz and Wackernagel, 1994; Nielsen et al., 2000) where microbes can readily exchange genetic information in the environment (Amábile-Cuevas and Chicurel, 1992; Salyers and Amabile-Cuevas, 1997; Stewart, 1989). This can occur through horizontal gene transfer (HGT) or intrinsic mutation transfer (Madigan et al., 2010). Since indigenous microbial communities are better adapted to survive in the environment, there is also a potential for transferred resistance characteristics to persist in the environment (Mackie et al., 2006b). ARGs are often linked with plasmids, transposons, and integrons (mobile genetic elements) which can be shared through HGT mechanisms between all microorganisms (Levy and Marshall, 2004) through:

i. Transduction, defined as gene transfer mediated by bacteriophages;
ii. Conjugation, defined as gene transfer via cell-to-cell contact, which can have a large effect on the spreading of ARGs (von Wintersdorff et al., 2016); and
iii. Transformation, defined as the uptake of extracellular DNA.

There are several studies that report an increase of ARGs and AMR in manure-amended soils (Cadena et al., 2018; McKinney et al., 2018; Ruuskanen et al., 2016; Sun et al., 2019; Wepking et al., 2017). For instance, a recent study conducted by McKinney et al. (2018) revealed that the abundance of clinically relevant ARGs significantly increased through the application of dairy manure to soil when compared with soils that received inorganic

fertilizers or those soils that did not receive any manure. Through their study, it was concluded that (McKinney et al., 2018):

i. Manure application increases ARG abundance in soil;
ii. The rate of manure application has a higher impact on increasing ARG abundance than repeated annual applications of manure at a similar rate;
iii. The higher the amount of manure applied, the higher the concentration of ARGs released into the soil; and
iv. The abundance as well as occurrence of ARGs can decrease with increasing the soil depth.

McKinney et al. (2018) performed a literature review and identified a need for more studies to determine the potential impact of long-term application of dairy manure on the abundance of ARGs in soils.

2.4.1 *Transport of AMR and ARGs from Manure-Amended Soils to Tile Drainage Systems*

Manure-associated contaminants, including antibiotic residues, microorganisms, AMR, and ARGs, can attach to the soil particles, suspend in water at soil surfaces, as well as other colloidal surfaces (Wegst-Uhrich et al., 2014). Xenobiotics and microorganisms suspending in solution or attaching to soil surfaces are likely to move from agricultural land via surface and subsurface transport (Figure 2.2). Since the application of manure is a hotspot for the spread of manure-associated contaminants (Westphal-Settele et al., 2018), it is essential to investigate the transport of AMR and ARGs in agricultural land receiving manure to minimize their potential impacts on public health (Casewell et al., 2003). There

are several studies confirming the transport of AMR and ARGs from manure-amended soils to tile drainage water, and, subsequently, to waterbodies (Garder et al., 2014; Garder et al., 2012; Luby et al., 2016; Rossow, 2018). Since both AMR and ARGs have been used to estimate manure-associated resistance; it is beneficial to understand the behavioral differences of AMR and ARGs in soil receiving manure (Rossow, 2018). This would assist in determining the elements driving shorter survival and how different measures of antibiotic resistance compare (Rossow, 2018).

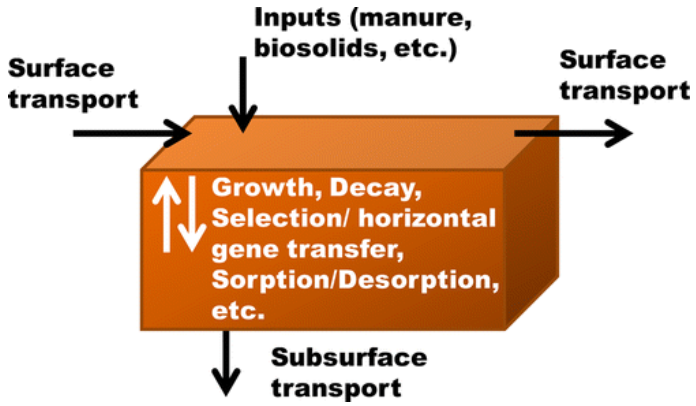

Figure 2.2 A schematic of transport mechanisms of manure-associated contaminations in the soil environment (Fahrenfeld et al., 2014).

Viable AMR microbes help to spread ARGs through two main approaches, mutation and HGT to adjacent microbes. ARGs are also present in the soil environment as part of extracellular DNA, and on mobile genetic elements, such as integrons, transposons, and plasmids, in viable and non-viable cells (Allen et al., 2010).

ARGs can exist in two main forms, including intracellular ARGs and extracellular ARGs (Dong et al., 2019). Intracellular ARGs can present as intracellular DNA and increase the spread of AMR through transduction due to infection of bacteriophages, or

through conjugation due to cell-to-cell contact (Dong et al., 2019). However, extracellular ARGs exist as extracellular DNA and have the potential to be assimilated by capable, non-resistant microbes through transformation, and thus lead to a rapid increase of antibiotic resistance (Liu et al., 2018). Transduction, transformation, and conjugation are the main mechanisms of HGT which are largely responsible for the spread of ARGs between microorganisms (Guo et al., 2018; Zhang et al., 2013). Extracellular DNA has the potential to spread in the environment more easily than intracellular DNA (Mao et al., 2013) and persist in the sediment and soil for an extended period of time, ranging from months to years (Pietramellara et al., 2009; Vlassov et al., 2007; Zhu, 2006). Extracellular ARGs can constitute a major part of the total concentration of ARGs in wastewater (Zhang et al., 2018) and are also expected in manure and soils (Rossow, 2018). Carini et al. (2017) showed that 40% of soil DNA was comprised of extracellular DNA, so the occurrence of ARGs as extracellular soil DNA in manure-amended soils is very probable (Rossow, 2018). Extracellular ARGs are able to disseminate in the soil environment via integrons (a mobile genetic element) of soil microorganisms (Gillings et al., 2008), which results in the risk of interaction with pathogenic microbes (Gillings et al., 2008). These microbes are able to be transmitted to humans via contaminated water, air, dust, and soil, as well as animals (Huijbers et al., 2015). Due to this risk, Pruden et al. (2006) considered ARGs as emerging contaminants for which mitigation is required to avoid extensive distribution.

Microorganisms carrying resistance genes have the same transport mechanisms as any microorganisms through physical movement in or on soil, water, air, humans, and animals (Allen et al., 2010). A literature review performed by Pachepsky et al. (2006) revealed that movement via water is the primary mechanism transporting manure-associated

contaminants in agricultural soils. In the soil environment, the rate of gene transfer can be affected by a variety of factors including rainfall, soil moisture, temperature, soil microbiota, existing microbial communities, presence of nutrients, donor and recipient strains, presence of nutrients, presence of selective pressures, as well as soil types (Cycoń et al., 2019; Hill and Top, 1998; Washington, 2017). Most of these factors follow seasonal trends which results in continuously changing conditions. The seasonal changes in conditions lead to differences in AMR and ARG persistence. Marti et al. (2014) and Fahrenfeld et al. (2014) approximated the persistence of ARGs in the soil environment and found the half-lives of ARGs ranged from 0.20 days to 68 days. Garder et al. (2014) and Luby et al. (2016) detected high concentrations of antibiotic residues and resistant microbes in soils which received swine manure for several weeks following manure application, which may provide donor strains, and nutrients, as well as antibiotic selective pressures (Rossow, 2018). Pachepsky et al. (2006) suggested that factors, such as availability of nutrients and predation which change between seasons, influence the decay levels of manure-associated AMR, ARGs, and antibiotic residues.

Manure application practices may affect the spread of ARGs and AMR into the environment (Mackie et al., 2006a; Onan and LaPara, 2003). For example, liquid manure applied to tile-drained agricultural lands can easily contaminate drainage tile water by rapidly moving through the soil matrix and entering SDSs. Injecting manure in agricultural fields can also transfer microbes and resistant genes directly into the soils, and thereby increase microbial sorption to soil particles (Unc and Goss, 2004). This was supported by Garder et al. (2014) who detected elevated concentrations of AMR and ARGs in manure injection bands within the soil following application of manure. Surface application of

manure might be the most efficient method of land application of manure to manage pathogens and resistant microbes, mainly due to desiccation which occurs once manure is exposed to solar radiation (Hoerter et al., 2005). The need for a deep understanding of microbial growth, die-off, infiltration, adsorption, and partitioning was suggested by Pachepsky et al. (2006) to assist with developing manure management practices which can minimize the potential risk of microbial transport off agricultural lands.

The SDSs in tile-drained agricultural fields have the potential to influence the survival and transport of manure-associated AMR and ARGs. Such SDSs reduce hydrologic retention which, in turn, results in the decreased retention time of AMR and ARGs in soils and provides a direct pathway to waterbodies (Rossow, 2018). A study by Fahrenfeld et al. (2014) determined that there is a very low transport of manure-associated AMRs and ARGs over the soil surface. However, ARG transport into tile water in agricultural lands after the application of manure is well documented (Garder et al., 2014; Luby et al., 2016; Rossow, 2018). For example, Luby et al. (2016) studied the transport of AMR and ARGs from soils with swine manure to tile-fed waterways and waterbodies, and suggested that HGT to indigenous soil microorganisms resulted in more frequent detection of ARGs in tile drainage relative to manure-associated AMR. Garder et al. (2014) determined that weather conditions played a significant role on the transport of AMR and ARGs in tile drainage, with lower occurrences of AMR and ARGs in tile effluent in years with below-average rainfall.

The presence of AMR and ARGs in the environment is a substantial threat to public and animal health due to the high risk of transmission of antibiotic resistance between organisms (Balabanova, 2017; Heuer et al., 2011; Igbinosa and Odjadjare, 2015).

Therefore, it is essential to better quantify and qualify the fate and transport of resistant microorganisms in manure-amended soils in agricultural ecosystems. This will help to design on-farm management practices that can decrease the exposure hazards (Ilhan et al., 2012). Furthermore, since the prevalence and types of antibiotic resistance in tile drainage water are not well understood, more research is required to identify the types of antibiotics as well as the microbes resistant to them in tile drainage water.

2.5 Improving Tile Drainage Water Quality

The discharge of contaminated tile drainage waters into waterbodies is a growing concern for the environment (Tanji and Kielen, 2002). Therefore, it is necessary to develop management practices that can reduce or prevent chronic problems caused by excessive concentrations of pollutants in tile drainage water as well as protect aquatic ecosystems and public health (Fritsch, 1997). Many in-field and edge-of-field management practices have been developed to decrease the amount of nitrogen in drainage and decrease the amount of drainage from agricultural fields. Some examples include improving the application rate and timing of fertilizers and manure and increasing the use and application of better crop rotations, cover crops, and wetlands (Dinnes et al., 2002). Controlled drainage and denitrification bioreactors also show promise for reducing the export of nitrogen from agricultural fields (Appelboom and Fouss, 2006). At some locations, a combination of two or more such practices are needed to satisfy water quality objectives (Dinnes et al., 2002; Randall and Sawyer, 2008).

Standard conservation practices may not be effective in treating tile water or may have restrictions. For instance, vegetated buffer strips rely mostly on infiltration to treat runoff (Cooke et al., 2001) and are not able to treat flow from SDSs because SDSs bypass the

management practice (Kovacic et al., 2000), and consequently are not appropriate for treating subsurface drainage water. Furthermore, although optimizing the application rate and timing of fertilizers and manure can substantially improve water quality (Lawlor et al., 2008, 2011; Randall and Mulla, 2001), in some cases, even a small amount of fertilizers or manure produce drainage nutrient concentrations above the MCL (Jaynes et al., 2001). Therefore, Randall and Sawyer (2008) suggested that the application of this option alone is not enough to meet water quality objectives in all cases.

A few management practices have been developed to address the issue of nutrients in tile drainage water, including controlled drainage systems and edge-of-field management practices such as wetlands, saturated buffers, and carbon-based bioreactors (Christianson et al., 2016a). Controlled drainage reduces the amount of drainage water and the associated nutrients leaving the field (Dinnes et al., 2002; Gilliam et al., 1979). This practice results in an average total nitrogen load decrease of 41% (Christianson et al., 2012a). Despite its high capability of reducing nitrogen loads, controlled drainage is limited to agricultural lands with low slopes (< 0.5% - 1.0%) (Dinnes et al., 2002). Constructed wetlands are another practice which has a high potential for nitrogen load reduction and offer a variety of benefits for wildlife and flood control (Iovanna et al., 2008). There are many successes with constructed wetlands (IDALS, 2009); however, they are costly to establish with estimates between $30,000 - $65,000 per acre (in 2004 dollars) (USEPA, 1999), making it difficult to implement this system broadly. Several management practices used in the wastewater industry could also be adapted for tile drainage systems, including passive bioreactors with biofilm, biological and chemical denitrification, reverse osmosis, packed or fluidized bed bioreactors, and lagoons (Cooke et al., 2001; Jaynes et al., 2008;

Tchobanoglous et al., 1991). For field-scale applications on agricultural land, engineering for biological denitrification is one of the most practical methods of reducing nitrate in SDSs because of its low expense and maintenance coupled with a relatively long lifespan (Cooke et al., 2001; Jaynes et al., 2008; Schipper et al., 2010b). The feasibility of agricultural treatment systems is a key factor which needs to be considered, particularly in the U.S. where the water quality of agricultural drainage is not regulated (Christianson, 2011).

2.6 Nitrogen Cycle and Denitrification Process

Increased application of nitrogen fertilizer has modified the nitrogen cycle throughout the past century and has led to global environmental impacts (Erisman et al., 2013; Gruber and Galloway, 2008). In the U.S., the application of nitrogen fertilizers increased by 2.4 kg/ha/yr from the 1960s to the 1990s (Dinnes et al., 2002). The changes in nitrogen inputs and the installation of SDSs throughout the past century have reduced the nitrogen-cycling efficiency in the environment (Dinnes et al., 2002). While these modifications have influenced the amount of nitrogen in waterbodies, it is worth mentioning that increased applications of inorganic nitrogen are not the only reason for high nitrogen loads in drainage water in the Midwestern U.S. (Christianson, 2011). Soil nitrogen mineralization, a direct result of microbial activity (Dinnes et al., 2002), also contributes to nitrogen leaching in the soil environment, due to the lack of synchrony between application time of nitrogen fertilizers and plant needs as well as microbial population dynamics (Dinnes et al., 2002).

Denitrification is the microbially facilitated reduction of nitrate to nitrogen gas (Christianson, 2011) (Equation 2.1) and is a major mechanism for reducing nitrate in the

soil environment (Lamb et al., 2014; Tiedje, 1994). The nitrogen cycle is impacted by SDSs which are capable of altering the hydrologic cycle in agricultural systems (Christianson, 2011). The SDSs facilitate the quick movement of drainage water to surface waters and, in turn, natural processes, like denitrification, do not have adequate time to occur (Kellman, 2005; Moorman et al., 2010). In most cases, carbon sources are also limited at deeper depths, which significantly reduces the capability of denitrifiers to denitrify soil nitrate before it enters SDSs (Moorman et al., 2010).

$$5C + 4\,NO_3^- + 2\,H_2O = 2\,N_2 + 4\,HCO_3^- + CO_2$$ (Equation 2.1, Christianson (2011))

Denitrification is a multi-step process which involves four enzymatic activities including nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) (Trivedi et al., 2012). Each enzyme results in an intermediate denitrification product (Figure 2.3) (Robertson and Groffman, 2007). In the first step, Nar acts as the catalyst to transform nitrate (NO_3^-) to nitrite (NO_2^-). In the next step, Nir functions as a catalyst and converts nitrite (NO_2^-) to nitric oxide (*NO*). The nitric oxide (*NO*) is then reduced to nitrous oxide (N_2O) via Nor. Finally, nitrogen gas (N_2) is produced as the last step by Nos (Sylvia et al., 2005).

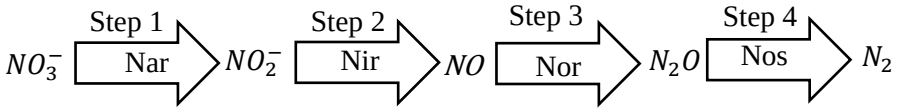

Figure 2.3 Denitrification as a multi-step process.

Necessary requirements for the denitrification process to proceed are anaerobic conditions, denitrifying bacteria, available carbon sources as electron donors, and a

nitrogen oxide, a binary compound of oxygen and nitrogen such as $NO_3^-, NO_2^-, N_2O,$ and $NO,$ as an electron acceptor (Korom, 1992). Anaerobic environments are necessary for the enzymes involved in the denitrification processes, since they are inhibited by dissolved oxygen (DO) (Hoover, 2012), resulting in an increase of some hazardous denitrification intermediates including nitrite and other nitrogen oxides (Ge et al., 2012; Her and Huang, 1995). Under anaerobic conditions, a diverse group of mostly facultative anaerobes, both autotrophic and heterotrophic denitrifiers (Patureau et al., 2000), the majority of which are heterotrophic organisms, convert nitrate into either nitrogen gas or nitrous oxide (Flores-Mireles et al., 2007; Sylvia et al., 2005; Verbaendert et al., 2011). Heterotrophic denitrifying bacteria, including *Achromobacter*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Chromobacterium*, *Corynebacterium*, *Flavobacterium*, *Hyphomicrobium*, *Moraxella*, *Paracoccus*, *Propionibacterium*, *Pseudomonas* (the most common bacteria), *Rhizobium*, and *Rhodopseudomonas* (Payne, 1983), are abundant in the environment , comprising 0.1% to 5.0% of the whole microbial community in the soil (Sylvia et al., 2005). The most common source of electrons for metabolic reactions in nature originate from organic matter which provides the most energy for these heterotrophs (Korom, 1992).

When nitrate is reduced nearly completely through denitrification, other anaerobic bacteria can become active (Christianson and Helmers, 2011). They can consume other available electron acceptors including sulfate (SO_4^{2-}), manganese (Mn), iron (Fe), and carbon dioxide (Korom, 1992).

Nitrogen gas (N_2), carbon dioxide (CO_2), and bicarbonate (HCO_3^-) are the last products of denitrification (Equation 2.1). However, incomplete denitrification can result

in the production of nitrous oxide, a harmful greenhouse gas (Korom, 1992). Many environmental factors can result in incomplete denitrification, including high DO, low pH, low temperature, as well as low carbon to nitrogen ratio (Chapin et al., 2002).

2.7 Enhanced Denitrification through Solid Carbon Materials

The denitrification process is a very effective approach to remove nitrate (Guo et al., 2017); however, an external carbon source is needed to sustain denitrification (Greenan et al., 2006) and increase nitrate removal. Gale et al. (1993) demonstrated a positive correlation between the concentrations of available carbon and the rates of denitrification which were supported by Lin et al. (2007), who found higher rates of denitrification in soils with diverse quantities of extractable organic carbon.

Advancements in water treatment systems have led to the improvement of permeable reactive barriers (PRBs) to treat waters with high concentrations of nitrate (Christianson, 2011). The PRBs are subsurface emplacements of materials through which contaminated water is moved, usually via a natural gradient (CLU-IN, 2019). Function of PRBs can be enhanced by adding carbon source materials to the system to provide carbon at a concentration whereby biological denitrification can easily occur and oxygen concentrations are reduced in the system via aerobic respiration (Schipper et al., 2005).

This novel technology has been used for over 20 years to remove nitrate in septic tank effluents, tile drainage water, and groundwater (Christianson et al., 2012b; Schipper et al., 2010b). Blowes et al. (1994) published the first research of this denitrification system, which consisted of two barrels filled with a combination of organic materials, including tree bark, woodchips, and leaf compost, placed in a streambank 100 m from a tile drainage

discharge outlet. The results confirmed the potential of the carbon materials to remove nitrate from influent having nitrate concentrations ranging from 2 mg/L - 6 mg/L to below 0.02 mg/L in the effluent. Shortly after this work, Robertson and Cherry (1995) conducted a similar work to investigate the passive treatment of septic wastewaters and revealed a substantial attenuation (between 60% to 100%) of input nitrate concentrations during one year. Robertson et al. (2000) summarized the results of six to seven years of operation of the systems used in the latter study (Robertson and Cherry, 1995) and reported a high and consistent nitrate removal (between 58% to 91%) for these systems. This method of nitrate removal was later trademarked by the University of Waterloo (Robertson et al., 2005) and first installed in Southern Ontario where it achieved an average nitrate removal of 96% over a period of five years (Robertson et al., 2005). Field-scale denitrification research commenced in New Zealand shortly after the initial work in Canada (Blowes et al., 1994) with the installation of a denitrification wall to treat groundwater (Schipper and Vojvodić-Vuković, 1998). Schipper et al. (2010b) introduced denitrification bioreactors as a method for decreasing loads of nitrate to receiving waters and provided insights into the efficiency of these systems on removing nitrate from different pollutant waters, including tile drainage water, wastewater, and groundwater. A series of studies by Cameron and Schipper (2010), Warneke et al. (2011a), and Warneke et al. (2011b) helped determine the optimum denitrification fill material and aided understanding of the processes within denitrification beds.

2.8 Types of Denitrification Bioreactors

Denitrification bioreactors are installed to intercept nitrate enriched waters and remove excessive nitrate (Schipper et al., 2010b). They are filled with an organic carbon energy

source, usually woodchips, to support the activity of denitrifying microorganisms and increase the magnitude of denitrification processes (Christianson et al., 2012b; Schipper et al., 2010b). Traditional bioreactor designs include beds which intercept concentrated discharge, layers which intercept soil leachate, or walls which intercept shallow groundwater (Schipper et al., 2010b). Denitrification walls are installed to remove nitrate from groundwater prior to surface water recharge (Robertson et al., 2000; Schipper et al., 2005; Schipper et al., 2010b). Denitrifying beds are containers or trenches used to remove nitrate from wastewaters (Robertson et al., 2005) or subsurface drainage systems from agricultural fields (Blowes et al., 1994; Robertson and Merkley, 2009; Robertson et al., 2009). Some denitrification beds are placed into drainage ditches or existing streambeds and are referred to as stream-bed bioreactors (Schipper et al., 2010b) (Figure 2.4). Lastly, denitrification layers are horizontal layers of carbonaceous materials, receiving nitrified effluent from above (Schipper and McGill, 2008; Schipper et al., 2010b).

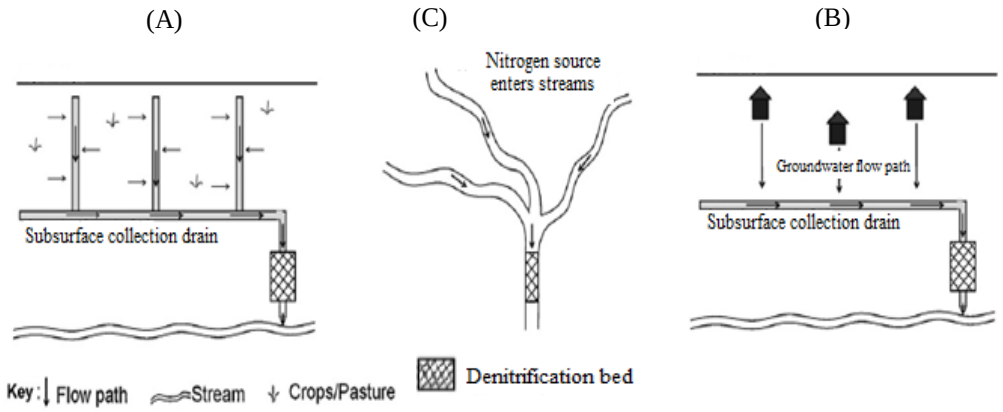

Figure 2.4 Schematic of different denitrification beds installed to intercept tile drainage water from agricultural lands (A), drainage water leaching from septic tanks (B), and installed into base of stream (C) (Schipper et al., 2010b).

2.8.1 *Drainage Denitrification Bioreactors*

While initial denitrification bioreactor studies focused on groundwater and septic water treatments, they have since been conducted for use in treating agricultural tile drainage water. Table 2.2 summarizes laboratory and field-scale studies that evaluate the performance of denitrification bioreactors for removing nitrate from agricultural drainage water. This simple and cost-efficient technology has a high capacity for removing nitrogen from agricultural drainage water, with nitrate removal efficiencies reaching up to 100% (Table 2.2). Field studies have reported denitrification bioreactors usually have hydraulic retention times (HRTs) ranging from 26 minutes to 22 days, and have resulted in nitrate removal rates between 3% to nearly 100%. Laboratory studies had HRTs ranging from 1.7 hours to 17.4 days, and resulted in nitrate removal rates from 8% to 100% (Table 2.2). Nitrate-nitrogen concentrations from field denitrification bioreactor influents were between 1 mg/L to 180 mg/L (Table 2.2) which was much higher than MCL of 10 mg/L, in most cases.

In South Dakota, the initial agricultural drainage denitrification bioreactors for research were installed in 2012 near Baltic and Montrose, in 2013 near Arlington, and in 2014 near Hartford. Several bioreactor laboratory and field studies have been conducted in South Dakota including the effectiveness of the installed woodchip bioreactors on nitrate removal (Partheeban et al., 2014a), the potential of woodchip bioreactors and steel filters for removing nutrients from tile drainage water (Hua et al., 2016), and the characterization of the leached dissolved carbon from woodchip bioreactors (Abusallout and Hua, 2017).

Table 2.2 Review of laboratory and field studies on evaluating of denitrification bioreactors for removing nitrate from agricultural drainage water.

Source	Type of study	Location	Media	Hydraulic retention time (HRT)	Inflow nitrate-nitrogen concentration	Removal (load or concentration)	Removal rate
Blowes et al. (1994)	Field study	Ontario, Canada	Woodchips, grow-bark, sand, and composted leaf	1 - 6 day	2 - 6 mg/L	~ 100% concentration	NA
Cooke et al. (2001)	Laboratory study	Illinois, U.S.	Woodchips	8 hours (at 25 °C)	25 mg/L	~ 100% concentration	NA
Wildman (2002)	Field study	Illinois, U.S.	Woodchips	NA	1 - 18 mg/L	~ 100% concentration	NA
					4 - 16 mg/L	~ 100% concentration	
Doheny (2003)	Laboratory study	Illinois, U.S.	Woodchips	10 hours (at 10 °C)	25 mg/L	60% concentration	NA
Van Driel et al. (2006)	Field study	Ontario, Canada	Fine and coarse wood media	9 hours	11.8 mg/L	33% concentration	0.95 - 2.5 gN/m3/day
Jaynes et al. (2008)	Field study	Iowa, U.S.	Woodchips	NA	19.1 - 25.3 mg/L	40 - 65% load	0.62 gN/m3/day
Chun et al. (2009)	Laboratory study	Illinois, U.S.	Woodchips	2.6 - 12.0 hours	10.4 - 33.7 mg/L	10 - 40% concentration	NA
				> 15 hours		100% concentration	
Robertson et al. (2009)	Field study	Ontario, Canada	Woodchips and sawdust	0.41 - 2.2 day	4.8 mg/L	NA	2 - 16 gN/m3/day
Greenan et al. (2009)	Laboratory study	Iowa, U.S.	Woodchips	2.1 - 9.8 day	50 mg/L	30 - 100% concentration	2.94 - 4.51gN/m3/day
Moorman et al. (2010)	Field study	Iowa, U.S.	Woodchips	24 hours	20 - 25 mg/L	50 - 60% concentration	NA
Chun et al. (2010)	Field study	Illinois, U.S.	Woodchips	4.4 hours	269.9 g mass	47% load	NA
Verma et al. (2010)	Field study	Illinois (Deland),	Woodchips	NA	3 - 16 mg/L	42 - 48% load	NA

		U.S.					
		Illinois (Decatur), U.S.			5 - > 20 mg/L	81 - 98% load	
		Illinois (Decatur), U.S.			4 - 12 mg/L	54% load	
Woli et al. (2010)	Field study	Illinois, U.S.	Woodchips and pruned limb	26 min - 2.8 hours	2.8 - 18.9 mg/L	23 - 50% load	6.4 gN/m3/day
Rodriguez (2010)	Laboratory study	California, U.S.	Woodchips	4.2 hours	10 - 11 mg/L	39% concentration	NA
				6.3 hours		76% concentration	
				8 hours		96% concentration	
Ranaivoson et al. (2010)	Field study	Minnesota (Claremont), U.S.	Woodchips	32 hours	11 - 28 mg/L	18 - 47% load	NA
		Minnesota (Dundas), U.S.		NA	7 - 14 mg/L	35 - 45% load	
Christianson et al. (2011b)	Pilot scale study	Iowa, U.S.	Mixed hardwood chips	4 - 8 hours	10.1 mg/L	30 - 70% concentration	3.8 - 5.6 gN/m3/day
Christianson et al. (2011c)	Pilot scale study	New Zealand	Woodchips	1.5 - >15 hours	7.7 - 35.6 mg/L	14 - 37% load	2.1 - 6.7 gN/m3/day
Christianson et al. (2012a)	Field studies	Iowa (Pekin), U.S.	Woodchips	NA	1.23 - 8.54 mg/L	22 - 74% load	0.38 - 3.78 gN/m3/day
		Iowa (NERF), U.S.			9.9 - 13.2 mg/L	12 - 14% load	0.86 - 1.56 gN/m3/day
		Iowa (Greene Co.), U.S.			7.7 - 12.8 mg/L	27 - 33% load	0.41 - 7.76 gN/m3/day
		Iowa (Hamilton Co.), U.S.			7.7 - 9.6 mg/L	49 - 57%load	0.42 - 5.02 gN/m3/day
Zoski et al. (2013)	Laboratory study	Saskatchewan, Canada	Woodchips	NA	58 mg/L	8 - 60% concentration	NA
			Woodchips and water treatment residuals			1 - 54% concentration	
Christianson et al. (2013)	Field study	Iowa, U.S.	Woodchips	7.5 – 79 hours	11.64 - 18.4 mg/L	7 – 100% load	0.38 – 1.06 gN/m3/day
Partheeban et al. (2014a)	Field study	South Dakota, U.S.	Woodchips	4.9 hours	~ 15 - 45 mg/L	51 - 96% concentration	0.98 - 12.58 gN/m3/day

Bock et al. (2015)	Laboratory study	Virginia Tech, U.S.	Woodchips and biochar	18 - 72 hours	35 mg/L	86 - 97% concentration	NA
			Woodchips			13 - 75% concentration	
Healy et al. (2015)	Laboratory study	Galway, Ireland	Lodgepole pine woodchips	3.7 - 17.4 days	20 - 29.6 mg/L	57.7 - 99.7% concentration	Maximum 3.5 gN/m3/day
			Cardboard	3.5 - 10.2 days		99.4 - 99.8% concentration	
			Lodgepole pine needles	3.6 - 11.6 days			
			Barley straw	3.5 - 21.7 days			
Bell et al. (2015)	Field study	Illinois, U.S.	Woodchips	2 - 8 hours	< 0.1 - 17 mg/L	20 - 98% concentration	5 - 30 gN/m3/day
Goodwin et al. (2015)	Laboratory study	Illinois, U.S.	Woodchips and iron turnings	4.45 - 5.25 hours for woodchips, 1.69 - 2 hours for steel	11.72 - 13.12 mg/L	~ 44.6 - 88.8% concentration	NA
Camilo (2016)	Technical study	Germany	Straw of common wheat and bark mulch of pine tree	0.43 days (at 9 °C)	100 mg/L	8% concentration	3 gN/m3/day
				0.43 days (at 21 °C)		82% concentration	30 gN/m3/day
Feyereisen et al. (2016)	Laboratory study	Minnesota, U.S.	Woodchips, shredded corn stover, corn cobs	12 hours (at 1.5 °C)	50 mg/L	4 - 16% load	1.6 - 7.4 gN/m3/day
				12 hours (at 15.5 °C)		5 - 73% load	1.4 - 35 gN/m3/day
Hoover et al. (2016)	Laboratory study	Iowa, U.S.	Woodchips	1.7 - 21.2 hours (at 10°C)	10 - 30 mg/L	15 - 64% concentration	5.7 - 8.9 gN/m3/day
				1.7 - 21.2 hours (at 15°C)	30 mg/L	15 - 64% concentration	11 - 13.8 gN/m3/day
				1.7 - 21.2 hours (at 20°C)	30 mg/L	48 - 56% concentration	19 - 23 gN/m3/day
				1.7 - 21.2 hours (at	10 - 30 mg/L	17 - 93% concentration	9.1 - 20.4 gN/m3/day

				21.5°C)			
Bock et al. (2016)	Field study	Virginia Tech, U.S.	Woodchips	~ 7 days	0.1 - 33.3 mg/L	53 - 99% concentration	0.25 - 6.06 gN/m3/day
			Woodchips and biochar		0.1 - 22.2 mg/L		
Hua et al. (2016)	Laboratory study	South Dakota, U.S.	Woodchips and steel filters	6 – 24 hours	20 mg/L	53.5 – 100% concentration	10.1 - 21.6 gN/m3/d
				24 hours	50 mg/L	75% concentration	18.9 gN/m3/d
David et al. (2016)	Field study	Illinois, U.S.	Woodchips	NA	~ 30 mg/L	3 - 81% concentration	0.7 - 116 gN/m3/day
Gottschall et al. (2016)	Field study	Ontario, Canada	Woodchips	< 3 hours	35 - 80 mg/L	33% concentration	NA
			Woodchips and drinking water treatment plant residuals			74% concentration	
Hartz et al. (2017)	Field study	California, U.S.	Woodchips	2 days	60 - 180 mg/L	NA	6.4 - 8 gN/m3/day
	Laboratory study		Enriched woodchips with methanol		160 mg/L	~ 100% concentration	NA
	Laboratory study		Enriched woodchips with clycerin		161 mg/L	~ 100% concentration	NA
Hassanpour et al. (2017)	Field study	New York, U.S.	Woodchips	0.5 - 2.8 days	0.7 - 21 mg/L	42 - 68% concentration	4.7 - 13.5 gN/m3/day
			Woodchips and biochar	0.3 - 2.3 days		55 - 66% concentration	4.7 - 15.1 gN/m3/day
Christianson et al. (2017)	Laboratory study	West Virginia, U.S.	Woodchip, mine drainage treatment residuals, steel slag	7.6 min - 51 hours	20 - 40 mg/L	18 – 95% concentration	8.0 – 18 gN/m3/day
Husk et al. (2017)	Field study	Quebec, Canada	Woodchips	~ 14.1 hours	~ 22 mg/L	99% load	NA
Rosen and Christianson (2017)	Field study	Maryland, U.S.	Woodchips	NA	4.65 - 13.46 mg/L	9 - 62% concentration	0.21 - 5.36 gN/m3/day
Bock et al. (2018)	Field study	Maryland, U.S.	Woodchips and biochar	4 - 10 hours	2.8 - 4.6 mg/L	9.5% concentration	0.56 ± 0.25 gN/m3/day

Soupir et al. (2018)	Laboratory study	Iowa, U.S.	Woodchips	12 - 24 hours (at 21.5 °C)	30 mg/L	67 - 96% concentration	14.6 - 22.5 gN/m3/day
				12 - 24 hours (at 10 °C)		29 - 48% concentration	7.7 - 8.8 gN/m3/day
Martin et al. (2019)	Laboratory study	Iowa, U.S.	Woodchips	2 hours	103.3 mg/L	9% concentration	9 ± 4.01 gN/m3/day
				8 hours	26.4 mg/L	32.1% concentration	8.5 ± 2.42 gN/m3/day
				16 hours	14.8 mg/L	53.8% concentration	7.4 ± 2.52 gN/m3/day
Hassanpour et al. (2019)	Laboratory study	New York, U.S.	Woodchips	4 - 72 hours	1.5 and 11.5 mg/L	65 - 100% concentration	NA
Rivas et al. (2019)	Field study	New Zealand	Woodchips	5 - 22 days	NA	48 - 99% concentration	NA
Šereš et al. (2019)	Laboratory study	Czech Republic	Woodchips, birch, and gravel	4.1 days	102 mg/L	96% concentration	NA
Coleman et al. (2019)	Laboratory study	Virginia Tech, U.S.	Woodchips and biochar	3 - 12 hours	4.5 - 16 mg/L	15 – 98% concentration	11.0 gN/m3/day

2.8.2 *Phosphorus-Sorbing Filters and Dual-Nutrient Systems*

The success in nitrate removal achieved with woodchip denitrification bioreactor technology has raised interest in expanding its capabilities for removing other pollutants by using other types of media like phosphorus-sorbing filters, or combining other media with woodchips to remove a variety of pollutants including nitrate, phosphorus, and pesticides (Bock et al., 2016; Goodwin et al., 2015; Inyang and Dickenson, 2015; King et al., 2010; Pluer et al., 2016).

