PROMOTING DENITRIFICATION IN RESTORED WETLANDS: THE IMPORTANCE OF SOIL CARBON QUALITY

by

Si Qi (Cindy) Yao

A Paper Presented to the

Faculty of Mount Holyoke College

in Partial Fulfillment of the Requirements for

the Degree of Bachelors of Arts

with Honor

Department of Environmental Studies

South Hadley, MA 01075

May 2015

This paper was prepared under the direction of
Professor Kate Ballantine for eight credits.

Acknowledgements

I am very grateful to have had the opportunity to do research with Professor Kate Ballantine, who has been my independent study advisor since my sophomore year. Her restored wetland sites offer a wealth of knowledge for both the science and the practice of ecological restoration, and I am honored to have participated in this exciting project through my thesis work. It has been wonderful to be able to discuss biogeochemical processes, scientific writing, and presentation tips with someone so knowledgeable, and I will always remember our meetings with fondness.

I would also like to acknowledge my thesis committee and lab group for providing so much help this past year. Through her course as well as individual meetings, Professor Janice Gifford enabled me to both understand my data conceptually and carry out statistical analyses correctly. Todd Anderson gave me detailed feedback to improve my drafts and constructive comments to strengthen my presentations. It was also very helpful to have conversations with my insightful fellow thesis-writer, Erin Pierce.

Thanks are also due to Peter Groffman and Lisa Martel at the Cary Institute of Ecosystem Studies, who supervised me during my first experience working in a lab. Because of them, I was able to learn a great deal about useful soil analysis techniques as well as gain valuable lab experience.

Finally, I would like to thank my friends and family for supporting me throughout this entire process.

Table of Contents

List of Figures

List of Tables

Abstract

Wetlands contribute a wide variety of ecosystem functions, one of which is the improvement of water quality through denitrification. In order to offset the negative environmental impact of destroying wetlands for the sake of development, the U.S. has a policy in which destroyed wetland area must be compensated for through the restoration of a new wetland somewhere else. However, these restored wetlands are estimated to require decades to achieve equivalent levels of functioning to natural wetlands. This study evaluates the efficacy of using carbon amendments during wetland restoration to promote denitrification potential in four restored wetlands in Ithaca, NY. The amendments used during restoration were straw, topsoil, and biochar, which have differing levels of carbon lability and thus different rates of decomposition by soil microbes. Soil samples analyzed in this study were collected six years after the restoration of the four wetland sites. These samples, in addition to samples from a natural reference wetland, were analyzed for denitrification potential as well as for a suite of associated soil properties, including organic carbon, respiration, microbial biomass, forms of nitrogen, potential net N mineralization and nitrification, and pH. Denitrification potential was significantly higher in plots with the carbon amendments than in plots where no amendment was added, but there were no significant differences between amendment types. Denitrification potential was significantly positively correlated with soil organic carbon as well as microbial biomass nitrogen. This demonstrates that the availability of organic carbon sources in restored wetlands is vital for supporting populations of microbes that carry out ecosystem functions such as denitrification, and that the incorporation of carbon amendments can help provide this important requirement. However, denitrification potential in the natural wetland was at least 50 times higher than in the restored wetlands, highlighting the limitations of using wetland restoration to compensate for the destruction of natural wetlands.

Introduction

  Wetland ecosystem functions

  Extensive research has revealed numerous key contributions made by wetlands to their surrounding ecosystems, including water quality improvement, flood abatement, biodiversity support, and carbon sequestration (Sather and Smith 1984; Richardson 1994; Zedler and Kercher 2005). Wetlands are characterized by the presence of water above the surface of the soil, resulting in anaerobic soil conditions, and the ability of wetlands to carry out such important functions is mediated by their unique hydrology. Overall, wetlands provide 40% of annual renewable ecosystem functions worldwide, though their area covers only 3% of the globe (Costanza *et al.* 1997). One of these important functions is water quality improvement, because wetlands have been found to reduce concentrations of nutrients in groundwater (Verhoeven *et al.* 2006). Additionally, the flood abatement properties of wetlands are increasingly relevant to questions of development because of increased human settlement into floodplains (Zedler and Kercher 2005). The ability of wetlands to take up and store large quantities of extra water enables them to be effective in mitigating flood damage when located upstream of areas in which flooding would be costly (Hey *et al.* 2002). Another property of interest is the high degree of biodiversity support offered by wetlands as a result of their unique habitat qualities. This is illustrated by the fact that many animal species are dependent on wetlands (McAllister *et al.* 2000).

Wetlands and climate change

Wetlands play a significant role in mediating the global carbon cycle and are especially relevant to questions of how climate change will impact the future of our planet. One important role of wetlands is as carbon sinks, meaning that carbon dioxide, a greenhouse gas, is sequestered and stored in wetland soils and vegetation. It has been estimated that 20 to 30% of the organic carbon stored in soils worldwide are specifically in wetland soils (Mitsch *et al.* 2010). At the same time, wetlands are the largest natural source of methane, which is a more potent greenhouse gas than carbon dioxide (Bridgham *et al.* 2013). This methane is generated by microbes in wetland soils and subsequently released into the atmosphere by diffusion, ebullition, or transport through aerenchymateous tissue of vascular plants, and these pathways are associated with varying rates of emission (Joabsson *et al.* 1999). To determine the net effect of wetlands on climate change, we must consider the balance between net carbon sequestered by wetlands that would otherwise be present in the atmosphere as carbon dioxide and net methane put out into the atmosphere by wetlands, taking into account that methane has a global warming potential 25 times higher than that of carbon dioxide (Mitsch *et al.* 2013). After analyzing historical carbon dioxide and methane emission data from North American wetlands, Bridgham *et al.* (2006) determined that these wetlands have a net cooling effect over a period of 100 years, though they mention that the result is probably not significantly different from zero because of large errors in estimates used for the calculations. Mitsch *et*

al. (2013) developed a carbon balance model which accounts for the degradation

of methane in the atmosphere and it was found that most wetlands are still carbon

sinks even when methane emissions are considered.

Another key interaction between wetlands and climate is that climate

change poses a threat to the productivity and survival of wetlands. Wetlands are

sensitive to changes in hydrology and temperature, and thus climate change is

likely to affect wetlands through these avenues (Erwin 2009). Specifically,

changes in local precipitation, temperature, and humidity brought on by climate

change could alter wetland water depth and hydroperiod, which could pose risks

to wetlands. It is predicted that climate change will decrease the number of

wetlands in most regions as well as cause the geographic location of wetlands to

shift (Erwin 2009). As another dramatic example of the potential harm of climate

change to wetlands, models have predicted that coastal wetlands in certain regions

could disappear as a result of rising sea-levels associated with global warming by

2080 (Nicholls *et al.* 1999).

The mutual influence that wetlands and climate have on each other is

further complicated by the possibility that a changing climate will alter the rate of

carbon flux in wetlands. There is evidence that methane emissions in wetlands

have responded to yearly climate fluctuations in the past few decades, which

suggests that methane fluxes will also respond to temperature changes brought on

by climate change (Bridgham *et al.* 2013). As temperatures increase and wetland

water levels drop, there is likely to be a higher rate of decomposition and carbon

oxidation in wetland soils, leading to increased release of carbon by wetlands

(Chmura *et al.* 2003). In the specific case of tropical wetlands, which make up 30%

of all wetlands, a warming climate could change them from completely flooded

wetlands into wetlands with periods of dryness and wetness, resulting in higher

levels of methane generation (Mitsch *et al.* 2010).

   Nitrate and nitrate pollution

   The role of wetlands in water quality improvement will serve as the focus

of our investigation. One pollutant that threatens the quality of water is nitrate,

and one of the major ways nitrate is introduced into groundwater is through

nutrient leaching from agricultural systems. The green revolution started a

worldwide shift in agricultural practice by introducing chemical fertilizers

containing nitrogen, the use of which largely has replaced the traditional method

of using manure to enrich nutrients in the soil (Tillman 1998). The resulting influx

of nitrogen fertilizers has increased crop yields to support the rapid growth in

human population, but has also caused a high degree of nitrate pollution

worldwide (Tillman 1998). Nitrate is water soluble, highly mobile, and persists in

the groundwater for decades (Martin *et al.* 1998). These chemical properties

facilitate the accumulation of nitrate in groundwater after leaching out from its

initial agricultural setting. In the United States, nitrate is a federally regulated

contaminant due to its acute toxicity (Spalding and Exner 1993). From a human

health standpoint, the presence of nitrate in sources of drinking water can cause a

condition called methemoglobinemia in which the blood oxygen-carrying

capacity is reduced, which is linked to brain damage and suffocation in infants (Prasad and Power 1995). Many cases of high nitrate concentrations in drinking water wells have been observed around the world, sometimes reaching levels higher than the critical World Health Organization value of 11.3 mg/L in sources of drinking water (Spalding and Exner 1993; Richter and Roelcke 2000).

In addition to harmful effects on human health, nitrate also plays a role in large-scale environmental quality. Eutrophication causes nitrogen-limited populations of phytoplankton bloom in response to an influx of excess nitrogen (Turner and Rabalais 1994). This phenomenon may result in hypoxia, which is the condition of low concentrations of dissolved oxygen in sub-surface water levels (Diaz 2001). Hypoxia is caused by a combination of water column stratification and decomposition of organic matter in the bottom water, both of which are exacerbated by increased levels of nitrogen into the system (Diaz 2001). The resulting decrease in dissolved oxygen creates an inhospitable environment, leading to decreases in fish populations, which is not only harmful from a biodiversity standpoint but also negatively impacts the yields of commercial fisheries (Diaz 2001). Thus, excess nitrogen has a wide scope of water quality implications, and it is crucial to reduce the amount of nitrate in groundwater.

Role of denitrification in nitrate removal

Studies have shown that natural wetlands are a sink for nitrogen pollutants, with a wide range of observed efficiencies (Seitzinger 1994). Several processes are responsible for removing nitrogen from water, including denitrification,

ammonia volatilization or adsorption, anammox, and organic nitrogen burial

(Vymazal 2007). The primary process for nitrate removal is denitrification, which

is the microbe-mediated process by which nitrate is reduced into nitrogen gases.

The denitrifying bacteria reduce nitrate into nitrous oxide and then atmospheric

nitrogen, N_2, which is released into the atmosphere (Verhoeven *et al.* 2006).

Because denitrifying bacteria favor reducing oxygen rather than nitrate when

oxygen concentrations are high enough to allow them to do so, denitrification can

only occur in low oxygen environments. Wetland soils are mostly anaerobic as a

result of being submerged in water, and thus, a key site for denitrification. Nitrate

dissolved in water flowing from agricultural systems to the stream can be

removed completely from the aquatic system in the form of harmless N_2 gas when

it passes through wetlands (Verhoeven *et al.* 2006). In riparian wetlands, it has

been estimated that denitrification can remove over 50% of the incoming nitrate

(Hanson *et al.* 1994). That the end-product of denitrification is N_2 is a notable

advantage of nitrate removal through denitrification as compared to removal

through assimilation into plant matter, because N_2 is truly removed from the

system and cannot return through mineralization (Hanson *et al.* 1994).

Loss and degradation of natural wetlands

Given the importance of wetland functions such as denitrification, there is

concern for the historical and projected losses in wetland area around the world.

The tension between development and preservation is a key factor influencing the

amount of wetland area in regions populated by humans. One estimate states that

around half of wetland area worldwide has been lost due to human activities,

primarily due to drainage for agriculture (IUCN 1996). It is estimated that the

contiguous United States has lost 53% of its original wetlands in the 200 years

since colonization (Dahl 1990). Most of this loss results from conversion to

agriculture and other land uses, especially in the Midwest (Brinson and Malvarez

2002). Additionally, urban development has also led to the destruction of

wetlands, and it is estimated that 10-15% of wetlands originally located in what

are now urban areas remain (Kusler 1988). In addition to the direct dredging,

draining, and filling of wetlands, humans are also mediating indirect threats to

wetland area through the effects of global warming, as discussed earlier.

Apart from outright destruction, wetland degradation may also hinder the

ability of wetlands to perform ecological functions (Zedler and Kercher 2005).

Geomorphic and hydrologic changes caused by dam construction, for example,

affect wetlands by changing their shape and hydrologic conditions (Brinson and

Malvarez 2002). Additionally, excessive nutrient and contaminant loading is often

caused by chemical fertilizers in large-scale practices and can alter species

compositions and cause eutrophication in wetlands (Brinson and Malvarez 2002).

Because different plant species are more competitive in certain nutrient levels

than in others, changes in the latter can impose selection pressure, changing

wetland species compositions (Bedford *et al.* 2001). Eutrophication may also

compromise biodiversity because lower amounts of available oxygen pose

selective pressures. Critical loads of nutrients like nitrogen and phosphorous are

input rates below which a system remains almost unchanged, but above which dramatic changes in species dominance and ecosystem functioning take place (Verhoeven *et al.* 2006). Surpassing these critical loads leads to degradation in a variety of wetland properties.

History of U.S. wetland regulation

While agricultural and urban development is needed to sustain a growing population, the goal of environmental protection is also important. There has been a long history in the United States of regulatory policy aimed to bring the two simultaneously important but mutually competing goals into balance. In 1972, Section 404 of the Clean Water Act established a means of regulating the discharge of dredged or fill material into U.S. waters through a permitting program. In making decisions regarding whether and under what conditions a permit should be issued, the aim was to mitigate adverse effects caused by the proposed activity. The term mitigation has been defined and redefined in different guidelines throughout the course of the program, but the main idea is that environmental impact should be avoided, minimized, or compensated for. Whereas these mitigation approaches were meant to be applied sequentially such that compensation is the last option, increased focus went to compensation due to the 1989 "no net loss" policy. Because of the concept of "net" loss, emphasis was shifted from impact avoidance and minimization to compensating for the impact such that there is no cumulative environmental impact (Hough and Robertson 2009).

Compensatory mitigation methods

Means of compensatory mitigation include the establishment of a new wetland site, the restoration of a preexisting site, the enhancement of function in a preexisting site, and the preservation of a preexisting site (Hough and Robertson 2009). Restoration is a key aspect of wetland mitigation policy, and approaches to restoration depend on the complex conditions of each specific site. Hydrological conditions are essential to wetland structure and function, so one aspect of restoration is to restore the natural water levels by removing dikes, blocking drainage sources, or adding water control structures (Zedler and Kercher 2005). Topography is also an important structural factor, and artificially flattened topography can be restored into natural slopes (Larkin *et al.* 2005). Restoration of damaged soil conditions can be done through adding soil amendments, while soils with excess nutrients can be defertilized by removing soil (Zedler and Kercher 2005). Additionally, soil transplants can be applied to sites that have lost soil. In addition to restoration of these abiotic factors, aspects such as vegetation, animal populations, and microorganisms are also considered during wetland restoration.

Problems with noncompliance

Despite the development of both mitigation policy and restoration techniques with the aim of no net loss, this goal has not been achieved. In 2001, an extensive National Research Council evaluation of compensatory mitigation policy concluded that the mitigation program did not succeed in meeting the goal of no net loss. This assessment was based upon literature review of studies

showing that many restored wetlands were not performing at levels specified by permits, and in cases wherein mitigation was performing as specified, the restored wetlands were not supporting functions equivalent to natural sites (NRC 2001). In 2005, a Government Accountability Office report attributed this failure in achieving no net loss to ineffective oversight and enforcement (GAO 2005). Tied in to this issue is that while permits granted to allow wetland destruction included mitigation requirements, these were not enforced and carried out to acceptable degrees (Turner *et al.* 2001). In part due to noncompliance with compensatory mitigation requirements, it has been estimated that rather than no net loss, there was an 80% net loss of wetland area under the Clean Water Act permitting program (Turner *et al.* 2001).

An analysis of wetland restoration compliance in Massachusetts found similar results (Brown and Veneman 2001). Through an analysis of project files and restored wetlands in the state, it was found that the compliance rate was around 40%, with the largest single reason for noncompliance being failure to build a replication site at all (Brown and Veneman 2001). In the cases in which a wetland was constructed, the main reasons for noncompliance were failure to achieve 75% wetland vegetation cover within two growing seasons and failure to construct a wetland at least the same size as the original natural wetland (Brown and Veneman 2001).

A more recent study of compensatory mitigation analyzed the criteria by which restored wetlands are evaluated. In analyzing the restoration criteria

imposed on 76 wetlands, it was found that a majority of the performance goals focused on wetland vegetation, whereas only one site was evaluated for a dynamic process, specifically sediment accumulation rate (Matthews and Endress 2008). However, vegetation parameters such as plant cover do not necessarily reflect the overall quality of restored wetlands, so the fact that a wetland was deemed to be compliant with permit criteria does not mean that it has successfully replaced the functions of the original natural wetland (Matthews and Endress 2008). The main conclusion from this study was that the criteria used to evaluate compensatory mitigation wetlands should be expanded to include properties like soil quality and hydrology, and that these should be based on comparisons to natural reference sites instead of being set arbitrarily (Matthews and Endress 2008).

Loss of function in restored wetlands

In addition to the overall insufficiency in regulatory policy for the program, there is the issue of evaluating wetland function instead of just area. Turner *et al.* (2001) found that even in cases in which there is compliance with mitigation requirements, the goal of no net loss in wetland function is often not met. Whereas aspects of compensatory mitigation policy such as mitigation requirements and monitoring periods were based on the assumption that structure and function of restored wetlands would reach natural levels in five to ten years, long-term sampling of both soil and vegetative properties has revealed that this is not the case (Zedler and Callaway 1999; Ballantine and Schneider 2009). It was

found that the development of wetland properties is unpredictable due to lack of strong directional trends in measured properties and high variability between years due to factors like flooding (Zedler and Callaway 1999). This study concluded that better estimates for the time needed to achieve functional replacement in restored wetlands would lie between 20 to 100 years, though compensation sites may never fully replace natural wetland functions (Zedler and Callaway 1999). Ballantine and Schneider (2009) found that for a chronosequence of restored freshwater depressional wetlands ranging in age from 3 – 55 years after restoration, soil organic matter, which is considered an indicator of wetland function, did not reach even half of natural levels after five decades.

Factors affecting denitrification

Restored wetlands have lower levels of functioning than natural wetlands despite having the same area because ecosystem functions, such as denitrification, are mediated by complex biogeochemical factors that depend on soil properties rather than wetland size. Two of the most direct factors are oxygen concentration and nitrate availability. Anaerobic bacteria responsible for denitrification require an anoxic environment in order to reduce nitrate instead of oxygen, and denitrification has been found to be inhibited when dissolved oxygen concentrations in soil water exceed 0.2 mg/L (Nichols 1983). The direct relationship between nitrate concentrations and denitrification rates in wetlands has been supported by observed increases in the latter as nitrate concentrations were experimentally increased incrementally (Seitzinger 1994). Additionally, a

study comparing denitrification rates in two riparian wetland sites differing only in nitrate enrichment level also found higher denitrification rates in the more enriched site (Hanson *et al.* 1994).

Another key property affecting denitrification is the presence of microbial communities and the abiotic factors supporting them in wetland soils (Sirivedhin and Gray 2006). In order to fuel respiration in populations of denitrifying bacteria, the soil must provide a source of carbon in the form of organic matter, and it has been shown that organic matter availability in wetland soils is correlated with increased denitrification rates (Burchell *et al.* 2007). The rate at which organic matter can be used by bacteria depends on carbon lability of the organic matter source, so carbon quantity as well as lability affect denitrification rates (Schipper *et al.* 1994). Wetland vegetation also influences denitrification, as was observed in a study comparing denitrification rates among wetlands with different vegetation treatments (Bachand and Horne 2000). This experiment suggested that different types of vegetation contributed organic matter to the wetlands at different rates wherein certain plants were integrated into the water column and became available to denitrifying bacteria more quickly than others (Bachand and Horne 2000). It has been proposed that vegetation also enhances denitrification through the induction of nitrate transport from the water column to anaerobic soil zones as they draw water toward their roots (Martin and Reddy 1997). There has also been evidence of periphytic algae having a role in increasing denitrification rates. It has been hypothesized that organic carbon produced by these algae is

very labile and thus can be easily used by denitrifying bacteria (Sirivedhin and Gray 2005).

In addition to these factors, wetland denitrification rates are also affected by redox potentials, temperature, pH, and soil type. A study comparing denitrification rates under a range of redox conditions found that bacterial denitrification was dominant in strongly reducing conditions (Seo and DeLaune 2010). There is a positive correlation between temperature and denitrification rates up to $60 - 75$ °C (Hernandez and Mitsch 2007a; Faulwetter *et al.* 2009). pH levels of $6 - 8$ optimize denitrification, whereas rates are low at pH 5 and absent at pH levels below 4 (Vymazal 2007). In terms of soil type, denitrification rates have been shown to be higher in peaty soil than in sandy loam soil (Davidsson and Stahl, 2000).

In a wetland system, the suite of relevant factors discussed above vary according to seasonal conditions as well as position in the wetland, thus creating temporal and spatial variability of denitrification rates (Seitzinger 1994). Denitrification rates have been found to be higher in the summer than in the winter, which may be due to differences in temperature or vegetation between the two seasons (Christensen and Sørensen 1986; Xue *et al.* 1999; Hernandez and Mitsch 2007a). Another temporal variation is the increase in denitrification as a wetland, particularly a newly restored wetland, ages, which is due to accumulation in organic matter over time (Hernandez and Mitsch 2007b). With regards to spatial distribution, it was found that nitrate was characterized by

diminishing mass removal as it passed through the wetland system (Martin and Reddy 1997). Another study also observed denitrification rates to be higher near the inflow part of the wetland as compared to the outflow (Hernandez and Mitsch 2007a).

Importance of soil organic matter

Restored wetlands may not achieve functional equivalency to natural wetlands because there is not enough organic matter accumulated in the soil (Gale *et al.* 1993). Soil organic matter is a key parameter of soil structure and function, and is an indicator of overall soil quality (Bruland and Richardson 2004). In particular, organic matter contains organic carbon, which affects denitrification rates because denitrifying microbes require carbon for both energy and cellular synthesis (Burchell *et al.* 2007). Assuming other factors are not limiting, increased levels of organic matter in wetland soils leads to larger populations of denitrifying bacteria, resulting in higher rates of denitrification. Additionally, organic matter addition not only improves denitrification rates, but also supports the development of above-ground biomass, which is a renewable carbon source important to long-term wetland function (Burchell *et al.* 2007).

Use of carbon amendments in restored wetlands

On the basis that organic carbon plays a large role in wetland function, research has been done to determine the effects of adding different sources of carbon to wetland soils. These additions can be referred to as soil amendments, which are used routinely in agriculture to improve physical properties of the soil.

For example, carbon was introduced to restored salt marshes through the addition of litterbags filled with straw, and it was found that $50 - 66\%$ of the nitrogen was lost in the first two weeks (Gibson *et al.* 1994). The researchers theorized that two possible explanations for the quick nitrogen loss were either high rates of leaching or high rates of denitrification.

In a study investigating the effects of hydrology and topsoil amendments on restored wetlands, Bruland and Richardson (2004) found that soil moisture, water holding capacity, and P sorption index were in some cases higher in sites amended with topsoil than in control sites. From these results, the researchers concluded that in cases of wetland restoration in which excavation exposes nutrient-poor, high density subsoil, it may be necessary to add topsoil or organic matter to improve soil properties and support development of plant and microbial communities (Bruland and Richardson 2004).

Biochar, a form of carbon derived from heating biomass in the absence of oxygen, has gained much interest in recent years in an agricultural context due to several unique chemical and physical properties. It has been shown to increase cation exchange capacity (CEC) due to an abundance of charged groups, as well as soil surface area as a result of its porous structure (Liang *et al*. 2006). When biochar is added to soil, its unique properties influence functions of the soil. For example, soil amended with biochar has increased water-holding capacity and lower amounts of nutrient leaching (DeLuca *et al.* 2009). Biochar can also affect soil microbial communities by acting as a habitat for bacteria, and the soil

bacterial composition arising from biochar addition may result in changes in soil processes such as denitrification (Pietikäinen *et al*. 2000).

Agricultural field studies have shown that biochar may increase soil fertility and crop yields. For example, a study spanning four years after biochar addition to the soil found increases up to 140% in maize grain yield in plots amended with biochar over the control (Major *et al*. 2010). The study attributed these increases in yield to the effect of biochar increasing soil CEC and making limiting nutrients such as calcium and magnesium more available (Major *et al.* 2010). A laboratory study using four biochars and two types of soil found that biochar treatments consistently decreased N_2O emissions (Singh *et al.* 2010). This effect may be explained by the increase in soil pH associated with biochar addition, which may increase the conversion of N_2O to N_2, the last step of denitrification (Van Zwieten *et al.* 2009). From these studies, it can be noted that biochar affects soil properties and function through a variety of complex mechanisms. Lacking in the literature is research aimed specifically at studying the effects of biochar on denitrification in wetland environments.

