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The effects of carbon on nitrogen transformations in restored wetland and wastewater soils

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THE EFFECTS OF CARBON ON NITROGEN TRANSFORMATIONS IN RESTORED WETLAND AND WASTE WATER SOILS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Oceanography and Coastal Sciences

by
Jared M. Theriot
B.S., Louisiana State University, 2010
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ABSTRACT

Since the industrialization of the Haber-Bosch process in the 1940's, anthropogenic activity has nearly doubled the Earth's nitrogen fixation. Furthermore, nitrate has become the number one groundwater contaminant in the United States and has harmful effects such as eutrophication, algal blooms, and pollution of drinking water. Soils from two sites influenced by high nitrate loading were examined to determine their biogeochemical integrity. First, the Loosahatchie Bar, located northwest of Memphis, Tennessee, is influenced by excess surface water nitrate loading by the Mississippi River. The Loosahatchie Bar is a newly restored wetland that now has similar hydrologic influence to an upstream control site. The upstream control site and the restored bar sites are both bottomland hardwood forest but exhibit very dissimilar soil properties and microbial functions. Significant differences ($P < 0.05$) between the control and restored sites were observed for moisture content, bulk density, total carbon, nitrogen, and phosphorus, microbial biomass nitrogen, potentially mineralizable nitrogen, and potential denitrification. Second, the Tallahassee Wastewater Treatment Plant, located just southeast of Tallahassee, Florida, receives high nitrate loads to spray field pivot soils from Tallahassee, Florida's municipal wastewater. Although the intended function of the spray field pivots is to remove excess nitrate from the wastewater, there has been observed eutrophication in Wakulla Springs 17.5 km south of the treatment facility. Soil analysis was conducted to compare the pivot soils with an up gradient control site. Significant differences ($P < 0.05$) were observed for moisture content, percent organic matter, total carbon, nitrogen, and phosphorus, and denitrifying enzyme activity. Carbon amendment experiments were conducted on the pivot soils with residual biosolids and corn plants. There were no significant differences ($P >$

0.05) observed for potential denitrification with either carbon amendment. More research should be done to understand water retention at these sites and the microbial communities involved in denitrification.

CHAPTER 1:

DIFFERENCES IN SOIL AND MICROBIAL PROPERTIES BETWEEN RECENTLY RESTORED AND NATURAL MISSISSIPPI RIVER RIPARIAN WETLANDS

1.1 Wetlands

Historically, wetlands have been thought to possess little or no value. However, wetlands have been discovered to have major value with the advancement of technology. Physical benefits provided by wetlands are a natural floodwater buffer system, a natural habitat for wildlife and fisheries, improvement of water quality, stabilization of river banks, and protection of the shoreline from erosion. All are qualities the United States federal government spends hundreds of millions of dollars protecting. Wetlands also provide critical chemical and biogeochemical functions that are necessary for life on earth. Within these wetlands are microbial populations that are responsible for many of these physical and chemical processes. Microbes are single-celled organisms that cannot be seen with the naked eye due to their extremely small size (about 1 micron). The propagation of these microscopic organisms within wetlands promotes many biochemical functions such as decomposition, fermentation, and critical biotransformations. These biotransformations include the conversion of gases from one form to another. These gaseous processes such as nitrification and denitrification, convert forms of nitrogen unusable to animals, plants, and other microbes to a different chemical structure and composition that can be utilized by these organisms (Reddy and Delaune 2008). Wetlands also function as a useful method of carbon sequestration, helping to alleviate the increasing carbon dioxide levels, and further reducing the effects of global warming (Beran 1995, Choi and Wang 2004).

Wetlands are defined by an area of land meeting three specific parameters. First, the land must be frequently inundated or saturated with water. Second, the land must have soils that reflect this specific hydrologic activity. Third, the land must have plant species that are adapted to living in flooded conditions. These three points are outlined by the 1977 Clean Water Act

(Section 404). There are several variations of the definition of a wetland; however, almost all versions of the definition satisfy these three critical parameters in one form or another.

Along with the historical ideology of valueless land, wetlands function as a sink for excess nutrients and are advantageous for conversion to agricultural land. After drainage has occurred for conversion from wetland to upland land for agricultural use, it will no longer be able to function as a nutrient sink (Orr et. al. 2007). Reduction of wetlands compounded by massive fertilizer usage has resulted in increased outflow of excess nutrients. In the case of the Mississippi River and its drainage basin, the excess nutrients flow into the Gulf of Mexico and consequently lead to eutrophication. These eutrophication events are responsible for the massive annual hypoxia events known as the Dead Zone (Rabalais et. al. 2002). Since scientists have discovered the ecological value of these wetland areas and the negative impacts of their removal, lawmakers have legally protecting these wetland areas and funds have been allocated for wetland restoration projects across the United States.

Wetland restoration projects are conducted by reintroducing a viable watershed to a previously wetland area. After water restoration occurs, the sites are monitored for several years. Most restoration projects are assessed by the frequent inundation or saturation by water. After hydrologic restoration of the restoration site, the criteria for a successful wetland restoration project are determined by only one other part of the definition of a wetland: the area of land must have vegetation that is suited for living in flooded conditions (Jarman et. al. 1991). Consequently, one critical part of the definition of a wetland is almost completely ignored, hydric soils. Hydric soils are distinguished from non-hydric soils by specific guidelines outlined in *Field Indicators of Hydric Soils in the United States*. Monitoring hydric soil properties would help give an idea of a timeline of how long it would take for these wetland

restoration projects to return to its original wetland function or function similar to a natural wetland site. Although these restored wetlands may have similar vegetation, hydrologic activity, and look very similar (Figure 1.1.1; Figure 1.1.2). Soil analysis can reveal very different soil properties and function.



Figure 1.1.1 Photograph of Loosahatchie Bar restored site.

In order to assess the soil function, one can look at the soil properties to help understand what an expected timeline for complete restoration may be. There are many measurable soil properties used to help interpret the functions and qualities of the soil. Some examples of measurable



Figure 1.1.2 Photograph of Loosahatchie Bar natural site.

physical soil properties are bulk density, moisture content, and soil organic matter content. The percentage of soil organic matter is important for supplying nutrients, contributing to cation exchange, and improving soil structure (Schulte 1988). The soil organic matter has been found to be much lower in newly restored wetland sites than in the natural wetland sites (Clewett and Lea 1989). Reduced soil organic matter is caused by oxidation of the drained soils which results in a decrease of soil carbon storage (Bruland et al. 2006). Some measurable soil microbial properties include microbial biomass carbon, microbial biomass nitrogen, potentially mineralizable nitrogen, potential denitrification, denitrifying enzyme assay, and reduction/oxidation potential.

Microbial biomass carbon and nitrogen is a measure of the amount of carbon and nitrogen locked away in the biomass of microbes. Potentially mineralizable nitrogen is representative of the fraction of nitrogen that is able to be utilized by vegetation (Bonde et. al. 1988). Potential denitrification and denitrifying enzyme assay are both measures of the ability of the microbial community to carry out the denitrification portion of the nitrogen cycle. As discussed earlier, denitrification is important for the reduction of excess nutrients in the watershed. Lastly, reduction/oxidation potentials which are measures of the electron availability and an indirect measure of whether the soils are under oxidizing or reducing conditions (Reddy and DeLaune 2008). These potentials can help understand whether the microbial community will be able to complete the nitrogen cycle.

1.2 Denitrification

1.2.1 Denitrification Introduction

Since preindustrial times, humans have dramatically altered the global nitrogen cycle. The rate of nitrogen fixation, through fertilizer production, has doubled and the rate of nitrogen deposition has increased nearly tenfold (Smil 1990; Smil 1991; Vitouesk and Matson 1993; Ayers et. al 1994; Galloway et. al. 1995; Vitousek et. al. 1997). In most ecosystems, nitrogen is often cited as the limiting nutrient (Ryther and Dunstan, 1971; Boynton et al. 1982; Gerhart and Likens 1975; Vitousek and Howarth 1991). Changing the global balance of limiting nutrients can have a drastic effect on ecosystem response and may also affect cycling other nutrients and elements in the ecosystem. As the amount of cycled nitrogen increases so does the nitrogen cycle's expression of eutrophication and production of nitrous oxide, a potent greenhouse gas.

A variety of organic and inorganic nitrogen forms are found within wetland soils (Reddy and Delaune 2008). The inorganic forms are the most important nutrients due to their immediate availability to vegetation and wetland soil microbes (McClelland and Valiela 1998). The chemical reduction of inorganic form of nitrogen, nitrate, to a chemically different form of nitrogen, nitrogen gas, is denitrification (Payne 1973). Denitrification is an important wetland soil biogeochemical process for the removal of nitrate, returning it back to the atmospheric pool (Groffman and Hanson 1997).

Denitrification is a process linked to microbial respiration (Reddy and Delaune 2008). More specifically, the addition of electrons to nitrate or nitrite (as the terminal electron acceptor), leads to the reduction of nitrogen to nitrous oxide or nitrogen gas, a change of the oxidation state of nitrogen from +5 to 0 (Seitzinger 1988; Reddy and DeLaune 2008, Figure 1.2.1). There are several intermediate steps producing intermediate compounds such as nitric oxide, nitrogen oxide, and nitrous oxide involved in the denitrification pathway (Ye et. al. 1994; Hollocher and Hibbs 1996).

1.2.2 Biology of Denitrification

Denitrifying organisms are able to use nitrogen oxides in the place of oxygen as electron acceptors and are both heterotrophic and autotrophic (Knowles 1982). The *Pseudomonas* genera is the most common worldwide and possibly the most active denitrifying bacteria isolated in soils (Heitzer and Ottow 1976; Gamble, et. al. 1977). Denitrifying species of the *Pseudomonas* genera include *P. aerogenes*, *P. auerofacines*, *P. caryophylli*, *P. choloroaphis*, *P. denitrificans*, *P. fluorescens*, *P. lemoignei*, *P. mallei*, *P. mendocina*, *P. perfectomarinus*, *P. picketti*, *P. pseudoalcaligenes*, *P. pseudomallei*, *P. solonacearum*, and *P. stutzeri* (Allen and Neil 1952;

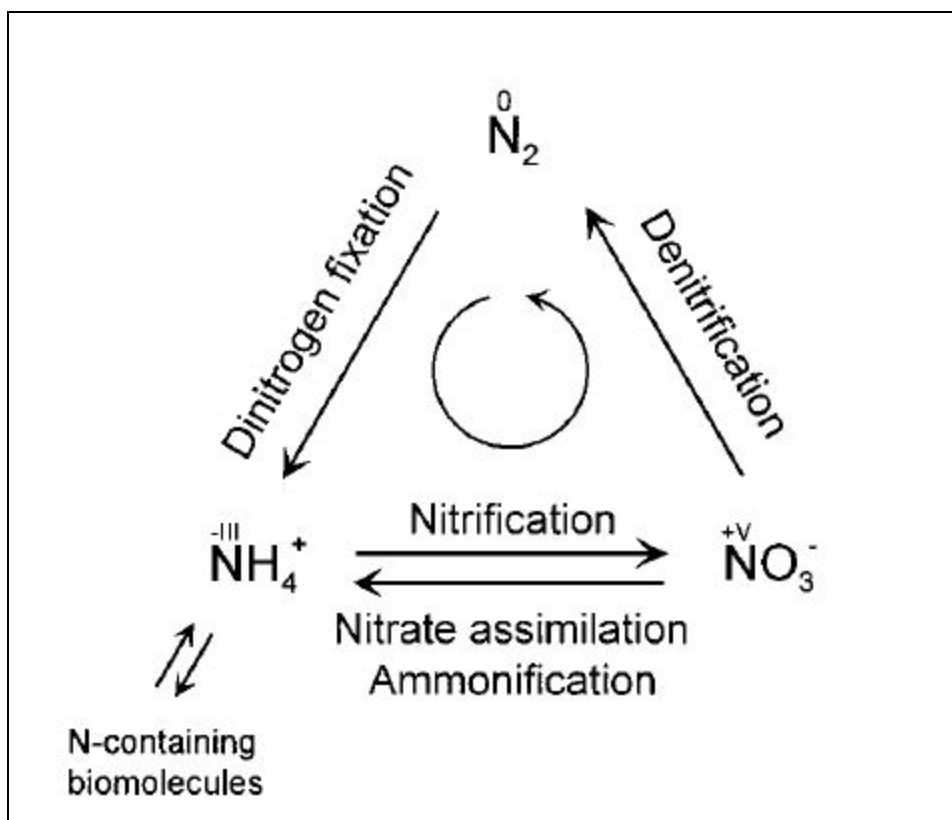


Figure 1.2.1 General nitrogen cycle diagram. Roman numerals denote formal oxidation state (Zumft 1997).

Buchanan and Gibbons 1974; Gamble et. al. 1977; Greenberg and Becker 1977; Pichinoty et. al. 1977). The second most common worldwide denitrifying genera is *Alcaligenes* includes species *A. faecalis*, *A. eutrophus*, *A. denitrificans*, *A. odorans* (Pfitzner and Schlegel 1973; Gamble et. al. 1977). Less common genera include *Achromobacter*, *Agrobacterium*, rice field abundant *Bacillus*, *Chromobacterium*, *Corynebacterium*, *Flavobacterium*, *Halobacterium*, *Moraxella*, *Paracoccus* (*Micrococcus*), *Thiobacillus*, and *Xanthomonas* (Meschner and Wuhrmann 1963; Hart et. al. 1965; Iwasaki and Matsubara 1972; Davies 1973; Williams and Evans 1975; Pichinoty et. al. 1976; Gamble et. al. 1977; Garcia 1977; Grant and Payne 1981).

The genes or loci responsible for regulation of denitrification are found as nitrate reductase systems (NRS) in many different forms and in genetically dissimilar organisms (Tiedje

et. al. 1981; Zumft 1997). Although mostly prokaryotic, these genes or loci for denitrification are found in genera of multiple kingdoms and exist in almost all soils. The nitrate reductase systems found within the organisms will only code for the organism's ability to either nitrify or denitrify, but not both (Zumft 1997).

Bacterial enzymes such as nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase facilitate the movement of electrons as organic carbon is oxidized (Hochsetin and Tomlinson 1988, Figure 1.2.2). The enzyme breakdown is mediated by facultative bacteria and is triggered by low oxygen availability the presence of a nitrogen oxide, temperature, and available organic matter (Burford and Bremner 1975; Reddy et. al. 1982; White and Reddy 1999; Cooper 1990; Wang et. al. 2007).

1.2.3 Greenhouse gases

Denitrification plays a critical role in the removal of excess fertilizer nitrogen and the removal of nitrogen-rich animal waste (Knowles 1982). However, incomplete denitrification reactions have been shown to have negative effects. In wetland soils, Nitrous oxide (N_2O) is a greenhouse gas and a biologically produced intermediate of the nitrogen cycle. Nitrous oxide has a 120 year atmospheric lifetime and is approximately 320 times as strong a greenhouse gas as carbon dioxide on a mole basis (IPCC 1997). Approximately 94% of nitrous oxide is converted to nitrogen gas under normal anaerobic, denitrifying conditions (Blackmer and Bremner 1978). Under slightly aerobic conditions, oxygen will inhibit nitrous oxide reductase from converting nitrous oxide to nitrogen gas. Nitrous oxide reductase inhibition will stop denitrification at nitrous oxide, releasing nitrous oxide into the atmosphere and decreasing the percentage of nitrous oxide converted to nitrogen gas (Knowles 1982). In ephemerally flooded systems, such

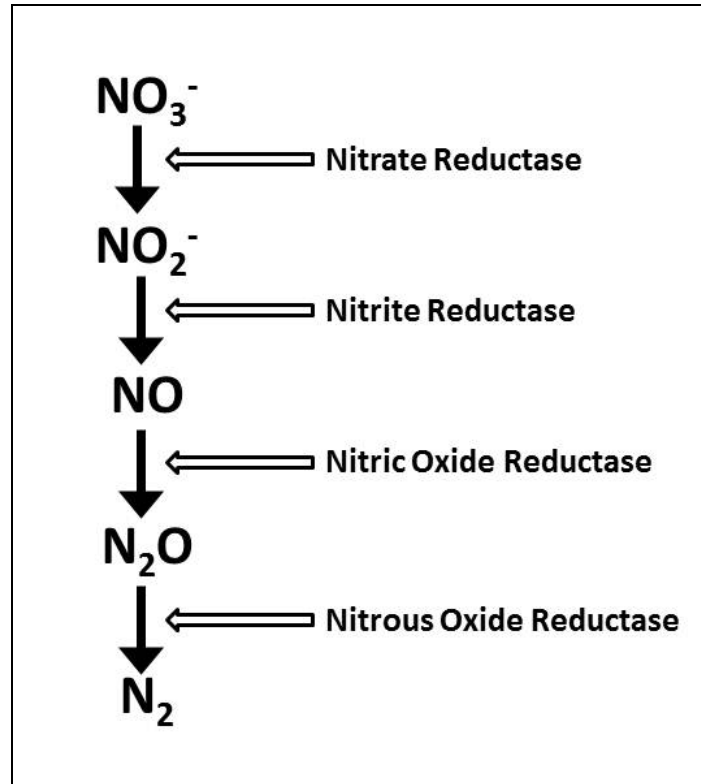


Figure 1.2.2 Denitrification pathways and enzymes involved
(Adapted from Hochstein and Tomlinson, 1988).

as agricultural land, the recurring alternation between flooded anaerobic conditions and dry aerobic conditions allows for nitrous oxide gases to escape into the atmosphere. In systems that experience longer periods of flooding, nitrous oxide gas and other gases move approximately 100 times slower within water compared to air. Slow movement throughout the water column allows sufficient time for reduction of nitrous oxide to inert nitrogen gas. Under aerobic conditions, the denitrifiers' aerobic metabolism is metabolically advantageous. The structural conformation of the enzyme receptors used for denitrification with change. The receptor's conformational change blocks the substrates needed for denitrification, and stops the reduction of nitrate. As with the amount of nitrogen cycled on the earth, the amount of nitrous oxide released into the atmosphere has also dramatically increased (Galloway et. al. 1995). Nitrous oxide is and

increasing at a rate of $4.5 \pm 0.6 \text{ Tg N yr}^{-1}$, an increase of approximately 0.25% per year (Khalil and Rasmussen 1992). Reduction of global nitrogen levels may help mediate the production of this harmful greenhouse gas.

1.2.4 Changes in Denitrification over Time

Anthropogenic alteration of the global nitrogen cycles through the combustion of fossil fuels, usage and production of nitrogen fertilizers, agricultural cultivation of nitrogen-fixing legumes, and other human causes have resulted in a dramatic increase in the amount of nitrogen cycled (Galloway et. al. 1995). As mentioned earlier, the rate of nitrogen fixation has doubled since pre-industrial times and the rate of nitrogen deposition has increased nearly tenfold (Smil 1990; Smil 1991; Vitousek and Matson 1993; Ayers et. al. 1994; Galloway et. al. 1995; Vitousek et. al. 1997; Figure 1.2.3). To put that into numbers, anthropogenic sources cause the fixation of approximately 140 Tg of new nitrogen per year in terrestrial ecosystems and mobilizes approximately 70 Tg more nitrogen per year (Galloway et. al. 1995). The increasing nitrogen also directly affects aquatic systems and water chemistry. Although there are no long term historical records for nitrate concentrations, the amount of nitrate has doubled in the Mississippi River since 1965 (Turner and Rabalais 1995, Justic et. al. 1995). The most well understood consequence of anthropogenic increase of nitrate is eutrophication (Howarth 1988; Justic et. al. 1995; Nixon 1995; Nixon et. al. 1996). Eutrophication, an overabundance of limiting nutrients, can lead to hypoxia (low oxygen concentration) and anoxia (zero oxygen concentration). The Mississippi River and Atchafalaya River are the primary inflows to the Northern Gulf of Mexico, responsible for 91% of the nitrate load (Goolsby 1999). This loading results in annual hypoxic and anoxic events over areas as large as 20,700 km² in the northern Gulf of Mexico (Rabalais 2002). Abnormally low oxygen conditions result in reduced fisheries, benthic fauna, and

bottom-dwelling species (Rabalais 2001). Decreased available food resources, altered trophic level interactions, and disrupted migration patterns were also observed along with physiological, reproductive, developmental, and growth abnormalities of affected species. Economic losses from a single hypoxic event can exceed \$2 billion (Rabalais et. al. 2010). Another pernicious effect of eutrophication is the explosive growth of nuisance algae and harmful algal blooms (Anderson and Garrison 1997). Under eutrophic conditions, concentrations of algal species can exceed a threshold and begin to cause problems such as shading, toxicity, and hypoxia. Once algal blooms begin causing unfavorable conditions for other biology, they are termed harmful algal blooms. With high nutrient influxes, shifts of the dominant algal species from harmless to toxic species have been observed (Bargu et. al. 2011). The incidence of these harmful algal blooms has increased within the past decade and is directly linked eutrophication (Hallegraeef 1993; Shumway1990,).

Other effects observed from increasing available nitrogen are reduction of biological diversity in affected ecosystems, acidification of soils, depletion of soil minerals, alteration of freshwater and marine ecosystems, increased acid rain, and promotion of the greenhouse effect (Timan 1987; Berendse et. al. 1993; Aber et. al. 1995; Likens et. al. 1996; Nixon et. al. 1996; Chameides et. al. 1994; Albritton et. al. 1995).

1.2.5 Effects of organic matter on Denitrification

Denitrifying microbes must have an available energy source to carry out denitrification. Generally, this source of energy is organic carbon. Organic carbon in the form of simple organic compounds is directly available to the denitrifying community and has an influence on denitrifying capabilities of soils (Starr and Gillham 1993; Cornwell et. al. 1999; Greenan et. al.

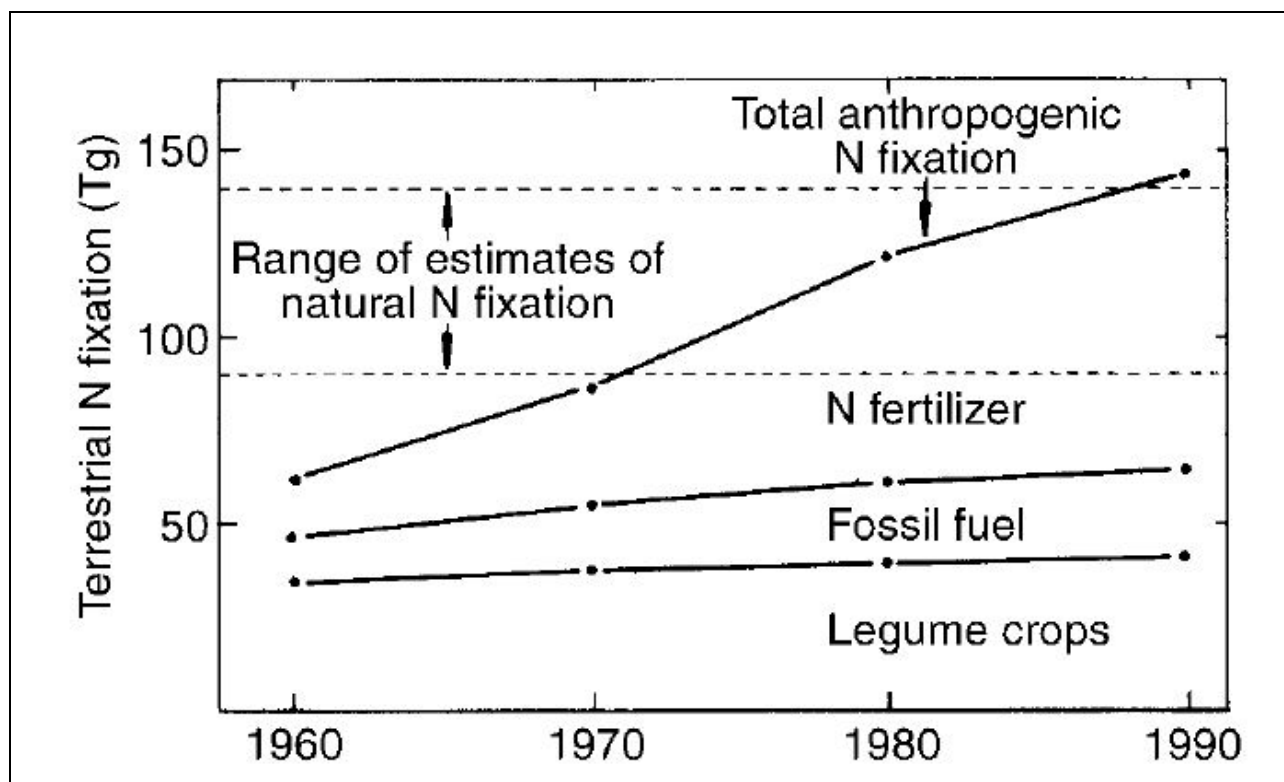


Figure 1.2.3 Anthropogenic nitrogen fixation in terrestrial ecosystems over time compared with natural biological nitrogen fixation on land. (Modified from Galloway et. al. 1995).

2006; Hill et. al. 2000; Figure 1.2.3). There is also evidence that a higher soil organic matter content will also lead to an increase in denitrification potential (Bijay-Singh et. al. 1988; Gale et. al. 1993). Carbon amendment experiments have been conducted on carbon-limited soils using glucose as the carbon source. The denitrifying bacteria responded with a drastic increase in denitrification rates (Garcia-Montiel 2003). In a separate experiment, glucose was added to a high organic matter forest soil and substantially lower organic matter grassland soil. The high organic matter forest soil microbial community responded with an increased denitrification rate compared to the lower organic matter grassland soil. These experiments suggest that although carbon may be present in the form of organic matter, not all of the organic matter available to the

microbial community can be utilized (Hill and Cardaci 2004). These studies also suggest that different qualities of organic carbon may be important even in soils that are not carbon limited.

1.2.6 Measuring Denitrification

Denitrification has been assessed through many different methods. Kaplan et. al. (1997) estimated denitrification by measuring nitrogen production by the use of *in situ* domes in a salt marsh. Mass balance calculations have been used to approximate the denitrification occurring (Dierberg and Brezonik 1983; Brinson et. al. 1984; Bowden 1986). Other studies have measured nitrous oxide in nitrate added homogenous soil slurries to approximate potential denitrification (Muller et. al. 1980; Gordon et. al. 1986; Westermann and Ahring 1987; Koerselman et. al. 1989). Other studies similarly measured nitrous oxide in nitrate added to whole sediment cores to approximate potential denitrification (Dierberg and Brozonik 1983). These nitrate addition experiments usually utilize acetylene to inhibit nitrification (Dierberg and Brozonik 1983; Urban et. al. 1988; Zak and Grigal 1991; Merrill and Zak 1992; Hynes and Knowles 1978). Nitrification of mineralized ammonia provides a source of nitrate utilized in denitrification reactions (Patrick and Reddy 1976; DeBusk and Reddy 1987; Reddy et. al. 1989). Denitrification approximations have also been made from homogenized sediment slurries with no nitrate added (Hemond 1983; Westermann and Ahring 1987; Koerselman et. al. 1989).

1.3 Site Description

The study site on the Mississippi River is located at the Loosahatchie Bar. The Loosahatchie Bar, named after the mainly east to west Loosahatchie River, begins in Hardeman County, Tennessee and merges with the Mississippi River. The two rivers join just north of Memphis, Tennessee near Mud Island in Shelby County. The Loosahatchie Bar is between river

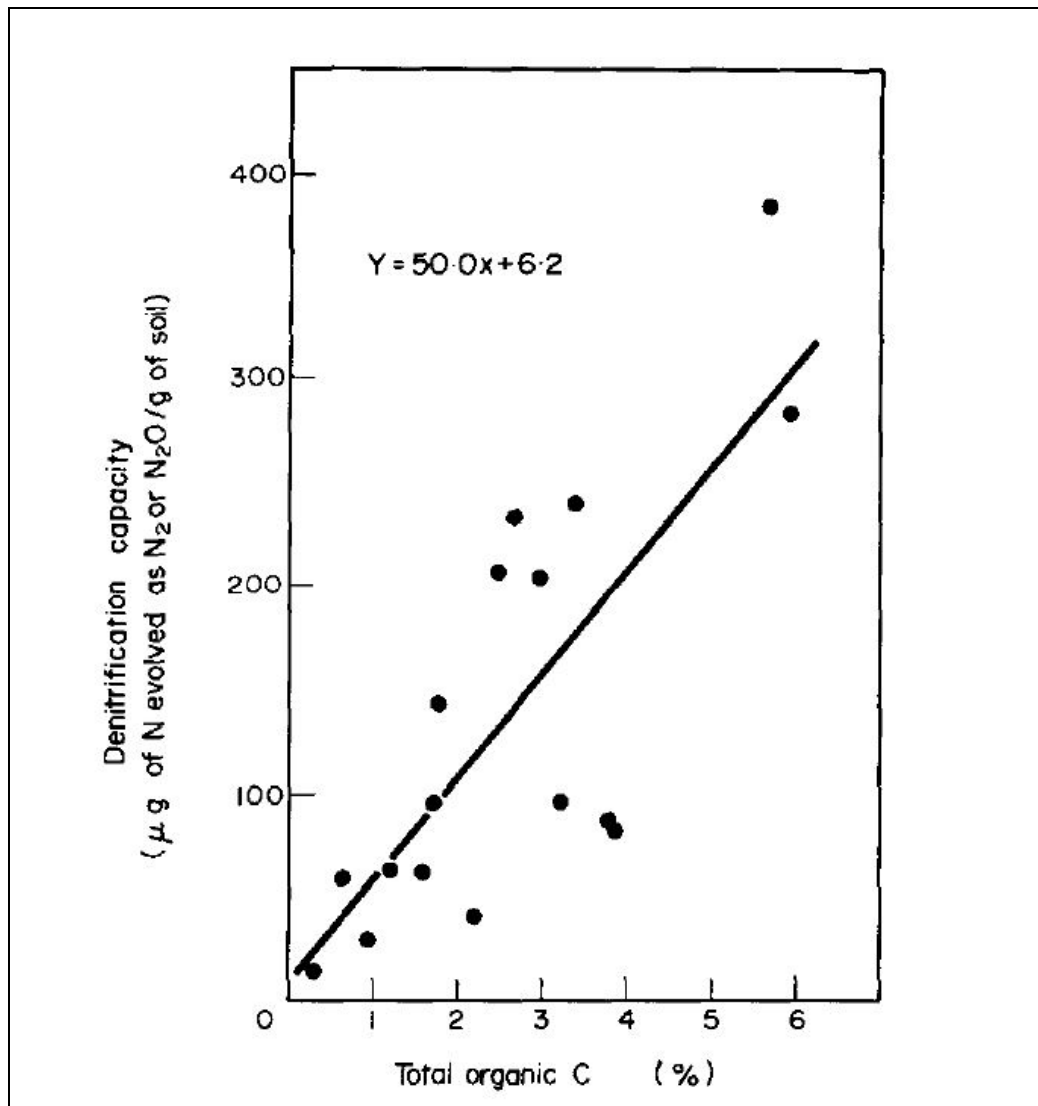


Figure 1.2.4 Relationship between denitrification capacity and total organic carbon (Burford and Bremner 1975).

miles 736.5 and 742.8 on the western (Arkansas) side of the Mississippi River. In 1960, the US Army Corps of Engineers diverted flow away from the Redman Point of the Loosahatchie Bar secondary channel into the Mississippi River in an effort to divert as much water as possible into the river for safe navigation at river low stages. This was accomplished using stone dikes to ensure proper flow of the river water.

In 2008, the Army Corps of Engineers began a wetland restoration project. As part of this project, 12 Army Corps designed notches were added to the existing nine stone dikes to help restore flow in over 11 miles of secondary channels in the bar. This site was sampled to better understand the quality of wetland restoration at riverine restoration sites.