Phosphorus-sorbing filters are currently used to capture excess phosphorus from aquaculture wastewater and tile-drained agricultural fields through low-cost natural filters (Hua et al., 2016; Li et al., 2017; McDowell et al., 2005; Moore, 2016; Penn et al., 2007; Penn et al., 2012; Sibrell and Kehler, 2016). These filters may include industrial byproducts (e.g., fly ash, steel materials, and acid mine drainage residue) or minerals (e.g., limestone, gypsum, zeolite, and goethite) (Chardon et al., 2012; Erickson et al., 2012; McDowell et al., 2008; Penn et al., 2007; Yao et al., 2011). Phosphorus-sorbing filters are rich in aluminum, calcium, and iron (metal cations) which, in combination, enhances phosphorus sorption and results in high phosphorus removal through the treatment systems (Penn et al., 2016; Penn et al., 2011; Penn et al., 2014). Steel by-products (e.g., slag, turnings, and wool), produced during metal processing, have a high capacity for phosphate adsorption, mainly because of their high iron content (Erickson et al., 2012) which can bind with dissolved phosphorus and form un-dissolvable compounds (Lyngsie et al., 2014; Weng et al., 2012).

There are also several studies assessing the effects of combining phosphorus-sorbing

filters with woodchips as dual-nutrient reduction systems to remove nitrate and phosphorus (Bock et al., 2016; Goodwin et al., 2015; Inyang and Dickenson, 2015; King et al., 2010; Pluer et al., 2016). In some cases, biochar has been added to denitrifying bioreactors to promote denitrification (Bock et al., 2016; Pluer et al., 2016) and increase phosphorous removal (Bock et al., 2015). In other cases, industrial waste (like steel by-products) or phosphorous-immobilizing materials (like water treatment residual) were combined/paired with woodchips to remove both nitrate and phosphorus from the nutrient-laden waters (Goodwin et al., 2015; Hua et al., 2016; Zoski et al., 2013). Pairing denitrification woodchip bioreactors with separate, downstream phosphorus-sorbing filters is a promising technique (Coleman et al., 2019) to enhance phosphorus removal relative to woodchips alone, especially when using iron-based filters such as steel-byproducts (Goodwin et al., 2015; Hua et al., 2016), acid mine drainage treatment residuals (Zoski et al., 2013), and calcium-based filters such as fly ash pellets and steel slag (Christianson et al., 2017; Li et al., 2018).

Biochar, a carbon-dense product of thermal biomass decomposition (Lehmann et al., 2011), changes the nitrogen cycle via boosting microbial activities (Anderson et al., 2014; Xu et al., 2014) by providing a colocation for food (carbon), microbes, and nutrients which results in an elevated carbon consumption efficiency as well as microbial activities (Lehmann et al., 2011). Based on a review study by Stenström (2017), biochar showed a removal capacity of 62% – 88% for total nitrogen and a removal capacity of 32% – 89% for total phosphorus. Phosphorous adsorption to biochar is extremely dependent on its mother material (Stenström, 2017) and production methods (Ogonek, 2016) and can occur through physical (adsorption through forces between the phosphate ions and the surface)

and/or chemical (binding phosphorous on the surface) reactions (Stenström, 2017).

2.9 Factors Impacting Denitrification Bioreactor Performance

Many factors affect denitrification processes, and consequently the performance of denitrification bioreactors to remove nutrients. They include HRT (Rodriguez, 2010; Schipper et al., 2010b), type of carbon source media (Gibert et al., 2008; Greenan et al., 2006), DO (Gómez et al., 2002; Healy et al., 2006), pH (Rivett et al., 2008), microbial community (Feng et al., 2017; Jang et al., 2019; Zhao et al., 2018), initial nitrate concentration (Schipper et al., 2010a; Schipper et al., 2010b), and temperature (Cameron and Schipper, 2010; Van Driel et al., 2006).

2.9.1 *Carbon Source Media*

The type of carbonaceous material can greatly influence the denitrification process (Christianson, 2011). A number of carbonaceous materials have been examined to stimulate denitrification processes including different types of woodchips (soft/hardwoods) (Cameron and Schipper, 2010; Peterson et al., 2015); glucose (Shah and Coulman, 1978); sawdust (Warneke et al., 2011a); sucrose (Sison et al., 1995); maize cob (Warneke et al., 2011b); corn (Fay, 1982); alfalfa (Vogan, 1994); methanol (Reising and Schroeder, 1996; Wang et al., 1995); shredded newspaper (Volokita et al., 1996); mixed organic substances like grow-bark, woodchips, and leaf compost with sand (Blowes et al., 1994); cornstalks, cardboard fibers, woodchips with soybean oil (Greenan et al., 2006); and a mixture of woodchips and gravel (Wildman, 2002). Warneke et al. (2011b) found maize cobs and woodchips as the best carbon source media for denitrifying bioreactors, with nitrate removal rates of 6.2 gN/m^3/day and 1.3 gN/m^3/day, respectively. However, since maize cobs showed some adverse effects, like releasing dissolved nitrous oxide, woodchips were

suggested as the preferred media (Warneke et al., 2011b).

In the Midwestern U.S., woodchips have been used as the primary carbon source media in bioreactors (Soupir et al., 2018) with a typical size in the range of ¼" (6.35 mm) to 1" (25.40 mm) (Christianson and Helmers, 2011), mainly because of their high degree of nitrate removal (Greenan et al., 2006), their long functional lifespan (up to 15 years), and their minimal maintenance requirements (Schipper et al., 2010b).

Hardwoods (e.g., oak) and softwoods (e.g., pine) have both been used successfully in-situ denitrification bioreactors (Jaynes et al., 2008; Schipper and Vojvodić-Vuković, 1998; Van Driel et al., 2006). Studies have demonstrated that softwoods have higher nitrate removal potential than hardwoods (Cameron and Schipper, 2010; Gibert et al., 2008). However, a more recent study by Peterson et al. (2015) found that hardwoods (willow oak and red maple) removed more nitrate than softwoods.

While a few studies have examined the impact of woodchip size on nitrate removal, the results have been inconsistent (Cameron and Schipper, 2010; Peterson et al., 2015). Peterson et al. (2015) assessed the impact of woodchip particle size on denitrification bioreactors and found that smaller woodchip particle sizes (5 mm) achieved higher nitrate removal than the larger particle sizes (9.5 mm and 13 mm). This was attributed to the higher surface area of smaller particles per unit mass which, in turn, provides more accessible organic carbon for denitrifying microbes, possibly resulting in higher nitrate removal (Peterson et al., 2015). In contrast to this, Cameron and Schipper (2010) measured an increase in nitrate removal with an increase of woodchip particle size which was attributed to the effect of large woodchip particle sizes on the reactor porosity. Larger woodchip

particle sizes may contribute to higher porosity with greater internal pore structures which can result in greater water holding capacity in a bioreactor system (Lopez-Ponnada et al., 2017). More research is required to determine the effect of woodchip particle sizes on the efficiency of denitrification bioreactors.

2.9.2 *Hydraulic Retention Time*

Hydraulic retention time (HRT) is another key factor influencing denitrification bioreactor performance. It is calculated by dividing the total volume of water passing through the reactor by the influent flow rate (Equation 2.2). Therefore, it is controlled through the incoming flow rate (Schipper et al., 2010b) along with design factors including the volume and media porosity of bioreactor (Christianson et al., 2011b).

$$HRT = \frac{V \times n}{Q} \qquad \text{(Equation 2.2)}$$

Where, n is the media porosity, V is the active volume of the bioreactor (mL), and Q is the influent flow rate (mL/unit time). Woodchip bioreactor porosity has been measured to be 0.7 on average (Chun et al., 2010; Van Driel et al., 2006; Woli et al., 2010).

An adequate HRT is needed to achieve the desired denitrification processes within woodchip bioreactors (Christianson et al., 2012b). Moorman et al. (2015) summarized these estimations and concluded that an HRT of 6 – 24 hours is essential to reach the target efficiency of 50% nitrate removal for influent concentrations of 10 mg/L – 25 mg/L. In a more recent study by Bock et al. (2018), it was determined that an average HRT of 10 hours would be adequate to reach an efficiency of 25% to 45% nitrate removal in a biochar-amended bioreactor.

Long HRTs yield a high nitrate removal (Christianson and Helmers, 2011; Hoover,

2012; Rodriguez, 2010; Schipper et al., 2010b), whereas very low HRTs may not be enough to decrease the influent DO concentrations to a concentration allowing denitrification to proceed (Christianson et al., 2012b). Field-scale woodchip bioreactors have shown a varied range of HRTs between under 3 hours up to 22 days (Table 2.2), resulting in nitrate removal rates ranging from 3% to 100%. As examples, Rodriguez (2010) conducted a laboratory column study under three different HRTs (4.2, 6.3, and 8.0 hours) and concluded that nitrate removal in bioreactors is an HRT dependent reaction, since the nitrate removals increased with increasing HRTs, with nitrate removals of 39%, 76%, and 96% for HRTs of 4.2, 6.3, and 8.0 hours, respectively. Accordingly, he suggested long HRTs to achieve nitrate concentrations below the USEPA's MCL requirement (10 mg/L). In another study, Chun et al. (2009) detected lower nitrate removals (10% - 40%) at low HRTs (< 5 hours) while complete nitrate removal (100%) was detected at long HRTs (15.6 and 19.2 hours). This matches the findings of Greenan et al. (2009), where HRTs of 2.1 days and 9.8 days resulted in nitrate removal rates of 30% and 100%, respectively. Christianson et al. (2012a) and Woli et al. (2010) confirmed a correlation between increased nitrate removal efficiency and increased HRT with field-scale studies in Iowa and Illinois.

Despite the benefits of long HRTs on the removal of nitrates, long HRTs in bioreactors may result in unwanted reactions (Christianson and Helmers, 2011) including the production and release of CH_4, mercury methylation, and hydrogen sulfide gas (H_2S) (Herbstritt, 2014; Hudson and Cooke, 2011). This results from microbes which begin to utilize other electron acceptors when nitrate is depleted in the bioreactors (Christianson and Helmers, 2011; Korom, 1992).

2.9.3 *Dissolved Oxygen*

The presence of dissolved oxygen (DO) in denitrifying bioreactors inhibits nitrate removal by limiting denitrifying microbial activities (Gómez et al., 2002) and causing the accumulation of nitrite and nitrous oxide as unwanted and toxic denitrifying intermediates in bioreactors (Elgood et al., 2010; Gómez et al., 2002). For example, Elgood et al. (2010) stated that high influent DO concentrations (e.g., 12 mg/L) resulted in incomplete denitrification that, in turn, increased the production of dissolved nitrous oxide. Inhibition of nitrate removal occurs when oxygen is present in denitrifying bioreactors, since it can compete with nitrate, serving as the terminal electron acceptor (Healy et al., 2006; Rivett et al., 2008). Oxygen consumption is much easier than nitrate consumption by microorganisms, therefore, the available oxygen is depleted by aerobic bacteria after which the decrease of nitrate (as another electron acceptor) becomes energetically favorable for denitrifiers (Rivett et al., 2008). When oxygen concentrations fall to below 2 mg/L, denitrifying microbes become active (Fahrner, 2002; Rivett et al., 2008) and start consuming the available carbon, resulting in the removal of nitrate as a part of their respiration process.

The presence of high DO is more likely to be problematic under short HRTs (Schipper et al., 2010b). Laboratory and field studies have indicated that around one hour is required to deplete DO in water passing through two-year-old woodchip media (Robertson, 2010) and woodchip particle reactors (Down, 2001; Robertson et al., 2009). Healy et al. (2006) attributed poor nitrate removal in the denitrification beds with various wood materials to high DO concentrations (3.7 mg/L – 7.3 mg/L) and short HRTs. To maximize nitrate removal through the systems under the presence of DO, it has been recommended to add

an additional carbon substrate to the system which can increase the demand for electron acceptors, and subsequently cause more nitrate reduction as it is used as an electron acceptor (Gómez et al., 2002).

A clear DO inhibition threshold for denitrifying bioreactors has not been identified. Oh and Silverstein (1999) found denitrification rates were decreased by 85% in a laboratory-scale study at DO concentration of 2 mg/L; however, Gómez et al. (2002) and Healy et al. (2006) reported no inhibition in denitrification below DO concentrations of 4.5 mg/L and 3.7 mg/L, respectively, in a denitrification bed. Warneke et al. (2011a) also detected no decreases in nitrate removal with average DO concentrations between 0.35 mg/L and 1.7 mg/L.

2.9.4 *Microbial Community*

Since denitrifying microbes are abundant in the natural environment, there is no need for inoculating drainage denitrification bioreactors (Schipper et al., 2010b). Denitrifying microbes can easily enter a denitrification bioreactor via tile drainage water, and colonize the interior of bioreactors. Although denitrifiers are thought to be the major denitrification vehicle, the role of fungi is also important to consider due to their ability to release soluble carbon and thereby provide an important enhancement for denitrification processes (Appleford et al., 2008). The presence of denitrifiers has been detected on both the surface of woodchip media as well as in the bioreactor solution (Appleford et al., 2008). Denitrification may not be restricted to the surface of the woodchips (Christianson, 2011), as Robertson et al. (2000) noticed that large wood particles had dark-colored rims that penetrated several millimeters into the wood particles, indicating the denitrification zone also penetrated several millimeters into the wood media (Robertson et al., 2000).

The variation of microbial communities (e.g., denitrifiers, fungi, and total microbes) within denitrifying biofilters has been documented through DNA techniques. Deeper depths show higher microbial variation compared with upper layers, suggesting the high potential for depth to influence microbial community structure within the denitrifying biofilters (Andrus et al., 2010). In addition to denitrifiers, the presence of biofilms has been documented in woodchip bioreactors (Chun et al., 2009; Damaraju et al., 2015), which can have different microbial compositions, including denitrifiers, and can cause clogging in the control structures or other parts of the bioreactor system (Christianson, 2011). Biofilms can also wash-off at high flow rates or slough-off as they thicken due to attachment weakness, even at moderate flow rates (Christianson et al., 2016b; Chun et al., 2009; Volokita et al., 1996). Therefore, it is possible that washing- or sloughing-off could limit the type and amount of microbes present in the biofilms, including denitrifiers; however, this has not been verified.

In spite of frequent transport of soil microbes from the agricultural lands to the bioreactors through tile drainage water, the microbial communities in bioreactors are distinct from their source communities (agricultural lands) as demonstrated through a study conducted by Hathaway et al. (2015). Therefore, the authors suggested that the contemporary environmental conditions have a substantial effect on the microbial community composition in the bioreactors (Hathaway et al., 2015). This may be used to further optimize denitrifying bioreactors through improvement in design and management characteristics influencing environmental conditions present in the bioreactor, rather than a modification of the microbial communities in the bioreactors or inoculation (Hathaway et al., 2015).

The abundance of denitrifying genes is highly impacted by the carbon sources used in the denitrification bioreactor (Healy et al., 2015). An examination of four bioreactors containing different carbon sources (see Healy et al. (2015) in Table 2.2) showed that the reactors containing cardboard had the highest abundance of denitrifying genes, but the lowest number of denitrification genes as a proportion of total microbes when compared with other bioreactors filled with lodgepole pine woodchips and lodgepole pine needles. The latter carbon sources, however, had the greatest number of denitrification genes, suggesting that a greater proportion of carbon was used by denitrifiers in these bioreactors, while non-denitrifying microbes, like fungi or yeasts, were responsible for consuming a greater proportion of carbon in the bioreactors containing cardboard (Healy et al., 2015).

Although a few studies assessed the microbial communities in woodchip bioreactors (Hathaway et al., 2015; Healy et al., 2015; Porter et al., 2015; Warneke et al., 2011b), the specific microbes in charge of removing nitrate through woodchip bioreactors have not been identified (Jang et al., 2019). This may partially be attributed to difficulties in denitrifying microbial detection (Jang et al., 2019). Since non-denitrifying and denitrifying strains are able to exist in the same genus, it is hard to recognize denitrifying microbes relying solely on taxonomic evidence (Jang et al., 2019). Jang et al. (2019) noted that identification of denitrifying microbes is further complicated by the fact that denitrifying microbes in different taxa can have nearly the same denitrification functional gene sequences (Ishii et al., 2009; Philippot, 2002).

Recently, Jang et al. (2019) published the first report on microbes responsible for denitrification in small-scale woodchip bioreactors in comparatively cold conditions (at 15 $^{\circ}$C) through comparative 16S rRNA and culture isolation techniques. The identified

microbes included *Cellulomonas* spp., *Polaromonas* spp., and *Pseudomonas* spp (Jang et al., 2019). Since *Cellulomonas* spp. is able to degrade cellulose and other complicated polysaccharides of wood media (Thayer et al., 1984), they may provide carbon for themselves as well as other denitrifiers in denitrification bioreactors (Jang et al., 2019). The major microbial community structure in denitrifying woodchip bioreactors was also assessed for a woodchip-based solid-phase denitrification system, and included *Bacteroidetes*, *Proteobacteria*, and *Firmicutes* as the major phyla (Zhao et al., 2018). This was in agreement with a previous study by Feng et al. (2017), where *Bacteroidetes* and *Proteobacteria* were identified as important microbial phyla that played a large role in denitrification bioreactors by removing nitrate and degrading lignocellulose.

Additional laboratory and field-scale studies are needed to help enhance woodchip bioreactor conditions by identifying the microbes responsible for denitrification and the major type and structure of microbial communities in denitrification bioreactors under various conditions (e.g., different temperatures, HRTs, designs, etc.). This information could be used to support the development of an effective design for higher nutrient removal using denitrifying bioreactors. In addition, the understanding of microbial communities within denitrifying bioreactors would further help to understand the impact of bioreactors on altering microbial communities in tile drainage water, and consequently the potential of WBs to release or treat microbial contamination in tile drainage water.

Once tile water passes through denitrification bioreactors, it may facilitate cell-to-cell contact inside the bioreactors, resulting in genetic transfer (HGT) mostly through conjugation (Madigan et al., 2010). Biofilm formation, such as is found on bioreactor woodchips (Chun et al., 2009; Damaraju et al., 2015), may also provide “hotspots” for

HGT (Nesse and Simm, 2018), leading to a potential increase in ARGs and AMR released into the environment. In addition to this, bioreactors can alter the concentration of different microbial communities through high carbon and nutrient concentrations found within the bioreactors which provide favorable conditions for microbes to grow (Madigan et al., 2010). Furthermore, denitrification bioreactors promote nutrient rich environments, which, in turn, may promote cell reproduction and result in an overall increase in the copies of ARGs leaving the bioreactor systems in tile drainage water. On the other hand, removal mechanisms, which may include physical, chemical, and microbial mechanisms, present in denitrification bioreactors may result in reduced concentrations of unwanted microbial populations prior to being released into the environment (Alufasi et al., 2017; Haig et al., 2015; Liao et al., 2015; Stevik et al., 2004). Therefore, it is essential to detect and quantify the potential effect of denitrification bioreactors on the type and structure of microbial compositions to improve the efficiency of systems to remove nitrate and support the development of microbial contaminant removal from waters passing through these systems.

2.9.5 *Temperature*

Temperature is another factor that plays a significant role in removing nitrate from waters passing through denitrification bioreactors. The capacity of denitrification can be increased by maintaining temperatures that result in higher biological reaction rates, specific enzyme kinetics, and growth rates of denitrifying microorganisms (Liu et al., 2013; Saleh-Lakha et al., 2009; Schipper et al., 2010b). Higher temperatures also likely provide greater available carbon for denitrifying microorganisms through the decomposition of wood particles (Warneke et al., 2011a).

Denitrification occurs at temperatures between 5 °C and 30 °C (Blowes et al., 1994; Timmermans and Van Haute, 1983) with the optimum temperature range from 25 °C to 35 °C (Gibert et al., 2008). Previous studies have found a positive correlation between nitrate removal rates and temperature (Cameron and Schipper, 2010; Hoover, 2012; Robertson et al., 2008; Robertson et al., 2009). Examination of field bioreactors in Canada revealed that bioreactors continued to remove nitrate (up to 2 g N/m^3 day) even at low temperatures ranging from 1 °C – 5 °C (Elgood et al., 2010; Robertson et al., 2009). Elgood et al. (2010) determined that a streambed bioreactor could continue to remove nitrate during the winter despite the periodic freezing of the stream surface. To maintain higher nitrate removal rates at lower temperatures, longer retention times are recommended (Robertson et al., 2005; Volokita et al., 1996).

The relationship between nitrate removal rate and temperature can be described with a temperature coefficient (Q_{10}) by which nitrate removal rates change with every 10-degree change in temperature (Davidson and Janssens, 2006) (Equation 2.3). This coefficient depends on nitrate and carbon availability, and denitrifying microbial population dynamics (Hoover, 2012) and usually acts as a tool to approximate the change in denitrification rate at different temperatures observed in the field (Davidson et al., 2006).

$$Q_{10} = \left[\frac{R2}{R1}\right]^{\frac{10}{t2-t1}} \qquad \text{(Equation 2.3)}$$

Where R_2 and R_1 are the nitrate removal rates at temperatures 2 (t_2) and 1 (t_1), respectively and $t_2 > t_1$.

For a laboratory woodchip column, a Q_{10} of 1.7 was reported at temperatures ranging from 14.0 °C to 23.5 °C (Cameron and Schipper, 2010); however, for an in-situ

denitrification bed the Q_{10} was slightly higher at 2.0 with a similar temperature range of 15.5 °C to 23.7 °C (Warneke et al., 2011a). Elgood et al. (2010) also found a Q_{10} of 2 for a streambed denitrifying bioreactor.

2.9.6 *pH*

The denitrification process can also be significantly impacted by pH since the activities of enzymes involved in the process of denitrification are pH-dependent (Partheeban et al., 2014b). A pH range between 5.5 and 8.0 is usually preferable for denitrifying microbes (Rivett et al., 2008) while the optimum pH for denitrification is between 6 and 8 (Bremner and Shaw, 1958).

When denitrification occurs, bicarbonates (HCO_3^-) and hydroxyl ions (OH^-) can be produced (Rivett et al., 2008; Rust et al., 2000), which consequently leads to an increase in pH. This does not have any adverse impact on the denitrifying microbes because they can tolerate a pH between 6 and 9 (Dinçer and Kargı, 2000). However, when the organic material is decomposed, hydrogen ions (H^+) are produced and subsequently result in a reduction of pH (Robertson et al., 2007). Most studies that measured pH reported an increase in pH from the influent to the effluent of denitrification bioreactors (Bock et al., 2016; Damaraju et al., 2015; Nordström and Herbert, 2017; Reddy et al., 2014; Warneke et al., 2011a). However, there are also a few studies that measured a decrease in pH at the outflow of woodchip denitrification reactors (Goodwin et al., 2015; Robertson et al., 2005; Van Driel et al., 2006).

2.9.7 *Initial Nitrate Concentration*

The influent nitrate concentration, whether it is high or low, directly affects the

denitrification processes (Francis and Mankin, 1977; Robertson et al., 2000; Schipper et al., 2005). If influent nitrate concentration exceeds the capacity of denitrifying microbes for removing nitrate, the removal rate remains constant (Hoover et al., 2016). However, under very high influent nitrate concentrations (> 6 g/L), the denitrification processes will be inhibited (Francis and Mankin, 1977). Limitations in removing nitrate at high nitrate concentrations are attributed to the availability of carbon, denitrifier biomass (Moorman et al., 2010), or as a result of the toxicity of nitrite accumulation (Chen et al., 1991; Glass, 1998).

2.9.8 *Nitrate Removal Kinetics in Denitrification Bioreactors*

The reactions in the denitrification process are enzyme catalyzed reactions and are likely to follow Michaelis–Menten kinetics (Ghane et al., 2015). The Michaelis-Menten equation considers the concentration and accessibility of substrates, in this case nitrate, involved in denitrification (Sylvia et al., 2005) and takes the following form with nitrate as the substrate (Fogler, 2010):

$$r_{NO3} = \frac{V_{max}\, C_i}{K_m + C_i} \qquad \text{(Equation 2.4)}$$

Where $V_{max}$is the maximum rate of nitrate removal (mg/L/hour), $K_m$is the Michaelis-Menten constant (mg/L) defined as the concentration of nitrate at which the nitrate removal rate is half of V_{max}, C_i is the influent concentration of nitrate (mg/L), and r_{NO3} is the nitrate removal rate (mg/L/hour) defined as the difference between the bed influent and effluent nitrate concentrations (mg/L) divided by the time (hours) it takes for the nitrate removal to occur (Ghane et al., 2015).

High influent nitrate concentration ($C_i \gg K_m$) may saturate denitrifying microbes and

consequently result in zero-order kinetics in the systems. In contrast to this, at low influent nitrate concentrations ($C_i \ll K_m$), nitrate is limiting and denitrification rates are proportional to the concentration of nitrate, resulting in first-order kinetics (Ghane et al., 2015).

Nitrate removal kinetics in denitrification bioreactors are complex (Schipper et al., 2010b) and controversial (Christianson et al., 2012b). They are complex because several factors can affect nitrate removal including the inherent capacity of the microbial community for denitrification, the HRT in the systems (Schipper et al., 2010b), and the availability of dissolved organic carbon (Robertson, 2010).

Nitrate removal kinetics are still controversial because there is no agreement regarding the exact type of nitrate removal kinetics (zero-order or first-order) in denitrification bioreactors. While it was reported that denitrifying bioreactors obey zero-order approaches as they receive higher initial nitrate concentration than the $K_m$of denitrifying microbes (Barton et al., 1999), first-order kinetics have also been reported in several cases (Chun et al., 2009; Leverenz et al., 2010; Moorman et al., 2015), especially when the concentration of nitrate falls to less than 1 mg/L (Robertson, 2010). For example, in a small-scale study which assessed whether or not denitrifying bioreactors operate under zero-order kinetics, Robertson (2010) tested a series of woodchip columns under successive runs at increasing influent nitrate concentrations in a range between 3.1 mg/L and 49 mg/L. The results showed that increasing influent nitrate concentrations did not result in higher rates of nitrate removal, and consequently concluded that zero-order approaches would apply with this wide range of nitrate concentrations. Hoover et al. (2016) confirmed that nitrate-saturation occurs in a range between 30 mg/L and 50 mg/L, which is suggestive of Michaelis–Menten

approaches. Hua et al. (2016) recently determined that when nitrate concentration becomes limited (< 3 mg/L) nitrate removal kinetics switched from zero-order to first-order kinetics. Understanding the mechanism governing nitrate removal in denitrification bioreactors is necessary to enhance the development of effective design for denitrifying bioreactors.

2.10 Longevity of Denitrification Bioreactors

Denitrification bioreactors need to have adequate longevity to be economically viable solutions for managing nitrate (Robertson et al., 2000). Many factors influence the longevity of denitrification bioreactors including the type of material, mass of reactive material, physical changes in the porosity and permeability of media, the reaction rate, flow conditions, uniformity, and saturation level in the bioreactors (Blowes et al., 2000; Robertson and Cherry, 1995; Schipper et al., 2010b).

Most of the longevity estimations for denitrification bioreactors have been done for the treatment of groundwater or tile drainage water, and the bioreactors were estimated to last a minimum of 10 years (e.g., Blowes et al. (1994): 72 years, Long et al. (2011): 66 years, Moorman et al. (2010): 37 years, Robertson and Cherry (1995): 20 years, and Christianson et al. (2012b): at least 10 years (through empirical data)). Although the precise longevity of drainage denitrification systems is unknown, drainage denitrification bioreactors are typically designed based on a minimum lifespan of 10 years (USDA-NRCS, 2009).

Estimating the lifespan of denitrifying bioreactors treating drainage water is complicated due to fluctuations of their flow depth as well as water saturation (Christianson et al., 2012b). When flow fluctuates in the systems, woodchips near the surface of denitrification systems become less consistently saturated compared with those in the

deeper parts (Christianson et al., 2011a; Moorman et al., 2010), which may impact the carbon deterioration and result in quicker carbon degradation. There are several studies demonstrating limited carbon deterioration of wood particles that were consistently saturated during the first years of operation (Moorman et al., 2010; Robertson et al., 2000; Schipper and Vojvodić-Vuković, 2001). Based on this information, Schipper et al. (2010b) suggested that the slow degradation of wood particles under anoxic conditions could support a long lifespan of denitrification bioreactors (up to 15 years) without additional maintenance or carbon replacement.