Molecular structure determines carbon lability

From the studies described above of how straw, topsoil, and biochar additions influence denitrification in soils, all three amendments seem to be effective to some extent in stimulating denitrification. However, because these studies were done under different conditions, the results are not directly comparable to each other. Therefore, it is unclear how these potential

amendments would compare in their influence on denitrification in restored

wetlands. Potential differences would likely result from differences in the

chemical structure of the amendments themselves. For example, the cellulose in

straw is a polysaccharide with many hydroxyl groups extending from the sugar

rings. This is dramatically different from the structure of biochar, which is

composed of sheets of aromatic carbon rings. Whereas the chemical structure of

straw allows it to be digested by bacteria relatively quickly, biochar is a more

stable form of carbon. In other words, the carbon in straw is more labile than the

carbon in biochar. Because the structure of the amendment affects how microbes

can use the organic carbon contained, differences in lability resulting from the

structure may lead to different effects on denitrification between amendments.

Project goals and hypotheses

The aim of this project was to investigate how different organic carbon

amendments affect denitrification rates in restored freshwater wetlands. The three

amendments of interest were straw, topsoil, and biochar, listed here in order of

decreasing carbon lability. We were interested in the effect of each amendment on

denitrification as well as how these effects differ across the different amendments,

either due to carbon lability or other chemical factors. In order to understand how

carbon type mediates changes in denitrification, we also analyzed other soil

factors, including organic carbon, respiration, microbial biomass, forms of

nitrogen, and pH, and examined their correlations with denitrification.

Additionally, we aimed to compare denitrification rates in restored wetlands

versus a reference wetland in order to gain insight into how restored wetlands function with respect to natural ones.

Experimentally, soil taken from plots amended with each of these materials were analyzed and compared against soil taken from control plots in order to determine the effects of different soil amendments on denitrification potential. We expected that the addition of straw, topsoil, or biochar increases denitrification potential in restored wetland soil samples six years after the restoration of the wetland. These three materials are sources of carbon, and carbon availability is necessary for optimal denitrification. Therefore the addition of amendments should have a positive effect on denitrification (Burchell *et al.* 2007). Specifically, comparing the different amendments against one another was expected to reveal that increase in denitrification is different depending on the type of amendment, with biochar providing the greatest increase. Because biochar has been shown to increase CEC and soil surface area, these effects may increase denitrification beyond the effect of carbon availability alone (Lehmann *et al.* 2006).

In order to compare denitrification in restored and natural wetlands, we collected samples from a reference site, a natural wetland ecologically comparable to a neighboring restored site. Comparisons of the restored wetland soils against natural wetland soils were expected to reveal that denitrification potential of soils from both control and treatment plots in the restored wetlands is still lower than that of soils taken from the natural wetland. Because soils are

estimated to take decades to develop, their functioning probably does not measure

up to that of natural wetlands after only six years, even with the addition of

amendments (Zedler and Callaway 1999).

Methods

Site Description

Four restored wetlands within 120 km of Ithaca, New York were our experimental sites (Figure 1). The wetlands were all restored in July 2007 on land previously used for agriculture by removing topsoil and subsequently constructing a flood control berm. The four sites are similar in topography, size, and land use history, but different in soil type and hydrology. The four wetlands are all classified as palustrine emergent depressional wetlands (Cowardin *et al.* 1979). In addition to these experimental sites, a neighboring ecologically comparable natural wetland provided reference samples. The geographic locations of the restored wetland sites as well as the natural wetland are given in Table 1.

Experimental design

In each restored wetland, twenty-five 2 m by 2 m plots were set up in rows such that the plots were separated from each other by 2 m. Five replicates of each treatment were randomly assigned to the plots. Apart from the controls in which no amendment was added, the three treatments examined in this experiment were straw, topsoil, and biochar. The amount of organic carbon added to a plot through each amendment was equalized at 8 kg for all treatments. The straw treatment used dry stalks of *Triticum aestivum* subsp. *spelta* grown organically at Oescher Farm in Newfield, New York. The topsoil treatment for each site used homogenized topsoil taken from the site itself. The biochar treatment used biochar produced from fast pyrolysis of a mixture of hardwoods at 450 °C with a retention

time of less than five seconds (Dynamotive, Vancouver, Canada). The set-up of the experiment is the same as in previous research published using data from these sites (Ballantine *et al.* 2012).

Sample collection

Soil samples were collected in May 2013, using a 2.5 cm diameter soil corer to take 10 cm-deep cores. At Sites 1, 2, and 3, samples were taken from every plot, whereas at Site 4, samples were taken from only three of the five plots of each treatment type due to inclement weather. For each plot, approximately four cores of soil were placed into a Ziploc bag. At the natural wetland, samples were taken by hand rather than with a corer. All soil samples were then transported in a cooler before being stored at 4 °C prior to analysis.

Denitrification enzyme activity assay

Denitrification potential was quantified via denitrification enzyme activity (DEA) assay (Smith and Tiedje 1979). In this method, excess amounts of factors that limit denitrification are added to a soil sample such that the amount of end product depends only on the amount of enzyme in the sample, thus quantifying denitification potential (Groffman *et al.* 1999). Because the main product of denitrification, N_2, is difficult to measure, acetylene is used to inhibit the last reduction step so that the end product is nitrous oxide, N_2O (Groffman *et al.* 1999). For the samples gathered in this experiment, soils were combined with 40 mg/kg of glucose and 100 mg N/kg of KNO_3, as well as 10 mg/kg of chloramphenicol to prevent enzyme production. These soil mixtures were

prepared in flasks, which were then evacuated and filled with N_2 to create anaerobic conditions. Subsequently, acetylene was added, and the samples were incubated on an orbital shaker. Samples of gas in the flask headspace taken at 30 and 90 minutes after the start of shaking were stored in pre-evacuated glass vials and analyzed for N_2O levels through gas chromatography, using a Shimadzu GC-14 with thermal conductivity detector. The raw data of parts per million of N_2O were converted to nanograms of nitrogen produced per gram of dry soil per hour, which is used to quantify denitrification potential.

Chloroform fumigation-incubation method

The chloroform fumigation-incubation method (CFIM) was used to measure respiration, microbial biomass carbon and nitrogen, nitrate and ammonium levels, potential net N mineralization, and potential net nitrification (Jenkinson and Powlson 1976). Soil samples were fumigated with chloroform in order to kill and break open microbial cells, and these fumigated samples were then inoculated with a small amount of unfumigated soil, which introduced microbes into the soil. These samples, as well as control samples which did not undergo fumigation, were incubated for 10 days. After incubation, gas samples were drawn from the headspace of the incubation containers and analyzed for carbon dioxide using a Shimadzu GC-14, which was used to calculate respiration, a proxy for carbon lability. Soil extracts were prepared using KCl, and extracted nitrate and ammonium was quantified using the Lachat Quikchem 8100 flow injection analyzer. The difference in respiration between fumigated and control

soil samples was used to calculate microbial biomass carbon and microbial biomass nitrogen, which are measures of how much carbon and nitrogen are contained within the soil microbial population. Potential rates of N mineralization and nitrification were obtained to supplement understanding of nitrogen cycle processes linked to denitrification.

Other measurements

In addition to the data collected using the methods described above, carbon content, nitrogen content, and pH were quantified for the same soil samples in another laboratory. Levels of organic and inorganic carbon were measured using RC 612 from LECO Corporation.

Data analysis

Using SPSS (IBM Corp, Version 21.0), ANOVA analyses were carried out in order to identify differences between treatments and sites for the four restored wetlands. In addition, linear regressions were done to gain insight into which factors mediate the effect of carbon amendments on denitrification. Data were checked for normality and transformed using a log transformation if required. Restored sites were compared to the natural reference wetlands quantitatively rather than using ANOVA due to the differences in experimental design and sampling regime between them.

Results

In the restored wetlands, amendments had a significant effect on denitrification potential as well as many associated soil properties. Denitrification potential was higher in plots amended with straw, topsoil, and biochar than in the unamended control plots. Denitrification potential was significantly correlated with total carbon, organic carbon, and microbial biomass nitrogen. However, denitrification potential in natural reference wetlands was still at least 50 times higher than in amended wetland plots.

Denitrification potential in restored wetlands

Averaged across the four restored wetland sites, there was a strong treatment effect on denitrification potential ($p<0.001$). Denitrification potential was significantly higher in plots where carbon amendments were added than in control plots ($p=0.031$ for straw, $p<0.001$ for both topsoil and biochar), and denitrification potential was marginally higher in biochar plots than in straw plots ($p=0.071$) (Figure 2a). In comparison to control plots, denitrification potential was approximately 3 times higher in straw plots, 8 times higher in topsoil plots, and 11 times higher in biochar plots. In addition to the treatment effect, denitrification potential also differed significantly across sites when values were averaged across the treatments in each site ($p=0.003$) (Figure 2b). Specifically, denitrification was lower in Site 1 than in Sites 2 and 3 ($p=0.022$ and $p=0.007$, respectively).

Taking each restored wetland site separately, treatment effects were significant in some sites but not in others. In Sites 1 and 2, there were no

significant differences in denitrification potential across the treatments (Figure 3). In Sites 3 and 4, denitrification potential was significantly higher in biochar plots than in control plots (p=0.027 for Site 3, p=0.003 for Site 4) (Figure 3).

Carbon in restored wetlands

There was a strong treatment effect on total carbon (p<0.001). Total carbon was higher in topsoil plots than in control and straw plots (p<0.001 and p=0.045, respectively), and highest in biochar plots relative to any other plot type (p<0.001 for both control and straw, p=0.001 for topsoil) (Figure 4a). Total carbon also differed significantly across sites (p<0.001). Specifically, total carbon was lower in Site 3 than in Sites 1 and 2 (p<0.001 for both), and lowest in Site 4 relative to all the other sites (p<0.001 for Sites 1 and 2, p=0.010 for Site 3) (Figure 4b).

Linear regression analysis showed a positive correlation between total carbon and denitrification potential when data from all treatments and sites were pooled (p=0.001, r^2=0.166) (Figure 5). Analyzing the sites separately yielded varying levels of significance (Table 2). There was no significant correlation between total carbon and denitrification potential in Site 1.

When looking at organic and inorganic carbon separately, organic carbon showed similar trends to total carbon, whereas inorganic carbon was quite different. For organic carbon, there were significant differences by treatment and site (p<0.001 for both). Like total carbon, organic carbon was higher in topsoil plots than in control plots and straw plots (p=0.007 and p=0.002, respectively), as

well as higher in biochar plots than in any other plot type ($p<0.001$ for control and straw; $p=0.007$ for topsoil) (Figure 6a). Organic carbon was lower in Site 3 than in Sites 1 and 2 ($p<0.001$ and $p=0.012$, respectively) and lower in Site 4 than in Sites 1 and 2 as well ($p=0.001$ and $p=0.023$, respectively) (Figure 6b).

As with total carbon, there was a positive correlation between organic carbon and denitrification potential for the overall pooled data ($p=0.001$, $r^2=0.169$) (Figure 7). Analyzing the sites separately yielded varying levels of significance (Table 3). There was no significant correlation between organic carbon and denitrification potential in Site 1.

There was a significant treatment effect on inorganic carbon ($p=0.028$). Inorganic carbon was either significantly or marginally significantly higher in straw plots than in other treatments ($p=0.007$ for control, $p=0.054$ for topsoil, and $p=0.090$ for biochar) (Figure 8a). There were also significant site differences ($p<0.001$). Inorganic carbon was lower in Site 4 than in all other sites ($p<0.001$ for all), and the levels were so low that Site 2, which had the highest level, had 40 times more inorganic carbon than Site 4. Site 2 had higher levels of inorganic carbon than all other sites ($p<0.001$ for all) (Figure 8b). There was no correlation between inorganic carbon and denitrification potential ($p=0.576$) (Figure 9).

Respiration in restored wetlands

There were no significant differences in respiration between treatments or sites ($p=0.569$ and $p=0.192$, respectively) (Figure 10). There was also no correlation between respiration and denitrification potential ($p=0.413$) (Figure 11).

Microbial biomass carbon and nitrogen in restored wetlands

There were no significant differences in microbial biomass carbon between treatments or sites (p=0.933 and p=0.467, respectively) (Figure 12). There was also no correlation between microbial biomass carbon and denitrification potential (p=0.151) (Figure 13).

Microbial biomass nitrogen differed significantly by both treatment and site (p=0.002 and p<0.001, respectively). Microbial biomass nitrogen was higher in topsoil plots than in control and straw plots (p=0.012 and p=0.018, respectively) (Figure 14a). Microbial biomass nitrogen was higher in Site 4 than in the other sites (p=0.041 for Site 1, p=0.002 for Site 2, and p<0.001 for Site 3), as well as lower in Site 3 than in all other sites (p<0.001 for Sites 1 and 4; p=0.003 for Site 2) (Figure 14b).

There was a positive correlation between microbial biomass nitrogen and denitrification potential for the overall pooled data (p=0.024, r^2=0.078) (Figure 15). Analyzing the sites separately yielded varying levels of significance (Table 4). There was no significant correlation between microbial biomass nitrogen and denitrification potential in Site 1.

There was also a positive correlation between total organic carbon and microbial biomass nitrogen for the overall pooled data (p=0.001, r^2=0.150) (Figure 16). Analyzing the sites separately yielded varying levels of significance (Table 5). There was no significant correlation between total organic carbon and microbial biomass nitrogen in Site 1.

Nitrogen in restored wetlands

There were no significant differences in total nitrogen between treatments or sites (p=0.567 and p=0.148, respectively) (Figure 17). There was also no correlation between total nitrogen and denitrification potential (p=0.693) (Figure 18).

When looking at nitrate specifically, there was no treatment effect (p=0.894) but there was a significant site effect (p<0.001) (Figure 19). Nitrate levels were higher in Site 3 than in any other site (p<0.001 for all), and higher in Site 2 than in Site 4 (p=0.046). There was no overall linear relationship between nitrate and denitrification potential (p=0.163) (Figure 20).

When looking at ammonium specifically, there were significant treatment and site differences (p<0.001 for both). Ammonium levels were higher in straw plots than in control and topsoil plots (p=0.002 and p=0.005, respectively), as well as higher in biochar plots than in control and topsoil plots (p=0.002 and p=0.004, respectively) (Figure 21a). Ammonium levels were higher in Site 1 than in Sites 2 and 3 (p<0.001 for both), as well as higher in Site 4 than in Sites 2 and 3 (p<0.001 for both) (Figure 21b). There was no linear relationship between ammonium and denitrification potential (p=0.785) (Figure 22).

C:N ratio in restored wetlands

The ratio between total carbon and total nitrogen, the C:N ratio, differed significantly by both treatment and site (p=0.002 and p=0.003, respectively). C:N ratio was significantly higher in biochar plots than in straw and topsoil plots

(p=0.016) (Figure 23a). C:N ratio was significantly higher in Site 2 than in Site 1 as well as higher in Site 1 than in Site 3 (p=0.011 and p=0.003 , respectively) (Figure 23b). There was no linear relationship between C:N ratio and denitrification potential (p=0.212) (Figure 24).

Other nitrogen cycling processes

There were significant treatment differences for potential net N mineralization (p=0.036), but no significant site differences (p=0.938) in the absolute rate. Despite the significant overall treatment effect, there were no significant post-hoc pair-wise differences in rate between treatments. However, potential net N mineralization was around zero in control plots, positive in straw plots, and negative in topsoil and biochar plots (Figure 25a). Potential net N mineralization was positive in Site 1, around zero in Site 2, and negative in Sites 3 and 4 (Figure 25b). There was no linear relationship between the absolute rate of potential net N mineralization and denitrification potential (p=0.159) (Figure 26).

For potential net nitrification, there were no significant treatment or site differences in the absolute rate (p=0.903 and p=0.621, respectively). Potential net nitrification was slightly negative in the topsoil plots (Figure 27a) and slightly negative in Site 3 (Figure 27b). There was no linear relationship between the rate of potential net nitrification and denitrification potential (p=0.631) (Figure 28).

pH in restored wetlands

pH was not significantly different across treatments (p=0.215) (Figure 29a), but there were significant site differences (p<0.001). Site 1 had a near

neutral pH averaging 6.94, Sites 2 and 3 were slightly basic with average pH

values of 7.30 and 7.24 respectively, and Site 4 was acidic with an average pH of

4.58. These differences were significant: Site 4 had a lower pH than any other site

($p<0.001$ for all), and Site 1 had a lower pH than Sites 2 and 3 ($p=0.001$ and

$p=0.004$, respectively) (Figure 29b). Linear regression found no correlation

between pH and denitrification potential ($p=0.264$) (Figure 30).

Comparisons against the natural reference wetland

Denitrification potential was much higher in the natural wetland than in all

the restored wetland treatments. Averaged across sites, denitrification potential in

the natural wetland was over 600 times higher than in control plots, 200 times

higher than in straw plots, 74 times higher than in topsoil plots, and 55 times

higher than in biochar plots (Figure 31).

Averaged across sites, total carbon was around 10 times higher in the

natural wetland than in control plots and 5 times higher than in biochar plots,

which had the highest total carbon (Figure 32a). For organic carbon, these values

were similar, with organic carbon around 15 times higher in the natural wetland

than in control plots and 6 times higher than in biochar plots (Figure 32b). The

natural wetland had around one-fifth of the total inorganic carbon as compared to

the restored treatment plots (Figure 32c).

Respiration was over 10 times higher in the natural wetland than in the

restored treatment plots (Figure 33). The wetland had around 10 times the

microbial biomass carbon (Figure 34a), but at least 30 times the microbial

biomass nitrogen as restored treatment plots (Figure 34b). These were highest when compared to just the control plots: 12 times for microbial biomass carbon, and 60 times for microbial biomass nitrogen.

Higher levels of nitrogen were observed in the natural wetland as well. Total nitrogen was at least 5 times higher in the natural wetland than in the restored treatment plots (Figure 35a). Whereas nitrate levels were not much higher in the natural wetland (Figure 35b), ammonium levels were around 20 times higher in the natural wetland than in straw or biochar plots and around 50 times higher than in the control or topsoil plots (Figure 35c).

The ratio of total carbon to total nitrogen was lower in the natural wetland than in the restored wetland plots (Figure 36). C:N ratio was around 14 in the natural wetland, around 17 in control plots averaged across sites, and around 28 in biochar plots averaged across sites.

The absolute rates of potential nitrogen cycle processes were much higher in the natural wetland soils than in samples from restored wetlands. Potential net N mineralization was close to 500 times higher in the natural wetland than in control plots and around 40 times higher than in biochar plots, which had the highest rate (Figure 37a). Potential nitrification was almost 90 times higher in the natural wetland than in control plots and close to 60 times higher than in biochar plots (Figure 37b).

The natural wetland had an acidic pH of 5.17 (Figure 38).

Discussion

Treatment effect on denitrification potential

The addition of carbon amendments to wetland soils increased denitrification potential significantly, when compared to untreated soils. Plots treated with biochar showed the highest improvements, with 11 times higher denitrification potential than in control plots (Figure 2a). Additionally, denitrification potential was 8 times higher in topsoil plots than in control plots and 3 times higher in straw plots than in control plots (Figure 2a). The addition of carbon amendments to soil is a promising technique for wetland restoration, because the treated plots have higher potential for carrying out biogeochemical functions, in this case denitrification. In a previous study on nitrogen removal processes in these same wetlands one and three years after restoration, it was found that denitrification potential was highest in topsoil plots, higher in biochar plots than in straw and control plots, and the same in straw and control plots (Ballantine *et al.* 2014). In this current study, we find different results, demonstrating that amendment effects can change over time. In order to investigate how the carbon amendments mediate improvements in denitrification potential, we must analyze treatment differences in basic soil properties and find correlations between these properties and denitrification potential.

Treatment effect on soil carbon

Treatment differences in total carbon and total organic carbon resembled those in denitrification potential. Plots treated with biochar or topsoil had

significantly higher total (Figure 4a) and organic (Figure 6a) carbon levels than

untreated control plots. Plots treated with straw did not have significantly

different levels of total or organic carbon when compared to the control plots.

Organic carbon accounted for most of the total carbon measured, but some

inorganic carbon was present as well (Figure 8). Plots treated with straw had

higher levels of inorganic carbon than the other treatments, with significant to

marginally significant differences.

Because the amount of amendment used during initial wetland restoration

was determined such that the same mass of carbon added was the same for each

plot, the differences in soil carbon content observed here are due to differences in

carbon lability, that is, how easily carbon in each amendment is used by microbes.

The carbon amendments were chosen such that there were different levels of

lability represented, with straw being the most labile and biochar being the least

labile. This intended trend was observed in earlier analyses of these wetland plots:

an average of data from one and three years after wetland restoration showed that

straw plots had the highest carbon lability, and topsoil, biochar, and control plots

were not significantly different from each other, though the mean lability for

topsoil was higher than that of biochar (Ballantine *et al.* 2014). However, the

present study shows that six years after restoration, there were no significant

differences in respiration by treatment (Figure 10a). Respiration as quantified by

the rate at which carbon dioxide is evolved from the soil serves as a proxy for the

pools of labile carbon present in soil. Comparing directly to values from the

previous study, changes in relative carbon lability between treatments was due to decreases in pools of labile carbon in both straw and topsoil plots, whereas there was no change in control and biochar plots. This suggests that as microbes have used up more labile elements in the straw and topsoil for cellular respiration, they have left behind less labile fractions that take longer to be decomposed. Perhaps the lack of change in control and biochar plots indicate that the carbon in these plots are of relatively homogenous lability, such that there are no fractions that are more labile than others, or that the most labile parts were already consumed before the first study. The low lability of biochar carbon is due to its stable aromatic structure, which is composed of carbon that is unavailable to microbes (Anderson *et al.* 2011). In our restored wetland plots, this stability has allowed it to remain in the soil at higher levels than in the other treatments, as seen from the total carbon data. In contrast, the labile parts of straw have probably been used by microbes for respiration and thus removed from the soil as carbon dioxide, resulting in low levels of total carbon similar to that in control plots, even though the same amount of carbon was originally added to all three treatment plot types.

Because there were no significant differences in denitrification potential among the three types of amended plots (Figure 2a), we cannot say that the enhancement of denitrification potential depended on the lability of the amendments. However, we see by comparing our results to those obtained at previous points in the development of the wetlands that the relative denitrification potentials between amended plots are shifting. For example, the previous study

found that topsoil plots had significantly higher denitrification potential than biochar plots, which in turn had significantly higher denitrification potential than straw plots (Ballantine *et al*. 2014). Therefore, continued monitoring of how denitrification potential changes with time for the different amendments may reveal differences attributable to carbon lability.

As originally expected, we found that total carbon (Figure 5), and specifically total organic carbon (Figure 7), was correlated with denitrification potential. The treatments introduced additional carbon into the soil, and in plots where more organic carbon was present, higher denitrification potential was observed. This relationship between organic carbon and denitrification potential is consistent with results from the literature. For example, it was found in a study of Louisiana coastal wetlands that total organic carbon was the only soil property studied to have a significant positive correlation with denitrification potential, suggesting that in those wetlands, organic carbon acted as the dominant factor controlling denitrification (Dodla *et al.* 2008). By adding different amounts of compost in a restored wetland, Sutton-Grier *et al.* (2009) found a significant positive correlation between soil organic matter, part of which is organic carbon, and denitrification potential.

Treatment effect on microbial biomass

One explanation for the relationship between organic carbon levels and denitrification potential is that microbial communities that carry out denitrification require sources of organic carbon, so higher levels of organic

carbon can support more denitrifying microbes. This explanation is supported by

a highly significant correlation between total organic carbon and microbial

biomass nitrogen (Figure 16), as well as a significant correlation between

microbial biomass nitrogen and denitrification potential (Figure 15). Microbial

biomass nitrogen is a measure of how much of the soil nitrogen is associated with

microbes, such as in microbial proteins and DNA, and is thus an indicator of the

size of microbial communities. In our restored wetlands, treatment differences in

microbial biomass nitrogen were similar to those seen for organic carbon, namely

that microbial biomass nitrogen was significantly higher in topsoil plots than in

control and straw plots, and it was intermediate in biochar plots (Figure 14a). The

correlation between microbial biomass nitrogen and denitrification shows that

larger microbial communities have the ability to carry out higher rates of

denitrification as compared to smaller communities. Past analysis of these

restored wetlands found a similar significant correlation between microbial

biomass nitrogen and denitrification, suggesting that this relationship has been

important over the course of development for these soils (Ballantine *et al*. 2014).

This correlation is consistent with Groffman *et al.* (1996), who found a significant

positive correlation between microbial biomass nitrogen and denitrification

potential in different wetland types.