The soils were classified according the United States Department of Agriculture's National Resources Conservation Service Web Soil Survey. The soils at the natural wetland site are classified as Bowdre silty clay and are often inundated. Soils from a depth of 0-5 in CEC are

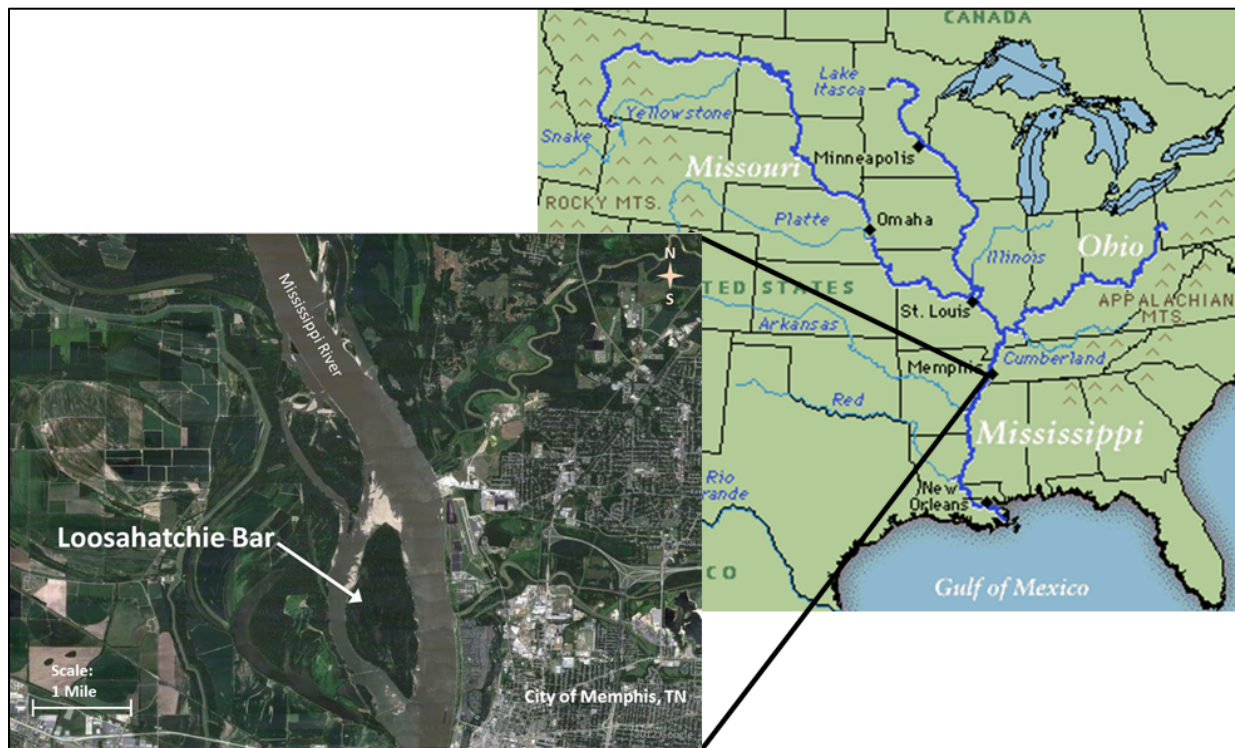


Figure 1.3.1 Satellite and map view of Loosahatchie Bar study site (Images: Google 2012)

25-45 meq/100g with a pH of 5.6-7.3. Soils from a depth of 5-17 in CEC are 22-40 meq/100g with a pH of 5.6-7.3. Soils from a depth of 17-42 in CEC are 7.0 meq/100g with a pH of 6.1-8.4.

Soils from a depth of 42-60 in CEC are 5.0-18 meq/100g with a pH of 6.1-8.4. Only the soil at the mid site was classified for the restored wetland sites. The soils at the mid restored site are classified as Crevasse fine sand. Soils from a depth of 0-8 in CEC are 1.4-6.1 meq/100g with a pH of 5.6-8.4. Soils from a depth of 8-60 in CEC are 1.4-6.1 meq/100g with a pH of 5.6-8.4.

1.4 Sample Collection and Preparation

Triplicate soil cores were collected from a prepared plot 25m² at the four sites along the Mississippi River at the Loosahatchie Bar site (Figure 1.4.1). Three sites are representative of a hydrologically reconnected wetland area. These three sites are labeled relative to their location to the start of the reconnected waterway. The near site is located approximately 500m (35°12'00.04"N, 90°04'34.55"W, Elevation: 61.75 m) from the start of the reconnected waterway, the mid site approximately 2.25km (35°11'05.04"N, 90°05'14.56"W, Elevation: 61.84 m), and the far site approximately 4.5km (35°09'57.44"N, 90°04'42.58"W, Elevation: 62.94 m). The fourth site is an undisturbed natural wetland area located upstream approximately 10km (35°15'14.26"N, 90°06'51.23"W, Elevation: 62.64 m). The soils were taken for the top 10cm of soil profile depth.

Soil core samples were collected using the push core method using a core of 10 cm diameter, 15 cm depth and 1117.5 cm³ volume. The top 15 cm of soil was removed in the field. The samples were placed in zip-lock bags, placed on ice and brought back to the laboratory. Once back to the lab, large root fragments were manually extracted and the remaining sample was homogenized and stored at 4°C in polyethylene containers. This sampling scheme was repeated four times in one year in the months of December 2009, March 2010, June 2010, and August 2010.

1.5 Soil Characterization and Microbial Analysis

1.5.1 Soil Characterization

Collected soils were homogenized and prepared in the laboratory for soil analyses with triplicates included for every ten samples for all measured parameters. Moisture content, dry weight, bulk density, total carbon (TC), total nitrogen (TN), total phosphorus (TP), potentially mineralizable nitrogen (PMN), microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), and potential denitrification of these soils were determined (Figure 2).

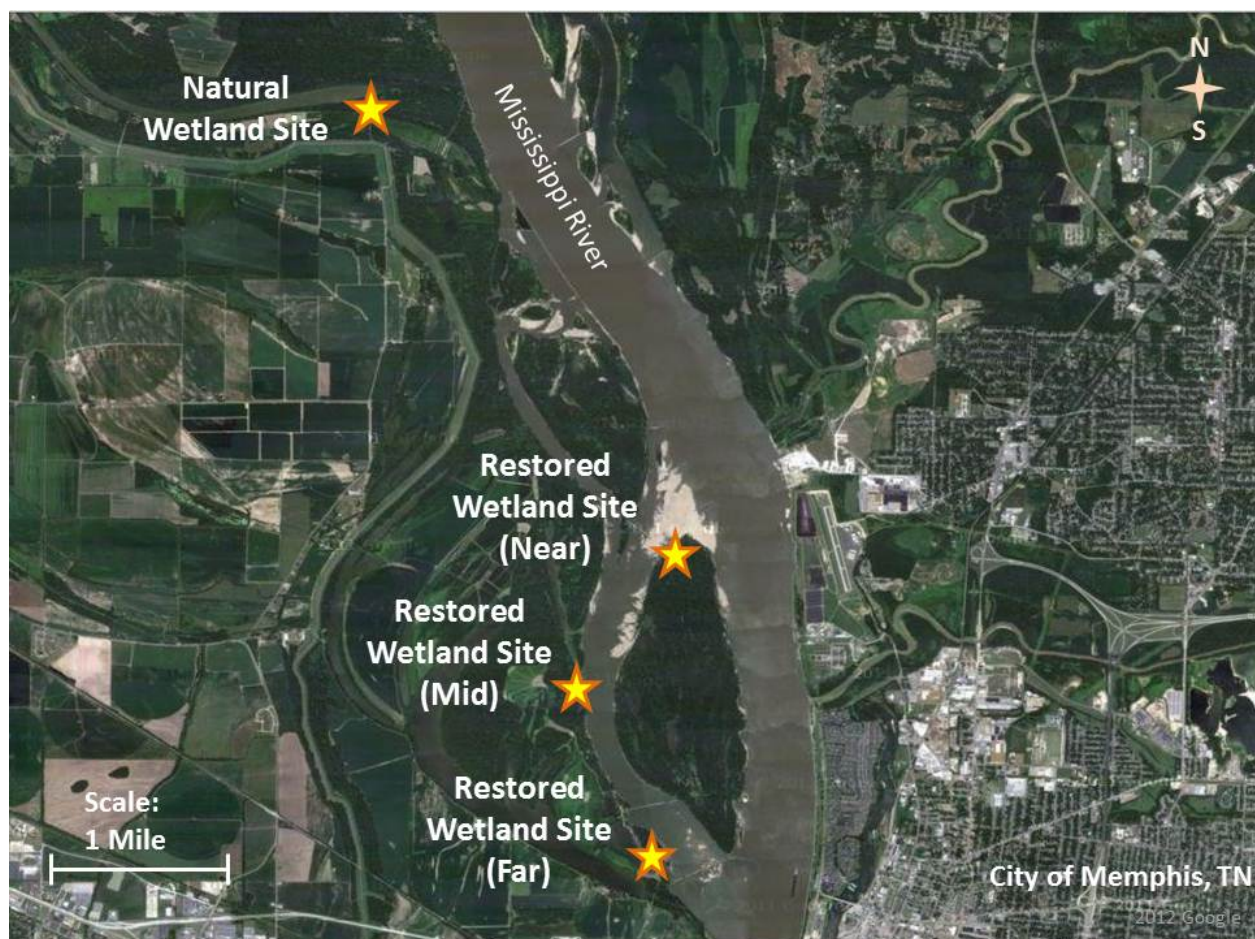


Figure 1.4.1 Satellite view of study site and sampling locations. (Google 2012)

Moisture content of the soils was determined by weighing before and after drying of the subsamples 70°C for 72 hours, or until completely dry. From the moisture content, dry weight of each sample could be calculated. Bulk density (Blake and Harge, 1986) was measured from the complete soil core. Bulk density was calculated by dividing the dry soil weight by the volume of the soil core used and is expressed in units of g cm^{-3} for the 10 cm soils cores. Dried, ground subsamples were analyzed for total carbon and total nitrogen using an Elemental Combustion System with a detection limit of 0.005 g kg^{-1} (Costech Analytical Technologies, Inc., Valencia, CA). Total phosphorus was determined by using the ashing method. 0.5 g of soil were weighed and placed into a 50mL beaker. The beaker and sample was then placed in a muffle furnace at 250°C for 30 minutes, followed by 550°C for 4 hours, then allowed to cool to room temperature. The samples and beakers were weighed once again to determine the loss on ignition of each sample. After weighing, the remaining sample ash was moistened with distilled deionized water to avoid loss of sample. Then, 20mL of 6.0 M HCl was slowly added to each beaker. The samples were transferred to a hot plate located under a fume hood. The samples were then heated on the hotplate on a medium-low setting (100-120°C) until dry. Once the beakers were dry, the hot plate temperature was raised to high for 30 minutes. After 30 minutes, the samples were removed from the hot plate and allowed to cool. Once cool, the samples were remoistened with 2-3mL of distilled deionized water then 2.25mL of 6.0 M HCl was added. The samples were returned to the hotplate on the high setting and brought to a boil then immediately removed. After cooling once more, the samples were filtered through Whatman #41 filter paper into a 50mL volumetric flask. The beakers and filter paper were rinsed with distilled deionized water three times each. Next, the volumetric flasks were filled with distilled deionized water to 50mL total volume, covered with parafilm and inverted to mix 10 times. Twenty milliliters of

each digestate was transferred to a 20ml plastic scintillation vial and stored at room temperature until analysis on an AQ2 Automated Discrete Analyzer (SEAL Analytical Inc., Mequon, Wisconsin).

1.5.2 Microbial Biomass Carbon and Nitrogen

Microbial biomass carbon and nitrogen were determined using the fumigate-extraction technique. Subsamples were measured to 5.0g, placed in centrifuge tubes, and separated into fumigate and Non-fumigate sets. 0.5mL of pure chloroform were added to each centrifuge tube of the fumigate set. The fumigate set was then placed in a desiccator along with a wet paper towel and a 50mL beaker of pure chloroform and 5-10 boiling stones. The desiccator was vacuumed to -40kPa allowing the beaker of pure chloroform to boil (maybe bubble vigorously), and then released to be re-filled with room air three consecutive times. The desiccator was then vacuumed to -40kPa once more, sealed off, and placed in fume hood for 24 hours. After 24 hours, the 50mL beaker of pure chloroform and boiling stones was removed. The desiccator was resealed and vacuumed to -40kPa and then released to be refilled with room air seven consecutive times. The fumigate and non-fumigate sets were then extracted in a similar manner. Twenty-five mL of 0.5 M K_2SO_4 was added to every sample, shaken for 30 minutes on a longitudinal shaker, then centrifuged at 6000 rpm for 10 minutes at 10°C. Both sets of samples were vacuum filtered using 0.45 μ m membrane filters. Samples were stored at 4°C until analysis for total organic carbon and nitrogen (Shimadzu Scientific Instrument-VCSN, Columbia, MD). Measurements for the non-fumigate dissolved organic carbon were subtracted from the fumigate dissolved organic carbon measurements to calculate the microbial biomass carbon. The values were then divided by 1000. Measurements for microbial biomass nitrogen were calculated in a similar manner.

1.5.3 Potentially Mineralizable Nitrogen (PMN)

Potentially mineralizable nitrogen assay is utilized to quantify the net nitrogen mineralization rates in soils by anaerobic bottle incubation by measuring the release of ammonium (NH_4^+). To determine potentially mineralizable nitrogen, two sets labeled as time zero and incubate samples were weighed to 0.5 grams dry weight equivalent of soil and transferred to 50mL centrifuge tubes and 50mL glass serum bottles, respectively. For the time zero samples, 25mL of 2.0 *M* KCl was added to the centrifuge tubes. The centrifuge tubes were capped then shaken in a reciprocating shaker for 1 hour, then centrifuged for 10 minutes at 6000rpm and 10°C. The extract was then filtered through Whatman #41 filter paper into 20mL plastic scintillation vials and stored at 4°C until $\text{NH}_4\text{-N}$ analysis by AQ2 Automated Discrete Analyzer (SEAL Analytical Inc., Mequon, Wisconsin) (EPA Method 351.2, 1983). For the incubate samples, 5mL of distilled deionized water was added to bottles. The bottles were also capped with a butyl rubber stopper and crimped with an aluminum crimp top. All the samples were vacuumed to -40kPa, and then purged with 99.99% pure N_2 gas for 5 minutes. The anaerobic serum bottles were then incubated without light at 40°C for 10 days. After 10 days, the serum bottles were removed and cooled for 30 minutes to room temperature. The serum bottles were injected with 20mL of 2.0 *M* KCL using an outlet needle, and then shaken on a reciprocating shaker for 1 hour. After 1 hour, the bottles were opened and its contents were transferred to 50mL centrifuge tubes and centrifuged for 10 minutes at 6000 rpm at 10°C. The extract was then filtered through Whatman #41 filter paper into 20mL plastic scintillation vials and stored at 4°C until $\text{NH}_4\text{-N}$ analysis by AQ2 Automated Discrete Analyzer (SEAL Analytical Inc., Mequon, Wisconsin) (EPA Method 351.2, 1983).

1.5.4 Potential Denitrification

Potential denitrification was determined using the acetylene inhibition method. Five grams of each subsample were weighed and placed in a 160mL serum bottle. Each bottle was sealed with a rubber septa and aluminum crimp cap. The bottles were then vacuumed to -40kPa. Then the bottles were purged with 99.99% pure N₂ gas for 10 minutes to remove all oxygen from the headspace. Calcium carbide rocks were combined with water in a separate vacuumed bottle to create pure acetylene gas. Sixteen mL of acetylene gas was injected into each serum bottle containing sample to represent 10% headspace of the bottle. The bottles were shaken on a longitudinal shaker for 10 minutes. While shaking the bottle, 500mL of distilled deionized water was purged with 99.99% pure N₂ gas. A KNO₃ solution was prepared using previous data to approximate 10 times the maximum nitrogen usage observed. The maximum rate observed in a similar experiment (Dolda et. al. 2008) approximately 2.0 mg-N kg⁻¹ day⁻¹ was multiplied by 5 as a cushion factor, resulting in 10 mg-N per 5 mL injection of a prepared 14.4 g/L KNO₃ solution. The samples were then injected with 10mL of the N₂ enriched distilled deionized water, followed by 5mL of the prepared KNO₃ solution. The pressures of the bottles were increased to 50kPa with 99.99% pure N₂ gas. Headspace gas samples were taking at 2, 12, 24, 36, 48, 50, 62, and 70 hours to determine the 3 day short term denitrification rates and were shaken after each headspace sampling. Gas samples were extracted using 1mL BD disposable insulin syringes and analyzed on a Shimadzu GC-8A equipped with an electron capture detector (Shimadzu Scientific Instruments, Columbia, MD, detection limit 0.006 mg N₂O-N kg⁻¹ hr⁻¹).

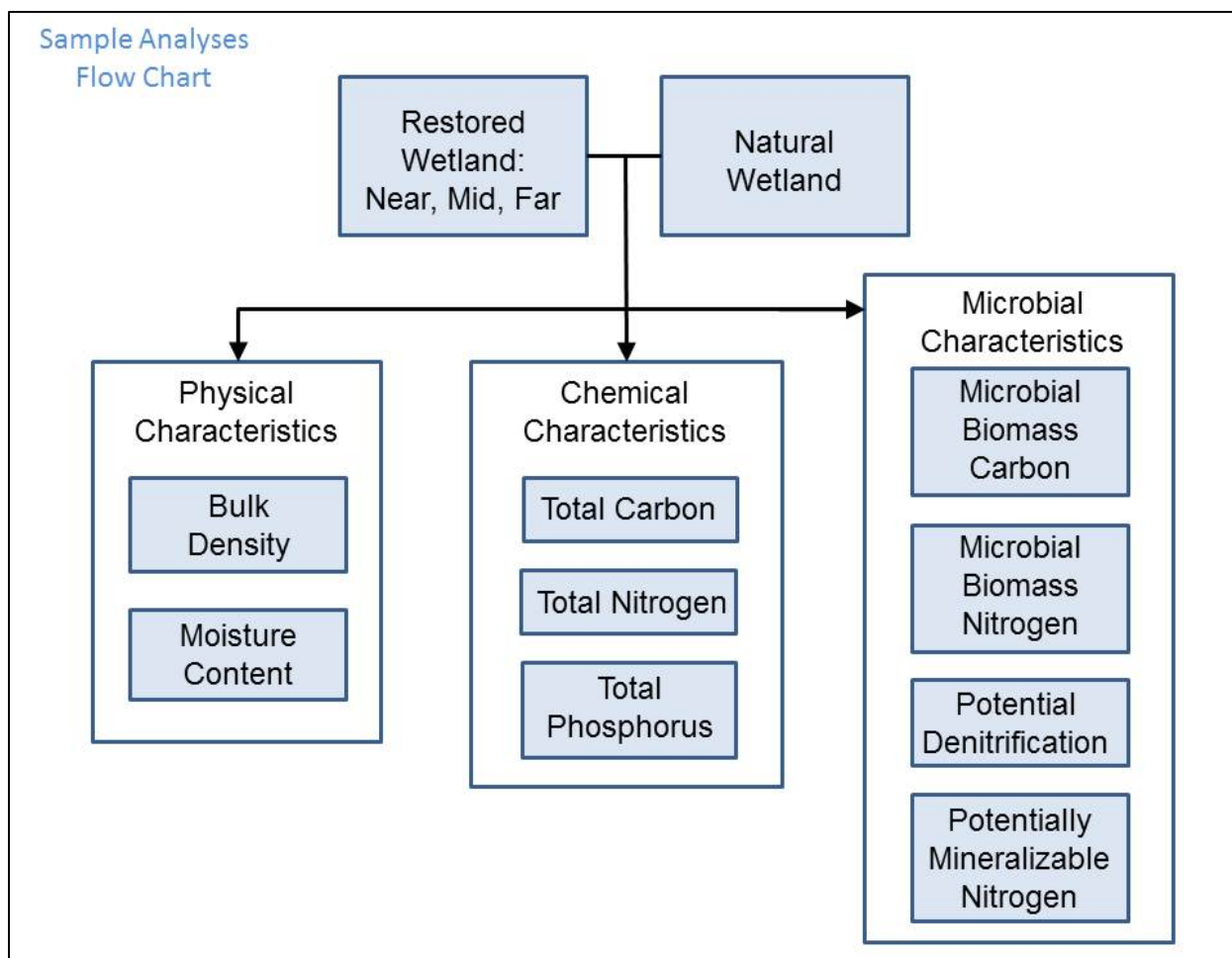


Figure 1.5.1 Sample analysis flow chart of Loosahatchie Bar soil samples.

1.6 Data Analysis

A total of 60 soil samples were analyzed for correlation between variables. The relationship between sampling location, sampling event, and measured soil and microbial parameters were analyzed using SAS 9.3 statistical Software (SAS Institute Inc., Cary, NC). Data was tested for normality using the K-S Test at $\alpha = 0.05$ and logarithmically transformed when necessary. Once data normality was determined, F-tests were conducted to determine the homogeneity of variance. Sample comparisons with $P \geq 0.05$ were assumed to have equal variance. Sample comparisons with $P < 0.05$ were assumed to have unequal variance, while a

two sided unpaired T-test ($P < 0.01$) was applied to determine differences between separate sampling locations, while a two sided paired t-test was used to identify relationships between sampling events. Variation among restored sites was determined using a one way ANOVA ($P < 0.05$).

1.7 Results

1.7.1 Bulk Density

Bulk density in the restored sites ranged from $0.51 - 1.13 \text{ g cm}^{-3}$ with a mean value of $0.81 \pm 0.11 \text{ g cm}^{-3}$, while bulk density of the natural site ranged from $0.32 - 0.69 \text{ g cm}^{-3}$ with a mean value of $0.52 \pm 0.11 \text{ g cm}^{-3}$. The mean bulk density of the restored sites was significantly higher ($P < 0.01$) than the mean bulk density of the natural site. When comparing the reference to individual restored sites within a sampling event, only the Mid site in June and the Far site in both June and August were not statistically significant from the reference site ($P > 0.05$). There was no significant difference ($P > 0.05$) between the means of each of the three restored sites or across sampling events. The mean bulk density of the soils at each site increased throughout the sampling period and was significantly correlated with time for the Near, Far and reference sites (Table 1). When examining the mean of the three restored sites at each sampling event, August was significantly greater than December ($P < 0.05$).

1.7.2 Moisture Content

Moisture content of the restored sites ranged from 8.27 to 46.3 percent with a mean value of 28.8 ± 27.3 percent, while the natural sites ranged from 31.6 to 48.1 percent with a mean value of 41.4 ± 2.06 percent (Figure 1.7.3). The mean moisture content of the restored sites was

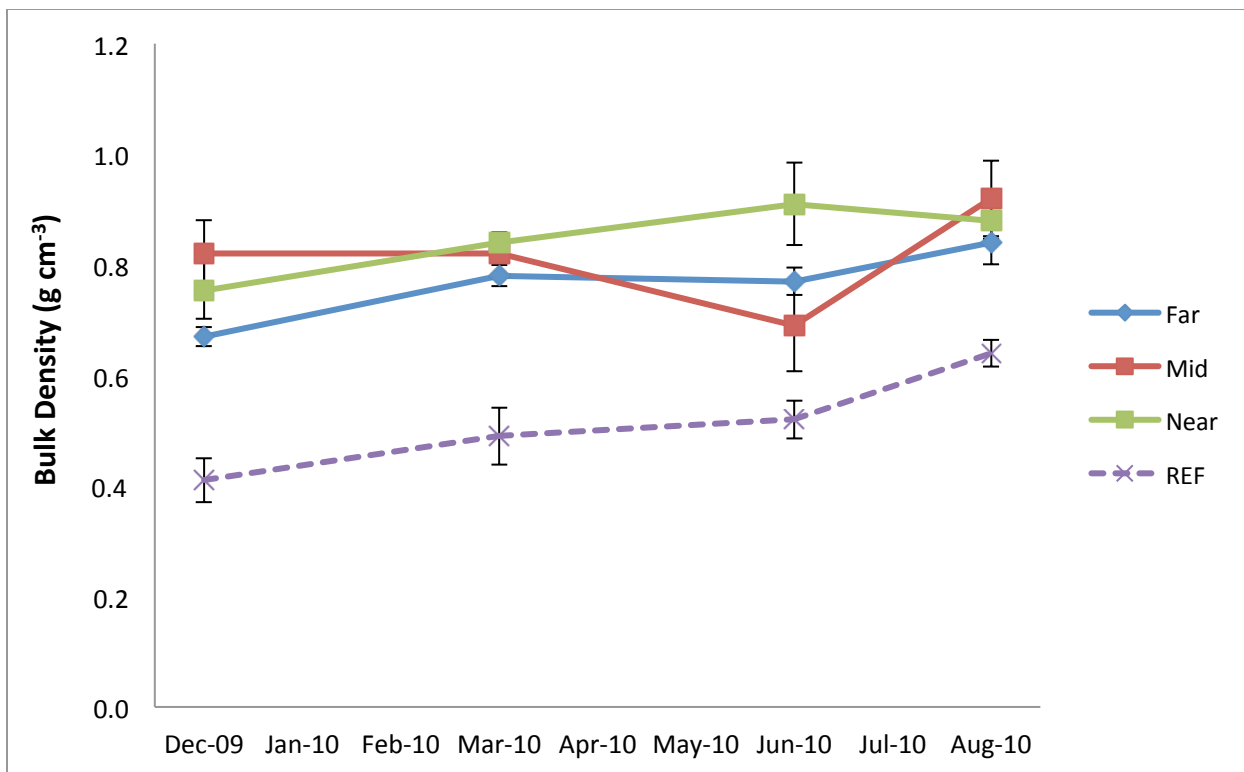


Figure 1.7.1 Bulk density of Near, Mid, Far, and Reference Sites over four seasonal sampling periods. Data are mean \pm one standard deviation.

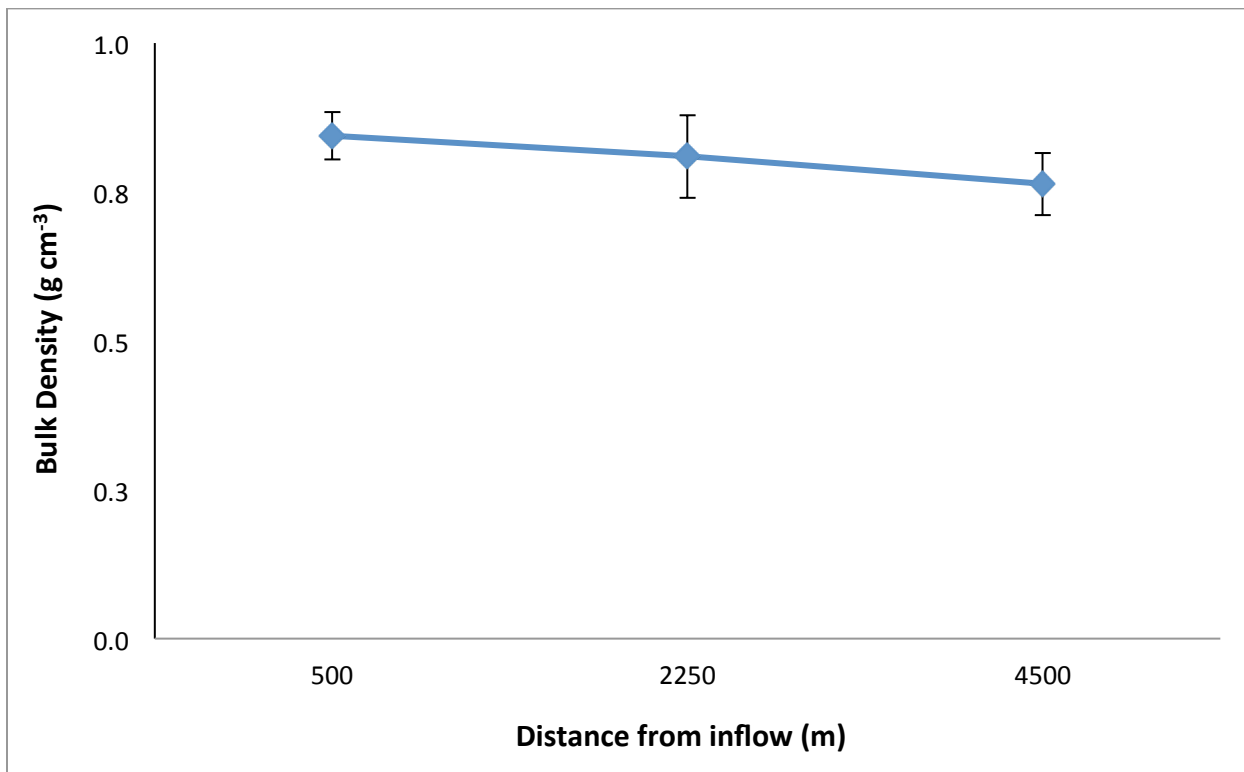


Figure 1.7.2 Bulk density of Near, Mid, and Far restored sites. Data are mean \pm one standard deviation.

significantly less ($P < 0.01$) than that mean value of the natural sites. There was no significant moisture content ($P > 0.05$) difference between the three restored sites (Figure 1.7.4). There was a significant decrease in moisture content between the June 2010 and August 2010 sampling events. There was no significant difference ($P > 0.05$) between the first and last sampling events of the restored sites. However there was significantly ($P < 0.05$) less moisture content observed in the last sampling event compared to the first sampling event.

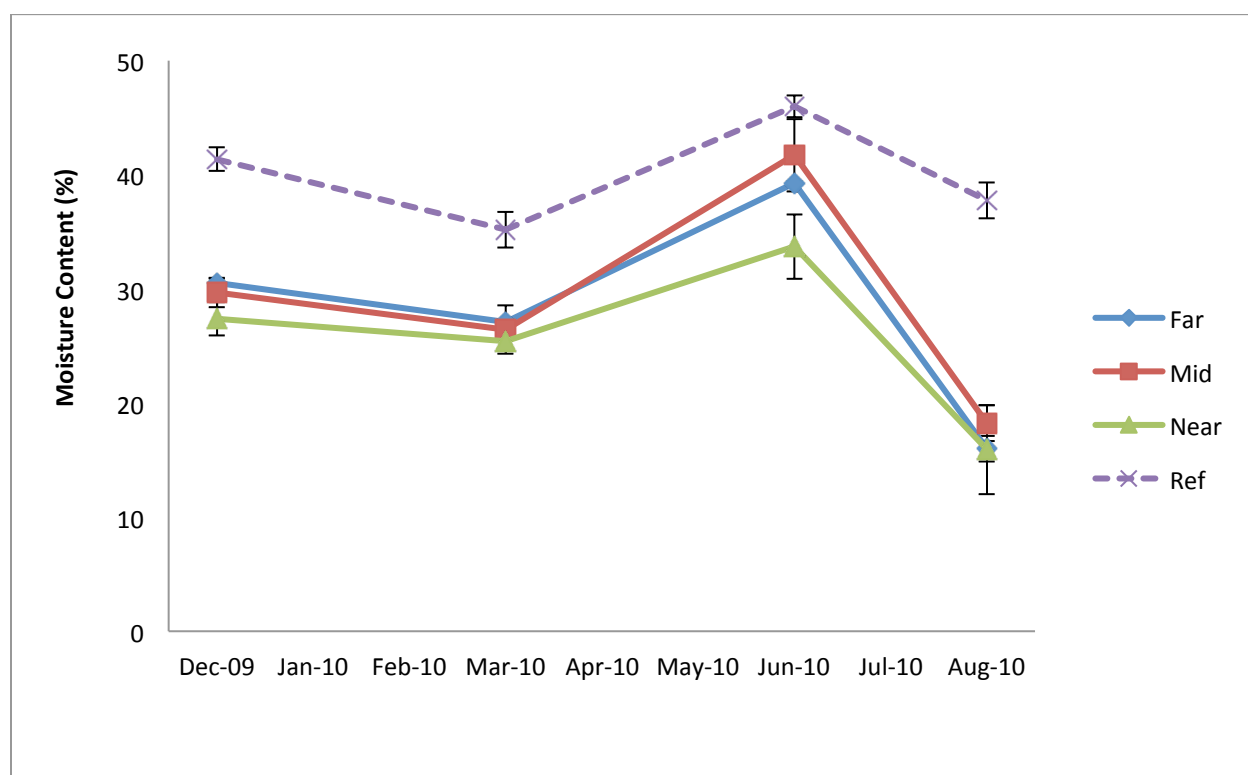


Figure 1.7.3 Moisture content of Near, Mid, Far, and Reference Sites over four seasonal sampling periods. Data are mean \pm one standard deviation.

