WOODCHIP-AMENDED RAPID INFILTRATION BASINS FOR ENHANCED REMOVAL OF NITRATE FROM SECONDARY EFFLUENT

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TABLE OF CONTENTS

LIST OF TABLES ............................................................................................................ vi
LIST OF FIGURES .......................................................................................................... vii
ABSTRACT ........................................................................................................................ ix

Chapter

1 INTRODUCTION AND OBJECTIVES ................................................................. 1

2 LITERATURE REVIEW .......................................................................................... 5

2.1 Why Remove Nitrate? ......................................................................................... 5

2.1.1 Public Health Considerations ....................................................................... 5

2.1.2 Ecological Considerations ............................................................................ 7

2.2 Denitrifying Microbes ......................................................................................... 8

2.2.1 Summary of Denitrification .......................................................................... 8

2.2.2 Genetic Basis ................................................................................................... 8

2.2.3 Identification Methods .................................................................................. 10

2.2.4 Natural and Artificial Enrichment ................................................................. 11

2.3 Rapid Infiltration Basins for Nitrogen Removal .............................................. 12

2.3.1 Summary of Treatment ................................................................................. 12

2.3.2 Focus on Nitrification .................................................................................... 13

2.3.3 Soils Selection ................................................................................................ 14

2.3.4 pH and Temperature Effects ......................................................................... 15

2.3.5 Importance of Drying Cycle ......................................................................... 15

2.3.6 Organic Carbon Limitations .......................................................................... 16

2.4 Carbon Consumption ........................................................................................ 17

2.4.1 Viable Carbon Sources ................................................................................. 17

2.4.2 Carbon Lifespan Estimates .......................................................................... 18

2.5 Project Justification ............................................................................................ 19
LIST OF TABLES

Table 3.1. PCR Primers for Amplification of DNA Extracts................................. 34
Table 4.1. Characteristics of RIB Feed Water (Phase-1)................................. 38
Table 4.2. Percent Removal Measurements for 3-Foot Depth (Phase-1)............ 49
Table 4.3. Percent Removal Measurements for 6-Foot Depth (Phase-1).......... 49
Table 4.4. Characteristics of RIB Feed Water (Phase-2)................................. 51
Table 4.5. Percent Removal Measurements for 3-Foot Depth (Phase-2).......... 59
Table 4.6. Percent Removal Measurements for 6-Foot Depth (Phase-2).......... 59
Table 4.7. Characteristics of RIB Feed Water (Phase-3)................................. 61
Table 4.8. Percent Removal Measurements for 6-Foot Depth (Phase-3).......... 70
Table 4.9. Groundwater Monitoring Parameters............................................. 85
LIST OF FIGURES

Figure 2.1. Denitrification Reaction Diagram ........................................... 9
Figure 3.1. Schematic of the RIB Site in Middletown, DE ............................. 22
Figure 3.2. Configuration of Laboratory Columns ........................................ 28
Figure 4.1. Nitrate Concentration in 3-Foot Sampling Depth (Phase-1) ............. 41
Figure 4.2. Nitrate Concentration in 6-Foot Sampling Depth (Phase-1) .......... 42
Figure 4.3. Nitrate Concentration in 3-Foot Sampling Depth (Phase-1, abbrev.) .. 45
Figure 4.4. Nitrate Concentration in 6-Foot Sampling Depth (Phase-1, abbrev.) .. 46
Figure 4.5. Nitrate Concentration in 3-Foot Sampling Depth (Phase-2) .......... 54
Figure 4.6. Nitrate Concentration in 6-Foot Sampling Depth (Phase-2) .......... 55
Figure 4.7. Unoxidized Ammonia in Lysimeter Samples (Phase-3) ............... 63
Figure 4.8. Nitrate Concentration in 3-Foot Sampling Depth (Phase-3) .......... 66
Figure 4.9. Nitrate Concentration in 6-Foot Sampling Depth (Phase-3) .......... 67
Figure 4.10. Acclimation Period for Column Experiments ............................ 72
Figure 4.11. Variable Loading Period for Column Experiments ...................... 74
Figure 4.12. Feed Denitrification During Column Experiments ........................ 76
Figure 4.13. Restart Period and Final Stages of Column Experiments ............... 78
Figure 4.14. COD Concentration in Column Effluent During Final Stages ........ 81
Figure 4.15. RIB-1 Gel Image (PCR Amplification of narG) ......................... 89
Figure 4.16. RIB-2 Gel Image (PCR Amplification of narG) ......................... 89
Figure A.1. Percent Removal and Linear Fits for 3-Foot Depth (Phase-1).................. 103
Figure A.2. Percent Removal and Linear Fits for 6-Foot Depth (Phase-1)............... 104
Figure A.3. Percent Removal for 3-Foot Depth (Phase-2).................................. 105
Figure A.4. Percent Removal for 6-Foot Depth (Phase-2)................................. 106
Figure A.5. Percent Removal and Linear Fits for 6-Foot Depth (Phase-3)............. 107
ABSTRACT

In order to provide additional discharge capacity to the rapidly expanding Town of Middletown, DE, Rapid Infiltration Basins (RIBs) are being considered for the disposal and further nitrogen treatment of secondary lagoon effluent. A pilot-scale RIB system consisting of four test basins (25 by 60 feet) was constructed in the buffer areas of an existing spray irrigation field. The test basins contained 0, 10, 20, or 30% by volume of finely graded woodchips in the top 1-foot of soil, which acted as an external carbon source. Three woodchip-amended laboratory columns were also operated to simulate the RIBs and act as a model for predicting carbon depletion in the field. Between May 2014 and March 2015, lagoon effluent was loaded into the basins in volumes of 7,100-41,000 gallons per day for 1-9 days, with resting periods (no loading) of 6-13 days. Suction lysimeters were installed at 3 feet and 6 feet below the surface for the collection of samples following infiltration. After some initial flushing effects, the system was shown to remove significant amounts of nitrate, with the highest removal (≥50% under some conditions) observed in the basin amended with 30% woodchips. Improved performance was observed for increased sampling depth and increased woodchip volume. The short (1-2 day) loading plan was shown to be superior to the extended (9 day) loading. During the winter months the 6-foot samples continued to show substantial treatment, while some operational concerns were raised from excessive ice buildup. The laboratory column study predicted a woodchip lifespan of 2-3 years. Recommendations for the full-scale operation of the RIB system for enhanced denitrification were suggested based on the results of this study.
Chapter 1
INTRODUCTION AND OBJECTIVES

The use of Rapid Infiltration Basin (RIB) systems for the treatment of wastewater has been heavily investigated as a relatively low-technology option for removing nitrogen compounds from plant effluent. This method offers major advantages over alternative technologies by operating at a low cost and requiring a small space with simple installation. The nitrate removal process of rapid infiltration relies on biological reduction of nitrate to nitrogen gas, a process called denitrification. It is widely understood that this nitrogen transformation is carried out by microbes under anoxic conditions. However in natural soils denitrification is a slow process, inadequate to treat the large fluxes of nitrate that may be introduced during effluent discharge. The addition of a carbon source has proven to be stimulating for microbial denitrification, and many studies have demonstrated the feasibility of using carbon amendments to promote denitrification in the soil. These carbon amendments range from easily degraded compounds such as acetate and methanol, to more recalcitrant materials including reeds, leaves, and woodchips. This second category may be advantageous due to its low cost and extended lifespan in the soil during degradation.

The infiltration process for nitrate removal offers an alternative to proven conventional methods such as reverse osmosis and ion exchange technologies. RIB denitrification systems operate at a low cost while maintaining high selectivity for nitrate, and they do not require a system for sludge disposal. Locally sourced agricultural and industry byproducts can be incorporated into soils to function as
electron donors. These inexpensive amendments support the growth of facultative denitrifiers, which use nitrate as the terminal electron acceptor. For comparison, a drinking water nitrate removal facility operating in Des Moines, Iowa was reported in 2009 to be maintained at an average daily cost of $3,000 (Greenan et al., 2009). 

RIB systems are a sensible option for Land-Based Wastewater Disposal (LBWD) because they avoid discharging directly into surface waters. It is widely assumed that wastewater quality is improved by physical processes and biological degradation during infiltration and transport within the subsurface. Rapid infiltration also recharges groundwater reserves, and requires a much smaller land footprint than other LBWD options such as spray irrigation. However, when operated at high loading rates, there is concern that insufficient contact times between soils (with associated microbes) and influent will be achieved for adequate treatment. Rapid flow velocities may also lead to nutrient leaching and water table mounding. These questions may be particularly justified in sandy soils with high hydraulic conductivity.

The protection of groundwater quality is of major importance locally, nationally, and worldwide. In southern Delaware, most drinking water is provided by wells, and groundwater delivers the majority of stream flow (Andres and Sims, 2013). Agricultural fertilizers have introduced large quantities of nitrate into the soil. This nitrate may discharge into surface waters and lead to algal blooms and hypoxia in downstream waterways. Denitrification systems have been tested for the in-situ treatment of groundwater discharged into drinking reservoirs, and technologies similar to RIBs have been used for the treatment of residential effluent and stormwater runoff.

Existing research has produced mixed results as to the effectiveness of using the RIB system for final treatment. Concern exists that RIBs may in fact reduce the
groundwater quality below the basins. The effectiveness of the RIB system relies on the system’s ability to maintain conditions suitable for biological denitrification, while providing ample contact time between microbes, a carbon source, and nitrogen species. Complicating the issue, several forms of nitrogen are usually present in RIB influent, including ammonia, nitrate and nitrite, and organic nitrogen. Ammonia is rapidly converted to nitrate under oxic conditions by a process called nitrification. Organic nitrogen (such as excreted waste or biomass) may be converted to ammonia during decomposition, through a transformation called ammonification. The effectiveness of RIB systems relies on balancing multiple design considerations, including the natural soil characteristics, carbon amendments, influent quality, and loading schedule. Seasonal effects are also poorly understood, but it is likely that the performance is affected by temperature changes because it is biologically driven. While many studies have examined each of these factors with the use of laboratory scale columns, very few large scale RIB denitrification studies have been conducted.

The Town of Middletown is a growing community in Delaware. It currently uses a lagoon system to treat municipal wastewater, and it discharges lagoon effluent using large spray irrigation fields. Rapid infiltration is one alternative disposal method being considered. A field-scale RIB system consisting of four basins was constructed at the spray irrigation field in Middletown and amended with varying amounts of finely graded woodchips as an external carbon source. The basins were operated under a variety of loading and resting plans, using lagoon effluent as the feed solution. Laboratory columns were operated at the University of Delaware which simulated the field system under high loading rates. These two elements aimed to supplement several areas of existing RIB research, examined later in the Literature Review.
The objectives of this study are to:

1. Compare nitrate removal by RIB systems based on the volume of woodchips incorporated in the surface soil.

2. Compare nitrate removal by RIB systems operating under alternative schedules (flooding vs drying time) and loading volumes.

3. Characterize nitrate removal within the RIBs and operational feasibility across seasonal variation.

4. Estimate the lifespan of woodchips in the subsurface in order to effectively schedule woodchip replacement in field RIBs.

5. Compare the performance of the pilot-scale RIB system to the behavior predicted by the bench-scale experiments.
Chapter 2

LITERATURE REVIEW

2.1 Why Remove Nitrate?

Nitrate can be introduced as a drinking water contaminant from a variety of sources, including fertilizer runoff, septic or sewage leaks, and natural erosion (USEPA, 2009). Land based wastewater disposal systems such as spray irrigation or rapid infiltration basins represent additional risks for nitrate contamination if operated improperly. Consumption of nitrate laden groundwater may cause some health risks, and high nitrate inputs can have detrimental effects on environmental quality.

2.1.1 Public Health Considerations

The current primary drinking water regulation is listed as 10 mg/L NO$_3$-N for the protection of public health (USEPA, 2009). The most widely advertised risk for nitrate exposure is methaemoglobinemia, more commonly called blue-baby syndrome, which has been regularly cited as a health concern for infants during the past 70 years (APHA, 1950; Comly, 1945; Powlson et al., 2008; Ward et al., 2005).

Some review of the data has suggested that a nitrate regulation of 10 mg/L may be overly conservative. Previous research focusing on well water may have overlooked the co-occurrence of bacterial contaminants in wells which caused the onset of blue-baby syndrome (Powlson et al., 2008). The original research asserts that nitrate was responsible for contraction of the disease, while acknowledging that the drinking wells were located in close proximity to outhouses or barnyards and “the
water was not tested bacteriologically” (Comly, 1945). Infant illness was attributed to nitrate, while bacterial infection may have played an equally large role due to contamination with animal or human waste (Powlson et al., 2008).

Infants are more susceptible to this illness because their stomachs are less acidic than adults, meaning nitrate reducing bacteria can survive in the gut in large numbers (APHA, 1950; Camargo and Alonso, 2006). The reduced nitrogen species produced by these bacteria can convert hemoglobin to methaemoglobin, a form which doesn’t effectively carry oxygen to the body (Alonso and Camargo, 2003; Camargo and Alonso, 2006; Meade and Watts, 1995; Powlson et al., 2008). In many instances, infants that consumed water with high levels of nitrate were unaffected by blue-baby syndrome, indicating that nitrate alone may not cause the illness (APHA, 1950; Powlson et al., 2008).

Even if the drinking water regulation of 10 mg/L NO$_3$-N is overly conservative for blue-baby syndrome, some populations may be more susceptible than others, and other chronic health effects may also be severe (Ward et al., 2005). Nitrate could play a role in several serious diseases, including bladder, ovarian, and digestive cancers, as well as diabetes and reproductive defects (Camargo and Alonso, 2006; Ward et al., 2005). Still, reviews of the data have pointed to a lack of convincing evidence (Powlson et al., 2008) and draw attention to some of the health benefits of consuming nitrate in the diet. Nitrate can be reduced to nitric oxide in the digestive system, providing pathogen protection in the mouth and stomach (Powlson et al., 2008; Ward et al., 2005). While the human safety risks of nitrate consumption are debatable, other factors related to environmental health must be considered.
2.1.2 Ecological Considerations

Elevated nitrate levels can have severe effects on the health of ecosystems when discharged into surface waters. High nitrogen loading to an open water body can cause competitive phototrophic organisms to grow rapidly while consuming this precious resource (James et al., 2005). This results in the eutrophication of the aquatic system, a condition characterized by algal blooms which can shade out vegetation and starve organisms of oxygen as they decompose (Camargo and Alonso, 2006; James et al., 2005; Scavia et al., 2003). Two common examples of hypoxia, a detrimental oxygen-starved condition, are found in the northern Gulf of Mexico (Scavia et al., 2003) and Chesapeake Bay (Bricker et al., 2014).

