University of Nevada, Reno

Improving denitrification bioreactor efficacy on the microbial, property, and regional scales

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by

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Abstract

The Santa Fe River watershed in North Central Florida has a significant nitrogen contamination problem that is causing eutrophication in both the river and the many springs in the area. The watershed is dominated by agriculture, which has been identified as the main source of the eutrophication. One solution is to build a denitrification bioreactor or denitrification wall that removes nitrogen in situ. The objective of this study was to install a 320-meter denitrification wall to remove property-scale nitrogen loads coming off of a commercial nursery. This study analyzed efficacy of this reactor on three different scales: on the microbial level to understand of community composition and to develop optimization strategies; on the well transect level to understand the reactor’s impact on water quality, and regionally using GIS to identify suitable locations where these walls could be placed. Based on water quality analysis from 6 well transects, nitrate-nitrogen levels decreased, on average, from 7.1±3.2 mg/L to 1.3±0.6 mg/L, which is a nitrate load reduction of 3,000 kg-N/yr. The reactor is also cost effective with a total installation cost of $50,000, which is $1.73/kg-N over an estimated 20 year lifespan. This wall had lower overall removal efficiency rates around 70-75% compared to previous reactors that had removal efficiencies of 90% and above, which was likely due to lower hydraulic conductivity in this wall. The installation of the wall caused a 6% increase in microbial diversity and was largely dominated by anaerobic genera, including known denitrifiers. Additionally much of the variability was explained by environmental factors like total organic carbon, nitrate concentrations, and conductivity. The spatial assessment tool identified close to 40 square kilometers across the Santa Fe River watershed as suitable locations.
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# Table of Contents

Abstract ................................................................................................................................. i
Acknowledgements .................................................................................................................. ii

## Chapter 1: Introduction
The Problem With Nitrogen................................................................................................. 1
Nitrogen and the Nitrogen Cycle ......................................................................................... 2
Nitrogen Treatment Options ............................................................................................... 7
Site Background .................................................................................................................... 13

## Chapter 2: Efficacy of a Property Scale Denitrification Wall
Holly Factory Denitrification Wall ......................................................................................... 14
Methods ............................................................................................................................... 16
Wall Construction .............................................................................................................. 16
Wall Monitoring ................................................................................................................. 18
Load and Cost Calculations ............................................................................................... 19
Data Management and Statistical Methods ....................................................................... 20

## Chapter 3: Impact of Excavation and Carbon Addition on Native Microbial Communities
Introduction ......................................................................................................................... 42
Hypotheses ............................................................................................................................ 48

## Chapter 4: Development of a Spatial Tool for Denitrification Tool Placement
Introduction ............................................................................................................................ 64

## Chapter 5: Conclusion


Overall Effectiveness ................................................................. 73
Further Work .......................................................... 75

Appendix .................................................................................. 78
Nitrate Statistics ........................................................................ 78
Ammonia Statistics .................................................................. 82
Total N Statistics ..................................................................... 84
Organic Nitrogen Statistics....................................................... 88
Total Organic Carbon Statistics ................................................ 91
Dissolved Oxygen Statistics ...................................................... 95
pH Statistics ............................................................................. 97
Shannon Diversity .................................................................. 101
RDA .......................................................................................... 104
All Data ................................................................................... 104
Only 2018 RDA ......................................................................... 105

References ............................................................................... 106
Table of Tables

Table 1: Average YSI Probe Values .................................................................34
Table 2: Cost Analysis Table for Nitrogen Removal Options ..........................37
# Table of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Denitrification wall schematic</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Hydraulic Conductivity across the wall</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>The hydraulic loading rate of the treatment sections</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>Nitrate concentrations in the treatment section</td>
<td>23</td>
</tr>
<tr>
<td>5</td>
<td>Nitrate concentrations in the control sections</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>Ammonia concentrations in the treatment sections of the wall</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>Ammonia concentrations in the control sections of the wall</td>
<td>26</td>
</tr>
<tr>
<td>8</td>
<td>Organic Nitrogen in the treatment sections</td>
<td>27</td>
</tr>
<tr>
<td>9</td>
<td>Organic Nitrogen in the control sections</td>
<td>27</td>
</tr>
<tr>
<td>10</td>
<td>Total Nitrogen concentrations in the treatment sections</td>
<td>29</td>
</tr>
<tr>
<td>11</td>
<td>Total nitrogen concentrations in the control sections</td>
<td>29</td>
</tr>
<tr>
<td>12</td>
<td>Total organic carbon concentrations in the treatment sections</td>
<td>31</td>
</tr>
<tr>
<td>13</td>
<td>Total organic carbon concentrations in the control sections</td>
<td>32</td>
</tr>
<tr>
<td>14</td>
<td>Dissolved Oxygen Concentrations in the treatment sections</td>
<td>33</td>
</tr>
<tr>
<td>15</td>
<td>pH of treatment sections</td>
<td>34</td>
</tr>
<tr>
<td>16</td>
<td>Oxidation Reduction Potential of treatment sections</td>
<td>34</td>
</tr>
<tr>
<td>17</td>
<td>Shannon diversity demonstrating diversity increase based on gradient</td>
<td>49</td>
</tr>
<tr>
<td>18</td>
<td>Shannon diversity based on treatment and gradient</td>
<td>50</td>
</tr>
<tr>
<td>19</td>
<td>Principle Coordinate Analysis of community data using a Bray-Curtis distance matrix based on gradient</td>
<td>52</td>
</tr>
<tr>
<td>20</td>
<td>Redundancy Analysis on both the 2009 wall and the 2018 wall. Only TOC was a statistically significant factor influencing variability across the entire denitrification wall.</td>
<td>53</td>
</tr>
<tr>
<td>21</td>
<td>Redundancy Analysis on the 2018 wall. NO3, TOC, and Conductivity were all significant environmental factors influencing bacterial community variability.</td>
<td>54</td>
</tr>
<tr>
<td>22</td>
<td>Heatmap based on relative abundance calculated using ANCOM</td>
<td>56</td>
</tr>
<tr>
<td>23</td>
<td>Agriculture land use selected from Florida State Land Cover</td>
<td>63</td>
</tr>
<tr>
<td>24</td>
<td>Clay layer selected from the geology layer with the watershed layer</td>
<td>64</td>
</tr>
<tr>
<td>25</td>
<td>Santa Fe River watershed with wetland areas and rivers highlighted</td>
<td>66</td>
</tr>
<tr>
<td>26</td>
<td>Possible locations for a denitrification wall based on agriculture, rangeland, clay, and wetland data</td>
<td>67</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction
The Problem With Nitrogen

One of the greatest challenges facing the human race is the ability to produce enough food to feed the growing population while also minimizing environmental impact. Many innovations that have increased food production have had unintended consequences to the environment. For example, yields would decrease between 40-60% without nitrogen fertilizer inputs [Vitousek et al., 1997]. The production of nitrogen fertilizer produces more available nitrogen than total global bacterial nitrogen fixation and all other nitrogen fixation sources combined [Schlesinger and Bernhardt, 2013]. While this process tripled crop yields, it also increased nutrient loads to the surrounding environment because plants do not use the entire amount of fertilizer that is applied. Resultant consequences include the formation of algal blooms, anoxic zones in the Gulf of Mexico, increased eutrophication, and threatened marine fisheries [Ricklefs, 2010]. Balancing agriculture with water quality highlights the need to create innovative fertilization techniques and to improve nitrogen pollution mitigation methods.

Anthropogenic nitrogen is one of the main causes of eutrophication. Because nitrate is an anion, it is not easily bound by negatively charged soil particles. As a result, nitrate frequently reaches and contaminates groundwater and surface water. Once in the aqueous environment, nitrate is utilized rapidly in nitrogen limiting environments, stimulating the growth of algae, cyanobacteria, and other microbes. Algae growth can produce toxins and cause eutrophication, whereby decomposing algae consume oxygen. This process has occurred often in the Gulf of Mexico, San Francisco Bay, the Black Sea, Lake Erie and many more smaller water bodies, resulting in areas considered dead zones.
because they are so anoxic [Anderson et al., 2015]. Elevated nitrate levels pose a major threat to marine and aquatic ecosystems and water quality in general.

High environmental nitrogen levels also pose a risk to human health. The toxins that some algae produce can be harmful to humans and have caused beaches and waterways to be shut down due to the presence of toxins [United States Environmental Protection Agency, 2017]. In addition to nitrate fertilizer byproducts, direct nitrate contamination in groundwater poses a threat to human health, particularly infants. Nitrate contaminated water can put infants at risk for methemoglobinemia, which prevents babies from getting enough oxygen, which can lead to coma and even death [Knobeloch et al., 2000]. Due to the risks that nitrate and other nitrogen compounds pose to human health, the quantities of nitrate and nitrite present in drinking water are regulated by the United States Environmental Protection Agency. Nitrate has a limit of 10 mg/L and nitrite has a limit of 1 mg/L, both as nitrogen. These commonly accepted standards are shown to be safe for human consumption [Knobeloch et al., 2000]. States that have had frequent algal blooms and high levels of eutrophication, like the state of Florida, have implemented more stringent standards than federal requirements. The focus of this project is the Santa Fe River in Florida, which has a monthly average of 0.35 mg/L nitrate-N, two orders of magnitude below federal standards (Florida Department of Environmental Protection 2014).

**Nitrogen and the Nitrogen Cycle**

Nitrogen is one of the key nutrients for life [Ricklefs, 2010]. Nitrogen is a key component in amino acids that form proteins, nucleic acids, cell walls, and any other
biomolecules, organic, and inorganic molecules. As a result of the necessity of nitrogen, it is cycled through the planet on the micro scale, the regional scale, and on the global scale. Broadly, nitrogen is cycled between nitrogen gas (N\textsubscript{2}), ammonium (NH\textsubscript{4}\textsuperscript{+}), and nitrate (NO\textsubscript{3}\textsuperscript{-}), with various intermediaries. The majority of nitrogen is in the atmosphere as gaseous N\textsubscript{2}, has a very stable triple bond, and is considered the elemental form. A significant source of environmental nitrogen occurs with the breaking of the triple bond of nitrogen through microbial nitrogen fixation, which transforms nitrogen gas to organic nitrogen. Breaking the triple bond of nitrogen gas is very energy intensive, requiring 16 moles of ATP per mole of N, and the reaction proceeds as shown in Equation 1

\[
\text{N}_2 + 8\text{H}^+ + 8\text{e}^- + 16\text{ATP} \xrightarrow{\text{enzyme}} 2\text{NH}_3 + \text{H}_3 + 16\text{ADP} + 16\text{P}_i
\]  

[1]

There is a diverse group of bacteria including cyanobacteria that can fix nitrogen gas, utilizing the nitrogenase enzyme. Overall, global biological nitrogen fixation accounts for 122 million tons annually [Herridge et al., 2008]. Nitrogen that is incorporated into organisms through fixation or assimilation can be released through the ammonification process to ammonium (NH\textsubscript{4}\textsuperscript{+}). There are multiple biochemical pathways for ammonium. First, ammonium can lose a proton and become ammonia (NH\textsubscript{3}). Additionally, ammonia can be volatilized, or assimilated into plant material and other organic forms. Ammonia is the most biologically available form of nitrogen because it provides the most favorable energetics to organisms, partially because of its reduced state. As such, ammonia is the most common form of nitrogen fertilizer applied to plants. However, since ammonia is not charged, but ammonium is positively charged, it tends to interact with negatively charged clay particles. This interaction means that the ammonium does not commonly
contaminate water because it does not easily move through soil. While ammonia does not commonly pose a direct threat to water quality, further transformations in the nitrogen cycle do pose a threat, particularly nitrate. Nitrification transforms immobile ammonium to highly soluble and mobile nitrate (NO$_3^-$).

The nitrification reaction is a two-step, bacterially-mediated reaction performed most commonly by the *Nitrosomonas* and *Nitrobacter* genera. These two genera are very common in soils and are often found together. *Nitrosomonas* transforms ammonium to nitrite (NO$_2^-$) in a three-step reaction with electrons going through the electron transport processes. The ammonium is first oxidized to hydroxylamine (NH$_2$OH) with the enzyme ammonia monooxygenase (AMO). The hydroxylamine is further oxidized to nitrite with water being formed from the free hydrogen, oxygen, and electrons following Equation 2.

$$NH_3 + O_2 + 2H^+ \rightarrow NH_2OH + H_2O \rightarrow NO_2^- + 5H^+$$  \[2\]

*Nitrobacter* then oxidizes the nitrite to nitrate (NO$_3^-$) with a two-step enzymatic reaction. The nitrite is first oxidized to nitrate using the nitrite-oxidizing enzyme (NXR) with hydrogens and electrons as a byproduct. The second reaction forms water from the free oxygen, hydrogen, and electrons from the first step Equation 3 and so on.

$$2NO_2^- + 2H_2O \rightarrow 2NO_3^- + 4H^+ + 4e^- + O_2 \rightarrow 2H_2O$$  \[3\]

The energetics of this process are not well understood to date and research is underway to characterize the mechanism behind this reaction [Ward, 2013]. While there is still much that is not understood about this process, the ecological impacts of nitrification are significant. Nitrification is a common natural process in aerobic conditions. Nitrate is not as readily bioavailable or as energetically favorable as ammonia, but it is still an
important source of nitrogen for organisms [Allan and Castillo, 2007; Ricklefs, 2010; Schlesinger and Bernhardt, 2013].

Nitrate produced from nitrification can be incorporated into organic tissues, leach through the soil into groundwater, become immobilized in soil organic matter, or go through the denitrification process. Denitrification is a microbially-mediated process that transforms nitrate back to gaseous forms (N₂, N₂O). Like nitrification, denitrification is a common environmental reaction. However, there are limits to the amount of denitrification that can take place in the environment. These limitations are usually due to lack of other resources, primarily carbon, but also phosphorus and other trace nutrients [Elser et al., 2007]. So while much of the nitrate added to the environment is naturally remediated, there are still significant amounts left in the ecosystem that contaminate waterways. This highlights the need for further remediation to limit the environmental impact from anthropogenic nitrogen additions.

The denitrification reaction reduces nitrate to nitric oxide (NO), to nitrous oxide (N₂O), and finally to nitrogen gas (N₂). There is a significant amount of metabolic variation among denitrifying bacteria but in general they are heterotrophs, meaning they use nitrite and nitrate as electron acceptors with organic matter functioning as both the carbon and energy source. While most of the identified species are heterotrophs, there are species that are autotrophs, which use other inorganic ions as their electron donor and carbon source. Denitrifying bacteria tend to be facultative aerobic organisms, meaning that they will use oxygen if it is available, but they are also capable of using nitrate as an electron acceptor. Therefore, this reaction is highly dependent on the amount of oxygen in the environment and will not proceed when high levels of oxygen are present, as
oxygen is the more energetically favorable electron acceptor. In aquatic ecosystems and saturated terrestrial systems, the available electron acceptors leads to a microbial community gradient following the energetics of the electron acceptor with aerobes being at and near the soil surface, followed by denitrifiers, then iron reducers, and so on. Oxygen and other electron acceptor availability in the soil control the community gradient, making it easier to predict the type of bacteria present in different sections of soil [Voroney and Heck, 2015]. Most denitrifying species are facultative, giving them a wide distribution. They are known to develop abundant communities in anaerobic, nitrate-rich soils, which improves detection [Robertson and Groffman, 2015]. The most common denitrifiers are species within the Pseudomonas, Bacillus, Paracoccus families, but there are many others [Andalib et al., 2012].

The denitrification reaction reduces nitrate to nitrite and then to nitrogen gas through a series of half reactions shown in Equations 4-7 with the final combined reaction shown in Equation 8.

\[
\begin{align*}
2e^- + NO_3^- + 2H^+ &\rightarrow NO_2^- + H_2O \quad [4] \\
e^- + NO_2^- + 2H^+ &\rightarrow NO + H_2O \quad [5] \\
2e^- + 2NO + 2H^+ &\rightarrow N_2O + H_2O \quad [6] \\
2e^- + N_2O + 2H^+ &\rightarrow N_2 + H_2O \quad [7] \\
10e^- + 2NO_3^- + 12H^+ &\rightarrow N_2 + 6H_2O \quad [8]
\end{align*}
\]

These reactions are the most common pathways that microbes take to reduce nitrate to nitrogen gas, but there are many other possible pathways depending on the particular species and the particular environmental situation. Many of these bacteria are opportunistic and will use the nitrogen in the way that best matches their survival needs,
with most of their responses being controlled by the carbon to nitrogen ratio [Andalib et al., 2012]. This can be monitored by and predicted through microbial analysis and nutrient analysis of the soils [Voroney and Heck, 2015].

The nitrogen cycle takes different pathways and has different time scales depending on the environment and conditions. For example, nitrogen compounds can become incorporated into recalcitrant organic matter and undergo sedimentation, travel through soil matrices and into groundwater, and ultimately through the marine nitrogen cycle, or in the case of nitrous oxide, stay in the atmosphere for 100 years or more. Additionally, further advances in genetic analysis and the discovery of new organisms have revealed much greater complexity within the nitrogen cycle than previously thought. Nitrogen rarely goes directly from nitrogen gas to ammonia to nitrate and back to nitrogen gas. Comprehension of the nitrogen cycle is constantly changing and evolving as more information becomes available about the microbial community interactions [Andalib et al., 2012]. The nitrogen cycle has a significant impact on local, regional, and global environments. It is important to study the fate and transport of nitrogen on different scales and in different environments so that the cycle can be better understood, human impacts can be fully realized and mitigated, and to allow for human manipulation of the cycle. [Ricklefs, 2010].

Nitrogen Treatment Options

There are many technologies currently available that either help reduce the amount of nitrogen applied or help remediate polluted watersheds. Wastewater treatment plants are capable of removing nitrogen from the water supply. However this method is
expensive (with removal costing around $40 per kilogram of nitrogen) and only remediates the water from sewer systems or after the contaminated water has already been in the stream or in the groundwater [Schmidt and Clark, 2011]. There is developing technology that uses GIS to more effectively target fertilizer application. However, even after applying more conservative fertilizing methods, there are still water contamination issues [Passeport et al., 2013]. Artificial treatment wetlands have also shown to be effective at removing nitrogen, but are not as effective as natural wetlands and are not very effective at cleaning up contaminated groundwater. Treatment wetlands generally have a nitrogen removal rate of around 45%, but are much more cost effective than municipal treatment approaches [Passeport et al., 2013]. A treatment method that has been shown to be effective particularly at cleaning up contaminated groundwater is permeable reactive barriers [Schmidt and Clark, 2011].

Permeable reactive barriers like denitrification walls and denitrification beds are effective treatment options for cleaning up contaminated groundwater because they are efficient and cost effective. Denitrification walls and beds rely on materials that have high carbon to nitrogen ratios to help trigger a variety of processes that take up the excess nitrogen in the water; either through assimilation, dissimilatory nitrate reduction to ammonium (DNRA), or most commonly, denitrification. Permeable reactive barriers are dependent on groundwater temperature, groundwater flow rate, nitrate concentration, and other factors, meaning that they are not suitable for every location. However, for areas where permeable reactive barriers are well suited, they are extremely cost effective (costing between $0.79-15.17 per kilogram of nitrogen removed depending on materials), and are easy to implement using materials and equipment that many agricultural
properties already have on hand [Schipper et al., 2010; Schmidt and Clark, 2011]. Denitrification walls are placed directly in the groundwater flow path, while denitrification beds are large containers filled with woodchips placed underneath agricultural tile drains, septic systems, or other effluent from pipe flow [Addy et al., 2016]. These systems often have removal rates of around 50 - 90% depending on the environmental conditions.