2.11 Installation Costs of Denitrification Bioreactors

Denitrification bioreactors must be cost-effective to be widely used for managing nitrate. The cost of bioreactors per kilogram of nitrate removed ranges from about US$2.39 to US$15.17 (Table 2.3). For the farmers with access to wood media and a backhoe, the total annual cost per kilogram nitrate removal would be around US$2.39 per kg nitrate (Schipper et al., 2010b). This cost compares favorably with the estimated costs of other technologies for managing nitrate (Table 2.3, provided by Schipper et al. (2010b)). Schipper et al. (2010b) described the bioreactors as a cost-efficient alternative for removing nitrate when they are compared with other management methods for reducing nitrate (Table 2.3).

Table 2.3 A comparison between the approximate cost of bioreactors with other agricultural practices for managing nitrate (Schipper et al. (2010b)).

Practices	US $ (per kg nitrate)	Source
Bioreactor	2.39 – 15.17	Schipper et al. (2010b)
Soil testing and side dressing N fertilizer	1.15	Saleh et al. (2007)
Drainage water management	2.71	Jaynes and Thorp (2008)
Wetlands	3.26	Hyberg (2007)
Fall cover crops	11.06	Saleh et al. (2007)

In Iowa, the installation cost of bioreactors was estimated to be in the range of $7,000 to $10,000 to treat drainage water from approximately 30 acres to 100 acres (Christianson and Helmers, 2011). There is a cost-sharing program for roughly half the installation cost of bioreactors, provided by the Environmental Quality Incentive Program (EQIP), which reduces the installation cost to about $4,000 (Christianson and Helmers, 2011). Similarly, Van Driel et al. (2006) and UMN Extension (2011) have reported installation costs of Can$2,000 and US$3,200, respectively.

In South Dakota, the total cost of four installed bioreactors was more expensive and ranged from US$7,914 to US$10,414 (Partheeban et al., 2014a). These costs are similar to the installation costs reported in Iowa without considering the cost-sharing program. The annual cost per kg nitrate removal was also estimated to be $11 to $61 (Thapa, 2017). These costs were within the estimated range by Schipper et al. (2010b) for two of the four bioreactors reported (Arlington and Hartford); however, the costs exceeded the range for the other two bioreactors (Baltic and Montrose).

2.12 Concerns with Denitrification Bioreactors

While denitrification bioreactors are effective at removing nitrate from tile drainage water (Table 2.2), there are also several major concerns regarding the application of these systems, including the flushing of organic matter during the start-up phase, the release of CH_4 and nitrous oxide (N_2O) as greenhouse gases, the emission of H_2S, and mercury methylation (Herbstritt, 2014; Rivas et al., 2019; Schipper et al., 2010b; Weigelhofer, 2015).

2.12.1 *Organic Flushing*

The flushing of organic matter is one possible problem occurring during the start-up phase of bioreactors filled with woody carbonaceous material. Flushing of organic matter is problematic because it increases the total organic carbon (TOC) (Gibert et al., 2008), dissolved organic carbon (DOC) (McLaughlan and Al-Mashaqbeh, 2009), biological oxygen demand (BOD) (Schipper et al., 2010b), ammonium (NH_4^+) (Cameron and Schipper, 2011; Lepine et al., 2016), and total Kjeldahl nitrate (TKN) (Zoski et al., 2013) in the effluent of bioreactors, which can impact downstream water quality. The initial dark colored effluent of woodchip bioreactors is an indicator that early DOC may have concentrations of hundreds of milligrams per liter (Schipper et al., 2010b). However, DOC concentrations can be stabilized at lower levels over time (Robertson et al., 2005).

To mitigate the side effect of organic matter, the carbon materials are often pre-washed/flushed in the laboratory-scale studies (Diaz et al., 2003); however, this method is logistically difficult at the field-scale (Schipper et al., 2010b). Instead, flushing of organic matter may be mitigated via starting the bioreactors under high flow conditions in the field, with an understanding that the organic flushing effect will not be eliminated from the systems (Schipper et al., 2010b).

2.12.2 *Greenhouse Gases*

Nitrous oxide (N_2O) and methane (CH_4) are greenhouse gases which can be produced as by-products in these bioreactors. The N_2O has received greater attention due to its higher impact on depleting the ozone layer (Ravishankara et al., 2009). The N_2O is released either as a gas from the surface or dissolved in the liquid effluent of denitrification bioreactors (Christianson, 2011). Many environmental factors are involved in increased N_2O

production, including high DO, low pH, and a low ratio of total carbon to nitrogen (Chapin et al., 2002). High DO can be of particular concern for drainage bioreactors (Christianson, 2011). The presence of high DO concentrations (aerobic conditions) in the systems causes incomplete denitrification, which can result in the production of N_2O (Elgood et al., 2010; Greenan et al., 2009). To mitigate the release of N_2O, it has been proposed to design the bioreactors to remove 100% of nitrate (Elgood et al., 2010); however, this may cause other problems such as sulfate reduction and mercury methylation.

Another by-product of these bioreactors can be CH_4 formed by archaea known as methanogens under anaerobic conditions (Elgood et al., 2010; Healy et al., 2012). Elgood et al. (2010) measured the production of dissolved CH_4 in a stream-bed denitrifying bioreactor and reported an elevated concentration of dissolved CH_4 when nitrate concentrations were almost depleted. In contrast to this study, Healy et al. (2012) detected no dissolved CH_4 and low CH_4 emissions from denitrification bioreactors due to the presence of high nitrate concentrations in the bioreactors, which caused the methanogenic archaea to be out-competed by denitrifying microbes for available carbon sources.

N_2O and CH_4 are recognized as substantial contributors to global radiative forcing (Myhre et al., 2014). Their production has been detected through laboratory-scale denitrification bioreactors at different HRTs (Bock et al., 2018; Greenan et al., 2009; Healy et al., 2012); however, the scale of the bioreactors were relatively small (less than 0.001 m^3). A recent pilot-scale study confirmed the production of N_2O and CH_4, mostly in the dissolved form, in the effluents of nine pilot-scale bioreactors (6.38 m^3) at different HRTs (2, 8, and 16 hours) (Davis et al., 2019). Their results showed that the shortest HRTs (2 hours) had the highest impact on the production of N_2O and CH_4, releasing the highest

N_2O and the lowest CH_4. Their study suggested that managing HRT at between 6 hours and 8 hours could decrease the total greenhouse gas production and increase nitrate removal through denitrification bioreactors.

2.12.3 *Sulfate Reduction and Mercury Methylation*

Sulfate reduction occurs when there is nearly complete nitrate removal through denitrification systems, mostly at low flow rates (long HRTs) (Van Driel et al., 2006). When nitrate is completely depleted in the systems, sulfate-reducing microbes out-compete denitrifying microbes for available carbon sources which eventually results in the conversion of naturally present sulfate to hydrogen sulfide gas (H_2S). This process is concerning for several reasons. First, the consumption of carbon through sulfate-reducing microbes may jeopardize denitrification processes through the loss of carbon. Second, H_2S is a noxious gas (harmful to living things); and third, the microbiological processes of sulfate-reducing microbes can lead to the production of mercury methylation, as a common form of mercury (Hudson and Cooke, 2011; Krabbenhoft and Rickert, 2009). Mercury methylation can jeopardize human health through direct inhalation of elemental mercury or the ingestion of accumulated methyl mercury (CH_3Hg^+) in aquatic creatures (Krabbenhoft and Rickert, 2009). Therefore, it is necessary to design and manage bioreactors to minimize the process of sulfate reduction, possibly through retaining low levels of nitrate in the denitrification bioreactor effluent (Robertson and Merkley, 2009). The production of H_2S can be recognized by a detectable rotten egg smell (Herbstritt, 2014) around the outflow control structure, and can be managed by lowering the stop logs in the control structure to facilitate the movement of water (Christianson, 2011).

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CHAPTER 3: THE EFFECT OF WOODCHIP BIOREACTORS ON MICROBIAL CONCENTRATION IN SUBSURFACE DRAINAGE WATER AND THE ASSOCIATED RISK OF ANTIBIOTIC RESISTANCE DISSEMINATION

A paper under revision

Abstract

Woodchip bioreactors (WBs) are a promising technology capable of decreasing nitrate concentration in subsurface (tile) drainage system (SDS) effluent. Although they are effective in removing nitrate, little is known regarding the potential impact on microbes and antimicrobial resistance (AMR) in subsurface drainage waters. Therefore, the main objective of this study was to measure how bioreactors influence microbial concentrations and AMR concentrations in tile drainage water with different flow conditions and microbial communities. A column study was conducted to simulate an existing, field denitrifying woodchip bioreactor. Four synthetic waters were spiked with different combinations of microbes isolated from the influent of a field-scale bioreactor and pumped through the columns during the experiment. Steady and wet-dry flow conditions were examined. The results of this work demonstrated the potential for denitrifying bioreactors to reduce *E. coli* concentrations within SDSs under different flow conditions, which expands the potential benefits of WBs. However, the results also illustrated that WBs have the potential to increase phenotypic antibiotic-resistant microbes in the subsurface drainage waters. Applying different microbial communities had significant effects on culturable microbial concentrations, as columns receiving more microbes with a greater diversity had a greater increase in culturable microbial concentrations. A statistically significant and consistent increase in phenotypic AMR concentrations was also found within woodchip

bioreactors after assessing the sensitivity of recovered isolates to five different antibiotics. Additional laboratory and future in-field studies are warranted to improve the prediction of pathogen removal and AMR changes in tile drainage water, as well as to support the development of an effective design for microbial contaminant removal from waters passing through denitrifying bioreactors.

Keywords: Agriculture, Column study, Tile drainage, Water quality, *Escherichia coli*, Antimicrobial resistance.

3.1 Introduction

Subsurface (tile) drainage systems (SDSs) are installed in agricultural lands to remove excess water from the root zone and decrease the water table in poorly drained soils. However, these systems are a direct pathway for many pollutants including nitrate, microbes (e.g., fecal indicator organisms), and antimicrobial resistance (AMR) via antibiotic resistance genes (ARGs) to enter surface water (Hoang et al., 2013; Jaynes et al., 1999; Kay et al., 2005; Washington et al., 2018). The application of fertilizers, including manure, to agricultural land is the primary source of these pollutants (Goolsby and Battaglin, 2000; Heuer et al., 2011; Reddy et al., 1981). The pollutants leach through the soil and move into the tile drainage water mainly by infiltration via soil macro-pores (Hruby et al., 2016; Kay et al., 2004, 2005; Kladivko et al., 1999). The nutrient-laden tile drainage waters are then transported to surface waters through SDSs, where it negatively impacts water quality (Pinheiro et al., 2013; Skaggs et al., 1994).

In the Midwestern U.S., significant quantities of nitrate are delivered to streams and rivers through SDSs (Gedlinske, 2014). Nitrate concentrations detected in SDSs are often

higher than the maximum contaminate level of 10 mg/L set by the Environmental Protection Agency (EPA) for drinking water (Jaynes et al., 1999; Kalita et al., 2006; USEPA, 2002a). Transport of nitrate via SDSs is a widespread concern due to its profound effect on eutrophication and hypoxic conditions in waterbodies, such as in the Gulf of Mexico (Dale et al., 2007; Rabalais et al., 1996).

The transport of pollutants through SDSs needs to be managed to reduce the possible water quality impairments in streams receiving tile drainage water. One effective and cost-efficient management practice to remove nitrate in tile drainage water is denitrifying woodchip bioreactors (WBs) (Christianson et al., 2012; Partheeban et al., 2014; Robertson, 2010; Robertson et al., 2008; Schipper et al., 2010). WBs consist of a trench that is filled with wood media which tile drainage water is diverted through. Woodchips serve as a carbon and energy source for microbial growth, and the systems are designed to utilize these microbes to remove nitrate from drainage water through denitrification. In denitrification, a diverse group of mostly facultative anaerobes (Patureau et al., 2000), most of which are heterotrophic organisms (Korom, 1992), convert nitrate into either nitrogen gas or nitrous oxide gas under anoxic micro-environment conditions (Flores et al., 2007; Sylvia et al., 2005; Verbaendert et al., 2011). Heterotrophic denitrifying microbes (including *Bacillus*, *Corynebacterium*, and *Pseudomonas*), which are abundant in the natural environment (Sylvia et al., 2005), enter denitrifying WBs via tile drainage water, and colonize the interior of bioreactors. They consume carbonaceous components of the wood as carbon source (food) and energy source (electron donor), and reduce/convert the nitrate (electron acceptor) as a part of their anaerobic respiration process (Christianson and Helmers, 2011). Denitrification is influenced by environmental parameters including the

form of carbon available, dissolved oxygen, pH, and temperature (Cameron and Schipper, 2010; Xu et al., 2009).

While denitrifying WBs are effective at removing nitrate from tile drainage water (Christianson et al., 2012; Schipper et al., 2010), little consideration has been given to the fate of fecal indicator organisms (e.g., *E. coli*), ARGs, and AMR in WBs. A few studies assessed the ability of WBs to reduce microbial contaminants from wastewaters (Rambags et al., 2016; Robertson et al., 2005; Tanner et al., 2012). For example, Rambags et al. (2016, 2019) examined the impact of bioreactors on *E. coli* concentrations in wastewaters, and demonstrated the border versatility of WBs on microbial contaminant removal (*E. coli*, total coliforms, and viruses) for wastewater treatment. Additionally, Soupir et al. (2018) focused on bacteria removal in drainage from manure-amended agricultural lands, and demonstrated the potential for WBs to remove enteric bacteria. However, no work has been done that examines the impact of these systems on AMR and ARGs in the drainage waters which subsequently move into the environment.

The goal of this study was to detect and quantify the potential effect of WBs on microbial concentrations and phenotypic AMR microbe concentrations in tile drainage water to understand the potential for WBs to reduce unwanted microbes (e.g., *E. coli*) and their potential to alter AMR microbe concentrations in subsurface drainage waters. In this experiment, four laboratory-scale bioreactors containing woodchips were used to simulate existing, field denitrifying bioreactors.

3.2 Materials and Methods

3.2.1 *Material Description*

Cottonwood woodchips, the same variety as typically used in field bioreactor installations in the region, were purchased from a supplier in Sioux Falls, South Dakota (SD). The woodchips were washed to remove dirt and fine particles and air-dried before use to prevent the fine particles from blocking flow within the column. The woodchip size was determined using 500 g of the dried woodchips, fractionated by ASTM standard sieves in a shaker for 5 min (ANSI/ASAE, 2007). Sizes ranged from 0.1 - 6 cm in length and 0.1 - 2 cm in width (Table 1). Woodchip porosity was also measured based on the methods described by Christianson et al. (2010). Briefly, distilled water was added to 1-L bottles packed with woodchips (3 repetitions) until the pore volumes were filled with distilled water and the bottles reached saturation. The bottles were capped and left to absorb the distilled water for 24 - 48 hours, and refilled to saturation. The average woodchip porosity was calculated based on dividing the average total volume of water added to the bottles by the average volume of bottles. The average woodchip bulk density was also calculated based on the total volume of the columns and the average mass of the woodchips added to the columns. The moisture content of the woodchip particles at the time the columns were packed was calculated as a percent of water mass loss from woodchip samples oven-dried at 70 ºC. The pH of woodchips was measured by using the hot water extract method described by Sithole (2005). 50 grams of woodchip samples (oven-dried overnight at 105 ºC) were placed in a 2 L beaker before 180 mL distilled water and 20 mL of 2M NaCl solution were added to the beaker. The beaker was covered with aluminum foil and boiled for 10 minutes on a hot plate. The pH of the solution was measured after cooling to room

temperature (about 23 ºC).

Table 3.1 Characteristics of woodchip media.

Material	Woodchip
Type	Cottonwood
pH	6.8
TOC	212 mg/L
Porosity	52%
Bulk density	0.29 g/cm^3
Moisture content	10.24%
Size (distribution% by weight)	10% small (0.1 – 1.0 cm wide, 0.1 – 1.0 cm long)
	58% medium (0.5 – 1.5 cm wide, 1 – 3 cm long)
	32% large (0.5 – 2.0 cm wide, 3 – 6 cm long)

3.2.2 *Bioreactor Setup*

Four acrylic, up-flow columns were used to simulate an existing, field denitrifying bioreactor (Figure 3.1). Perforated Plexiglas plates were fitted to both ends of the columns to diffuse the flow of synthetic solution into the columns. Two ports, with tubing attached, were also applied at both ends of the columns to allow for inflow and outflow of the solution. In each cap, a 6-inch-long temperature sensor was also installed to measure the temperature inside the columns throughout the experiment. Water samples were taken from the influents, effluents, and the midpoints by three sampling ports (1, 2, and 3) consisting of tubing and an attached valve (Figure 3.1).

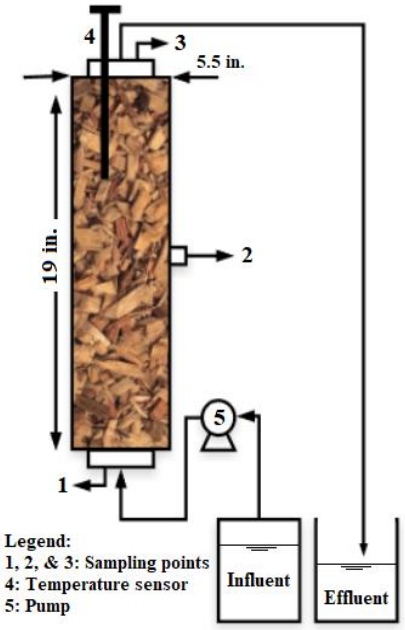

Figure 3.1 Schematic of the laboratory woodchip reactors. A peristaltic pump (5) was used to pump synthetic water up through the column, creating saturated conditions. Samples were collected at ports 1, 2, and 3.

Woodchips smaller than 2.36 mm were removed via sieving to prevent clogging in the columns. The rest of the woodchips were manually compressed into the columns and tamped incrementally to decrease void space via using a steel rod. Approximately 2,085 ± 40 g of woodchips were used to fill each column. The columns had an average total volume of 7,340 cm^3 and an estimated average pore volume of 3,817 cm^3.

3.2.3 *Flow Rate*

Four separate influent containers holding up to 40 L of solution provided synthetic tile drainage water to each column. The columns were operated at room temperature (23 °C). Water was pumped up through the columns using Masterflex FH100 variable speed peristaltic pumps (Cole-Parmer, Vernon Hills, IL), one for each column. The target hydraulic retention time (HRT) of 12 hours was achieved by controlling pump speed (revolutions per minute: 46) as well as silicone tubing size (0.08 mm ID). The silicone tubing was changed periodically to prevent its excessive wear and tear. The flow rate was calculated to be 5.3 mL/min (Equation 3.1) and verified via periodic measurements.

$$Q = \frac{V \times n}{60 \times HRT} \qquad \text{(Equation 3.1)}$$

Where V is the volume of the column (mL), and n is the porosity of the woodchip media (%).

3.2.4 *Microbial Isolates*

Before the experiment, water samples were taken from the influent and effluent of a field-scale woodchip bioreactor (WB) located near Baltic, SD. *E. coli* as well as the general culturable microbial community were enumerated using standard membrane filtration

(USEPA, 2002b) and serial dilution methods (Ridout, 2014), respectively. The most common, morphologically distinct microbial colonies (in terms of colony shape, size, and pigmentation), hereafter referred to as “indigenous microbes”, were recognized, recorded, and recovered from the samples. Ten separate *E. coli* colonies taken from the influent and 17 indigenous microbes taken from the influent (seven colonies) or the effluent (10 colonies) were isolated and used in this experiment. All indigenous microbes were grown in 10 mL of Reasoner's 2A (R2A) broth and incubated with agitation at 25 °C until dense growth occurred, ranging from 1 to 3 days depending on the isolate. Each dense broth culture was subjected to a serial 10-fold dilution to determine the concentration of each microbe in the growth media. The optical density (OD) of the resulting suspension was recorded by using a spectrophotometer at 600 nm (GENESYS™ 20 Visible Spectrophotometer, Thermo Scientific, Waltham, MA.). All dilutions were plated onto R2A agar plates and incubated at 35 ± 0.5 °C for 72 ± 2 hours. All resulting colonies were counted, and the concentrations of the indigenous microbes per mL were determined. All results were recorded in a table showing the approximate concentration of each indigenous microbe at different dilutions and OD_{600}. Frozen stocks of all strains were maintained at −80 °C in the appropriate broth containing 15% (v/v) glycerol until use.

3.2.5 *Inoculating the Reactors*

The woodchip bioreactor columns were inoculated with the indigenous microbes isolated from bioreactor effluent water prior to the experiment. Four similar synthetic waters were prepared to inoculate the reactors, one for each column. Nutrients were added to the inoculation waters to maintain isotonic conditions for the microbial cells and decrease the potential impact of micronutrient deficiencies on the denitrifier microbial

populations (Hoover, 2012). The nutrients were added to 12 L of Reverse Osmosis (RO) water to obtain final concentrations of: 4.0 mM $CaCl_2$, 2.0 mM KH_2PO_4, 1.0 mM K_2SO_4, 1.0 mM $MgSO_4$, 25 μM H_3BO_3, 2.0 μM $MnSO_4$, 2.0 μM $ZnSO_4$, 0.5 μM Na_2MoO_4, and 0.5 μM $CuSO_4$ (Hoover, 2012; Nadelhoffer, 1990).

Ten indigenous microbes with different colony morphologies from the field WB effluent were revived from frozen stocks, and grown in 10 mL of R2A broth in an incubator with agitation at 25 °C. The OD_{600} of the resulting broth culture was measured and used to estimate the concentration of each culture. Since OD is directly proportional to the cell concentration (Dubey, 2014; Madigan et al., 2010), the concentration of each culture was estimated using the OD_{600} and the concentration-OD_{600} relationship for each indigenous microbe determined using methods discussed in section 3.2.4. Afterward, around 0.1 mL of each culture was immediately added to 20 mL of phosphate buffered saline solution (PBS) and diluted to achieve the desired concentration of approximately 2×10^{1} CFU/mL of each microbe to reach a total concentration of 2×10^{2} CFU/mL before being added to the inoculation solution. The solution with cells was recirculated through the reactors for 10 days at 2 mL/min to inoculate the reactors with microbes and promote the growth of denitrifying microbes. After the inoculation period, each reactor was flushed with 12 L of the RO water and micronutrients for 2 days to restore anaerobic conditions and refresh microbial activity. The initial flow rate used to flush the columns was 2 mL/min and was then gradually increased to the experimental flow rate of 5.3 mL/min to minimize the possible disturbance to microbial communities.

3.2.6 *Synthetic Tile Water Preparation*

Four different synthetic waters were used for the experiment, one for each column.

Each synthetic water had a base composition of RO water with added micronutrients (see recipe above) and 30 mg/L (0.3 mM) of potassium nitrate. The microbes, including seven indigenous microbes and 10 *E. coli* isolates from the WB influent, were revived from frozen stocks and grown in 10 mL of R2A or tryptic soy broths, respectively, with agitation at 25 °C for one to two days prior to mixing the synthetic waters. The OD_{600} of each broth culture was measured to estimate the number of microorganisms contained in each broth. The OD_{600}–concentration relationship for each indigenous microbial isolate was determined as described above; however, the *E. coli* isolates were assumed to have a concentration of 8×10^8 cells/mL when the OD_{600} was 1.0 (Loehrer et al., 2016; Pumphrey, 2000). The synthetic waters (Table 3.2) of columns A and C were spiked with 2×10^{2} CFU/mL of *E. coli* and 2×10^{2} CFU/mL of indigenous microbial isolates, respectively, while both sets of microorganisms were added to synthetic water of column B. No microbes were added to the synthetic water of column D, which was used as a control treatment. The synthetic waters were made every five days and pumped into each column throughout the experiment. Homogeneous mixing of the synthetic water solution was achieved by applying an agitator attached to a small aquarium pump in each container which ran throughout the experiment.

Table 3.2 Synthetic water characteristics.

Column	Synthetic Water Makeup
A	Nitrate + micronutrients + *E. coli*
B	Nitrate + micronutrients + *E. coli* + indigenous microbes
C	Nitrate + micronutrients + indigenous microbes
D	Nitrate + micronutrients

3.2.7 *Experimental Procedure*

Peristaltic pumps were used to pump the synthetic solutions through the columns which ran continuously for 60 days. Water samples were taken 10 times over the course of the

experiment, on days 1, 2, 3, 4, 5, 10, 15, 20, 30, and 60. Samples were collected from the influent, midpoint, and effluent (Figure 3.1). Sampling tubes were changed after each sampling to avoid contamination. After 60 days, two sets of wet-dry flow experiments were conducted. The columns were allowed to drain via gravity flow for 24-hours before a pulse of water was pumped through each column and samples collected at the influents, effluents, and midpoints. The 24-hour draining and subsequent water pulse were conducted three times. This set of wet-dry experiments was repeated with a 48-hour draining time. The same flow rate was applied in both flow conditions (5.3 mL/min).

At the end of the experiment, the woodchips from each column were removed and examined for biofilm. Each column was divided into three equal depths, and labeled as inlet (starting from the inlet at 0 inches to 6.3 inches of the column's length), middle (6.3 to 12.6 inches), and outlet (12.6 to 19 inches). Woodchip biofilm samples, which were visually detected, were taken separately from each depth (inlet, middle, and outlet) and washed with PBS two times to remove superficially associated microbes before being stored in PBS solutions for further analysis. The biofilm samples were prepared based on the methods described in Fischer et al. (2012) and then used for photo-documentation by scanning electron microscopy (SEM) at 15 kV. Biological specimens were fixed on the woodchip samples by glutaraldehyde (GA) and osmium tetroxide (OsO_4). The samples were subsequently dehydrated with ethanol (ETOH) and dried with hexamethyldisilazane (HMDS) to be used with gold sputter coating for SEM imaging (JEOL JSM-7001F, JEOL USA, Inc.). Woodchip samples were also placed in PBS, sonicated, and vortexed to detach biofilms from the samples (Kobayashi et al., 2009). For each depth, 40 g of woodchips covered with biofilms were placed into four centrifuge tubes containing 30 mL of PBS

(~10 g sample for each tube), and vortexed for 30 s, sonicated at 40 kHz for 10 minutes, and vortexed again for 30 s at room temperature. One of the aqueous samples was immediately used to culture *E. coli* while the others were stored in the freezer for future DNA extraction using a DNeasy PowerSoil Kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of DNA in each extract was determined using a Qubit 3.0 Fluorometer (Invitrogen, Burlington, ON, Canada).

3.2.8 *Sample Analysis*

Water samples were analyzed for *E. coli* concentrations, total culturable microbial concentrations (aerobic or facultative anaerobic microbes), Gram-negative and positive wall characteristics of microbes, and phenotypic antibiotic resistance of the microbes. At the end of the experiment, the biofilm's makeup in the columns was characterized using SEM and 16S rRNA gene amplicon sequencing. The samples were processed for *E. coli* (influents, midpoints, and effluents) and the general microbial community (influents and effluents), using standard membrane filtration with culturing on modified mTEC ager (USEPA, 2002b) and serial dilution methods (Ridout, 2014) with culturing on R2A ager, respectively, within 6 hours of sample collection.

For standard membrane filtration, water samples were filtered through a sterile, 0.45-μm-membrane filter. The samples were plated in triplicate (with two dilutions, 1 and 10 mL) on modified mTEC agar, placed in a water bath at 35 ± 0.5 °C for 2 ± 0.5 hours, and incubated at 44.5 ± 0.2 °C for 22 ± 0.5 hours. Concentrations of *E. coli* per mL water were determined from averaging triplicate plates containing 30 – 300 CFU. One to 10 *E. coli* isolates were streaked on tryptic soy ager (TSA) plates and subjected to phenotypic antibiotic susceptibility testing using a modified Kirby-Bauer method (Bauer et al., 1966;

CLSI, 2011). Five antibiotics that have been detected in the aquatic and terrestrial environments (Carvalho and Santos, 2016; Kemper, 2008; Kim et al., 2011; Kümmerer, 2009) were tested, including tetracycline (30 µg), ampicillin (10 µg), penicillin (10 U), sulfisoxazole (0.25 mg), and erythromycin (2 µg), with disc concentrations shown in brackets. The disc concentrations were selected according to the usual concentrations used in Kirby-Bauer testing for environmental isolates (CLSI, 2011; Helt, 2012; Muñoz-Atienza et al., 2013; Nasreen et al., 2015).

Antibiotic-sensitivity discs were placed on Mueller-Hinton agar plates inoculated with a microbial isolate and incubated for 24 - 48 hours at 37 ± 0.5 °C. After incubation, the inhibition zone diameters were measured and classified as resistant, intermediate, or sensitive using reference levels determined by the Clinical and Laboratory Standards Institute (CLSI, 2011) and National Committee for Clinical Laboratory Standards (NCCL, 1984) for bacterial strains. Intermediate strains were combined with the resistant category since those isolates are also somewhat resistant to the antibiotic (Łuczkiewicz et al., 2010; Reinthaler et al., 2003).

For serial dilutions, the water samples were diluted (five 10-fold dilutions) in PBS, plated on R2A plates, and incubated at 35 ± 0.5 °C for 72 ± 0.5 hours. All samples were processed in duplicate. Concentrations of culturable microbes per mL water were determined from averaging dilution plates containing 30 – 300 CFU. The characteristics of morphologically distinct microbial colonies (shape, size, and pigmentation) were documented and phenotypic antibiotic susceptibility testing was conducted on each distinct isolate to assess their sensitivity to the antibiotics. Gram-negative and Gram-positive strains were determined by using the KOH (3%) string test (Carlone et al., 1982).

Finally, the woodchip biofilm samples from the bioreactors were processed for *E. coli* concentrations, SEM analysis, and 16S rRNA gene amplicon sequencing. 16S rRNA gene amplicons were sequenced using MiSeq Illumina 2 × 300 bp chemistry using the primers 515Ff and 806rB targeting V4 hypervariable region of bacterial and archaeal 16S SSU rRNA gene sequences by the University of Minnesota Genomics Center. Each sample was sequenced once. Post sequence processing was performed within the mothur (ver. 1.37.6) sequence analysis platform (Schloss et al., 2009) following the MiSeq SOP (Kozich et al., 2013) as described previously (Hamilton and Havig, 2017). Amplicon libraries ranged in size from 46,207 to 246,009 total sequences following quality control. Sequences were binned into operational taxonomic units (OTUs) based on a sequence similarity of 97.0% and classified using a Bayesian classifier within mothur against the Silva (v132) reference taxonomy. Within sample diversity (alpha diversity) and relative abundance were calculated and visualized using Phyloseq (McMurdie and Holmes, 2013). For analysis of alpha diversity, each sample was rarefied to an even depth. Replicate samples were pooled for visualization. All sequence data, including raw reads with quality scores for this study, have been deposited in the NCBI Sequence Read Archive (SRA) database under with the BioProject number PRJNA513338.

3.2.9 *Statistical Analysis*

E. coli reduction and culturable microbial increase was calculated as the average difference in concentration (CFU/mL) between the influent and effluent of each column at each flow condition, and the difference was expressed as a percentage of influent concentration. To analyze the data, R (version 1.1.442) software was used with a significance level of 0.95 ($\alpha = 0.05$). Continuous data were tested for normal distribution

using Shapiro–Wilk's W test. For normally distributed data, parametric tests were applied (paired t-test and Tukey HSD), while for non-normally distributed data, non-parametric tests (Wilcoxon signed-rank test and Dunn test) were conducted. For categorical data like antimicrobial resistance results, the chi-square test was used to determine changes in resistance for different microbial compositions or flow conditions.

3.3 Results and Discussion

3.3.1 Changes in *E. coli* and Culturable Microbial Concentrations

3.3.1.1 *Changes in Culturable Microbial Concentrations*

A significant increase was found in culturable microbial concentrations from the influents to the effluents of all columns for all flow conditions (Figure 3.2). The results were expected given the temperature (24 ºC) along with high carbon and nutrient concentrations found within the bioreactors, providing favorable conditions for mesophilic microbes to grow (Madigan et al., 2010). Higher microbial concentrations with more diverse microbial communities in the inflows (columns B and C) led to significantly more culturable microbial concentrations in the effluents under both flow conditions than those columns with limited concentrations and lower diversity (columns A and D) (Figure 3.3.I). Culturable microbial concentrations increased by 3.8, 4.5, 4.7, and 5.4 times on average in the effluents of columns A, B, C, and D, respectively. No additional microbes were added to the synthetic water provided to column D, and proportionally, a much larger increase in microbial concentration was observed between the influent and effluent concentrations. However, the overall culturable microbial population in column D was significantly lower than columns B and C at both the influent and effluent (Figure 3.2), suggesting the high impact of influent microbial concentrations on the effluent microbial communities.