1.7.3 Total Carbon

Total carbon (TC) in the restored sites ranged from 11.5 – 19.7 g-C kg⁻¹ with a mean value of 15.3 \pm 2.35 g-C kg⁻¹, while the natural site ranged from 40.9 – 77.1 g-C kg⁻¹ with a mean value of 50.6 \pm 10.6 g-C kg⁻¹ (Figure 1.7.5). The mean total carbon of the restored sites was

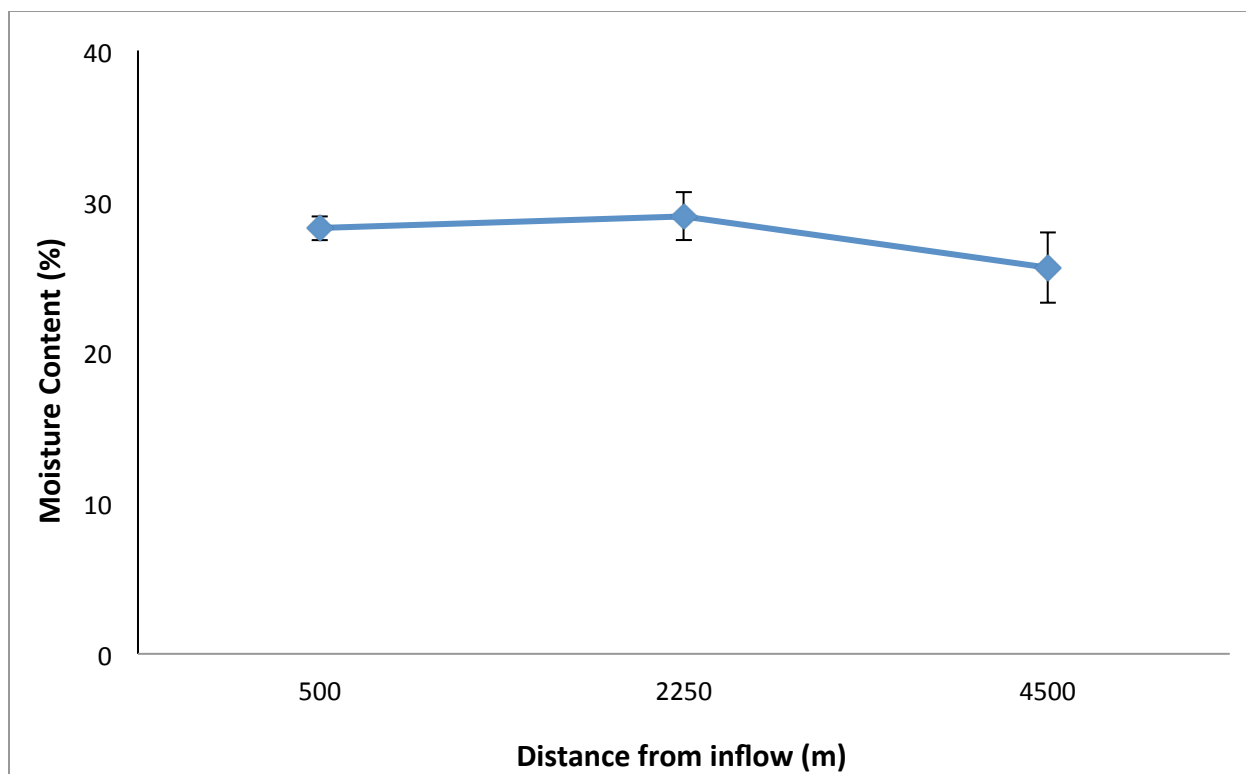


Figure 1.7.4 Moisture content of Near, Mid, and Far restored sites. Data are mean \pm one standard deviation.

significantly lower ($P < 0.001$) than the mean total carbon of the natural site. When comparing the individual restored sites with the Reference site within each sampling event, the Reference site has significantly greater soil carbon ($P < 0.001$; Figure 1.7.6) when compared with all three restored sites. The TC concentrations had little variation throughout the year for all of the restored sites, both individually and as a whole. There was a significant increase from the Near to Far restored sites ($P < 0.05$), but no significant difference between the Near and Mid along with the Far and Mid sites ($P > 0.05$).

1.7.4 Total Nitrogen

Total nitrogen (TN) in the restored sites ranged from $0.58 - 2.08 \text{ g kg}^{-1}$ with a mean value of $1.15 \pm 0.37 \text{ g kg}^{-1}$, while the total carbon of the natural site ranged from $3.18 - 4.87 \text{ g kg}^{-1}$

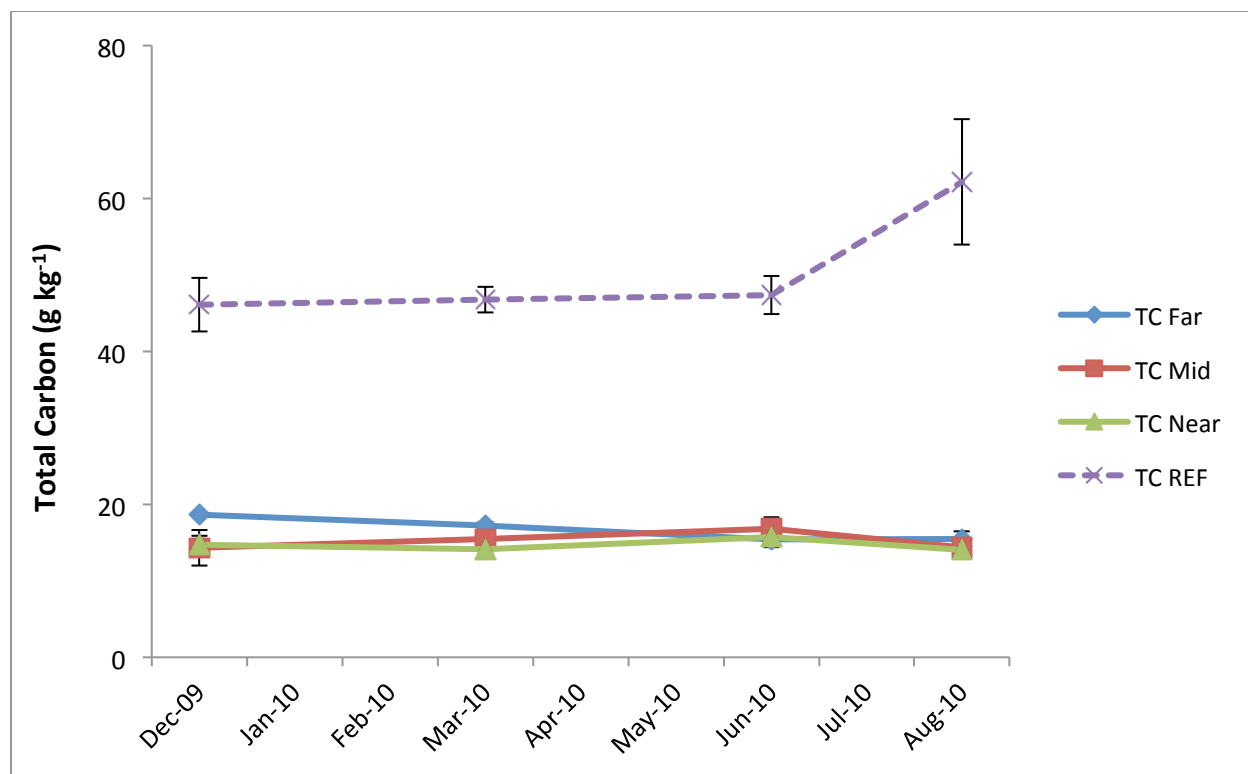


Figure 1.7.5 Total carbon (TC) of Near, Mid, Far, and Reference Sites over four seasonal sampling periods. Data are mean \pm one standard deviation.

with a mean value of $3.87 \pm 0.54 \text{ g kg}^{-1}$ (Figure 1.7.7). The mean total nitrogen of the restored sites was significantly lower ($P < 0.001$) than the mean total nitrogen of the natural site. When comparing the individual restored sites with the Reference site within each sampling event, the Reference site is significantly larger ($P < 0.001$; Figure 1.7.8). There was a significant increase from the Near to Far restored sites ($P < 0.05$, but not at $P < 0.1$), but no significant difference between the Near and Mid along with the Far and Mid sites ($P > 0.05$). The TN concentrations at the restored sites were not significantly different in the first and last sampling event (December 2009 to August 2010; $P > 0.05$). However, there was a temporary significant increase of TN in June compared to December and August in both the natural and restored sites ($P < 0.05$). While the spike in TN at the restored sites was temporary, the TN concentrations at

the reference site were significantly higher in both June and August compared to March ($P < 0.05$).

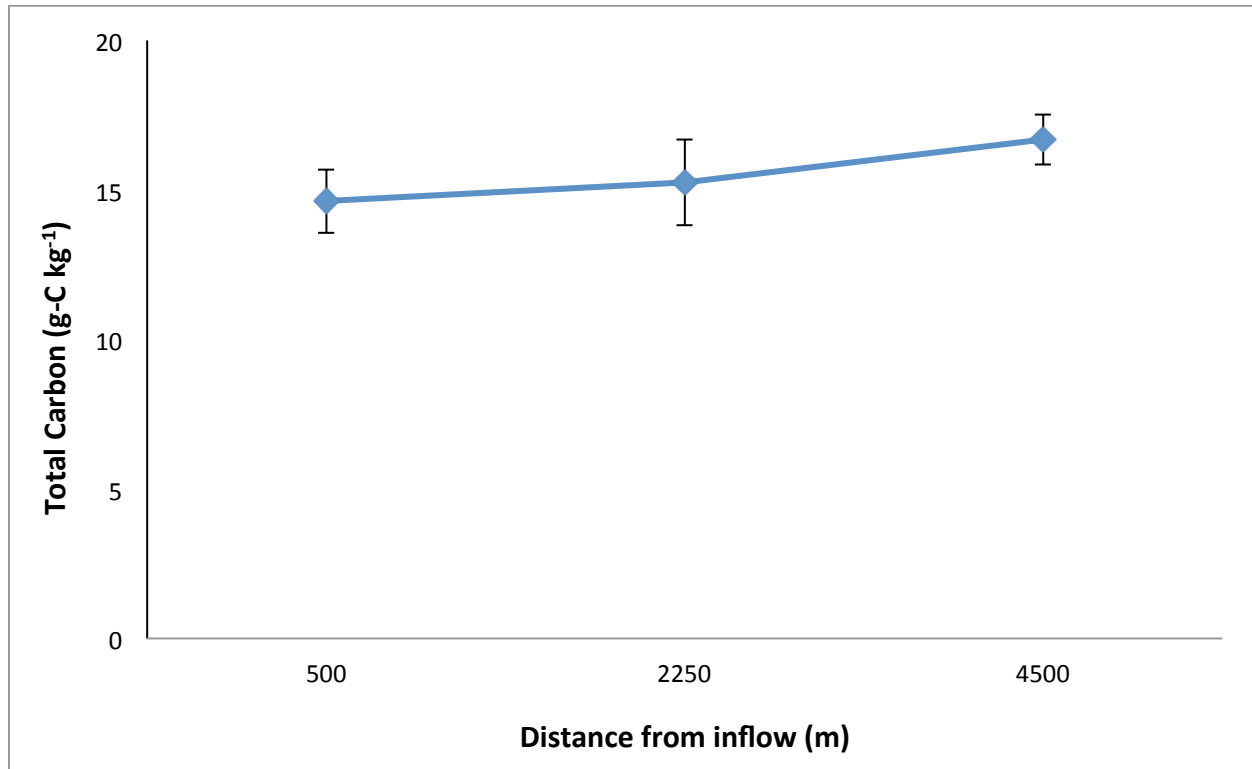


Figure 1.7.6 Total carbon (TC) of Near, Mid, and Far restored sites. Data are mean \pm one standard deviation.

1.7.5 Total Phosphorus

Total phosphorus (TP) in the restored sites ranged from 421 – 721 mg kg⁻¹ with a mean value of 566 ± 73.1 mg kg⁻¹, while total phosphorus of the natural site ranged from 935 – 1170 mg kg⁻¹ with a mean value of 1070 ± 68.5 mg kg⁻¹ (Figure 1.7.9). The mean total phosphorus of the restored sites was significantly lower ($P < 0.001$) than the mean total phosphorus of the natural site. When comparing the individual restored sites with the Reference site within each sampling event, the Reference site is significantly larger ($P < 0.001$; Figure 1.7.10). When comparing the

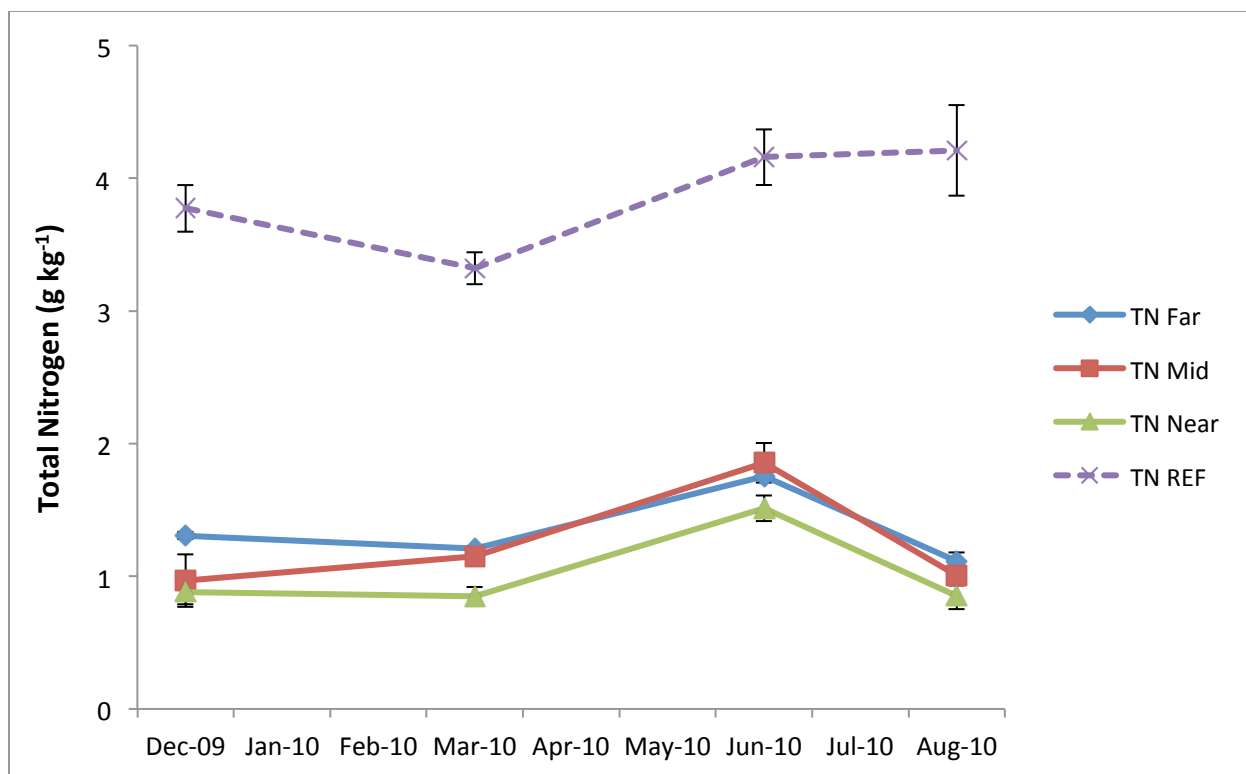


Figure 1.7.7 Total nitrogen (TN) of Near, Mid, Far, and Reference Sites over four seasonal sampling periods. Data are mean \pm one standard deviation.

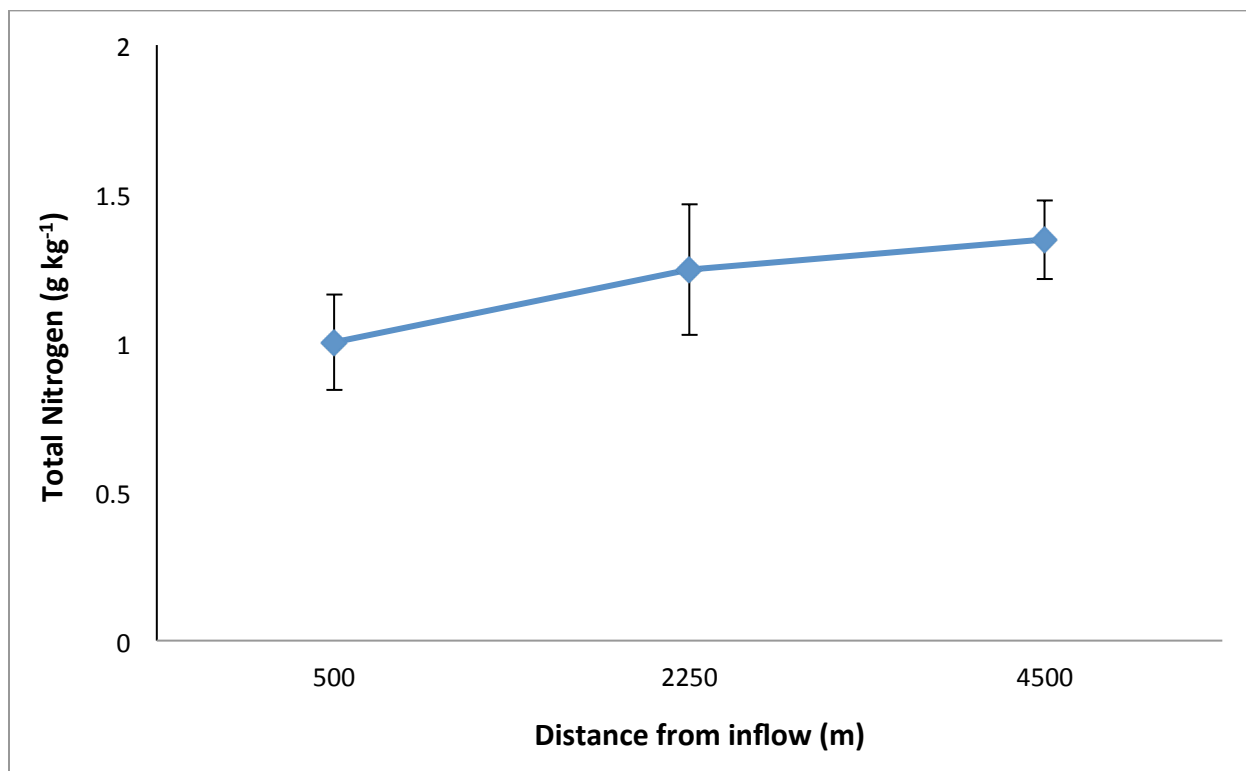


Figure 1.7.8 Total nitrogen (TN) of Near, Mid, and Far restored sites. Data are mean \pm one standard deviation.

restored sites, the near site is significantly different ($P < 0.001$) than that of the Mid and Far sites. However, there was no difference between the Mid and Far sites ($P > 0.05$). The mean TP at each restored site exhibited no significant increase or decrease in concentrations between the first and last sampling period (December 2009 to August 2010; $P > 0.05$). However, the March sampling period was significantly lower ($P < 0.05$) than the December, June and August sampling periods for the restored sites combined means of the restored values.

1.7.6 Microbial Biomass Carbon

Microbial biomass carbon (MBC) in the restored sites ranged from 235 – 5440 mg kg⁻¹ with a mean value of 1630 ± 1400 mg kg⁻¹, while microbial biomass carbon of the natural sites ranged from 854 – 4950 mg kg⁻¹ with a mean value of 2410 ± 1590 mg kg⁻¹ (Figure 1.7.11). The mean microbial biomass means of the restored sites was significantly lower ($P < 0.05$) than the natural sites only after a logarithmic transformation with restored sites and natural site values of 3.05 ± 0.39 and 3.3 ± 0.26 , respectively. Individual restored sites were significantly different ($P < 0.05$) than the natural site for all three restored sites. There was no significant differences ($P > 0.05$) when comparing the three restored sites (See Figure 1.7.12). All sites sampled showed a significant increase ($P < 0.05$) between the first three sampling periods from December 2009 sampling until June 2010 sampling, followed by a dramatic decrease between the June and August sampling periods. The August sampling was significantly less ($P < 0.05$) than all three previous samplings.

1.7.7 Microbial Biomass Nitrogen

Microbial biomass nitrogen (MBN) the restored sites ranged from 6.41 – 60.1 mg kg⁻¹ with a mean value of 21.1 ± 12.8 mg kg⁻¹ (Figure 1.7.13), while microbial biomass nitrogen of the natural site ranged from 37.2 – 113 mg kg⁻¹ with a mean value of 69.7 ± 27.2 mg kg⁻¹. The

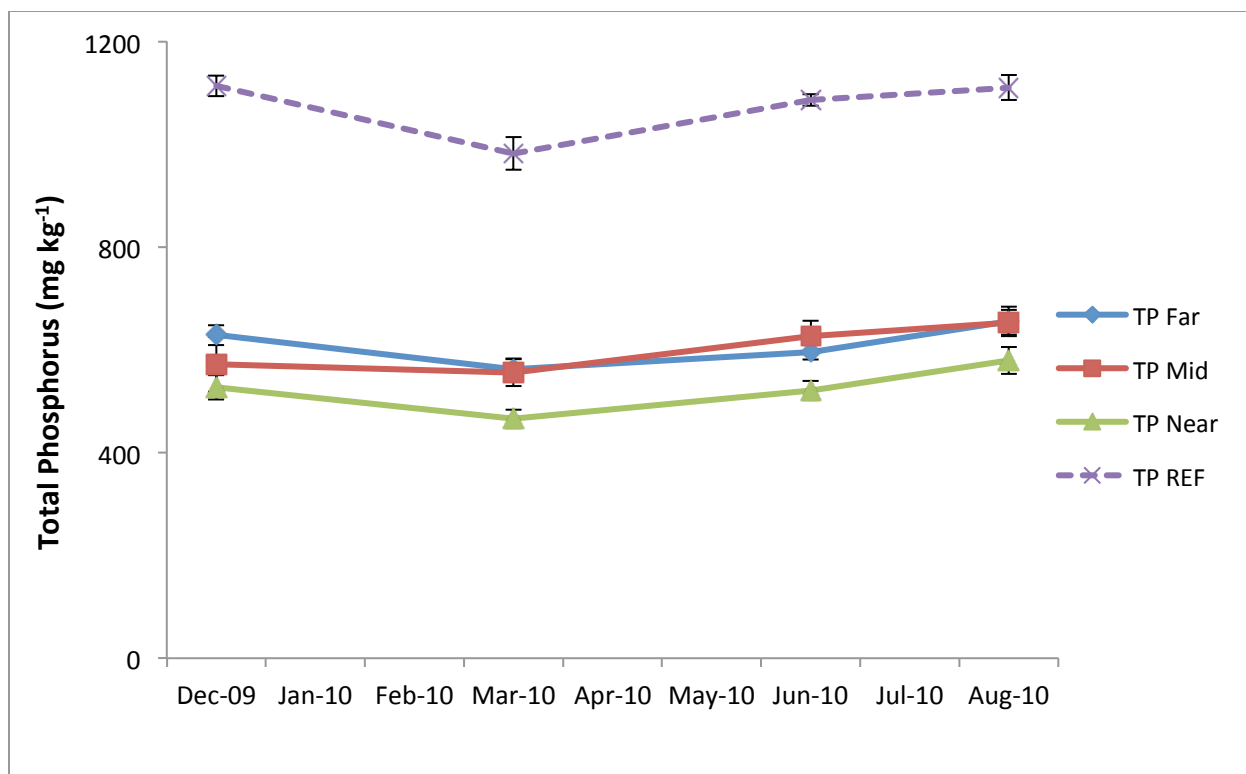


Figure 1.7.9 Total phosphorus (TP) of Near, Mid, Far, and Reference Sites over four seasonal sampling periods. Data are mean \pm one standard deviation.

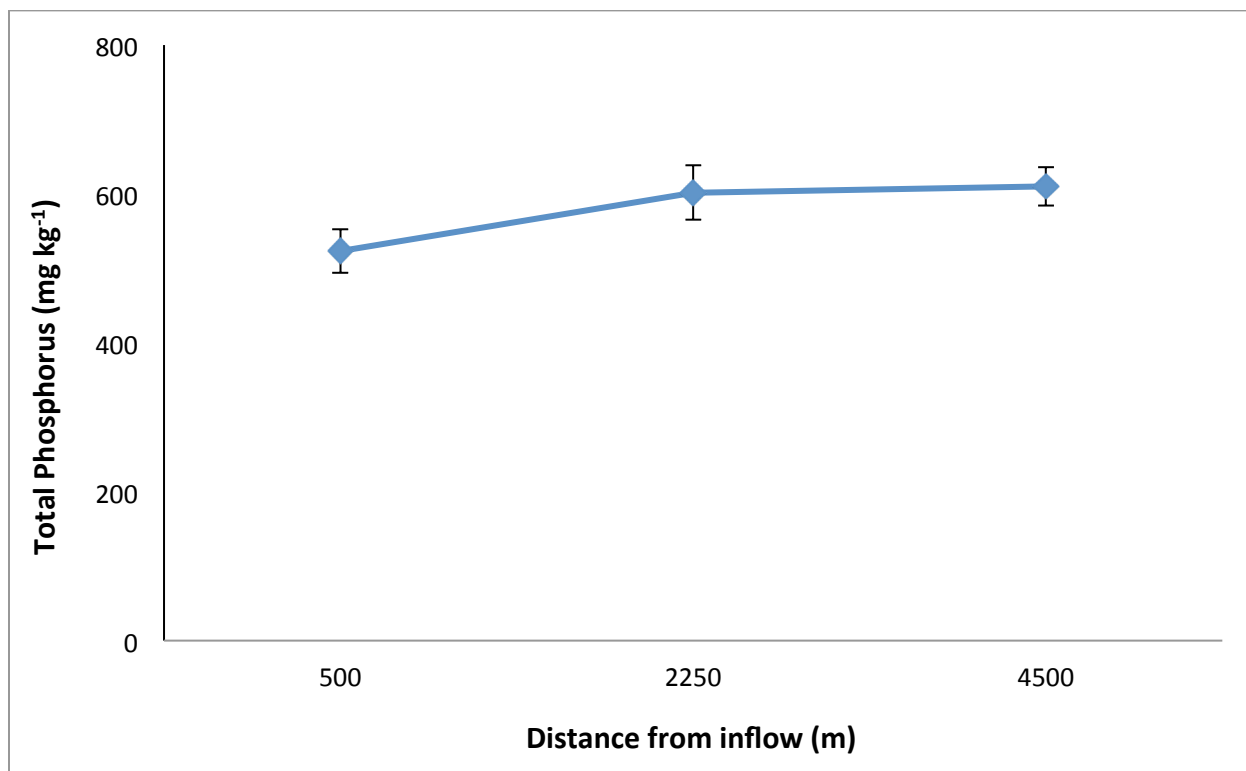


Figure 1.7.10 Total phosphorus (TP) of Near, Mid, and Far restored sites. Data are mean \pm one standard deviation.

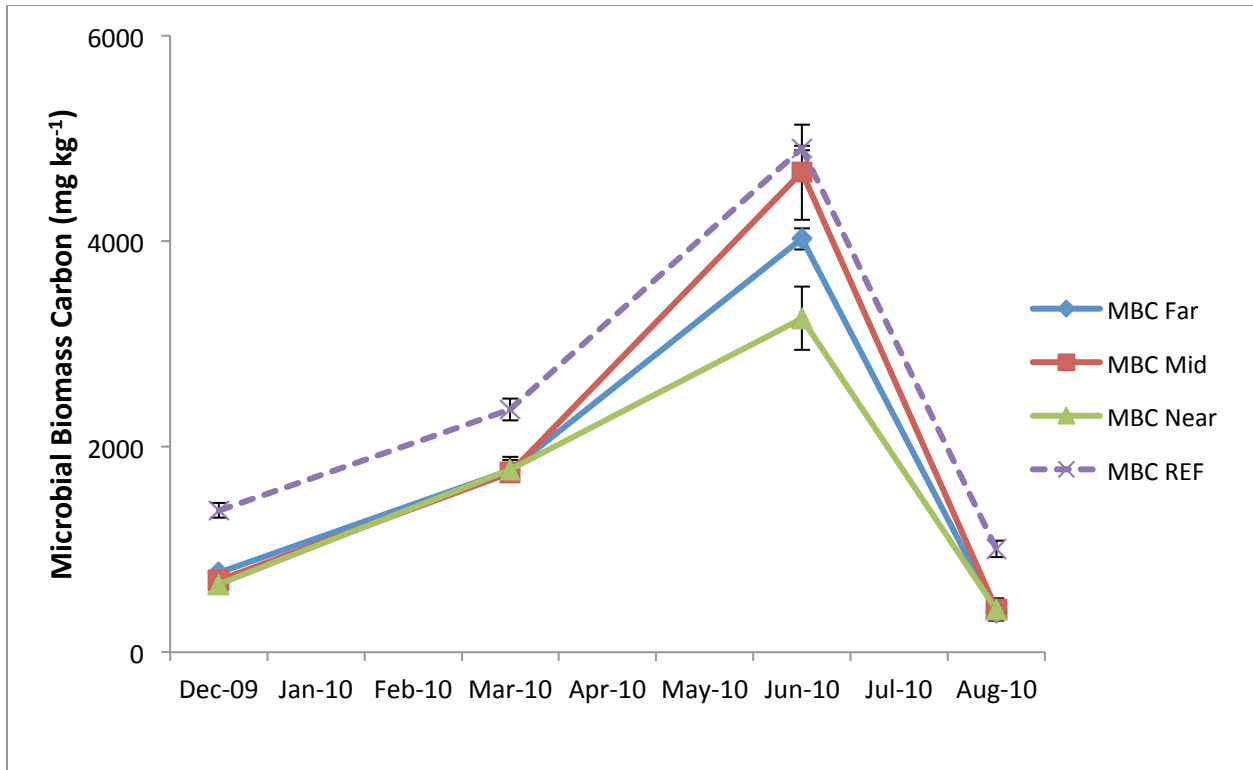


Figure 1.7.11 Microbial biomass carbon (MBC) of Near, Mid, Far, and Reference Sites over four seasonal sampling periods. Data are mean \pm one standard deviation.

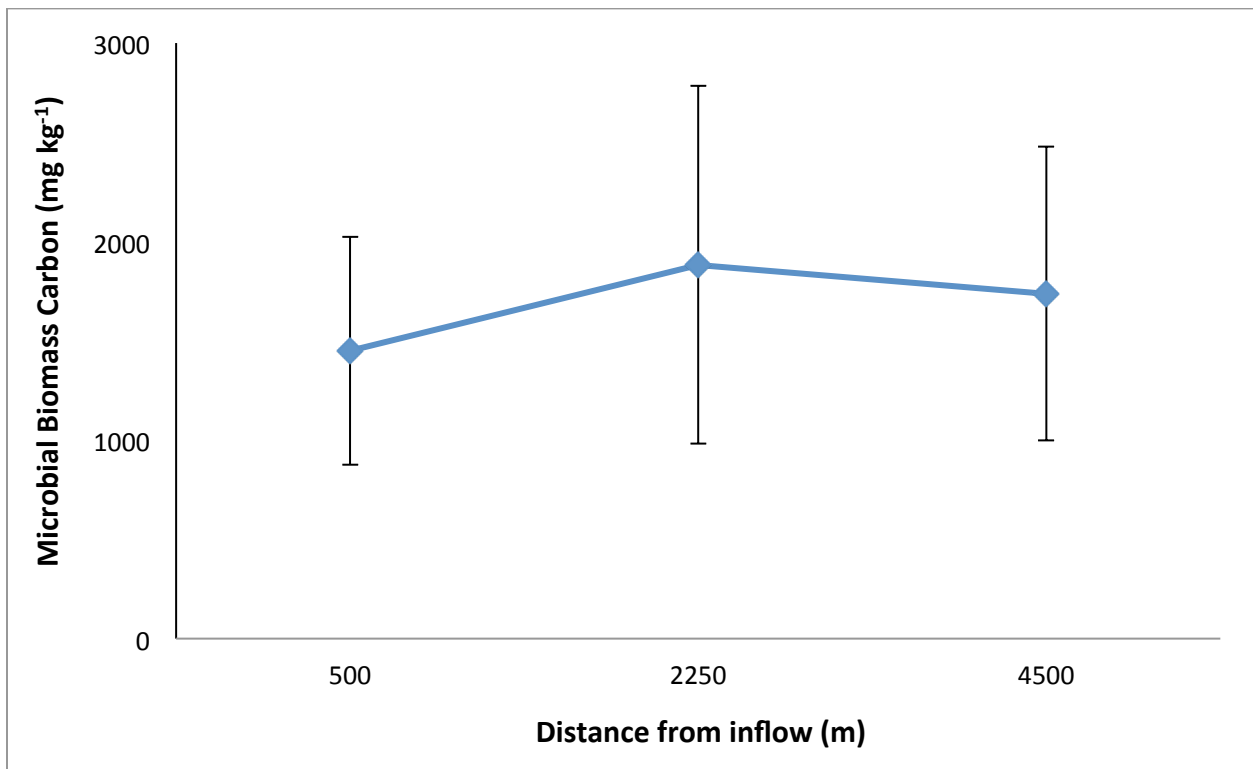


Figure 1.7.12 Microbial biomass carbon (MBC) of Near, Mid, and Far restored sites. Data are mean \pm one standard deviation.

mean microbial biomass nitrogen of the restored sites were significantly lower ($P < 0.001$) than the mean microbial biomass nitrogen of the natural site. Additionally, all of the individual restored sites were significantly lower than the reference sites within each sampling event ($P < 0.05$). There were no significant differences between the three restored sites within each sampling event ($P > 0.05$; Figure 1.7.14). The mean MBN values for the restored and reference sites were lower in August compared to December. However, only at the Near site in December was the MBN significantly higher than the other sampling events ($P < 0.01$).