Excessive nutrients are often provided to large water bodies by surface runoff into their upstream tributaries, but nitrates can also discharge from groundwater aquifers into rivers or directly into a marine or estuarine system (Ouyang, 2012; Rao and Charette, 2012). Some nitrate that passes through an aquifer may be denitrified at the seepage face if conditions are favorable (Rao and Charette, 2012), but additional nutrients will export into the water body. These export effects may be the most severe during periods when the groundwater table is high, due to increased flow into surface waters as a result of the subsurface hydraulic gradients that form (Ouyang, 2012). The identity of nitrogen as a limiting nutrient in marine, estuarine, and freshwater ecosystems raises the importance of denitrification research.
2.2 Denitrifying Microbes

Facultative anaerobes are known to carry out the process of denitrification, in which nitrate is converted to nitrogen gas. A diverse collection of microbes perform nitrogen transformations, relying on specific genes to carry out each step. These genes can be utilized in a variety of molecular biology techniques to identify denitrifying metabolisms in the environment. The microbes responsible for these processes can become enriched in engineered systems as they remove specific target compounds.

2.2.1 Summary of Denitrification

The process of denitrification relies on a collection of enzymes which reduce nitrate in a series of sequential steps. In its most basic configuration, nitrate is metabolized as a terminal electron acceptor and transformed into less oxidized forms of nitrogen. Nitrate is first reduced to nitrite, followed by stepwise reduction to gaseous nitric oxide, nitrous oxide, and nitrogen gas (Henry et al., 2006; Levy-Booth et al., 2014; Seenivasagan et al., 2014; Zumft, 1997). Each step is catalyzed by different enzymes carried by a variety of microbes in soil. The individual enzymes responsible for these processes are widely described, and the genes which encode these enzymes are often used to identify the presence of metabolisms in the environment. The most commonly used markers are described below.

2.2.2 Genetic Basis

Nitrate reduction is facilitated by the genes narG or napA. The narG gene, which encodes membrane bound nitrate reductase, is thought to be the more important than the periplasmic napA for facultative anaerobes under hypoxic conditions (Ji et al., 2012a; Lopez-Gutierrez et al., 2004; Mellor et al., 1992). Nitrite reduction genes nirK and nirS encode enzymes producing the first gaseous product in the denitrification
cycle, nitric oxide. This gas is subsequently converted to nitrous oxide by enzymes coded by the *norB* gene. The final conversion from nitrous oxide to nitrogen gas is carried out by *nosZ* coded enzymes, and the product is released.

These numerous enzymes may be membrane bound or soluble in the bacterial cell, depending on their specific function (Adav *et al*., 2010; Ji *et al*., 2012b; Levy-Booth *et al*., 2014; Mellor *et al*., 1992; Zumft, 1997). Although most of these reactions are catalyzed by bacteria, some archaea are also capable of catalyzing denitrification (Zumft, 1997) or nitrification reactions (Levy-Booth *et al*., 2014). A simple reaction diagram showing the enzymes responsible for denitrification is presented in Figure 2.1.

![Figure 2.1. Denitrification Reaction Diagram](image.png)
2.2.3 Identification Methods

As an assay to microbial activity, these genes have often been identified and quantified in soil and water systems using molecular biology approaches such as quantitative PCR and in-situ hybridization (Henry et al., 2006; Ji et al., 2012a; Ji et al., 2012b; Levy-Booth et al., 2014). Molecular techniques such as these work by carefully identifying nucleotide sequences expressed by the functional gene of interest. These sequences match up with specially designed primers which allow for the identification of genes in field or laboratory samples. By confirming the presence of the gene in a soil or water sample, it becomes apparent that a specific metabolism is being expressed. Quantitative techniques make it possible to explore the amount of expression in the soil, for instance how many copies of the gene are active.

Just as dominant metabolisms can be found in the soil and groundwater, individual microbes can be identified. This can be accomplished by similar molecular techniques as those above, or with culture dependent methods such as using selective media (Seenivasagan et al., 2014). Several studies have identified microbes involved in various denitrification reactions, but the collection is highly diverse (Zumft, 1997). Included regularly are microbes belonging to the genus *Bacillus*, genus *Corynebacterium*, genus *Pseudomonas*, and family Enterobacteriaceae (Blowes et al., 1994; Huang et al., 2014; Seenivasagan et al., 2014; Zumft, 1997). Environmental conditions may influence which communities are dominant in a particular system. In a field-scale RIB test for nitrate removal, we would expect microbial communities to become heavily enriched with both nitrifying and denitrifying species.
2.2.4 Natural and Artificial Enrichment

Identification of dominant metabolisms in a sample can often indicate if conditions are favorable for particular bioprocesses. Within natural and engineered systems, microbial footprints are influenced by environmental conditions and substrate availability. In reactor experiments, communities can become enriched with microbes adept at processing specific substrates (Ginige *et al.*, 2009). Microbial communities adapt to consume the resources available in their environments, and this is true across large scales in areas historically contaminated with nitrate (Seenivasagan *et al.*, 2014) or more toxic compounds such as petroleum constituents (Ortega-Gonzalez *et al.*, 2013; Owsianiak *et al.*, 2009). Metabolism identification has also been used in engineered subsurface infiltration systems (Ji *et al.*, 2012b) and constructed wetlands (Ji *et al.*, 2012a), with experimental attempts to pair certain metabolisms with specific soil depths in treatment units.

In many cases, experimental media are enriched with microbes taken from the natural world or active treatment systems. Inoculated treatment systems experience shorter startup times and may achieve better removal efficiencies due to the presence of adapted microbes. Denitrifiers are widespread in anaerobic systems, and the introduction of an inoculum provides a concentrated dose for the initial colonization. Many of the seed media are derived from natural environments, including soils and aquatic sediments (Blowes *et al.*, 1994; Greenan *et al.*, 2009; Qian *et al.*, 2011). Alternatively, recycled or dewatered activated sludge can provide a diverse collection of microbes from engineered systems that are already operational (Ginige *et al.*, 2009; Li *et al.*, 2013).

In experiments where no concentrated inoculum is indicated, it is likely that the system relies on the presence of denitrifiers in the treatment influent, or those
naturally present in on-site soils. Denitrifiers in the influent stream should become attached and grow on surfaces in the treatment units. Communities will grow rapidly with the new influx of nutrients and favorable metabolic substrates.

2.3 Rapid Infiltration Basins for Nitrogen Removal

Rapid infiltration has been investigated as a low cost, simple treatment option for over 100 years. As of 2003, greater than 350 rapid infiltration systems were used in the United States, primarily as a polishing step for secondary effluent and for the replenishment of groundwater (USEPA, 2003). The majority of past research has focused on the nitrification process, but the system can be adjusted to support denitrification. Several factors play a role in the successful implementation of a rapid infiltration system, including the soil physical and chemical characteristics.

2.3.1 Summary of Treatment

Infiltration basins take advantage of several natural processes, including filtration, ion exchange, adsorption, precipitation, and microbial activity, which improve wastewater quality as it percolates through the soil matrix (Akhavan et al., 2013; Andres and Sims, 2013; USEPA, 2003). Each of these processes contributes to the overall improvement in the effluent quality beneath the basin. Besides having a strong disinfection capability for unwanted fecal coliforms (Bali et al., 2010; USEPA, 2003), rapid infiltration basins have the potential to remove 95-99% of BOD, 95-99% of total suspended solids and 25-90% of total nitrogen by the coupled nitrification-denitrification pathway (USEPA, 2003).
2.3.2 Focus on Nitrification

Infiltration basins and a variety of related technologies have been studied widely for the transformation of ammonia to nitrate. The transformation of ammonia to nitrate creates a less toxic form of nitrogen, but does not remove it from the environment. The biological lethal concentration of ammonia, nitrate, and nitrite differ based on the organism of toxic study (Alonso and Camargo, 2003; Meade and Watts, 1995), but certain taxonomic groups (such as crustaceans) may share similar toxic effects (Meade and Watts, 1995). Since tolerance differs based on species, this helps explain how some organisms may be able to thrive in polluted waterways. An example can be found with the invasive aquatic snail *Potamopyrgus antipodarum*, which has a much higher tolerance for nitrogen compounds than most aquatic invertebrates (Alonso and Camargo, 2003).

While the oxidation of ammonia helps reduce its toxicity, nitrate in extreme concentrations is a toxicant in its own right (Alonso and Camargo, 2003; Meade and Watts, 1995; Romano and Zeng, 2007). Furthermore, either of these inorganic forms of nitrogen are still highly bioavailable for plant uptake in terrestrial (Hynes and Germida, 2012), wetland (Tanner *et al*., 2002), and aquatic systems (Kurten *et al*., 2010). Significant risk remains for algal blooms and hypoxia in the environment, since bioavailability is maintained when ammonia is traded for nitrate within a treatment system.

Several case studies have examined the removal of ammonia from wastewaters in which this is the primary nitrogen constituent. Ammonia is rapidly oxidized to nitrite and then nitrate in the aerobic vadose zone. Conversions of ammonia regularly exceed 90% (Li *et al*., 2013; Mottier *et al*., 2000; Bali *et al*., 2010). Although these studies focus on the removal of ammonia, a net reduction in total nitrogen was also
observed in the results, indicating that denitrification likely played a role within the
depth of the treatment units. Since denitrification relies on facultative anaerobes
(Blowes et al., 1994; Huang et al., 2014; Lopez-Gutierrez et al., 2004; Zumft, 1997),
specific design considerations must be met to produce the conditions required for this
microbial process.

2.3.3 Soils Selection

Conditions for denitrification are closely related to the physical and chemical
characteristics of the soils underlying the basin, thus site selection is critical. The soils
selected for installation of a rapid infiltration system are recommended to be coarse
sands or sandy gravels with a permeability of at least 0.6 in/hr and a depth of at least
10 to 15 feet (USEPA, 2003). For systems where carbon compounds may be oxidized
in the upper soil, higher pore velocities can preserve organic material for lower layers
where denitrification is favored (Akhavan et al., 2013). As the system ages, soil pores
can become clogged with algae from upstream treatment processes (USEPA, 2003) or
microbial cells and metabolites (Akhavan et al., 2013; Li et al., 2013). As a
consequence, occasional or annual clearing of the top layer of the soil may be required
to maintain infiltration capacity (USEPA, 2003). As a contrast, soils which have a
high infiltration rate may not allow sufficient contact time between influent and
denitrifying microbes capable of removing nitrate from the wastewater (Andres and
Sims, 2013). Similarly, soils which have channels or large pores may allow short-
circuiting past denitrification sites (Willems et al., 1997). Rapid soil velocities may
also contribute to groundwater mounding below the basin (Akhavan et al., 2013;
Mottier et al., 2000).
2.3.4 pH and Temperature Effects

Since denitrification is a microbially-mediated process, factors such as pH and temperature can play a role in the success of a denitrification system. Most denitrifiers are best suited to a neutral pH (Akhavan et al., 2013; Shahabi and Naeimpoor, 2014) ranging from about 6 to 8. Temperature variations in the short and long term can cause the metabolic rate of the microbes to change, as well as the operational infiltration rate. In field-scale and laboratory systems, effluent quality was improved when temperature was increased (Bali et al., 2010; Robertson et al., 2000; Willems et al., 1997), suggesting an increase in microbial activities during the summer months.

The draining rate of a basin is also highly impacted by temperature. The viscosity of water increases by about 2% per degree Celsius within the range of normal environmental temperatures, causing large differences between winter and summer infiltration characteristics (Lin et al., 2003). The infiltration rate of a basin can fluctuate by greater than 50% between months of operation in the summer and winter (Braga et al., 2007; Lin et al., 2003). The draining rate may also be affected by changes in the influent salinity which alter the density of the fluid, although these variations are generally negligible (Lin et al., 2003). If ponding occurs within the basin higher than approximately 10 cm, the infiltration rate will increase as a result of the hydraulic head (Braga et al., 2007).

2.3.5 Importance of Drying Cycle

The recommended application period of wastewater into a basin is between 4 hours and several days, but the duration of the drying cycle is equally important for basin operation (USEPA, 2003). The drying cycle allows for aerobic conditions to return to the upper horizons of the soil, facilitating oxidation of ammonia if it is
present in the influent (Bali et al., 2010; Mottier et al., 2000). Dissolved oxygen is also introduced to the system by the influent wastewater (Tanner et al., 2002). When the basin is drained, oxygen is replenished most rapidly near the surface. The resulting oxygen gradients cause nitrification to proceed mainly in the upper soil layers, with denitrifying microbes more prevalent at lower depths (Li et al., 2013). The drying also allows for organic material to dehydrate and decompose in the upper soil, providing bioavailable carbon to the subsurface (USEPA, 2003). The drying cycle can vary in length and typically lasts at least as long as the loading period, but often continues much longer (USEPA, 2003).

2.3.6 Organic Carbon Limitations

The presence of organic matter is required for denitrification, and bioavailable organic materials can be limiting in natural soils or influent wastewater if it is not added by some external sources (Zheng et al., 2013). The incorporation of long-lasting organic materials into the soil can provide electron donors for nitrate reducing microbes. Numerous small-scale studies have shown that a wide variety of carbon compounds (discussed later) are suitable as substrates for the growth of denitrifiers. With carbon additions, denitrification was observed under a variety of reactor configurations and loading rates, with upwards of 90% nitrate removal in several cases (Greenan et al., 2009; Palomo et al., 2013; Qian et al., 2011). The potential exists for a scale-up of these carbon amendments in infiltration systems.
2.4 Carbon Consumption

Since organic carbon can be naturally limited in the subsurface, the consumption of supplementary carbon is an important factor in the effectiveness of a rapid infiltration system. Many different labile and recalcitrant carbon compounds have been shown to support denitrification metabolisms. The lifespan of these carbon materials in the environment is important for sustaining the treatment ability over long periods of time.