Unlike with microbial biomass nitrogen, there were no significant

treatment differences in microbial biomass carbon (Figure 12a), and no

correlation was observed between microbial biomass carbon and denitrification

potential (Figure 13). This aspect is not consistent with the Groffman *et al.* study (1996), in which microbial biomass carbon, in addition to microbial biomass nitrogen, was significantly correlated with denitrification potential.

The role of carbon in supporting microbial communities

The mechanisms by which microbial communities benefit from the carbon amendments establish the connection between carbon availability and denitrification potential. Heterotrophic denitrifying bacteria require carbon for energy production, harvesting electrons by breaking down organic carbon during anaerobic respiration (Knowles 1982). Theoretically, the amendments would all have provided these bacteria with additional organic carbon beyond what was present in the unamended restored wetland soils, and thus more resources were available to support the development of larger microbial populations. Straw and topsoil both contain phenolic substances which can be readily used by microbes (Ballantine *et al.* 2014). However, most of the carbon in biochar has low lability because it is part of stable aromatic systems and thus unavailable as an electron source for microbial respiration. Therefore, biochar must be mediating increased denitrification potential by some other mechanism.

The unique structure of biochar gives rise to properties that can influence soil biogeochemistry and in turn promote microbial activity. One of the major properties of biochar is its ability to increase the CEC of soil, because biochar contains many negatively charged groups that can associate with cations through electrostatic interactions (Liang *et al.* 2006). Soils with higher CEC can adsorb

greater amounts of positively charged soil nutrients, thus making them more available to microbes. For example, biochar can adsorb organic molecules through electrostatic interactions, which may serve to make these molecules more available to microbes (Joseph *et al.* 2010). Additionally, biochar has been found to remove ammonium from solution through adsorption (Yao *et al.* 2012). In our study, we do find that ammonium levels are significantly higher in biochar plots than in control plots (Figure 21a), which may illustrate the ability of biochar to adsorb ammonium and therefore promote nitrification and subsequent denitrification. Another mechanism by which biochar is thought to benefit microbes is the ability of its porous structure to act as a habitat which microbes can colonize (Joseph *et al.* 2010). A recent study investigating changes in soil microbial communities in response to biochar addition found that the largest relative abundance increase was for two families of bacteria which can carry out denitrification, suggesting that biochar amendments can promote denitrification by selecting for denitrifying bacteria (Anderson *et al.* 2011). Given all these different mechanisms, we see that though the stable carbon in biochar is not directly available to microbes as food, it can provide other benefits which are able to promote denitrification in wetland soils. Under this interpretation, the correlation found in this study between organic carbon and microbial biomass nitrogen does not necessarily identify a simple relationship in which higher levels of carbon feed larger microbial communities. Rather, the structure and properties

of the added carbon can influence soil chemistry and promote the growth and functioning of microbes.

Treatment effect on nitrogen and other nitrogen processes

Denitrification is one of the many microbial nitrogen transformation processes that take place in soil and thus can be understood in the context of the various forms of nitrogen as well as other nitrogen cycle processes. While soil carbon is a key factor influencing denitrification, soil nitrogen must be considered as well. This is illustrated by a study finding that the addition of organic carbon alone, in the form of glucose, to wetland soils does not increase denitrification rates (Merrill and Zak 1992). In our experiment, there were no significant differences in either total nitrogen (Figure 17a) or nitrate (Figure 19a) among the treatments. This was somewhat surprising given that there was a strong treatment effect on denitrification potential, a process closely related to nitrate. In one way, it is reasonable that the different treatments plots in each site would receive similar amounts of nitrate from the water, since they all share the same wetland. But on the other hand, one might expect plots that carry out denitrification at higher rates to have lower nitrate levels because more is converted to nitrous oxide or N_2 gas. It does not seem to be the case that amended plots start out with a higher level of nitrate than control plots as a result of higher nitrification rates, because nitrification potential was not significantly different among treatments (Figure 27a). So, the fact that we did not observe lower nitrate levels in plots amended with carbon sources than in control plots may illustrate the gap between

the denitrification potentials quantified in the laboratory and the actual denitrification rates in the wetland soil.

This disparity has been quantified previously for wetland soils: one study found that potential denitrification rates measured after nitrate enrichment ranged from 1 to 1100 μmol $N/m^2/h$ whereas rates measured without nitrate addition were generally under 1 μmol $N/m^2/h$ (Seitzinger 1994). In our experiment, it is likely that while the addition of carbon amendments led to the establishment of larger microbial communities that were able to carry out higher rates of denitrification when both glucose and nitrate were supplied, these microbes are not actually carrying out such high rates of denitrification at the wetland sites because of limiting factors such as low levels of nitrate, which often controls denitrification rates in wetlands (Seitzinger 1994). In our restored wetlands, the mean nitrate levels measured for each type of treatment were around 1 μg N/g dry soil, which may be limiting denitrification. Therefore, actual rates of denitrification in the wetlands may not be as high as the denitrification potentials measured, and the consistent low level of nitrate among treatments may indicate that denitrification rates are limited by soil nitrate levels.

While there were no treatment differences in nitrate, ammonium levels were different, with soil ammonium being more than twice as high in straw and biochar plots than in control and topsoil plots (Figure 21a). Ammonium is produced from mineralization, which is the microbial decomposition of organic nitrogen, for example in proteins, into the inorganic form ammonium. The reverse

process is immobilization, in which ammonium is taken up and incorporated by microbes into organic forms. Averaged across the restored wetland sites, potential net N mineralization was positive in straw plots and negative in topsoil and biochar plots (Figure 25a), indicating that more ammonium is being generated than incorporated in straw plots, whereas the reverse is true in topsoil and biochar plots. It is surprising that straw and biochar plots both had similarly high levels of ammonium, even though potential net N mineralization was dominant in straw plots while potential immobilization was dominant in biochar plots. This demonstrates the complex interactions between nitrogen cycle processes and associated compounds. As with denitrification potential, potential net N mineralization values may not reflect the actual rates in the wetland soils, because the laboratory measurements were taken in aerobic conditions, and mineralization rates are faster in aerobic conditions than in anaerobic ones (Vymazal 2007).

Ammonium is ultimately related to denitrification, because ammonium can be converted to nitrate through the microbial process of nitrification. In fact, it has been suggested that the major source of nitrate for denitrification is nitrification of ammonium (Seitzinger 1994). In our study, there was no significant treatment effect on either potential nitrification (Figure 27a) or nitrate levels (Figure 19a), though straw and biochar treatments had higher ammonium levels than the other plots. Nitrification is dependent on factors beyond ammonium availability, including temperature, pH, and dissolved oxygen

(Vymazal 2007). So, it is likely that nitrification rates were limited not by ammonium concentration, but by another factor.

C:N ratio as an indicator for nitrogen availability

The ratio of total carbon to total nitrogen was significantly higher in biochar plots than in straw and topsoil plots, while control plots had an intermediate C:N ratio (Figure 23a). Microbes require carbon for energy as well as nitrogen for synthesizing cellular components such as proteins. Therefore, if there is not enough nitrogen relative to the available carbon, microbes tend to immobilize nitrogen rather than carry out mineralization (Robertson and Groffman 2007). Therefore, denitrification is promoted by soil with low C:N ratio, because this promotes mineralization of nitrogen into inorganic forms which can then be transformed into nitrate and used for denitrification. This was the case in the previous study of these wetland sites, in which topsoil plots had the lowest C:N ratio, positive potential net N mineralization, and the highest denitrification potential (Ballantine *et al.* 2014). In this current study, biochar plots had the highest C:N ratio and also had a high level of negative potential net N mineralization, i.e. potential immobilization (Figure 25a). This result is consistent with the model of how C:N ratio controls the balance of mineralization and immobilization described above. However, it is surprising, given the high potential immobilization, that denitrification potential was also the highest in biochar plots, because if nitrogen is incorporated into microbial biomass, it cannot be used for denitrification.

Denitrification potential varied by site

There were significant site differences, with denitrification potential being higher in Sites 2 and 3 than in Site 1 while Site 4 had an intermediate denitrification potential not significantly different from any of the other sites (Figure 2b). Measurements taken previously from these same restored wetlands showed that denitrification potential was higher in Sites 4 and 3 than in Site 2, and Site 1 had an intermediate denitrification potential (Ballantine *et al.* 2014). That denitrification potential is different between the four restored wetland sites is not particularly surprising, because the sites differ in key properties such as baseline carbon levels, hydrology, and soil type (Ballantine *et al.* 2012). As we have seen in investigating differences in denitrification potential by treatment, carbon is an important factor affecting denitrification potential. Therefore, it makes sense that differences in baseline carbon levels between sites would also lead to differences in denitrification potential in the four restored wetlands. By analyzing how soil properties differ between sites, we can try to understand the observed variations in denitrification potential by site.

pH varied by site

One major site difference was soil pH (Figure 29b). Averaging across treatments, Sites 2 and 3 had pH values slightly above neutral at 7.30 and 7.24 respectively, Site 1 had a lower, neutral pH of 6.94, and Site 4 had a much lower pH of 4.58. Denitrification is optimized at a pH range of 6 to 8, so the acidity of Site 4 soil may account for the lower denitrification potential observed there.

However, Site 1 had the lowest denitrification potential even though its acidity was near neutral, suggesting that factors other than pH are needed to explain the low denitrification potential levels in Site 1.

When denitrification potential was determined for each treatment-by-site combination (Figure 3), it was found that in the acidic Site 4, control soils had no measurable denitrification potential, and straw and topsoil soils had lower denitrification potential than their counterparts in the other sites. Surprisingly, biochar soils in Site 4 actually had the second highest denitrification potential out of all the possible treatment-by-site combinations. This peculiar feature of biochar plots in the acidic restored wetland may be an illustration of the pH buffering capacities of biochar. Previous studies have found that the CEC of biochar enables it to serve as a buffer, because the oxygen-containing functional groups on the biochar surface can associate with protons in acidic conditions and dissociate with protons, hence releasing them into solution, in basic conditions (Yuan *et al.* 2011, Xu *et al.* 2012). In Site 4 biochar plots, the mean pH measured was 4.73, whereas it was around 4.5 in the other plot types, although this difference was not statistically significant, and 4.73 is still quite acidic. It may be the case that the method used to measure pH does not accurately reflect the ability of biochar to act as a buffer, or perhaps biochar is providing some other means of maintaining high levels of denitrification potential.

Carbon varied by site

Total carbon was highest in Sites 1 and 2, significantly lower in Site 3, and significantly lower still in Site 4 (Figure 4b). Total organic carbon was significantly higher in Sites 1 and 2 than in Sites 3 and 4 (Figure 6b), and total inorganic carbon was highest in Site 2, significantly lower in Sites 1 and 3, and almost zero in Site 4 (Figure 8b). Given the strong correlation we found of total organic carbon with denitrification potential, it is surprising that Site 1 had the lowest denitrification potential of all the sites even though it contained the most organic carbon of all the sites. In fact, when linear regression analyses were performed on each site separately, Site 1 was the only site that did not show a significant correlation between total organic carbon and denitrification potential (Table 3). Whereas regression analysis found high r^2 values indicating that variation in total organic carbon accounted for half, a third, or three-fourths of the variation in denitrification potential in Sites 2, 3 and 4, respectively, the correlation was not significant in Site 1. It seems that in general, total organic carbon is a very important factor affecting denitrification potential, but Site 1 strangely does not demonstrate this relationship and has lower denitrification potential than would be expected from its high organic carbon levels. In order to examine this incongruity further, we can examine differences in microbial biomass amongst the restored sites.

Microbial biomass nitrogen varied by site

Just as there were no significant differences in microbial biomass carbon by treatment, it also did not differ significantly by site (Figure 12b). However, the restored wetland sites had different levels of microbial biomass nitrogen: it was highest in Site 4, significantly lower in Sites 1 and 2, and significantly lower still in Site 3 (Figure 14b). Again, Site 1 was the only site in which there was no significant correlation between microbial biomass nitrogen and denitrification potential, whereas variation in microbial biomass nitrogen accounted for large portions of the variation in denitrification potential in the other three sites (Table 4). In determining whether total organic carbon correlated with microbial biomass nitrogen, we found again that Site 1 was the only site for which no significant correlation was present (Table 5). In all of these cases, the lack of correlation in Site 1 had the effect of depressing the r^2 value when the overall correlation was analyzed for the entire pooled data containing all sites.

Site 1 does not follow trends consistent in other sites

The high degree of variation among our experimental wetland sites is illustrated by the idiosyncrasies of Site 1 described above. The correlations of organic carbon and microbial biomass nitrogen to denitrification potential were consistent in the other three sites, allowing us to confidently identify these properties as important factors governing denitrification potential. This highlights the importance of having several different sites to analyze in this type of experiment, because important trends may not necessarily be present in each

individual restored wetland. Despite the results from the linear regression that show no significant correlation of organic carbon and microbial biomass nitrogen in regards to denitrification potential, the scatterplots do still show the hint of correlation in Site 1 data points (Figure 16). So, it seems like these factors would be correlated with denitrification potential in Site 1 if it were not for some source of interference, that is, some factor that represses denitrification potential even at high levels of organic carbon and microbial biomass nitrogen. At present, it is unclear what this limiting factor might be, because while denitrification potential was lowest in Site 1, none of the other soil properties we analyzed were lowest here.

Nitrate varied by site

There were significant differences in nitrate levels by site: Site 3 had the highest nitrate level, Site 2 had a significantly lower nitrate level, Site 1 had an intermediate level between Sites 2 and 4 that was not significantly different from either, and Site 4 had the lowest nitrate level (Figure 19b). Previously, it has been shown in both field and laboratory experiments that higher nitrate levels promote higher rates of denitrification. In comparing wetlands in a nutrient-enriched residential site and an undeveloped control site, it was found that denitrification levels were higher in the enriched site (Hanson *et al.* 1994). Another study compared denitrification in wetland soils before and after nitrate addition to the overlying water and found that denitrification rates increased as a result (Seitzinger 1994). In a laboratory experiment in which soils were given controlled

nitrate concentrations at different concentrations, it was also found that denitrification potential was higher in samples with higher nitrate (Sirivedhin and Gray 2006). However, we did not observe a correlation between soil nitrate levels and denitrification potential (Figure 20).

Denitrification potential in the natural wetland

While carbon amendments, especially biochar, have increased denitrification potential in comparison to control plots, denitrification potential was still at least 50 times higher in the natural wetland than in restored wetland amended plots (Figure 31). When considering only control plots, denitrification potential was 600 times higher in the natural wetland than in unamended restored wetland plots. This disparity in denitrification potential between restored and natural wetlands is of a larger magnitude than those found in other studies. Hunter and Faulkner (2001) found that for bottomland hardwood wetlands, denitrification potential was was 657 ng N/g soil/hour for natural wetlands and 167 ng N/g soil/hour for restored wetlands. Peralta *et al.* (2010) compared floodplain forest wetlands and found that denitrification potential was around 10 times higher in the natural wetland than in high-elevation restored wetlands and around 3 times higher in the natural wetland than in low-elevation restored wetlands. The results from our current study are consistent with the literature in that denitrification potential is higher in the natural wetland than in the restored wetlands, but the magnitude of this difference is very large compared to similar comparisons in other studies.

Other soil properties in the natural wetland

Analyzing correlations of various soil properties to denitrification potential has revealed that organic carbon and microbial biomass nitrogen greatly influence denitrification potential. In terms of actual denitrification rates, ammonia and nitrate concentrations are important factors as well. Thus, it is consistent with the difference observed in denitrification potential that the natural reference wetland had higher levels of many other soil parameters when compared to the restored wetlands. Most notably, it had 15 times the organic carbon (Figure 32b) and almost 60 times the microbial biomass nitrogen (Figure 34b) as control plots in the restored wetlands. Given the correlations of these properties to denitrification potential, these differences may explain why denitrification potential is so much higher in the natural wetland than in the restored sites. Most of the other soil properties analyzed in this experiment were also higher in the natural wetland than in the restored sites. Two exceptions are that C:N ratio was lowest in the natural wetland (Figure 36), and that the natural wetland had a pH of 5.17 (Figure 38). The low C:N ratio illustrates that the natural wetland has not only more carbon, but also more nitrogen than the restored wetlands, and that there is proportionally more nitrogen per unit carbon in the natural wetland than in the restored wetlands. The acidity observed in the natural wetland may be due to the generation of organic and inorganic acids as organic matter decomposes.

One property that we have not yet analyzed is microbial community structure, which is potentially a very important aspect. In a study done by Peralta *et al.* (2010), it was found that variation in the composition of denitrifying bacteria between restored wetlands and natural reference wetlands accounted for 40% of the variation in denitrification potential between these wetlands, thus explaining why dentrification potential was higher in the natural wetland. Therefore, it is possible that a similar difference exists in the wetlands we studied and contributes to the large difference in denitrification potentials observed.

The role of amendments in achieving functional equivalency

Using denitrification as an example that may be generalized to wetland functions in general, it is clear that the restored wetlands, six years after restoration, are not carrying out ecosystem functions to the same extent as a comparable natural wetland. This result is not surprising, because previous studies have found that it could take decades for many properties in restored wetlands to reach equivalency to natural wetlands (Zedler and Callaway 1999; Ballantine and Schneider 2009). However, the fact that we found denitrification potential to be up to 11 times higher in the amended plots than in the control plots suggests that the use of organic carbon amendments is promising way to enhance function in restored wetlands and accelerate their functional equivalency to natural levels. Six years after restoration, there are no significant differences in denitrification potential among the three different amendment types, though quantitatively, biochar plots have the highest denitrification potential. With continued monitoring,

we may be able to identify which of the amendments used is the most effective in promoting wetland functions.

Future directions

The most essential continuation of this research is continued sampling and analysis over time, such that changes in treatment effects over time can be identified. The chemical structure of the amendments will continue to change as they are digested and weathered, therefore, they may have different effects on soil properties and denitrification potential in the future. Additionally, being able to plot the trajectory of the development of these restored wetlands would provide insight into the effectiveness of carbon amendments in helping restored wetland soils reach functional equivalency to natural wetlands.

Though denitrification potential values gathered from DEA are useful for comparing different treatments and sites and are correlated with actual rates, they do not necessarily reflect actual rates, particularly when wetlands are limited by a nutrient, for example nitrate. In order to better understand the dynamics of the restored wetlands, it will be important to measure actual rates of denitrification and other nitrogen cycling processes.

Another key aspect of this research is understanding the mechanisms of how the three different carbon amendments influence soil properties and functions. Perhaps a fruitful way of understanding how microbial communities are using and interacting with the different amendments would be to chemically analyze soil samples to identify specific carbon moieties (Dodla *et al.* 2008). This would be

especially beneficial to studying how biochar promotes denitrification, because it could identify functional groups that play an important role in adsorbing cations and other nutrients. Comparing CEC among treatments may also provide insight into the role of biochar in the restored wetland soil.

Because microbial community structure has been found to be relevant to biochar amended soils as well as to the disparity between restored and natural wetlands, a promising direction of inquiry would be to identify and compare the microbial communities present in our wetlands. If we do find that microbial communities differ between restored sites and the natural wetland, it may be worthwhile to consider amending restored wetlands with soil transplanted from a natural wetland.

Conclusion

Through this investigation of denitrification and related soil properties in restored wetlands, we found that the incorporation of carbon soil amendments during initial restoration resulted in significantly higher values of denitrification potential compared to unamended plots six years later. Denitrification potential was significantly correlated with organic carbon as well as with microbial biomass nitrogen, which suggests that it is important for carbon sources to be present in large enough quantities so that they can support the respiration of microbial communities which can carry out denitrification. However, there are complex interactions between microbes and carbon in that organic carbon amendments can provide other benefits to the soil, such as the ability of biochar to increase soil CEC. Denitrification potential as well as many of the soil properties studied varied by site, and correlations were not consistent among all four sites. The high levels of site variability highlight the importance of analyzing multiple similar replicate sites. While we saw that carbon amendments successfully increased the function of restored wetlands compared to control plots, soil properties and processes in even the amended plots are still much lower than those in a natural reference wetland. This disparity illustrates the limitations of wetland restoration in generating wetlands that are functionally equivalent to natural ones. But, through continued tracking of how the amended restored wetland plots develop over time, we can gain insight into the long-term efficacy of carbon amendment addition and suggest improvements to restoration practices.

References

Anderson, C. R., L. M. Condron, T. J. Clough, M. Fier, A. Stewart, R. A. Hill, and R. R. Sherlock. 2011. Biochar induced soil microbial community change: Implications for biogeochemical cycling of carbon, nitrogen and phosphorus. Pedobiologia **54**:309-320.

Bachand, P. A. M. and A. J. Horne. 2000. Denitrification in constructed free-water surface wetlands: II. Effects of vegetation and temperature. Ecological Engineering **14**:17-32.

Ballantine, K. A., P. M. Groffman, J. Lehmann, and R. L. Schneider. 2014. Stimulating nitrate removal processes of restored wetlands. Environmental Science and Technology **48**:7365-7373.

Ballantine, K. and R. Schneider. 2009. Fifty-five years of soil development in restored freshwater depressional wetlands. Ecological Applications **19**:1467-1480.

Ballantine, K., R. Schneider, P. Groffman, and J. Lehmann. 2012. Soil properties and vegetative development in four restored freshwater depressional wetlands. Soil Science Society of America Journal **76**:1482-1495.

Bedford, B. L., D. J. Leopold, and J. P. Gibbs. 2001. Wetland ecosystems. Encyclopedia of Biodiversity **5**:781-804.

Bridgham, S. D., J .P. Megonigal, J. K. Keller, N. B. Bliss, and C. Trettin. 2006. The carbon balance of North American wetlands. Wetlands **26**:889-916.

Bridgham, S. D., H. Cadillo-Quiroz, J. K. Keller, and Q. Zhuang. 2013. Methane emissions from wetlands: biogeochemical, microbial, and modeling perspectives from local to global scales. Global Change Biology **19**:1325-1346.

Brinson, M. M. and A. I. Malvarez. 2002. Temperate freshwater wetlands: types, status, and threats. Environmental Conservation **29**:115-133.

Brown, S. C. and P. L. M. Veneman. 2001. Effectiveness of compensatory wetland mitigation in Massachusetts, USA. Wetlands **21**:508-518.

Bruland G. L. and C. J. Richardson. 2004. Hydrologic gradients and topsoil additions affect soil properties of Virginia created wetlands. Soil Science Society of America Journal **68**:2069-2077.

Burchell M. R.,II, R. W. Skaggs, C. R. Lee, and S. Broome. 2007. Substrate organic matter to improve nitrate removal in surface-flow constructed wetlands. Journal of Environmental Quality **36**:194-207.

Chmura, G. L., S. C. Anisfeld, D. R. Cahoon, and J .C. Lynch. 2003. Global carbon sequestration in tidal, saline wetland soils. Global Biogeochemical Cycles **17**:22.1-22.12

Christensen, P. B. and J. Sørensen. 1986. Temporal variation of denitrification activity in plant-covered, littoral sediment from Lake Hampen, Denmark. Applied and Environmental Microbiology **51**: 1174-1179.

Costanza, R., R. d'Arge, R. de Groot, S. Farber, M. Grasso, B. Hannon, K. Limburg, S. Naeem, R. V. O'Neill, J. Paruelo, R. G. Raskin, R. Sutton, and M. van den Belt. 1997. The value of the world's ecosystem services and natural capital. Nature **387**:253-260.

Cowardin, L. M. 1979. Classification of wetlands and deepwater habitats of the US. DIANE Publishing.

Dahl, T. E. 1990. Wetland losses in the United States, 1780's to 1980's. Report to the Congress.

Davidsson, T. E. and M. Stahl. 2000. The influence of organic carbon on nitrogen transformations in five wetland soils. Soil Science Society of America Journal **64**:1129–1136.

DeLuca, T. H., M. D. MacKenzie, and M. J. Gundale. 2009. Biochar effects on soil nutrient transformations. Biochar for Environmental Management: Science and Technology 251-270.

Diaz, R. J. 2001. Overview of hypoxia around the world. Journal of Environmental Quality **30**:275-281.

Dodla, S. K., J. J. Wang, R. D. DeLaune, and R. L. Cook. 2008. Denitrification potential and its relation to organic carbon quality in three coastal wetland soils. Science of the Total Environment **407**:471-480.

Erwin, K. L. 2009. Wetlands and global climate change: the role of wetland restoration in a changing world. Wetlands Ecology and Management **17**:71-84.

Faulwetter, J. L., V. Gagnon, C. Sundberg, F. Chazarenc, M. D. Burr, J. Brisson, A. K. Camper, and O. R. Stein. 2009. Microbial processes influencing performance of treatment wetlands: A review. Ecological Engineering **35**:987-1004.

Gale, P. M., I. Devai, K. R. Reddy, and D. A. Graetz. 1993. Denitrification potential of soils from constructed and natural wetlands. Ecological Engineering **2**:119-130.