Permeable reactive barriers are simple by design, but take advantage of multiple natural processes, making them cost effective and efficient. They utilize carbon-rich media like wood chips or sawdust to create a nitrogen-limited environment so that whenever nitrate is added to the system it is utilized by soil microbes quickly, particularly under anoxic conditions. When under anoxic conditions, the nitrate can be converted to nitrogen gas through denitrification. By engineering an environment where denitrification can occur, the excess nitrogen can be removed from the system to limit the negative impacts. Denitrification walls rely on Darcian flow principles and passive groundwater flow into the bioreactor, therefore requiring a strong understanding of site hydrology to achieve the best results. This technology relies on high water tables with consistently saturated soils, a confining layer or a layer with low hydraulic conductivity, and an existing high conductivity soil that the media can be mixed with. The carbon substrate is usually mixed with existing soil or soil that is brought in and then placed vertically into shallow groundwater perpendicular to the flow (Figure 1). The high porosity of the carbon substrate-soil mixture gives it a high hydraulic conductivity which, according to Darcian flow, causes the groundwater to have preferential flow paths through the denitrification wall [Schipper et al., 2010].
These types of denitrification walls are effective over both the long and short term, and maintain high levels of nitrate removal throughout the life of the wall, which is usually estimated to be around 15-20 years [Long et al., 2011]. Denitrification beds are more complicated in their design than denitrification walls, as they are lined and have inflow and outflow pipes. Denitrification beds require high volumes of agricultural effluent so that the bed can be saturated and maintain anoxic conditions [Christianson and Schipper, 2016]. These high base flow volumes generally lead to lower hydraulic retention time and lower overall nitrate reduction rates in denitrification beds over denitrification walls. However, denitrification beds can be designed to optimize hydraulic retention times [Christianson et al., 2013; Addy et al., 2016].

One important aspect of permeable reactive barriers is the carbon source, which can range from readily-available glucose to lignocellulosic material like woodchips and sawdust. The latter carbon sources are preferred, because of a greater longevity in the
environment. Multiple studies have examined various types of lignocellulosic carbon media to identify the most suitable material based on carbon amount, longevity, and overall nitrate removal. Overall, the lignocellulosic carbon source does not substantially impact overall nitrate removal rates [Cornwell et al., 2009; Cameron and Schipper, 2010; Robertson, 2010; Warneke et al., 2011; Schmidt and Clark, 2013]. While the amount of carbon between softwoods and hardwoods does not impact nitrogen removal rates, it is important to consider the type of carbon material for longevity. Softer woods like pine are more likely to degrade faster due to higher levels of oxygen to carbon to hydrogen ratios [Cornwell et al., 2009]. While hardwoods are less likely to degrade, they have more variable densities and can be harder to obtain large volumes for field scale projects [Schipper et al., 2010]. There have been some studies that have used other carbon materials like maize cobs, wheat straw, general green waste, biochar, and others [Cameron and Schipper, 2010; Warneke et al., 2011; Bock et al., 2016]. These materials have high nitrate removal rates, particularly maize cobs, but they have not been tested beyond laboratory tests and small pilot scales. Additionally these materials have not been tested for longevity, so more study is necessary to determine the feasibility [Addy et al., 2016].

Permeable reactive barriers are installed using backhoes and similar construction type equipment. Denitrification beds are often retrofitted to a preexisting system, like a tile drain or a septic system and require lining and outflow and inflow plumbing [Robertson et al., 2008; Christianson et al., 2013; Hoover et al., 2017]. Denitrification walls require the wall to be placed directly in the path of groundwater and ideally keyed into a confining layer. The functional requirements for a denitrification wall make it
harder to find suitable locations to place them, whereas tile drains can often be installed to guide water to denitrification beds. Additionally, denitrification walls will not be successful in all locations in the world, or even within a single watershed due to variations in geology, hydrology, and nitrogen concentration. There has been little research on methods for providing guidance on suitable locations for denitrification wall installation. One aspect of this study is using spatial analysis and geographic information systems to identify potential locations for denitrification walls.

Once a suitable denitrification wall location is targeted, it is important to scale the treatment system appropriately. This technology was first tested at the laboratory scale in the mid to late 1990s and the first pilot scales tests of denitrification walls were not started until the late 1990s [Robertson and Cherry, 1995; Schipper and Vojvodić-Vuković, 1998]. To date, the majority of studies have been bench or column studies or pilot studies [Christianson et al., 2013; Li et al., 2014; Weigelhofer and Hein, 2015; Bock et al., 2016; Hoover et al., 2017]. Additionally, the majority of studies have been denitrification bed studies, so there is a large gap in denitrification wall data [Addy et al., 2016]. Not only have there been few denitrification wall field tests, they have also been relatively small, with the largest reaching 150 meters long [Schipper, 2013]. These tests have treated small volumes of contaminated groundwater, but have not made significant impacts on the pollution. Treating large amounts of contaminated groundwater is possible through strategic targeting on each individual property based on areas with the highest nitrogen loads and areas that best meet the denitrification wall functional requirements. In order for a denitrification wall to be effective at treating the overall nitrogen load coming off a property it needs to be scaled based on the property size and total nitrogen load.
Site Background

Florida is an optimal location to test a large-scale version of a denitrification wall due to shallow groundwater contamination, suitable geology, and hydrology. Because of a series of algal blooms and nitrogen contamination of groundwater, the state of Florida is working to reduce the nitrogen load on its waterways. This led to statewide numeric standards for nitrogen in 2014 of 0.35 mg/L nitrate-N [Florida Department of Environmental Protection, 2018]. Geologically, Florida is a limestone plateau that was formed from deposits from a shallow sea that has since receded. Due to the amount of limestone, Florida is dominated by karst geology, which is famous for sinkholes and springs. However, northeastern Florida has an additional layer from the Miocene era known as the Hawthorn group made up of largely impervious phosphatic clay that covers the karst formations [Scott, 1983]. This clay layer creates a perched aquifer and a high water table leading to the area having many wetlands, rivers and streams. Utilizing geographical methods to find these clay layers could help to target denitrification walls to broader areas. The Santa Fe and Suwannee watersheds are two interconnected watersheds partially covered by the Hawthorn Formation. The majority of the land use in the area is agricultural. In the years since Florida passed nitrogen and phosphorus regulations, plans have been developed to reduce the nitrogen loading. Most of these plans have dealt with encouraging best management practices and decreasing the total maximum daily load (TMDL) from wastewater treatment plants. However, the state has shown interest in restoration projects as well as innovative treatment technologies, such as denitrification walls [Florida Department of Environmental Protection, 2018]. In this study, I implemented a property scale bioreactor for remediation, examined the microbial
communities involved in the remediation process, and developed a spatial tool to identify locations for these types of reactors.

**Objectives**

**Overall Objective:** Reduce nitrogen loading to the Santa Fe River to contribute to meeting Total Maximum Daily Load requirements.

1. Gain a better understanding of microbial communities within and surrounding denitrification walls and how they change over time.
2. Construct a cost-effective and functional field scale denitrification wall.
3. Construct a spatial tool to identify other suitable locations for installing large-scale denitrification walls in the Santa Fe Watershed.

**Chapter 2: Efficacy of a Property Scale Denitrification Wall**

**Holly Factory Denitrification Wall**

Denitrification walls have been established as effective treatment technologies on a pilot and laboratory scale, commonly having over 90% nitrogen removal rates [Cameron and Schipper, 2010; Schmidt and Clark, 2011]. However, there has been very little work examining if denitrification walls could make an impact on the pollution coming off of entire properties. It is unclear if denitrification walls would still have the same removal rates if they are scaled up or if scaling up would be a cost effective option. There are also concerns regarding the negative environmental impacts from scaling the wall up [Weigelhofer and Hein, 2015]. For example, it is possible that denitrification
walls are producing high levels of nitrous oxide, so scaling up the wall could increase the output of a negative greenhouse gas. In order to understand if denitrification bioreactors could be effective at treating property scale pollution, it is important to test a large scale denitrification wall in an area that has already shown to be effective for this type of technology.

In 2009, a denitrification wall was installed on a 65-hectare nursery called the Holly Factory in the Santa Fe River Watershed. This nursery grows wholesale trees and shrubs, like holly trees, crepe myrtle, wax myrtle and others shipped across the East Coast. The grower grows his plants in pots partially submerged in the ground, irrigates mostly through drip irrigation with some small sections using overhead irrigation, and fertilizes semi-annually with slow-release fertilizer. The grower uses approximately 4 million liters per day to irrigate all of his plants, keeping the water table close to the surface year round. The average nitrate-N concentration in surface waters discharging the property is $7.5 \pm 0.73 \text{ mg/L}$, although groundwater concentrations as high as 80 mg/L have been observed [Schmidt and Clark, 2012a].

A denitrification wall was placed on the edge of the Holly Factory to test if this would be an effective method at removing the excess nitrogen discharging from the property. This wall was installed in 2009 using a 1:1 sawdust mixture and excavation equipment to the dimensions of 55 m long, 1.7 m wide, 1.8 m deep (168 m$^3$). The wall was monitored for a three-year period and during that monitoring time the groundwater had a residence time of 1.7-1.9 days and a rapid pore velocity with nitrate removal efficiencies of around 90%. These nitrogen removal rates have stayed consistent to the present day [Schmidt and Clark, 2012a; Schmidt, 2018]. However, due to the scale of the
property, the 2009 wall is only treating around 10% of the water impacted by the nursery. Additionally, the grower has dramatically expanded his operations highlighting a need for further treatment.

The Holly Factory was effective for a pilot scale study of denitrification wall technology. Due to the high fertilization rates, consistently high water table, and warm temperatures, it is also likely a suitable location for a large-scale version of a denitrification wall. This property also has streams that drain directly into the Santa Fe River, so a large-scale denitrification wall on this property could also help evaluate if permeable reactive barriers are an effective treatment technology for reducing non point source pollution. Additionally, testing a cost-effective and functional property scale version of a bioreactor was one of the primary objectives of the study. All of these factors led to the selection of the Holly Factory for a property scale denitrification wall.

**Methods**

**Wall Construction**

The wall was installed during the week of March 4th, 2018 at a cost of $50,000. The 320-meter wall was installed using a 1:1 sawdust and native soil mixture. The 1:1 sawdust to native soil mixture was chosen based on multiple constant head hydraulic conductivity experiments with varying ratios of sawdust to soil. The sawdust was a very fine pine mixture that was mixed with native soil excavated from the trench. Portions of the trench were dug with a trench box to help maintain the integrity of the walls and to maintain a consistent width of the wall. The average width of the wall was 1.5 meters and the depth varied along the length of the wall depending on the depth to the Hawthorn clay layer with it averaging 2 meters in depth across the 320-meter total length of the wall.
After the trench was dug to the Hawthorn layer, it was immediately filled with the media mixture. The trench was filled to 0.6 meters below the ground surface. There were two control sections incorporated into the wall, with the first section starting at 120 meters and ending at 137 meters, and the second section starting at 301 meters and ending at 320 meters. The control sections were dug to the Hawthorn layer and the soils were mixed and backfilled without the inclusion of sawdust. After the completion of the wall, media samples and GPS points were taken at 15-meter intervals. Single ring infiltrometer tests were also done on a control section, media section, and on a native soil following the NRCS method from the 2014 Soil Survey Field and Laboratory Methods Manual [Soil Survey Staff, 2014].

The week of March 25, 2018, 6 monitoring well transects were inserted into the wall. The wells were installed upgradient, within, and downgradient from the wall. There was a transect placed in each control section and the remaining 4 transects were inserted at 37 meters, 62 meters, 196 meters, and 232 meters as shown in Image 1.
Wall Monitoring

The 6 well transects inserted shortly after the wall installation were sampled on a biweekly basis from May to the end of September 2018. They were sampled for nitrate-N, ammonia-N, Total Kjeldahl N (TKN), groundwater temperature, pore water velocity, dissolved oxygen, redox, pH, and conductivity. Total organic carbon was also tested biweekly to better understand the pulse of carbon that is being introduced to the environment and the overall environmental impact. Slug tests were also performed and were used to calculate saturated hydraulic conductivity, calculated using Hvorslev’s method. Effective porosity of the wall was also calculated in the lab using recreated cores.
of soil and sawdust in the same ratios and bulk density of the wall in triplicate. Effective porosity was calculated as the water volume difference between saturation and field capacity (33 kPa).

The sampling procedure involved purging two well volumes using a peristaltic pump, and collecting a sample. Nitrate and ammonia samples were filtered using a 0.45 µm membrane filter, TOC and TKN were unfiltered and all samples were preserved with sulfuric acid and immediately placed under ice and transported to the lab. The filtered samples were tested for nitrate-N using the EPA method 353.2 and EPA method 350.1 for ammonia-N and the unfiltered samples were tested for Kjeldahl N using the EPA method 351.2. The pore water velocity and temperature were measured using an In-Situ Inc. Rugged TROLL 200 transducer. The dissolved oxygen, redox, pH, conductivity were tested with an YSI probe. Total organic carbon was determined using EPA method 415.3.

**Load and Cost Calculations**

Nitrogen load reduction rates were calculated using mass of nitrate-N loss per volume of treatment media [Schipper and Vojvodić-Vuković, 2001]:

\[
N_r = \frac{v A \Delta_n}{V_s}
\]

Where \( N_r \) represents the nitrate mass loss per volume of treatment media (g-N m\(^{-3} \) d\(^{-1} \)), \( v \) is the porewater velocity (m/d), \( A \) is the cross sectional area times the effective porosity (m\(^2 \)), \( \Delta_n \) is the change in total nitrogen concentration (g/m\(^2 \)), and \( V_s \) is the media volume of the wall (m\(^3 \)).

Cost efficiencies (\( C_o \)) were calculated by dividing the cost of the wall ($50,000) by the nitrogen reduction rate times a 20-year estimated lifespan. This cost efficiency was
compared to other treatment system costs calculated from a literature review of other treatment technologies.

\[ C_e = \frac{C}{N_r \times 20} \]

**Data Management and Statistical Methods**

Statistical tests and analysis were run using JMP 14.0 (SAS Inc., Cary, NC). One-way ANOVAs were run on the average of all water quality results between location (upgradient, center, downgradient wells) and control versus treatment sections. Statistical significance was calculated using an alpha value of 0.05. After running the initial ANOVA tests, the results were further analyzed for significance using Tukey’s HSD method. To determine if the time since treatment had an impact on the water quality results, a paired test was run on the water quality data and the days since treatment. Full statistics are listed in Appendix 1.

**Results and Discussion**

**Hydrology**

The treatment sections had an average hydraulic conductivity of 1.71±0.4 cm/hr in the upgradient section and 1.35±0.3 cm/hr in the excavated and back-filled center section. While the control section had an average hydraulic conductivity of 1.84±0.3 cm/hr in the upgradient section and 0.70±0.8 cm/hr in the center section (Figure 2). The hydraulic conductivity decreased within the wall both in the treatment and the control sections highlighting that the decrease is resulting from the excavation and backfilling process, and not the treatment media. The effective porosity also decreased from 50% to 12% from the upgradient to center sections. The decrease in hydraulic conductivity in
both the treatment and control sections is likely due to the excavation of the wall and the destruction of soil structure, which has been shown to reduce hydraulic conductivity [Fetter, 2001; Hillel, 2008; Kirkham, 2014]. The decrease in hydraulic conductivity suggests that there will be some short circuiting of the bioreactor [Christianson et al., 2016]. While some level of short-circuiting should be expected, because of the size of the wall, the level of nitrate treatment should still be significant. Further work could be done on the groundwater flow paths to calculate the amount of short-circuiting and the impact that it has on the efficiency of the bioreactor. Additionally, the denitrification wall is treating around 30 million liters of water per year (Figure 3). The total discharge from the property is around 300-1000 million liters per year based on monitoring from the 2009 reactor. There is not data from the property for 2018, so there is a possibility that the discharge has changed over time. Even if there was a change in the total discharge, the volume being treated by the denitrification wall is relatively small in comparison to the total volume coming off of the property, further highlighting the problem of the decreased hydraulic conductivity, and significantly impacting the effectiveness of the treatment system.
Figure 2: Saturated Hydraulic Conductivity for the control and treatment sections

Figure 3: The hydraulic loading rate of the treatment sections
Nitrate Nitrogen

Nitrate concentrations significantly decreased from 7.1±3.2 mg/L on average in the upgradient sections to 1.3±0.6 mg/L on average in the center sections, although there was no significant difference between the upgradient transects and downgradient transects (6.0±1.3 mg/L) nitrate concentrations (Figure 4, One way ANOVA, df=2, F=48.9, P=0.0001; Tukey’s HSD, P=0.0001). There was no difference in the nitrate concentration over time in the treatment sections (Matched Pairs, P=0.89). This indicates that there is consistent reduction in nitrate over time and high nitrate removal rates can be expected over the life of the wall. However, it would be necessary to monitor concentrations for a longer period of time to confirm that there is no difference over time.

The control section also had a significant decrease in the center of the wall when compared to above and below the wall (Figure 5, One way ANOVA, df=2, F=4.75, P=0.02; Tukey’s HSD, P=0.0163). On average, the nitrate concentrations above the wall were 8.65±2.85 mg/L and decreased to 6.93±0.78 mg/L within the center of the wall and then increased again to 7.31±0.49 mg/L below the wall on average. The difference in nitrate concentrations between the control sections is likely due to the excavation of the wall. This was further supported by the fact that towards the end of the study, the control transect nitrate concentrations in the upgradient (8.55±1.52 mg/L), center (8.04±0.27 mg/L), and downgradient wells (8.85±0.98 mg/L) were very similar. This stabilization by the end of the study suggests that the change in the control section, while statistically significant, is unlikely to be a significant source of nitrate load reduction over the life of the denitrification wall. However, to confirm that the excavation is not causing significant denitrification further sampling would be necessary. Additionally, even though there was
a significant difference between the control section gradients, there was also a significant
decrease between the treatment and the control sections.

Overall, the wall stimulated denitrification, causing an 18% decrease of nitrate-N on average between the upgradient wells and the center wells. This decrease translates to a nitrate load reduction of over 3,000 kg/yr. The increase of nitrogen in the downgradient wells is further evidence, beyond lower hydraulic conductivity values, that there is short-circuiting of the bioreactor and that the downgradient wells contain some untreated water. It is not useful to calculate the treatment efficacy of the wall using the difference between the upgradient and downgradient results because it does not represent the water that has actually been treated. Additionally, due to the shorter monitoring time, it is possible that the amount of nitrate reduction could be greater than the initial testing as the microbial communities become more established and as clearer ground water flow paths are established in the treatment media.
Figure 4: Nitrate concentrations in the treatment section

Figure 5: Nitrate concentrations in the control sections
Ammonia Nitrogen

The majority of nitrogen discharged from the Holly Factory is nitrate, however, ammonia does make up a small component of the total groundwater nitrogen concentration. We monitored ammonia in this study to ensure that denitrification was occurring and not dissimilatory nitrate reduction to ammonium (DNRA) or other microbial transformations that produce other bioavailable nitrogen products. The ammonia concentrations were significantly higher in the center sections than the other sections (Figure 6, One way ANOVA, df=2, F=13.2, P=0.0001; Tukey’s HSD, P=0.0001), but there was no difference between the treatment and the control sections (Figure 7, One way ANOVA, df=1, F=0.317, P=0.579). The ammonia increased from an average of 0.09±0.01 mg/L in the upgradient sections to an average of 0.19±0.04 mg/L in the center sections and then decreased to an average of 0.11±0.03 mg/L in the downgradient sections. This fluctuation in the center wells is likely due to the excavation of the wall, especially because there was no difference between the treatment and the control sections. It also appears that the ammonia concentration decreased over time (Matched Pairs, P=0.025), indicating that while there was an ammonia spike it does not appear to have a long lasting impact on the wall and does not appear to be a major byproduct.
Figure 6: Ammonia concentrations in the treatment sections of the wall. There was a clear spike in ammonia concentrations, likely due to the excavation, which decreased over time to background levels.