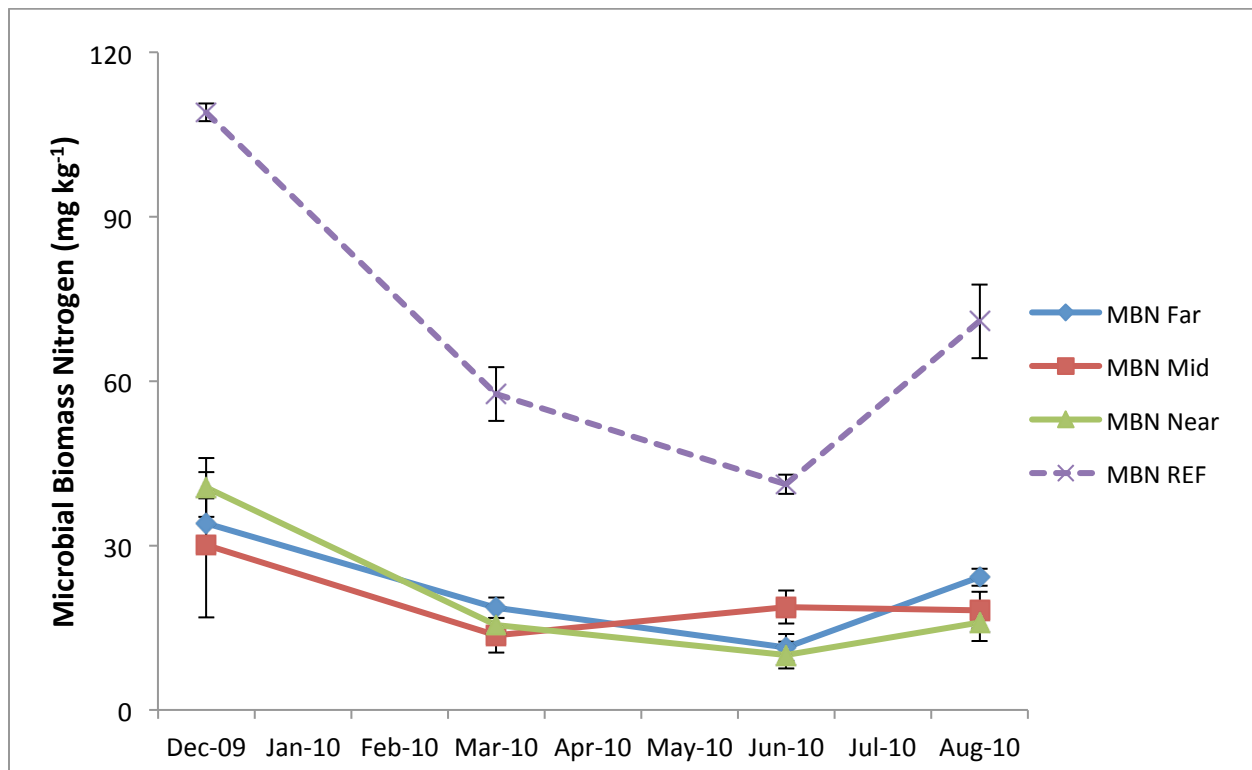


Figure 1.7.13 Microbial biomass nitrogen (MBN) of Near, Mid, Far, and Reference Sites over four seasonal sampling periods. Data are mean \pm one standard deviation.

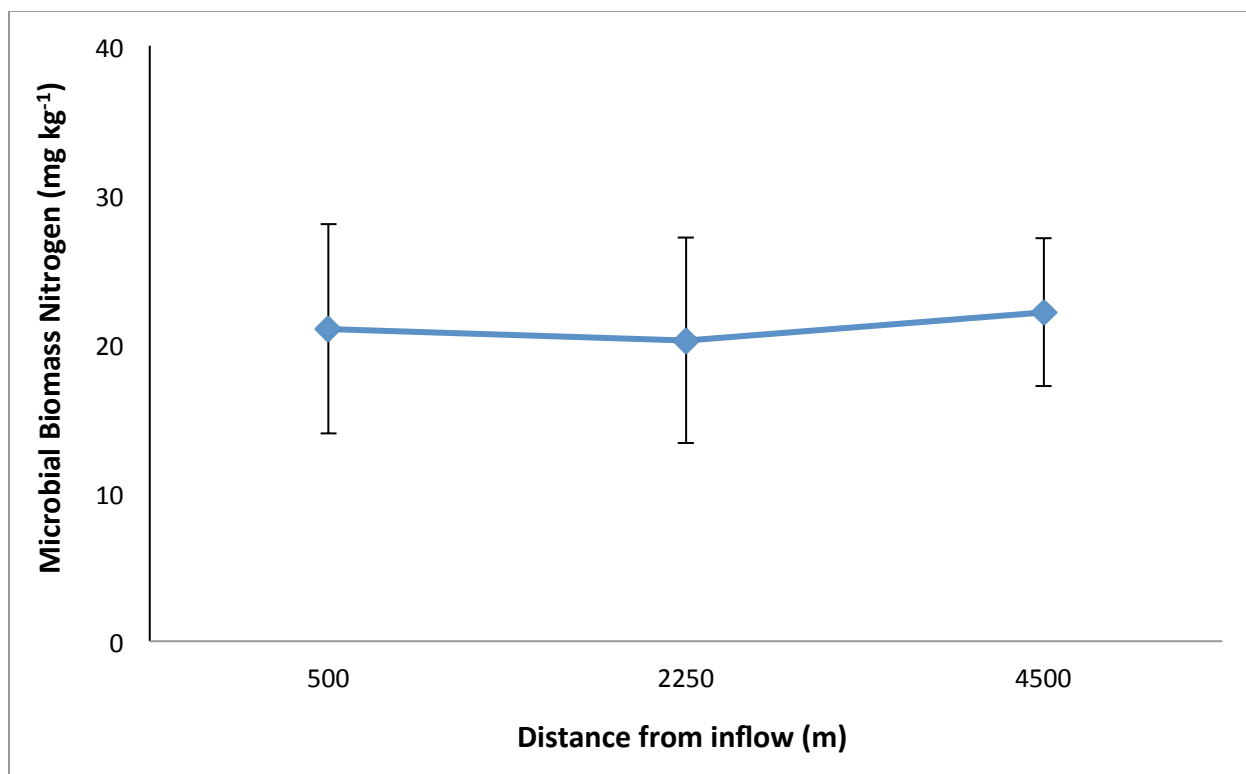


Figure 1.7.14 Microbial biomass nitrogen (MBN) of Near, Mid, and Far restored sites. Data are mean \pm one standard deviation.

1.7.8 Potentially Mineralizable Nitrogen

Potentially mineralizable nitrogen (PMN) in the restored sites ranged from 11.6 – 91.5 mg-N kg⁻¹ day⁻¹ with a mean value of 40.6 ± 18.2 mg-N kg⁻¹ day⁻¹, while potentially mineralizable nitrogen of the natural site ranged from 57.0 – 148 mg-N kg⁻¹ day⁻¹ with a mean value of 106 ± 32.0 mg-N kg⁻¹ day⁻¹ (Figure 1.7.15). The mean potentially mineralizable nitrogen of the restored sites was significantly lower ($P < 0.001$) than the mean PMN values of natural sites. When comparing the individual restored sites with the reference site within each sampling event, the reference site is significantly larger ($P > 0.05$), with the exception of the Near site in December ($P < 0.05$; Figure 1.7.16). There were no significant differences ($P > 0.05$) in PMN concentrations between the restored sites at each sampling event. The mean PMN values for the individual restored sites were lower ($P < 0.05$) in August than December, while the reference site

was not significantly different between December and August ($P > 0.05$). There was a significant increase in PMN in June over December and August ($P < 0.05$) and March over August ($P < 0.05$) when examining the mean of all the restored sites. A similar trend was seen with the reference site where June and March were significantly higher than December and August ($P < 0.05$).

1.7.9 TC:TN Ratios

TC:TN in the restored sites ranged from 10.1 – 23.4 with a mean value of 16.5 ± 3.61 , while the TC:TN ratio of the natural site ranged from 13.2 – 21.1 with a mean value of 15.3 ± 2.29 (Figure 1.7.17). The mean TC:TN of the restored sites was not significantly different ($P > 0.05$) than the mean TC:TN of the natural site.

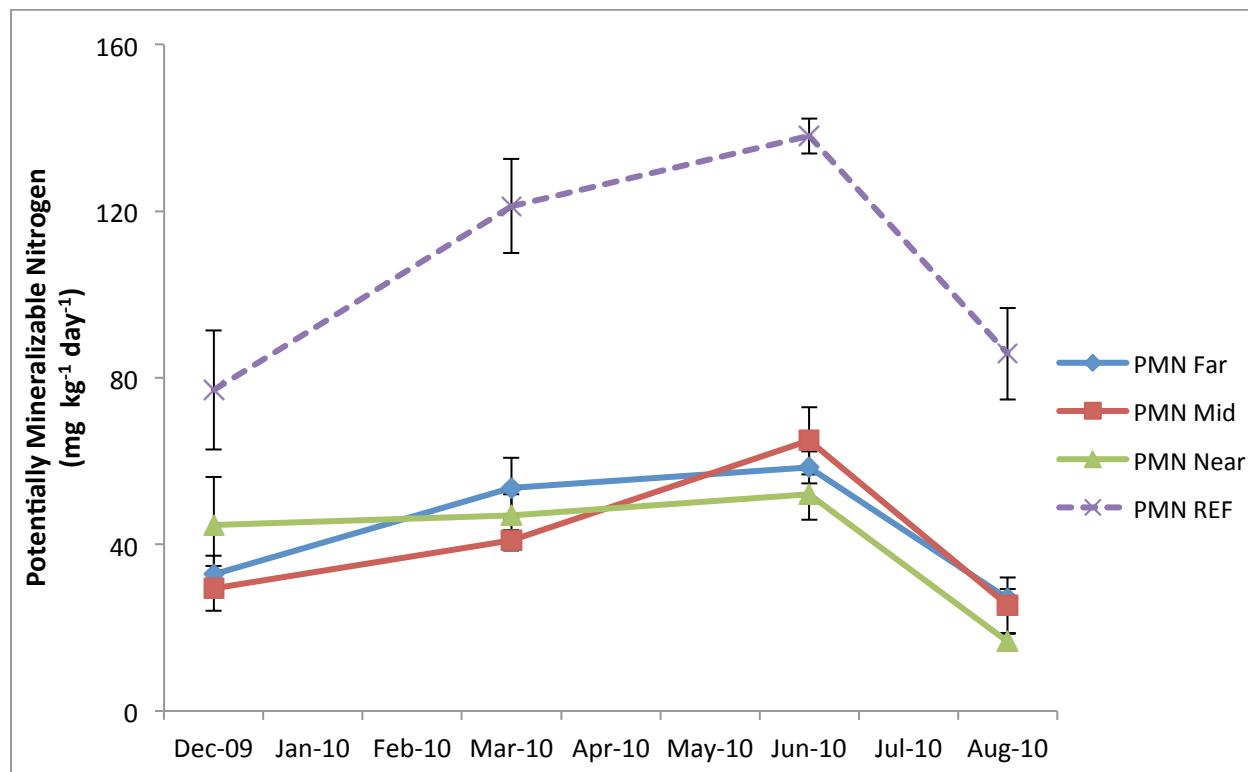


Figure 1.7.15 Potentially mineralizable nitrogen (PMN) of Near, Mid, Far, and Reference Sites over four seasonal sampling periods. Data are mean \pm one standard deviation.

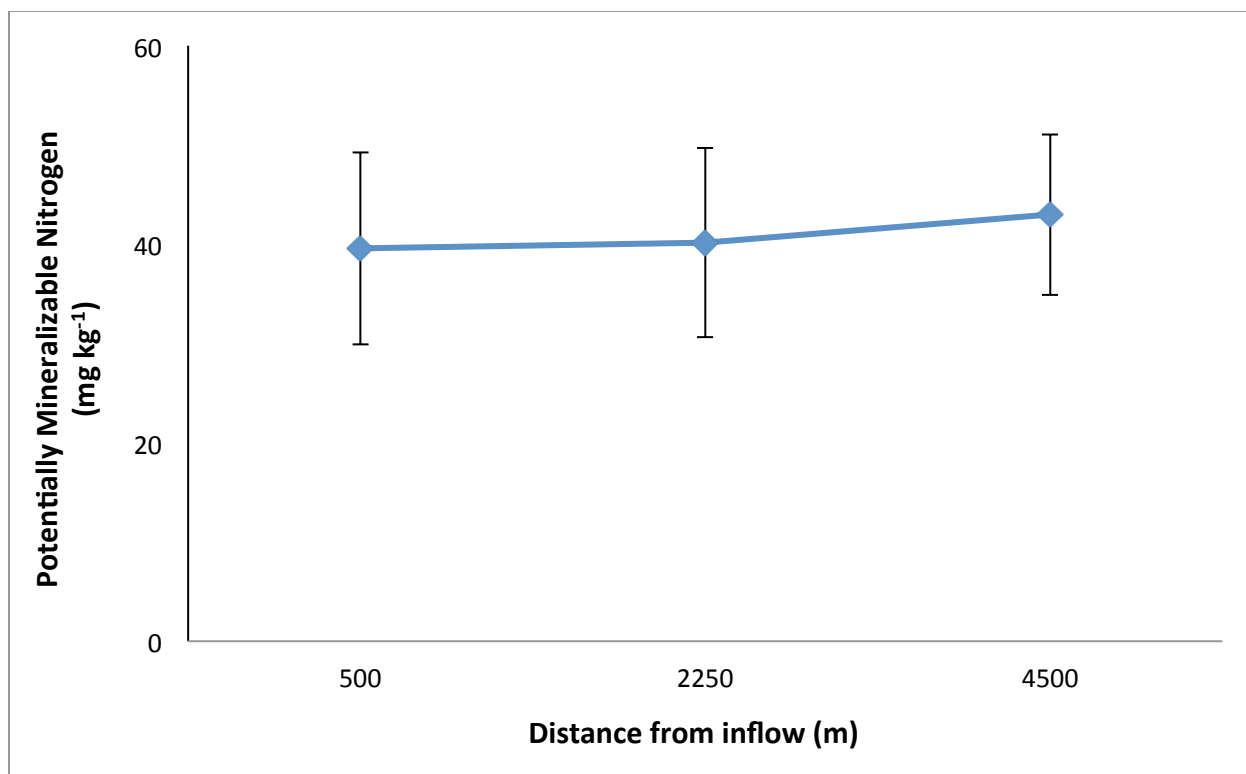


Figure 1.7.16 Potentially mineralizable nitrogen (PMN) of Near, Mid, and Far restored sites. Data are mean \pm one standard deviation.

However, the TC:TN ratio among the reference and individual restored sites was significantly different ($P < 0.05$) in December. Furthermore, when comparing the individual restored sites within each sampling event, only the Near site in December had a significantly higher TC:TN ratio than the reference site ($P < 0.001$). There was a strong positive correlation ($R^2 = 0.928$) between total carbon and total nitrogen (Figure 1.7.18). There were no significant differences among the restored sites within each sampling event. However, when comparing restored sites across sampling events, the TC:TN ratios for June were significantly lower ($P < 0.05$) than the restored sites across all sampling events.

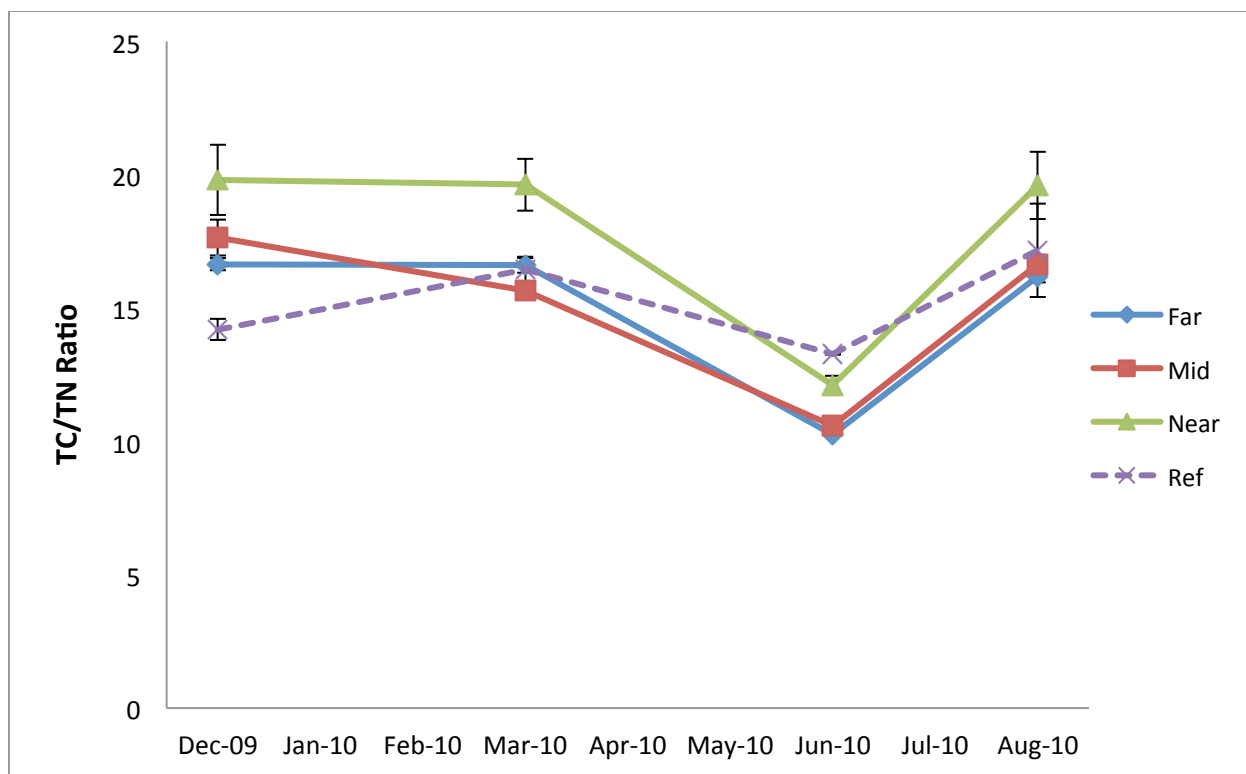


Figure 1.7.17 Total carbon (TC) to total nitrogen (TN) ratio of Near, Mid, Far, and Reference Sites over four seasonal sampling periods. Data are mean \pm one standard deviation.

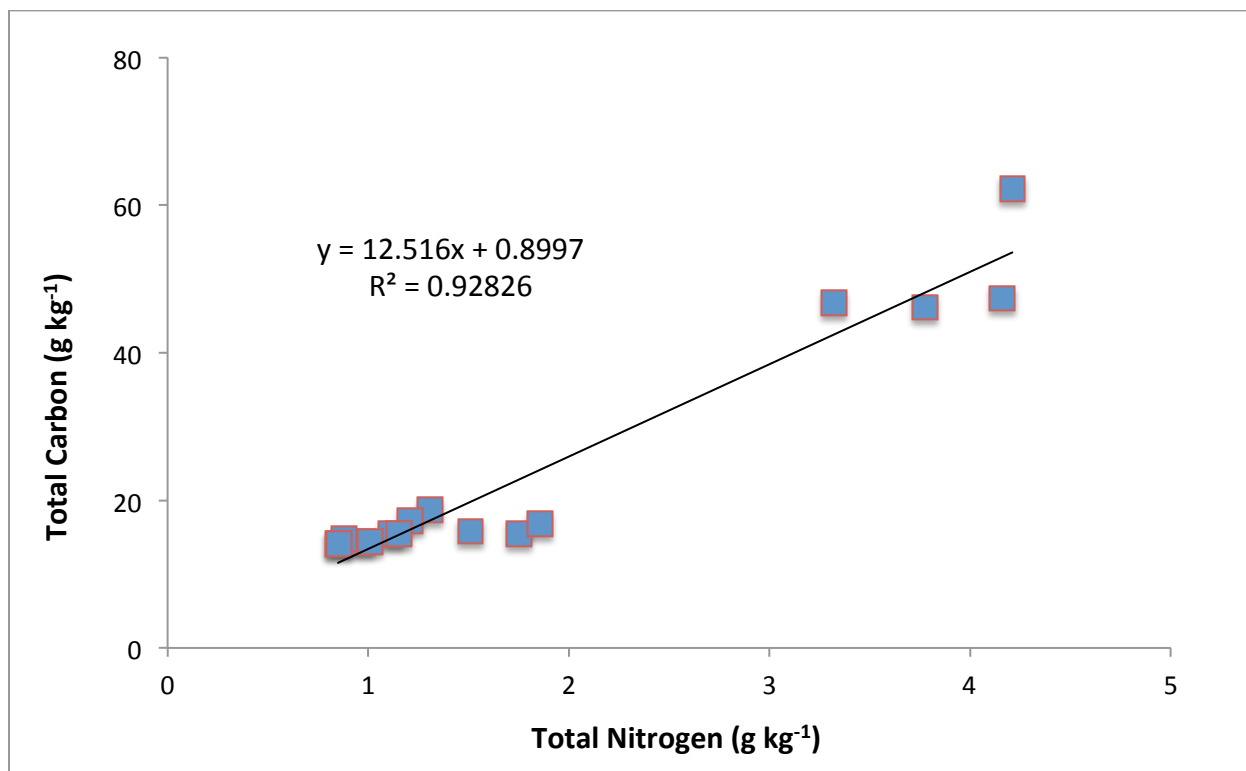


Figure 1.7.18 Linear regression of total carbon (TC) versus total nitrogen (TN) of Reference, Near, Mid, and Far sampling locations.

1.7.10 TC:TP Ratios

TC:TP in the restored sites ranged from 50.6 – 90.7 with a mean value of 70.3 ± 9.87 , while the TC:TP in the natural site ranged from 91.1 – 185 with a mean value of 122 ± 27.0 (Figure 1.7.19). The mean TC:TP of the restored site was significantly lower ($P < 0.001$) than the mean TC:TP of the natural sites. When comparing the natural site with individual restored sites, all restored sites were significantly lower ($P < 0.05$) than the reference site. The ratios decreased in August, where the combined experimental mean in March and June was significantly higher ($P < 0.05$). There were no significant differences when comparing restored sites within a sampling event and across sampling events. A strong positive correlation ($R^2 = 0.920$) between total carbon and total phosphorus was observed (Figure 1.7.20).

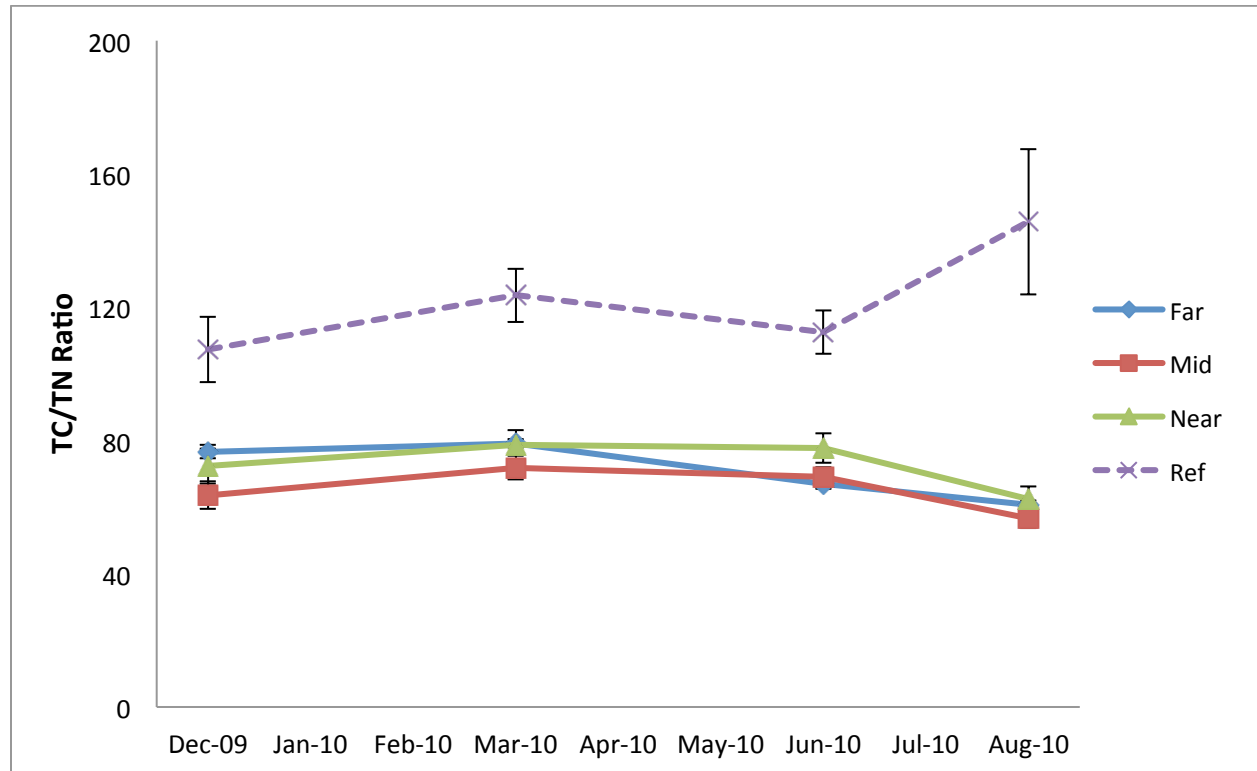


Figure 1.7.19 Total carbon (TC) to total phosphorus (TP) ratio of Near, Mid, Far, and Reference Sites over four seasonal sampling periods. Data are mean \pm one standard deviation.

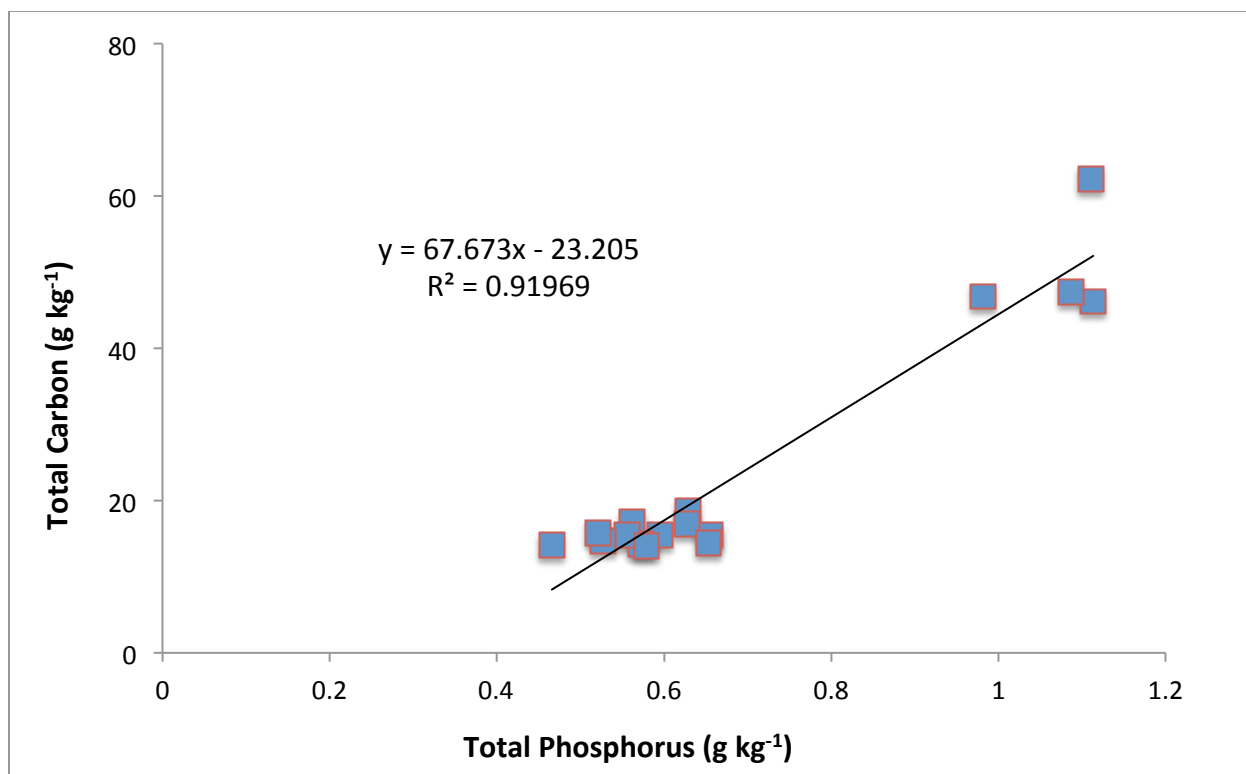


Figure 1.7.20 Linear regression of total carbon (TC) versus total phosphorus (TP) of Natural, Near, Mid, and Far sampling locations.

1.7.11 Potential Denitrification

Potential denitrification rates were only examined for each site during the August 2010 sampling event. Potential denitrification rates in the restored sites ranged from 1.32 – 162 mg N₂O-N m⁻² day⁻¹ with a mean value of 62.2 ± 52.8 mg N₂O-N m⁻² day⁻¹, while the potential denitrification rates of the natural soils ranged from 2130 – 3110 mg N₂O-N m⁻² day⁻¹ with a mean value of 2520 ± 520 mg N₂O-N m⁻² day⁻¹ (See Table 1.7.2). The mean potential denitrification rates of the restored site were significantly lower ($p < 0.001$) than mean potential denitrification rates of the natural site. The potential denitrification rates for the restored sites were only 1-3% of the reference site (Table 2). The potential denitrification were significantly lower ($P < 0.05$) at the near site than at the Mid and Far sampling sites. Additionally, the Mid and Far sampling sites were not significantly different ($P > 0.05$; Figure 1.7.21).

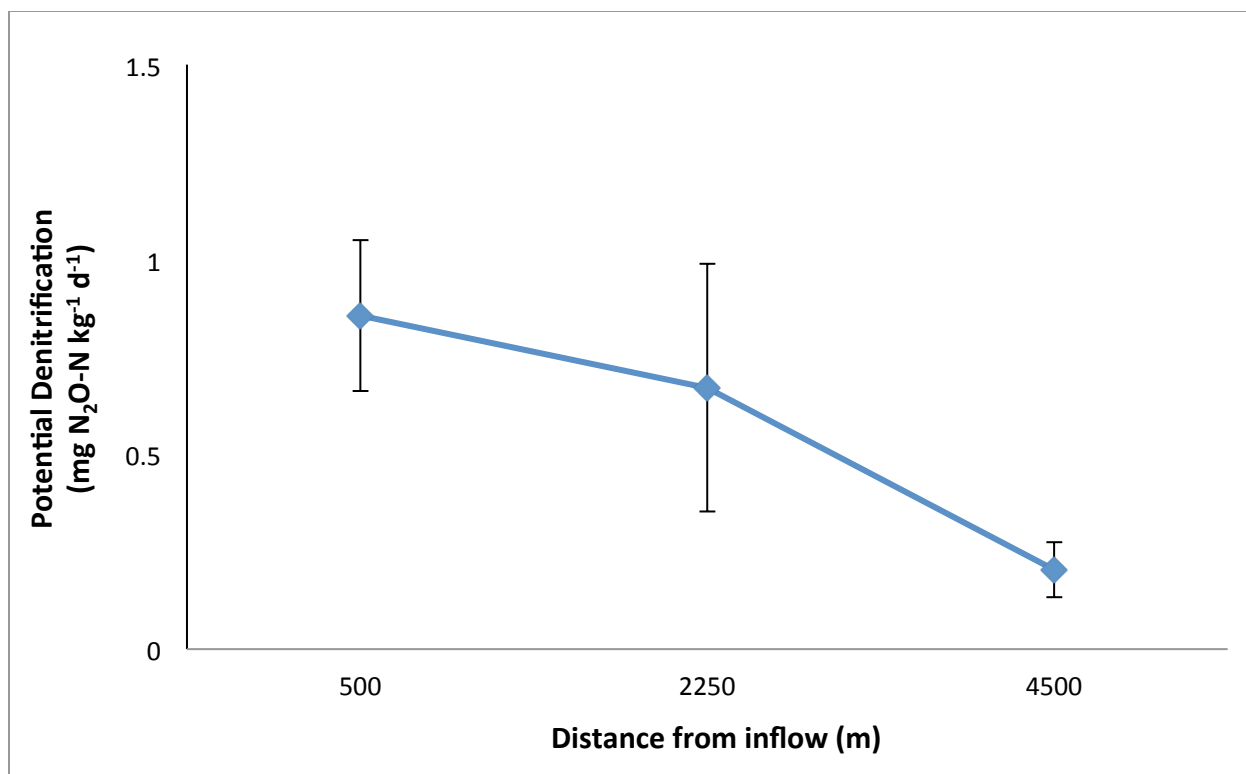


Figure 1.7.21 Combined mean potential denitrification of Near, Mid, and Far restored sites. Data are mean \pm one standard deviation.