GAO. 2005. Corps of Engineers does not have an effective oversight approach to ensure that compensatory mitigation is occurring. Report GAO-05-898. GAO, Washington DC.

Gibson, K. D., J .B. Zedler, and R. Langis. 1994. Limited response of cordgrass (Spartina foliosa) to soil amendments in a constructed marsh. Ecological Society of America **4**:757-767.

Groffman, P. M., G. C. Hanson, E. Kiviat, and G. Stevens. 1996. Variation in microbial biomass and activity in four different wetland types. Soil Science Society of America Journal **60**:622-629.

Groffman, P. M., E. A. Holland, D. D. Myrold, G. P. Robertson, Z. XiaoMing, D. C. Coleman, C. S. Bledsoe, and P. Sollins. 1999. Denitrification. Standard soil methods for long-term ecological research: 272-288.

Hanson, G. C., P. M. Groffman, and A. J. Gold. 1994. Denitrification in riparian wetlands receiving high and low groundwater nitrate inputs. Journal of Environmental Quality **23**:917-922.

Hernandez, M. E. and W. J. Mitsch. 2007a. Denitrification in created riverine wetlands: influence of hydrology and season. Ecological Engineering **30**:78-88.

Hernandez, M. E. and W. J. Mitsch. 2007b. Denitrification potential and organic matter as affected by vegetation community, wetland age, and plant introduction in created wetlands. Journal of Environmental Quality **36**:333-342.

Hey, D. L., D. McGuiness, M. N. Beorkrem, D. R. Conrad, and B. D. Hulsey. 2002. Flood damage reduction in the Upper Mississippi River Basin: an ecological means. McKnight Foundation, Minneapolis, MN.

Hough, P. and M. Robertson. 2009. Mitigation under Section 404 of the Clean Water Act: where it comes from, what it means. Wetlands Ecology and Management **17**:15-33.

Hunter, R. G. and S. P. Faulkner. 2001. Denitrification potentials in restored and natural bottomland hardwood wetlands. Soil Science Society of America Journal **65**:1865-1872.

IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.

IUCN. 1996. Guidelines for aid agencies for improved conservation and sustainable use of tropical and sub-tropical wetlands. Paris: Organ. Econ. Co-op. Dev.

Jenkinson, D. S. and D. S. Powlson. 1976. The effects of biocidal treatments on metabolism in soil: A method for measuring soil biomass. Soil Biology and Biochemistry **8**:209–213.

Joabsson, A., T. R. Christensen, and B. Wallén. 1999. Vascular plant controls on methane emissions from northern peatforming wetlands. Trends in Ecology and Evolution **14**:385-388.

Joseph S. D., M. Camps-Arbestain, Y. Lin, P. Munrow, C. H. Chia, J. Hook, L. van Zwieten, S. Kimber, A. Cowie, B. P. Singh, J. Lehmann, N. Foidl, R. J. Smernik, and J. E. Amonette. 2010. An investigation into the reactions of biochar in soil. Australian Journal of Soil Research **48**:501-515.

Knowles, R. 1982. Denitrification. Microbiological Reviews **46**:43-70.

Kusler, J. A. 1988. Urban wetlands and urban riparian habitat: battleground or creative challenge for the 1990s. In Proceedings of the National Wetland Symposium, Urban Wetlands. Association of Wetland Managers, Berne, NY, USA.

Larkin, D. J., G. Vivian-Smith, and J. B. Zedler. 2005. Topographic heterogeneity theory and applications to ecological restoration. In Foundations of Restoration Ecology. Ed. D Falk, M Palmer, JB Zedler, Washington DC: Island. In press.

Lehmann J., J. Gaunt, and M. Rondon. 2006. Bio-char sequestration in terrestrial ecosystems – a review. Mitigation and Adaptation Strategies for Global Change **11**:403-427.

Liang B., J. Lehmann, D. Solomon, and J. Kinyangi. 2006. Black carbon increases cation exchange capacity in soils. Soil Science Society of America Journal **70**:1719-1730.

Major, J., M. Rondon, D. Molina, S. J. Riha, and J. Lehmann. 2010. Maize yield and nutrition during 4 years after biochar application to a Colombian savanna oxisol. Plant Soil **333**:117-128.

Martin, J. F. and K. R. Reddy. 1997. Interaction and spatial distribution of wetland nitrogen processes. Ecological Modelling **105**:1-21.

Martin, T. L., N. K. Kaushik, J. T. Trevors, and H. R. Whiteley. 1998. Review: denitrification in temperate climate riparian zones. Water, Air, and Soil Pollution **111**:171-186.

Matthews, J. W. and A. G. Endress. 2008. Performance criteria, compliance success, and vegetation development in compensatory mitigation wetlands. Environmental Management **41**:130-141.

McAllister, D. E., J. F. Craig, N. Davidson, S. Delaney, and M. Seddon. 2000. Biodiversity impacts of large dams. A contributing paper to the World Commission on Dams.

Merrill, A. G. and D. R. Zak. 1992. Factors controlling denitrification rates in upland and swamp forests. Canadian Journal of Forest Research **22**:1597-1604.

Mitsch, W. J., A. Nahlik, P. Wolski, B. Bernal, L. Zhang, and L. Ramberg. 2010. Tropical wetlands: seasonal hydrologic pulsing, carbon sequestration, and methane emissions. Wetlands Ecology and Management **18**:573-586.

Mitsch, W. J., B. Bernal, A. M. Nahlik, U. Mander, L. Zhang, C. J. Anderson, S. E. Jørgensen, and H. Brix. 2013. Wetlands, carbon, and climate change. Landscape Ecology **28**:583-597.

Nicholls, R. J., F. M. J. Hoozemans, and M. Marchand. 1999. Increasing flood risk and wetland losses due to global sea-level rise: regional and global analyses. Global Environmental Change **9**:S69-S87.

Nichols, D. S. 1983. Capacity of natural wetlands to remove nutrients from wastewater. Journal of the Water Pollution Control Federation **55**:495-505.

NRC. 2001. Compensating for wetland losses under the Clean Water Act. National Academy Press, Washington DC.

Peralta, A. L., J. W. Matthews, and A. D. Kent. 2010. Microbial community structure and denitrification in a wetland mitigation bank. Applied and Environmental Microbiology **76**:4207-4215.

Pietikäinen, J., O. Kiikkilä, and H. Fritze. 2000. Charcoal as a habitat for microbes and its effect on the microbial community of the underlying humus. Oikos **89**:231-242.

Prasad, R. and J. F. Power. Nitrification inhibitors for agriculture, health, and the environment. Advances in Agronomy **54**:233-281.

Richardson, C. J. 1994. Ecological functions and human values in wetlands: a framework for assessing forestry impacts. Wetlands **14**:1-9.

Richter, J. and M. Roelcke. 2000. The N-cycle as determined by intensive agriculture – examples from central Europe and China. Nutrient Cycling in Agroecosystems **57**:33-46.

Robertson, G. P. and P. M. Groffman. 2007. Nitrogen transformation. Pages 341-364 in E. A. Paul, ed. Soil Microbiology, Biochemistry, and Ecology. Springer, New York, New York, USA.

Sather, J. H. and D. Smith. 1984. An overview of major wetland functions and values. U.S. Fish and Wildlife Service, Washington, D.C.

Schipper, L. A., C. G. Harfoot, P. N. McFarlaine, and A. B. Cooper. 1994. Anaerobic decomposition and denitrification during plant decomposition in an organic soil. Journal of Environmental Quality **23**:923-928.

Seitzinger, S. B. 1994. Linkages between organic matter mineralization and denitrification in eight riparian wetlands. Biogeochemistry **25**:19-39.

Seo, D. C. and R. D. DeLaune. 2010. Fungal and bacterial mediated denitrification in wetlands: Influence of sediment redox condition. Water Research **44**:2441-2450.

Singh, B. P., B. J. Hatton, B. Singh, A. L. Cowie, and A. Kathuria. 2010. Influence of biochars on nitrous oxide emission and nitrogen leaching from two contrasting soils. Journal of Environmental Quality **39**:1224-35.

Sirivedhin, T. and K. A. Gray. 2006. Factors affecting denitrification rates in experimental wetlands: field and laboratory studies. Ecological Engineering **26**:167-181.

Smith, M. S. and J. M. Tiedje. 1979. Phases of denitrification following oxygen depletion in soil. Soil Biology and Biochemistry **11**:262-267.

Spalding, R. F. and M. E. Exner. 1993. Occurrence of nitrate in groundwater – a review. Journal of Environmental Quality **22**:392-402.

Sutton-Grier, A. E., M. Ho, and C. J. Richardson. 2009. Organic amendments improve soil conditions and denitrification in a restored riparian wetland. Wetlands **29**:343-352.

Tillman, D. 1998. The greening of the green revolution. Nature **396**:211-212.

Turner, R. E. and N. N. Rabalais. 1994. Coastal eutrophication near the Mississippi river delta. Nature **368**:619-621.

Turner, R. E., A. M. Redmond, and J. B. Zedler. 2001. Count it by acre or function: mitigation adds up to net loss of wetlands. National Wetlands Newsletter **23**:5-16.

Van Zwieten, L., B. Singh, S. Joseph, S. Kimber, A. Cowie, and K. Y. Chan. 2009. Biochar and emissions of non-CO2 greenhouse gases from soil. Biochar for Environmental Management: Science and Technology 227-249.

Verhoeven, J. T. A., B. Arheimer, C. Yin, and M. M. Hefting. 2006. Regional and global concerns over wetlands and water quality. Trends in Ecology and Evolution **21**:96-103.

Vymazal, J. 2007. Removal of nutrients in various types of constructed wetlands. Science of the Total Environment **380**:48-65.

Xu, R.-k., A.-z. Zhao, J.-h. Yuan, and J. Jiang. 2012. pH buffering capacity of acid soils from tropical and subtropical regions of China as influenced by incorporation of crop straw biochars. Journal of Soils and Sediments **12**:494-502.

Xue, Y., D. A. Kovacic, M. B. David, L. E. Gentry, R. L. Mulvaney, and C. W. Lindau. 1999. In situ measurements of denitrification in constructed wetlands. Journal of Environmental Quality **28**:263-269.

Yao, Y., B. Gao, M. Zhang, M. Inyang, and A. R. Zimmerman. 2012. Effect of biochar amendment on sorption and leaching of nitrate, ammonium, and phosphate in a sandy soil. Chemosphere **89**:1467-1471.

Yuan, J.-H., R.-K. Xu, and H. Zhang. 2011. The forms of alkalis in the biochar produced from crop residues at different temperatures. Bioresource Technology **102**:3488-3497.

Zedler, J. B. and J. C. Callaway. 1999. Tracking wetland restoration: do mitigation sites follow desired trajectories? Restoration Ecology **7**:69-73.

Zedler, J. B. and S. Kercher. 2005. Wetland resources: Status, trends, ecosystem services, and restorability. Annual Review of Environment and Resources **30**:39-74.