2.4.1 Viable Carbon Sources

Even when populations of denitrifiers are established within nitrate treatment systems, reliable carbon sources must be provided. The process of denitrification in infiltration systems is primarily carried out by heterotrophic microbes, which consume carbon-based materials as electron donors. In many reactor and column studies, simple carbon compounds such as methanol, ethanol, acetate, and succinate were examined as the primary carbon source (Adav et al., 2010; Ginige et al., 2009; Palomo et al., 2013; Shahabi and Naeimpoor, 2014). While these rapidly degraded compounds may work in tank or batch systems, this application would not be ideal for RIB systems because of the costs associated with frequent replenishment. For low-maintenance infiltration systems, a long-lasting carbon source is a better alternative. In denitrification walls and small scale studies, long-lasting materials have shown a strong capacity to support denitrification over extended periods. Some examples include the use of sawdust (Robertson et al., 2000; Schipper and Vojvodic-Vukovic, 2001), leaf compost (Blowes et al., 1994; Robertson et al., 2000), reed and rice stalks (Qian et al., 2011), tree bark (Blowes et al., 1994), and woodchips or mulch (Blowes et al., 1994; Greenan et al., 2009; Palomo et al., 2013; Robertson et al., 2000).
2.4.2 Carbon Lifespan Estimates

Wood-based carbon sources can provide substrates for long time periods as they decompose. Some studies have shown that cellulose based materials may support denitrification for several years in groundwater permeable barriers (Robertson et al., 2000; Schipper and Vojvodic-Vukovic, 2001). Stoichiometric estimates and extrapolation from field-data have shown the potential for woody materials to last over a decade, although the nitrate loading rates have obvious effects on the carbon consumption (Robertson et al., 2000; Schipper and Vojvodic-Vukovic, 2001). These estimates are based on the redox coupling of nitrate and organic carbon (Akhavan et al., 2013; Blowes et al., 1994):

\[
\frac{4}{5}\text{NO}_3^- + \text{CH}_2\text{O} \rightarrow \frac{2}{5}\text{N}_2(\text{g}) + \text{HCO}_3^- + \frac{1}{5}\text{H}^+ + \frac{2}{5}\text{H}_2\text{O} \tag{1}
\]

This equation can also be written alternatively as (Robertson et al., 2000):

\[
\frac{4}{5}\text{NO}_3^- + \text{CH}_2\text{O} \rightarrow \frac{2}{5}\text{N}_2(\text{g}) + \text{CO}_2 + \frac{4}{5}\text{OH}^- + \frac{3}{5}\text{H}_2\text{O} \tag{2}
\]

The form of the equation depends on the dissolution of carbon dioxide in water, but the denitrification reaction results in a net release of hydroxyl ions, which raise the pH (Shahabi and Naeimpoor, 2014).

Other forms of the stoichiometric balance exist for specific carbon sources such as acetate and ethanol (Shahabi and Naeimpoor, 2014). This redox reaction proceeds rapidly in the absence of oxygen, because nitrate is an energy-efficient electron acceptor under anaerobic conditions. Other competing ions in the soil, such as manganese, iron, and sulfate, are less thermodynamically advantageous (Blowes et al., 1994). Thus, it is expected that nitrate should be readily consumed once oxygen is depleted. This preference for nitrate as the next most beneficial oxidizing agent is the mechanism for its removal within the soils underlying the infiltration basin.
2.5 Project Justification

The RIB system located in the Town of Middletown, Delaware offers an example of a field-scale system for nitrate removal. Rapid infiltration has been widely shown to be effective for the transformation of ammonia to nitrate, but the removal of nitrate has not been the focus of these nitrogen removal studies. Denitrification with the use of woody carbon substrates has been heavily studied in column and bench-scale experiments, but these treatments have not been quantified in larger systems. While nitrate removal has been convincing in column studies, the scale-up process raises questions about the treatment efficiency in the field. While carbon materials have a long lifespan in denitrification walls, the fate of carbon under the heavy loading of a rapid infiltration basin has not been examined.

This project allows for the observation of nitrate removal on a large scale using the same woody materials which have proved highly effective under controlled conditions. The effects of woodchip mass, operation schedule (flooding vs drying time), influent loading volume, and seasonal temperature changes were observed over the course of the study from May 2014 to March 2015. Long term column studies in our lab aimed to categorize the treatment lifespan of the woodchips, by exposing them to the high volumetric loadings typically experienced in an operational rapid infiltration system. These data supplement current research on rapid infiltration basins and related technologies.
Chapter 3

METHODS AND MATERIALS

3.1 RIB Field Site Description

Four pilot scale Rapid Infiltration Basins (RIBs) with dimensions of 25 by 60 feet were constructed at the Von Croy Farm site located in the Town of Middletown, Delaware. (The basins had hand-measured lengths of 62 to 63 feet, and widths of 25 to 27 feet. Including a small concrete slab surrounded by crushed stone, each basin had an infiltration area of just over 1,500 square feet). The RIBs were surrounded by 3-foot high berms with 3:1 side slopes. Four of the berms were covered with plastic sheeting to prevent excessive seepage to open areas of the site not intended for rapid infiltration. The RIB site occupied a small portion of the spray irrigation facility currently used by the Town of Middletown. For providing effluent to the basins, a temporary transmission line and flow meter were added to the existing transmission piping from the Middletown lagoon wastewater treatment facility. The transmission piping provided secondary lagoon effluent to each basin during a daily loading period. The volume loaded depended on the selected loading plan for the RIB case study.

Three of the basins were amended with varying amounts of woodchips, which were tilled into the upper 12 inches of the RIB soils. RIB-0 containing no woodchips was a control basin for some loading cycles of the study. RIB-1, RIB-2, and RIB-3 each contained 10, 20, and 30% by volume of finely graded woodchips, respectively, as an external carbon source. After incorporation of the woodchips, the basins were allowed to rest unused for approximately one month (May 2014) to allow for
compaction of the soil and stabilization of the woodchips. According to data available from the U.S. Department of Agriculture, the soil underlying the site was classified as a Reybold Silt Loam. This soil type is described as a naturally well-drained soil with moderate water storage capacity. Typically, this soil has a 30 inch upper horizon of silty loam with an underlying layer of more than 50 inches of gravelly coarse sandy loam (USDA, 2015).

Each of the four basins contained six suction lysimeters for the purpose of sampling the water passing through the soil. Three of the lysimeters in each basin were installed at 3 feet below the surface, and the other three were set to a depth of 6 feet. As lagoon effluent passed through the system, the lysimeters drew a small quantity of water, which could be manually extracted for analysis. The site also contained three wells below the water table for the purpose of periodic (monthly) monitoring. Well-1 was located on the outside of RIB-0, down-gradient of the site (as it relates to the prevailing direction of groundwater flow). Well-2 was located in the middle of the site between RIB-1 and RIB-2. Well-3 was located on the outside of RIB-3, up-gradient of the site. A metal tube which extended roughly 3 feet above the ground surface indicated the location of the well. A schematic diagram of the experimental site is presented in Figure 3.1.
Figure 3.1. Schematic of the RIB Site in Middletown, DE
3.2 Middletown Lagoon System

The Town of Middletown uses a lagoon system operated by Artesian Resources to treat municipal wastewater and some industrial wastewater. The lagoon system is located approximately 1.2 miles from the RIB field site, and transmission piping transported lagoon effluent to the RIBs located adjacent to the existing spray irrigation fields. This lagoon system provided 7,100 to 41,000 gallons to each of the RIBs during the different experimental phases. The amount of time to load these volumes into the basins was approximately 15 to 100 minutes. With each loading cycle, a sample of lagoon effluent was obtained from plant operators as a feed sample to the RIBs. The lagoon effluent samples were taken by an automated composite sampler during the period while the RIBs were being loaded. The composite samples were collected and transported in plastic containers with a volume of 0.95 liters. These samples were analyzed and stored in the same manner as the RIB lysimeter samples. In addition, Artesian provided a 0.24 liter sample which had been lightly acidified with H$_2$SO$_4$. This acidified sample was used for ammonia analysis (with the acidification improving accuracy).

3.3 RIB Loading Schedules and Volumes

During this field study, the basins were loaded with different loading/resting schedules, in order to characterize the effectiveness of the RIBs under different operation conditions. The flooding and drying times were varied, as well as the volume of lagoon effluent loaded onto the basins. These loading schedules can be grouped into distinct phases, the details of which are discussed below.
3.3.1 Phase-1 (Summer)

The first phase of the RIB study began on May 29, 2014 and continued through August 27, 2014. This is labelled as the summer phase of the project, and consisted of a short loading period and high loading volume. The basins were loaded for 2 days with 35,000 gallons of lagoon effluent loaded into each basin each day. This loading period was followed by 10 days of basin resting, when drying took place. Lysimeters were sampled in the days following the basin loading. A total of 8 cycles were completed during Phase-1.

3.3.2 Phase-2 (Fall)

The second phase of the RIB study began on September 24, 2014 and continued through December 8, 2014. This is labelled as the fall phase of the project, and consisted of a long loading period and relatively low loading volume. RIB-0 (containing no woodchips) was not operated during this phase due to draining issues encountered during Phase-1. The remaining basins were loaded for 9 consecutive days. RIB-1 and RIB-2 were loaded with 14,200 gallons during each loading day, while RIB-3 was loaded with only 7,100 gallons due to slow draining concerns. This extended loading period was followed by 13 days of basin resting. Lysimeters were sampled on day 3 and day 5, just before the loading began on each day, and again after the final day of loading for each cycle. Variation in the actual sampling days was necessary when lysimeters remained submerged due to slow draining within the basins. A total of 4 extended cycles were completed during Phase-2.

3.3.3 Phase-3 (Winter)

The third phase of the RIB study began on January 14, 2015 and continued through March 23, 2015. This is labelled as the winter phase of the project, and
consisted of a short loading period and high loading volume. As in Phase-2, RIB-0 was not operated due to draining concerns. All of the remaining basins were loaded for 1 day with 41,000 gallons of lagoon effluent. This loading period was followed by 6 days of basin resting. Lysimeters were sampled in the days following the basin loading. Due to weather restrictions and freezing conditions from February 12 to 28, several lysimeters were unsuccessfully sampled during the fifth cycle of treatment, and two loading periods on February 18 and February 25 were missed entirely. A total of 8 loading cycles were completed during Phase-3.

3.4 Lysimeter Sampling

As previously stated, each basin contained six suction lysimeters, with three installed at 3 feet and three installed at 6 feet below the ground surface. The lysimeters sampled the basin effluent as it flowed vertically downward through the soil. Each lysimeter consisted of a perforated screen at the bottom and a body tube with an inner diameter of approximately 1.84 inches. The lysimeter extended slightly above the ground surface (2 to 6 inches), and was covered by a rubber stopper which fit tightly into the open end. This cap was fitted flexible vacuum tubing and sealed with an adjustable plastic clamp.

Water was drawn into the lysimeter by a vacuum pressure, which was manually installed. Prior to a loading period, a hand pump was used to set the lysimeter vacuum. The rubber stopper was removed and any residual liquid in the lysimeter was extracted (there was rarely any residual liquid except in the case of a prior lysimeter failure). High vacuum grease, which was found to sustain the vacuum over longer periods, was spread around the rubber stopper when it was reinserted into the top of the lysimeter. The hand pump was fitted to the vacuum tubing and pumped
to achieve a suction pressure within the lysimeter. The plastic clamp was then secured and left for the duration of a loading period as soil pore water accumulated.

After loading was completed the lysimeters were sampled, usually 2 to 5 days after loading ended. The cap was removed and the hand pump was used to extract the volume of water present in the lysimeter. The water samples were placed into 300 mL flasks during Phase-1, or 50 mL centrifuge tubes during Phase-2 and Phase-3. These flasks and centrifuge tubes were used for the transportation and storage of samples before and after analysis. Samples were transported from the field site at ambient temperature and stored at 4°C at the University of Delaware. The volume of water collected (range 25 to 1400 mL, typically 300 to 700 mL) varied between individual lysimeters. Greater volume was collected for longer loading periods.

3.5 Well Depth Measurement and Sampling

The three monitoring wells located at the RIB field site were sampled on a monthly basis during periods when the basins were operated. Well-3 (up-gradient), Well-2 (middle), and Well-1 (down-gradient) were installed to examine the subsurface water quality below the RIB site. Water table measurements were taken using a depth-measurement tape. The tape was lowered into the well housing until an indicator beeped when it contacted the water. The depth was then taken from the tape, and the height of the well housing was subtracted from the measurement. After the depth reading was recorded, a groundwater sample was extracted from the well using the same hand pump apparatus as in the lysimeter sampling. The tubing was lowered slowly into the water column, and kept above the well base to avoid disturbing any silt at the bottom of the well. A volume of 0.5 to 1.0 liters was removed from the well, and 50 to 300 mL were stored at 4°C before analysis.
3.6 Laboratory Column Construction

Along with the field-scale RIB study, laboratory columns were operated in parallel to investigate the degradation of woodchips over long operation periods. Three up-flow columns were constructed, corresponding to RIB-1 (10% woodchips), RIB-2 (20% woodchips), and RIB-3 (30% woodchips). Each column was constructed from 32-inch long clear plastic tubing with an inner diameter of 1.46 inches. An influent solution was fed into the bottom of each column using a peristaltic pump. Within each column, the bottom 2.5 inches were filled with inert glass beads to distribute the inflow across the area of the column. The overlying 12 inches consisted of soils amended with varying amounts of woodchips, which had been sieved between a #10 and #18 standard mesh to give a size distribution between 1.0 and 2.0 mm. Column-1 contained a mix of 10% woodchips by volume within this 12 inches, with Column-2 and Column-3 containing 20% and 30% woodchips, respectively. The soils used in this mix were taken from the RIB field site in June 2014 and allowed to dry in the lab, making them easier to mix uniformly. Above the woodchip layers, 15 inches of soil were added without any carbon addition. At the top of the columns was another 2.5 inches of inert glass beads. Tubing ports were located near the top of the columns for sampling the effluent.

Each of the columns was fed with influent solution from a shared reservoir. The feed containers were sparged with nitrogen gas to remove residual oxygen from the column influent. The N\textsubscript{2} gas sparging also promoted mixing in the tanks. An aquarium aeration stone was located in the bottom of each tank, with a gas release line (not shown) installed at the top. A diagram of the column configuration is presented in Figure 3.2.
Figure 3.2. Configuration of Laboratory Columns
3.7 Laboratory Column Operation and Sampling

The column feed solution was made up of a mixture of secondary effluent from the Middletown lagoon wastewater treatment facility and synthetic nitrate solution. Approximately every two weeks, secondary effluent was collected from the Middletown facility to supplement the feed tanks when they became low (< 2 gallons). In addition to the lagoon effluent, an equal volume of synthetic nitrate solution was added to the feed tanks. The synthetic nitrate solution was prepared by dissolving NaNO₃ in deionized water, and the concentration was adjusted to maintain the nitrate concentration in the column feed between 15 and 25 mg/L NO₃-N. The solutions were prepared at the end of a week of operation (usually on a Friday) to allow the feed reservoir to mix before column operation resumed during the following week.

The columns were operated during the period from July 15, 2014 to April 17, 2015 with the exception of December 20, 2014 to February 8, 2015 when the columns were shut down due to laboratory restrictions. During this shutdown period, the columns were sealed at each end to prevent oxygen from diffusing inside. Initially, the flowrate into each of the columns was adjusted to 50 mL/hr. Column operation usually started on Monday mornings and continued until Friday afternoons, giving a continuous loading period of 102 hours during a normal week. This operating period used a volume of 5.1 L per week per column. During each day of operation, the nitrogen gas was used to degas the feed tanks for a minimum of 30 minutes. After 4 months of operation, the column flowrate was increased to 60 mL/hr, where it remained for the duration of the study. This increased flowrate resulted in a feed volume of 6.1 L per week per column.