Table 1.7.1 Mean values and standard deviation for the soil properties measured at each site and sampling event.

Bulk Density (g cm⁻³)				
	Far	Mid	Near	Reference
December 2009	0.67 ± 0.03	0.82 ± 0.12	0.75 ± 0.10	0.41 ± 0.08
March 2010	0.78 ± 0.04	0.82 ± 0.08	0.84 ± 0.03	0.49 ± 0.10
June 2010	0.77 ± 0.05	0.69 ± 0.17	0.91 ± 0.15	0.52 ± 0.07
August 2010	0.84 ± 0.08	0.92 ± 0.14	0.88 ± 0.07	0.64 ± 0.05
TN (g kg⁻¹)				
December 2009	1.31 ± 0.05	0.97 ± 0.40	0.88 ± 0.19	3.77 ± 0.35
March 2010	1.21 ± 0.04	1.15 ± 0.13	0.85 ± 0.14	3.32 ± 0.24
June 2010	1.75 ± 0.02	1.86 ± 0.30	1.51 ± 0.29	4.16 ± 0.42
August 2010	1.11 ± 0.13	1.01 ± 0.15	0.85 ± 0.20	4.21 ± 0.68
TC (g kg⁻¹)				
December 2009	18.7 ± 0.24	14.3 ± 4.65	14.7 ± 2.24	46.1 ± 6.99
March 2010	17.2 ± 0.78	15.4 ± 1.71	14.2 ± 1.37	46.8 ± 3.41
June 2010	15.4 ± 0.26	16.9 ± 2.88	15.8 ± 2.89	47.4 ± 4.94
August 2010	15.5 ± 1.98	14.4 ± 2.50	14.0 ± 2.22	62.2 ± 16.4
TP (mg kg⁻¹)				
December 2009	629 ± 39.3	572 ± 107	527 ± 47.5	1110 ± 40.2
March 2010	562 ± 42.9	556 ± 52.9	466 ± 36.2	982 ± 63.2
June 2010	595 ± 28.0	627 ± 59.8	520 ± 68.5	1090 ± 23.0
August 2010	655 ± 57.3	653 ± 47.7	579 ± 52.8	1110 ± 49.1
TC:TN				
December 2009	16.4 ± 0.46	17.6 ± 1.34	19.8 ± 2.62	14.2 ± 0.80
March 2010	16.6 ± 0.56	15.7 ± 0.58	19.6 ± 1.94	16.4 ± 0.95
June 2010	10.3 ± 0.09	10.6 ± 0.47	12.1 ± 0.77	13.3 ± 0.05
August 2010	16.2 ± 0.44	16.7 ± 0.72	19.6 ± 2.52	17.2 ± 3.49
TC:TP				
December 2009	76.7 ± 4.00	63.6 ± 8.26	72.3 ± 10.4	107 ± 19.6
March 2010	79.2 ± 2.65	71.8 ± 7.10	78.7 ± 8.85	123 ± 16.0
June 2010	66.9 ± 2.80	69.0 ± 6.12	77.7 ± 8.08	113 ± 13.0
August 2010	60.7 ± 2.39	56.6 ± 5.94	62.4 ± 7.74	146 ± 43.6

Table 1.7.2 Mean values and standard deviation for the microbial properties measured at each site and sampling event.

Sampling Date	Far	Mid	Near	Reference
MBC (mg kg⁻¹)				
December 2009	773 ± 25.8	698 ± 104	659 ± 106	1380 ± 146
March 2010	1770 ± 196	1750 ± 39.1	1779 ± 243	2360 ± 214
June 2010	4021 ± 211	4670 ± 923	3250 ± 873	4900 ± 46.2
August 2010	387 ± 80.4	417 ± 118	415 ± 216	1010 ± 160
MBN (mg kg⁻¹)				
December 2009	34.1 ± 9.10	30.2 ± 26.5	40.6 ± 10.8	109 ± 3.24
March 2010	18.6 ± 3.69	13.63 ± 6.31	15.5 ± 6.38	57.6 ± 9.76
June 2010	11.4 ± 4.83	18.81 ± 6.07	10.0 ± 6.18	41.2 ± 3.53
August 2010	24.2 ± 3.14	18.20 ± 6.65	15.9 ± 6.64	70.9 ± 13.5
PMN (mg-N kg⁻¹ day⁻¹)				
December 2009	32.8 ± 9.01	29.4 ± 10.7	44.7 ± 23.0	77.0 ± 28.6
March 2010	53.6 ± 14.6	40.95 ± 4.83	47.0 ± 9.99	121 ± 22.6
June 2010	58.5 ± 7.79	64.9 ± 16.2	52.0 ± 13.6	138 ± 8.36
August 2010	27.0 ± 4.54	25.4 ± 13.4	16.7 ± 3.83	85.8 ± 22.0
Potential Denitrification (mg N₂O-N m⁻² d⁻¹)				
August 2010	27.6 ± 19.1	87.0 ± 72.6	106 ± 41.0	2520 ± 520

Table 1.7.3 The percent difference (based on weight basis) between the measured soils properties at each of the three restored sites compared to the reference site. The values in the “All Sites” column are the average of the three restored sites at each sampling event compared to the reference site.

Sampling Date	Far	Mid	Near	All Sites
Bulk Density % of Reference site				
December 2009	162%	198%	183%	181%
March 2010	158%	166%	171%	165%
June 2010	148%	133%	176%	152%
August 2010	132%	144%	138%	138%
TN % of Reference site				
December 2009	35%	26%	23%	28%
March 2010	36%	35%	26%	32%
June 2010	42%	45%	36%	41%
August 2010	26%	24%	20%	24%
TC % of Reference site				
December 2009	40%	31%	32%	34%
March 2010	37%	33%	30%	33%
June 2010	33%	36%	33%	34%
August 2010	25%	23%	23%	24%
TP % of Reference site				
December 2009	56%	51%	47%	52%
March 2010	57%	57%	47%	54%
June 2010	55%	58%	48%	53%
August 2010	59%	59%	52%	57%
TC:TN				
December 2009	117%	139%	124%	127%
March 2010	101%	119%	95%	105%
June 2010	77%	91%	80%	83%
August 2010	94%	114%	97%	102%
TC:TP				
December 2009	71%	67%	59%	66%
March 2010	64%	64%	58%	62%
June 2010	59%	69%	61%	63%
August 2010	42%	43%	39%	41%

Table 1.7.4 The percent difference (based on weight basis) between the measured microbial properties at each of the three restored sites compared to the reference site. The values in the “All Sites” column are the average of the three restored sites at each sampling event compared to the reference site.

Sampling Date	Far	Mid	Near	All Sites
MBC % of Reference site				
December 2009	56%	51%	48%	52%
March 2010	75%	74%	75%	75%
June 2010	82%	95%	66%	81%
August 2010	38%	41%	41%	40%
MBN % of Reference site				
December 2009	31%	28%	37%	32%
March 2010	32%	24%	27%	28%
June 2010	28%	46%	24%	33%
August 2010	34%	26%	22%	27%
PMN % of Reference site				
December 2009	43%	38%	58%	46%
March 2010	44%	34%	39%	39%
June 2010	42%	47%	38%	42%
August 2010	32%	30%	19%	27%
Potential Denitrification % of Reference site				
August 2010	1%	2%	3%	2%

1.8 Discussion and Conclusion

Wetland restoration projects are becoming increasingly undertaken for protection from floodwaters, stabilization of river banks, and facilitation of the removal of excess fertilizers used in agricultural processes. The Loosahatchie Bar wetland restoration project was designed to convert uplands area back into functional riparian wetlands by reintroduction of Mississippi River water in 2007. Frequent inundation by the river water has changed the soil and microbial properties of the soil at these sites. Over time, the converted upland site has and will become more similar, biogeochemically, to the natural wetland site. Flooding should result in an increase of organic matter accretion rates (Mitsch and Gosselink 2000). It is important to note

that there are significant differences between the natural and control soil texture types. The soils of the natural wetlands contain greater organic matter. The natural wetlands are finer particle sizes such as silts and clays, while the restored site's soils are larger and contain larger silts and sand. It is important to note that just the differences in soil texture may result in differences observed. The natural soils had a greater moisture content, which is directly related to the particle size.

Since the start of the Loosahatchie Bar Wetland restoration project, the upland has been converted to a bottomland hardwood forest that receives frequent flooding from the Mississippi River. Compared to the upriver, natural wetland site, there are no obvious major physical differences between the two sites and include hydrophilic vegetation along with frequent inundation by water. Upon examination of soil biogeochemical properties, significant differences were noted in both soil characteristics and microbial properties between the natural and restored wetland sites. Bulk density of the restored wetland soils were significantly higher than those of the natural wetland site over the four sampling periods due to decreased organic matter within the soil profile. Soil characteristics such as total carbon, total nitrogen, and total phosphorus were significantly lower in the restored wetlands than the natural wetlands. There were no measured differences in either primary productivity or ephemeral productivity between the restored sites and the natural site (Koontz et. al. 2012).

Since there was no difference in productivity between the sites, the differences in nutrients may be due to the effects of multiple inundation events in the early part of the year by the Mississippi River (Figure 1.8.1). Flooding may remove newly formed organic matter within both the natural and restored sites. This will leave behind the previously mineral soils at the restored sites and organic matter at the natural site. Loss on ignition measurements for the

restored and natural wetland sites were significantly greater ($P < 0.001$) for the natural site's soils. The higher loss on ignition is indicative of higher organic matter within the soil. Microbial properties such as microbial biomass nitrogen, potentially mineralizable nitrogen, and potential denitrification were significantly greater ($P < 0.05$) in the natural wetland sites than in the restored wetland sites. Although microbial biomass carbon was not significantly different ($P > 0.05$), after a logarithmic transformation, the data was significantly different ($P < 0.05$). However, similarities within the MBC data may be from a malfunctioning vacuum pump used to remove the chloroform (a carbon containing substance). This may have resulted in an incomplete removal of additional carbon added by the chloroform. Potentially mineralizable nitrogen differences between the restored and natural sites are critical. Nitrogen mineralization is the conversion of organic nitrogen to the plant-available form of nitrogen ammonium. Higher availability of ammonium to the plant community results in increased plant biomass. Increased plant results in an increased decaying plant organic matter rich in organic carbon and nitrogen. This cyclic process occurs at a benefit to the microbial community utilizing the decomposing organic matter.

When all three restored sites over the four sampling times are averaged and compared with the average of the four sampling times of the natural site, the mean of the natural sites is significant greater ($P < 0.05$) than the mean of the restored sites (Table 1.8.1). The largest disparity in data is found within the potential denitrification measurements. The natural sites mean are 54 times greater than the mean of the restored sites (Figure 1.8.2). Lower potential denitrification rates observed within the restored wetland may be due an inability of the denitrifying community to completely carry out denitrification at similar rates of the natural

wetland. It is also possible the community of denitrifiers may be unable to access sufficient carbon and nitrogen need for denitrification within the restored wetland.

As mentioned earlier, some differences in the data such as potential denitrification may be due to the size of the soil particles. The small soil particle size may help slow the received Mississippi River water allowing for a longer interaction with the denitrifying community. The natural soils' small particle size may skew the data. However, with such large differences, the results observed still present strong conclusions of significantly greater potential denitrification occurring within the natural wetland.

When analyzing the data spatially, little to no significant variation was observed in measured soil and microbial parameters measured between the Near, Mid, and Far restored sites, except for potential denitrification. Potential denitrification increased from the near restored site to the far restored site. Low variation between sites indicates that these three sites are statistically similar and can be grouped for statistical comparison with the natural wetland site.

With a length of restoration time of five years, the trajectory of completely restoring the soil characteristics and microbial properties may take a very long time. Extrapolation of the current data would provide little reliable estimates of future restoration progress. The important function of water quality improvement through denitrification is not occurring within the restored wetlands at a rate comparable to the natural wetlands. Future research to increase the denitrification rate should be done.

It is also very important to consider the role of legacy in biogeochemical stationarity when trying to understand future water quality improvement (Basu et. al. 2010). Nutrient sources have been created within landscape elements over long-term accumulation of excessive

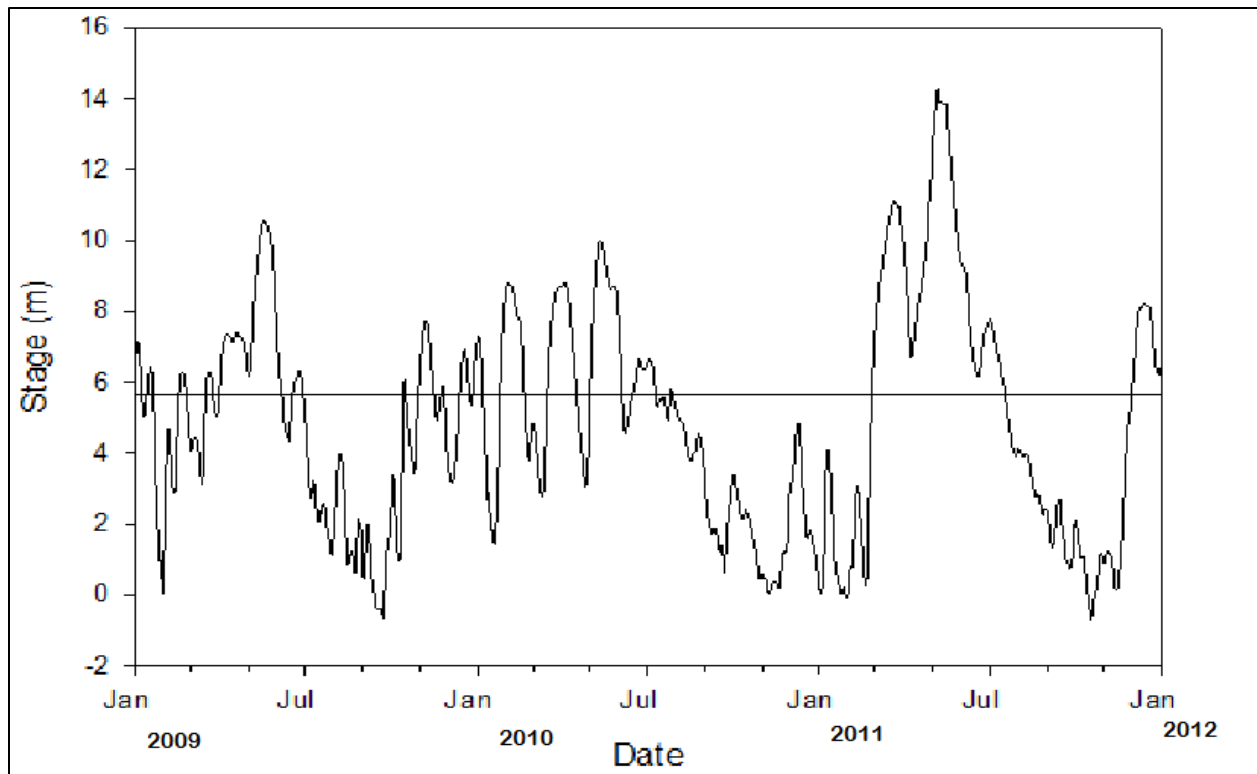


Figure 1.8.1 Mississippi River stage measurements made at Memphis, Tennessee at the Weather Bureau Gage from late January 2009 through January 2012. Gage zero is 56.06 m. Sites are inundated at approximately 5.5 m, indicated by the line.

fertilizer usage. If all additional sources of nutrient input were halted, a legacy from the landscape elements would be observed. Similar biogeochemical inputs and functions would still occur with stabilized nutrient loads for possibly decades after the original inputs have subsided. Therefore, there must be a restoration of biogeochemical integrity within the restored wetlands.

To understand the changes over the one year of sampling, data were compared between the first and last sampling periods. Very little change from the first sampling in December 2009 to last sampling in August 2010 indicates that the Loosahatchie Bar wetland restoration sites indicates a need for continued monitoring for at least several more years or decades to better understand the success of this and other wetland restoration projects. This may also be related to

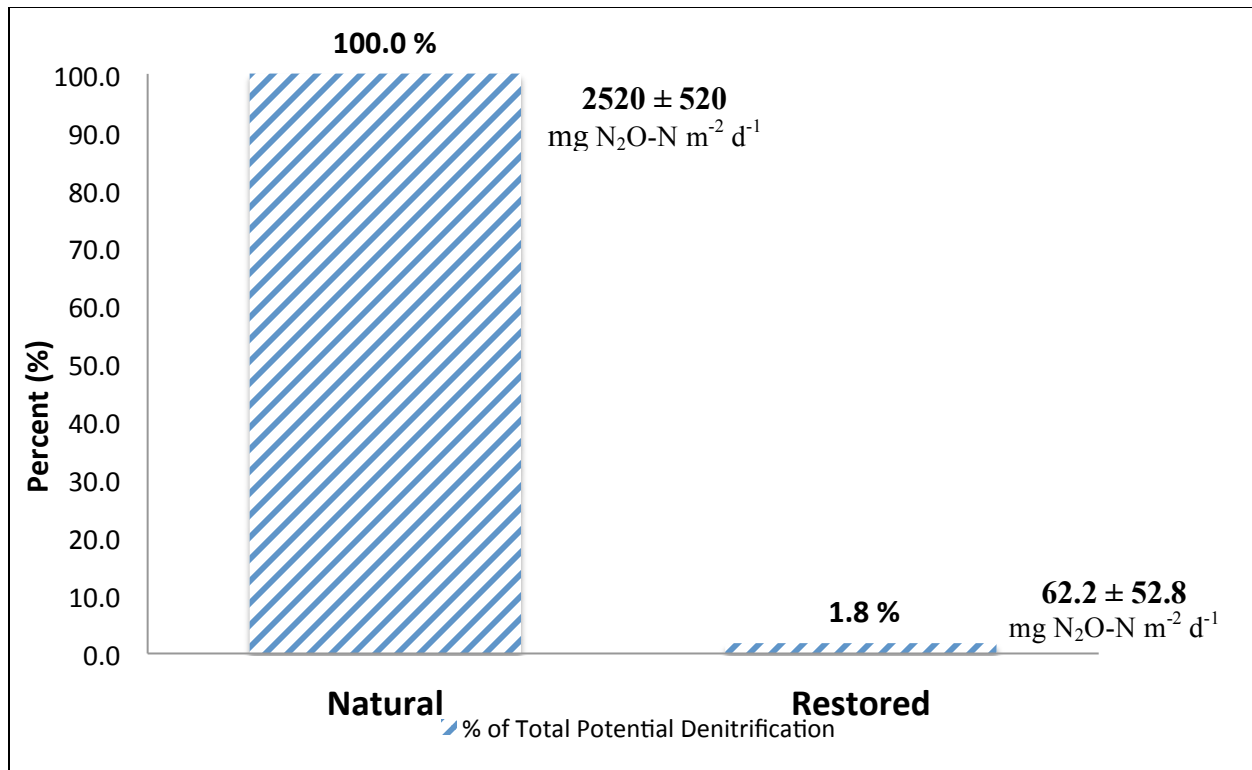


Figure 1.8.2 Percent potential denitrification and mean \pm one standard deviation within the restored wetland of the natural wetland sites.

reduced floodwater without extrapolation of current data, there is no sufficient method to predict the future of the wetland restoration project. Natural events such as a change in biological activity, climate, river flooding and other anthropogenic influences may rapidly change the current trend of slow recovery of the restored sites. It is possible that other methods may help increase the rate of recovery of the restored wetland sites. Organic matter addition to the soil may increase denitrification rates and may decrease the projected complete restoration time. Increasing the inflow of the Mississippi River water, resulting in a greater rate of organic matter accretion may also have a positive effect on the recovery of the restored wetlands. With the high cost of wetland restoration projects along with many uncertain factors for wetland restoration projects there is a dire need for more research and continual monitoring of these sites.

Several approaches have been proposed to help offset the increasing nitrogen loading into the Mississippi River and consequently, the Gulf of Mexico. The use of restored and created of riparian wetlands has been used to effectively reduce the overabundance runoff nitrate contained within the Mississippi River water. The restorations of these wetlands are very expensive and must be extensively planned; furthermore, construction of these wetlands in areas that were not previously wetlands cost about four time more than restoration. Estimates have predicted that to offset the hypoxia observed within the Gulf of Mexico, about 7%, or 200,000 km² of the Mississippi River Basin would need to be converted to bottomland hardwood forests. This estimate would require a 2500% increase of restoration projects (Mitsch et. al. 2001). Alternatively, development of methods to increase the effectiveness of denitrification within current wetlands may help offset the need for a 2500% increase in wetland restoration projects.

Factors regulating denitrification such as soil oxygen content, denitrifying enzyme availability, and temperature satisfied the requirements for denitrification to occur. However, another important factor, the supply of electron donors (carbon), may possibly be limiting within this system. Carbon amendment experiments could be done within the restored sites to see if the addition of carbon may increase denitrification. It is also possible that with the swiftly moving Mississippi River water, the residence time of the water may not be long enough for all the steps of denitrification to completely occur (Figure 1.1.19). ADCP monitors to observe the water flow would be useful to understand the rate of water movement through the restored sites.

At the time of data collection, these sites were restored for 3-4 years (Since 2007). It is possible that after large pulse flooding events and considerable time has passed, these soils may have soil and microbial properties similar to those of the natural wetland. More sampling and soil analysis ten year and possibly twenty years after the restoration completion may provide a

good temporal analysis of restoration projects. The importance of wetland restoration projects cannot be underestimated. With increasing anthropogenic nitrogen fixing and dramatic loss of wetlands, there is an impending doom for the Earth's wetlands if nothing is done to help counteract the effect of humans.

Table 1.8.1 Measured soil characteristics, microbial properties, and percent restoration estimate of natural and restored wetlands from all sampling dates. * denotes significant difference between columns ($P < 0.01$).

Soil Characteristic	Restored	Natural	Percent Restoration
Bulk Density (g cm ⁻³)	0.81 ± 0.11*	0.52 ± 0.11*	63%
Moisture Content (%)	0.29 ± 0.03*	0.41 ± 0.02*	70%
Total Nitrogen (g kg ⁻¹)	1.15 ± 0.37*	3.87 ± 0.54*	30%
Total Carbon (g kg ⁻¹)	15.3 ± 2.35*	50.6 ± 10.6*	30%
Total Phosphorus (mg kg ⁻¹)	566 ± 73.1*	1073 ± 68.5*	53%
Microbial Biomass Carbon (g kg ⁻¹)	1.63 ± 1.40	2.41 ± 1.59	68%
Microbial Biomass Nitrogen (mg kg ⁻¹)	21.1 ± 12.8*	69.7 ± 27.2*	30%
Potentially Mineralizable Nitrogen (mg kg ⁻¹)	40.6 ± 18.2*	106 ± 32.0*	38%
Potential Denitrification (mg N ₂ O-N m ⁻² day ⁻¹)	62.2 ± 52.8*	2520 ± 520*	2%

CHAPTER 2:

CARBON LIMITATIONS ON POTENTIAL DENITRIFICATION OF WASTEWATER PIVOT SOILS, TALLHASSEE, FLORIDA

2.1 Introduction

As the world population grows exponentially, so does the demand for fresh water. The aquifers tapped for freshwater are rapidly reducing due to overuse and anthropogenic and natural contamination. Wastewater treatment plants are being utilized to help alleviate the dependence on these aquifers by treating wastewater and providing water reuse for irrigation and other uses. Three main types of wastewater treatment are primary, secondary, and tertiary treatment. Primary treatment is a temporary hold of the wastewater allowing solids to settle out. Secondary treatment is the removal of suspended and dissolved biological matter. Tertiary treatment is anything more than primary or secondary treatment for sufficient cleaning for agricultural uses or groundwater discharge. Tertiary treatment is often utilized to remove nutrient concentrations above a legal discharge threshold.

The Tallahassee, Florida wastewater treatment facility is located just southeast of Tallahassee, Florida. The wastewater treatment facility utilize Wastewater spray field pivots and biogeochemical processes of soils for the denitrifying ability and primary producers for their ability to uptake excess nutrients. Allowing the wastewater to slowly percolate through the soils helps create a transient state where the soil microbial community can facilitate denitrification and uptake by crops to prevent eutrophication of the ground water. The crops are harvested removing excess nutrients from the system.

Incomplete removal of nitrate is occurring at the Tallahassee Wastewater Treatment Plant. The excess nitrate is entering the groundwater and traveling 17.5 miles southwest to Wakulla Springs. Eutrophication of Wakulla springs has been directly related to the wastewater

treatment plant through stable isotope analysis. Improving denitrification at the wastewater treatment plant may help reduce the eutrophication of the nearby springs.

2.1.1 Denitrification Introduction

Since preindustrial times, humans have dramatically altered the global nitrogen cycle. The rate of nitrogen fixation, through fertilizer production, has doubled and the rate of nitrogen deposition has increased nearly tenfold (Smil 1990; Smil 1991; Vitouesk and Matson 1993; Ayers et. al 1994; Galloway et. al. 1995; Vitousek et. al. 1997). In most ecosystems, nitrogen is often cited as the limiting nutrient (Ryther and Dunstan 1971; Boynton et al. 1982; Gerhart and Likens 1975; Vitousek and Howarth 1991). Changing the global balance of limiting nutrients can have a drastic effect on ecosystem response and may also affect cycling other nutrients and elements in the ecosystem. As the amount of cycled nitrogen increases so does the nitrogen cycle's expression of eutrophication and production of nitrous oxide, a potent greenhouse gas.

A variety of organic and inorganic nitrogen forms are found within wetland soils (Reddy and Delaune 2008). The inorganic forms are the most important nutrients due to their immediate availability to vegetation and wetland soil microbes (McClelland and Valiela 1998). The chemical reduction of inorganic form of nitrogen, nitrate, to a chemically different form of nitrogen, nitrogen gas, is denitrification (Payne 1973). Denitrification is an important wetland soil biogeochemical process for the removal of nitrate, returning it back to the atmospheric pool (Groffman and Hanson 1997).

Denitrification is a process linked to microbial respiration (Reddy and Delaune 2008). More specifically, the addition of electrons to nitrate or nitrite (as the terminal electron acceptor), leads to the reduction of nitrogen to nitrous oxide or nitrogen gas, a change of the oxidation state of nitrogen from +5 to 0 (Seitzinger 1988; Reddy and DeLaune 2008; Figure 2.1.1). There are

several intermediate steps producing intermediate compounds such as nitric oxide, nitrogen oxide, and nitrous oxide involved in the denitrification pathway (Ye et. al. 1994; Hollocher and Hibbs 1996).

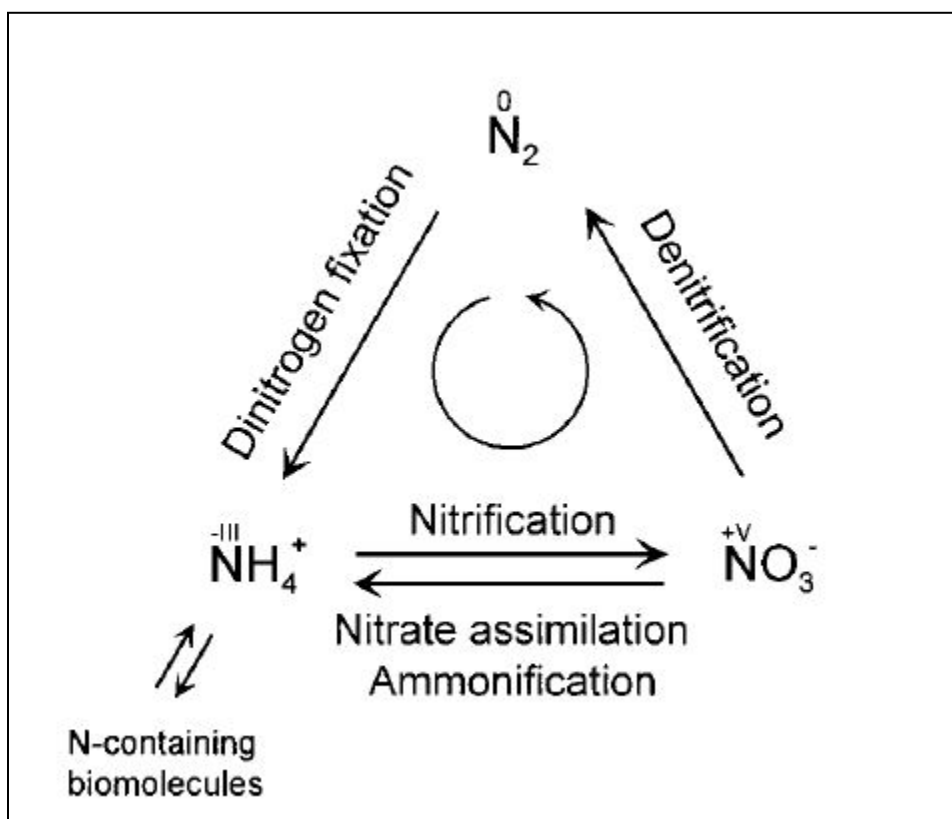


Figure 2.1.1 General nitrogen cycle diagram. Roman numerals denote formal oxidation state (Zumft 1997).

2.1.2 Biology of Denitrification

Denitrifying organisms are able to use nitrogen oxides in the place of oxygen as electron acceptors and are both heterotrophic and autotrophic (Knowles 1982). The *Pseudomonas* genera is the most common worldwide and possibly the most active denitrifying bacteria isolated in soils (Heitzer and Ottow 1976; Gamble et. al. 1977). Denitrifying species of the *Pseudomonas* genera include *P. aerogenes*, *P. auerofacines*, *P. caryophylli*, *P. choloroaphis*, *P. denitrificans*, *P. fluorescens*, *P. lemoignei*, *P. mallei*, *P. mendocina*, *P. perfectomarinus*, *P. picketti*, *P.*

pseudoalcaligenes, *P. pseudomallei*, *P. solonacearum*, and *P. stutzeri* (Allen and Neil 1952; Buchanan and Gibbons 1974; Gambe et. al. 1977; Greenberg and Becker 1977; Pichinoty et. al. 1977). The second most common worldwide denitrifying genera is *Alcaligenes* includes species *A. faecalis*, *A. eutrophus*, *A. denitrificans*, *A. odorans* (Pfitzner and Schlegel 1973; Gamble et. al. 1977). Less common genera include *Achromobacter*, *Agrobacterium*, rice field abundant *Bacillus*, *Chromobacterium*, *Corynebacterium*, *Flavobacterium*, *Halobacterium*, *Moraxella*, *Paracoccus*(*Micrococcus*), *Thiobacillus*, and *Xanthomonas* (Meschner and Wuhrmann 1963; Hart et. al. 1965; Iwasaki and Matsubara 1972; Davies 1973; Williams and Evans 1975; Pichinoty et. al. 1976; Gamble et. al. 1977; Garcia 1977; Grant and Payne 1981).

The genes or loci responsible for regulation of denitrification are found as nitrate reductase systems (NRS) in many different forms and in genetically dissimilar organisms (Tiedje et. al. 1981; Zumft 1997). Although mostly prokaryotic, these genes or loci for denitrification are found in genera of multiple kingdoms and exist in almost all soils. The nitrate reductase systems found within the organisms will only code for the organism's ability to either nitrify or denitrify, but not both (Zumft 1997).

Bacterial enzymes such as nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase facilitate the movement of electrons as organic carbon is oxidized (Hochsetin and Tomlinson 1988, Figure 2.1.2). The enzyme breakdown is mediated by facultative bacteria and is triggered by low oxygen availability the presence of a nitrogen oxide, temperature, and available organic matter (Burford and Bremner 1975; Reddy et. al. 1982; White and Reddy 1999; Cooper 1990; Wang et. al. 2007).