Figures
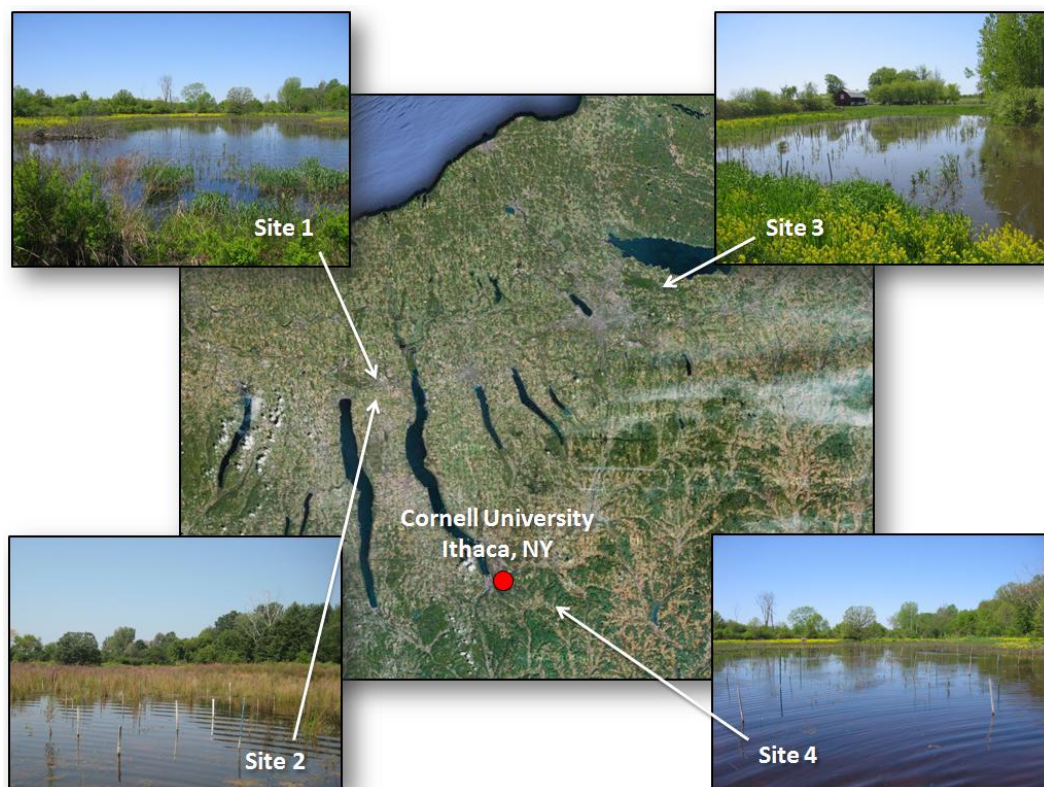
Figure 1. Photographs and locations of the four restored wetland sites studied.
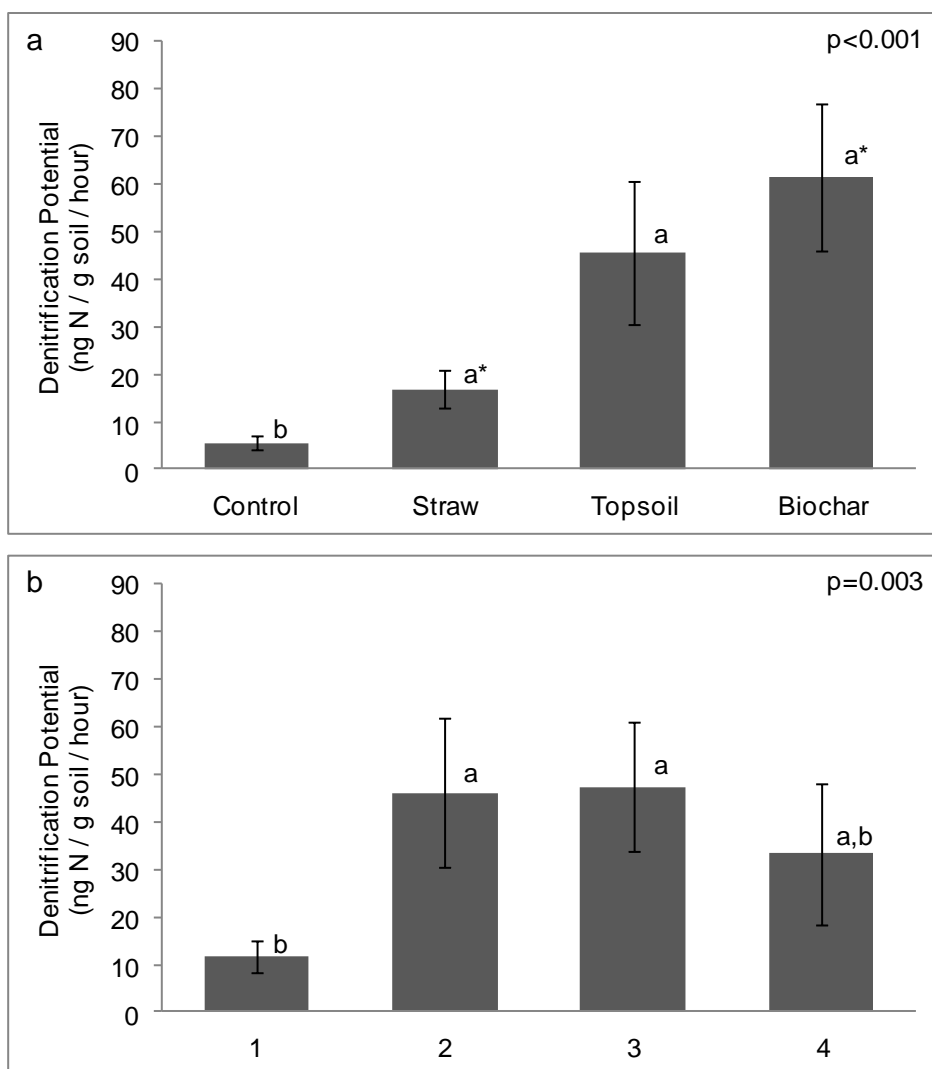
Figure 2. Differences in denitrification potential across (a) treatments and (b) sites. Tukey HSD post hoc comparisons are summarized by the letters above each bar. Bars that do not share a letter are significantly different from each other. Asterisks indicate a marginally significant difference between straw and biochar. ANOVA and post hoc analyses were done using transformed data. Error bars show standard error.
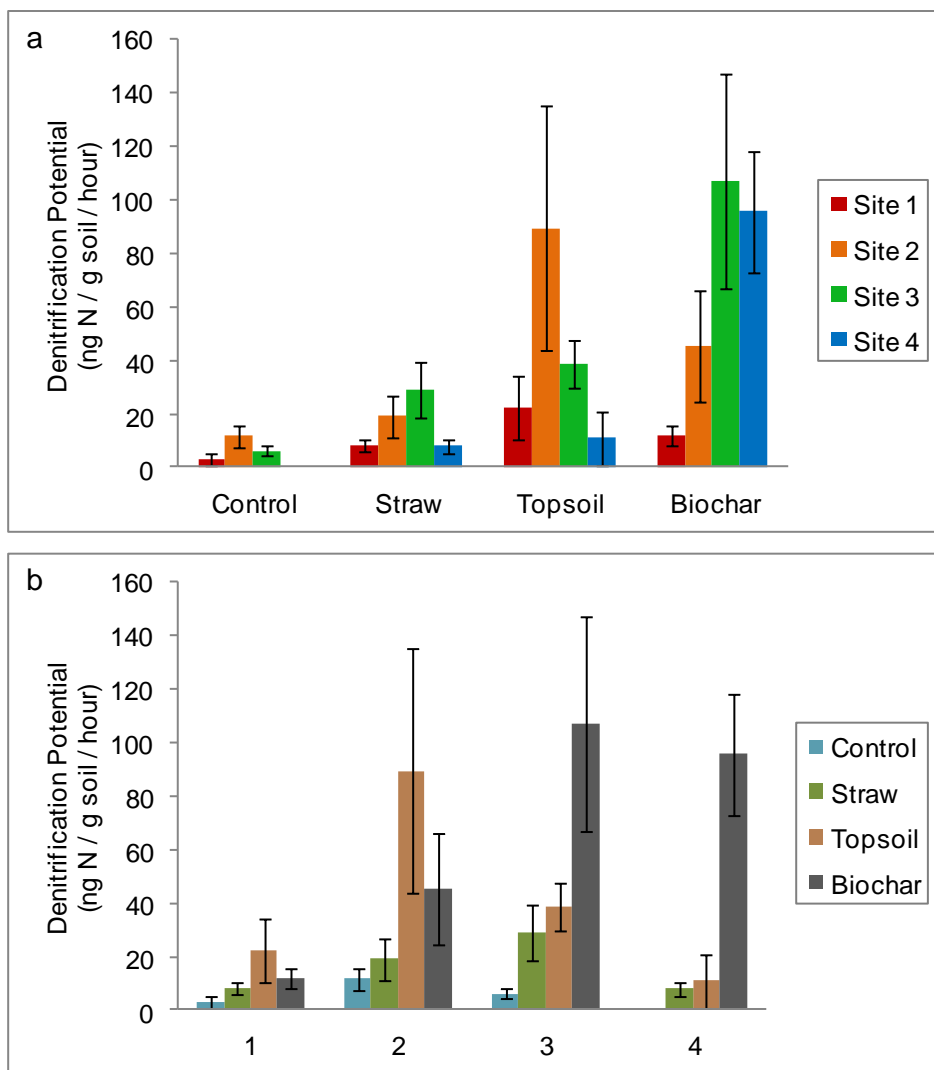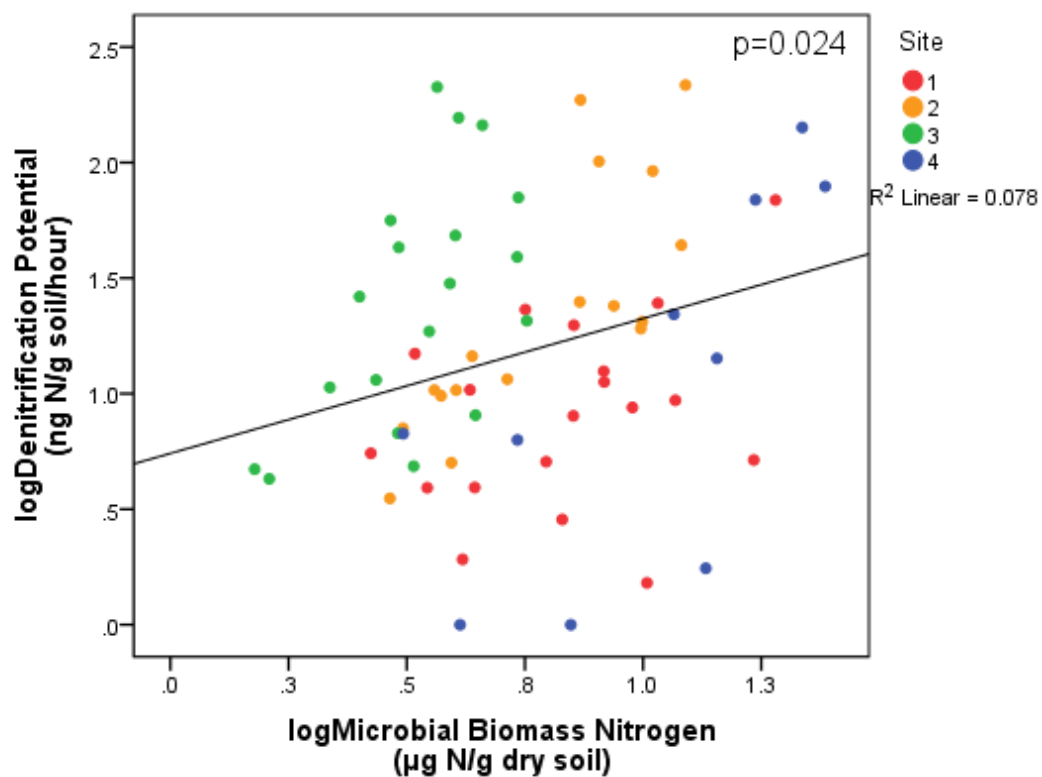
Figure 3. Denitrification potential for each treatment-by-site-combination. The same data is organized differently in r(a) and (b). Error bars show standard error.
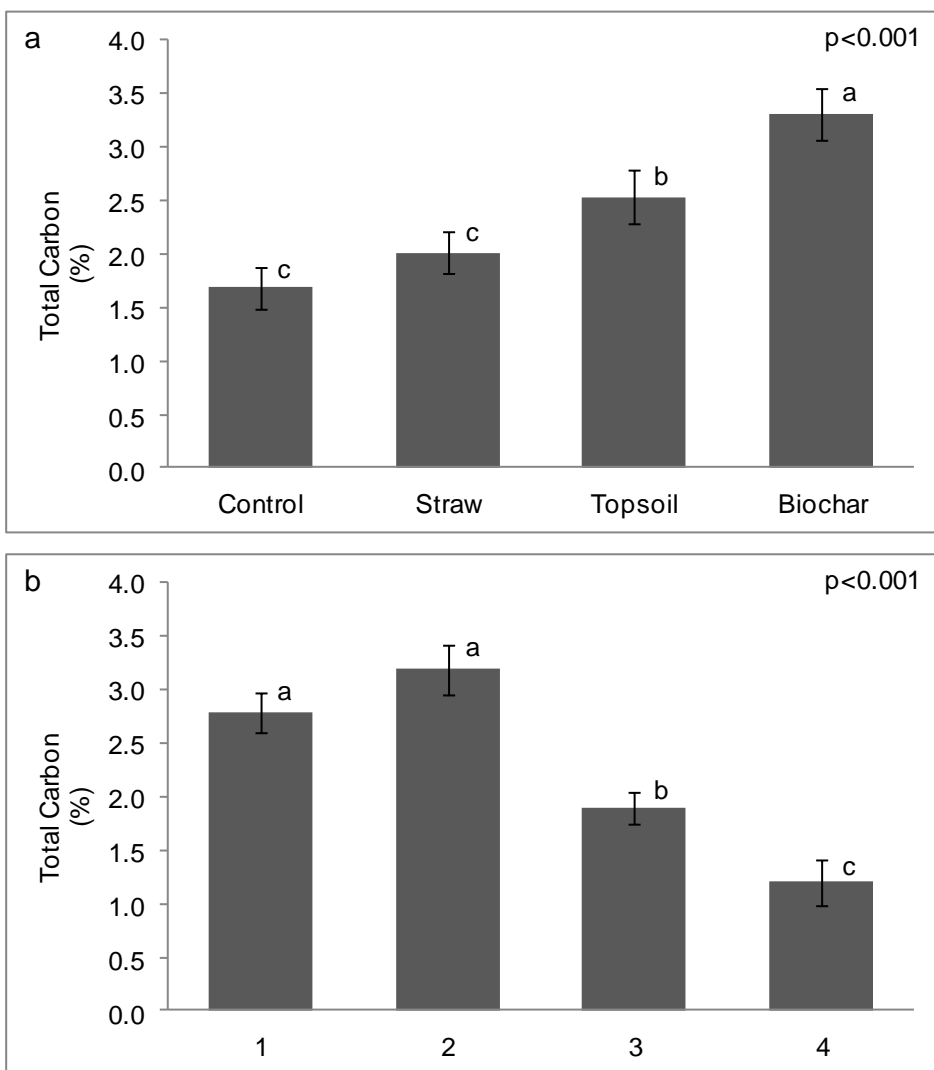
Figure 4. Differences in total carbon across (a) treatments and (b) sites. Tukey HSD post hoc comparisons are summarized by the letters above each bar. Bars that do not share a letter are significantly different from each other. Error bars show standard error.
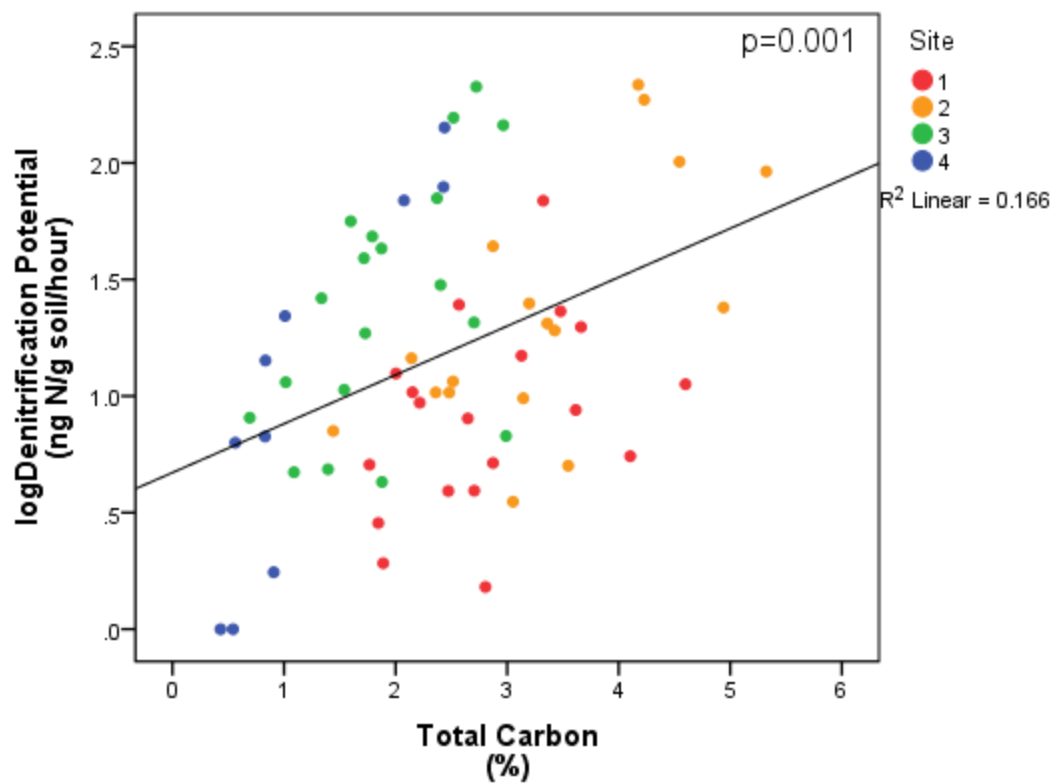
Figure 5. Scatterplot and linear fit showing a positive relationship between total carbon and denitrification potential. Pooled data is labeled according to site.
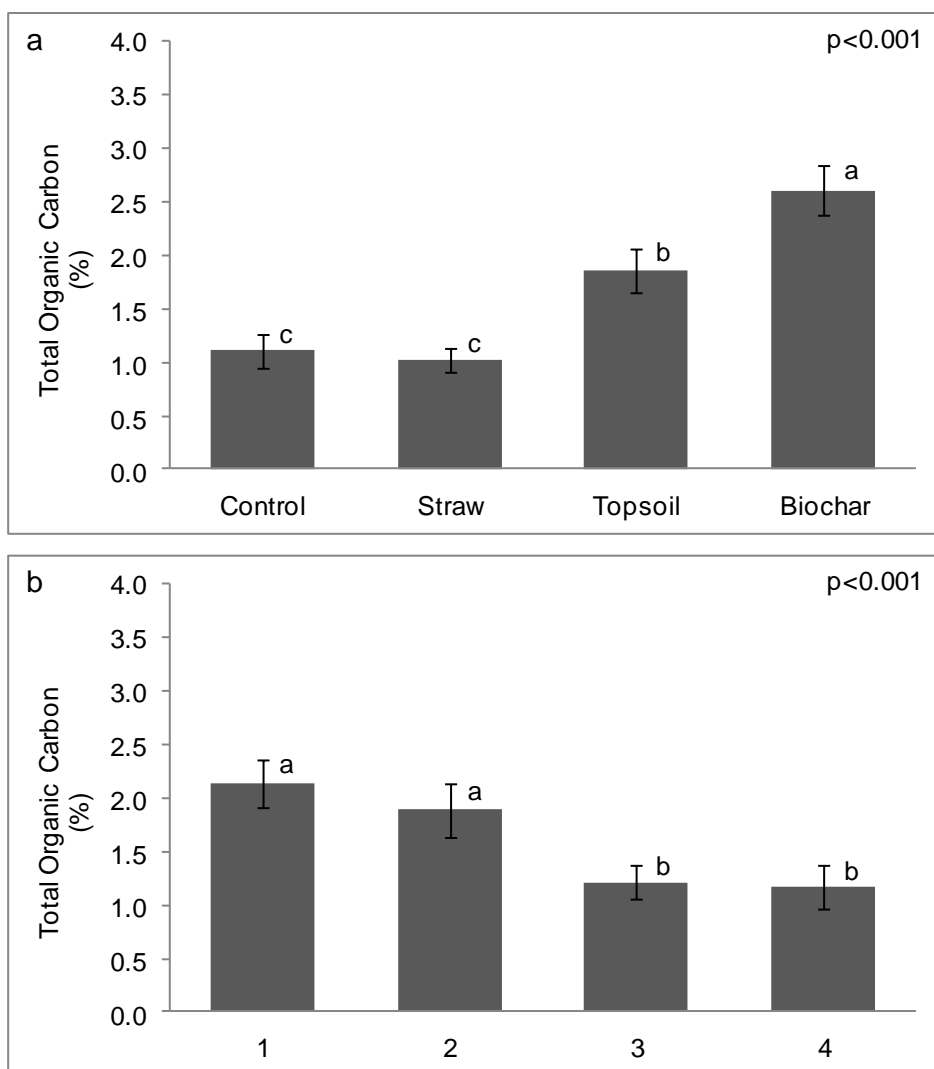
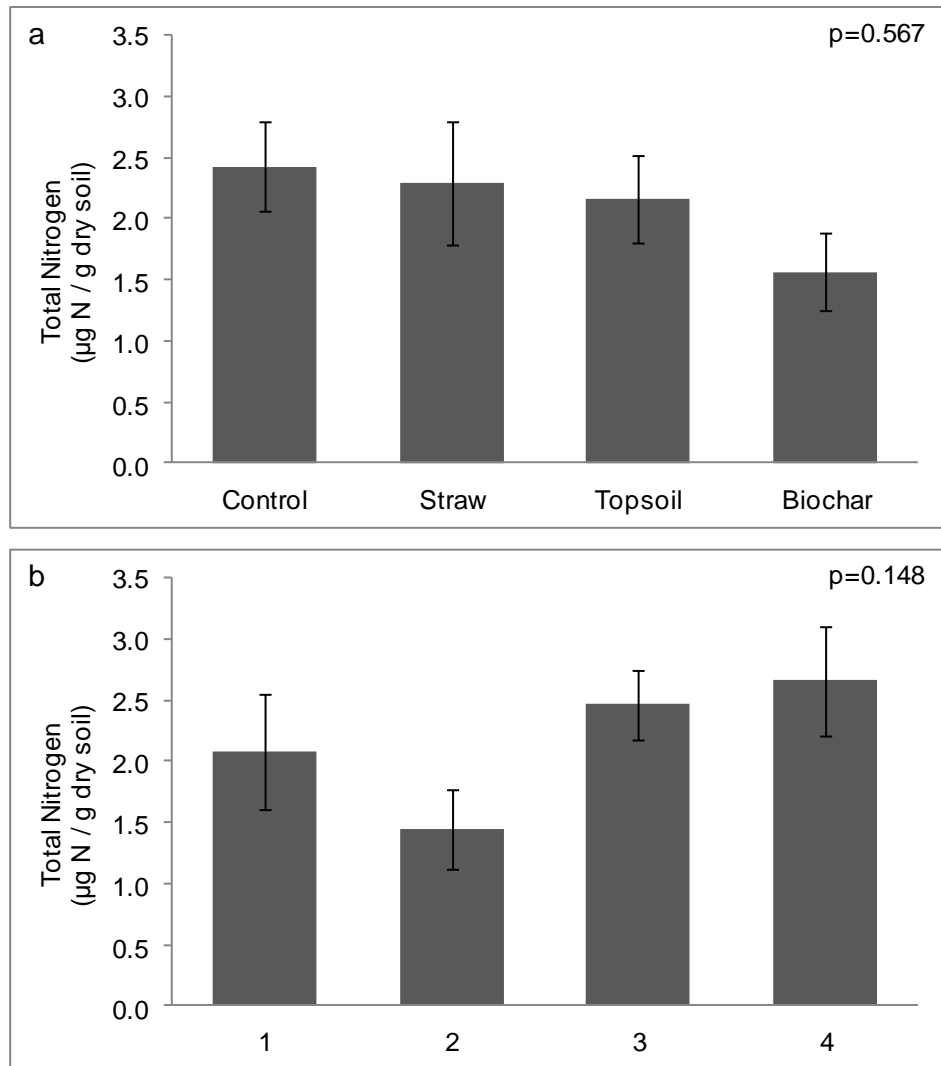Figure 6. Differences in total organic carbon across (a) treatments and (b) sites. Error bars show standard error. Tukey HSD post hoc comparisons are summarized by the letters above each bar. Bars that do not share a letter are significantly different from each other.
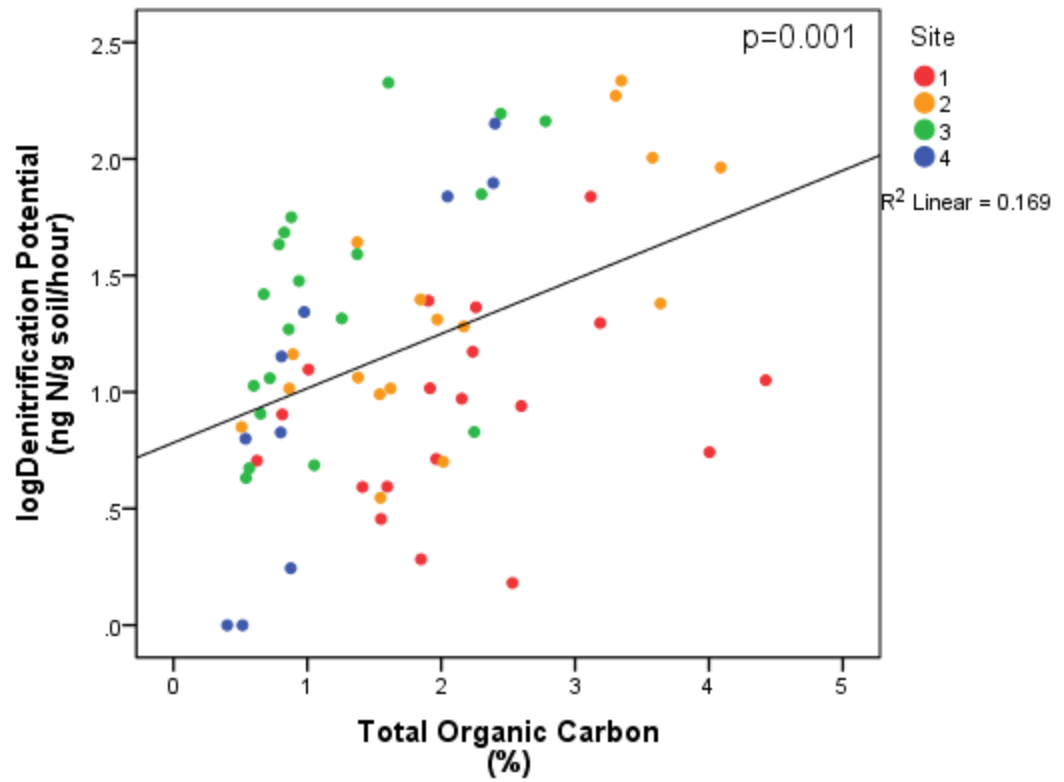
Figure 7. Scatterplot and linear fit showing a positive relationship between organic carbon and denitrification potential. Pooled data is labeled according to site.
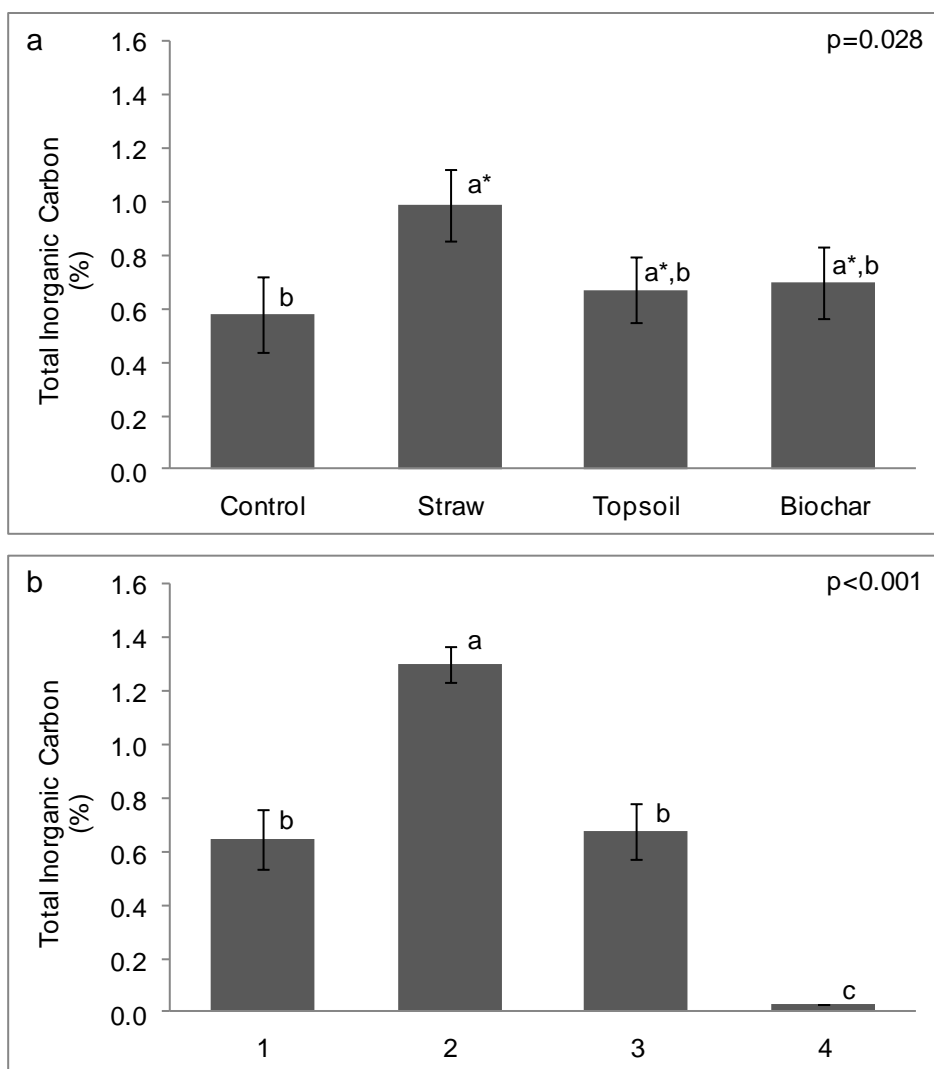
Figure 8. Differences in total inorganic carbon across (a) treatments and (b) sites. Error bars show standard error. Tukey HSD post hoc comparisons are summarized by the letters above each bar. Bars that do not share a letter are significantly different from each other. Asterisks indicate marginally significant differences between straw and topsoil as well as between straw and biochar.
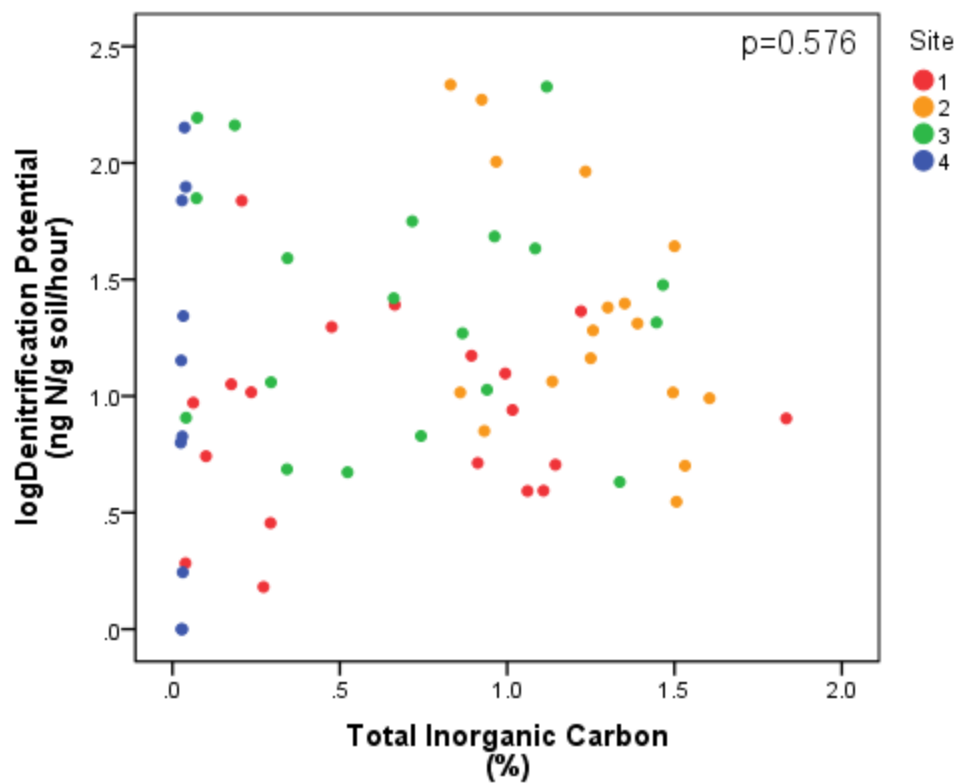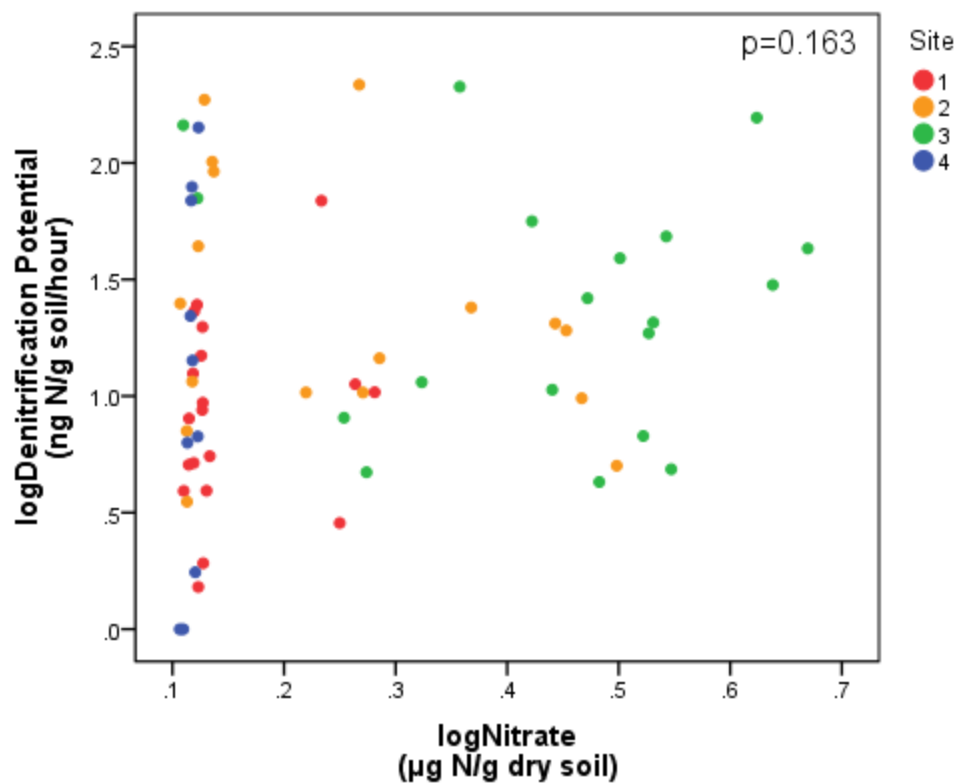
Figure 9. Scatterplot showing the absence of a linear relationship between inorganic carbon and denitrification potential. Pooled data is labeled according to site.
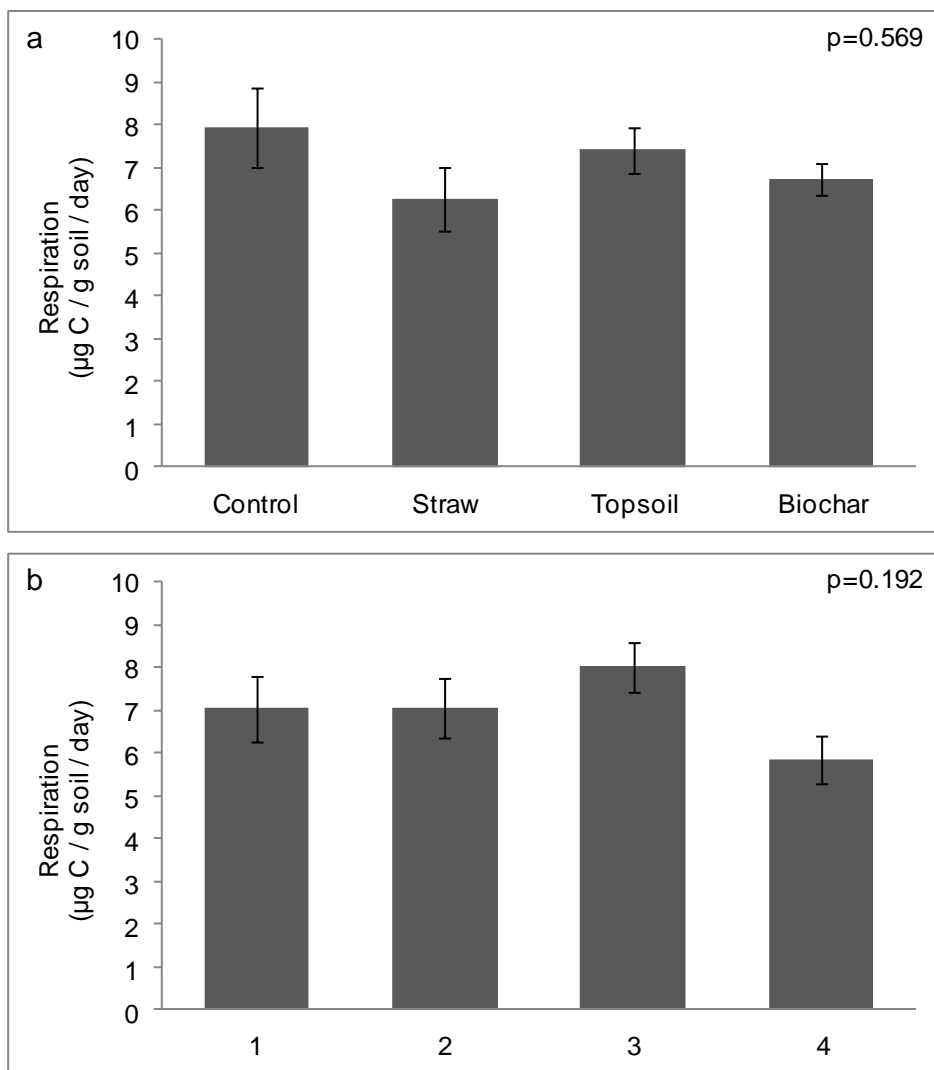
Figure 10. Respiration was not significantly different between (a) treatments and (b) sites. Error bars show standard error.
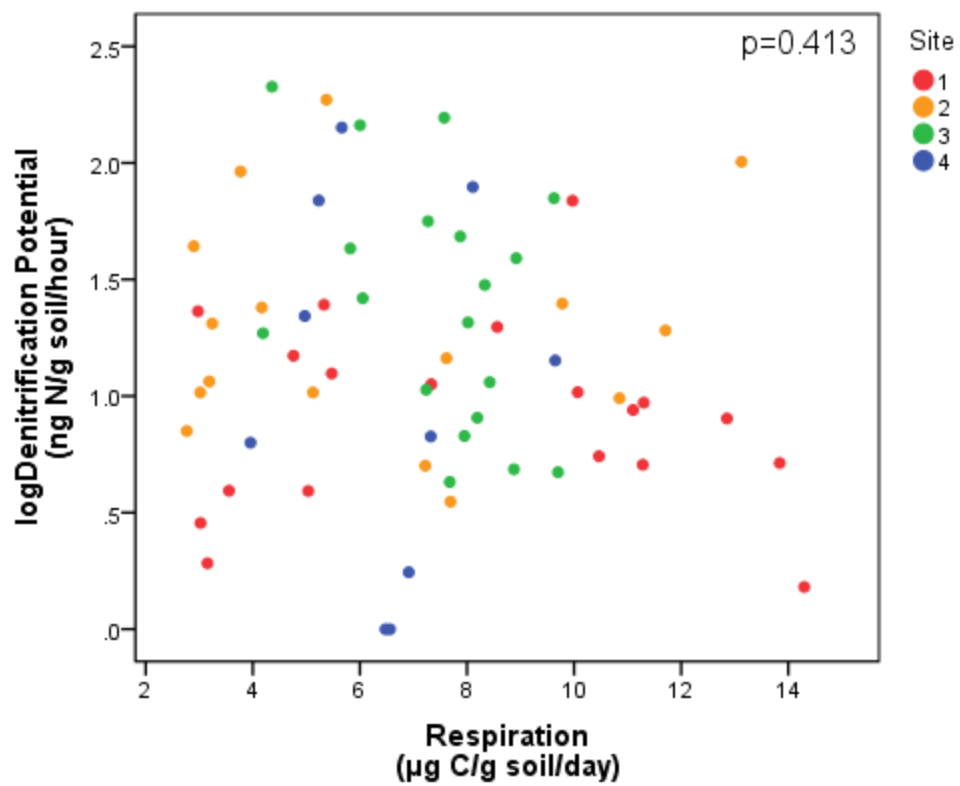
Figure 11. Scatterplot showing the absence of a linear relationship between respiration and denitrification potential. Pooled data is labeled according to site.
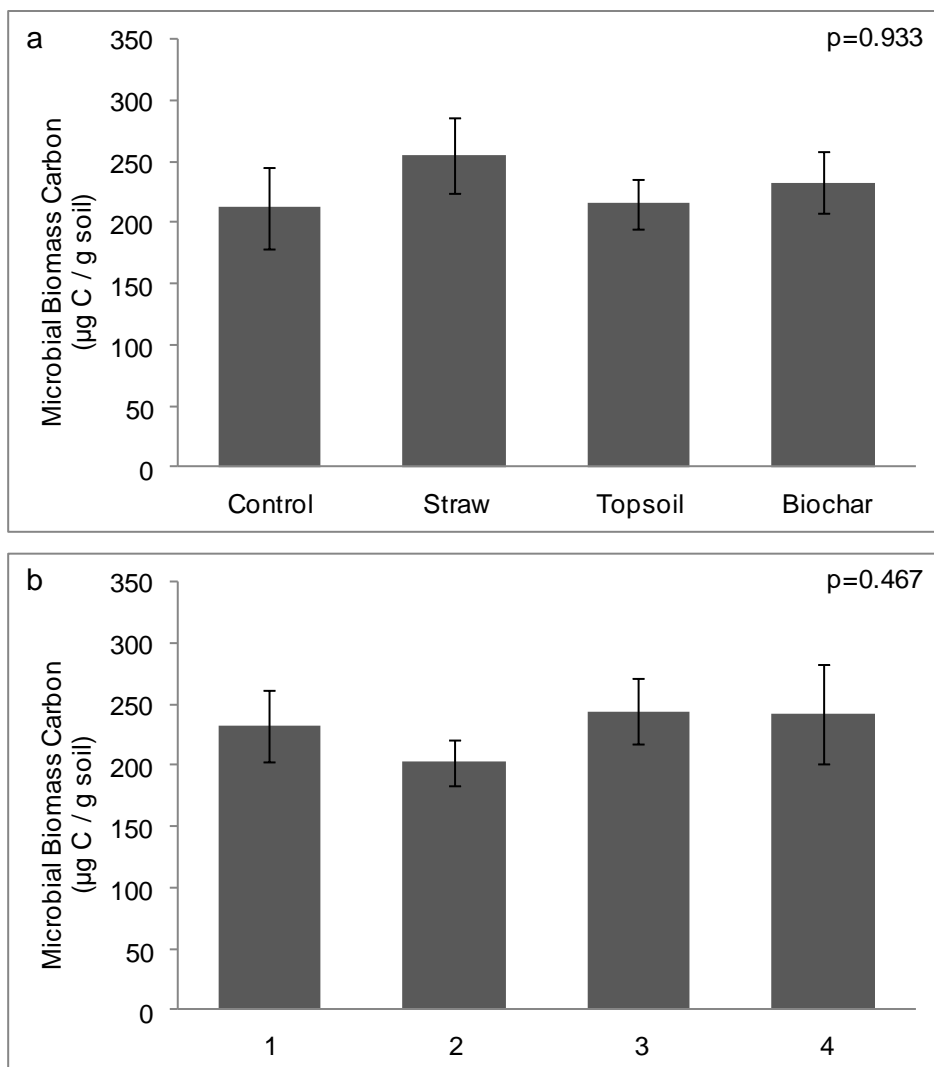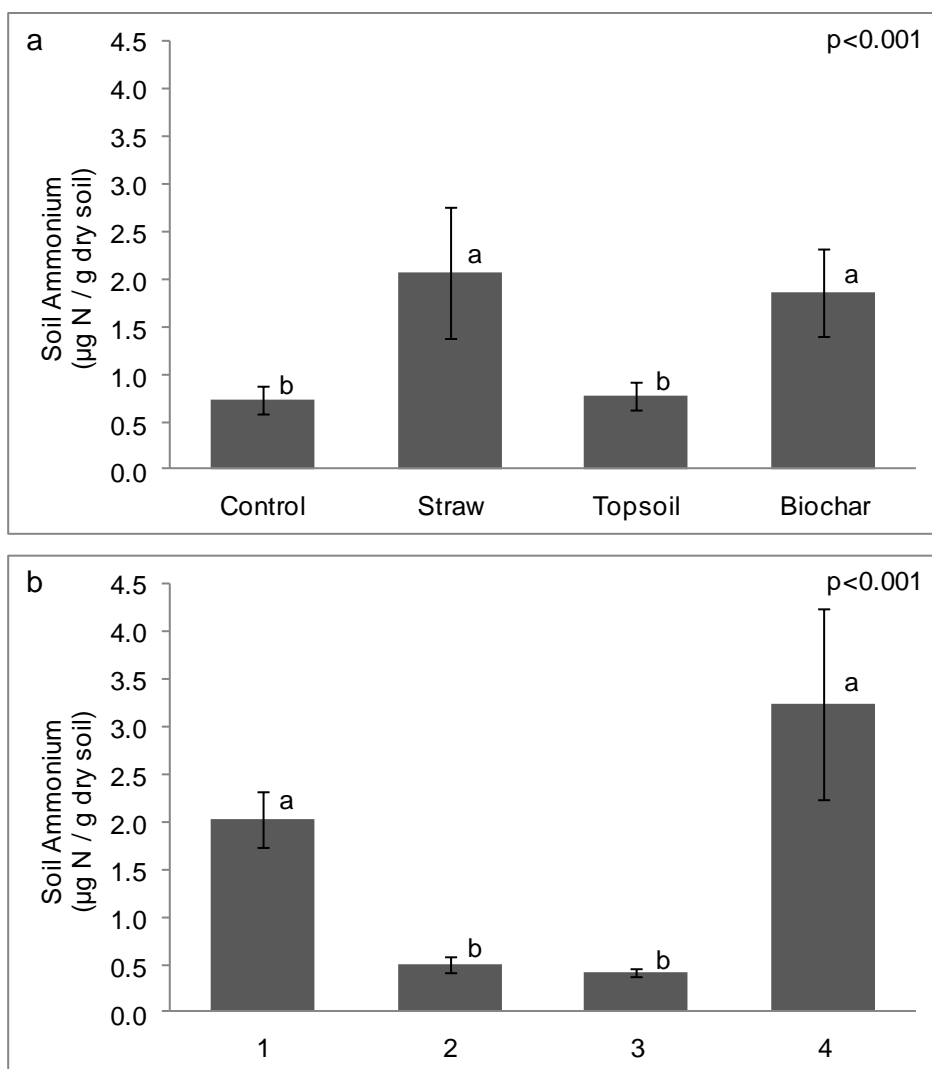
Figure 12. Microbial biomass carbon was not significantly different between (a) treatments and (b) sites. ANOVA and post hoc analyses were done using transformed data. Error bars show standard error.
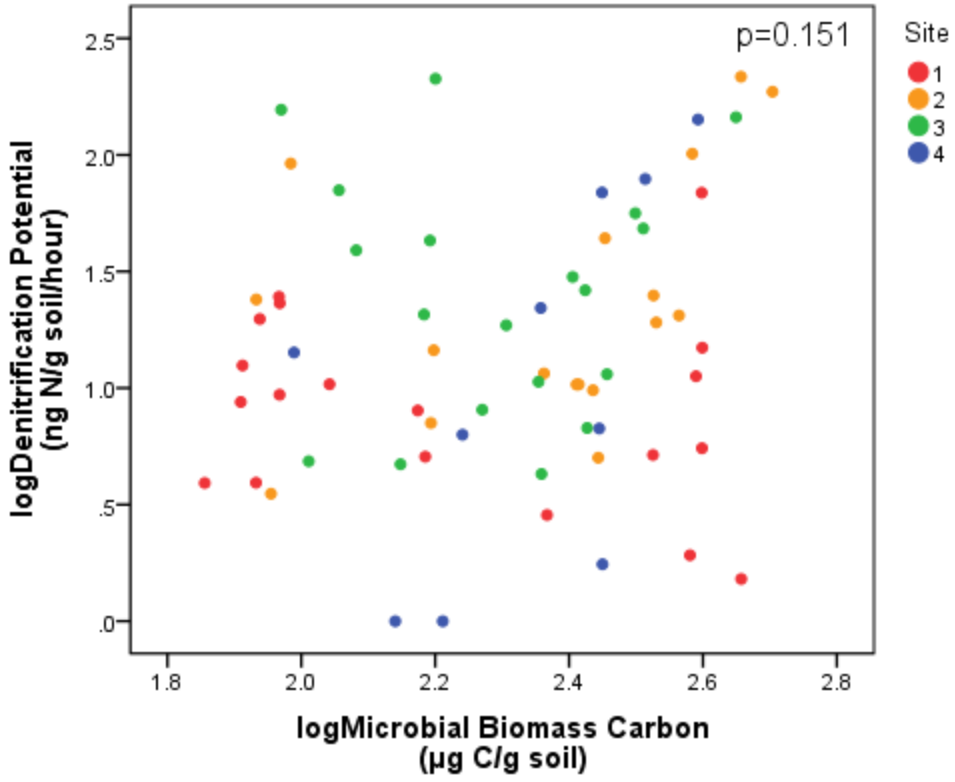
Figure 13. Scatterplot showing the absence of a linear relationship between microbial biomass carbon and denitrification potential. Pooled data is labeled according to site.
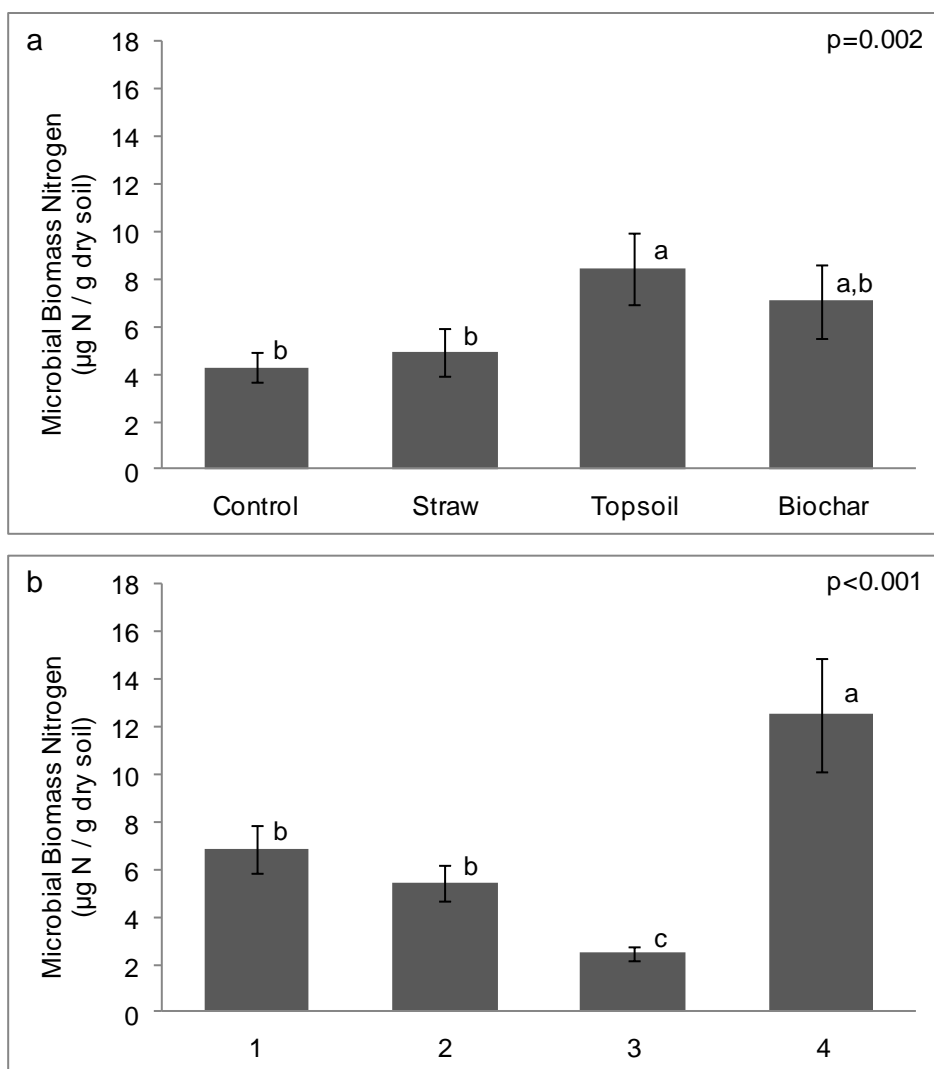
Figure 14. Differences in microbial biomass nitrogen across (a) treatments and (b) sites. Tukey HSD post hoc comparisons are summarized by the letters above each bar. Bars that do not share a letter are significantly different from each other. ANOVA and post hoc analyses were done using transformed data. Error bars show standard error.