Samples were generally taken from each of the experimental columns every 2 weeks. In special cases, the columns were sampled more frequently. During a period
to investigate denitrification occurrence in the feed reservoir, column effluent samples were gathered in 2 day intervals. On dates when the columns were sampled, a volume of influent from the feed tank was also collected for comparison. All samples were collected in 50 mL centrifuge tubes and stored at 4°C prior to analysis.

3.8 Physicochemical Analyses

A variety of analytical methods were used to characterize the physicochemical properties of the lysimeter samples, lagoon effluent samples, groundwater well samples, and laboratory column samples. These measurements included nitrate, nitrite, ammonia, total nitrogen, organic nitrogen, pH, total suspended solids, and chemical oxygen demand. The methods to analyze each of these parameters are discussed below.

3.8.1 Nitrate and Nitrite

Nitrate and nitrite were analyzed using both ion chromatography and spectrophotometric methods. A standard curve for a Dionex ion chromatograph (IC) was constructed with concentrations of 1, 5, 10, 50, and 100 mg/L NO$_2$/NO$_3$. Standards were diluted from stock solutions containing solid NaNO$_3$, solid NaNO$_2$, and distilled water. Using these standards and given operating conditions, nitrate and nitrite were analyzed during a single chromatography run, with nitrite eluted from the column at 9.9 minutes and nitrate following at 13.9 minutes. Prior to IC analysis, the samples were passed through a 1.6 micron Whatman glass microfiber filter using a suction pump. The filtered sample was then poured into a 5 mL Dionex autosampler tube, and capped after removing any air bubbles. The prepared samples were then inserted into the autosampler and analyzed according to a 20 minute elution program.
As a confirmation step and alternative method, samples were analyzed using a Hach spectrophotometer. NitraVer and NitiVer reagents were used to determine the nitrate and nitrite concentrations, respectively. Filtered samples were analyzed according to Hach procedures for the specified reagents. All samples (lysimeter samples, lagoon effluent samples, groundwater well samples, and laboratory column samples) were analyzed using these methods. Nitrite was tested only sparingly in some cases, when concentrations were found to be negligible.

### 3.8.2 Ammonia

The Hach spectrophotometer method was used to measure ammonia content in unfiltered samples. Each acidified lagoon effluent sample was analyzed according to the Hach procedure for AmVer High Range Ammonia reagents. Groundwater wells were tested on a monthly basis. For RIB lysimeter samples, ammonia tests were used sparingly in certain cases, to confirm that ammonia was negligible in the samples and to meet monitoring requirements.

### 3.8.3 Total Nitrogen

The Hach spectrophotometer method was used to analyze unfiltered samples for their total nitrogen content. Total Nitrogen Acid Solution/Hydroxide reagent sets were used according to the Hach procedure, to analyze lagoon effluent samples approximately once per month. The groundwater monitoring wells were also analyzed monthly to meet monitoring requirements. For RIB lysimeter samples, total nitrogen contents were measured intermittently, to meet monitoring requirements and to allow for the calculation of organic nitrogen (discussed below).
3.8.4 Organic Nitrogen

Organic nitrogen was calculated using a mass balance approach with the nitrate, nitrite, ammonia, and total nitrogen values. Organic nitrogen values were calculated as the difference between the measured total nitrogen (spectrophotometry) and the sum of the other measured nitrogen species (both chromatography and spectrophotometry). This value was computed for all of the groundwater well samples, as well as monthly lagoon effluent and select RIB lysimeter samples.

3.8.5 pH

The sample pH was monitored using a calibrated Cole Parmer pH probe. The probe was inserted into the sample and allowed to equilibrate for roughly 5 minutes until the reading stabilized. Throughout the study, pH was recorded for all lagoon effluent samples and groundwater samples taken from the monitoring wells. During Phase-1, the pH was recorded for each lysimeter sample taken from the RIBs. This measurement frequency was reduced during Phase-2 and Phase-3 due to a lack of variation observed in the pH data.

3.8.6 Total Suspended Solids

The total suspended solids (TSS) were measured for each of the lagoon effluent samples. A 1.6 micron Whatman glass microfiber filter was weighed to the closest 0.0001 grams, and then a 100 mL sample of lagoon effluent was passed through the filter using a vacuum pump. The filter and the collected solids were allowed to dry for at least 4 hours in a 105°C oven. The dried filter was then reweighed, and the additional mass deposited on the filter was used to calculate the TSS per liter of lagoon effluent.
3.8.7 Chemical Oxygen Demand

The Hach spectrophotometer method was used to analyze the chemical oxygen demand (COD) of the laboratory column effluent and feed solution. Samples were filtered through a 1.6 micron Whatman glass microfiber filter and processed with COD Digestion Solution vials according to the recommended Hach procedure.

3.9 Soil Microbial Sampling

Soil samples were gathered from the Middletown field site before and during the RIB study to examine the changing microbial communities during basin operation. Soil samples were collected before the first loading of Phase-1, during May 2014. Additional samples were collected following the final loading of Phase-2, on December 8, 2014. Three soil cores were collected from each of the woodchip-amended RIBs (RIB-0 was not used in the microbial analysis). One replicate was gathered near the north end of each RIB, one near the south end, and one in the middle. Soil was collected using a handheld soil auger, equipped with a thin-walled tube sampler with an inner diameter of 0.75 inches and a length of 16 inches. The auger was inserted into the soil to a depth of 8 to 12 inches, and an intact core of soil was extracted which was then transferred to a zipped plastic bag. Samples were transported from the field site and stored at -15°C before DNA extraction.

3.10 DNA Extraction and Amplification

Microbial DNA was extracted from the 18 soil samples using a Fisher BioReagents SurePrep Soil DNA Isolation Kit. The soil samples were allowed to thaw at room temperature, and a subsample of 0.25 grams of soil from each core was extracted according to the SurePrep procedure. Extracted DNA was frozen at -15°C prior to polymerase chain reaction (PCR) amplification. Two amplifications were
performed with selective primers for the *narG* gene and a universal microbial primer. For the PCR reactions, primers and thermocycler protocols were obtained from previous publications which had investigated denitrifying microbes (Lopez-Gutierrez *et al.*, 2004; Ji *et al.*, 2012a). The primers used in the reactions are listed in Table 3.1.

For each sample, the reaction mixture included 1.0 μL DNA, 0.5 μL PCR primers, 0.5 μL dNPT, 2.5 μL 10X PCR buffer, 0.3 μL Taq polymerase, and sterilized DI to bring the total volume to 25 μL. After preparation, the samples were processed in a BIO-RAD T-100 Thermocycler. The temperature settings differed between the two primer sets, and were modeled from the publications mentioned above. For the universal primers, the program began with a 5 minute denaturation period at 94°C. This was followed by 30 cycles (15 seconds at 95°C denaturation, 30 seconds at 60°C annealing, 30 seconds at 72°C extension) for amplification, and a final 8 minute extension period at 72°C. The *narG* program also began with a 5 minute denaturation at 94°C, but was followed by 45 cycles (15 seconds at 95°C denaturation, 30 seconds at 63°C to 58°C annealing, 30 seconds 72°C extension) for amplification, before a final 8 minute extension period at 72°C. During the annealing step for *narG*, the temperature was lowered from 63°C in increments of 1°C for each of the first 5 cycles, and a temperature of 58°C was used in each of the final 40 cycles. Both protocols were followed by a storage period at 12°C before removal.

<table>
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<tr>
<th>Target</th>
<th>Primer Name</th>
<th>Sequence</th>
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<td></td>
<td>2050m2r</td>
<td>5’-CGTAGAAGAACCTGCTGCTT-3’</td>
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<tr>
<td></td>
<td>518R</td>
<td>5’-CCATACGGGAGGCAGGCAG-3’</td>
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3.11 Microbial Gel Analysis

After completing the PCR amplification, gel electrophoresis was used to visualize the amplified environmental DNA. An individual gel was used to analyze the set of samples from each RIB, because there were too many samples to include all of the RIBs in a single run. Gels containing 0.8% agarose in 1X TAE buffer were heated and mixed on a stir plate until clear. The melted gel was then poured into a comb mold where it was allowed to set for roughly 30 minutes. The combs were removed and the gel was placed in an electrophoresis chamber and submerged under 1X TAE buffer. A pipette was used to mix an 8 μL aliquot of amplified DNA with 2 μL of 6X loading dye, and the sample was injected into one of the wells created by the comb mold. The *narG* samples were injected into the top row of wells, and the universal primer samples were inserted into another line of wells located further down the gel. A negative control and DNA ladder were included on either end of the environmental samples.

After pipetting the samples into the gel, the electrophoresis chamber was covered and activated. Each gel run was completed at 90 volts for 35 minutes. After completion, the gels were removed from the buffer and stained for 30 to 45 minutes in ethidium bromide. They were then removed and visualized under UV illumination. Digital photographs were converted to negatives, and the brightness and contrast were altered using imaging software to aid in identifying faint bands.

3.12 Statistical Tools

The primary statistical tool used in this study was the two way ANOVA with replication. Separate statistical tests were used in Phase-1, Phase-2, and Phase-3 to characterize the differences in the nitrate percent removal across the three woodchip
treatments and between subsequent loading cycles. In each case, SAS programming was used to perform the analysis, and significance was determined at $\alpha=0.05$. Two way ANOVA is an appropriate test to use when the experimental data has two nominal variables and one measurement variable (McDonald, 2014). In this study, the two nominal variables were the identity of the RIB and the cycle number used in each phase. The 3-foot and 6-foot lysimeters were tested separately, because the inclusion of this information would create a third nominal variable in the data.

The three lysimeters located at each depth were treated as replicates, hence the two way ANOVA with replication was used. To perform a two way ANOVA, the number of data points in each variable group must be equal, and in several cases the lysimeters did not successfully produce samples. This created an issue for the statistical test, and a reasonable way to replace this data was needed. When necessary, several methods of data estimation can be used to substitute for missing values.

One way to substitute missing data is to use mean substitution, which simply replaces the missing value with the mean of the other replicates observed in the study. This technique is usually not recommended if a better alternative exists, primarily because it artificially lowers the variance of the data (Howell, 2008; Osborne, 2013). A better technique for data estimation is to fit a function, such as a straight line, to the available data in order to project a reasonable value for the missing replicate (Dallal, 1998; Howell, 2008). Another common solution is the Last Observation Carried Forward method, which is often used in medical research (Dallal, 1998; Howell, 2008). In this technique, if a respondent does not finish a treatment, the remaining sampling dates are updated with the most recent observation of that individual. It is
important to note that any replacement of missing data reduces the reliability of the statistical test. The methods behind all data replacement are fully disclosed as follows:

During Phase-1, a linear projection was used to forecast for missing values between the fifth and eighth cycles of operation. During Phase-2, each cycle of operation had several sampling days. The final day of each cycle was used in the statistical analysis because it was the most complete. The Last Observation Carried Forward technique was primarily used for substituting missing values, because there were not enough data points collected during each cycle to fit a linear trend line. In one case, a mean substitution was necessary because no prior observations were recorded. Phase-3 used a linear regression fit across the entire set of available data. The estimated data points for the statistical analysis are clearly marked in Chapter 4, and additional information is presented for each of the phases.
RESULTS AND DISCUSSION

4.1 Phase-1 (Summer) RIB Loading

During the first phase of the RIB study (May 29, 2014 to August 27, 2014) the test RIBs were loaded according to a 2-day loading and 10-day resting period. Each RIB received the same loading volume of 35,000 gallons per day during the summer tests. The characteristics of the feed solution are presented in Table 4.1.

Table 4.1. Characteristics of RIB Feed Water (Phase-1)

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<th>pH (standard)</th>
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<th>NO$_2$-N (mg/L)</th>
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<td>6.4</td>
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</tr>
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<td>8/21</td>
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<td>5.2</td>
<td>5.2</td>
</tr>
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<td>8/22</td>
<td></td>
<td>9</td>
<td>10.10</td>
<td>0.0</td>
<td>0.0</td>
<td>5.6</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*Sum of NH$_3$-N, NO$_2$-N, and NO$_3$-N
As seen in Table 4.1, the ammonia concentrations decreased over the duration of the study, and completely disappeared from the feed solution by the end of July. As ammonia decreased during the month of July, this trend was opposed by an increase in the nitrite and nitrate concentrations of the feed solution, indicating that the wastewater lagoon was undergoing nitrification during the warmest summer months. However, by the end of the July the nitrite and nitrate concentrations also began to decrease. This may have been a result of denitrification in the lagoon sediments. The listed total nitrogen concentration was determined as the sum of all nitrogen species in the table. The total nitrogen in the feed water decreased over the course of Phase-1, with the lowest concentration occurring during August.

It was assumed that any ammonia or nitrite in the feed solution would be rapidly oxidized to nitrate during the infiltration process. Effectively, all nitrogen in the feed solution was loaded as nitrate during the warm summer months due to this rapid conversion of nitrogen species. This assumption was reasonable based on nitrite and ammonia analyses during Phase-1, which showed very low concentrations of these species in the lysimeter samples.

The pH values of the feed solutions increased over the 3 months of the study, but this seemed to have minimal effects on the pH of the lysimeter samples, indicating that the soil chemistry was resistant to changes in the overall pH. (The lysimeter samples remained in the pH range of 6 to 7 over the course of the study.) The elevated pH in the lagoons would form un-ionized ammonia, which could volatilize from the lagoons and contribute to the observed disappearance of ammonia from the feed samples. The TSS fluctuated during the study, with the highest suspended solids of this phase occurring in late July and early August. The maximum values for TSS were
in the range of 17 to 27 mg/L, indicating significant algal growth in the wastewater lagoons. This was evident in visual examination of the samples. Organic nitrogen (data not shown) was determined monthly. This value was calculated to be minimal (positive or negative values) in the feed and lysimeters samples, indicating that organic nitrogen was a minor component of the overall nitrogen balance. Small instrumental errors likely played a role in the negative calculated values, but these were not deemed to be significant compared to the overall total nitrogen values.