Although there is no data regarding the change of general microbial populations from the influent to the effluent of WBs treating tile drainage water, an increase of total coliforms in the effluents of woodchip reactors treated with tile drainage water was found under different flow rates (Zoski et al., 2013).

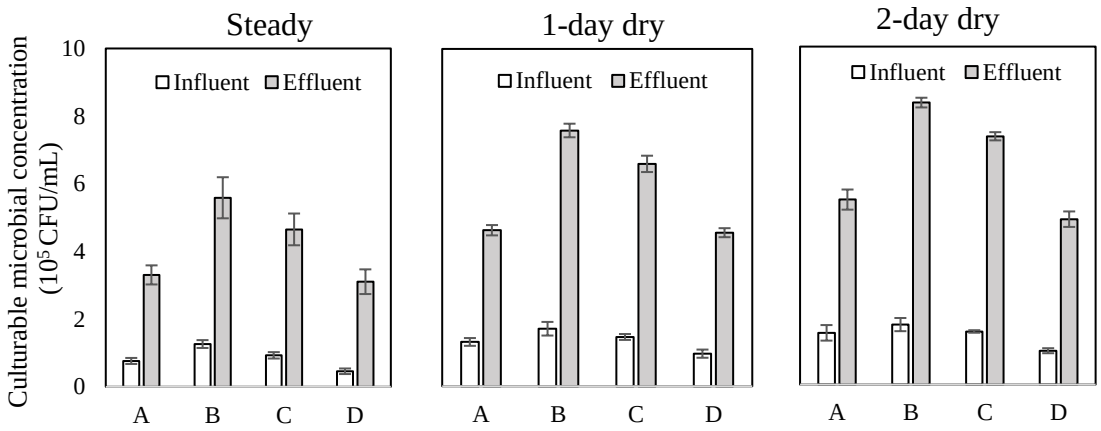

Figure 3.2 Culturable microbial concentrations obtained under different flow conditions and microbial communities (error bars indicate standard deviation on 10, 3, and 3 samples in steady, 1-day dry, and 2-day dry flow conditions, respectively).

3.3.1.2 *The Effect of Wet-Dry Cycles on Culturable Microbial Concentrations*

Wet-dry cycles resulted in a significant increase in the culturable microbial concentrations in the effluents of all columns (Figure 3.2). While evaluating the mechanistic reasons behind the culturable microbial changes was beyond the scope of this project, there are several possible explanations for this increase. The wet-dry cycles may have encouraged sloughing of cells from the microbial biofilms on the woodchip surfaces, due in part to expansion/contraction of biofilm matrices. Also, the shift from water-saturated conditions to more aerobic conditions during the dry cycle likely encouraged more aerobic metabolism during the dry cycle, possibly involving fungal activity, more so

than during wet cycles. If microbial degradation of the woodchips increases during dry cycles, the degradation may lead to increases in decomposer populations, many of which could have exited the bioreactor in the ensuing wet cycle.

Effluent concentrations were not significantly different between columns D and A under either flow condition (Figure 3.3.I), indicating that *E. coli* alone did not have an impact on culturable microbial concentrations in the bioreactor effluent. However, significant differences were found between the effluents of column comparisons B: A, C: A, C: B, D: B, and D: C under both flow conditions in the majority of circumstances, highlighting the significant impact of the quantity and diversity of microbial communities in the influent of WBs on the microbes releasing from the bioreactors. Additionally, for all columns, the increases in culturable microbial populations were significantly greater for the 2-day dry flow conditions as compared to steady state conditions (Figure 3.3.II), suggesting a significant impact of dry periods (at least 2 days) on microbial release by denitrification bioreactors. No significant difference was found between the two wet-dry cycles under different microbial treatments (Figure 3.3.II), suggesting that short dry cycles might not have as much impact as longer dry cycles do on releasing microbial cells compared to the steady flow. More studies are needed to determine if longer dry cycles have a significant impact on microbial communities within and effluent of denitrification bioreactors as well as the mechanisms behind the changes.

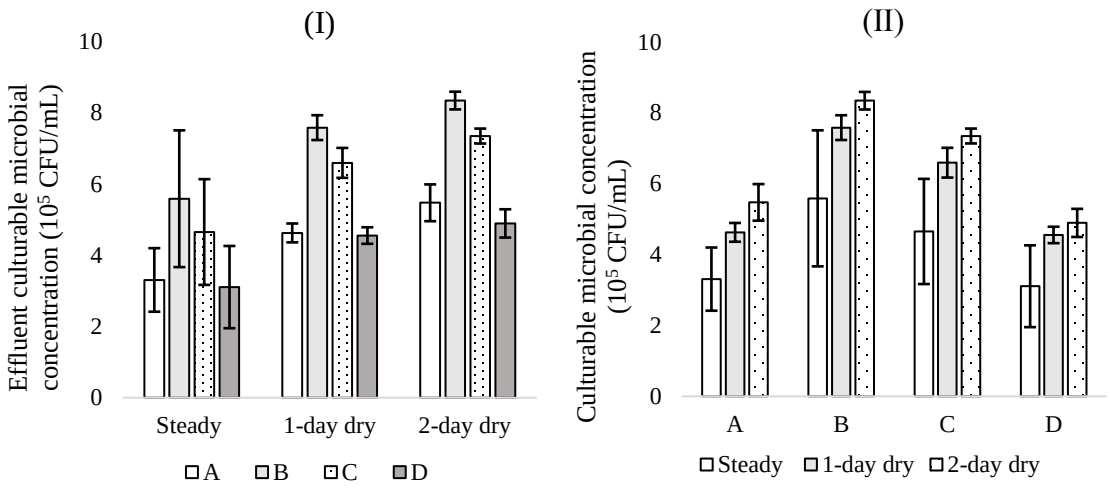

Figure 3.3 Comparison between effluent culturable microbial concentrations obtained under different flow conditions (I) and microbial communities (II) (error bars indicate standard deviation on 10, 3, and 3 samples in steady, 1-day dry, and 2-day dry flow conditions, respectively).

Another comparison between the last three effluent culturable microbial concentrations under the steady flow condition (on days 20, 30, and 60) and the samples taken in wet-dry cycle conditions (1-day dry and 2-day dry) showed no significant difference between effluent culturable microbial concentrations under different flow conditions and microbial compositions. This suggests that low effluent culturable microbial concentrations at the beginning of the experiment had a significant effect on the results. More studies are required to determine the stability of the effluent microbial concentrations over a longer period of time and to monitor microbial communities of a newly installed denitrification bioreactor over a long period.

3.3.1.3 *Changes in E. coli Concentrations*

The synthetic waters for columns A and B were spiked with 2×10^{2} CFU/mL of *E. coli*. However, the *E. coli* died off quickly, and counts decreased to $< 6 \times 10^{1}$ CFU/mL in

the synthetic water for the experimental runs. The denitrifying columns achieved a steady-state *E. coli* removal within reactors A and B (column B > column A) for both flow conditions (Figure 3.4), indicating the potential for woodchip bioreactors to provide an additional benefit of removing fecal indicator microorganisms (e.g., *E. coli*) from tile drainage water. Significant reductions in *E. coli* were found between the influents and midpoints as well as influents and effluents of columns A and B, while no *E. coli* was found in the sampling points of columns C and D where no *E. coli* was added to the synthetic waters. Removal of *E. coli* in column B increased more rapidly and was higher than that in column A for both flow conditions (Figure 3.4), with removal rates of 63%, 68%, and 77% on average for column B and 49%, 65%, and 68% on average for column A under steady, 1-day dry, and 2-day dry flow conditions, respectively (Table 3.3). Removal of *E. coli* in column B was higher than that in column A under both flow conditions; however, the differences were not significant. Many mechanisms, such as natural *E. coli* decay, competition, and predation, might be involved resulting in a greater capacity for *E. coli* removal in column B as compared to column A; however, identifying these mechanisms was beyond the scope of this study.

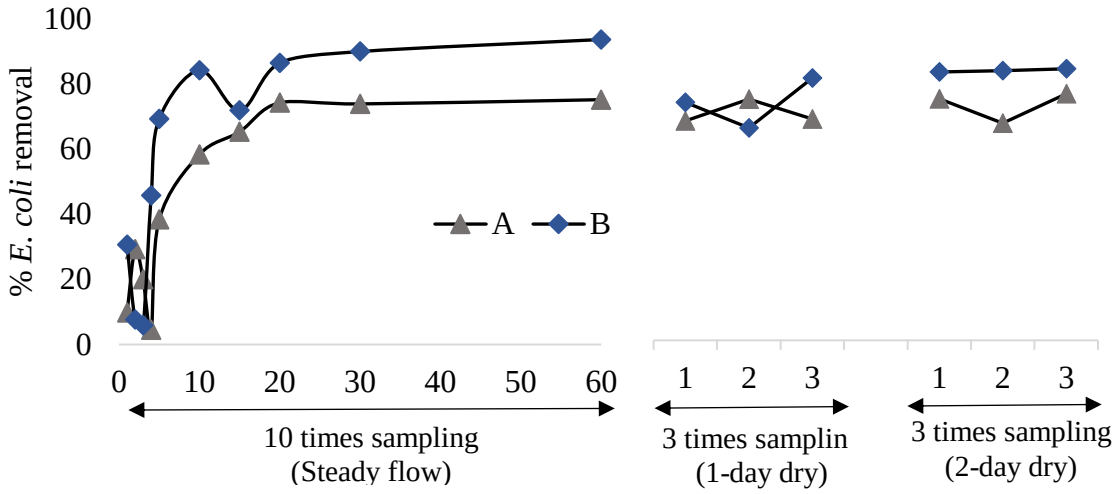

Figure 3.4 Percent *E. coli* removal in columns A & B obtained under different flow conditions and microbial communities.

Table 3.3 *E. coli* reduction by columns obtained under different flow conditions and microbial communities.

Reduction*					
Columns		A		B	
Flow conditions		CFU/mL	%	CFU/mL	%
Steady flow		20.9 (15.0)	49%	21.8 (14.2)	63%
Wet-dry cycles	1-day dry	29.2 (7.6)	65%	21.9 (5.4)	68%
	2-day dry	27.2 (8.1)	68%	31.1 (2.7)	77%

*Reduction is defined as the mean difference in concentration between column influents and effluents (standard deviation in parentheses), or that difference indicated as a percentage of influent concentration.

Substantial reductions of *E. coli* within denitrifying bioreactors have been reported in previous studies (Rambags et al., 2016, 2019; Robertson et al., 2005; Soupir et al., 2018; Tanner et al., 2012). For example, Robertson et al. (2005) found that *E. coli* concentrations were consistently reduced to near zero ($< 1 \times 10^1$ CFU/100 mL) in wood media filters treating water from wastewater treatment systems, indicating that *E. coli* was attenuated in the wood filter. In other studies by Rambags et al. (2016, 2019), significant reductions in *E. coli* were achieved between the influents and effluents of denitrifying woodchip

bioreactors treating wastewaters. Zoski et al. (2013) also found 97%, 67%, and 61% removal for *E. coli* in a reactor amended with wood shavings at different flow rates (1, 2, and 4 mL/s, respectively) when the influent water was spiked with 1.80×10^{2} CFU/mL of *E. coli*. In terms of experimental conditions, this experiment has more similarity to a study by Soupir et al. (2018), where a significant reduction in *E. coli* (91% - 96%) was achieved between the influent and effluent of laboratory columns treated with flows from synthetic agricultural drainage at 21.5 °C. In the current study, most of the *E. coli* removal occurred within the first half of the column, between the influent and midpoint (sampling point 2, Figure 3.1), indicating the possible capacity of the columns to manage higher microbial loads with shorter HRTs. This result is similar to that of Rambags et al. (2016) who found that most of the *E. coli* load reduction occurred within the first 5% of the reactor distance.

3.3.1.4 *The Effect of Wet-Dry Cycles on E. coli Removal*

Although there was not any significant difference in *E. coli* removal between two different flow conditions, columns A and B consistently exhibited higher *E. coli* removal in wet-dry flow conditions than in the steady flow condition (Table 3.3).

A lower *E. coli* removal was observed in the initial "warm-up" period of 0 - 20 days (Figure 3.4). A comparison between the *E. coli* removal rates of the last three samples under the steady flow condition (days 20, 30, and 60) and the *E. coli* removals under wet-dry cycle conditions indicated that the highest *E. coli* removals were found once column B removal rates reached steady-state under constant flow (Figure 3.4). A reduction of *E. coli* removal was found in wet-dry cycles in comparison with the last three removal rates under steady flow, but it was not significant. This suggests that bioreactors are highly capable of removing *E. coli* after passing their "warm-up" period, and this is not significantly

impacted by varying flow conditions (i.e. steady flow versus wet-dry cycling). Additional information is required to validate the impact of the "warm-up" period for bioreactors on removing fecal indicator microbes, and to determine the duration of this period under different circumstances (e.g., design, carbon media, flow conditions, etc.).

The efficiency of woodchip bioreactors to remove pathogenic microbes can be improved with a better understanding of the main removal mechanisms. This would assist with identifying practical methods to improve prediction of pathogenic contaminant removal in denitrifying bioreactors and defining standards for effective bioreactor designs for microbial contaminant removal (Rambags et al., 2016). The removal mechanisms may include a variety of processes including chemical, physical, and biological, alone or in combination. Physical mechanisms include filtration of microorganisms through the attachment to the substrate, sedimentation, and interaction of *E. coli* with woodchip surfaces as influenced by cell surface charge, surface structures (e.g., fimbriae and flagella), hydrophobicity and extracellular polymeric substances (Liao et al., 2015). *E. coli* can also be inactivated through adsorption to organic matter and oxidation. Predation by other organisms, natural die-off, and competition for resources may constitute biological factors for *E. coli* inactivation in the woodchip bioreactors (Alufasi et al., 2017; Haig et al., 2015; Stevik et al., 2004). More research on the removal mechanisms of *E. coli* is needed to evaluate how these mechanisms change with bioreactor age, and how they would be affected by factors such as influent microbial community, loading rate, HRT, and seasonality.

3.3.2 Antimicrobial Resistance (AMR) of Isolates

3.3.2.1 *AMR of Culturable Microbial Isolates*

A total of 610 isolates from the broader microbial community within the influents and effluents of the bioreactors were recovered (164, 170, 147, and 129 isolates from the columns A, B, C, and D, respectively) and tested for their sensitivity to five antimicrobials (Table 4). Out of the 295 isolates from the influents, 251 (85%) were phenotypically resistant to at least one of the antibiotics tested, while 37 (13%) demonstrated phenotypic resistance to a single antibiotic, and 214 (72%) of the isolates demonstrated phenotypic resistance to multiple antibiotics. Out of the 315 isolates from the effluents, 283 (90%) were resistant to at least one of the antibiotics tested, and 235 (75%) were resistant to multiple antibiotics.

Table 3.4 Number of *E. coli* and culturable microbial isolates tested for AMR.

Columns			A		B		C		D	
Microbes	Flow conditions		Inflow	Outflow	Inflow	Outflow	Inflow	Outflow	Inflow	Outflow
E. coli	Steady		91	90	89	86	-	-	-	-
	Wet-dry cycles	1-day dry	20	14	18	20	-	-	-	-
		2-day dry	20	15	20	20	-	-	-	-
Total isolates			130	119	127	126	-	-	-	-
Other culturable microbes	Steady		54	57	56	57	50	51	37	48
	Wet-dry cycles	1-day dry	12	13	16	16	11	12	10	12
		2-day dry	14	14	13	12	11	12	11	11
Total isolates			80	84	85	85	72	75	58	71

The ratio of AMR, or the total number of isolates resistant to at least one antibiotic per the total isolates examined, was calculated for all columns. The results showed that the AMR ratio often increased from the influents to the effluents; however, these increases were not consistently significant throughout the experiment (Figure 3.5). AMR microbial concentrations were estimated by multiplying the AMR ratio by the culturable microbial concentrations. The statistically similar AMR ratios coupled with a significant increase of

culturable microbial concentrations in the effluents suggests a substantial increase in phenotypic AMR concentrations and the associated risk of antibiotic resistance dissemination in the environment. This is supported by the significant increases in AMR microbial concentrations at the effluents of all columns as compared to the influents.

The mechanisms behind the increase in AMR concentrations was beyond the scope of this study. One possible explanation is that the biofilms formed in WBs (Chun et al., 2009; Damaraju et al., 2015) may provide "hotspots" for horizontal gene transfer (HGT) (Nesse and Simm, 2018) and lead to an increase in AMR concentration released in the effluents of all columns. Another explanation is that as the culturable microbes multiplied within the reactors, the associated antibiotic resistance traits also multiplied, resulting in a significant increase of AMR concentrations in the effluents. Also, conditions inside the bioreactors may have favored retention and spread of ARGs, due to production of antibiotics by some fungi or bacteria at microsites in the bioreactor acting as selection pressures for antibiotic resistance.

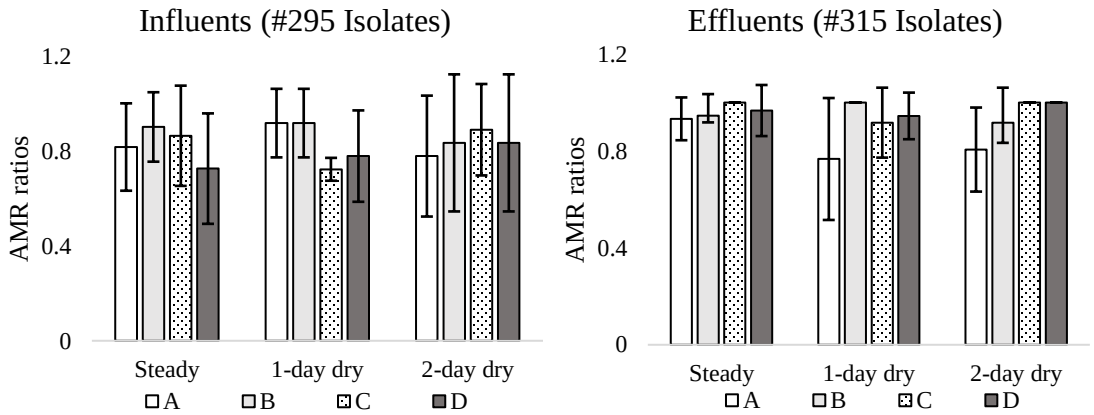

Figure 3.5 Ratio of AMR obtained under different flow conditions and microbial communities (error bars indicate standard deviation on 10, 3, and 3 samples in steady, 1-day dry, and 2-day dry flow conditions, respectively).

In the current study, columns B and C showed significantly higher effluent AMR concentrations in comparison with columns A and D under both flow conditions (Figure 3.6.I), suggesting the potential for higher release of AMR concentrations through bioreactors receiving more microbes with a greater diversity. The effluent AMR concentrations were not significantly different between columns A and D or columns B and C, where the lowest and highest microbial communities were added to the synthetic waters, respectively. The 2-day dry condition had significantly higher AMR concentrations than the steady flow condition for columns C and D; however, most flow conditions did not show significant differences (Figure 3.6.II).

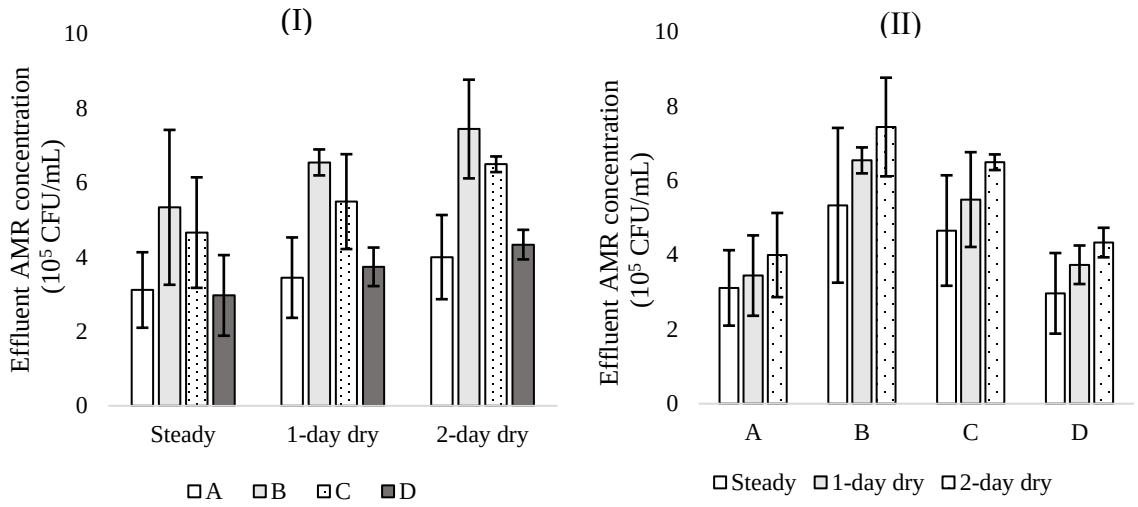

Figure 3.6 Comparison between effluent AMR concentrations obtained under different flow conditions (I) and microbial communities (II) (error bars indicate standard deviation on 10, 3, and 3 samples in steady, 1-day dry, and 2-day dry flow conditions, respectively).

A higher percentage of microbial isolates tested were resistant to erythromycin, penicillin, ampicillin, and sulfisoxazole than to tetracycline (Figure 3.7). Similar patterns of antibiotic resistance of culturable microbes were found in the influents and effluents of the columns (Figure 3.7), possibly due to the genetic similarity of microbes used in this

project and the exposure of the microbes to the same antibiotic agents. The microorganisms isolated from the effluents often had a higher percentage that was resistant to the antibiotics tested in comparison with those in the influents, particularly under the steady flow. The Gram-negative and Gram-positive characteristics of all isolates also indicated that although the proportion of Gram-negative and Gram-positive isolates from the influents was almost the same, more of the isolates in the effluents were Gram-negative (57%). As Gram-negative microbes have an outer membrane which often makes them less susceptible to antibiotics, some of the observed antibiotic resistance increase may be explained by the higher proportion of Gram-negative microbes. The effectiveness of the antibiotics tested in this experiment also needs to be considered when interpreting the results, since they might target some microbial groups more than others. For example, penicillin and erythromycin are generally most effective against Gram-positive microbes. Further studies need to focus on examining genetic resistance of microbes to quantify the impact of WBs on antibiotic resistance of microbes; however, this was beyond the scope of the current work.

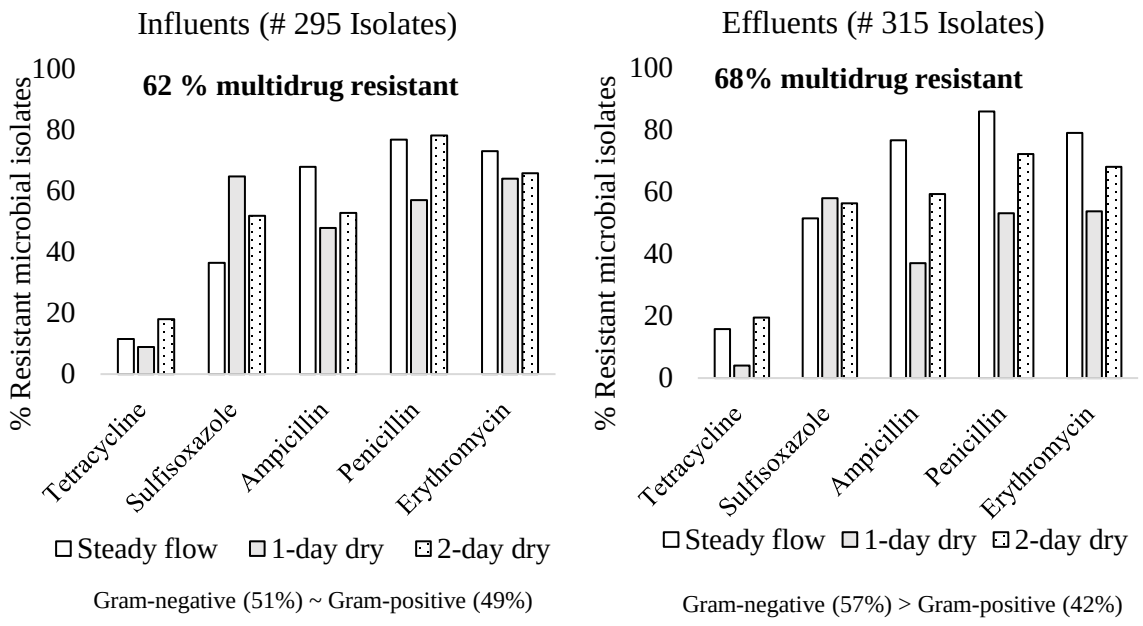

Figure 3.7 Average percentage of resistant microbial isolates in the influents and effluents obtained under different flows.

A comparison between phenotypic antibiotic susceptibility of the microorganisms isolated from the influents and effluents of the columns showed a difference of antibiotic resistance proportion (sometimes significant) throughout the experiment (Figure 3.8). This suggests that the microbial communities developing in some of the WBs (in columns A and C) have phenotypic antibiotic resistance. Since there is no data regarding the antimicrobial-resistant patterns of microbes from WB effluents compared to the influents, there is abundant room for further studies in determining the potential of WBs to increase the concentrations of antibiotic resistant microbes and genes.

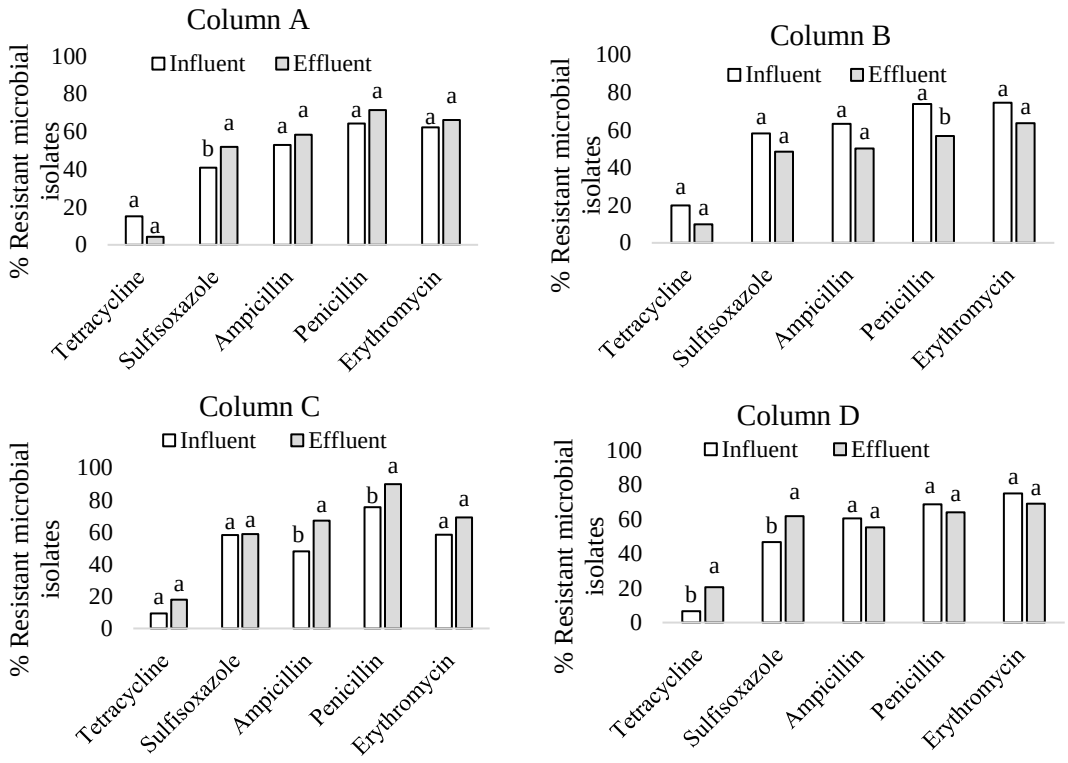

Figure 3.8 Average percentage of resistant culturable microbial isolates in the influents and effluents obtained using different microbial communities throughout the experiment (lower-case letters indicate significant differences between the influent and effluent for each antibiotic).

3.3.2.2 *AMR of E. coli Isolates*

A total of 502 *E. coli* isolates from the bioreactors were recovered (249 and 253 isolates from columns A and B, respectively) and tested for their sensitivity to five antimicrobials (Table 4). Similar patterns of *E. coli* resistance were found in the influents and effluents of the columns (Figure 3.9). All of the isolates were resistant to more than one of the antibiotics in both inflow and outflow, which is expected given that *E. coli* are Gram-negative and not very susceptible to some antimicrobials, including penicillin. Nearly all of the *E. coli* isolates from both the influents and effluents were resistant to ampicillin, penicillin, and erythromycin while a large portion were susceptible to tetracycline and

sulfisoxazole (Figure 3.9). This is consistent with previous work, which has shown the rates of resistance among *E. coli* isolates in the aquatic environment to ampicillin and sulfisoxazole were higher than tetracycline (Watkinson et al., 2007). The multidrug phenotypic resistance of *E. coli* found herein is similar to that found in previous studies (Kinge et al., 2010; Lin et al., 2004). For example, a large proportion (75.2%) of enteric bacteria isolated from the environment (Mhlathuze river) had multidrug resistance while 94.7% were resistant to at least one class of antibiotic (Lin et al., 2004).

No change was found between the ratio of AMR in *E. coli* isolates between the influents and effluents of columns A and B under different flow conditions. Since a substantial *E. coli* reduction was found in columns A and B, substantially lower AMR *E. coli* concentrations are expected in the bioreactor effluent.

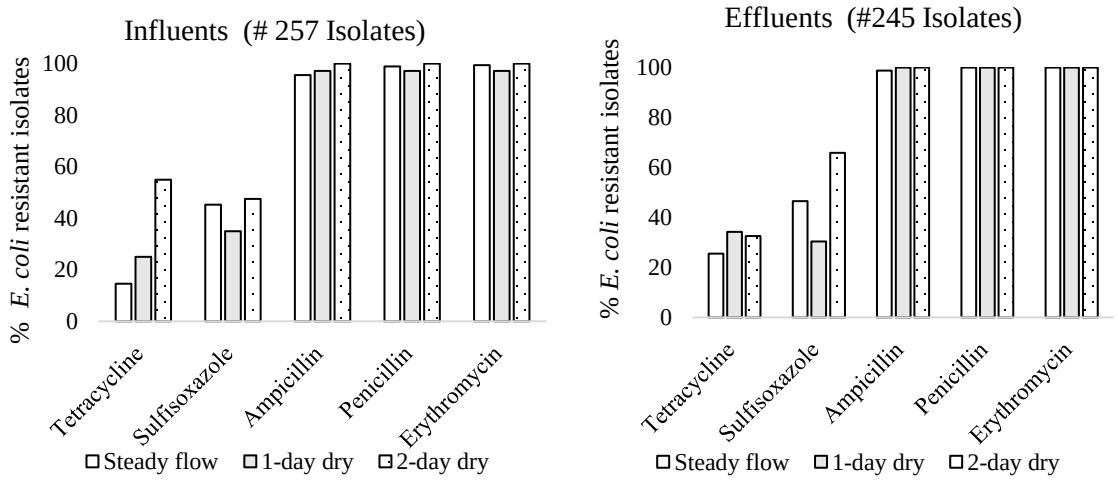

Figure 3.9 Average percentage of resistant *E. coli* isolates in the influents and effluents obtained under different flow conditions.

The proportion of *E. coli* isolates resistant to tetracycline and sulfisoxazole was generally higher in the effluents in comparison to the influents under different flow

conditions; however, this difference was not significant. Column A had a significantly higher percentage of *E. coli* resistant to sulfisoxazole during the 1-day dry flow condition as compared to column B, while other changes were not significant.

Other studies have shown a high level of AMR in environmental microbes. *Enterococcus* spp. bacteria recovered from surface and groundwaters impacted by a concentrated swine feeding operation had a high level of erythromycin resistance (Sapkota et al., 2007). Martinez (2003) found that 20% of microbial strains originating from seawater were resistant to at least five antibiotics, and 90% of those were resistant to more than one antibiotic. *E. coli* isolates from surface water in the Red Cedar watershed in Michigan were resistant only to cephalothin (Sayah et al., 2005), while 100% of bacterial isolates were resistant to penicillin and erythromycin in water samples from a river impacted by an urban wastewater treatment plant and antibiotic-production plant discharges (Sidrach-Cardona et al., 2014).