Figure 15. Scatterplot and linear fit showing a positive relationship between microbial biomass nitrogen and denitrification potential. Pooled data is labeled according to site.
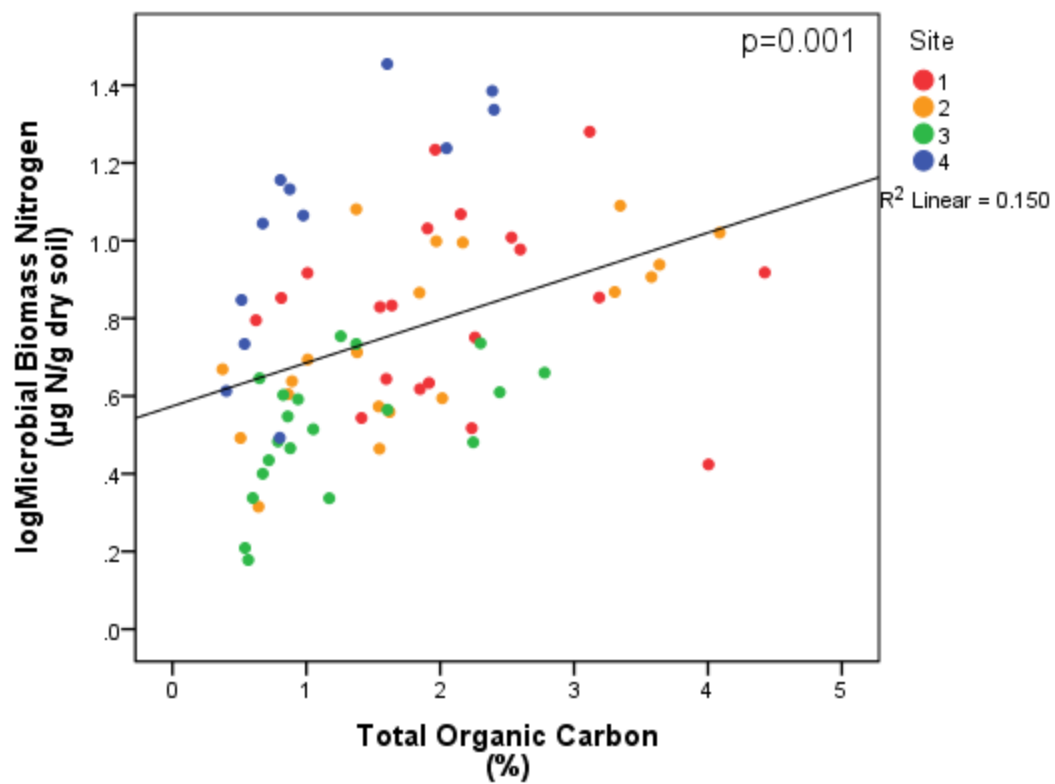
Figure 16. Scatterplot and linear fit showing a positive relationship between total organic carbon and microbial biomass nitrogen. Pooled data is labeled according to site.

Figure 17. Total nitrogen was not significantly different across (a) treatments and (b) sites. Error bars show standard error.
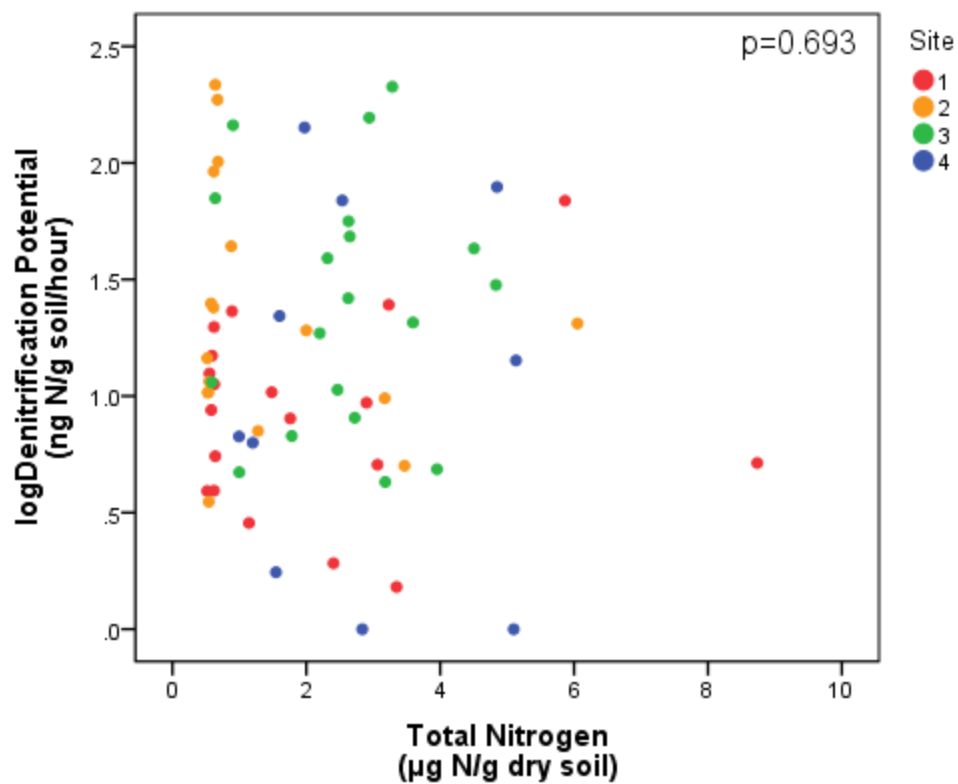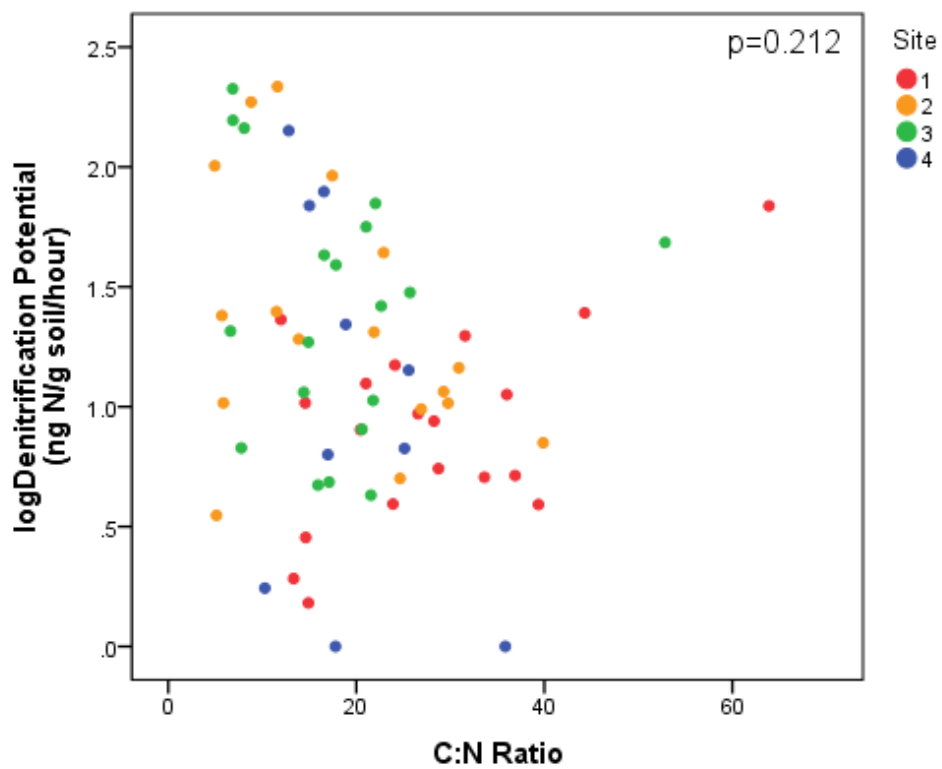
Figure 18. Scatterplot showing the absence of a linear relationship between total nitrogen and denitrification potential. Pooled data is labeled according to site.
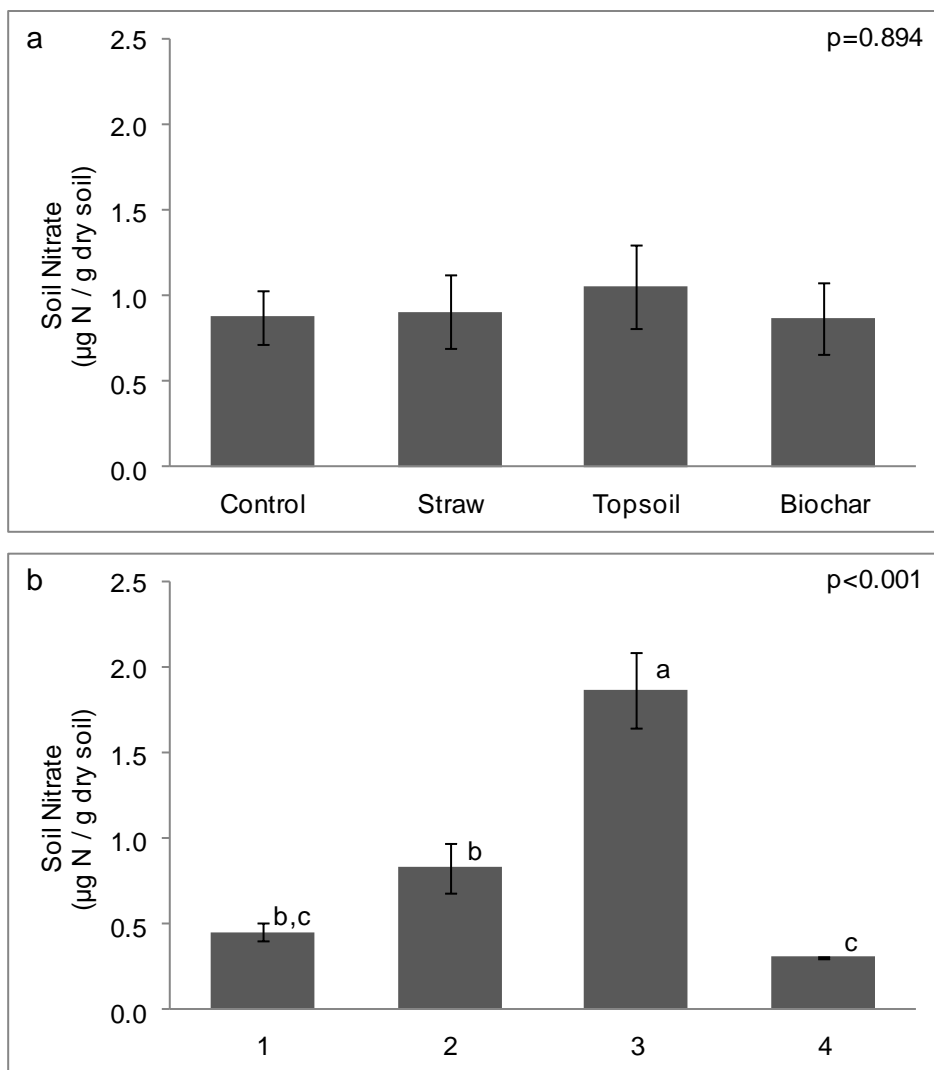
Figure 19. Soil nitrate was not significantly different across (a) treatments.
Differences across (b) sites are shown by Tukey HSD post hoc comparisons
summarized by the letters above each bar. Bars that do not share a letter are
significantly different from each other. Error bars show standard error. ANOVA
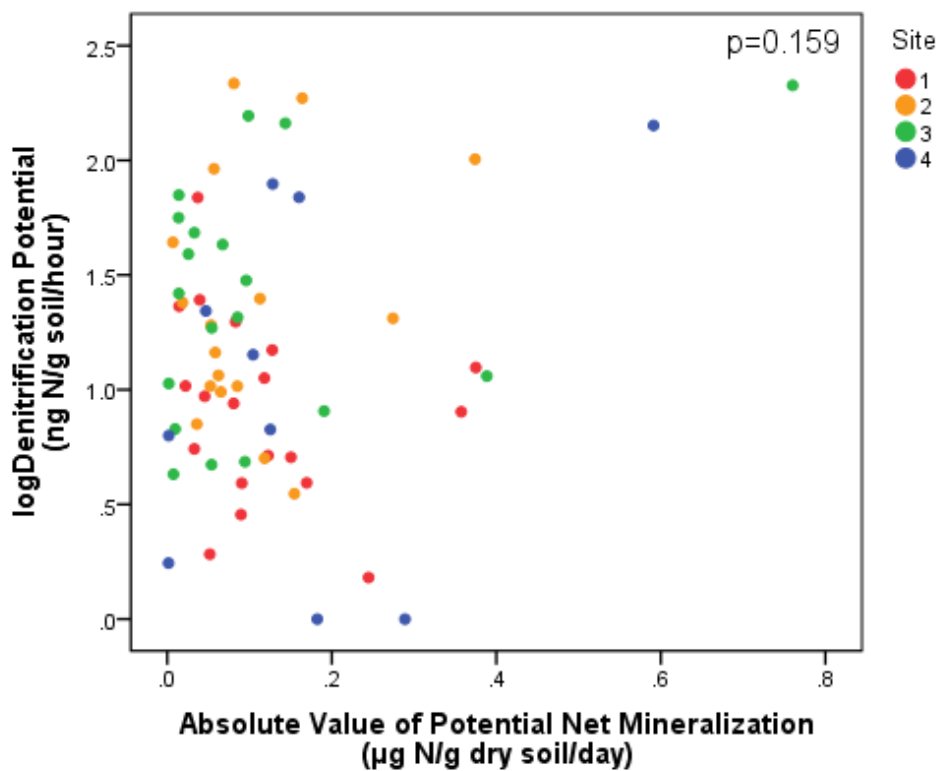and post hoc analyses were done using transformed data.