The feed solution was loaded into the woodchip-amended basins and percolated to the lysimeter ports located 3 and 6 feet below the surface. These samples were gathered and average nitrate concentrations were determined for each basin at the given depths. The nitrate concentrations for the entire phase at the 3-foot and 6-foot sampling depths are shown in Figure 4.1 and Figure 4.2, respectively. In these figures, the concentration in the feed solution is shown as the total nitrogen concentration converted to nitrate, as previously explained. It is an average value of the first and second day of loading for each cycle. The error bars represent the sample standard deviation for the 3 lysimeters (or in some cases only 2 lysimeters) sampled after each loading period. The absence of error bars for a RIB average indicates that only 1 lysimeter was successfully sampled following the loading cycle.
Figure 4.1. Nitrate Concentration in 3-Foot Sampling Depth (Phase-1)
Figure 4.2. Nitrate Concentration in 6-Foot Sampling Depth (Phase-1)
The first cycle of operation, loaded on May 29 and May 30, 2014, resulted in significant removal of nitrate in both the 3-foot and 6-foot lysimeters. Over 50% removal was observed for each RIB. It is likely that much of the observed nitrate removal during the first loading period was due to sorption and other physical processes in the soil, as opposed to microbial denitrification. It was hypothesized that a denitrifying population would take longer than one loading cycle to grow and become enriched in the basin soils.

During subsequent weeks of operation, spikes in the nitrate concentration were observed in the lysimeter samples. The nitrate concentrations measured in the lysimeters far exceeded the feed concentration, demonstrating that a source of nitrogen was present in the basins. The surplus of nitrogen is too excessive to be explained simply as a result of analytical or sampling errors. Since the basins were not operated for several months prior to the current loading study, organic materials accumulated in the soil may have provided this source of excess nitrogen. Decomposing plant growth in the basins and nitrogen carried over from the past studies are two probable reasons for these high nitrate levels.

The pattern of excess nitrogen in the experimental data suggests that residual nitrate was flushed out of the system over time. The nitrate excess became apparent in the 3-foot lysimeters during the second cycle of operation, and seemed to be flushed out of the system by the fifth cycle. In the 6-foot lysimeters, the nitrate excess was delayed. The nitrate levels in the lysimeter samples did not exceed the feed solution during the second cycle of operation, but excessive levels were observed during the following cycle. This observation is consistent with our hypothesis of a flushing of nitrate which was sorbed to the soils near the surface of the basins.
lagoon wastewater drew this nitrate downward through the soil, where it first appeared in the 3-foot lysimeter samples, and then the 6-foot samples. As evident in the figures, the concentration of excess nitrate was lower in the 6-foot samples than the 3-foot samples, suggesting that some nitrate present at 3 feet deep may have been denitrified before reaching the 6-foot lysimeters. As with the 3-foot samples, the nitrate flushing appeared complete by the fifth cycle.

Due to the flushing effects, the first four cycles of operation from May 29 to July 5 have limited value in evaluating the effectiveness of the RIB system. The remaining operation time of Phase-1 occurred after the excess nitrate was flushed out of the system. Figure 4.3 and Figure 4.4 show the average nitrate concentrations for cycles five to eight of Phase-1 for the 3-foot and 6-foot sampling depths, respectively.
Figure 4.3. Nitrate Concentration in 3-Foot Sampling Depth (Phase-1, abbrev.)
Figure 4.4. Nitrate Concentration in 6-Foot Sampling Depth (Phase-1, abbrev.)
The removal of nitrate by the basins differed based on the woodchip amendment and sampling depth. Overall, the average nitrate concentrations showed a positive nitrate removal by the system. In the RIBs with 20% woodchips (RIB-2) and 30% woodchips (RIB-3), significant removal was observed in the 6-foot sampling ports. The concentrations of nitrate in the lysimeter samples were lower than the feed solution in every case involving RIB-3, suggesting that the woodchip addition was effective in providing electron donors to the soil. In the case of RIB-2, the 3-foot sampling ports did not show a reduction in nitrate, but the 6-foot samples showed a substantial nitrate reduction. This implies that the contact time between the feed water and subsurface microbes was important in determining the extent of denitrification. It also suggests that additional denitrification may occur deeper in the soil, further protecting groundwater quality. Since the woodchips were only present in the top 12 inches of the soil, the enhancement of denitrification between the 3-foot and 6-foot depth indicates that electron donors migrated downward into the soil.

In RIB-0 (no woodchip amendment), it was anticipated that only a minimal amount of nitrate would be denitrified in the subsurface. However, substantial amounts of nitrate removal were observed in both the 3-foot and 6-foot samples. A possible explanation for the observed trend is the presence of organic material in the soil as a result of plant decomposition, which may have provided the electron donors for denitrification. RIB-0 was also observed to be the slowest draining basin, which allowed for a longer contact time with the soil. The continuous ponding would also sustain the anaerobic conditions necessary for denitrification. RIB-1 appeared to have a minimal effect on the nitrate concentration. Despite the unexpected behavior of
RIB-0, the other basins followed the hypothesized trend that increasing the woodchip amount would enhance denitrification in the subsurface.

In order to analyze the treatment differences more quantitatively, the individual lysimeter measurements were analyzed statistically using a two way ANOVA with replication. The three lysimeters acted as replicates, and the percent removal was used to account for the changing feed solution during the final cycles. The individual removal efficiencies are shown in Table 4.2 and Table 4.3 for the 3-foot and 6-foot sampling depths, respectively. Measurements marked with an asterisk (*) are interpolated values, as explained in Chapter 3. The percent removal measurements for both depths are shown in Figure A.1 and Figure A.2, along with the linear trend lines used for calculating the missing values.
Table 4.2. Percent Removal Measurements for 3-Foot Depth (Phase-1)

<table>
<thead>
<tr>
<th>Location</th>
<th>Cycle 5</th>
<th>Cycle 6</th>
<th>Cycle 7</th>
<th>Cycle 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIB-0</td>
<td>38</td>
<td>35</td>
<td>43</td>
<td>43*</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>32</td>
<td>38</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>57</td>
<td>51</td>
<td>71</td>
</tr>
<tr>
<td>RIB-1</td>
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<td>-40</td>
<td>-42</td>
<td>-29</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0</td>
<td>-6</td>
<td>-17</td>
</tr>
<tr>
<td></td>
<td>40*</td>
<td>38</td>
<td>19</td>
<td>26</td>
</tr>
<tr>
<td>RIB-2</td>
<td>11</td>
<td>5</td>
<td>1*</td>
<td>-3</td>
</tr>
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<td>6*</td>
</tr>
<tr>
<td></td>
<td>-105</td>
<td>-61</td>
<td>-27</td>
<td>-25</td>
</tr>
<tr>
<td>RIB-3</td>
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<td>36</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
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<td>79</td>
</tr>
<tr>
<td></td>
<td>-48</td>
<td>-39</td>
<td>-1</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 4.3. Percent Removal Measurements for 6-Foot Depth (Phase-1)

<table>
<thead>
<tr>
<th>Location</th>
<th>Cycle 5</th>
<th>Cycle 6</th>
<th>Cycle 7</th>
<th>Cycle 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIB-0</td>
<td>27</td>
<td>72</td>
<td>64</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>-23</td>
<td>-105</td>
<td>-62</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>19</td>
<td>8</td>
<td>66</td>
</tr>
<tr>
<td>RIB-1</td>
<td>4*</td>
<td>-5</td>
<td>-22</td>
<td>-27</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-16</td>
<td>-30</td>
<td>-22</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>15</td>
<td>-1</td>
<td>3</td>
</tr>
<tr>
<td>RIB-2</td>
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<td>32</td>
</tr>
<tr>
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<tr>
<td></td>
<td>29</td>
<td>33</td>
<td>20</td>
<td>39</td>
</tr>
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<td>RIB-3</td>
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</tr>
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<td>-5</td>
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<td></td>
<td>51</td>
<td>36</td>
<td>47</td>
<td>67</td>
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</tbody>
</table>
Using the table values, the two way ANOVA with replication showed that the removal efficiency differed significantly based on the test RIBs, and the removal efficiencies were similar across the different cycles. The interaction effects were not significant for either the 3-foot depth (p=0.981) or 6-foot depth (p=0.607) results, showing that the effect of basin identity was consistent across the different cycles of operation. If the interaction term had been significant, this would mean the p-values generated for the two main effects of cycle number and woodchip treatment could be misleading. Since there is no significant interaction, there is little risk in interpreting the results of the two way ANOVA for cycle number and basin identity.

For the 3-foot samples, the effect of the cycle number was not significant (p=0.683), but the effect of the RIB identity was significant (p=0.001). Likewise, the percent removals of the 6-foot samples were not differentiated by cycle number (p=0.576), but the basins were found to be statistically different (p=0.046). These results provide additional evidence that the woodchip amendment played a role in the removal efficiency of the basin. By observation of Figures 4.3 and 4.4, the removal efficiencies were similar across all of the cycles, but it is clear that certain basins were more effective in treating the lagoon effluent.

While the startup effects were severe during Phase-1 of the project, the latter part of the RIB study was not heavily influenced. The woodchips appeared to play a role in the enhanced denitrification in RIB-2 and RIB-3 at the 6-foot depth, and electron donors were shown to migrate downward through the soil.
4.2 Phase-2 (Fall) RIB Loading

During the second phase of the RIB study (September 24, 2014 to December 8, 2014) the RIB system was loaded according to a 9-day loading and 13-day resting period. During this phase, RIB-3 received only 50% of the 14,200 gallons loaded to RIB-1 and RIB-2 each day. RIB-0 was not loaded during this time period due to the significant ponding observed during Phase-1 of this study. In terms of the nitrogen load, the feed solution grew gradually stronger throughout the fall loading period.

Nearly all of the nitrogen in the lagoon effluent was present as nitrate, with only very minor contributions from ammonia and nitrite. The characteristics of the basin feed solution during this period are presented in Table 4.4. Since samples were normally provided on day 1 and day 9 of the loading cycle, an average value was used to estimate the feed concentrations during the middle of the 9-day loading period.

Table 4.4. Characteristics of RIB Feed Water (Phase-2)

<table>
<thead>
<tr>
<th>Date</th>
<th>Operation Cycle #</th>
<th>TSS (mg/L)</th>
<th>pH (standard)</th>
<th>NH$_3$-N (mg/L)</th>
<th>NO$_2$-N (mg/L)</th>
<th>NO$_3$-N (mg/L)</th>
<th>Total N* (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9/24</td>
<td>1</td>
<td>28</td>
<td>7.06</td>
<td>0.0</td>
<td>0.1</td>
<td>5.3</td>
<td>5.4</td>
</tr>
<tr>
<td>10/2</td>
<td></td>
<td>10</td>
<td>7.26</td>
<td>0.0</td>
<td>0.1</td>
<td>4.9</td>
<td>5.0</td>
</tr>
<tr>
<td>10/16</td>
<td>2</td>
<td>21</td>
<td>6.91</td>
<td>0.7</td>
<td>0.3</td>
<td>8.0</td>
<td>9.0</td>
</tr>
<tr>
<td>10/24</td>
<td></td>
<td>42</td>
<td>7.13</td>
<td>0.6</td>
<td>0.4</td>
<td>11.9</td>
<td>12.9</td>
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<tr>
<td>11/7</td>
<td></td>
<td>33</td>
<td>7.40</td>
<td>0.4</td>
<td>0.4</td>
<td>10.0</td>
<td>10.8</td>
</tr>
<tr>
<td>11/11</td>
<td>3</td>
<td>34</td>
<td>7.50</td>
<td>0.1</td>
<td>0.4</td>
<td>11.4</td>
<td>11.9</td>
</tr>
<tr>
<td>11/15</td>
<td></td>
<td>39</td>
<td>7.81</td>
<td>0.1</td>
<td>0.4</td>
<td>12.1</td>
<td>12.6</td>
</tr>
<tr>
<td>11/29</td>
<td>4</td>
<td>11</td>
<td>7.65</td>
<td>0.1</td>
<td>0.0</td>
<td>16.0</td>
<td>16.1</td>
</tr>
<tr>
<td>12/7</td>
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<td>9</td>
<td>7.37</td>
<td>0.0</td>
<td>0.0</td>
<td>16.2</td>
<td>16.2</td>
</tr>
</tbody>
</table>

*Sum of NH$_3$-N, NO$_2$-N, and NO$_3$-N
As seen in Table 4.4, the ammonia and nitrite concentrations were significantly lower than during Phase-1 of the study. These concentrations were similar to those in the latter half of the summer loading period, but did not show any high values similar to those observed in the feed during the May through early July experiments. As in Phase-1, total nitrogen was determined as the sum of the nitrogen species listed in the table, and was used as the nitrogen load value during analysis of RIB samples. This value for total nitrogen trended upward during the fall. During the summer months, the total nitrogen was used to simulate the rapid oxidation of the other nitrogen species to nitrate. During Phase-2, virtually all of the nitrogen was already present as nitrate, so it is reasonable to use this value as the RIB feed during this phase. The organic nitrogen level in the feed solution was minimal.

The pH values of the feed solution remained in the neutral range during the 3 months of fall testing, and fluctuated only slightly between values of 6.9 and 7.8. The pH of the lysimeter samples remained unaffected. The TSS of the feed water varied greatly during the fall study, with the highest suspended solids occurring in late October and persisting through November. During this period TSS measurements reached as high as 33 to 42 mg/L, indicating excessive algal growth in the lagoons. This loading of elevated TSS into infiltration basins may have contributed to some of the slow draining observed in the RIBs during the fall study, as algal cells clogged pores in the soil matrix. While the excessive levels of algae in the feed water may have decreased the infiltration rates of the basins, the drop in seasonal temperatures likely played a larger role. With temperature reduction, the infiltration rate of soils can be reduced substantially due to the increased viscosity and other property changes of the water (refer to section 2.3.4).
The lysimeters were sampled on day 3 and day 5, just before the loading began on each day, and again after the completion of loading for each cycle. This loading pattern allowed for the evaluation of nitrate removal during three different periods of the extended cycles. The sampling schedule allowed for a comparison of the performance early in the cycle, in the middle of the cycle, and again during the last few days. There was some sampling adjustment during the middle of November when the lysimeter ports were submerged, but the beginning-middle-end sampling plan was maintained through this interruption. It was anticipated that denitrification would be enhanced during later sampling days. As the basins remained saturated with longer loading periods, anoxic conditions would persist in the subsurface. These sustained anoxic conditions late in the cycle would be more favorable for denitrification.

Furthermore, samples taken early in the cycle might collect the initial flow-through of nitrate before the metabolisms of facultative anaerobes adjusted to respire nitrate.

Figure 4.5 and Figure 4.6 show the average nitrate concentrations during the Phase-2 fall loading period for the 3-foot samples and 6-foot samples, respectively. The error bars represent the sample standard deviation for the lysimeter replicates, and the absence of error bars for a RIB average indicates that only 1 lysimeter sample was obtained. The averages for each sampling date are grouped according to the cycle of operation, and presented in chronological order.
Figure 4.5. Nitrate Concentration in 3-Foot Sampling Depth (Phase-2)
Figure 4.6. Nitrate Concentration in 6-Foot Sampling Depth (Phase-2)
During the first sampling period of Phase-2, excess nitrate was present in the system, which mostly dissipated by the end of the first loading cycle. The nitrate removal in the RIB samples followed the expected trend as the performance improved with increased amounts of woodchips in the soil. This is clearly evident in the 6-foot samples, in which the average nitrate concentration decreased between RIB-1, RIB-2, and RIB-3 in every sampling period during the final three cycles. A similar trend was also observed for the 3-foot samples, with few exceptions. Significant treatment was observed in RIB-3 for both the 3-foot and 6-foot samples although the deviation of the samples was usually larger in the shallower locations. Greater than 50% removal was regularly achieved in RIB-3.