Figure 7: Ammonia concentrations in the control sections of the wall. There was a clear spike in the ammonia concentrations, likely due to the excavation, which decreased over time to background levels.
Organic Nitrogen

Organic nitrogen, such as that found in nucleic acids, proteins, and other biomolecules, could increase due to the addition of organic material like the sawdust added to create the bioreactor. There was a minor increase in organic nitrogen in the center well sections with an average of $0.78\pm0.12$ mg/L compared to $0.04\pm0.21$ mg/L in the upgradient wells and $0.13\pm0.33$ mg/L in the downgradient wells (Figure 8, One way ANOVA, df=2, F=27.6, P=0.0001; Tukey’s HSD 0.0001). However, there was no difference between the control wells (Figure 9, One way ANOVA, df=2, F=1.48, P=0.24). This indicates that the bioreactor is a source of organic nitrogen, but that it is a small addition to the overall system and that addition appeared to decrease over time.
Figure 8: Organic Nitrogen in the treatment sections

Figure 9: Organic Nitrogen in the control sections

Total Nitrogen

The total nitrogen highlights the overall effectiveness of the bioreactor, because it incorporates nitrate decreases, and increases in other forms. While denitrification systems
can increase ammonia and organic nitrogen, the majority of nitrogen present was in the form of nitrate, resulting in a significant reduction in total nitrogen as well. The total nitrogen decreased from an average of 7.22±3.12 mg/L in the upgradient wells to 2.04±0.67 mg/L in the center wells and then increased again to 6.13±1.38 mg/L in the downgradient wells (Figure 10, One way ANOVA, df=2, F=41.16, P=0.0001; Tukey’s HSD, P=0.0001). There was also a small decrease in the control section, where total nitrogen decreased from an average of 8.62±2.78 mg/L in the upgradient wells to 7.19±0.87 mg/L in the center wells and then increased again to 7.35±0.46 mg/L in the downgradient wells (Figure 11, One way ANOVA, df=2, F=4.27, P=0.023; Tukey’s HSD, P=0.0308). The 72% decrease in nitrogen over the length of the wall shows the effectiveness over a large area and its potential as a property scale treatment option.
Figure 10: Total Nitrogen concentrations in the treatment sections

Figure 11: Total nitrogen concentrations in the control sections
Dissolved Organic Carbon

Dissolved organic carbon (DOC) is known to leach when organic matter is in contact with groundwater. The carbon released increases the chemical oxygen demand in receiving waters, which decreases the dissolved oxygen (DO). In the 2009 wall, there was a significant TOC pulse that lowered the DO and caused a sulfur bacterial growth in the streams down from the property for 30 days post installation [Schmidt and Clark, 2012b]. In order to understand environmental impacts from the bioreactor, we monitored TOC over the course of this study. TOC concentrations were initially high in the center sections; with an initial concentration of 200±26 mg/L 23 days post installation and then increasing to 600±170 mg/L 55 days post installation and eventually decreasing to 12±3.0 mg/L on average (Figure 12). The TOC was slightly elevated in the downgradient wells (average 23±20 g/L) as compared to the upgradient wells (average 12±9.0 mg/L); however, the upgradient wells and downgradient wells were not significantly different between control and treatment sections (One way ANOVA, df=2, F=6.09, P=0.007). There was no increase in carbon in the control section as groundwater passed through the wall, which had an average of 11±3 mg/L along the entire well (Figure 13, One way ANOVA, df=2, F=2.49, P=0.10).

The TOC peak was slower to emerge and lasted longer in the 2018 wall than in the denitrification wall constructed in 2009. The 2009 wall peaked after 37 days, while the 2018 wall did not peak until 55 days and did not start to decrease from 600±120 mg/L until after 85 days post installation. This is likely due to the difference in hydraulic conductivity and slower groundwater velocities. However, the peak declined and did not have a significant, lasting impact on the environment downstream from the bioreactor.
However, it was not possible to see if there was a difference over time in the TOC concentrations due to the fact that there were only two center well samples that were collected throughout the study and the matched pairs test using all of the well locations did not show significance (Matched Pairs, P=0.15). While the difference in TOC concentrations was not statistically significant over time, it does not mean that there was not a decrease in TOC. Denitrification walls do cause a significant increase in TOC, but it is a relatively minor spike relative to the life of the wall. This is promising for any future reactors of a similar size, as there are few lasting negative impacts due to the increase in carbon.

Figure 12: Total organic carbon concentrations in the treatment sections
Environmental Metrics

While there were clear trends in the nitrogen concentrations, the trends from the YSI probe data were more variable (Table 1). There was no statistical difference between the control and treatment sections for dissolved oxygen and the dissolved oxygen was below 1.50 for all locations within the well transects in both the treatment and control sections (Figure 14, One way ANOVA, df=1, $F=0.143$, $P=0.71$). There was also no statistical difference between the pH of the control sections and the treatment sections (Figure 15, One way ANOVA, df=1, $F=1.93$, $P=0.18$). The oxidation-reduction potential was significantly lower in the center treatment sections than the above and below the wall, with an average of -66±11 mV, demonstrating that the center of the reactor is a reducing environment (Figure 16, One way ANOVA, df=2, $F=59.2$, $P=0.0001$; Tukey’s HSD, $P=0.0001$). There was also a significant difference within the control section between the upgradient (35±26 mV), center (85±42 mV), and downgradient wells.
(109±33 mV (One way ANOVA, df=2, F=11.2, P=0.0002; Tukey’s HSD, P=0.0002).
While there was a difference in ORP between different locations in the well transect, all of these differences had a positive absolute value, indicating a more oxidizing environment across the control section. This is in contrast to the treatment sections where the ORP reading was negative inside the wall and positive above and below the wall, signifying a reducing environment within the treatment system. The reducing environment is another indicator that denitrification is occurring within the bioreactor.
YSI probe readings of dissolved oxygen were less sensitive to changes in microbial metabolism.

Table 1: Average ± standard deviation values from YSI probe

<table>
<thead>
<tr>
<th></th>
<th>DO (mg/L)</th>
<th>Turbidity</th>
<th>ORP</th>
<th>pH</th>
<th>Conductivity (uS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment Upgradient</td>
<td>0.96 ± 0.27</td>
<td>7.16 ± 4.58</td>
<td>75.1 ± 44.11</td>
<td>5.84 ± 0.36</td>
<td>244.6 ± 71.96</td>
</tr>
<tr>
<td>Treatment Center</td>
<td>1.1 ± 0.28</td>
<td>7.73 ± 2.7</td>
<td>-66.31 ± 11.19</td>
<td>5.64 ± 0.17</td>
<td>209.1 ± 15.43</td>
</tr>
<tr>
<td>Treatment Downgradient</td>
<td>0.95 ± 0.45</td>
<td>10.55 ± 7.62</td>
<td>68.84 ± 51.7</td>
<td>5.35 ± 0.49</td>
<td>190.8 ± 30.56</td>
</tr>
<tr>
<td>Control Upgradient</td>
<td>1.09 ± 0.57</td>
<td>5.15 ± 2.83</td>
<td>35.47 ± 26.16</td>
<td>6.35 ± 0.43</td>
<td>269.7 ± 32.4</td>
</tr>
<tr>
<td>Control Center</td>
<td>0.95 ± 0.37</td>
<td>17.9 ± 9.44</td>
<td>84.69 ± 41.94</td>
<td>5.43 ± 0.57</td>
<td>200.0 ± 26.09</td>
</tr>
<tr>
<td>Control Downgradient</td>
<td>1.21 ± 0.48</td>
<td>8.38 ± 5.93</td>
<td>109.08 ± 32.95</td>
<td>5.33 ± 0.6</td>
<td>178.7 ± 16.57</td>
</tr>
</tbody>
</table>
Figure 14: Dissolved Oxygen Concentrations in the treatment sections

Figure 15: pH of treatment sections
Figure 16: Oxidation Reduction Potential of treatment sections

**Cost Analysis**

The construction cost of the wall was $50,000. Based on previous work [Schmidt and Clark, 2012a], we will be using a conservative 20-year lifespan. Dividing the construction cost by the yearly nitrogen removal rate times the 20-year life span yields a cost of $1.76 per kg of nitrogen removed. It is difficult to compare nitrogen treatment options due to the fact that many studies do not report the cost of the system. If a study does report the cost it is often only the total cost and not the cost per unit removed. Cost calculations are also difficult to directly compare because there is not one standardized unit of measurement across studies. Despite the difficulty in comparing treatment technologies, this denitrification wall is a particularly cost effective denitrification treatment (Table 2).
<table>
<thead>
<tr>
<th>Reactor Type</th>
<th>Subtype</th>
<th>Nitrate Removal (%)</th>
<th>Cost</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced Septic Systems&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td>Recirculating systems (i); sequential systems (ii)</td>
<td>40-70% (i); &gt;90% (ii)</td>
<td>$15-30 K (includes design and installation)</td>
<td>Additional costs: $100/year for media filter and $200-$600/year for maintenance</td>
</tr>
<tr>
<td>Permeable Reactive Barrier (PRBs)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2009 Denitrification Wall 2018 (ii) Denitrification Wall (ii)</td>
<td>&gt;90%</td>
<td>$2.94/kg-N over 20 yr (i); $1.76/kg-N over 20 yr (ii)</td>
<td>Estimated 20 year lifespan</td>
</tr>
<tr>
<td>Permeable Reactive Barrier (PRBs)&lt;sup&gt;4,5&lt;/sup&gt;</td>
<td>Denitrification Bed</td>
<td>&gt;90 %</td>
<td>Variable $2-$15/kg N removed</td>
<td>No additional maintenance costs</td>
</tr>
<tr>
<td>Treatment wetlands&lt;sup&gt;6,7,8,9&lt;/sup&gt;</td>
<td>FWS wetlands (i); horizontal sub-SWF wetlands (ii); vertical sub-SWF wetlands (iii)</td>
<td>40-44% on average</td>
<td>Variable: $0.001-$0.1/m&lt;sup&gt;2&lt;/sup&gt; (i); $0.03-$1/m&lt;sup&gt;2&lt;/sup&gt; (ii)</td>
<td>Works best with C:N&gt;5:1 and permanently saturated conditions plus long residence time (e.g. type ii)</td>
</tr>
<tr>
<td>Riparian zones&lt;sup&gt;10,11&lt;/sup&gt;</td>
<td>Coarse sand and gravel soil (i); other soil (ii)</td>
<td>40-100% (i); 90-100% (ii)</td>
<td>Variable $0.0262/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>70-90% removal may be achieved in ≥ 25 in many riparian zones</td>
</tr>
<tr>
<td>Artificial lakes and reservoirs&lt;sup&gt;12,13&lt;/sup&gt;</td>
<td>None</td>
<td>10-100%</td>
<td>A few thousands of dollars to millions of dollars</td>
<td>N removal rate strongly positively correlated to residence time</td>
</tr>
<tr>
<td>Stream restoration&lt;sup&gt;14,15,16,17&lt;/sup&gt;</td>
<td>Organic matter additions (i); channel reconstruction (ii); floodplain reconnection (iii); artificial geomorphic features (iv); bank stabilization</td>
<td>5-40%; 11% during baseflow; 24% during high flows</td>
<td>$15-812 K/project; $520-$1526/m restored stream</td>
<td>Many restored streams contain &quot;hot spots&quot; of N removal, but overall effect on water quality with respect to N at the reach scale remains uncertain</td>
</tr>
</tbody>
</table>
Adapted from Passeport (2013)
2 Water Environment Research Foundation (2010)
7 Davis, B. M., S. Wallace, and R. Wilson (2009)
9 BMP database (2010)
13 Passeport, E., P. Vidon, K. J. Forshay, and others (2013)
14 Bernhardt, E. S. (2005)
16 Kaushal, S. S. et al. (2015)
17 United States Environmental Protection Agency (2006)
Conclusions

Based on the nitrate reduction and other water quality data, the bioreactor is an effective way to remove excess nitrogen in situ on a property scale, which fulfills the main objective of reducing the overall nitrogen load from the property. The wall was also cost effective, costing only $1.73 per kg-N, which fulfills objective 2. However, this wall only had 70-75% nitrate removal rates, which is less effective than the 2009 wall (90%) and other permeable reactive barriers. Part of the reason for this is the increase in scale because it covers a wider area with differing levels of nitrate concentrations and groundwater flow.

The main reason for the decreased efficiency appeared to be due to the lower hydraulic conductivity of the wall, which caused short-circuiting of the wall. Based on the fact that the control sections had a larger decrease in hydraulic conductivity than the treatment sections, it is likely that the excavation caused most of the reduction in hydraulic conductivity. Further testing would be necessary to confirm the change in hydraulic conductivity and if the hydraulic conductivity would increase over time. It would also be important to calculate how much water is not being treated due to the short-circuiting of the wall. The wall is only treating 30 million liters of water per year, which is much smaller than the total discharge from the property (300-1000 million liters per year from 2009). However, it would be important to calculate total discharge coming off of the property today to be able to calculate the overall volume being treated vs. the total discharge. Calculating the volume of water being treated and the amount going untreated would be vital to understanding if denitrification walls are effective on the property scale. While the wall is removing 70% of the nitrate-nitrogen that goes through
the wall, it will not have much of an impact if it is only treating 20% or less of the volume. Short-circuiting has been a major problem with permeable reactive barriers and is a challenge that needs further work to address [Christianson et al., 2013]. It is difficult to address problems with hydraulic conductivity due to excavation because it would be impossible to install the denitrification system without the excavation process. Having a narrower wall might help with the hydraulic conductivity decrease because it would be doing less damage to the soil structure overall, however there would still be a decrease in the center section. Ensuring that the wall is completely connected to the confining layer could also prevent some of the short-circuiting. Further research would need to be done on the hydraulics of these systems to try to maximize the amount of treatment and minimize the amount of groundwater not being treated. It would also be important to monitor if the lower hydraulic conductivity has any other negative impacts, such as water backing up on the property.

The excavation also appeared to have an impact on the ammonia concentrations. There was a spike in the ammonia concentrations in the center wells in both control and treatment sections that appeared to be caused by the construction of the wall. This could be due to introducing organic matter, like turf and other plant material, into the denitrification wall. However, this increase was not true for organic nitrogen; there was only a spike in the treatment sections for organic nitrogen. This could indicate that nitrification was stimulated by the excavation, which led to the lower concentrations of organic nitrogen, while still having a spike in ammonia. There are also many other possible explanations, such as a smaller amount of organic material in some of the control sections, or that the turf had a high amount of ammonia fertilizer, which could
explain the lower concentration of organic nitrogen compounds and the higher ammonia concentrations. The center of the control section also had a slight reduction in nitrate and a minor carbon spike, which are both suggestive of an excavation effect.

While the data for this study was from a relatively short monitoring period, the continued nitrate removal of the 2009 wall suggests that the wall in 2018 will also have continued nitrate reduction over time. More monitoring would need to be done to confirm the long-term efficacy of the reactor, especially due to the increased size of the 2018 wall in comparison the 2009 wall. There are also still many questions regarding the gas evolution from a denitrification wall, especially with such a large wall. More work could also be done to verify that denitrification is occurring through monitoring gas evolution of the wall in conjunction with continued microbial sampling of the wall.

Chapter 3: Impact of Excavation and Carbon Addition on Native Microbial Communities

Introduction

Improved understanding of microbial interactions is a crucial step needed to reduce nitrogen contamination, and to design remediation approaches. Advancements in genomic testing have helped scientists better understand the complex ecology of microbial systems, and these tools could be useful for reducing the impact of excessive nitrogen in agriculture. While there have been many advancements, there are many large gaps in the knowledge base of the microbial world. This is particularly true of large biogeochemical cycles like the nitrogen cycle. For example anaerobic ammonium oxidation (anammox), a whole new process in the nitrogen cycle, was only discovered in 1999. Previous to the discovery of anammox bacteria, it was thought that ammonium oxidation
oxidation was only possible under aerobic conditions [Ward, 2013]. Another example of an overlooked aspect of the nitrogen cycle is dissimilatory nitrate reduction (DNRA), which is an anaerobic respiration process where microbes use nitrate as an electron acceptor, reducing it to nitrite and then ammonium [Ward, 2013]. These types of paradigm-shattering discoveries are becoming commonplace with the advances in genetic technology. However, there is still much work to be done.

The key to all life is with nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA stores the genetic information, and RNA uses the genetic code for protein creation. There have been multiple technologies that have helped further our understanding of microbial ecology through DNA analysis. The keystone of these technologies is the polymerase chain reaction (PCR). PCR is an enzymatic reaction used to generate copies of a target DNA strand through a series of temperature cycles. Before Karry Mullis developed PCR [Pepper et al., 2015], scientists relied on gene probes to try to study genetic material, but the signal was often too weak to elucidate any usable information. Each PCR cycle is able to double the amount of target DNA. Multiple PCR cycles exponentially increase the amount of DNA, allowing it to be studied and quantified accurately. PCR uses DNA polymerase to copy the strands and then repeats the process until there is enough DNA to quantify. However, in order for DNA polymerase to work it has to have a 3’-OH group. Researchers are able to manipulate this characteristic to develop oligonucleotides, which are short strands of single-stranded DNA that starts with a 3’-OH which is used by DNA polymerase to complete the strands of DNA produced in the PCR process. These oligonucleotides are called primers, which allow researchers to target specific areas of DNA instead of amplifying the entire
Researchers can design primers so that DNA polymerase starts synthesizing at the regions that they most want to amplify.

Ideally, PCR would be used to sequence whole the genome of the bacteria, but this is difficult because of the vast amount of information it yields and because it is a much more expensive process. Instead of sequencing whole genomes, researchers target specific areas of DNA that have enough information to differentiate between species, but also enough in common that the researcher knows what they are designing the primer for. One of the easiest sections of DNA to sequence for bacteria is the 16S rRNA subunit. The 16S rRNA is part of the 30S prokaryotic ribosome gene and is highly conserved across all bacteria. Additionally, nearly all bacteria have the gene because ribosomes are required for protein synthesis, making the 16S rRNA an easy gene to create a universal primer for. The 16S rRNA gene has nine hyper variable regions, each flanked by a highly-conserved region. This means that not only is it possible to create a primer that can work for nearly all bacterial genera, but it is also possible to differentiate between the different genera because of the hypervariable regions [Pepper et al., 2015]. The 16S rRNA represents the gold standard for bacterial community analysis due to the ease of access and because of the large library that has been created for the different genera, thus making it easier for researchers to match sequences to known sequences and taxonomic classifications [Vierheilig et al., 2015]. 16S rRNA analysis has been helpful in understanding the nitrogen cycle by identifying the microbial ecology and microbial controls on the nitrogen cycle [Francis et al., 2007; Fierer et al., 2012b, 2012a].

The PCR process has provided insight into the diversity of microscopic life and has generated a more comprehensive picture of gene functions and metabolic pathways
that can provide insight into global biogeochemical cycles like the nitrogen cycle. This is particularly true for engineered systems. For example, genetic analysis in wastewater treatment plants have allowed plant managers to adjust nutrient levels, temperatures, and other factors to optimize the nitrogen removal from the wastewater [Lu et al., 2014]. Molecular techniques like PCR have helped target specific microbes in order to make nitrogen removal in wetlands more efficient [Yan et al., 2018]. However, microbial research in bioengineered nitrogen removal systems is limited. There has been very little work done on the community structure, defined here as the composition and abundance of microbes grouped based on ecosystem function, of these bioengineered systems and how these systems could be optimized [Graham et al., 2016].