Figure 20. Scatterplot showing the absence of a linear relationship between TIN and denitrification potential. Pooled data is labeled according to site.

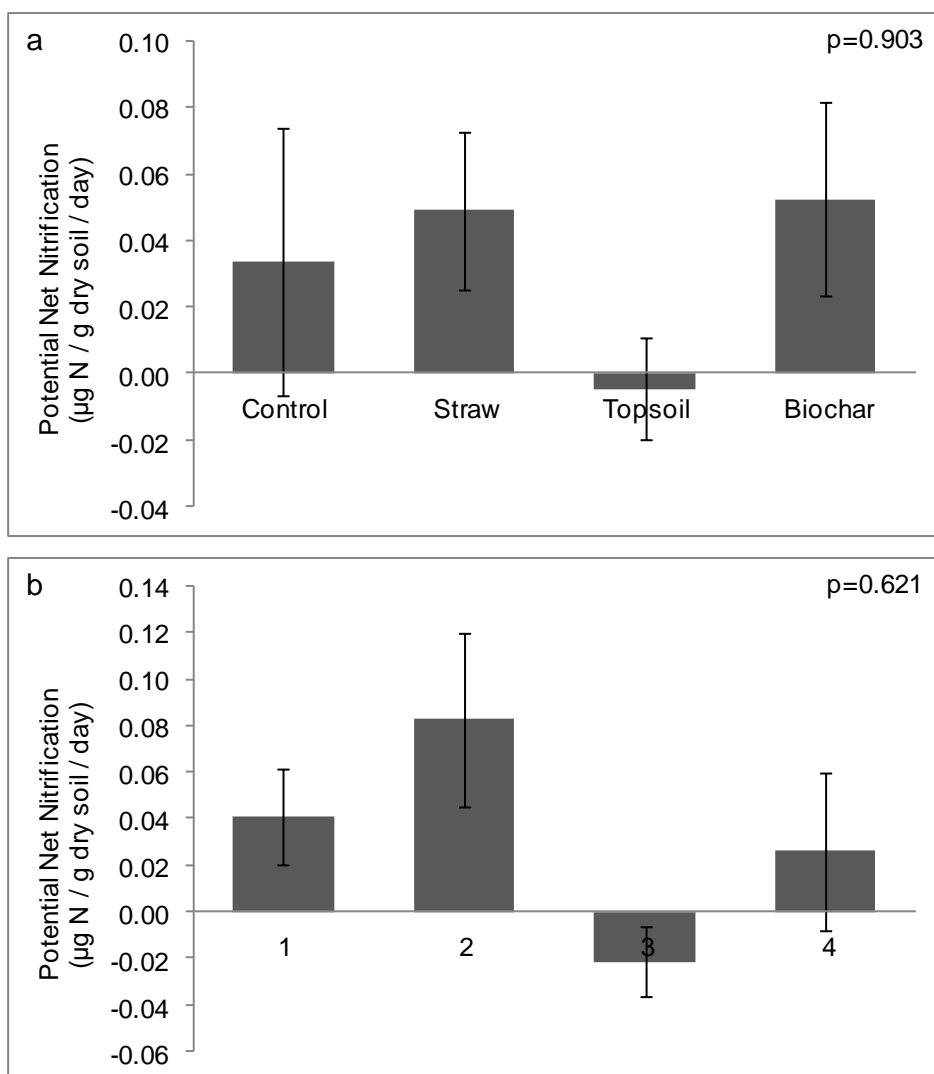Figure 21. Differences in soil ammonium across (a) treatments and (b) sites. Tukey HSD post hoc comparisons are summarized by the letters above each bar. Bars that do not share a letter are significantly different from each other. ANOVA and post hoc analyses were done using transformed data. Error bars show standard error.
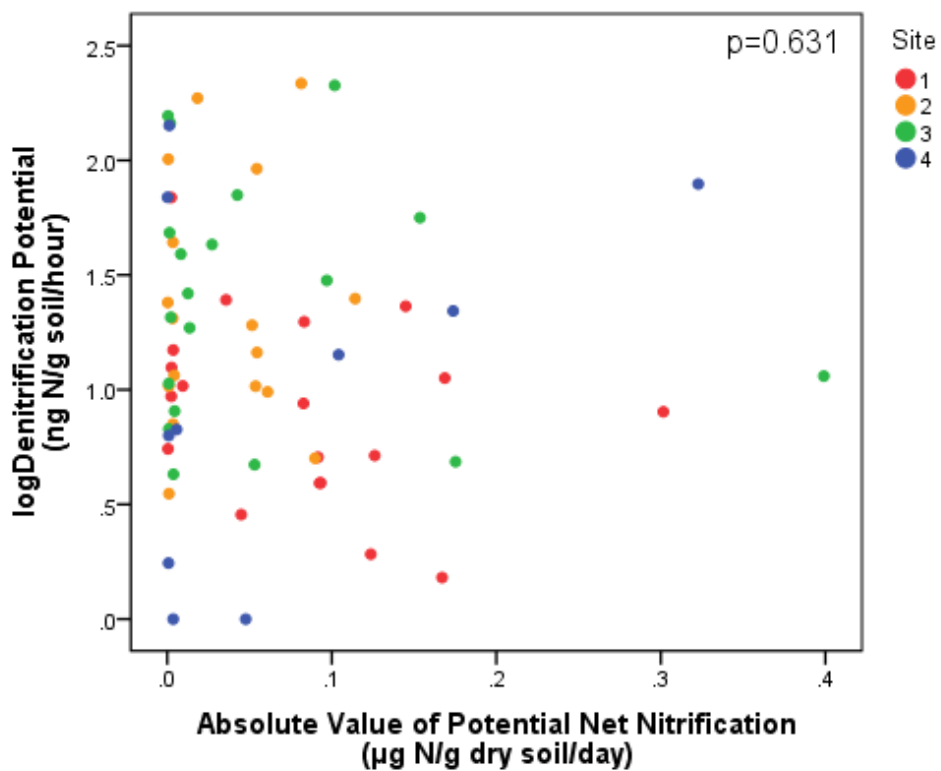
Figure 22. Scatterplot showing the absence of a linear relationship between ammonium and denitrification potential. Pooled data is labeled according to site.
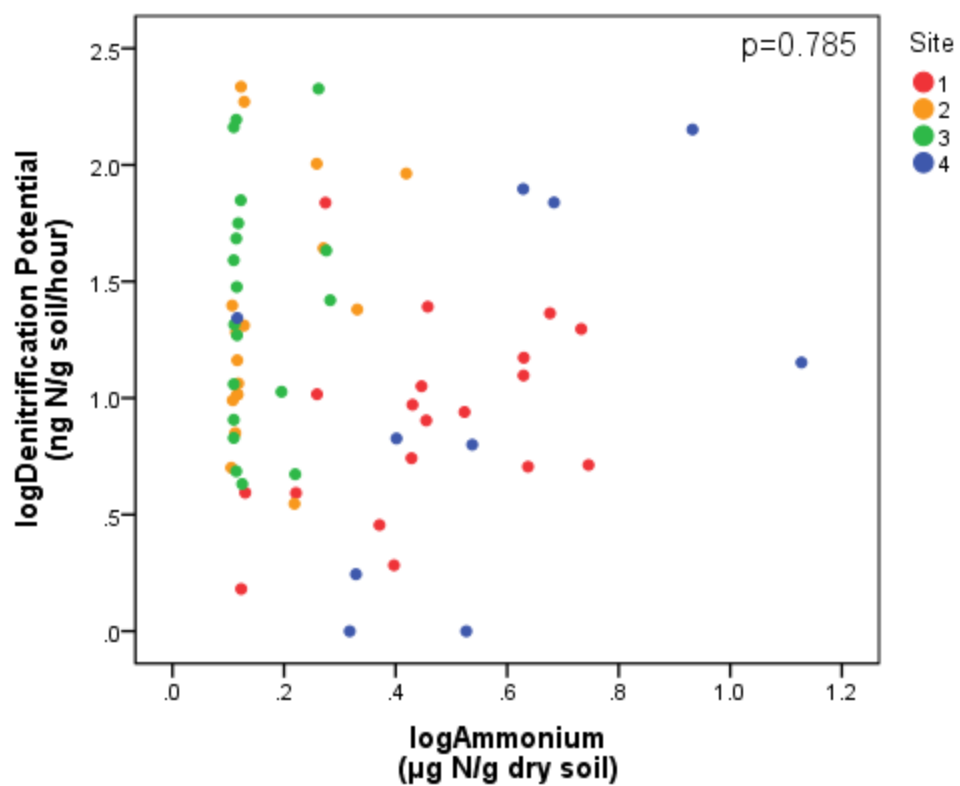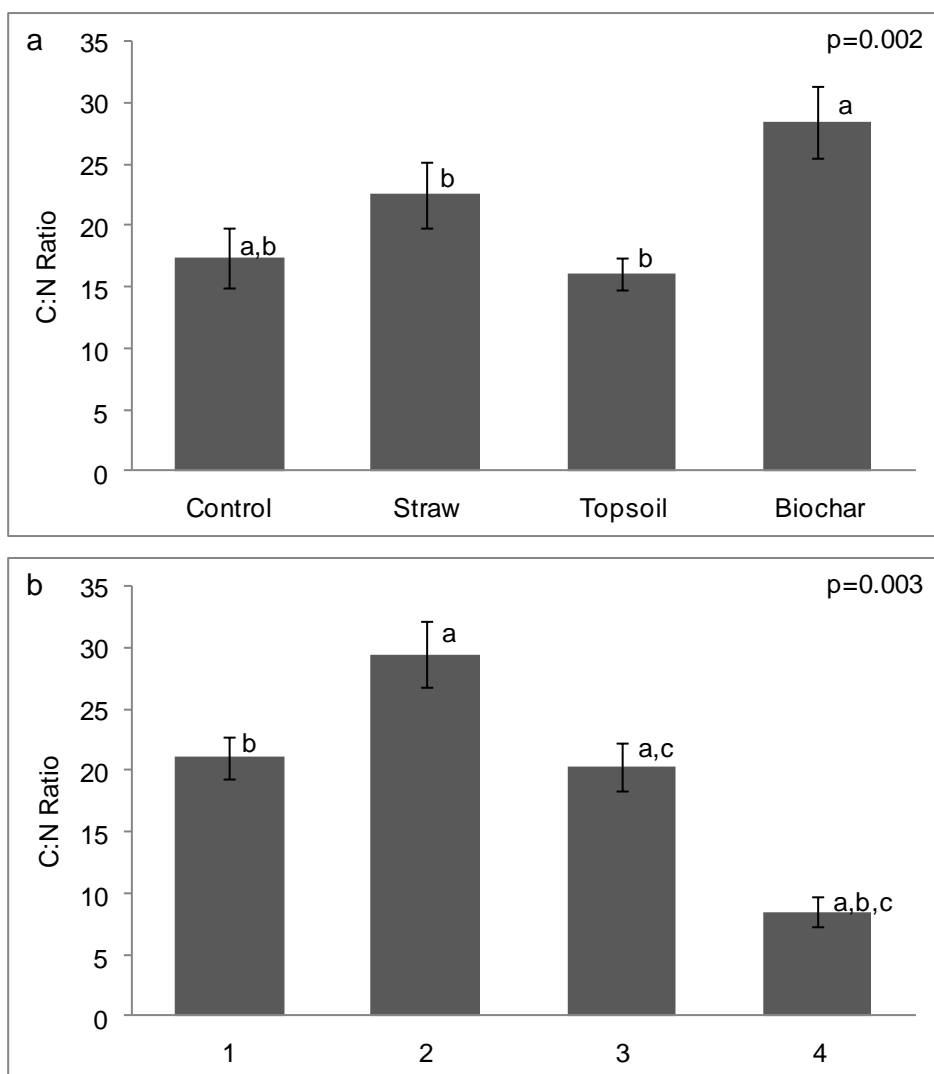
Figure 23. Differences in C:N ratio across (a) treatments and (b) sites. Tukey HSD post hoc comparisons are summarized by the letters above each bar. Bars that do not share a letter are significantly different from each other. Error bars show standard error.

Figure 24. Scatterplot showing the absence of a linear relationship between C:N ratio and denitrification potential. Pooled data is labeled according to site.
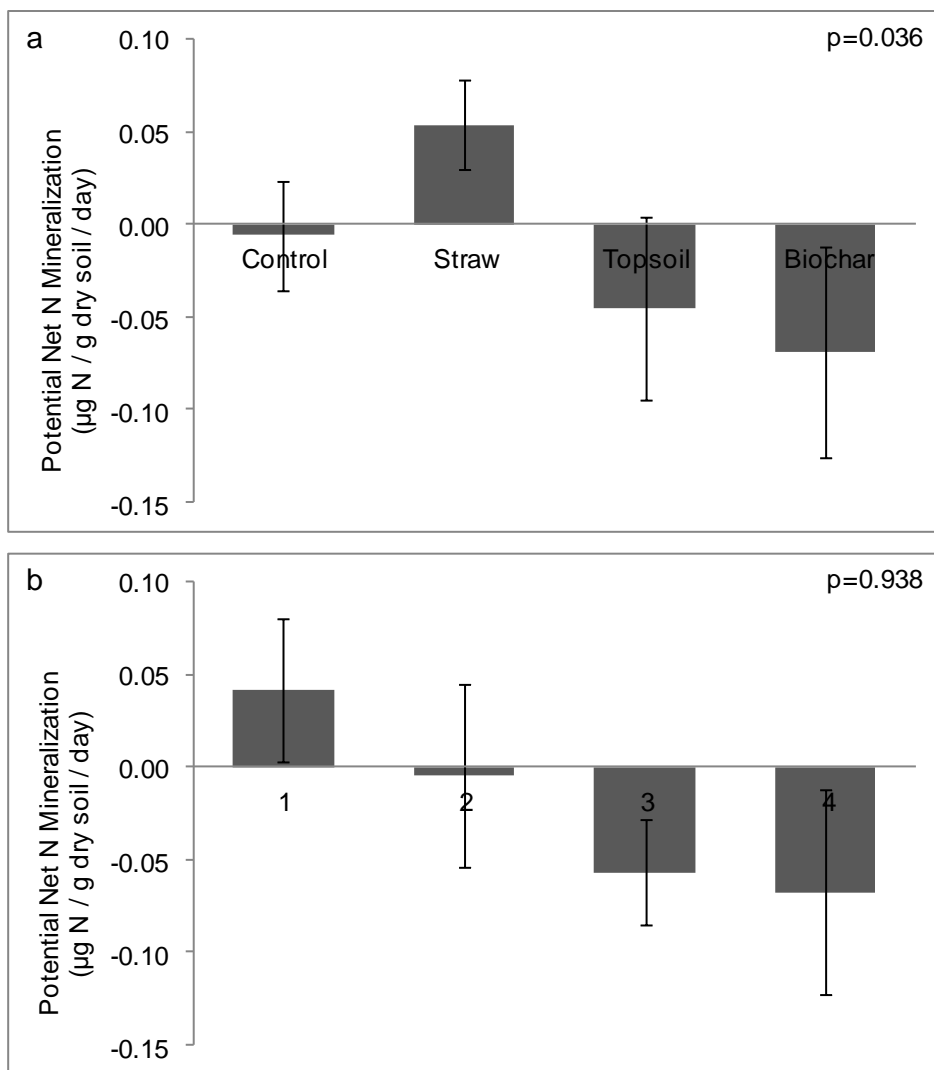
Figure 25. Differences in potential net N mineralization between (a) treatments and (b) sites. Despite overall significance for treatment, no significant pair-wise differences were found with Tukey HSD. Error bars show standard error.

Figure 26. Scatterplot showing the absence of a linear relationship between potential net N mineralization and denitrification potential. Pooled data is labeled according to site.

Figure 27. Differences in potential net nitrification between (a) treatments and (b) sites. Error bars show standard error.