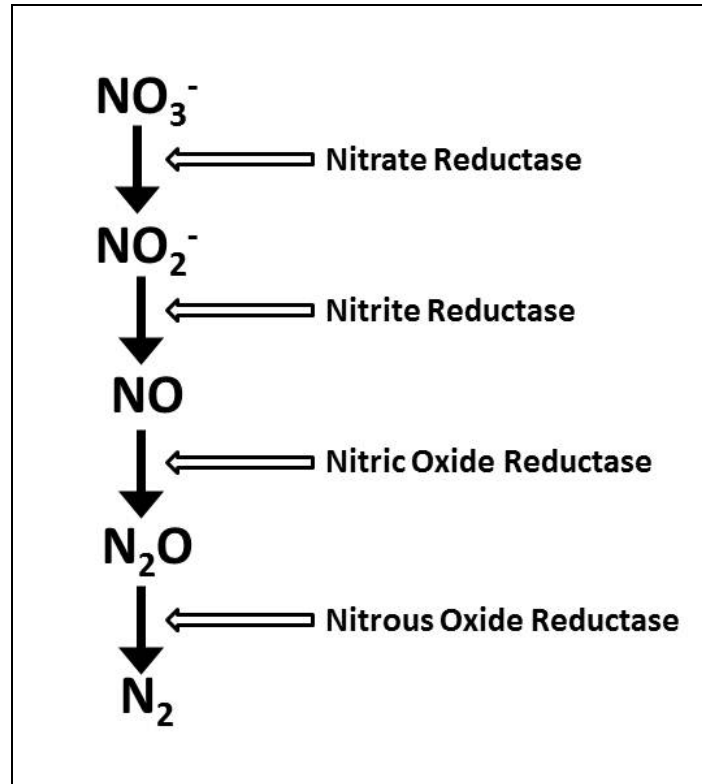


Figure 2.1.2 Denitrification pathways and enzymes involved
(Adapted from Hochstein and Tomlinson, 1988).

2.1.3 Greenhouse gases

Denitrification plays a critical role in the removal of excess fertilizer nitrogen and the removal of nitrogen-rich animal waste (Knowles 1982). However, incomplete denitrification reactions have been shown to have negative effects. In wetland soils, Nitrous oxide (N_2O) is a greenhouse gas and a biologically produced intermediate of the nitrogen cycle. Nitrous oxide has a 120 year atmospheric lifetime and is approximately 320 times as strong a greenhouse gas as carbon dioxide on a mole basis (IPCC 1997). Approximately 94% of nitrous oxide is converted to nitrogen gas under normal anaerobic, denitrifying conditions (Blackmer and Bremner 1978). Under slightly aerobic conditions, oxygen will inhibit nitrous oxide reductase from converting nitrous oxide to nitrogen gas. Nitrous oxide reductase inhibition will stop denitrification at

nitrous oxide, releasing nitrous oxide into the atmosphere and decreasing the percentage of nitrous oxide converted to nitrogen gas (Knowles 1982). In ephemerally flooded systems, such as agricultural land, the recurring alternation between flooded anaerobic conditions and dry aerobic conditions allows for nitrous oxide gases to escape into the atmosphere. In systems that experience longer periods of flooding, nitrous oxide gas and other gases move approximately 100 times slower within water compared to air. Slow movement throughout the water column allows sufficient time for reduction of nitrous oxide to inert nitrogen gas. Under aerobic conditions, the denitrifiers' aerobic metabolism is metabolically advantageous. The structural conformation of the enzyme receptors used for denitrification with change. The receptor's conformational change blocks the substrates needed for denitrification, and stops the reduction of nitrate. As with the amount of nitrogen cycled on the earth, the amount of nitrous oxide released into the atmosphere has also dramatically increased (Galloway et. al. 1995). Nitrous oxide is increasing at a rate of $4.5 \pm 0.6 \text{ Tg N yr}^{-1}$, an increase of approximately 0.25% per year (Khalil and Rasmussen 1992). Reduction of global nitrogen levels may help mediate the production of this harmful greenhouse gas.

2.1.4 Changes in Denitrification over Time

Anthropogenic alteration of the global nitrogen cycles through the combustion of fossil fuels, usage and production of nitrogen fertilizers, agricultural cultivation of nitrogen-fixing legumes, and other human causes have resulted in a dramatic increase in the amount of nitrogen cycled (Gallow et. al. 1995). As mentioned earlier, the rate of nitrogen fixation has doubled since pre-industrial times and the rate of nitrogen deposition has increased nearly tenfold (Smil 1990; Smil 1991; Vitousek and Matson 1993; Ayers et. al 1994; Galloway et. al. 1995; Vitousek et. al. 1997; Figure 2.1.3). To put that into numbers, anthropogenic sources cause the fixation

of approximately 140 Tg of new nitrogen per year in terrestrial ecosystems and mobilizes approximately 70 Tg more nitrogen per year (Galloway et. al. 1995). The increasing nitrogen also directly affects aquatic systems and water chemistry. Although there are no long term historical records for nitrate concentrations, the amount of nitrate has doubled in the Mississippi River since 1965 (Turner and Rabalais 1995, Justic et. al. 1995). The most well understood consequence of anthropogenic increase of nitrate is eutrophication (Howarth 1988; Justic et. al. 1995; Nixon 1995; Nixon et. al. 1996). Eutrophication, an overabundance of limiting nutrients, can lead to hypoxia (low oxygen concentration) and anoxia (zero oxygen concentration). The Mississippi River and Atchafalaya River are the primary inflows to the Northern Gulf of Mexico, responsible for 91% of the nitrate load (Goolsby 1999). This loading results in annual hypoxic and anoxic events over areas as large as 20,700 km² in the northern Gulf of Mexico (Rabalais 2002). Abnormally low oxygen conditions result in reduced fisheries, benthic fauna, and bottom-dwelling species (Rabalais 2001). Decreased available food resources, altered trophic level interactions, and disrupted migration patterns were also observed along with physiological, reproductive, developmental, and growth abnormalities of affected species. Economic losses from a single hypoxic event can exceed \$2 billion (Rabalais et. al. 2010). Another pernicious effect of eutrophication is the explosive growth of nuisance algae and harmful algal blooms (Anderson and Garrison 1997). Under eutrophic conditions, concentrations of algal species can exceed a threshold and begin to cause problems such as shading, toxicity, and hypoxia. Once algal blooms begin causing unfavorable conditions for other biology, they are termed harmful algal blooms. With high nutrient influxes, shifts of the dominant algal species from harmless to toxic species have been observed (Bargu et. al. 2011). The incidence of these harmful algal

blooms has increased within the past decade and is directly linked eutrophication (Hallegraef 1993; Shumway1990,).

Other effects observed from increasing available nitrogen are reduction of biological diversity in affected ecosystems, acidification of soils, depletion of soil minerals, alteration of freshwater and marine ecosystems, increased acid rain, and promotion of the greenhouse effect (Timan 1987; Berendse et. al. 1993; Aber et. al. 1995; Likens et. al. 1996; Nixon et. al. 1996; Chameides et. al. 1994; Albritton et. al. 1995).

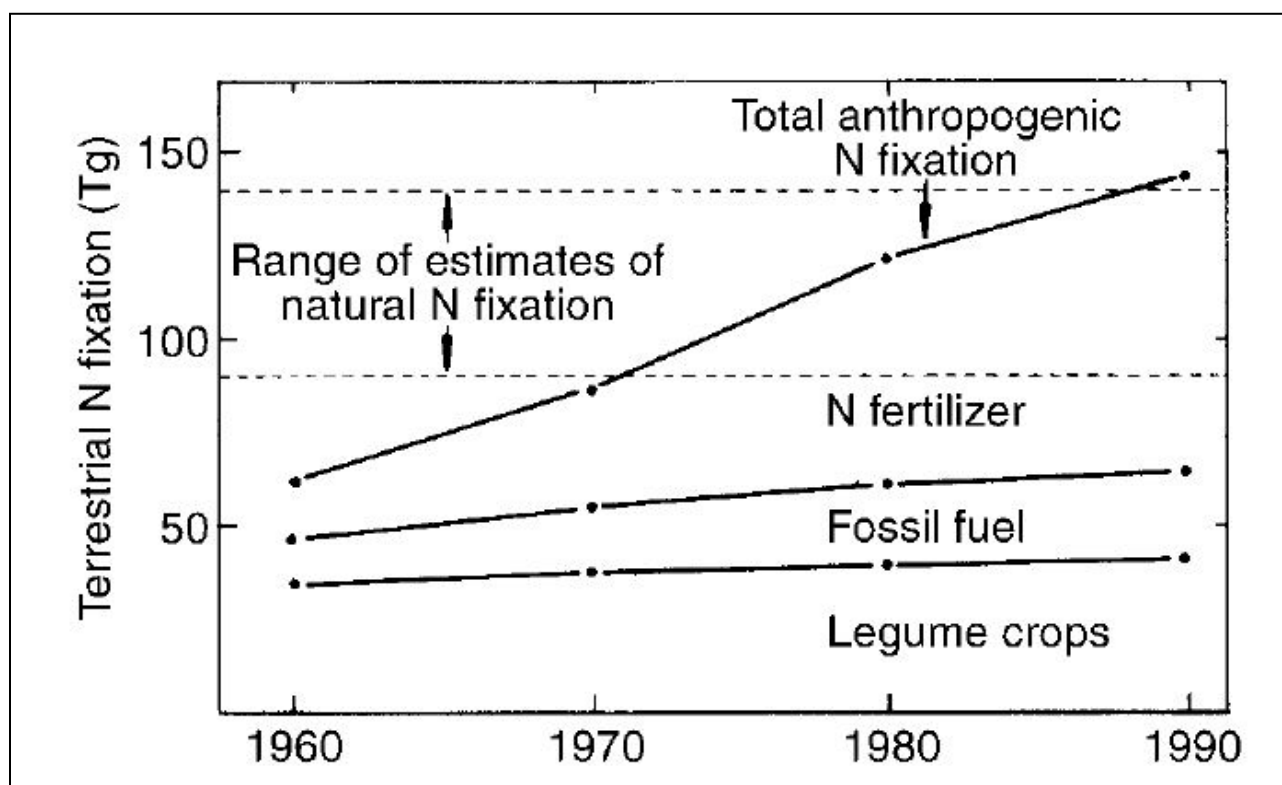


Figure 2.1.3 Anthropogenic nitrogen fixation in terrestrial ecosystems over time compared with natural biological nitrogen fixation on land. (Modified from Galloway et. al. 1995).

2.1.5 Effects of organic matter on Denitrification

Denitrifying microbes must have an available energy source to carry out denitrification. Generally, this source of energy is organic carbon. Organic carbon in the form of simple organic compounds is directly available to the denitrifying community and has an influence on denitrifying capabilities of soils (Starr and Gillham 1993; Cornwell et. al. 1999; Greenan et. al. 2006; Hill et. al. 2000; Figure 2.1.4). There is also evidence that a higher soil organic matter content will also lead to an increase in denitrification potential (Bijay-Singh et. al. 1988; Gale et. al. 1993). Carbon amendment experiments have been conducted on carbon-limited soils using glucose as the carbon source. The denitrifying bacteria responded with a drastic increase in denitrification rates (Garcia-Montiel 2003). In a separate experiment, glucose was added to a high organic matter forest soil and substantially lower organic matter grassland soil. The high organic matter forest soil microbial community responded with an increased denitrification rate compared to the lower organic matter grassland soil. These experiments suggest that although carbon may be present in the form of organic matter, not all of the organic matter available to the microbial community can be utilized (Hill and Cardaci 2004). These studies also suggest that different qualities of organic carbon may be important even in soils that are not carbon limited.

2.1.6 Measuring Denitrification

Denitrification has been assessed through many different methods. Kaplan et. al. (1997) estimated denitrification by measuring nitrogen production by the use of *in situ* domes in a salt marsh. Mass balance calculations have been used to approximate the denitrification occurring (Dierberg and Brezonik 1983; Brinson et. al. 1984; Bowden 1986). Other studies have measured nitrous oxide in nitrate added homogenous soil slurries to approximate potential denitrification

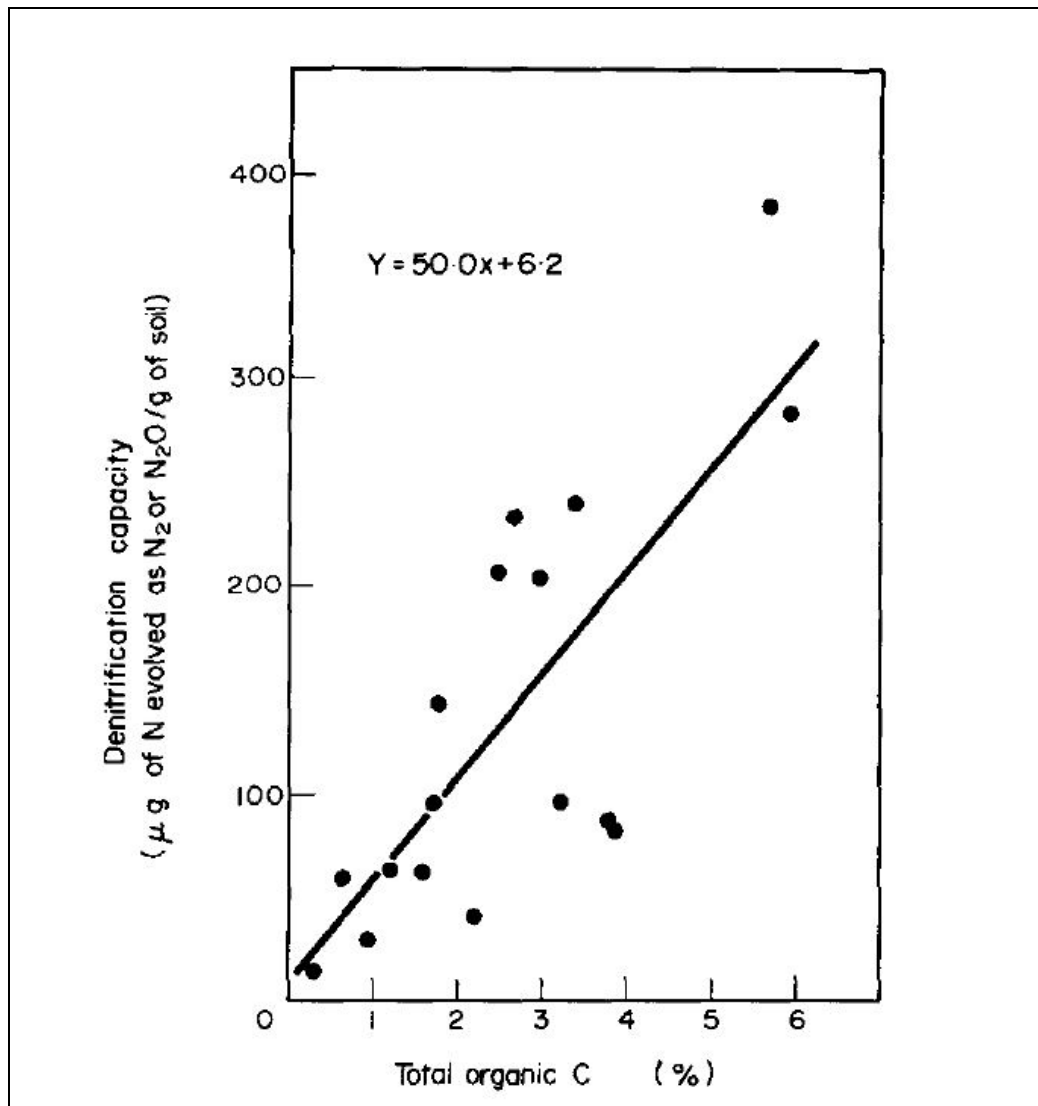


Figure 2.1.4 Relationship between denitrification capacity and total organic carbon (Burford and Bremner 1975).

(Muller et. al. 1980; Gordon et. al. 1986; Westermann and Ahring 1987; Koerselman et. al. 1989). Other studies similarly measured nitrous oxide in nitrate added to whole sediment cores to approximate potential denitrification (Dierberg and Brozonik 1983). These nitrate addition experiments usually utilize acetylene to inhibit nitrification (Dierberg and Brozonik 1983; Urban et. al. 1988; Zak and Grigal 1991; Merrill and Zak 1992; Hynes and Knowles 1978).

Nitrification of mineralized ammonia provides a source of nitrate utilized in denitrification

reactions (Patrick and Reddy 1976; DeBusk and Reddy 1987; Reddy et. al. 1989).

Denitrification approximations have also been made from homogenized sediment slurries with no nitrate added (Hemond 1983; Westermann and Ahring 1987; Koerselman et. al. 1989).

2.2 Methods

2.2.1 Site Summary

The city of Tallahassee, Florida draws approximately 25 million gallons of water every day from the Karstic Upper Floridian aquifer, through the operation of 29 separate wells. The generated wastewater is then sent to the city's wastewater treatment plant for primary, secondary, and tertiary treatment. Once the water has been treated at the treatment plant, it is pumped 8 miles to the Southeast farm. The Southeast farm facility receives approximately 64.5 million liters per day. The received, treated wastewater is applied to 16 sprinkler systems rotating around a central pivot point throughout 774 ha of farmland. This agricultural land at the Southeast farm facility is used to grow fodder crops such as canola, corn, soybeans, hay, and sorghum. The treated wastewater received at the Southeast facility is also used to irrigate several commercial developments, road medians, and the golf courses and athletic facilities of Florida State University.

2.2.2 Experimental Design

Five Tallahassee, Florida wastewater pivot sites of varying locations throughout the wastewater treatment plant were analyzed three times over two years (January 2009, May 2010, and February 2011) (Figure 2.2.1). Two sites that are up gradient of the water flow and one site that is down gradient were chosen as control sites and sampled similarly to the pivot sites (Figure 2.2.2). None of the control sites are spray field pivots; however, after soil characterization, it

was determined that control site 3 incurred similar wastewater to that of the pivot sites and was determined to be too similar to be used as an adequate control site. Triplicate soil samples were collected at each of the eight pivot sites during the three sampling periods. The soil samples were homogenized, and analyzed for soil characteristics including moisture content, total carbon, total nitrogen, and total phosphorus. Microbial biomass measurements were taken for the first two sampling periods (Figure 2.2.4). Of these eight treatment sites one site (Pivot 6) was chosen for a carbon amendment experiment.

One hundred grams of homogenized Pivot 6 soil was weighed and placed in a 1 liter container and was repeated for three sets treatments. Based on the total carbon of the soil, carbon amendments were added according to a percentage of the total carbon. The first of the three treatments was homogenized pivot soil without any amendment. The second of the three treatments was divided into three subsets of pivot soil samples and each subset was amended with 0.25%, 1.0%, and 2.0% total mass ground corn plant amendment, essentially the control site. The third of the three treatments was divided into three subsets of pivot soil and each subset was amended with 0.25%, 1.0% and 2.0% total mass of dried ground biosolids (residual primary wastewater treatment byproduct) amendment. Each subset was further divided into three time steps: 1 week, 1 month, and 3 months, to be sampled at their appropriate times. Additionally, each of the three time steps were divided into three replicates to reduce statistical variability (Figure 2.2.3).

2.2.3 Soil Characterization

Collected soils were homogenized and prepared in the laboratory for soil analyses with triplicates included for every ten samples for all measured parameters. Moisture content,



Figure 2.2.1 Map of location of Tallahassee, Florida wastewater treatment facility.

organic matter, total carbon (TC), total nitrogen (TN), total phosphorus (TP), potentially mineralizable nitrogen (PMN), microbial biomass carbon (MBC), denitrifying enzyme activity (DEA), and potential denitrification of these soils were determined (Figure 2.2.4).

Moisture content of the soils was determined by weighing before and after drying of the subsamples 70°C for 72 for hours, or until completely dry. From the moisture content, dry weight of each sample could be calculated. Bulk density (Blake and Harge 1986) was measured from the complete soil core. Bulk density was calculated by dividing the dry soil weight by the volume of the soil core used and is expressed in units of g cm^{-3} . Dried, ground subsamples were analyzed for total carbon and total nitrogen using an Elemental Combustion System with a

detection limit of 0.005 g kg^{-1} (Costech Analytical Technologies, Inc., Valencia, CA). Total phosphorus was determined by using the ashing method. 0.5 g of soil were weighed and placed into a 50mL beaker. The beaker and sample was then placed in a muffle furnace at 250°C for 30 minutes, followed by 550°C for 4 hours, then allowed to cool to room temperature. The samples and beakers were weighed once again to determine the loss on ignition of each sample. After weighing, the remaining sample ash was moistened with distilled deionized water to avoid loss of sample. Then, 20mL of 6.0 M HCl was slowly added to each beaker. The samples were transferred to a hot plate located under a fume hood. The samples were then heated on the hotplate on a medium-low setting ($100\text{-}120^{\circ}\text{C}$) until dry.

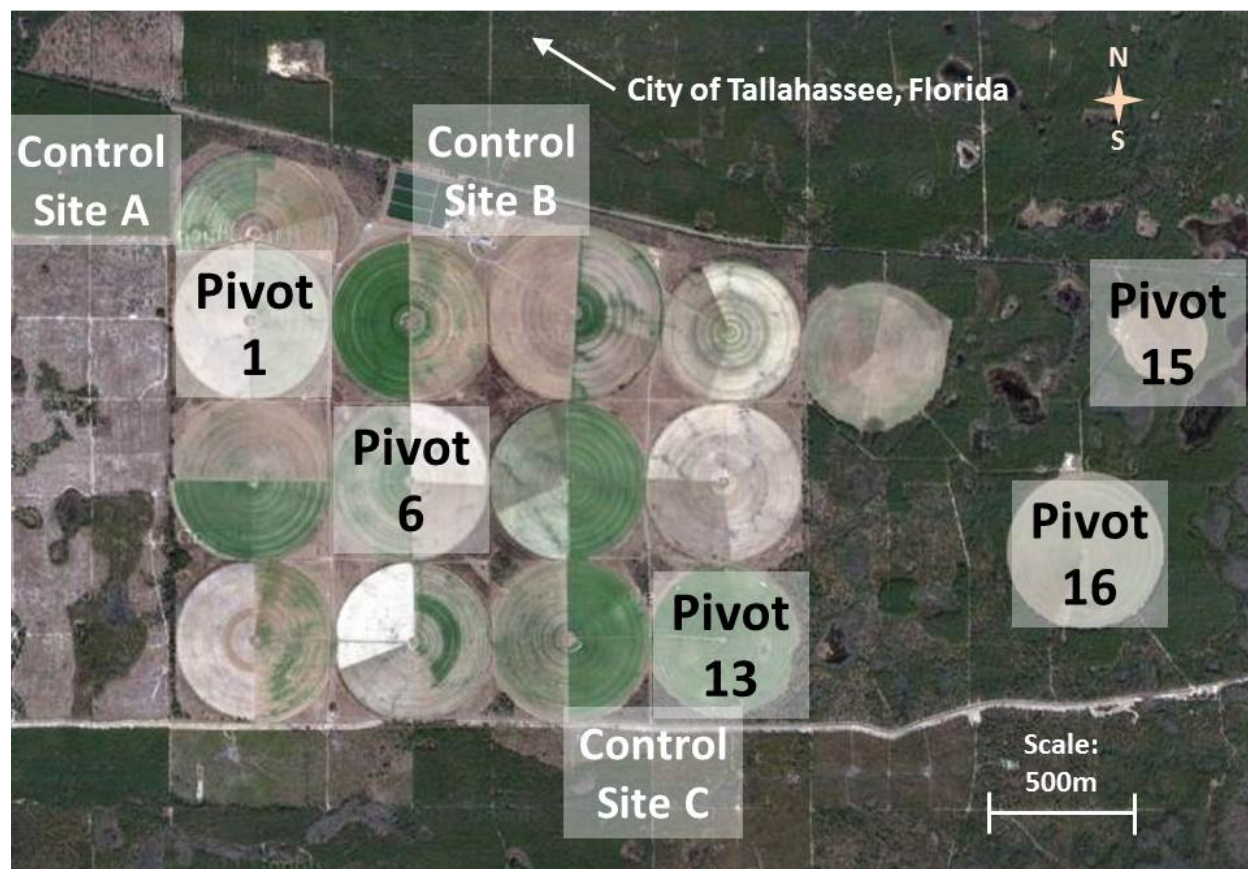


Figure 2.2.2 Satellite image showing Florida spray field sampling sites (Google 2012).

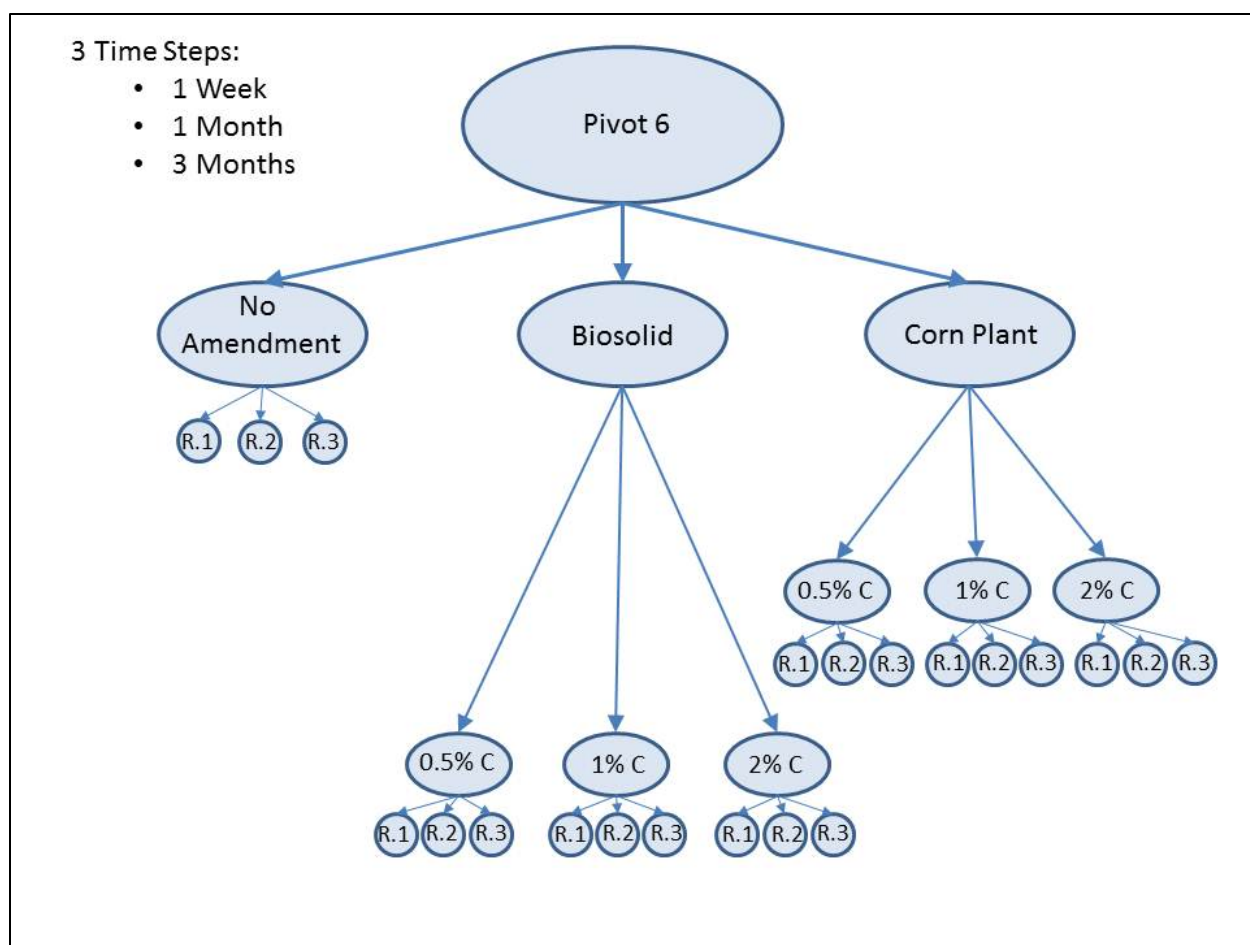


Figure 2.2.3 Flow chart of Tallahassee, Florida spray field carbon amendment experimental design.

Once the beakers were dry, the hot plate temperature was raised to high for 30 minutes. After 30 minutes, the samples were removed from the hot plate and allowed to cool. Once cool, the samples were remoistened with 2-3mL of distilled deionized water then 2.25mL of 6.0 M HCl was added. The samples were returned to the hotplate on the high setting and brought to a boil then immediately removed. After cooling once more, the samples were filtered through Whatman #41 filter paper into a 50mL volumetric flask. The beakers and filter paper were rinsed with distilled deionized water three times each. Next, the volumetric flasks were filled with distilled deionized water to 50mL total volume, covered with parafilm and inverted to mix

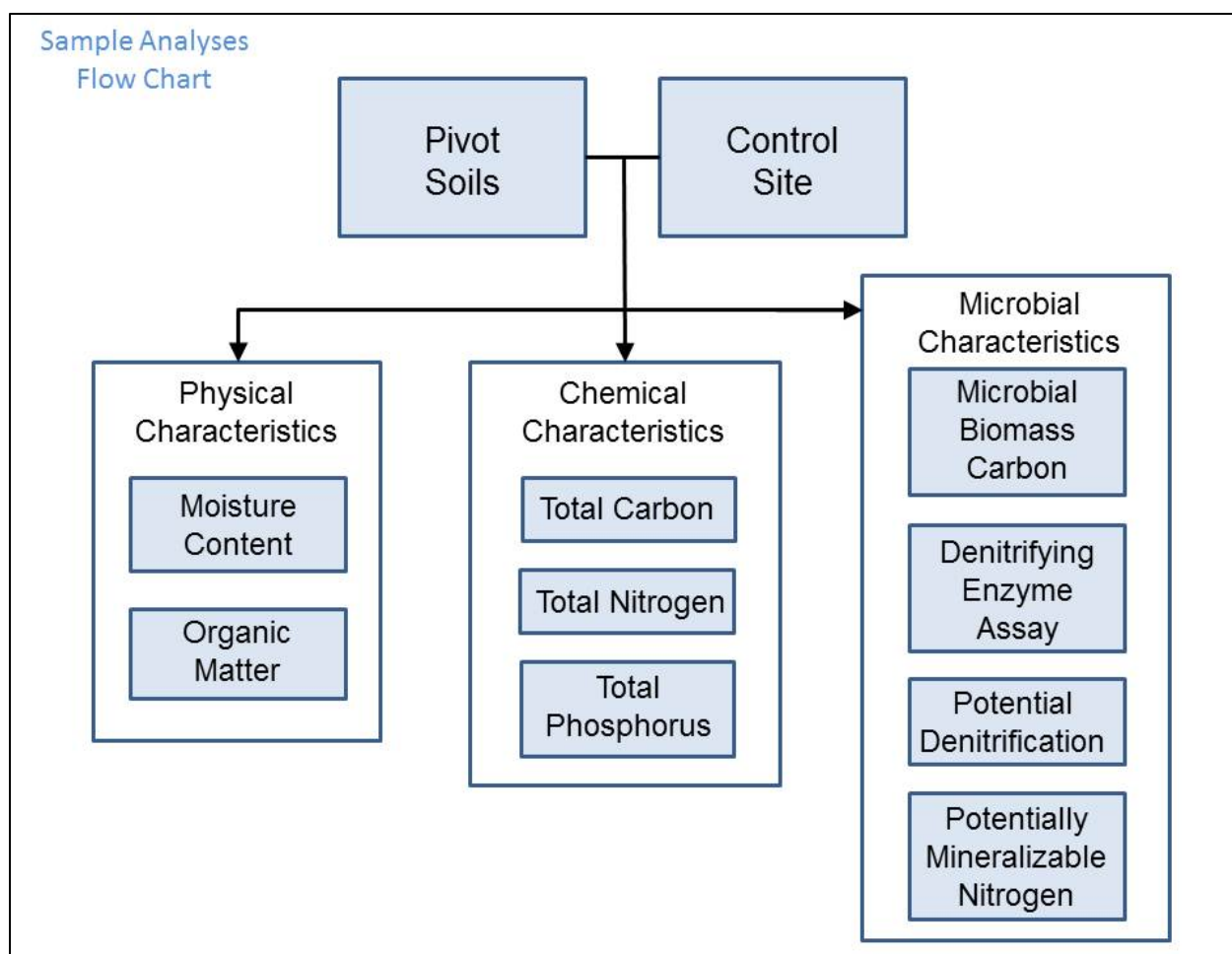


Figure 2.2.4 Sample analyses flow chart for Tallahassee, Florida wastewater treatment plant soils.

ten times. Twenty milliliters of each digestate was transferred to a 20ml plastic scintillation vial and stored at room temperature until analysis on an AQ2 Automated Discrete Analyzer (SEAL Analytical Inc., Mequon, Wisconsin).