While there is still much to be learned about engineered systems, there is a wider knowledge base regarding natural microbial communities that can be applied to some aspects of engineered systems. One of the most common metrics that microbial ecologists use to measure microbial communities is diversity. Diversity is a common metric because it indicates higher ecosystem productivity, efficiency, and stability [Ricklefs, 2010]. This is a particularly important characteristic for an engineered system, as a more stable and productive community leads to a more efficient bioreactor. However the relationships that govern microbial diversity are complex and are not well understood. Community structure, the various types and numbers of different species, and community interactions, the way that those species interact with each other, are one key factors for understanding how communities function and change over time [Pepper et al., 2015]. Soil microbial communities are generally highly diverse, but they are also highly stratified based on the environmental conditions. Soil microbial community composition is largely controlled by
carbon substrate, pH, and salinity and tends to be stratified based on those environmental constraints [Lozupone and Knight, 2007; Kopecky et al., 2012; Llorens-Marès et al., 2015]. Additionally, nutrient additions cause diversity to decline because it changes species abundance, often allowing for dominance of a few species [Aanderud et al., 2018]. However, microbial diversity is not controlled purely by environmental conditions, it is also impacted by bacterial interactions and community structure. Some functional taxa (nitrifiers, decomposers, etc.) are able to control nutrient type and availability and thereby much of the growth of the rest of the microbial community. Using these principles of community structure and diversity it can be possible to predict biogeochemical processes, particularly nitrogen and carbon cycling. Microbial community data has been particularly useful in explaining some of the variation in denitrification rates not explained by environmental data alone [Graham et al., 2016].

Studying microbial communities can give insights into biogeochemical cycles like the nitrogen cycle and can be particularly valuable for engineering treatment systems to increase longevity and efficiency. However, studying specific microbial functional roles in an ecosystem can have its challenges. This is particularly true for studying denitrification on the microbial level because there is a diverse group of bacteria capable of denitrification. Unlike nitrogen fixation, nitrate reduction, and other steps of the nitrogen cycle, there is not one set microbial guild that performs the reactions. Conversely, bacteria capable of nitrogen fixation are well known and classified. There is only one major known enzyme that is capable of nitrogen fixation, nitrogenase, making it easier to identify within diverse microbial communities. In addition, nitrogen fixation has
also been studied since Beijerinck discovered this biological process in 1901. Neither of these is true for denitrification.

Denitrification is a catch-all term used to describe multiple reactions that transform nitrate into nitrous oxide and molecular nitrogen. Bacteria known as denitrifying bacteria mediate all of these reactions. These denitrifying bacteria are estimated to make up around 15% of environmental bacteria (soil, water, etc) [Mrkonjic Fuka et al., 2007]. Additionally, more than 50 genera are capable of denitrification, with most belonging to the Proteobacteria phylum. Furthermore, there are many other denitrifiers found in other phylum. These bacteria are nearly all facultative aerobes, meaning that they will use oxygen as their terminal electron acceptor if available, but will switch to nitrate if not enough oxygen is present. Various enzymes catalyze the denitrification reactions: nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS). Due to the diverse nature of denitrifying bacteria, microbial denitrification is measured by the presence and quantity of the enzymes and not the microbial composition. Microbial denitrification can be quantified by measuring the quantity of the enzymes using quantitative PCR (qPCR). These reactions can then be used to determine the amount of total denitrification and ratios of enzymes can be used to estimate the amount going to nitrogen gas and the amount going to nitrous gas [Wu et al., 2018]. However, the qPCR reactions do not account for interactions between individuals. So while it is possible to determine how much enzyme is present for a particular part of the reaction, it is not helpful in determining the overall rate of denitrification across the ecosystem [Ashton et al., 2010; Cadotte et al., 2011; Graham et al., 2016]. Understanding the community structure can
also give insights to other reactions and interactions that might be impacting the
denitrification rate. For example, the presence of obligate aerobes would indicate that
there is too much oxygen for the denitrification reaction to occur, even if denitrification
enzymes are present [Aanderud et al., 2018].

Linking microbes to ecosystem processes and rates has been shown to be a
promising area of study to help explain variation from environmental data alone [Ashton
et al., 2010; Cadotte et al., 2011; Graham et al., 2016]. While this is a growing field in
environmental microbiology, the research is still limited on engineered systems. Further
research combining environmental data and microbial data in engineered systems could
help improve the engineered systems design and efficiency. This project involved
examining the microbial community structure and diversity of both a denitrification wall
installed in 2009 and a wall installed in 2018, which was to built to fulfill one of the main
objectives of this study: To gain a better understanding of the microbial community
within, and surrounding denitrification walls and how these communities change over
time.

**Hypotheses**

1. The microbial community will evolve from a diverse set of microbes to be
dominated by anoxic bacterial families, most of which will be denitrifiers.

2. The 2009 wall will have a more established denitrification community and there
will be higher numbers of denitrifiers present than in the 2018 wall.

3. The wall will transform the microbial composition downgradient from the wall to
the stream to include more anaerobic genera.
Methods

Microbial Sampling

For the genetic analysis, soil samples were collected once along each well transect at the approximate location of the wells by excavating to the middle of the denitrification wall using a Dutch auger. Samples were collected two feet upgradient, within the denitrification bioreactor, and two feet downgradient. Samples were collected in nine transect locations including the bioreactor installed in 2009. Samples were taken following Joux’s method for environmental sampling of microorganisms in the Environment [Joux et al., 2015]. Samples consisted of 2 g from the middle of the soil core. Samples were removed from the auger with a soil knife and put in 60 mL sterile Rnase/Dnase free transport bag from Fisher Scientific and then placed into a cooler and shipped with dry ice the same day to Omega Bioservices in Norcross, GA. After each sample the auger and soil knife were disinfected with 70% denatured ethanol.

After shipping the samples, they were tested for 16S rRNA to help give better understanding of the microbial community present. Omega Bioservices in Norcross, GA was responsible for the extraction and purification of nucleic acid, genetic library preparation, genetic library normalization and flow cell loading, sequencing library on appropriate Illumina platform, data delivery, and quality control testing. The samples were extracted following Omega Bioservices standard protocol and using the E.Z.N.A. Total DNA soil extraction kit [Omega Bio-Tek Inc Norcross, GA]. The extraction process involved washing each sample with Tris-EDTA (TE) buffer to help stabilize pH and remove common organic soil particles. The samples were then vortexed with TE buffer and phenol-chloroform-isoamyl alcohol and then centrifuged at 2700 g at 4°C with
the supernatant collected after 10 minutes. The DNA was then precipitated using isopropanol and sodium acetate and then centrifuged and subsequently washed with cold 70% ethanol. Next the samples were purified and quantified using a NanoDrop. Following the extraction and purification the lab used standard Illumina 16S primers covering the V3 and V4 regions [Illumina, 2013]. The samples were loaded into the Illumina MiSeq for PCR analysis with the primers, deoxyribonucleotide triphosphates (dNTPs), which are nucleotides containing triphosphate groups, and the DNA polymerase, Tag polymerase. The thermal cycle consisted of a denaturation step at 95°C for 3 minutes followed by 25 denaturation cycles of 95°C for 30 seconds, annealing at 55°C for 30 seconds, and amplifying at 72°C for 30 seconds with a final amplification cycle at 72°C for 5 minutes. The amplicons were then purified using AMPure XP beads (Agencourt AMPure XP PCR Purification Beckman Coulter Inc., Brea, CA, USA), pooled, and then sequenced [Illumina, 2013]. The sequences were trimmed, cleaned, analyzed and matched to known sequences and taxonomic classifications using mothur following the MiSeq standard operating procedure [Schloss et al., 2009; Kozich et al., 2013; Pepper et al., 2015]. After the trimming and cleaning of the sequences, UCHIME was used to remove chimeras and reference sequences from the Ribosomal Database Project (RDP) were used to remove irrelevant lineages, such as chloroplast, mitochondrial, archaeal, and eukaryotic lineages [Edgar et al., 2011; Cole et al., 2014]. The sequences were then aligned using the SILVA reference files and were given taxonomic classification using the RDP taxonomy reference files to create and identify operational taxonomic units (OTUs) [Quast et al., 2013; Cole et al., 2014].
Further statistical analysis was completed using R and R studio with the phyloseq R package following sample code developed by Dr. Zachary Aanderud and Erin Jones from the Brigham Young University Environmental Microbiology Lab \cite{McMurdie and Holmes, 2013; Jones and Aanderud, 2018; R Development Core Team, 2018}. Heatmaps, Principal Coordinates Analysis (PCoA), Shannon Diversity, and Redundancy Analysis (RDA) were all run using the phyloseq and vegan packages in R \cite{McMurdie and Holmes, 2013; Oksanen et al., 2013}. Principal Coordinate Analysis (PCoA) was based on a Bray-Curtis distance matrix to compare community composition along age, gradient, and treatment. Redundancy Analysis also used a Bray-Curtis distance matrix to visualize the impact of environmental factors on community composition. Shannon Diversity was used to summarize the diversity of the community. ANOVA, Tukey’s method, and PERMANOVA (Permutational Multivariate Analysis of Variance using distance matrices) using the \textit{adonis} function were run on the Shannon Diversity results to determine statistical significance between the control and treatment sections and between the old wall and the new wall. Further PERMANOVA were run on the RDA data to calculate variance in the community based on environmental data. Relative abundance was calculated using analysis of composition of microbiomes or ANCOM based, using gradient and age, as they were the significant factors from the PERMANOVA analysis. ANCOM was run using the ANCOM functions developed by Mandal \cite{Mandal et al., 2015}. ANCOM was used to analyze relative abundance over other methods because it does not assume independence between different factors unlike other relative abundance methods. Considering most environmental data has multiple interconnected factors it is important to not assume independence. Additionally it does not assume the operational
taxonomic units (OTUs) are evenly distributed. This helps detect changes in community data and helps to normalize sequence depth [Mandal et al., 2015]. ANCOM abundances were used to select 30 families for a heatmap. The families were selected from the ANCOM run using gradient and age because they were statistically significant from the PERMANOVA analysis and the families were selected for the heatmap at the 0.8 significance level. Full statistics are listed in Appendix 1.

**Results and Discussion**

**Impact on Diversity**

Shannon diversity index was used to calculate diversity. The diversity was analyzed based on age of the wall, treatment type (control vs treatment), and location above, within, and below the wall (gradient) to determine if any of these factors had an impact on diversity. Treatment and gradient both significantly influenced the bacterial diversity and richness, while the age of the bioreactor had no significant impact on diversity. Specifically, gradient caused a 9.31% increase in diversity (3-way ANOVA age × gradient × treatment, df=2, F=9.6, P=0.01) while treatment caused a 6.02% increase in diversity (3-way ANOVA, age × gradient × treatment, df=1, F=0.88, P=0.05) based on the Shannon diversity. Based on a Tukey’s HSD test, center sections were significantly different from the upgradient sections and treatment was significantly different from control. Diversity was highest in the center gradient of the wall and was higher in the control center sections than the treatment center sections (Figure 17 and Figure 18). The center control section of the bioreactor had a 19.32% increase in diversity on average, while the center treatment section had a 5.94% increase in diversity on average. Further statistics for Shannon diversity calculations are in Appendix 1.
The fact that the control sections had a greater increase in diversity shows that the increase in diversity might be due to the excavation of the trench, causing the aeration.
and mixing of different soil horizons rather than the carbon nutrient addition of the woodchips. This also suggests that the carbon addition, and resulting biogeochemical changes, dampened the impact of the excavation and decreased the overall diversity of these sections when compared to the control center sections. Regardless of the reason for the increase in diversity, it indicates that the microbial community is both more robust and more resilient to change than a community with low diversity [Lozupone and Knight, 2007; Ricklefs, 2010]. If the diversity increases were due to the excavation, further monitoring of the diversity would be necessary to confirm that high levels of diversity will be maintained throughout time. While the diversity increase may have been due to the excavation, the diversity remained high even after 10 years with the 2009 reactor as evidenced by the fact that that age was not significant. This demonstrates that denitrification walls can sustain a high level of diversity. This likely positively impacts long term sustainability of denitrification rates due to the fact that higher diversity is linked to increased denitrification [Graham et al., 2016]. These results are confirmed by the stability of denitrification rates over an almost 10-year period. A community with high diversity increases the likelihood that there will be multiple species of bacteria capable of performing denitrification. Having multiple types of denitrifiers present reduces the risk that the reactor will fail if one community is disrupted. In a diverse population, there are multiple other denitrifiers that can replace a disrupted community and keep denitrification rates high. Furthermore, diversity enhances denitrification rates because there are other positive microbial interactions that can assist the denitrifiers. For example, various decomposers transform carbon from the woodchips into labile carbon
accessible to the denitrifiers and other microbes that the denitrifiers rely on to complete their reactions.

**Community Structure**

The results suggest that age and gradient were the main drivers of bacterial community variation, while treatment did not have a significant impact on community variation. This conclusion is based on a PERMANOVA run using the *adonis* function in vegan and a Principle Coordinate Analysis (PCoA) both based on a Bray-Curtis distance matrix (Figure 19). The PCoA showed a clustering along the x-axis based on age explaining 20.2% of the variation and showed a clustering along the y-axis based on gradient explaining 12.6% of the variation. This conclusion was supported by the PERMANOVA results that showed that age and gradient were significant, while treatment was not significant (age, df=1, $R^2=0.134$, $P=0.001$; gradient df=2, $R^2=0.165$, $P=0.001$; treatment df=1, $R^2=0.047$, $P=0.078$).
The fact that age significantly impacted the community structure, but not the diversity, shows that the community does evolve with time. However, those changes are not decreasing the overall diversity of the system. The number of species present was similar across samples, but the actual species making up the communities were different. Gradient impacting community structure could be caused by a variety of environmental factors, as evidenced by the fact that treatment did not have a significant impact on the community structure. It could be due to a natural change across the width of the wall that was present before the installation of the wall. It could also be explained by the excavation itself, which appears to have also impacted the diversity.
Community Structure and Environmental Variables

The differences in community structure were further explored through a Redundancy Analysis (RDA) to examine the impact of environmental factors on the bacterial community. This analysis was done on the entire dataset (Figure 20) and on the dataset from only the 2018 wall section (Figure 21). The RDA was run on community data with a Bray-Curtis distance matrix and the following environmental data: nitrate, ammonia, pH, total organic carbon, dissolved oxygen, temperature, conductivity, turbidity, and oxidation-reduction potential. The RDA on the entire dataset showed that total organic carbon was significant in explaining bacterial variation (TOC, df=1, F=2.41, P=0.01). The RDA on only the 2018 wall showed that nitrate, TOC, and conductivity explained part of the community variation (NO₃, df=1, F=2.363, P=0.040; TOC, df=1, F=1.91, P=0.050; Cond, df=1, F=3.31, P=0.015).

Figure 20: Redundancy Analysis on both the 2009 wall and the 2018 wall. Only TOC was a statistically significant factor influencing variability across the entire denitrification wall.
Figure 21: Redundancy Analysis on the 2018 wall. NO$_3$, TOC, and Conductivity were all significant environmental factors influencing bacterial community variability.

Overall, the bacterial community was most impacted by the carbon content, which follows nutrient stoichiometry because nutrients govern community structure [Aanderud et al., 2018]. The areas most impacted by the carbon were the new (2018) center sections. The 2018 wall community variability was more significantly impacted by environmental factors like conductivity, TOC and NO$_3$ than the 2009 wall. The conductivity explained the most variability in the new wall. The conductivity explained 68.8% of the variation in the bacterial community for the upgradient samples of the new wall section. Variation in community populations with changing levels of conductivity have been tied to changing gradients and soil type, particularly soils with high clay content [Geyer et al., 2013; Xue et al., 2018]. However, the reason behind the change due to conductivity is unclear. Nitrate explained 17.8% of the bacterial variation of the 2018 wall. It was most strongly connected to the upgradient and center sections of the wall. This matches the water quality data where the nitrate levels were the highest in the upgradient sections but
decreased along the wall. This shift shows that there is some nitrogen transformation, likely denitrification that is impacting the bacterial communities.

**Family Abundance**

Further conclusions can be drawn from the most relatively abundant bacterial families as shown in the heatmap (Figure 22). The heatmap was created using relative abundance calculated using ANCOM, specifically analyzing abundance based on differences in age and gradient. The first major grouping evident from the heatmap is the grouping of the majority of the upgradient samples in the middle of the heatmap; they are very similar to each other based on the close branching of the dendrograph at the top of the heatmap. This indicates that these sites were not disturbed and are behaving like the untreated communities. This is also what the communities would have likely been composed of if the reactor had not been installed. The families dominant in this region of the heatmap are common soil bacteria like *Rhizobiaceae* and *Cystobacteraceae* and *Nitrospiraceae*. These families are known for nitrate oxidation (nitrification), nitrogen fixation, iron oxidation, and sulfur reduction [Dos Santos et al., 2013; Carrareto Alves et al., 2014; Daims, 2014].
Figure 22. Heatmap based on relative abundance calculated using ANCOM. Labels describe one or more samples based on location, age and treatment.

Beyond the upgradient samples grouping together, there are two other major groupings. The first grouping is both the center and downgradient sections of the old wall (2009). These samples are dominated by sulfur reducing families like *Selenomondales*, *Synthrophobacteraceae*, *Syntrophaceae*, and *Desulfo bacteraceae*. Common methanogenic bacteria *Methyl coccaceae* and *Anaerolineaceae* were also dominant in these samples. This further supports previous findings that complete denitrification has already occurred before the center well removing all nitrate, and that the old
denitrification wall was oversized [Schmidt and Clark, 2012a; Bowman, 2014; Kuever, 2014; Rosenberg et al., 2014].

The last major grouping in the heatmap is primarily made up of the new wall center and downgradient samples as can be seen in the right-hand side of the heatmap. This grouping dominated by Proteobacteria like Burkholderiales and Comamonadaceae. These bacterial families have genera that are common denitrifiers [Willems, 2014]. Additionally, the Planococcaceae family was abundant in this grouping. The Planococcaceae family is known for being part of a functional group that degrades lignocellulose [Shivaji et al., 2014]. This likely signifies the presence of bacteria breaking down the sawdust material. This section also had nitrogen fixing bacteria and nitrate reducers present. The control samples were also found in this grouping, although with smaller abundance values than the treatment sections. This indicates that the installation of the wall had a significant impact on the microbial communities even when carbon was not added to the system. However, the control sections had a lesser abundance of denitrifying bacteria and a higher abundance of common obligate anaerobes that do not reduce nitrogen or sulfur. The downgradient control sample even had the highest relative abundance of Methyloccaceae, which is a methanogen. While the control samples had some similarities to the treatment sections, there is a functional difference between the most relative abundant taxa.

It is useful to compare the samples from the old wall to the new wall as the old wall is now primarily dominated by sulfur reducers, indicating that the community of the reactor has stratified based on electron acceptor availability. The denitrifiers are likely further upgradient than the area that was sampled, while the new wall does not yet have
signs of this stratification. In order to confirm that the reactor is indeed stratifying based on electron acceptor availability further testing would be necessary on both the 2009 reactor and the 2018 reactor. It would be important to sample the 2009 reactor in between the upgradient well and the center gradient well to determine if denitrification occurs in that section. It would also be important to sample the 2018 reactor as time goes on to see if the community stratifies.