3.3.3 Study on Biofilms

3.3.3.1 *Physical Characteristics*

At the end of the wet-dry experiments, each column was divided into three equal depths and woodchip samples were collected in triplicate from each depth. Woodchips close to the inlets of the bioreactors, particularly those from columns receiving more diverse microbial communities, were darker in appearance than those close to the outlets of the columns, suggesting wood particles might decompose and age faster near the inlet. The same observation was also found in a previous study by Moorman et al. (2010) who suggested that the uneven aging of woodchips may affect the overall nitrate reduction rate of a field reactor, and should be taken into account. The darkened coloration of some

woodchips may have been due to iron sulfide deposition on the wood during the experiment. The production of hydrogen sulfide gas was also evident by a detectable rotten egg smell (Herbstritt, 2014; Nordström and Herbert, 2017) from the effluents of all columns in this current study over the course of the experiment, expected to be a result of sulfate reduction (Davies, 2003; Elgood et al., 2010; Robertson, 2010). It is assumed that longer HRT designs might maximize nitrate removal efficiency, but may also result in sulfate reduction/sulfide production when nitrate is almost completely removed (Elgood et al., 2010; Lepine et al., 2016; Robertson, 2010; Robertson et al., 2009).

The scanning electron microscope (SEM) images revealed that all samples generally had fiber detachment and cell wall collapse, a sign of degradation (Figure 3.10). This could be a result of two processes inside the WB columns. The first is when degrading microbes hydrolyze the woodchips and other facultative anaerobes (denitrifying bacteria) consume these degradation products. The second is the fermentation of organic matter in an anaerobic environment that can result in the release of carbohydrates as well as acids (Guo et al., 2017; Zhao et al., 2018).

3.3.3.2 *E. coli concentrations in the Biofilm Matrix*

Analysis of the woodchip biofilm samples indicated that *E. coli* was not part of the biofilm matrix in any of the columns. This would suggest that *E. coli* was not incorporated into the biofilm microbial community, or that a longer time is needed for *E. coli* to colonize the biofilm. Biofilms wash-off at high flow rates or slough-off as they thicken due to attachment weakness, even at moderate flow rates (Chun et al., 2009); therefore, it is possible that washing- or sloughing-off could limit the type and amount of microbes present in the biofilms, including *E. coli*. It is also possible that the physical structure of

biofilms were not favorable for the adhesion of *E. coli* cells, as biofilm structure can affect adhesion of this bacterium (Janjaroen et al., 2013). As biofilms provide both physical and chemical protection to their resident microbes, the absence of *E. coli* in the biofilms may also represent a short-term presence of this fecal indicator bacterium in the bioreactor system. Additional studies on older biofilms may be necessary to determine if *E. coli* can assimilate into the biofilm matrix given a longer time period.

3.3.3.3 *Microscopic Studies*

Biofilm is a term used to describe a matrix of microbial cells as well as exopolymeric substances (Daniels and Cherukuri, 2005). The formation of biofilm on the surface of the materials can modify the physiochemical properties of the materials by changing the roughness, hydrophobicity, and electrokinetic properties of materials when extracellular polymeric substances are present (Clement et al., 1996; Taylor et al., 1990). This would affect the surface interactions between the materials and suspended particulates and alter the capacity of the filter media to remove pollutants (Dai and Hozalski, 2002; Torkzaban et al., 2007). The presence of biofilm can also enhance the chances of microbes to survive in the environment and may promote the spread of antibiotic resistance in the environment (Chen and Wen, 2011; Davies, 2003). The presence of biofilms has been documented in in-situ woodchip bioreactors by Chun et al. (2009) and in a column study by Damaraju et al. (2015).

In this experiment, biofilm formation was observed by SEM using 5,000 × magnification. The protocol used for sample preparation almost certainly removed water from the extracellular polymeric substance of the biofilms, so that the biofilm matrix was dehydrated but visible as thin layers, strands or granular material on the wood surfaces.

Microbial cells had regular shapes indicating that cell walls remained intact. Based on SEM observation, woodchip biofilm samples from different depths within a bioreactor differed in appearance; and biofilm samples from the same depth in different bioreactors also differed in appearance. Biofilms appeared to have mixed composition, with more than one cell type/species present (Figure 3.10). Unicellular bacteria were the most common type of cells observed; but some larger filaments or hyphae were also observed, indicating the presence of fungi. Although SEM confirmed that biofilm was present on the woodchip media taken from different depths of each column; and that different levels/strata of column had biofilm of differing appearance judged by SEM, there were not enough photos and fields of view photographed for a good quantitative SEM study of numbers of cells of different types. In addition, because it was unlikely that all of the cell types seen in SEM could be cultured using agar plates, we combined SEM and 16S rRNA approaches to provide the most information about the microbial community in our samples.

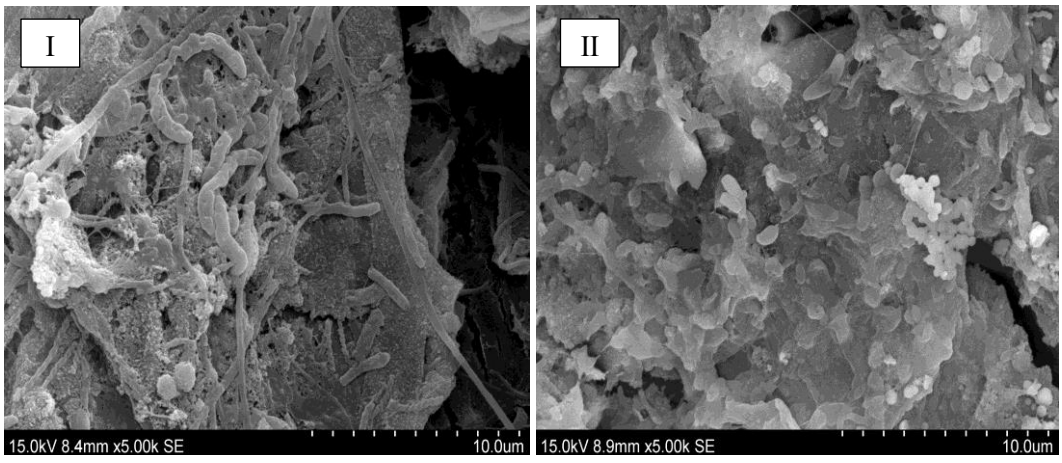

Figure 3.10 SEM images of biofilm samples taken from the middle of column C (I) and the outlet of column A (II).

3.3.3.4 *DNA Analysis*

General characterization of the microbial community contained in the biofilm was conducted at the phylum level (Figure 3.11). Proteobacteria (Gram-negative cells), Firmicutes (Gram-positive cells), and Bacteroidetes (Gram-negative cells) were the main phyla in all samples. This is in agreement with Zhao et al. (2018) who found Bacteroidetes, Proteobacteria, and Firmicutes were the major phyla in microbial community structures of a woodchip-based solid-phase denitrification bioreactor. Feng et al. (2017) found Bacteroidetes and Proteobacteria played a significant role in a denitrification bioreactor. In addition, the presence of Firmicutes would suggest the presence of fermentation processes (Madigan et al., 2010) in bioreactors. These data support the coexistence of denitrifiers and fermentative microbes inside the WBs, as previously suggested by Zhao et al. (2018).

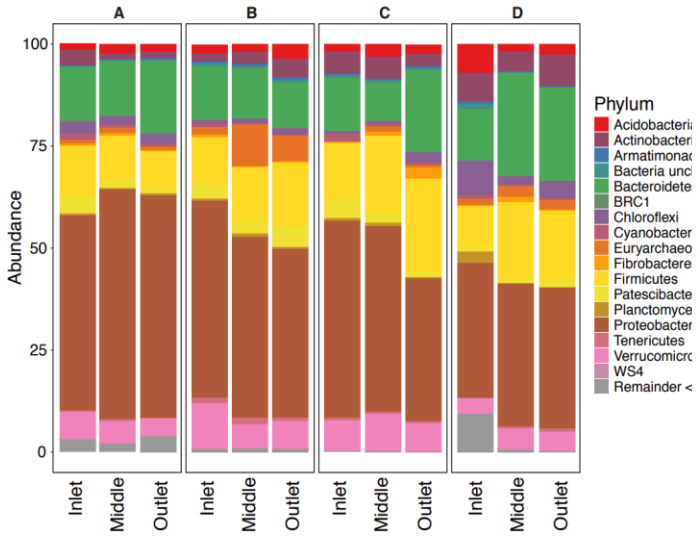

Figure 3.11 Taxonomic composition at the phylum level. Inlet, middle, and outlet are related to the samples taken from the inlet (starting from the inlet (0 inch) to 6.3 inch), middle (6.3 to 12.6 inch), and outlet (12.6 to 19 inch) of the column's length.

Column B, which received both *E. coli* and indigenous microbes, had biofilms with the highest microbial diversity (Figure 3.12). In contrast, the biofilms made in column D had the lowest alpha diversity, where it was amended with no microbes. Biofilms in column A, which recived only *E. coli*, had a higher Chao1 diversity than those in column C, where

amended with only indigenous microbes, while biofilms in column C had higher levels of Simpson diversity (Figure 3.12). Higher Chao1 diversity in column A suggests that a higher richness of biofilms in this column while higher values of Simpson diversity in column C suggests more diversity of the microbial population in the biofilms of this column. In column A, alpha diversity of biofilms increased between inlet, middle, and outlet samples whereas in column B, higher alpha diversity of biofilms was observed in the inlet and outlet samples compared to the middle sample (Figure 3.12). In column D, the highest alpha diversity of biofilms was observed in the middle sample (Figure 3.12). Higher diversity in the biofilms of column B is expected given the addition of nutrients and a variety of microbes (both *E. coli* and indigenous microbes) in the influent of this column. However, lower diversity of biofilms in the middle section of column B may suggest that some of these microbes pass through the bioreactor rather than establishing robust populations in situ.

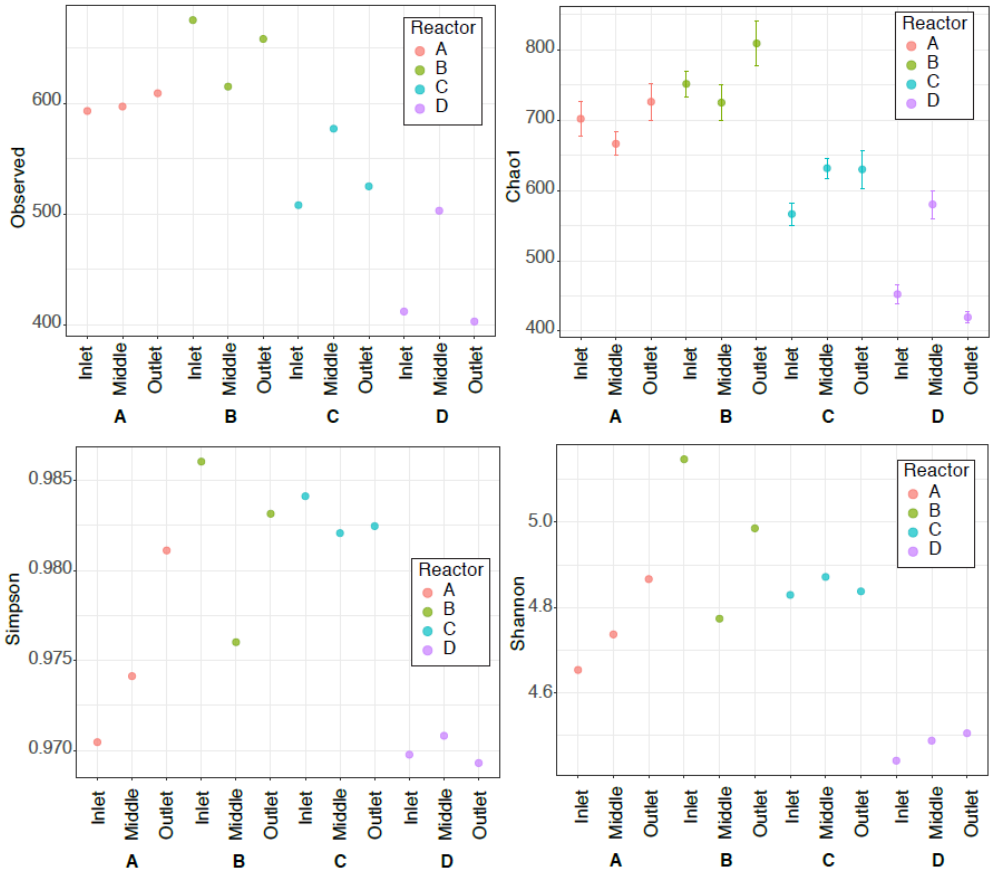

Figure 3.12 Alpha diversity measures for all samples. Inlet, middle, and outlet are related to the samples taken from the inlet (starting from the inlet (0 inch) to 6.3 inch), middle (6.3 to 12.6 inch), and outlet (12.6 to 19 inch) of the column's length.

In general, these results suggest that WBs receiving more microbes with a greater diversity have a higher potential to make biofilms with greater microbial diversity than those receiving low microbial communities with a less diversity. This might, in turn, impact on capacity of wood media on removing pollutants and persistence of microbes in the system where biofilms can provide "hot spots" for HGT and result in a potential increase in AMR concentration released to the environment, which need to be assessed further.

3.4 Conclusions

Previous studies have shown that denitrifying wood bioreactors (WB) are effective at removing nitrate from subsurface drainage systems (SDSs) (Christianson et al., 2012; Schipper et al., 2010); however, microbial contaminants are also transported as agricultural pollutants in SDSs, especially in manure-amended lands (Hruby et al., 2016; Pappas et al., 2008), and are problematic in water resources due to their direct effects on terrestrial and aquatic health (Ng and Gin, 2019; Sanganyado and Gwenzi, 2019). In this study, four laboratory scale bioreactors, receiving synthetic waters with different microbial communities, were designed to assess the potential effects of denitrifying bioreactors on microbial populations (*E. coli* and other culturable microbes) and phenotypic antimicrobial resistance (AMR) in subsurface drainage water under steady and wet-dry flow conditions.

The results demonstrated that WBs were capable of significantly removing *E. coli* and increasing culturable microbial concentrations. Applying different microbial communities also had significant effects on culturable microbial concentrations, as WBs receiving more microbes with a greater diversity had a greater increase in culturable microbial concentrations. Once the initial "warm-up" period (0 - 20 days) for the steady state flow condition was achieved, removal rates were steady and not significantly different from the wet-dry flow conditions. Additionally, the recovered culturable isolates from the influents and effluents had similar ratios of AMR for all columns. This combined with the increased culturable microbial population indicates the potential for increased concentrations of AMR microbes in tile drainage water when these waters pass through a WB; however, future work is required to quantify the change in antimicrobial resistance genes. The recovered isolates from the influents and effluents were more frequently phenotypically

resistant to ampicillin, penicillin, erythromycin, and sulfisoxazole, while little phenotypic resistance was found to tetracycline. *E. coli* isolates demonstrated a high phenotypic antibiotic susceptibility to tetracycline and sulfisoxazole, but were found to be nearly 100% resistant to ampicillin, penicillin, and erythromycin.

To improve the prediction of pathogen removal and AMR changes in tile drainage water, as well as to support the development of an effective design for removing microbial contamination using denitrifying woodchip bioreactors, more research is needed on:

i. The longevity of *E. coli* removal with different HRTs, substrate, seasonality, temperatures, as well as fluctuating water levels;

ii. The dominant mechanisms for microbial contaminant removal;

iii. The effect of microbial influent loading rate on microbial contaminant removal and AMR changes in tile drainage water;

iv. *E. coli* removal and AMR of microbes collected from field-scale WBs in agricultural land under different management practices; and

v. The change in AMR gene concentrations in tile drainage water passing through WBs.

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CHAPTER 4: THE CHANGES OF PHYSICOCHEMICAL WATER QUALITY PARAMETERS IN DENITRIFICATION WOODCHIP BIOREACTORS TREATING SUBSURFACE DRAINAGE WATER AND THEIR RELATIONSHIPS WITH MICROBIAL POPULATIONS

A paper in preparation

Abstract

Denitrification bioreactors are a promising technology to treat nitrate from tile drainage water. While these treatment systems are largely assessed for nitrate removal under different conditions, not much is known regarding the change of tile drainage microbial concentrations (e.g., pathogenic microbes and general microbial community) through these treatment systems. In addition, it is not clear if there is any relationship between microbial populations and physicochemical water quality parameters. Therefore, the main objectives of this study were to assess the potential effect of denitrification bioreactors on microbial concentrations and the physicochemical water quality parameters, and determine the relationships between microbial populations and other water quality attributes. To achieve this goal, a column study was conducted to simulate a field denitrification bioreactor. Four columns were filled with wood media and received four different influent microbial suspensions including *E. coli* only, *E. coli* and indigenous microbes, indigenous microbes only, and no microbes. Microbial isolates were taken from the influent of an in-situ denitrification bioreactor and pumped throughout the columns under two different flow conditions: steady and wet-dry flow. The results revealed significant relationships between pH as well as electerical conductivity (EC) and the microbial populations in denitrification bioreactor effluents. Furthermore, there was a statistically significant negative correlation

betweee the microbial community and *E. coli* concentrations in the effluents. More studies are suggested to examine the potential impact of microbial diversity on survival of *E. coli* in denitrification bioreactors and to identify microorganisms that can promote the decrease of pathogenic *E. coli*, which might be used in the removal systems to decrease the survivability of pathogenic microbes.

Key words: Column study, Tile drainage water, Pathogenic microbes, Correlation, and Microbial population.

4.1 Introduction

Subsurface drainage systems (SDSs) play a substantial role in agriculture by altering the hydrology of the field, lowering the water table, and allowing for highly productive cropping systems (Skaggs et al., 1994). Nitrate, contained in fertilizers used on agricultural lands and produced by natural events in the nitrogen cycle, is highly soluble, mobile, and prone to leach through soil and enter groundwater (Böhlke, 2002; Nolan et al., 2002; Spalding and Exner, 1993) or subsurface drainage water (Jaynes et al., 2001; Jaynes et al., 1999; Kladivko et al., 2004; Letey et al., 1977), depending on hydrogeological locations, seasonal variations, and anthropogenic activities (Yakovlev et al., 2015). Between 20% and 40% of nitrate applied annually to agricultural fields in the Midwest is lost through SDSs (Dinnes et al., 2002; Gentry et al., 2009).

In addition, manure is used as an organic fertilizer and contains a variety of pollutents including nutrients, organic matter, heavy metals, microbes (Chadwick and Chen, 2002), antibiotics, and resistant microbes (Heuer et al., 2011). Leaching of these components can contaminate water resources (Dinnes et al., 2002; Kanwar et al., 1987; Pinheiro et al.,

2013), and can cause problems for both terrestrial and aquatic health (Fritsch, 1997).

There are many management practices to decrease constituent transport from agricultural fields and from entering surface waters. Denitrification bioreactors are one such practice that are economical and increasingly popular, particularly in the Midwestern U.S. (Christianson et al., 2013; Elgood et al., 2010; Schipper et al., 2010). Denitrification bioreactors usually consist of a trench excavated at the edge of an agricultural field and filled with a solid carbon source. Drainage water is then diverted through the system. Biological processes are recognized as the predominant mechanism in converting nitrate into nitrogen gas and, thereby, removing nitrate from drainage water (Flores-Mireles et al., 2007; Sylvia et al., 2005; Verbaendert et al., 2011). Past studies have demonstrated a wide range of nitrate removal in both laboratory and field settings over the past two decades, from 10% to 100% (Christianson et al., 2012).

While previous studies have mostly focused on these treatment systems for nitrate removal, little attention has been given to the potential impacts of these systems on changing microbial populations in subsurface drainage waters. Only a few studies have examined the ability of denitrification bioreactors to reduce microbial contaminants from wastewaters (Rambags et al., 2016; Robertson et al., 2005; Tanner et al., 2012). However, not much is known about the impact of these systems on altering microbes from subsurface drainage water (Soupir et al., 2018; Zoski et al., 2013). In addition, it is not well documented how bioreactors impact other water quality parameters, and if there is any significant relationship between these parameters and microbial concentrations in denitrification bioreactors. Therefore, the main objectives of this study were to i) evaluate the changes of water quality parameters along the length of denitrification bioreactors, and

ii) determine the correlation between microbial populations and physicochemical water quality attributes. In this experiment, we simulated a field denitrification bioreactor treating synthetic tile drainage water, and measured the changes of microbial concentrations along with other water quality parameters including pH, electrical conductivity (EC), dissolved oxygen (DO), total organic carbon (TOC), nitrate, and turbidity. A companion paper (Mardani et al., Under revision), presents the details of microbial changes in the tile drainage water, while this publication focuses on the physiochemical characteristics and their relationship with the microbial populations.

4.2 Materials and Methods

Four clear acrylic tubes (19" H, 5.5" dia) were used to simulate denitrification bioreactor systems (Figure 4.1). Two ports were attached at both ends of the columns for inflow, outflow, and water sample collection. Each cap contained an access point for a temperature sensor. Each column contained woodchips (cottonwood) as the carbon source and was operated at room temperature, about 23.5 °C. Prior to filling the columns, the woodchips were washed, air-dried, and sifted through a shaker, removing particles smaller than 2.36 mm to prevent clogging in the columns. The woodchips were then weighed (2,085 ± 40 g) and compressed into the columns using a steel rod. For more detailed information on the woodchip media

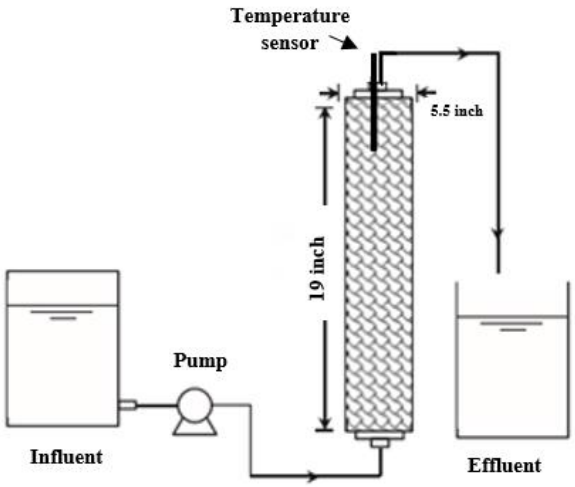

Figure 4.1 Schematic of one of woodchip bioreactor columns.

used in this experiment, see the companion paper (Mardani et al., Under revision).

Four separate containers were used to pump four different synthetic waters, one through each column. The columns used an up-flow design, and the water was pumped through the system using separate Masterflex peristaltic pumps (FH100 variable speed, Cole-Parmer, Vernon Hills, IL). The desired hydraulic retention time (12 hours) was achieved by controlling the pump speed (RPM = 46) and tubing size (0.08 mm ID). The flow rate was determined using a desired hydraulic retention time of 12 hours, the measured porosity of the woodchips (Mardani et al., Under revision), and the volume of the column (7.34 L) and was 5.3 mL min^{-1}.

Prior to the experiment, water samples were taken from the influent and effluent of an in-situ denitrification woodchip bioreactor located near Baltic, South Dakota, and processed for *E. coli* and the general culturable microbial community, as described by the companion paper (Mardani et al., Under revision). Briefly, 10 separate *E. coli* colonies taken from the influent and 17 most common, morphologically distinct microbial isolates taken from the influent (seven colonies) or the effluent (10 colonies) were isolated and stored at –80 °C until used in this experiment. The colonies taken from the effluent of the bioreactor were used to inoculate the columns, while the colonies taken from the influent of the bioreactor were used for synthetic water preparation as follows.

Two weeks prior to the experiment, the woodchip columns were inoculated with the 10 most common, morphologically distinct microbial isolates taken from the effluent of an in-situ denitrification bioreactor, as described by Mardani et al. (Under revision). Briefly, these isolates were added to a synthetic solution that contained micronutrients at a

concentration of 2×10^2 CFU mL^{-1}. The solution was pumped directly from the influent containers to each column and recirculated through the columns for 10 days at a flow rate of 2 mL min^{-1} to allow for the microbes to colonize and establish within the columns. After the inoculation period, each reactor was flushed for 2-days to restore anaerobic conditions and refresh microbial activity.

Four different synthetic waters were used for this experiment, one for each column. Each synthetic water had a base of reverse osmosis water with micronutrients, which were added to reduce the potential effect of micronutrient deficiencies (Hoover, 2012), as well as 30 mg L^{-1} of potassium nitrate (0.3 mM KNO_3), to simulate nitrate concentrations within SDSs. However, the synthetic solutions were different in terms of microbial communities. In this experiment, the seven most common, morphologically distinct microbial isolates, hereafter referred to as "indigenous microbes", and 10 separate *E. coli* colonies isolated from the influent of an in-situ denitrification bioreactor were used for the synthetic solutions. The synthetic influents consisted of *E. coli* only (column A), *E. coli* and indigenous microbes (column B), indigenous microbes only (column C), and no microbial additions (column D). Approximately 2×10^2 CFU mL^{-1} of each microbial isolate used in this study (indigenous and *E. coli* isolates) were prepared in the laboratory and added to the solutions, where appropriate.

Peristaltic pumps were used to pump the synthetic waters through the bioreactor columns at a constant rate of 5.3 mL min^{-1}, resulting in a hydraulic retention time of 12 hours. Two different flow conditions were applied during this experiment, steady flow and wet-dry flow conditions. During steady flow, the pumps ran continuously for 60 days, and water samples were collected 10 times over the course of the experiment on days 1, 2, 3,

4, 5, 10, 15, 20, 30, and 60. After the steady flow experiment was completed, two sets of wet-dry flow experiments were conducted. The columns were drained for 24-hours before a pulse of water was pumped through each column. The 24-hour draining and subsequent water pulse were conducted three times, and a single water sample was collected during each pulse. The wet-dry flow experiment was repeated with a 48-hour draining time.

All samples were collected from the influent and effluent and analyzed for a range of water quality variables including culturable microbes, *E. coli*, nitrate, pH, electrical conductivity (EC), dissolved oxygen (DO), turbidity, and total organic carbon (TOC). Nitrate was analyzed using an AQ2 Discrete Analyzer (Seal Analytical Inc, Mequon, WI). TOC was measured with a DR2800 spectrophotometer (Hach, Loveland, CO). A portable field probe (HI98193, Hanna Instruments, Smithfield, RI) was used to measure DO immediately following sample collection to minimize changes in oxygen concentrations. A laboratory probe was used to measure pH and EC (AB15, Fisher Scientific, Hampton, NH) while a field colorimeter kit (TN400, Apera, Columbus, OH) was used to measure turbidity. The room temperature and bioreactor temperature were also recorded on days when samples were collected.

To analyze the data, R (1.1.442) was used, and tests were conducted with a significance level of 0.95 ($\alpha = 0.05$), where appropriate. The Shapiro-Wilk's W test of normality was conducted prior to evaluating significant differences to identify appropriate statistical tests. Parametric tests (paired t-test and Tukey HSD) were used for normally distributed data, while non-parametric tests (Wilcoxon signed-rank and Dunn test) were applied to data that were not normally distributed. Additionally, Spearman rank-order correlation, a non-parametric correlation analysis, was used to analyze relationships between microbial

concentrations (e.g., *E. coli* and culturable microbes) and physicochemical water quality variables (e.g., pH, EC, DO, TOC, turbidity, and temperature).

4.3 Results and Discussion

4.3.1 pH

The desired pH range for denitrification processes is between 5.5 and 8.0 (Rivett et al., 2008), and the pH in the influent and effluent of all columns remained within this range, indicating acceptable pH conditions within the bioreactor columns for denitrification. The pH increased from a range of 6.4 – 6.9 in the influent to 6.7 – 7.8 in the effluent. During the steady flow experiment, all columns demonstrated a significant increase, while three out of four columns (A, B, and D) demonstrated a significant increase under the 1-day dry flow condition. However, the pH increases were not significant for the 2-day dry condition.

Increasing pH in tile drainage water passing through bioreactors has been documented in several studies (Damaraju et al., 2015; Nordström and Herbert, 2017; Warneke et al., 2011); however, this trend is not universal. Other studies have measured a decrease in the pH of tile drainage water passing through woodchip bioreactor systems (Mardani et al., In preparation-c; Robertson et al., 2005; Van Driel et al., 2006). The release of bicarbonates (HCO_3^-) and hydroxyl ions (OH^-) during denitrification as well as the utilization of some organic acids produced from fermentation and biodegradation of the woodchip material are possible explanations for the increase in pH along the length of the columns (Rivett et al., 2008; Rust et al., 2000; Zhao et al., 2018). Bacteria carrying out dissimilatory reduction of nitrate to ammonium under anaerobic conditions could also be involved in causing a pH increase (Rütting et al., 2011; Semenov et al., 2019). Furthermore, the growth of microbes inside the bioreactors, as demonstrated in the companion paper (Mardani et al., Under

revision), might have led to dramatic changes in pH. Microbes can increase the pH (Collins, 1987) or lower the pH (Solé et al., 2000), which may be favorable or harmful for their own growth (Ratzke and Gore, 2018). For example, *Corynebacterium ammoniagenes* cells can increase the pH, where at the same time, this increase can have positive feedback on their own growth (Ratzke and Gore, 2018) since they prefer higher pH levels (Collins, 1987). The potential impact of a single bacterial species on the change of pH has been demonstrated in a study by Ratzke and Gore (2018), where the greatest change was linked with higher nutrient concentrations.

In general, columns B and C, which had more diverse communities in their synthetic influents, often had higher effluent pH levels as compared with columns A and D. This difference was only significant during the 1-day dry flow condition (Figure 4.2). This might be attributed to the higher microbial growth and activity in the columns that received more diverse microbial communities in the influent, as demonstrated by the increased concentration of culturable microbes in the effluent (see companion paper, Mardani et al. (Under revision)).

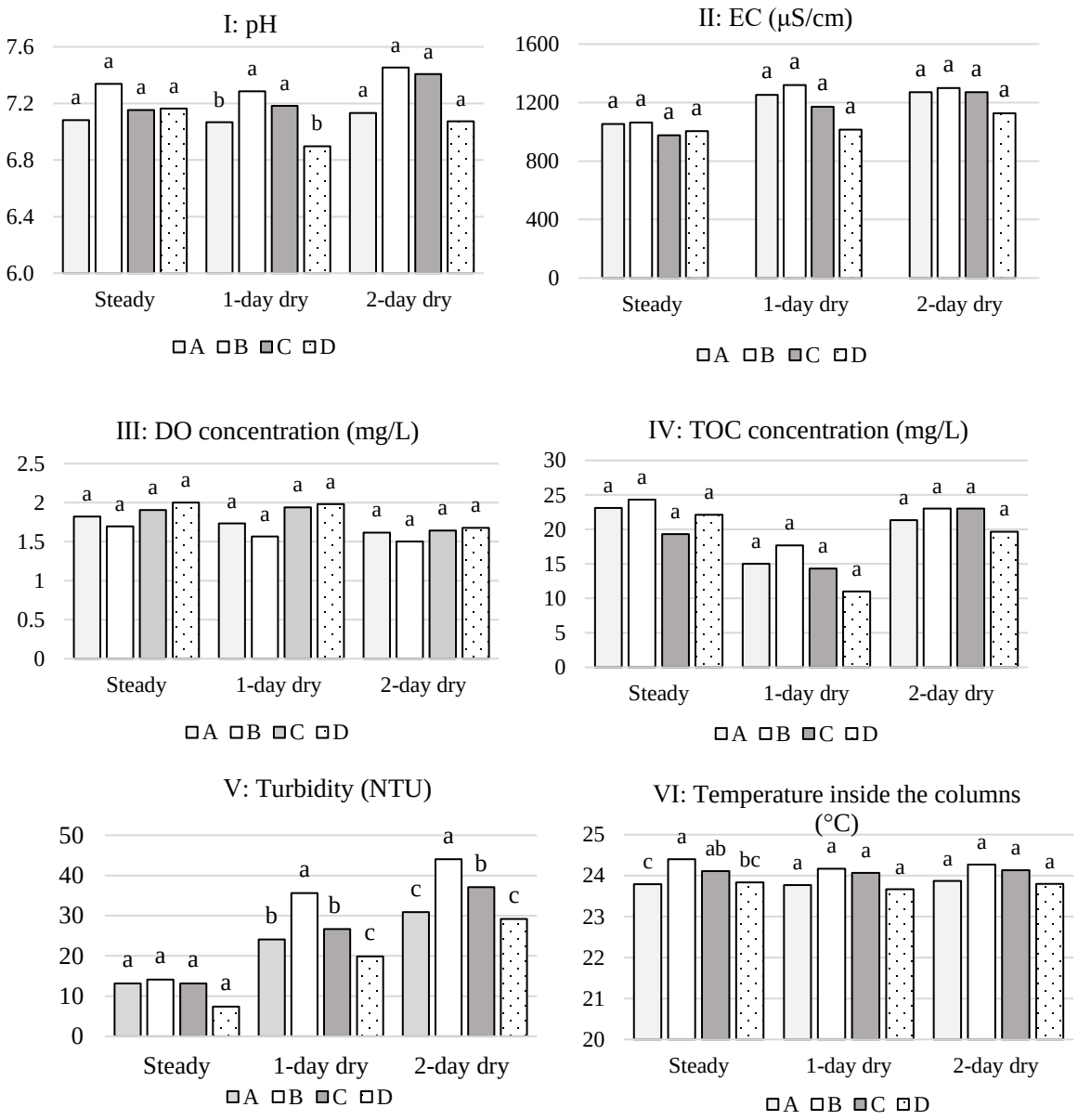

Figure 4.2 The average effluent pH (I), EC (II), DO (III), TOC (IV), and turbidity (V) as well as average temperature inside the columns (VI) obtained under different flow conditions. Lower-case letters indicate significant differences between the effluents for each flow condition.