Figure 28. Scatterplot showing the absence of a linear relationship between potential net nitrification and denitrification potential. Pooled data is labeled according to site.
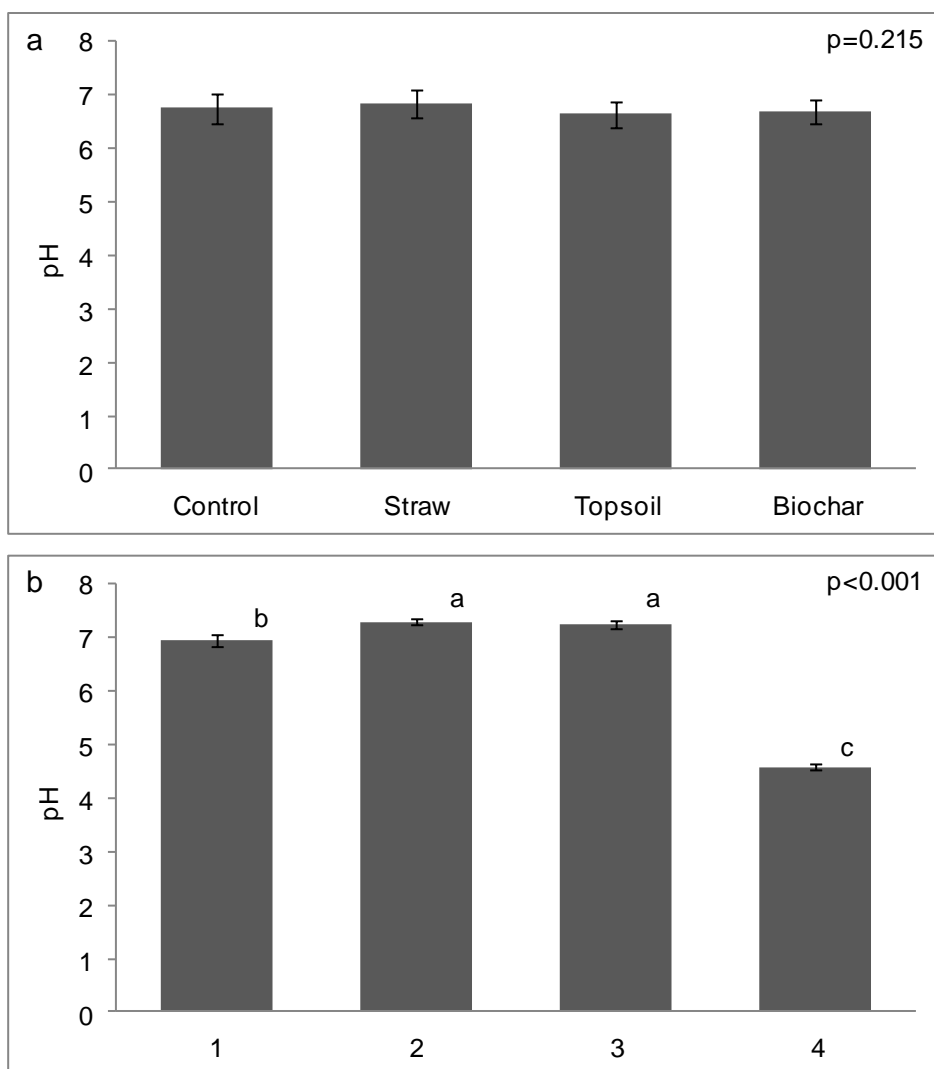
Figure 29. pH was not significantly different across (a) treatments. Differences across (b) sites are shown by Tukey HSD post hoc comparisons summarized by the letters above each bar. Bars that do not share a letter are significantly different from each other. Error bars show standard error.
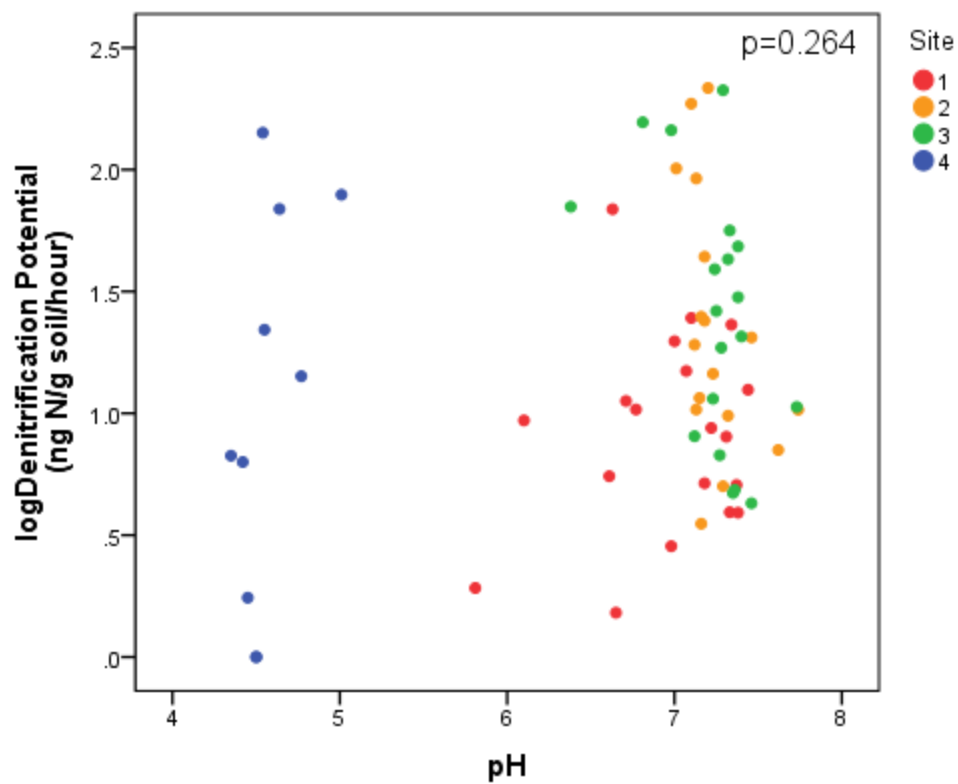
Figure 30. Scatterplot showing the absence of a linear relationship between pH and denitrification potential. Pooled data is labeled according to site.
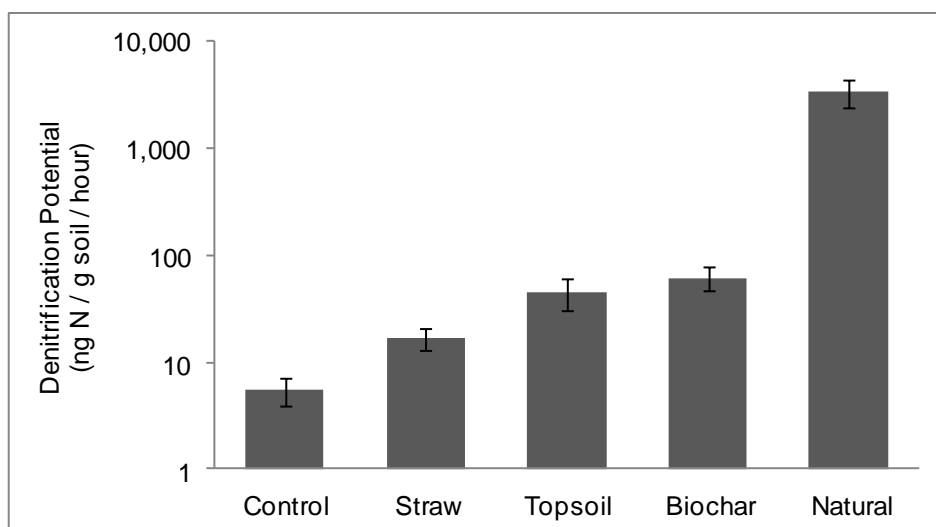
Figure 31. Differences in denitrification potential across treatments in comparison to the natural wetland. The y-axis is in log scale for clarity. Error bars show standard error.
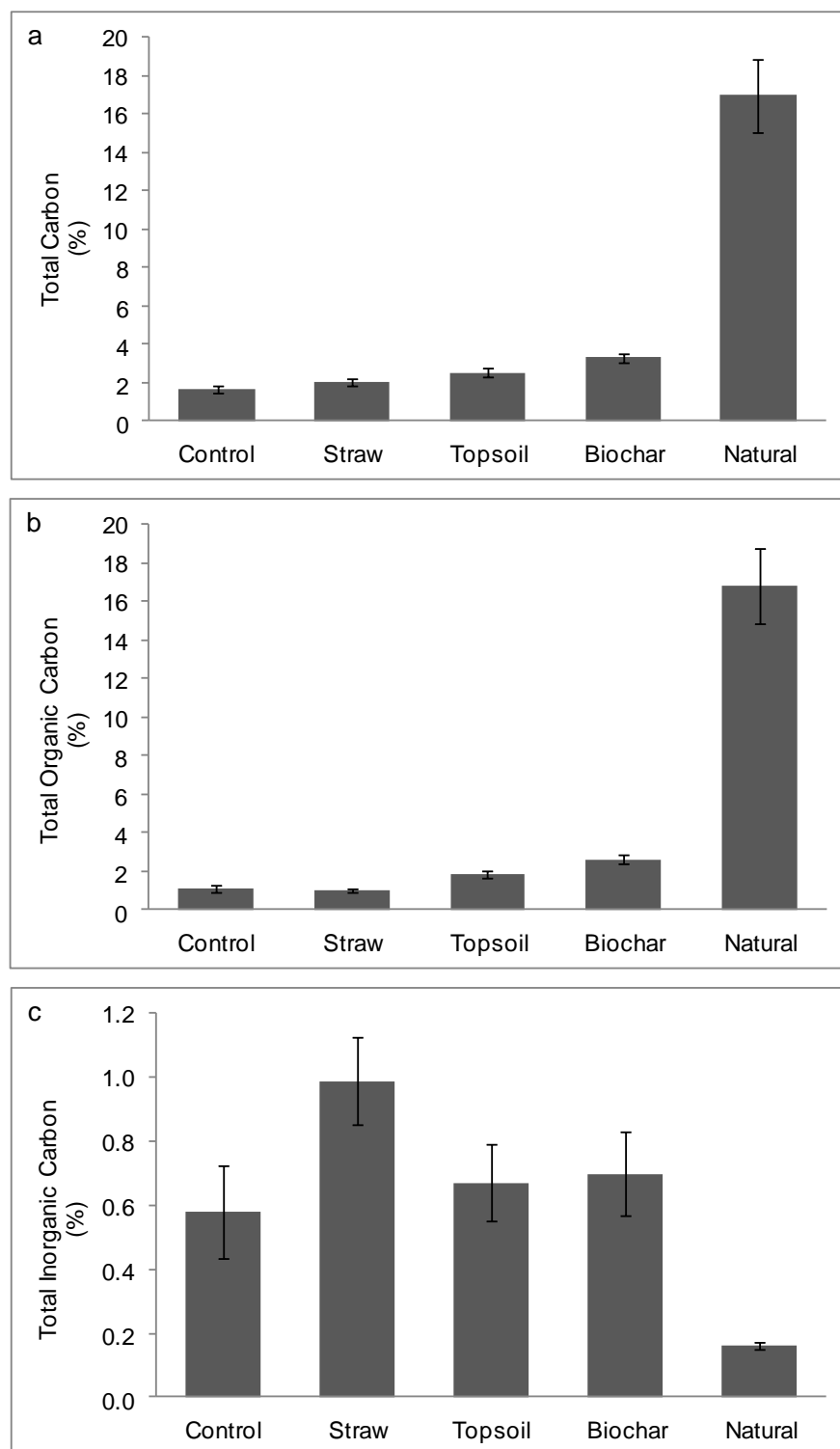
Figure 32. Differences in (a) total, (b) organic, and (c) inorganic carbon across treatments in comparison to the natural wetland. Error bars show standard error.
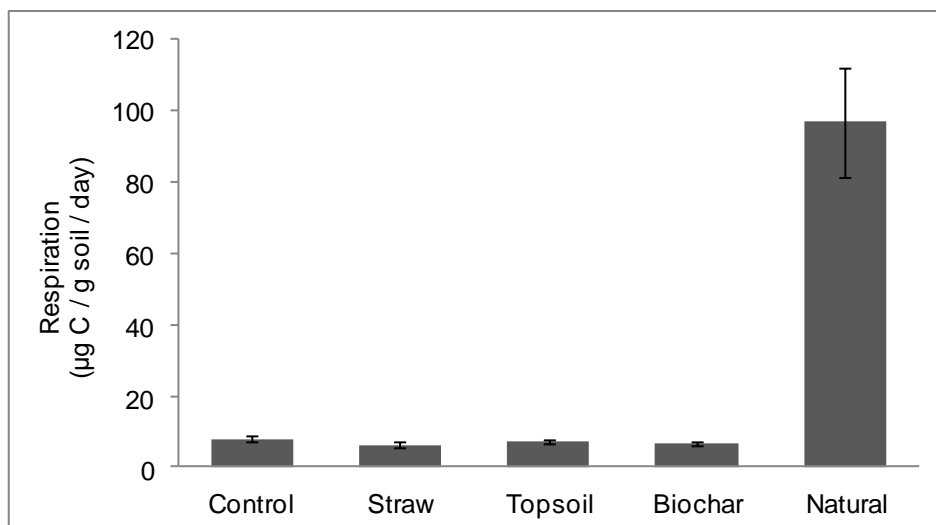
Figure 33. Differences in respiration across treatments in comparison to the natural wetland. Error bars show standard error.
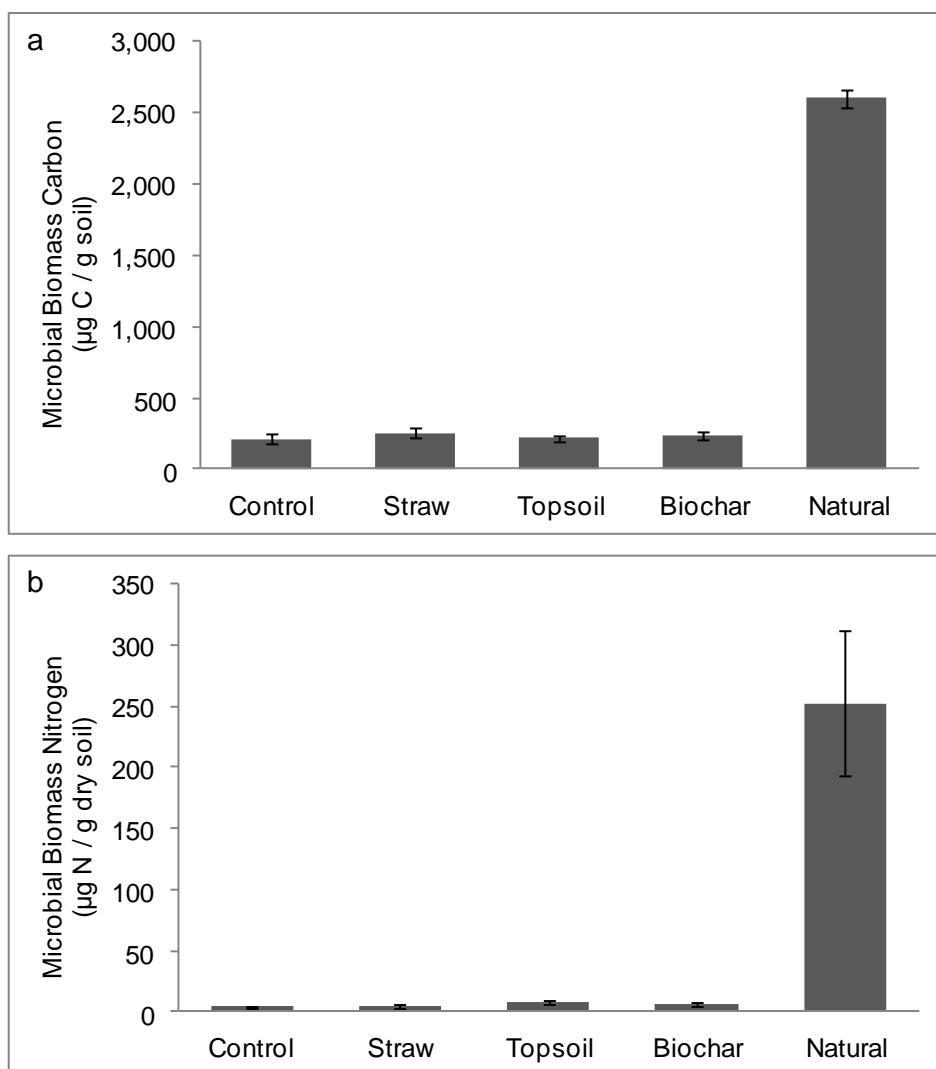
Figure 34. Differences in (a) microbial biomass carbon and (b) microbial biomass nitrogen across treatments in comparison to the natural wetland. Error bars show standard error.
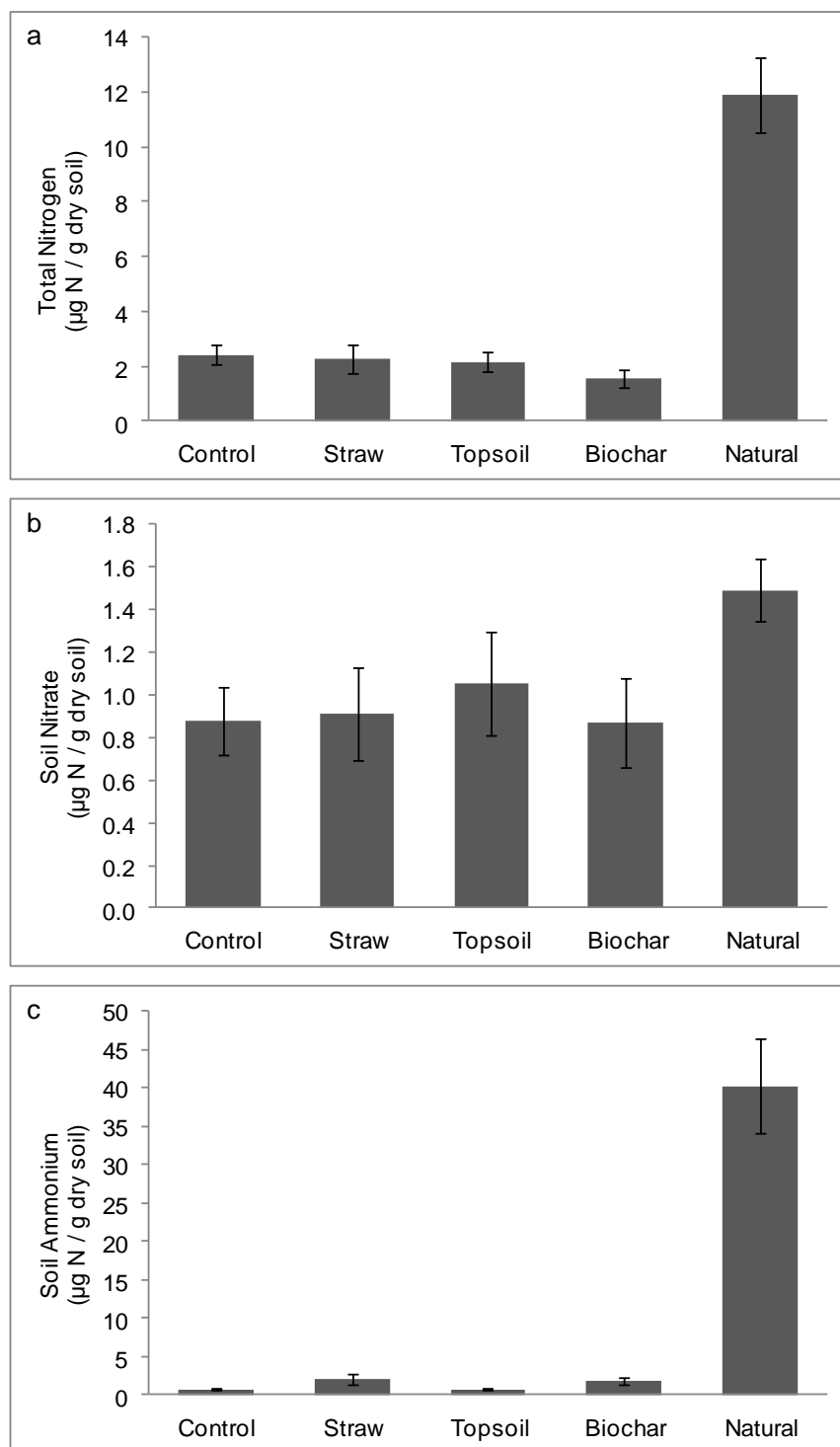
Figure 35. Differences in (a) total nitrogen, (b) soil nitrate, and (c) soil ammonium across treatments in comparison to the natural wetland. Error bars show standard error.
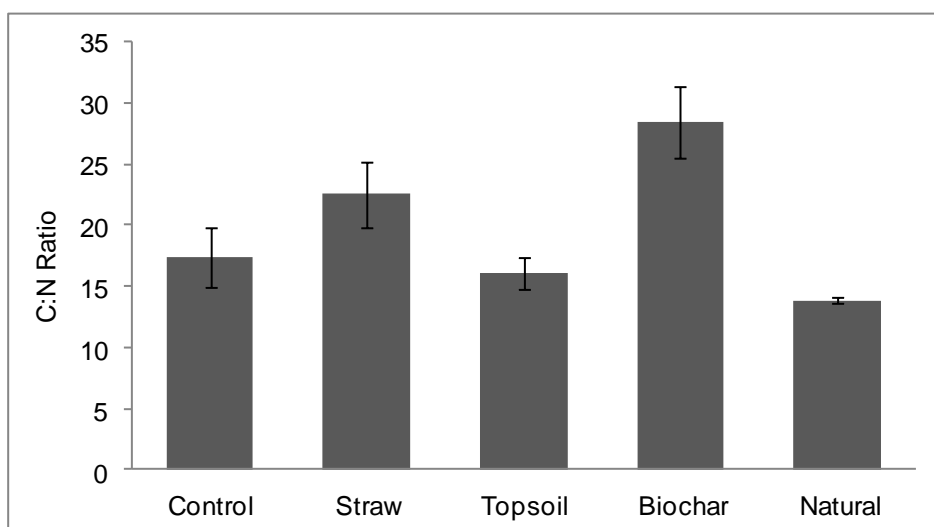
Figure 36. Differences in C:N ratio across treatments in comparison to the natural wetland. Error bars show standard error.
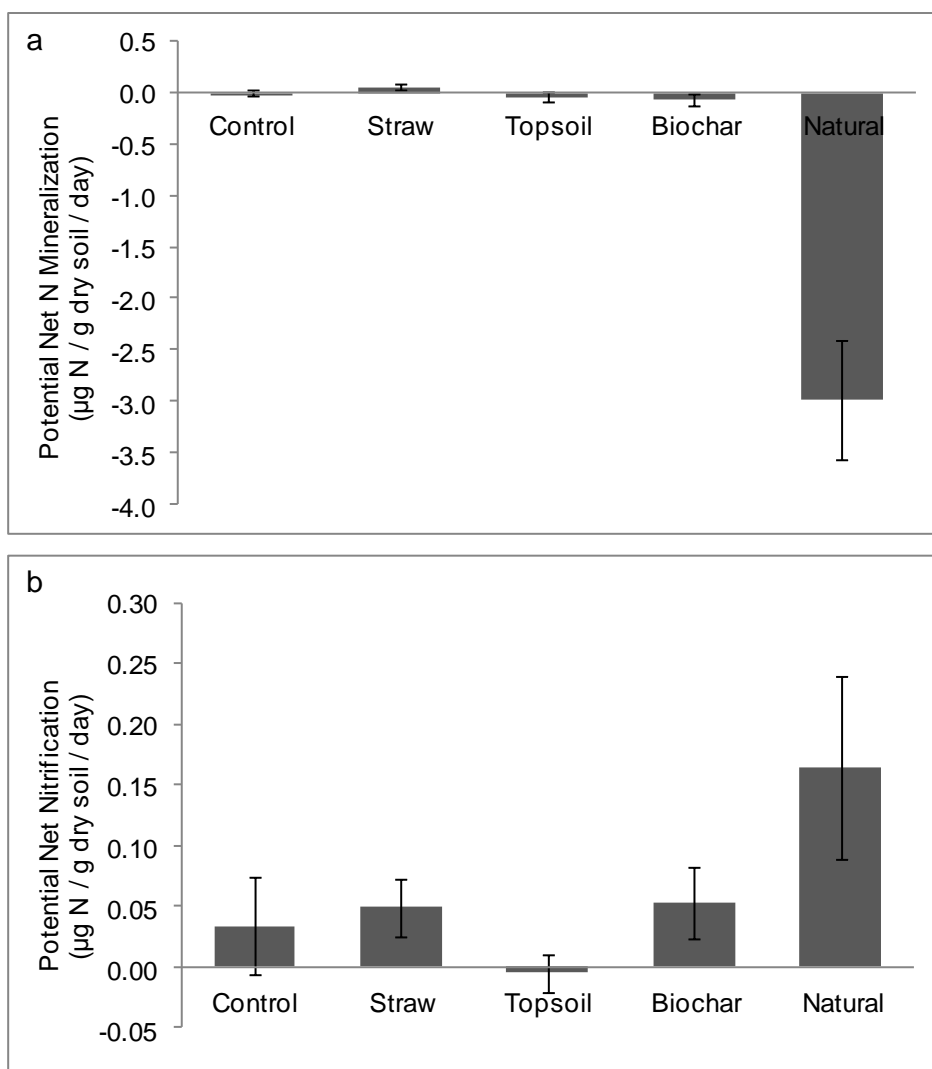
Figure 37. Differences in (a) potential net N mineralization and (b) potential net nitrification across treatments in comparison to the natural wetland. Error bars show standard error.
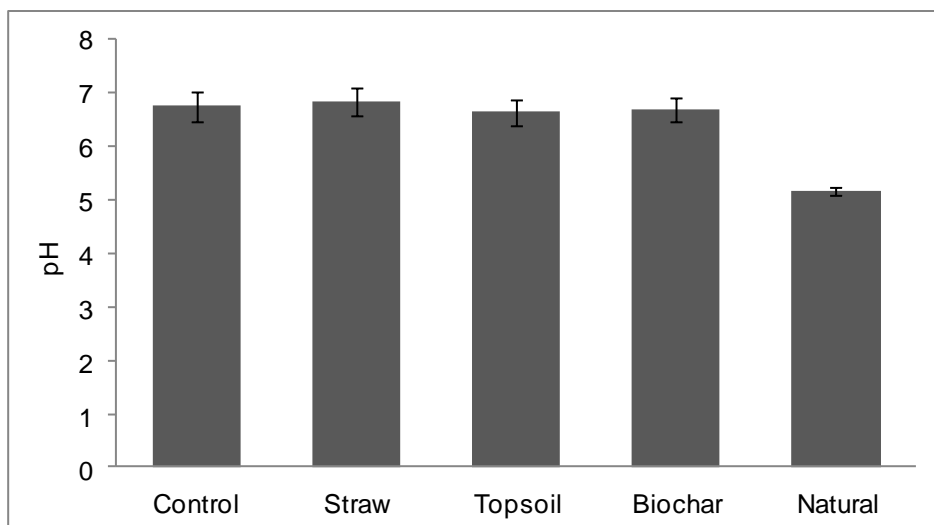
Figure 38. Differences in pH across treatments in comparison to the natural wetland. Error bars show standard error.

Tables

Table 1. Geographical coordinates of the restored wetland sites and the natural reference wetland.

Site	Location
Restored Wetland Site 1	42°55′39″N 76°51′31″W
Restored Wetland Site 2	42°55′37″N 76°51′22″W
Restored Wetland Site 3	42°23′11″N 76°18′17″W
Restored Wetland Site 4	43°10′11″N 75°56′04″W
Natural Wetland	42°23′12″N 76°18′29″W

Table 2. Summary of p and r^2 values associated with the linear regression relationship between total carbon and denitrification potential.

	p	r^2
Overall	0.001	0.166
Site 1	0.110	-
Site 2	0.005	0.423
Site 3	0.018	0.286
Site 4	0.001	0.785

Table 3. Summary of p and r^2 values associated with the linear regression
relationship between total organic carbon and denitrification potential.

	p	r^2
Overall	0.001	0.169
Site 1	0.264	-
Site 2	0.001	0.548
Site 3	0.010	0.333
Site 4	0.001	0.786

Table 4. Summary of p and r^2 values associated with the linear regression relationship between microbial biomass nitrogen and denitrification potential.

	p	r^2
Overall	0.024	0.078
Site 1	0.163	-
Site 2	<0.001	0.626
Site 3	0.011	0.321
Site 4	0.022	0.501

Table 5. Summary of p and r^2 values associated with the linear regression relationship between total organic carbon and microbial biomass nitrogen.

	p	r^2
Overall	0.001	0.150
Site 1	0.822	-
Site 2	0.001	0.457
Site 3	0.017	0.279
Site 4	0.004	0.577