While there were multiple denitrifying bacteria found in the samples, there were only two major families that were among the most abundant. Searching through all of the OTUs, there were multiple genera of Proteobacteria, which are the most common type of denitrifiers found in wastewater treatment plants along with *bacillus*, *thiobacillus*, *pseudomonas*, *acinetobacter*, and along with many other genera that are known denitrifiers [Wang et al., 2018]. However, aside from the Proteobacteria, many of these families or genera were not among the most abundant families or genera in the samples. This can be seen from the heatmap that shows the most abundant families based on ANCOM calculations. Even though common denitrifiers do not appear to be significantly abundant, that does not mean that denitrification is not occurring. Firstly, the vast majority of the bacteria in the sample could not be classified, meaning that there might be major denitrifiers that were not identified based on lack of information about them in the genetic database. Secondly, denitrifiers are capable of reducing nitrate in significant amounts even if they are not a large part of the bacterial community [Mrkonjic Fuka et al., 2007]. This means that even though the denitrifiers are not present in a significant abundance, they are still likely having a significant impact on the denitrification in the system.
While the community structure and diversity data have given insights into the microbial processes, it would be interesting to pair these data with functional gene analysis to better understand what denitrification pathway was the most common: nitrogen gas, nitrous gas, or some other constituent. While, these questions cannot be answered from the current information, it is possible to infer that there is a healthy population of denitrifying bacteria based on the chemical analysis of the walls. Additionally, this population will likely be sustained in the new wall because of the high levels of diversity, making the population more stable. This helps indicate the sustainability of the wall, because there is no single genera that performs denitrification, or associated metabolic processes. The more diverse the community the more likely that there will be genera that can perform denitrification over a long period of time.

Conclusion

Overall, the microbial data suggests that denitrification is occurring within the denitrification wall. Hypothesis one which states, “the microbial community will evolve from a diverse set of microbes to be dominated by anoxic bacterial families, most of which will be denitrifiers,” was partially correct. Anoxic bacterial communities dominated the system, but the number of denitrifiers could not be quantified, although there were some present. The community was also more diverse in the center sections, likely due to the excavation of the wall, but the treatment had a slightly lower diversity than the control sections. The decrease in the treatment sections was likely due to the nutrient addition, which has been shown to decrease overall biodiversity and allow for the community to be dominated by a few species [Bobbink et al., 2010; Leff et al., 2015; Lee
While diversity was high, that does not necessarily mean that denitrification will be high as evidenced by the fact that diversity was the highest diversity and was also the section where there was minimal nitrate reduction. Diversity does not indicate denitrification levels, but instead indicate the health and stability of the overall community. It is not currently possible to conclude for hypothesis two or three that the 2009 wall will have a more established denitrification community or that there will be higher numbers of denitrifiers present than in the 2018 wall or that the wall will transform the microbial composition downgradient from the wall to the stream to include more anaerobic genera. While anaerobic families dominated the downgradient sections, it is unclear if the denitrification wall was the source of the anaerobic dominance or if that was already how the community was structured. The microbial data can be used further to confirm denitrification and can be used in proper sizing of denitrification walls. The microbial data also indicates that the wall will be likely be sustainable for long periods of time due to high microbial diversity within the system across both the 2009 wall and the 2018 wall.

Chapter 4: Development of a Spatial Tool for Denitrification Tool Placement

Introduction

Geographic information systems (GIS) have been used in nearly every field to help maximize resources and efficiency. There have been many examples using GIS to help manage natural resources more effectively [Baker and Miller, 2013; Kulkarni et al., 2014; Albano et al., 2015; Pickard et al., 2015; Saha and Eckelman, 2015]. GIS is also commonly applied to help make agriculture more efficient, particularly with fertilizer application and irrigation [Todorovic and Steduto, 2003; Fortes et al., 2005; Liu, 2009;
Geospatial analysis has been shown to have significant power to not only preserve resources, but where to best use them. For example, GIS has been used in agriculture to determine what areas of a field have the best soil type for particular plants and to develop the most effective planting strategies [Paz, 2009]. This same concept can also be applied to placing bioreactors and other environmental tools. One of the biggest obstacles to widespread use of denitrification bioreactors is determining installation locations. Denitrification walls will not be effective in all locations, so identifying suitable locations is key to their success. Denitrification bioreactors are dependent on having accessible groundwater. For example, Central California routinely has groundwater nitrate concentrations exceeding 90 mg/L, but the depth to groundwater in Central California is often greater than 25 meters, which makes it unfeasible for a denitrification wall bioreactor [Shrestha and Luo, 2018]. In places like California, where the groundwater is too deep or there is another barrier to bioreactor use, using other remediation methods would be more effective. For example, pump and treat remediation, or increasing fertilizer application efficiency, and other sustainable nitrogen management strategies have all been shown to be effective treatment methods [Hansen et al., 2017]. Using GIS and information about the functional requirements of denitrification walls it is possible to narrow down locations where the technology could be most effective. Building this preliminary spatial tool has also been helpful in calculating if denitrification walls would be effective in helping reduce the nitrate load to the Santa Fe River basin. If there is not a significant enough area where denitrification walls could be built within the watershed, then it is not a viable treatment option for the watershed and other remediation techniques should be explored.
Methods

Denitrification walls require high nitrogen levels, a confining layer, like the Hawthorne formation in Florida, and consistently high water tables, among other factors. Areas that meet all the functional requirements can be identified using spatial analysis and geographic information system software. The first task to determine land that would be suitable for a denitrification wall was to gather suitable GIS layers that could be manipulated and analyzed to isolate functional locations. A digital elevation model (DEM) of the watershed, a watershed area shape file, wetland area shape file, geology shape file, and a land cover shape file for the state of Florida were all downloaded from various government databases such as the USGS, US Fish and Game Service, and the Florida Department of Environmental Protection [Homer et al., 2015; Protection, 2016]. These files were imported into ESRI’s ArcMap version 10.4 and clipped down to the Santa Fe watershed level to narrow the search area.

As a further step, agricultural land and rangeland were selected from land cover data to determine land where this technology could be best implemented. Agricultural lands, either on a commercial scale or on a smaller scale are the best land cover type due to high usage of nitrogen fertilizer and high irrigation rates (Figure 23). Additionally, agricultural landowners are likely to have the equipment and ready access to woodchips or other high carbon media.
After selecting for agriculture, the next step was to isolate the clay layer to identify areas that could have a perched aquifer. Clay was selected for by clipping the Hawthorne Clay Layer attribute from Florida Department of Environmental Protection (FL-DEP) datasets. However, this layer did not cover the entire watershed, and so the clay layer from a national geology dataset from the USGS was also included (Figure 24).
Clay Layer Data from National Geology vs Hawthorne Clay Layer from Florida DEP

Figure 24. Clay layer selected from the geology layer with the watershed layer

The next step was to select for the wetland areas that would be indicative of saturated soils, high water tables, and long hydroperiods (Figure 25). Ponded water on the surface of a wetland is not always indicative of continuously saturated soils or wetlands that are groundwater fed. Seepage wetlands are wetlands that have continually saturated soils that are likely fed from low-lying groundwater. Seepage wetlands are the dominant type of wetlands treated in the present study at the Holly Factory for instance. Seepage wetlands form from groundwater that is so close to the surface that it rises up through the soil to the surface. Additionally, emergent wetlands are wetlands that are frequently dominated by perched aquifers. Emergent wetlands are dominated by emergent macrophytes, which can only grow in continually saturated soils or in shallow water. Wetlands that have shallow water ponded at the surface are most often dominated by
groundwater that is close to the surface as deeper aquifers usually provide more water depth and wetlands fed by surface flows often fluctuate in water depth and amount. This means that wetlands that are consistently dominated by emergent macrophytes are most likely fed by shallow groundwater [Carter, 1997]. In addition to seepage and emergent wetlands, riparian areas are likely to have consistently high water tables throughout the year [Mitsch et al., 2015]. Therefore in selecting areas that are best suited hydrologically for a denitrification wall it was important to select for emergent, seepage, and riverine wetlands from the wetland layer. Each isolated wetland area was buffered by 45 meters to find the most suitable locations for potential walls. 45 meters was chosen as the buffer distance because it is the minimum distance away from a wetland for construction due to state regulations for distance of construction between a wetland or other water source. Therefore 45 meters is the closest that a wall could be constructed in this area, and will increase the likelihood of a high water table, the treatment of a larger groundwater profile, and a reduction in excavation depths and costs.
After selecting for the wetland layer, the next step was to intersect all of the areas to determine suitable locations for the denitrification wall. Clay, agricultural, and rangeland layers were intersected followed by the buffered wetland data to narrow down possible locations to a smaller geographical area.

**Results and Discussion**

Intersecting the clay, agricultural, rangeland, and wetland layers yielded 37 square kilometers as shown in Figure 26. This area is small enough that it could be field verified and tested for denitrification wall suitability. There is still work that would need to be done at each site to determine if the location would be effective for this type of technology. Field testing could include gathering well samples from the locations, calculating the clay layer depth, soil testing, surveying landowners about fertilization use,
and overall nitrogen contamination in those areas. Doing so would not only narrow locations based on functional requirements for the denitrification wall, but it would also narrow locations based on actual nitrate-nitrogen contamination.

![Potential Wall Areas for Denitrification Walls in the Santa Fe Watershed](image)

Figure 26. Possible locations for a denitrification wall based on agriculture, rangeland, clay, and wetland data

Additionally, further work could be done to verify the clay layer data to make sure that it is accurate across the watershed. Florida DEP had a thorough Hawthorne Clay Formation data set, but it did not cover the entire watershed. The area identified by the national USGS geology layer is fairly well established as being part of the Hawthorne Clay Formation in Florida, but it is unclear if the boundaries of the data layer match the actual extent of the clay layer. Additionally, both data sets do not include clay layer depth, making it difficult to site denitrification walls without additional field testing. The
presence of the specific type of wetland chosen in this study does indicate continued saturation, but does not guarantee a confining layer that a denitrification wall can be keyed in to.

A further aspect of study would be to verify the wetland data. Much of the wetland data seems to be accurate, but it would be important to physically visit a random sample of the identified wetlands to make sure that the search criteria holds for the entire watershed and not just the research site. For example it would be important to test emergent wetlands to make sure that the groundwater feeding it is not from the Floridian aquifer, but from the perched aquifer. It would also be important to verify the riverine wetlands and that these areas have high water tables that would be suitable for denitrification walls.

One important aspect of field testing before denitrification wall deployment would be a determination of the hydraulic conductivity at each site. Making sure that the hydraulic conductivity is suitably high at each location would be vital to ensure for proper groundwater flow and treatment. Without conductive soil this technology could cause cementation and potentially cause flooding and infiltration problems on the property. However, this testing is something that would need to be done once a location was narrowed down further as hydraulic conductivity is highly variable across the landscape.

Conclusion

Spatial analysis is an effective tool for identifying locations where this technology could be implemented. Many of the functional requirements for these reactors can be
modeled spatially, making it easy to select for these attributes and narrow the search for the location to 36 square kilometers. Further work can be done to verify the established parameters. Additionally, it would be necessary to calculate the potential nitrate-nitrogen load reduction within the Santa Fe River watershed to see whether this technology has regional scale value. This calculation would require more accurate knowledge of the nitrate contamination levels across the watershed and if the areas that have high contamination values match with the areas identified by the spatial tool. With continued support from land owners, land managers, the Florida Department of Environmental Protection, the EPA, and researchers it is possible to have widespread implementation of this technology.

**Chapter 5: Conclusion**

**Overall Effectiveness**

The denitrification bioreactor is an effective treatment, not only on a pilot scale, but also on a property scale. This is demonstrated through the 3,360 kg/yr nitrate load reduction from the upgradient to the center well sections and the cost effectiveness of $1.73 per kg-N. The lower reduction rate of 70-75% is likely due to the lower hydraulic conductivity of the denitrification wall that may have been due to the excavation of the wall. This short-circuiting of the denitrification wall is a concern for this reactor and any future reactors. It is important to try to minimize the short-circuiting as much as possible, so that larger volumes of groundwater can be treated. It would be important to calculate the volume of water not being treated by the denitrification bioreactor, so that the impact on the pollution coming off of the property can be properly evaluated. The reactor is treating a relatively small volume of water (30 million liters per year compared to over
300 million liters of total discharge), but it is removing high levels of nitrogen, which makes it hard to gauge what kind of broad impact the reactor is having on the watershed. Evaluating the level of short-circuiting would be necessary before further installation of property scale walls. If the volume of water treated is not treating a significant percentage of the pollution coming off of the property then it would probably be better to install a smaller, targeted denitrification wall for the areas of the property with the highest nitrate concentrations in conjunction with other treatment technologies to minimize nitrate concentrations coming off of individual properties. There are also few negative environmental impacts from these bioreactors. There is a carbon pulse that can impact chemical oxygen demand in surrounding rivers and other water bodies. However, this pulse is short-lived and does not have a lasting negative impact on the environment. Overall, this type of technology could be very useful in helping landowners and regulatory agencies meet TMDL requirements, as it is cost effective, efficient, and requires no maintenance after installation.

The microbial communities within and surrounding the wall gave insights into the functionality and processes going on in the wall. The diversity of the microbial communities increased within the wall likely due to the disturbance caused by the installation process. High levels of diversity are good indicators of stability of the community and the likelihood of continued denitrification. This was supported by the 2009 wall, which still had high levels of diversity despite the amount of time between the disturbance and the sampling. However, in order to make firm conclusions about the diversity and stability of the reactor it would be important to complete further microbial testing. There were some denitrifying genera present, but they were not among the most
abundant genera. However, this does not mean that denitrification is not occurring in high amounts, as the bulk of the bacterial sequences could not be matched to a currently known sequence. Carbon was also a key driver of the community structure. Overall, the microbial data provided insights into the stability, general community structure, and confirmed how denitrification walls can be sized.

Suitable locations for denitrification walls are limited by a variety of factors. Therefore, it is important to check that installation sites have all of the necessary functional requirements and that enough field testing is completed before installation. Denitrification walls will not work everywhere, so isolating the places where they will work based on hydrology, level of nitrate-nitrogen concentration, depth to groundwater, presence of a confining layer, and other considerations is important. All of these considerations can be used to isolate areas where denitrification bioreactors could be effective using geographic information systems. By using GIS to calculate total potential areas for a denitrification wall, it can be possible to determine if denitrification bioreactors would make enough of a difference on the total nitrate concentrations in a watershed to be a suitable treatment option on a regional scale. However, while the spatial tool can give broad regional insights, it would need individual field testing to confirm that the area identified by the GIS model matches the necessary functional requirements.

Further Work

Further monitoring could be done on both the 2009 and 2018 bioreactors to confirm nitrate removal rates over time, and other chemical constituents could be examined to confirm that the wall is not a source of other nitrogen compounds. It would
also be interesting to monitor the 2018 wall to see how the hydraulic conductivity changes with time as groundwater flow paths that were destroyed during excavation are reestablished. This could determine if this changes the amount of groundwater that is treated by the denitrification wall. To date, phosphorus has not been monitored, but monitoring phosphorus could give additional insights into nutrient interactions. It would also be interesting to see if the denitrification wall is having an impact on the phosphorus levels leaving the property, especially because phosphorus is naturally high in Alachua County, Florida.

There is also plenty of opportunity for further work on the microbial level. In order to confirm many of the conclusions about community structure and diversity of the wall over time, more data would be needed. There is also more opportunity to tie the microbial communities to ecological processes, like denitrification, nitrification, and other microbially mediated processes, that would require further work and analysis [Cadotte et al., 2011]. Further microbial analysis could also give more insight into the most common denitrification pathway. It could also help further maximize denitrification and long-term stability by better understanding how to foster a diverse denitrifying bacterial community. There could also be more work on the microbial interactions with the carbon source to determine how microbes respond to different types of carbon media. It would also be interesting to monitor how the level of phosphorus is impacting the microbial community. Study of these processes could give insights to the amount of carbon that the microbes need in relation to the amount of nitrogen, according to the Redfield Ratio [Aanderud et al., 2018].
The spatial tool also needs field verification to ensure that the geospatial analysis was accurate. After field testing, it would be important to encourage landowners with property highlighted by the spatial tool to implement this technology. It would also be important to estimate the amount of nitrate load reduction to the watershed if more bioreactors were installed in the area identified as suitable locations for denitrification walls. This estimate would require accurate data on nitrate concentrations and how those nitrate concentrations are distributed across the county. This type of effort would require not only assistance from academia, but also from regulatory agencies, local governments, and landowners in the area. Further outreach and education could not only help this technology become more widespread, but also help reduce the overall nitrate load in the Santa Fe River Basin.

Overall, denitrification walls have the potential to be effective long-term treatments for an entire property. Denitrification walls are not only cost effective, but also have high treatment efficiencies, making them a good option for landowners looking to reduce the overall nitrate concentration leaving their property. They are also easy for a landowner to install with some technical guidance. The ease of implementation, and low maintenance requirements, and low cost make it a good option for landowners. Further research into the hydraulic conductivity, excavation impacts, and the microbial community would maximize nitrate treatment and make this technology more broadly applicable.
Appendix

Nitrate Statistics

Oneway Analysis of NO3 By Gradient Treatment=Control

Oneway Anova
Summary of Fit
Rsquare 0.223535
Adj Rsquare 0.176476
Root Mean Square Error 1.432568
Mean of Response 7.631389
Observations (or Sum Wgts) 36
Analysis of Variance

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<th>Source</th>
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<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
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Means for Oneway Anova

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<th>Lower 95%</th>
<th>Upper 95%</th>
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Std Error uses a pooled estimate of error variance

Oneway Analysis of NO3 By Gradient Treatment=Treatment

Oneway Anova
Summary of Fit
Rsquare 0.747761
Adj Rsquare 0.732474
Root Mean Square Error 1.590426
Mean of Response 4.771111
Observations (or Sum Wgts) 36
Analysis of Variance

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Means for Oneway Anova

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<th>Upper 95%</th>
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Std Error uses a pooled estimate of error variance

Means Comparisons
Comparisons for all pairs using Tukey-Kramer HSD
Confidence Quantile
q* Alpha
2.45379 0.05
HSD Threshold Matrix
Abs(Dif)-HSD

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Positive values show pairs of means that are significantly different.

Connecting Letters Report
Level | Mean
-----|------
Up    | A 7.1483333
Down  | A 6.0466667
Center| B 1.1183333

Levels not connected by same letter are significantly different.

Ordered Differences Report

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Oneway Analysis of NO3 By Treatment Gradient=Center

Oneway Anova
Summary of Fit
Rsquare 0.836261
Adj Rsquare 0.828818
Root Mean Square Error 1.343947
Mean of Response 4.02625
Observations (or Sum Wgts) 24
Pooled t Test
Treatment-Control
Assuming equal variances
Difference -5.8158 t Ratio -10.6
Std Err Dif 0.5487 DF 22
Upper CL Dif -4.678 Prob > |t| <.0001
Lower CL Dif -6.9537 Prob > t 1
Confidence 0.95 Prob < t <.0001
Analysis of Variance
Source | DF | Sum of Squares | Mean Square | F Ratio | Prob > F |
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Means for Oneway Anova
Level | Number | Mean | Std Error | Lower 95%  | Upper 95% |
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Std Error uses a pooled estimate of error variance
Means Comparisons
Comparisons for all pairs using Tukey-Kramer HSD
Confidence Quantile
q* Alpha
2.07388 0.05
HSD Threshold Matrix
Abs(Dif)-HSD

Control  Treatment
Control  -1.1379  4.678
Treatment  4.678  -1.1379

Positive values show pairs of means that are significantly different.

Connecting Letters Report

Level     Mean
Control   A  6.9341667
Treatment B  1.1183333

Levels not connected by same letter are significantly different.