4.3.2 Electrical Conductivity

Electrical conductivity (EC) is a measure of dissolved salts in the water (Ebong and Etuk, 2017) and is affected by the concentration of inorganic dissolved solids, including

those from the environment, such as soil and sediment, or from discharge, such as runoff, tile drainage water, and wastewater. Generally, the EC increased from the influent to the effluent in all columns. Under steady-state flow, all columns showed significant increases, while two out of the four columns (B and C) showed significant increases under 1-day dry flow condition. Under 2-day dry flow, three out of four columns (A, C, and D) also showed significant increases. The range for EC was between 605 – 818 μS cm^{-1} for the influent and increased to 833 – 1,587 μS cm^{-1} in the effluent, above the tolerable EC range of 150 – 500 μS cm^{-1} for most aquatic creatures (USEPA, 2012). This result was in contrast with the results obtained from an in-situ denitrification bioreactor in which EC in the tile drainage water generally decreased as it passed through the denitrification bioreactor, ranging from 1,189 – 4,210 μS cm^{-1} in the influent and 1,161 – 3,200 μS cm^{-1} in the effluent (Mardani et al., In preparation-c).

The increase of EC along with denitrification bioreactor might be attributed to the ions that are released during denitrification, and decomposition and biodegradation of the bioreactor's organic substances. It might also be impacted by microbial growth, which can change the EC concentrations (Krishnamurti and Kate, 1951). Comparing EC from the effluent of different columns and different flow conditions showed no significant differences (Figure 4.2). A slight increase of EC was often detected in the wet-dry cycles compared to the EC concentrations in the steady flow, which was only significant in column C between steady flow and 2-day dry condition.

4.3.3 Dissolved Oxygen

The dissolved oxygen (DO) varied from 5.34 to 7.58 mg L^{-1} in the influent and 1.18 to 2.52 mg L^{-1} in the effluent under all flow conditions. Though the reduction of DO from the

influents to the effluents was significant under all conditions, the columns were not completely anaerobic; however, the effluent concentrations were generally within the acceptable range for denitrification. Gómez et al. (2002) reported no inhibition of denitrification below DO concentrations of 4.5 mg L^{-1}, and Healy et al. (2006) found denitrification inhibition above DO concentrations of 3.7 mg L^{-1} in a denitrification bed.

Column B, which was provided with the highest diversity and concentration of microbes in the influent water, had the lowest effluent DO out of all the tested influent waters; however, it was not significantly different from the other columns (Figure 4.2). Lower, but not significantly different, DO was also observed in the effluent from the wet-dry flow conditions when compared to the steady flow condition, which may be attributed to a burst of microbial activity after the columns were allowed to dry, as evidenced by the increased culturable microbial population (see companion paper, Mardani et al. (Under revision)).

4.3.4 Total Organic Carbon

Total organic carbon (TOC) concentrations in the effluent gradually decreased over the course of the steady-state flow experiment in all columns. The highest concentrations of TOC were largely found at the initial stages of operation, which is similar to the results found in previous work that demonstrated the removal of organic carbon from woodchip bioreactors (Gibert et al., 2008; Hoover et al., 2016; Zhao et al., 2018). At the beginning of the experiment, TOC concentrations ranged from 45 – 68 mg L^{-1} and decreased to 8 – 30 mg L^{-1} at the end of the steady flow experiment. Higher TOC concentrations were found in the effluents from column B, where the most microbes were added to the synthetic water; however, the TOC concentrations were not significantly higher than the other columns

(Figure 4.2). TOC concentrations were often lower under wet-dry conditions than for steady flow (Figure 4.2); however, the differences were not significant. This result was different from Hoover et al. (2016), who hypothesized higher percentages of woodchip mass loss with unsaturated woodchip bioreactors.

4.3.5 *Turbidity*

Elavated turbidity can be indicative of the presence of high microbial numbers in the water (USEPA, 2017). Fairly clear water in the environment has a turbidity below 10 NTU; however, during storm events or snow melt, turbidity can increase substantially (e.g., 100 NTU) (Wetzel, 2001). The turbidity measured at the influent and effluent of the columns significantly increased as the water passed through the woodchip media. This is unsurprising and likely caused by fine particulate organic matter derived from microbial decomposition of the wood media as well as the microbes themselves, which significantly increased from the influents to the effluents (see companion paper, Mardani et al. (Under revision)). In addition, the wet-dry flow had greater turbidity than the steady flow condition (Figure 4.2), possibly due to increased microbial activity under the wet-dry flow conditions when compared to the steady flow, as evidenced by the increased microbial population in the effluent under wet-dry flow conditions (Mardani et al., Under revision).

Column B, which had the largest, most diverse microbial populations added to the influent water, had significantly higher turbidity in the effluent than all the other columns under wet-dry flow conditions. Column C, which had the next most diverse microbial population, had also significantly higher turbidity than columns A and D during the 2-day dry flow condition. Wet-dry flow conditions had a greater impact on turbidity levels at the effluents of all columns compared to those under steady flow. This difference was

significant between the effluent turbidity of all columns in the steady flow and 2-day dry flow conditions. Additionally, column D experienced statistically higher turbidity under the 1-day dry condition compared to steady flow, and also under 2-day dry flow compared to 1-day dry flow. This may be attributed to the increased microbial activity under wet-dry flow conditions compared to the steady flow (Mardani et al., Under revision) encouraging microbes to "chew-up" the woodchip media at a higher rate, thereby releasing higher amounts of organic matter.

4.3.6 Temperature within the Column

The temperature inside the reactors ranged from 23.1 °C to 24.9 °C throughout the experiment, which is within the ideal range (5 °C to 30 °C) for the denitrification process (Timmermans and Van Haute, 1983). The highest temperature was inside column B during steady flow, which was significantly higher than both columns A and D, where they had statistically lower effluent microbial concentrations. No significant differences were found during the wet-dry flow experiments. Applying different flow conditions did not significantly change the temperature within the columns; however, a slight decrease in temperatures during wet-dry cycles was often detected compared to the steady flow.

4.3.7 Nitrate

In this experiment, a steady-state nitrate removal of > 90% was achieved within all columns operated under different flow conditions and microbial communities. No significant differences in nitrate removal were detected between the different columns or flow conditions, possibly due to the relatively long retention time (12 hours), which has been shown to remove most (~ 99%) of the nitrate from tile drainage water in the field (Husk et al., 2017).

The highest and lowest nitrate removal rates were found in column B and column D, respectively (Table 4.1), where the highest and lowest microbial concentrations were observed in the influent and effluent waters (Mardani et al., Under revision). All columns demonstrated the higher nitrate removal rates during wet-dry flow conditions, possibly due to the larger microbial communities shown by Mardani et al. (Under revision). These high nitrate removal rates during wet-dry cycling were also observed by Hua et al. (2016), who found 100% removal of nitrate after a 3-day dry period and suggested that microbes in denitrification bioreactors can be reactivated quickly after a dry period. Drainage systems often experience very low flow rates or dry periods during an active drainage season (Christianson et al., 2012), and bioreactor activity after dry periods is not problematic (Van Driel et al., 2006). Woli et al. (2010) and Hua et al. (2016) linked the high nitrate removals detected for several low retention time events (i.e., high flow events) to the dry cycles preceding each of these events, which may have resulted in an increased nitrate removal capacity of the bioreactor. It has been postulated that throughout the drying cycle, the soluble carbon from the carbon media was made more accessible, allowing for greater denitrification potential (Christianson et al., 2017).

Table 4.1 Nitrate removal by columns obtained under different flow and microbial conditions.

Removal (%)*		Columns			
Flow conditions		A	B	C	D
Steady flow		96.6	98.1	97.8	94.7
Wet-dry cycles	1-day dry	97.5	98	97.6	96.7
	2-day dry	99.3	99.7	99.4	99.5

*Removal (%) calculated based on a percentage of the average difference between influent and effluent concentrations over influent concentration.

While significant differences were not observed in the bioreactors treated with

different microbial communities in this experiment, further work should be conducted with lower retention times, to determine if the trends observed with the different microbial communities and flow conditions in this study are significant with higher flows/lower retention times, which are often used in field-scale bioreactors. Peralta et al. (2010) found that 40% of the variation in denitrification potential within restored and reference wetlands is due to the microbial community composition, whereas nitrate, ammonium, the ratio of carbon to nitrate, and pH combined only accounted for 32% of the variation in denitrification potential. Given the importance of the microbial community in denitrification potential of other aquatic systems, it is feasible that, with less time for treatment (i.e., lower retention time), the microbial community may significantly impact nitrate removal.

4.3.8 Microbial Concentrations and Physicochemical Water Quality Variables

The microbial changes, presented in the companion paper (Mardani et al., Under revision), demonstrated the potential for woodchip bioreactors to remove *E. coli* and increase culturable microbial populations in synthetic subsurface drainage waters. Applying different microbial communities had significant effects on culturable microbial concentrations, where columns receiving more microbes with greater diversity had a greater increase in culturable microbial population. In addition, it resulted in a greater capacity for *E. coli* removal in the column receiving more microbes with greater diversity as compared to the column receiving limited microbes with less diversity. To detect the possible significant relationships between the effluent microbial concentrations and the effluent water quality variables in denitrification bioreactors, a correlation analysis was performed for each column under both flow conditions. The correlation analysis revealed

a strong relationship ($p \leq 0.05$) under the steady flow condition; however, the relationships were mostly weak ($p > 0.05$) under the wet-dry cycles, potentially due to the limited sample size.

Table 4.2 Correlation between effluent microbial concentrations (*E. coli* and culturable microbes) and physicochemical water quality variables under steady flow.

Column	A	B	C	D	All columns combined
Correlation	Culturable microbes	Culturable microbes	Culturable microbes	Culturable microbes	Culturable microbes
pH	**0.84**	**0.92**	**0.72**	**0.81**	**0.73**
EC	**0.76**	**0.90**	0.47	**0.7**	**0.59**
DO	-0.26	-0.19	-0.06	0.18	-0.24
TOC	0.21	-0.57	-0.38	-0.25	-0.23
Turbidity	0.49	0.52	0.43	0.74	**0.52**
Nitrate	-0.18	-0.56	-0.33	-0.55	**-0.47**
Temperature	0.05	-0.13	-0.14	-0.22	**0.49**
E. coli	**-0.81**	**-0.74**	-	-	**-0.84**

*Correlations resulted from a Spearman rank-order correlation test; bold values are significantly correlated at 5%.

The results revealed a significant positive correlation between the effluent culturable microbial concentrations and the effluent pH as well as the effluent EC for most of the columns (Table 4.2). Microbes are able to alter their environment, like pH conditions, through consuming available resources and excreting metabolites (Ratzke and Gore, 2018); therefore, understanding how microbes change and react to the environment can help to understand and even predict the interaction between the microbes themselves and between microbes and their environment (Ratzke and Gore, 2018).

Correlations between turbidity and the culturable microbial concentrations were positive for all columns, and the relationships were moderate for all columns and significant when all the data were combined (Table 4.2). The increased microbial population in the columns might encourage microbes to "chew-up" the woodchip particles

at a higher rate, consequently releasing higher amounts of organic matter and resulting in higher turbidity.

Furthermore, the correlations between nitrate concentrations and culturable microbial concentrations were negative for all columns, suggesting greater nitrate removal capacity with greater microbial populations. The relationship between nitrate and microbial populations was not significant for the individual columns, possibly due to the large retention time which resulted in high (> 90%) nitrate removal in all instances and, therefore, little variation for analysis. However, a significant relationship between nitrate concentration and the microbial population was observed when the data were combined.

Moreover, a statistically significant negative relationship was detected between *E. coli* concentrations and culturable microbial concentrations in the effluents of both columns A and B (Table 4.2). This suggests that although carbon sources might be readily available for *E. coli* in denitrification bioreactors due to cellulose- and lignin-degrading organisms, microbial populations might have a negative effect on *E. coli* survival potentially due to factors such as predation and substrate competition (Jiang et al., 2002; Unc et al., 2006; Wanjugi, 2013). These biotic factors have been shown to impact *E. coli* survival in aquatic systems (González et al., 1990; Menon et al., 2003; Rhodes and Kator, 1988). An overriding effect of the natural microbiota on the survival of *E. coli* was previously documented by Jiang et al. (2002) and Unc et al. (2006), and the cumulative impact of the total native microflora on *E. coli* survival was often negative likely due to predation, substrate competition, and antagonism. Several studies have shown the negative impacts of natural microbiota on fecal indicator bacteria survival (Anderson et al., 1983; González et al., 1990; Menon et al., 2003; Rhodes and Kator, 1988).

The diversity of the microbial population is another important factor which can regulate the population of *E. coli* (Van Elsas et al., 2006). Environments with a greater degree of microbial diversity are more resistant to perturbations than those with a lower biodiversity (Tilman, 1997; Trevors, 1998), suggesting that the environments with a higher level of diversity would be less susceptible to invasion by *E. coli* than where a lower diversity exists (Van Elsas et al., 2011). More studies are suggested to examine the potential impact of microbial diversity on survival of *E. coli* in denitrification bioreactor and identify microorganisms that can promote the decrease of pathogenic *E. coli*, which might be used in the removal systems to decrease the survivability of pathogenic microbes.

4.4 Conclusions

This study assessed the potential changes of tile drainage water quality parameters flowing through denitrification bioreactors. A laboratory study was conducted with four columns operating under different influent microbial communities and flow conditions. The results of this study showed the high impact of influent microbial communities on the change of water quality parameters through denitrification bioreactors. Column B, which was provided with the highest diversity and concentration of microbes in the influent water, often had the highest pH, electerical conductivity (EC), total organic carbon (TOC), and turbidity in its effluent in most circumstances. However, column D, which was not treated with microbes in the influent, often had the lowest pH, EC, TOC, and turbidity in its effluent. In addition, the highest and lowest nitrate removal rates were found in column B and column D in most circumstances, respectively. Furthermore, wet-dry cycles often increased EC, turbidity, and nitrate removal as compared to the steady flow.

The results revealed significant relationships between pH as well as EC and the

microbial populations in the effluent of all denitrification bioreactor columns. When the data were combined for all columns, a significant negative relationship was also found between cultural microbial populations and nitrate concentrations. Furthermore, the impact of microbial community on *E. coli* concentration was significant, as statistically negative correlations were detected between these two microbial indices. This suggests the impact of biotic factors on removing pathogenic microbes, possibly due to predation, substrate competition, and antagonism which needs to be determined by further experiments.

4.5 Acknowledgements

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CHAPTER 5: EVALUATING NUTRIENT FILTER MATERIALS ON REDUCING *E. COLI* UNDER DIFFERENT INFLUENT *E. COLI* CONCENTRATIONS

A paper in preparation

Abstract

Nutrient filter materials are usually used to remove the excess nitrate and phosphorus from nutrient-laden waters (e.g., tile drainage water or wastewaters). While much attention has been given to the evaluation of nutrient filter materials for removing nutrients, little consideration has been given to the potential impact of these filter materials on microbial fluxes into the environment. Therefore, the main objective of this study was to evaluate the impact of nutrient filter materials on the concentration of unwanted microbes (e.g., *E. coli*) in the treated water. In this study, four lab-scale nutrient treatment beds were used to evaluate the changes of *E. coli* concentrations in synthetic water when passed through different nutrient filter materials, including woodchips only, woodchips followed by steel turnings, steel turnings only, and woodchips mixed with biochar. A synthetic solution was spiked with *E. coli* isolates and pumped through the beds at low and high concentrations (2×10^1 and 2×10^3 CFU mL^{-1}, respectively). Each set of experiments lasted for 60 days, during which water samples were collected from the inlets and outlets of all beds and processed for *E. coli* concentrations. All filter materials were capable of significantly removing *E. coli* from water passing through these removal systems. Higher concentrations of *E. coli* in the influent decreased the efficiency of these systems to remove the *E. coli*. Steel turnings showed the lowest *E. coli* removal rates under both high and low influent *E. coli* concentrations. Moreover, woodchips followed by steel turnings and woodchips mixed with biochar, resulted in higher removal efficiency with high concentrations of influent *E.*

coli, suggesting these configurations might be promising alternatives to woodchip only systems to foster *E. coli* reductions in waters with high *E. coli* concentrations.

Key words: Water quality, Treatment systems, Tile drainage, Wastewater, Microbial removal, *E. coli*.

5.1 Introduction

Over the past few decades, nutrient loading has become one of the largest water quality concerns in the U.S. (USEPA, 1990). It has negatively impacted recreation (Dodds et al., 2008); ecosystem, animal and human health (Hilborn et al., 2007); and aquatic industries (Benayache et al., 2019) due to algal blooms, and hypoxic conditions (Diaz and Breitburg, 2009; Heisler et al., 2008). Agricultural sources have been recognized as a major pathway for nutrients to enter waterways, such as the Mississippi River (Alexander, 2008; USGS, 2014). Nutrients can be transported both through runoff and subsurface drainage systems.

Subsurface drainage systems are critical for agricultural production in areas with excess moisture to remove water from the soil below the surface (root zone) and provide favorable conditions for plant growth. However, nutrients, applied as fertilizer, can leach through the soil and move into tile drainage water where they are rapidly moved to surface waters, bypassing natural removal by plant uptake and denitrification (Kovacic et al., 2000; Tanji and Kielen, 2002). Management practices have been developed to reduce subsurface drainage flow and/or nutrient concentrations in subsurface drainage water, thereby reducing the overall load from subsurface drainage waters.

Denitrifying bioreactors are one promising management practice for nitrate removal in subsurface drainage as well as wastewater treatment systems. They are typically a trench

that is filled with carbonaceous material and installed at the edge of an agricultural field or downstream of a primary or secondary septic tank. The nutrient-laden water is diverted through the system, which results in saturated conditions necessary for denitrification. In the Midwestern U.S., woodchips have been the primary carbon source tested and used in denitrifying bioreactors (Soupir et al., 2018). Nitrate removal in these systems can reach nearly 100% for nutrient-laden drainage waters (Bell et al., 2015; Mardani et al., In preparation-a; Wildman, 2002).

There has been recent interest in examining similar ideas for other agricultural pollutants such as phosphorus-sorbing, in-line filters (Penn et al., 2007; Thapa, 2017), as well as assessing the effectiveness of other types or combinations of media on removing nitrate, phosphorus, organic contaminants, and pesticides (Bock et al., 2016; Goodwin et al., 2015; Inyang and Dickenson, 2015; King et al., 2010; Pluer et al., 2016). For example, biochar has been added to denitrifying bioreactors to act as dual-nutrient removal systems by promoting denitrification (Bock et al., 2016; Pluer et al., 2016), and increasing phosphorus removal (Bock et al., 2015). In other cases, industrial waste, such as steel by-products (Christianson et al., 2017; Goodwin et al., 2015; Hua et al., 2016), or phosphorous-immobilizing materials, such as water treatment residuals (Zoski et al., 2013), were combined or paired with woodchip media to remove both nitrate and phosphorus from nutrient-laden waters, including subsurface drainage water.

Despite the extensive research done on these nutrient removal systems, little consideration has been given to the potential effect of the filter materials and dual-nutrient removal systems on other contaminants, including bacteria. Biological processes play a crucial role in treating nitrate through woodchip denitrifying bioreactors (WBs). However,

the application of carbonaceous materials, like woodchips and biochar, in these systems may also provide opportunities for undesirable microbial growth and increases in undesirable microbial characteristics, such as antibiotic resistance. Fecal indicator bacteria (FIB), including *E. coli*, have the capability of growing in the environments external to a host given adequate conditions which include temperature, pH, availability of water, nutrients, and energy sources (Van Elsas et al., 2011). On the other hand, lack of selection pressure for antimicrobial resistance may result in a decrease in these traits (Austin et al., 1999), and filtration, competition, or predation may result in reduced concentrations of undesirable microbial populations (Alufasi et al., 2017; Haig et al., 2015; Liao et al., 2015; Stevik et al., 2004).

A few studies have demonstrated the ability of WBs to reduce *E. coli* populations (Mardani et al., Under revision; Rambags et al., 2016, 2019; Robertson et al., 2005; Soupir et al., 2018; Zoski et al., 2013), and increase (Mardani et al., Under revision; Zoski et al., 2013) or decrease (Rambags et al., 2016, 2019) populations of other microorganisms. Biochar can increase total microbial activity (Lehmann et al., 2011; Yan-li et al., 2013) and, denitrifying bacteria abundance (Anderson et al., 2011; Kolb et al., 2009) and when used as a soil amendment, can aid in removing *E. coli* (Abit et al., 2012; Afrooz and Boehm, 2016; Bolster and Abit, 2012). However, to our knowledge, no work has been conducted exploring the change of microbial concentrations or FIB using a woodchip bioreactor mixed with biochar. Similarly, the capability of steel by-products for removing *E. coli* from storm water has been examined at a small scale (Dai, 2019; Hooshyari, 2017); however, there is considerable ambiguity in the change of FIB populations as the systems are scaled up, and there is no information about how these systems impact microbial populations.

The goal of this study was to evaluate the impact of nutrient filter materials on reducing *E. coli* concentrations in bacterial-laden waters under different influent *E. coli* concentrations. To reach this goal, four laboratory-scale beds were filled with different nutrient filter materials, including woodchips, woodchips followed by steel turnings, steel turnings, and woodchips mixed with biochar and used to evaluate the change of *E. coli* populations in the water passing through these nutrient filter materials under two different influent *E. coli* concentrations.

5.2 Materials and Methods

5.2.1 *Bed Design*

Four horizontal, lab-scale flow-through nutrient treatment beds were used to evaluate the changes in *E. coli* concentration in synthetic water when passed through different media. The beds consisted of four glass aquariums (12.5" W × 36" L × 16.5" D): one filled with woodchips (A), one filled with woodchips followed by steel turnings (B), one filled with steel turnings (C), and one filled with a combination of

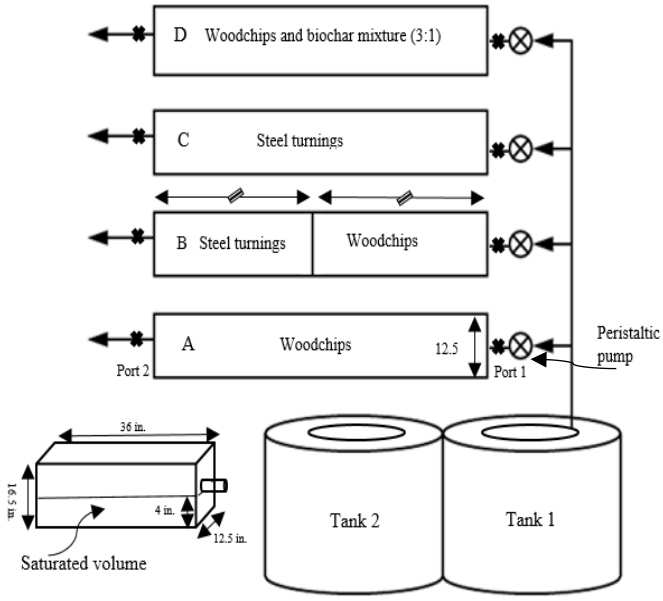

Figure 5.1 Schematic of the nutrient removal beds. Bed A contains woodchips, bed B contains woodchips in half the bed followed by steel turnings in the downstream half, bed C contains steel turnings only, and bed D contains a 3:1 mixture of woodchips and biochar.

woodchips and biochar at a 3:1 ratio (D) (Figure 5.1). Two ports were fixed at either end of the beds to allow for inflow and outflow of the synthetic water as well as to collect water samples. The beds were kept at room temperature which ranged from 23 °C to 25 °C for the duration of the experiments. Water samples were taken prior to entering the beds (Port 1 in Figure 5.1) and after exiting the beds (Port 2 in Figure 5.1).

5.2.2 *Bed Materials*

Cottonwood was used for the woodchip material. It is the same variety typically used in field bioreactor installations in the north-central U.S. The steel turnings consisted of AISI 1018 carbon steel provided by a metal factory in Sioux Falls, South Dakota (SD). The biochar used in this study was derived from ponderosa pine (pine feedstock), and had a surface area of 297.5 m^2 g^{-1}, and was provided from a supplier located in Texas.

Prior to the experiment, the woodchips and biochar were washed with tap water to remove fine particles and mitigate the impact of initial losses of total organic carbon on the experiment (Christianson and Schipper, 2016; Fenton et al., 2016). The materials were then air-dried. Some of the dried woodchips and biochar were mixed by alternately adding woodchips and biochar at a rate of 75% and 25%, respectively, resulting in a volume ratio of 3:1. The materials were mixed to create a relatively homogenous media prior to the experiment.

The steel turnings were also washed to eliminate the oils present on the surface of the steel as a result of the manufacturing process. The procedure involved washing the steel media in a solution of hot tap water and phosphate-free soap and rinsing with hot tap water before being air-dried. Based on visual appearance, some oxidation of the steel occurred

during the washing and drying process.

The porosity and particle size of the materials used in this experiment were measured to better describe them (Table 5.1). The material porosities were calculated based on methods described by Christianson et al. (2010). Briefly, one liter bottles were packed with the air-dried materials: woodchips, steel turnings, and the mixture of woodchips and biochar, with three repetitions each. Distilled water was added until the pore volumes were filled and the bottles reached saturation. The bottles were capped and their contents left to absorb the distilled water for 24 - 48 hours, then re-filled to saturation. The average media porosity was calculated based on the quotient of the average total volume of water added to the bottles over the average volume of the bottles.

The particle size of the materials was determined using 500 g of the dried materials fractionated by ASTM standard sieves in a shaker for five minutes (ANSI/ASAE, 2007). The materials primarily consisted of medium-sized particles, ranging from 1.8 – 10 cm in length and 0.2 – 1.5 cm in width (Table 5.1).

5.2.3 *Packing the Beds*

All the experimental beds had a similar internal volume, and approximately half of this volume was filled with packing material. The beds had a four inch flow depth resulting in a saturated volume of 29 L and an unsaturated volume of 30 L. This is based on a common bioreactor design in which an unsaturated layer of woodchips is used as a top layer to provide an additional carbon source for microbes to replace woodchips that are consumed or settle out (Christianson and Schipper, 2016). A tight matrix was achieved through incremental additions and tamping the materials with a steel rod. After packing the beds,

they were covered with three layers of aluminum foil and taped to minimize external influences on the beds.

The bed with woodchips followed by steel turnings was separated by steel mesh and mimicked an arrangement previously assessed by Goodwin et al. (2015), where the placement of a horizontal column with steel turnings downstream of a horizontal column with woodchips resulted in considerable orthophosphate removal.

Table 5.1 Bed characteristics.

Bed	A	B	C	D
Material	Woodchips only	Half woodchips, half steel turnings	Steel turnings only[a]	A mix of woodchips and Biochar (3:1)
Size (distribution % by weight)	22% small (0.1 - 3 cm long, 0.1 - 1cm wide)	-	5% small (0.1 - 2 cm long, 0.2 cm thick)	13% small (0.1 - 3 cm long, 0.1 - 1cm wide)
	61% medium (3 - 5 cm long, 0.5 - 1.5 cm wide)		83% medium (1.8 - 10 cm long, 0.2 cm thick)	75% medium (3 - 5 cm long, 0.5 - 1.5 cm wide)
	17% large (5 - 8 cm long, 0.5 - 3 cm wide)		12% large (5 - 12 cm long, 0.2 cm thick)	12% large (5 - 8 cm long, 0.5 - 2 cm wide)
Porosity (%)	60	73[b]	87	53
Flow rate (mL min^{-1})	48.4	58.8	70.0	42.7
Pump speed (Revolutions per minute)	10	11	13	8

[a] Contained C (0.14% - 0.20%); Fe (98.81% - 99.26%); Mn: 0.60% - 0.90%; P ≤ 0.04%; sulfur ≤ 0.05%) (Hua et al., 2016).
[b] Calculated by averaging the porosities in woodchips and steel turnings.

5.2.4 *Inoculating the Beds*

Inoculation water was mixed and circulated through the beds for 10 days at 18 mL min^{-1}. The mixture consisted of tap water, nutrients, and concentrated microbes indigenous to woodchip bioreactors. The nutrients included 2.0 μM $MnSO_4$ and 2.0 mM KH_2PO_4 as described by Nadelhoffer (1990). The nutrients were added to 500 L of tap water to

decrease the potential impact of micronutrient deficiencies on the denitrifier microbial communities (Hoover, 2012). The 10 most common, morphologically distinct microbial isolates, hereafter referred to as “indigenous microbes”, were previously isolated from the effluent of a field-scale WB located near Baltic, SD, and stored at –80 °C until use.

To inoculate the beds, the indigenous microbes were revived from the frozen stocks and grown in R2A broth. The concentration of each isolate grown in the broth was estimated based on previously identified relationships between the optical density (OD) at 600 nm and concentrations determined by viable plate count. After determining cell concentration, the microbes were diluted by using phosphate buffer saline solution (PBS) to achieve the desired concentration of 2×10^1 CFU mL^{-1} and added to the inoculation solution. After the inoculation period, all beds were flushed with 500 L of tap water and micronutrients for two days, to restore anaerobic conditions and refresh microbial activity. The flow rate used to flush the columns started at the speed used for inoculation (18 mL min^{-1}) and gradually increased to the experimental flow rate for each bed to minimize the possible disturbance to the microbial communities.

5.2.5 *Synthetic Water*

Synthetic water was prepared using a basal solution of tap water and nutrients (e.g., 2.0 mM KH_2PO_4 and 1.0 mM $MgSO_4$) to support microbial growth. The water was also spiked with a final concentration of 0.3 mM KNO_3 (30 mg L^{-1} potassium nitrate) and a mix of 10 *E. coli* isolates at two levels: i) a high concentration of approximately 2×10^3 CFU mL^{-1} used for the first set of experiments and ii) a low concentration of approximately 2×10^1 CFU mL^{-1} used for the second set of experiments. Ten *E. coli* isolates were taken from the influent of a field-scale WB located near Baltic, SD. The isolates were stored at –80 °C

until use. They were revived and grown in tryptic soy broth (Difco) one day prior to mixing the synthetic waters. The OD_{600} of each broth culture was measured to estimate the number of microorganisms contained in each broth. It was assumed that an OD_{600} was associated with a concentration of 8×10^8 cells mL^{-1} (Loehrer et al., 2016; Pumphrey, 2000).

The broth was diluted with PBS to achieve the desired *E. coli* concentration. For the high concentration, the broth culture was diluted to an estimated concentration of 2×10^2 CFU mL^{-1} for each isolate and mixed with 950 L of prepared water to achieve the desired concentration of 2×10^3 CFU mL^{-1}. For the low concentration, the broth culture was diluted to an estimated concentration of 2×10^0 CFU mL^{-1} for each isolate and mixed with 950 L of prepared water to achieve the desired concentration of 2×10^1 CFU mL^{-1}.

Synthetic water was made every three days throughout the experiment. Homogeneous mixing of the synthetic water solution was achieved by applying an agitator attached to a small aquarium pump in the tank which ran throughout the experiment.

5.2.6 *Flow Rate*

Two 1000 L polyethylene tanks were used to mix and store the synthetic water. Four separate peristaltic pumps (Masterflex FH100, Cole-Parmer, Vernon Hills, IL) were used to pump the synthetic water through the beds. The flow rate was adjusted for each individual bed based on the material porosity to achieve a target hydraulic retention time of six hours (Table 5.1). The silicone tubing (6.4 mm ID) was changed periodically throughout the course of the experiment to prevent variability caused by wearing. The flow rate for each bed was calculated using Equation 5.1.

$$Q = \frac{V_{active} \times \phi}{60 \times HRT} \quad \text{(Equation 5.1)}$$

Where Q is the flow rate (mL min^{-1}), V_{active} is the active volume of the bed (mL), ϕ is the porosity (%), and HRT is the hydraulic retention time (hour). The flow rate was verified at the outlet of each bed every two days.