Similar to the results obtained during Phase-1, the average concentration of nitrate in RIB-2 was lower in the 6-foot samples than the 3-foot samples, providing further evidence that electron donors migrate downward in the soil and contribute to denitrification at depth. On the other hand, the nitrate removal in RIB-1 was minimal, particularly during the final two cycles. During these periods, the average nitrate concentration was usually within 1 mg/L of the feed nitrogen concentration, indicating poor treatment. This was the case for both sampling depths. Low removal achieved in RIB-1 during both Phase-1 and Phase-2 of the study suggests that the volume of woodchips added to the basin did not provide sufficient bioavailable carbon to promote substantial denitrification. Moreover, the lower density of woodchips in the soil could provide more pathways for wastewater to travel through the basin matrix while circumventing woodchip deposits. The higher density of woodchips in RIB-2 and RIB-3 would increase the likelihood that wastewater would encounter these substrates and carry electron donors to denitrification sites. In other words, the
physical distribution of woodchips in the basin may play a role in the potential for nitrate removal.

The expected trend of increasing removal efficiency with the longer loading cycle was not evident with the data presented in Figures 4.5 and 4.6. The average nitrate concentration in the samples often trended upward over time in both of the sampling depths. In some cases this increase may simply be due to the increasing nitrate concentration in the influent. In particular, the nitrogen load from the wastewater lagoon was rapidly increased during cycles two and three, resulting in rising nitrate concentrations in several of the lysimeter samples during those periods. It is important to note that some of the most noticeable decreases in treatment efficiency may be due to sampling bias. These biases would not reflect the actual treatment within the system. For example, a missing value from a lysimeter which typically exhibits strong nitrate removal produces an artificially poor removal efficiency, and a missing value from a poor denitrifying location biases the average towards strong treatment. Since there are only three data points for each average value from a RIB, these values make a noticeable difference. Specifically, the 3-foot samples taken from RIB-3 during the final two loading cycles show a considerable upward trend in effluent concentration, but these averages were heavily biased by missing values. In summary, the increases in the lysimeter concentration over time may be due to factors other than inefficient treatment, but there is certainly a lack of conclusive evidence that the treatment efficiency was improved by elongating the loading cycle. For this reason, a lengthier loading cycle may not be advantageous for basin operation, particularly during cold periods when operation can be complicated by increased drainage time or icing concerns (discussed later).
Although the loading volume was reduced in RIB-3 as compared to the other basins, the draining times remained similar. There was often residual ponding in both RIB-2 and RIB-3 24 hours after a single loading period. The ponding indicated that these basins were subjected to the longest residence time for the RIB feed water. It is not known whether the woodchips play a direct role in the reduced infiltration capacity of the basins, but the combination of the longer residence time and larger woodchip amounts appeared to work in parallel to enhance nitrate removal. The removal is further improved in the deeper lysimeter samples because there is a longer contact time between subsurface microbes and the denitrification substrates.

For a quantitative analysis, the individual lysimeter measurements were analyzed statistically using a two way ANOVA with replication, as in Phase-1. Again, the three lysimeters acted as replicates, and the percent removal was used to account for the changing feed solution between cycles. Since there were three sampling days associated with each operation cycle, the final day of sampling was used in the analysis. Although the highest treatment was not always achieved on the final day, this set was the most complete and therefore deemed the most appropriate for statistical analysis. The individual removal efficiencies for this final day are shown in Table 4.5 and Table 4.6 for the 3-foot and 6-foot sampling depths, respectively. Measurements marked with an asterisk (*) are Last Observation Carried Forward values, used to substitute for missing data as explained in Chapter 3. The estimate marked with a double asterisk (**) is an average of the other group values. The full set of percent removal values for each depth are found in Figure A.3 and Figure A.4.
Table 4.5. Percent Removal Measurements for 3-Foot Depth (Phase-2)

<table>
<thead>
<tr>
<th>Location</th>
<th>Cycle 1</th>
<th>Cycle 2</th>
<th>Cycle 3</th>
<th>Cycle 4</th>
</tr>
</thead>
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<tr>
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<td>6</td>
<td>1</td>
</tr>
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<td>-4</td>
</tr>
<tr>
<td></td>
<td>-51</td>
<td>20</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>RIB-2</td>
<td>1</td>
<td>9</td>
<td>2</td>
<td>0*</td>
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<td>-157</td>
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Table 4.6. Percent Removal Measurements for 6-Foot Depth (Phase-2)

<table>
<thead>
<tr>
<th>Location</th>
<th>Cycle 1</th>
<th>Cycle 2</th>
<th>Cycle 3</th>
<th>Cycle 4</th>
</tr>
</thead>
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<tr>
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<td>16</td>
<td>54</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>78</td>
<td>58*</td>
<td>18</td>
</tr>
<tr>
<td>RIB-3</td>
<td>-25</td>
<td>69</td>
<td>61</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>34</td>
<td>12</td>
<td>23</td>
</tr>
</tbody>
</table>
The results of the two way ANOVA showed differentiation of the data based on the cycle as well as the basin. As with the previous phase, the interaction effects were not statistically significant for the 3-foot depth ($p=0.367$) or 6-foot depth ($p=0.070$), although the p-values were much lower than in the prior tests. In looking at the main effects for the 3-foot samples, the effect of the cycle was found to be significant ($p=0.004$), but the effect of the RIB identity was not significant in this case ($p=0.120$). In the 6-foot dataset, the percent removal was found to be influenced by both of the main effects. Cycle number ($p=0.001$) and basin identity ($p<0.0001$) were each found to be highly influential on the observations.

Overall, the treatment efficiency was unusually low in the first cycle, but moderate to high during the remaining cycles of operation. The effects of the basin may not have been significant in the 3-foot samples due to the poor treatment in RIB-3 during the first cycle. As an extension, the tests were repeated without the inclusion of cycle one, and in this case the effects of cycle number and basin were both found to be significant for each sampling depth. The sets of data analyzed in Phase-2 had fewer true observations than the previous tests, and less reliable methods for substituting the missing values. For this reason, the statistics used here have more limited value.

As in Phase-1, there seemed to be some startup effects which added excess nitrate to the samples, but the impacts were much less severe. RIB-2 and RIB-3 showed promising treatment, particularly in the 6-foot samples, while RIB-1 made minimal improvements to the sample quality. Compared to the short loading plan, the extended loading did not clearly enhance denitrification. There is some concern about the practicality of this schedule during extended freezing periods (as in Phase-3).
4.3 Phase-3 (Winter) RIB Loading

During the third and final phase of the RIB field study (January 14, 2015 to March 23, 2015) the system was loaded according to a 1-day loading and 6-day resting schedule. Each basin was loaded with 41,000 gallons of feed water. During Phase-3, significant amounts of ammonia were present in the feed solution, increasing over time. The total nitrogen loading to the field site also reached a maximum value during Phase-3. The characteristics of the feed solution are presented in Table 4.7. A short interruption occurred in February as a result of sustained freezing conditions, which created a thick layer of ice over the surface of the RIB system, inhibiting sampling efforts. The freezing also had major impacts on cycles five and six, with the ice causing lysimeter damage and then diluting samples when temperatures warmed.

Table 4.7. Characteristics of RIB Feed Water (Phase-3)

<table>
<thead>
<tr>
<th>Date</th>
<th>Operation Cycle #</th>
<th>TSS (mg/L)</th>
<th>pH (standard)</th>
<th>NH₃-N (mg/L)</th>
<th>NO₂-N (mg/L)</th>
<th>NO₃-N (mg/L)</th>
<th>Total N* (mg/L)</th>
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<tr>
<td>1/14</td>
<td>1</td>
<td>1</td>
<td>7.43</td>
<td>4.1</td>
<td>0.6</td>
<td>14.2</td>
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<tr>
<td>1/21</td>
<td>2</td>
<td>15</td>
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<td>5.7</td>
<td>0.6</td>
<td>12.1</td>
<td>18.4</td>
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<tr>
<td>1/28</td>
<td>3</td>
<td>13</td>
<td>7.19</td>
<td>6.6</td>
<td>0.4</td>
<td>11.3</td>
<td>18.3</td>
</tr>
<tr>
<td>2/4</td>
<td>4</td>
<td>13</td>
<td>7.14</td>
<td>6.8</td>
<td>0.5</td>
<td>10.4</td>
<td>17.7</td>
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<tr>
<td>2/11</td>
<td>5</td>
<td>4</td>
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<td>7.2</td>
<td>0.5</td>
<td>9.9</td>
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</tr>
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<td></td>
<td>Suspended Operations</td>
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<td>12.9</td>
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<td>7.81</td>
<td>10.8</td>
<td>0.3</td>
<td>7.9</td>
<td>19.0</td>
</tr>
</tbody>
</table>

*Sum of NH₃-N, NO₂-N, and NO₃-N
As seen in Table 4.7, the pH of the feed solution remained close to neutral during Phase-3 of this study. Testing of the lysimeter samples revealed similar pH values as the previous summer and fall tests. The subsurface samples remained in a slightly acidic range above 6.0, which should not have an inhibitory effect on the denitrifying activity of the soils. The TSS showed some variation in the feed solution, with the highest values in January and February. However, these loads were much lower than during Phase-2, meaning pore clogging was less of a concern.

As in earlier phases, total nitrogen was determined by the sum of the nitrogen species listed in the table, and was used as the nitrogen load value during the analysis of RIB samples. High concentrations of total nitrogen were maintained during the entire duration of the phase, and a maximum value was reported in early March following the two week shutdown period. The nitrite concentration represented a small fraction of the overall nitrogen balance. Organic nitrogen again had only a minor impact on the total nitrogen value, and was neglected.

As mentioned earlier, the ammonia contribution increased over time, with the nitrate concentration decreasing in a corresponding fashion. Ammonia had not been present in the feed solution during Phase-2, and lysimeter samples had shown nearly complete oxidation of ammonia at both lysimeter depths during Phase-1. However, this nitrification was not complete during Phase-3 of the loading experiments as some ammonia was still present in the lysimeters. Pooled ammonia samples taken from each sampling depth are presented in Figure 4.7 for the five most complete datasets.
Figure 4.7. Unoxidized Ammonia in Lysimeter Samples (Phase-3)
Each bar represents an average concentration of ammonia at the given depth across all of the RIBs. The bars represent pooled data with at least one replicate from RIB-1, RIB-2, and RIB-3 (whenever available). This pooling should give a more accurate representation for the average concentration of nitrate at the listed depths. This combination of data across the different RIBs is reasonable because the process of nitrification should not be affected by the mass of woodchips added to the soil. In nitrification, the electron donor ammonia is coupled with oxygen. Carbon is not utilized as an electron donor, so the woodchip treatment in the soil should not have an effect on the observed nitrification. The concentration of ammonia remained high in the 3-foot lysimeters, but was completely (or nearly completely) removed by the time it reached the 6-foot sampling depth.

This fate of ammonia is important for characterizing the treatment efficiency of the field system. While low nitrate concentrations were observed in the 3-foot sampling depths, it is important to note that much of the ammonia in the feed water had not been oxidized. The 6-foot depth had negligible concentrations of ammonia, indicating that complete oxidation of the ammonia fraction had occurred by the time the feed solution percolated to the deeper lysimeters. As seen in Figure 4.7, the 6-foot samples taken on February 2 and March 11 showed some evidence of ammonia, but these bars were each effected by an outlying data point. More specifically, 13 of the 15 tested lysimeters at the 6-foot depth had less than 0.6 mg/L of ammonia, and values were typically in the range of 0.0 to 0.3 mg/L (below the recommended detection limit for the analytical method).

Since the concentrations of ammonia in the 6-foot samples were considered to be insignificant, the data are shown as in Phase-1 and Phase-2, with the ammonia
concentration in the lysimeters ignored. However, the ammonia fraction in the 3-foot samples was clearly substantial. The average ammonia concentration measured in the samples is included in the treatment figure. The ammonia remaining in the samples could be oxidized to nitrate as it infiltrated within the basin, and represents additional nitrate to that which was directly measured. Although only the five most complete datasets are shown in Figure 4.7, lysimeter replicates were also analyzed for ammonia in the other three cycles to give an accurate representation during the entire phase. Ammonia was only found to be negligible during the first loading cycle, when the concentration in the feed was also the lowest.

The average nitrate concentrations during the Phase-3 winter loading period are shown in Figure 4.8 for the 3-foot samples along with underlying bars which represent the measured ammonia concentration. Figure 4.9 presents nitrate data from the 6-foot samples, with the ammonia contribution omitted. Nitrite measurements were minimal compared to the nitrate and ammonia values. The error bars represent the sample standard deviation for the nitrate concentrations in the lysimeter replicates. The absence of error bars for a RIB average indicates that only 1 lysimeter sample was recorded for that sampling date.
Figure 4.8. Nitrate Concentration in 3-Foot Sampling Depth (Phase-3)
Figure 4.9. Nitrate Concentration in 6-Foot Sampling Depth (Phase-3)
During the first cycle of Phase-3, there did not seem to be significant startup issues compared to those seen in the previous two phases. The five most complete datasets (as seen in Figure 4.7) were not clearly differentiated by the woodchip treatment during the winter experiments. In the 3-foot samples, the treatment was moderate. In January and during late March, the overall removal of nitrogen was relatively low, but better removal was observed during early March when the nitrogen load was highest in the feed water. The treatment efficiency was still much lower than that observed in the 6-foot samples. Surprisingly, each of the basins showed very strong treatment in the deeper sampling ports regardless of the woodchip volume, with over 50% removal regularly observed. The RIB performance was especially notable near the end of the Phase-3 when the nitrogen loads were highest.