Ordered Differences Report

Level   - Level Difference   Std Err Dif   Lower CL   Upper CL   p-Value
Control  Treatment  5.815833  0.5486641  4.677972  6.953694  <.0001

Oneway Analysis of NO3 By Treatment Gradient=Down

Oneway Anova
Summary of Fit
Rsquare 0.256418
Adj Rsquare 0.222618
Root Mean Square Error 1.1242
Mean of Response 6.67875
Observations (or Sum Wgts) 24
Pooled t Test
Treatment-Control
Assuming equal variances
Difference -1.2642  t Ratio -2.75436
Std Err Dif 0.459  DF 22
Upper CL Dif -0.3123  Prob > |t| 0.0116
Lower CL Dif -2.216  Prob > t 0.9942
Confidence 0.95  Prob < t 0.0058

Analysis of Variance
Source   DF   Sum of Squares   Mean Square   F Ratio   Prob > F
Treatment 1   9.588704   9.5887   7.5865   0.0116
Error    22  27.806158   1.26392
C. Total 23 37.394863

Means for Oneway Anova
Level  Number  Mean   Std Error   Lower 95%   Upper 95%
Control  12  7.31083  0.32454  6.6378  7.9839
Treatment 12  6.04667  0.32454  5.3736  6.7197

Std Error uses a pooled estimate of error variance

Means Comparisons
Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile
q*  Alpha 2.07388 0.05

HSD Threshold Matrix
Abs(Dif)-HSD

Control  Treatment
Control  -0.95184  0.31232
Treatment  0.31232  -0.95184
Positive values show pairs of means that are significantly different.

Connecting Letters Report

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Levels not connected by same letter are significantly different.

One-way Analysis of NO3 by Treatment Gradient=Up

One-way Anova

Summary of Fit

| Rsquare | 0.139088 |
| Adj Rsquare | 0.099955 |
| Root Mean Square Error | 1.94987 |
| Mean of Response | 7.89875 |
| Observations (or Sum Wgts) | 24 |

Pooled t Test

Treat-Control

Assuming equal variances

| Difference | -1.5008 | t Ratio | -1.88528 |
| Std Err Dif | 0.7961 | DF | 22 |
| Upper CL Dif | 0.1501 | Prob > | t | 0.0727 |
| Lower CL Dif | -3.1518 | Prob > | t | 0.9637 |

Confidence | 0.95 | Prob < | t | 0.0363 |

Analysis of Variance

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Means for One-way Anova

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Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

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HSD Threshold Matrix

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<td>Control</td>
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<td>Treatment</td>
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Positive values show pairs of means that are significantly different.

Connecting Letters Report

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<td>Treatment</td>
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Levels not connected by same letter are significantly different.
Ammonia Statistics

Oneway Analysis of NH3 By Gradient Treatment=Control
Oneway Anova
Summary of Fit
Rsquare 0.109897
Adj Rsquare 0.055951
Root Mean Square Error 0.092116
Mean of Response 0.130556
Observations (or Sum Wgts) 36
Analysis of Variance
Source    DF    Sum of Squares    Mean Square    F Ratio    Prob > F
Gradient 2   0.03457222  0.017286   2.0372  0.1465
Error 33 0.28001667  0.008485
C. Total 35 0.31458889

Means for Oneway Anova
Level    Number    Mean    Std Error    Lower 95%    Upper 95%
Center 12 0.1675 0.02659 0.1134 0.2216
Down 12 0.1325 0.02659 0.0784 0.1866
Up 12 0.091667 0.02659 0.03757 0.14577

Std Error uses a pooled estimate of error variance

Oneway Analysis of NH3 By Gradient Treatment=Treatment
Oneway Anova
Summary of Fit
Rsquare 0.444887
Adj Rsquare 0.411244
Root Mean Square Error 0.052815
Mean of Response 0.127778
Observations (or Sum Wgts) 36
Analysis of Variance
Source    DF    Sum of Squares    Mean Square    F Ratio    Prob > F
Gradient 2 0.07377222  0.036886 13.2237 <.0001
Error 33 0.09205  0.002789
C. Total 35 0.16582222

Means for Oneway Anova
Level    Number    Mean    Std Error    Lower 95%    Upper 95%
Center 12 0.190833 0.01525 0.15981 0.22185
Down 12 0.105833 0.01525 0.07481 0.13685
Up 12 0.086667 0.01525 0.05565 0.11769

Std Error uses a pooled estimate of error variance

Means Comparisons
Comparisons for all pairs using Tukey-Kramer HSD
Confidence Quantile
q* Alpha
2.45379 0.05
HSD Threshold Matrix
Abs(Dif)-HSD

<table>
<thead>
<tr>
<th></th>
<th>Center</th>
<th>Down</th>
<th>Up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
<td>-0.052910</td>
<td>0.03209</td>
<td>0.05126</td>
</tr>
<tr>
<td>Down</td>
<td>0.03209</td>
<td>-0.052910</td>
<td>-0.03374</td>
</tr>
<tr>
<td>Up</td>
<td>0.05126</td>
<td>-0.03374</td>
<td>-0.05291</td>
</tr>
</tbody>
</table>

Positive values show pairs of means that are significantly different.

Connecting Letters Report
<table>
<thead>
<tr>
<th>Level</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center A</td>
<td>0.19083333</td>
</tr>
<tr>
<td>Down B</td>
<td>0.10583333</td>
</tr>
<tr>
<td>Up B</td>
<td>0.08666667</td>
</tr>
</tbody>
</table>

Levels not connected by same letter are significantly different.

**Ordered Differences Report**

<table>
<thead>
<tr>
<th>Level</th>
<th>Level Difference</th>
<th>Std Err Dif</th>
<th>Lower CL</th>
<th>Upper CL</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center Up</td>
<td>0.1041667</td>
<td>0.0215615</td>
<td>0.051259</td>
<td>0.1570742</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Center Down</td>
<td>0.085</td>
<td>0.0215615</td>
<td>0.032092</td>
<td>0.1379075</td>
<td>0.0011</td>
</tr>
<tr>
<td>Down Up</td>
<td>0.0191667</td>
<td>0.0215615</td>
<td>-0.033741</td>
<td>0.0720742</td>
<td>0.651</td>
</tr>
</tbody>
</table>

**Oneway Analysis of NH3 By Treatment Gradient=Center**

**Oneway Anova**

Summary of Fit

<table>
<thead>
<tr>
<th>Rsquare</th>
<th>Adj Rsquare</th>
<th>Root Mean Square Error</th>
<th>Mean of Response</th>
<th>Observations (or Sum Wgts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.014192</td>
<td>-0.03062</td>
<td>0.10156</td>
<td>0.179167</td>
<td>24</td>
</tr>
</tbody>
</table>

Pooled t Test

Treatment-Control

Assuming equal variances

<table>
<thead>
<tr>
<th>Difference</th>
<th>Std Err Dif</th>
<th>Upper CL Dif</th>
<th>Lower CL Dif</th>
<th>Confidence</th>
<th>Prob &gt; t</th>
<th>Prob &lt; t</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02333</td>
<td>0.04146</td>
<td>0.010932</td>
<td>-0.06265</td>
<td>0.95</td>
<td>0.5793</td>
<td>0.3094</td>
</tr>
</tbody>
</table>

**Analysis of Variance**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>0.00326667</td>
<td>0.003267</td>
<td>0.3167</td>
<td>0.5793</td>
</tr>
<tr>
<td>Error</td>
<td>22</td>
<td>0.22691667</td>
<td>0.010314</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>23</td>
<td>0.23018333</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means for Oneway Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>0.1675</td>
<td>0.02932</td>
<td>0.1067</td>
<td>0.2283</td>
</tr>
<tr>
<td>Treatment</td>
<td>12</td>
<td>0.190833</td>
<td>0.02932</td>
<td>0.13003</td>
<td>0.25163</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance

**Oneway Analysis of NH3 By Treatment Gradient=Down**

**Oneway Anova**

Summary of Fit

<table>
<thead>
<tr>
<th>Rsquare</th>
<th>Adj Rsquare</th>
<th>Root Mean Square Error</th>
<th>Mean of Response</th>
<th>Observations (or Sum Wgts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.046895</td>
<td>0.003572</td>
<td>0.062783</td>
<td>0.119167</td>
<td>24</td>
</tr>
</tbody>
</table>

Pooled t Test

Treatment-Control

Assuming equal variances

<table>
<thead>
<tr>
<th>Difference</th>
<th>Std Err Dif</th>
<th>Upper CL Dif</th>
<th>Lower CL Dif</th>
<th>Confidence</th>
<th>Prob &gt; t</th>
<th>Prob &lt; t</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.026671</td>
<td>0.02563</td>
<td>0.02649</td>
<td>-0.07982</td>
<td>0.95</td>
<td>0.3094</td>
<td>0.1547</td>
</tr>
</tbody>
</table>

**Analysis of Variance**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>0.00326667</td>
<td>0.003267</td>
<td>0.3167</td>
<td>0.5793</td>
</tr>
<tr>
<td>Error</td>
<td>22</td>
<td>0.22691667</td>
<td>0.010314</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>23</td>
<td>0.23018333</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Treatment 1 0.00426667 0.004267 1.0825 0.3094
Error 22 0.08671667 0.003942
C. Total 23 0.09098333
Means for Oneway Anova
Level Number Mean Std Error Lower 95% Upper 95%
Control 12 0.1325 0.01812 0.09491 0.17009
Treatment 12 0.105833 0.01812 0.06825 0.14342
Std Error uses a pooled estimate of error variance
Oneway Analysis of NH3 By Treatment Gradient=Up
Oneway Anova
Summary of Fit
Rsquare 0.00256
Adj Rsquare -0.04278
Root Mean Square Error 0.051537
Mean of Response 0.089167
Observations (or Sum Wgts) 24
Pooled t Test
Treatment-Control
Assuming equal variances
Difference -0.005 t Ratio -0.23764
Std Err Dif 0.02104 DF 22
Upper CL Dif 0.03863 Prob > |t| 0.8144
Lower CL Dif -0.04863 Prob > t 0.5928
Confidence 0.95 Prob < t 0.4072
Analysis of Variance
Source DF
Treatment 1
Error 22
C. Total 23
Means for Oneway Anova
Level Number
Control 12
Treatment 12
Std Error uses a pooled estimate of error variance
Sum of Squares Mean Square F Ratio Prob > F
0.00015 0.00015 0.0565 0.8144
0.05843333 0.002656
0.05858333
Mean Std Error Lower 95% Upper 95%
0.091667 0.01488 0.06081 0.12252
0.086667 0.01488 0.05581 0.11752

Total N Statistics

Oneway Analysis of Total N By Gradient Treatment=Control

Oneway Anova
Summary of Fit
Rsquare 0.205514
Adj Rsquare 0.157364
Root Mean Square Error 1.311363
Mean of Response 7.7225
Observations (or Sum Wgts) 36
Analysis of Variance
Source | DF | Sum of Squares | Mean Square | F Ratio | Prob > F 
---|---|---|---|---|---
Gradient | 2 | 14.67965 | 7.33982 | 4.2682 | 0.0225 
Error | 33 | 56.749225 | 1.71967 | 
C. Total | 35 | 71.428875 | 

Means for Oneway Anova 

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
<td>12</td>
<td>2.00917</td>
<td>0.42956</td>
<td>1.1352</td>
<td>2.8831</td>
</tr>
<tr>
<td>Down</td>
<td>12</td>
<td>6.12583</td>
<td>0.42956</td>
<td>5.2519</td>
<td>6.9998</td>
</tr>
<tr>
<td>Up</td>
<td>12</td>
<td>7.24167</td>
<td>0.42956</td>
<td>6.3677</td>
<td>8.1156</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance 

Means Comparisons 

Comparisons for all pairs using Tukey-Kramer HSD 

Confidence Quantile 

<table>
<thead>
<tr>
<th>q*</th>
<th>Alpha</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.45379</td>
<td>0.05</td>
</tr>
</tbody>
</table>

HSD Threshold Matrix 

<table>
<thead>
<tr>
<th>Abs(Dif)-HSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up</td>
</tr>
<tr>
<td>Up</td>
</tr>
<tr>
<td>Down</td>
</tr>
<tr>
<td>Center</td>
</tr>
</tbody>
</table>

Positive values show pairs of means that are significantly different.

Connecting Letters Report 

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up</td>
<td>A</td>
</tr>
<tr>
<td>Down</td>
<td>A</td>
</tr>
<tr>
<td>Center</td>
<td>B</td>
</tr>
</tbody>
</table>

Levels not connected by same letter are significantly different.

Ordered Differences Report 

<table>
<thead>
<tr>
<th>Level</th>
<th>- Level</th>
<th>Difference</th>
<th>Std Err Dif</th>
<th>Lower CL</th>
<th>Upper CL</th>
<th>p-Value</th>
</tr>
</thead>
</table>
| Up | Center | 1.4275 | 0.1138 | 2.741168 | 0.0308 | ++++

| Up | Down | 1.2675 | -0.04617 | 2.581168 | 0.06 |

| Down | Center | 0.16 | -1.15367 | 1.15367 | 0.15 |

Oneway Analysis of Total N By Gradient Treatment=Treatment 

Oneway Anova 

Summary of Fit 

Rsquare 0.713848 
Adj Rsquare 0.696506 
Root Mean Square Error 1.488035 
Mean of Response 5.125556 
Observations (or Sum Wgts) 36 

Analysis of Variance 

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradient</td>
<td>2</td>
<td>182.28434</td>
<td>91.1422</td>
<td>41.1617</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>33</td>
<td>73.07015</td>
<td>2.2142</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>35</td>
<td>255.35449</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means for Oneway Anova 

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
<td>12</td>
<td>2.00917</td>
<td>0.42956</td>
<td>1.1352</td>
<td>2.8831</td>
</tr>
<tr>
<td>Down</td>
<td>12</td>
<td>6.12583</td>
<td>0.42956</td>
<td>5.2519</td>
<td>6.9998</td>
</tr>
<tr>
<td>Up</td>
<td>12</td>
<td>7.24167</td>
<td>0.42956</td>
<td>6.3677</td>
<td>8.1156</td>
</tr>
</tbody>
</table>
Std Error uses a pooled estimate of error variance
Means Comparisons
Comparisons for all pairs using Tukey-Kramer HSD
Confidence Quantile
$q^*\quad$ Alpha
2.45379 0.05
HSD Threshold Matrix
Abs(Dif)-HSD

<table>
<thead>
<tr>
<th></th>
<th>Up</th>
<th>Down</th>
<th>Center</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up</td>
<td>-1.4906</td>
<td>-0.3748</td>
<td>3.7419</td>
</tr>
<tr>
<td>Down</td>
<td>-0.3748</td>
<td>-1.4906</td>
<td>2.626</td>
</tr>
<tr>
<td>Center</td>
<td>3.7419</td>
<td>2.626</td>
<td>-1.4906</td>
</tr>
</tbody>
</table>

Positive values show pairs of means that are significantly different.

Connecting Letters Report

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up A</td>
<td>7.241667</td>
</tr>
<tr>
<td>Down A</td>
<td>6.1258333</td>
</tr>
<tr>
<td>Center</td>
<td>2.0091667</td>
</tr>
</tbody>
</table>

Levels not connected by same letter are significantly different.

Ordered Differences Report

<table>
<thead>
<tr>
<th>Level - Level</th>
<th>Difference</th>
<th>Std Err Dif</th>
<th>Lower CL</th>
<th>Upper CL</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up Center</td>
<td>5.2325</td>
<td>0.6074876</td>
<td>3.74185</td>
<td>6.72315</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Down Center</td>
<td>4.11667</td>
<td>0.6074876</td>
<td>2.62602</td>
<td>5.607316</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Up Down</td>
<td>1.115833</td>
<td>0.6074876</td>
<td>-0.37482</td>
<td>2.606483</td>
<td>0.1735</td>
</tr>
</tbody>
</table>

Oneway Analysis of Total N By Treatment Gradient=Center

Oneway Anova
Summary of Fit
Rsquare 0.841209
Adj Rsquare 0.833991
Root Mean Square Error 1.176264
Mean of Response 4.60125
Observations (or Sum Wgts) 24
Pooled t Test
Treatment-Control
Assuming equal variances
Difference  -5.1842  t Ratio -10.7957
Std Err Dif  0.4802  DF  22
Upper CL Dif -4.1883  Prob > |t|  <.0001
Lower CL Dif -6.1801  Prob > t  1
Confidence  0.95  Prob < t <.0001

Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>161.2535</td>
<td>161.254</td>
<td>116.5465</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>22</td>
<td>30.43916</td>
<td>1.384</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>23</td>
<td>191.69266</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means for Oneway Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>7.19333</td>
<td>0.33956</td>
<td>6.4891</td>
<td>7.8975</td>
</tr>
<tr>
<td>Treatment</td>
<td>12</td>
<td>2.00917</td>
<td>0.33956</td>
<td>1.305</td>
<td>2.7134</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance
Means Comparisons
Comparisons for all pairs using Tukey-Kramer HSD
Confidence Quantile
q* Alpha
2.07388 0.05
HSD Threshold Matrix
Abs(Dif)-HSD

<table>
<thead>
<tr>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-0.9959  4.1883</td>
</tr>
<tr>
<td>Treatment</td>
<td>4.1883  -0.9959</td>
</tr>
</tbody>
</table>

Positive values show pairs of means that are significantly different.

Connecting Letters Report

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>A 7.1933333</td>
</tr>
<tr>
<td>Treatment</td>
<td>B 2.0091667</td>
</tr>
</tbody>
</table>

Levels not connected by same letter are significantly different.

Oneway Analysis of Total N By Treatment Gradient=Down

Summary of Fit

<table>
<thead>
<tr>
<th>Rsq</th>
<th>Adj Rsq</th>
<th>Root Mean Square Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.292134</td>
<td>0.259959</td>
<td>0.99786</td>
</tr>
</tbody>
</table>

Mean of Response 6.739583

Observations (or Sum Wgts) 24

Pooled t Test

Treatment-Control

Assuming equal variances

<table>
<thead>
<tr>
<th>Difference</th>
<th>t Ratio</th>
<th>Std Err Dif</th>
<th>DF</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1.2275</td>
<td>-3.0132</td>
<td>0.4074</td>
<td>22</td>
</tr>
</tbody>
</table>

Upper CL Dif -0.3827  Prob > |t| 0.0064
Lower CL Dif -2.0723  Prob > t 0.9968
Confidence 0.95  Prob < t 0.0032

Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>9.040538</td>
<td>9.04054</td>
<td>9.0793</td>
<td>0.0064</td>
</tr>
<tr>
<td>Error</td>
<td>22</td>
<td>21.905958</td>
<td>0.99573</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>23</td>
<td>30.946496</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means for Oneway Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>7.35333</td>
<td>0.28806</td>
<td>6.7559</td>
<td>7.9507</td>
</tr>
<tr>
<td>Treatment</td>
<td>12</td>
<td>6.12583</td>
<td>0.28806</td>
<td>5.5284</td>
<td>6.7232</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile
q* Alpha
2.07388 0.05

HSD Threshold Matrix
Abs(Dif)-HSD

<table>
<thead>
<tr>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-0.844840.38266</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.38266  -0.84484</td>
</tr>
</tbody>
</table>

Positive values show pairs of means that are significantly different.