5.2.7 *Experimental Procedure*

The experiment was conducted in two parts over about a five-month period under different influent *E. coli* concentrations (Table 5.2). The first set of experiments was conducted using a high *E. coli* concentration (2×10^3 CFU mL^{-1}). The pumps ran continuously at a constant rate for 60 days. The high concentration was selected to reflect elevated *E. coli* populations in municipal wastewaters (Kay et al., 2008; Lubello et al., 2004; Reinthaler et al., 2003). Water samples were taken 10 times over the course of the experiment from the influents and effluents of the beds. All samples were processed for *E. coli* and water chemistry parameters including pH and dissolved oxygen (DO).

Table 5.2 Summary of experiments.

Days from the beginning	Initial *E. coli* concentration	Material replacement	Inoculating/flushing the reactors	Period applied for data analysis
Set 1	2×10^3 CFU mL^{-1}	-	day 0 - 12	day 12 - 72 (n* = 10)
Set 2	2×10^1 CFU mL^{-1}	day 73 - 92	day 92 - 104	day 104 - 164 (n = 13)

* n is the number of samplings per each part.

The second set of experiments was conducted using a low influent *E. coli* concentration (2×10^1 CFU mL^{-1}). To eliminate the impacts of the high *E. coli* concentrations used in the first set of experiments, all materials were replaced and inoculated using the same procedure. Samples were collected 12 times throughout the 60 day duration of the experiment. The water samples were collected from the inlets and outlets of the beds and

processed for the same parameters as the first set of experiments.

5.2.8 *Sample Analysis*

Samples were collected in 300 mL, autoclaved bottles, and 30 mL, washed bottles, for microbiological and water chemistry analyses, respectively. Standard membrane filtration was used to process samples for *E. coli* concentration in triplicate (USEPA, 2002). According to this method, water samples were filtered through 0.45 μm filters. The filters were then plated on modified mTEC agar, housed in a water bath for 2 ± 0.5 hours at 35 ± 0.5 °C prior to incubation. After removing the plates from the water bath, the samples were incubated for 22 ± 0.5 hours at 44.5 ± 0.2 °C.

DO was measured using a portable field water analysis probe (model HI98193, Hanna Instruments, Smithfield, RI) immediately after collecting the sample. The pH of the water samples was also measured (Hach sensION^{TM+}, Hatch, Loveland, CO).

5.2.9 *Statistical Analysis*

To statistically analyze the data, R software (version 1.1.442) was used with a significance level of 0.95 ($\alpha = 0.05$). Non-parametric tests were used to compare the variables between the influent and effluent of the beds (Wilcoxon signed-rank test), among the effluents of the beds under different influent *E. coli* concentrations (Dunn test after Kruskal-Wallis test), and under different influent *E. coli* concentrations (Mann-Whitney U test).

5.3 Results and Discussion

5.3.1 *E. coli Concentration*

All beds and influent concentrations resulted in significant reductions in *E. coli*

concentrations (Table 5.3), suggesting that all filter materials have the capability of removing *E. coli* from water passing through these systems. Different influent *E. coli* concentrations had significant effects on the ability of the removal beds to reduce *E. coli* in water passing through these filters. Low influent *E. coli* concentrations resulted in significantly higher *E. coli* removal (80% - 97%) in all beds when compared with the removal rates under high influent *E. coli* concentrations (43% - 72%), indicating that *E. coli* removal efficiency decreased when the filter materials received sufficiently high influent *E. coli* concentrations. *E. coli* removal efficiency of these filter materials under a high influent *E. coli* concentration decreased by 35%, 22%, 46%, and 24% for the beds with woodchips, woodchips followed with steel turnings, steel turnings, and a mixed of woodchips and biochar, respectively compared to when the filters received a low influent *E. coli* concentration. The reduction in *E. coli* removal efficiency with increasing influent *E. coli* concentration is documented with other materials (Hooshyari, 2017; Mohanty and Boehm, 2014). Mohanty and Boehm (2014) evaluated *E. coli* removal through a sand filter amended with biochar and found a significant decrease (6.2%) in efficiency when influent *E. coli* concentrations increased from 10^6 to 10^7 CFU mL^{-1}. Similarly, Hooshyari (2017) found that steel chips had reduced *E. coli* removal efficiency by 2.4% when the influent *E. coli* concentration was increased from 10 to 10^4 MPN mL^{-1}.

Table 5.3 *E. coli* reduction under different influent *E. coli* concentrations.

Bed	A (Woodchips only)		B (Half woodchips, half steel turnings)		C (Steel turnings only)		D (A mix of woodchips and biochar)	
Influent *E. coli* concentrations	10^3 CFU mL^{-1}	%	10^3 CFU mL^{-1}	%	10^3 CFU mL^{-1}	%	10^3 CFU mL^{-1}	%
2×10^3 CFU mL^{-1}	94* (19)	63	99 (24)	72	56 (20)	43	95 (26)	72
2×10^1 CFU mL^{-1}	1.5 (0.3)	97	1.4 (0.3)	92	1.2 (0.3)	80	1.5 (0.3)	95

*Reduction is defined as the mean difference in concentration between column influents and effluents (standard deviation in parentheses), or that difference indicated as a percentage of influent concentration.

E. coli removal rates increased by nearly 10% when using woodchips combined with either biochar or steel turnings in the presence of a high influent *E. coli* concentration when compared to woodchips only. Although this increase was not significant, it may indicate that these configurations can be promising alternatives to woodchip only systems to increase *E. coli* reductions in waters with high bacterial concentrations.

Under both influent *E. coli* conditions, steel turnings (bed C) had the lowest *E. coli* removal rate. Low influent *E. coli* concentrations had a reduction of 80% while high influent concentrations only saw a 43% reduction in *E. coli* when steel turnings were used. This might be attributed to the attachment sites on steel media being blocked by *E. coli* more rapidly in the presence of a high influent *E. coli* concentration than a low influent *E. coli* concentration, as previously suggested by Dąbroś and Van de Ven (1982) and Camesano and Logan (1998). The removal rate for the steel filter in this study was lower than results by Hooshyari (2017) which demonstrated the high adsorption capacity of steel chips resulting in *E. coli* removal rates ranging from 94.0% to 96.3% under different influent concentrations (10 - 10^4 MPN mL^{-1}).

The beds achieved a steady-state *E. coli* removal rate under most combinations of material and influent *E. coli* concentrations (Figure 5.2). High influent *E. coli*

concentrations resulted in significantly lower *E. coli* removal during an initial warm-up period (days 0 - 20) compared with the removal rates experienced at the end of the experiment (days 20 - 60). However, this difference was not observed in the low influent *E. coli* experiments.

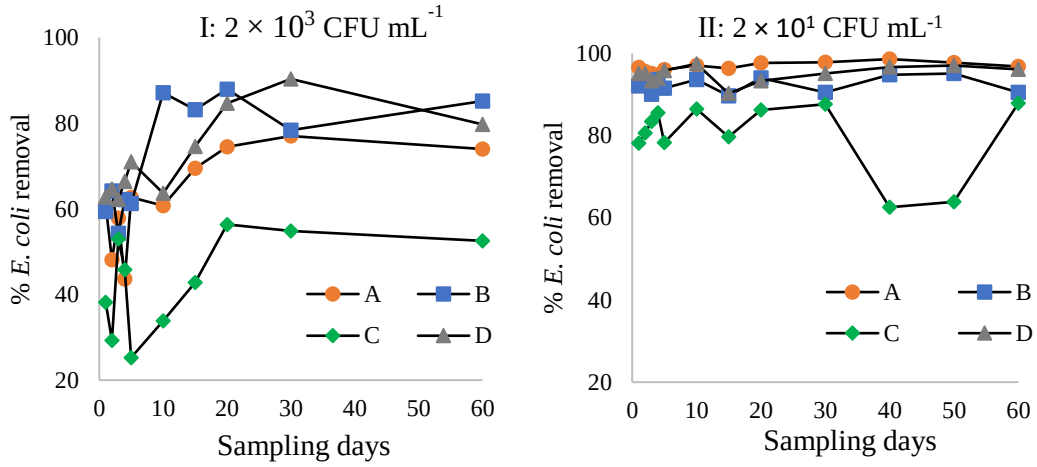

Figure 5.2 Percent *E. coli* removal in the removal beds operated under high (I) and low (II) influent *E. coli* concentrations.

The ability of woodchip bioreactors to remove *E. coli* has been demonstrated in previous works (Mardani et al., Under revision; Soupir et al., 2018; Zoski et al., 2013), where removal rates were evaluated for influent *E. coli* concentrations ranging from 1.84 × 10^2 CFU mL^{-1} to 1.13 × 10^3 CFU mL^{-1} and resulted in *E. coli* reductions from 49% to 97%. In the present study, the removal rate for woodchips operated under a low influent *E. coli* concentration was similar to the results of Zoski et al. (2013), which demonstrated a high removal capacity of woodchips (97%) under an influent *E. coli* concentration of 1.8 × 10^2 CFU mL^{-1} and a flow rate of 1 mL s^{-1}. However, it was higher than results by Mardani et al. (Under revision), which detected *E. coli* removal rates ranging from 49% to 68% under an influent *E. coli* concentration of 2 × 10^2 CFU mL^{-1} and different flow conditions.

Furthermore, the removal rate for woodchip filters under a high influent *E. coli* concentration was lower than the results of Soupir et al. (2018), which presented a high removal capacity of woodchips resulting in *E. coli* removal rates ranging from 78.0% to 96.3% under influent *E. coli* concentrations between $1.84 \times 10^2 – 1.13 \times 10^3$ CFU mL^{-1} and different temperature conditions (10 °C and 21.5 °C).

There are several possible removal mechanisms with the different materials, including chemical, physical, and biological, alone or in combination. Physical mechanisms include interaction of *E. coli* with the media surfaces as influenced by surface structures and cell surface charge, as well as filtration and adsorption of *E. coli* through attachment to solid substrates (Liao et al., 2015). The irregular shape and rough surface of a material may also encourage the attachment of microbes through straining (Bradford et al., 2006). Predation by other organisms, natural die-off, and competition for resources may constitute biological factors for *E. coli* inactivation (Alufasi et al., 2017; Haig et al., 2015; Stevik et al., 2004). Carbonaceous materials, like woodchips and biochar, enhance microbial activity (Yan-li et al., 2013), which, in turn, may increase competition and predation within the microbial community (Haig et al., 2015). Changes in pH can also control the ability of microbes to adsorb to materials (Guber et al., 2005; Hooshyari, 2017; Scholl and Harvey, 1992). For example, the external surfaces of biochar will have a net negative charge in a high pH aqueous environment, whereas a low pH environment will result in a net positive charge (Mukherjee et al., 2011) that will be more prone to adsorb negatively charged microbes, like *E. coli*, through electrostatic interactions. The formation of biofilm on the surface of the materials can also modify the physiochemical properties of the materials by changing the roughness, hydrophobicity, and electrokinetic properties of materials when

extracellular polymeric substances are present (Clement et al., 1996; Janjaroen et al., 2013; Taylor et al., 1990). This would affect the surface interactions between the materials and suspended particulates, and alter the capacity of the filter media to remove pollutants (Dai and Hozalski, 2002; Torkzaban et al., 2007).

5.3.2 *Water Chemistry*

During the course of the experiment, pH values ranged from 6.3 to 6.9 in the influent and 6.1 to 8.7 in the effluent of the removal beds. The pH in the denitrification removal beds (i.e., those with carbonaceous components) was within the desired pH range for denitrification, between 5.5 and 8.0 (Rivett et al., 2008). An increase in pH was observed in the effluent as compared to the influent of all the removal beds, indicating that the water became more alkaline when passing through the materials. While it was beyond the scope of this experiment to determine the mechanism responsible for this increase, the possible explanations include the release of ferrous hydroxide as a result of steel corrosion (USGS, 1962), the release of bicarbonates and hydroxyl anions during the denitrification process, or the release and later utilization of organic acids produced from fermentation and biodegradation of the carbon media (Rivett et al., 2008; Rust et al., 2000; Zhao et al., 2018). Also, bacteria activity as dissimilatory reducers of nitrate to ammonium may have been present (Rütting et al., 2011; Semenov et al., 2019). The increases in pH were most significant when the water passed through the steel turnings, possibly due to the oxidizing of iron in the presence of water (USGS, 1962).

The highest effluent pH was detected in the removal beds with or combined with steel turnings, beds B and C, under both influent *E. coli* concentration operations (Figure 5.3). The effluent from the removal bed containing steel turnings had significantly higher pH

than the effluent from the removal beds containing woodchips and woodchip-biochar mixture under both influent conditions.

The results of this experiment are similar to previous studies which found pH increases in water passing through denitrification bioreactors packed with carbonaceous materials (Bock et al., 2016; Damaraju et al., 2015; Mardani et al., In preparation-a; Nordström and Herbert, 2017; Reddy et al., 2014; Warneke et al., 2011), steel slag filters (Barca et al., 2012; Lee et al., 2010; Neville, 2019; Weber et al., 2007), and steel turnings (Goodwin et al., 2015). While many previous studies demonstrated an increase in pH, a few studies examining denitrifying woodchip bioreactors showed a decrease in the pH of the water passing through the system (Mardani et al., In preparation-c; Robertson et al., 2005; Van Driel et al., 2006).

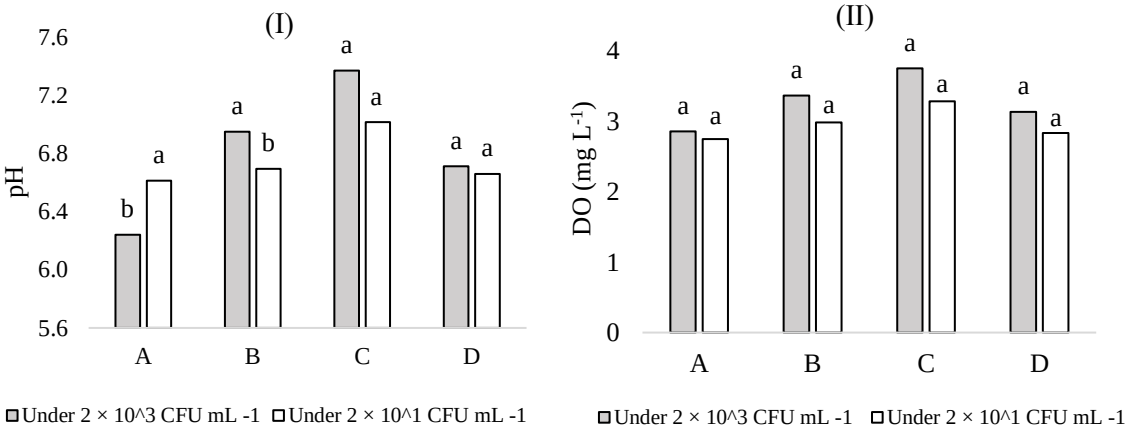

Figure 5.3 The average of effluent pH (I) and dissolved oxygen (II) (DO) levels under high and low influent *E. coli* concentrations (2×10^3 and 2×10^1 CFU mL^{-1}, respectively) (lower-case letters indicate significant differences between effluents receiving different *E. coli* concentrations; beds with the same letters are not significantly different at 5% level in different influent *E. coli* concentrations).

DO concentrations ranged from 6.2 to 8.2 mg L^{-1} in the influent and 1.2 to 4.7 mg L^{-1}

in the effluent of the removal beds. While there was a significant reduction in the DO concentration as the water flowed through the removal beds, the beds were not anaerobic. The highest effluent DOs were detected in the beds with or combined with steel turnings (beds C and B), while the lowest effluent DO concentrations were in the beds containing carbonaceous materials (beds A and D) under both influent *E. coli* concentration operations (Figure 5.3).

DO concentrations in the denitrification removal beds were within the allowable concentration range for denitrification. For example, Gómez et al. (2002) found no denitrification inhibition below 4.5 mg L^{-1}, while Healy et al. (2006) found no inhibition below 3.7 mg L^{-1}. No significant differences were detected between effluent DO concentrations with different initial *E. coli* concentrations (Figure 5.3).

5.4 Conclusions

Previous studies have largely focused on evaluating nutrient removal from phosphorus-sorption materials, carbon media, and dual-nutrient reduction systems. However, little consideration has been given to the potential effect of these systems on microbes, which are grown in/on and/or pass through these systems. In this study, four laboratory-scale removal beds receiving similar synthetic waters were designed to assess the potential effects of nutrient filters on *E. coli* concentrations in the water passing through the media. Four nutrient filter materials were evaluated, including woodchips, woodchips followed by steel turnings, steel turnings, and woodchips mixed with biochar. Two influent *E. coli* concentrations were evaluated: a high concentration of 2×10^3 CFU mL^{-1} and a low concentration of 2×10^1 CFU mL^{-1}.

The results demonstrated that all materials were capable of significantly removing *E. coli* from water passing through these systems. Higher concentrations of *E. coli* in the influent reduced the efficiency of these systems to remove the *E. coli*. Steel turnings had the lowest *E. coli* removal rates under both high and low influent *E. coli* concentrations. Moreover, the dual-nutrient systems, of woodchips followed by steel turnings and woodchips mixed with biochar, resulted in higher removal efficiency with high concentrations of influent *E. coli*, suggesting these are promising arrangements to achieve higher removal in systems with elevated *E. coli* concentrations. More studies are suggested to assess the longevity of *E. coli* removal with different hydraulic retention times, varying temperatures, and fluctuating water levels through different nutrient filter materials and arrangements.

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CHAPTER 6: THE EFFECT OF IN-SITU WOODCHIP BIOREACTORS ON MICROBIAL CONCENTRATION IN SUBSURFACE DRAINAGE WATER AND THE ASSOCIATED RISK OF ANTIBIOTIC RESISTANCE DISSEMINATION

A paper in preparation

Abstract

Denitrification bioreactors are a best management practice for nutrient reduction that have proven to be effective at reducing nitrate in tile drainage water. However, not much is known about the potential effect of these systems on the microbes and antimicrobial resistance (AMR) within tile drainage water, particularly at the field-scale. Therefore, the main objective of this study was to evaluate the effect of an in-situ woodchip bioreactor on microbial concentrations in subsurface drainage water, and provide preliminary insights into the possible associated risk of antimicrobial resistance dissemination to the environment. In this study, an in-situ denitrification bioreactor was monitored for a five-month period during the 2018 field season. Water samples were collected from influent and effluent on a weekly basis during the study period and processed for *E. coli* concentrations, total culturable microbe concentrations, and phenotypic antibiotic resistance of the microbes. The results showed the effluent often had an increase in general microbial concentrations and *E. coli* concentrations, though the increases were not significant. The culturable microbial concentration increased by 116% on average. The increases in *E. coli* concentrations ranged from 2% to 1700% while reductions in *E. coli* occurred less frequently and ranged from 54% to 89%. An increase in phenotypic AMR concentrations was also found within the woodchip bioreactor after assessing the sensitivity of recovered isolates to five different antibiotics; however, it was not significant.

To increase our understanding of microbial fluxes and AMR in water flowing through denitrification bioreactors, more in-situ studies are required to quantify the change in numbers of undesirable microbes, such as *E. coli*, and undesirable traits, such as AMR, under different environmental conditions.

Keywords: Agriculture, Field study, Tile drainage, Water quality, *Escherichia coli*, Antimicrobial resistance.

6.1 Introduction

Nutrient loading is one of the top water quality concerns in the U.S. (USEPA, 1990), due to its impacts on eutrophication of surface waters and its role in causing the large hypoxic zones, as seen in the Gulf of Mexico (David et al., 2010; Rabalais et al., 1996; USGS, 2000). Agricultural sources are recognized as a major contributor of nutrients entering the Mississippi River, which ultimately delivers these nutrients to the Gulf of Mexico (Alexander, 2008; USGS, 2014).

Subsurface tile drainage practices in the Midwestern U.S. region result in a direct pathway for these nutrients to reach surface water (Jaynes and James, 2007). The application of fertilizers, including manure, to agricultural land is the main source of these pollutants (Puckett, 1995). Manure usually contain elevated levels of nutrients, organic matter, heavy metals, a variety of microbes (Chadwick and Chen, 2002), antibiotics, and resistant microbes (Heuer et al., 2011), which can leach through the soil and move into tile drainage water, mainly by infiltration via soil macropores. These pollutant-laden waters are then discharged to surface waters (Pinheiro et al., 2013; Skaggs et al., 1994).

Many in-field and edge-of-field management practices have been developed to

decrease nutrient loads from agricultural fields. Denitrification bioreactors are one such practice that has proven to be economical (Christianson et al., 2009; Schipper et al., 2010) and effective in reducing nitrate in tile drainage water (Christianson et al., 2012; Schipper et al., 2010). The denitrification bioreactor systems traditionally consist of a lined trench at the edge of an agricultural field that is filled with carbonaceous media, typically woodchips (Blowes et al., 1994). The tile drainage water is diverted through the system from the agricultural fields, treating between 23% and 98% of the annual nitrate load passing through tile drainage systems (Verma et al., 2010; Woli et al., 2010).

While extensive research has demonstrated the efficiency of denitrification bioreactors in removing nitrate from tile drainage water (Addy et al., 2016; Christianson et al., 2012; Schipper et al., 2010), few studies have examined the potential effect of these systems on the microbial populations within tile drainage water, particularly at the field-scale. The purpose of denitrification bioreactors is to create an environment for denitrifiers to reduce nitrate to nitrogen gas (Schipper et al., 2010). However, these high carbon and nutrient environments have the potential to impact microbial communities in the drainage water as well. While the denitrification bioreactor systems are designed to promote the growth of microbes for denitrification, they also have the potential to treat *E. coli* found in tile drainage water through physical and/or biological processes. Physical mechanisms may include the filtration of microorganisms through their attachment to the substrate and other interactions of *E. coli* with woodchip surfaces (Liao et al., 2015). Biological processes for *E. coli* inactivation in the woodchip bioreactors may include predation by other organisms, natural die-off, and competition for resources (Alufasi et al., 2017; Haig et al., 2015; Stevik et al., 2004). A few studies have demonstrated the capability of woodchip bioreactors to

decrease *E. coli* concentrations (Mardani et al., Under revision; Rambags et al., 2016, 2019; Robertson et al., 2005; Soupir et al., 2018; Tanner et al., 2012), suggesting an added benefit of installing these systems in the field to treat both tile drainage water and wastewaters. While denitrification bioreactors treating wastewaters have been studied at both pilot- and field- scales (Rambags et al., 2016, 2019; Robertson et al., 2005; Tanner et al., 2012), denitrification bioreactors treating agricultural tile drainage water were only studied at the laboratory-scale (Mardani et al., Under revision; Soupir et al., 2018; Zoski et al., 2013).

In addition to altering the concentration of *E. coli* in tile drainage water, bioreactors can alter the concentration of other microbes (Mardani et al., Under revision; Zoski et al., 2013) due to their high carbon and nutrient concentrations which provide favorable conditions for microbes to grow (Madigan et al., 2010). A previous laboratory-scale study has shown an increase of general culturable microbial concentrations in synthetic tile drainage waters passing through woodchip denitrification bioreactors (Mardani et al., Under revision). Zoski et al. (2013) also found an increase in total coliforms (90% to > 3000%) in the effluents of woodchip bioreactors used to treat synthetic tile drainage water when compared to the inlet concentrations. On the other hand, Rambags et al., (2016, 2019) demonstrated the potential of bioreactors to treat microbial contaminants in wastewaters, resulting in a decrease in total coliforms.

Despite the recent research examining microbes and bioreactors, no work has been conducted on the impact of in-situ denitrification bioreactors on the concentration of unwanted microbial characteristics in tile drainage water, such as antimicrobial resistance (AMR). As tile drainage water passes through a denitrification bioreactor, it may facilitate cell-to-cell contact between the microbial communities within the bioreactor itself,

resulting in the potential for genetic transfer, primarily through conjugation (Madigan et al., 2010). Biofilm formation, which has been observed on bioreactor woodchips (Chun et al., 2009; Damaraju et al., 2015), may also provide "hotspots" for horizontal gene transfer (Nesse and Simm, 2018), leading to a potential increase in AMR microbes released into the environment. In addition, denitrification bioreactors are nutrient-rich environments which, in turn, promote cell reproduction and may lead to an overall increase in the abundance of AMR microbes, with associated antimicrobial genes. These microbes can then be transported from the bioreactor systems via tile drainage water, and are subsequently passed to the environment.

It is important to understand the potential impacts of denitrification bioreactors on undesirable microbes, such as *E. coli,* and undesirable traits, such as antimicrobial drug resistance, to support the development of effective designs for microbial contaminant removal and reduce the ancillary impacts of denitrifying bioreactors on the environment. Therefore, the goal of this study was to conduct a case study evaluating the potential effect of an in-situ denitrification bioreactor on the microbial populations (e.g., *E. coli*, general culturable microbes, and antibiotic resistance traits) in the tile drainage water passing through the system and provide preliminary insights into the possible associated risk of antimicrobial resistance dissemination to the environment. This goal was achieved through monitoring a field-scale bioreactor installed in South Dakota (SD) during the 2018 field season.

6.2 Materials and Methods

6.2.1 *Site Description*

A six year old, in-situ denitrification bioreactor was selected for monitoring for microbial indices during the 2018 field season. The bioreactor was installed in November 2014 near Hartford, South Dakota (SD), to treat tile drainage water from 8.1 acres. The bioreactor was located downstream of a livestock operation. Corn was grown during the study period. The primary soil type was silty loam soil.

6.2.2 *Bioreactor Description*

The bioreactor was a woodchip-filled trench designed with an estimated hydraulic retention time of 6.33 hours and the ability to pass 18% of the peak flow through the bioreactor. Details of the tile drainage system, bioreactor characteristics, and media characteristics can be found in Table 6.1.

Table 6.1 Site drainage, bioreactor, and media characteristics.

Tile drainage characteristics	Tile size (cm)	15.2
	Velocity in pipe (cm s^{-1})	41
	Tile grade (%)	0.4
	Peak flow from tile size (L min^{-1})	453.8
Bioreactor characteristics	Length (m)	38.1
	Width (m)	3.0
	Depth of media (m)	1.21
Woodchip characteristics	Type	Cottonwood
	Particle size (cm)	0.60 - 5.10
	Porosity %	70
	Hydraulic conductivity of wood media (cm s^{-1})	9.5

Two inline water-level control structures (AgriDrain Corp. Adair, Iowa) were installed at the inlet and outlet of the bioreactor (Figure 6.1). The structures were fitted with V-notch boards (45°) for more accurate flow measurements. The inlet control structure was a three chamber design, that allows for flow into the bioreactor as well as bypass flow to avoid excess moisture conditions in the field during high drainage flow periods, and minimizes the development of preferential flow pathways in the bioreactor (Soupir et al., 2018). The

outlet control structure was a two chamber design, connecting the bioreactor flow to the overflow pipe (Figure 6.1). The water depths were measured by two pressure transducer sensors (Decagon CTD-10, Decagon Devices, Pullman, WA), one at the inlet control structure and one at the outlet control structure (Figure 6.1). Precipitation and temperature gauges were also installed at the site. The sensor data were recorded and stored every 10-minutes by connecting the sensors to data loggers at the control structures.

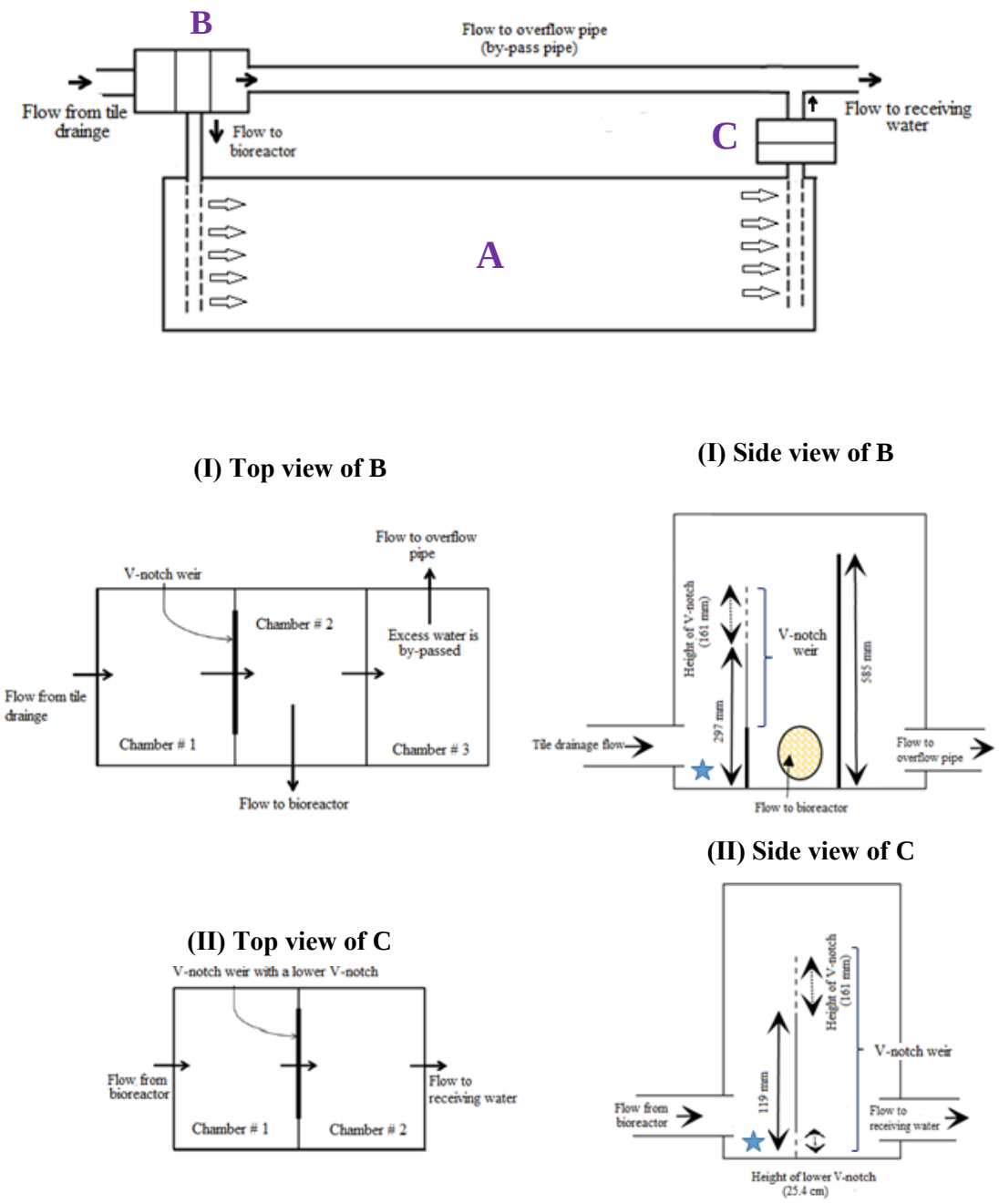

Figure 6.1 A schematic of the denitrification bioreactor (A), inflow and outflow control structures (B and C, respectively), top and side views of inflow control structure (**I**), top and side views of outflow control structure (**II**). Blue star signs show the location where pressure transducers were installed at both control structures.

6.2.3 *Flow Rate*

The transducer located in the inflow and outflow control structures provided an estimate of the volume of water bypassing through the overflow pipe and the discharge by the bioreactor, respectively (Chun and Cooke, 2008; Partheeban et al., 2014). Daily flow rates were calculated by taking a daily average of flows calculated for the recorded depths at 10-minute intervals for both control structures throughout the monitoring period.

6.2.4 *Hydraulic Retention Time*

The in-situ hydraulic retention time (HRT) was estimated using the following Equation (Christianson et al., 2011).

$$HRT = \frac{V_s \times n}{Q} \qquad \text{(Equation 6.1)}$$

Where HRT is the in-situ hydraulic retention time, Q is the bioreactor flow, n is the porosity of the woodchip media, and V_S is the saturated volume of the bed calculating as a product of the average observed inflow and outflow water depths, the length of the bioreactor, and the width of bioreactor.

6.2.5 *Sampling Procedure*

Influent and effluent samples were collected on a weekly basis during a five-month period (June – October) in 2018 while there was flow. Samples were collected in a 300 mL sterilized laboratory bottle attached to a steel rod. The samples were immediately placed on ice for transport back to the laboratory and subsequent analysis. The samples were processed for microbiological data within eight hours of sample collection.