2.2.4 Microbial Properties Analysis

Microbial biomass carbon and nitrogen were determined using the fumigate-extraction technique. Subsamples were measured to 5.0g, placed in centrifuge tubes, and separated into fumigate and Non-fumigate sets. 0.5mL of pure chloroform were added to each centrifuge tube

of the fumigate set. The fumigate set was then placed in a desiccator along with a wet paper towel and a 50mL beaker of pure chloroform and 5-10 boiling stones. The desiccator was vacuumed to -40kPa allowing the beaker of pure chloroform to boil (maybe bubble vigorously), and then released to be re-filled with room air three consecutive times. The desiccator was then vacuumed to -40kPa once more, sealed off, and placed in fume hood for 24 hours. After 24 hours, the 50mL beaker of pure chloroform and boiling stones was removed. The desiccator was resealed and vacuumed to -40kPa and then released to be refilled with room air seven consecutive times. The fumigate and non-fumigate sets were then extracted in a similar manner. Twenty-five mL of 0.5 M K_2SO_4 was added to every sample, shaken for 30 minutes on a longitudinal shaker, then centrifuged at 6000 rpm for 10 minutes at 10°C. Both sets of samples were vacuum filtered using 0.45 μm membrane filters. Samples were stored at 4°C until analysis for total organic carbon and nitrogen (Shimadzu Scientific Instrument-VCSN, Columbia, MD). Measurements for the non-fumigate dissolved organic carbon were subtracted from the fumigate dissolved organic carbon measurements to calculate the microbial biomass carbon. The values were then divided by 1000. Measurements for microbial biomass nitrogen were calculated in a similar manner.

Potentially mineralizable nitrogen assay is utilized to quantify the net nitrogen mineralization rates in soils by anaerobic bottle incubation by measuring the release of ammonium (NH_4^+). To determine potentially mineralizable nitrogen, two sets labeled as time zero and incubate samples were weighed to 0.5 grams dry weight equivalent of soil and transferred to 50mL centrifuge tubes and 50mL glass serum bottles, respectively. For the time zero samples, 25mL of 2.0 M KCl was added to the centrifuge tubes. The centrifuge tubes were capped then shaken in a reciprocating shaker for 1 hour, then centrifuged for 10 minutes at

6000rpm and 10°C. The extract was then filtered through Whatman #41 filter paper into 20mL plastic scintillation vials and stored at 4°C until NH₄-N analysis by AQ2 Automated Discrete Analyzer (SEAL Analytical Inc., Mequon, Wisconsin) (EPA Method 351.2, 1983). For the incubate samples, 5mL of distilled deionized water was added to bottles. The bottles were also capped with a butyl rubber stopper and crimped with an aluminum crimp top. All the samples were vacuumed to -40kPa, and then purged with 99.99% pure N₂ gas for 5 minutes. The anaerobic serum bottles were then incubated without light at 40°C for 10 days. After 10 days, the serum bottles were removed and cooled for 30 minutes to room temperature. The serum bottles were injected with 20mL of 2.0 M KCL using an outlet needle, and then shaken on a reciprocating shaker for 1 hour. After 1 hour, the bottles were opened and its contents were transferred to 50mL centrifuge tubes and centrifuged for 10 minutes at 6000 rpm at 10°C. The extract was then filtered through Whatman #41 filter paper into 20mL plastic scintillation vials and stored at 4°C until NH₄-N analysis by AQ2 Automated Discrete Analyzer (SEAL Analytical Inc., Mequon, Wisconsin) (EPA Method 351.2, 1983).

Potential denitrification was determined using the acetylene inhibition method. Five grams of each subsample were weighed and placed in a 160mL serum bottle. Each bottle was sealed with a rubber septa and aluminum crimp cap. The bottles were then vacuumed to -40kPa. Then the bottles were purged with 99.99% pure N₂ gas for 10 minutes to remove all oxygen from the headspace. Calcium carbide rocks were combined with water in a separate vacuumed bottle to create pure acetylene gas. Sixteen mL of acetylene gas was injected into each serum bottle containing sample to represent 10% headspace of the bottle. The bottles were shaken on a longitudinal shaker for 10 minutes. While shaking the bottle, 500mL of distilled deionized water was purged with 99.99% pure N₂ gas. A KNO₃ solution was prepared using previous data to

approximate 10 times the maximum nitrogen usage observed. The maximum rate observed in a similar experiment (Dolda et. al. 2008) approximately $2.0 \text{ mg-N kg}^{-1} \text{ day}^{-1}$ was multiplied by 5 as a cushion factor, resulting in 10 mg-N per 5 mL injection of a prepared 14.4 g/L KNO_3 solution. The samples were then injected with 10mL of the N_2 enriched distilled deionized water, followed by 5mL of the prepared KNO_3 solution. The pressures of the bottles were increased to 50kPa with 99.99% pure N_2 gas. Headspace gas samples were taking at 2, 12, 24, 36, 48, 50, 62, and 70 hours to determine the 3 day short term denitrification rates and were shaken after each headspace sampling. Gas samples were extracted using 1mL BD disposable insulin syringes and analyzed on a Shimadzu GC-8A equipped with an electron capture detector (Shimadzu Scientific Instruments, Columbia, MD, detection limit $0.006 \text{ mg N}_2\text{O-N kg}^{-1} \text{ hr}^{-1}$).

Denitrifying enzyme activity (DEA) was determined by the method outlined by Tiedje in 1982, with adaptations by White and Reddy (1999). Five grams of each subsample were weighed and place in a 190 mL glass serum bottle. Each bottle was sealed with a rubber septa and aluminum crimp cap. The bottles were then vacuumed to -75 kPa, purged with 99.99% pure N_2 gas for one minute. The samples were then injected with 8 mL of N_2 enriched distilled deionized water. Approximately 15% of the serum bottle headspace was replaced with acetylene gas (C_2H_2 ; Yoshinari and Knowles 1976). All bottles were shaken on a longitudinal shaker for 30 minutes to distribute added acetylene gas. Eight mL of a prepared solution of $56 \text{ mg KNO}_3\text{-N l}^{-1}$, $288 \text{ mg dextrose-C l}^{-1}$, and $2 \text{ mg chloramphenicol l}^{-1}$ was added. Chloramphenicol was used as an enzyme inhibitor to prevent *de novo* enzymes from synthesizing during incubation (Smith and Tiedje 1979). Headspace gas samples were taken at 30, 60, 90, and 120 minutes. Samples were continually agitated throughout headspace sampling. Gas samples were analyzed for N_2O on a Shimadzu GC-8A ECD (Shimadzu Scientific Instruments, Colombia, MD, detection limit

0.006 mg N₂O-N kg⁻¹ hr⁻¹). Nitrous oxide production was calculated with consideration for portions of the product in an aqueous phase using the Bunsen absorption coefficient (0.544) (Tiedje 1982). The DEA of the subsamples was calculated as the slope of the line when mg N₂O-N kg soil⁻¹ was compared versus time.

2.2.5 Data Analysis

A total of 60 soil samples were analyzed for correlation between variables. The relationship between sampling location, sampling event, and measured soil and microbial parameters were analyzed using SAS 9.3 statistical Software (SAS Institute Inc., Cary, NC). Data was tested for normality using the K-S Test at $\alpha = 0.05$ and logarithmically transformed when necessary. Once data normality was determined, F-tests were conducted to determine the homogeneity of variance. Sample comparisons with $P \geq 0.05$ were assumed to have equal variance. Sample comparisons with $P < 0.05$ were assumed to have unequal variance, while a two sided unpaired T-test ($P < 0.01$) was applied to determine differences between separate sampling locations, while a two sided paired t-test was used to identify relationships between sampling events. Variation among restored sites was determined using a one way ANOVA ($P < 0.05$). Linear regressions were performed to determine the relationship between total carbon and total nitrogen along with total carbon and total phosphorus.

Differences between pivot soils and the effect of the two carbon amendments on potential denitrification were determined using a one-way ANOVA model ($P < 0.05$) and the Tukey's Studentized (HSD) post-hoc test. Homogeneity and normality were determined using an F-test and K-S test, respectively.

2.3 Results

2.3.1 Moisture Content

Moisture content of the pivot soils ranged from 6.06 to 16.2 percent with a mean value of 9.16 ± 1.77 percent, while the control sites ranged from 3.88 to 15.5 percent with a mean value of 7.57 ± 3.11 percent (Figure 2.3.1; Table 2.3.1). The mean moisture content of the pivot soils was significantly greater ($P < 0.01$) than the mean moisture content of the control sites. There was no significant difference ($P > 0.05$) between the three control sites; however, there was a significant ($P < 0.001$) difference between the pivots.

2.3.2 Organic Matter

Percent organic matter of the pivot soils ranged from 0.00 to 5.10 percent with a mean value of 1.73 ± 1.01 percent, while the control sites ranged from 0.00 to 2.98 percent with a mean value of 1.17 ± 1.23 percent (Figure 2.3.2; Table 2.3.1). The mean percent organic matter of the pivot sites were significantly greater ($P < 0.001$) than the mean percent organic matter of the control sites.

2.3.3 Total Carbon

Total carbon of the pivot soils ranged from 2.90 to 22.3 g kg⁻¹ with a mean value of 8.98 ± 4.32 g kg⁻¹, while the control sites ranged from 2.00 to 28.4 g kg⁻¹ with a mean value of 6.37 ± 4.41 g kg⁻¹ (Figure 2.3.3; Table 2.3.1). The mean total carbon of the pivot sites were significantly greater ($P < 0.001$) than the mean values of the control sites.

2.3.4 Total Nitrogen

Total nitrogen of the pivot soils ranged from below detection to 1.25 g kg⁻¹ with a mean value of 0.551 ± 0.258 g kg⁻¹, while the control sites ranged from below detection to 1.67 g kg⁻¹

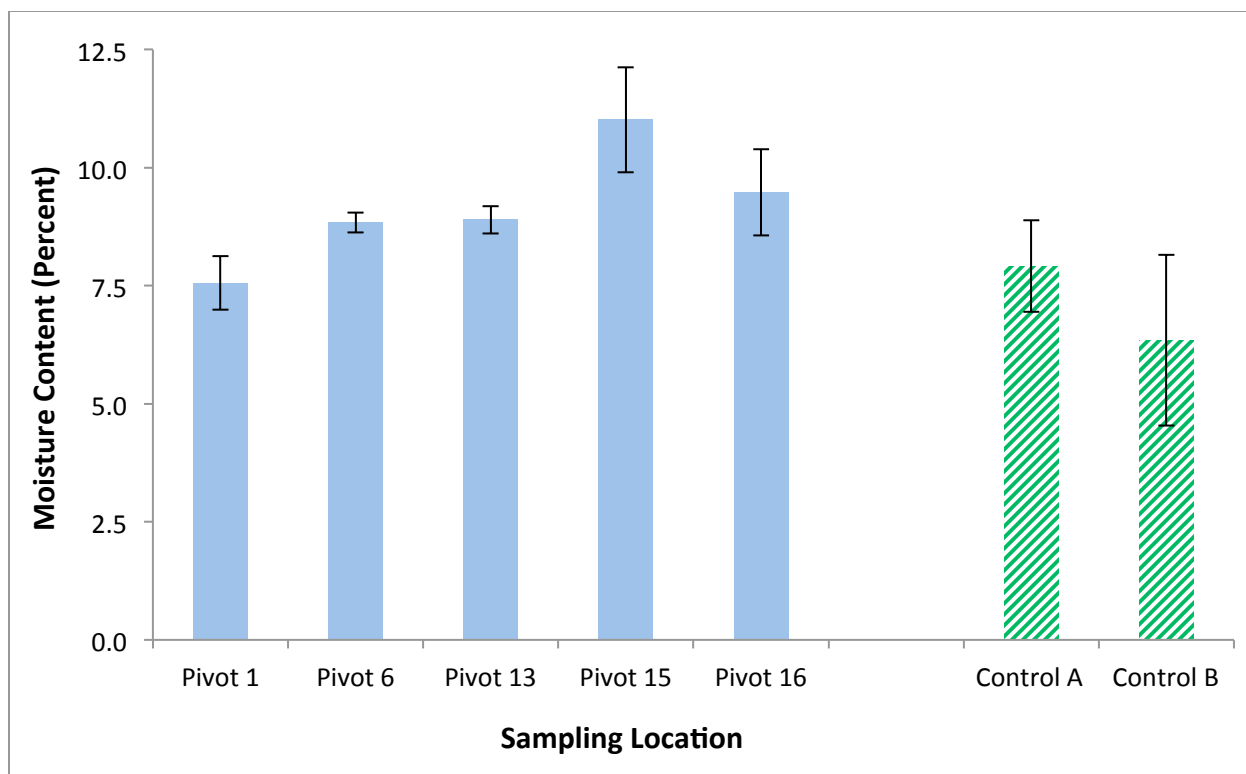


Figure 2.3.1 Moisture content of pivot and control sampling locations. Data is mean \pm one standard deviation.

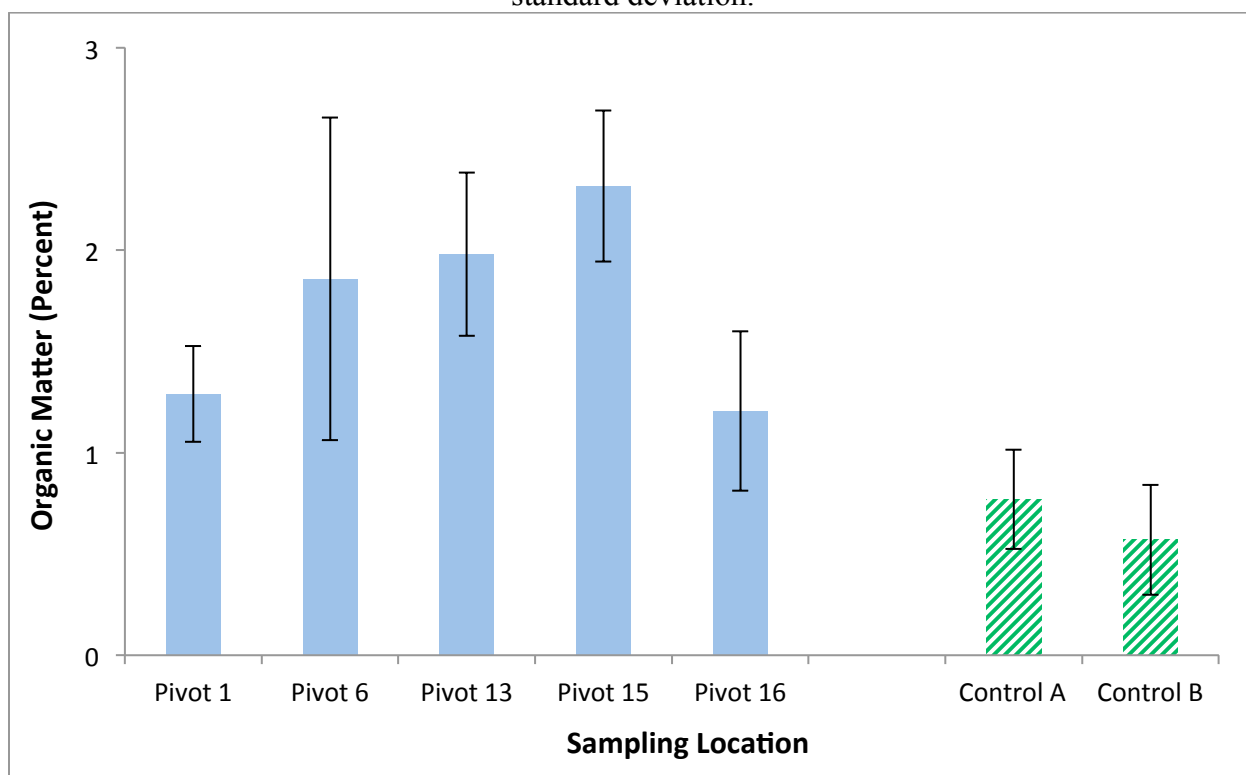


Figure 2.3.2 Percent organic matter of pivot and control sampling locations. Data is mean \pm one standard deviation.

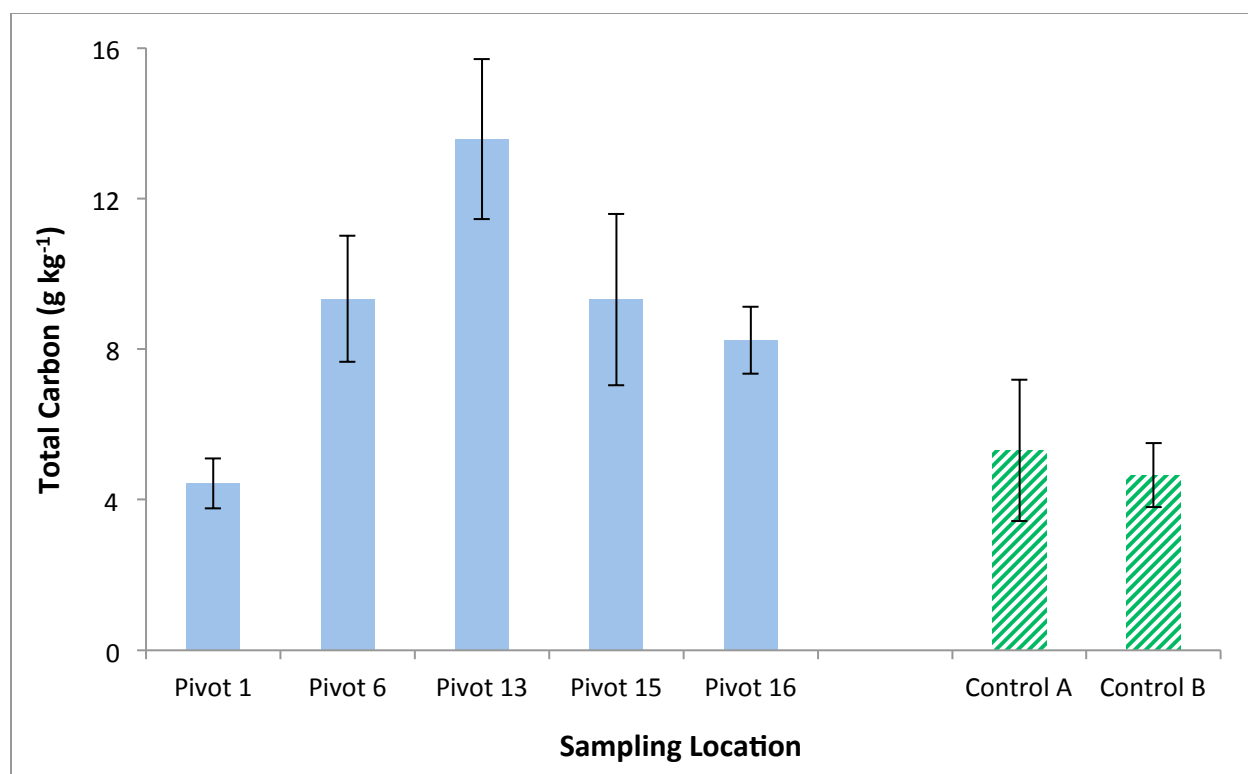


Figure 2.3.3 Total carbon (TC) of pivot and control sampling locations. Data is mean \pm one standard deviation.

with a mean value of $0.327 \pm 0.317 \text{ g kg}^{-1}$ (Figure 2.3.4; Table 2.3.1). The mean total nitrogen of the pivot sites were significantly greater ($P < 0.001$) than the mean values of the control sites.

2.3.5 Total Phosphorus

Total Phosphorus of the pivot soils ranged from below detection 141 to $1,350 \text{ mg kg}^{-1}$ with a mean value of $310 \pm 282 \text{ mg kg}^{-1}$, while the control sites ranged from 36.9 to $1,140 \text{ mg kg}^{-1}$ with a mean value of $194 \pm 209 \text{ mg kg}^{-1}$ (Figure 2.3.5; Table 2.3.1). The mean total phosphorus values of the pivot sites were significantly greater ($P < 0.001$) than the mean values of the control sites.

2.3.6 Microbial Biomass Carbon

Microbial biomass carbon was only sampled for the first two sampling events (January

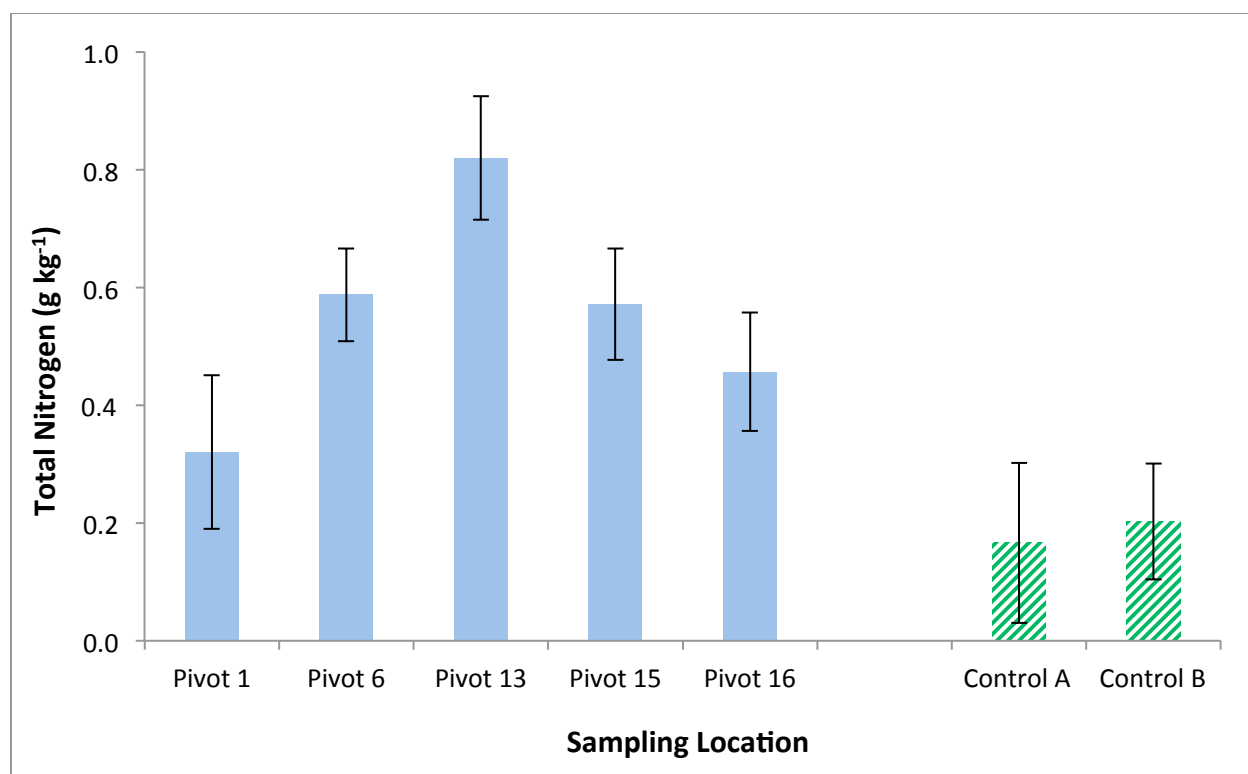


Figure 2.3.4 Total nitrogen (TN) of pivot and control sampling locations. Data is mean \pm one standard deviation.

2009 and May 2010). The microbial biomass carbon of the pivot soils ranged from 186 to 1378 mg kg⁻¹ with a mean value of 715 ± 477 mg kg⁻¹, while the control sites ranged from 192 to 1310 mg kg⁻¹ with a mean value of 701 ± 468 mg kg⁻¹ (Figure 2.3.6; Table 2.3.1). The mean microbial biomass carbon of the pivot sites were not significantly different ($P > 0.05$) than the mean values of the control sites.

2.3.7 Denitrifying Enzyme Activity

Denitrifying enzyme activity for the pivot soils ranged from 0.13 to 0.67 $\mu\text{g-N kg}^{-1} \text{ hr}^{-1}$ with a mean value of 0.30 ± 0.15 $\mu\text{g-N kg}^{-1} \text{ hr}^{-1}$, while the control sites ranged from 0.20 to 0.63 $\mu\text{g-N kg}^{-1} \text{ hr}^{-1}$ with a mean value of 0.40 ± 0.23 $\mu\text{g-N kg}^{-1} \text{ hr}^{-1}$. The mean value of the pivot sites was significantly greater ($P < 0.001$) than the mean value of control sites 1 and 2. However,

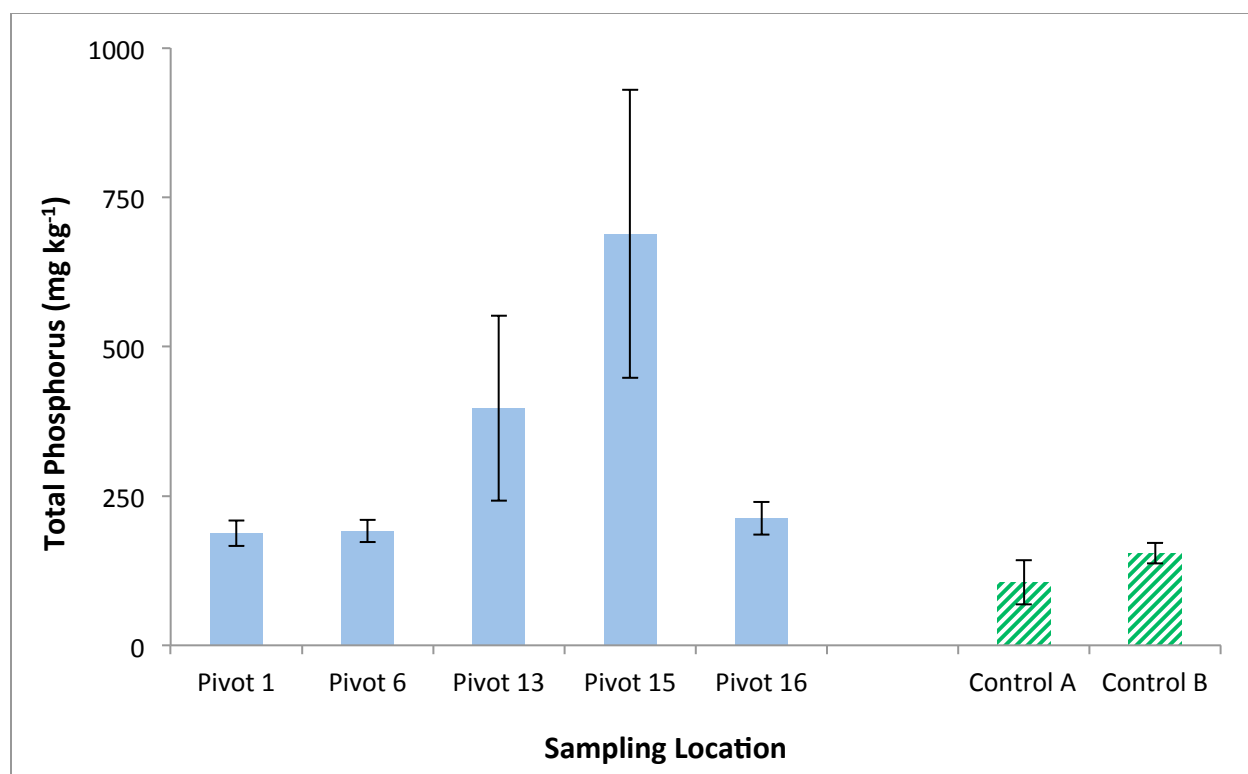


Figure 2.3.5 Total phosphorus (TP) of pivot and control sampling locations. Data is mean \pm one standard deviation.

there was no significant difference ($P > 0.05$) when comparing the mean value of the pivot sites with the mean value of the previously excluded control site 3. This is probably due to an inflow of runoff wastewater from the pivot soils.

2.3.8 Carbon Amendment Experiment Potential Denitrification

Only pivot 6 soil was used for carbon amendment experimentation to reduce statistical variability between amendment samples. The corn plant amendment had a total carbon of 477 g kg⁻¹. The biosolids amendment had a total carbon of 377 g kg⁻¹. The maximum potential denitrification rate of the corn plant amendment ranged from below detection to 4.19 mg N₂O-N kg⁻¹ d⁻¹ with a mean value of 1.18 ± 1.33 N₂O-N kg⁻¹ d⁻¹ (See Table 2.3.2). The 0.25% corn plant amendment maximum potential denitrification rates ranged from below detection to 1.38 N₂O-N kg⁻¹ d⁻¹ with a mean value of 0.761 ± 0.47 N₂O-N kg⁻¹ d⁻¹. The 1.0% corn plant

amendment maximum potential denitrification rates ranged from 0.08 to 4.19 $\text{N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$ with a mean value of $1.99 \pm 1.73 \text{ N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$. The 2.0% corn plant amendment maximum potential denitrification rates ranged from below detection to 3.49 $\text{N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$ with a mean value of $0.792 \pm 1.18 \text{ N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$. The maximum potential denitrification rate of the biosolid amendment ranged from 0.268 to 5.70 $\text{N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$ with a mean value of $3.42 \pm 1.60 \text{ N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$. The 0.25% biosolid amendment maximum potential denitrification rates ranged from 1.89 to 5.53 $\text{N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$ with a mean value of $4.20 \pm 1.27 \text{ N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$. The 1.0%

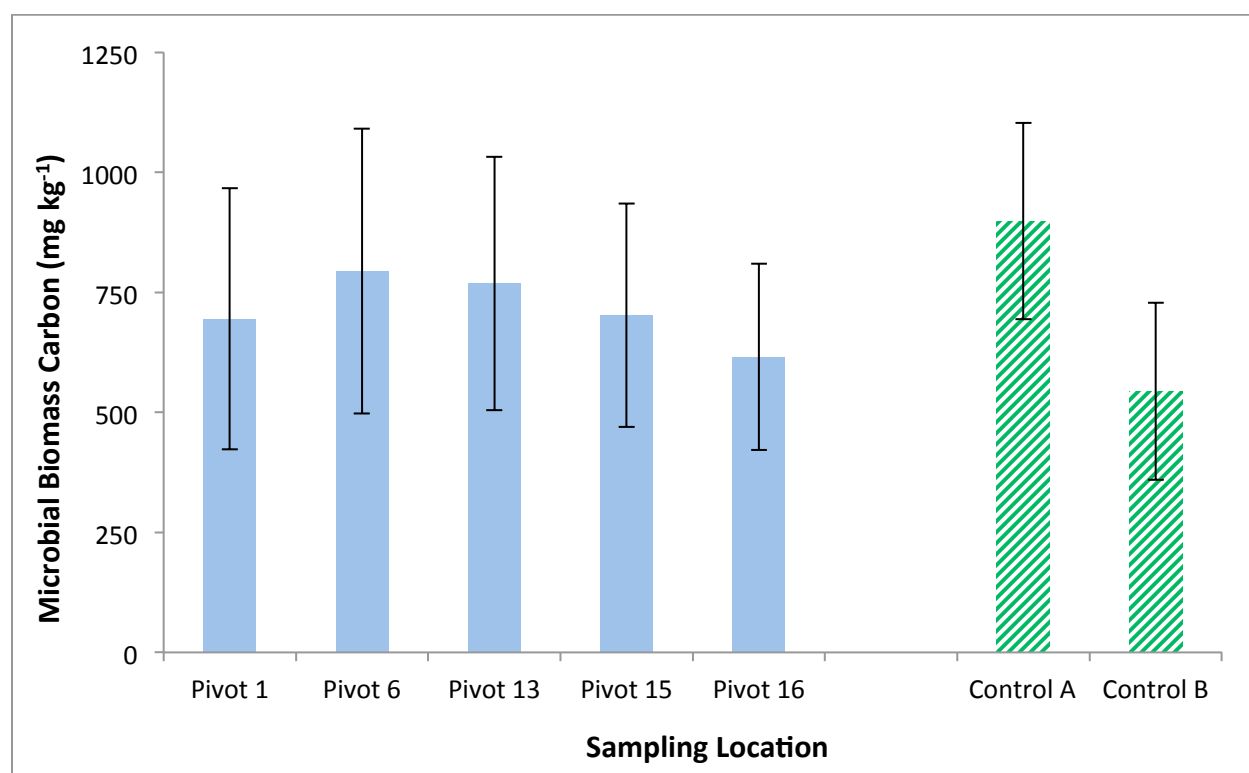


Figure 2.3.6 Microbial biomass carbon (MBC) of pivot and control sampling locations. Data is mean \pm one standard deviation.

Table 2.3.1 Mean values plus/minus one standard deviation for the soil properties measured at each site. Microbial biomass carbon units are mg kg^{-1} . **Denotes omitted control site due to similarity in DEA to pivot sites and similar landscape position.