Connecting Letters Report

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>A 7.3533333</td>
</tr>
</tbody>
</table>

"Connecting Letters Report"
Treatment B 6.125833
Levels not connected by same letter are significantly different.
Oneway Analysis of Total N By Treatment Gradient=Up
Oneway Anova
Summary of Fit
Rsquare 0.128395
Adj Rsquare 0.088776
Root Mean Square Error 1.876581
Mean of Response 7.93125
Observations (or Sum Wgts) 24
Pooled t Test
Treatment-Control
Assuming equal variances
Difference -1.3792 t Ratio -1.80022
Std Err Dif 0.7661 DF 22
Upper CL Dif 0.2097 Prob > t| 0.0856
Lower CL Dif -2.968 Prob > t 0.9572
Confidence 0.95 Prob < t 0.0428
Analysis of Variance
Source DF Sum of Squares Mean Square F Ratio Prob > F
Treatment 1 11.412604 11.4126 3.2408 0.0856
Error 22 77.474258 3.5216
C. Total 23 88.886863
Means for Oneway Anova
Level Number Mean Std Error Lower 95% Upper 95%
Control 12 8.62083 0.54172 7.4974 9.7443
Treatment 12 7.24167 0.54172 6.1182 8.3651
Std Error uses a pooled estimate of error variance
Means Comparisons
Comparisons for all pairs using Tukey-Kramer HSD
Confidence Quantile
q* Alpha
2.07388 0.05
HSD Threshold Matrix
Abs(Dif)-HSD
Control Treatment
Control -1.5888 -0.2097
Treatment -0.2097 -1.5888
Positive values show pairs of means that are significantly different.
Connecting Letters Report
Level Mean
Control A 8.620833
Treatment A 7.241667
Levels not connected by same letter are significantly different.

Organic Nitrogen Statistics

Oneway Analysis of Organic N By Gradient Treatment=Control

Oneway Anova
Summary of Fit
Rsquare 0.082548
Adj Rsquare 0.026944
Root Mean Square Error 0.261958
Mean of Response 0.035833
Observations (or Sum Wgts) 36

Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradient</td>
<td>2</td>
<td>0.20375</td>
<td>0.101875</td>
<td>1.4846</td>
<td>0.2413</td>
</tr>
<tr>
<td>Error</td>
<td>33</td>
<td>2.264525</td>
<td>0.068622</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>35</td>
<td>2.468275</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means for One way Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
<td>12</td>
<td>0.14</td>
<td>0.07562</td>
<td>-0.0139</td>
<td>0.29385</td>
</tr>
<tr>
<td>Down</td>
<td>12</td>
<td>0.0025</td>
<td>0.07562</td>
<td>-0.1514</td>
<td>0.15635</td>
</tr>
<tr>
<td>Up</td>
<td>12</td>
<td>-0.035</td>
<td>0.07562</td>
<td>-0.1889</td>
<td>0.11885</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance

Oneway Analysis of Organic N By Gradient Treatment=Treatment

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD
Confidence Quantile
$q^*$ Alpha
2.45379 0.05

HSD Threshold Matrix

<table>
<thead>
<tr>
<th>Center</th>
<th>Down</th>
<th>Up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
<td>-0.239060</td>
<td>0.42844</td>
</tr>
<tr>
<td>Down</td>
<td>0.33677</td>
<td>-0.23906</td>
</tr>
<tr>
<td>Up</td>
<td>0.42844</td>
<td>-0.1474</td>
</tr>
</tbody>
</table>

Positive values show pairs of means that are significantly different.

Connecting Letters Report

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
<td>0.7025</td>
</tr>
<tr>
<td>Down</td>
<td>B</td>
</tr>
<tr>
<td>Up</td>
<td>B</td>
</tr>
</tbody>
</table>

Levels not connected by same letter are significantly different.

Ordered Differences Report
### Oneway Analysis of Organic N By Treatment Gradient=Center

#### Oneway Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Difference</th>
<th>Std Err Dif</th>
<th>Lower CL</th>
<th>Upper CL</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center Up</td>
<td>0.6675</td>
<td>0.0974263</td>
<td>0.428436</td>
<td>0.906564</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Center Down</td>
<td>0.5758333</td>
<td>0.0974263</td>
<td>0.336769</td>
<td>0.8148974</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Down Up</td>
<td>0.0916667</td>
<td>0.0974263</td>
<td>-0.147397</td>
<td>0.3307307</td>
<td>0.6187</td>
</tr>
</tbody>
</table>

#### Summary of Fit

- Rsquare: 0.382637
- Adj Rsquare: 0.354575
- Root Mean Square Error: 0.373133
- Mean of Response: 0.42125
- Observations (or Sum Wgts): 24

#### Pooled t Test

- Treatment-Control
  - Assuming equal variances
  - Difference: 0.5625
  - t Ratio: 3.692617
  - Std Err Dif: 0.152331
  - Upper CL Diff: 0.878415
  - Prob > |t|: 0.0013
  - Lower CL Diff: 0.246585
  - Prob > t: 0.0006
  - Confidence: 0.95

#### Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>1.8984375</td>
<td>1.89844</td>
<td>13.6354</td>
<td>0.0013</td>
</tr>
<tr>
<td>Error</td>
<td>22</td>
<td>3.063025</td>
<td>0.13923</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>23</td>
<td>4.9614625</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Means for Oneway Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>0.14</td>
<td>0.10771</td>
<td>-0.0834</td>
<td>0.36339</td>
</tr>
<tr>
<td>Treatment</td>
<td>12</td>
<td>0.7025</td>
<td>0.10771</td>
<td>0.4791</td>
<td>0.92589</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance.

#### Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

<table>
<thead>
<tr>
<th>q*</th>
<th>Alpha</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.07388</td>
<td>0.05</td>
</tr>
</tbody>
</table>

HSD Threshold Matrix

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>-0.315920.24658</td>
</tr>
<tr>
<td>Control</td>
<td>0.24658 -0.31592</td>
</tr>
</tbody>
</table>

Positive values show pairs of means that are significantly different.

#### Connecting Letters Report

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>A</td>
</tr>
<tr>
<td>Control</td>
<td>B</td>
</tr>
</tbody>
</table>

Levels not connected by same letter are significantly different.

### Oneway Analysis of Organic N By Treatment Gradient=Down

#### Oneway Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Difference</th>
<th>Std Err Dif</th>
<th>Lower CL</th>
<th>Upper CL</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center Up</td>
<td>0.0916667</td>
<td>0.0974263</td>
<td>-0.147397</td>
<td>0.3307307</td>
<td>0.6187</td>
</tr>
</tbody>
</table>

#### Summary of Fit

- Rsquare: 0.382637
- Adj Rsquare: 0.354575
- Root Mean Square Error: 0.373133
- Mean of Response: 0.42125
- Observations (or Sum Wgts): 24
Pooled t Test
Treatment-Control
Assuming equal variances
Difference  0.12417  t Ratio  1.603499
Std Err Dif  0.07743  DF  22
Upper CL Dif  0.28476  Prob > |t|  0.1231
Lower CL Dif -0.03642  Prob > t  0.0615
Confidence  0.95  Prob < t  0.9385

Analysis of Variance
Source  DF  Sum of Squares  Mean Square  F Ratio  Prob > F
Treatment  1  0.09250417  0.092504  2.5712  0.1231
Error  22  0.79149167  0.035977
C. Total  23  0.88399583

Means for Oneway Anova
Level  Number  Mean  Std Error  Lower 95%  Upper 95%
Control  12  -0.035  0.03311  -0.1037  0.03366
Treatment  12  0.035  0.03311  -0.0337  0.10366

Std Error uses a pooled estimate of error variance

Oneway Analysis of Organic N By Treatment Gradient=Up
Oneway Anova
Summary of Fit
Rsquare  0.092221
Adj Rsquare  0.050958
Root Mean Square Error  0.114693
Mean of Response  -4.60E-18
Observations (or Sum Wgts)  24
Pooled t Test
Treatment-Control
Assuming equal variances
Difference  0.07  t Ratio  1.494981
Std Err Dif  0.04682  DF  22
Upper CL Dif  0.16711  Prob > |t|  0.1491
Lower CL Dif -0.02711  Prob > t  0.0746
Confidence  0.95  Prob < t  0.9254

Analysis of Variance
Source  DF  Sum of Squares  Mean Square  F Ratio  Prob > F
Treatment  1  0.0294  0.0294  2.235  0.1491
Error  22  0.2894  0.013155
C. Total  23  0.3188

Means for Oneway Anova
Level  Number  Mean  Std Error  Lower 95%  Upper 95%
Control  12  -0.035  0.0331  -0.1037  0.03366
Treatment  12  0.035  0.0331  -0.0337  0.10366

Std Error uses a pooled estimate of error variance

Total Organic Carbon Statistics

Oneway Analysis of TOC By Gradient Treatment=Control

Oneway Anova
Summary of Fit
Rsquare 0.15583
Adj Rsquare 0.093299
Root Mean Square Error   13.21112
Mean of Response         10.46867
Observations (or Sum Wgts)  30

Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradient</td>
<td>2</td>
<td>869.8889</td>
<td>434.944</td>
<td>2.492</td>
<td>0.1016</td>
</tr>
<tr>
<td>Error</td>
<td>27</td>
<td>4712.4107</td>
<td>174.534</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>29</td>
<td>5582.2995</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means for One-way Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
<td>10</td>
<td>18.071</td>
<td>4.1777</td>
<td>11.499</td>
<td>26.643</td>
</tr>
<tr>
<td>Up</td>
<td>10</td>
<td>7.052</td>
<td>4.1777</td>
<td>1.52</td>
<td>15.624</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance

Missing Rows   6

Oneway Analysis of TOC By Gradient Treatment=Treatment

Oneway Anova
Summary of Fit
Rsquare 0.31079
Adj Rsquare 0.259737
Root Mean Square Error 138.552
Mean of Response 79.19567
Observations (or Sum Wgts) 30

Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradient</td>
<td>2</td>
<td>233724.81</td>
<td>116862</td>
<td>6.0876</td>
<td>0.0066</td>
</tr>
<tr>
<td>Error</td>
<td>27</td>
<td>518310</td>
<td>19197</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>29</td>
<td>752034.81</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Means for One-way Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
<td>10</td>
<td>203.822</td>
<td>43.814</td>
<td>113.9</td>
<td>293.72</td>
</tr>
<tr>
<td>Down</td>
<td>10</td>
<td>23.002</td>
<td>43.814</td>
<td>-66.9</td>
<td>112.9</td>
</tr>
<tr>
<td>Up</td>
<td>10</td>
<td>10.763</td>
<td>43.814</td>
<td>-79.1</td>
<td>100.66</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance

Means Comparisons
Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

<table>
<thead>
<tr>
<th>q*</th>
<th>Alpha</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.47942</td>
<td>0.05</td>
</tr>
</tbody>
</table>

HSD Threshold Matrix

<table>
<thead>
<tr>
<th>Abs(Dif)-HSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
</tr>
<tr>
<td>Center</td>
</tr>
<tr>
<td>Down</td>
</tr>
<tr>
<td>Up</td>
</tr>
</tbody>
</table>

Positive values show pairs of means that are significantly different.

Connecting Letters Report

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
<td>A</td>
</tr>
<tr>
<td>Down</td>
<td>B</td>
</tr>
<tr>
<td>Up</td>
<td>B</td>
</tr>
</tbody>
</table>
Levels not connected by same letter are significantly different.

### Ordered Differences Report

<table>
<thead>
<tr>
<th>Level</th>
<th>- Level</th>
<th>Difference</th>
<th>Std Err Dif</th>
<th>Lower CL</th>
<th>Upper CL</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center</td>
<td>Up</td>
<td>193.059</td>
<td>39.428</td>
<td>34.6896</td>
<td>0.0116</td>
<td></td>
</tr>
<tr>
<td>Center</td>
<td>Down</td>
<td>180.82</td>
<td>27.189</td>
<td>334.4506</td>
<td>0.0186</td>
<td></td>
</tr>
<tr>
<td>Down</td>
<td>Up</td>
<td>12.239</td>
<td>-141.392</td>
<td>165.8696</td>
<td>0.9787</td>
<td></td>
</tr>
</tbody>
</table>

### Means for Oneway Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>18.071</td>
<td>53.487</td>
<td>-94.3</td>
<td>130.44</td>
</tr>
<tr>
<td>Treatment</td>
<td>10</td>
<td>203.822</td>
<td>53.487</td>
<td>91.45</td>
<td>316.19</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance

### Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

<table>
<thead>
<tr>
<th>q* Alpha</th>
<th>HSD Threshold Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.10092</td>
<td>0.05</td>
</tr>
</tbody>
</table>

### Connecting Letters Report

Levels not connected by same letter are significantly different.

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>203.822</td>
</tr>
<tr>
<td>Control</td>
<td>18.071</td>
</tr>
</tbody>
</table>

Missing Rows 6

### Missing Rows

6

### Oneway Analysis of TOC By Treatment Gradient=Center

Oneway Anova Summary of Fit

<table>
<thead>
<tr>
<th>Rsquare</th>
<th>Adj Rsquare</th>
<th>Root Mean Square Error</th>
<th>Mean of Response</th>
<th>Observations (or Sum Wgts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.250942</td>
<td>0.209327</td>
<td>169.1419</td>
<td>110.9465</td>
<td>20</td>
</tr>
</tbody>
</table>

### Pooled t Test

Treatment-Control

<table>
<thead>
<tr>
<th>Difference</th>
<th>Std Err Dif</th>
<th>Upper CL Dif</th>
<th>Lower CL Dif</th>
<th>Confidence</th>
<th>Prob &gt;</th>
<th>t</th>
<th>Prob &gt; t</th>
</tr>
</thead>
<tbody>
<tr>
<td>185.751</td>
<td>75.643</td>
<td>344.67</td>
<td>26.832</td>
<td>0.95</td>
<td>0.0245</td>
<td>0.0122</td>
<td>0.9878</td>
</tr>
</tbody>
</table>

### Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>172517.17</td>
<td>172517</td>
<td>6.0302</td>
<td>0.0245</td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td>514961.64</td>
<td>28609</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Total</td>
<td>19</td>
<td>687478.81</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Means for Oneway Anova

<table>
<thead>
<tr>
<th>Level</th>
<th>Number</th>
<th>Mean</th>
<th>Std Error</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>18.071</td>
<td>53.487</td>
<td>-94.3</td>
<td>130.44</td>
</tr>
<tr>
<td>Treatment</td>
<td>10</td>
<td>203.822</td>
<td>53.487</td>
<td>91.45</td>
<td>316.19</td>
</tr>
</tbody>
</table>

Std Error uses a pooled estimate of error variance

Means Comparisons

Comparisons for all pairs using Tukey-Kramer HSD

### Confidence Quantile

<table>
<thead>
<tr>
<th>q* Alpha</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.10092</td>
</tr>
</tbody>
</table>

### HSD Threshold Matrix

<table>
<thead>
<tr>
<th>Abs(Dif)-HSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Control</td>
</tr>
</tbody>
</table>

Positive values show pairs of means that are significantly different.

### Connecting Letters Report

Levels not connected by same letter are significantly different.

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean</th>
</tr>
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<tbody>
<tr>
<td>Treatment</td>
<td>203.822</td>
</tr>
<tr>
<td>Control</td>
<td>18.071</td>
</tr>
</tbody>
</table>

### Missing Rows

4

### Oneway Analysis of TOC By Treatment Gradient=Down

Oneway Anova Summary of Fit
Rsquare 0.204862
Adj Rsquare 0.160687
Root Mean Square Error 17.36001
Mean of Response 14.6425
Observations (or Sum Wgts) 20
Pooled t Test
Treatment-Control
Assuming equal variances
Difference 16.719 t Ratio 2.153503
Std Err Dif 7.7636 DF 18
Upper CL Dif 33.0298 Prob > |t| 0.0451
Lower CL Dif 0.4082 Prob > t 0.0225
Confidence 0.95 Prob < t 0.9775
Analysis of Variance
Source DF Sum of Squares Mean Square F Ratio Prob > F
Treatment 1 1397.6248 1397.62 4.6376 0.0451
Error 18 5424.6562 301.37
C. Total 19 6822.281
Means for OneWay Anova
Level Number Mean Std Error Lower 95% Upper 95%
Control 10 6.283 5.4897 -5.25 17.816
Treatment 10 23.002 5.4897 11.47 34.535
Std Error uses a pooled estimate of error variance
Means Comparisons
Comparisons for all pairs using Tukey-Kramer HSD
Confidence Quantile
q* Alpha
2.10092 0.05
HSD Threshold Matrix
Abs(Dif)-HSD
Treatment Control
Treatment -16.311 0.408
Control 0.408 -16.311
Positive values show pairs of means that are significantly different.
Connecting Letters Report
Level Mean
Treatment A 23.002
Control B 6.283
Levels not connected by same letter are significantly different.
Missing Rows 4
OneWay Analysis of TOC By Treatment Gradient=Up
OneWay Anova
Summary of Fit
Rsquare 0.025456
Adj Rsquare -0.02869
Root Mean Square Error 12.10169
Mean of Response 8.9075
Observations (or Sum Wgts) 20
Pooled t Test
Treatment-Control
Assuming equal variances
Difference 3.711 t Ratio 0.685693
Std Err Dif 5.412 DF 18
Upper CL Dif 15.081 Prob > |t| 0.5016
Lower CL Dif -7.659  Prob > t 0.2508
Confidence 0.95  Prob < t 0.7492

Analysis of Variance

Source  DF  Sum of Squares  Mean Square  F Ratio  Prob > F
Treatment 1  68.8576 68.858   0.4702 0.5016
Error 18  2636.1164 146.451
C. Total 19  2704.974

Means for Oneway Anova

Level  Number  Mean  Std Error  Lower 95%  Upper 95%
Control  10  7.052  3.8269  -0.988 15.092
Treatment 10 10.763 3.8269  2.723 18.803

Std Error uses a pooled estimate of error variance

Missing Rows 4

Dissolved Oxygen Statistics

Oneway Analysis of DO By Gradient Treatment=Control
Oneway Anova
Summary of Fit
Rsquare 0.008974
Adj Rsquare -0.05937
Root Mean Square Error 0.72585
Mean of Response 0.999969
Observations (or Sum Wgts) 32

Analysis of Variance

Source  DF  Sum of Squares  Mean Square  F Ratio  Prob > F
Gradient 2  0.138354 0.069177  0.1313 0.8775
Error 29  15.278899 0.526859
C. Total 31  15.417253

Means for Oneway Anova

Level  Number  Mean  Std Error  Lower 95%  Upper 95%
Center 10  1.097  0.22953  0.44394 1.5631
Down 11  0.94786 0.21885  0.50026 1.3955
Up 11  0.96386 0.21885  0.51626 1.4115

Oneway Analysis of DO By Gradient Treatment=Treatment
Oneway Anova
Summary of Fit
Rsquare 0.018851
Adj Rsquare -0.04881
Root Mean Square Error 0.787873
Mean of Response 1.087656
Observations (or Sum Wgts) 32

Analysis of Variance

Source  DF  Sum of Squares  Mean Square  F Ratio  Prob > F
Gradient 2  0.34586 0.17293  0.2786 0.7589
Error 29  18.001589 0.620744
C. Total 31  18.347449

Means for Oneway Anova

Level  Number  Mean  Std Error  Lower 95%  Upper 95%
Center 10  0.9535  0.24915  0.44394 1.4631
Down 11  1.21045 0.23755  0.7246 1.6963
Up 11  1.08682 0.23755  0.60097 1.5727

Std Error uses a pooled estimate of error variance
Oneway Analysis of DO By Treatment Gradient=Center

Oneway Anova

Summary of Fit
Rsquare 0.007879
Adj Rsquare -0.04724
Root Mean Square Error 0.848679
Mean of Response 1.02525
Observations (or Sum Wgts) 20
Pooled t Test
Treatment-Control
Assuming equal variances
Difference -0.1435 t Ratio -0.37809
Std Err Dif 0.37954 DF 18
Upper CL Dif 0.65389 Prob > |t| 0.7098
Lower CL Dif -0.94089 Prob > t 0.6451
Confidence 0.95 Prob < t 0.3549

Analysis of Variance
Source DF Sum of Squares Mean Square F Ratio
Treatment 1 0.102961 0.102961 0.143
Error 18 12.964613 0.720256
C. Total 19 13.067574

Means for Oneway Anova
Level Number Mean Std Error Lower 95%
Control 10 1.097 0.26838 0.53316
Treatment 10 0.9535 0.26838 0.38966
Std Error uses a pooled estimate of error variance