While direct comparison between the two depths is difficult due to the ammonia contribution, it appears that the overall total nitrogen was significantly lowered between the 3-foot and 6-foot depths. Nearly all of the ammonia was nitrified within this depth range, and subsequent denitrification was observed. One reason for low nitrification activity above the 3-foot depth may be due to daily and seasonal temperature variation in the soil. At depths greater than 0.5 to 1 meter, the ground temperature is generally stable across short term temperature changes, and seasonal temperature fluctuations are mitigated (Florides and Kalogirou, 2004). Nitrification is a temperature dependent process, and the warmer soils between 3 and 6 feet may have facilitated nitrification (and denitrification) within this range. The ammonia concentration in the 3-foot lysimeters increased over the duration of the experiment, corresponding to the feed concentration.
Besides impacting the treatment efficiency at 3 feet, the cold winter temperatures created operational problems for sampling and loading. Sustained cold periods caused a thick ice layer (>2 inches) to form on the water surface. As the basins drained from below, the ice damaged several lysimeter caps which lost samples and needed to be serviced. Although the infiltration ability did not seem to be impacted by the ice, the loading apparatus could have potentially been damaged. Due to the ice formation two loading cycles were cancelled. The operational interruptions were relatively easy to manage with the short loading schedule, but an extended loading (i.e. Phase-2) would be more difficult to schedule during the winter.

A statistical analysis was performed only on the 6-foot data for this phase of the study. It was not reasonable to apply the pooled ammonia average to the individual lysimeter measurements in the 3-foot lysimeters, and this dataset was also more incomplete. As in the previous two phases, a two way ANOVA with replication was used on the individual lysimeter percent removals. Only the five most complete datasets were included in this analysis. The percent removal measurements for each of the 6-foot wells are shown in Table 4.8. Measurements marked with an asterisk (*) are interpolated values, as explained in Chapter 3. The full set of removal efficiencies and the trend line used for filling the missing values can be seen in Figure A.5.
Table 4.8. Percent Removal Measurements for 6-Foot Depth (Phase-3)

<table>
<thead>
<tr>
<th>Location</th>
<th>Cycle 2</th>
<th>Cycle 3</th>
<th>Cycle 4</th>
<th>Cycle 7</th>
<th>Cycle 8</th>
</tr>
</thead>
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<tr>
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<td>30</td>
<td>64</td>
<td>57</td>
</tr>
<tr>
<td></td>
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<td>55</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>27</td>
<td>28</td>
<td>62</td>
<td>53</td>
</tr>
<tr>
<td>RIB-2</td>
<td>17</td>
<td>28*</td>
<td>26</td>
<td>66</td>
<td>46</td>
</tr>
<tr>
<td></td>
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<td>22</td>
<td>28</td>
<td>33</td>
<td>73</td>
<td>64</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
<td></td>
<td>16</td>
<td>23</td>
<td>20</td>
<td>54</td>
<td>40</td>
</tr>
</tbody>
</table>

The results of the two way ANOVA confirmed that the removal efficiencies were similar between the different basins, but differed across the operation cycles. The interaction term was not statistically significant for the 6-foot depth (p=0.487). In looking at the main effects for the percent removal data, the effect of the cycle was found to be highly significant (p<0.0001), but the effect of the RIB identity was not important during this phase (p=0.378). The data show that the treatment efficiency of the basins increased over time, but the basins showed similar nitrate concentrations across all of the woodchip treatments.

Each RIB showed similar removal ability, and the basins did not convincingly differentiate by the woodchip volume incorporated into the soil. The persistence of ammonia in the 3-foot sampling depth was probably caused by low temperatures near the soil surface which inhibited microbial activity. While overall removal was moderate in the 3-foot depth, the deeper samples showed high removal efficiencies (often above 50%), which improved during sequential cycles.
4.4 Laboratory Column Operation

Laboratory columns were constructed in July of 2014 to simulate the consumption of woodchips in the RIB field site. With the carbon amendment supporting denitrification by subsurface microbes, field woodchips are expected to disappear over time and eventually need replacement. Three columns were constructed to correspond to RIB-1, RIB-2, and RIB-3. Each of these columns contained 12 inches of soil amended with a prescribed volume of woodchips and an overlying 15 inches of unaltered soil. Column-1, Column-2 and Column-3 contained 10%, 20%, and 30% woodchips by volume in those 12 inches. The columns removed significant amounts of nitrate from the feed solution, which consisted of a mixture of lagoon effluent and synthetic nitrate. After an acclimation period, the performance of the columns became clearly differentiated according to the volume of woodchips in the soil mixture.

While the RIBs were operated on a weekly or semi-weekly schedule, the columns were operated continuously during the week. This operation schedule allowed the columns to process a much larger volume of water per unit surface area, thus aging the woodchips prematurely. The increased exposure to nitrate simulated an accelerated version of the field system, as the woodchips were exposed to several times the equivalent volume of the field system. The increased loading rate also created a shorter residence time for nitrate removal compared to the slower draining field system. Even during the early weeks of testing, the columns were able to remove appreciable amounts of nitrate from the influent, as shown in Figure 4.10.
Figure 4.10.  Acclimation Period for Column Experiments
During the acclimation period shown in Figure 4.10, all three of the columns removed a similar amount of nitrate. The columns were able to remove substantial amounts of nitrate but the treatment did not differ by the volume of woodchips added to the soil. In the early samples, microbial populations were being established within the columns under the nutrient rich feeding conditions. Column-3 contained the most woodchips, and therefore should support the largest population of denitrifiers. However, the growth of microbes took time because the column matrix was starting from nearly sterile conditions, unlike the field site. Even though the high loading rates of nitrate into the columns should increase the rate of carbon consumption, the denitrifiers needed time to colonize. In other words, the increased loading of nitrate would not accelerate the establishment of denitrifiers in the columns.

Unlike the field study, there was no evidence of startup effects and spikes of nitrate early in the study. This supports the hypothesis that excess nitrate in the early lysimeter samples was caused by additional field inputs such as plant decomposition or nitrate carried over from past studies. It is unlikely that the woodchip amendments were the source of the nitrate spikes seen in the field samples, because they showed no impacts in the columns. Although the nitrate removal was notable during these early weeks, greater removal was anticipated as microbial density increased. Removal performance improved drastically in the following weeks of operation, as seen in Figure 4.11. This figure represents a longer time series of operation under variable nitrate loading conditions.
Figure 4.11. Variable Loading Period for Column Experiments
After the acclimation period and development of denitrifying communities in the columns, the removal of nitrate from the laboratory columns followed the expected trends of nitrate removal. As seen in Figure 4.11, the woodchip addition in the columns had a clear impact on the extent of treatment. Nitrate removal was the most substantial in Column-3, with Column-2 and Column-1 following in treatment efficiency. This trend was maintained across the entire range of sampling days, indicating a strong relationship between the availability of the carbon source and subsequent denitrification. While Column-1 and Column-2 typically removed 15 to 60% of nitrate, Column-3 performed significantly better and regularly denitrified greater than 90% of the nitrate present in the feed solution. This implies that greater soil amendments may be necessary to achieve the desired level of nitrate removal in the field system. Heterogeneity in the field would certainly decrease the effectiveness of the treatment compared to the controlled conditions of the laboratory.

In addition to demonstrating the denitrification potential of the woodchips, the observed data also established the capability of the treatments under highly varied loading conditions. As seen in Figure 4.11, the feed solution varied by nearly 15 mg/L of nitrate. Despite the fluctuations, the treatment efficiency was sustained at a high level, especially in Column-3. Column operation continued until December 20, 2014, when the column experiments were shut down temporarily.

It is important to note that most of the column sampling during this period occurred after two weeks of steady loading. Some denitrification could have occurred in the feed reservoir itself during this time interval, meaning the average feed concentration was actually higher than the value reported. Denitrification in the feed tank was investigated during late December, as shown in Figure 4.12.
Figure 4.12. Feed Denitrification During Column Experiments
The investigation in late December showed possible denitrification in the column feed reservoir during the study. This removal was likely due to denitrifying microbes which were attached to the inner surfaces of the feed tanks. Low oxygen conditions were established in the tanks by the daily bubbling of nitrogen through the feed solution, which stripped excess oxygen from the liquid. The carbon source necessary for denitrification would have been provided as organic compounds present in the diluted lagoon effluent, or else the microbes may have relied on endogenous decay for energy sources. (Endogenous decay is the oxidation of internal storage molecules or cell tissues.)

The denitrification in the feed tank suggests that the measured nitrate values in the feed solution may not represent the actual loading concentration. The average concentration of nitrate in the column feed may have been several mg/L higher than the measured concentration, since denitrification occurred during the two weeks of steady operation. Based on observation of the sampling dates and measured feed values, loss of nitrate in the feed tank was probably not an issue early in the experiment but may have become more severe over time. During late December, the columns were thoroughly cleaned and dried, resetting this cycle and temporarily eliminating feed denitrification. After the winter resting period which lasted until early February, the columns quickly regained their treatment ability to previous levels. This restart period is shown in Figure 4.13. The figure continues through the final stages of column operation.
Figure 4.13. Restart Period and Final Stages of Column Experiments
After seven weeks of inactivity, the columns again began to receive feed solution in early February. As seen in Figure 4.13, the performance of the columns was immediately differentiated by the woodchip amendment. The columns quickly regained their previous treatment capacities, although there was an initial delay. The first sampling date had a reduced performance compared to the pre-winter nitrate removal, but the removal improved rapidly. This preservation of treatment capacity after long periods of inactivity had implications for the field system. It suggested that periods of inactivity due to maintenance such as surface scraping or periods of low wastewater demand should not cause a serious decline of the system. The initial establishment of the microbial communities took a longer amount of time, but the reactivation of the denitrification metabolism seemed to be quicker. This may be true for disturbances lasting well over a month. However, the data does suggest that continuous operation will keep the system performing at the highest level.

The nitrate removal efficiency of the system was maintained or improved over the duration of the experiment (with a temporary decline in early February after the winter shutdown). However, at the first sampling date in April the removal efficiency in each of the columns decreased for the first time. The reduction in performance was most acute in Column-3, which fell from greater than 95% nitrate removal to just over 50% removal. The decline in performance of Column-2 was also severe during this time period. The subsequent sampling date showed that the decline in the treatment efficiency was consistent, as an additional week of operation did not show treatment recovery. Treatment would have been expected to recover if the initial decline had been a result of some disturbance such as a toxic shock to the column due to residual chlorine or other compounds. The replenishment of the feed solution would have
alleviated this condition and allowed the microbial biomass to recover. Column-1 and Column-2 decreased even further during this extra week of operation, and Column-3 remained at a similar removal efficiency (well below the normal level).

The decline in nitrate removal efficiency could indicate that the energy source provided by the woodchips had become depleted by this point in the study. While some resistant material might have still been present in the columns, the concentration of readily bioavailable carbon compounds may have dropped to a limiting value. It is important to note that each of the columns began to reach carbon exhaustion at a similar point in time. The larger volume of woodchips present in Column-3 would support the largest denitrifying community, which would in turn consume the woodchips at a faster rate. While Column-1 supported the smallest population, it also experienced the slowest rate of nitrate reduction. If the size of the microbial population was limited by the woodchip volume (or surface area in particular), we would expect each of the columns to become drained of carbon in a similar period.

COD measurements were used as a rough indicator of either carbon or nitrate limited conditions. These COD tests had narrow value, because effluent measurements are not representative of the conditions within the columns. However, we would expect the COD to drop near 0 mg/L if bioavailable carbon was fully exhausted in the columns, and we would expect a high COD if nitrate was limited in the system (i.e. near 100% removal of nitrate). Several samples were analyzed near the end of column operation to characterize the carbon conditions in the effluent. The results of this late-term COD analysis are presented in Figure 4.14.
Figure 4.14. COD Concentration in Column Effluent During Final Stages
As seen in Figure 4.14, the COD values did not correlate with the expected trends for nitrate removal in the final stages of column operation. The data points in the COD figure correspond to the sampling dates shown in Figure 4.13. On April 13, we would expect the COD to be very high in Column-2 and Column-3 because of the low nitrate concentration remaining in the effluent. The system would have been nitrate limited, with excess carbon passing through the column. In fact, Column-1 had the highest COD on this date. The nitrate removal in this column may be limited by the microbial biomass rather than the carbon concentration. As performance decreased in April, we expected the COD to drop abruptly in Column-3. However, the measured value was actually highest in Column-3 over the final two sampling dates. Overall, the COD measurements seemed fairly scattered and random between the different columns. COD tests do not give any information about the bioavailable fraction of the carbon in the sample, since all of the compounds are oxidized chemically during the analysis. The carbon measured in the COD test could represent a fraction of the woodchips which could not be used by the denitrifying microbes. Bioavailable material may have reached a limiting value.

Assuming that this is the case, the decrease in nitrate removal performance allowed us to roughly predict a schedule for the replenishment of woodchips in the field RIB system. Due to the denitrification within the feed solution, it became difficult to accurately compute the mass of nitrate removed during the column lifetime. The infrequency of sampling dates in two week intervals meant the nitrate loading experienced during the full operation period was not catalogued. However, it can be seen that the nitrate concentration in the column feed was generally higher than the total nitrogen in the RIB feed water (See Table 4.1, Table 4.4, and Table 4.7).
This is particularly true given that denitrification occurred in the feed tanks prior to sampling. Furthermore, the removal efficiency was much higher in the columns, meaning carbon was depleted at a much higher rate than in the field where denitrification was less effective. The columns were characterized in terms of the volume of water which was treated, a value which should be conservative for predicting the treatment lifetime of the woodchips.

Each laboratory column was operated from July 16 until November 17 at a rate of 1.35 gallons per week. The flowrate was subsequently adjusted upward to roughly 1.62 gallons per week for the remainder of the study (excluding the winter shutdown). Accounting for these flowrates and some schedule variations, each column processed approximately 41 gallons during the experimental period from startup until the performance drop-off in early April. Adjusting for the infiltration surface area of the columns (1.67 square inches) compared to the field system (1,500 square feet), this equates to a RIB processing 5.3 million gallons before treatment reduction.

Accounting for the resting phases, the overall loading rates were similar across each of the experimental seasons. Phase-1, Phase-2, and Phase-3 each received between 5,810 and 5,860 gallons per day. At these rates, the 5.3 million gallon treatment capacity is equivalent to over 900 days of RIB operation. In the field, it may be necessary to replace woodchips every 2 to 3 years in order to maintain available carbon sources for denitrification. As discussed earlier, this estimate was based on the volume of wastewater rather than the overall nitrogen mass removed, and may vary based on the field loading conditions. A longer interval between woodchip replacements may be feasible if carbon is depleted more slowly in the field system.
4.5 **Groundwater Monitoring**

In accordance with the monitoring requirements listed in the RIB permit, groundwater samples were obtained periodically from the monitoring wells located at the field site. Groundwater flowed from the west end of the site near RIB-3 towards RIB-0 at the east end. Well-3 was the most up-gradient well, Well-2 was located in the middle of the site, and Well-1 was the most down-gradient well. The layout of the monitoring wells can be reviewed in Figure 3.1. The wells were designed to sample pure groundwater as it entered the site and effluent water which travelled off-site.