Pivot	Moisture Content (%)	Total Carbon (g/kg)	Total Nitrogen (g/kg)	Total Phosphorus (mg/kg)	Microbial Biomass Carbon
1	7.56 ± 1.13	4.43 ± 1.32	0.321 ± 0.261	188 ± 42.4	695 ± 544
6	8.84 ± 0.430	9.34 ± 3.35	0.587 ± 0.158	191 ± 36.8	794 ± 593
13	8.90 ± 0.58	13.6 ± 4.25	0.820 ± 0.211	397 ± 309	769 ± 528
15	11.0 ± 2.23	9.32 ± 4.55	0.572 ± 0.190	689 ± 483	702 ± 465
16	9.48 ± 1.82	8.23 ± 1.77	0.457 ± 0.201	212 ± 55.0	616 ± 388
Average	9.16 ± 1.77	8.98 ± 4.32	0.551 ± 0.258	310 ± 282	715 ± 477
Control A	7.91 ± 1.94	5.31 ± 3.76	0.166 ± 0.272	105 ± 74.3	899 ± 409
Control B	6.35 ± 3.62	4.66 ± 1.70	0.203 ± 0.197	154 ± 35.3	544 ± 369
** Control C	6.99 ± 0.517	10.7 ± 8.19	0.586 ± 0.565	405 ± 436	738 ± 542
Average	7.57 ± 3.11	6.37 ± 4.41	0.327 ± 0.317	194 ± 209	701 ± 468

biosolid amendment maximum potential denitrification rates ranged from 0.861 to 5.70 $\text{N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$ with a mean value of $3.04 \pm 1.62 \text{ N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$. The 2.0% biosolid amendment maximum potential denitrification rates ranged from 0.268 to 5.08 $\text{N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$ with a mean value of $3.01 \pm 1.74 \text{ N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$. The non-amended soils maximum potential denitrification rates ranged from 0.864 to 3.16 $\text{N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$ with a mean value of $3.16 \pm 1.51 \text{ N}_2\text{O-N kg}^{-1} \text{ d}^{-1}$.

The corn amendment was significantly less ($P < 0.001$) than those of the biosolid and without amendment when comparing the mean value of potential denitrification rates among all concentrations combined. The biosolid was not significantly different ($P > 0.05$) from the soils without amendment.

The one week incubation had significantly greater ($P < 0.005$) potential denitrification rates than after four weeks of incubation, but was not significantly different ($P > 0.05$) after twelve weeks when comparing the potential denitrification rates over time for the corn plant amended soils. The 0.25% concentration of corn plant amendment was significantly less ($P < 0.05$) than the 1.0% concentration of corn plant amendment, but not significantly different ($P > 0.05$) than the 2.0% concentration of corn plant amendment when comparing the potential denitrification all three sampling periods. The varying corn plant amendment concentrations were not statistically compared over the three sampling periods due to small sample size, but were illustrated in Figures 2.3.8 - 2.3.10.

For the biosolid amendment among all concentrations, the one week incubation had significantly less potential denitrification rates than after four weeks ($P < 0.001$) and twelve weeks ($P < 0.05$) of incubation and the four week incubation was significantly greater ($P < 0.05$) than the twelve week incubation. The 0.25% concentration of biosolid amendment was not significantly different ($P > 0.05$) than either the 1.0% or the 2.0% concentrations of amendments, nor were the 1.0% and the 2.0% statistically different ($P > 0.05$). At four weeks, the mean potential denitrification rates at all three concentrations of amendments were significantly greater ($P < 0.001$) than the mean rates of the non-amended soil. The varying biosolid amendment concentrations were not statistically compared over the three sampling periods due to small sample size, but were illustrated in Figures 2.3.11 - 2.3.13.

For the non-amended soils, there was no significant difference ($P > 0.05$) between any of the three sampling periods for potential denitrification rates.

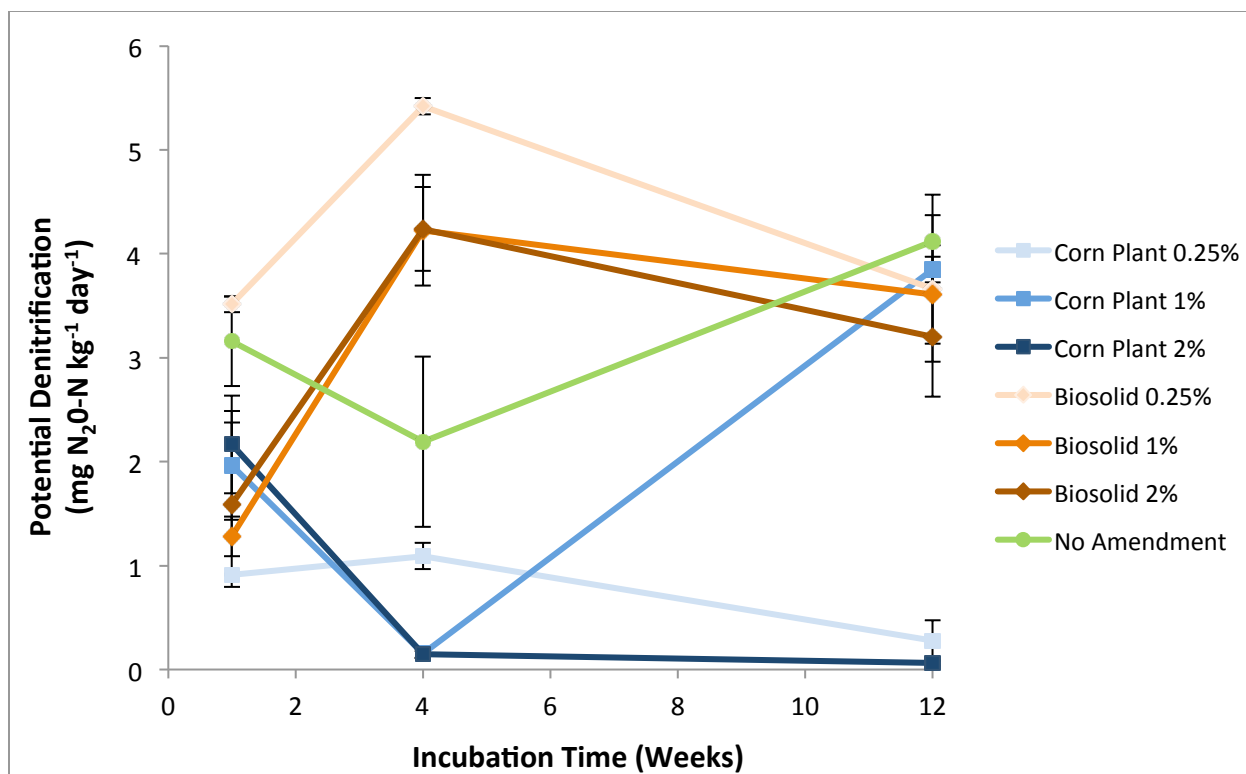


Figure 2.3.7 Maximum potential denitrification rates of all carbon amendments over 12 weeks of incubation.

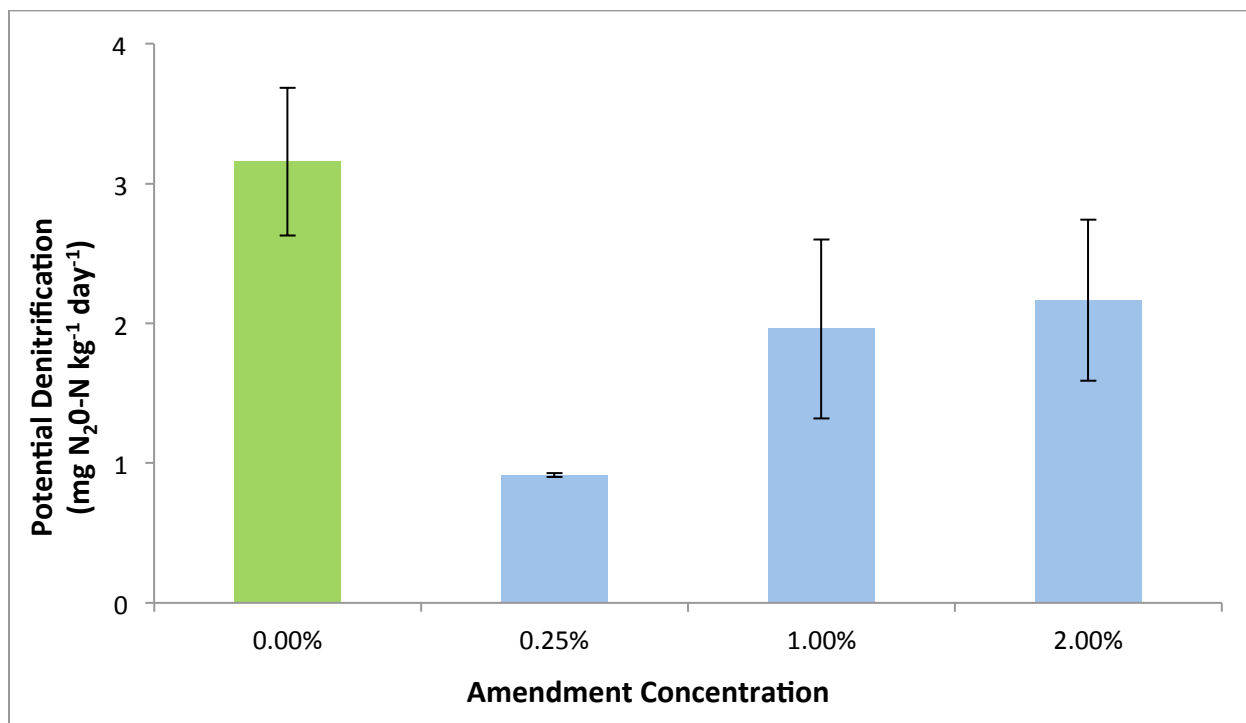


Figure 2.3.8 Maximum potential denitrification rates of varying corn amendment concentrations after one week of incubations. Data is mean value \pm one standard deviation. No Amendment has been labeled 0.00%.

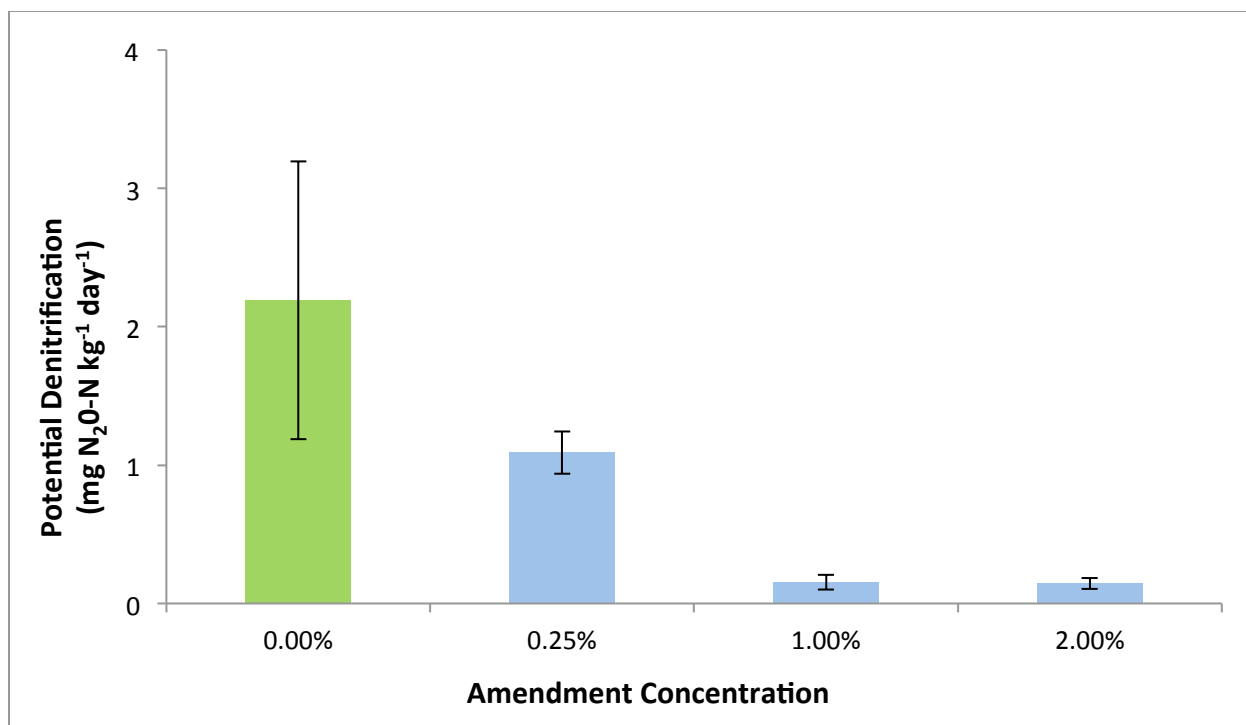


Figure 2.3.9 Maximum potential denitrification rates of varying corn amendment concentrations after four weeks of incubations. Data is mean value \pm one standard deviation. No Amendment has been labeled 0.00%.

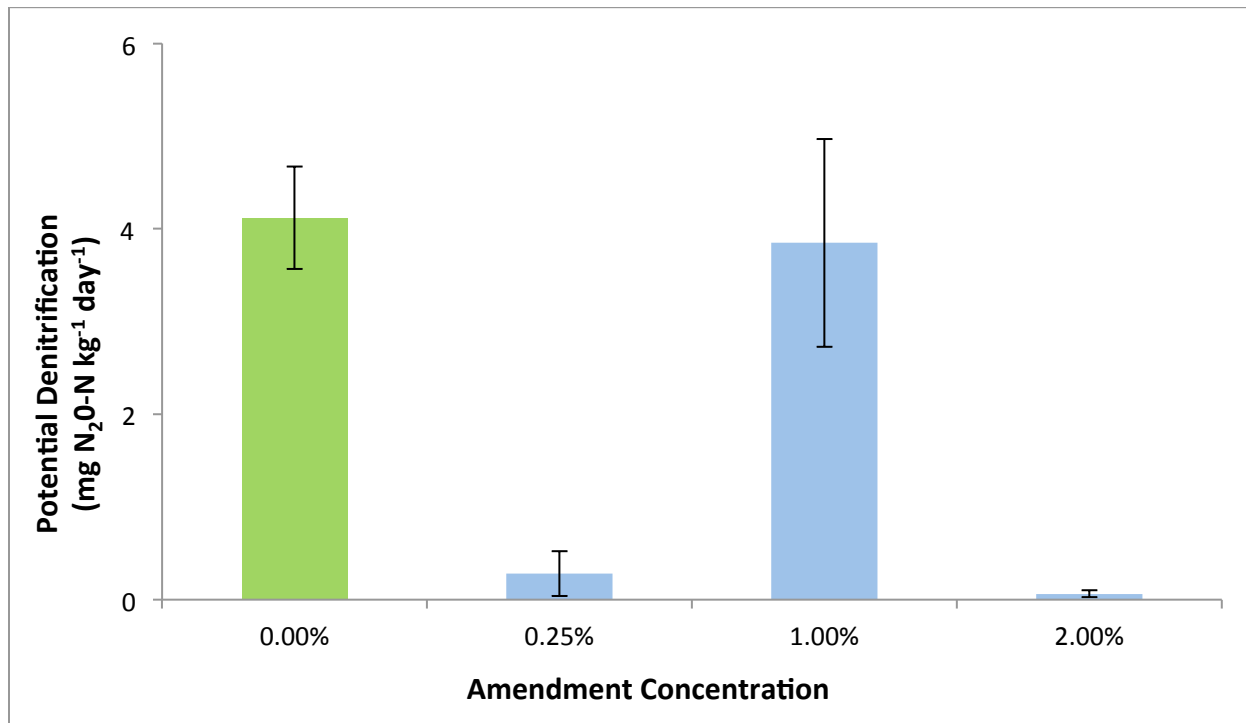


Figure 2.3.10 Maximum potential denitrification rates of varying corn amendment concentrations after twelve weeks of incubations. Data is mean value \pm one standard deviation. No Amendment has been labeled 0.00%.

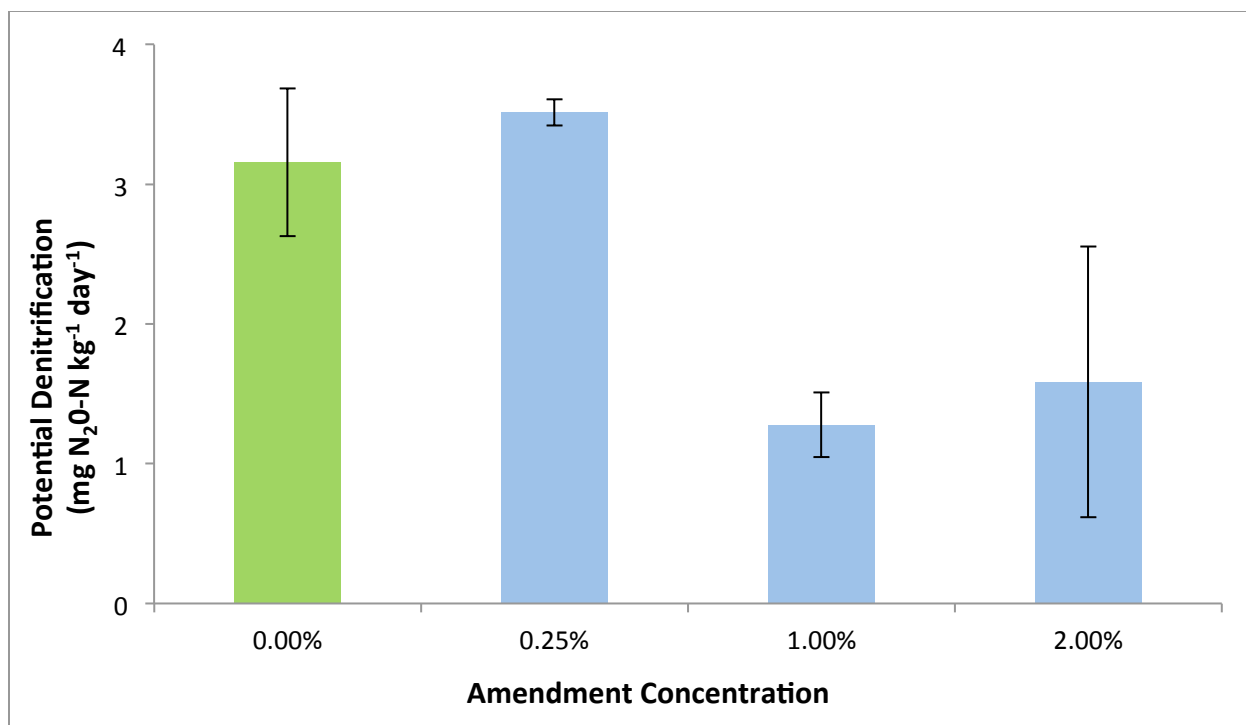


Figure 2.3.11 Maximum potential denitrification rates of varying biosolid amendment concentrations after one week of incubations. Data is mean value \pm one standard deviation. No Amendment has been labeled 0.00%.

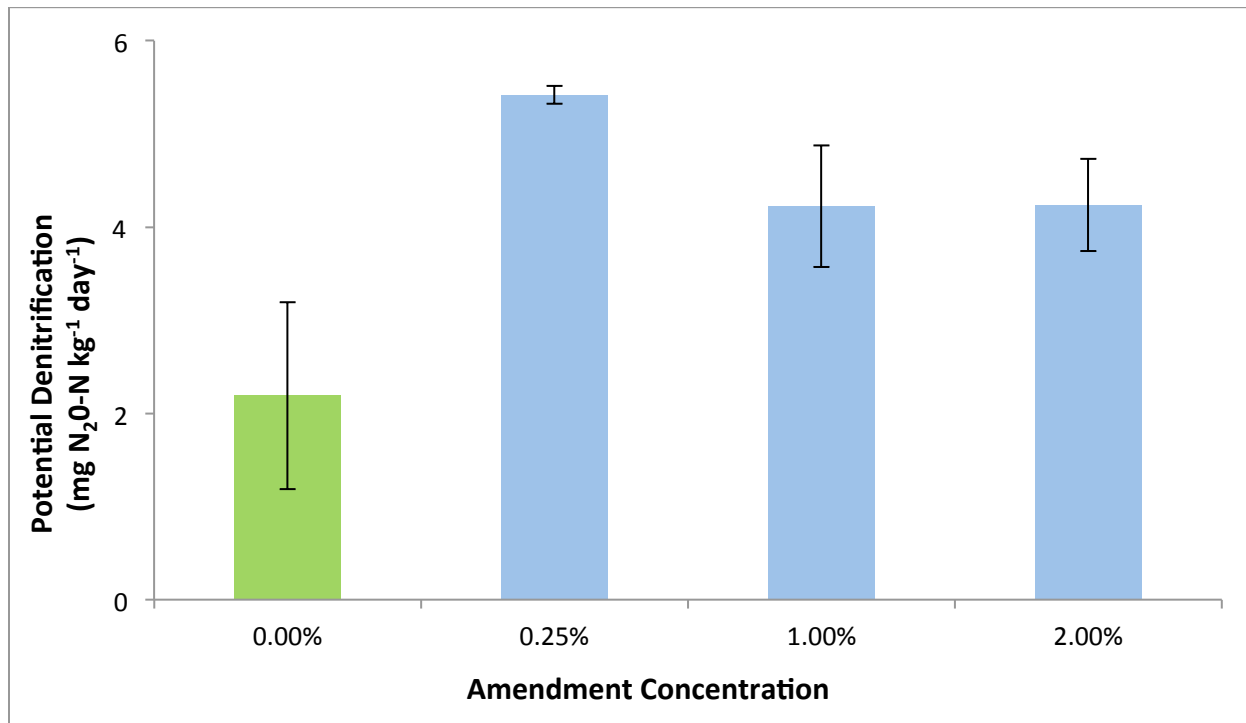


Figure 2.3.12 Maximum potential denitrification rates of varying biosolid amendment concentrations after four weeks of incubations. Data is mean value \pm one standard deviation. No Amendment has been labeled 0.00%.

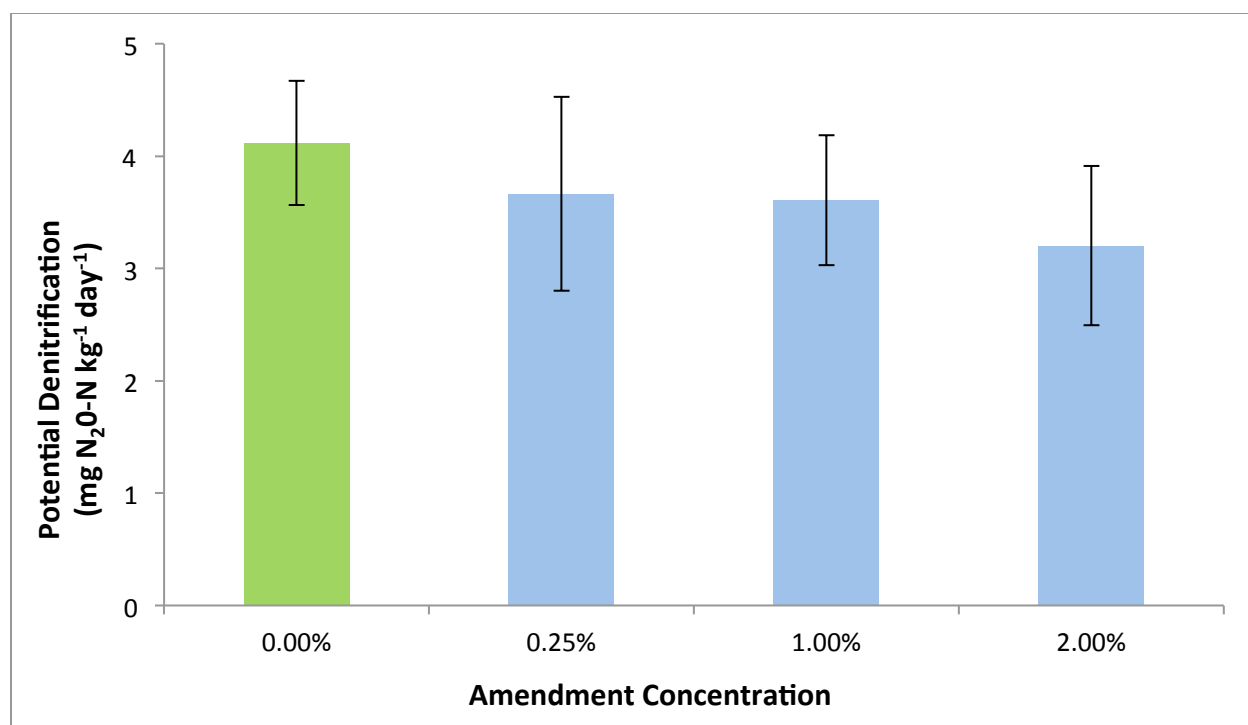


Figure 2.3.13 Maximum potential denitrification rates of varying biosolid amendment concentrations after twelve weeks of incubations. Data is mean value \pm one standard deviation. No Amendment has been labeled 0.00%.

Table 2.3.2 Maximum and average potential denitrification rates of organic amendment soils over the 12 week incubation period. Data is expressed as mean value plus/minus one standard deviation. Units are mg N₂O-N kg⁻¹ day⁻¹

	1 Week	4 Weeks	12 Weeks	Average
Corn Plant 0.25%	0.91 \pm 0.02	1.09 \pm 0.25	0.28 \pm 0.39	0.76 \pm 0.35
Corn Plant 1%	1.96 \pm 1.05	0.15 \pm 0.09	3.85 \pm 0.25	1.99 \pm 1.51
Corn Plant 2%	2.17 \pm 0.94	0.15 \pm 0.06	0.06 \pm 0.06	0.79 \pm 0.97
Biosolid 0.25%	3.52 \pm 0.15	5.42 \pm 0.16	3.67 \pm 1.41	4.2 \pm 0.86
Biosolid 1%	1.28 \pm 0.38	4.23 \pm 1.07	3.61 \pm 0.95	3.04 \pm 1.27
Biosolid 2%	1.59 \pm 1.58	4.24 \pm 0.81	3.20 \pm 1.16	3.01 \pm 1.09
No Amendment	3.16 \pm 0.86	2.19 \pm 1.64	4.12 \pm 0.9	3.16 \pm 0.79

2.4 Discussion and Conclusion

All soil characteristics measured showed significant differences between the pivot sites and the control sites. The differences are most likely due to differences in agriculture land use and irrigation in the spray field pivots. The wastewater nitrate concentration ranged from 4.5 – 7.6 mg/L and had significantly greater nitrate the runoff water received by the control sites (Katz et. al. 2009). However, there was no significant difference in microbial biomass carbon. Denitrifying enzyme activity was used to determining the activity of the microbial denitrifiers. Higher DEA rates are indicative of more denitrification occurring at the site. High DEA rates at the down gradient control site indicate that nitrate-rich water is entering the control site 3 and provided a basis for removal of site 3 as a control site. The DEA rates for control site 1 and 2 were below detection. Since nitrate-rich water is entering the down gradient control site, but neither of the up gradient control sites, there is not a complete removal of the nitrate within the wastewater spray field.

The nitrate runoff does not stop at the pivots and bordering locations. The wastewater has been shown to travel from the Upper Floridan aquifer to Wakulla springs (Davis et. al. 2011). The increase in nitrate loading has resulted in damaging effects to aquatic ecosystems, including accelerated algal growth and a proliferation of nuisance aquatic vegetation (Florida Springs Task Force 2000). It was estimated about 40% of the nitrate input to Wakulla Springs were from the Tallahassee wastewater treatment plant (Chelette et. al. 2002).

In an effort to increase denitrification to offset the inadequate wastewater nitrate removal by the pivot soils, two types of soil amendments were combined with pivot soils. These two amendments were chosen due to their practicality and high carbon content. Corn plant hulls

which are burned away for removal from the field and primary wastewater treatment residual biosolids were chosen. These two amendments would generate only an additional labor cost for application for its use. The residual solid waste biosolids and byproduct corn plant hulls were dried and ground and added on a mass basis. 0.25, 1.0, and 2.0 percent soil mass of the amendments were added to the soil treatments. The addition of the carbon amendments to the pivot soil showed mixed results. The mean values of the corn plant amendment of all three concentrations showed significantly less potential denitrification than that of the non-amended soil, while the mean values of the biosolid amendment at the three concentrations showed no statistical difference with the non-amended soil. With a C:N ratio of 25.5 for the corn plant and 5.94 for the biosolids, the soil microbes will be pressured to scavenge the soil to obtain nitrogen. The scavenging of nitrogen will deplete the soil's supply of soluble nitrogen, possibly delaying the decay of organic matter. It is likely that the potential denitrification peaks observed in Figure 2.3.7 for the biosolid amendments after four weeks of incubation were due to remaining nitrogen availability at four weeks, while the non-amended soils may have taken longer to deplete the available nitrogen or consumed all of it prior to the four week sampling.

Potential denitrification rates of the amended soils ranged from below detection to 4.19 mg N₂O-N kg⁻¹ d⁻¹ with a mean value of 1.2 ± 1.33 mg N₂O-N kg⁻¹ d⁻¹. These measurements are very similar to a separate shallow water aquifer which was continuously treated for sewage for 55 years, which had potential denitrification rates estimated to be between 0.30 – 2.2 mg N₂O-N kg⁻¹ d⁻¹ (Smith and Duff 1988). In another study of soils with a high nitrate groundwater inflow potential denitrification rates ranged from 0.17 – 10.4 mg N₂O-N kg⁻¹ d⁻¹ (Bradley et. al. 1992). With the two carbon amendments chosen there was no discernible advantage to its use to increase denitrification. It is possible that with high nitrate levels in the wastewater applied to

the amendment slurry, there may have been inhibiting accumulations of nitrites produced during denitrification (Thomsen et. al. 1994).

A companion study is currently being conducted at Florida Agricultural & Mechanical University by Denis Wafula under the direction of Dr.Ashvini Chauhan. Notable microbial community population shifts have been observed by an ARISA (automated ribosomal intergenic spacer analysis), even though there was very little significant differences in the microbial activity after the addition of organic amendments. There were significant changes not only with the varying organic amendments, but also over the 12 weeks of incubation.

Although there was no statistical increase in potential denitrification by the addition of carbon amendments, addition of carbon should not be excluded as a viable method for increasing denitrification. The Tallahassee wastewater treatment facility distributes 117 million L/day over 16 spray field pivots. The addition of carbon amendments may aid in retardation of the rapid percolation of wastewater through the sandy pivot soils. Although the addition of carbon may have little effect on the potential denitrification rate, slowing the wastewater movement through the soils may help increase the denitrification by allowing more time for the wastewater to interact with the denitrifying community, similar to the bottle incubations. To increase denitrification, addition of carbon amendments and an increase in the rotational speed of the pivots may be a viable method to increase wastewater-soil interaction time within the pivots.

It is possible that other more labile forms of carbon could be added to enhance denitrification. Methane has been shown to be a viable source of carbon for denitrifiers (Thalasso et. al 1997). Ethanol (Blaszczyk 1993; Chang et al. 1992; Hancher et. al. 1978; Schugerl 1989; Constantin and Fick 1997) and acetic acid (Almeida et. al. 1995; Akuna et. al.

1993; Feuerhake and Jordening 1993; Francis and Hancher 1986; Gonzales et. al. 1992; Kitsos et. al. 1992; Wilderer et. al. 1987; Constantin and Fick 1997) were studied as a carbon source for denitrification at both high and low concentrations. However, since these sources are not waste material, these sources would be very costly for large scale applications. Future research should be done with other types of available carbon to better understand the effects of varying types of carbon amendments on the microbially-facilitated denitrification and focus on slowing production without creating flooding conditions in the field.

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VITA

Jared Theriot was born and raised in Houma, Louisiana, with his parents Glenn and Lori, and younger siblings, Jude, Ryan, and Tiffany. Growing up in southern Louisiana, Jared gained a deep respect for the value of the ocean. He spent many early mornings out on the water fishing, crabbing, and shrimping.

Jared attended Louisiana State University in Baton Rouge, Louisiana and graduated with a Bachelor of Science degree in biology in May 2010. He was a member of the city and college tennis programs, was a member of LSU Ambassadors recruiting and orientation group, and worked part time in the work study program during his undergraduate career. At Louisiana State University, Jared enrolled in an introductory oceanography class that opened his eyes to how dependent biology is to oceanography. Further, Jared realized and how important oceanography and wetlands are to his home, Southern Louisiana.

After graduation, Jared continued learning about the importance of wetlands with Dr. John White at Louisiana State University in Baton Rouge, Louisiana. He became a teacher's assistant for the same introductory oceanography class that had once inspired him. The second year of his master's program he accepted a fellowship and continued his research. Jared's master's research has revealed to him the importance of research and hard work.