6.2.6 *Sample Analysis*

Samples were processed for microbiological parameters including *E. coli*

concentrations, total culturable microbe concentrations (aerobic or facultative anaerobic microbes), and phenotypic antibiotic resistance of the microbes. Other water quality indices analyzed included pH, electrical conductivity (EC), and dissolved oxygen (DO).

Weekly samples were analyzed for *E. coli* via standard membrane filtration (USEPA, 2002). Briefly, water samples were filtered through a sterile, 0.45 μm filter. The samples were plated in triplicate on modified mTEC agar and placed in a water bath at 35 ± 0.5 °C for 2 ± 0.5 hours. The plates were then placed in an incubator 44.5 ± 0.2 °C for 22 ± 0.5 hours. The averages of the triplicate values are reported in this publication.

Culturable microbe concentrations and phenotypic antibiotic resistance were evaluated roughly biweekly throughout the monitoring season. The culturable microbe concentration was determined using plate count (Ridout, 2014). Briefly, five 10-fold dilutions using phosphate buffer saline solution were plated on R2A agar and incubated at 35 ± 0.5 °C for 72 ± 0.5 hours. All samples were processed in duplicate. Concentrations of culturable microbes per milliliter of water were determined by averaging the values of duplicate dilution plates.

Phenotypic antibiotic resistance was assessed using a modified Kirby-Bauer method (Bauer et al., 1966; CLSI, 2011). One to 10 common colonies from the culturable microbe plates were isolated through streaking each colony onto a separate R2A agar plate. The isolates were then tested for phenotypic antibiotic resistance to five antibiotics that have been detected in aquatic and terrestrial environments (Carvalho and Santos, 2016; Kemper, 2008; Kim et al., 2011; Kümmerer, 2009), including tetracycline (30 μg), ampicillin (10 μg), penicillin (10 U), sulisoxazole (0.25 mg), and erythromycin (2 μg). Disc

concentrations were selected based on previous environmental testing concentrations (CLSI, 2011; Helt, 2012; Muñoz-Atienza et al., 2013; Nasreen et al., 2015). The discs were placed on Mueller-Hinton agar that had been inoculated with a microbial isolate. The plates were then incubated for 24 – 48 hours at 37 ± 0.5 °C. After incubation, the inhibition zone diameters were measured and classified as resistant, intermediate, or sensitive using reference levels determined by the Clinical and Laboratory Standards Institute (CLSI, 2011) and the National Committee for Clinical Laboratory Standards (NCCL, 1984) for bacterial strains. Strains classified as intermediate were combined with the resistant classification, as those isolates are somewhat resistant to the antibiotic (Łuczkiewicz et al., 2010; Reinthaler et al., 2003)

Water samples were also analyzed for DO (HI98193 field probe, Hanna Instruments, Smithfield, RI), pH, and EC (Hach sensION^{TM+}, Hach, Loveland, CO). The DO was measured immediately after sample collection to minimize changes in oxygen concentrations. In addition, water temperature was continuously measured along with flow using the CTD sensors installed in the control structures.

6.2.7 *Data Analysis*

Changes of the measured variables were calculated as the average difference in concentration between the influent and effluent of the bioreactor. The difference was expressed as a percentage of influent concentration. For statistical analyses, R (version 1.1.442) statistical software was used with a significance level of 0.95. A non-parametric test (Wilcoxon signed-rank test) was conducted to compare the values between the influent and effluent of the bioreactor. For categorical data like antimicrobial resistance results, the chi-square test was used to determine changes in resistance during the course of this study.

6.3 Results and Discussion

6.3.1 *Rainfall, Bioreactor Discharge, and Bypass Flow*

The total rainfall for 2018 at the bioreactor site was 679 mm, with about 542 mm of rainfall occurring during the monitoring period, June to October 2018 (Figure 6.2). The previous 10-year average precipitation recorded by the closest weather station to the bioreactor site was around 442 mm in the monitoring period (June to October), which was 100 mm less than the precipitation that occurred at the bioreactor site in 2018 during the study period. The above normal rainfall conditions led to several flow events where the tile flow rate exceeded the bioreactor capacity. About 23% of the total flow bypassed the bioreactor during the course of the study via the overflow pipe. The high flows seen in 2018 resulted in flow through the bioreactor during the entire monitoring period.

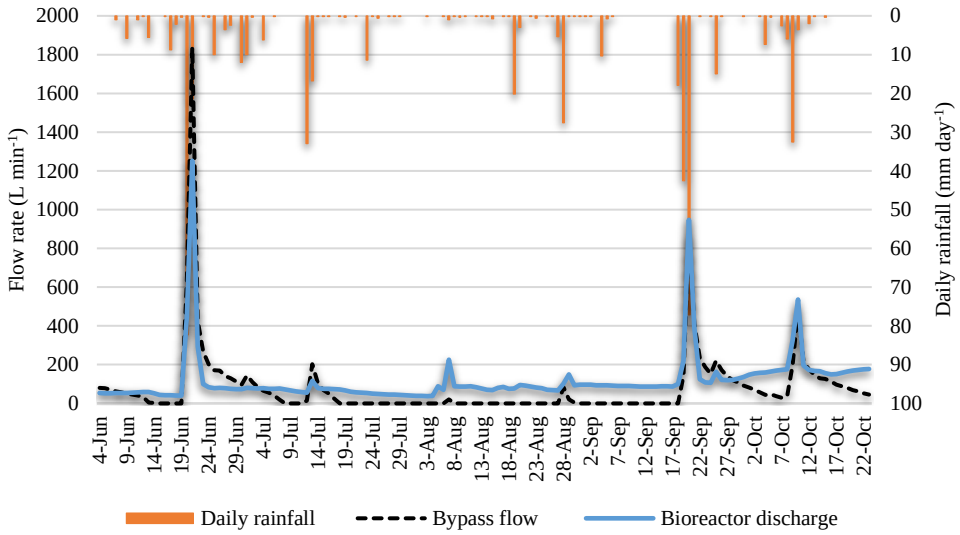

Figure 6.2 Discharge and bypass flow from the bioreactor near Hartford, SD.

The average daily bioreactor discharge varied between 37.4 and 1,252 L min^{-1} from June to October 2018, with an average discharge rate of 118 L min^{-1} over the course of the

monitoring period. Since the bioreactor had continuous discharge during the period of study, it was assumed that the bioreactor inflow was equal to the measured bioreactor discharge based on conservation of water within the lined denitrification bioreactor (Figure 6.1, A) (Rosen and Christianson, 2017). The continuous bioreactor discharge also implies wet conditions were continuously present within the bioreactor rather than being periodically dry, which does occur in this region during some seasons. Wet-dry cycling within this bioreactor commonly occurred from August through October in both 2016 and 2017.

Of note at the monitored bioreactor site is the elevation of the control structure at the inlet. Over time, substantial sediment buildup surrounding the control structure resulted in the structure having roughly the same elevation as the surrounding land. This led to occasional inflow due to runoff and, potentially along with the runoff, bacterial contamination from an adjacent feedlot. Direct inflow of surface water has been documented in previous studies by Hassanpour et al. (2017) and Pluer et al. (2016).

Elevated flow conditions can result in a number of changes to the bioreactor system as compared to typical moisture conditions. For one, elevated flows lead to a decrease in the retention time within the system (Greenan et al., 2009), resulting in less time for denitrification to occur (Christianson et al., 2012). The increased flow rate can also introduce water at different temperatures than baseflow, which can impact denitrification rates (Hoover et al., 2016). In addition, storm conditions can increase the volume of constituents, like nitrate, that leach from agricultural fields, thus impacting the load and concentration of the constituents in tile drainage water and, thereby, the amount passing through the bioreactor (Pluer et al., 2019). Water level fluctuations in the inflow control

structure can have both immediate and long-term impacts on denitrification activity, microbial community composition, and the stability of denitrification performance (Hathaway et al., 2017), probably in part through flushing organic content, biofilms, and enzymes out of the bioreactor.

6.3.2 *In-situ Hydraulic Retention Time*

The in-situ hydraulic retention time (HRT) ranged from 44 minutes to over 10 hours during the monitoring period (Figure 6.3). The average in-situ HRT for the season was 5.4 hours, nearly an hour shorter than the design HRT of 6.3 hours. This was likely due to the increased inflow observed during 2018. Shorter HRTs result in tile drainage water remaining within the system for a shorter duration, which may not provide denitrifying microbes sufficient time for denitrification (Chun et al., 2009; Robertson, 2010; Rodriguez, 2010). On the other hand, longer HRTs can cause unwanted chemical reactions, including sulfate reduction (Van Driel et al., 2006) and mercury methylation (Hudson and Cooke, 2011) within the bioreactor.

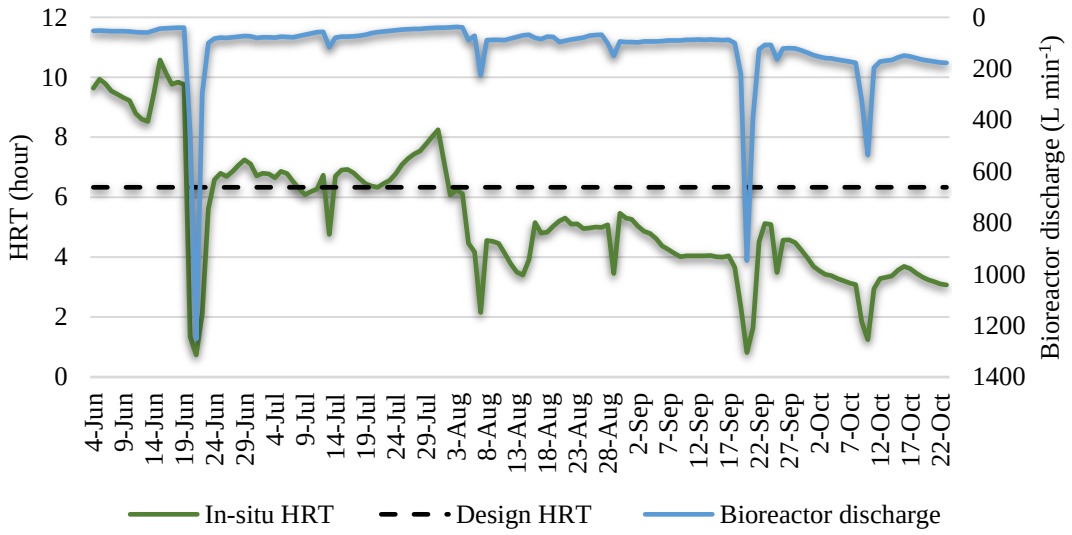

Figure 6.3 In-situ HRT, design HRT, and bioreactor discharge over the study period.

6.3.3 Water Chemistry

During the monitoring period, water samples were evaluated for pH, electrical conductivity (EC), and dissolved oxygen (DO) in addition to the microbial characteristics. The pH ranged from 7.1 to 7.7 in the influent and 7.0 to 7.7 in the effluent. There was a significant decrease in pH from the inlet to the outlet ($p = 0.05$) (Table 6.2). The pH measured was within the range previously reported for tile drainage water, which ranges from 7.0 to 7.6 (Fleming and VandeWeghe, 2003), as well as within the desirable range for the denitrification process which has an optimal range from 5.5 to 8.0 (Rivett et al., 2008).

The electrical conductivity (EC) in the tile drainage water generally decreased as it passed through the bioreactor, ranging from 1.2 – 4.2 mS cm^{-1} in the influent and 1.2 – 3.2 mS cm^{-1} in the effluent, but the decrease was not significant ($p = 0.23$) (Table 6.2). These measurements were higher than those previously reported in tile drainage water which is typically below 1.3 mS cm^{-1} (Kay et al., 2004; Patni and Hore, 1978; Patni et al., 1996).

Table 6.2 Summary of microbial and water chemistry values for the influent and effluent of the bioreactor over the course of study. Bold values indicate the median was used for the measure of central tendency and the 90th percentile for the measure of dispersion. All other measures of central tendency and dispersion were calculated using the mean and standard deviation, respectively.

Parameters	Influent		Effluent		% Change (reduction (R), Increase (I))	P-value*
	Mean or median	Standard deviation or 90th percentile	Mean or median	Standard deviation or 90th percentile		
E. coli (10^3 × CFU 100 mL^{-1})	**0.45**	**4.20**	**0.43**	**9.63**	46.0% I	0.150
Culturable microbes (10^6 × CFU 100 mL^{-1})	**4.83**	**11.0**	**13.9**	**17.90**	116.0% I	0.062
AMR (10^6 × CFU 100 mL^{-1})	**4.31**	**9.68**	**13.3**	**21.20**	127.0% I	0.063
AMR ratio	0.87	1.0	0.90	1.0	3.40% I	1.000
pH	7.39	0.15	7.29	0.19	1.31% R	0.050
EC (mS cm^{-1})	2.45	0.76	2.33	0.57	4.63% R	0.230
DO (mg L^{-1})	6.32	1.38	4.17	1.24	34.0% R	< 0.001

* P-values obtained with Wilcoxon signed-rank test.

Dissolved oxygen (DO) concentrations varied from 4.5 to 9.3 mg L^{-1} in the influent and 2.2 to 6.9 mg L^{-1} in the effluent of the bioreactor during the course of the study. As the DO enters the bioreactor, it can be removed through microbial metabolism; however, the efficiency of denitrification might be decreased as a result (Greenan et al., 2009). The DO concentrations were significantly reduced as the tile water passed through the bioreactor ($p < 0.001$) (Table 6.2), but the water was not anaerobic. The presence of high DO in the denitrifying bioreactor can inhibit nitrate removal by limiting denitrifying microbial activity (Gómez et al., 2002), as well as cause the accumulation of nitrite and nitrous oxide as unwanted intermediates in the bioreactor (Elgood et al., 2010; Gómez et al., 2002). While there is no definitive threshold of DO which inhibits denitrifying bioreactor

performance, Gómez et al. (2002) and Healy et al. (2006) reported no inhibition in denitrification below DO concentrations of 4.5 mg L^{-1} and 3.7 mg L^{-1}, respectively.

6.3.4 Microbial Characteristics

6.3.4.1 E. coli Concentrations

The geometric mean for *E. coli* concentration in the influent was 365 CFU 100 mL^{-1}, which is about three times higher than the current water quality criteria for primary contact recreation, which is 126 CFU 100 mL^{-1} (USEPA, 2012). Elevated *E. coli* concentrations in the influent were largely observed around times of elevated flows, when flows exceeded bioreactor capacity (Figure 6.4). These elevated concentrations in the influent may have been due to increased infiltration into the tile drainage or runoff from a nearby feedlot directly entering the inlet control structure during precipitation events. Heavy rainfall can increase the volume of contamination leaching from the agricultural field, which might impact the load and concentration of contamination in tile drainage water (like nitrate) and thereby the amount of pollutants in the inflow of the denitrification bioreactor (Pluer et al., 2019). During sampling events, animal grazing and piles of manure were often observed less than 200 m from the bioreactor site, which likely also contributed to increasing *E. coli* concentrations in the inlet control structure from runoff during rainfall events.

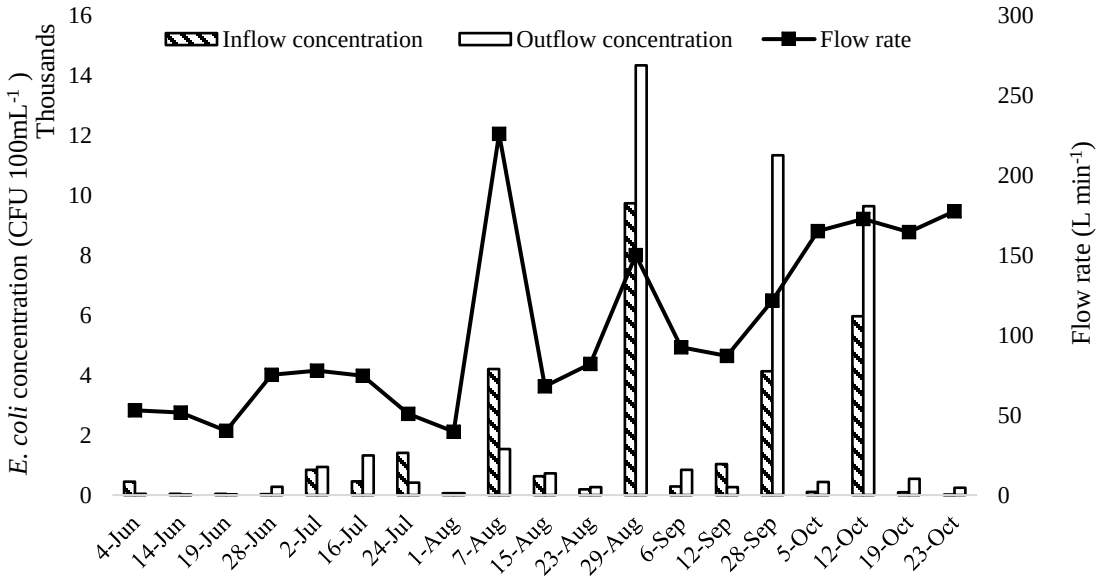

Figure 6.4 Influent and effluent *E. coli* concentrations demonstrated both increasing and decreasing concentrations as the tile drainage water passed through the bioreactor system. Elevated concentrations were largely associated with elevated flows.

E. coli concentrations varied greatly over the monitoring period, from 1.4×10^1 to 9.7-$\times 10^3$ CFU 100 mL^{-1} in the influent and 1.4×10^1 to 1.4×10^4 CFU 100 mL^{-1} in the effluent. *E. coli* concentrations increased from the influent to the effluent in 13 out of 19 samples collected (Figure 6.5). *E. coli* increases ranged from 2% to 1700%, while reductions in *E. coli* occurred less frequently and ranged from 54% to 89%. This is not consistent with previous laboratory studies which have demonstrated the capability of denitrifying woodchip bioreactors to consistently reduce *E. coli* concentrations. Previous literature has reported reductions in *E. coli* ranging from 49% to 97% from woodchip bioreactors treating synthetic tile drainage waters (Mardani et al., Under revision; Soupir et al., 2018; Zoski et al., 2013); however, these were at laboratory scale where the variables (e.g., temperature and flow) were controlled. To our knowledge, no in-situ study on changes in *E. coli* concentrations in drainage water have been published. Consistent reductions in *E. coli*

numbers (ranging from 1.2 – 2.2log_{10}) have also been reported from denitrification bioreactors treating wastewaters in pilot and full-scale studies (Rambags et al., 2016, 2019; Tanner et al., 2012).

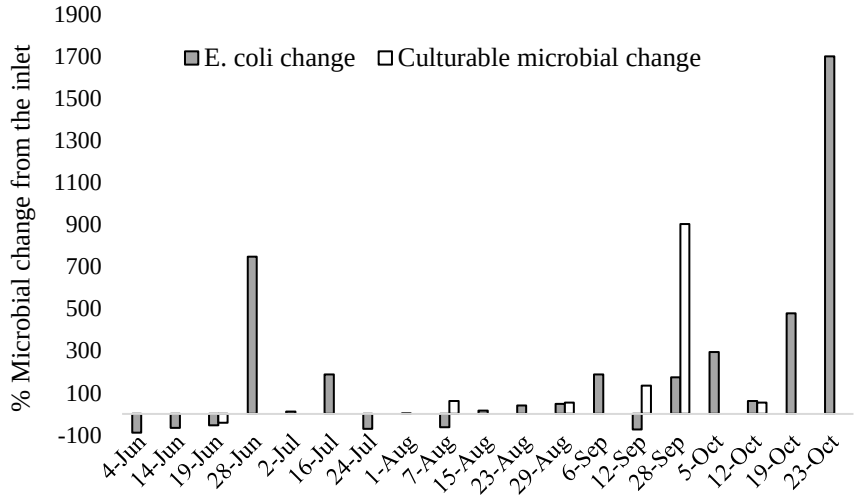

Figure 6.5 Percent microbial change (*E. coli* and culturable microbes) from the influent to the effluent over the course of study.

The mechanisms behind the increase in *E. coli* concentrations were beyond the scope of this work. However, it is possible that the bioreactors provided a favorable environment for *E. coli* growth, including high carbon and nutrient concentrations. These conditions have led to the growth of *E. coli* in the soil and aquatic environments (Ishii et al., 2006; 2009; NandaKafle et al., 2018; Suzuki et al., 2019). In addition, *E. coli* cells can form biofilms (Chekabab et al., 2013; Dewanti and Wong, 1995; Somers et al., 1994) within the bioreactor, which can wash-off at high flow rates or slough-off as they mature and thicken due to attachment weakness, even at moderate flows (Chun et al., 2009). These processes of washing- or sloughing-off can also increase the quantity of *E. coli* in the effluent.

6.3.4.2 Culturable Microbial Concentrations

Monitoring the culturable microbe populations in the influent and effluent was limited in extent; however, most (5 of 6 samples) demonstrated increases in the culturable microbial concentrations from the influent to the effluent (Figure 6.5). Culturable microbial concentrations fluctuated from 2.0×10^6 to 1.4×10^7 CFU 100 mL^{-1} in the influent and from 1.9×10^6 to 3.0×10^7 CFU 100 mL^{-1} in the effluent (Figure 6.6). Greater microbial populations in the effluent were anticipated given the high carbon and nutrient concentrations within the bioreactor, along with internal bioreactor temperatures ranging from 10 – 17 °C, which provide favorable conditions for psychrotophs and mesophiles to grow (Madigan et al., 2010).

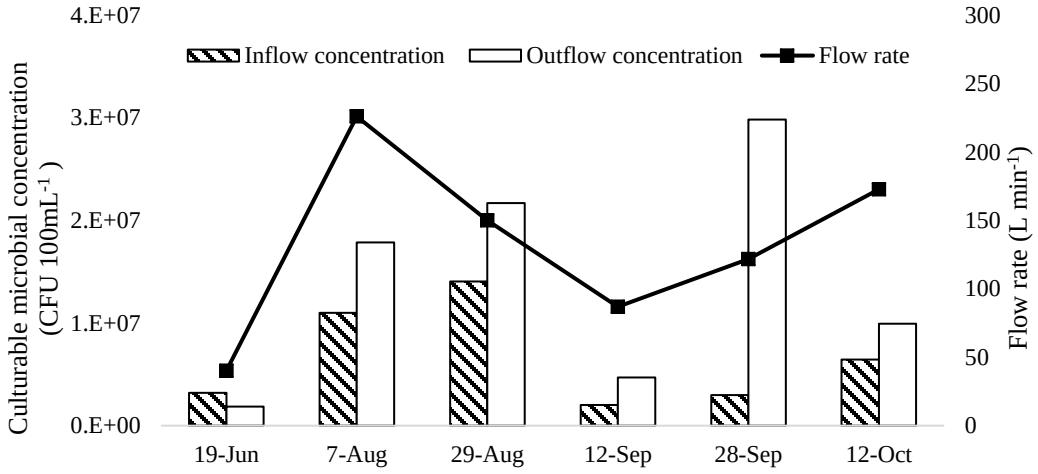

Figure 6.6 Influent and effluent culturable microbial concentrations demonstrated increases in the culturable microbial population as the drainage water passed through the bioreactor.

The culturable microbial concentration increased by 116% on average, which is less than a previous laboratory study which demonstrated increases ranging from 250% to 573% depending on flow conditions and influent microbial communities (Mardani et al.,

Under revision). Zoski et al. (2013) also found an increase in total coliforms in the effluents of woodchip reactors treated with tile drainage water under different flow rates. This suggests the capability of bioreactors to alter the concentration of microbes in tile drainage water passing through these removal systems.

6.3.4.3 Antimicrobial Resistance

A total of 126 isolates from the broader culturable microbial community within the influent and effluent of the bioreactor were recovered and tested for their resistance to five antimicrobials, including tetracycline, ampicillin, penicillin, sulfisoxazole, and erythromycin. Out of the 51 isolates from the influent, 44 (86%) were phenotypically resistant to at least one of the antimicrobials tested, and 34 (66%) of the isolates demonstrated phenotypic resistance to multiple antibiotics. Out of the 75 isolates from the effluent samples, 65 (87%) of the isolates were resistant to at least one of the antibiotics tested, and 51 (68%) of the isolates were resistant to multiple antibiotics.

A higher percent of microbial isolates tested were phenotypically resistant to erythromycin, penicillin, and ampicillin than were phenotypically resistant to tetracycline and sulfisoxazole (Figure 6.7.I). Similar patterns of phenotypic AMR of culturable microbes were found within the influent and effluent of the bioreactor, possibly due to genetic similarity of microbes entering and exiting the bioreactor, and the exposure of the microbes to the same antibiotic agents.

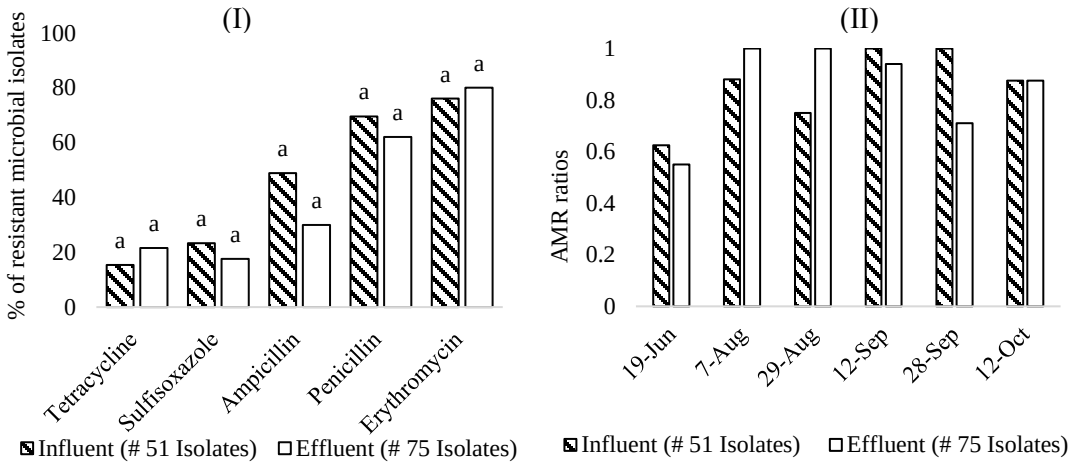

Figure 6.7 Average percentage of resistant culturable microbial isolates in the influent and effluent (I) (where lower-case letters indicate significant differences between the influent and effluent for each antibiotic) and ratio of AMRs for influent and effluent of bioreactor over the sampling period (II).

The ratio of antimicrobial resistance (AMR) was calculated for the influent and effluent during each sampling event by using the total number of isolates resistant to at least one antimicrobial compared to the total isolates examined (Figure 6.7.II). There was no significant change in the AMR ratio from the influent to the effluent (Table 6.2).

The AMR microbial concentrations were estimated by multiplying the AMR ratio by the culturable microbe concentration. Given that the AMR ratio and culturable microbial concentration did not demonstrate significant differences between the influent and effluent (Table 6.2), it is unsurprising that, although there was increase in phenotypic AMR microbial concentrations, the increase was not significant ($p = 0.062$). The limited number of isolates tested may have limited the ability to detect any significant difference. Further studies are required to examine genetic resistance of isolates to a variety of antibiotics, and to clarify the potential effect of denitrification bioreactors on AMR being released into the

environment via tile drainage water.

6.4 Conclusions

While extensive research has shown the efficiency of denitrification bioreactors on removing nitrate from tile drainage water, little attention has been given to the impacts of bioreactors on microbial characteristics in tile drainage water, especially at the field-scale. In this study, the potential impacts of denitrifying bioreactors on the microbial characteristics of tile drainage water were examined, and preliminary insights into the possible associated risk of antimicrobial resistance (AMR) dissemination to the environment were provided by monitoring a field-scale bioreactor installed in SD. The results showed an increase in *E. coli* concentrations, the general microbial concentrations, and phenotypically antibiotic resistant microbes, though the increases were not significant ($0.15 < p < 0.06$), potentially due to the small number of samples, 19 samples for *E. coli*, 6 samples for culturable microbial concentrations, and 51 and 75 isolates for phenotypic AMR for influent and effluent microbes, respectively.

E. coli increases ranged from 2% to 1700% while reductions in *E. coli* occurred less frequently and ranged from 54% to 89%. In addition, the culturable microbial concentration increased by 116%. Moreover, the recovered culturable isolates from the influent and effluent samples had similar ratios of phenotypic AMR; however, when combined with the increased culturable microbial population in the effluent, there is a potential for increased AMR in tile drainage water when these waters pass through a denitrification bioreactor. More work is required to assess the changes in AMR and ARGs in water passing through bioreactors. The recovered isolates from the influent and effluent were more frequently phenotypically resistant to ampicillin, penicillin, and erythromycin

while little phenotypic resistance was found for tetracycline and sulfisoxazole. To increase our understanding of microbial fluxes and AMR in water flowing through denitrification bioreactors, more in-situ studies are required to quantify the change in microbes, the microbial communities, the impact on undesirable microbes such as *E. coli*, and changes in AMR, including antibiotic resistance genes under different environmental conditions.

6.5 Acknowledgements

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CHAPTER 7: CONCLUSIONS AND FUTURE WORK

Three sets of experiments were conducted to quantify the potential effect of edge-of-field nutrient management practices on microbial concentrations (e.g., *E. coli* and general microbial community) and antimicrobial resistance (AMR) in tile drainage water. Two laboratory studies were conducted to evaluate the impacts of different influent microbial communities, influent microbial concentrations, and filter materials on effluent microbial populations. In addition, one in-situ woodchip bioreactor was monitored to evaluate changes in the microbial community in tile drainage water passing through woodchip bioreactors.

The results of this research demonstrated the capability of woodchip bioreactors (WBs) to alter the concentration of microbes in tile drainage passing through these removal systems. Both increases and decreases in *E. coli* concentrations in tile drainage water were observed. The results revealed that WBs were capable of significantly removing *E. coli* (49% - 77%) and increasing culturable microbial concentrations (250% - 573%) from synthetic tile drainage water. However, the results of monitoring an in-situ WB had varied results with the majority of samples collected (13 out of 19) resulting in an increase in *E. coli* (2% - 1700%), and five out of six samples processed for general microbial concentrations resulted in an increased general microbial concentrations (53% - 902%), though the increases were not significant. Different influent microbial communities also had significant effects on culturable microbial concentrations, as laboratory WBs receiving more microbes with a greater diversity had a greater increase in culturable microbial concentrations. Additionally, the recovered isolates from the influents and effluents had similar ratios of AMR for all WBs examined in the laboratory and field. The estimated

AMR concentrations did significantly increase in the synthetic tile drainage water from the inlets to the outlets of all laboratory WBs due to the significant change in culturable microbial population. However, the estimated AMR concentrations did not significantly increase in the tile drainage water from the inlet to the outlet of the in-situ WB due to the lack of significant change in AMR ratios as well as culturable microbial population. The recovered isolates from general microbial communities in the influents and effluents of laboratory WBs were more frequently phenotypically resistant to ampicillin, penicillin, erythromycin, and sulfisoxazole while little phenotypic resistance was found to tetracycline. The same trend was detected for the recovered isolates from the general microbial population in the in-situ WB influent and effluent samples, with the exception of sulfisoxazole which had little resistance. In addition, *E. coli* isolates recovered from the influents and effluents of laboratory WBs demonstrated a high phenotypic antibiotic susceptibility to tetracycline and sulfisoxazole, but were found to be nearly 100% resistant to ampicillin, penicillin, and erythromycin.

The impact of different nutrient removal materials on reducing *E.coli* concentrations for two different influent *E. coli* concentrations (high and low) was also evaluated. The filter materials assessed included woodchips, steel turnings, woodchips followed by steel turnings, and woodchips combined with biochar. This set of experiments showed the capability of all nutrient removal materials to significantly remove *E. coli* (43% - 97%) from water passing through the systems. Higher concentrations of *E. coli* in the influent decreased the efficiency of these systems to remove the *E. coli*. Steel turnings showed the lowest *E. coli* removal rates under both high and low influent *E. coli* concentrations. Moreover, dual-nutrient removal systems, including woodchips followed by steel turnings

and woodchips mixed with biochar, resulted in higher removal efficiency with high concentrations of influent *E. coli*, suggesting these configurations might be promising alternatives to woodchip-only systems to foster *E. coli* reductions in waters with high *E. coli* concentrations.

More research is needed to improve the prediction of pathogen removal and AMR changes in tile drainage water, as well as determine the mechanisms of removal to support the development of effective design for microbial contaminant removal using WBs and nutrient filter materials.