The goal of the groundwater monitoring was to determine whether the groundwater was impacted by the overlying infiltration system. Depth to groundwater measurements were recorded to document any groundwater mounding due to the RIB loading. The wells were monitored for pH and all of the nitrogen species previously discussed. The ammonia, nitrite, and nitrate species were summed to determine the total nitrogen of each well sample, in addition to an independent measurement of total nitrogen. The difference between the measured total nitrogen and calculated total nitrogen represents the organic nitrogen content of the samples. As with feed and lysimeter samples, the values of organic nitrogen were relatively small (either positive or negative), indicating small errors in the analytical methods. Nitrate was the primary nitrogen compound in every case. The concentrations of the measured nitrogen species and other groundwater parameters are listed in Table 4.9.
Table 4.9. Groundwater Monitoring Parameters

<table>
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<th>Experimental Phase</th>
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<th>Sample ID</th>
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<th>NH₃-N (mg/L)</th>
<th>NO₂-N (mg/L)</th>
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<th>Total N (mg/L)</th>
<th>Total N (mg/L)</th>
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</thead>
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<td>6.07</td>
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<td>0.0</td>
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*Sum of NH₃-N, NO₂-N, and NO₃-N
In every set of groundwater monitoring data, the depth to the water table was the shallowest in the middle of the site, indicating that Well-2 was impacted by RIB-1 and RIB-2 which were both located directly adjacent to the well. As water infiltrated, it altered the hydraulic gradient of the native groundwater. A linear gradient was drawn between the groundwater depths listed for Well-3 and Well-1, giving a prediction for the groundwater depth in the middle of the site without any loading to the infiltration basin. The observed depths to groundwater were 2.0 to 2.5 feet higher than expected, indicating some moderate mounding effects. It is also noteworthy that the well samples were gathered generally 2 to 5 days following the RIB loading. This means the mounding effects may have been more severe than observed in the depth measurements, but dissipated within this time frame.

Well-3 was intended to intercept upstream groundwater travelling onto the site, but may have been moderately impacted by mounding near RIB-3. It is possible that some effluent from beneath the RIB traveled up-gradient and affected the measured values. The effects appear to be relatively small, considering the low total nitrogen concentrations observed in the well across all of the sampling dates, with values ranging between 0.4 and 1.5 mg/L.

Well-1 initially showed the highest impacts from the RIB system, evident in the maximum total nitrogen concentration observed on July 31, 2014. This well would have been impacted by all of the upstream RIBs, but the primary reason for the severe impacts on this date was likely its proximity to RIB-0, which was operated during Phase-1 only. This RIB showed high nitrate concentrations in the 6-foot lysimeter samples in late July, as seen in Figure 4.2. Since RIB-0 did not receive a woodchip amendment, electrons donor would not be available below the 6-foot depth,
and no further treatment would be likely. Once the operation of RIB-0 was discontinued during Phase-2 and Phase-3, the two down gradient wells had comparable total nitrogen values.

Well-2 was located between RIB-1 and RIB-2, and likely had effects from both of these basins as demonstrated by the moderate groundwater mounding previously discussed. The concentrations of total nitrogen in this monitoring well ranged from 1.9 to 6.1 mg/L. These values are significantly lower than the concentrations observed in the 3-foot and 6-foot lysimeter samples from the adjacent RIBs over the duration of the study. It is expected that dilution in the native groundwater accounted for the observed concentrations, but it is also possible that further treatment by denitrification occurred below the 6-foot depth before the effluent reached the groundwater. As shown earlier, the treatment often improved between the 3-foot and 6-foot sampling range, meaning further nitrate removal is probable at greater depth. With the groundwater located 10.2 to 12.2 feet below the ground surface, the residence time in the full soil profile is nearly twice as long as in the 6-foot samples.

The pH of the samples tended to be higher in Well-1 and Well-2, which may have been a result of the denitrification reaction affecting the soil or may have been caused by natural variability between the well locations. The pH differences were not extremely large between the samples, and the values remained within a reasonable range for microbial activity to proceed. Nitrite was never detected in the samples, and only one sample in July showed significant ammonia. This implies that all of the nitrogen constituents were fully nitrified before reaching the water table, and there is potential for further denitrification.
4.6 Microbial Investigation

As previously detailed, soil samples were obtained from each basin during May 2014 and December 2014 to give a representation of the microbial communities both before and after the loading with lagoon wastewater. The microbial gel analysis showed evidence of denitrifiers present in the field system. RIB-1 showed two faint bands in the narG amplified region of the gel (one before loading, one after). The RIB-2 gel contained three clearly amplified bands (one before loading, two after). There may have been some evidence of amplicon in other samples from RIB-2, but the resulting bands were too weak to recognize definitively. Unlike the other two basins, RIB-3 did not show any amplicon using the narG primers. Since the PCR and staining were successful in the other two gels, this absence of bands was probably due to a variety of biases in the soil sampling and DNA manipulation (discussed later).

No bands were visible in the regions of the gel containing the samples amplified with the universal microbial primers. This lack of PCR products is likely due to errors in the reaction protocol. Since the universal primers contain some mismatched base pairs (nucleotides that do not match the exact microbial sequence due to natural variation), a lower annealing temperature may have been necessary to amplify the full set of environmental species represented in the DNA extraction. In addition, the reduced number of cycles in the universal amplification (30) compared to the narG amplification (45) may have produced too low of a nucleic acid concentration to be seen by the staining process. Due to the absence of universal bands across all of the RIBs and sampling dates, these regions of the gel were excluded in the enhanced images. Figure 4.15 and Figure 4.16 show the stained gel images for the narG amplification of the RIB-1 and RIB-2 replicates, respectively. The DNA ladder served only as a positive indicator of the staining procedure.
Figure 4.15. RIB-1 Gel Image (PCR Amplification of narG)

Figure 4.16. RIB-2 Gel Image (PCR Amplification of narG)
The two gel images could not definitively show an enrichment of denitrifiers within the basins. The number of positive results was similar both before and after the periods of loading. Comparing band strengths is not a reliable quantitative measure, and regardless there were no noticeable differences between the observed brightness of the bands before and after treatment. Perhaps the most interesting result was the lack of bands in the gel run with the RIB-3 soil extracts and several of the December replicates taken in the other basins. This lack of band appearance was most likely due to sampling bias in the original soil cores. It was hypothesized that denitrifying microbes would become more enriched deeper within the soil, and the lysimeter nitrate samples supported this expectation. The microbial soil sampling was performed using a hand agar which only collected soil from upper layers.

The growth of denitrifiers would be limited due to the shallow depth (although some enrichment would still be expected). Furthermore, the small sample that was used for the DNA extraction, about 0.25 grams, was unlikely to be representative of the bulk soil. This necessitated the gathering of triplicate samples from each RIB, but biodiversity could still be missed if denitrifiers were not a dominant fraction of the overall biomass. Although the density of denitrifiers near the surface would be expected to be low, they could propagate in anaerobic microenvironments. Since denitrification is carried out by facultative anaerobes, these microbes would be able to survive in an oxygen-rich environment, although the denitrifying metabolism would not be expressed. This could explain the positive identification of denitrifiers prior to the first loading cycle. Since denitrifiers are widespread in the natural environment, this finding was anticipated.
Another issue with the microbial analysis may have resulted from the DNA processing. Natural humic acids or ethanol from the extraction process could have interfered with the amplification reactions. Again this would have affected the samples on an individual basis, so some samples could have failed to amplify while others went unaffected. Since there were several potential biases in this analysis, a more complete microbial study would be beneficial. Samples would be more representative if they were extracted from a greater depth, and PCR protocols could be improved to attain a better amplification. As an extension, sequencing tests would allow for the documentation of the microbial populations responsible for denitrification in the system. This could provide an opportunity to further optimize the field basins to become more favorable to specific communities, consequently enhancing treatment.

Overall, the gels succeeded in showing the presence of the denitrification metabolism in the soil samples. The positive band formation showed that the PCR amplification of the narG gene was effective in several samples, meaning the selective primers, PCR reaction mixture, and thermocycler program can be used in additional studies. While the microbial analysis had limited value in this study, it could be beneficial to characterize the microbial community in order to verify the cause of the observed nitrate removal. The treatment efficiency of the RIBs might also be improved with a fuller understanding of the microbial activity in the system.
Chapter 5
CONCLUSIONS AND RECOMMENDATIONS

The overall goal of this study was to demonstrate the woodchip-amended RIB system as a practical treatment option to remove nitrate from lagoon effluent before it percolated to the groundwater table. Two main elements of this study were the woodchip-amended field system and corresponding laboratory columns. Although the pilot-scale system did not remove as much nitrate as the column studies, significant denitrification was still observed. This was particularly true in RIB-3, which was consistently able to remove several mg/L of nitrate. The results were successful in supplementing current research on the use of RIBs for denitrification treatment.

The column results clearly showed the effect of different woodchip volumes on the treatment efficiency. Column-3 regularly denitrified greater than 90% of the nitrate in the feed solution. The column study provided strong evidence that increased woodchip amounts improve the treatment performance by providing electron donors. Over all experimental phases, the nitrate removal was lower in the field than in the laboratory columns. Soil is a highly heterogeneous environment, and the distribution of woodchips in the upper soil layer did not provide a uniform infiltration area. While there was large variability in treatment efficiencies, the correlation between woodchip mass and nitrate removal was demonstrated during the summer and fall field tests.

The 6-foot samples generally had more efficient removal of nitrate than the shallower samples. Since woodchips were placed in the upper 12 inches of soil, this indicated that electron donors from the woodchip layer could migrate downward in the
soil profile and provide metabolic substrates to denitrifying microbes below the 6-foot lysimeter ports. This creates further protection for the natural groundwater, as the residence time for the lagoon effluent would increase nearly twofold between the sampling ports and the water table.

Surprisingly, the basins all showed similar treatment at the 6-foot depth during the winter testing, as all of the RIBs achieved high nitrate removal efficiencies. One possible reason for the higher nitrate removal may be the reduced infiltration rate observed during the winter. The lowered drainage characteristics would provide a longer residence time for microbial activity.

The reduced infiltration rate observed in RIB-3 may have indirectly added to the treatment capacity of the basin by increasing the hydraulic residence time. While the longer draining time caused some operational problems, the basin was always dry before the start of the next loading cycle. The infiltration rates of all basins may be increased by occasional scraping of the soil surface to remove clogging materials such as algal deposits, although the nitrate removal performance might also be affected.

In order to investigate the effects of different loading schedules, two short loading periods (Phase-1, Phase-3) and one extended loading period (Phase-2) were tested. There was a lack of conclusive evidence that the treatment efficiency was improved by elongating the loading cycle. The increased resting time may have been excessive for maintaining the denitrification metabolism in the subsurface. As seen in the laboratory column studies, the performance temporarily declined following the winter shutdown period. This implied that shorter resting times may be advantageous.

During the winter, decreased soil temperatures caused a reduction in ammonia removal close to the surface. Due to the reduced microbial activity, much of the
ammonia in the feed solution was conserved in the 3-foot samples, but the ammonia was nearly fully oxidized before reaching the lower sampling depth. This suggested that ammonia would be unlikely to impact groundwater quality, even in the winter. All of the basins showed high nitrate removal in the 6-foot samples and demonstrated the capability of the RIBs to be operated during the full range of seasonal variation.

From a maintenance and operation standpoint, a shorter loading cycle such as that used during Phase-3 may be beneficial during the winter. There were some concerns about damage to the loading apparatus as a result of ice formation. A shorter cycle during winter operation could be easily adjusted if weather conditions were expected to drop significantly below freezing. Extended loading cycles would be difficult to modify around freezing conditions.

Groundwater monitoring data showed that nitrate was the main nitrogen species that reached the water table. Nearly all of the ammonia and nitrite were removed by the infiltration process. The measured nitrate concentrations were significantly lower than those observed in the lysimeters. The lower concentrations may be attributed to dilution with the native groundwater and further treatment by denitrifying populations below the 6-foot sampling depth. There was some groundwater mounding evident from the well depth measurements. In every case, the depth to the water table was the shallowest in the middle of the site, indicating some moderate effects from the loading volume.

The column studies were not fully conclusive in determining the exhaustion of the woodchips, but performance decreased in early April (after 150 operational days). While COD measurements of the effluent did not characterize the depletion of bioavailable carbon within the columns, the decrease in denitrification suggested that
the amended woodchips were approaching their limit. By scaling up to the area of the field-system, each column processed a volume equivalent to over 900 days of RIB loading. A replenishment of woodchips every 2 to 3 years may be necessary in order to maintain bioavailable carbon sources for denitrification. A longer interval between woodchip replacements may be possible if carbon is depleted more slowly in the field.

Based on the findings of this study, the recommendations for operation of the woodchip-amended RIB system are as follows:

- Inclusion of 30% woodchips by volume in the soil surface. 
  *Increased amounts of woodchips in the soil were shown to support a higher denitrification capacity in both the soils underlying the basins and the laboratory columns.*

- Use of a short loading cycle (such as the Phase-3 schedule). 
  *The short loading cycles performed equally well or better than the extended loading schedule used during Phase-2. The shorter loading cycles may be easier for maintenance and operation.*

- Avoidance of basin loading/ponding during freezing temperatures. 
  *The short loading period can be easily scheduled around cold temperatures to avoid forming excessive layers of ice, which could damage the loading and sampling apparatus during the winter.*

- Replenishment of soil woodchips (≥ 12 inches) every 2 to 3 years. 
  *The column studies suggested that bioavailable material from the woodchips may become limiting after roughly 900 days of scheduled operation. This estimate may be conservative, and woodchip depletion may be slower in the field system.*

- Scraping of the soil surface to remove fines (when necessary). 
  *Algal deposits, sediments, and microbial biomass may clog the surface of the basins over time, reducing the infiltration rate below an acceptable level.*

- Continued sampling and extension of the groundwater well system. 
  *Monitoring of the local groundwater quality is necessary for identifying any major impacts below the basins.*
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Appendix

SUPPLEMENTARY FIGURES

Figures showing the percent removal measurements of the individual lysimeter ports during Phase-1, Phase-2, and Phase-3 are included in the following pages. These figures were used for the estimation of missing lysimeter values for inclusion in the statistical analysis, as explained in the relevant sections of Chapter 3 and Chapter 4. Phase-1 and Phase-3 were fitted using linear fits with the available data points, and the Last Observation Carried Forward methodology was primarily used to model missing values from Phase-2.
Figure A.1. Percent Removal and Linear Fits for 3-Foot Depth (Phase-1)
Figure A.2. Percent Removal and Linear Fits for 6-Foot Depth (Phase-1)
Figure A.3. Percent Removal for 3-Foot Depth (Phase-2)
Figure A.4. Percent Removal for 6-Foot Depth (Phase-2)
Figure A.5. Percent Removal and Linear Fits for 6-Foot Depth (Phase-3)