Missing Rows 2

Oneway Analysis of DO By Treatment Gradient=Down

Oneway Anova

Summary of Fit
Rsquare 0.036694
Adj Rsquare -0.01147
Root Mean Square Error 0.705555
Mean of Response 1.079159
Observations (or Sum Wgts) 22
Pooled t Test
Treatment-Control
Assuming equal variances
Difference 0.26259 t Ratio 0.87283
Std Err Dif 0.30085 DF 20
Upper CL Dif 0.89015 Prob > |t| 0.3931
Lower CL Dif -0.36497 Prob > t 0.1966 Prob > F
Confidence 0.95 Prob < t 0.7098

Analysis of Variance
Source DF Sum of Squares Mean Square F Ratio
Treatment 1 0.379247 0.379247 0.7618
Error 20 9.95617 0.497808 Upper 95%
C. Total 21 10.335417 1.6608

Means for Oneway Anova
Level Number Mean Std Error Lower 95%
Control 11 0.94786 0.21273 0.50411
Treatment 11 1.21045 0.21273 0.7667
Std Error uses a pooled estimate of error variance
Oneway Analysis of DO By Treatment Gradient=Up
Oneway Anova
Summary of Fit
Rsquare 0.007962
Adj Rsquare -0.04164
Root Mean Square Error 0.719712
Mean of Response 1.025341
Observations (or Sum Wgts) 22
Pooled t Test
Treatment- Control
Assuming equal variances
Difference 0.12295 t Ratio 0.400652
Std Err Dif 0.30689 DF 20
Upper CL Dif 0.76311 Prob > |t| 0.6929
Lower CL Dif -0.5172 Prob > t 0.3465 Prob > F
Confidence 0.95 Prob < t 0.6535 0.3931
Analysis of Variance
Source DF Sum of Squares Mean Square F Ratio Prob > F
Treatment 1 0.083148 0.083148 0.1605
Error 20 10.359706 0.517985
C. Total 21 10.442854 1.3916
Means for Oneway Anova
Level Number Mean Std Error Lower 95% Upper 95%
Control 11 0.96386 0.217 0.51121
Treatment 11 1.08682 0.217 0.63416
Std Error uses a pooled estimate of error variance

ph Statistics
Oneway Analysis of pH By Gradient Treatment=Control

Oneway Anova
Summary of Fit
Rsquare 0.325592
Adj Rsquare 0.279081
Root Mean Square Error 0.310255
Mean of Response 5.607813
Observations (or Sum Wgts) 32
Analysis of Variance
Source DF Sum of Squares Mean Square F Ratio Prob > F
Gradient 2 1.347682 0.673841 7.0003 0.0033
Error 29 2.791489 0.096258
C. Total 31 4.139171
Means for Oneway Anova
Level Number Mean Std Error Lower 95% Upper 95%
Center 10 5.637 0.09811 5.4363 5.8377
Down 11 5.34818 0.09355 5.1569 5.5395
Up 11 5.84091 0.09355 5.6496 6.0322
Std Error uses a pooled estimate of error variance
Means Comparisons
Comparisons for all pairs using Tukey-Kramer HSD
Confidence Quantile
q* Alpha
2.46966 0.05
HSD Threshold Matrix
Abs(Dif)-HSD

<table>
<thead>
<tr>
<th></th>
<th>Up</th>
<th>Center</th>
<th>Down</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up</td>
<td>-0.32672</td>
<td>-0.13088</td>
<td>0.16601</td>
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<tr>
<td>Center</td>
<td>-0.13088</td>
<td>-0.34267</td>
<td>-0.04597</td>
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<tr>
<td>Down</td>
<td>0.16601</td>
<td>-0.04597</td>
<td>0.32672</td>
</tr>
</tbody>
</table>

Positive values show pairs of means that are significantly different.

Connecting Letters Report
Level | Mean
--- | ---
Up    | A 5.8409091
Center | A B 5.637
Down  | B 5.3481818

Levels not connected by same letter are significantly different.

Ordered Differences Report

<table>
<thead>
<tr>
<th>Level</th>
<th>- Level</th>
<th>Difference</th>
<th>Std Err Dif</th>
<th>Lower CL</th>
<th>Upper CL</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up</td>
<td>Down</td>
<td>0.4927273</td>
<td>0.1322932</td>
<td>0.166007</td>
<td>0.8194471</td>
<td>0.0024</td>
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<tr>
<td>Center</td>
<td>Down</td>
<td>0.2888182</td>
<td>0.1355602</td>
<td>-0.04597</td>
<td>0.6236064</td>
<td>0.1012</td>
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<tr>
<td>Up</td>
<td>Center</td>
<td>0.2039091</td>
<td>0.1355602</td>
<td>-0.130879</td>
<td>0.5386973</td>
<td>0.3038</td>
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Missing Rows 1

Oneway Analysis of pH By Gradient Treatment=Treatment

Oneway Anova
Summary of Fit
Rsquare 0.634255
Adj Rsquare 0.609031
Root Mean Square Error 0.369385
Mean of Response 5.713906
Observations (or Sum Wgts) 32

Analysis of Variance
Source | DF | Sum of Squares | Mean Square | F Ratio | Prob > F
--- | --- | -------------- |-------------|---------|----------
Gradient | 2 | 6.861866 | 3.43093 | 25.1451 | <.0001 |
Error | 29 | 3.956921 | 0.13645 |
C. Total | 31 | 10.818787 |

Means for Oneway Anova
Level | Number | Mean | Std Error | Lower 95% | Upper 95%
--- | --- | --- | ------- | -------- | --------
Center | 10 | 5.4315 | 0.11681 | 5.1926 | 5.6704 |
Down | 11 | 5.33318 | 0.11137 | 5.1054 | 5.561 |
Up | 11 | 6.35136 | 0.11137 | 6.1236 | 6.5791 |

Std Error uses a pooled estimate of error variance

Means and Std Deviations
Level | Number | Mean | Std Dev | Std Err Mean | Lower 95% | Upper 95%
--- | --- | --- | ------- | ----------- | -------- | --------
Center | 10 | 5.4315 | 0.297686 | 0.0941366 | 5.2185483 | 5.6444517 |
Down | 11 | 5.33318 | 0.3181373 | 0.095922 | 5.1194542 | 5.5469094 |
Up | 11 | 6.35136 | 0.4633848 | 0.1397158 | 6.0400575 | 6.6626698 |

Means Comparisons
Comparisons for all pairs using Tukey-Kramer HSD

Confidence Quantile

<table>
<thead>
<tr>
<th>q*</th>
<th>Alpha</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.46966</td>
<td>0.05</td>
</tr>
</tbody>
</table>

HSD Threshold Matrix
Abs(Dif)-HSD
Up  Center  Down
Up  -0.388990  0.52127  0.62919
Center  0.52127  -0.40797  -0.30028
Down  0.62919  -0.30028  -0.38899
Positive values show pairs of means that are significantly different.

Connecting Letters Report
Level  Mean
Up  A  6.3513636
Center  B  5.4315
Down  B  5.3331818
Levels not connected by same letter are significantly different.

Ordered Differences Report
Level  - Level Difference  Std Err Dif  Lower CL  Upper CL  p-Value
Up  Down  1.018182  0.1575064  0.629194  1.40717  <.0001
Up  Center  0.919864  0.1613961  0.52127  1.318458  <.0001
Center  Down  0.098318  0.1613961  -0.300276  0.496912  0.8163
Missing Rows  1

Oneway Analysis of pH By Treatment Gradient=Center

Oneway Anova
Summary of Fit
Rsquare 0.097011
Adj Rsquare 0.046845
Root Mean Square Error 0.330438
Mean of Response 5.53425
Observations (or Sum Wgts) 20
Pooled t Test
Treatment-Control
Assuming equal variances
Difference  -0.2055  t Ratio  -1.39061
Std Err Dif  0.14778  DF  18
Upper CL Dif  0.10497  Prob > |t|  0.1813
Lower CL Dif  -0.51597  Prob > t  0.9094
Confidence  0.95  Prob < t  0.0906
Analysis of Variance
Source  DF  Sum of Squares  Mean Square  F Ratio  Prob > F
Treatment  1  0.2111512  0.211151  1.9338  0.1813
Error  18  1.965125  0.10919
C. Total  19  2.1765638
Means for One-way Anova
Level  Number  Mean  Std Error  Lower 95%  Upper 95%
Control  10  5.637  0.10449  5.4175  5.8565
Treatment  10  5.4315  0.10449  5.212  5.651
Std Error uses a pooled estimate of error variance
Missing Rows  2

Oneway Analysis of pH By Treatment Gradient=Down

Oneway Anova
Summary of Fit
Rsquare 0.000627
Adj Rsquare  -0.04934
Root Mean Square Error 0.313924
Mean of Response: 5.340682
Observations (or Sum Wgts): 22

Pooled t Test
Treatment-Control
Assuming equal variances
Difference: -0.015  
Std Err Dif: 0.13386  DF: 20
Upper CL Dif: 0.26422  Prob > |t|: 0.9119
Lower CL Dif: -0.29422  Prob > t: 0.5441
Confidence: 0.95  Prob < t: 0.4559

Analysis of Variance
Source  DF  Sum of Squares  Mean Square  F Ratio  Prob > F
Treatment: 1  0.0012375  0.001238  0.0126  0.9119
Error: 20  1.9709648  0.098548
C. Total: 21  1.9722023

Means for Oneway Anova
Level  Number  Mean  Std Error  Lower 95%  Upper 95%
Control: 11  5.34818  0.09465  5.1507  5.5456
Treatment: 11  5.33318  0.09465  5.1357  5.5306

Std Error uses a pooled estimate of error variance

Oneway Analysis of pH By Treatment Gradient=Up
Oneway Anova
Summary of Fit
Rsquare: 0.337587
Adj Rsquare: 0.304466
Root Mean Square Error: 0.374969
Mean of Response: 6.096136
Observations (or Sum Wgts): 22

Pooled t Test
Treatment-Control
Assuming equal variances
Difference: 0.510455  
Std Err Dif: 0.159887  DF: 20
Upper CL Dif: 0.843974  Prob > |t|: 0.0046
Lower CL Dif: 0.176936  Prob > t: 0.0023
Confidence: 0.95  Prob < t: 0.9977

Analysis of Variance
Source  DF  Sum of Squares  Mean Square  F Ratio  Prob > F
Treatment: 1  1.4331011  1.4331  10.1926  0.0046
Error: 20  2.812033  0.1406
C. Total: 21  4.2451341

Means for Oneway Anova
Level  Number  Mean  Std Error  Lower 95%  Upper 95%
Control: 11  5.84091  0.11306  5.6051  6.0767
Treatment: 11  6.35136  0.11306  6.1155  6.5872

Std Error uses a pooled estimate of error variance

Comparisons for all pairs using Tukey-Kramer HSD
Confidence Quantile
q*  Alpha: 2.08596  0.05
HSD Threshold Matrix
Abs(Dif)-HSD

Treatment  Control
Treatment  -0.333520.17694
Control  0.17694 -0.33352
Positive values show pairs of means that are significantly different.

Connecting Letters Report
Level     Mean
Treatment A   6.3513636
Control B    5.8409091
Levels not connected by same letter are significantly different.

**Shannon Diversity**

3-way ANOVA - age*treatment*gradient

<table>
<thead>
<tr>
<th></th>
<th>Df</th>
<th>Sum Sq</th>
<th>Mean Sq</th>
<th>F value</th>
<th>Pr(&gt;F)</th>
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<tbody>
<tr>
<td>age</td>
<td>1</td>
<td>0.0340</td>
<td>0.0340</td>
<td>0.310</td>
<td>0.5860</td>
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<td>0.8867</td>
<td>0.8867</td>
<td>8.080</td>
<td>0.0123</td>
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<tr>
<td>gradient</td>
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<tr>
<td>treatment:gradient</td>
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<td>0.2735</td>
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<td>Residuals</td>
<td>15</td>
<td>1.6460</td>
<td>0.1097</td>
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<td></td>
</tr>
</tbody>
</table>

---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 1

Tukey multiple comparisons of means
95% family-wise confidence level

Fit: aov(formula = Shannon ~ age * treatment * gradient, data = richnesstest)

S‘age’

diff  lwr      upr     p adj
old -new -0.0828037 -0.3998856 0.2342782 0.5860011

S‘treatment’

diff  lwr      upr     p adj
treat-control -0.4068802 -0.7126115 -0.1011488 0.0125001

S‘gradient’

diff  lwr      upr     p adj
Down-Center -0.3665437 -0.7967580 0.06367070 0.1012587
Up-Center  -0.7065698 -1.1367841 -0.27635541 0.0018400
Up-Down    -0.3400261 -0.7702405 0.09018825 0.1338346
**S' age:treatment'**

<table>
<thead>
<tr>
<th></th>
<th>diff</th>
<th>lwr</th>
<th>upr</th>
<th>p adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>old :control-new:control</td>
<td>0.3764733</td>
<td>-0.4030607</td>
<td>1.15600730</td>
<td>0.5229592</td>
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<tr>
<td>new:treat-new:control</td>
<td>-0.2417619</td>
<td>-0.7263059</td>
<td>0.24278203</td>
<td>0.4964749</td>
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<tr>
<td>old :treat-new:control</td>
<td>-0.4855224</td>
<td>-1.0636403</td>
<td>0.09259552</td>
<td>0.1156107</td>
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<tr>
<td>new:treat-old :control</td>
<td>-0.6182352</td>
<td>-1.3521422</td>
<td>0.11567176</td>
<td>0.1141354</td>
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<tr>
<td>old :treat-old :control</td>
<td>-0.8619957</td>
<td>-1.6607803</td>
<td>-0.06321102</td>
<td>0.0324242</td>
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<tr>
<td>old :treat-new:treat</td>
<td>-0.2437604</td>
<td>-0.7587036</td>
<td>0.27118277</td>
<td>0.5389999</td>
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</tbody>
</table>

**S' age:gradient'**

<table>
<thead>
<tr>
<th></th>
<th>diff</th>
<th>lwr</th>
<th>upr</th>
<th>p adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>old :Center-new:Center</td>
<td>-0.25110438</td>
<td>-1.0370801</td>
<td>0.53487134</td>
<td>0.8976599</td>
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<tr>
<td>new:Down-new:Center</td>
<td>-0.41652585</td>
<td>-1.0682225</td>
<td>0.23517079</td>
<td>0.3487194</td>
</tr>
<tr>
<td>old :Down-new:Center</td>
<td>-0.55185181</td>
<td>-1.4523001</td>
<td>0.34859650</td>
<td>0.3907614</td>
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<tr>
<td>new:Up-new:Center</td>
<td>-0.77578963</td>
<td>-1.4274863</td>
<td>-0.12409299</td>
<td>0.0155515</td>
</tr>
<tr>
<td>old :Up-new:Center</td>
<td>-0.83416486</td>
<td>-1.7346132</td>
<td>0.06628345</td>
<td>0.0773264</td>
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<tr>
<td>old :Down-old :Center</td>
<td>-0.16542146</td>
<td>-0.9264392</td>
<td>0.59559625</td>
<td>0.9782666</td>
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<tr>
<td>old :Down :Center</td>
<td>-0.30074743</td>
<td>-1.2832171</td>
<td>0.68172222</td>
<td>0.9126633</td>
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<tr>
<td>new:Up-old :Center</td>
<td>-0.52468525</td>
<td>-1.8570300</td>
<td>0.23633247</td>
<td>0.2765481</td>
</tr>
<tr>
<td>old :Up-old :Center</td>
<td>-0.58306048</td>
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<tr>
<td>old :Down-new:Down</td>
<td>-0.13532597</td>
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<tr>
<td>new:Up-new:Down</td>
<td>-0.35926379</td>
<td>-0.9806322</td>
<td>0.26210458</td>
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<tr>
<td>old :Up-new:Down</td>
<td>-0.41763901</td>
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<tr>
<td>new:Up-old :Down</td>
<td>-0.22393782</td>
<td>-1.1026854</td>
<td>0.65480975</td>
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<tr>
<td>old :Up-old :Down</td>
<td>-0.28231305</td>
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<tr>
<td>old :Up-new:Up</td>
<td>-0.05837523</td>
<td>-0.9371228</td>
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<td>0.9999202</td>
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</tbody>
</table>

**S' treatment:gradient'**

<table>
<thead>
<tr>
<th></th>
<th>diff</th>
<th>lwr</th>
<th>upr</th>
<th>p adj</th>
</tr>
</thead>
<tbody>
<tr>
<td>treat:Center-control:Center</td>
<td>-0.86882766</td>
<td>-1.7475752</td>
<td>0.009919913</td>
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<tr>
<td>control:Down-control:Center</td>
<td>-0.77705624</td>
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<td>0.29918534</td>
<td>0.2359238</td>
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<td>-1.10311190</td>
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<tr>
<td>control:Up-control:Center</td>
<td>-1.02409405</td>
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<td>control:Down-treat:Center</td>
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<td>-1.3207389</td>
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<td>treat:Down-control:Down</td>
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<td>-1.2048032</td>
<td>0.552691904</td>
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<td>control:Up-control:Down</td>
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<td>treat:Up-control:Down</td>
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treat:Up-treat:Down -0.39174374 -1.0864547 0.302967209 0.4759875

Tukey multiple comparisons of means
95% family-wise confidence level

Fit: aov(formula = Shannon ~ treatment, data = richnesstest)

S’ treatment’

<table>
<thead>
<tr>
<th>diff</th>
<th>lwr</th>
<th>upr</th>
<th>p adj</th>
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</thead>
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<tr>
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<td>-0.4120554</td>
<td>-0.8218501</td>
<td>-0.002260668 0.0488541</td>
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Call:
adonis(formula = community_bray ~ age * treatment * gradient, data = communitydf)

Permutation: free
Number of permutations: 999

Terms added sequentially (first to last)

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<thead>
<tr>
<th>Df</th>
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<th>MeanSqs</th>
<th>F.Model</th>
<th>R2 Pr&gt;F</th>
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<td>0.23440</td>
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<td>Total</td>
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<td>1.00000</td>
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Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Homogeneity of multivariate dispersions

Call: betadisper(d = community_bray, group = age)

No. of Positive Eigenvalues: 23
No. of Negative Eigenvalues: 0

Average distance to median:

<table>
<thead>
<tr>
<th>new</th>
<th>old</th>
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</thead>
</table>

Eigenvalues for PCoA axes:
(Showing 8 of 23 eigenvalues)

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<tr>
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<th>PCoA3</th>
<th>PCoA4</th>
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<tbody>
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Df  Sum Sq Mean Sq  F value  Pr(>F)

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<th>Df</th>
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<th>Mean Sq</th>
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<td>6782089</td>
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Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Df  Sum Sq Mean Sq  F value  Pr(>F)

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<th>Df</th>
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<th>Mean Sq</th>
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> tukey6 = TukeyHSD(aov, conf.level=0.95)

Fit: aov(formula = Observed ~ age, data = richnesstest)

`S`age`

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<tr>
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<th>lwr</th>
<th>upr</th>
<th>p adj</th>
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<td>old -new</td>
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<td>old -old</td>
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**RDA**

**All Data**

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<td>- TOC</td>
<td>1 116.11</td>
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**Only 2018 RDA**

<p>| | | | |</p>
<table>
<thead>
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<tr>
<td><strong>Df</strong></td>
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<table>
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<td>NH4</td>
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<table>
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References


Fu, W., H. Tunney, and C. Zhang (2010), Spatial variation of soil nutrients in a dairy farm and its implications for site-specific fertilizer application, Soil Tillage Res.,


Protection, F. D. of E. (2016), STATemap Geology,


Schmidt, C. A. (2018), Personal Communication,


